IN VITRO STUDIES ON ANTHER CULTURE OF ANTIRRHINUM MAJUS L.

By

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Thesis submitted to the Haryana Agricultural University in partial fulfilment of the requirements for the degree of :

MASTER OF SCIENCE

in

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HISAR

1984

DEDICATED TO

MY ESTEEMED
MOTHER FOR HER
BLESSINGS AND
CONSTANT INSPIRATION

CERTIFICATE I

This is to certify that this thesis entitled, "*In vitro* studies on anther culture of *Antirrhinum majus* L." submitted for the degree of Master of Science in Botany of the Haryana Agricultural University, is a bonafide research work carried out by Ranjana Sharma under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

Sudesh Babbar

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CERTIFICATE II

This is to certify that the thesis entitled, "*In vitro* studies on anther culture of *Antirrhinum majus* L." submitted by Ranjana Sharma to the Haryana Agricultural University in partial fulfilment of the requirements for the degree of Master of Science in Botany has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.

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INTRODUCTION

The significance of haploids in the field of genetics and plant breeding has been realized for a long time. However, they are of rare occurrence in nature ranging from 0.01 to 0.001 per cent and hence their exploitation is not feasible.

The success of Guha and Maheshwari (1964, 1966) and Bourgin and Nitsch (1967) in evolving haploid plants in *Datura* and *Nicotiana* respectively pave way to utilize it in other plants for evolving haploids. Since then anthers and pollen grains of more than 30 genera and 70 species of angiosperms have been triggered to produce haploid plants. In these plants the technique of anther culture, therefore, can be routinely and profitably used for raising haploids and thereafter homozygous diploids.

Besides the utility of haploids in plant breeding programmes they form excellent material for studying mutations as they contain only one set of chromosomes.

Although nutritive requirements for evolving anther haploids are known for a good number of plants today but the morphogenetic steps involved in regeneration and the biochemical changes associated with androgenesis in most of plants are insufficiently understood.

Among Scrophulariaceae the technique of anther culture had been successful only in _Digitalis purpurea_ (Corduan and Spix, 1975). The present investigation is, therefore, an attempt to find out the possibility of evolving androgenic plants under _in vitro_ conditions from anthers of _Antirrhinum majus_ L. a popular ornamental species and effort has also been made to find out the morphogenetic steps associated with regeneration and some of the biochemical changes underlying differentiation.

REVIEW OF LITERATURE

Blakeslee (1922) for the first time reported the haploid mutant in Datura stramonium and discussed the importance of such mutants in plant improvement programme.

In 1957 Tulecke showed that haploid tissue can be successfully induced from pollen grains of a gymnosperm Ginkgo biloba under in vitro conditions. Guha and Maheshwari (1964,1966) succeeded in raising embryoids and complete plantlets from immature pollen grains of excised anthers of Datura innoxia in culture medium. Following this Bourgin and Nitsch (1967) and Nakata and Tanaka (1968) achieved success in raising haploid plants of Nicotiana tabacum. These experiments in Datura and Nicotiana attracted the attention of scientists all over the world and since then concerted efforts have been made by a large number of scientists to produce haploid plants through anther and pollen culture of several angiosperms. Todate, it has been possible to produce androgenic plants in about 30 genera and 70 species of angiosperms.

Nutritional requirement

Culture media and its composition are basic requirements for successful anther culture. There are various media like White's basal medium (1943), Murashige and Skoog's medium (1962), Nitsch and Nitsch's (1969) with or without slight modifications used in anther culture.

In Datura and Nicotiana a simple medium containing sucrose and without any vitamin or plant growth substances will suffice for raising haploids from anthers (Nitsch, 1969, 1971).

Sucrose is the essential ingredient in culture medium for induction of androgenic callus (Nitsch, 1971). Normally, 2-4 per cent of sucrose is used in the medium (Nitsch, 1969) but in which pollen is shed at trinucleate stage 5-15 per cent of sucrose in culture medium is found to be effective (Clapham, 1971; Ouyang et al., 1973).

Gamborg et al. (1970) observed that rise in sucrose level increased the root formation in Nicotiana tabacum while in Lycopersicon esculentum it affected only cell proliferation, also reported by Sharp *et al.* (1971). High sucrose concentration increased callus formation and also subsequent organ formation in *Oryza* (Chi-Chang Chen, 1978). In rice, the medium containing 9 per cent sucrose differentiated to more of albino plants than that with low concentration of sucrose.

A proper balance of auxin and cytokinin and addition of nutritive substances like casein hydrolysate, yeast extract and coconut milk was found to be important for inducing androgenic callus (Nakata and Tanaka, 1968; Guha *et al.*, 1970; Sunderland, 1971; Iyer and Raina, 1972).

Gamborg *et al.* (1970) reported that exogenous growth hormone supplements have no obvious effects on the induction of callus from anthers of *Nicotiana*.

Gresshoff and Doy (1972) raised haploids ~~in~~ of *Lycopersicon* using formulated defined basal medium and reported that exogenous level of growth hormones could control differentiation both in light as well as in dark.

Dunwell and Sunderland (1973) reported in *Solanum tuberosum* that high level of growth regulators cause callus formation from the somatic tissue. Addition of high concentration of auxin or cytokinin led to indirect androgenesis instead of direct (Guha *et al.*, 1970) and also caused proliferation of diploid somatic cells of anther wall and connective (Raina and Iyer, 1972; Rashid and Street, 1973).

Sopory and Maheshwari (1976) studied the effect of hormones, auxin and kinetin and their interaction on the growth of callus in Datura.

Iron is an essential ingredient in culture medium. According to Nitsch (1971) without iron androgenesis could be initiated in tobacco but the development of proembryos was suppressed beyond the globular stage. With the supplementation of iron in the culture medium as ferric salt of ethylene diamine di-o-hydroxy phenylacetic acid, the frequency of androgenesis was found to be high (Rashid and Street, 1973). Ethrel could induce callusing but not embryogenesis (Mac Donald and Grant, 1975).

Nakamura and Itagaki (1973) reported that the addition of activated charcoal could effect the development of plantlets from pollen in tobacco. Chu et al. (1975) reported that $(NH_4)_2SO_4$ in combination with KNO_3 at low concentration was of advantage to pollen callus in Oryza. High concentration of $(NH_4)_2SO_4$ inhibited callus formation. Thus, from the available literature and recent researches, it is clear that nutritional requirements are not common for all plants but vary from species to species and also depends on the experimental techniques and conditions used for the experimentation.

Stage of anther inoculation

Selection of anthers at right stage of development is an essential requirement in raising androgenic plants. The pollen can be diverted from their normal gametophytic to a 'sporophytic' mode of development only if these are cultured within a certain period of development (Nakata and Tanaka, 1968; Nitsch and Nitsch, 1969; Sunderland and Wicks, 1969). This specific period starts with the formation of spore tetrad (Sunderland, 1971) and ends with the deposition of starch early at the gametophytic phase (Dunwell and Sunderland, 1973; Collins and Sunderland, 1974; Raghvan, 1975).

Nakata and Tanaka (1968) observed that embryoids could be differentiated from pollen at tetrad stage in Datura whereas in Nicotiana tabacum anthers at varying developmental stages ranging from tetrad to the mature pollen with bicellular stage could form embryoids (Sunderland and Wicks, 1971).

Engvild et al. (1972) demonstrated that ploidy level of embryoids from anther culture in Datura innoxia was very dependent on the developmental stage of flower bud and also observed that anthers from small bud of 3 cm long gave rise to haploids while those from buds of 5 cm long gave rise to diploids. Still larger buds, when selected formed source for triploids. In Petunia binucleate microspores when cultured gave rise to triploids (Engvild, 1973).

In Atropa belladona the frequency of haploid embryoids was more when anthers selected were of smaller size than sepals. At this stage, anthers embodied predominantly uninucleate microspores (Rashid and Street, 1973). Dunwell and Sunderland (1973) reported that in Solanum tuberosum the best stage of inoculation of anthers is at or just prior to the first pollen mitosis. Ivers et al. (1974) inter-related the bud length, corolla development and anther development and classified buds into five stages. Bud stage three was found to be most suitable for cultures as it contained uninucleate microspores and was devoid of starch.

Sangwan and Norrel (1977) also reported that anthers containing microspores in the early uninucleate to first mitosis stages are best studied for haploid formation.

Factors affecting androgenesis

The environmental and physiological conditions under which the plants are grown considerably influence the production of haploids. Engvild et al. (1972) explained that productivity of 'pollen plants' also depends on the seasonal variations.

Light and temperature also have considerable effect on androgenesis. Light is not known to be essential for the induction in any species but in Nicotiana tabacum it was reported to be beneficial for growth after induction of callus and also for increasing the yield of plantlet (Sunderland, 1971; Corduan, 1973).

In *Vitis vinifera* when the cultures were kept under light during the first 24 h of incubation, there was higher induction of androgenic callus (Gresshoff and Doy, 1974). There was a better response when cultures were shifted from dark to light in *Datura innoxia* as well (Sopory and Maheshwari, 1976).

A temperature ranging from 25 to 30°C has been found to be optimal for most of the species. In *Nicotiana* 25°C favoured haploid production (Sunderland, 1971) while in *Solanum* sp. 20°C was ideal (Irikura, 1975). In *Datura innoxia* there was an increase in embryoid production with increase in culture temperature from 20-30°C (Sopory and Maheshwari, 1976).

Keller *et al.* (1979) reported an increase in yield of microspore derived embryos when the temperature was 10°C above the optimal temperature in *Brassica campestris*. Genovesi *et al.* (1979) observed that uninucleate stage gave much higher frequency of callus induction in rice when the floral buds are subjected to cold shock, prior to inoculation. Huang and Sunderland (1982) reported that pretreatment of excised spikes was more effective than that of excised tillers at 4°C and 25°C. Nitsch and Norrel (1972) increased the number of embryoids by exposing tobacco flower buds to low temperature prior to culturing the anthers.

Anagnostakis (1974) reported that in *Nicotiana tabacum* the frequency of production of haploids can be increased by using activated charcoal. Johanson and Erikson (1977) proposed that in anther culture of several Anemone sp. activated charcoal induced the embryo formation while Abscissic acid (ABA) inhibited embryo formation in pollen. Tyagi *et al.* (1980) showed that there is enhancement of pollen embryo formation in *Datura innoxia* on addition of charcoal in the medium.

When anthers were taken from inflorescence in rice and treated with 2, chloroethylphosphoric acid there was increased embryogenic activity (Wang *et al.*, 1974).

It is presumed that these pretreatments bring~~s~~ about certain nuclear and cytoplasmic disturbances which stimulated mitotic divisions ultimately resulting to callus formation.

Androgenesis in anther culture

Sunderland and Wicks (1971) reported that embryoid formation took place easily in pollen grains which had undergone the first asymmetric pollen mitosis to give a small generative and a large vegetative cell. A number of structural changes take place during the early stages of pollen embryogenesis in *Nicotiana tabacum* under *in vitro* conditions (Dunwell and Sunderland, 1974; Sunderland and Wicks, 1971). In 7 to 8 old days cultures, some multivesiculate bodies appeared in the cytoplasm of the vegetative cell and these resembled lysosomes. Such areas in the cell turned completely devoid of starch contents by 12 days (Dunwell and Sunderland, 1974b). Ribosomes apparently disappeared during this period, accompanied by change in number and structure of other cell organelles. Therefore, it is evident that most of gametophytic cytoplasm present in the vegetative cell degraded before the first mitotic division of sporophytic embryoid.

Two types of pollen grains are present in anthers. One type has deeply stained cytoplasm and abundant starch content. Such pollen grains are non-embryogenic. The second type has light stained cytoplasm with less starch content and vacuoles and are embryogenic (Sunderland and Wicks, 1971).

Associated with the division of the vegetative nucleus, formation of new ribosome complement took place which rapidly filled the structurally empty zones in the cytoplasm. The two daughter cells with dense cytoplasm were formed accompanied by other multiplied cell organelles (Dunwell and Sunderland, 1975).

The plane of division in Datura innoxia was constant and it was parallel to the preceding division of microspore

(Dunwell and Sunderland, 1976). In pollen embryoid development, first asymmetric division was not an essential step. However, in some plant species microspore divided into two equal cells and formed embryoids by further cell divisions in one or both of these cells (Narayanswamy and George, 1972; Rashid and Street, 1973).

Pollen embryoids usually lack suspensor. Information on the divisional sequence leading to the callus formation from uninucleate microspore in cereals was given by Ouyang *et al.* (1973) and Sun Wang and Chu (1974).

Ploidy level of androgenic plants

Anther culture does not give rise to haploid plants only. Usually non haploids are more in number than haploids in the androgenic callus. This non-haploidy is observed both in callus and embryo producing species.

