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TISSUE CULTURE STUDIES ON *CITRUS* AND *WELWITSCHIA*

by

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CONTENTS

ABSTRACT

TISSUE CULTURE STUDIES ON *CITRUS* AND *WELWITSCHIA*

Part I. *IN VITRO* CULTURE OF CITRUS EMBRYOS AND NUCELLAR ISOLATES

Zygotic embryos of the Ellendale mandarin, a monoembryonic variety of citrus, were cultured on modified basal media of Murashige and Skoog (BM_1), and White (BM_2), supplemented with various growth regulators and nutrient additives. The growth of immature embryos was greatly enhanced by the addition of 400 mg/l casein hydrolysate (CH) to the basal media. Coconut milk (CM) and malt extract (ME) enhanced growth to a lesser extent, while the addition of indoleacetic acid (IAA) and kinetin (KIN) at the concentrations used, was in no way beneficial.

Nucellar isolates excised from abortive and normal Ellendale mandarin ovules eight to 20 weeks after anthesis, were cultured on BM_1 and BM_2 in the presence of various concentrations and combinations of IAA, indolebutyric acid, naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), KIN, CM, benzyl adenine, 6-dimethylallylamino purine, yeast extract (YE), ME, CH, adenine (AD), adenine sulphate (AS), ascorbic acid (AA), and benzylthiazole-2-oxyacetic acid. Some of the isolates which remained alive for four months did develop callus but no

differentiation of embryoids or other structures occurred.

Unfertilized ovules from 8-12-week-old Washington Navel orange fruits provided nucellar isolates which were cultured on media similar to those upon which mandarin nucelli were unsuccessfully cultured. In the case of Navel orange nucelli however, BM_1 + 400 mg/l filter-sterilized ME, and BM_1 + 40 mg/l AD yielded numerous pseudobulbils which later developed embryoids. Adenine (10 mg/l) was more effective than 20 mg/l which in turn was more effective than 30 mg/l. Adenine was more effective than its equivalent amount supplemented in the sulphate form except at 10 mg/l where the two forms were equally effective. Zeatin (ZE) at 0,2 mg/l did induce some pseudobulbils and embryoids, but all these treatments were less effective than 400 mg/l ME.

When transferred to BM_1 + GA_3 (1 mg/l), embryoids developed roots and later, shoots. It was necessary to remove plantlets from the GA_3-supplemented medium shortly after the first foliage leaves developed in order to prevent the development of weak, spindly plants. Plantlets were transferred from BM_1 + GA_3 to BM_1 only, and then after careful conditioning they were planted out in soil. This appears to be the first successful attempt at inducing adventive embryogenesis in the nucellus of unpollinated, unfertilized citrus ovules *in vitro*.

Part II. EMBRYO AND FREE-CELL CULTURE OF *WELWITSCHIA MIRABILIS*

Welwitschia embryos, cultured on BM_1 supplemented with CH, and low levels of IAA and KIN, germinated and developed leaves but not roots. Embryos cultured on BM_1 with 5,0 and 10,0 mg/l NAA produced an abundance of friable callus from the hypocotyl root axis. This callus was used for starting suspension cultures aimed at inducing vegetative embryogenesis. A number of nutritional additives and hormones were used alone and in combination at various concentrations. Cells of numerous shapes and sizes were observed but no organogenesis was apparent in either suspension cultures or in cell colonies plated out on semi-solid agar media. A closer study of cell aggregates formed in suspensions supplemented with CM + 2,4-D revealed that internal division occurred in approximately 40 per cent of the larger cells.

It is suggested that this internal division may constitute the first step in embryogenesis of *Welwitschia* cells in suspension culture. It is also tempting to speculate that this process, which has been reported by other researchers, is the first step in embryogenesis of free cells in general. Although this attempt at inducing adventive embryogenesis in cell cultures of *Welwitschia* was unsuccessful, some encouraging results were obtained on potentially suitable media and possible initial stages in the organization of embryoids.

ACKNOWLEDGEMENTS

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PART I

IN VITRO CULTURE OF CITRUS EMBRYOS AND NUCELLAR ISOLATES

INTRODUCTION

Potentially important techniques in crop-improvement programmes include meristem cultures (Morel, 1964, and others), embryo culture (Street, 1969), the culture of diploid plants from single somatic cells (Steward, Mapes, and Ammirato 1969), the derivation of haploid plants from anthers (Guha and Maheshwari, 1967; Sunderland and Wicks, 1971), and the differentiation of plant cell protoplasts (Bourgin, 1971). In addition to these, the establishment of plants of nucellar origin is of great potential significance since such plants are genetically identical to the seed-parent and are presumably virus-free. From a more basic point of view, *in vitro* culture of cells, tissues such as the nucellus, and organs provides an invaluable method of studying developmental morphology and the responses of cultured isolates to preselected environmental and nutritional conditions.

In citrus, the nucellus surrounds the embryo sac and is itself surrounded by an inner and an outer integument. It functions as a nurse tissue for the developing endosperm, and probably the zygotic embryo also, and is well adapted to nutrient conduction (Schneider, 1968).

Nearly a century ago, Strasburger (1878) showed that the supernumerary embryos found in many citrus varieties are formed *in vivo* by proliferation of nucellar cells surrounding the embryo sac. These embryos are located in the micropylar region and are initiated

at varying intervals after fecundation (Frost and Soost, 1968). Such nucellar embryos generally mature and germinate under natural conditions, and have been the origin of many commercial citrus clones. The hereditary trait of monoembryony as opposed to polyembryony is controlled by an allele pair; polyembryony being dominant and monoembryony recessive (Furusato, 1960). Thus many citrus clones may be rejuvenated through these naturally-occuring nucellar embryos. However there are a number of monoembryonic clones and seedless clones which cannot be rejuvenated in this way. Such varieties do produce ovules with normal nucelli which are potentially capable of producing adventive embryos *in vitro* but not *in vivo*. There are reports of isolated cases of "identical twins" (Frost, 1926) and "identical triplets" (Ozsan and Cameron, 1963). These are zygotic seedlings derived from a single zygote which has undergone multiple fission after fertilization.

It is generally agreed that pollination and fertilization of an ovule are probably essential prerequisites for the induction of adventive embryos in the nucellus whether *in vivo* or *in vitro* (Strasburger, 1907; Frost, 1926; Wardlaw, 1965; Nygren, 1967; Frost and Soost, 1968). Pollination, and more specifically fertilization, appear to provide an essential stimulus to the nucellus via the zygotic embryo, inducing it to produce adventive embryos. Once this triggering stimulus has been received by the nucellus it appears that the subsequent abortion of the zygotic embryo does not affect further development of the nucellar embryos since zygotic embryos of most polyembryonic varieties rarely germinate. Exactly what constitutes this triggering stimulus is not known, but it is probably hormonal.

After fertilization the nucellus enlarges greatly (Schneider, 1968) followed by a surge in growth of the triploid endosperm. As the ovule develops further, the expanding endosperm and later the embryo(s) compress the remaining few cell layers of the nucellus against the inner integument. Rangaswamy (1961) found that most of the nucellar tissue in 10-12-week-old fertilized ovules had been consumed, whereas Rangan, Murashige and Bitters (1968; 1969) were able to excise relatively large portions of nucellar tissue from the microplylar regions of 17-week-old fertilized ovules. They found no signs of nucellar degeneration at this stage.

Attempts have been made to culture and induce differentiation in ovules, nucellar isolates, zygotic and nucellar embryos at various stages of development, as well as in other citrus flower and fruit parts (Stevenson, 1956; Ohta and Furusato, 1957; Rangaswamy, 1958a, 1958b; 1959; 1961; Sabharwal, 1962; Ozsan and Cameron, 1963; and Rangan *et al.* 1968, 1969). Of these tissues and organs, only embryos and nucellar isolates from fertilized ovules have been successfully differentiated into plants. No attempts appear to have been made at culturing either entire unfertilized ovules or their nucelli.

Rangaswamy (1958a,b) cultured micropylar halves of nucelli from developing, fertilized ovules of polyembryonic varieties in which nucellar proembryos had been initiated. He found that when placed on a modified White's medium supplemented with 400 mg/l casein hydrolysate and solidified with 0,8 per cent agar, these isolates developed callus and then pseudobulbils. The addition of 2 mg/l

kinetin to the supplemented medium further enhanced pseudobulbil formation. He (1958b) found that adenine at 20 or 40 mg/l not only enhanced pseudobulbil formation but also their subsequent differentiation into plantlets, while gibberellin at 1 mg/l inhibited budding of pseudobulbils but enhanced root formation. These responses to gibberellin appear strange, but similar effects in *Capsella* embryo cultures (Raghavan and Torrey, 1964) and *Phlox* callus cultures (Konar and Konar, 1966) have been reported. Schooler (1960) found that potassium gibberellate enhanced both root and shoot growth in embryos excised from *Hordeum vulgare*.

It is generally accepted that the more immature an embryo, the greater is its degree of heterotrophy (Street, 1969). Thus the diverse responses of cultured embryos of different species to various media, nutritional supplements and growth regulators is not surprising. The requirements for inducing embryogenesis in somatic tissues such as the nucellus of citrus ovules or *Welwitschia* callus is likewise probably extremely specific, especially in terms of growth regulators. According to Street (1969), the actions of substances such as auxins, gibberellins and cytokinins when added singly to the medium, may be misleading since embryo development may well depend upon the balance between hormonal factors. For example, Raghavan and Torrey (1963) successfully cultured globular *Capsella* embryos only after incorporation of indoleacetic acid, kinetin and adenine sulphate in the basal medium.

Sabharwal (1962) was unable to induce development of nucellar embryos *in vitro* unless these had been initiated before excision of the nucelli. Rangan *et al.* (1968, 1969) appear to be

the only workers so far who have succeeded in triggering the initiation of adventive embryos in cultured nucellar isolates of three citrus varieties. They cultured nucellar isolates excised from fertilized ovules of 100-120-day-old fruits pollinated with pollen of *Poncirus trifoliata* which acted as a marker. Due probably to two dominant genes in the *P. trifoliata* pollen, all zygotic seedlings should possess the trifoliate characteristic of *P. trifoliata* and not the typical monofoliate leaves of *Citrus* (Cameron and Frost, 1968). During the excision, detectable zygotic embryos were removed before placing the nucellar isolates on a modified Murashige and Skoog's medium, gelled with one per cent agar. These workers were able to induce nucellar embryos by supplementing the basal medium with either 500 mg/l malt extract or a combination of 5 per cent orange juice, 25 mg/l adenine sulphate and 0,5 mg/l α-naphthaleneacetic acid. Malt extract proved the most effective supplement, yielding up to 20 per cent successful cultures. In contrast to the experiments of Rangaswamy (1961) who used a polyembryonic variety, Rangan *et al.*'s nucellar isolates from monoembryonic varieties did not form any callus or pseudobulbils but embryoids arose directly from the nucellus.

Both Rangaswamy and Rangan *et al.* appreciated the significance of isolating and culturing zygotic embryos of polyembryonic varieties in citrus breeding programmes, since these embryos seldom germinate naturally. Rangaswamy (1959, 1961) succeeded in culturing proembryos as well as more mature embryos on White's medium supplemented with 400 mg/l casein hydrolysate. He found that embryo growth proceeded normally, differentiation took place and seedlings developed. Cotyledons exhibited morphological deviations although leaves were normal.

differentiation as opposed to "maturation" of meristematic cells. Use of the word "differentiation" in this sense is disapproved of by Halperin (1969) who prefers to limit its meaning to cytological changes.

The term "embryoid' has been used by Vasil and Hilderbrandt (1966a) to describe "vegetatively produced embryo-like structures formed in tissue and organ cultures". Vasil and Hilderbrandt's definition has been adhered to in this thesis but has been further restricted to describe embryo-like structures having a definite axis. Prior to this ontogenetic stage, the term "pro-embryoid" has been used. This distinction was deemed necessary since in tissue cultures, not all globular "pro-embryoids' will develop into "embryoids" with definite morphological axes.

The disadvantages of using complex nutritional supplements such as malt extract, yeast extract, casein hydrolysate and coconut milk in studying embryogenesis (Mitra, 1967) and growth are accepted, and attempts have been made in this study to use definable synthetic media wherever possible.

A major problem encountered in tissue culture experiments is the difficulty of expressing results in absolute terms and of subjecting such results to statistical analysis. This problem is primarily a result of variability within treatments and the belated appearance of contaminants in cultures. Delayed contamination is not critical in cultures where results are of an all-or-nothing nature or where no subculturing is required. However is does impose a great restriction on the presentation and statistical processing of results from experiments on organization and development which do require subculturing.

1.3. *Culture media*

The two basal media used in this study comprised the inorganic salts of (a) Murashige and Skoog, and (b) White (Rangan *et al.*, 1969), to which the following organic substances were added (in mg/l): glycine, 5,0; thiamine hydrochloride, 0,3; pyridoxin hydrochloride, 0.05; niacin, 1,0; calcium pantothenate, 0,03; myo-inositol, 100; sucrose, 4×10^4 and agar, 10^4. These basal media, consisting of both inorganic and the above organic components have been abbreviated to BM_1 and BM_2 respectively. All chemicals used in making up the media were of analytical grade or chemically pure. All additives except malt extract (ME) and yeast extract (YE) were incorporated in the BM prior to adjusting the pH to 5,6, adding the agar, and autoclaving for 15 minutes at 1,05 kg/cm^2. Malt and yeast extracts were filter-sterilized and added to the cooled BM after autoclaving since these supplements have been shown to lose activity if autoclaved (Solomon, 1950).

Two basal media were used because of the controversial views on the effects of ammonium, nitrate, and reduced organic nitrogen on embryogenesis and subsequent embryo development (Wardlaw, 1965; Reinert, Backs and Krosing, 1966; Mitra, 1967; Norstog and Rhamstine, 1967; Reinert, 1967; Sussex and Frei, 1968; and Street, 1969). White's medium contains only NO_3^--nitrogen while that of Murashige and Skoog includes both NO_3^-- and NH_4^+-nitrogen. Reduced organic nitrogen was supplied mainly in the form of casein hydrolysate (CH) which has been

regularly used in culturing tissue with the aim of inducing embryogenesis, and in culturing immature embryos (Rangaswamy, 1961; Johri and Bhojwani, 1967; Rangan *et al.*, 1968; and Steward, 1969).

Subcultured tissue isolates were transferred to specimen tubes containing the fresh medium, while plantlets were transferred to wide-mouthed erlenmeyer flasks of the appropriate size, containing only the inorganic salts of BM_1 in one per cent agar.

1.4. *Culture conditions*

All tissue isolates were initially cultured in 70 X 25 mm, flat-bottomed, pyrex specimen tubes fitted with aluminium caps lined with non-absorbent cotton wool. These were placed in culture rooms at 25± 2°C, under a 16-hour photoperiod supplied by Gro-Lux fluorescent tubes at ca. 1 000 lux.

2. EMBRYO CULTURE

2.1. *Procedure*

Embryos from ovules of mature and later, immature fruits of the Ellendale mandarin and Djeroek shaddock were cultured on both basal media alone, and with the following supplements: coconut milk (CM) (15% v/v); CH (400 mg/l); ME (500 mg/l); CH + ME; CH + indoleacetic acid (IAA) (2 mg/l) + kinetin (KIN) (1 mg/l); CH + IAA (1 mg/l) + KIN (2 mg/l). Although not the major area of investigation, this embryo culture was undertaken to familiarise myself with tissue culture techniques and to find a suitable medium (or media) for embryo growth so that, in the event of adventive embryos being induced, these could subsequently be grown on this medium. Plantlets derived from cultured embryos were used in establishing a method of conditioning them to survive in the external environment.

In addition to culturing embryos of unknown pollen parentage, 150 immature embryos of the Ellendale mandarin X *Poncirus trifoliata* hybrid (see 3.1.) were used to check the efficacy of the marker pollen. These embryos were cultured on both the basal media supplemented with 400 mg/l CH.

In all cases, the embryo isolates (Fig. 1) consisted of the hypocotyl root axis and the plumule, but excluded the cotyledons.

2.2. *Results and discussion*

Mature embryos of mandarin and shaddock developed roots and leaves within two weeks of culturing on both the basal media, with and without additives.

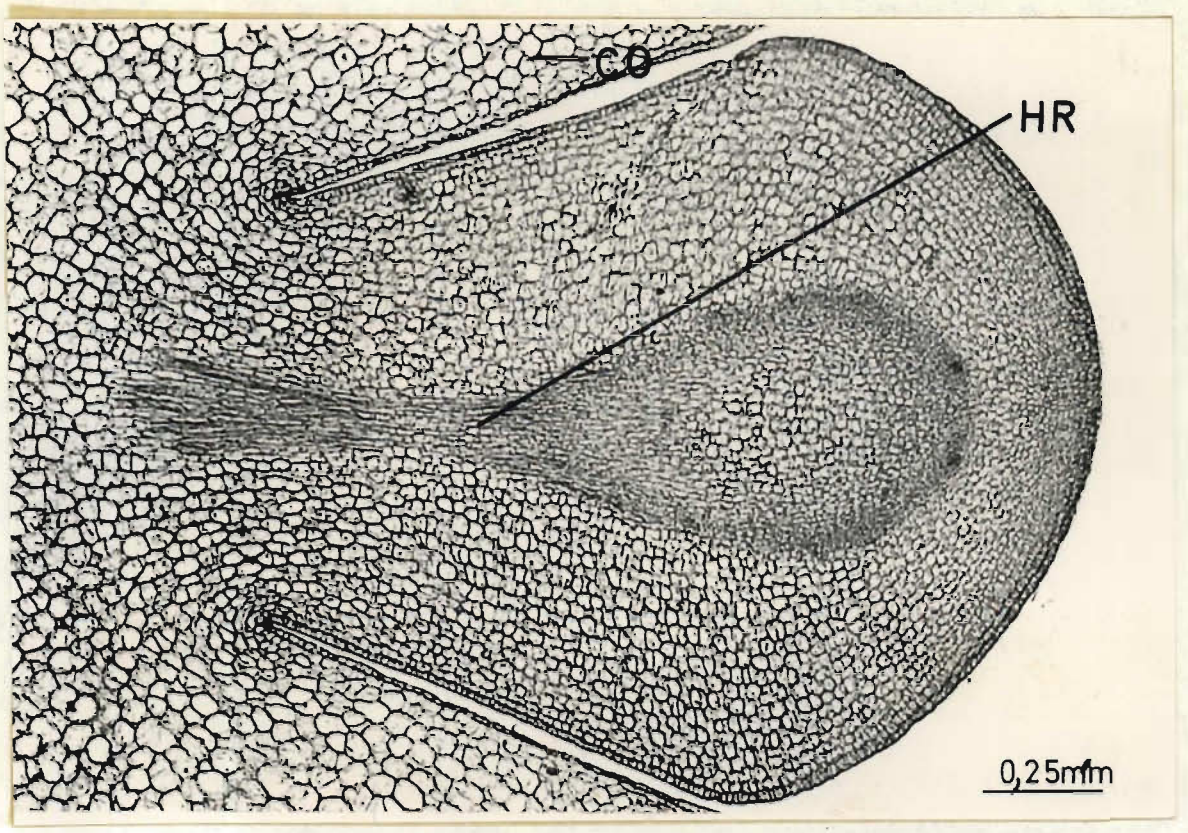

Fig. 1. Approximately median L/S through a typical mature Ellendale mandarin X *P. trifoliata* embryo showing the hypotoyl-root axis (HR) and a part of the cotyledons (CO).

