

PROTOPLAST ISOLATION AND REGENERATION STUDIES IN SELECTED SOLANACEOUS CROPS

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**DEPARTMENT OF CROP PHYSIOLOGY
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PROTOPLAST ISOLATION AND REGENERATION STUDIES IN SELECTED SOLANACEAOUS CROPS

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Affectionately Dedicated to
My Beloved Parents

DEPARTMENT OF CROP PHYSIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE

CERTIFICATE

This is to certify that the thesis entitled "Protoplast isolation and regeneration studies in selected solanaceous crops" submitted in partial fulfilment of the requirement for the degree of DOCTOR OF PHILOSOPHY(Agriculture) in Crop Physiology to the University of Agricultural Sciences, Bangalore is a record of research work carried out by Mr. PRAKASH, A.H. under my guidance and supervision and that no other degree, diploma, associateship, fellowship or other similar titles.

Bangalore
Feb. 1996



(K. Sankara Rao)
Major advisor

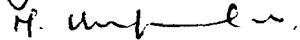
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INTRODUCTION

I. INTRODUCTION:

For centuries man has been trying to improve the quality and yield of cultivated plants by conventional breeding and selection. The outcome of these efforts have led to the "Green revolution" of 1960's.

Although the history of plant tissue culture goes back to the turn of the century (Haberlandt, 1902), it is only since 1950's that practical applications of this science are being considered. Plant tissue culture uses many different techniques viz. callus culture, anther culture, embryo culture, meristem culture, some of which have long been functionally applied in agriculture and horticulture. A good example is meristem tip culture of cardomum for virus eradication and subsequent micropropagation of the virus free propagules.

The word Plant Tissue Culture denotes generally all cells, tissues and organs and employs, the basic dogma of totipotency. The technique of tissue culture involves the separation of a cell, tissue or organ from a plant under aseptic conditions and growing it in an artificial medium in a suitable container under controlled environmental conditions.

The aseptic culture of plant cells has emerged in recent years as a powerful tool not only for the study of cell differentiation, but also for plant improvement and agriculture. Among the fast emerging areas of

biotechnology, Protoplast culture is one such important area to work with.

Protoplasts are defined as cells with cell wall stripped off by mechanical or enzymatic process and removed from the proximity of their neighbouring cells. The protoplasts are also referred as the spherical plasmolysed content of a plant cell enclosed in the plasmalemma and set free of the covering rigid cell wall. The naked protoplasts so obtained constitute an ideal "free cell" developmental system because protoplasts as separate entities are capable of reforming cell wall and regenerate into a whole plant. The advantage of obtaining clones, and large scale propagation of desired plants become more efficient.

The isolated plant protoplast is a single cell, bounded by the plasmalemma and when first formed, containing all the cell components, although being separated completely from its cell wall. From a physiological view point, however, the protoplasts cannot be regarded simply as a cell lacking wall, since the mechanism of isolation, in conjunction with environmental factor, undoubtedly influence its metabolism and elicit subtle ultrastructural changes. The absence of a functional cell wall affects the permeability of the cell membrane and lead to a general leakage of solutes from the protoplast.

The isolated protoplasts are dynamic bodies in a state of flux and yield to various genetic manipulation such as somatic hybridization, transformation and organelle transfer.

Protoplasts are rapidly gaining recognition as an important research tool in a variety of developmental, physiological and biochemical investigations; some important application in cell biology are

Method

Application

Protoplast isolation

- 1) Structure and chemistry of the plasmamembrane (Burgess and Linstead, 1977)
- 2) Cytoplasmic organelles associated with the plasmamembrane (van der Valk and Fowke, 1981)
- 3) Uptake and membrane transport by protoplast (Fowke and Gamborg, 1980)
- 4) Primary metabolism (Photosynthetic parenchyma) (Galun, 1981)
- 5) Secondary metabolism (Idioblasts) (Saunders and Conn, 1978)
- 6) Organelle isolation
 - i- Nuclei (Lorz and Potrykus, 1978)
 - ii- Chloroplast (Quail, 1979)
 - iii- Vacuoles (Nishimura and Beevers, 1979)

II) Protoplast culture

- 1) Cell wall formation (Fowke and Gamborg, 1980)
- 2) Plant regeneration (Vasil and Vasil, 1980)

III) Protoplast fusion

- 1) Plasmamembrane interaction (Fowke et al., 1977)
- 2) Behaviour of organelles in heterokaryocytes and hybrid cells (Fowke and Gamborg, 1980)
- 3) Cell cycle parameter (Szabados and Dudits, 1980)

To Study the basic processes on permeability, transport of ions, solutes, photosynthates, mechanism of action of plant hormones, phytochrome action and maintenance of totipotency, the first step is to obtain good, large quantity of viable protoplasts repeatedly.

Once regeneration of protoplasts are worked out, transformation of plant cells can be tried for crop improvement with Ti plasmid transformation or intra/interspecific hybridization by protoplast fusion. By these methods it is also possible to improve the nutritional value of crop, either by improving the photosynthetic efficiency or by modifying the quality of the food component like storage protein and disease resistance.

Plant regeneration from protoplasts has been viewed as a phenomenon restricted to *family solanaceae*. Nearly 38 solanaceous species have been regenerated from

cultured protoplasts. These include species of 17 Nicotiana, 6 Petunia and 6 Solanum. While most of the species have no economic value, some economically important crops (like tobacco, potato and egg plant) and ornamentals (Petunia, Salpiglossis and Nicotiana) have been regenerated from protoplasts quite easily.

Unfortunately, even among the Solanaceae, where most efforts has been diverted, only few cultivars of tomato and Bell pepper have been regenerated from protoplasts repeatedly. Most reports on plant regeneration, detailed experiments on composition of medium, donor tissue status and environmental conditions are not completely understood. Hence generalised procedures for isolation and regeneration are not available on these crops.

In Solanaceae, the flower is bisexual, regular or zygomorphic, hypogynous, sepal persists even after enlargement of fruits. Leaves are generally alternate in the vegetative parts and paired in the flowering parts. Fruit is a berry or capsule. Three genera considered for the work include Capsicum having 31 species widely cultivated for fleshy berried fruit, Lycopersicon with 9 species and Nicotiana with 66 species. Eventhough all the three belongs to same family Solanaceae, there has not been a single standardized method by which all these three crops could

be efficiently cultured. Even to develop interspecific or intergeneric crosses by protoplast fusion and compatability of species, it is necessary to have an efficient regeneration procedure.

Taking into consideration, the lack of a single standardized method of regeneration in vitro for three species, the present study was undertaken to find an efficient and also a general procedure of protoplast isolation and culture for the three crop species with the following objectives :

- i) Identification and preparation of explant suited for enzymatic digestion of cell wall
- ii) Standardization of incubation medium for enzyme digestion of cell wall and optimization of conditions for release of greater number of protoplasts, and
- iii) Testing the viability and callus forming capacity of protoplasts by culturing in vitro wherein media constituents and environmental conditions are standardized
- iv) Testing totipotency of protoplast derived calli.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

A distinguishing feature of the higher plant cell is that it possesses a rigid wall which forms a formidable physical boundary but does not represent a physiological barrier. It offers support and prevents the outer membranes against changing osmotic pressures and prohibit entry of microorganisms. Since it interferes with most cellular genetic modifications, a great deal of effort has been directed towards isolation of plant protoplasts.

Hanstein (1880) first used the term Protoplast (Protos meaning first, platos meaning being formed). Plant protoplasts are obtained either from intact tissues such as root tips, coleoptiles, epidermis and leaf mesophyll, or from calli and cell suspension cultures.

METHOD OF ISOLATION:

Initially protoplasts were isolated by mechanical method where the plant tissues are plasmolysed and the cell wall was cut and protoplasts were instantaneously released (Hanstein, 1880). Klercker (1892) experimented on water warrior (Stratiotes aloides), and observed the extrusion of protoplasts. Later, attempts were made to refine the technique and improve protoplast yield (Rechinger, 1893; Haberlandt, 1902).

New callus tissues obtained from expanding leaves of Saintpaulia ionantha (Bilkey and Cocking, 1982), had thin cell wall due to controlled conditions. The protoplasts were isolated by gently teasing the callus tissues apart in liquid culture medium with dissecting needle (Bilkey and Cocking, 1982). This can be used in studies where deleterious effects of the wall degrading enzymes have to be avoided.

Due to some limitation in mechanical isolation, alternate methods were tried. The use of cell wall degrading enzymes was soon recognised as a preferred method to release large number of uniform plant protoplasts. Cocking (1960), first demonstrated the enzymatic isolation of protoplasts from root tips of tomato (Lycopersicon esculentum) by using concentrated solution of Myrothecium verrucosa cellulase. This method was widely adapted by large number of workers as it facilitated use of any tissue and obtaining large number of uniform protoplasts with less osmotic shock and shrinkage (Ruesink, 1971). But Patnaik et al., (1982) reported that cell wall degrading enzymes are toxic to varying degrees and might affect the physiological process of the cells.

Later combinations of cellulose and pectin degrading enzymes were used to dissolve middle lamella and cell wall. Cellulase (Onozuka R-10), partially purified from the mold Trichoderma reesei (Patil et al.,

1994; Sankara Rao and Prakash, 1995) and Macerozyme R-10 obtained from fungi *Rhizopus* (Montagno et al., 1991) are commonly used enzymes (Ruesink, 1980). The cellulase appears to be a complex with multiple distinct activities. Two components have been separated using column chromatography. C₁ component is essentially a chain separating enzyme, while the C_x is responsible for subsequent depolarization to glucose. The two components act synergistically to digest cellulose (Selby, 1973).

A hemicellulase, Rhozyme-HP-150 obtained from *Aspergillus niger* is used for recalcitrant tissues (Fowke et al., 1973; Kao and Michayluk, 1980). There are many other potential enzymes used for protoplast isolation such as: Pectolyase Y-23 which in combination with cellulase, release tobacco protoplasts within 25 minutes (Nagata and Ishii, 1979); Helicase, derived from snails, in combination with macerase and cellulase release protoplasts from potato tubers (Lorenzini, 1973), Similarly, Zymolase, a crude preparation from *Arthrobacter luteus*, is effective in releasing protoplasts from pollen tetrads (Wakasa, 1973).

Plant protoplasts isolated by enzymes, when cultured under appropriate conditions regenerate cell wall and some of the cells thus formed have the

potential for cell division, callus formation and regeneration of entire plant. They may also lose the viability during the process of enzymatic isolation, as most of the commercial enzyme preparation contain toxic substances like ribonucleases, proteases, lipases and various other enzymes, phenolics and salts (Eriksson, 1977; Cocking, 1972). Tseng and Mount (1974), observed that purified protease and phospholipase from culture filtrate of Erwinia carotovora could lyse the protoplasts of cucumber.

There are reports of improved protoplast yield and viability using desalted enzyme preparation (Kao et al., 1971; Vasil et al., 1975). Schenk and Hilderbrandt (1969), purified commercial cellulase to eliminate toxic compounds but very highly purified cellulase were less effective in the release of protoplasts (Vasil and Vasil, 1980; Eriksson, 1985). Partial purification of enzymes by dialysis enhanced division of protoplasts of carrot suspension cultures (Slabas et al., 1980). Similarly BioGel (Santos et al., 1980; Arcioni et al., 1982) purified enzymes were essential for colony formation from medicago mesophyll protoplasts.

The concentration of the enzymes required depends on a number of factors - old cell suspensions yield very little or no protoplast at all, whereas young cultures, especially embryogenic ones, are easily vulnerable for protoplast formation as observed in rice (Abdullah et

al., 1986). Similarly, in vitro grown plants (Bajaj, 1972), young tissue and explants such as root tips (Xu et al., 1982), hypocotyl (Glimelius et al., 1974), cotyledon (hammatt et al., 1987) and shoots (Russell and McCown, 1986) require low concentration of enzymes and a relatively short period of incubation as compared to large leaves from old or mature plant parts.

Enzymatic isolation of plant protoplasts can be performed in two different ways

- 1) **One step process:** where tissues are subjected to a mixture of enzymes including cellulase and macerozyme (Power and Cocking, 1969; Patil et al., 1994). This method is frequently used as it is easy to handle cultures. However, mixture of enzymes are not effective in all situations, as in Avena coleoptile, the pectinase inhibits the release of protoplasts by cellulase (Ruesink and Thimann, 1966).

- 2) **Two step/ Sequential method:** where the tissues are first treated by pectinase/macerozyme which releases individual cells by degrading the middle lamella (Takebe et al., 1968; Nagata and Takebe, 1970). The more or less freed cells are then treated with cellulase which releases the protoplast. Eventhough it is a tedious process, it reduces the negative effects of long exposure to cell wall degrading enzymes.