The species in which non-haploids outnumbered haploids are *Oryza sativa* (Nishi and Mitsuoka, 1969), *Solanum nigrum* (Harn, 1971), *Atropa belladona* (Zenkteler, 1971), *Petunia hybrida* and *Petunia axillaria* (Raquin and Pilet, 1972; Engvild, 1973) and *Hyoscyamus niger* (Corduan, 1975). In some species viz., *Brassica campestris* (Keller *et al.*, 1975) and *Secale cereale* (Thomas and Wenzel, 1975) only non haploids are formed.

Narayanaswamy and Chandy (1971) observed a dominance of diploids in *Datura metel*. Seventy per cent were diploids, 6 per cent were haploids and 24 per cent were the triploids. In Datura innoxia the ploidy level varied from haploids to hexaploids (Engvild, 1972; Sunderland et al., 1974).

In Nicotiana tabacum cv. Burley when the anthers containing pollen grains which have undergone first pollen mitosis were cultured they gave rise to haploids (Sunderland, 1971). This was also true in N.knightiana, N.raimondii and N.attenuata (Collins and Sunderland, 1974). When anthers containing mature binucleate pollen grains were cultured diploids were obtained. The frequency of plantlets produced was upto 20 per cent (Engvild, 1974).

Doubling of chromosome number in haploids has been achieved by different methods. Chromosome doubling could be often accomplished by the use of colchicine. Burk et al. (1972) devised a method for colchicine treatment to seedlings. Lanolin paste containing 4 per cent colchicine was applied to the axillary buds in tobacco as detailed by Tanaka and Nakata (1969) or 0.1 per cent solution applied to the whole inflorescence for 1-2 days (Nakamura, 1974). To achieve the objective a second method involves the use of callus cultures of leaf or stem segment of haploid plants (Kasperbauer and Collins, 1972). Endomitosis in the callus cells may also lead to the spontaneous formation of the homozygous diploids.

Jensen (1974) reviewed various techniques used for chromosome doubling to derive diploid condition from haploids.

Tabata et al. (1977) observed that callus cultures obtained from haploid embryoid of Datura innoxia regenerate diploids. This might be due to spontaneous chromosome doubling occurred at the time of subculturing.

A mixploidy of cells of anther cultures in various strains of tomato was observed by Levenko et al. (1977). Among these aneuploidy predominated.

Morphogenetic studies

Morphogenetic steps involved in the development of plant organs in the anther culture are not properly understood. However, some details are available in other tissue culture systems.

The callus culture exhibited morphological heterogeneity and there was remarkable accumulation of starch in the differentiating callus (Halperin and Wethrell, 1964). According to Bergmann (1964) kinetin increases the general level of lignification and the percentage of tracheids in parenchymatous cells in the callus of Nicotiana.

The formation of meristematic nodules was found to be a common feature in developing cultures (Gautheret, 1959). Either they remained undifferentiated or formed centres of further cell proliferation (Yeoman Dyer and Robertson, 1965) by transferring into vascularized nodules from the perivascular region of which root and shoot primordia differentiated (Vasil and Hidebrandt, 1966). A proper balance of auxin/cytokinin ratio determines the organogenesis in Nicotiana tabacum pith tissue in vitro (Skoog and Miller, 1957). Higher concentration of kinetin caused bud initiation whereas more of auxin favoured rooting in Lycopersicon (Gresshoff and Doy, 1972b).

The site of organ initiation and the type of organ to be formed are controlled by phyllotactic and organogenic factors respectively which are supposed to be qualitatively alike but quantitatively different (Cutter, 1958).

The initiation of organogenesis took place within a group of undifferentiated cells or a meristemoid (Torrey, 1966). The cells of this group resembled apical meristem in several respects. These were supposed to be sites which received stimuli and had undergone organ formation depending upon the direction of the stimuli.

Thorpe and Murashige (1968) revealed a strong correlation between starch accumulation and shoot initiation. They concluded that starch accumulation was a prerequisite rather than a result of organ initiation.

Sometimes androgenic callus failed to form organs but showed prolific growth (Ivers et al., 1974). Capacity of callus to differentiate decreased with the age (Torrey, 1967).

Shizuka et al. (1978) found that shoot differentiated from callus originated from the superficial tissue and organ formation capacity was dependent on the level of exogenous hormones.

Uses of haploids

The principal use of haploids is in the production of fertile, homozygous diploid plants in large numbers through diploidization and clonal propagation in a single generation rather than taking years that are required to produce pure lines through inbreeding.

The haploids provide potentially useful material for the study of various fundamental as well as applied problems. Various mutants and recessive characters can be easily identified in such individuals.

Haploid cell lines have been shown to be useful in the induction and isolation of auxotrophic mutants in tobacco (Carlson, 1970). Haploid cell cultures have also been used to obtain streptomycin resistant cell lines of Petunia hybrida (Binding et al., 1970) and Nicotiana tabacum (Maliga et al., 1973). Maliga et al. (1973b) obtained 5-bromodeoxyuridine resistant cell lines of Nicotiana tabacum.

Such mutants are important in parasexual hybridization and in somatic cell genetics (Smith, 1974).

Haploids have their importance in transformation studies. Doy et al. (1972, 1973a,b) used androgenic haploid callus tissues of *Arabidopis thaliana* and *Lycopersicon esculentum* for the transfer and subsequent expression of three systems of genes from bacterium *E. coli*.

The anther culture technique is also important in clonal propagation of horticultural plants (Bajaj and Pierik, 1974). Various workers provided excellent reviews on the uses of androgenic haploids (Vasil and Nitsch, 1975; Collins, 1977).

Carbohydrate metabolism during morphogenesis

Carbohydrates play a significant role in controlling the morphogenetic pathways not only in lower vascular plants (Kato, 1965 and Kshirsagar and Mehta, 1978) but also in higher plants (Tren Thanh Van, 1977).

Thorpe and Murashige (1968, 1970) observed a heavy accumulation of starch in shoot forming tissues. This preceded any organ formation and was mainly in regions which ultimately gave rise to primordia. They opined the starch served as readily available source of energy for the process of organogenesis. This view was later on supported by Ross and Thorpe (1973). A strong correlation between the starch content of the tissue, its rate of respiration and shoot formation has been reported by Thorpe and Meier (1972). However, Nitsch and Lance-Nougarede (1967) failed to observe any accumulation of starch associated with organogenesis in *Nicotiana tabacum* and is perhaps the only report of this kind.

Halperin and Wetherell (1964) observed the accumulation of granular starch in 16 celled proembryos of *Daucus carota*.

Thorpe and Meier (1973a) observed that activities of soluble and insoluble invertase and sucrose synthetase increased significantly within first 3 days of culture during shoot formation. Thereafter, soluble invertase activity declined, while the activities of the insoluble invertase and sucrose synthetase remained relatively unchanged.

Palmer (1976) studied the activities of UDP-glucose pyrophosphorylase and ADP-glucose pyrophosphorylase and concluded that starch accumulation during shoot formation may not be regulated at the level of sugar nucleotide synthesis.

Ross and Thorpe (1973) opined that physiological gradient of substances like starch and also the rate of respiration in tissues may be the operative factors promoting organ initiation.

Thorpe (1974) observed that callus survived on sorbitol (alcoholic sugar) medium but did not produce shoots. It indicated that starch and free sugars were essential during shoot initiation. However, glycerol and myo-inositol supplemented medium induced tracheary element formation in cultured explants of Lactuca sativa but appearance of tracheary elements was delayed in glycerol supplemented medium as compared to glucose or myo-inositol containing medium (Roberts and Baba, 1982).

Brossard (1977) observed that root initiation in Crepis capillaris was accompanied by increase in starch content of the explants and a strong starch positive zone was always detected in parenchyma underlying their sites of initiation.

Accumulation of starch before the initiation of organs was followed by increase in amylase activity (Jain, 1978; Subhadra, 1981) and perhaps these factors are responsible for root and shoot initiation.

Repression of organogenesis especially of shoot differentiation in callus has been attributed to high endogenous GA_3 pool (Ross and Thorpe, 1973; Thorpe and Meier, 1973b).

Nucleic acid and protein metabolism

Direct and indirect evidences are available to show that DNA, RNA and protein synthesis occur during organ formation (Thorpe and Murashige, 1970). Bavrina et al. (1974) reported increase in both RNA and protein content during bud formation in tobacco callus. Vasseur (1972) using leaf sections of Chichorium intybus observed that initial high rate of RNA synthesis during callus formation was followed by rise in UMP/CMP ratio associated with shoot formation. This favoured the proposal that there was a change in the type of RNA being synthesized. Kovacs (1971) observed that shoot formation in tobacco callus followed increase in RNA/DNA and Histone/DNA ratio.

Sekiya and Yamada (1978) noted that DNA-dependent RNA polymerases (RPases) which catalyze the first step of transcription changes during shoot formation in tobacco.

Bhojwani et al. (1973) found that increase in DNA content was nearly double in embryogenic pollen. On the other hand, RNA content was reduced to about one-third and while the total protein remained unchanged. Bavrina et al. (1974) found that DNA content was more than double in regenerating stem buds of Trapezoid tobacco.

Maeda (1980) indicated that rice callus produced from seeds had 80 per cent more DNA content compared to that from the root tip cells.

Dougall (1962) using labelled tyrosine in tobacco callus and Syono (1965) using chloramphenicol in carrot callus, obtained evidence that protein synthesis occurred during shoot formation. Syono (1972) used an inhibitor that reduced the concentration of proteins. By doing so he could prevent organogenesis without reducing the growth of cells. It indirectly suggested that specific proteins were necessary for organogenesis. This was further supported by Sekiya and Yamada (1974). They also showed that these proteins were similar to those found in the leaves of green house grown plants.

Hasagawa et al. (1977a) using bud forming and non-bud forming cultures of Douglas fir cotyledons found that there were significant changes in protein composition during adventitious bud formation. These proteins were enzymatic in nature as also suggested by Rob~~p~~erts et al. (1966).

Thorpe (1978) indicated that during the initiation of shoot primordia in tobacco epidermal explants, there was a decrease in the rate of protein synthesis.

Shoot forming tissue maintained higher amounts of total nitrogen, protein nitrogen and nitrite nitrogen but amino nitrogen content was almost identical to non-shoot forming tissue (Thorpe, 1982).

Simola and Sopanen (1970) reported that leucine and alanine amino peptidases specific activity in cell suspension of Acer pseudoplatanus was maximum on 9th and 19th day of incubation which decreased gradually with the ageing of suspension and also reported that protein content was more in the cells grown with naphthalene acetic acid than in those cells cultured with naphthoxy acetic acid. Similarly, Simola (1973) observed that the clumps with roots showed higher activity of this enzyme which indicated effective turn over of amino acid and proteins in undifferentiated parts of clumps in cultured root cells of <u>Atropa belladona</u>. Werner and Gogolin (1970) observed that activity of aspartate amino transferase did not change significantly during root initiation.

MATERIAL AND METHODS

Cultivated variety of Antirrhinum majus Linn. has been used for experimentation.

Floral buds of different sizes i.e. 0.40, 0.5, 0.55, 0.6, 0.65 and 0.7 cm long were taken from the Botanical Garden of Botany Department, Haryana Agricultural University, Hisar during the months of January, February and March between 8.00 a.m. to 9.00 a.m. These buds were fixed in F.A.A. and dehydration and embedding were done by conventional method and microtomed at 10 u thickness. The sections were stained with safranin and fast green to examine the development of sporogenous tissue in relation to the size of bud. Observations were made on the basis of the study of 20 buds of different sizes collected from plants of different ages during flowering period.

To compare the bud size in relation to the stage of development of the sporogenous tissue, cytological preparations were also made. For this purpose, buds of different sizes were fixed in acetic alcohol (1 acetic acid: 3 alcohol) for 24 h and then preserved in 70 per cent alcohol. Squash preparations of anthers from buds at various stages were made using 0.5 per cent acetocarmine stain.

Methodology

Floral buds were collected in the morning hours during the months of January to March and were subjected to surface sterilization by absolute alcohol for 10 seconds and then washed twice with autoclaved double distilled water. Anthers were aseptically removed and planted on the agar solidifying media. The following were the basal media* which were used for the initial studies:

1. Murashige and Skoog's medium (1962) MS.
2. Gresshoff and Doy's medium (1972b) DG.
3. Nitsch and Nitsch's medium (1969) NN.

Each supplied with 2 mg/l naphthalene acetic acid (NAA) and 1 mg/l kinetin.

Besides this these media were supplemented with 2 mg/l dichlorophenoxyacetic acid (2,4-D). pH was adjusted to 5.8

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* Compositions of media given in appendices I, II and III.

with NaOH before the addition of agar-agar. Anthers from a single bud were put in each flask and 100 such culture flasks were set up. After inoculation the culture vessels were incubated at 26 ± 2°C in dark.