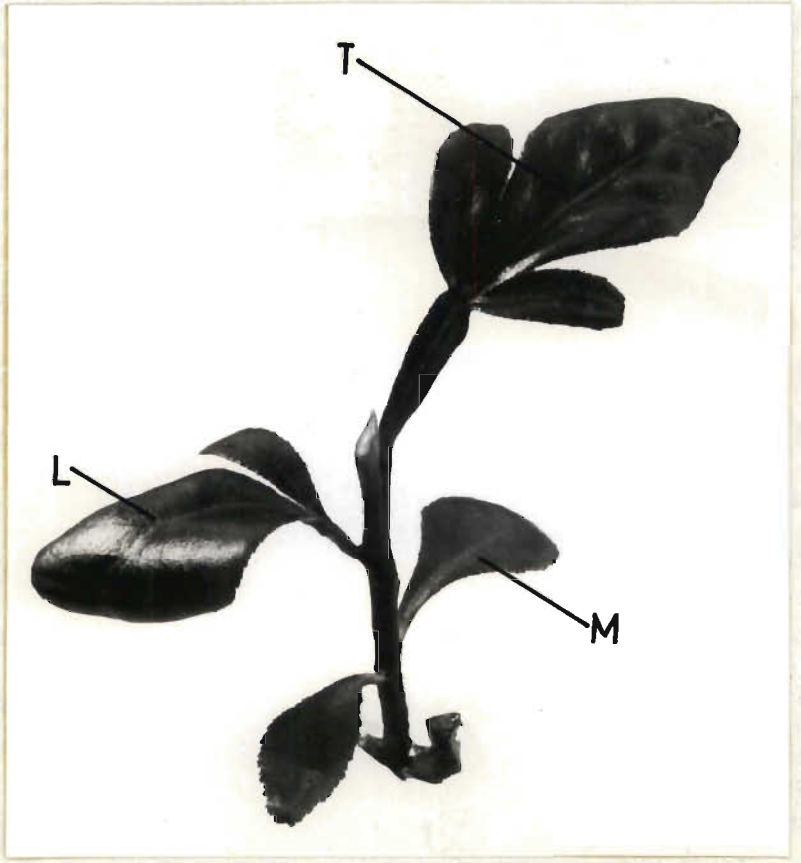

Fig. 2. Young hybrid plantlet showing monofoliate (M), lobed (L), and trifoliate (T) leaf forms.

on basal media only. The addition of CM, ME, and especially CH, greatly enhanced the degree of success. These embryos and subsequent plantlets invariably thrived better on BM_1 than on BM_2 whether or not nutritional supplements were added. The addition of IAA and KIN appeared in no way to be beneficial.

The hybrid embryos germinated and developed well on BM_1 but very poorly on BM_2. The first true leaves of the developing plantlets were monofoliate and not trifoliate as was expected. This caused some concern but leaves developed subseuqently first showing varying degrees of lobing and then later the true trifoliate character (Fig. 2). As was expected, all hybrid plantlets were trifoliate.

These results on embryo culture confirm the general consensus that the younger the embryo, the more heterotrophic it is, and that CH is an important nutritional supplement in culturing immature but not mature embryos. The active fraction is not known but it may be due to either the unspecific supplementation of reduced organic nitrogen or to the supply of some specific amino acid(s) or vitamin(s) (Street, 1969). Improved germination and growth of embryos on BM_1 does indicate that nitrogen should be supplied in the ammonium as well as in the nitrate form.

Although hormones such as KIN, IAA, naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D) have been implicated in embryogenesis in cell and tissue cultures, they are apparently not important in subsequent embryo development, especially not in concentrations exceeding 10^{-2} mg/l.

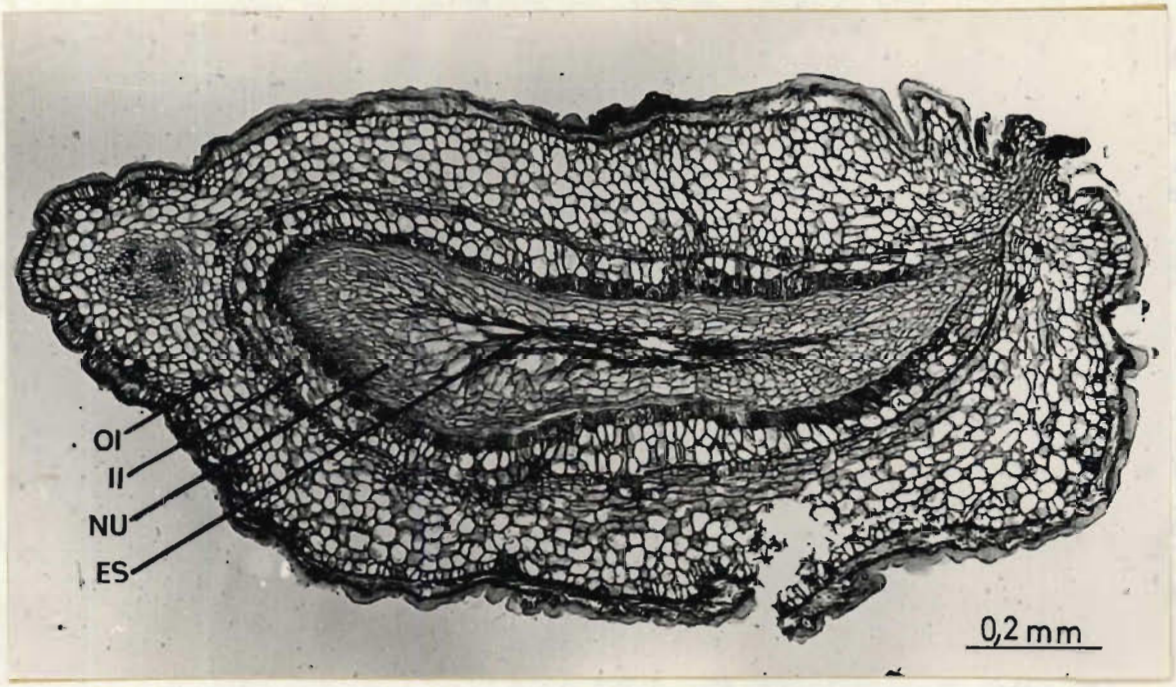

Fig. 3. Median L/S through an abortive Ellendale mandarin ovule about 20 weeks after anthesis, showing the outer (OI) and inner integuments (II), the nucellus (NU), and the degenerate embryo sac (ES).

	Gibberellic acid (GA_3)						
Auxin (IAA)	mg/l	0	0,1	0,5	1,0	5,0	10,0
	0						
	0,001						
	0,01						
	0,1						
	1,0						
	5,0						

	Gibberellic acid (GA_3)						
Cytokinin (Kinetin)	mg/l	0	0,1	0,5	1,0	5,0	10,0
	0						
	0,01						
	0,1						
	1,0						
	5,0						
	10% CM(v/v)						

	Cytokinin (Kinetin)						10% CM
Auxin (IAA)	mg/l	0	0,01	0,1	1,0	5,0	(v/v)
	0						
	0,001						
	0,01						
	0,1						
	1,0						
	5,0						

Fig. 4. Diagramatic layout of the two-dimensional grid experiments used in attempts to stimulate growth of nucellar isolates.

3. THE CULTURE OF NUCELLAR ISOLATES FROM FERTILIZED AND UNFERTILIZED OVULES OF THE ELLENDALE MANDARIN

3.1. *Procedure*

With the commencement of this research in February 1970, a number of open-pollinated fruits were brought to Pietermaritzburg from Nelspruit. Close examination revealed that at this stage - about 20 weeks after anthesis - only small amounts of nucellar tissue remained at the chalazal ends of developing ovules. There were, however, many abortive ovules present (Fig. 3), where the nucellus was still intact although cells of this tissue were highly vacuolated with only small amounts of what appeared to be relatively inactive cytoplasm.

Initially a number of two-dimensional grid experiments (Fig. 4) were carried out in attempts to stimulate growth of the small nucellar fragments removed from the fertilized ovules. When it became evident that these isolates were not amenable to culture, nucellar isolates from abortive ovules were used in a second series of grid experiments because the nucellus in these ovules was larger than that from fertilized ovules. Grids were used as it was felt that such experimental designs would quickly yield information on the effects of various hormones, alone at their different concentrations as well as in numerous combinations. Any growth-promoting or inhibitory effects could indicate interactions. These would then be followed up in smaller, more detailed experiments. Initially, 10 replicates per treatment were used, but in view

of the low incidence of contamination (<2%) and the size of the experiments, five replicates were considered adequate for such pilot experiments.

In addition to grid experiments, various auxins - NAA, indolebutyric acid (IBA) and 2,4-D; cytokinins - benzyl adenine (BA), and 6-dimethylallylamino purine (2-iP), gibberellin - (GA_3); nutritional supplements including ME, YE, CH, adenine (AD), ascorbic acid (AA) and benzylthiazole-2-oxyacetic acid (BTOA), were used at varying concentrations. Apart from the proliferation of small amounts of callus in isolated cases, no growth was observed in any of these cultures. The causes of these failures were probably due to: (1) the very small size of the nucellar isolates from both fertilized and unfertilized ovules, namely ca. 0,3 mm^3; and (2) the fact that these isolates were derived either from the chalazal ends of fertilized ovules - which Rangaswamy (1958a,b) also found non-amenable to culture - or from abortive ovules where nucellar degeneration had apparently proceeded to an irreversible degree. Degeneration was not anticipated in view of Rangan *et. al's* finding that nucellar isolates from ovules of approximately similar age were amenable to culture. Consequently it was decided that in future work only micropylar portions of nucelli from fertilized ovules would be used, and that the excision of such portions as well as entire nucelli from unfertilized ovules would be carried out earlier.

During the blossom period in September, 1970, a large number of *Poncirus* flower buds were picked from trees at the Fruit Research Institute, Nelspruit. Stamens were removed and placed in beakers over silica gel inside a vacuum desiccator. Drying under vacuum was necessary to liberate the pollen which was either used immediately

or refrigerated in the desiccator. Pollen viability was checked on each occasion before use. Some 2 000 Ellendale mandarin flower buds were cross-pollinated after petals and stamens had been removed from balloon-stage, terminal buds. Fruits set as a result of this treatment would contain ovules with nucelli which presumably had received the stimulus believed necessary for inducing nucellar embryos. In addition, plants derived from zygotic and nucellar embryos would be distinguishable. Bagging was not considered necessary in view of the sticky, entomophilous nature of both *Citrus* and *P. trifoliata* pollen (Frost and Soost, 1968), and the observation that insects rarely visit flowers whose petals have been removed (Furr, Carpenter and Hewitt, 1963).

A concentrated water extract of pollen, and a mixture of 5 mg/l 2,4-D plus 15 mg/l gibberellic acid (GA_3) were both applied separately to some 500 pistils in place of actual pollen in an attempt to simulate the apparent hormonal effects of pollination without fertilization. Pistils and adjacent leaves were lightly sprayed with the extract or hormonal mixture after petals and stamens had been removed. The auxin/GA_3 mixture was used because of its ability to increase Navel orange fruit set when sprayed at full-bloom (unpublished results). In order to maximise fruit set, only terminal blossoms were treated and tagged. All other blossoms on the same young branch were removed

to minimise competition (Cameron and Frost, 1968).

It was hoped that fruits set using these sprays would contain only unfertilized ovules from which nucellar isolates amenable to culture, could be obtained over a longer period than fertilized ovules with fast-developing endosperm and embryos. Tagged fruits were harvested eight weeks after anthesis and kept refrigerated at 6°C until used. Twenty-five nucellar isolates from normal ovules of cross-pollinated fruits and the same number from unfertilized ovules of hormone treated fruits were transplanted individually in tubes on the culture media listed in Table 1.

TABLE 1: Culture media for nucellar isolates from ca. eight-week-old normal and abortive Ellendale mandarin ovules and abortive Navel orange ovules.

Treatment Number	Composition of Medium	Treatment Number	Composition of Medium
1	BM_1	11	BM_1 + CM + AA
2	BM_1 + CM (10% v/v)	12	BM_1 + CM + CH
3	BM_1 + GA_3 (0,2 mg/1)	13	BM_1 + CM + ME
4	BM_1 + KIN (0,2 mg/1)	14	BM_1 + CM + GA_3 + AD
5	BM_1 + AD (40 mg/1)	15	BM_1 + CM + AD + CH
6	BM_1 + AA (40 mg/1)	16	BM_1 + CM + AA + CH
7	BM_1 + CH (400 mg/1)	17	BM_1 + CM + AD + AA + CH
8	BM_1 + ME (400 mg/1)	18	BM_1 + CM + GA_3 + AD + CH
9	BM_1 + CM + GA_3	19	BM_1 + CM + GA_3 + AD + AA + CH
10	BM_1 + CM + AD	20	BM_1 + CM + GA_3 + AD + AA + CH + ME

On the basis of observations during the first three weeks of culturing nucellar isolates on the media listed in Table 1, a further series of media (Table 2) were constituted. Benzothiazole-2-oxyacetic acid (BTOA) was included since Steward *et al.* (1969) found this substance effective in inducing cell division in a number of tissues. In view of the controversy in the literature concerning the effects of NO_3^- and NH_4^+-nitrogen on embryogenesis and subsequent embryo development, both BM_1 and BM_2 were used, thus doubling the number of treatments indicated in Table 2 for each tissue source.

TABLE 2: Culture media for nucellar isolates from 12 to 15-week-old normal and abortive Ellendale mandarin ovules and abortive Navel orange ovules.

Treatment Number	Composition of Medium	Treatment Number	Composition of Medium
1	BM + CM (15%)	11	BM + CM + AS* + AA
2	BM + AD (40 mg/l)	12	BM + CM + AS* + ME
3	BM + AA (40 mg/l)	13	BM + CM + AA + ME
4	BM + ME (400 mg/l)	14	BM + AS* + AA + ME
5	BM + CM + AD	15	BM + CM + AS* + AA + ME
6	BM + CM + AA	16	BM + BTOA (0,05 mg/l)
7	BM + CM + ME	17	BM + BTOA (0,5 mg/l)
8	BM + AS* + AA	18	BM + BTOA (5,0 mg/l)
9	BM + AS* + ME	19	BM + CM + BTOA (0,5 mg/l)
10	BM + AA + ME	20	BM + CM + AA + BTOA (0,5 mg/l)

AS* due to the temporary unavailability of adenine, adenine sulphate was used to supply the equivalent amount of adenine.

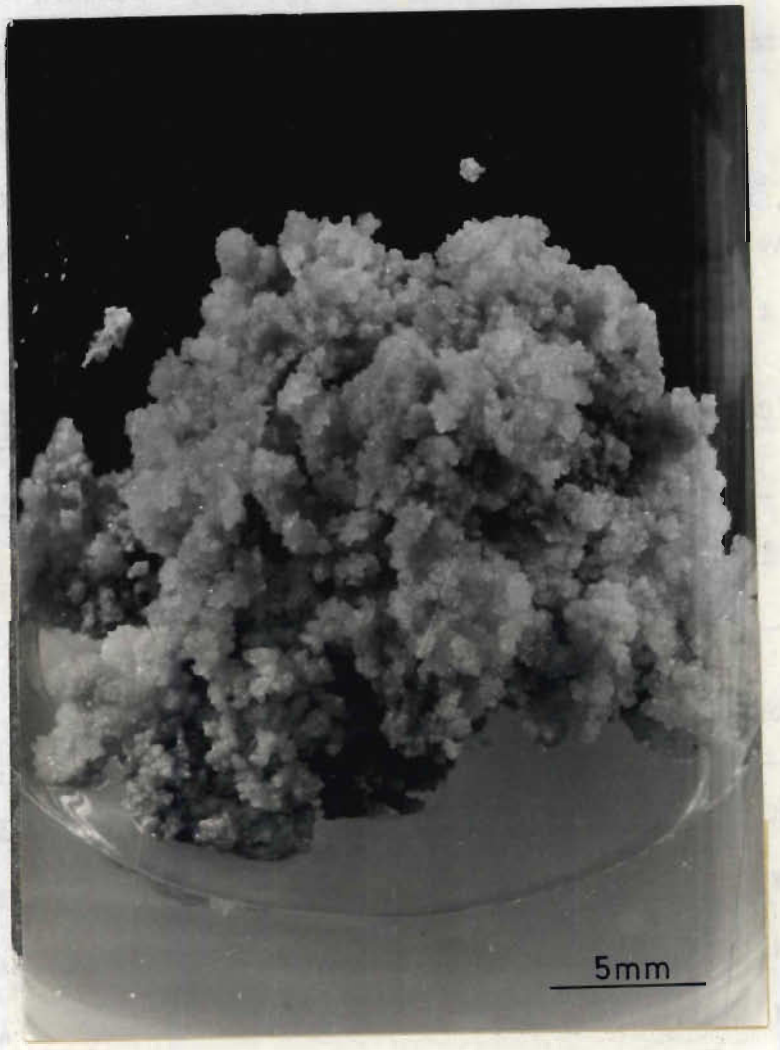

Fig. 5. Callus growth developed from a nucellar isolate of a fertilized Ellendale mandarin ovule after four months in culture.