OSMOTIC CONDITIONS:

Protoplast released directly into basal cell culture medium will burst. Hence, the pressure that is mechanically supported by the plant cell wall must be replaced with an appropriate osmotic pressure. The osmotic pressure within the cell and media must be balanced or even transfer of cells to plasmolysing solution will induce stress on the plant cells. It has been observed that when osmotic pressure is too high, metabolism and growth are impaired as observed by reduced amino acid uptake across the plasma membrane (Ruesink, 1978) and reduced cell wall regeneration (Pearce and Cocking, 1973).

Osmotic pressure is manipulated by adding various sugars or sugar alcohols to isolating solution. Cocking (1960) introduced the use of sucrose, Ruesink and Thimann (1965) used mannitol, and Eriksson and Jonasson (1969) used sorbitol as osmotic stabilizers. Since sugar alcohols are metabolically inert and their infusion into protoplasts is rather slow, they are most often used singly or in combination. It is used at a range of 0.3 to 0.7 M based on the tissue osmotic pressure at the time of isolation. Kao and Michayluk (1974) used glucose as an alternate to hexitols for isolation. Even mineral salts, particularly potassium chloride and calcium chloride (Horine and Ruesink, 1972) has been used to maintain osmolarity instead of sugar alcohols.

Endogenous cell osmotic pressure are markedly influenced by environmental conditions (Shepard and Totten, 1975), and can be manipulated by dark pretreatment of plants and use of young leaf tissues.

DONOR TISSUES:

Since protoplasts isolated by enzymes were first regenerated successfully from tobacco mesophyll cells (Takebe et al., 1971), leaves have been continued to be the material of choice, as large number of uniform sized protoplasts are obtained.

Protoplasts are also isolated from other explants like aleurone layer of Hordium (Taiz and Jones, 1971), coleoptiles of Avena (Reusink and Thimann, 1965), crown gall of Parthenocissus (Scowcroft et al., 1973), fibers of Gossypium (Beasley et al., 1979), placental tissues of tomato fruit (Gregary and Cockings, 1965), guard cells of Allium (Zeiger and Helper, 1976), flower petals of Nemesia (Hess and Endress, 1973), pollen tetrads of Datura (Rajashekar, 1973), roots of Phaseoleus (Xu et al., 1981), shoot tips of Pisum (Gamborg et al., 1978) and tubers of potato (Lorenzini, 1973).

When protoplasts are isolated from tissues of intact plant, the growth conditions of the plant are crucial to the success of the protoplast culture. Watts et al., (1974), demonstrated that conditioning of the plant are critical for successful culturing of the leaf

protoplasts of Nicotiana tabacum. The plants must be under rapid growth with ideal conditions of 22⁰C, 15 hours daylight of 10,000 to 20,000 lux and weekly fertilized with nitrogen. 40-60 days old plant were ideal for protoplast isolation. Similarly, Shepard and Totten (1977), demonstrated that potato plants are to be grown under controlled conditions with 12 hours light (15,000 lux) period until 4-10 days prior to protoplast isolation, and then expose to low light (7,000 lux) with a 6 hour period at 24⁰C and 75-80 per cent relative humidity throughout the growth period. Kao and Michayluk, (1980) established optimum conditions for protoplast isolation from alfalfa. Bhatt and Fassuliotin (1981) gave importance to pre-isolation growth conditions of egg plant for release of viable protoplasts. Only young plants with four or less leaves, grown at 7500 lux with 16 hour illumination and 26⁰C provide viable protoplast in egg plant. Deka and Sen (1976) showed that young plants are ideal for viable protoplast isolation from rice leaves.

Tomato, where recalcitrant problem exists, the importance is given for growth condition of explants. Shepard (1981) developed procedure similar to that followed in potato plant (Shepard and Totten, 1977) for optimum release of protoplasts from tomato explants when maintained at high light intensity with 12 hour photoperiod, 24⁰C and 70-75 per cent relative humidity.

Cassells and Barlass (1978), also emphasised that seven days prior to isolation the light intensity should be reduced to 500 lux and routinely supplied with calcium nitrate. Tal and Watts (1979) observed optimum protoplast release when the plants were grown in 82 per cent relative humidity and maintained at low temperature.

Preincubation of tissues and pre-conditioning of donor plants are generally followed to increase the release of viable protoplasts. Kao and Michayluk (1980), observed enhanced yield of alfalfa protoplasts if leaflets with lower epidermis removed and placed in culture medium prior to exposure to digestive enzymes. Dark preincubation of leaflets for 36-48 hours and culture medium enriched with glucose, xylose and growth regulators such as 2,4-D and zeatin riboside enhanced the plating efficiency (Kao and Michaylik, 1980).

To obtain source tissue under aseptic conditions, Binding (1974, 1976) used shoot tip culture of Petunia and tobacco. Schieder (1977) used shoot cultures of Datura species for routine protoplast isolation, that are capable of frequent plant regeneration. Negrutus and Mouseau (1980) established that Nicotiana sylvestris yielded viable protoplasts when shoot tips were maintained on MS medium with 0.1 μ M NAA and 0.03 M

(1977) described successful pretreatment of suspension cultures of Haplopappus gracilis with subculturing every 2-3 days with high NAA and shaking at 69rpm and maintained at 28⁰C. Even substitution of sucrose to glucose and addition of cysteine, methionine and 2-mercaptoethanol during subculturing changed the cell wall composition and hence less enzyme is required to release protoplasts and there by reducing toxic effect of enzymes.

Fukunaga and King (1978) observed that cell growth was optimum in potassium nitrate medium and poor in ammonium sulphate. But protoplast release was good when cells were grown in ammonium sulphate as in alfalfa, flax, rice, soybean and tobacco, and concluded that in cells grown in nitrate medium, cell wall is resistant to enzyme activity.

In order to minimise the detrimental effect of enzymes, leaf tissues are often plasmolysed prior to enzyme treatment (Cocking, 1972) by which the recovery of viable protoplasts are enhanced. Simultaneously, preplasmolysis reduces the leakage of electrolytes (Cocking, 1972), that would otherwise occur during protoplast isolation and also prevents uptake of exogenous enzymes into the cytoplasm during isolation (Ruesink, 1980). By chilling cotyledons of Cyamopsis tetragonoloba at 7⁰C for 12 hours before isolating

protoplast, the division frequency of protoplasts increased.

INCUBATION METHODS:

Many factors have been identified that influence the quantity and quality of protoplast release, including pH, light intensity, time and type of incubation, concentration and type of osmoticum and use of protecting chemicals.

The commercially available enzyme preparations contain many hydrolytic enzymes that are capable of damaging the isolated protoplasts. The damage can be minimised by varying the digestion time, viz. when incubation temperature is increased the digestion time is reduced. If temperature is reduced to 10⁰C, the incubation time is prolonged (Butenko, 1979). Potrykus et al., (1977), demonstrated that corn internode section when exposed to enzymes at 12⁰C takes 16 hours to release protoplasts but only 6 hours at 32⁰C. Similarly, Vasil and Vasil (1980) observed that corn internodes release protoplast one hour at room temperature but take 19 hours at 14⁰C.

Generally protoplast are isolated in dark (Gill et al., 1981) or in low light intensity (Chellappam et al., 1980). The pH of the incubating solution also varies from 5.4 to 6.2. Pelcher et al., (1974) suggested that pH6.0-7.0 is most favourable to release protoplast from

mesophyll tissues of Phaseolus and pH5.8 in case of mesophyll protoplasts of Glycine (Schwenk et al., 1981).

A gentle shaking is used for release of protoplast from cell suspension cultures, while stationary culture is usually used to isolate mesophyll protoplast (Kao, 1975).

Calcium chloride has been added at higher concentration of 6.0 mM as osmotic stabilizer (Gamborg, 1976) to increase membrane stability of isolated protoplasts. Magnesium chloride also has a positive effect on the release of stable protoplast as Kao and Machayluk (1980) demonstrated that these salts reduce the shock to protoplasts by direct dissolution of enzyme in the culture medium.

Phosphate buffer or 2-(N-Morpholine)ethane sulfonic acid (MES) buffer is used to minimise the shift in pH (Gamborg, 1976). The cells that are damaged during isolation release hydrolytic enzymes which will damage other healthy cells, hence compounds like 0.5% potassium dextran sulphate (Passiatore and Sink, 1981) are added which not only reduces protein contaminants present in crude enzyme preparation but also hydrolytic enzymes released by damaged protoplasts.

PURIFICATION OF PROTOPLASTS:

Following digestion, a mixture of undigested cells, components of broken or burst cells and

protoplasts are obtained. The mixture should be partially purified to eliminate broken and undigested cells. A number of techniques have been used, with varying degree of success. The frequently used purification technique is filtration-centrifugation. The protoplast mixture is passed through a filter, generally 40-100um pore size, to remove undigested cells, cell clumps, and vascular tissues from protoplasts. The separated protoplasts and cell fragments recovered are then centrifuged. The speed of centrifugation, usually 100 X g, should be sufficient to precipitate protoplasts while cell fragments continue to float (Sankara Rao and Gunasekari, 1991). The pellet is resuspended and cultured in protoplast culture medium. Filtration has the advantage over other purification techniques as the same osmotic solution is used during the entire purification process. Protoplasts are susceptible to breakage during passage through filters and therefore filters should be avoided for delicate protoplast preparation (Gamborg et al., 1981). Alternatively, floatation has been used to purify protoplasts (Gamborg et al., 1981) where protoplasts which has relatively low density compared to organelles or undigested cells when placed on a gradient and centrifuged will float and debris will settle down as pellets. Sucrose at 0.3 M (Shepard and Totten, 1977) to 0.6 M (Day et al., 1981) is commonly used for

floatation. For Solanum and Nicotiana species (Shepard and Totten, 1977; Wilson et al., 1980) used 0.3 M sucrose and centrifuged at 350 X g for 3-10 minutes, but for tomato (Zapata and Sink, 1981) it was at reduced speed (40-80 X g). Even sorbitol has been used as gradient for Arabidopsis (Somerville et al., 1981) and Maize (Day et al., 1981).

Filtration in combination with floatation has been followed in Nicotiana (Sankara Rao and Gunasekari, 1991; Passiatore and Sink, 1981). Even Gel-filtration is used along with floatation (Halim and Pearce, 1980). Similarly, Ficoll gradient was followed in Daucus carota (Gosch et al., 1975; Larkin, 1976) and percoll in Hyoscyamus muticus (Wernicke et al., 1979). Kanai and Edwards (1973) used a dextran-polyethylene glycol two phase system to recover intact protoplasts of many species. Even Sepharose J539 beads are used for purification as in Lolium (Keller and Stone, 1978). In case of root protoplasts (Xu et al., 1981), it is difficult to obtain pure preparation without considerable loss of material, since meristematic protoplasts generally sink in most osmolytica. In these cases, protoplasts along with free floating cell wall debris are cultured without purification.

CULTURE MEDIA COMBINATION:

As nutritional requirements of cultured plant cells and protoplasts are very similar, it is not

surprising that protoplast media are usually modifications of frequently used cell culture media. It has been proposed that concentrations of iron, zinc and ammonium in the standard cell culture medium may be too high for some protoplasts (von Arnold and Eriksson, 1977). Ammonium, in particular, has been found detrimental to protoplast survival. Culture media's have been devised for many species such as potato (Upadhyya, 1975; Shepard and Totten, 1977). Tomato (Zapata and Sink, 1981) and tobacco (Caboche, 1980), devoid of ammonium and replaced by organic nitrogen in the form of glutamine and serine. Calcium concentration should be increased 2-4 times over the concentrations normally used for cell cultures (Eriksson, 1977) as it is required for membrane stability.

Uchimiya and Murashige (1976) have shown that tobacco protoplasts grew equally well on sucrose, cellobiose or glucose as carbon source. For tomato, sucrose and glucose are mixed in a 2:1 ratio (Zapata and Sink, 1981). Kao and Michayluk (1975), found addition of secondary carbohydrate ribose is beneficial in alfalfa.

Numerous organic nutrients have been added to protoplast culture media. von Arnold and Eriksson (1977) suggested addition of folic acid stimulates division during Pea mesophyll protoplasts cultures. Kao and

Michayluk, (1974) added several vitamins, organic acids, sugars, sugar alcohols, casamino acids and casein hydrolysate for low density plating of Vicia hajastana (Vetch) protoplasts. Uchimiya and Murashige (1976) eliminated Casein hydrolysate and concluded^{that} it is not beneficial as against the suggestion of Constabel (1975) who stressed the need of Casein hydrolysate for protoplast culture.

Type and concentration of growth regulators are the most varied parameters based on the crop. Uchimiya and Murashige (1976) observed a higher rate of cell division in tobacco in NAA than when cultured with 2,4-D or IAA. Even protoplasts derived from hormone-independent crown gall tumors require hormones during the early stages of culture to initiate division (Scowcroft et al., 1975), before recovering their endogenous growth substances independently. But in Citrus, auxin and cytokinin are detrimental to protoplast growth (Vardi et al., 1982). von Arnold and Eriksson (1977) reported both auxin (2,4-D) and cytokinin (2ip) are required to induce cell division in pea mesophyll protoplast, whereas Uchimiya and Murashige (1976) in tobacco and Grambow et al., (1972) for carrot report that cytokinins are not required for cell division.

