OVULE AND OVARY CULTURE

IN

DORITIS PULCHERRIMA

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ABSTRACT

Pollination resulted in an increase in size of ovary, the development of the ovules and fertilization in Doritis pulcherrima. The growth curve of percentage increase in total growth of ovary width was bimodal. The first dip was at 40 days after pollination which was correlated with the time of megaspore formation inside the ovary. The growth curve of increase in percentage of ovary length was trimodal. These growth curves did not show a strong relationship with the development of ovule and embryo. However, the incremental growth in width and length of ovary was highest during rudimentary ovule formation and before embryo sac maturation. The mature pod dehisced at about 210 days after pollination, while the incremental growth in width and length stopped at about 90 to 110 days after pollination.

Wilting of perianth occurred 2 days after pollination, which is the first observable morphological change in Doritis. The placental ridge started to proliferate at about 20 days after pollination. Approximately 60 days was required to form the mature embryo sac and almost half of that time was occupied in placental growth.

Although the pollen tubes existed around developing ovules at 10 days after pollination, they entered the micropyle after the embryo sac was fully formed at about 60 days after pollination. Fertilization occurred soon after the embryo sac was fully formed containing mature egg at 60-65 days after pollination. Therefore, approximately 1/4 of the period between pollination and pod maturity was required for fertilization. Between 40 to 50 days after pollination, the megaspore mother cell underwent meiosis. After the first division, the two dyads

were formed, and after the second division two megaspores and the degenerating dyad was formed. The megaspore enlarged and formed the 8-nucleate embryo sac at about 60 days after pollination.

At about 65 days after pollination, the zygote and the endosperm initial cell were formed through double fertilization. However, the endosperm initial cell degenerated soon thereafter. At about 70 days after pollination, the zygote devided to form a 2-celled embryo which consisted of a terminal cell and a basal cell. Thin middle and suspensor initial cells were produced from the basal cell. The seed coat was also formed at about 70 days after pollination from the integuments. At about 80 days after pollination, the suspensor initial cell produced eight suspensor cells, and the terminal cell produced a multi-celled embryo.

Histochemical study showed that deoxyribonucleic acid (DNA), ribonucleic acid (RNA), total proteins and total carbohydrates decreased during megasporogensis. An interesting point was that RNA was more dense in the terminal cell than in the basal cell, which suggested that the initial differentiation had already occurred in the 2-celled embryo.

This is the first case of obtaining seedlings of orchids directly from ovules through ovary and ovule culture with ovules collected prior to the occurrence of fertilization. Approximately 150 days were reduced to obtain seedlings of Doritis pulcherrima from pollination through ovary and ovule culture over the traditional method of sowing "mature" seeds. Seedlings were obtained from ovules 45 days prior to normal fertilization time through ovary and ovule culture, which suggested that fertilization must have occurred in vitro during ovary and

ovule culture to obtain seedlings.

In the 20-day-old ovule culture, young seedlings were obtained from the treatments of sucrose; naphthaleneacetic acid (NAA) + coconut water; and NAA + 6-benzlaminopurine (BA) + coconut water. However, the growth rate of ovules during culture was not high in these treatments. In the 40-day-old ovule culture, the treatments of sucrose; coconut water; and NAA + BA + sucrose + coconut water were highly effective on both ovule growth rate and seedling formation. In the 60-day-old ovule culture, seedlings were obtained from all treatments except NAA; BA; and NAA + BA. Good growth of ovules did not always induce good seedling formation.

Seedlings were obtained from 20-, 40- and 60-day-old ovule cultures if media contained sucrose or coconut water. Sucrose must be necessary for seedling formation during ovule culture, since coconut water contains sucrose. However, if we compare the time required to obtain seedlings, coconut water promoted faster growth than sucrose alone in ovule culture. Maleic hydrazide, tryptophan, casein hydrolysate, NAA and BA were not effective on seedling formation without sucrose or coconut water.

The best hormonal conditions for obtaining seedlings in ovary culture were 1.0 ppm BA in 20-day-old ovary culture, 1.0 ppm NAA in 40-day-old ovary culture, and 1.0 ppm NAA in 60-day-old ovary culture. BA was more effective than NAA in forming seedlings in early stage (20 days after pollination) during ovary culture. Coconut water was also effective in ovary culture to obtain seedlings. However, it also increased callus formation rate.

Sucrose was necessary in ovule culture to obtain seedlings. However, even without sucrose seedlings were obtained through ovary culture with NAA or BA treatments. Therefore, sucrose might be necessary for ovule development and seedling formation, but in the case of ovary culture sucrose might be obtained from the ovary wall tissues. Sucrose was also necessary for seed germination, but coconut water can be substituted effectively for seed germination and protocorm formation. Sucrose appears to have a very important role in ovule and embryo developments and seedling formation.

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I. INTRODUCTION

Doritis pulcherrima Lindl., a member of the sub-tribe Vandinae of the family Orchidaceae, is widely distributed in Malaysia, Indochina, Thailand, Burma and Sumatra (Seidenfaden and Smitinand, 1963). This monopodial orchid has an erect habit of growth, and its oblong to round, leathery leaves are arranged in two rows along a central stem. The erect to sub-erect inflorescence is sometimes branched and carries numerous flowers about 2 to 3 centimeters across.

Doritis pulcherrima has been hybridized with members of the genus Phalaenopsis to form the intergeneric hybrid, Doritaenopsis. Some outstanding pink intergeneric hybrids have resulted from crossing Doritis pulcherrima and large-flowered tetraploid Phalaenopsis hybrids. However, the production of these intergeneric hybrids has not been easily accomplished.

The ovule culture technique which has been applied to various genera (Withner, 1943, 1955; Poddubnaya-Arnoldi, 1960) may be a useful tool for successfully obtaining intergeneric hybrids involving Doritis. This technique may provide additional advantages such as reducing the time for producing seedlings, and protecting the mother plant from being exhausted through the continued drain on its reserves while nuturing the ovaries and ovules.

The successful culture of ovaries in vitro, sometime after pollination but before fertilization, should further reduce the drain on the maternal parent. In addition, numerous crosses can be performed since the ovaries are detached from the maternal plant.

In order to successfully culture ovules and ovaries of Doritis

pulcherrima, gross morphology and anatomy of ovary and ovule development must first be established. Following this, some factors important to the successful culture of ovules and ovaries in vitro must be investigated.

II. LITERATURE REVIEW

A. Culture of embryo, ovule and ovary

Embryo culture:

The first embryo culture was carried out on Raphanus and Cochlearia (Cruciferae) in an artificial medium by Hanning in 1904. Further progress in embryo culture was made by Laibach (1925), who demonstrated the possibility of using the embryo culture technique in genetic studies to obtain viable hybrids from crosses which are otherwise unsuccessful, and to overcome dormancy in seeds. Tukey and Olav (1938) applied this technique in securing hybrids of peach. Lammerts (1942) showed that embryo culture may be useful in shortening the breeding cycle of deciduous fruit trees like apricot, nectarine and peach, and increasing the germiability of hybrid embryos.

Knudson (1922) first showed that orchid seeds which lack endosperm can be germinated asymbiotically and grown to mature plants on aseptic nutrient agar media containing sucrose. Ernst (1975) reported on seed germination of Phalaenopsis and Paphiopedilum in vitro. Curtis (1947) investigated the undifferentiated growth of Vanda tricolor embryos on media containing barbituric acid derivatives. The possibility of germination of orchid seeds from premature pods was demonstrated by Tsuchiya (1954). Examples of successful cultures of excised embryos are found in Cattleya (Raghavan and Torrey, 1963; 1964) and in Cymbidium and Phalaenopsis (Takagi, 1962). Ayers (1964) and Sagawa (1962) cultured the embryos of Phalaenopsis.

Ovule culture:

The first attempt to culture ovules was made by La Rue (1942), who was able to obtain some growth of the ovules of Erythronium. Kanta et al. (1962) observed fertilization and subsequent maturation of whole ovules in vitro. Enlargement of excised unfertilized ovules in the presence of indoleacetic acid (IAA) or gibberellic acid (GA) or both was observed by Beasley (1973). Maheshwari (1958) succeeded in culturing ovules of Papaver somniferum excised after fertilization, and obtained viable seeds on Nitsh's medium supplemented with 0.4 ppm kinetin. Kappor (1959) obtained similar results with Zephyranthes. Beasley and Ting (1974) and Lintilhac and Jensen (1974) observed the growth and fiber development in cotton.

Using Cypripedium, Withner (1943) investigated ovule culture as a new method for starting orchid seedlings. He was able to shorten the time required for the production of seedlings. He used this technique with Vanilla to avoid chemical or mechanical disadvantages for Vanilla seed germination in 1955. Poddubnaya-Arnoldi (1959, 1960) studied embryogenesis in orchids in excised ovules placed on a slide in a drop of water or 10% solution of sucrose. She followed the embryogeny from the entry of the pollen tube to the globular embryo stage in Calanthe, Cypripedium, Dendrobium and Phalaenopsis. Valmayor and Sagawa (1967) reported a comparison of the interval between pollination, fertilization and subsequent growth from embryo cultures in 37 species of orchids. They pointed out that in all orchid species, growth from ovule cultures was obtained only after fertilization had been accomplished.

Ovary culture:

The culture of flowers and ovaries was introduced by La Rue (1942).

He found that pollinated flowers of tomato, Kalanchoe, Forsythia and Caltha cultured in a mineral salt medium remained alive and grew appreciably in size during the culture period, resulting in normal fruits. Nitsch (1949, 1951) cultured the flowers of gherkin, bean, strawberry, tobacco and tomato which were excised from plants before and after pollination. He found that ovaries of pollinated flowers of gherkin and tomato, cultured in a relatively simple medium containing sucrose and mineral salts, formed fruits which ripened and produced seeds. Seedless fruits of tomato were obtained from unpollinated flowers on media containing auxins. Maheshwari and Lal (1958) cultured ovaries of Iberis amara, and studied the effects of several chemicals on the development of the pericarp as well as the ovules. The addition of kinetin alone, as well as in combination with indoleacetic acid, seemed to improve the growth of the ovaries only slightly. Jansen and Bonner (1949) obtained tomato fruits with pericarp and gelatinous placental tissue in the locules, in which ovules had not matured.

Ito (1958, 1960) found that the ovaries of Dendrobium developed well on Nitsch's medium containing only inorganic salts and sugar. Israel (1963) was able to obtain Dendrobium seedlings through aseptic culture of ovaries. However, he did not attempt to determine the optimal post-pollinated interval for starting ovary culture or the optimal NAA concentration of the culture medium. Niimoto and Sagawa (1962) poined out that the zygote must be formed before culturing if seedlings are to be obtained. On the other hand, Magli (1958) suggested that the development of immature ovules prior to fertilization may be induced and cultured in vitro.

B. Nutrition and hormones for cultures:

Nutrition:

Raghavan and Torrey (1964) found that Cattleya embryos are unable to utilize the ion NO_3^- during germination and early stages of growth. In embryo culture of cotton, Stewart and Hsu (1977) found that when the medium was supplemented with NH_4^+, more than 50% of the ovules produced mature embryos. Bahme (1949) found nicotinic acid to be a growth factor for embryos of Cattleya and Vanda. Better differentiation and germination of embryos were observed in a medium with tomato juice by Vacin and Went (1949). Viability and growth of Allium cepa flowers was observed when cultured 2 days after pollination (Guha and Johri, 1966). Spoerl (1948) found arginine and aspartic acid satisfactorily supported growth of unripe and mature orchid seeds, respectively. Ito (1960) observed that the germination of orchid seeds and the growth of protocorms were unsatisfactory unless peptone was present in the medium. Raghavan (1964, 1966) reported that certain organic nitrogen compounds were effective on growth in vitro of Cattleya seedlings.

For the cultures of ovaries of Dendrobium, Torigata (1976) found that sucrose is necessary for the early development of the ovary. Maheshwari and Lal (1961) showed that maleic hydrazide promoted ovary growth.

Hormones:

Sachar and Guha (1962) showed that in the ovary culture of Ranunculus sceleratus the growth of embryo was enhanced by supplementing with IAA or GA. Ten to 15 days after pollination, viable seeds were produced inside of excised ovaries of Reseda odorata on the medium

containing IAA and kinetin (Raghavan, 1976). Pierik and Steegmano (1972) demonstrated that a BA concentration of 1 to 10 ppm was very effective in producing a number of plantlets and protocorms from a single explant of Cattleya aurantiaca. Indoleacetic acid and gibberellic acid promoted ovule growth and fiber development of cotton, and kinetin promoted only ovule growth (Beasley and Ting, 1974). Cionin et al. (1976) reported that GA_3 concentration of 10^{-8} to 10^{-6} M can replace the suspensor in heart-shaped and early cotyledonary embryo of Phaseolus coccineus.

C. Ovule and embryo development

There is considerable information on gametogenesis and embryogenesis in Petunia (Rangaswamy and Shivanna, 1967), Capsella (Schulz and Jensen, 1968), rye (Hallam, 1972a,b), sunflower (Newcomb, 1973a,b), Plumbago capensis (Cass, 1972), Rhizohora mangle (Sussex, 1975), Antinhinum majus (Sangwan and Harada, 1975), and cotton (Schulz and Jensen, 1977).

Electron microscopic studies of embryo development were reported for Zea mays (Diboll and Larson, 1966; Diboll, 1968), Quercus gambelli (Mogensen, 1972; 1975), Stellaria media (Newcomb and Fowke, 1974), and Aquielegia formosa (Vijayaraghavan et al., 1972).

Several workers investigated gametogenesis and embryogenesis in orchids (Sharp, 1912; Carson, 1940; Spoerl, 1948; Swamy, 1949; Johansen, 1950; Wirth and Withner, 1959; Niimoto and Sagawa, 1961; Sagawa and Israel, 1964; Olsson, 1967; Cocucci and Jensen, 1969; Rodkiewicz and Bednara, 1976).

The normal sequence of ovule development was determined for

Dendrobium (Niimoto and Sagawa, 1961; Sagawa and Israel, 1964), Phalaenopsis (Niimoto and Sagawa, 1962), and Epidendrum (Cocucci and Jensen, 1969a,b).

Placental proliferation and ovule formation generally occurred after anthesis and required pollination to stimulate development (Withner, 1959). The orchid embryo develops in a sac without an endosperm; this has generally been interpreted as the cause of the rudimentary state of the embryo. The endosperm does not usually form in orchids, either due to a lack of fusion of the second sperm nucleus with the polar nuclei, or because of the immediate degeneration of the nucleus of the endosperm if double fertilization does take place (Withner, 1974).

D. Histochemical study

Many histochemical studies of the embryo have been made, such as those of Pollock et al. (1961) on Capsella, Koul (1964) on Coix, Zinger and Poddubnaya-Arnoldi (1959, 1966) on orchids, Cass and Jensen (1970) on barley, Haskell and Postlethwait (1971) on Acer saccharym, Caponetti (1972) on Osumunda cinnamonea, Panchaksharapa and Rudramuniyappa (1973) on millets, and Sehgal and Gifford (1979) on Nicotiana rustica.

Pritchard (1964) observed that in Stellaria media, the cell walls in the archesporium, megaspore mother cell, megaspore, and 2- and 4-nucleate embryo sacs were PAS-positive. He observed that DNA stainability in these tissues was low. However, RNA and protein content of these tissues varied. RNA and protein content was high in the archesporium and cytoplasmic RNA decreased markedly in the megaspore mother cell and increased in the megaspore. The protein content of the

megaspore was low, while 2- and 4-nucleate embryo sacs were rich in RNA and proteins.

Alvarez and Sagawa (1965) determined qualitative changes in the concentration and distribution of DNA, RNA, total proteins, histones, and insoluble polysaccharides in the developing embryo of Vanda. Total proteins and RNA were found in high concentrations in the embryo initial and low concentration in the suspensor initial. However, DNA and histones were present in higher concentrations in the large nuclei of the parenchymatous cells than in the small nuclei of the meristematic cells of the protocorm. Total protein and RNA concentrations were equal in the cells of both these tissues.

Polysaccharide constitution of the reproductive structures, especially the ovule and embryo sac, was investigated by Pritchard, 1964; Alvarez and Sagawa, 1965; Schulz and Jensen, 1968; Cass and Jensen, 1970; Panchaksharappa and Rudramuniyappa, 1973; Panchaksharappa and Hegde, 1974.

Panchaksharappa and Rudramuniyappa (1973) observed rich starch storage in the ovule primordium, while the archesporium, megaspore mother cell, megaspore and young embryo sac showed a low PAS-positive tinge in the cytoplasm.

III. MATERIALS AND METHODS

Flowers of diploid (2n=38) Doritis pulcherrima grown in the greenhouse of Department of Horticulture at the upper Manoa Campus of the University of Hawaii were self-pollinated. The growth of pods (length and width) was measured to the nearest 0.1 mm with a Vernier caliper at 10 day intervals up to 120 days after pollination.

To trace the development of ovules and embryos following pollination, paraffin sections of pods collected at 10 day intervals up to 120 days were prepared as follows: fixation in Craf (Jensen, 1962), followed by dehydration through graded series of tertiary butyl alcohol (Jensen, 1940) in Autotechnicon, embedding in paraffin, sectioning 10 microns thick with a Spencer rotary microtome, and staining with Safranin-Fast Green (Jensen, 1962). Sections were examined and photographed with a Zeiss photomicroscope.

Squashes were made of 60-day-old pods fixed in Craf utilizing Gomori's hematoxylin technique (Melander and Wigstand, 1953), in order to observe the fully formed embryo sac.

For histochemical studies, pods were collected at 10 day intervals up to 120 days. Qualitative changes in concentration and distribution of DNA (deoxyribonucleic acid), RNA (ribonucleic acid), total proteins and total carbohydrates were examined in the developing ovules and embryos. Tissues used for nucleic acid study were fixed 4 hours in Carnoy's solution consisting of 3 parts absolute alcohol and 1 part glacial acetic acid (Marvin and Sagawa, 1965), while those for total proteins and carbohydrates were fixed for 24 hours in FAA solution

consisting of 90 parts 60% ethanol, 5 parts glacial acetic acid and 5 parts 40% formaldehyde (Jensen, 1962). DNA, RNA, total proteins and total carbohydrates were stained with the Feulgen Method (Gomori, 1952), Methyl Green and Pyronic Method (Jensen, 1962), respectively.

Aseptic culture were grown on various types of media. Basal medium was Vacin and Went (Vacin and Went, 1949) modified by removal of sucrose. After adjustment of pH of all media to 4.8, all components of the media were autoclaved at 120 °C at 15 psi for 15 minutes.

Pods were collected at 20 day intervals over a period of 60 days from pollination for both ovule and ovary cultures. Pods were sterilized by dipping them into 95% ethyl alcohol for a few minutes and flaming briefly. The distal and proximal ends of the pod were then cut with a sterilized scalpel.

For ovule culture, pods were sectioned longitudinally and then ovules and placental ridges were removed from pod. To investigate the hormonal and nutritional effects on ovule development in vitro, NAA at 10, 20 and 30 ppm; BA at 10, 20 and 30 ppm; sucrose at 2%; coconut water (CW) at 15, 25, 35 and 45%; tryptophan at 100, 500 and 1000 ppm; casein hydrolysate at 100, 500 and 1000 ppm and maleic hydrazide at 100, 500 and 1000 ppm were used as supplements.

For ovary culture, immature ovaries were placed in an inverted position on media (Israel, 1963). To investigate the hormonal and nutritional effects on ovule development inside of ovary in vitro, NAA at 0.1, 1.0, 5.0 and 10.0 ppm; BA at 0.1, 1.0, 5.0 and 10.0 ppm; sucrose at 1, 2 and 4%; and 15% coconut water were used as supplements.

As each ovary reached an age of one year from the time of polli-

nation, the ovary was sectioned with a knife, and the size of seeds in it were examined.

For seed culture, pods 130 days after pollination were collected and sterilized by dipping the pods into 95% ethanol and flaming. Pods were cut with a scalpel under aseptic conditions, and seeds were removed and cultured on basic media with 2% sucrose; 10 ppm BA; 10 ppm NAA and 15, 25, and 35% coconut water as supplements. At 20 days after sowing seeds the germination of 100 seeds was examined. Protocorm number per vial was examined at 30 days and 40 days after sowing seeds on media.

All excised tissues were cultured in 11 x 2.5cm vials, 9.5 x 2.5cm vials or 13 x 1.5cm test tubes at 26 $\pm$ 3 $^{\circ}$C under 200 foot candles of continuous illumination from G. E. cool white flourescent lamps (Power Groove).

IV. RESULTS

The results are presented in four sections:

A. Morphological and histochemical study of ovule and ovary development.

B. Ovary culture.

C. Ovule culture.

D. Seed culture.

A. Morphological and histochemical study of ovule and ovary development

1. Morphological changes which occur following pollination

Flowers of Doritis pulcherrima are shown in Figure 1, and the ovaries at 40 days after pollination are shown in Figure 2, while Figure 3 shows the growth curve of ovaries expressed as the actual increase of ovary width. The width of ovary increases continuously until 80 days after pollination with the maximum increase observed at 10 days after pollination. The growth curve is bimodal with the first peak at 10 days and the second peak at 50 days after pollination. Figure 4 shows the growth curve expressed as the actual increase (mm) in ovary length. The length of ovary increases up to 100 days after pollination. The growth curve is trimodal with the first peak at 20 days, the second peak at 60 days and the third peak at 90 days after pollination.

2. Development of ovule and embryo following pollination

The time sequence of ovule and embryo development is shown in Table 1. Placental proliferation and ovule development occur after pollination. Wilting and drying of the perianth occur 2 days after pollination. Pollen tubes germinate, go down the style canal into the ovary, and produce masses of pollen tubes around the placental ridge

Fig. 1. Flowers of Doritis pulcherrima.

Fig. 2. Ovaries at 40 days after pollination.