3.2 *Results and discussion*

3.2.1. The culture of nucellar isolates

Some 10 per cent of the nucellar isolates from eight-week-old ovules formed callus when placed on media (Table 1) supplemented with CM alone or together with ME, AD or CH. Isolates on all the other supplemented media remained alive but dormant for four months, while those on BM_1 only, turned brown within two weeks. No nucellar isolates differentiated into either pseudobulbils or embryoids.

Nucellar isolates placed on the media listed in Table 2 were from fruits harvested on two occasions: mid-December, that is ca. 12 weeks after anthesis, and three weeks later. Unfortunately, due to the vast amount of work involved in dissecting out nucellar isolates from mandarin and orange fruits (since this work was carried out concurrently), this series of experiments had to be carried out over a period of three weeks. Thus some fruits were refrigerated for longer periods while others were harvested three weeks after the first batch.

Nucellar isolates were never as large as those depicted by Rangan *et al.* (1968, Fig. 2). By mid-December, ca. 12 weeks after anthesis, the nucellus appeared to be almost non-existent towards the micropylar end, while in ovules from fruits harvested three weeks later the nucellus was present only towards the chalazal end.

No differentiation occurred in any of these mandarin nucellar cultures although varying amounts of cream- to brown-coloured callus (Fig. 5) did develop in isolated cases after four

to eight weeks of culture on media supplemented with CM, AD, and ME ascorbic acid and BTOA did not enhance growth in any visible way.

The failure of this series of treatments was probably due to the fact that the fertilized ovules had developed too far, while abortive ones had degenerated. The fact that Rangan *et al.* (1968) were able to remove and culture relatively large pieces of nucellar tissue from ovules of 100- to 120-day-old fruits in California, while the nucellus in Ellendale mandarin ovules of comparable age, grown in South Africa had by then been largely consumed, is probably the result of differences in climate and cultivars. However, it is difficult to explain why none of the nucelli, especially those from fertilized ovules of eight-week-old fruits which were placed on media supplemented with ME and/or AD, showed any noteworthy growth.

It is intended to repeat this work using nucellar isolates excised from fertilized and unfertilized ovules of younger Ellendale mandarin fruits since it appears that even eight-week-old ovules were too advanced. This is strange in the light of Rangan *et al.'s* success with 20-week-old ovules. Weekly or fortnightly harvesting for 12 weeks from anthesis is visualized. The number of supplements used will be decreased and will include a number of levels of ME, AD and AS which have been effective in inducing embryogenesis in the unfertilized ovules of the Washington Navel. By increasing the number of replicates over fewer treatments, more reliable results should be obtained.

3.2.2. Effects of cross-pollination and sprays on fruit-set, fruit size, and seediness

These results are presented in Table 3.

TABLE 3: Effects of pollination and certain sprays on fruit-set, fruit size, and seediness of Ellendale mandarins

Treatment	Approximate fruit set (%)	Mean values from 25, ten-month-old fruits from Pietermaritzburg		
		Diameter (mm)	Normal seeds/fruit	Shrivelled seeds/fruit
Open pollinated*	<2	63,2	3,7	2,7
Cross-pollinated	30	67,8	8,0	6,5
2,4-D + GA_3 Spray	12	64,4	0	0
Pollen extract spray	0,01	Insufficient data available		

*Without removal of competing flowers on the same young twig.

Approximately 30 per cent of the cross-pollinated flowers set fruit, compared with less than two per cent of those which were open-pollinated. This great increase in fruit set was probably due indirectly to the combined effects of ample pollen and the removal of competition from all other flowers on the same twig of new growth. The 2,4-D + GA_3 spray increased fruit set, but not to the same extent as cross-pollination while the pollen extract spray decreased fruit set.

Cross-pollination and the 2,4-D + GA_3 spray treatments resulted in larger fruits than untreated ones. This was undoubtedly due to the elimination of nearby competition for available nutrients,

enhanced possibly by an increased magnitude of the sink (Crane, 1964) as a result of an accelerated metabolism stimulated by hormonal activity.

Cross-pollination markedly increased the seediness of Ellendale mandarin fruits. Similar results have been reported for cultivars such as the Shamouti orange and Kara mandarin (Frost and Soost, 1968) and may be due to low viability of pollen from the maternal variety. The occurrence of more shrivelled seeds (fertilized but not fully-developed) in cross-pollinated fruits is noteworthy. Their abortion at a relatively late stage of development may well have been the result of competition for available nutrients.

The expected, complete absence of seeds in fruit set by the 2,4-D + GA_3 spray did provide ovules from which nucelli could be removed over a longer period of time than in the case of fertilized ones. These unfertilized ovules remained small (1-2 mm long) and difficult to work with. The thickness of the outer integument almost doubled as the fruit enlarged, while nucellar cells became increasingly vacuolated and later degenerated.

4. CULTURE OF NUCELLAR ISOLATES FROM UNFERTILISED OVULES OF THE WASHINGTON NAVEL ORANGE

4.1 *Procedure*

In view of the potential advantages of using nucellar isolates from unfertilized ovules for rejuvenating monoembryonic citrus clones, it was decided to investigate the response of similar isolates derived from a polyembryonic clone. The Washington Navel was selected, as fruit of this clone were readily available and the chances of pollination or fertilization having taken place were highly unlikely since no pollen is produced by this clone and degeneration of the megasporocyte occurs frequently (Frost and Soost, 1968). The chances of cross pollination were remote because of the absence of pollen-producing *Citrus* species in the vicinity and the fact that most ovules used were from Navel fruits which had set out-of-season. A number of ovules were sectioned to ascertain whether or not any zygotic or nucellar embryos existed in the ovules used in the experiments.

4.1.1. Initiation and development of nucellar embryoids

Twenty-five nucellar isolates from ovules of ca. 8 to 12-week-old fruits were placed individually in culture tubes on each of the media given in Tables 1 and 2.

Based on results from the previous work a small, detailed experiment was laid out to test the efficacy of three levels of AD and AS in inducing adventive embryogenesis. Murashige and Skoog's basal medium was supplemented with AD at 10, 20 and 30 mg/l. Adenine sulphate was supplemented at rates calculated to contain similar concentrations of adenine. Four out-of-season fruits with diameters of 25 to 35 mm were used to supply the 25 nucellar isolates for each treatment.

Fig. 6. Embryoids (EM) which in most cases appeared to develop from pseudobulbils (PB).

Fig. 7. Two embryoids after subdivision from an embryoid mass such as in Fig. 6.

Nitsch *et al's* (1967) work on the role of adenine in bud differentiation in which the importance of adenine *per se* and the synergistic interaction between adenine and cytokinins, especially zeatin (ZE) is stressed, suggested that zeatin might possibly enhance the effect of AD in differentiating embryoids in nucellar cultures. In view of this, the efficacy of AD (40 mg/l), ZE (0,2 mg/l) and AD + ZE on embryoid induction was compared with that of ME (400 mg/l). Out-of-season fruits ranging from 30 to 50 mm in diameter were used to obtain the 25 nucellar isolates per treatment. Equal numbers of nucellar isolates from each fruit were allocated to each treatment to eliminate the effect of fruit size.

4.1.2. Differentiation of embryoids into plantlets

The embryoid masses such as shown in Fig. 6 were sub-divided as far as possible into single embryoids (Fig. 7), as many as 10 being obtained from a single original nucellar isolate. Individual embryoids were aseptically transferred to a second set of media. Embryoids derived from each original culture were distributed equally to all treatments of the second set of media so as to minimize any carryover of tissue or media effects. This second set of media consisted of four treatments: (1) BM_1 only (control); (2) BM_1 + 0,1 mg/l IAA (presumably to initiate roots); (3) BM_1 + 0,1 mg/l KIN (presumably to initiate shoots) and (4) BM_1 + 0,1 mg/l GA_3 (presumably to enhance germination or general growth).

4.1.3. Conditioning of plantlets

Plantlets were transferred to small pots containing soil, vermiculite or sand, either directly from BM_1 + GA_3, or

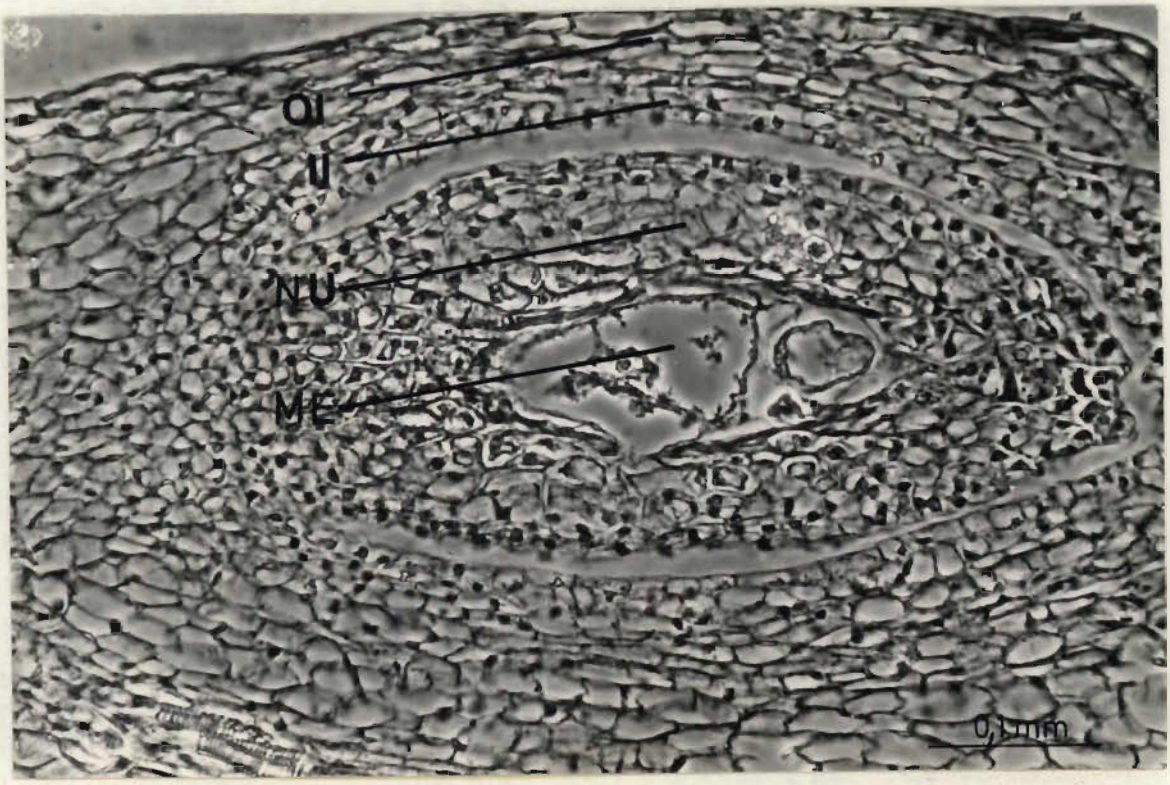

Fig. 8. Approximately median L/S through an unfertilized ovule excised eight weeks after anthesis showing the outer (OI) and inner (II) integuments, nucellus (NU), and degenerate megasporocyte (ME). Section stained with chlorazol Black-E, and photographed under phase contrast.

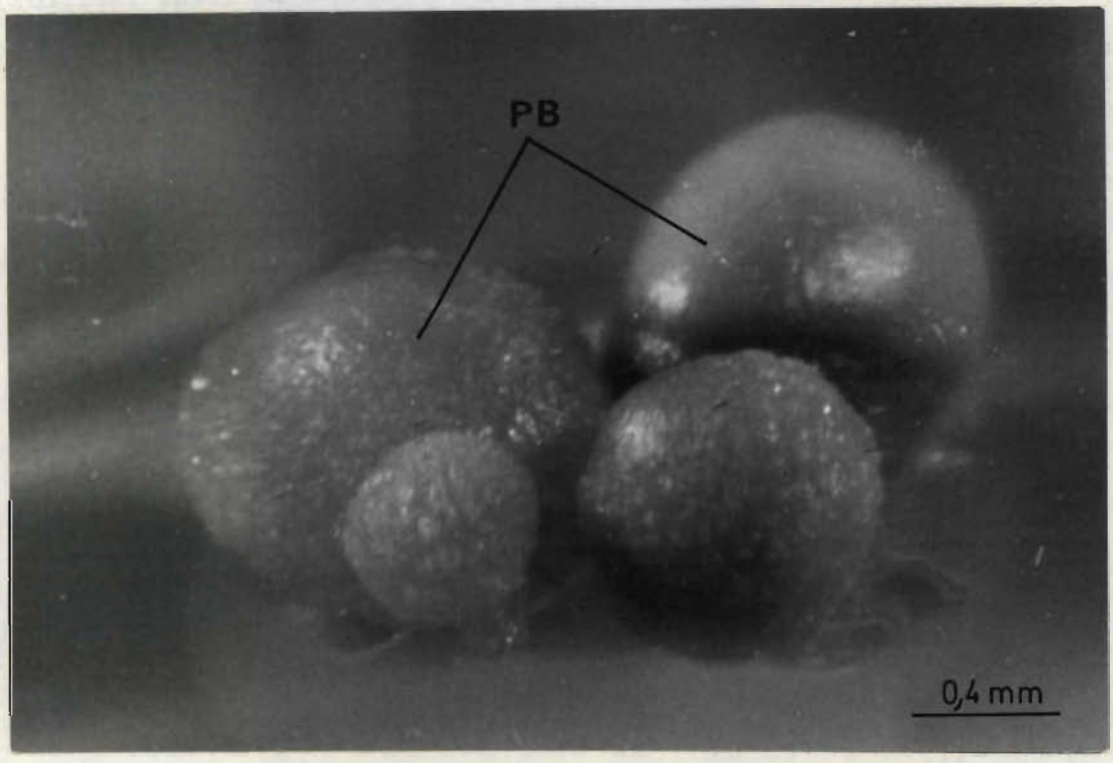

Fig. 9. Pseudobulbils (PB) which were the first visible signs of growth in cultured nucellar isolates from unfertilized ovules of a Washington Navel orange.

after they had been grown for a month in a medium containing only the inorganic constituents of BM_1 gelled with 2% agar. Pots were enclosed to varying degrees with polythene bags to maintain high relative humidity. The soil was moistened with tap water while the vermiculite and sand was regularly moistened with a solution of the inorganic constituents of BM_1. Hydroponic culture in Hoagland's solution was also attempted. A growth cabinet was used for some of this work to allow careful control of temperature, light and relative humidity.

4.2. *Results and discussion*

Although flowers were not bagged, the chances of cross-pollination and fertilization having taken place are considered to be negligible for the following reasons: (1) There is little overlap in the flowering period of Navel and Valencia types as the former tend to flower somewhat earlier than the Valencia types. (2) Wind-pollination is unlikely in view of the sticky texture of citrus pollen. (3) There were no pollen donors in the vicinity of the Navel orange tree used in these experiments. (4) Most of the experiments were carried out using ovules from fruits set out of season when the chances of cross-pollination would be even more remote. (5) Megasporocyte degeneration occurs frequently in the Navel orange. (6) Microscopic examination of 50 ovules revealed no signs of either zygotic or adventive embryo development (Fig. 8). Self-pollination can be ruled out since this cultivar produces no pollen.

4.2.1. Initiation and development of nucellar embryoids

The first visible signs of growth in these nucellar cultures were the appearance of pseudobulbils (Fig. 9) after about four weeks in culture. These originated from proembryo-like cell aggregates in the nucellar isolates (Fig. 10). Unlike Rangaswamy's (1961) nucellar cultures of *Citrus microcarpa*, these pseudobulbils arose

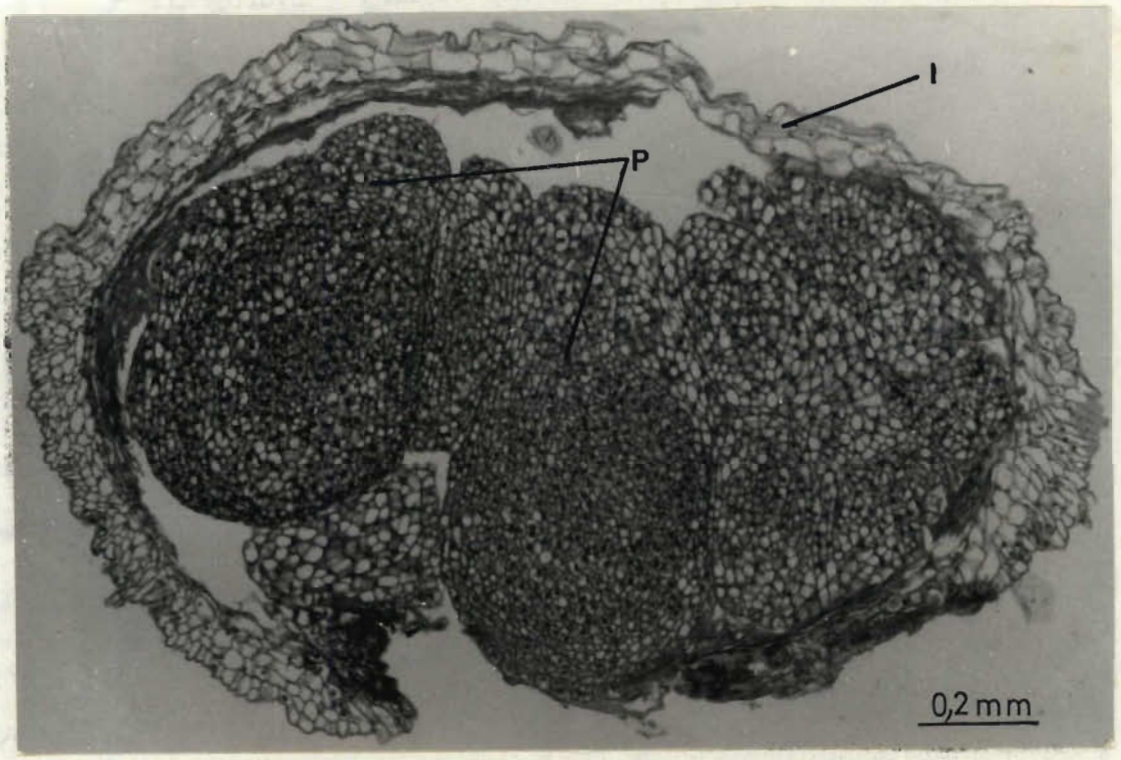

Fig. 10. Section through a nucellar isolate after two weeks in culture showing proembryo-like structures (P) within a degenerating integument (I).

directly from the nucellus without the prior appearance of callus. From one to 15 embryoids developed from the pseudobulbils of each isolate (Fig. 6). These embryoids appeared to originate as outgrowths from the pseudobulbils, although in isolated cases embryoids did appear to develop directly from the nucellar isolate. The latter form of embryoid ontogeny was observed by Rangan *et al.* (1968) in their nucellar cultures where neither callus nor pseudobulbils were formed.