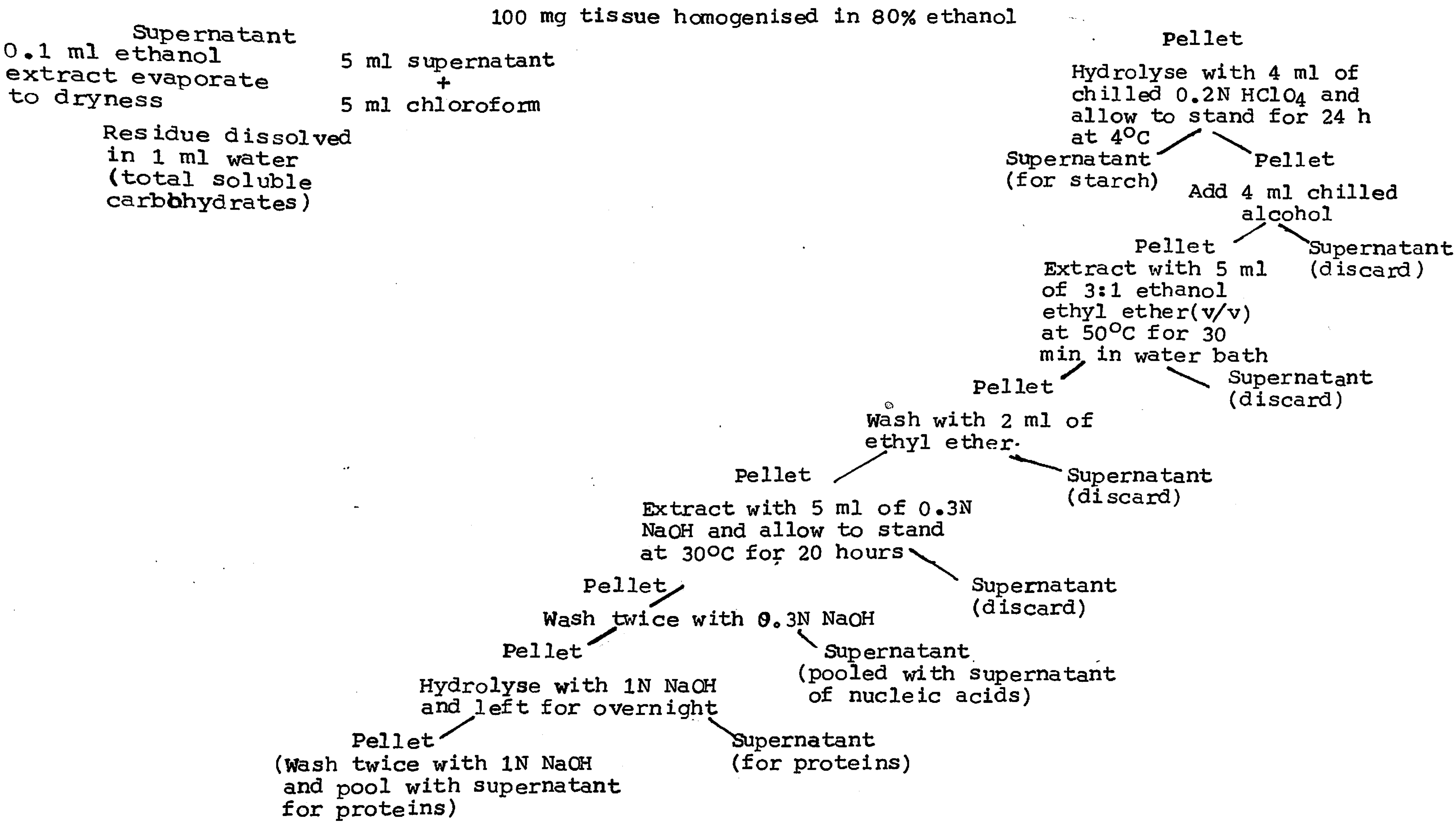
For the study of the behaviour of anthers at various stages of development in nutritive media, cultured anthers were periodically taken out before and shortly after the formation of callus fixed in FAA and dehydration and embedding were carried out and sections were cut at 10 u thickness with the help of microtome. The sections were stained with safranin and fast green. Cytological preparations were also made from the material.

Since the callus formation resulted only from anthers at uninucleate stage of development in culture media further inoculations were done with anthers only at this stage i.e. 0.55-0.65 cm long sized bud. Since MS medium was found to be best for callus induction, this medium was used for further growth and subculturing.

For studying the initial pattern of division in the microspore, anthers were periodically taken out. Squash preparations were made using acetocarmine stain.

Various media used for differentiation are:

i) MS + 2.0 mg/1 NAA + 1 mg/1 kinetin - M_1
ii) MS + kinetin 2 mg/1 + NAA (1 mg/1) - M_2
iii) MS + 8.0 mg/1 NAA + 1 mg/1 kinetin - M_3



Flow diagram: Fractionation of the sample for total soluble carbohydrates, free amino acids, phenols, starch, nucleic acids.

For differentiation, 16 h light and 8 h dark cycle was given to the cultures and were incubated at 26 ± 2°C.

For morphogenetic studies, the calli at different stages of development were fixed in FAA for 24 h and passed through a conventional series of dehydration and infiltration. The sections were cut at 10 u and stained with safranin and fast green (Johansen, 1940).

Biochemical estimations

Estimation of different metabolites viz., total soluble carbohydrates, starch, nucleic acids and proteins was done at three stages i.e. 14 days, 28 days and 49 days old callus henceforth also denoted as stage I, stage II and stage III.

For each sample one hundred mg of callus was homogenized in 80 per cent ethanol (v/v) using a pinch of acid washed sand as an abrassive. The homogenate was refluxed for 15 min on a water bath and centrifuged. The residue was further refluxed thrice with ethanol. The supernatants were combined and the fractions pellet were further partitioned according to procedure shown in the diagram flow.

Carbohydrate content

Estimation of total soluble carbohydrates was done by the following method of Yemm and Willis (1954).

0.1 ml of the ethanol extract was evaporated to dryness in a test tube. After cooling, the residue was dissolved in 1 ml of water (distilled) and to this 4 ml of anthrone reagent was added. The mixture was then heated on a water bath for 10 min. After cooling, O.D. was taken at a wavelength of 620 nm against reagent blank.

Standard curve was prepared using graded concentration of D-glucose.

Starch content

Estimation of starch was done using the method of Hassid and Neufeld (1964).

To 0.1 ml of the aliquot prepared for starch estimation added 1 ml of distilled water. To this added 4 ml of anthrone reagent. The mixture was heated for 10 min on a water bath then after cooling to room temperature, O.D. was taken at a wavelength of 620 nm against the reagent blank.

Calibration curve was prepared by using graded concentrations of D-glucose. Starch content was calculated by multiplying equivalent by 0.9.

Protein estimation

Protein content was determined by the method of Lowry et al. (1951).

To 1.0 ml of aliquot taken from protein extract prepared in 1 N NaOH, 5 ml of alkaline $CuSO_4$ was added and mixed well. The mixture was allowed to stand for 10 min and then 0.5 ml of Folin's reagent was added and mixed vigorously. After 30 minutes, O.D. was recorded at 750 nm against reagent blank.

Standard curve was prepared using graded concentrations of bovine serum albumin.

Nucleic acids

Nucleic acid extraction was done by alkali hydrolysis by the following method of Niemann and Poulson (1962).

Five ml of the supernatant obtained for nucleic acid estimation was taken and to it 3 ml of 1N $HClO_4$ was added so as to lower the pH of extract to 1. The mixture was kept at 4°C for 40 min. After centrifugation, the supernatant constituted RNA fraction 1. The sediment was resuspended in 2 ml of 1N $HClO_4$ and after centrifugation supernatant was mixed with RNA fraction 1 and volume was made 10 ml. The residue after the above extraction was extracted with 5 ml of 0.5 N $HClO_4$ at 70°C for 15 min and centrifuged after cooling it to 4°C that constitutes DNA fraction. The precipitate was resuspended with 2 ml of 0.5 N $HClO_4$ and extracted at 70°C. The supernatant was pooled with previous DNA fraction and the volume was made

10 ml. RNA and DNA contents were determined by recording O.D. of each at 260 and 290 nm on UV Spectrophotometer. The differences between O.D. at 260 and 290 nm were used for calculations. The calibration curve for RNA and DNA were made using graded concentrations of yeast RNA and calf thymus gland DNA respectively.

Histochemical analysis

The preserved material, after washing thoroughly with 70 per cent alcohol was dehydrated and cleared through ethanol-xylene series and embedded in paraffin wax. Serial transections were cut at 10 u thickness. Haupt's adhesive (Johansen, 1940) was used for affixing the paraffin ribbons to the slides. Various histochemical stains were tried for the localization of protein, starch and nucleic acids.

Localization of starch

Starch was histochemically localized by following the method of Johansen (1940).

Sections were deparaffinised in xylene and then brought to water. Sections were stained in I-KI solution for 10 min. These were then washed in water thoroughly and were mounted in DPX mountant after passing through the graded series of alcohol and xylene. Starch appeared blue to purple.

Localization of proteins

Proteins were localized by mercuric bromophenol blue method (Mazia *et al.*, 1953).

After deparaffinizing in xylene and bringing the sections to water, they were stained in mercuric bromophenol blue solution for 15 min (Mercuric bromophenol blue solution was prepared by dissolving 100 mg of bromophenol blue and 10 g of mercuric chloride in 100 ml of 95 % ethanol). Sections were washed in 0.5 per cent acetic acid for 20 min and then rinsed with water for 3 min. These were mounted in DPX mountant after passing through the graded series of alcohol and xylene.

Proteins were stained blue.

RNA localization

Brachet's method was employed for RNA localization (1953).

The sections were deparaffinised in xylene and brought to water after passing through alcohol series. Sections were placed in methyl green pyronin solution for 20 min and then were passed quickly through graded series of alcohol and xylene then mounted in DPX. RNA containing structures stained red.

Methyl green pyronin solution was prepared by dissolving 0.15 g of methyl green and 0.25 g of pyronin Y in 100 ml of acetate buffer pH 4.7. The buffer solution was prepared by mixing 45.5 ml of 0.2 M acetic acid and 54.5 ml of 0.2 M sodium acetate. The mixture of methyl green pyronin was extracted with chloroform in a separatory funnel till the chloroform did not take colour.

DNA localization

For the localization of DNA in the transection of callus Fuelgen method was employed (Gomori, 1952).

The sections were deparaffinised and were brought to 95 per cent ethanol. They were coated with 0.5 per cent celloidin solution. This coating prevented sections from floating during hydrolysis. The sections were hydrolysed in 1N HCl at 58-62°C for 10 min. After a complete washing in water the sections were stained in Schiff's reagent for 20 min and then rinsed in 2 per cent sodium metabisulphite for 2 min and washed in running water for 10 min. After dehydration and cleaning these were mounted in DPX.

DNA stained dark violet in preparation.

The Schiff's reagent was prepared by dissolving 0.5 gm of basic Fuschin and 0.5 gm of potassium metabisulphite in 100 ml of 0.15 N HCl. Mixture was shaken at intervals of 2-3 h till the dye was converted into Fuschin sulphurous acid. Filtration process was repeated till the filtrate was clear and colourless.

RESULTS

Floral buds of *Antirrhinum majus* of different sizes i.e. 0.40, 0.5, 0.55, 0.6, 0.65 and 0.7 cm long, were sectioned to study the development of sporogenous tissue in relation to the size of bud at the time of culturing. Transverse section of 0.4-0.5 cm long bud contained anthers in which microspore mother cell had undergone meiotic division~~s~~ to form tetrahedral tetrads. Tapetum started showing signs of degeneration at this stage. In the buds of 0.55-0.65 cm long the microspores were released from callose wall of the tetrad. At this stage microspores were uninucleate (Fig. 2) with a centrally placed nucleus and dense cytoplasm. The tapetum had fully degenerated by the time (Fig. 3) and middle layers also showed their

Fig. 1 Transverse section of microsporangium showing uninucleate microspores x 280.

Fig. 2 Showing isolated microspores and pollen grains x 800.

Fig. 3 Transverse section of microsporangium showing degenerated tapetum x 800.

dT - degenerated tapetum
M - Microspores
P - Pollen grains.