PLATING DENSITY: Generally, protoplasts are plated at 5000 to 10000 protoplasts ml⁻¹ (Sankara Rao and Prakash,

1995; Takebe et al., 1971). Kao and Michayluk, (1975) conducted extensive studies on nutritional requirements of cultured cells and protoplast at different plating densities in Vicia hajastana and concluded that a relatively simple B5 medium could support growth for >250 protoplasts ml^{-1} and B5 with 2,4-D, zeatin and NAA and a number of organic acids, supplemental sugars and undefined growth additives could support growth at <10 protoplasts ml^{-1} . Gleba (1978) found that culturing of protoplasts at high density for upto three days and then cultured as hanging drop culture at low density, increased the plating efficiency.

CULTURE METHODS:

Several methods like culturing in liquid media, semi-solid media, combination of liquid and solid media and different feeder techniques are reported.

Culturing in liquid media In liquid cultures the protoplast are suspended in a small volumes of liquid media in petridishes and sealed by parafilm. The protoplasts are maintained in a shallow depth (Binding, 1974). In drop culture developed by Kao et al., (1971); small drops of 40-100 μl of protoplast suspension are placed on the inner side of the lid of petridish and it is convenient for microscopic observation (Constabel, 1975).

Hanging drop method (Potrykus, 1976) allows culturing of fewer protoplasts per spot than the droplet method as in Zea mays. Multiple drop array technique is similar to drop culture method except that the size of droplets is very small (40 μ l) and this is generally used to test different media combinations. In micro droplet the size of the droplet is reduced to 0.25 to 0.50 μ L, that each droplet contains only one protoplast (Gleba, 1978).

Semisolid culture It involves agar as gelling agent as in Tobacco mesophyll protoplast culture (Nagata and Takebe, 1970, 1971), agarose (Lorz et al., 1983) or alginate (Brodelius and Nilsson, 1980) are the other gelling agents used for semisolid culture.

Combination of liquid and solid media: Protoplasts are embedded in agarose bead and these are cultured in liquid media (Shillito et al., 1983). In semisolid media for liquification, the protoplasts will be initially cultured in semisolid media with alginate as gelling agent and once the microcalli are formed, this is liquified to release the microcolonies (Brodelius and Nilsson, 1980).

There are other culture techniques followed in protoplast culture viz., Feeder layer (Raven et al., 1973), Nurse culture technique as in nitrate reductase deficient mutant of tobacco (Hein et al., 1983), Filter

paper discs on agar medium (Arcioni et al., 1982) and Agar drop method (Arcioni et al., 1982).

In Co-culturing technique a reliable, fast growing protoplast preparation like tobacco is mixed in varying ratios with protoplasts of a more recalcitrant species. By this the fast growing species will provide the growth factors and undefined diffusible chemicals which aid in regeneration of cell wall and cell division of the slow growers (Evans, 1979).

PROTOPLAST VAIBILITY TEST:

The most frequently used staining methods for assessing protoplast vaibility are fluorescein diacetate (FDA) (Widholm, 1972), phenosafranine (Widholm, 1972) and calcofluor white (Nagata and Takebe, 1970; Galbraith, 1981).

As FDA accumulates in cell membrane, viable intact protoplasts exhibits fluoresce yellow-green with in 5 minutes when observed under fluorescence microscope (FM). The chlorophyll from broken protoplast fluores red. The FDA gets dissociated from the membrane within 20 minutes, therefore observations on viabilty should be done within 15 minutes of FDA treatment.

Phenosafranine is specific for dead protoplasts. Immediately after mixing, dead protoplasts stain red and healthy ones will not takeup the stain.

Calcofluor white is most commonly used stain to ascertain viability also cell wall formation. The calcofluor white binds to B-linked glucosides of cell wall and fluoresce around the plasmalemma as a ring.

The other methods used to determine the viability are cyclosis (Pelcher et al., 1974), exclusion of evans blue dye (Kanai and Edwards, 1973) and Trypan blue (Glimelius et al., 1974), variation in protoplast size with osmotic changes (Kanai and Edwards, 1973), oxygen uptake studies (Taiz and Jones, 1971) and photosynthetic activity (Kanai and Edwards, 1973). Protoplast viability test are important tools when biochemical studies are to be taken.

REGENERATION:

There are conflicting reports set for the initiation of cellulose fiber formation varying between 10 minutes (Williamson et al., 1977) to 72 hours (Burgess and Fleming, 1974) or even later. In Convolvulus arvensis protoplasts (Horine and Ruesink, 1972), the budding was mistaken as indication for the preparation of protoplasts for cell division and wall formation. Burgess et al (1978) showed that in tobacco, fiber appeared after 10 hours after culturing, as observed under scanning electron microscope, These fibers could be removed by cellulase and cell wall formation continued only after cellulase enzyme was removed.

Biochemically cell wall components of protoplast differ from the intact cells (Asamizu and Nishi, 1980). The arabinose in the noncellulosic fraction was lower, while mannose, xylose and glucose were higher in cultured protoplasts. They also release large quantity of polysaccharides into the medium (Hanke and Northcole, 1974) as studied by the ^{14}C -glucose and ^3H -myo inositol labelled pulse chase technique. Takeuchi and Komamine (1978), reported that suspension cultured protoplasts and intact cells have different cell wall constituents.

The first step in regeneration of protoplasts, is cell wall formation. Tobacco requires 30-36 hours for cell wall formation (Nagata and Takebe, 1970). Staining with calcofluor white or Tinopol B.O.P.T. (Bilkey and Cocking, 1982) cell wall synthesis could be confirmed. Other methods primarily used for monitoring the cell wall synthesis are Gas-Liquid chromatography (Takeuchi and Komamine, 1978), incorporation of radioactive precursor (Klein et al., 1981), electron microscopy (Lloyd et al., 1980), thin sectioning and positive staining (Cocking, 1966) Negative staining (Burgess and Linstead, 1977) and Freeze etching techniques (Willison and Cocking, 1975).

Schilde-Rentschler (1977) found that in tobacco, cell wall regeneration is a pre-requisite for nuclear and cell division, but Meyer and Herth (1978) demonstrated that by inhibiting cell wall synthesis only cytokinesis

and not nuclear division is inhibited as there was formation of multinucleated cells.

The rate and regulation of cell wall regeneration depends on the plant species and the state of differentiation of the donor cells used for protoplast isolation. Protoplasts from mesophyll cells of Nicotiana, Petunia, Datura and Brassica form new cell wall within 24 hours after culturing, but in cereals and legumes it may require four days for wall regeneration (Giles, 1972).

Protoplast budding occurs due to non-incorporation of pectin into the new cell wall (Hanke and Northcole, 1974) and is the result of bulging of plasmamembrane from weakened areas in the newly synthesised cell wall (Fowke and Gamborg, 1980).

Not only genetic factor but culture medium composition also effects cell wall synthesis, as sucrose concentration in excess of 0.3 M and Sorbitol (0.5M) inhibit cell wall formation (Shepard and Totten, 1977). But growth regulators like 2,4-D and zeatin are important for regulation of cell division than cell wall synthesis (Takeuchi and Komamini, 1978). Many factors are important in initiation of cell division including genotype, culture media and condition of the donor tissue used for protoplast isolation. Protoplast isolation from cell culture containing rapidly growing

cells often undergo first cell division sooner than leaf mesophyll protoplasts (Vasil, 1976). The other factors which influence cell division are pretreatment and age of the plant, fertilization of the plants and application of pesticides to donor plant.

The plating efficiency varies from 1-90 per cent, while Banks and Evans (1976) reported 1 per cent plating efficiency for Nicotiana sylvestris mesophyll protoplast, Nagy and Maliga (1976) using modified procedures reported 60-90 per cent plating efficiency for the same species.

Incomplete cytokinesis has been frequently observed during first division resulting in spontaneous fusion and production of multinucleated protoplasts (Brar et al., 1979) and in most cases these multinucleated protoplasts will not undergo continued growth.

In most cases, first cell division occurs within 2-7 days after healthy protoplasts are cultured but in some cases there may be a long lag period as in case of cotton protoplasts of upto 25 days after culturing (Bhojwani et al., 1977). Subsequent division occurs more rapidly and in 2-3 weeks multicellular clumps will be visible.

MATERIAL AND METHODS

III. MATERIALS AND METHODS

This chapter describes the materials used and methodology adapted to assess and standardise protoplast isolation and regeneration potential of protoplasts in three solanaceous species - Tobacco (Nicotiana tabacum), Tomato (Lycopersicon esculentum) and Bell pepper (Capsicum annuum).

Plant material

Seeds of Tobacco (N. tabacum cv. Thompson) were procured from Dr. K. Sankara Rao, IISc, Bangalore. Seeds of Tomato (Arka Vikas and Arka Saurabh) and Bell pepper (Arka Lohit) was procured from Indian Institute of Horticultural Research, Bangalore, and Bell pepper cv California wonder was bought from MAHYCO seeds company. The seeds were aseptically germinated and shoots with cotyledonary leaves were cut and maintained as shoot tip cultures. Experiments were conducted in five replicated trials .

Chemicals and glassware:

All the chemicals and glassware used in the preparation of culture media and for analytical purposes were of research grade. Chemicals used for the preparation of culture media and assay solution were of research grade purity supplied by Merck, Glaxo, BDH, Loba, Sigma and other reputed companies. Glasswares like Erlenmeyer flasks, test tubes, beakers, pipettes used for experiments and analytical purposes were of

borosilicate glass. The disposable petridishes and culture plates were of Laxbro make. The enzymes used were from Biocon, Bangalore; Novo industries, Denmark and Yakult industries, Japan.

Preparation of glassware:

The glasswares were cleaned using 5% chromic acid, water and labolene (Glaxo) followed by thorough rinsing with tap water and double distilled water. They were dried in hot air oven.

Composition of culture medium:

The composition of the culture medium was essentially that of Murashige and Skoog, (1962) [MS] and Tomato culture medium [TM] (Shahin, 1985). The inorganic salts and organic constituents were grouped as shown in Table-1. Different inorganic constituents of each group were weighed separately in a single pan analytical balance and dissolved in sterile double distilled water. These solutions were mixed in volumetric flasks and volume was madeup to obtain stock solutions with required concentration of each constituent of the group. Similarly, required quantity of each of the organic constituents were weighed individually, dissolved in sterile double distilled water, mixed together and then volume was madeup.

Indole-3-acetic acid (IAA), 1-naphthene acetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D)

Table-1: Composition of media for axenic shoot cultures and protoplast culture

Composition	Shoot tip culture		Protoplast culture		Macrocalli culture		Shooting medium		Rooting medium	
	1/2 MS (mgL ⁻¹)	liquid medium, CM-1 & CM-2 (mgL ⁻¹)	CM-1 solid medium (mgL ⁻¹)	CM-3 (mgL ⁻¹)	CM-4 (g L ⁻¹)	CM-5 (mgL ⁻¹)				
Macro										
NH ₄ NO ₃	825	-	1500	-	1650	825	1900	950		
KNO ₃	950	1500	370	1500	270		185			
MgSO ₄	185	370	170	370	170		85			
KH ₂ PO ₄	85	170								
CaCl ₂	220	440	440	440	440		220			
Micro										
H ₃ BO ₄	3.1	6.20	6.20	6.20	6.20		3.1			
MnSO ₄	11.15	22.30	22.30	22.30	22.30		11.15			
ZnSO ₄	4.3	8.60	8.60	8.60	8.60		4.3			
KI	0.42	0.38	0.38	0.38	0.38		0.42			
Na ₂ MOO ₄	0.13	0.25	0.25	0.25	0.25		0.13			
CuSO ₄	0.013	0.025	0.025	0.025	0.025		0.013			
CoCl ₂	0.013	0.025	0.025	0.025	0.025		0.013			
Vitamin										
Inositol	50.0	100	100	100	100		50.0			
Nicotinic acid	0.25	2.50	5.00	5.00	5.00		0.25			
Pyridoxine. HCl	0.25	1.00	0.50	0.50	0.50		0.25			
Thiamine.HCl	0.05	10.00	0.50	0.50	0.50		0.05			
Glycine	1.00	0.50	2.50	2.50	2.00		1.00			
Casein hydrolysate	500	150	100	100	100		500			
Biotin	-	0.50	0.50	0.50	-		-			
Ascorbic acid	-	1.00	1.00	1.00	-		-			
FeSO ₄	14.00	14.00	14.00	14.00	28.00		14			
Na ₂ .EDTA	18.6	18.6	18.6	18.6	37.0		18.6			
Others										
MES	-	97.60	97.60	97.60	-		-			
PVP	-	1000.0	1000.0	1000.0	-		-			
Mannitol	-	9% & 6%	2.4%	2.4%	-		-			
Sucrose	2.0%	0.5%	1.4%	1.4%	2.0%		2%			
Sucrose (LM)	-	1000	1000	1000	-		-			
Agar	6000	-	6000	6000	6000		6000			

were dissolved seperately in minimal quantity of redistilled ethyl alcohol and volume was madeup with double distilled water. Gibberellic acid (GA_3), kinetin (kin), zeatin (zea) and benzyl adenine (BA) was dissolved in minimal quantity of 0.1N hydrochloric acid OR 0.1N sodium hydroxide and volume madeup with double distilled water. These stock solution of known concentrations were stored in refrigerator at $5-6^{\circ}C$, known quantity of the stock solution was pipetted out at the time of preparation of medium.