No growth was observed on either of the two basal media alone nor when supplemented with only AA or BTOA, however when on BM + AD or BM + AS or BM + ME, growth was rapid with numerous embryoids developing from the pseudobulbils. Basal medium + CM and BM + KIN were not as effective, but when used in combination with AD, AS and/or ME, they did increase the incidence of pseudobulbil and embryoid formation. Basal $medium_1$ + AD + ME yielded the best results by not only initiating the largest number of pseudobulbils but also by producing most embryoids per nucellar isolate. From these treatments it appeared that AD was more effective in initiating pseudobulbils than AS and that BM_1 was generally superior to BM_2.

The effects of three levels of AD and AS on the induction of pseudobulbils and embryoids is presented in Table 4.

TABLE 4: Efficacy of three concentrations of adenine and their equivalents supplied as adenine sulphate in inducing embryoids in nucelli from abortive Navel orange ovules

Treatment	Percentage six-week-old cultures with embryoids
BM_1 + 10 mg/l AD	75
BM_1 + 27 mg/l AS	75
BM_1 + 20 mg/l AD	60
BM_1 + 54 mg/l AS	50
BM_1 + 30 mg/l AD	43
BM_1 + 81 mg/l AS	17

From this Table it appears that the lowest concentrations of AD and AS were equally effective at inducing embryoids. With increasing levels the efficacy of AS dropped considerably faster than that of AD. These results indicate that the lower concentrations of both forms of adenine were the most effective and that with increasing the concentrations, the frequency of embryoid induction decreased concomitantly. This, of course, is at variance with Rangaswamy's (1961) finding that 40 mg/l of AS gave a more pronounced response than did 20 mg/l.

It is clear that ME was more effective at inducing pseudobulbils and embryoids than AD, ZE or AD + ZE (Table 5).

TABLE 5: Effects of malt extract, adenine, zeatin and adenine + zeatin on inducing pseudobulbils and embryoids after 6 weeks in culture

Treatment	Percentage of cultures with pseudobulbils	embryoids
BM_1 + ME (400 mg/1)	28	44
BM_1 + AD (40 mg/1)	4	26
BM_1 + ZE (0,2 mg/1)	29	21
BM_1 + AD + ZE	4	30

Zeatin alone was more effective than anticipated, especially in inducing pseudobulbils, which almost invariably give rise to embryoids. Adenine was not as effective as in other experiments and there appeared to be no synergistic effect between AD and ZE. From the results presented in Table 4, it is likely that the concentration of AD (40 mg/1) was excessively high and may have masked any interaction between

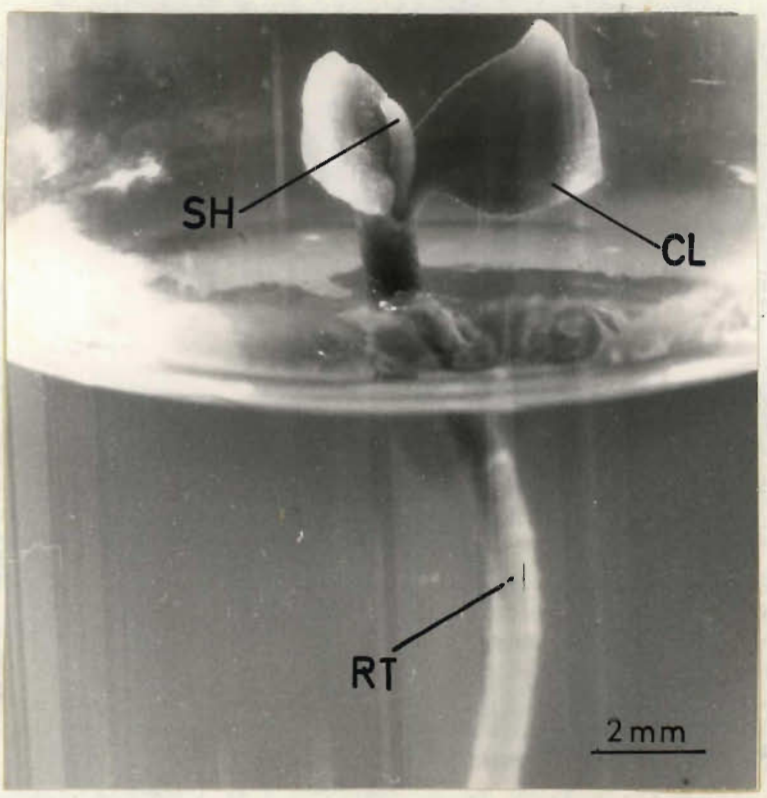

Fig. 11. Germinating embryoid two weeks after transfer to BM_1 + GA_3 (1 mg/l) showing the cotyledonary leaves (CL), root (RT) and shoot (SH).

Fig. 12. An example of an off-type plantlet.

AD and ZE.

Although numerous embryoids had developed in response to various treatments, none showed any signs of differentiation into plantlets even after 12 weeks in culture. It was obvious that some different form of stimulation was required for their differentiation.

4.2.2. Differentiation of embryoids into plantlets

Within two weeks of transferring to the second set of media, 62 per cent of the embryoids on BM_1 + GA_3 had formed roots (Fig. 11). This was somewhat surprising but a similar response to gibberellic acid was noted by Rangaswamy (1961) in his nucellar cultures. The response may be due to the ability of GA_3 to promote normal embryo germination where the radicle appears first, and not due to a direct stimulation of root formation.

The first true leaves emerged about three weeks after the radicle. In most cases these were typical citrus leaves but some off-type plantlets were also observed (Fig. 12).

Although embryoids placed on the other three media did occasionally develop roots and subsequently even whole plants, the medium supplemented with 1 mg/l GA_3 was undoubtedly the most effective. Single embryoids invariably differentiated quicker and more readily than did aggregates of more than one embryoid.

Experience showed that plantlets had to be removed from the GA_3 medium immediately after the first true leaves appeared in order to avoid the development of weak, spindly plants (Fig. 13). They were transferred to the third medium consisting of only the inorganic salts of BM_1 . Doubling the concentration of chelated

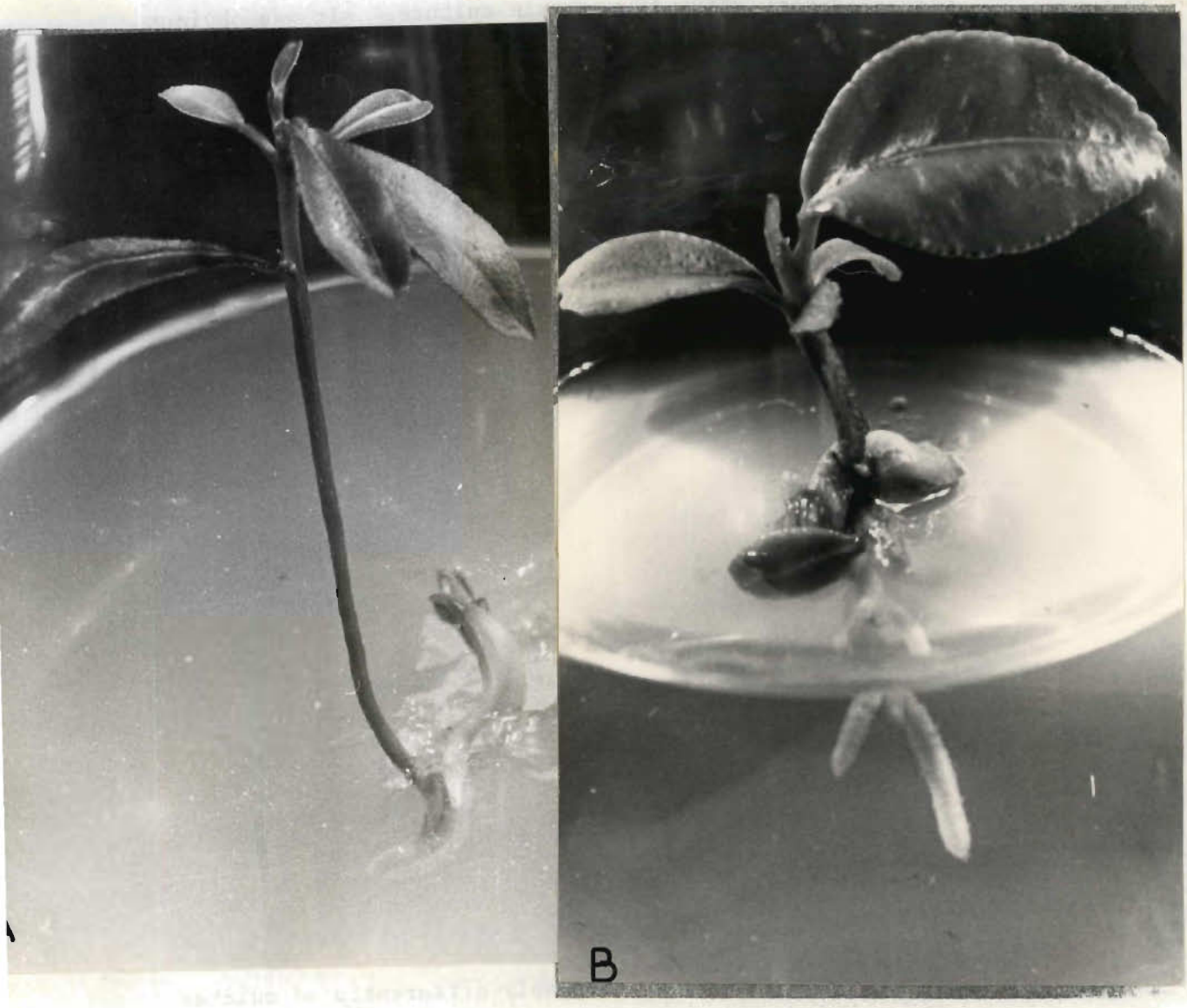

Fig. 13. The development of a weak, spindly plant (A) when left too long in the GA-supplemented medium compared with a more vigorous plant (B) which was transferred to a medium containing only the inorganic salts of BM_1 as soon as the first leaves developed.

iron in this medium prevented the appearance of iron-deficiency symptoms noted when the normal amount was applied.

4.2.3. Conditioning of plantlets

These plantlets grown under very humid, sterile conditions, on extremely rich media, required careful conditioning to enable them to grow in soil. This task was made more difficult by the absence of root hairs which are so important in the absorption of water and nutrients.

Plantlets transferred directly to pots of soil, wilted and died within 2 to 14 days. When pot and plant were entirely enclosed in a ploythene bag, the humidity remained high but fungi soon attacked and killed the plants within 14 days. Partial covering did prolong the life of plants by about two weeks but none grew successfully. Similar results were obtained when plants were transferred to vermiculite or sand which was regularly moistened with a solution of the inorganic constituents of BM_1. Attempts at growing plantlets in hydroponic culture also failed, primarily because of the inadequate root system and the inability of leaves to adapt to the low atmospheric humidity.

The following technique did prove successful: once plantlets became established in flasks containing the inorganic medium only, they were placed in a growth cabinet under high light intensity (4 400 lux) at 28°C for four days. The cotton bungs were then removed from the flasks, the relative humidity maintained at 85 per cent, and the light intensity reduced by half. The plants were held under these conditions for two days before sub-

jecting them again to 4 400 lux. The relative humidity was then slowly reduced to 45 per cent over the course of a week. Plants were removed from the flasks with as much of the remaining agar as possible, planted in pots of sandy soil, and watered well. Pots were again placed under the lower light intensity for four days before transferring them to the greenhouse (Fig. 14).

Fig. 14. A young Navel orange plant, originally from the nucellus of an unfertilized ovule, now thriving in soil.

isolates from both fertilized and unfertilized ovules of the Ellendale mandarin failed on the medium used by Rangan *et al.* supplemented with malt extract. Numerous other supplements, alone and in various combinations, also failed to result in embryogenesis. This complete failure to induce adventive embryos in the Ellendale mandarin nucellar isolates is difficult to explain after considering Rangan *et al.'s* success. It is possible that the development of fertilized ovules had progressed too far, while degeneration had proceeded to an irreversible degree in the unfertilized ovules.

Whereas adventive embryos have been successfully induced in nucellar isolates of fertilized ovules, this appears to be the first report on attempts to culture nucellar isolates from the numerous abortive (unfertilized) ovules found in all citrus fruits. The practical importance of inducing nucellar embryogenesis in such isolates, especially of monoembryonic and seedless varieties, is that the parentage of any plantlet developing is indisputable, since no zygotic embryo exists, and hence the necessity of hand pollination with marker pollen is eliminated. In addition, the apparently critical period during which nucelli can be excised and cultured may be extended by the use of unfertilized ovules. Furthermore, there are a number of seedless citrus cultivars, both monoembryonic and polyembryonic which could be rejuvenated by culturing abortive ovules.

More basically, such work could help establish the nature of the stimulus which apparently occurs with pollination and fertilization and which is believed to be essential for the induction of nucellar embryos. From this study it appears that either the nucelli of Washington Navel ovules do not require the 'stimulation' afforded by

pollination and/or fertilization in order to produce adventive embryos, or that malt extract, adenine and zeatin can substitute for pollination and fertilization. However, this does not appear to apply to ovules of the Ellendale mandarin. The different responses shown by these two cultivars may be related to the ability of the Navel orange to set fruit parthenocarpically whereas fertilization is probably essential for the setting of Ellendale mandarin fruits under natural conditions. Whether pollination and fertilization can be replaced by chemicals such as adenine and zeatin in other parthenocarpic citrus cultivars remains to be seen.

The possibility of extending the technique of using nucellar isolates from abortive ovules, to other citrus varieties, especially monoembryonic and seedless varieties, is particularly attractive as a practical means of freeing proven clones from viral diseases and rejuvenating them.

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PART II

EMBRYO CALLUS AND FREE-CELL CULTURE OF

WELWITSCHIA MIRABILIS

INTRODUCTION

It is generally accepted that Haberlandt in 1902 was the first to visualize the innate totipotency of somatic plants cells, but it was not until more than 60 years later that Steward and co-workers (Steward, Mapes and Ammirato, 1969; and references cited) succeeded in differentiating a carrot plant from free cells suspended in a nutrient medium. Until this event, much speculation existed as to what distinguished the zygote from any other somatic cell. In the zygotic nucleus, formed by the fusion of gametic nuclei, the normal diploid chromosome complement is reconstituted; but apart from the recombination of genes, this cell is intrinsically similar to any other somatic cell. Wardlaw (1965) emphasise the involvement of genetic factors, while Stewart *et al.* (1969) consider that it is the surrounding medium and environment which induces zygote development *in vivo*. In fact the differentiation of a zygote and of isolated somatic cells is undoubtedly due to the interaction of "intrinsic" and "extrinsic" factors. It is accepted that the younger the embryo (or embryoid), the greater the degree of heterotrophy. For this reason it is not surprising that Steward *et al.*

viewed their success at supplying the necessary nutritional and environmental requirements as somewhat fortuitous. It is also not surprising that although free cells of many plant tissues have been effectively proliferated in culture, those of relatively few have been successfully differentiated into plantlets. Most of the work on differentiation in callus and cell suspensions has involved dicotyledonous plants while very little success has been achieved using monocotyledons, especially woody ones. Fridborg (1971) considers this to be due to the difficulty of obtaining satisfactory callus from monocotyledonous plants in general.

After many years of research, Steward and co-workers formulated the general conditions which result in the realization of totipotency of carrot cells. They maintain that the cells must be grown (a) free, and (b) in or on a suitable medium under environmental conditions conducive to morphogenesis. This may require more than one step, such as high auxin and coconut milk to induce active growth, followed by a medium supplemented with coconut milk but without auxin to induce embryogenesis. Work on other plant species and other varieties of carrot have supported the theory that cells must first be grown free before they can exhibit totipotency.

Vasil and Hilderbrandt (1966b) point out that although differentiation of plants such as the carrot from single cells follows the course of natural embryogenesis closely, other plants such as *Petroselinum hortense* first form round masses of tissue

before roots and shoots are differentiated. Thus it appears that isolated cells may give rise directly to embryoid structures with morphological póles or they may first produce spherical cell masses before morphological poles are established. The most variable factors required to induce inherent totipotency are the composition of the media and the sequences in which the components of the media are used. Steward and co-workers concluded from their work on carrot cells, that coconut milk contained certain embryogenic factors which they were unable to substitute with chemically-defined substances. These properties of coconut milk have been disputed (Halperin and Wetherell, 1964; Vasil and Hilderbrandt, 1966a). Halperin and Wetherell (1965) consider totipotency to be dictated predominantly from within the cell and attach relatively little importance to the composition of the external medium. Since then, ammonium nitrogen (Halperin and Wetherell, 1965), ammonium and nitrate nitrogen (Reinert, 1967), and other unknown substances from conditioned media (Kato and Takeuchi, 1963) have been implicated in embryogenesis of isolated cell cultures.

It has been found that isolated cells maintained for a long time in an undifferentiated state were less likely to exhibit totipotency than similar cells transferred to the embryogenic medium shortly after separation from the callus mass (Syono, 1965; Nickell and Maretzki, 1969; Steward *et al.*, 1969). These results are disputed by Reinert (1959) who observed no deterioration in the ability of carrot cells to form embryoids after being

cultured for a year in an undifferentiated state. The decline in totipotency of cells cultured free for a long time, as observed by some workers, is probably the result of either increased chromosomal aberrations (since generally only normal diploid cells appear totipotent) or some physiological changes which take place (Nag and Johri, 1969; Thomas and Street, 1970).

Little appears to be known about the quantitative and qualitative effects of environmental factors such as the intensity and duration of light and concentrations of oxygen and carbon dioxide on morphogenesis of single cells.