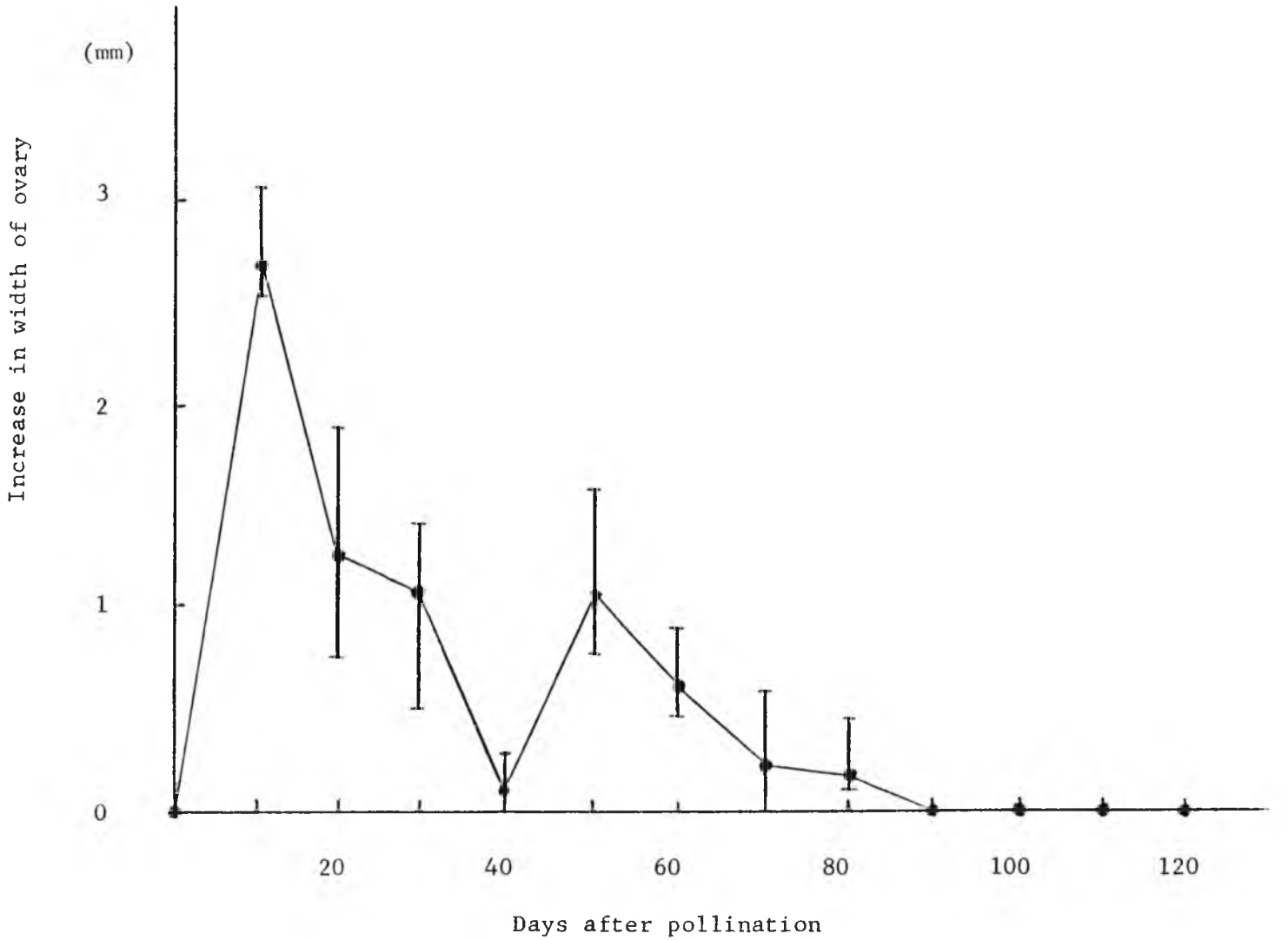

Figure 3. Growth curve of increase in ovary width.

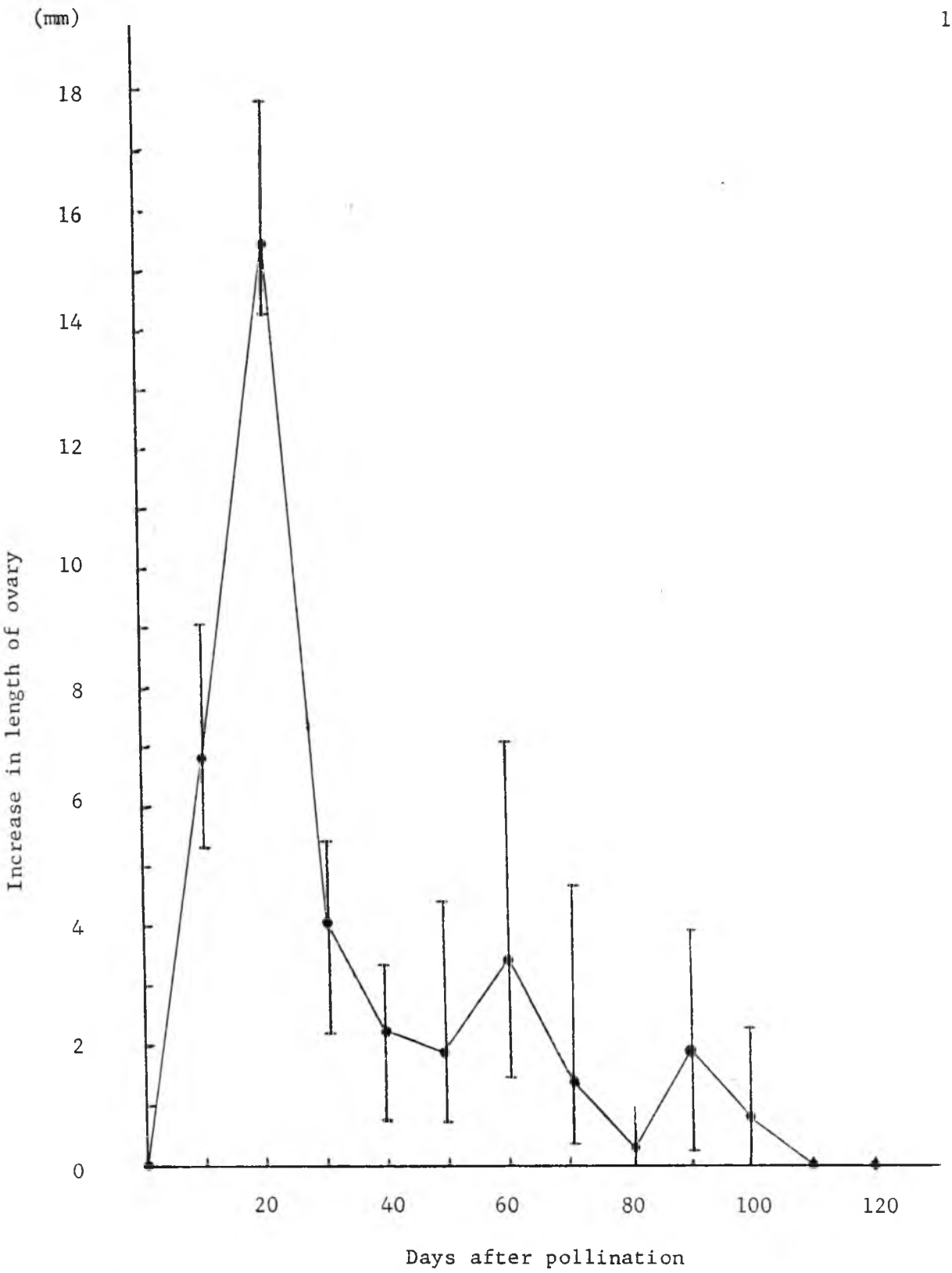

Figure 4. Growth curve of increase in ovary length.

Table 1. Time sequence of ovule and embryo development in Doritis pulcherrima.

Stages of ovule and embryo development	Days after pollination
1. Wilting of perianth	2 days
2. Pollen tube in ovary	10 days
3. Placental proliferation	10 days
4. Filamentous row of six or seven nucellar cells	30 days
5. Differentiation of archesporial cell	30 days
6. Ovules anatroposed, megaspore mother cell	40 days
7. Meiosis occurs, elongation of inner and outer integuments	40-50 days
8. Mature embryo sac	60 days
9. Fertilization	60-65 days
10. Zygote, endosperm initial cell	60-65 days
11. 2-celled embryo	70 days
12. Suspensor cells	80 days
13. Multi-celled embryo	90-210 days
14. Mature pod splits	210 days

in the ovary within 10 days after pollination (Figure 5). The ovule of the Doritis flower at anthesis is undeveloped. Figure 6 shows the undeveloped placental ridges. The slender locule of the ovary consists of three longitudinal rudimentary placental ridges oriented parietally. The placental ridge begins to proliferate in 10 days (Figure 7, 8, 9), and branches (Figure 10, 11) with a filamentous row of six or seven nucellar cells surrounded by a single layered epidermis in 30 days (Figure 12). The terminal cell of the nucellar column enlarges and forms the archesporial cell at 30 days after pollination (Figure 13). At about forty days after pollination, the archesporial cell functions directly as the megaspore mother cell (Figure 14). The ovule becomes recurved and anatropous with the primary and secondary integuments (Figure 15). The megaspore mother cell forms two dyads consisting of micropylar and chalazal cells at 50 days (Figure 16). The micropylar dyad degenerates, but the chalazal dyad divedes to form two megaspores (Figure 17). Between 50 to 60 days, the functional chalazal megaspore produces 2-nucleate embryo sac (Figure 18, 19). Sixty days after pollination, 8-nucleate embryo sac is formed through two successive divisions (Figure 20, 21, 22). Between 60 to 65 days, pollen tube enters the embryo sac through the micropyle (Figure 23, 24). Soon after the entrance of the pollen tube into the embryo sac, one of the sperm fuses with the egg to form a zygote, and the other sperm fuses with the polar nuclei to form an endosperm initial cell. Therefore, double fertilization occurs (Figure 25, 26). The endosperm initial cell degenerates and only the zygote can be observed (Figure 27). At about 70 days after pollination, the zygote divides (Figure 28, 29) to

form a 2-celled embryo which consists of a terminal cell and a basal cell (Figure 30). The middle and suspensor initial cells are produced from the basal cell (Figure 31). The suspensor initial cell divides longitudinally to form a 4-celled embryo (Figure 32). At 70 days, the seed coat has formed from the integuments (Figure 33). At 80 days, two subsequent divisions of the suspensor initial cell have produced eight suspensor cells (Figure 34). Successive divisions of the terminal cell produce a multi-cellular embryo (Figure 35). The embryo continuously develops with pod maturation (Figure 36).

3. Histochemical study

Table 2 shows the results of histochemical study to determine changes in DNA, RNA, total proteins and total carbohydrates at various stages of ovule and embryo development. Total carbohydrates are high in placental ridge at placental proliferation stage (Figure 37), then decrease continuously until the embryo sac is fully formed. At 40 days, total carbohydrates are low in the megaspore mother cell (Figure 38), in the megaspore (Figure 39) and in the zygote (Figure 40). During embryogenesis, they are still low (Figure 41, 42). Total proteins are almost constantly high during ovule and embryo development. Total proteins are high in the placental ridge (Figure 43), in the archesporial cell (Figure 44), in the megaspore (Figure 45) and in the multi-cellular embryo (Figure 46). RNA is high in the placental ridge (Figure 47), in the zygote (Figure 48) and in the multi-cellular embryo (Figure 49) through ovule and embryo development except in the megaspore mother cell (Figure 50) and in the megaspore (Figure 51). RNA is higher in the terminal cell than in the basal cell at 2-celled embryo stage

(Figure 52). DNA concentration is high in the placental ridge, moderate during megasporogenesis and high again during embryogenesis (Figure 53, 54, 55).

Fig. 5. Masses of pollen tubes (arrow) around the placental ridge in the ovary at 10 days after pollination (x 102.4).

Fig. 6. Placental ridge (arrow) before proliferation at 5 days after pollination (x 128).

Fig. 7. Placental ridge at 10 days after pollination (x 256).

Fig. 8. Proliferation of placental ridge. Cross section of pollen tubes (arrow) are observed at 10 days after pollination (x 256).

Fig. 9. Proliferation of placental ridge at 20 days after pollination (x 102.4).

Fig. 10. Branches of proliferated placental ridge at 20 days after pollination (x 102.4).

Fig. 11. Branches of proliferated placental ridge at 20 days after pollination (x 128).

Fig. 12. Filamentous row of seven nucellar cells surrounded by a single layered epidermis at 30 days after pollination (x 640).

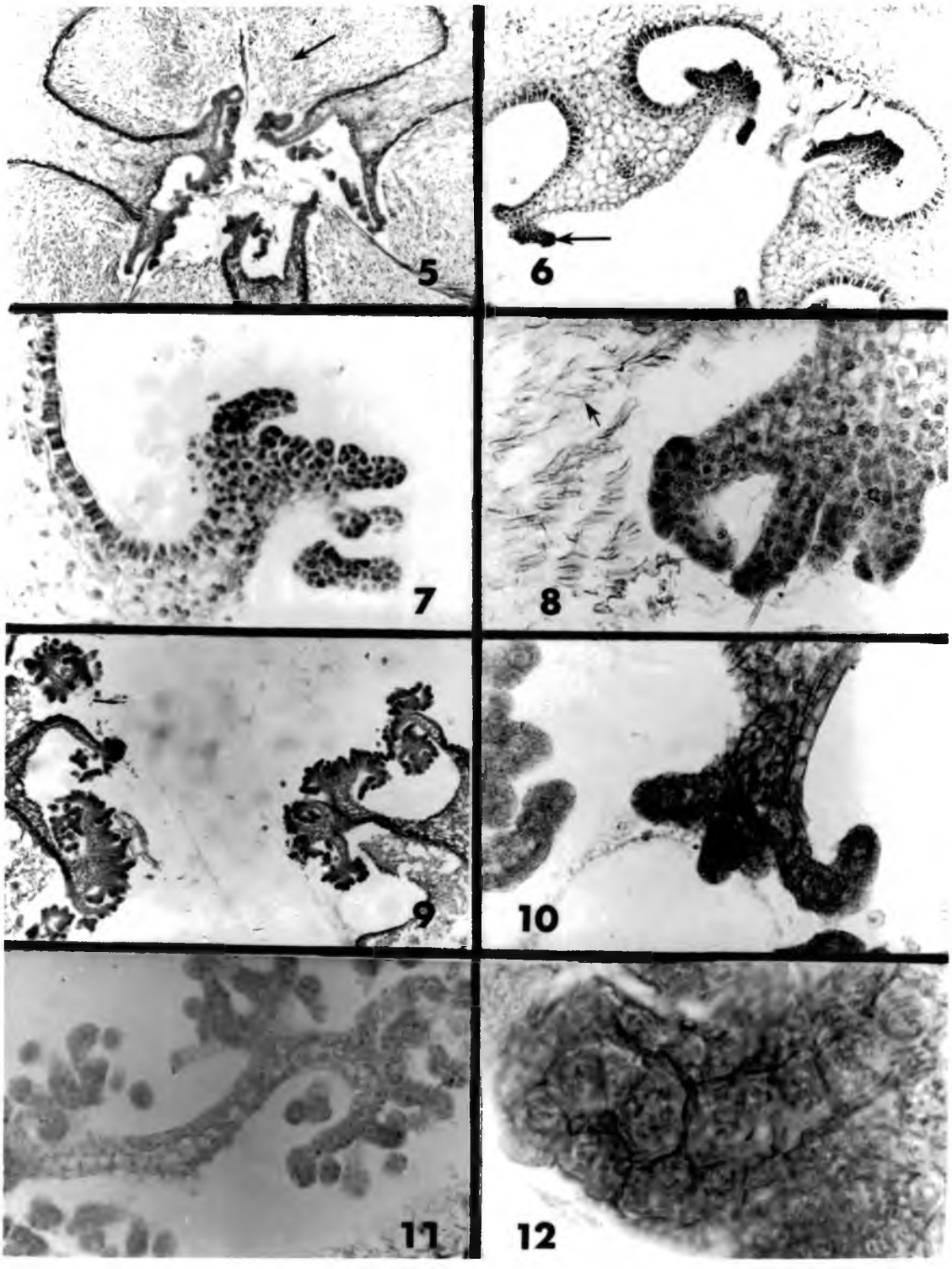
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Fig. 13. Archesporial cell (arrow) at 30 days after pollination (x 256).

Fig. 14. Enlarged megaspore mother cell (arrow) at 40 days after pollination (x 102.4).

Fig. 15. Recurved and anatropous ovules with primary (arrow) and secondary integuments at 40 days after pollination (x 256).

Fig. 16. Two dyads (arrow) at 50 days after pollination (x 640).

Fig. 17. Two megaspores (arrow) at 50 days after pollination (x 320).

Fig. 18. 2-nucleate embryo sac (arrow) at 50 days after pollination (x 256).

Fig. 19. 2-nucleate embryo sac (arrow) at 50 days after pollination (x 2560).

Fig. 20. 8-nucleate embryo sac (arrow) at 60 days after pollination (x 256).

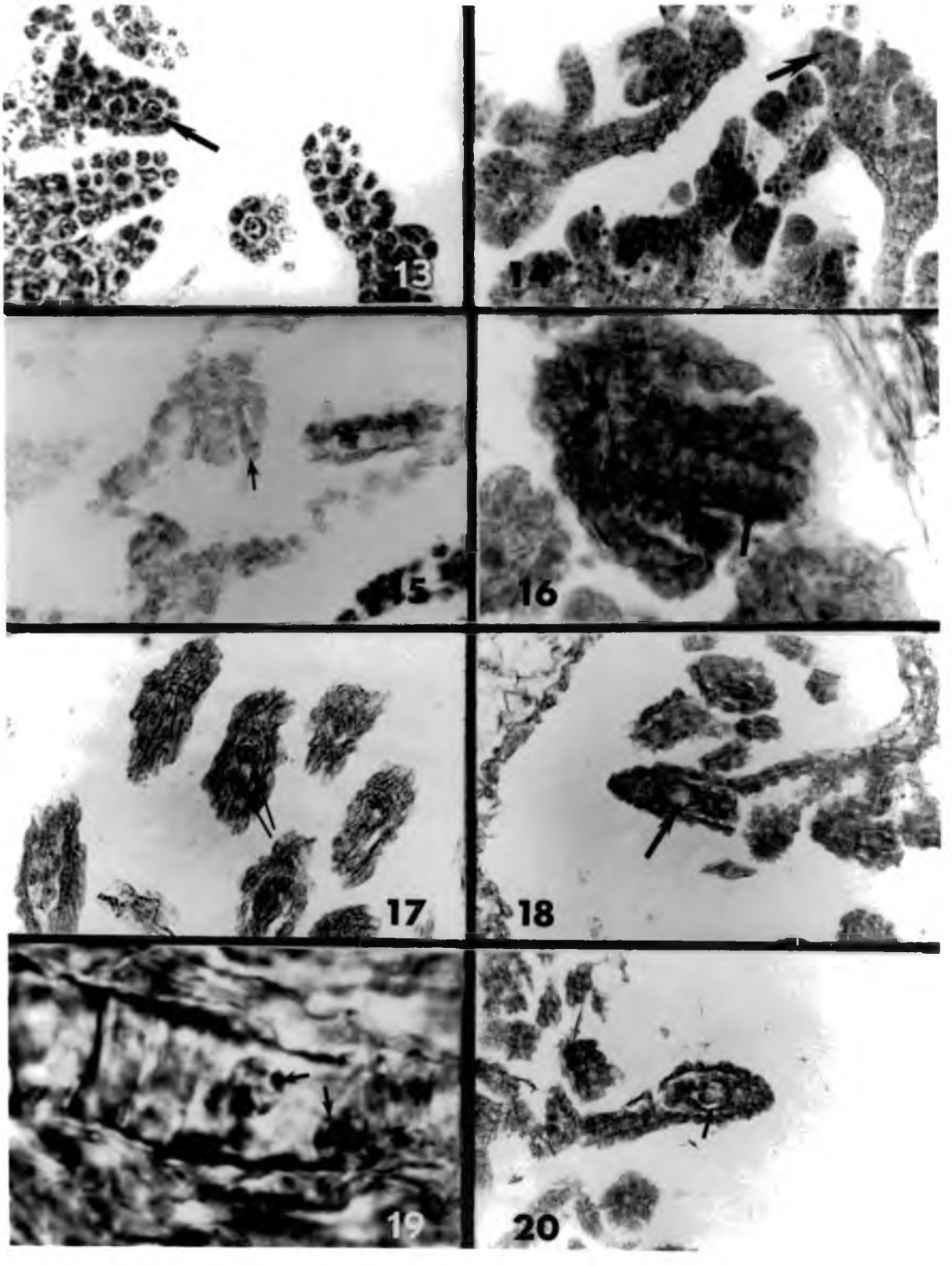
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Fig. 21. 8-nucleate embryo sac at 60 days after pollination (x 256).

Fig. 22. 8-nucleate embryo sac (arrow) (x 640).

Fig. 23. Pollen tube (arrow) enters the embryo sac through micropyle at 60 days after pollination (x 320).

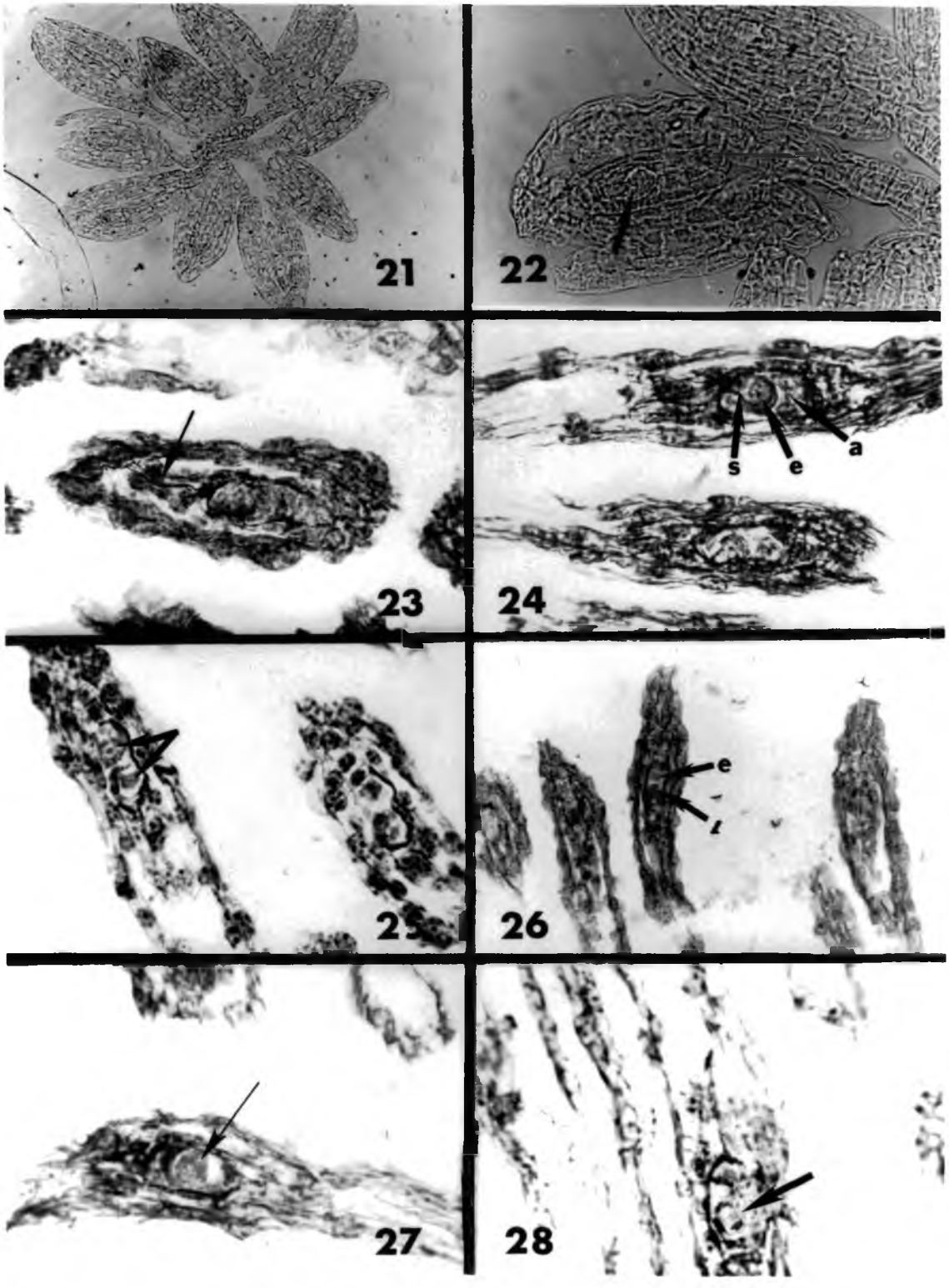
Fig. 24. Pollen tube enters the embryo sac through micropyle at 65 days after pollination (x 320). Egg (e)(arrow), antipodal cells (a)(arrow) and synergids (s)(arrow) are visible.