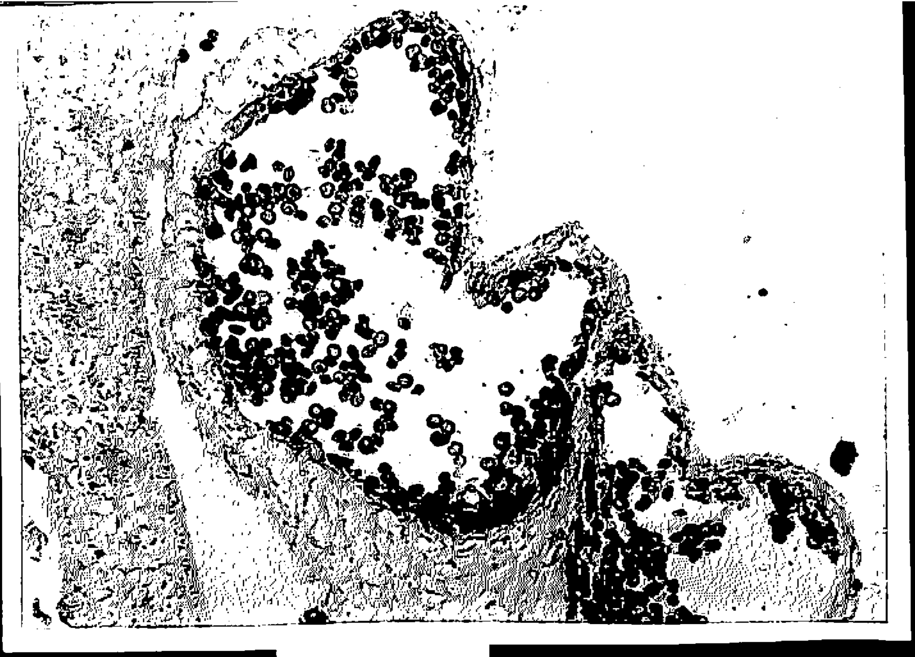


Fig. 1

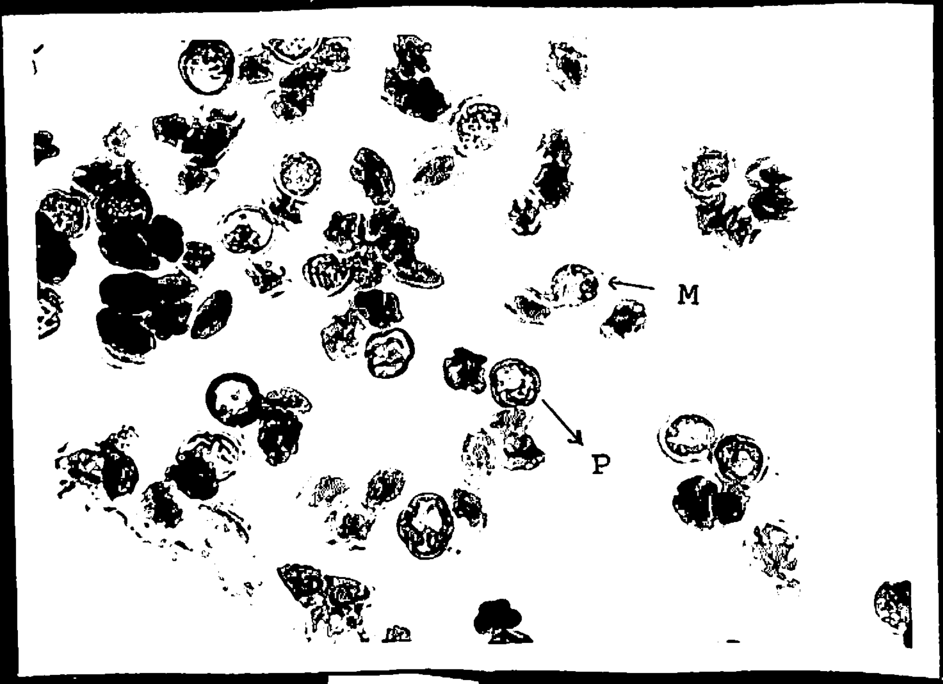
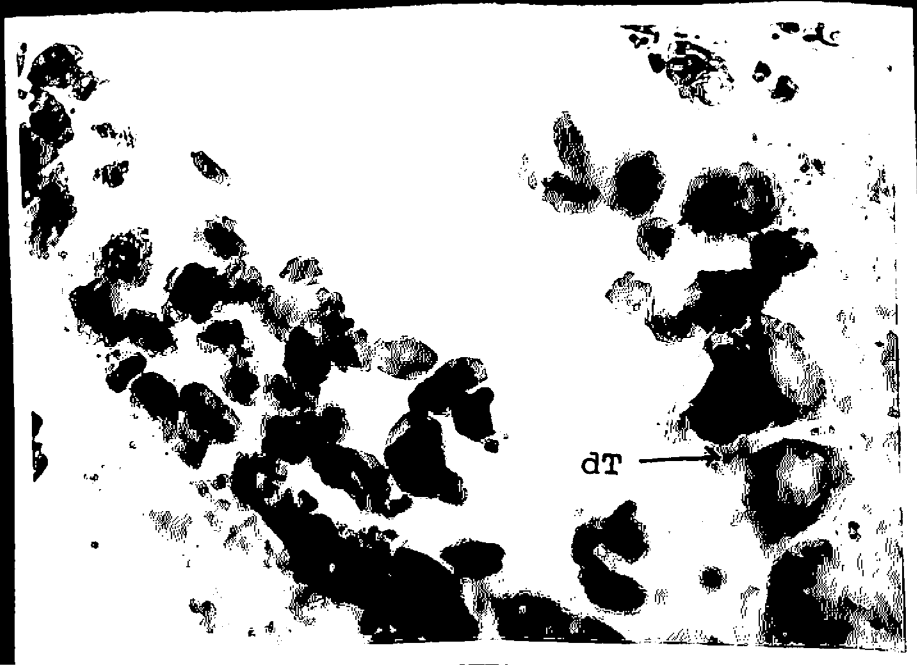


Fig. 2



disappearance with the result cavity was widened and filled with a large number of uninucleate microspores (Fig. 1). When bud attained a size of 0.65-0.70 cm anthers contain mature pollen grains. The anthers taken from the floral bud of Antirrhinum were cultured on three basal media namely, MS, DG and MN. The cytological preparations of cultured anthers revealed that anthers cultured at tetrad stage of development could not produce callus and the individual cells of tetrad group failed to get released themselves from the callose wall of tetrad. So this stage was not suitable for culturing.

Anthers cultuted at uninucleate microspore stage of development were highly responsive to the cultural conditions for androgenic callus formation. Histological preparation revealed that somatic tissue of anther remained inactive.

The anthers at mature stage were also unfit for culturing.

Establishment of callus

After 4 days of culturing of anthers on MS medium, the anthers increased in size and showed swelling. After one week of culturing microspores started callusing, the anthers dehisced longitudinally. At the beginning microspore divided into two equal cells (Fig. 4) followed by unequal divisions (Fig. 5) resulting into the callus formation.

Fig. 4 Showing initial division in the microspore x1200.

Fig. 5 Showing subsequent divisions in the microspore for callus formation x1200.

dM - dividing microspore.

Fig. 4

Fig. 6-8 Callus on MS medium (NAA 2 mg/1, kinetin 1 mg/1)

Fig. 6 14 days old callus on MS medium.

Fig. 7 Callus showing root initiation after 28 days of subculturing.

Fig. 8 Callus showing profused rooting after 56 days of subculturing.

Fig. 9 Callus on MS medium with NAA 8 mg/1, kinetin 1 mg/1 showing rooting.

R - Root.

Fig. 6

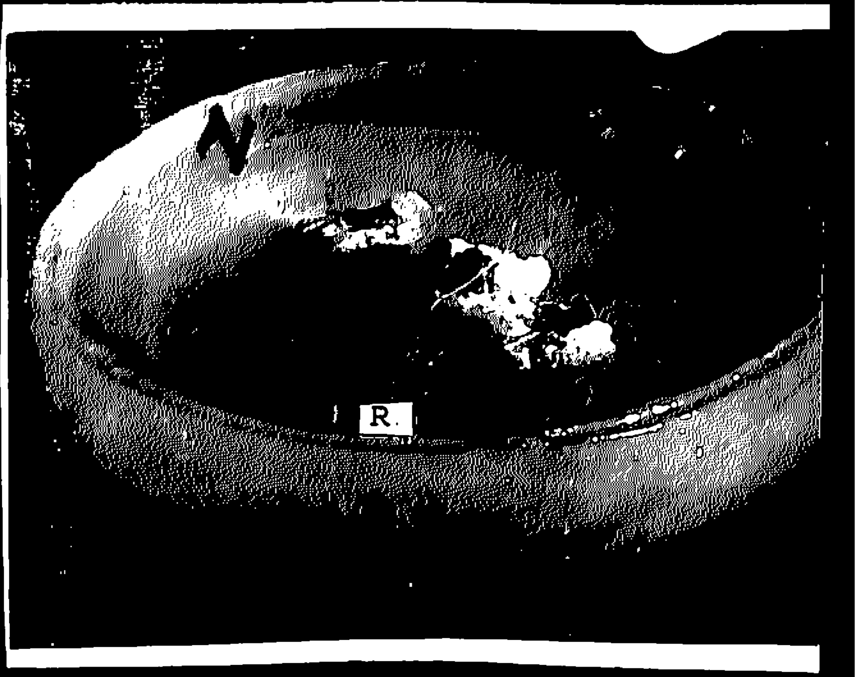
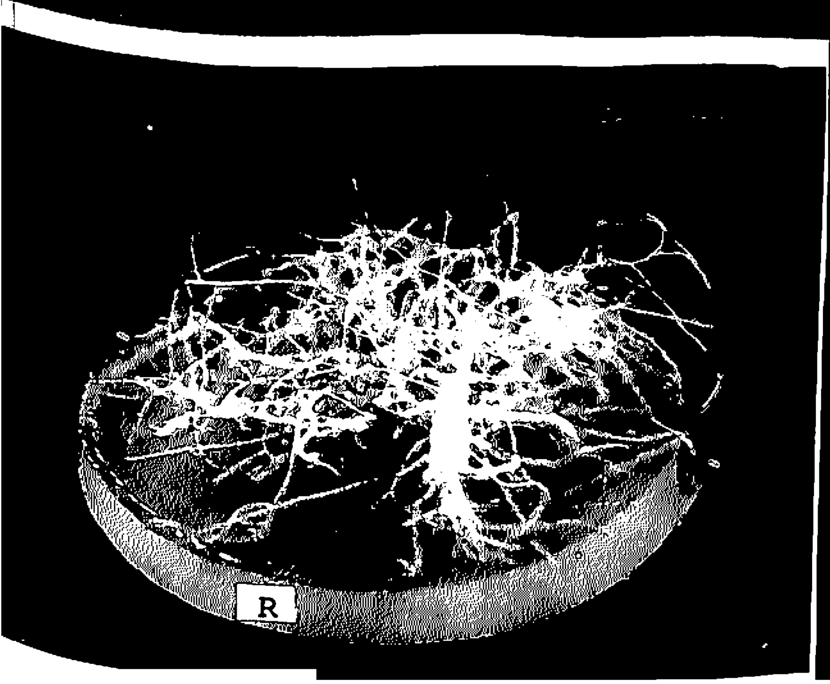


Fig. 7



Differentiation

Callus maintained on MS and DG medium was subcultured after 21 days of inoculation. When the cultures were kept in continuous dark root developed on MS medium containing NAA (2 mg/l) and kinetin (1 mg/l). Root initiation was first observed in this medium after 28 days of subculturing (Fig. 7). There was profused rooting on MS medium after 56 days of subculturing (Fig. 8). When concentration of NAA was increased in MS medium by keeping the kinetin concentration 1 mg/l the root~~ing~~ became more stout (Fig. 9). On DG medium, however, no rooting was observed after 28 days of subculturing (Fig. 10). After 56 days of subculturing very less rooting was observed on DG medium (Fig. 11). However, on MS and DG medium containing 2,4-D no differentiation was observed even in 50 days old callus (Figs. 12-14).

Morphogenetic studies

The study of various morphogenetic steps involved in its development and differentiation at successive stages of callus were made with the help of histological preparations.