Relationships between cell ultrastructure and morphogenesis are far from conclusively resolved despite active research in this field (Israel and Steward, 1966; Sutton-Jones and Street, 1968; Tulett, Bagshaw and Yeoman, 1969; Davey and Street, 1971). From these investigations it appears that quiescent cells are generally characterised by large vacuoles, a very thin layer of parietal cytoplasm containing few small spheroid mitochondria with swollen cristae, few indistinct dictyosomes, few large plastids with little internal structure, and nuclei of varying sizes. As cultured quiescent cells become active, they start to divide. Many of the large cells divide internally into three, four, or more daughter cells, each surrounded by its own cell wall inside the wall of the original cell. Daughter cells are released when the wall of the mother cell is ruptured. Further divisions tend to be normal. Active cells generally contain a large proportion of relatively dense cytoplasm, interupted by smallish vacuoles which are traversed by

thick strands of actively-streaming cytoplasm. The cytoplasm contains numerous mitochondria of various sizes with well-developed cristae. Some mitochondria are abnormally long. Occasional ribosomes as well as numerous dictyosomes with well-defined vesicles appear. Many plastids contain starch grains or develop lamellae. Nuclei are generally large in relation to cell size.

Good fixation and embedding appears to be a general problem encountered when working with cultured cells. Fixing and especially embedding over a long period of time seems to be essential.

Little success has been achieved in efforts to explain why some species or even varieties of a single species readily exhibit totipotency while others are extremely stubborn. Dicotyledonous plants, especially herbaceous ones, are generally more easily differentiated from isolated cells than are monocotyledons. The intrinsic ability of the former class to regenrate cambial cells has been put forward to account for this difference (Steward, Ammirato and Mapes, 1970). These authors also suggest that plants which rely heavily on embryo suspensors during their early nutrition *in vivo* are probably difficult to derive from single cells since steps in the development of such plants are essentially the same as in the case of a normal zygote. The suspensor is believed to modify the supplied nutrients in some subtle way, rendering them suitable for the developing zygote.

Free-growing cells are generally obtained from callus masses derived from different plant parts such as roots of carrot (Steward *et al.*, 1969) and *Atropa* (Thomas and Street, 1970), stems of asparagus (Steward and Mapes, 1971), embryos (Steward *et al.* 1969;

1970), anthers (Guha and Maheshwari, 1966; Nitsch and Nitsch, 1969) and megagametophytes (Norstog and Rhamstine, 1967). Callus growth is generally stimulated by supplementing the basal medium with an auxin at 5-10 mg/l alone or in combination with coconut milk. Steward *et al.* (1969) are convinced that free-cell cultures established from callus derived from immature embryos are more likely to exhibit totipotency than those derived from mature embryos because of the lesser degree of organization in the former and the possible presence of totipotency-inhibiting factors in the latter.

Once sufficient callus is formed, portions are subcultured in liquid media. Pectinases to digest middle-lamellae, or low-calcium concentrations to avoid formation of rigid intercellular cementing materials have been used to obtain free cells. Steward and co-workers have reported best results from placing actively-growing callus pieces in special flasks which are rotated on a wheel at 1 rpm. These flasks contain a small amount of the liquid nutrient medium and are constructed to allow optimum aeration of the tissue. Horizontal or rotary shaking, especially if violent, results in the growth of filamentous structures and much cell debris (Blakely and Steward, 1964). Rajasekhar, Edwards, Wilson and Street (1971) report that the rate of orbital shaking markedly affected cell proliferation in suspension cultures of *Acer pseudo-platanus* but they made no mention of its effect on cell shapes and debris. Nishi and Sugano (1970) found that auxin species also influenced cell shape in suspension cultures of carrot root phloem.

They found that 1 mg/l of 2,4-D promoted two-dimensional growth with the formation of cell tetrads and spherical single cells with a mean diameter of 29 μm, while IAA at the same concentration encouraged the growth of filamentous structures and elongated single cells up to 200 μm in length.

When sufficient free cells and cell aggregates have formed, they are transferred to agarified or liquid media which contain little or no auxin, but various concentrations of coconut milk or cytokinins. High auxin concentrations (>1 mg/l) invariably prevent organogenesis.

During the course of experiments, Steward and co-workers, and many others, have reported morphological features of free cells in suspension culture which are associated with incipient totipotency. Generally such suspensions comprise more or less isodiametric cells of varying sizes, some filamentous cells, pseudotracheids, groups of small cells, and large cells which often develop papillae and divide by budding as do yeast cells.

Differentiation of callus and suspended cells of dicotyledonous plants, especially from non-woody genera, has been achieved in numerous cases (Fridborg, 1971). However, only a limited number of plants have been successfully differentiated from similar cultures derived from monocotyledonous plants (Ziv, Halevy, and Shilo, 1970, and references cited; Fridborg, 1971).

Virtually all successfully cultured monocotyledonous tissue has been on the basal media of Murashige and Skoog (1962) or Linsmaier and Skoog (1965). These media are very similar but differ from other basal media in

that they are much higher in their mineral salt content, particularly nitrogenous and potassium salts. Basal media are generally supplemented by a large number of vitamins, inositol, and sucrose. Auxin is usually supplied as either NAA or 2,4-D. In some cases e.g. Ball (1950), and Sheridan (1968), IAA was used.

Very little work appears to have been done on culturing Gymnospermous tissues and cells. Ball (1950) cultured *Sequoia* callus derived from green shoots and placed on a half-strength Knop's medium supplemented with IAA.

A few attempts have been made at culturing *Pinus* tissues from seedling hypocotyls and root segments using various basal media including those of Heller, Knop, and Murashige and Skoog (Loewenberg and Skoog, 1952; Barnes and Naylor, 1958; Brown and Lawrence, 1968). Numerous supplements were incorporated into the media by these workers with inconsistent results. Auxin was supplied in the form of NAA and 2,4-D and Brown and Lawrence (1968) included KIN as well. On a Murashige and Skoog basal medium supplemented with niacin, pyridoxin, thiamin, 2,4-D, KIN, asparagine, inositol, and sucrose, Brown and Lawrence (1968) succeeded in growing callus of other genera of Gymnosperms including *Cunninghamia*, *Cupressus*, *Araucaria*, *Cycas*, *Thuja*, *Larix*, and *Picea*. *Cycas* tissue was also successfully cultured on a modified Linsmaier and Skoog (1965) basal medium by Norstog and Rhamstine (1967) who found White's medium to be unsuitable for this tissue.

Of all the Gymnospermous tissues cultured, most work

Fig. 15. A female *Welwitschia mirabilis* plant growing in the Namib Desert. Note the unbranched, woody stem (S), and the single pair of strap-like leaves (L).

appears to have been carried out on normal and tumerous tissue of *Picea* (Reinert, 1956; Reinert and White, 1956; De Torok and Thimann, 1961; Steinhardt, Standifer, and Skoog, 1961; Risser and White, 1964; White and Risser, 1964; White and Gilbey, 1966; White, 1967). The majority of these workers used modifications of Linsmaier and Skoog's basal medium which appears to be more suitable for Spruce tissue cultures than does White's medium. Ammonium-nitrogen which is present in Linsmaier and Skoog's medium but not in White's medium, appears to be necessary and can replace amino- nitrogen supplementation to some extent (White and Gilbey, 1966). Argenine, asparagine, cystine, and glutamine all appear to be important sources of amino-nitrogen in Spruce callus cultures. Vitamin B_{12}, thiamin, biotin, niacin, ascorbic acid and pantothenic acid have all been reported to enhance callus growth. Auxin in the form of 2,4-D is generally used and is more effective than IAA (Risser and White, 1964). Kinetin has been incorporated in media in some cases but has been shown to retard callus growth even at low concentrations of 0,01 mg/l (Risser and White, 1964). Sucrose is generally used as a carbohydrate source.

Welwitschia mirabilis (Fig. 15) is regarded as the most remarkable living plant (McClean and Ivimey-Cook, 1951). There is only one species in the genus and it is confined to the central and northern parts of the Namib Desert in South West Africa and parts of Angola (Rodin, 1953). The genus *Welwitschia* is included under the Gnetales in the class Gymnospermae.

Among the unique characteristics found in *Welwitschia*, a

woody perennial, are an unbranched stem resulting in a closed system of growth; a shoot apex which dies shortly after germination of the seed; a single pair of strap-like leaves that persist for the life-time of the plant; and a dioescious habit (McClean and Ivimey-Cook, 1951).

This research was aimed at studying the nutrient and hormonal requirements of callus and isolated cells derived from hypocotyls of *Welwitschia* embryos with the ultimate object of differentiating whole plants from isolated cells. This research was further motivated by the general paucity of information on the culture of Gymnospermous tissues and cells, and the unique nature of the plant material used.

Unbeknown to us at the time, Steward *et al.* (1970) had successfully generated callus from embryos of *Welwitschia mirabilis* and obtained a culture of free cells exhibiting features associated with incipient totipotency. An article on this topic (Button, Bornman and Carter, 1971) had already been accepted for publication by the time that Steward *et al.'s* paper appeared in print.

No literature review on embryos culture or the economic importance of totipotency has been included in this introduction as it is considered to have been adequately covered in the introduction to *Part I*. Abbreviations used in *Part II* are the same as those used in *Part I*.

While *Part I* deals with attempts at inducing organogenesis in isolates of nucellar tissue, this part is concerned with similar

attempts using callus and cell suspensions of *Welwitschia mirabilis*. Although the two plant taxa used were very different, the common aim and experimental technique warrants incorporating the two parts into a single thesis.

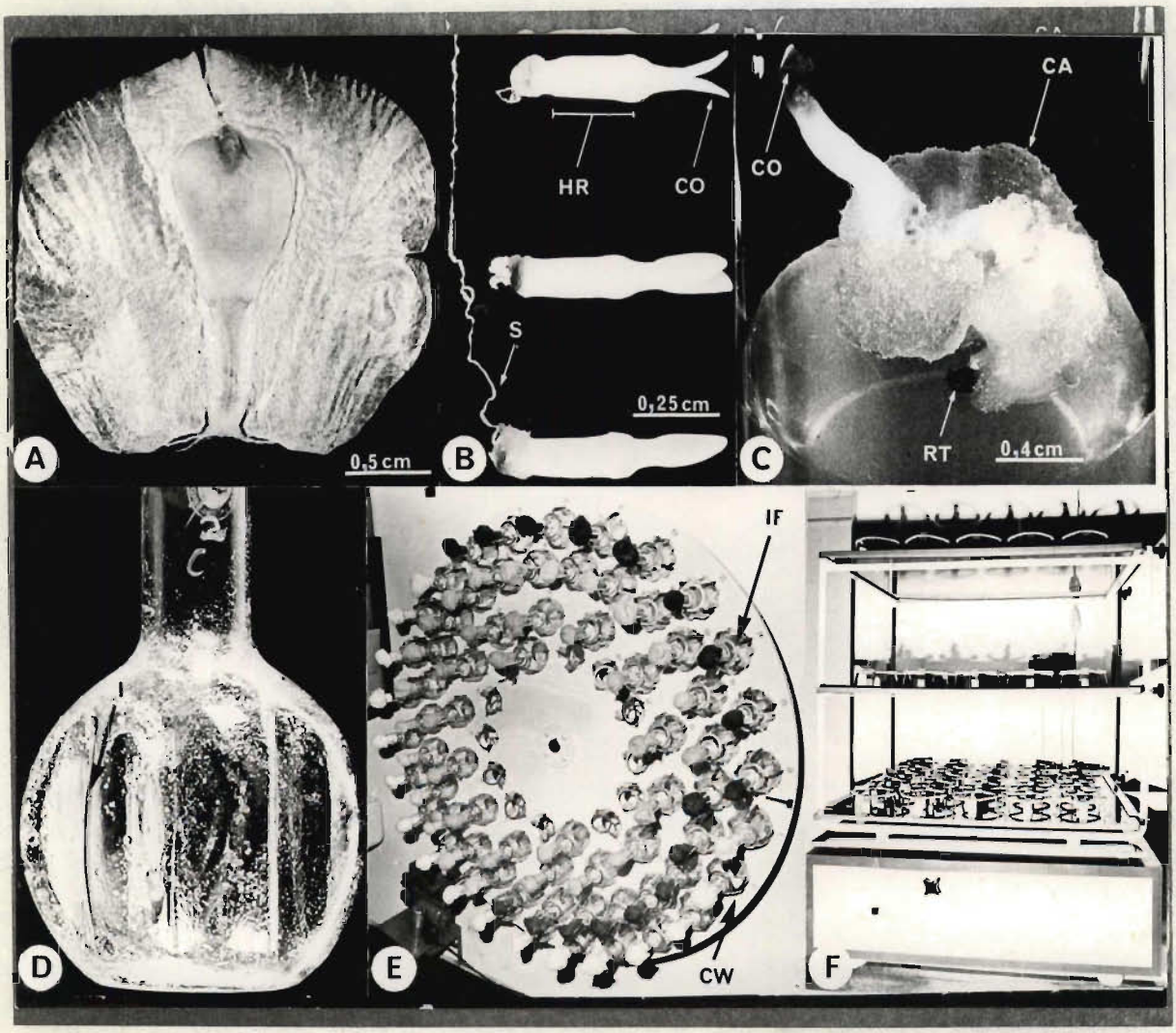


Fig. 16. A. Winged seed of *Welwitschia mirabilis*. B. Mature excised embryos showing cotyledons (CO), hypocotyl-root axis (HR) and suspensor (S). C. Amorphous, friable callus (CA) developing from the hypocotyl-root axis of a cultured embryo - root tip (RT) and cotyledons (CO) are indicated. D. Indented (I) culture flask containing cell suspension. E. Indented flasks (IF) attached to culture wheel (CW). F. Orbital shaker.

1. MATERIALS AND METHOD

1.1. *Embryo culture*

The wings were removed from selected, plump seed units (Fig. 16,A), and the seeds were then allowed to imbibe sterile, distilled water overnight. After surface-sterilizing with 3 per cent sodium hypochlorite in 70 per cent ethanol for 5 minutes, and rinsing 10 times with sterile distilled water, the embryos (Fig. 16, B) were aseptically excised and placed on agarified BM_1 supplemented with 400 mg/l CH. The effects of 0; 0,01; 0,1; 1,0; 5,0 mg/l NAA alone, and in combination with 0; 0,01; 0,1; 1,0 5,0 mg/l KIN on embryo growth was studied.

1.2. *Culture of isolated cells and cell aggregates*

Amorphous pieces of friable callus (Fig. 16, C) were obtained from embryos cultured on BM_1 with 1,0 and 5,0 mg/l NAA alone, or together with various levels of KIN. Portions of this auxin-induced callus about 0,5 cm^3 were transferred aseptically to specially constructed 500 ml tumbling flasks (Fig. 16, D) containing 10 ml liquid medium. These flasks were attached to a culture wheel (Fig. 16, E), and rotated at 1 rpm. The nutrient medium, pH 5,6, consisted of BM_1 with the following additives alone, and in combination with one-another:
CM (10%, v/v); ME (500 mg/l); YE (500 mg/l); and CH (400 mg/l).

After six weeks in culture, free cells and small cell aggregates were plated out in petri dishes on agarified (0,6%) BM_1 supplemented with CM (10%. v/v); ME (500 mg/l); CH (400 mg/l);

and CM + ME + CH.

A second batch of embryos was placed on BM_1 supplemented with 5,0 and 10,0 mg/l of IAA, IBA, NAA, and 2,4-D in order to determine which concentration and species of auxin would yield callus most suitable for cell suspension cultures.

Large pieces of friable callus obtained from the two NAA treatments were subdivided and cultured in tumbling flasks containing 10 ml BM_1 supplemented with the following: 400 mg/l CH; CH + 10% (v/v) CM; CM + 400 mg/l ME; CH + CM + ME; CH + CM + ME + 40 mg/l AD.

Eight weeks later, isolated cells and small clusters of cells were plated out in 250 ml, wide-mouthed erlenmeyer flasks and centrifuge tubes on agarified (0,6%) BM_1 with CM (10%); CM + 400 mg/l CH; CM + CH + 400 mg/l ME. The cultures in centrifuge tubes were subjected to 10Xg for 30 minutes every 12 hours during one week, in an attempt to establish polarity.

In a later experiment, callus pieces derived from embryos grown on a medium containing 5 mg/l NAA were placed in 500-ml tumbling flasks, as well as in 500-ml erlenmeyer flasks. The former were tumbled in the normal way while the latter flasks containing 15 ml medium each, were secured to an orbital shaker revolving at 80 rpm (Fig. 16, F). The following liquid media were used in both types of flask, 10 flasks of each type being used per treatment: BM_1 + 10% CM + 400 mg/l CH;

BM_1 + CM + 1 mg/l 2,4-D

BM_1 + CM + 1 mg/l IAA

BM_1 + CM + 0,1 mg/l 2,4-D

BM_1 + CM + 0,1 mg/l IAA

The object of this experiment was to investigate the effects of the different culture regimes on cell form and proliferation in the presence of different levels of IAA and 2,4-D.