Fig. 25. Double fertilization at 65 days after pollination (x 320).

Fig. 26. Double fertilization (arrow) at 65 days after pollination (x 256 Zygote (z)(arrow) and endosperm initial cell (e)(arrow) are visi

Fig. 27. Zygote (arrow) at 65 days after pollination (x 320).

Fig. 28. First division (arrow) of zygote at 70 days after pollination (x 320).


21
22
23
24
s
e
a
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26
e
l
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28

Fig. 29. Close up observation of first division of zygote (arrow) at 70 days after pollination (x 2560).

Fig. 30. 2-celled embryo (arrow) consisting of a terminal cell and basal cell at 70 days after pollination (x 2560).

Fig. 31. 3-celled embryo (arrow) with suspensor initial cell at 70 days after pollination (x 320).

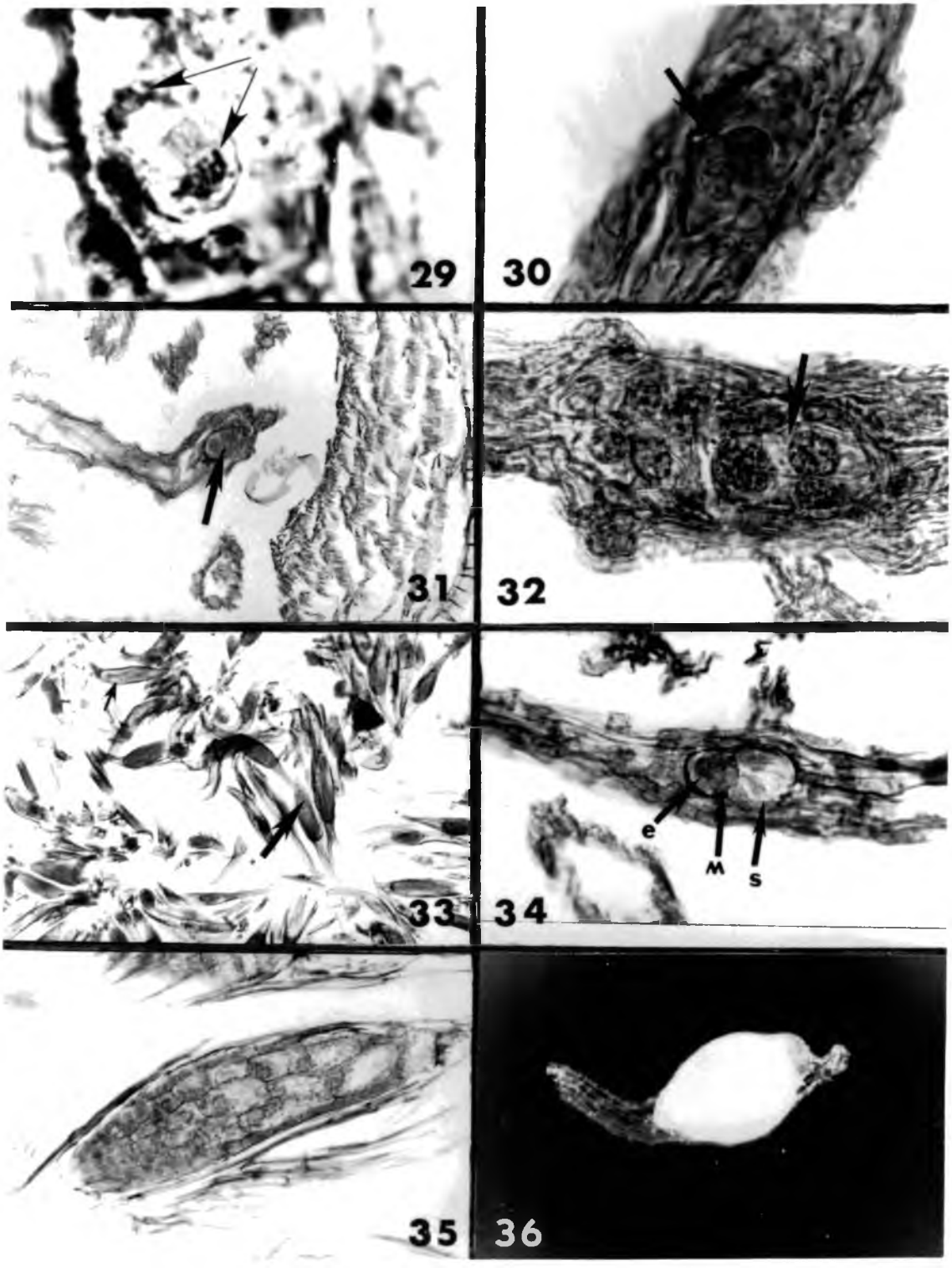
Fig. 32. 4-celled embryo (arrow) at 70 days after pollination (x 640).

Fig. 33. Seed coat (arrow) is formed from the integuments at 70 days aft pollination (x 128).

Fig. 34. Embryo with 8 suspensor cells at 80 days after pollination (x 3 Embryonal cell (e)(arrow), middle cell (m)(arrow) and 8 suspens cells (s)(arrow) are visible.

Fig. 35. Multi-celled embryo at 90 days after pollination (x 320).

Fig. 36. Multi-celled embryo of mature seed at 210 days after pollinatio (x 102.4)


29
30
31
32
33
e
M
S
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35
36

Table 2. Relative concentrations of DNA, RNA, total protein and total carbohydrates at various stages of development.

Compounds	Stages of development[z]							
	PP (0)	MMC (40)	M (50)	E (60)	Z (65)	2-celled EM (70)	EM & S (80)	MEM (120)
DNA	+++[y]	++	++	++	+++	+++	+++	+++
RNA	+++	++	++	+++	+++	+++	+++ & ++	+++
protein	+++	+++	++	+++	+++	+++	+++	+++
carbo-hydrates	+++	+	+	+	+	+	+	+

[z] The stages of development: PP - placental proliferation, MMC - megaspore mother cell, M - megaspore, E - egg, Z - zygote, 2-celled EM - 2-celled embryo, EM & S - embryo & suspensor and MEM - multi-celled embryo. Figures in parenthesis indicate number of days after pollination.

[y] Relative concentrations are designated as + low; ++ moderate; +++ high.

Fig. 37. Total carbohydrates in placental ridge at 5 days after pollinat
(x 128).

Fig. 38. Total carbohydrates in megaspore mother cell (x 320).

Fig. 39. Total carbohydrates in megaspore (x 320).

Fig. 40. Total carbohydrates in zygote (x 2560).

Fig. 41. Total carbohydrates in 2-celled embryo (x 256).

Fig. 42. Total carbohydrates in multi-celled embryo (x 640).

Fig. 43. Total proteins in placental ridge (x 256).

Fig. 44. Total proteins in archesporial cell (x 230).

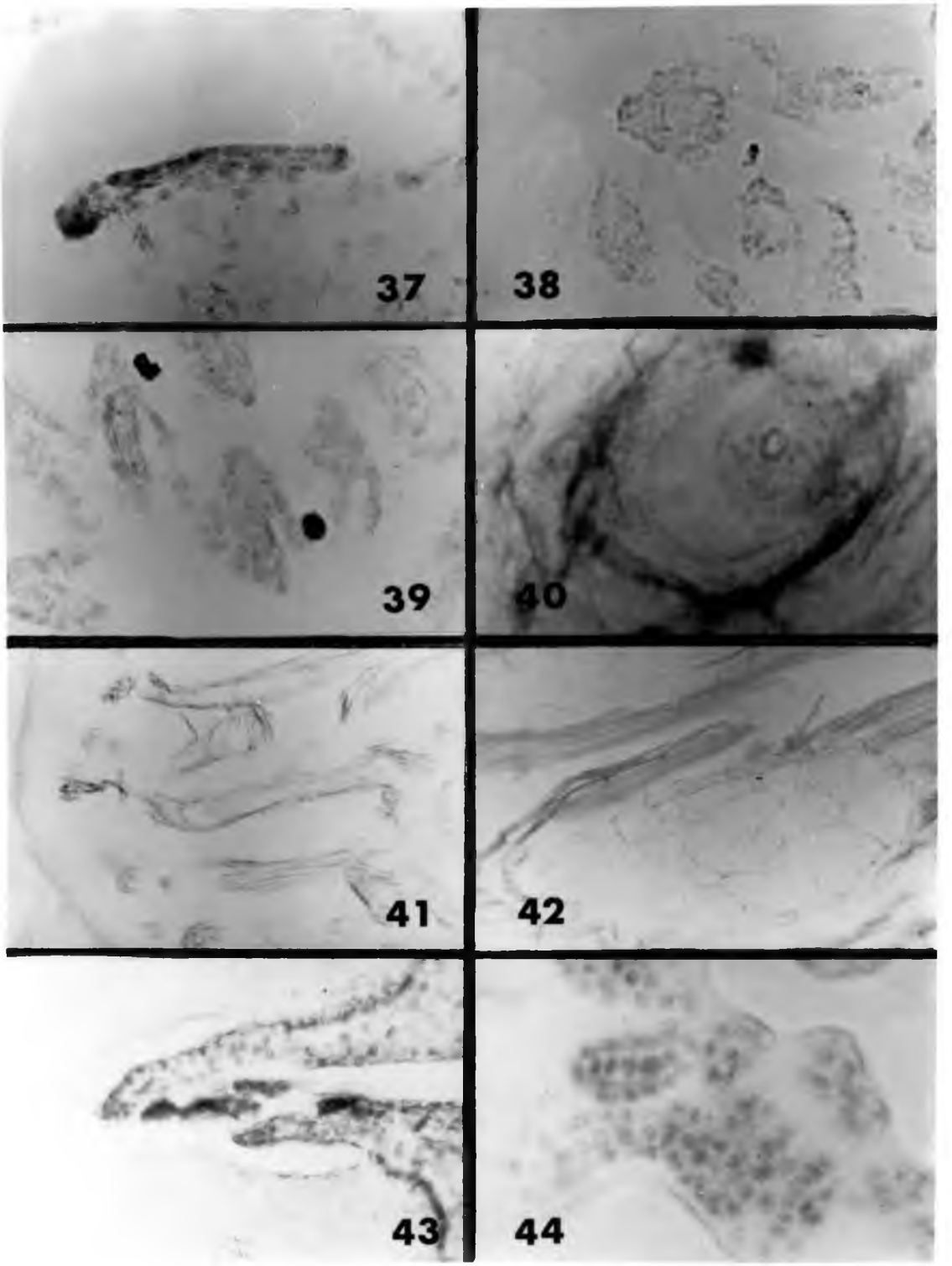
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Fig. 45. Total proteins in megaspore (x 230).

Fig. 46. Total proteins in multi-celled embryo (x 256).

Fig. 47. RNA in placental ridge (x 128).

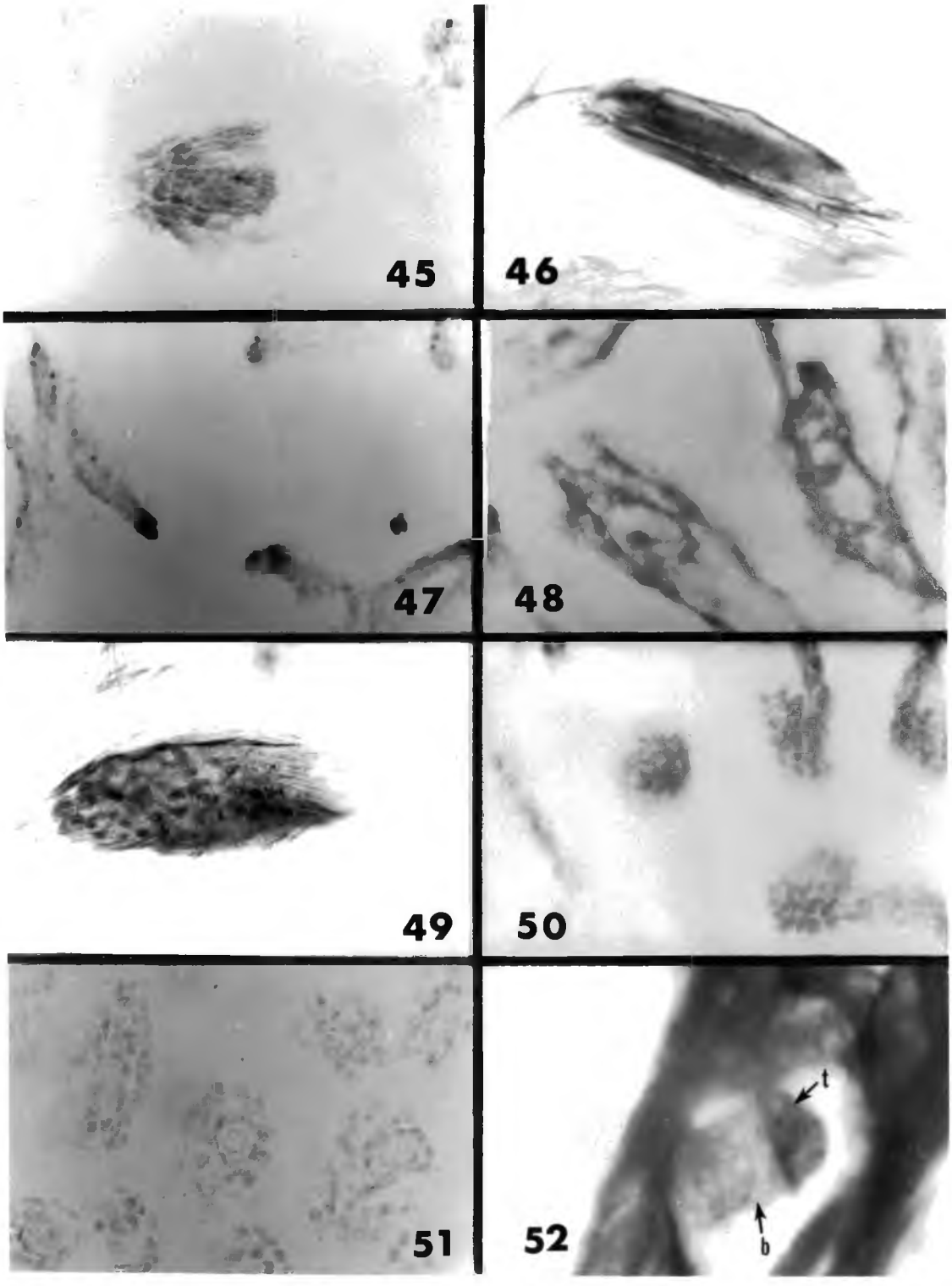
Fig. 48. RNA in zygote (x 320).

Fig. 49. RNA in multi-celled embryo (x 320).

Fig. 50. RNA in megaspore mother cell (x 128).

Fig. 51. RNA in megaspore (x 320).

Fig. 52. RNA in 2-celled embryo (x 2560). Terminal cell (t)(arrow) and basal cell (b)(arrow) are visible.


45
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51
52
t
b

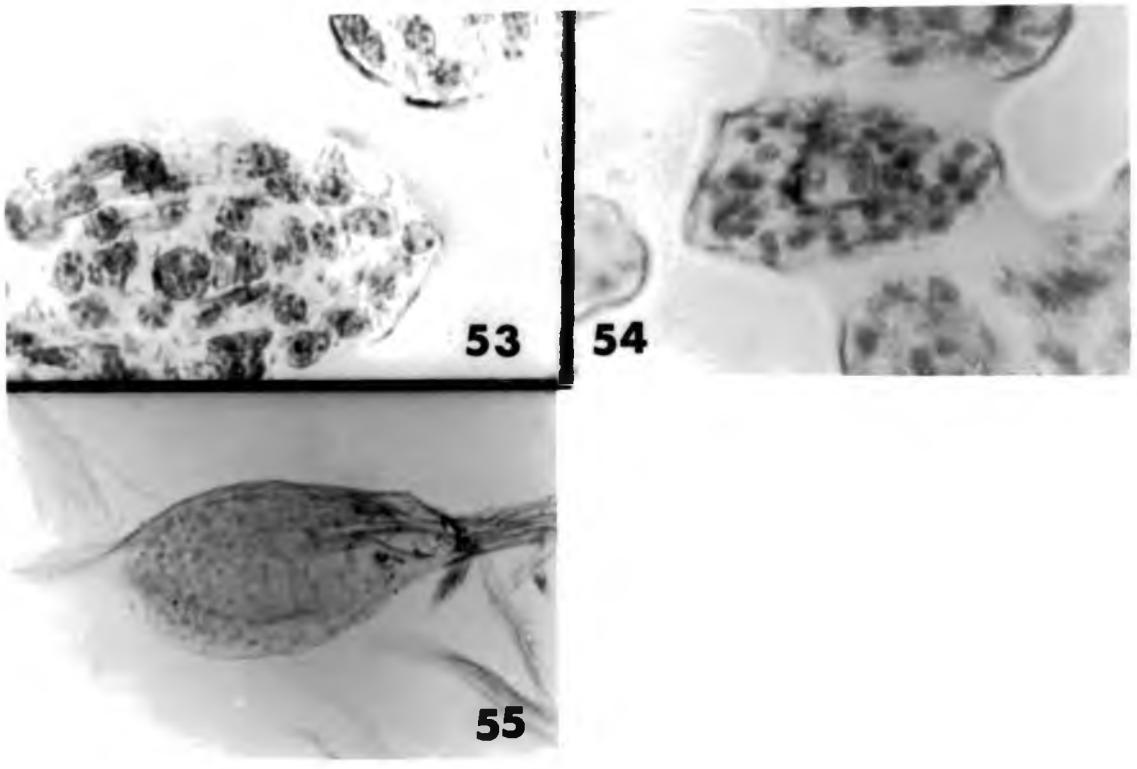


Fig. 53. DNA in megaspore (x 800).

Fig. 54. DNA in 4-celled embryo (x 320).

Fig. 55. DNA in multi-celled embryo (x 128).

B. Ovary culture

1. Hormonal effect

Hormonal effects of NAA and BA on the increase in width and increase in percentage of total width of 20-, 40- and 60-day-old ovaries after 3 months in culture, and statistical analyses of these data by ANOVA are shown in Table 3-8. The hormonal effects on the production of leaf and root, and the percentage of seedlings produced during one year of culture of ovaries are shown in Tables 9-11.

a. Culture of 20-day-old ovary

Table 3 shows hormonal effects on the increase in width and the increase percentage of total width of 20-day-old ovary cultured for 3 months. The maximum increase in actual width was 3.0 mm and the maximum increase percentage of width was 51% with 5 ppm NAA + 10 ppm BA. On the other hand, the minimum increase in width was 1.15 mm and increase percentage of total width was 15% in the control treatment (without hormone). Statistical analysis shows that treatment with hormones is highly significant, especially the interaction of NAA and BA, but the individual effect of NAA or BA is not significant (Table 4). The interaction is caused by the difference of NAA and BA in promoting rates on ovary growth. The hormonal effects on the production of leaf and root and the percentage of seedlings produced are shown in Table 5. The shortest period of culture and the hormonal conditions to obtain seedlings were 3 months and 1.0 ppm BA; 0.1 ppm NAA + 1.0 ppm BA; and 0.1 ppm NAA + 5.0 ppm BA. Excellent seedling production was obtained with 1.0 ppm BA. If the medium contained 1.0 ppm NAA, seedlings were obtained at 7 to 9 months after ovary culture regardless of BA

concentration.

Table 3. Hormonal effect on the increase of width and percentage of total width of 20-day-old ovary after 3 months in culture.

NAA (ppm) / BA (ppm)	0	0,1	1.0	5.0	10.0
0	ab[z] 1.15(15)[y]	h 2.00(40)	j 2.50(50)	gh 1.85(33)	bc 1.25(22)
0.1	h 2.00(35)	j 2.50(42)	g 1.75(34)	h 2.10(42)	gh 1.85(34)
1.0	j 2.35(47)	g 1.75(29)	a 1.05(18)	de 1.45(24)	gh 1.85(32)
5.0	ef 1.55(28)	bc 1.25(22)	ef 1.55(19)	fg 1.70(29)	j 2.40(44)
10.0	ef 1.55(31)	cd 1.35(22)	h 2.00(29)	k 3.00(51)	h 2.00(33)

[z] Duncan's multiple range test at 5% level.

[y] Increase of width in mm. Figures in parenthesis are the percentage increase of total width.

Table 4. ANOVA table of hormonal effect on the increase of width and percentage of total width of 20-day-old ovary after 3 months in culture.

	df	SS	MS	F
Rep.	1	14.58	14.58	0.264 NS[z]
Treatment	24	5555.18	231.47	4.201 **
NAA	4	33.28	8.32	0.151 NS
BA	4	603.28	150.82	2.737 NS
NAA x BA	16	4918.62	307.41	5.579 **
Error	24	1322.42	55.10	
Total	49	6892.18		

[z] NS = differences non-significant; ** = significant at 1% level.

Table 5. Hormonal effect on the leaf and root formation and percentage of seedling formation from 20-day-old ovary cultured for one year.

BA (ppm) \ NAA (ppm)	0		0.1		1.0		5.0		10.0	
0	z	y			1		1			
	x	w			9	100	8	100		
0.1					1					
					9	100				
1.0	4	4	2	2	2					
	3	100	3	100	7	100				
5.0	1		3	3	1	1				
	10	100	3	100	7	50				
10.0	1				3	2			1	
	10	50			7	50			10	50

[z] Degree of seedlings formed: 1 very few; 2 few; 3 moderate; and 4 many.