Asceptic techniques:

PREPARATION OF MEDIA:

Digestion mixture: Required quantity of mannitol was weighed and dissolved in double distilled water. Known volumes of stock solution of CPW salts were added. Osmolarity was checked using a cryoscopic osmometer (Osmomat 030-Gonotec). Cellulase and macerozymes were weighed and dissolved in the digestion mixture and madeup the volume to desired quantity. The enzyme digestion mixture was filter sterilized using membrane filter of 0.22 μ pore size.

Washing medium: To known volume of basal MS/TM media weighed quantities of mannitol and Kinetin (0.20 mg/L) were added. The osmolarity was checked and pH adjusted to 5.8 using either 0.1N sodium hydroxide OR 0.1N hydrochloric acid. The medium was dispensed in 125ml reagent bottle, autoclaved and stored at $4^{\circ}C$.

Floating medium: To known volume of basal MS/TM media weighed quantity of sucrose (20%) was added, pH adjusted to 5.8, autoclaved and stored at 4⁰C.

Culture medium: Based on the media composition varied levels and combinations of hormones were provided. Required quantity of sucrose was weighed and dissolved in double distilled water. Then known volume of stock solution of different groups were sequentially pipetted out into it. Before making up the final volume required quantity of mannitol was added. The osmolarity was checked using Osmomat. pH of the medium was adjusted using either 0.1N sodium chloride OR 0.1N hydrochloric acid. The media was dispensed in 125ml reagent bottle and autoclaved at a temperature of 121⁰C and 103.4KPa/cm² of pressure for 15 minutes and stored at 4⁰C. Growth hormones viz. IAA, GA₃ and zeatin were filter sterilized and added only at the time of culturing.

- Culture medium-1 (CM-1): Basal medium + 9% mannitol + 1.4% sucrose + hormones
- Culture medium-2 (CM-2): Basal medium + 6% mannitol + 1.4% sucrose + hormones
- Culture medium-3 (CM-3): Basal medium + 2.7% mannitol + 1.4% sucrose + Hormones + 0.6% Agar agar

Analytical procedures and methods: The following were the methods adopted to measure different parameters pertaining to protoplast viability, cell wall regeneration and plating efficiency.

Protoplast viability: Viability was determined using Fluorescein diacetate (FDA). A stock solution of 0.5% w/v FDA (Polyscience Inc., USA) was prepared in acetone. The diluted working solution of 0.01% v/v was prepared fresh from the stock in mannitol solution and used within few hours.

Equal quantities of freshly prepared protoplasts and FDA solutions were mixed and after 5 minutes number of protoplast that fluoresce was counted using UV microscope. Percentage viability of protoplast was calculated using formula

$$\frac{\text{No. of fluoresce protoplasts}}{\text{Total No. of protoplasts}} \times 100 = \text{Viability percent}$$

FDA is basically a non-fluorescent chemical, but can easily enter the cell. The living cell metabolise the dye and fluoresceine is liberated as the by-product, which being ionic in nature gets trapped inside the cell and under UV light it fluoresce, but non-viable protoplast do not fluoresce.

Wall regeneration: Generally calcofluor is used to determine the wall regeneration process. Here an indirect method of plasmolysis was used to determine the wall regeneration process.

0.80M mannitol solution was prepared using the basal MS medium. Equal quantity of 2-3 days old protoplast were mixed with 0.8 M mannitol solution and after 30

minutes observed for change in the configuration of the protoplasts. If cell wall is formed there would be a circular ring around the shrunken protoplasts, but where cell wall is not formed the circular ring was not observed.

Plating efficiency: is taken as percentage of protoplasts showing continuous division to the number of protoplasts plated.

The plating efficiency in tobacco and tomato was calculated on 15th day and bell pepper on 20th day after culture. On 15/20th day the plates were observed for number of protoplast dividing and at 6-10 cell stage and those not dividing per unit field of observation. To eliminate error 5-6 observations were taken per plate and later mean value was taken as plating efficiency and expressed as percentage.

EXPERIMENTAL DETAILS:

Experiment-1: Standardization of osmoticum for different species and genotypes for efficient protoplast isolation

Objective: The objective was to determine the tolerance level of different species and explants under study to osmotic conditions for protoplast isolation.

Material: Surface sterilized Tobacco, Tomato and Bell pepper seeds were allowed to germinate in sterile petriplate containing filter paper. The radical portion

was removed and only hypocotyl with cotyledonary leaves were cultured on half strength MS medium with kinetin at 0.2mg/L. Once the cotyledons are fully expanded (about 15 days) these cotyledons and hypocotyls were used for protoplast isolation. The axenic shoot cultures were maintained from which fully expanded leaves were used for protoplast isolation. A range of osmoticum from 0.0 to 0.65M mannitol was tried.

Observation: Protoplast yield and viability was recorded at a fixed level of cellulolytic enzymes (Cellulase- 2% and Macerozyme-0.5%)

Experiment-2: Testing different cell wall degrading enzymes on the efficiency of protoplast isolation

Objective: To determine the best source and combination of enzyme mixture to procure large quantity of viable protoplast

Material: Once the osmoticum was standardised, different sources of enzymes were tested

- 1) Cellulase and Pectinase (Liquid enzymes) from Biocon, Bangalore
- 2) Cellulase and Pectinase (Lyophilised powder) from Biocon, Bangalore
- 3) Celluclast and Pectinex (Liquid enzymes) from Novo industries, Denmark
- 4) Cellulase (Onozuka-R10) and Macerozyme-R10 (Yakult Biochem Co. Ltd. Japan)

The enzyme concentration varied from 0.1 to 5% cellulase and 0.1 to 2.0% pectinase.

Observation: Time taken for digestion, yield and viability was recorded

Expt-III: Testing the effect of constituent of digestion mixture, pH, temperature and pre-treatment of tissues on the rate and viability of protoplast

Objective: To determine the best environmental conditions for obtaining viable protoplasts and to minimize damage to protoplasts

Material: The explant was either kept in dark or light for 18 hours prior to protoplast isolation. The digestion mixture was added with ascorbic acid, MES and kinetin. Digestion was carried at two temperature regimes of $27 \pm 2^{\circ}\text{C}$ and room temperature ($30 \pm 2^{\circ}\text{C}$).

Observation: Rate of release, yield and protoplast viability was recorded

Expt-IV: Comparison of basal medium with different hormone concentrations on protoplast culture

Objective: To identify the appropriate culture medium for efficient protoplast culture in the species under study.

Material: Selectively pretreated plant materials were used in this experiment with 3 basal media and different hormonal combinations.

Observation: Plating efficiency, rate of callus development and regeneration potential was observed at regular interval.

Isolation and purification of protoplasts:

Based on the experiment, the tissue explants were pre-incubated in 18 hours dark OR light treatment at 4⁰C. The cotyledon and mesophyll explants were cut through the mid rib and later transversly into 2-3mm strips. About 0.5g tissue was incubated in 5ml filter sterilized enzyme mixture in 50 ml erlenmeyer flask at 27+2⁰C under dark without shaking during digestion.

The hypocotyl explant was also cut into small 2-3mm strips and incubated in filter sterilized enzymes for digestion.

Digested tissues were filtered through nylon mesh (Pore size-60um) into 15ml screw cap tubes. The filtrate was centrifuged at 60Xg (800 rpm) for 10 minutes. The pellet was resuspended in 5 ml washing medium and centrifuged for 5 minutes. The process was continued 3 times so that the cellulolytic enzymes are completely removed. Protoplasts were resuspended in 1 ml of washing medium and carefully layered on 9 ml of floating medium in 15ml screw cap tubes. The tubes were left undisturbed for 15 minutes and centrifuged at 75X g (1000 rpm) for 10 minutes. Intact protoplast formed a clear band at the interphase. The band was removed using pasteur pipette, kept in dark for 15 minutes, the yield and viability were recorded. The healthy protoplasts were cultured at $2 \times 10^4 \text{ ml}^{-1}$ plating density in liquid

medium (Table-1) and incubated in dark for two weeks. 200U1 of fresh 8E/TM-2 medium devoid of hormones were supplemented after 7days at three day interval. When the total volume of the protoplast culture had doubled, the cultures were divided over two dishes. Plating efficiency was determined on 15th day in tobacco and tomato and 20th day in bell pepper.

After two weeks the petridishes were exposed to cool daylight fluorescent light ($200\text{UEm}^{-2}\text{s}^{-1}$). After 20 days 50% of CM-I medium was replaced by equal volume of CM-II medium. In this medium the protoplasts formed microcalli and later transferred to CM-III solid media for macrocalli formation, where the rate of callus formation was enhanced. Organogenesis was initiated on CM-IV and rooted in CM-V media (Table-1).

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

In this chapter, the results of experiments performed are presented.

Experiment-1: Standardization of osmoticum for different species and genotypes for efficient protoplast isolation

Objective: To determine the tolerance level of different species and explants under study to osmotic conditions for protoplast isolation.

Treatment: Mesophyll explant of Nicotiana tabacum cv. Thompson, cotyledonary, hypocotyl and mesophyll explants of Lycopersicon esculentum cv. Arka saurabh and Capsicum annum cv. California wonder were tested for appropriate osmotic conditions for protoplast isolation. CPW salt solution with mannitol as osmotic stabilizer at an osmolarity ranging from 30 to 690 mOsmols respectively and cellulytic enzymes, Cellulase (2%) and Macerozyme(0.5%) were used.

Data on protoplast yield and viability at different osmolarity are presented for tobacco (Table-2), tomato (Table-3) and bell pepper (Table-4).

Tobacco: only mesophyll explant was tested for protoplast isolation as cotyledonary and hypocotyl tissues could not be obtained in sufficient quantity. At levels less than 0.4M mannitol, the cells bursted/ lysed and no protoplasts formed (Table-2). With increase in

Table-2: Standardization of osmotic conditions for optimum protoplast release* in Tobacco cv. Thompson

Osmoticum	Mesophyll	
Mannitol(M)	Yield/gFW	Viability (%)
0.00	Burst	-
0.40	Burst	-
0.45	2×10^5	70-75
0.50	$2-3 \times 10^5$	70-75
0.55	2×10^7	75-80
0.60	5×10^6	60
0.65	$2-3 \times 10^5$	60

*- Enzyme used: Cellulase (2%) and Macerozyme (0.5%)

osmolarity there was an increase in yield and reached a peak at 0.55M with 2×10^7 protoplasts ml^{-1} (Plate-1). With further increase in osmolarity there was a decline to 2.5×10^5 protoplasts ml^{-1} at 0.65M mannitol.

Similar trend was observed in viability of protoplasts also. At 0.45M the viability was around 75% and increased to 80% at 0.55M. Further increase in osmolarity reduced the viability to 60% at 0.60 and 0.65M resulted in protoplast collapse.

Tomato: Cotyledon, hypocotyl and mesophyll explants were tested for optimization of osmotic levels for protoplast isolation. Yield and viability was recorded only after purification of protoplasts (Table-3). Irrespective of the explants the cells lysed when exposed to less than 0.5M mannitol. At 0.5M level, the protoplast yield from cotyledon, hypocotyl and mesophyll was 4×10^3 , 100-200 and 1×10^4 protoplasts ml^{-1} , respectively. Maximum yield was obtained at 0.55M with 2×10^5 protoplasts ml^{-1} in cotyledon (Plate-7) but no change in hypocotyl and mesophyll tissues from 0.5M mannitol level.

The viability ranged from 65 to 70% in cotyledon and 70-80% in mesophyll protoplasts at the time of plating. The highest viability of 80% was obtained at 0.55M with mesophyll protoplasts. Viability was not recorded in hypocotyl protoplasts as working yield was not obtained.