Cell aggregates from these cultures were subcultured onto agarified media in 250-ml wide-mouthed erlenmeyer flasks. The latter were used as experience had shown that in them, evaporation was slower and contamination less than in petri dishes. Wherever possible, the donor tissue pieces were subdivided sufficiently to provide inoculum for one of 20 replicates of each of the following treatments:

BM_1 + 400 mg/l ME
BM_1 + 15% CM
BM_1 + 40 mg/l AD
BM_1 + 0,01 mg/l 2,4-D + 0,1 mg/l KIN

After two months in culture, tissue from these flasks was again subdivided in the same manner and placed in culture tubes on an agarified medium. Ten replicates per treatment were used in this grid experiment aimed at studying the effects of, and interactions between various concentrations of zeatin and 2,4-D in the presence and absence of 20 mg/l AD. The concentrations of the growth regulators were (mg/l):

Zeatin : 0; 0,001; 0,01; 0,1; 1,0
2,4-D : 0; 0,0001; 0,001; 0,01; 0,1

After one month on the above media, the pieces of callus were again subdivided and distributed among the 10 replicates per treatment of the following grid experiment using an agarified medium supplemented with CM and 2,4-D. The levels used were:

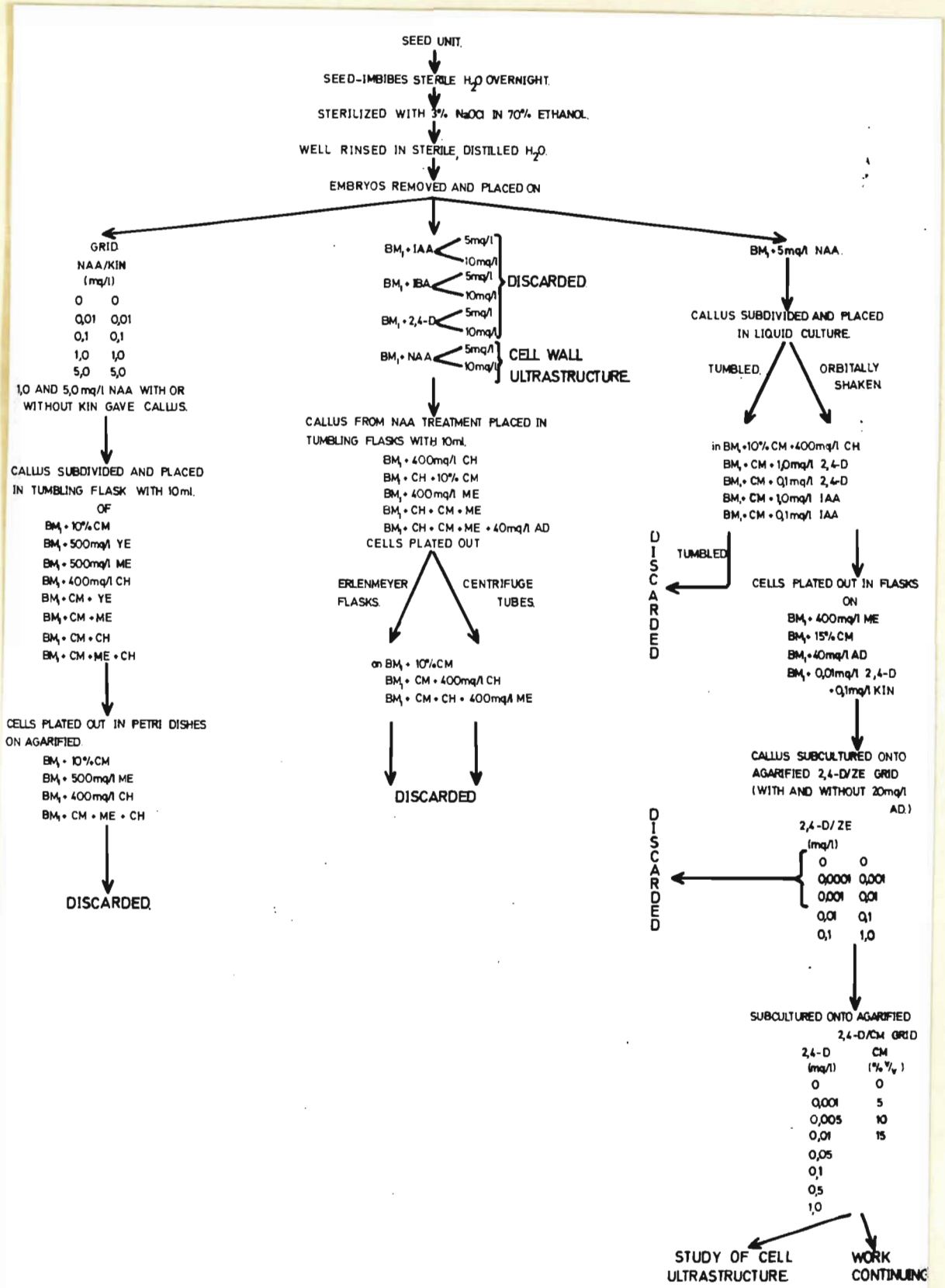


FIG. 17 FLOW-DIAGRAM SHOWING THE PROCEDURE, TREATMENTS, AND FATE OF CULTURED EMBRYOS, CALLUS, AND CELLS OF WELWITSCHIA.

CM (% v/v) : 0; 5; 10; 15;

2,4-D (mg/1) : 0; 0,001; 0,005; 0,01; 0,05; 0,1; 0,5; 1,0.

Figure 17 has been included to summarise the experimental procedure and to assist in tracing the source and fate of experimental material.

Throughout these experiments on *Welwitschia* cell culture, aliquots of cell suspensions were taken regularly, mounted on microslides in buffered methylene blue (Fink and Kühles, 1933), and examined under phase contrast. Frequency and types of cell abnormality, cellular vigour in terms of rates of cell division, cytoplasmic activity, and the ratios of living to non-living cells were observed and noted. These slides were also checked for any signs of cell aggregation and possible pro-embryoid structures.

1.3. *Fine structure of cultured cells*

An ultrastructural study of the cell-wall structure was undertaken using the method of Scott, Hamner, Baker and Bowler (1956) to examine the effect of species of auxin and concentration of auxin on wall microfibrillar structure and orientation. Tissue for this purpose was obtained from the experiment testing the efficacy of auxin species and concentration on generating callus from excised embryos.

In view of the reported difficulty in successfully fixing and embedding cultured free cells (Sutton-Jones and Street, 1968), it was decided to use the procedure which has given excellent results on phloem tissue of *Welwitschia* in our electron microscope unit. Free cells, cell aggregates, and loose pieces of callus were

collected from orbitally-shaken cultures supplemented with 2,4-D and CM, and killed in 6 per cent glutaraldehyde in sodium-cacodylate buffer at a pH of 7 for six hours and then fixed in 2 per cent OsO_4 in the same buffer for four hours. The material was dehydrated and embedded in Epon-Araldite following conventional procedures. Sections were stained with uranyl acetate followed by lead citrate.

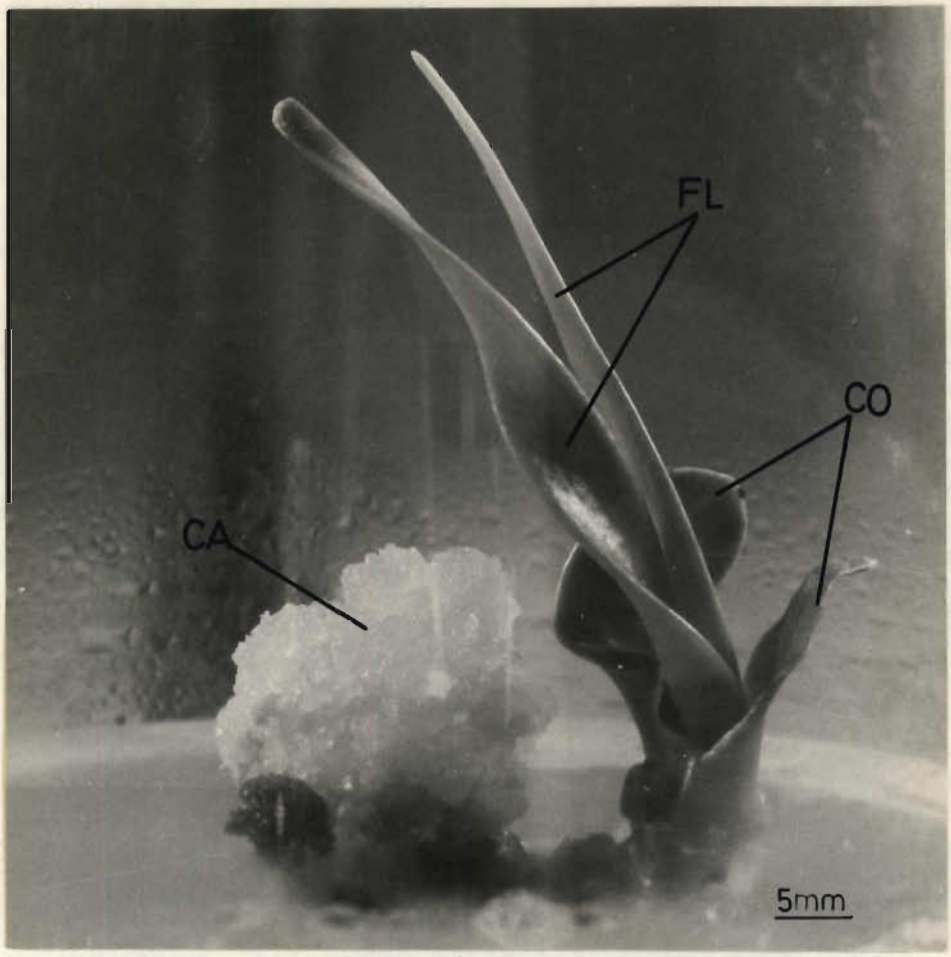


Fig. 18. *Welwitschia* plantlet in culture, showing cotyledons (CO), foliage leaves (FL), and callus (CA).

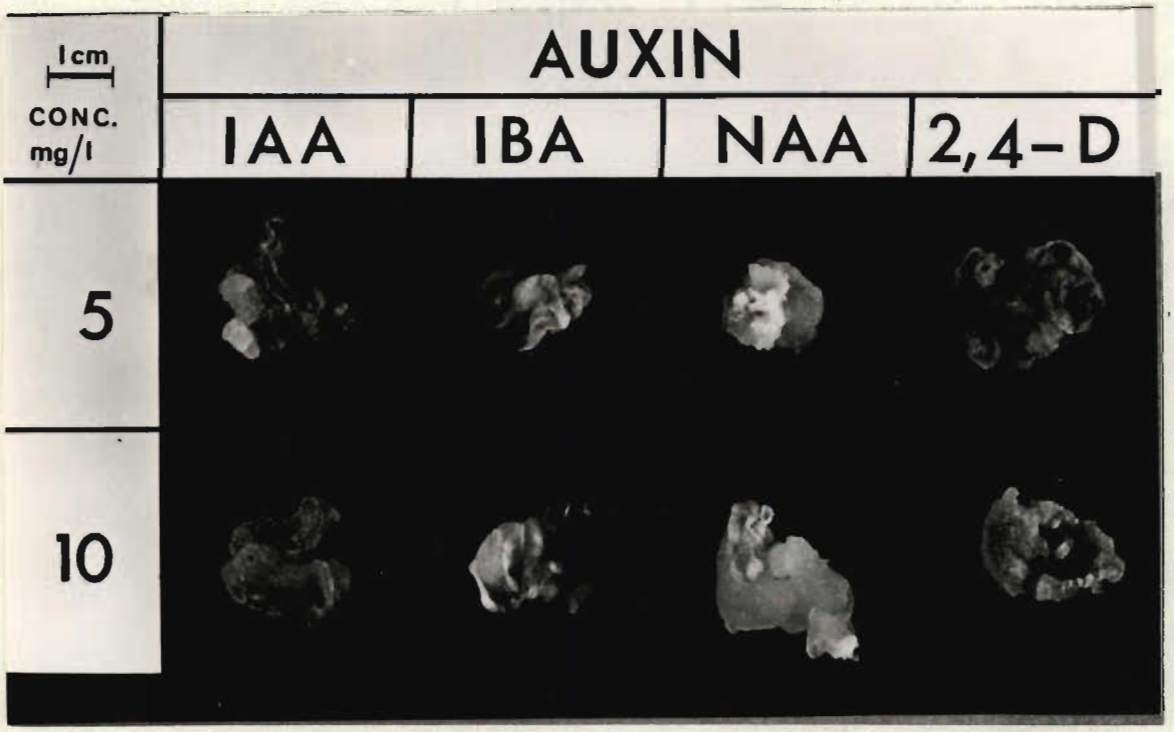


Fig. 19. Effect of auxin species and concentration on the development of callus from embryos of *Welwitschia* cultured for five weeks.

2. RESULTS AND DISCUSSION

2.1. *Embryo culture*

Embryos showed signs of growth during the first week in culture. Prolific callus development was observed in the treatments supplemented with NAA at 1 and 5 mg/l. The presence of KIN did not elicit a synergistic response with auxin.

Cotyledons and later, foliage leaves developed in many cultures but apart from high auxin promoting callus growth, responses to the growth regulators were very erratic. Leaves developed readily but roots were never successfully initiated although callus formed readily in the hypocotyl/root zone (Fig. 18). Further work on embryo culture was shelved because of the temptation to use the friable callus for experiments in free-cell suspension cultures.

2.2. *Culture of isolated cells and cell aggregates*

More callus was generated from embryo cultures by 10 mg/l NAA than by 5 mg/l (Fig. 19). This figure also shows that NAA was the most effective auxin species at inducing callus growth. The 10 mg/l NAA caused proliferation of excessively large, highly vacuolated cells which were up to 1 mm long and extremely weird in shape (Fig. 20). Although 5 mg/l also resulted in similarly shaped cells, their occurrence was less frequent. More cells from this treatment were roughly spherical with large nuclei surrounded by numerous plastids. The large vacuoles were tra-

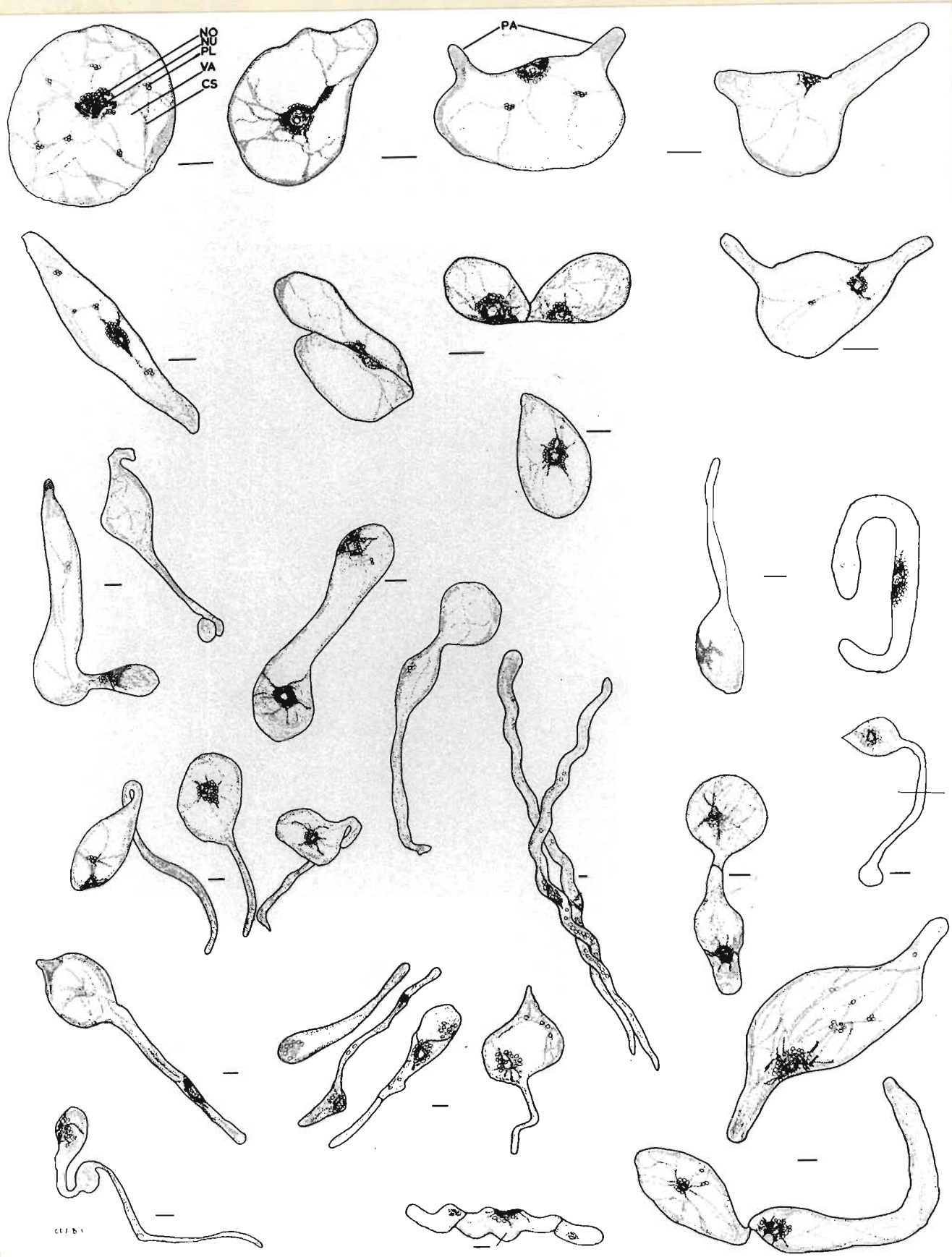


Fig. 20. Drawings of *Welwitschia* cells after culture in liquid media, showing the range in size, and shapes which varied from spherical (top left) through various stages of papilla development to extremely weird-shaped cells (lower half of figure). Outlines and shading was done by tracing cell images from photographic slides and negatives projected by a 35 mm enlarger. Not all cells are labelled as shading of inclusions is consistent throughout the figure. Nuclei (NU), nucleoli (NO), cytoplasmic strands (CS), and vacuoles (VA)

versed by thick cytoplasmic strands containing actively-streaming cytoplasm (Fig. 20).

Callus pieces placed in liquid media in flasks which were attached to the culture wheel soon disintegrated but cell proliferation continued slowly. No differentiation into recognisable pro-embryos or embryoids was noted in any of the media used. Proliferation was generally slow and the proportion of weird-shaped cells was great. Most of these cells died without dividing but some continued to divide into filamentous structures (Fig. 20). Many of the more spherical-shaped cells divided normally while others developed papillae (Fig. 20), which subsequently budded off the mother cell.

After 6-8 weeks in tumbled culture, aliquots of cell suspensions were subcultured on gelled media in petri dishes, wide-mouthed erlenmeyer flasks, and centrifuge tubes. Proliferation continued on these media but no sign of organization was observed. Growth was generally best on media containing CM + ME and CM + CH, but was never very prolific following tumbling. Cell aggregates invariably turned brown and were discarded. Petri dishes were unsuitable culture vessels as evaporation was great, unless sealed with masking tape, and contamination was frequent. Wide-mouthed erlenmeyer flasks proved to be much more suitable.

Regular examination and comparison of cells from orbitally-shaken and tumbled cultures revealed many interesting features. The rate of proliferation was invariably much greater in orbitally-shaken cultures where the highest and lowest yields were induced by BM_1 + CM + CH + 1 mg/l 2,4-D and BM_1 + CM + CH respectively.

Fig. 21. Macroscopic cell aggregates derived from free cells of *Welwitschia* which were orbitally-shaken in liquid BM_1 + 10% CM + 0,1 mg/l 2,4-D.