[y] Degree of roots formed: 1, 2, 3 and 4 same as above.

[x] Month(s) required for formation of seedlings.

[w] Percentage of seedling formation in 2 replicates.

b. Culture of 40-day-old ovary

Table 6 shows hormonal effects on increase of width and percentage of total width of 40-day-old ovary after 3 months in culture. The maximum increase in actual width was 3.25 mm and the maximum increase of percentage of width was 45% with 0.1 ppm NAA hormonal condition. On the other hand, the minimum increase in width was 0.2 mm and the minimum percentage increase was 3% in the control. Statistical analysis of these data shows that treatment with hormones is significantly different from control (without hormone) at the 5% level and the interaction of NAA and BA is significant at the 1% level, but the individual effect of NAA or BA is not significant (Table 7). The hormonal effects on the production of leaf and root, and the percentage of seedlings produced are shown in Table 8. In 40-day-old ovary culture, the shortest culture period and the hormonal condition to obtain seedlings are 2 months and 1.0 ppm NAA. The best seedling production was obtained under 1.0 ppm NAA. When the medium contained 1.0 ppm NAA, seedlings were obtained at 2 to 3 months after ovary culture regardless of BA concentration.

Table 6. Hormonal effect on the increase of width and percentage of total width of 40-day-old ovary after 3 months in culture.

NAA (ppm) / BA (ppm)	0	0.5	1.0	5.0	10,0
0	a[z] 0.20(3)[y]	i 3.25(45)	g 2.00(29)	d 0.90(18)	cd 0.85(10)
0.1	d 1.00(15)	d 1.05(12)	bc 0.60(18)	d 1.00(13)	bc 0.70(8)
1.0	g 2.00(31)	bc 0.60(8)	d 1.10(15)	d 1.00(15)	bc 0.60(7)
5.0	d 1.15(14)	g 2.00(25)	bc 0.65(8)	d 1.10(14)	b 0.55(7)
10.0	f 1.55(21)	h 2.25(28)	c 0.75(10)	e 1.35(18)	bc 0.60(8)

[z] Duncan's multiple range test at 5% level.

[y] Increase of width in mm. Figures in parenthesis are the percentage increase of total width.

Table 7. ANOVA table of hormonal effect on the increase of width and percentage of total width of 40-day-old ovary after 3 months in culture.

	df	SS	MS	F
Rep.	1	76.88	76.88	1.146 NS[z]
Treatment	24	4203.28	175.14	2.612 *
NAA	4	599.78	149.95	2.236 NS
BA	4	215.08	53.77	0.802 NS
NAA x BA	16	3388.42	211.78	3.159 **
Error	24	1609.12	67.04	
Total	49	5889.28		

[z] NS = differences non-significant; * = significant at 5% level; ** = significant at 1% level.

Table 8. Hormonal effect on the leaf and root formation and percentage of seedling formation from 40-day-old ovary cultured for one year.

BA (ppm) \ NAA (ppm)	0			0.1			1.0			5.0			10.0		
0	z	y	x				4		4	1					
	w	v	u				2	100		10	50				
0.1							4	1	3	1		3			
			1				2.5	100		3	50				
1.0	4		4				3		2				1		
	3	50					3	100					9	50	
5.0	2	1	2				2	2	2				3		3
	3	50					3	100					3	50	
10.0							3	1	3	2		3	3		4
							3	100		3	100		3	50	

[z] Degree of seedlings formed: 1 very few; 2 few; 3 moderate and 4 many.

[y] Degree of leaf formation: 1, 2, 3 and 4 same as above.

[x] Degree of root formation: 1, 2, 3 and 4 same as above.

[w] Month(s) required for formation of seedlings.

[v] Percentage of seedling formation in 2 replicates.

[u] Degree of mature seed formation: 1, 2, 3 and 4 same as above.

c. Culture of 60-day-old ovary

Table 9 shows hormonal effects on increase of width and percentage of total width of 60-day-old ovary after 3 months in culture. The maximum increase in width was 1.85 mm and the maximum percentage increase of total width was 24% with 10.0 ppm NAA + 5.0 ppm BA. Statistical analysis of these data (Table 10) shows that both NAA and BA treatments were highly significant, but the interaction of NAA and BA was not significant. The hormonal effects on production of leaf and root, and the percentage of seedlings are shown in Table 11. In the 60-day-old ovary culture, the shortest culture period and hormonal treatment to obtain seedlings was 2 months and 0.1 ppm NAA + 5.0 ppm BA; 1.0 ppm NAA + 0, 0.1, 1.0, 5.0 or 10 ppm BA; and 10.0 ppm NAA + 5.0 or 10 ppm BA. When the medium contained 1.0 ppm NAA, seedlings were obtained at 2 months after ovary culture regardless of BA concentration.

d. Comparison of cultures of 20-, 40- and 60-day-old ovaries

The cultures of 20-, 40- and 60-day-old ovaries in the media containing hormones (NAA or BA, or both) are shown in Figures 56 to 65. Some of the seedlings obtained from ovary culture are shown in Figures 66 to 82. The hormonal effects on the percentage increase of total width of 20-, 40- and 60-day-old ovary culture are shown in Figures 83, 84 and 85, respectively. In general, the percentage increase of width is highest in the 20-day-old culture of ovary and decreases with the age increase of ovary.

Table 9. Hormonal effect on the increase of width and percentage of total width of 60-day-old ovary after 3 months in culture.

NAA (ppm) / BA (ppm)	0	0.1	1.0	5.0	10.0
0	a[z] 0[y] (0)	a 0 (0)	ab 0.15(2)	bc 0.25(3)	a 0 (0)
0.1	d 0.50(7)	de 0.55(8)	i 1.45(22)	de 0.65(9)	d 0.50(7)
1.0	cd 0.40(6)	cd 0.35(5)	f 0.90(11)	g 1.10(13)	bc 0.30(3)
5.0	ef 0.85(11)	ef 0.85(11)	g 1.10(14)	de 0.65(9)	j 1.85(24)
10.0	e 0.70(9)	ab 0.15(2)	h 1.30(16)	fg 1.00(12)	cd 0.40(5)

[z] Duncan's multiple range test at 5% level.

[y] Increase of width in mm. Figures in parenthesis are the percentage of total width increase.

Table 10. ANOVA table of hormonal effect on the increase of width and percentage of total width of 60-day-old ovary after 3 months in culture.

	df	SS	MS	F
Rep.	1	72.00	72.00	1.475 NS [z]
Treatment	24	3899.00	162.46	3.330 **
NAA	4	1914.20	478.55	9.808 **
BA	4	934.40	233.60	4.788 **
NAA x BA	16	1050.40	65.65	1.346 NS
Error	24	1171.00	48.79	
Total	49	5142.00		

[z] NS = differences non-significant; ** = significant at 1% level.

Table 11. Hormonal effect on leaf and root formation and percentage of seedling formation from 60-day-old ovary cultured for one year.

BA (ppm) \ NAA (ppm)	0			0.1			1.0			5.0			10.0		
0	z	y	x				4		2						
	w	v	u				2	100				1			
0.1				1			3		2	2		2	1		
			1	8	100		2	100		3	50		11	50	
1.0	2		2	1	1	1	3		2						
	3	50		7	50		2	100				1			1
5.0	1	1		3		2	3	2	2	1			3	2	3
	6	100		2	50		2	100		3	50		2	100	
10.0	2	2	1	1		1	2		1				3		4
	3	50		8	100		2	100					2	50	

[z] Degree of seedlings formed: 1 very few; 2 few; 3 moderate and 4 many.

[y] Degree of leaf formation: 1, 2, 3 and 4 same as above.

[x] Degree of root formation: 1, 2, 3 and 4 same as above.

[w] Month(s) required for formation of seedlings.

[v] Percentage of seedling formation in 2 replicates.

[u] Degree of mature seed formation: 1, 2, 3 and 4 same as above.

Fig. 56. Ovaries from 20-day-old ovary cultured for 3 months with various NAA concentrations (0, 0.1, 1.0, 5.0, 10.0 ppm).

Fig. 57. Ovaries from 20-day-old ovary cultured for 3 months with various BA concentrations (0, 0.1, 1.0, 5.0, 10.0 ppm). Type 1 indicates that the arrengement of vials were in the same order as in Figure 57.

Fig. 58. Ovaries from 40-day-old ovary cultured for 3 months with various NAA concentrations (0, 0.1, 1.0, 5.0, 10.0 ppm). Type 1 indicates the same order in figure 56.

Fig. 59. Ovaries from 40-day-old ovary cultured for 3 months with various BA concentrations (0, 0.1, 1.0, 5.0, 10.0 ppm). Type 1 indicates the same order in Figure 56.

Fig. 60. Ovaries from 60-day-old ovary cultured for 3 months with various NAA concentrations (0, 0.1, 1.0, 5.0, 10.0 ppm). Type 1 indicates the same order in Figure 56.

Fig. 61. Ovaries from 60-day-old ovary cultured for 3 months with various BA concentrations (0, 0.1, 1.0, 5.0, 10.0 ppm). Type 1 indicates the same order in Figure 56.

Fig. 62. Ovaries from 20-day-old ovary cultured for 7 months with 1 ppm NAA + various BA concentrations (from left to right 0, 0.1, 1.0, 5.0, 10.0 ppm).

Fig. 63. Various types of seedlings and calluses arising from ovaries after 4 months of culture of ovaries with various hormonal conditions.

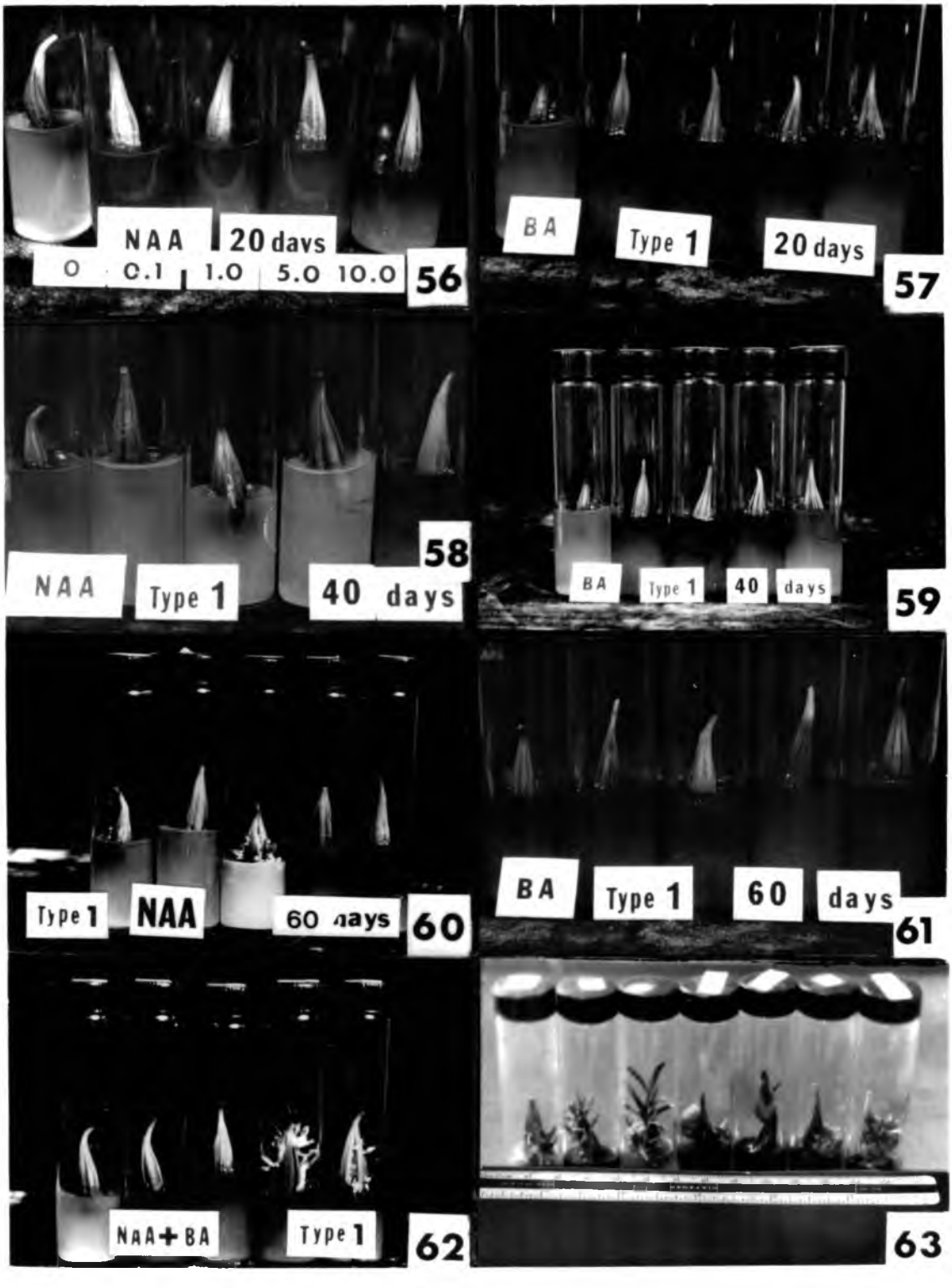

NAA 20 days
0 0.1 1.0 5.0 10.0
56
BA Type 1 20 days
57
58
NAA Type 1 40 days
BA Type 1 40 days
59
Type 1 NAA 60 days
60
BA Type 1 60 days
61
NAA+BA Type 1
62
63

Fig. 64. Callus and seedling(arrow) differentiated from callus which were obtained through ovary culture.

Fig. 65. Ovaries from 40-day-old ovary cultured for 3 months with 1 pp NAA + various BA concentrations (from left to right 0, 0.1, 1.0, 5.0, 10.0 ppm).

Fig. 66. Ovaries from 60-day-old ovary cultured for 2 months with 1 pp NAA + various BA concentrations (from left to right 0, 0.1, 1.0, 5.0, 10.0 ppm).

Fig. 67. Ovaries from 60-day-old ovary cultured for 3 months with 10 ppm NAA + various BA concentrations (from left to right 0, 0. 1.0, 5.0, 10.0 ppm).

Fig. 68. Seedlings obtained from 20-day-old ovary cultured for 8 month with 1 ppm NAA + 10 ppm BA.

Fig. 69. Seedlings obtained from 20-day-old ovary cultured for 8 month with 1.0 ppm BA.

Fig. 70. Seedlings obtained from 20-day-old ovary cultured for 9 month with 1.0 ppm NAA.

Fig. 71. Seedlings obtained from 40-day-old ovary cultured for 3 month with 1.0 ppm BA.


64
40 days
65
40 days
66
60 days
67
20 days
68
20 days
69
20 days
70
40 days
71

Fig. 72. Seedlings obtained from 40-day-old ovary cultured for 3 months with 5.0 ppm BA.

Fig. 73. Seedlings obtained from 40-day-old ovary cultured for 3 months with 1.0 ppm NAA + 0.1 ppm BA.

Fig. 74. Seedlings obtained from 40-day-old ovary cultured for 3 months with 1.0 ppm NAA.

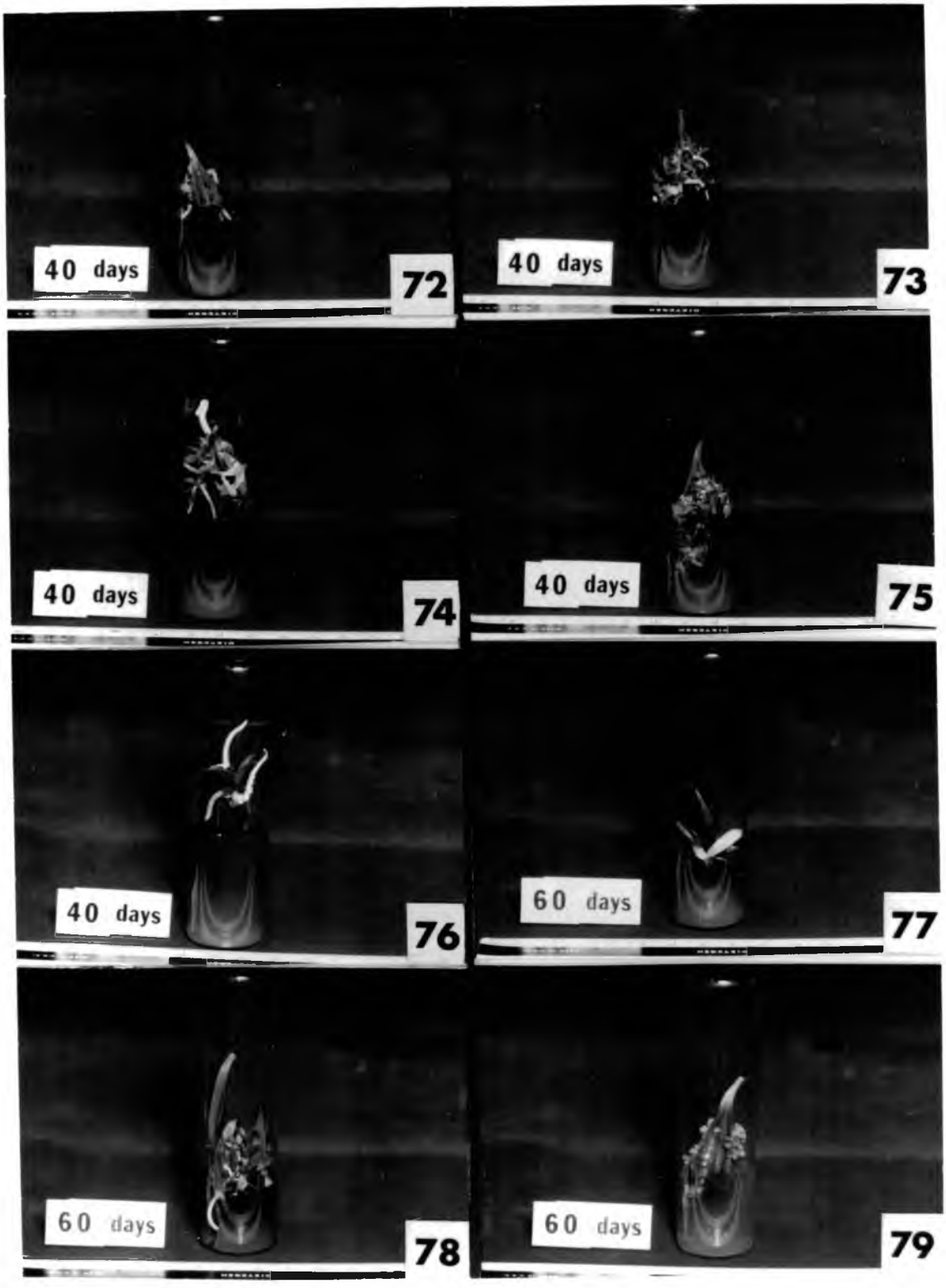
Fig. 75. Seedlings obtained from 40-day-old ovary cultured for 3 months with 10.0 ppm NAA + 10.0 ppm BA.

Fig. 76. Seedlings obtained from 40-day-old ovary cultured for 3 months with 5.0 ppm NAA + 0.1 ppm BA.

Fig. 77. Seedlings obtained from 60-day-old ovary cultured for 3 months with 1.0 ppm BA.

Fig. 78. Seedlings obtained after 60-day-old ovary culture for 3 months with 1.0 ppm NAA.

Fig. 79. Seedlings obtained from 60-day-old ovary cultured for 3 months with 5.0 ppm NAA + 5.0 ppm BA.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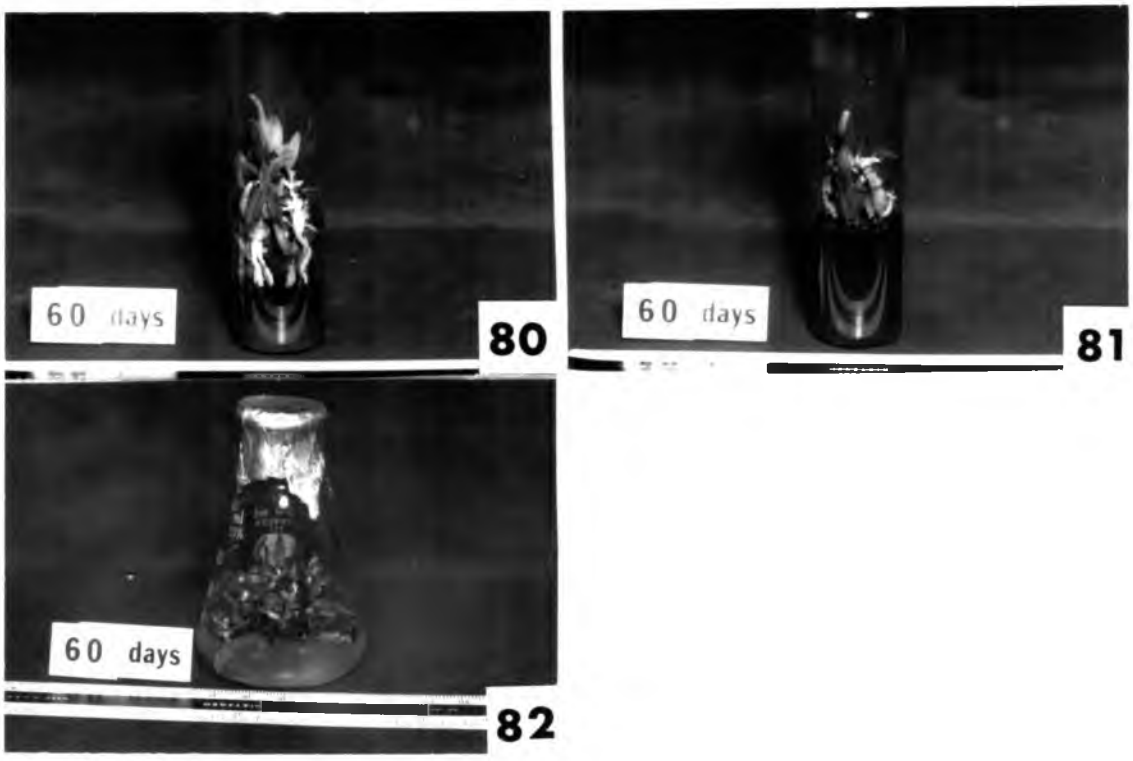

Fig. 80. Seedlings obtained from 60-day-old ovary cultured for 3 months with 1.0 ppm NAA + 5 ppm BA.

Fig. 81. Seedlings obtained from 60-day-old ovary cultured for 3 months with 1.0 ppm BA.

Fig. 82. Seedlings obtained from 60-day-old ovary cultured for 6 months and transflasked.