The activity and growth of cells of different regions of tissue also differed substantially. The steps of development were classified into various stages, for the sake of convenience. At stage I the callus was found to consist~~s~~ of heterogeneous mass of cells varying in shape, size,

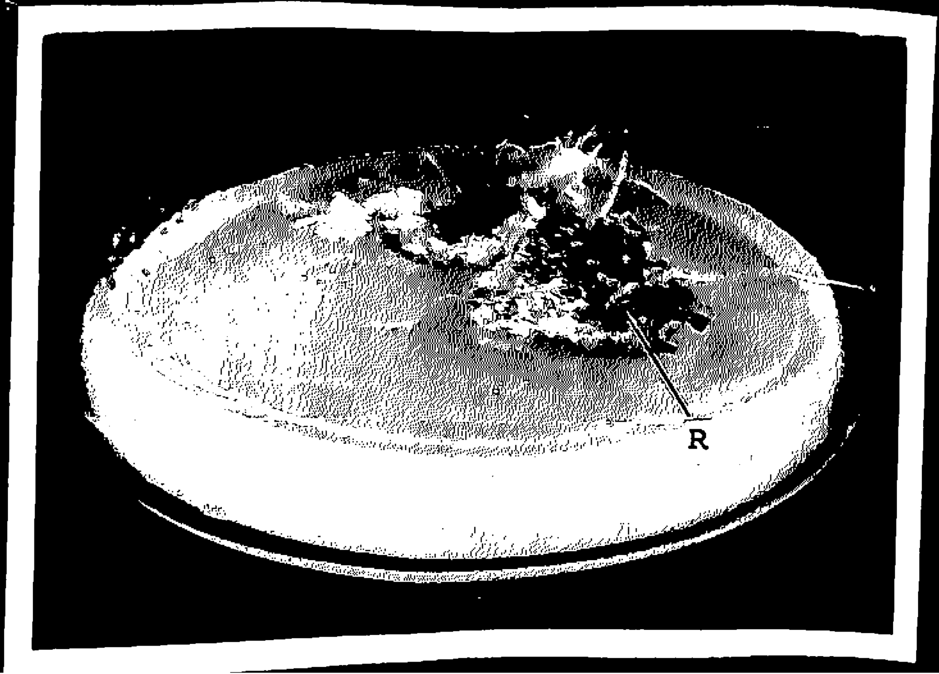
Fig. 10-11 Callus on DG medium (2 mg/1 NAA, 1 mg/1 kinetin)

Fig. 10 After 28 days of subculturing.

Fig. 11 After 56 days of subculturing.

R - Root.

Fig. 10



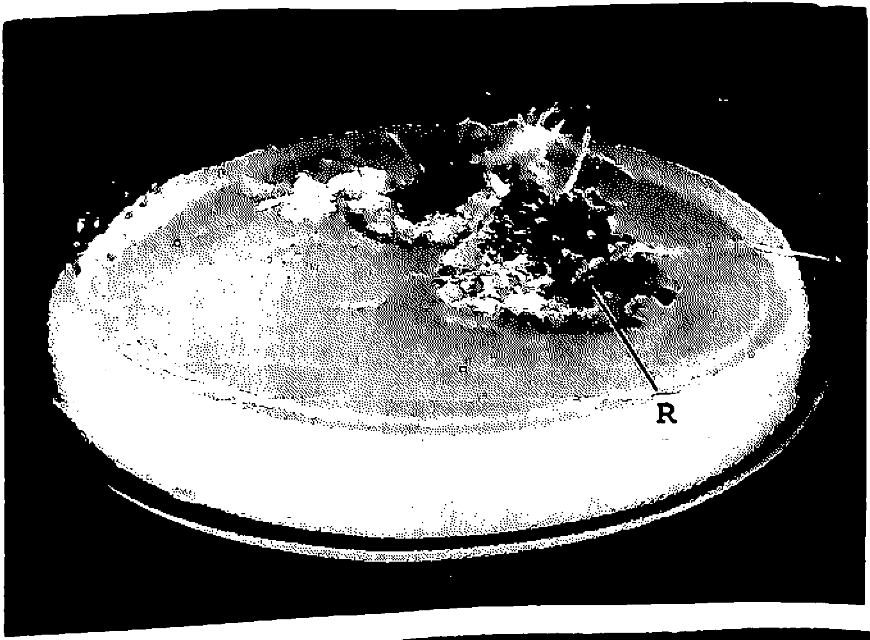

R

Fig. 12-14 Callus on MS and DG medium with 2,4-D (2 mg/l)

Fig. 12 25 days old callus on MS medium.

Fig. 13 50 days old callus on MS medium.

Fig. 14 50 days old callus on DG medium.

Fig. 12

Fig. 13

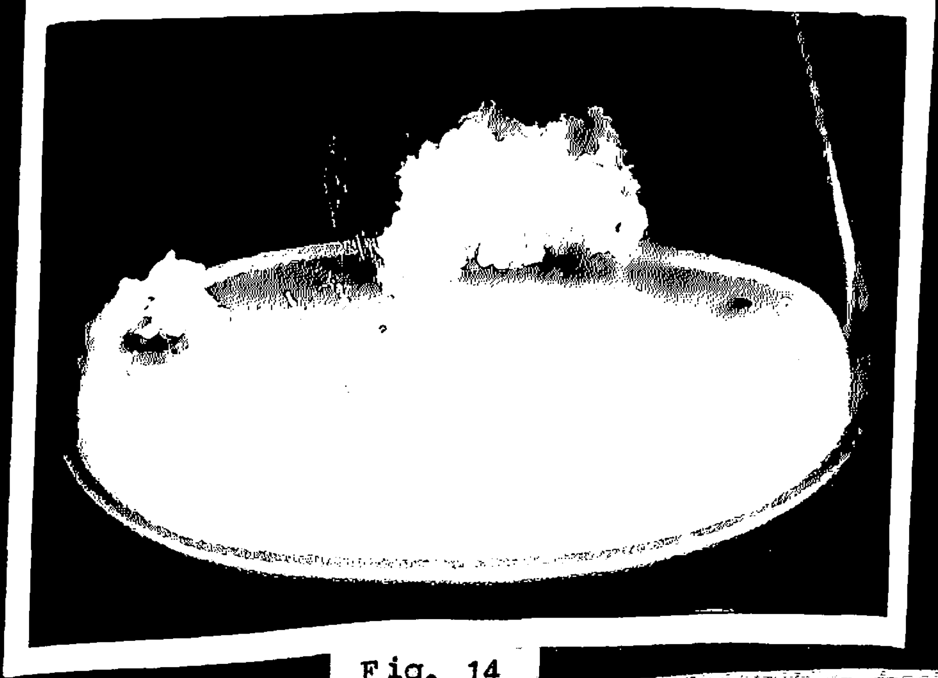


Fig. 14

construction of wall and contents. The callus showed a tendency of peripheral growth. The cells on the margin were smaller in size compar~~ing~~ed with the adjacent ones. They were compact, with dense cytoplasm, less vacuoles and conspicous nuclei.

A next step in development could be detected in somewhat older callus i.e. stage II (~~Fig.~~). This was marked by increasing cellular differentiation through expansion and maturation of cells. The positions where meristematic activity had prevailed, the development of tracheid was induced indiscriminately (Fig. 15) and later on, vessels were also formed (Fig. 16). The tracheids had mostly spiral thickenings.

At stage III of callus, such meristematic centres having cells with dense cytoplasm, less vacuoles and prominent nuclei were formed. Later on the cells of meristematic centre arranged themselves in a ring (Figs. ~~16,~~ 17 and 18). proceeding towards second step of differentiation. In the third step, vascular elements were formed in the meristematic centre and vascularized centres were formed.

In the beginning the vascular tissue was scattered but mostly arranged in interrupted ring. The vascular tissue later on formed a continuous pattern ~~(Fig.)~~. These structures gave rise to root or shoot. In the early stages, root or shoot primordia could not be detected but

Fig. 15 Showing formation of tracheids in the callus x 560.

Fig. 16 Showing tracheids and vessel the callus x 800.

Fig. 17 Showing formation of meristematic centre in the callus x 800.

Fig. 18 Showing meristematic centre prior to differentiation x 800.

T - tracheids
V - vessels
MC - meristematic centre.

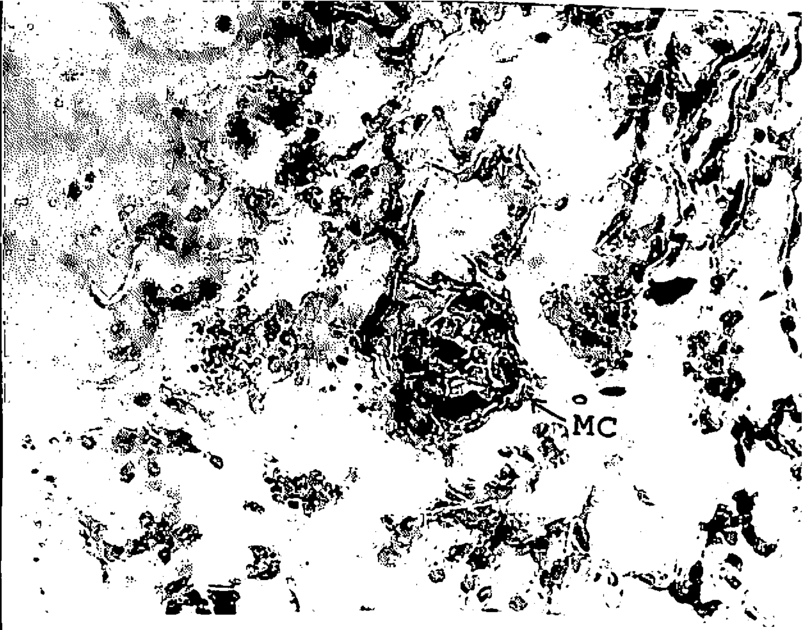
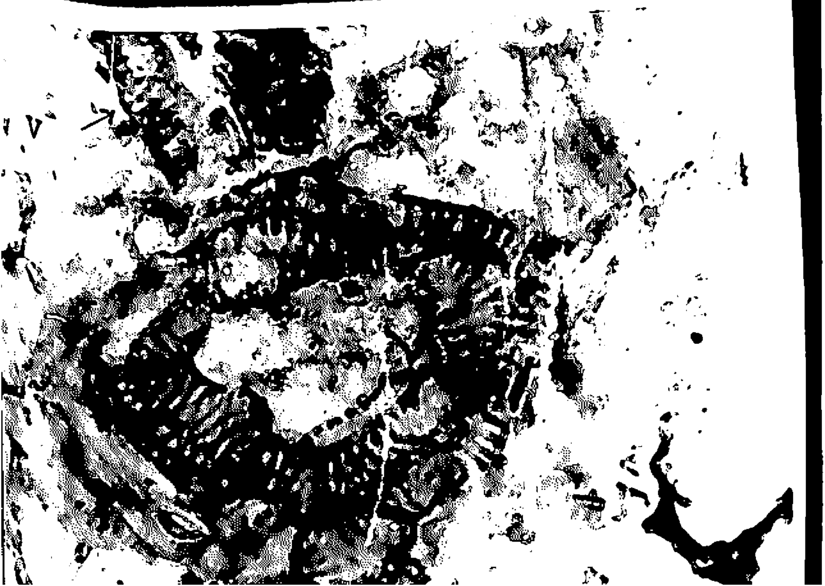


Fig. 15

Fig. 17



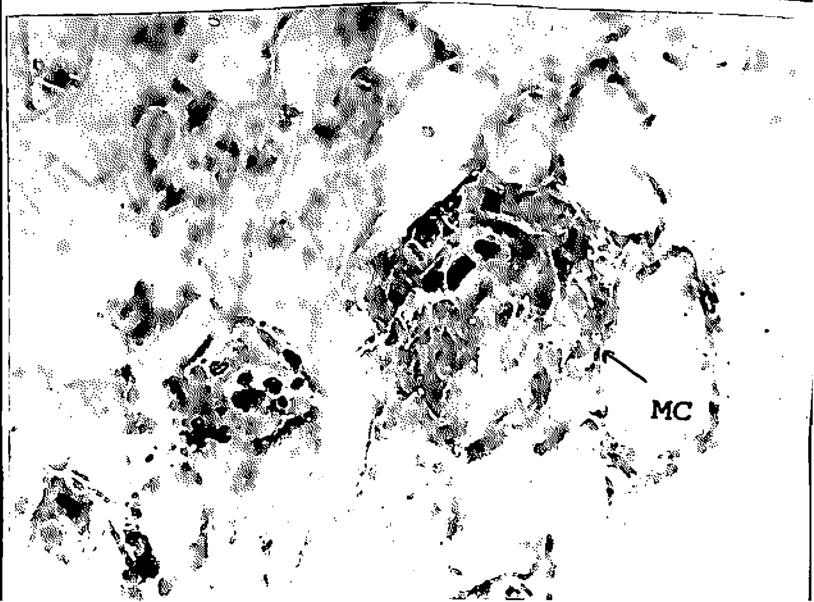


Fig. 19-21 Transverse section of callus showing RNA stain.

Fig. 19 Callus at Ist stage x 280.

Fig. 20 Callus at IInd stage showing prominence of meristematic centre x 800.

Fig. 21 Callus at IIIrd stage showing formation of vascular elements in meristematic centre x 280.

VE - Vascular elements
MC - Meristematic centre.