Cells were generally smaller and their shapes more regular in orbitally-shaken cultures than in tumbled cultures, where many irregular shapes and sizes were observed. This is somewhat surprising in view of the more violent nature or orbital-shaking, and the findings of Blakely and Steward (1964) that such shaking generally results in the development of filamentous structures and much debris. In general, IAA promoted filamentous type growth and the production of giant cells. These effects were most pronounced in tumbled cultures containing the higher level of IAA. In contrast, 2,4-D stimulated aggregation of relatively small, regular-shaped cells. This treatment was most effective at its lower concentration in orbitally-shaken cultures where some of the macroscopic aggregates (Fig. 21) turned light green or reddish, while others remained white. Pseudotracheids were also observed during microscopic examination of many of these cultures. The 2,4-D had little effect on promoting aggregation in tumbled cultures. These observations on the effects of the two auxin species on growth form are in agreement with similar observations on carrot cell suspensions (Nishi and Sugano, 1970).

When plated out on agarified BM_1 + ME; BM_1 + CM; BM_1 + AD and BM_1 + 2,4-D + KIN; cells and aggregates from orbitally-shaken cultures continued to proliferate. Tumbled cultures were discarded in view of the large proportion of dead and abnormally-shaped cells, and the general lack of vigour. In the plated cultures, growth was mostnoticeable on BM_1 + CM; and BM_1 + 2,4-D + KIN where numerous greenish, reddish or white nodules developed. Unfortunately no dif-

ferentiation occurred in these nodules which undoubtedly originated from the aggregates induced by the previous 2,4-D treatments. The absence of signs of differentiation after eight weeks in culture indicated that further subculturing was necessary.

In view of the promising signs of aggregation and chlorophyll formation in many of the cultures containing 2,4-D and KIN, it was decided that a 2,4-D, zeatin grid experiment might well include the balance between these hormones necessary for the differentiation of roots, shoots, or roots and shoots. Although growth continued on these media, no further differentiation took place during the four-week culture period.

It was then decided to investigate the effects of various levels of CM alone and in combination with a wide range in 2,4-D concentrations. It was hoped that the diversity of growth-promoting and embryogenic substances in CM would succeed where the supplements of cytokinins and myo-inositol had failed. For this reason, tissue pieces from the auxin/zeatin grid experiment were subcultured on gelled media supplemented with CM and 2,4-D.

A moderate rate of growth was induced by 2,4-D alone, especially at the higher levels of 0,5 and 1,0 mg/l. Supplementing with five per cent CM only, resulted in relatively vigorous callus growth. Proliferation was very good with 10 and 15 per cent CM and many cultures turned pale to dark green. Explosive callus growth was induced by 15 per cent CM and all levels of auxin, but this was especially marked at the higher auxin levels

Fig. 22. Explosive callus growth with dense white aggregates (AG) bristling with pseudothalli (PT).

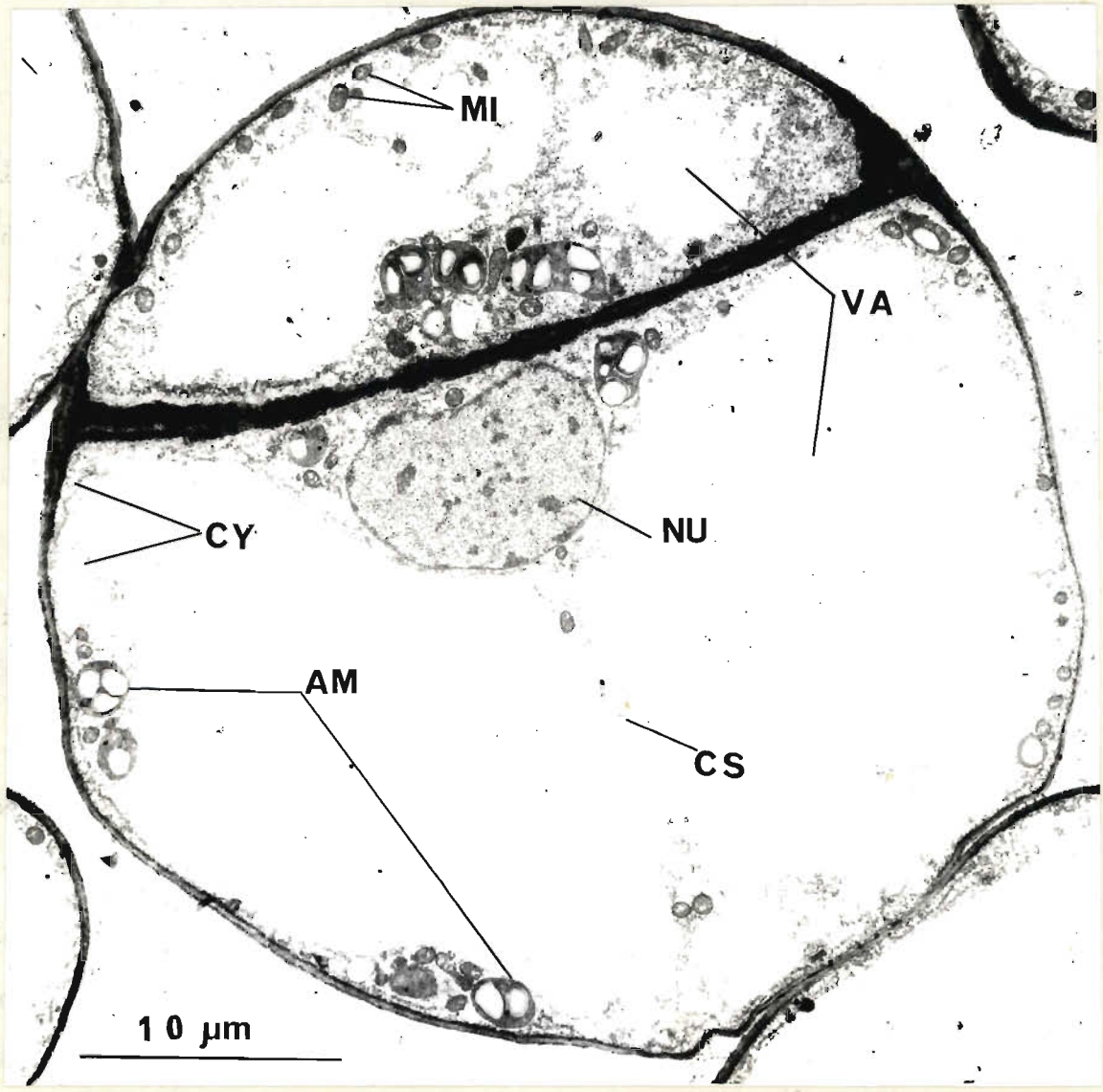


Fig. 23. Recently-divided giant cell showing a nucleus (NU), the thin layer of parietal cytoplasm (CY) with in-

where dense, white aggregates were formed, many of which were bristling with pseudothalli (Fig. 22). Still no signs of differentiation were visible at the time of writing.

2.3. *Fine structure of cultured cells*

Examination of the shadowed cell-wall preparations revealed little difference in microfibril structure and orientation. Species of auxin appeared to have no effect, while the highest levels generally resulted in a slightly more open arrangement of microfibrils than did the lowest levels. This difference in arrangement of microfibrils could be expected in view of the tendency for larger cells to be formed under the influence of high auxin concentrations than under low concentrations.

The method of fixing and embedding free cells and cell aggregates proved very successful. A wide range in size and type of cells was observed. Giant cells (Fig. 23), from 100 to 300 µm in diameter were found among the free cells. These cells were highly vacuolate, with a very thin parietal layer of cytoplasm which contained remarkably large numbers of mitochondria, storage plastids, and occasional dictyosomes. The nuclei of these cells varied in size from ca. 10 µm to ca 15 µm in diameter. Giant cells made up a large proportion of the cells originally inoculated onto the BM_1 + CM + 2,4-D media, but were relatively rare in the sections examined under the electron microscope.

Many of the large cells appear to divide internally into a number of discrete daughter cells, surrounded by the original wall of the mother cell (Fig. 24 A,B). This feature was also observed by Sutton-Jones and Street (1967). It is very likely

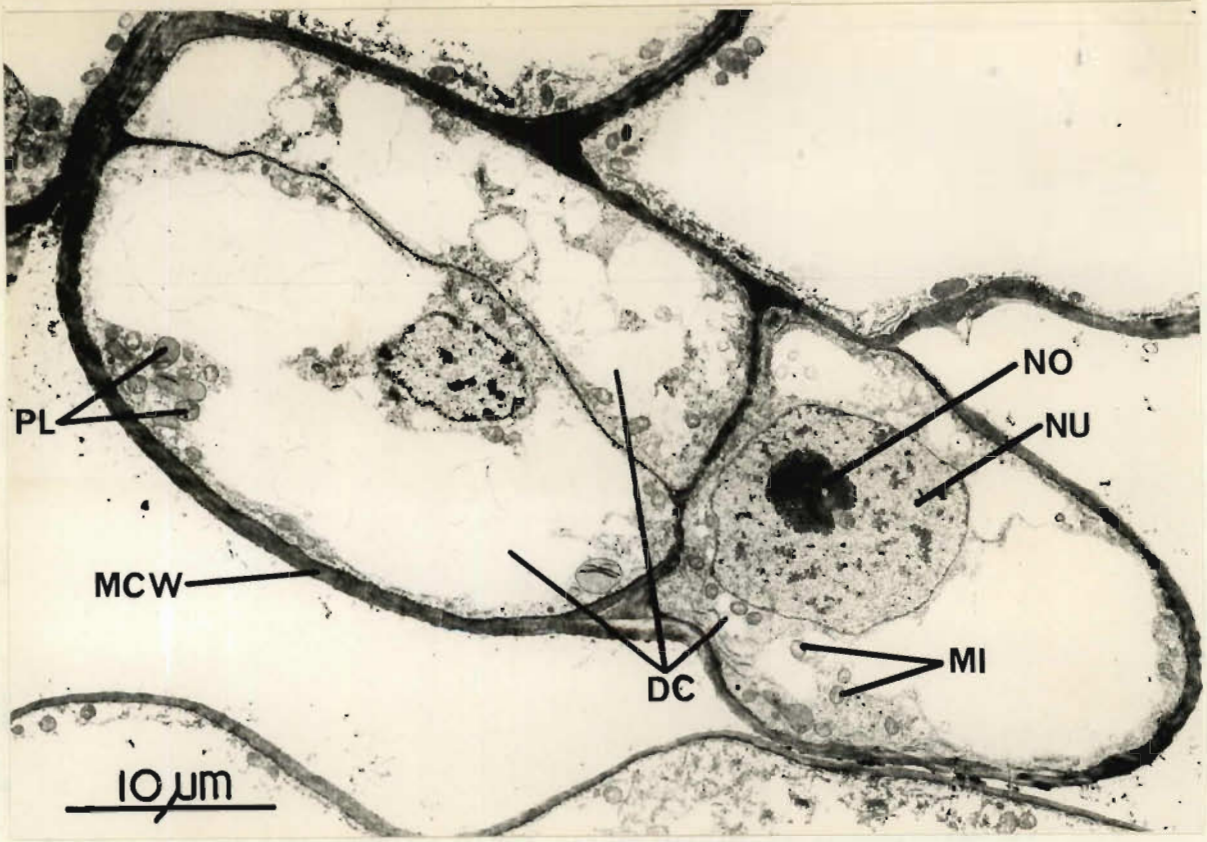


Fig. 24A. Giant mother cell, bounded by the mother-cell wall (MCW), which appears to have divided initially across the long axis and subsequently with the long axis to give three daughter cells (DC) with well-defined nuclei (NU), nucleoli (NO), mitochondria (MI), and plastids (PL).

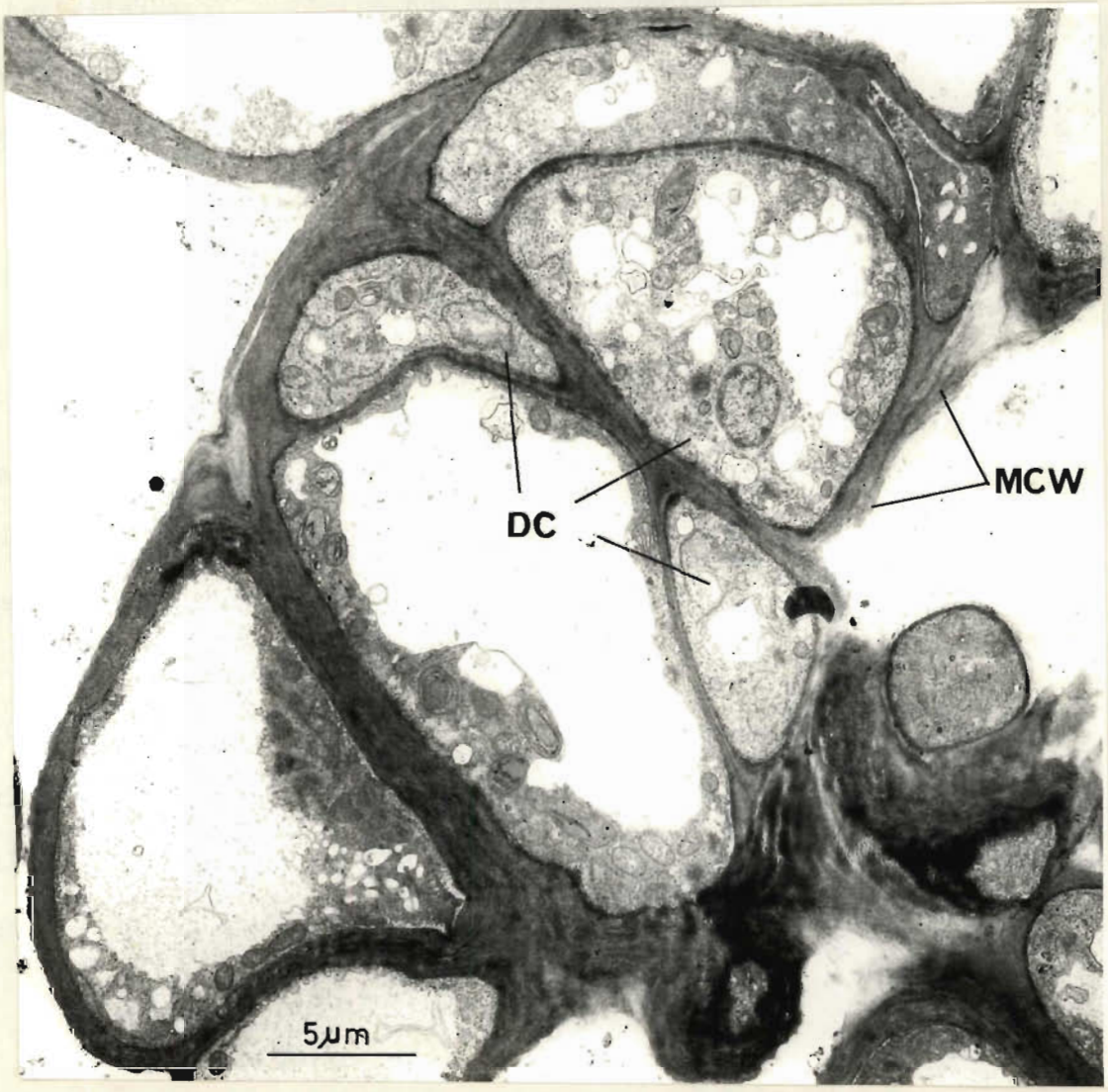


Fig. 24B. Daughter cells (DC) becoming liberated with the dissolu-

that these early internal divisions in fact result in the initiation of aggregates of cells which continue to divide, enlarge, and remain attached to one-another. I should like to suggest that in *Welwitschia* this type of division constitutes the first step in embryogenesis from free-cell cultures. Many illustrations of single cells, and cell aggregates resembling pro-embryos have been published, but the initial transformation from a free cell to a group of cells has not been clearly shown. This type of internal division does seem to be a logical first-step in embryogenesis, especially in liquid culture. In liquid culture, cells would tend to separate after normal division without the initial protection offered by the additional wall of the mother cell. The aggregate of daughter cells become liberated when the mother-cell wall disintegrates (Fig. 24,B) Steward *et al.* (1969), however, consider the first step in embryogenesis to be the development of "wing cells" on opposite sides of the original "mother cell". This would be conceivable on a semi-solid medium but is less likely to occur in shaken or tumbled liquid cultures which would tend to separate newly-divided "wing cells" from the "mother cell".