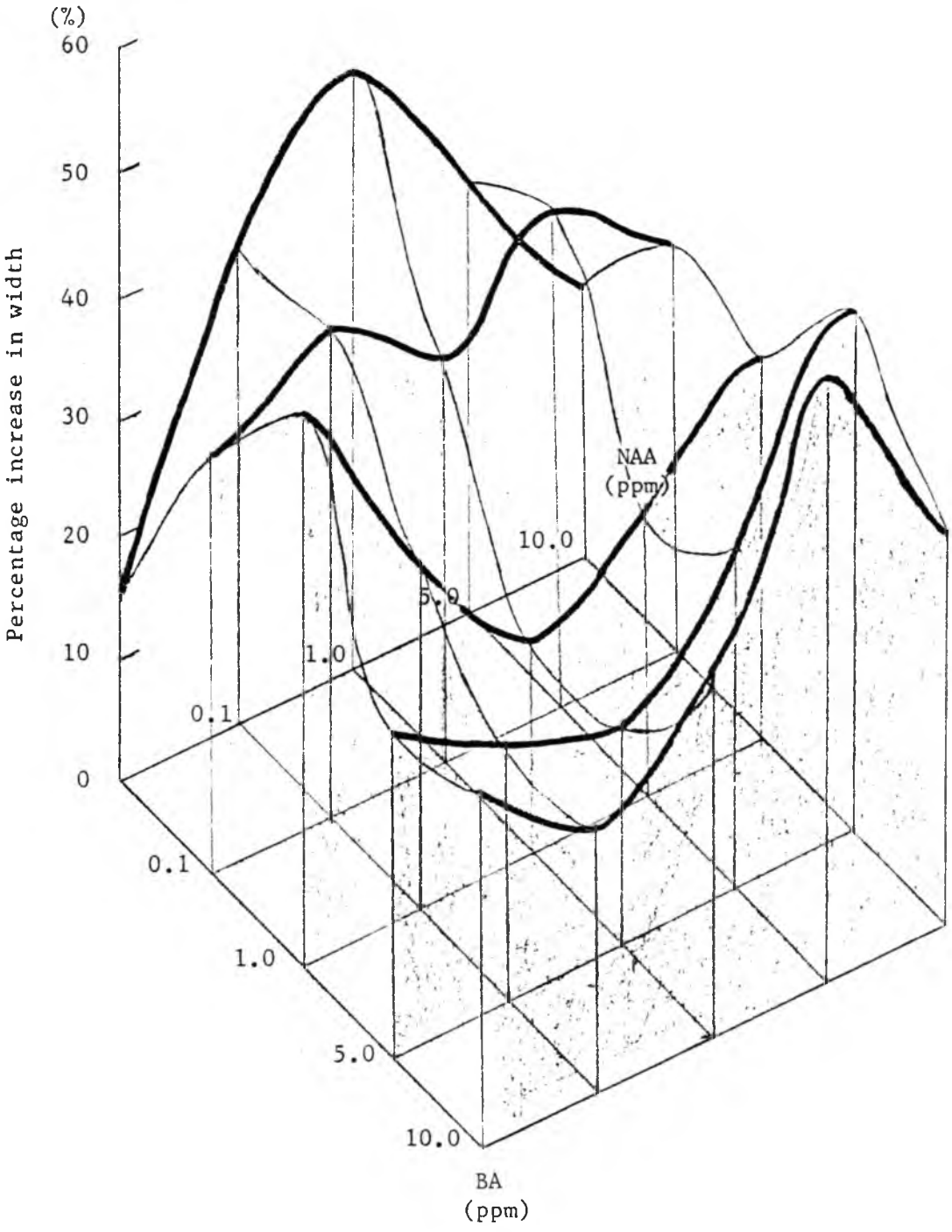

Figure 83. Hormonal effects on the increase percentage of total width of 20-day-old ovary after 3 months of culture.

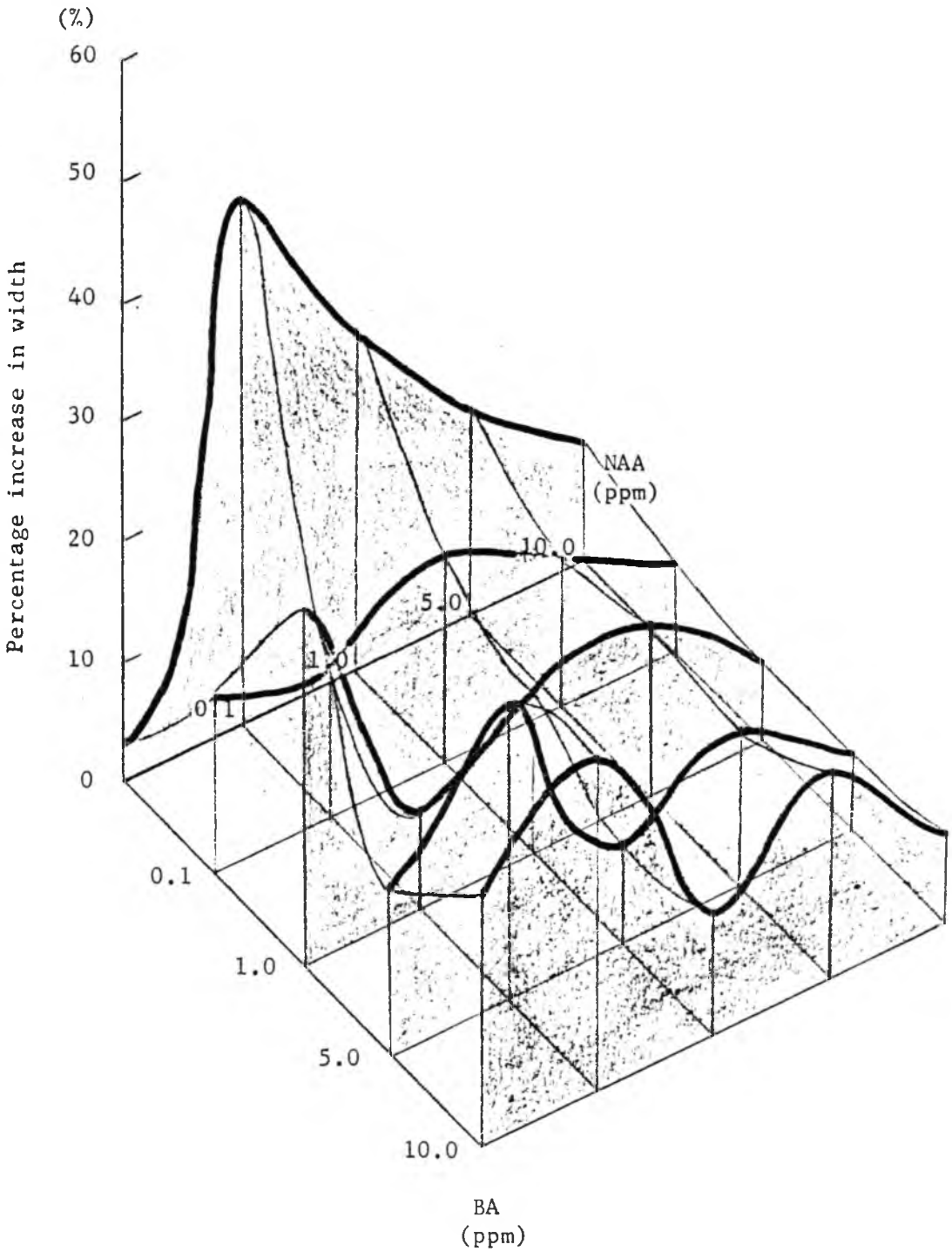

Figure 84. Hormonal effects on the increase percentage of total width of 40-day-old ovary after 3 months of culture.

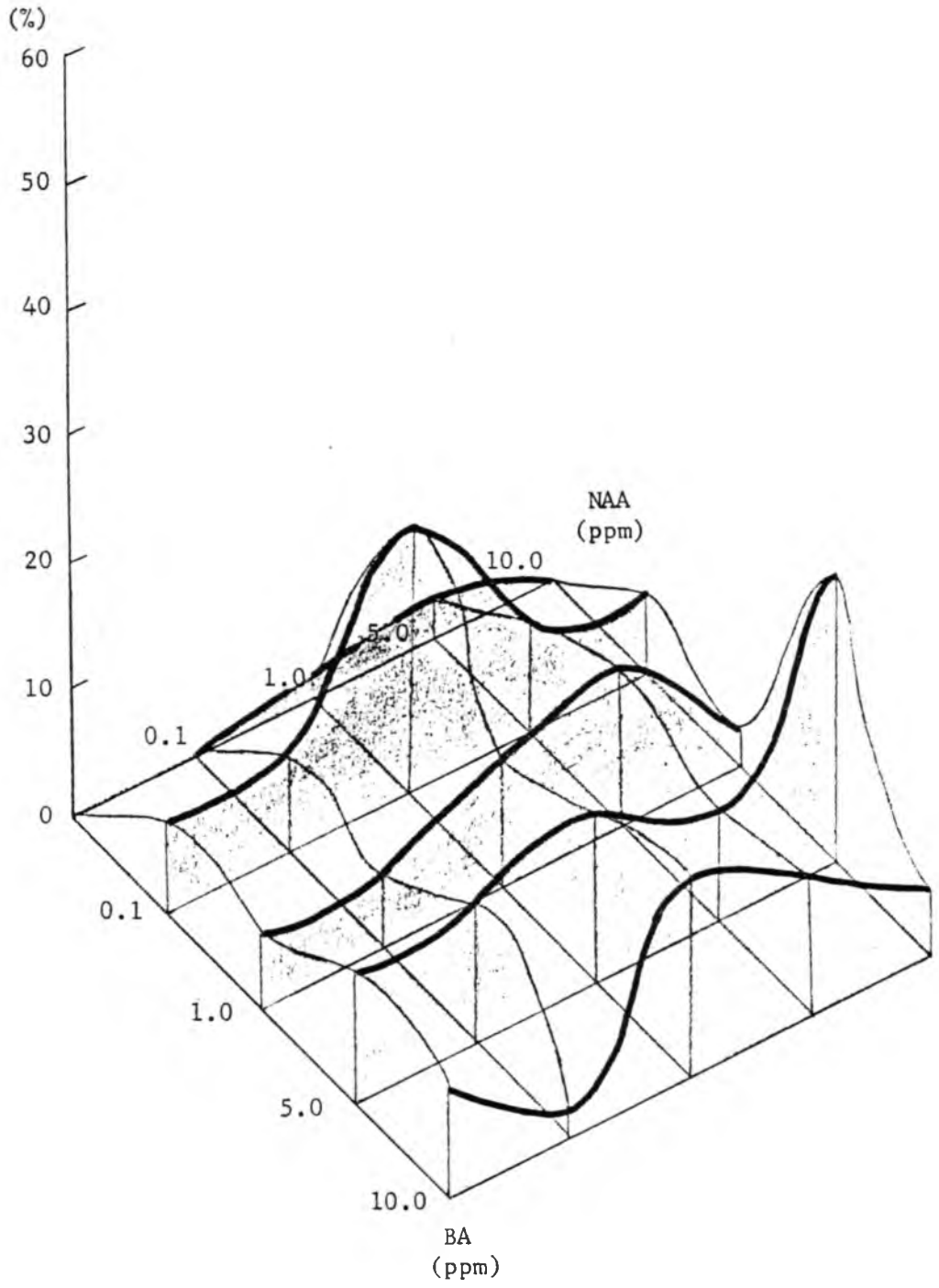

Figure 85. Hormonal effects on the increase percentage of total width of 60-day-old ovary after 3 months of culture.

2. Nutritional effects

The effects of coconut water and sucrose on increase of width and percentage of total width of ovary after 3 months in culture are shown in Table 12. No increase in width and percentage of total width are observed in the control (without coconut water and sucrose) with all ages of ovary. The greatest increase in width and percentage of total width are observed in 4 % sucrose on 20-day-old ovary and 4% sucrose + 15% coconut water on 40-, 60-, 70- and 80-day-old ovaries. Statistical analyses of these data are shown in Tables 13 to 17. Treatments were significant at the 1% level in 20-day-old ovary culture, and at the 5% level in 60-day-old and 70-day-old ovary culture. Therefore, coconut water and sucrose are effective in increasing width of 20-, 60- and 70-day-old ovaries. Treatments were non-significant in 40-day-old and 80-day-old ovary cultures. Sucrose was effective in all treatments, except 80-day-old ovary culture. Effect of coconut water was significant in 20-, 70- and 80-day-old ovary cultures. Sucrose and coconut water interaction is non-significant, except in 20-day-old ovary culture. Therefore, there is a difference between sucrose and coconut water in promoting width growth of 20-day-old ovary.

Nutritional effects on the production of leaf and root, and the percentage of seedlings produced are shown in Table 18. Seedlings were produced in 20-day-old ovary only with 15% coconut water, and in 40-day-old ovary only with 4% sucrose + 15% coconut water. In 60-, 70- and 80-day-old ovaries, seedlings were produced with 15% coconut water, 1% sucrose, separately and in combination. In 80-day-old ovary culture, seedlings were produced even without sucrose and coconut water. The

shortest period to obtain seedlings is 2 months in the culture of 60-day-old ovary under 1% sucrose + 15% coconut water. Culturing of 20-, 60-, 70- and 80-day-old ovaries on media containing coconut water or sucrose, or both is shown in Figures 86 to 90.

Observations on hormonal effects on ovary growth of 1-week and 2-week-old ovaries at different stages of development after 3 months in culture are shown in Table 19. In the 7-day-old ovary culture, the maximum growth in width is observed with 10 ppm NAA. Anatomical study shows that no seed or protocorms were produced in ovaries with all hormonal conditions. Statistical analysis of these data is shown in Table 20-a. Hormone treatments are not significant. In the 14-day-old ovary culture, the maximum growth in width was observed with 10 ppm NAA, 10 ppm NAA + 10 ppm BA and 30 ppm NAA + 10 ppm BA. Anatomical study showed that no protocorm was produced, seed was produced with 30 ppm NAA + 10 ppm BA; and megaspore mother cell was produced with 10 ppm NAA conditions. Statistical analysis of these data is shown in Table 20-b. Hormone treatments are not significant. Culturing of 7-day- and 14-day-old ovaries is shown in Figures 91 and 92.

Table 12. Effect of coconut water and sucrose on increase in width and percentage of total width of ovary after 3 months in culture.

Age of ovary used	sucrose / C. W.[z]	0%	1%	2%	4%
(20)[y]	0%	-0.75[x](-11.3)[w]	1.00(15.5)	1.50(19.9)	1.95(29.0)
	15%	0.70(10.0)	1.75(25.0)	1.80(30.0)	1.75(29.2)
(40)	0%	-0.50(6.7)	0.40(5.5)	0.95(13.4)	2.05(26.6)
	15%	0.50(6.9)	0.70(9.5)	0.80(11.2)	2.50(32.9)
(60)	0%	0 (0)	0.80(11.7)	0.50(7.9)	1.00(15.7)
	15%	0.25(3.0)	1.00(12.7)	0.50(6.0)	1.50(22.5)
(70)	0%	0 (0)	0.35(4.5)	0.35(4.0)	0.75(9.0)
	15%	0.60(7.0)	0.85(10.5)	0.35(4.5)	1.05(14.0)
(80)	0%	0 (0)	0.20(2.5)	0.30(3.5)	0.40(5.0)
	15%	1.05(14.0)	0.50(6.5)	0.90(11.5)	1.10(14.0)

[z] C. W. = coconut water.
[y] Figures in parenthesis are the days after pollination.
[x] Increase of width of ovary in mm.
[w] Figures in parenthesis are the percentage increase of total width.

Table 13. ANOVA table of nutritional effect on increase in width and percentage of total width in 20-day-old ovary culture.

	df	SS	MS	F		z
Rep.	1	12.25	12.25	1.657	NS	
Trt.	7	1141.75	163.11	22.072	**	
s	3	862.25	287.42	38.893	**	
cw	1	132.25	132.25	17.896	**	
s x cw	3	147.25	49.08	6.642	*	
Error	7	51.75	7.39			
Total	15	1205.75				

Table 14. ANOVA table of nutritional effect on increase width and percentage of total width in 40-day-old ovary culture.

	df	SS	MS	F		z
Rep.	1	100.0	100.0	1.226	NS	
Trt.	7	1260.0	180.0	2.207	NS	
s	3	1128.5	376.17	4.612	*	
cw	1	64.0	64.0	0.785	NS	
s x cw	3	67.5	22.5	0.276	NS	
Error	7	571.0	81.57			
Total	15	1931.0				

[z]NS = differences non-significant; * = significant at 5% level; ** = significant at 1% level.

Table 15. ANOVA table of nutritional effect on increase in width and percentage of total width in 60-day-old ovary culture.

	df	SS	MS	F	
Rep.	1	7.57	7.57	0.843	NS [z]
Trt.	7	320.44	45.78	5.098	*
s	3	285.19	95.78	10.590	**
cw	1	22.56	22.56	2.512	NS
s x cw	3	12.69	4.23	0.417	NS
Error	7	62.92	8.98		
Total	15	390.93			

Table 16. ANOVA table of nutritional effect on increase width and percentage of total width in 70-day-old ovary culture.

	df	SS	MS	F		
Rep.	1	22.57	22.57	5.670	*	z
Trt.	7	171.44	24.49	6.154	*	
s	3	98.19	32.73	8.224	*	
cw	1	52.57	52.57	13.204	**	
s x cw	3	20.68	6.89	1.732	NS	
Error	7	27.92	3.98			
Total	15	221.93				

z NS = differences non-significant; * = significant at 5% level; ** = significant at 1% level.

Table 17. ANOVA table of nutritional effect on increase in width and percentage of total width in 80-day-old ovary culture.

	df	SS	MS	F	
Rep.	1	0.07	0.07	0.006	NS
Trt.	7	237.44	33.92	2.955	NS
s	3	33.19	11.06	0.964	NS
cw	1	175.57	175.57	15.294	**
s x cw	3	28.68	9.56	0.833	NS
Error	7	80.42	11.48		
Total	15	317.93			

NS = differences non-significant; * = significant at 1% level.

Table 18. Nutritional effect on the production of leaf and root, and percentage of seedlings produced.

Age of ovary	coconut water	Sucrose											
		0%			1%			2%			4%		
(20)[z]	0%	y	x	w									
		v	u	t									
	15%	3	2										
		9	50										
(40)	0%												
	15%										3	2	2
											4	50	
(60)	0%				2	2	2						
					4	100							1
	15%	1	1	3	1	1	3						
		4	100		2	100							1
(70)	0%				1	1							
					4	100				1			2
	15%	2	1	3	2	2							
		3.5	100		3	50							
(80)	0%	1			2	2	1						
		10	50		4	50				1			1
	15%	3	2	2	2	1							
		3	100		3	50				1			

[z] Figures in parenthesis are the days after pollination.

[y] Degree of seedlings formed: 1 very few; 2 few; 3 moderate and 4 many.

[x] Degree of leaf formation: 1, 2, 3 and 4 same as above.

[w] Degree of root formation: 1, 2, 3 and 4 same as above.

[v] Month(s) required for formation of seedlings.

[u] Percentage of seedling formation in 2 replicates.

[t] Degree of mature seed formation: 1, 2, 3 and 4 same as above.

Fig. 86. Seedlings obtained from 20-day-old ovary cultured for 10 months with 15% coconut water.

Fig. 87. Ovaries from 60-day-old ovary cultured for 3 months with various nutritional conditions (from left to right 15% coconut water; 1%, 2% and 4% sucrose).

Fig. 88. Ovaries from 70-day-old ovary cultured for 3 months with various nutritional conditions (from left to right 15% coconut water; 1%, 2% and 4% sucrose).

Fig. 89. Ovaries from 70-day-old ovary cultured for 3 months with various nutritional conditions (from left to right 15% coconut water; 1%, 2% and 4% sucrose).

Fig. 90. Ovaries from 80-day-old ovary cultured for 3 months with various nutritional conditions (from left to right 1% sucrose + 15% coconut water; 15% coconut water; 1% sucrose; control).

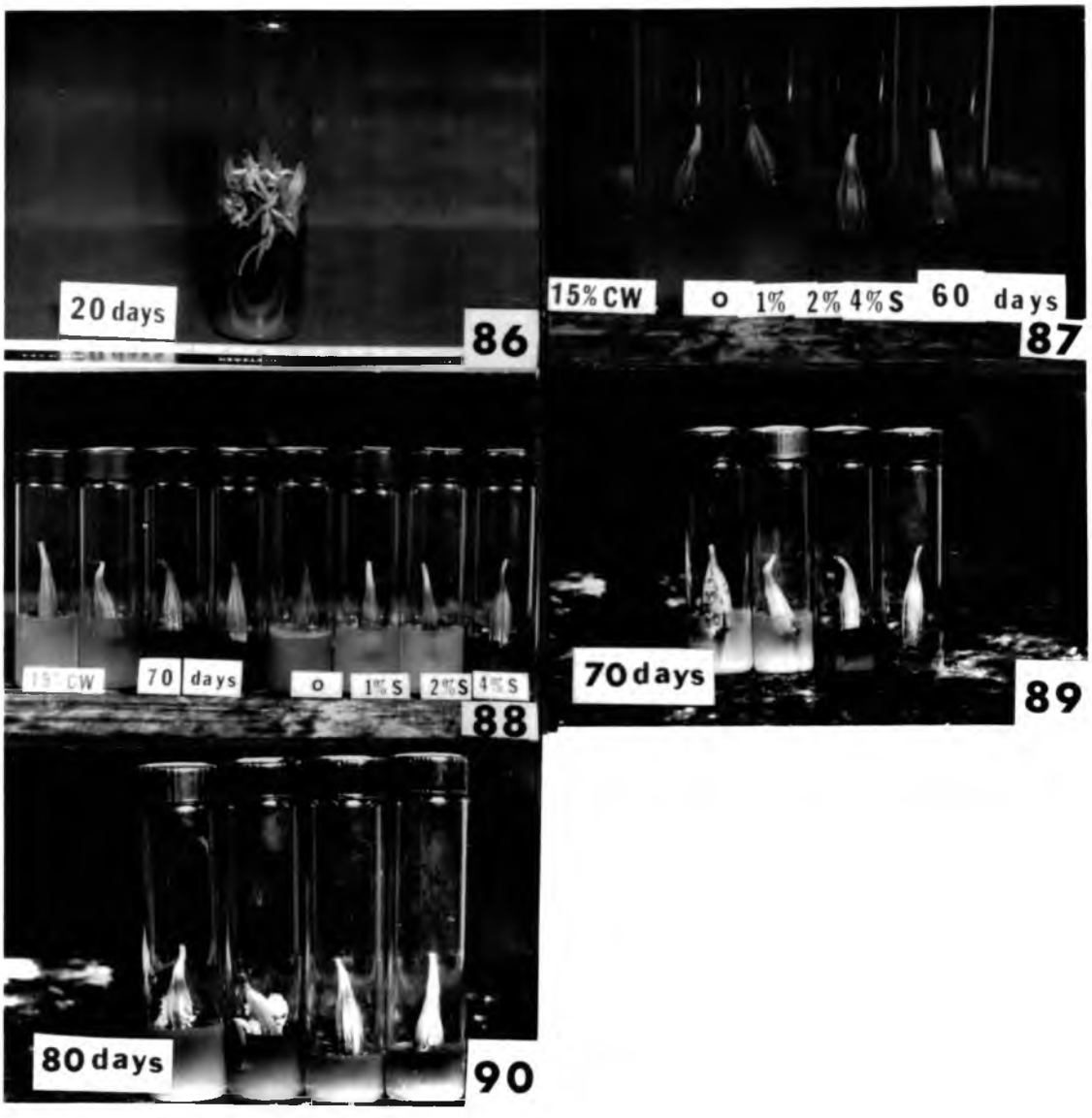
20 days
86
15% CW
0 1% 2% 4% S
60 days
87
15% CW
70 days
0 1% S 2% S 4% S
88
70 days
89
80 days
90

Table 19. Hormonal effect on ovary growth (width) of 7- and 14-day-old ovaries after 3 months of culture.