Table-3: Standardization of osmotic conditions for optimum protoplast release in Tomato cv. Arka saura at cellulase (2%) and Macerozyme (0.5%)

Osmoticum Mannitol(M)	Cotyledon		Hypocotyl		Mesophyll	
	Yield/gFW	Viability (%)	Yield/gFW	Viability (%)	Yield/gFW	Viability (%)
0.00	Burst	-	Burst	-	Burst	-
0.40	Burst	-	Burst	-	Burst	-
0.45	Burst	-	Burst	-	Burst	-
0.50	4X10 ³	65	2-3X10 ²	-	1X10 ⁴	70
0.55	2X10 ⁵	70	2-3X10 ²	-	1X10 ⁴	70
0.60	shrivelled	-	shrivelled	-	shrivelled	-
0.65	Shrivelled	-	Shrivelled	-	Shrivelled	-

Bell pepper: Of the three explants, hypocotyl yielded lowest with maximum of 500 protoplasts ml^{-1} at 0.5 and 0.55M, below 0.5M it lysed and 0.6M and above it collapsed (Table-4).

Cotyledonary cells could not tolerate less than 0.45M and lysed. At 0.45M, it produced $<1 \times 10^3$ protoplast ml^{-1} and maximum at 0.55M with 1×10^5 protoplasts ml^{-1} , but slightly reduced to 8×10^4 protoplasts ml^{-1} at 0.6M. At 0.65M the protoplasts collapsed. Similar trend was observed with mesophyll tissues also with maximum yield of 8×10^5 at 0.55M and 2×10^3 protoplasts ml^{-1} at 0.65M (Plate-IIIa).

Maximum viability of 70% was observed at 0.55M and reduced to 60% at 0.60M in cotyledonary protoplasts. Mesophyll protoplasts showed viability of 75% at 0.55M and reduced slightly to 70% at 0.60M and further reduced to 40% at 0.65M.

Experiment-II: Test the efficacy of different cell wall degrading enzymes on protoplast isolation

Objective: To determine the best combination of enzyme mixtures to procure large quantity of viable protoplasts.

Treatment: Four different sources of cellulolytic enzymes were used at varying concentration to standardize the concentration and duration of incubation

Table-4: Standardization of osmotic conditions for optimum protoplast release in Bell pepper cv. California wonder cellulase (2X) and Macerozyme (0.5X)

Osmoticum Mannitol (M)	Cotyledon		Hypocotyl		Mesophyll	
	Yield/gFW	Viability (%)	Yield/gFW	Viability (%)	Yield/gFW	Viability (%)
0.00	Burst	-	Burst	-	Burst	-
0.40	Burst	-	Burst	-	Burst	-
0.45	<1X10 ⁴	-	1-2X10 ²	-	<1X10 ³	-
0.50	2X10 ⁴	70	5X10 ²	-	2X10 ⁵	75
0.55	1X10 ⁵	70	5X10 ²	-	8X10 ⁵	75
0.60	8X10 ⁴	60	Shrivelled	-	5X10 ⁴	70
0.65	Shrivelled	-	Shrivelled	-	2X10 ³	40

for optimum protoplast yield. a) **Cellulase:** Liquid and lyophyllised powder of cellulase (0.5-5.0%) from Biocon, Bangalore; Celluclast (0.1-2.0%) from Novo industries, Denmark and Cellulase-Onazuka R-10 (0.5-2.0%) from Yakult Biochemicals, Japan.

b) **Pectinase:** Liquid and lyophyllised powder of pectinase (0.1-2.0%) from Biocon, Pectinex (0.1-0.5%) from Novo industries, Denmark and Macerozyme R-10 (0.1-0.5%) from Yakult Biochemicals, Japan. Data on protoplast yield in relation to enzymes and duration are presented in table-5, 6 and 7.

Tobacco: Cellulase and pectinase enzymes from Biocon were not effective in protoplast release. There was digestion of cell wall but in the process protoplasts also lysed completely.

Protoplast release was observed within four hours after incubation in Novo enzymes at 0.5% celluclast and 0.25% pectinex, but the tissue was not completely digested even after 12hours and yield was only 4×10^3 protoplasts ml^{-1} (Table-5). The maximum yield was obtained at 1.2% cellulase and 0.5% pectinex with an yield of 8×10^6 by 8 hours, but reduced to 4×10^6 protoplasts ml^{-1} by 12 hours and completely collapsed by 16 hours.

When Onazuka R-10 enzyme combination was used, with increase in concentration of enzymes the digestion

Table-5: Effect of different cell wall degrading enzymes on protoplast yield and time of release in tobacco cv. Thompson

Enzyme/concentration (%)		Source tissue			
		Mesophyll			
		4h	6h	12h	16h
<u>Nova enzymes</u>					
<u>Celluclast</u>	<u>pectinex</u>				
0.1	0.10	-	-	-	-
0.5	0.10	-	-	-	-
1.2	0.10	-	-	-	-
0.1	0.25	-	-	-	-
0.5	0.25	<1x10 ³	1x10 ³	4x10 ³	damaged
1.2	0.25	<1x10 ³	4x10 ⁵	5x10 ⁵	damaged
0.1	0.50	3x10 ³	5x10 ⁵	4x10 ⁶	damaged
0.5	0.50	4x10 ⁴	8x10 ⁶	4x10 ⁶	damaged
1.2	0.50	2x10 ⁴	4x10 ⁶	3x10 ⁵	damaged
<u>Onozuka-R-10</u>					
<u>Cellulase</u>	<u>Macerozyme</u>				
0.5	0.10	-	-	-	few
1.0	0.10	-	-	-	few
2.0	0.10	-	-	-	1x10 ³
0.5	0.25	-	-	-	3x10 ⁴
1.0	0.25	-	-	<1x10 ³	5x10 ⁵
2.0	0.25	-	-	4x10 ³	4x10 ⁶
0.5	0.50	-	-	5x10 ³	8x10 ⁴
1.0	0.50	-	-	3x10 ⁴	3x10 ⁶
2.0	0.50	-	-	2x10 ⁴	4x10 ⁷

was more pronounced and reached maximum at 2.0% cellulase and 0.5% Macerozyme with 4×10^7 protoplast ml^{-1} after 16 hours digestion, it did not show any deleterious effect even with further increase in digestion time. The Novo enzymes required lesser time than Onazuka enzymes.

In tomato, after preliminary experiments (Table-3) only cotyledon and mesophyll explants were used. The Biocon enzymes used here had a deleterious effect on the protoplast as it lysed the cells irrespective of the explants used.

Novo enzymes with a minimum of 0.5% Celluclast and 0.25% Pectinex (Table-6) is required to initiate digestion and protoplast release. The optimum yield of 7×10^4 protoplasts ml^{-1} in cotyledon and 3×10^4 protoplasts ml^{-1} in mesophyll tissues at 1.2% cellulase and 0.5% pectinex and the digestion was completed by 4-6 hours.

Similarly in Onazuka enzymes, minimum of 2.0% and 0.25% or 0.5% and 0.5% cellulase and macerozyme was required for initiation of digestion and optimum concentration being 2.0% cellulase and 0.5% macerozyme with an average yield of 2×10^5 and 4×10^4 protoplasts ml^{-1} in cotyledonary and mesophyll explants respectively.

Even in bell pepper explants, Biocon enzymes were not effective for protoplast isolation as there was lysis of cells.

With increase in concentration of Novo enzymes the digestion was effective. Individually either celluclast or pectinex was not effective for cell wall digestion (Table-7).

At very low levels of pectinex of 0.1% the digestion was not complete even in combination of higher levels of celluclast (1.2%) in cotyledonary tissues. At 1.2% celluclast and 0.25% pectinex, the digestion was complete with yield of 4×10^5 protoplasts ml^{-1} at 12 hours but further increase in digestion time to 16 hours completely damaged the protoplasts. The optimum concentration of enzyme was 0.5% celluclast and 0.5% pectinex with an average yield of 6×10^5 protoplasts ml^{-1} within 12 hours but with increase in celluclast to 1.2% the yield reduced to 2×10^4 protoplasts ml^{-1} (Table-7).

At 0.5% celluclast and 0.25% pectinase there was protoplast release in mesophyll tissues but time required was 16 hours. The optimum yield of 4×10^6 protoplasts ml^{-1} was obtained at 0.5% celluclast and 0.5% pectinex by 6 hours and further increase upto 12 hours reduced yield to 5×10^4 and by 16 hours the protoplasts appeared collapsed.

Table-7: Effect of different cell wall degrading enzymes on protoplast isolation in Bell pepper cv. California wonder

Enzyme/Time (%)	Source tissue							
	Cotyledon			Mesophyll				
	4h	6h	12h	16h	4h	6h	12h	16h
<u>Nova enzymes</u>								
<u>Celluclast</u>								
pectinex								
0.1	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	-	-	-
1.2	-	-	-	-	-	-	-	-
0.1	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	-	-	-
1.2	-	-	-	-	-	-	-	-
0.1	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	-	-	-
1.2	-	-	-	-	-	-	-	-
<u>Onozuka-R-10</u>								
<u>Cellulase</u>								
macerozyme								
0.5	-	-	-	-	-	-	-	-
1.0	-	-	-	-	-	-	-	-
2.0	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	-	-	-
1.0	-	-	-	-	-	-	-	-
2.0	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	-	-	-
1.0	-	-	-	-	-	-	-	-
2.0	-	-	-	-	-	-	-	-

0.10
0.10
0.10
0.25
0.25
0.25
0.50
0.50
0.50

3X10³
5X10⁵
4X10⁴

4X10⁵
6X10⁵
2X10⁴

damaged
damaged
damaged

1X10³
1X10³
1X10³

10³
3X10⁴
4X10⁶
8X10⁴

2X10³
4X10⁵
5X10⁴
damaged

1X10⁴
damaged
damaged

1X10⁴
5X10⁵
2X10⁵
6X10⁵

1X10⁴
3X10⁴
5X10⁵
8X10⁶

damaged
damaged
damaged

Onazuka enzymes at less than 1.0% cellulase and 0.25% macerozyme didnot yield protoplasts. The optimum yield of 6×10^5 in cotyledon and 8×10^6 protoplasts ml^{-1} in mesophyll was obtained at 2.0% cellulase and 0.5% macerozyme.

From the above few experiments it was found that Biocon enzymes were not suitable for protoplast isolation. Novo enzymes were required at low concentration and optimum being 1.2% celluclast and 0.5% pectinex and digestion time being 4-8hours. For onazuka enzymes it was 2.0% cellulase and 0.5% macerozyme with digestion time ranging from 6hours for tomato and 16 hours for tobacco and Bell pepper.

The same trend was observed in all the cultivars tested viz. Tomato (Arka vikas and Arka saurabh) and Bell pepper (Arka Lohit and California wonder). For further experiments only one cultivar of tobacco (cv. Thompson), tomato (cv. Arka saurabh) and Bell pepper (cv. California wonder) was used as handling of large quantity of materials was not feasible.

Experiment-III: Testing the effective combination of digestion mixture, temperature and plant pretreatment on the rate and viability of protoplast release.

Objective: To determine the best environmental condition for obtaining viable protoplasts and to minimize damage to protoplasts.

Table--8: Effect of pre-treatment of explant and incubation mixture on protoplast stability and viability

Explant	Light treatment		Dark treatment(18h)		CPW salts +
	DDW	CPW salts	CPW salts + *	DDW	
Tobacco mesophyll	Large no. of damaged pp	Good pp	Good pp	Good pp	very good pp very good pp
Tomato cotyledon	pp turned brown	pp turned brown	not healthy	pp turned brown	pp turned brown Good pp
Pepper mesophyll	pp turned brown	pp turned brown	not healthy	pp turned brown	pp turned brown Good pp
Pepper cotyledon	pp turned brown	pp turned brown	not healthy	pp turned brown	pp turned brown Good pp

Treatment: In this part of the experiment, pre-treatment of plant materials and various incubation mixtures were tried (Table-8) at a single temperature regime of $27 \pm 2^{\circ}\text{C}$ and protoplast status was analysed 24 hours after culture.

In general, when protoplasts were isolated in distilled water or CPW salts alone, the protoplasts turned brown due to high phenolic secretion, except in tobacco. The protoplasts collapsed when isolated in distilled water with out pretreatment of donor plant. When CPW salts with antioxidants (ascorbic acid) and kinetin was used in digestion mixture, there was no phenolic secretion by protoplasts. In light pretreated plants, the protoplasts were not looking healthy even though no phenolic secretion, but from dark pretreated tissues the protoplasts looked healthy. Hence in all other experiments, dark pretreatment with CPW salts and antioxidant was followed.

An experiment was conducted to study the enzymatic activity with time in two sets of enzymes viz. Novo and Onazuka enzymes, at optimum level based on previous experiments (Table-5, 6 & 7).

Two temperature regimes were maintained viz. $27 \pm 2^{\circ}\text{C}$ and room temperature ($30 \pm 2^{\circ}\text{C}$) and observed for rate of digestion.