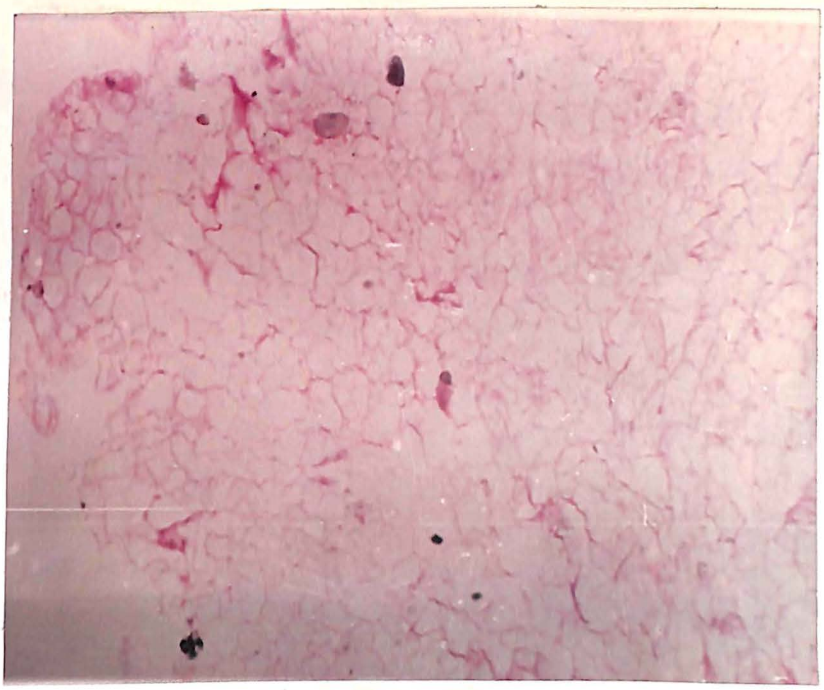


Fig.19

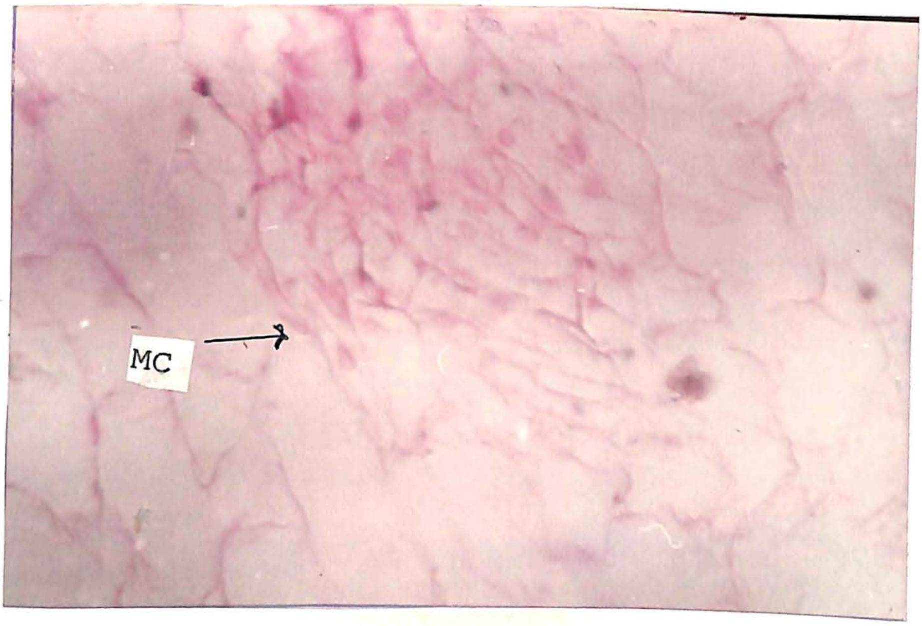


Fig. 20

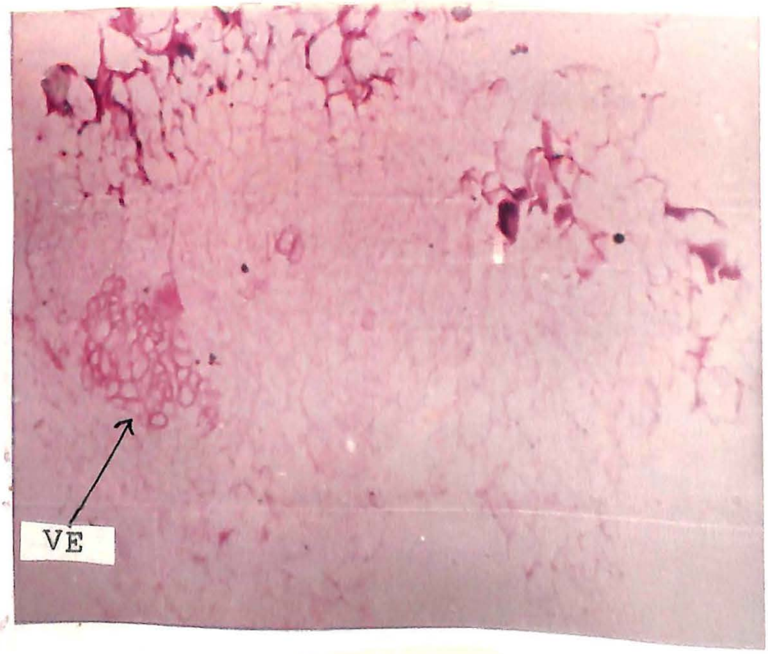


Fig. 21

Fig. 22-23 Transverse section of callus showing protein stain.

Fig. 22 Callus at Ist stage x 280.

Fig. 23 Callus at IInd stage showing prominence of meristematic centre x 800.

MC - Meristematic centre.

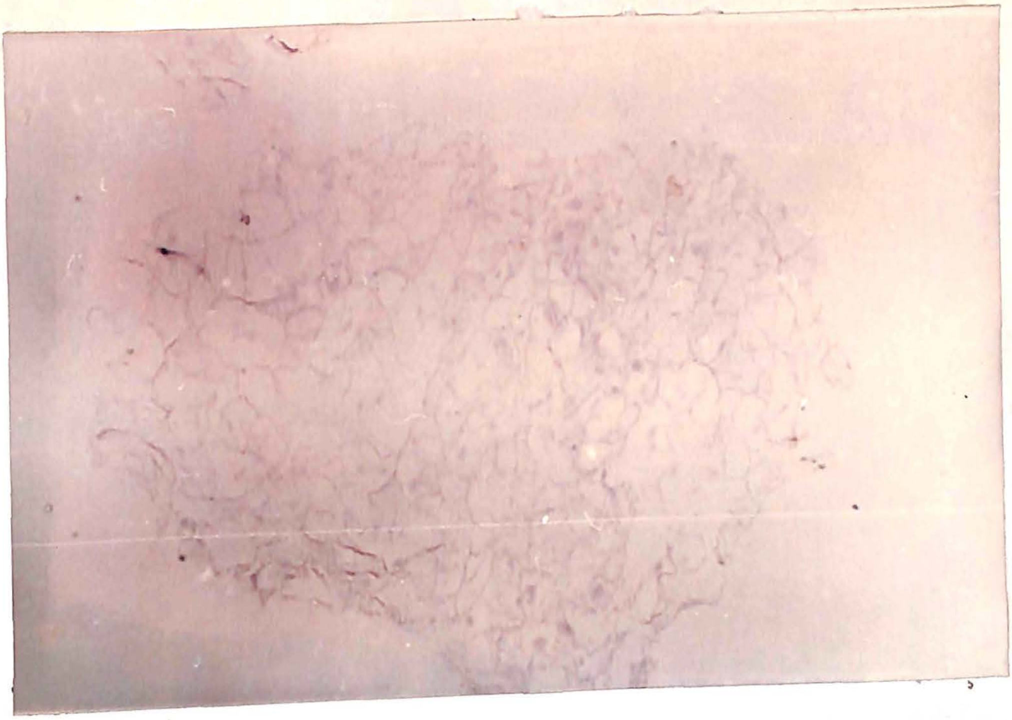


Fig. 22

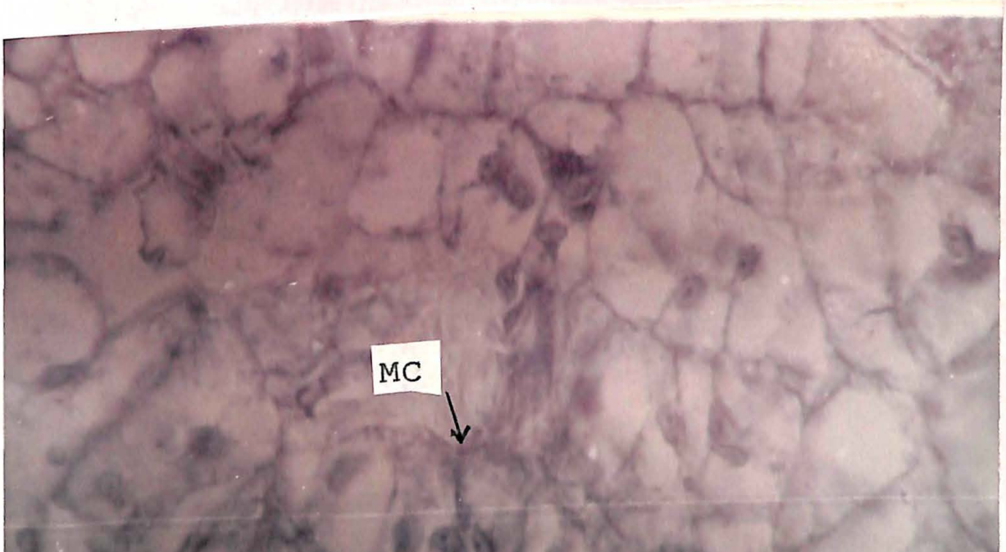


Fig. 23

further development of root confirmed that it was root meristem. The protoxylem at many places was found to be exarch -a feature which is found to be characteristic of root.

Histochemically, it has been found that concentration of RNA (Figs.19-21) protein (Figs.22-23) and starch is more in the meristemic centres of the callus as compared to the surrounding cells.

Metabolic changes

Soluble carbohydrates showed the tendency of continuous increase from stage I to stage III of callus on both MS and DG media, the increase being relatively more from stage II to stage III as compared to that from stage I to stage II (Fig. 24). However, content of total soluble carbohydrates was more at stage III on MS medium than on DG medium.

Starch content increased continuously after inoculation of Antirrhinum anther on MS medium. However, increase was more from stage I to stage II as compared to the same from stage II to stage III on MS medium. On the other hand, starch content was almost identical at stage I and stage II which thereafter increased tremendously on DG medium (Fig.24) but still values obtained were less as compared to those cultured on MS medium.

Fig. 24 Total soluble carbohydrates (mg g^{-1} fresh wt.) at different stages of development of callus, M, S, I denote CD at 5% LS for medium, stage and interaction, respectively.

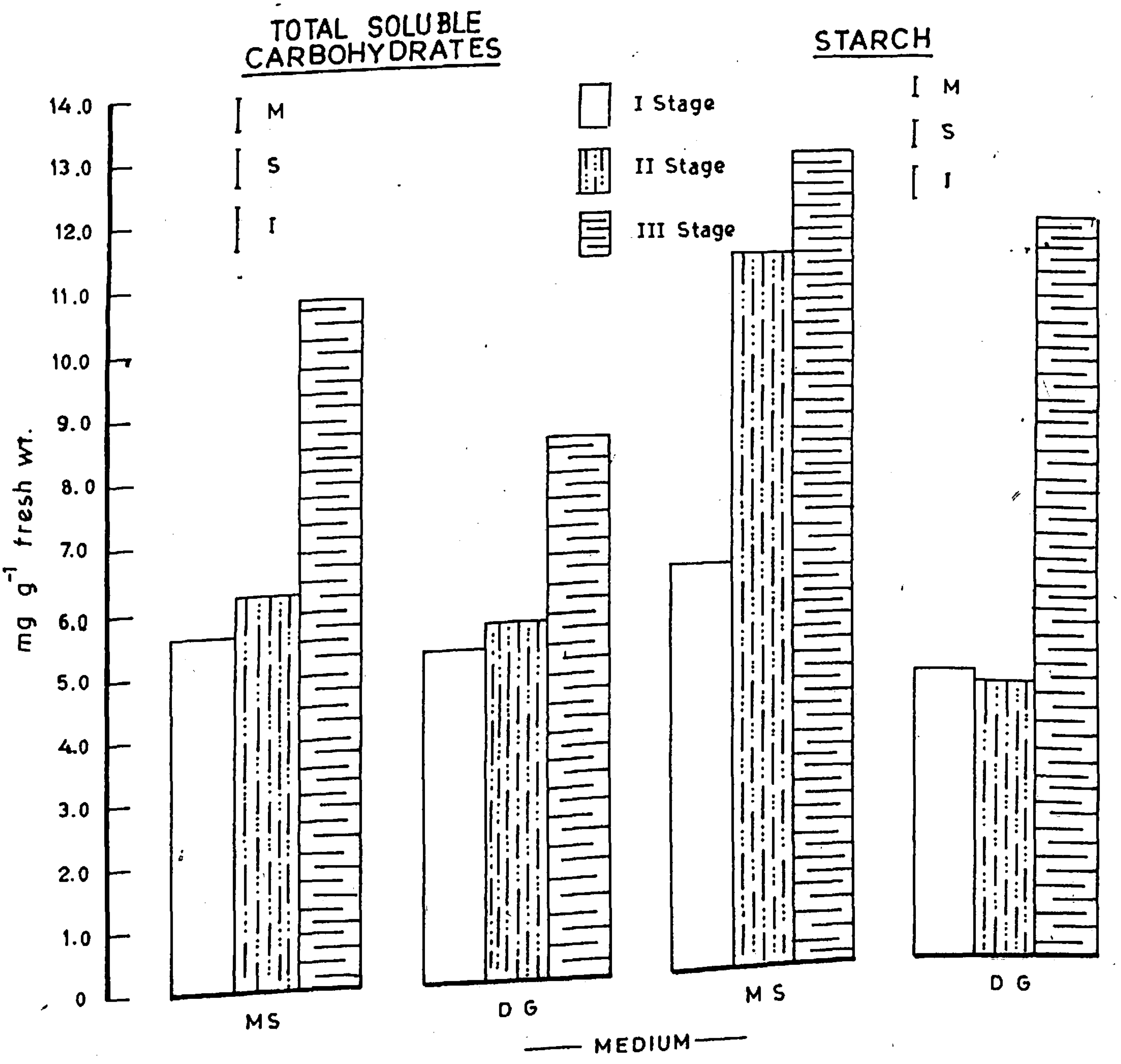

TOTAL SOLUBLE CARBOHYDRATES
STARCH
M
S
I
I Stage
II Stage
III Stage
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
mg g^{-1} fresh wt.
MS
D G
M S
D G
MEDIUM