Hormone	1 week-old ovary culture			2 week-old ovary culture		
	width increase (mm)	percentage of total width	stage of development	width increase (mm)	percentage of total width	stage of development
NAA (10ppm)	0.5	14.4	M.F.[z]	1.25	19.2	MMC[z]
NAA (10ppm)+ BA (10ppm)	0	0	M.F.	1.25	18.9	MMC
NAA (20ppm)+ BA (10ppm)	0.25	5.5	M.F.	0.25	3.7	M.F.
NAA (30ppm)+ BA (10ppm)	0.25	5.5	P.P.	1.15	16.9	S.
BA (10ppm)	0.25	7.1	M.F.	0.75	11.5	M.F.
control	0	0	D.	0	0	D.

[z] M.F. : many fibers; P.P : placental proliferation; MMC : megaspore mother cell; S : seed; D : dead.

Table 20-a. ANOVA table of hormonal effect on ovary growth (width) of 7-day-old ovary culture.

	df	SS	MS	F	
Between treatment	5	35.42	7.08	0.200	NS [z]
control vs hormone	1	10.42	10.42	0.715	NS
NAA vs BA, NAA + BA	1	15.63	15.63	1.072	NS
BA vs NAA + BA	1	1.05	1.05	0.072	NS
NAA + BA	2	8.32	4.16	0.285	NS
Within treatment	6	87.49	14.58		
Total	11	122.91			

[z] NS = differences non-significant.

Table 20-b. ANOVA table of hormonal effect on ovary growth (width) of 14-day-old ovary culture.

	df	SS	MS	F	
Between treatment	5	293.73	58.75	0.893	NS [z]
control vs hormone	1	144.15	144.15	2.192	NS
NAA vs BA, NAA + BA	1	25.60	25.60	0.389	NS
BA vs NAA + BA	1	2.67	2.67	0.041	NS
NAA + BA	2	121.31	60.66	0.923	NS
Within treatment	6	394.5	65.75		
Total	11	688.25			

[z] NS = differences non-significant.

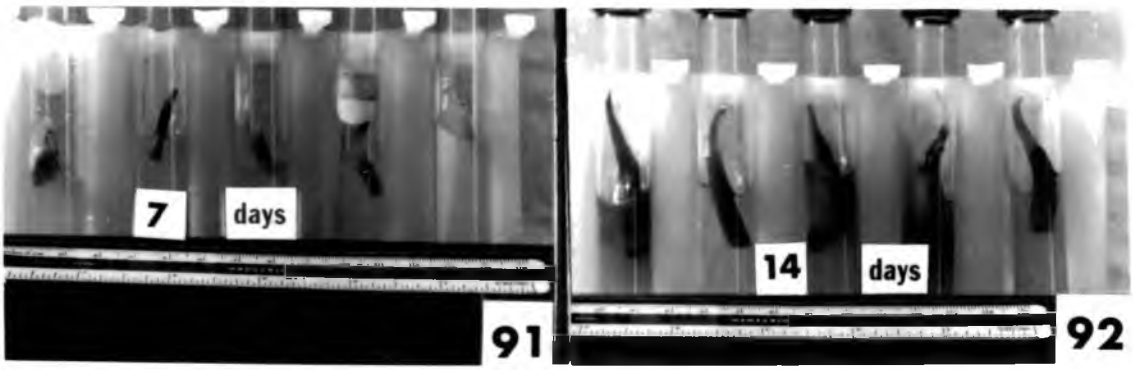

Fig. 91. Ovaries from 7-day-old ovary cultured for 3 months with various hormonal conditions.

Fig. 92. Ovaries from 14-day-old ovary cultured for 3 months with various hormonal conditions.

C. Ovule culture

1. Hormonal effect on ovule growth

Hormonal effects of NAA and BA are shown in Table 21. The maximum ovule growth was observed in 20-day-old ovule culture with 30 ppm NAA. Statistical analyses are presented in Table 22 (a to f). NAA effect on ovule growth was significant for all stages of ovule culture.

2. Nutritional effect on ovule growth

Nutritional effects (coconut water; sucrose; caseinhydrolysate; tryptophan and maleic hydrazide) are shown in Table 23. The maximum ovule growth was observed in 60-day-old ovule culture with 45% coconut water, and the minimum ovule growth was observed in the control (without nutritional supplements and hormones). Statistical analyses are shown in Table 24 (a to o). The effects of water and sucrose on ovule growth were significant for any ovule cultures. Casein hydrolysate effect on ovule growth was only significan in 60-day-old ovule culture. Tryptophan effect on ovule growth was significant in all ovule cultures. Maleic hydrazide effect on ovule growth was not significant in all ovule cultures.

Table 21. Hormonal effect of NAA and BA on ovule growth in ovule cultures for 4 months.

Hormone	Relative amount of ovule growth														
	20-day-old ovule					40-day-old ovule					60-day-old ovule				
	Replication					Replication					Replication				
	I	II	III	IV	mean	I	II	III	IV	mean	I	II	III	IV	mean
NAA 10ppm	8[z]	8	8	5	(7.25)[y]	4	4	8	5	(5.25)	8	4	8	4	(6.00)
20ppm	8	8	8	5	(7.25)	9	8	4	4	(6.25)	4	4	8	4	(5.00)
30ppm	12	8	12	6	(9.75)	8	8	8	5	(7.25)	12	12	4	4	(8.00)
BA 10ppm	8	8	8	5	(7.25)	13	8	5	7	(8.25)	10	8	4	4	(6.50)
20ppm	8	11	9	5	(8.25)	8	4	0	8	(5.00)	8	12	8	4	(8.00)
30ppm	7	10	7	5	(7.50)	6	4	0	4	(3.50)	4	4	4	4	(4.00)
control	0	0	0	0	(0)	0	0	0	0	(0)	0	0	0	0	(0)

[z] Numbers represent the sum of 4 months observations of the relative degree of ovule growth (0 = no growth; 1 = slight growth; 2 = moderate; 3 = good; 4 = excellent) in each month.

[y] Figures in parenthesis are means of relative ovule growth of 4 replications.

Table 22-a to f. ANOVA table of hormonal effect of NAA and BA on ovule cultures for 4 months.

Table 22-a. NAA (20-day-old ovule)[z]

	df	SS	MS	F
Rep.	3	24.0	8.0	4.371 *[y]
Trt.	3	205.5	68.5	37.431 **
Error	9	16.5	1.83	
Total	15	246.0		

Table 22-b. NAA (40-day-old ovule)

	df	SS	MS	F
Rep.	3	7.69	2.56	0.766 NS
Trt.	3	125.19	41.73	12.49 **
Error	9	30.56	3.34	
Total	15	163.44		

Table 22-c. NAA (60-day-old ovule)

	df	SS	MS	F
Rep.	3	19.0	6.33	0.78 NS
Trt.	3	139.0	46.33	5.72 *
Error	9	73.0	8.10	
Total	15	231.0		

Table 22-d. BA (20-day-old ovule)

	df	SS	MS	F
Rep.	3	25.19	8.397	5.79 *
Trt.	3	175.19	58.397	40.273 **
Error	9	13.06	1.45	
Total	15	213.44		

Table 22-e. BA (40-day-old ovule)

	df	SS	MS	F
Rep.	3	62.19	20.73	5.248 *
Trt.	3	140.69	46.90	11.872 **
Error	9	35.56	3.95	
Total	15	238.44		

Table 22-f. BA (60-day-old ovule)

	df	SS	MS	F
Rep.	3	22.75	7.58	1.886 NS
Trt.	3	146.75	48.92	12.17 **
Error	9	36.25	4.02	
Total	15	205.75		

[z] Figures in parenthesis are ages of ovules.

[y] NS = differences non-significant; * = significant at 5% level; ** = significant at 1% level.

Table 23. Nutritional effect of coconut water, sucrose, casein hydrolysate, tryptophan, and malic hydrazide on ovule growth in culture of ovules of 4 months.

Nutrition[z]	20 day-old ovule				40 day-old ovule				60 day-old ovule			
	Replication				Replication				Replication			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
C. W. 15%[y]	5	9	4	12	15	14	12	16	5	8	14	11
25%	4	4	5	10	11	10	10	16	8	8	15	15
35%	4	4	5	6	14	12	16	12	8	4	15	11
45%	8	8	9	5	16	12	16	16	16	16	16	11
S. 2%	4	9	13	11	12	15	4	8	4	8	15	8
C. 100ppm	4	4	8	8	10	9	4	4	8	8	6	6
500ppm	4	4	8	4	10	9	7	4	7	4	9	8
1000ppm	4	4	4	4	8	8	4	4	4	3	6	8
T. 100ppm	4	4	12	7	8	7	8	5	9	8	9	8
500ppm	5	5	8	4	9	8	4	4	4	6	6	8
1000ppm	4	4	4	4	8	4	4	1	8	8	4	4
M. 100ppm	0	0	4	4	2	0	4	4	2	2	4	4
500ppm	4	0	1	1	7	8	4	0	2	0	2	0
1000ppm	4	0	0	0	6	7	1	0	4	2	0	0
control	0	0	0	0	0	0	0	0	0	0	0	0

[z] C. W. is coconut water, S. is sucrose, C. is casein hydrolysate, T. is tryptophan, M. is maleic hydrazide.

[y] Numbers represent the sum of 4 months observations of the relative degree of ovule growth (0 =no growth; 1 = slight; 2 = moderate; 3 = good; 4 = excellent) in each month.

Table 24-a to o. ANOVA table of nutritional effect of coconut water, sucrose, casein hydrolysate, tryptophan and maleic hydrazide on ovule growth in ovules cultured for 4 months. The firgures in parenthesis represents the age of ovules.

Table 24-a. Coconut water (20)

	df	SS	MS	F
Rep.	3	16.6	5.53	1.09 NS [z]
Trt.	4	152.3	38.08	7.51 **
Error	12	60.9	5.07	
Total	19	229.8		

Table 24-b. Coconut water (40)

	df	SS	MS	F
Rep.	3	76.0	25.33	3.15 NS
Trt.	4	501.3	125.33	15.59 **
Error	12	96.5	8.04	
Total	19	673.8		

Table 24-c. Coconut water (60)

	df	SS	MS	F
Rep.	3	75.75	25.25	1.122 NS
Trt.	4	378.20	94.55	4.202 *
Error	12	207.00	22.50	
Total	19	660.95		

Table 24-d. Sucrose (20)

	df	SS	MS	F
Rep.	3	22.38	7.46	1.00 NS
Trt.	1	171.13	171.13	22.97 *
Error	3	22.37	7.45	
Total	7	215.88		

Table 24-e. Sucrose (40)

	df	SS	MS	F
Rep.	3	34.38	11.46	1.00 NS
Trt.	1	190.13	190.13	11.61 *
Error	3	34.37	11.45	
Total	7	258.88		

Table 24-f. Sucrose (60)

	df	SS	MS	F
Rep.	3	31.38	10.46	1.00 NS
Trt.	1	153.13	153.13	14.65 *
Error	3	31.37	10.45	
Total	7	215.88		

[z] NS = differences non-significant; * = significant at 5% level; ** = significant at 1% level.

Table 24-g. Caseinhydrolysate (20)

	df	SS	MS	F
Rep.	3	11.0	3.66	0.35 NS[z]
Trt.	3	83.0	27.66	2.65 NS
Error	9	94.0	10.44	
Total	15	111.0		

Table 24-h. Caseinhydrolysate (40)

	df	SS	MS	F
Rep.	3	47.25	15.75	0.75 NS
Trt.	3	141.25	47.08	2.25 NS
Error	9	188.50	20.94	
Total	15	619.00		

Table 24-i. Caseinhydrolysate (60)

	df	SS	MS	F
Rep.	3	7.19	2.39	0.41 NS
Trt.	3	104.69	34.89	5.98 *
Error	9	52.52	5.84	
Total	15	164.40		

Table 24-j. Tryptophan (20)

	df	SS	MS	F
Rep.	3	20.69	6.89	2.00 NS
Trt.	3	103.19	34.39	9.97 **
Error	9	31.02	3.45	
Total	15	154.90		

Table 24-k. Tryptophan (40)

	df	SS	MS	F
Rep.	3	29.25	9.75	3.95 *
Trt.	3	118.25	39.41	15.96 **
Error	9	22.25	2.47	
Total	15	169.75		

Table 24-l. Tryptophan (60)

	df	SS	MS	F
Rep.	3	1.25	0.416	0.16 NS
Trt.	3	156.75	52.250	19.87 **
Error	9	23.75	2.63	
Total	15	181.75		

[z] NS = differences non-significant; * = significant at 5% level; ** = significant at 1% level.

Table 24-m. Maleic hydrazide (20)

	df	SS	MS	F
Rep.	3	8.25	2.75	0.86 NS[z]
Trt.	3	8.75	2.92	0.91 NS
Error	9	28.75	3.19	
Total	15	45.75		

Table 24-n. Maleic hydrazide (40)

	df	SS	MS	F
Rep.	3	21.19	7.06	0.97 NS
Trt.	3	48.69	16.23	2.23 NS
Error	9	65.55	7.28	
Total	15	135.43		

Table 24-o. Maleic hydrazide (60)

	df	SS	MS	F
Rep.	3	2.75	0.92	0.51 NS
Trt.	3	18.75	6.25	3.45 NS
Error	9	16.25	1.81	
Total	15	37.75		

[z] NS = differences non-significant.

3. Effect of interaction on ovule growth

Interaction of hormones and nutritional supplements.

The interaction of hormones and nutritional supplements are shown in Table 25. The maximum ovule growth was observed in 40-day-old culture with 10 ppm NAA + 10 ppm BA + 2% sucrose + 25% coconut water, and the minimum ovule growth was observed in the control (without nutritional supplements and hormones). Statistical analyses of interactional effects of these hormones and nutritional supplements are shown in Table 26 to 28.

In 20-day-old ovule culture, interactional effects of treatments (BA + sucrose; BA + coconut water; NAA + BA + sucrose + coconut water) on ovule growth were significant (Table 26). In 40-day-old ovule culture, interactional effects of treatments (NAA + BA + coconut water; BA + sucrose + coconut water; NAA + BA + sucrose + coconut water) on ovule growth were significant (Table 27). In 60-day-old ovule culture, effects of interactions (sucrose + coconut water; NAA + BA + coconut water; NAA + BA + sucrose) on ovule growth were significant (Table 28). These results show that NAA, BA, sucrose and coconut water promote ovule growth in different rates, and these interactions are caused by that difference in promoting rates. Moreover, these interactions are observed only if coconut water or sucrose or both exist in medium.

Interaction of sucrose and nutritional supplements (casein hydrolysate; tryptophan; maleic hydrazide). The interaction of sucrose and nutritional supplements are shown in Table 29. The maximum ovule growth was observed in 40-day-old ovule culture with sucrose and in 60-day-old ovule culture with casein hydrolysate + sucrose. The

minimum ovule growth was observed in the control. Statistical analyses of interaction of sucrose and nutritional supplements were highly significant for all ovule cultures (Table 30a-c). Therefore, the promoting rates in ovule growth of sucrose and nutritional supplements are different. Nutritional effects vs control on ovule growth was significantly different at the 1% level. Sucrose vs casein hydrolysate + tryptophan + maleic hydrazide was not significant, except in the 20-day-old ovule culture. Casein hydrolysate + tryptophan vs maleic hydrazide, and casein hydrolysate vs tryptophan were not significantly different.

4. The production of leaf, root and protocorm, and protocorm formation percentage in ovule culture.

Results are shown in Table 31. In 20-day-old ovule culture, the shortest period to produce protocorms was observed with NAA + BA + coconut water after 3 months of culture which gave 50% protocorm formation. In 40-day-old ovule culture, the shortest period to form protocorm was observed with 15%, 25% and 35% coconut water, and NAA + BA + sucrose + 25% coconut water conditions after 2 months of culture with 25% protocorm formation. In 60-day-old ovule culture, the shortest period to form protocorm was observed with 25%, 35% and 45% coconut water; 2% sucrose; NAA + 25% coconut water; BA + 2% sucrose; BA + 25% coconut water; 2% sucrose + 25% coconut water; NAA + BA + 25% coconut water; NAA + 2% sucrose + 25% coconut water; BA + 2% sucrose + 25% coconut water; and NAA + BA + 25% coconut water after one month of culture. The highest protocorm formation percentage was 75% with BA + 25% coconut water and 2% sucrose + 25% coconut water. Some protocorms

and seedlings obtained from ovule culture are shown in Figures 93 to 109.

Table 25. Interactional effect of hormones and nutritional supplements on ovule growth in ovule cultured for 4 months.

Treatment[z]	20-day-old ovule				40-day-old ovule				60-day-old ovule			
	Replicates				Replicates				Replicates			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
N x S	4[y]	4	12	8	12	4	4	7	12	10	6	8
N x C	4	4	6	5	14	10	10	4	4	16	8	11
B x S	7	7	6	5	7	4	4	8	13	11	8	8
B x C	16	16	14	10	8	7	4	4	11	15	15	16
N x B	11	8	4	4	0	7	4	4	4	0	8	4
S x C	8	4	10	10	7	13	15	8	15	15	16	15
N x B x S	6	6	6	8	7	6	6	6	12	6	14	10
N x B x C	16	12	8	16	8	7	12	8	16	11	15	11
N x S x C	4	4	15	14	16	14	4	8	15	12	15	8
B x S x C	15	4	8	15	16	16	15	11	16	16	12	11
NxBxSxC	4	4	16	10	16	16	14	15	16	12	15	15
control	0	0	0	0	0	0	0	0	0	0	0	0

[z] N = NAA, B = BA, S = sucrose, C = coconut water.

[y] Numbers represent the sum of 4 months observations of the relative degree of ovule growth (0 = no growth; 1 = slight; 2 = moderate; 3 = good; 4 = excellent) in each month.

Table 26. ANOVA table of effects of hormones and nutrition, and their interaction on ovule growth in 20-day-old ovule cultured for 4 months.

	df	SS	MS	F	
Rep.	3	177.88	59.29	6.967	** [y]
Treatment [z]	15	633.00	42.20	4.959	**
N	1	1.00	1.00	0.117	NS
B	1	115.56	115.56	13.579	**
S	1	10.56	10.56	1.241	NS
C	1	138.06	138.06	16.223	**
N x B	1	18.06	18.06	2.122	NS
N x S	1	14.06	14.06	1.652	NS
N x C	1	14.06	14.06	1.652	NS
B x S	1	156.25	156.25	18.361	**
B x C	1	56.25	56.25	6.610	*
S x C	1	20.25	20.25	2.380	NS
N x B x C	1	1.00	1.00	0.117	NS
N x S x C	1	25.00	25.00	2.938	NS
B x S x C	1	5.06	5.06	0.689	NS
N x B x S	1	12.25	12.25	1.439	NS
N x B x S x C	1	45.56	45.56	5.354	*
Error	45	383.12	8.51		
Total	63	1194.00			

[z] N = NAA, B = BA, C = coconut water, S = sucrose.

[y] NS = differences non-significant; * = significant at 5% level; ** = significant at 1% level.

Table 27. ANOVA table of effects of hormones and nutrition and their interaction on ovule growth in 40-day-old ovule culture for 4 months.

	df	SS	MS	F	
Rep.	3	48.69	16.23	1.803	NS [y]
Treatment [z]	15	926.94	61.796	6.966	**
N	1	0.06	0.06	0.066	NS
B	1	4.00	4.00	0.444	NS
S	1	175.56	175.56	19.507	**
C	1	420.25	420.25	46.694	**
N x B	1	0	0	0	NS
N x S	1	3.06	3.06	0.351	NS
N x C	1	2.25	2.25	0.250	NS
B x S	1	4.00	4.00	0.444	NS
B x C	1	0.66	0.66	0.066	NS
S x C	1	4.00	4.00	0.444	NS
N x B x C	1	39.06	39.06	4.340	*
N x S x C	1	2.25	2.25	0.250	NS
B x S x C	1	175.56	175.56	19.507	**
N x B x S	1	20.25	20.25	2.250	NS
N x B x S x C	1	76.56	76.56	8.506	**
Error	45	405.30	9.01		
Total	63	1380.93			

[z] N = NAA, B = BA, C = coconut water, S = sucrose.

[y] NS = differences non-significant; * = significant at 5% level; ** = significant at 1% level.

Table 28. ANOVA table of effects of hormones, nutrition, and their interaction on ovule growth in 60-day-old ovule culture for 4 months.

	df	SS	MS	F	
Rep.	3	175.19	58.40	10.103	** [y]
Treatment [z]	15	1064.44	70.96	12.277	**
N	1	0.06	0.06	0.010	NS
B	1	49.00	49.00	8.478	**
S	1	210.25	210.27	36.375	**
C	1	625.00	625.00	108.131	**
N x B	1	4.00	4.00	0.692	NS
N x S	1	1.00	1.00	0.173	NS
N x C	1	20.25	20.25	3.503	NS
B x S	1	14.06	14.06	2.432	NS
B x C	1	0.06	0.06	0.010	NS
S x C	1	52.56	52.56	9.093	**
N x B x C	1	39.06	39.06	6.758	*
N x S x C	1	3.06	3.06	0.529	NS
B x S x C	1	4.00	4.00	0.692	NS
N x B x S	1	33.06	33.06	5.720	*
N x B x S x C	1	9.00	9.00	1.557	NS
Error	45	260.31	5.78		
Total	63	1499.94			

[z] N = NAA, B = BA, C = coconut water, S = sucrose.

[y] NS = differences non-significant; * = significant at 5% level; ** = significant at 1% level.

Table 29. Interactional effect of sucrose and nutritional supplements on ovule growth in ovule culture for 4 months.