Table-9: Effect of temperature on the rate of tissue digestion

Explant/Enzymes	Temperature	
	27±2°C	30±2°C
<u>Tobacco mesophyll</u>		
Celluloclast+Pectinex*	6-8 hours	<4 hours
Cellulase + Macerozyme**	16 hours	8 hours
<u>Tomato cotyledon</u>		
Celluloclast+Pectinex*	4 hours	3 hours
Cellulase + Macerozyme**	5 hours	3 hours
<u>Pepper cotyledon</u>		
Celluloclast+Pectinex*	8-10 hours	4-5 hours
Cellulase + Macerozyme**	16 hours	8-10 hours
<u>Pepper mesophyll</u>		
Celluloclast+ Pectinex*	8-10 hours	4-5 hours
Cellulase + Macerozyme**	16 hours	8-10 hours

*- 1.2 % celluclast + 0.5% pectinex

** - 2.0% cellulase + 0.5% macerozyme

For Novo enzymes at $27 \pm 2^{\circ}\text{C}$ it took 6-8 hours for digestion but reduced to 4 hours at room temperature in tobacco mesophyll tissues. Similarly with Onazuka enzymes the digestion time reduced from 16 hours to 8 hours with increase in temperature (Table-9). Similar trend was observed in tomato and bell pepper explants (Table-9).

From these set of experiments the final physiological and environmental conditions were formulated for further regeneration studies. All donor plants were dark pretreated for 18 hours at 4°C , used CPW salts with ascorbic acid and kinetin and cellulolytic enzymes. Onazuka enzymes -cellulase (2%) and macerozyme (0.5%) or Novo enzymes- celluclast (1.2%) and pectinex (0.5%) in digestion mixture.

Experiment IV: Comparasion of basal medium with different hormone concentration on protoplast culture

Objectives: To manipulate the media constituents and culture conditions so as to favour cell wall formation, cell division, callus formation and finally organogenesis in the species under study.

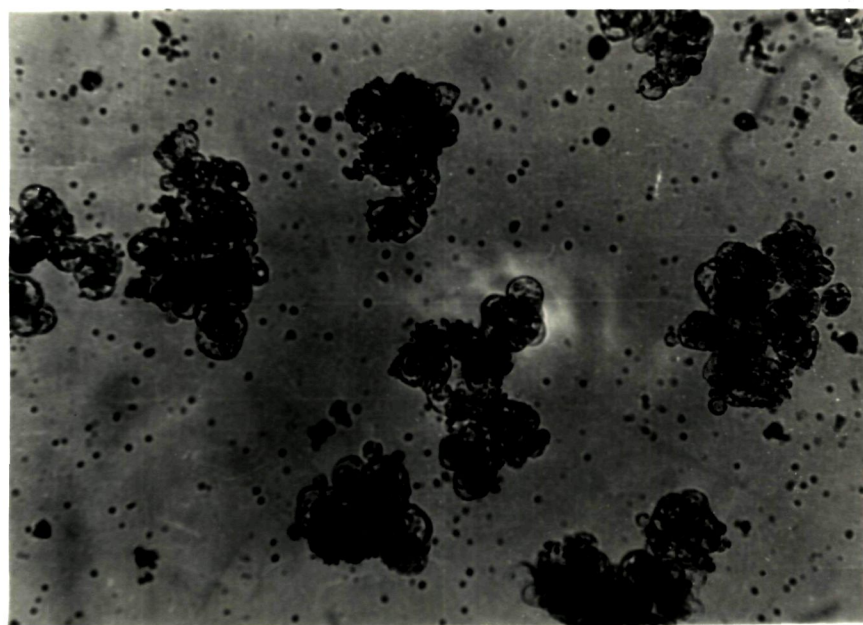
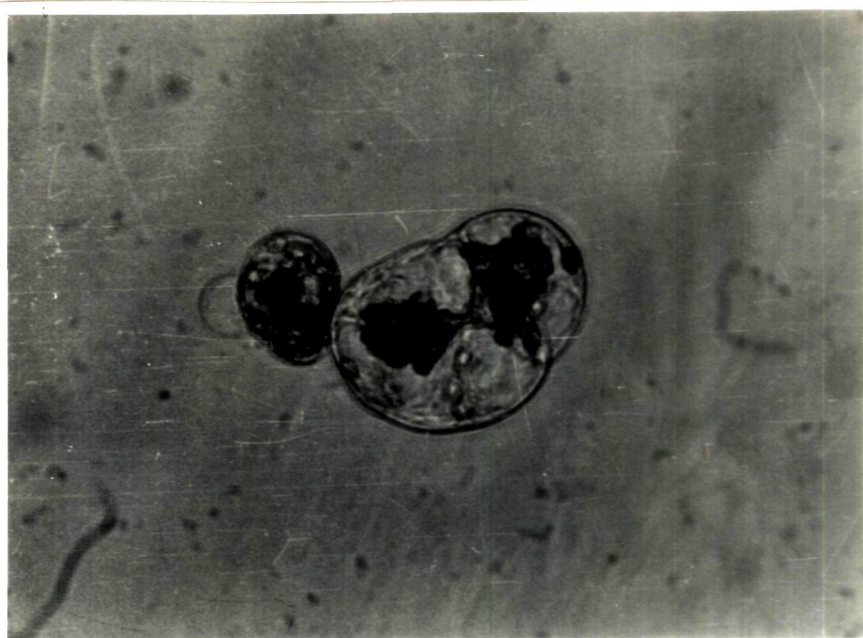
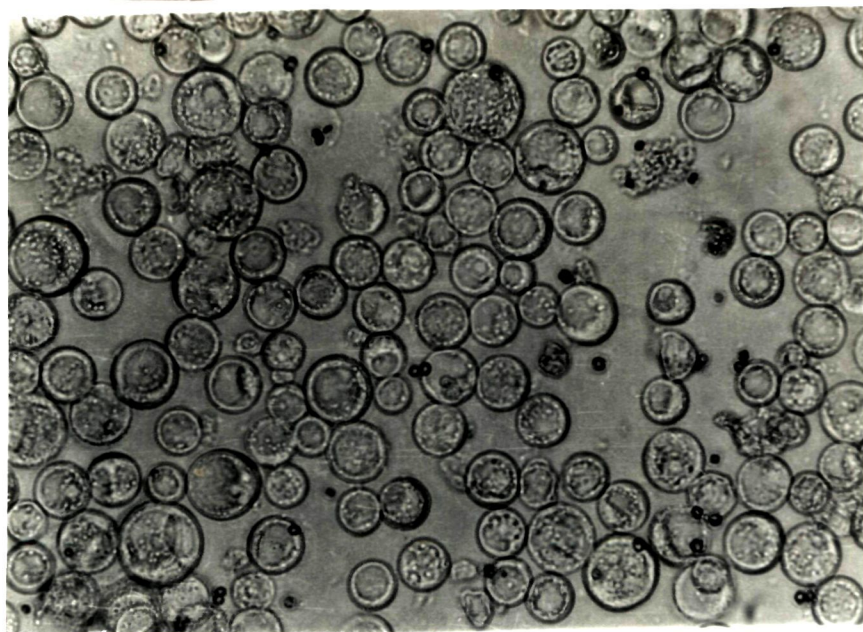
Treatment: Protoplasts from three experimental species were isolated based on previous experimental results. Tobacco protoplasts were cultured in modified 8E medium (Niedz et al., 1985) as reported by Sankara Rao and Gunasekari (1991). (1991). Tomato and bell pepper were

Protoplast isolation and culture of N. tabacum cv. Thompson

Plate-1: Freshly isolated protoplasts

Plate-2: First cell division

Plate-3: Microcalli formation



cultured in TM medium (Shahin, 1985) as preliminary experiments using modified 8E medium didnot give positive results. Once macrocalli was formed it was transferred to MS medium for organogenesis. The protoplast cultures were kept in dark or exposed to light to study the effect of light on protoplast culture. Plating efficiency was recorded on 15th day in tobacco and tomato, and on 20th day in bell pepper.

Tobacco mesophyll protoplasts were isolated using both the set of cellulytic enzymes and cultured in modified 8E medium containing NAA (1mgL^{-1}) + 2,4-D (1mgL^{-1}) + BA (0.5mgL^{-1}) + GA (0.5mgL^{-1}) and incubated in dark for two weeks, later transferred to diffused light. Once micro calli was formed it was kept in cool daylight ($200 \text{ UEin. m}^{-2}.\text{s}^{-1}$) with a light/dark period of 16/8 hours.

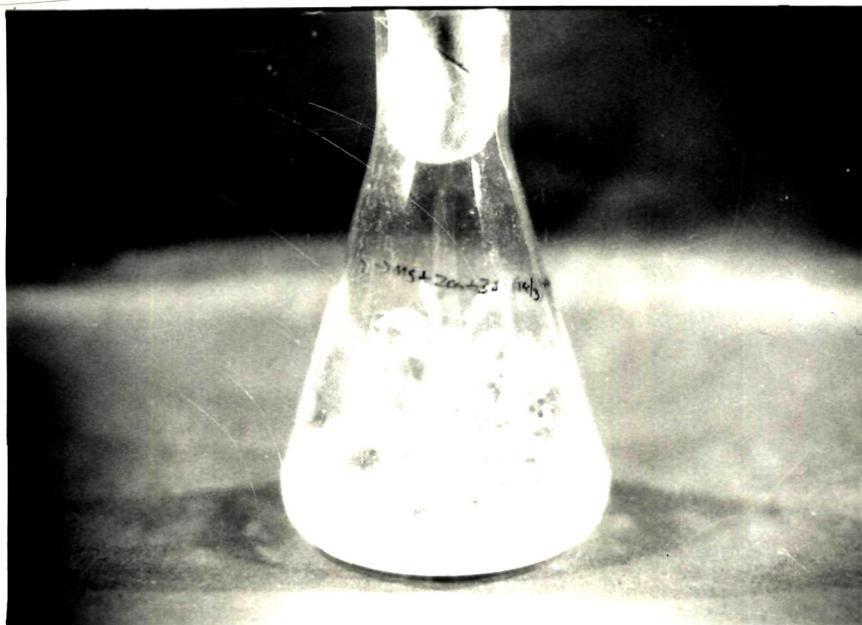
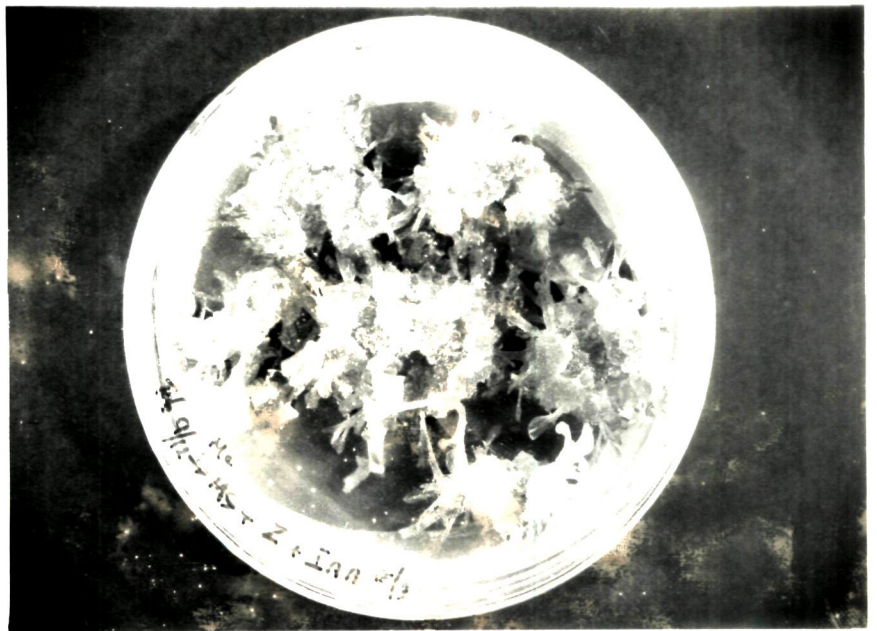
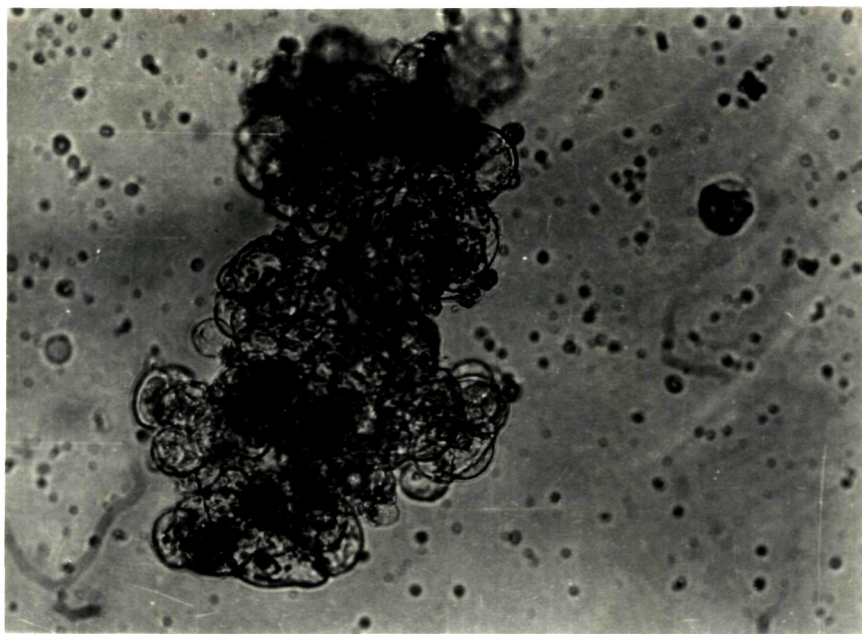
Freshly prepared protoplasts were cultured at 2×10^4 protoplasts ml^{-1} and incubated in dark. By two days cell wall formation was observed by a ring around the plasmolysed cell when the protoplasts were plasmolysed. By 3rd day first division observed (Plate-2) and 5th day the second division/quarterplet formed and continued regular cell division. After 7 days, 100ul of CM-1 medium was added every three days till 20th day. After 15 days plating efficiency was determined (Table-11). Irrespective of the enzyme sets