Fig. 24

Fig. 25 Protein content (mg g^{-1} fresh wt.) at different stages of development of callus. M, S, I denote CD at 5% LS for medium, stage and interaction, respectively.

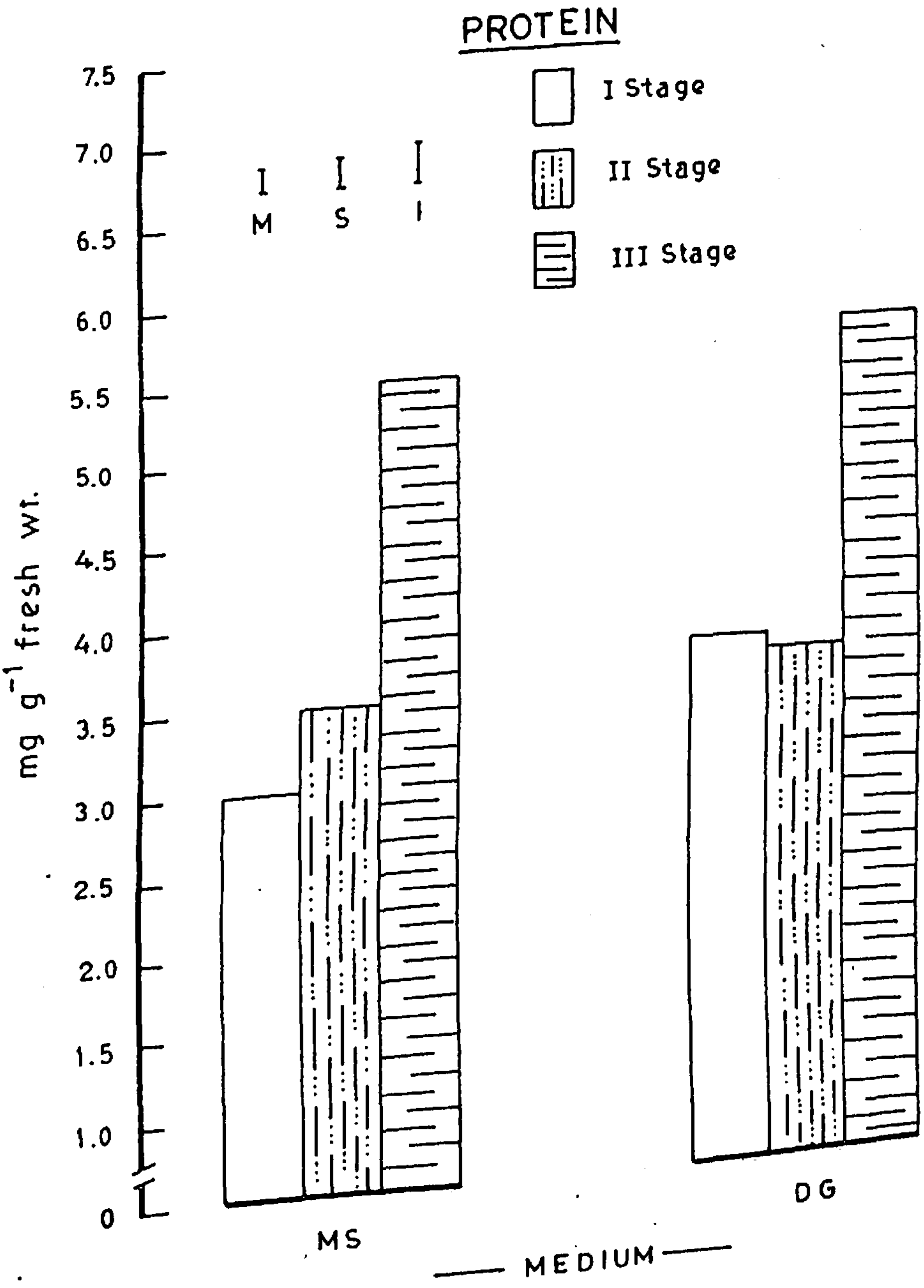

PROTEIN
I Stage
II Stage
III Stage
M
S
I
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0
mg g^{-1} fresh wt.
MS
DG
MEDIUM

Fig. 25

Fig. 26 RNA content (mg g^{-1} fresh wt.) at different stages of development of callus. M, S, I denote CD at 5% LS for medium, stage and interaction, respectively.

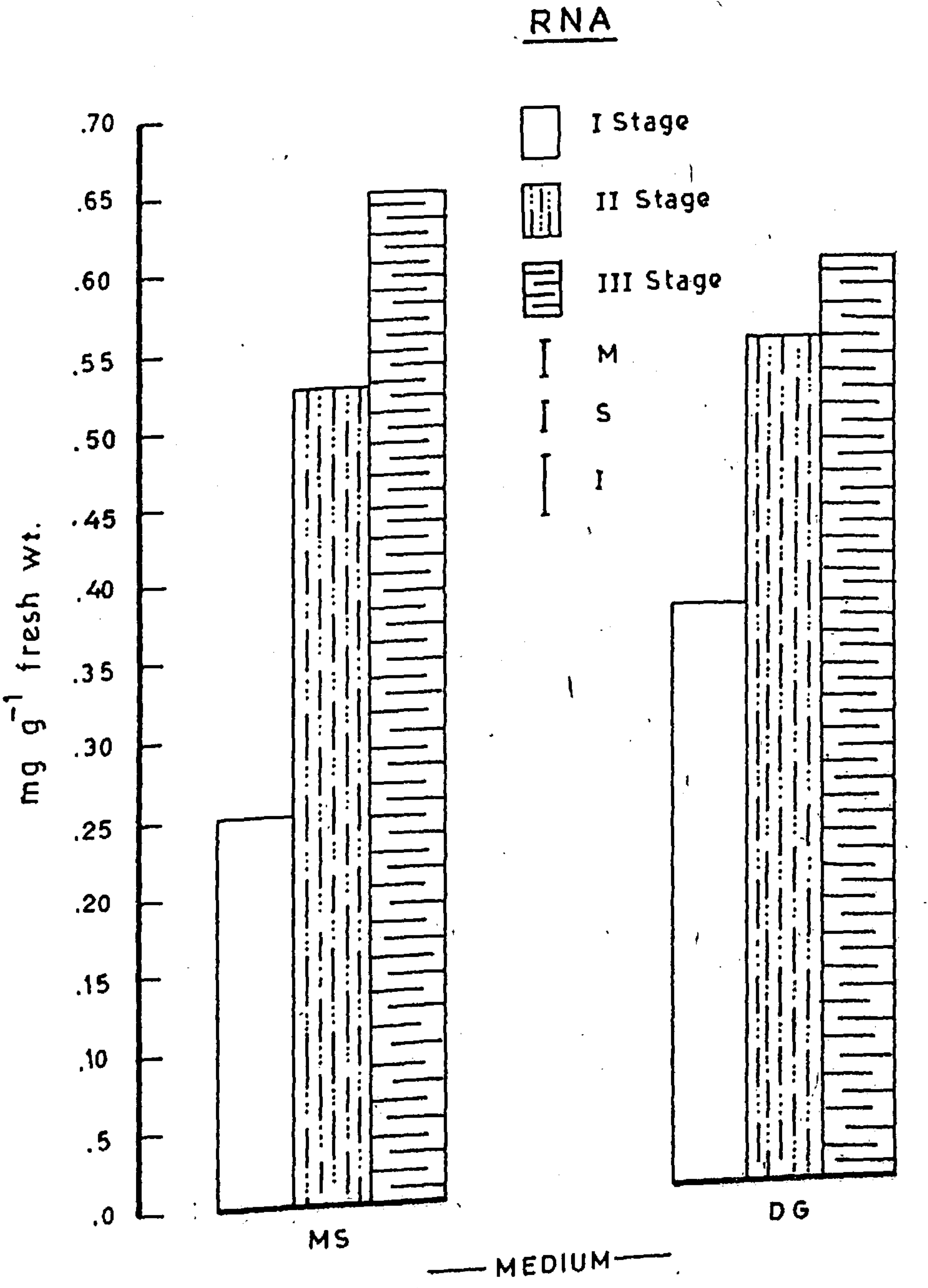

RNA
I Stage
II Stage
III Stage
M
S
I
mg g^{-1} fresh wt.
.70
.65
.60
.55
.50
.45
.40
.35
.30
.25
.20
.15
.10
.5
.0
MS
DG
MEDIUM

Fig. 26

Fig. 27 DNA content (ug g^{-1} fresh wt.) at different stages of development of callus. M, S, I denote CD at 5% LS for medium, stage and interaction, respectively.

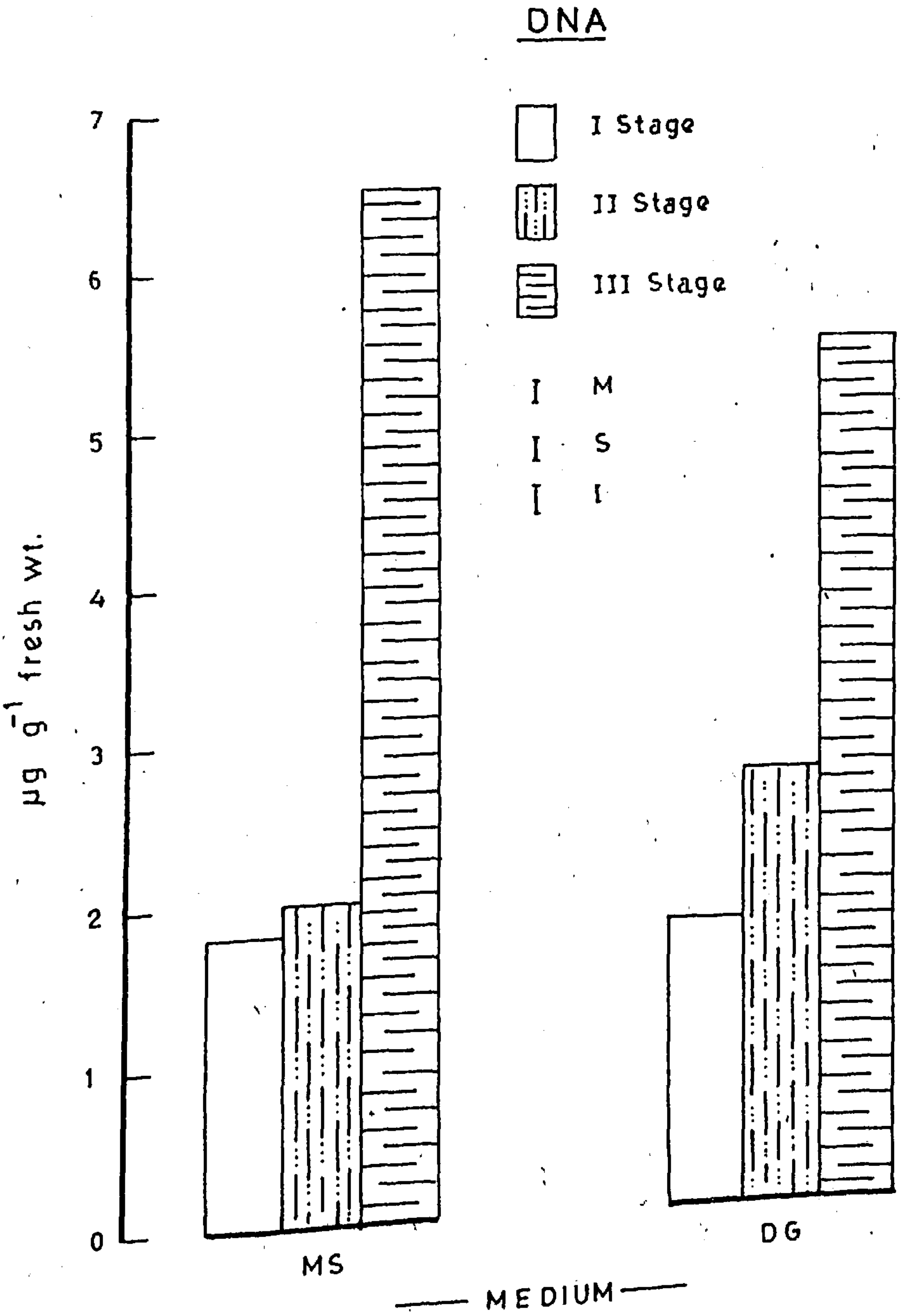

DNA
I Stage
II Stage
III Stage
I M
I S
I I
7
6
5
4
3
2
1
0
μg g^{-1} fresh wt.
MS
DG
MEDIUM

Fig. 27

Protein content of the callus cultured on both the media increased with the advancement of stage. Initially protein content was more in callus grown on DG medium than on MS medium. At stage II, protein content did not change significantly on DG medium but a marginal increase was evident on cultures grown on MS medium. Protein content at stage II was comparable for the callus samples drawn from the two media (Fig. 25).