Most daughter cells appear to be firmly attached to one-another, linked with profusely branched plasmadesmata which sever as the cells mature. Connections between the walls of adjoining mother cells tend to be loose (Fig. 25). Shapes of recently divided cells vary, and axes range from ca. 10 - 50 μm in length. Nuclei are usually large in relation to cell size and may occupy over 75 per cent of the cell volume. One, two or three prominent nucleoli may be seen in each nucleus. The cytoplasm is relatively dense and

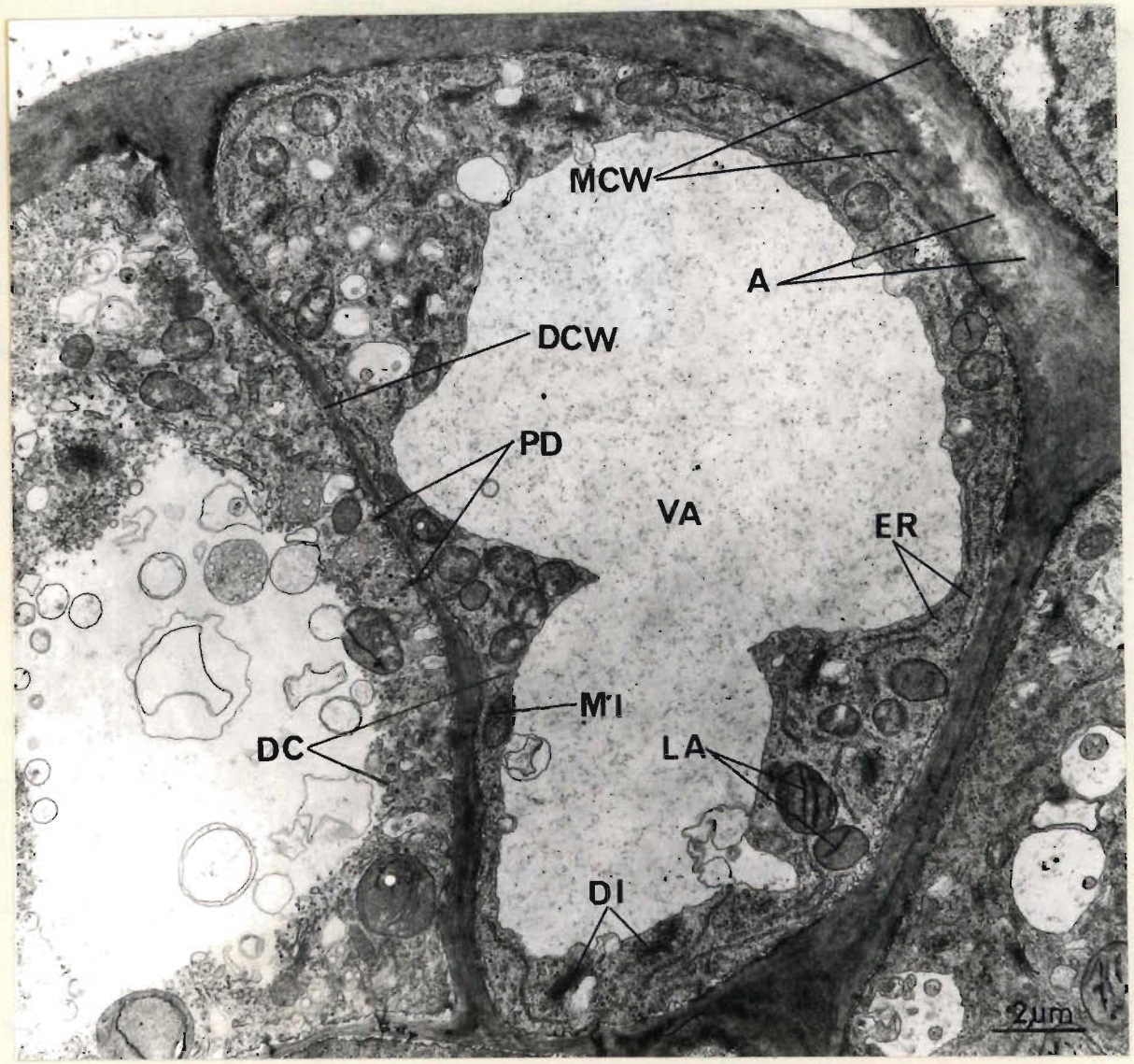
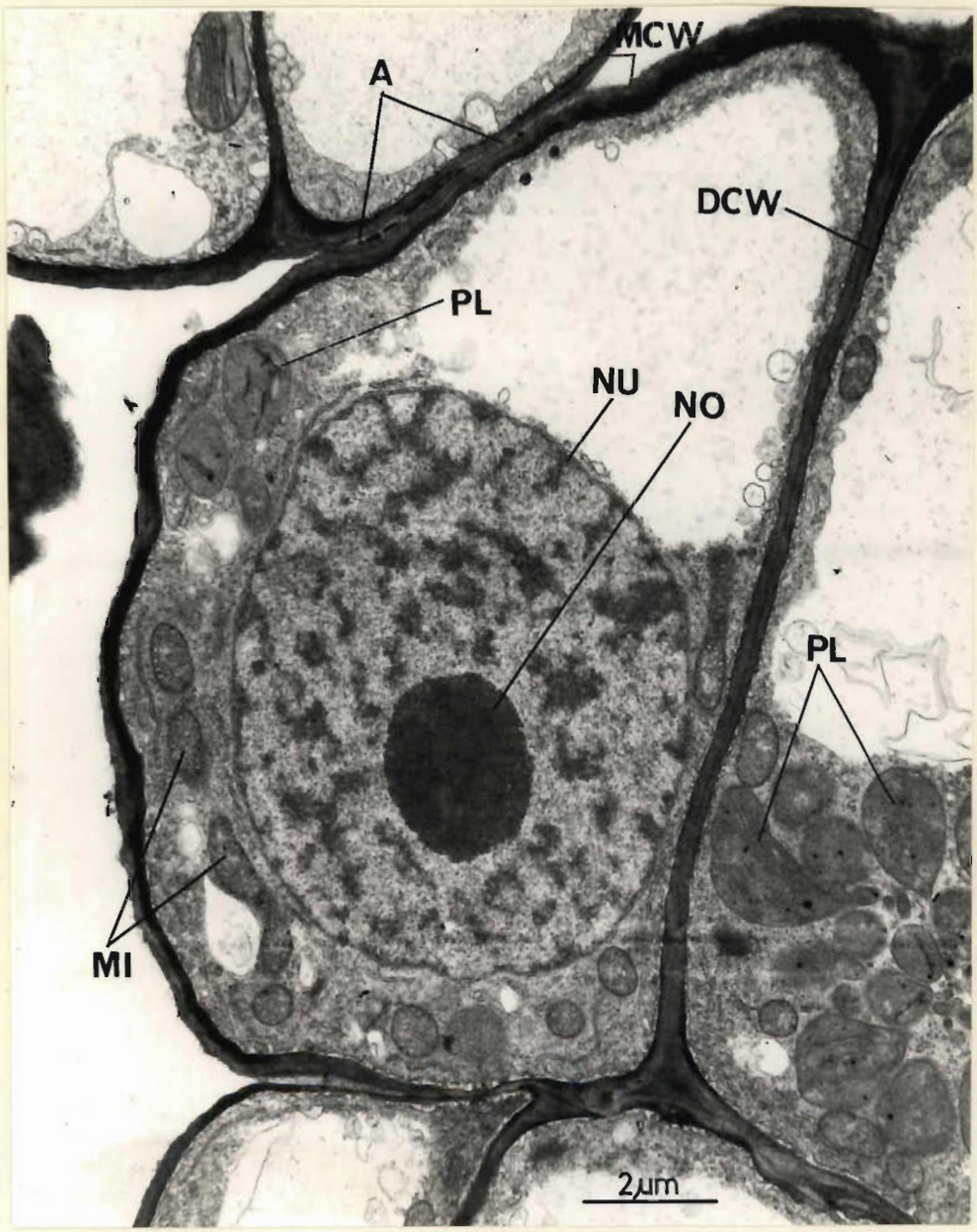


Fig. 25. Electron micrographs showing the intimate connection between daughter cells (DC) with plasmodesmata (PD) passing through the narrow cell wal (DCW), as opposed to the loose connection between the walls of adjoining mother-cells (MCW). These loose connections are probably due to dissolution of pectic substances in the region of the middle lamella between the adjoining mother-cells as is suggested at (A). Daughter cells generally have large vacuoles (VA), relatively large nuclei (NU) with nucleoli (NO), and cytoplasm with suspended mitochondria (MI) whi may be elongated, plastids (PL) which may have lamellae (LA), numerous dictyosomes (DI), and much endoplasmic reticulum (ER).


MCW
A
DCW
PL
NU
NO
PL
MI
2µm

contains numerous inclusions. In some cases the cytoplasm occupies a large proportion of the cell volume, but in others it forms a thin layer inside the plasmalemma and outside the nuclear membrane. The vacuole/s in these cells are traversed by cytoplasmic strands of ca. 1-2 µm in diameter. The degree of vacuolation in these cells varies greatly.

The plastids, which usually contain osmiophilic globules, are round to ovoid in section, and range from less than 1 µm to more than 3 µm in diameter. Chloroplasts appear to originate either from plastids which develop lamellae or from division of developing chloroplasts. Various stages in lamellar development from short, prolamellar bodies (Clowes and Juniper, 1968) through to discrete grana and stroma lamellae were observed (Fig. 26).

Mitochondria with dilated cristae abound and are generally somewhat longer than the generally accepted 1-2 µm (Clowes and Juniper, 1968). Lengths of up to 5,5 µm were recorded (Fig. 27).

Dictyosomes with well-defined vesicles are plentiful (Fig. 25) and much smooth and rough, endoplasmic reticulum with associated ribosomes was seen in most sections.

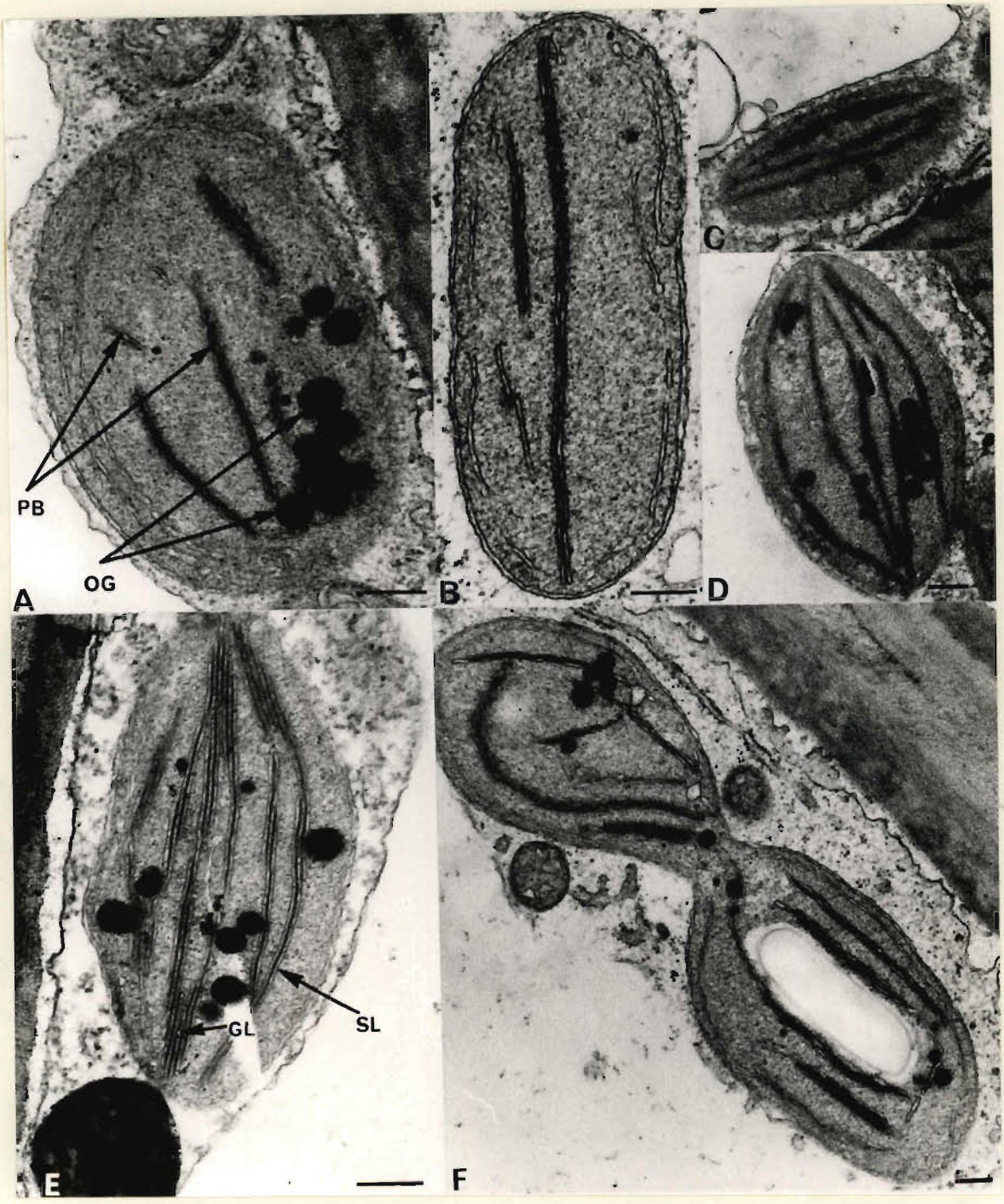


Fig. 26. Some stages in the development of chloroplasts in aggregates of cultured *Welwitschia* cells. Observed stages ranged from plastids containing osmiophilic globules (OG) and prolamellar bodies (PB) such as in A, through various stages of lamellar development (B,C,D) to chloroplasts with grana (GL) and stroma (SL) lamellae as in E. Division of developing chloroplasts (F) was also seen. Scale lines represent 0,25 µm.

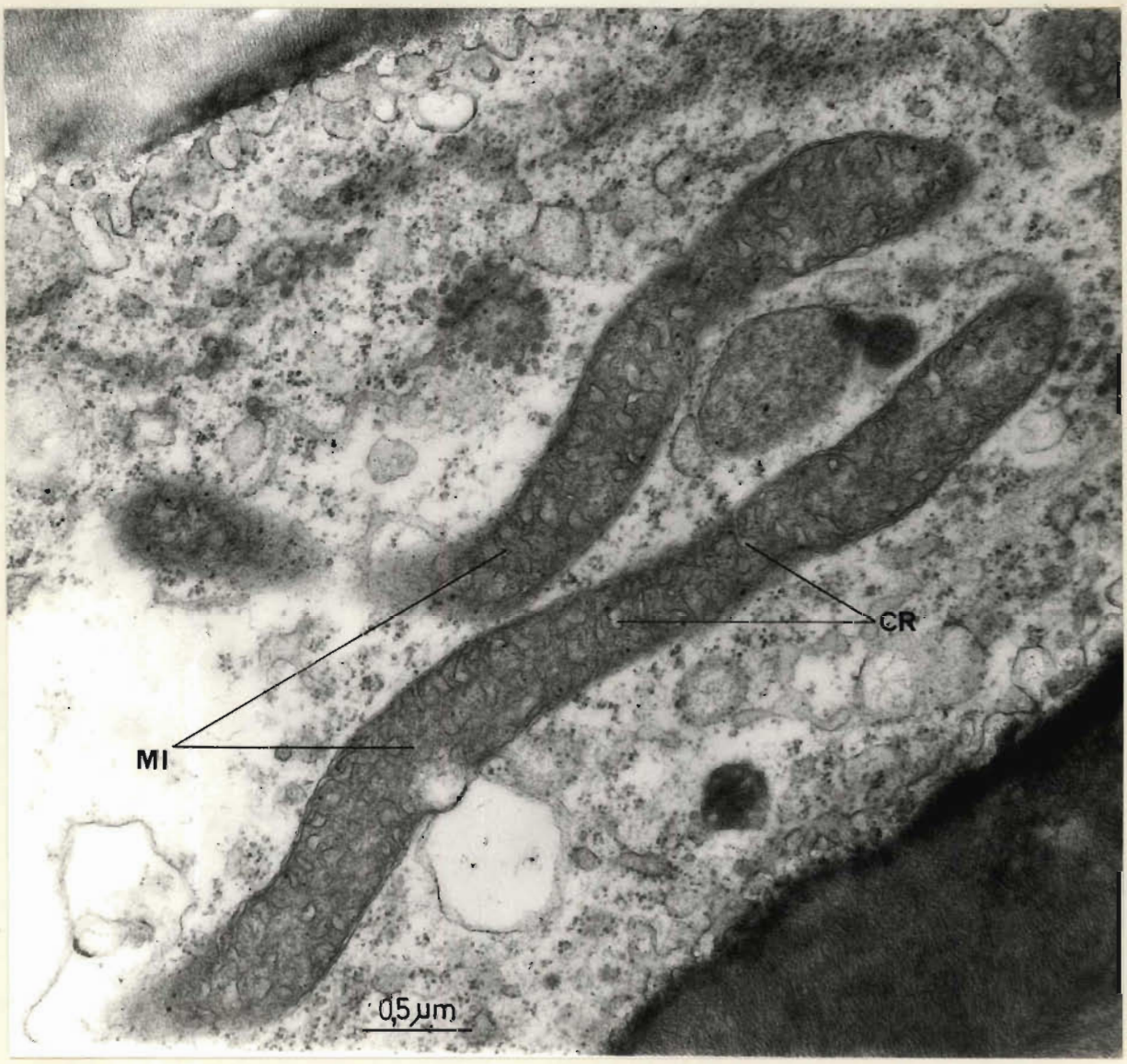


Fig. 27. Exceptionally long mitochondria (MI) with dilated cristae (CR).

3. CONCLUSIONS

Despite much research on free-cell cultures, to date no satisfactory explanation has been put forward to explain why isolated cells of some plants readily express their totipotency and develop into plants, while cells of other plants may proliferate easily but will not undergo adventive embryogenesis and subsequent differentiation. It has been fairly conclusively shown that complete, or nearly complete isolation from other cells is a prerequisite for complete expression of totipotency. In addition to this requirement, it is believed that correct nutrition is the prime, if not the only factor which must be satisfied. In this regard, it is unlikely that the inorganic constituents of a medium such as that of Murashige and Skoog would constitute the limiting factor. The organic constituents, especially hormones, vitamins, and amino acids are more likely to make up the critical fraction of the medium in determining totipotency.

Steward *et al.* (1970) consider that plants with embryos which do not rely on their suspensors for early nutrition, are probably among those which readily regenerate from single cells, while other plants, probably including *Welwitschia*, require the suspensor to subtly modify or supply the essential nutritional and hormonal balance to the young embryoid for its development. Thus although isolated cells of the latter type of plants are potentially totipotent, their requirements in terms of hormones, vitamins, amino acids

and possibly purines, are extremely specific. These requirements are likely to be quantitative as well as qualitative. Further work to study systematically the effects of individual amino acids, and combinations of amino acids and hormones on embryogenesis in isolated *Welwitschia* cells is to be undertaken. It is hoped that this approach will lead to a better understanding of the qualitative and quantitative nutritional requirements than the rather hit-and-miss approach using complex substances such as ME, YE, CH and CM.

Steward and co-workers prefer tumbling of cultures to reciprocal or orbital shaking for obtaining and differentiating free cells. With *Welwitschia* cell cultures, however, cell proliferation was much better in orbitally-shaken than in tumbled cultures. Orbital shaking yielded the greater percentage of regular, spherically-shaped cells which tended to divide more regularly and are generally considered to have greater totipotential than abnormally-shaped cells.

Cell aggregation appears to be stimulated by 2,4-D at concentrations of 1,0 mg/l and lower, but not by IAA. It is debatable as to whether 2,4-D stimulates internal cell divisions or whether it promotes division of cells round the periphery of an aggregate originally formed by internal division, or both these processes. This aspect is to be further investigated.

From the electron microscope study of cultured *Welwitschia* cells and aggregates it is tempting to propose that in general the initial formation of an aggregate form a single cell is due to multiple internal divisions rather than the formation of what would probably be

rather unstable "wing cells". It would be interesting to study nuclear divisions in dividing giant cells to see whether each nuclear division is followed by cell wall formation or if a multi-nucleate state exists for a time, as in a developing zygote *in vivo*. Indications from this study so far are that wall deposition follows each nuclear division.

From both a physiological and ultrastructural point of view, the totipotentiality of cultured free cells of *Welwitschia* offers great challenge for further work which could lead to a better general understanding of adventive embryogenesis *in vitro*.

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