Nutritional supplements[z]	20-day-old ovule				40-day-old ovule				60-day-old ovule			
	Replicates				Replicates				Replicates			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
C x S	10[y]	8	4	8	8	8	4	12	4	8	16	11
T x S	8	7	5	4	4	8	4	8	4	8	8	4
M x S	6	4	5	4	5	8	8	4	4	8	7	8
S	4	9	13	11	12	15	4	8	4	8	15	8
control	0	0	0	0	0	0	0	0	0	0	0	0

[z] C = casein hydrolysate, T = tryptophan, M = maleic hydrazide, S = sucrose.

[y] Numbers represent the sum of 4 months observation of the relative degree of ovule growth (0 = no growth; 1 = slight; 2 = moderate; 3 = good; 4 = excellent) in each month.

Table 30-a. ANOVA table of interactional effect of sucrose and nutritional supplement on ovule growth in 20-day-old ovule culture for 4 months.

	df	SS	MS	F	
Between nutrition	4	196.50	49.13	9.633	**[y]
control vs nutrition	1	151.25	151.25	29.657	**
S vs C + T + M[z]	1	30.08	30.08	5.898	*
C + T vs M	1	10.67	10.67	2.092	NS
C vs T	1	4.50	4.50	0.882	NS
Within nutritions	15	76.5	5.10		
Total	19	273.00			

Table 30-b. ANOVA table of interactional effect of sucrose and nutritions on ovule growth in 40-day-old ovule culture for 4 months.

	df	SS	MS	F	
Between nutrition	4	216.5	54.13	6.272	**[y]
control vs nutrition	1	180.0	180.0	20.857	**
S vs C + T + M[z]	1	27.0	27.0	3.129	NS
C + T vs M	1	1.5	1.5	0.174	NS
C vs T	1	8.0	8.0	0.927	NS
Within nutrition	15	129.5	8.63		
Total	19	346.0			

[z] S = sucrose, C = caseinhydrolysate, T = tryptophan, M = maleic hydrazide.

[y] NS = differences non-significant; * = significant at 5% level; ** = significant at 1% level.

Table 30-c. ANOVA table of interactional effect of sucrose and nutritions on ovule growth in 60-day-old ovule culture for 4 months.

	df	SS	MS	F	
Between nutrition	4	231.50	57.88	5.224	** [y]
control vs nutrition	1	195.31	195.31	17.627	**
S vs C + T + M [z]	1	4.69	4.69	0.423	NS
C + T vs M	1	3.38	3.38	0.305	NS
C vs T	1	28.12	28.12	2.538	NS
Within nutrition	15	166.25	11.08		
Total	19	397.75			

[z] S = sucrose, C = caseinhydrolysate, T = tryptophan, M = maleic hydrazide.

[y] NS = differences non-significant; * = significant at 5% level; ** = significant at 1% level.

Table 31. Production of leaf, root, protocorm and protocorm formation percentage in ovule cultures for 4 months.

Age of ovule	Medium[z]	months to get seedling	degree of producing leaf	root	protocorm	% protocorm formation
(20)[y]	S	5(months)			+	25 (%)
	N x C[x]	4			+	25
	N x B x C	3			+	50
(40)	C 15%	2	+	+	++	25
	C 25%	2			++	25
	C 35%	2	++++	+++	+++	25
	S	3	+++	++	++	25
	N x B x S x C	2			++	25
(60)	C 25%	1	++++	++	+++	50
	C 35%	1	++++	+	++++	25
	C 45%	1	++++	+++	++++	25
	S	1	+	+	+++	25
	N x S	2			++	50
	N x S[w]	2			++	50
	N x C	1	++	++	++	50
	B x S	1			++	25
	B x C	1	+++	++	++++	75
	S x C	1	+		++++	75
	N x B x C	1	++	++	++	50
	N x S x C	1			++	25
	B x S x C	1			+++	50
	N x B x S x C	1			++	50
	N x B x S	2			+	50
	Ca x S	4			+	25

[z] C = 25% coconut water, S = 2% sucrose, N = 10 ppm NAA, B = 10 ppm BA, Ca = 1000 ppm caseinhydrolysate.

[y] N x C = 20 ppm NAA + 25% coconut water.

[x] N x S = 30 ppm NAA + 2% sucrose.

[w] Figures in parenthesis represent the days after pollination.

Fig. 93. Seedlings obtained from 40-day-old ovule cultured for 2 months with 15% coconut water.

Fig. 94. Seedlings obtained from 20-day-old ovule cultured for 6 months with various conditions (2% sucrose; 20 ppm NAA + 25% coconut water; 10 ppm NAA + 10 ppm BA + 25% coconut water).

Fig. 95. Various stages of seedlings obtained from 60-day-old ovule cultured with various conditions.

Fig. 96. Protocorms obtained from 60-day-old ovule cultured for 1 month with various nutritional conditions (2% sucrose; 15%, 25%, 35% coconut water).

Fig. 97. Protocorms obtained from 60-day-old ovule cultured for 1 month with various conditions (10 ppm BA; 2% sucrose; 35% coconut water).

Fig. 98. Seedlings obtained from 60-day-old ovule cultured for 1 month with 10 ppm NAA + 10 ppm BA + 25% coconut water.

Fig. 99. Seedlings obtained from 60-day-old ovule cultured for 2 months with 45% coconut water.

Fig. 100. Protocorms obtained from 20-day-old ovule cultured for 3 month with 10 ppm NAA + 10 ppm BA + 25% coconut water.


40 days
93
20 days
94
60 days
95
60 days
2%S
15% 25% 35% CW
96
60 days
0 (vw) BA 2%S 35% CW
97
60 days
98
60 days
99
20 days
100

Fig. 101. Protocorms obtained from 40-day-old ovule cultured for 2 month with 15% coconut water.

Fig. 102. Protocorms obtained from 60-day-old ovule cultured for 1 month with 45% coconut water.

Fig. 103. Leaves produced from 60-day-old ovule cultured for 2 months with 10 ppm NAA + 25% coconut water.

Fig. 104. Leaves produced from 60-day-old ovule cultured for 2 months with 10 ppm BA + 25% coconut water.

Fig. 105. Leaves and roots are obtained from ovule culture for 3 months with 35% coconut water.

Fig. 106. Transflasked seedlings obtained from 60-day-old ovule cultured for 4 months.

Fig. 107. Seedlings obtained from ovule cultured aseptically and transplanted into 6 inch pod 210 days after pollination.

Fig. 108. Ovules growth at different stages *in vitro*.

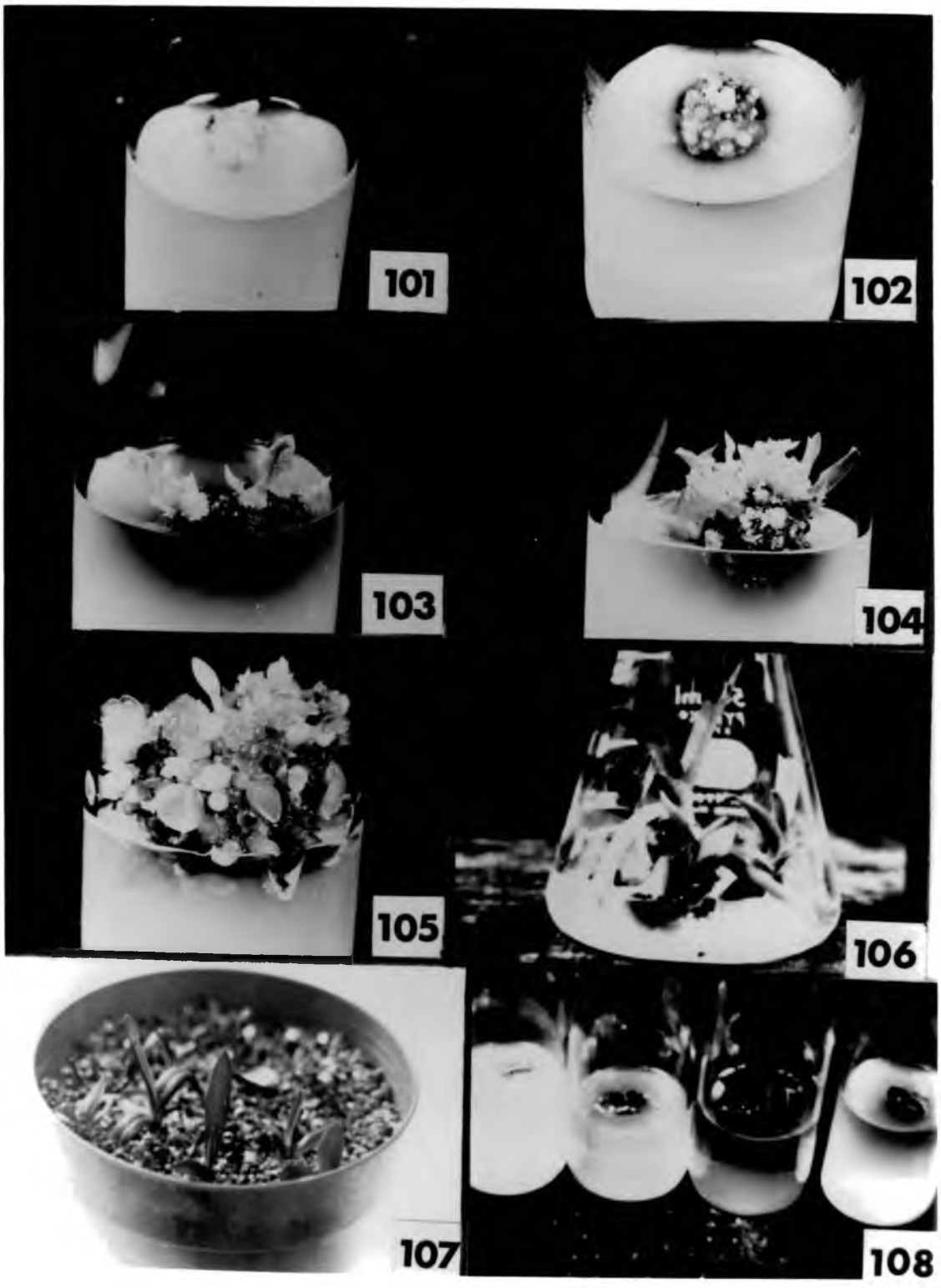
101
102
103
104
105
106
107
108

Fig. 109. Seedlings obtained from 40-day-old ovule cultured for 4 months with 2% sucrose.

D. Seed culture (hormones and nutritional supplement effects on seed germination and protocorm formation).

The germination rate per 100 seeds at 20 days after sowing seeds, and the protocorm number per vial at 40 days after sowing seeds are shown in Table 32. The highest germination rate and protocorm formation were observed in coconut water treatment. Statistical analysis of protocorm formation at 40 days after seed sowing is presented in Table 33. Treatment was highly significant. Effects of sucrose, coconut water and hormones were significant, and interactions of sucrose + coconut water and sucrose + coconut water + hormones were also significant. The interactions might be caused by the difference between sucrose and coconut water in promoting rate and the inhibiting effects of hormones on seed germination and protocorm formation. Some protocorms and seedlings obtained from seed culture are shown in Figure 110 and 111. Coconut water promotes seed germination and protocorm formation more than sucrose.

The comparison of days required for getting seedlings by normal seed germination, immature seed culture, ovary culture and ovule culture is shown in Figure 112.

Table 32. Hormonal and nutritional supplements effect on seed germination and protocorm formation.

Medium[z]	Germination rate per 100 seeds 20 days after sowing	Protocorm number 40 days after sowing
Control	0 [a y]	0 [a]
S	13 [b]	47 [c]
C 15%	24	62
C 25%	30 } 29.3 [e]	93 } 77.0 [d]
C 35%	34	76
S x C	33 [e]	64 [c]
N	0	0
B	3 } 1.3 [a]	0 } 0 [a]
N x B	1	0
B x S	21 } 17.5 [c]	15 } 9.0 [a]
N x S	14	3
B x C	25 } 21.5 [d]	47 } 32.5 [b]
N x C	18	18
B x S x C	32	52
N x S x C	30 } 32.3 [e]	47 } 42.1 [b]
N x B x S x C	35	27

[y] Duncan's multiple range test at 5% level.

[z] S = 2% sucrose, C = coconut water (25%), N = 10 ppm NAA, B = 10 ppm BA.

Table 33. ANOVA table of hormonal and nutritional effect on seed and protocorm formation.

	df	SS	MS	F	
Rep.	1	13.69	13.69	0.355	NS [y]
Treatment [z]	7	10472.27	1496.04	38.788	**
S	1	745.29	745.29	19.320	*
C	1	4977.30	4977.30	129.046	**
H	1	2299.20	2299.20	59.611	**
S + C	1	1169.64	1169.64	30.325	**
S + H	1	7.29	7.29	0.189	NS
C + H	1	131.10	131.10	3.399	NS
S + C + H	1	1142.44	1142.44	29.620	**
Error	7	269.98	38.57		
Total	15	10755.94			

[z] N = NAA, B = BA, C = coconut water, S = sucrose.

[y] NS = differences non-significant; * = significant at 5% level; ** = significant at 1% level.

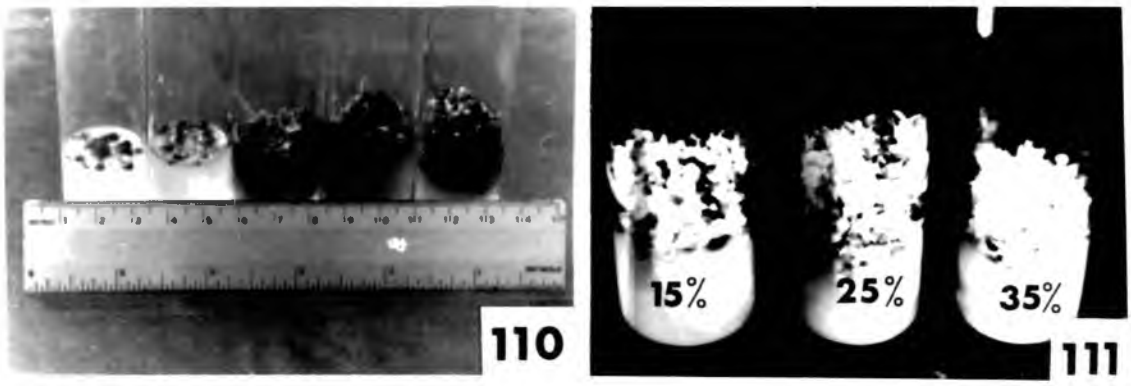

Fig. 110. Protocorms and seedlings at various stages obtained from immature seed culture.

Fig. 111. Seedlings obtained from immature seed cultured with 15%, 25% and 35% coconut water.

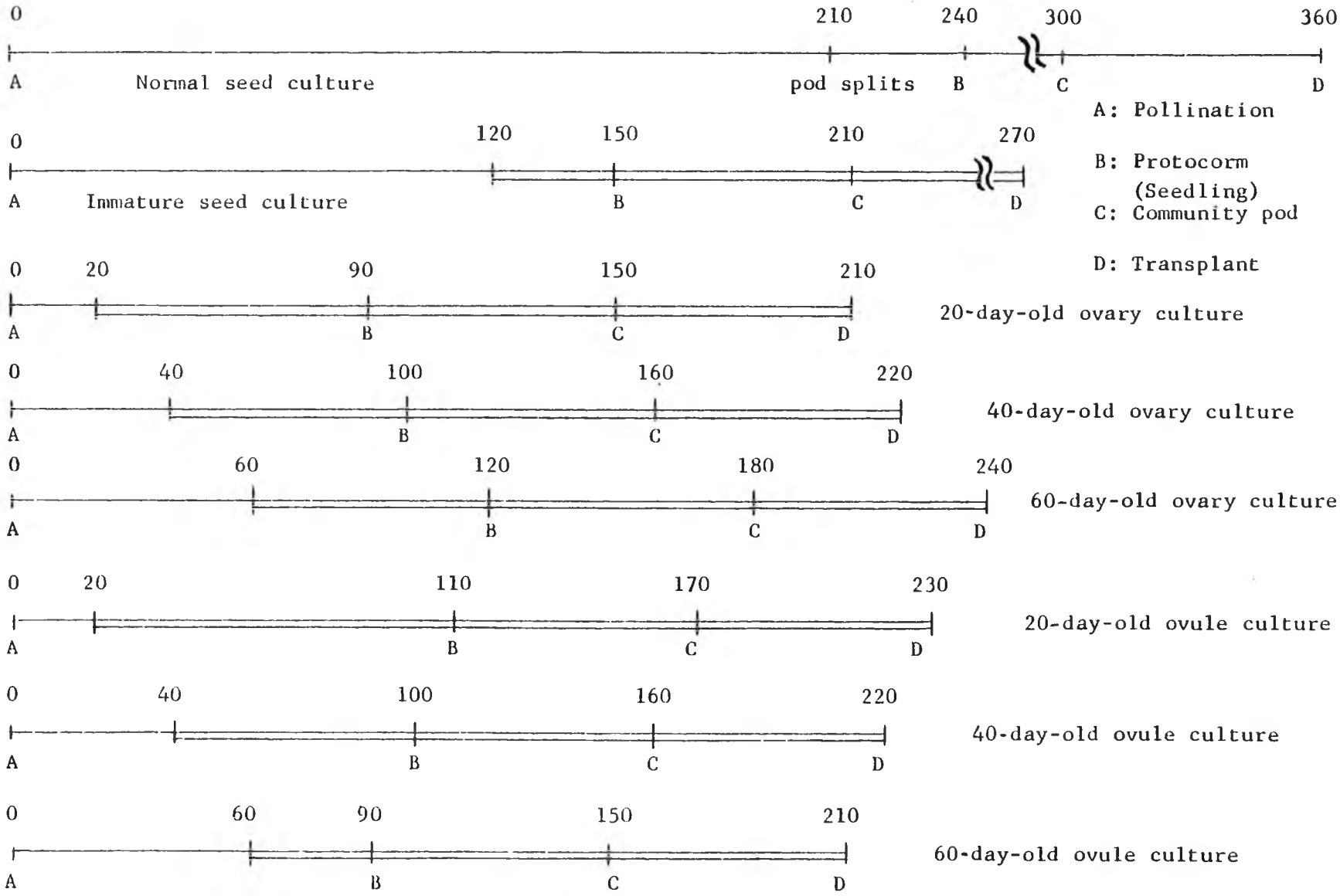

Figure 112. Comparison of days required for getting seedlings by normal seed germination, immature seed culture, ovary culture and ovule culture.

V. DISCUSSION

The discussion will be presented in two sections. Section I involves the morphological and histochemical studies of ovule and ovary development. Section II involves the culture of ovule, ovary and seed.

A. Morphological and histochemical studies of ovule and ovary development

The growth curve (Fig. 3) shown as the actual increase (mm) in ovary width is bimodal, with the first peak at 10 days and the second peak at 50 days after pollination. Pollination effects the increase in size of the ovary, the maturation of the ovules and fertilization. Duncan and Curtis (1942) studied the growth curve for orchid seed pods and correlated the increments with the internal developments of ovule development and fertilization, especially the maximum rate of macrosporogenesis and the maximum rate of fertilization. They suggested that the curve of the increments of growth in width consists of three component curves, which show the time sequence of ovule development inside ovary, namely, ovule rudiment formation, (placental proliferation), macrogametophyte maturation and embryo development. In Doritis pulcherrima, the growth curve of the increment in ovary width was not trimodal but bimodal. A dip is at 40 days in this curve, which is correlated with the time of the megasporogenesis inside the ovary. This is similar to the result shown by Duncan Curtis (1943). However, the maximum rate of fertilization occurs at 60 to 65 days after pollination, which is not correlated with the second dip. Therefore, it seems that Doritis does not fit the curve developed by Duncan and Curtis (1942) for phalaenopsis, a closely allied genera of Doritis.

Incremental growth in width and length of ovary was maximum during rudimental ovule formation (placental proliferation) before embryo sac maturation. Upon fertilization, which occurs 60 to 65 days after pollination, the ovary grows in width and length. The mature pod splits at about 210 days after pollination, while the incremental growth in width and length stops at about 90 to 110 days after pollination.

Wilting of the perianth occurred at 2 days after pollination, which is the first observable morphological change. Duncan and Curtis (1942) suggested that the auxin in the pollen might cause this wilting. Application of 10 ppm NAA on unpollinated flowers of Doritis caused the ovary wilt. Probably the 10 ppm concentration was too high for ovary development.

Embryogenesis in angiosperms was observed by a number of workers (Rondet, 1962; Konar and Nataraja, 1965; Diboll and Larson, 1966; Mukkada, 1969; Schulz and Jensen, 1969; Newcomb and Steeves, 1971; Brown and Mogensen, 1972; Lintilhac and Jensen, 1974; Rembert, 1977; Wilson and Mahlberg, 1978). The successive steps in megasporogenesis in angiosperms involve (a) attainment of haploidy, (b) onset of polarity, (c) elaboration phase, and (d) mature organization (Swamy and Krishnamurthy, 1975). In Orchidaceae a unique feature is that placental proliferation and ovule formation generally occur after anthesis and that pollination is required to stimulate ovule development (Withner, 1959). Doritis, like other orchids, begin placental proliferation after pollination.

The sequence of orchid ovule development and the length of time from pollination to fertilization have been reported by a number of

workers (Sharp, 1912; Carlson, 1940; Swamy, 1949; Joshansen, 1950; Olsson, 1967; Cocucci and Jensen, 1969). The present study shows that approximately 60 days are required to form the mature embryo sac and almost half of that time is occupied in placental growth and elaboration. This tendency is similar to other orchids like Dendrobium (Niimoto and Sagawa, 1961) and Phalaenopsis (Niimoto and Sagawa, 1962).

Although pollen tubes exist around developing ovules, they enter the micropyle after the embryo sac is fully formed, about 60 days after pollination. According to Cocucci and Jensen (1969) the entrance of the pollen tube into the embryo sac is a complicated, biochemically regulated process rather than the penetration by physical forces. Pollen tubes remain in the ovary for 50-55 days and are in constant contact with the developing ovules until they reach maturity. Fertilization occurs soon after the embryo sac is fully formed, containing mature egg at 60-65 days after pollination. The pod requires about 210 days to mature and dehisce. Therefore, approximately 1/4 of the period between pollination and pod maturity is required for fertilization. Fertilization in flowering plants is double fertilization (Jensen, 1973; Cass and Jensen, 1970; Poddubnaya-Arnoldi, 1959, 1960). The pollen tube penetrates one of the synergids, hence the gametes are not delivered by the pollen tube directly into the egg and the central cell but to the degenerating synergid (Cocucci and Jensen, 1969). In this study, the penetration of pollen tube into the embryo sac was observed.