Protoplast isolation and culture of N. tabacum cv.
Thompson

Plate-4: Protoplast derived calli

Plate-5: Shoot oragnogenesis
from protoplast deerived calli

Plate-6: Whole plant regeneration



used the plating efficiency was 75-80%. On 20th day the CM-I was partially removed equal amount of CM-II was added. At this stage 10-20 celled clumps (Plate-3) were observed. at weekly interval 100ul of fresh CM-II medium was added. By 6th week micro calli was carefully lifted and plated on to CM-III solid medium for macrocalli formation (Plate-4). The growth was vigorous and by 8th week the macro calli was formed. This was later exposed to different hormonal combination for organogenesis. By 10 weeks shoot initials were observed and by 12th week the shoots were ready to be rooted (Plate-5). By 14th week the plantlet was ready for planting-out (Plate-6).

Tomato cotyledonary protoplasts were isolated using Onozuka and Novo enzymes. But protoplasts isolated from Novo enzymes turned brown and died after 4-5 days in culture, irrespective of the culture medium.

The protoplasts were cultured in TM-medium and exposed to dark or light treatment. Generally, cell wall formation was observed. Eventhough cellwall formation was observed no further division was observed in protoplasts exposed to light and after few days they died due to phenolic production

In protoplasts incubated in dark, the protoplasts with new cell walls showed division only in few combinations. It required both NAA and 2,4-D to *initiate* cell division and in absent of any one the cell division

Table-10: Standardization of media composition for protoplast culture

Hormone combination in CM-1 (mg/L)	Tomato cotyledon		Explant source		Pepper mesophyll	
	Light	PE	Dark	PE	Light	PE
0.5 NAA + 0.5 BA	+	-	+	-	+	-
1.0 NAA + 0.5 BA	+	-	+	-	++	20
1.0 NAA + 1.0 BA	+	-	+	-	+	-
0.5 NAA + 0.5 Zea	+	-	+	-	+	-
1.0 NAA + 0.5 Zea	+	-	+	-	+	-
1.0 NAA + 1.0 Zea	+	-	+	-	+	-
0.5 2,4-D + 0.5 BA	+	-	+	-	+	-
1.0 2,4-D + 0.5 BA	+	-	+	-	+	-
1.0 2,4-D + 1.0 BA	+	-	+	-	+	-
0.5 2,4-D + 0.5 Zea	+	-	+	-	+	-
1.0 2,4-D + 0.5 Zea	+	-	+	-	+	-
1.0 2,4-D + 1.0 Zea	+	-	+	-	+	-
0.5 NAA + 0.5 Kin	-	-	-	-	-	-
1.0 NAA + 0.5 Kin	-	-	-	-	-	-
1.0 NAA + 1.0 Kin	-	-	-	-	-	-
0.5 2,4-D + 0.5 Kin	-	-	-	-	-	-
1.0 2, 4-D + 0.5 Kin	-	-	-	-	-	-
1.0 2, 4-D + 1.0 Kin	-	-	-	-	-	-
1.0 NAA + 1.0 2,4-D + 0.5 Kin	-	-	-	-	+	-
1.0 NAA + 1.0 2,4-D + 0.5 Zea	+	-	+++	20-25	++	-
1.0 NAA + 1.0 2,4-D + 0.5 BA	+	-	+++	20	++	20
1.0 NAA + 1.0 2,4-D + 0.5 BA	+	-	+++	-	++	-
+0.5 Zea	+	-	++	-	+	-

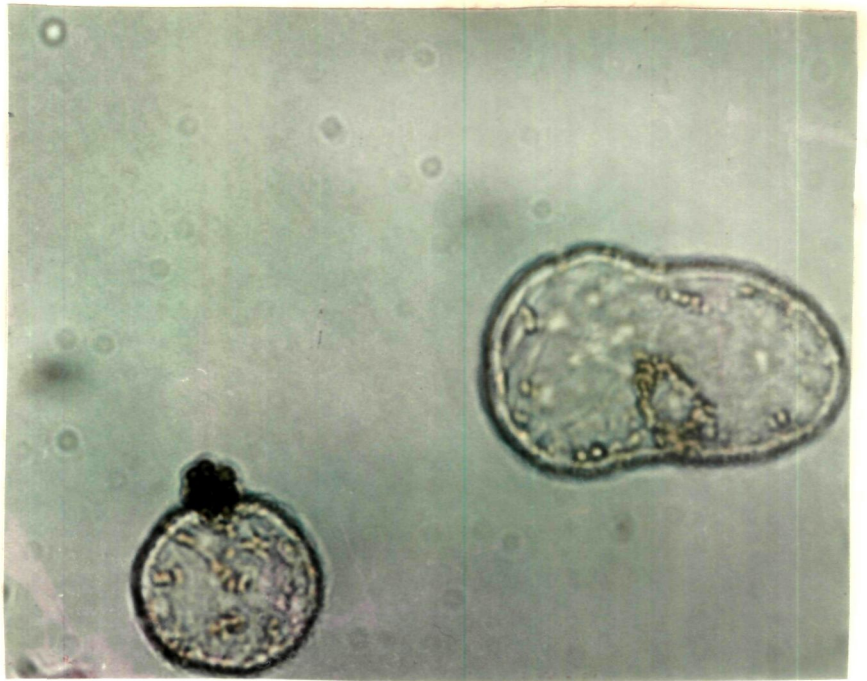
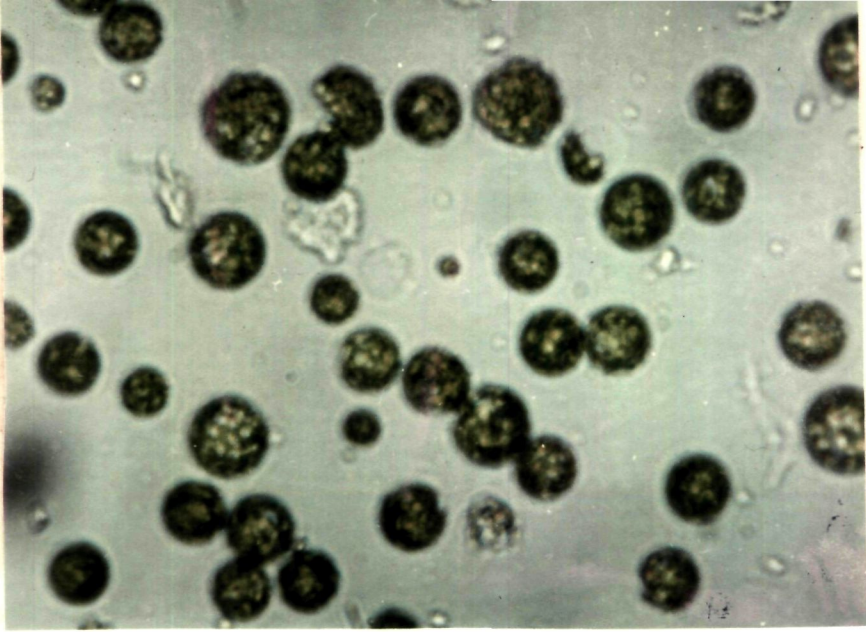
+ Double shaped protoplasts formed
 ++ First cell division only
 +++ Regular successive cell division

Protoplast isolation and culture of L. esculentumcv.
Arka Saurabh

Plate-7: Freshly isolated protoplasts

Plate-8: First cell division

Plate-9: Irregular cell division



was hampered. It was also observed that cytokinins- BA and zeatin in combination were harmful for cell division and phenolic secretion was observed after first division itself.

In two combination viz. NAA (1mgL^{-1}) + 2,4D (1mgL^{-1}) + zeatin (0.5mgL^{-1}) and NAA (1mgL^{-1}) + 2,4D (1mgL^{-1}) + BA (0.5mgL^{-1}) there was continuous cell division and showed a plating efficiency of 20-25% after 15 days (Table-10). In the final experiments to work out organogenesis, the isolated protoplasts were cultured in CM-I in petri dishes and incubated in dark. As followed in tobacco after 7 days every 3 days 100 μ l of fresh CM-I was added till 20th day. By second day cell wall formation was observed and first division by fourth day (Plate-8) and by 6th day second division and further division continued. The plating efficiency was 25-30 % after 15 days. After 20th day 50% of CM-I medium was replaced by equal quantity of CM-II. After few divisions the cells started showing irregular division and started forming single chains rather than a clump (Plate-9). Finally by 30 days the cell division stopped and no further growth was observed even after 40-45 days and slowly the cells started degenerating. Organogenesis could not be attempted as microcalli could not be obtained.

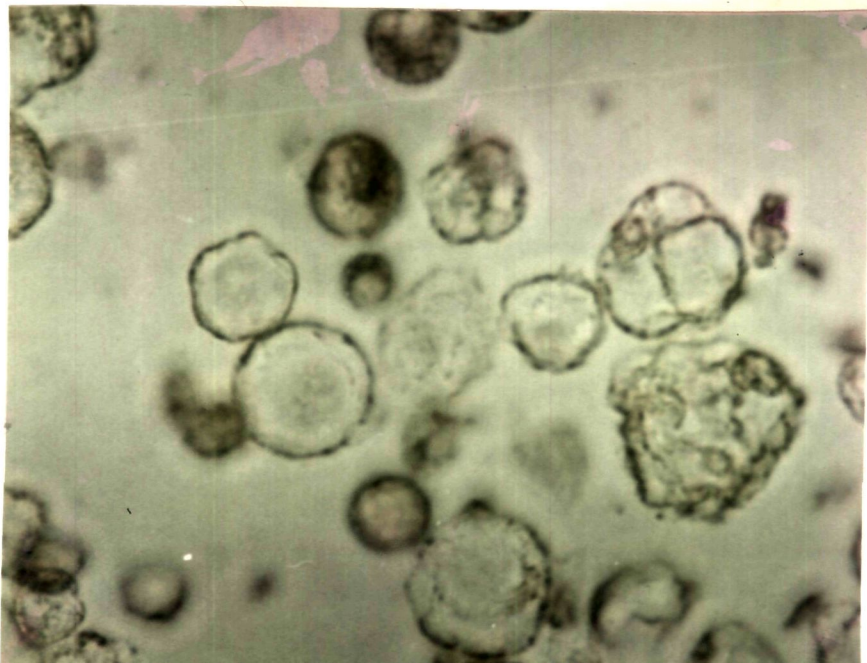
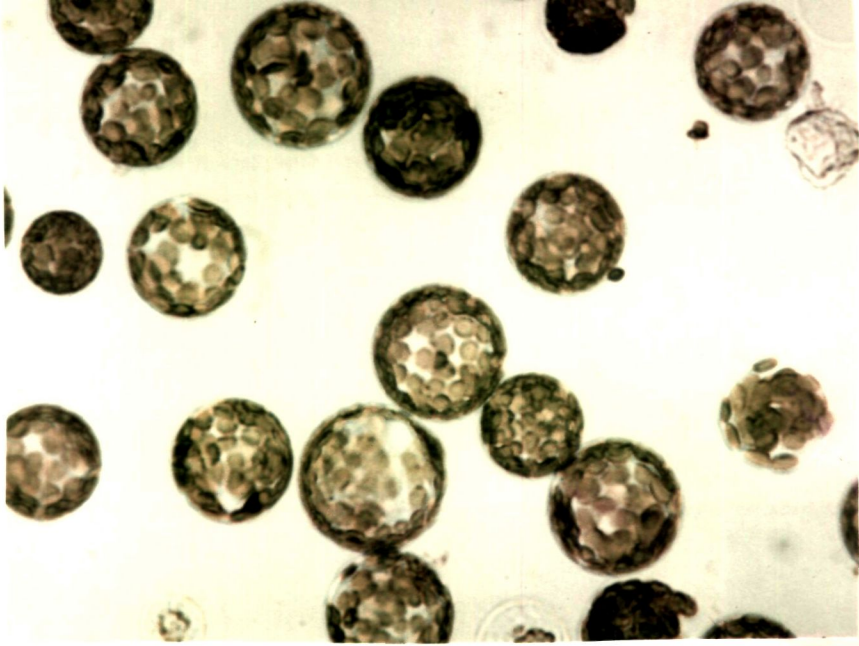
Bell pepper protoplasts were isolated from cotyledon and mesophyll tissues and a good yield was

Protoplast isolation and culture of C. annuum cv.
California wonder

Plate-10: Freshly isolated protoplasts

Plate-11: First cell division

Plate-12: Second cell division



obtained. Both Onazuka and Novo enzymes were used for isolation.