After a week's inoculation of Antirrhinum anther from both the media, RNA content was more on DG medium than on MS medium. With the advancement of stage of callus, it was observed that the rate of increase in RNA on MS medium was more and final values obtained with MS medium were slightly more with respect to their counters on DG medium (Fig. 26).

On MS medium, DNA contents of the callus did not change with the advancement of stage I to stage II and then it shot up to the values which were more than three times of stage I. However, on DG medium there was continuous increase in concentration of DNA with the advancement of stage; the increase being more during the period between stage II and stage III than that between stage I and stage II (Fig. 27).

DISCUSSION

The present investigation on anther culture of Antirrhinum majus L. was undertaken with the purpose of designing a suitable medium for the production of androgenic callus and its regeneration (ii) to find out the histological and morphogenetic steps involved in androgenesis and (iii) to collect information on certain biochemical changes associated with morphogenesis.

It was found that the anthers with uninucleate microspores were highly responsive to culture media i.e. MS and DG with NAA (2 mg/l) and kinetin (1 mg/l) as compared to the other stages of development of sporogenous tissue i.e. tetrad stage and mature pollen stage. However, the NN medium was found to be unsuitable for achieving androgenesis in the species.

At the uninucleate microspores contained dense cytoplasm and a centrally placed nucleus. Exine layer was not fully developed with the result the cell could easily expand, an essential requirement for the initial divisions during callus initiation. Uninucleate microspores were found to be best for culturing in (ii) Lycopersicon esculentum Mill. (iii)(Varghese and Yadav, 1984; (i)Misra, 1978) and in(ii)Solanum melongena also.

A special advantage in culturing anthers at or after release of microspores from microspore tetrad is that the chances of producing callus from the wall layers including tapetum are minimal, as all other tissues in the anther other than the microspores become'senescent' at this stage.

Out of three media used, namely MS, DG and NN, MS medium was found to be superior to DG medium with NAA 2 mg/l and kinetin (1 mg/l) for callus induction. The response of anthers for callus initiation was 55 per cent in MS medium whereas only 30 per cent in DG medium. This difference may probably be attributed to the high content of sugar and other salts in MS medium.

The callus initiation took place through the division of the microspores. The first division of the microspore was transverse resulting into two equal cells. Subsequent divisions are unequal resulting into the formation of callus. Similar mode of development has been reported also in Triticum (Ouyang *et al.*, 1973).

Calli maintained on media containing NAA and kinetin were different in texture from the calli maintaoned on media supplemented with 2,4-D. The callus on media with NAA and kinetin was composed of compactly arranged cells while that in media with 2,4-D had loosely arranged cells. The morphological difference may be due to the difference in nature of various growth hormones.

Of the two media MS and DG, root initiation was observed only in MS medium after 28 days of subculturing whereas no such initiation was observed in DG medium after the above duration. Observation after 56 days of subculturing revealed that root formation is profuse in cultures maintained on MS medium while there was less rooting in cultures on DG medium.

Morphogenetic studies were carried out to understand the various steps associated with androgenesis. The callus consisted of heterogeneous mass of cells differing in shape, size and contents of the cells and also the construction of cell wall. The growth of the callus was through the activity of meristematic cells located at the margin. These marginal cells were distinguished from central cells by their dense cytoplasm, smaller size, conspicuous nuclei and lesser vacuoles thus assuming the characteristic of meristematic cells. After a definite period of marginal growth of the meristem some of the meristematic cells located at the periphery became inactive and cell walls stained brown with safranin indicating the presence of suberin in the cell walls. The further growth of callus was irregular. It is difficult to explain why some groups of meristematic cells located at the margins became inactive while others continue to be active.

The differentiation of the callus was an essential pre-requisite for organogenesis. The visual sign of differentiation was the development of radially elongated cells resembling procambial tissue present deeper in the callus. The induction of tracheids took place from some of the cells distributed discriminately. The amount of tracheids was more when the media contained NAA and kinetin in the medium. The role of NAA and BAP in tracheid formation was reported in tuber culture of Helianthus tuberosum by Minocha and Halperin (1974,1976). Bergmann (1966) also reported that kinetin increased the general level of lignification and amount of tracheids in callus cultures. Tracheidal elements were mostly spirally thickened. The formation of vessels and phloem followed thereafter.

At the initial stage of differentiation some of the cells in groups attained meristematic activity, forming the centres of meristematic growth. These centres either remained undifferentiated or or got transformed into vascularized nodules. Such vascularised nodules developed to primordia for roots in the species. Gautheret (1959) also pointed that formation of growth centres was a common feature in rapidly growing callus cultures. The significance of meristematic nodules in callus cultures was also stressed by Torrey (1966).

The biochemical estimation of the growing androgenic callus revealed that starch contents increased from stage I to stage III. This evidently proposes that the starch synthesis reach to a maximum in the callus prior to root initiation. The present observation is in conformity with the views of Brossard (1977) who reported that the accumulation of starch in root forming callus of *Crepis capillaris*. In tobacco var. White burley a slight increase in starch was observed under root forming conditions (Misra *et al*., 1984). Similarly, soluble sugars also increased with the development of callus. In tobacco var. White burley increase in soluble sugars was also observed under root~~ing~~ forming conditions. Increased levels of sugars may also be due to increased uptake from the medium and degradation of sucrose by enzymes such as invertase and sucrose synthetase which showed increased activity during organogenesis (Thorpe and Meier, 1973a). Higher development through cellular metabolism may partially account for osmotic requirement for organogenesis (Brown et al., 1979).

Protein and RNA contents also increased during development of callus. In root forming callus total protein content was the maximum. Similar results were obtained by Guzman et al. (1971) and Chowdhury (1978). The present observation adduces support to the suggestion of Hasagawa et al. (1977a) that organ forming callus can be distinguished from control through the observation on protein content. DNA contents also increased in the growing callus significantly confirming the reports of Bavrina et al. (1970) and Kovacs et al. (1971).

Histochemical studies revealed that locations from where organogenesis took place in callus stained more for RNA, protein and starch as compared to the surrounding cells (Thorpe and Murashige, 1970). These observations suggest that these chemicals also play a predominant role in the process of organogenesis.

SUMMARY

Anthers of Antirrhinum majus L. were cultured in different nutritive media with the following objectives:

(i) to find out the right stage of anthers for inoculation to obtain androgenic callus,

(ii) to select the best nutritive media for the induction of callus, its growth and differentiation,

(iii) to study the various morphogenetic steps involved during callus formation and differentiation, and

(iv) to study qualitative and quantitative changes in various metabolites associated with androgenesis.

Anthers taken from the bud of 0.55-0.65 cm long were found to be suitable for culturing. At this stage anthers contained uninucleate microspores.

Out of three media used for callus induction viz., Murashige and Skoog's medium (MS), Nitsch and Nitsch's medium (NN) and Gresshoff and Doy's medium (DG), each supplemented with 2 mg/l NAA and kinetin 1 mg/l and separately with 2,4-D (2 mg/l) also. Percentage of callusing was 55 % in MS medium, 30% in DG medium whereas zero per cent in Nitsch medium. So out of these media used, MS proved to be the best since it showed an early response with enhanced proliferation of microspore.

In the beginning micropores had undergone transverse division to form two equal cells. Then both of these cells divided unequally to form callus. The established callus, when subcultured on various media, initiated rooting after 28 days on MS medium supplemented with NAA (2mg/l) and kinetin (1 mg/l) while no rooting was observed in DG medium after the above mentioned duration with same ratio of NAA and kinetin. After 56 days of subculturing profuse rooting was observed in MS medium (Naa/kinetin 2:1) whereas less rooting on DG medium (NAA (2 mg/l) and kinetin (1 mg/l).

Morphogenetic studies revealed that during early stages of development peripheral growth of callus was due to localized meristematic activity in the callus tissue.

The combination of NAA and kinetin enhanced the tracheid formation while no tracheid formation was observed with 2,4-D as growth regulator. Meristematic activity is marked by some of the individual cell or group of cells which differentiated themselves from the surrounding cells resulting into the formation of meristematic nodule. This was the first step towards differentiation. A large number of such meristematic nodules were found to be scattered throughout the callus cells. Later on, complete vascularization of these meristematic centres took place to initiate the organ formation.

Biochemical estimations revealed that total soluble carbohydrates and starch contents increased at the time of organogenesis. Similarly, proteins along with RNA and DNA increased with the development of callus as number of cells per unit area also increased. This increase in RNA, protein and starch has also been confirmed histochemically.

APPENDIX I

Murashige and Skoog's medium

Major elements	mg/l
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.H_2O$	440
$MgSO_4$	370
KH_2PO_4	170

Minor elements	mg/l
$MnSO_4$	22.3
KI	0.83
$CaCl_2$	0.025
$CuSO_4$	8.6
H_3BO_3	6.2
Na_2MoO_4	0.25

Vitamins	mg/l
Glycine	2.0
Myo-inositol	100.0
Thiamine HCl	0.1
Pyridoxine-HCl	0.5
Nicotinic acid	0.5
Na_2EDTA	37.3
$FeSO_4.7H_2O$	27.8
Sucrose	30,000
pH	5.8
Agar-Agar (Difco)	8,000

5 ml/l solution of $Na_2FeEDTA$ (dissodium ethylene diamine tetracetate and $FeSO_4.7H_2$) was added.

APPENDIX II

Gresshoff and Doy's medium

Major elements	mg/l
KH_2PO_4	300
KNO_3	1000
NH_4NO_3	1000
$Ca(NO_3)_2$	347
$MgSO_4$	35
KCl	65
Minor elements	**mg/l**
KI	0.8
$MnSO_4$	10.0
H_3BO_3	3.0
$ZnSO_4$	3.0
Na_2MoO_4	0.25
$CaCl_2$	0.25
Vitamins	**mg/l**
Nicotinic acid	0.1
Thiamine-HCl	1.0
Pyridoxine-HCl	0.1
Glycine	0.4
Myo-inositol	100
Na_2EDTA	37.3
$FeSO_4 \cdot 7H_2O$	27.8
Sucrose	20,000
pH	5.8
Agar-Agar (Difco)	8,000

$Na_2FeEDTA$ added 5 ml/l.

APPENDIX III

Nitsch and Nitsch's medium

Major elements	mg/l
KNO_3	950
NH_4NO_3	720
$MgSO_4.H_2O$	180
$CaCl_2.H_2O$	166
KH_2PO_4	68

Minor elements	mg/l
$MnSO_4.4H_2O$	25
H_3BO_3	10
$ZnSO_4.7H_2O$	10
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4$	0.025

Organic components	mg/l
Glycine	2
Myo-inositol	100
Nicotinic acid	5
Pyridoxine-HCl	0.5
Thiamine HCl	0.5
Biotin	0.5
Folic acid	0.5
Na_2EDTA	5 ml/1 g stock soluton.
Sucrose	20,000
Agar-Agar(Difco)	8,000
pH	5.5

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