Between 40-50 days after pollination, the megaspore mother cell undergoes meiosis. After the first division, a cell wall is formed between the two dyads, and after the second division two megaspores

and the degenerating dyad is formed. The megaspore enlarges and divides successively three times to form an 8-nucleate embryo sac. Therefore, the type of megasporogenesis of Doritis is monosporic, which is observed in the majority of orchids. According to Savina (1974), in orchids megasporogenesis may be completed with the formation of a tetrad of megaspores, a row of three cells (triad) or a dyad of two cells. Tetrads and triads are characteristic for the subfamily of Monoandrae and triads occur in orchids twice as often as tetrads. The formation of dyads is characteristic for the subfamily of Diandrae. Doritis belongs to Monandrae and forms tetrads in megasporogenesis.

The length of interval between pollination and fertilization is important in orchids from the taxonomic point of view. In general, the more primitive orchids often show a short interval (Olsson, 1967). Doritis is not a primitive orchid because of the relatively long interval from pollination to fertilization; fertilization in Doritis occurs 60-65 days after pollination. In Doritis the suspensor initial cell gives rise to 8 suspensor cells. Suspensor cells are an important character in the classification of embryogenesis of orchids. Doritis follows the Phalaenopsis grandiflora pattern of Johansen(1950).

The use of the histochemical method makes it possible to trace the localization and quantities of biochemicals in ovule and embryo. Raghavan (1976) summarized biochemical embryogenesis and pointed out that nucleus of mature egg cell of some ferns and flowering plants lack DNA and behave abberrantly when they are ready for fertilization, although in the early stages of their formation they are strongly Feulgen-positive. This present study also showed that DNA is rich in

the placental ridge at early placental proliferation, and is low in megaspore mother cell, megaspore and egg, while DNA is rich in the zygote, 2-celled embryo, 4-celled embryo with suspensor and multi-celled embryo. In Stellaria media, Pritchard (1964) observed that DNA stainability is low in archesporium, megaspore mother cell, megaspore, 2- and 4-celled embryo sacs. Low stainability of DNA was recorded in the egg nucleus of Stellaria media (Pritchard, 1964) and cotton (Jensen, 1965a,b). There were no marked differences in DNA concentrations between the suspensor and embryo proper cells in this study. Similar results were reported in Nicotiana rustica (Sehgal and Gifford, 1979) and in Vanda (Alvarez and Sagawa, 1965).

RNA was rich in the placental ridge at early placental proliferation stage. Pritchard (1964) reported that RNA was rich in the archesporium of Stellaria media. However, he also reported that RNA decreased markedly in the megaspore mother cell and increased in quantity in the megaspores. In this study, RNA concentration was about equal in the megaspore mother cell and megaspore. RNA was rich in the egg, zygote, 2-celled embryo and multi-celled embryo. High stainability of RNA was recorded in the egg of Stellaria media (Pritchard, 1964) and of Vanda (Alvarez and Sagawa, 1965). Rondet (1961) studied in detail the role of RNA in embryo development of Myosurus and Alyssium. In these plants zygote was strongly stained. In the 2-celled embryo, both cells were uniformly stained. After cell divisions, rich RNA content was confined to the embryo proper. In Vanda Alvarez and Sagawa (1965) reported that RNA was rich in 3- and 4-celled embryos. In 2-celled embryo of Stellaria media RNA was more dense in the basal than in the terminal

cell (Pritchard, 1964). On the other hand, in this study RNA was more dense in terminal cell than in basal cell similar to those observed by Jensen (1964) in cotton and Alvarez and Sagawa (1965) in Vanda. In all cases, the initial differentiation occurred in the two cells (terminal cell and basal cell). Jensen (1964) suggested that a functional differentiation between the terminal (embryonal) cell and the basal (suspensor initial) cell is possible on the basis of a large amount of RNA. This present study supports Jensen's hypothesis.

Protein is rich in the egg in this study which is in accord with the result in Stellaria media (Pritchard, 1964) and in Vanda (Alvarez and Sagawa, 1965). Alvarez and Sagawa (1965) reported that in Vanda protein is low in the suspensor cell but rich in the embryonal cell and multi-celled embryo. On the other hand, Pritchard (1964) reported that in Stellaria media protein is uniform in both embryonal cell and suspensor cell. In this study, protein was rich in embryonal cell.

Polysaccharides are absent from all stages except the synergids and nucellar cells during megasporogenesis and embryogenesis of Stellaria media (Pritchard, 1964). Polysaccharides were rich in the ovule primordium, while the archesporium, megaspore mother cell, megaspore and young embryo sac contained low amounts of carbohydrates in pulses (Panchaksharappa and Heggde, 1974) and in cereals (Panchaksharappa and Rudramniyappa, 1973). In this present study, total carbohydrates were very low during megasporogenesis and embryogenesis and were rich in the placental ridge at placental proliferation.

In Doritis all of the DNA, RNA, total proteins and carbohydrates decreased during megasporogenesis. These results are in accord with

those of Savina (1974) that the orchid embryo sac differed from the embryo sac of other angiosperm by its sharply decreased physiological activity, and depression of oxidative process.

B. Culture of ovule, ovary and seed

This study represents the first case of seedlings of orchids obtained directly from ovules through both ovary and ovule culture with ovules collected prior to the occurrence of fertilization. Many workers obtained seeds from ovules through ovary culture or ovule culture in orchids (Poddbnaya-Arnoldi, 1959; Niimoto and Sagawa, 1961, 1962; Israel, 1963; Valmayor and Sagawa, 1967). However, they did not get seedlings directly from the ovary, but obtained seeds which were sown on another media. They used ovules collected after the occurrence of fertilization. As a result of this study, obtaining seedlings of Doritis through both ovary and ovule culture is reduced by approximately 150 days.

Few studies have been conducted of hormonal effects on germination and protocorm formation in orchids. This present study suggests that hormones (NAA and BA) are effective on germination and protocorm formation as the inhibitors. However, seedlings can be obtained in vitro through ovary culture with hormones but not with sucrose. This shows the possibility of getting seedlings from ovules directly without symbiosis or sucrose but with ovary tissues through ovary culture.

Poddubnaya-Arnoldi (1959), Niimoto and Sagawa (1961, 1962), Israel (1963) and Valmayor and Sagawa (1967) demonstrated that the growth from ovule cultures was obtained only after fertilization in orchid. On the other hand, Magli (1958) suggested that the development of immature

ovules prior to fertilization may be induced and cultured *in vitro* by using growth regulators. This study showed that seedlings obtained through both ovule and ovary culturs were not haploid but diploid. Therefore, fertilization might have occurred *in vitro* during cultures. Ito (1960) succeeded in inducing viable seeds from ovules. He showed the possibility to get seeds aseptically from ovules. However, his method was to keep the peduncle inside of a vial and left the flowers outside. Withner (1955) succeeded in obtaining seedlings of *Vanilla* through culturing ovules from a 30-day-old pod. He did not check the fertilization time anatomically, but calculated it as 35 days after pollination from the growth curve of ovary diameter based on Duncan and Curtis (1942). Therefore, there is a possibility that he was able to get seedlings from immature ovules prior to fertilization through ovule culture. In this study, seedlings were obtained from ovules 45 days prior to fertilization through ovary and ovule culture. Fertilization must have occurred *in vitro* to produce the seedlings.

Nitsch (1949, 1951) cultured flowers of gherkin and tomato excised from plants before and after pollination. He found that ovaries of pollinated flowers of gherkin and tomato, which were cultured in a relatively simple medium containing sucrose and mineral salts, formed fruits containing seeds. In *Zea mays*, Gengenbach (1977) succeeded in getting seedlings through ovule culture by pollination *in vitro* and fertilization *in vitro*. He obtained 45% fertilization *in vitro*. Kanta, Swamy and Maheshwari (1962) were the first to report test-tube fertilization. The above studies strongly support the conclusion that seedlings obtained through ovary and ovule culture in this study

originated from fertilization and not from parthenogenesis.

In 20-day-old ovule culture, seedlings were obtained from treatments of sucrose; NAA + coconut water; and NAA + BA + coconut water. However, ovule growth rate was not high in these treatments. In 40-day-old ovule culture, treatments of sucrose; coconut water; and NAA + BA + sucrose + coconut water were highly effective on both ovule growth rate and seedling formation rate. In 60-day-old ovule culture, seedlings were obtained from all treatments except NAA; BA; and NAA + BA. There was no direct relationship between ovule growth rate and seedling formation rate. Seedlings were obtained from 20-, 40- and 60-day-old ovule cultures if the media contained sucrose or coconut water. Therefore, sucrose may be necessary for seedling formation from ovule culture, since coconut water contains sucrose. However, if we compare the time required to obtain seedlings, coconut water promoted faster growth than sucrose alone in 20- and 40-day-old ovule cultures. In 60-day-old ovule culture, coconut water and sucrose were almost equal in promoting seedling formation. At the 60-day-stage, the embryo sac is already fully formed in ovule. Therefore, the difference between these results suggest that other biochemicals included in coconut water are effective on embryo sac formation in 20- and 40-day-old cultures. For leaf and root formation, coconut water was more effective than sucrose, which indicates that not only sucrose but also many biochemicals are necessary for their formation. Kapoor (1959) showed that supplementing the basal medium with coconut water supported development of viable seeds from cultured ovules of Zephyranthes excised at the stage of the zygote. According to van Overbeek (1942), coconut water

contains at least three factors: (a) embryo factor, (b) leaf growth factor and (c) auxin. Raghavan (1976) showed that coconut milk contains several inorganic ions, amino acids, organic acids, vitamins, growth hormones, sugars, sugar alcohols and other substances. In this study, maleic hydrazide, tryptophan, casein hydrolysate and NAA, which are included in coconut water, were not effective on seedling formation without sucrose. This result differed with ovule culture of Papaver somniferum (Maheshwari and Lal, 1961), which showed the addition of kinetin, casein hydrolysate or yeast extract accelerated the initial rate of growth of proembryos in varying degrees.

The technique of culture of flowers and ovaries was introduced by La Rue (1942). Israel (1963) and Ito (1961) induced mature pods containing seeds in vitro through ovary culture. However, they did not get seedlings directly from ovaries, but instead sowed their seeds obtained through ovary cultures on another media. Thus, this present study is the first case of obtaining seedlings directly from ovaries by continuous culturing of ovaries in the same media.

The younger stages of ovary show greater responses to hormones than the older stages. NAA and BA promoted the production of seedlings in ovary culture. This is in accord with the results of Sankhla and Sankhla (1967) in Reseda odorate in which IAA and kinetin promoted the production of viable seeds in ovaries. On the other hand, combination of IAA and kinetin was inhibitory for embryo growth in the ovary of Ranunculus sceleratus (Sachar and Guha, 1962).

The best hormone treatments for obtaining seedlings in this present study were 1.0 ppm BA in 20-day-old ovary culture, 1.0 ppm NAA in

40-day-old ovary culture. Thus, BA was more effective than NAA in 20-day-old ovary. Up to 40-day-old ovary there was an interactional disappeared in 60-day-old ovary. The interaction might be caused by the difference between NAA and BA in promoting seedling formation.

Although suitability of coconut water for successful germination and growth of orchid embryo was reported by Niimoto and Sagawa (1961), little attention was paid to coconut water in ovary culture. This study indicates that coconut water is effective on seedling formation in ovary culture. However, callus formation rate was also increased by coconut water.

Sucrose is necessary in ovary and ovule culture for seedling formation. However, even without sucrose seedlings were obtained in ovary culture with NAA or BA treatments. This indicates that sucrose might be necessary for ovule development and seedling formation, but in the case of ovary culture sucrose can be provided from ovary tissue to ovule in some forms of carbohydrates.

Orchid embryos are able to utilize a wider range of carbohydrates than embryos of other angiosperms. Among the sugars tested, sucrose seems to be best for germination or growth of embryo (Breddy, 1953). Knudson (1922) showed that orchid seeds are able to germinate without symbiosis on medium containing sucrose. This study also shows that sucrose is necessary for seed germination, and coconut water can replace sucrose and is more effective than sucrose on seed germination and protocorm formation in seed culture.

Pierik and Steegmans (1972) investigated the effect of BA on growth and development of Cattleya seedlings. At high BA concentration, the

formation of numerous protocorm-like bodies (PLB) was observed. Growth of orchid embryos were definitly inhibited by IAA during the early stage development after germination (Curtis and Nichol, 1948). Withner (1955) has observed promotion of growth of embryos by IAA. Therefore, embryos of orchids vary considerably in their responses to hormones. In the present study, 1.0 ppm BA and 1.0 ppm NAA promoted seedling formation in ovary culture; 10 ppm BA and 10 ppm NAA did not promote seedling formation without sucrose in ovule culture; and 10 ppm BA and 10 ppm NAA did not promote seed germination in seed culture. More studies are necessary to determine the concentration of hormones and the time of use during ovule development and seedling formation. Dhindsa (1978) reported that IAA and GA regulate the enzymes of CO_2 fixation in unfertilized cotton ovules. It would be interesting to study the effects of hormones on ovule development and seedling formation from the enzymatical point of view.

Orchids have exalbuminous seeds, or seeds in which the endosperm is short lived and is used up during the growth of the embryo, or no endosperm is produced. Because of their lack of endosperm, orchid seeds require external energy source for germination. This study showed that sucrose was very important in ovule and embryo development. The development of ovule and embryo is a process of organ formation including cell division and growth, which require high energy. Therefore, sucrose might be one of the most important energy sources for ovule and embryo development in orchids. Harrison (1977) reported that neither dictyosomes nor rough endoplasmic reticulum was found in the cell of seedlings grown on sugarless medium.

In general, carbohydrates are the major energy source in many plants. The important role of starch accumulation in differentiation has been demonstrated in many reports (Wardlaw, 1968; Thorpe and Murashige, 1970; McWilliam et al., 1972). It is also well known that starch is produced from sucrose as a storage product, and that when the energy is needed starch is changed to sucrose again. Jeffs and Northcote (1967) pointed out that sucrose has a specific action in inducing differentiation into nodules containing xylem, phloem and meristematic activity. Smith (1973) suggested the possible role of high osmotic pressures in regulating embryo growth in culture. Therefore, there might be two possible roles of sucrose: in organ formation as the source of energy and the source of high osmotic pressure.

The present histochemical study showed that total carbohydrates decreased during megasporogenesis and embryogenesis. This suggests that total carbohydrates including sucrose are used as energy source during the development of ovule and embryo. In ovule, ovary and seed culture, sucrose was necessary for ovule development and seedling formation.

APPENDIXES

APPENDIX I

Modified Vacin and Went Basic Medium

Constituents:

Tricalcium phosphate	$Ca_3(PO_4)_2$	0.20 gm.
Potassium nitrate	KNO_3	0.525
Monopotassium acid phosphate	KH_2PO_4	0.25
Magnesium sulfate	$MgSO_4 \cdot 7H_2O$	0.25
Ammonium sulfate	$(NH_4)_2SO_4$	0.50
Manganese sulfate	$MnSO_4 \cdot H_2O$	0.068
Agar		8.00
Iron chelate		0.057
Water		1000 ml.

APPENDIX II

Composition of coconut milk

1.	Inorganic ions	(mg/100g)
	Potassium	312
	Chlorine	183
	Sodium	105
	Phosphorus	37
	Magnesium	30
	Sulfur	24
	Iron	0.10
	Copper	0.04
2.	Amino acids	(µg/ml of coconut milk)
	Aspartic acid	65
	Threonic acid	44
	Serine	111
	Asparagine and glutamine ca.	60
	Proline	97
	Glutamic acid	240
	Alanine	312
	Valine	27
	Methionine	8
	Isoleucine	18
	Leucine	22
	Tyrosine	16
	Phenylalanine	12
	-alanine	12
	-Aminobutyric acid	820
	Lysine	150
	Tryptophan	39
	Arginine	133
	Ornithine	22
	Histidine	trace
	Pipecolic acid	+
	Hydroxyproline	trace
	Glycine	13.9
	Homoserine	5.2
	Cystine (g/100g) dried protein	0.97-1.17

APPENDIX II (Continued)

Composition of coconut milk

3.	Other nitrogen compounds (mol/ml - analysis of Philippine coconut)	
	Dihydroxyphenylalanine	+
	Ethanolamine	0.01
	Ammonia	+
4.	Organic acids (meq/ml)	
	Malic acid	34.31
	Shikimic acid, quinic acid etc.	0.57
	Citric acid	0.37
	Pyrrolidine carboxylic acid	0.39
5.	Vitamins (mg/ml)	
	Nicotinic acid	0.64
	Pantothenic acid	0.52
	Biotin	0.02
	Riboflavin	0.01
	Folic acid	0.003
	Thiamine	trace
	Pyridoxine	trace
6.	Sugars (mg/ml)	
	Sucrose	9.18
	Glucose	7.25
	Fructose	5.25
	Mannitol	0.8%
7.	Growth substances (mg/ml)	
	Auxin	0.07
	Gibberellin	+
	Cytokinins	+
8.	Miscellaneous substances (mg/l)	
	1,3-Diphenylurea	5.8
	Leucoanthocyanin	+
	Phyllococosine	+
	Sorbitol	15
	myo-Inositol	0.1
	scyllo-Inositol	0.5
	Acid phosphatase	+
	Diastase	+
	RNA polymerase	+
	Dehydrogenase	+
	Peroxidase	+
	Catalase	+

APPENDIX III

Staining Methods
of anatomical and histochemical study

1. DNA - Feulgen Method (Berlyn & Mikshe, 1976)

xylene (5 min.) → xylene/absolute alcohol 1 : 1 (5min.) → absolute alcohol (5 min.) →

95% alcohol (5 min.) → 70% alcohol (5 min.) → 50% alcohol (5 min.) → water wash →

1N-HCl 60'C 10-15 min. → Dip water 4'C → Schiff's solution * 1-2 hours →

Bleach 3 times 10 min. → water 10 min. → water 10 min. → 50% alcohol rapid →

70% alcohol rapid → 95% alcohol rapid → xylene rapid → mount

* Schiff's solution (Leucobasic fuchsin)
Add 2 g of stain (basic fuchsin) + 3.8 g Na or K metabisulfite to 200 ml of 0.15 N-HCl in a flask and stire or shake on a mechanical shaker for 2 hours. Decolorizing by adding 1 g of activated charcoal; stire for 5 min.; filter the mixture using a Buchner funnel, side-arm flask and vaccum, and No.1 Whatman filter paper (Lillie, 1951).

* Feulgen bleach solution
Add 10 ml of 1N-HCl and 10 ml Na metabisulfite to 180 ml of water. The bleach solution should be made on the day it is to be used.

APPENDIX III (Continued)

Staining Methods
of anatomical and histochemical study

2. RNA - Methyl Green & Pyronine Method (Brachet, 1953)

xylene (5 min.) → xylene/absolute alcohol 1 : 1 (5 min.) → absolute alcohol (5 min.) →

95% alcohol (5 min.) → 75% alcohol (5 min.) → 50% alcohol (5 min.) → water →

Mixture* pH = 4.7 20 min. → running water until no red color → 50% alcohol rapid → 70% alcohol rapid →

95% alcohol rapid → absolute alcohol rapid → xylene rapid → mount

* Mixture solution
0.15 g of methyl green, 0.25 g of pyronine B, and 100 ml of acetate buffer at pH = 4.7. Make the buffer by adding 1.65 g of sodium acetate and 10 ml of HCl to 1000 ml of water. Test the pH, and dilute 4 times before using. Extract the methyl green with ether or chloroform in a separatory funnel before using in this solution. repeat extraction until fresh ether or chloroform does not take on a purple color.

APPENDIX III (Continued)

Staining Methods
of anatomical and histochemical study

3. Total proteins - Ninhydrin - Schiff's Reaction (Yasuma & Ichikawa, 1953)

xylene (5 min.) → xylene/absolute alcohol 1 : 1 (5 min.) → 0.5% ninhydrin in absolute alcohol 37'C 24 hours →

absolute alcohol rinse → absolute alcohol rinse → water →

Schiff's solution (10-30 min.) → water → 2% sodium bisulfate (1-2 min.) →

running tap water (10-20 min.) → 50% alcohol rapid → 70% alcohol rapid →

95% alcohol rapid → absolute alcohol rapid → xylene rapid → mount

* Deamination: Place the tissue in a mixture made up of 20 ml of 60% sodium nitrate and 60 ml of 1% acetic acid at room temperature for 1-24 hours.

Acetylation: Place the tissue in a 10% solution of acetic anhydride in pyridine at room temperature for 2-20 hours.

If color is obtained in either control the reaction is localizing some compound other than proteins having α-amino and α-carboxyl group in the constituent amino acids.

APPENDIX III (Continued)

Staining Methods
of anatomical and histochemical study

4. Total carbohydrates of insoluble polysaccharides
- Periodic Acid Schiff's Reaction (PAS)- (Jensen, 1962)

xylene (5 min.) → xylene/absolute alcohol 1 : 1 (5 min.) → absolute alcohol (5 min.) →

95% alcohol (5 min.) → 70% alcohol (5 min.) → 50% alcohol (5 min.) → water →

0.5% periodic acid solution at room temperature (5-30 min.) → running water (10 min.) →

Schiff's reagent (10-15 min.) → water → 2% sodium bisulfate (1-2 min.) →

running water → 50% alcohol (rapid) → 70% alcohol (rapid) →

95% alcohol (rapid) → absolute alcohol (rapid) → xylene (rapid) → mount

APPENDIX III (Continued)

Staining Methods
of anatomical and histochemical study

5. Safranin-Fast Green Method

xylene (5 min.) → xylene/absolute alcohol 1 : 1 (5 min.) → absolute alcohol (5 min.) →

95% alcohol (5 min.) → 70% alcohol (5 min.) → 50% alcohol (5 min.) → Safranin (1-10 min.) →

running water → 70% alcohol (rapid) → 95% alcohol (rapid) →

absolute alcohol (rapid) → Fast Green (2 min.) → Mixture* (5-15 min.) → Mixture (5-15 min.) →

xylene (15 min.) → xylene (15 min.) → xylene (15 min.) → mount

* Mixture: 50% clove oil, 25% absolute alcohol, 25% xylene.

Safranin: 1% safranin in 95% alcohol : water = 1 : 1.

Fast Green: 0.5% solution in 50% clove oil, 95% alcohol.

APPENDIX III (Continued)

Staining Methods
of anatomical and histochemical study

6. Squash Method - Gomori's hematoxylin technique (1953)

70% alcohol → 1N- HCl at 60'C → filter paper →
30 min. 10 min.

hematoxylin at 60'C → 45% acetic acid → Squash in a fresh drop of 45% acetic acid
30 min. rinse

* Hematoxylin solution: Equal parts of 1% aqueous hematoxylin and 3% aqueous chrom alum are mixed together, and to each 100 ml of the mixture, 2 ml of 5% aqueous potassium bichromate and 2 ml of 0.5 N sulfuric acid are added. The stain is allowed to ripen for at least 24 hours until a metallic film develops on the surface and filtered before use.

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