Irrespective of the culture medium tried the protoplast turned brown and degenerated by 4-5 days in protoplasts isolated using Novo enzymes.

The cotyledonary protoplasts isolated with Onazuka enzymes however, appeared healthy and irrespective of hormonal combinations used produced cell wall in two days. There was initiation of cell division in few combination where BA and Zeatin was used. Cell elongation, the prerequisite for cell division was observed by 5th day and formed dumble shape. There was no crosswall formation viz. cytokinesis, and hence further division process was hampered in cotyledonary protoplasts. Even after 15-20 days the cells were viable but later degenerated. This was observed in most of the combination tested.

Mesophyll protoplasts isolated using Onazuka enzymes were cultured in different hormonal combination and under two environmental conditions viz., light and dark incubation. Most of the combination except with kinetin showed symptoms of cell division by 4th day. When protoplasts was exposed to continuous light from the time of isolation, only in NAA (1mgL^{-1}) + 2,4-D (1mgL^{-1}) + Zea (0.5mgL^{-1}), the protoplasts showed first division (Table-10). Even in this the second and the subsequent divisions were not observed and the cells survived for

Table-11 : Standardization of culture conditions for development of Organogenesis from protoplast

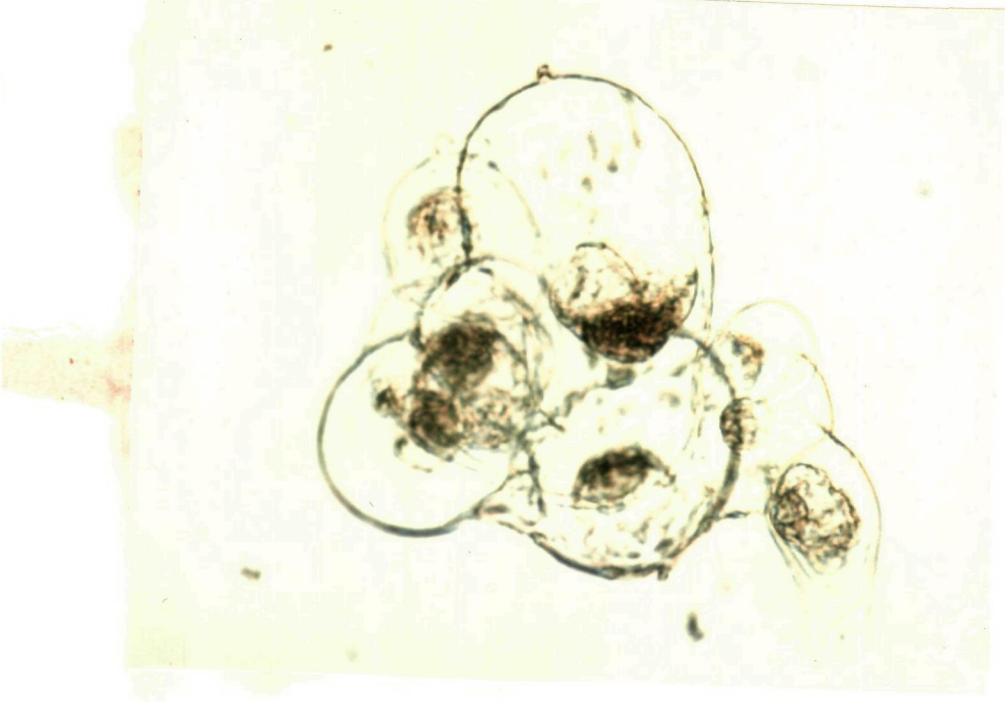
Species	Source Tissue	Protoplast culture		Micro calli culture		Macrocalli culture		Shooting medium		Rooting Culture period			
		Culture medium	Period of culture (day)	Plating efficiency(%)	Period of culture (day)	Medium	Period of culture (day)	Medium	Period of culture (day)				
<i>M. Tabacum</i> cv. Thompson	Mesophyll	CH-10	20	75-80	CHII	20	CHIII	20	CHIV-1	25	10-12	CHV	25
<i>esculentum</i> cv. Arka saurabh	Cotyledon	CH-1	20	25-30	CHII	15ss	-	-	-	-	-	-	-
<i>C. annuum</i> cv. California-wonder	Cotyledon	CH-1	30s	-	-	-	-	-	-	-	-	-	-
	Mesophyll	CH-1	25	20-25	CHII	20	CHIII	20	CHIV-1	25	-	Embryoid	-
												Calli	-
												2-5 shoots	CHV
												Calli	-
CH-10	MSmedium + 1.0mg/L BAA + 1.0mg/L 2,4-D + 0.5 mg/L BA + 0.5mg/L GA												
CH-1	TMmedium + 1.0mg/L BAA + 1.0mg/L 2,4-D + 0.5 mg/L BA												
CH-11	TMmedium + 2.0mg/L BAA + 0.5 mg/L BA												
CH-111	MSmedium + 2.0mg/L BAA + 0.5 mg/L BA + 0.6% agar												
CH-1V-1	MSmedium + 0.5mg/L IAA + 2.0mg/L Ica												
CH-1V-2	MSmedium + 0.5mg/L IAA + 1.0 mg/L BA												
CH-1V-3	MSmedium + 0.5mg/L IAA + 2.0mg/L GA + 10.0 mg/L BA												
CH-1V-4	MSmedium + 0.5mg/L IAA + 2.0mg/L GA + 10.0 mg/L Kin												
CH-V	1/2 MSmedium + 1.0mg/L BAA												
*	CAPSICUM cotyledonary protoplasts didnot divide												
**	Microcalli turned brown and degenerated												

Protoplast isolation and culture of C. annum cv.
California wonder

Plate-13: Microcalli formation

Plate-14: Protoplast derived
calli

Plate-15: Shoot organogenesis from
protoplast derived calli



20-25 days and later degenerated. In all other cases even though cell wall formation and cell elongation was observed, no division occurred, probably due to phenolic secretion from protoplasts and later degenerated.

The dark incubated protoplasts showed the shape and configuration that generally is noticeable before cell division occurs in all combinations except in kinetin (Table-10). In two combinations first division was observed but only in one the sustained cell division occurred (Table-10). The cells divided and formed microcalli, macrocalli and finally organogenesis occurred. Since only in this combination there was cell division this was used for organogenesis (Table-11). The cell wall formation was observed by 2nd day and only after 7-8 days the first division occurred (Plate-11). The initial process of cell division was very slow, but the second division occurred by 12th day (Plate-12). As in tobacco and tomato, every three days after 7th day after inoculation, 100ul of fresh medium was added till 25th day. On 20th day the plating efficiency was recorded as 20-25%. On 25th day, half the quantity of CM-I medium was removed and equal quantity of CM-II was added and the culture were exposed to light. The rate of cell division slightly improved and by 45th day microcalli was formed (Plate-13) and at this stage the micro calli was plated on to CM-III solid medium. By 65th day the macro calli was formed (Plate-14), which

Protoplast isolation and culture of C. annum cv.
California wonder

Plate-16: Whole plant regeneration



was exposed to CM-IV medium with different hormonal combination (Table-11). Based on the hormonal combinations the calli response varied from continued calli formation, embroid formation or shoot formation by 25th days after culture (Table-11). 4-5 shoots were obtained per calli which was transferred to rooting medium (CM-V) with IAA (0.5mg/L) (Plate-15). There was 30% rooting, and finally the whole plant was obtained (Plate-16). The whole cycle of protoplast isolation to plantlet regeneration took around 110-120 days.

DISCUSSION

V. DISCUSSION

The first set of experiments was conducted with the objective of determining the tolerance level of different species and explants under study to osmotic conditions for effective protoplast isolation. The second experiment was conducted to determine the best source and combination of cell wall degrading enzymes for protoplast isolation. The third experiment was conducted to assess the effect of biotic and abiotic factors for isolation of viable protoplasts. Finally, experiments were conducted to standardise the culture conditions for demonstrating the totipotency of protoplasts in relation with to explant source of all the three species tested.

In the present set of experiments the leaves from axenic shoot cultures were cut into small strips and exposed to enzymatic digestion. This method has been reported in Lycopersicon peruvianum (Muhlbach, 1980), L. esculentum (Tan et al., 1987), tobacco (Dorion et al., 1975; Bourgin et al., 1979) and Capsicum (Diaz et al., 1988). Before Dorion et al., (1975) observed that cut leaves are also efficient for protoplast isolation, workers used to peel the lower epidermies and then float the entire leaf over the enzyme solution (Nagata and Takebe, 1971; Morgon and Cocking, 1982; Koblitz and Koblitz, 1982a,b).

The pattern similar to the osmotic response observed for the crops under study was also reported in tomato by Cocking (1960). It was suggested that the protoplasts should be slightly plasmolysed during isolation. Excess osmotic pressure has been reported to impair cell metabolism (Reusink, 1978) and cell wall regeneration (Pearce and Cocking, 1973).

Mannitol and sorbitol are most frequently used, but mannitol is preferred for isolation of mesophyll protoplasts. The protoplasts tend to sink in osmotic solutions of hexitols and glucose, but float on sucrose. Hence protoplasts are generally cultured in inert hexitols (Eriksson, 1985). Osmotic potential changed with the source tissue used and varied between 0.3 to 0.7M .

In the present study, 0.55M mannitol with 10mM CaCl_2 was found to be beneficial for protoplast isolation, irrespective of the source tissues used. Eventhough the range of tolerance to osmotic levels varied with species and genotypes, tomato mesophyll tissues showed a narrow tolerance level of 0.5 to 0.55M, but tobacco and pepper showed tolerance to range of 0.4-0.6M mannitol concentration.

Similar observations has been reported by Sankara Rao and Gunasekari (1991) in tobacco, Muhlbach(1980), Tan et al.,(1987) and Gleddie et al.,(1989) in tomato.

Sankara Rao and Gunasekari (1991) used 9% mannitol in tobacco which has also been followed in the present study, but Diaz et al., (1988) used 13% mannitol to isolate Capsicum mesophyll protoplasts. Chung et al., (1986) reportedly used 0.4M mannitol as osmoticum to isolate protoplasts of L. esculentum and L. pimpinifolium cv. Tiny tim.

There are reports of sugars other than mannitol used for protoplast isolation viz. sorbitol at 0.4M in Capsicum (Niedz et al., 1987) and 0.7M in tomato (Zapata et al., 1977, Koblitz and Koblitz, 1982a,b) and sucrose at 0.3M for mesophyll tissues of tomato (O'Connell and Hanson, 1985; Shahin, 1985). Bellini et al., 1990, reportly used myo-inositol as an osmotic stabiliser in digestion mixture and observed enhanced viability of tomato protoplasts.

Besides the osmotic stabilisers, the other factors which are important for efficient protoplast isolation, are membrane stabilizers, antioxidants, buffers and media composition.

To isolate protoplasts in tomato and Capsicum, shahin (1985) and Donato et al., (1989), similarly used CPW salts, 5mM MES buffer, 0.5 mgL^{-1} ascorbic acid and 1.0% PVP and discussed their importance in protoplstat culture. Cocking, 1982; Koblitz and Koblitz, 1982a,b; 1983; Niedz, 1985; Tan et al., 1987; Shahin et al., 1985).

There are reports of accumulation of phenolics secreted by protoplasts during isolation and culture which affect protoplast viability and survival. This has been demonstrated in Capsicum suspension culture (Luenca et al., 1989) and tomato protoplast culture (Niedz et al., 1985). Therefore antioxidant (ascorbic acid) and polyphenol adsorber (PVP) was used in the protoplast isolation and culture media.

In the present study, mesophyll tissues from 2-3 week-old axenic cultures with 2% cellulase and 0.5% macerozyme were used for protoplast isolation. Earlier reports show that these enzymes were used for isolation of protoplasts from various species and genotypes viz. N. tabacum (Nagata and Takebe, 1971; Bourgin et al., 1979, Bourgin and Missonier, 1978), N. rustica (Gill et al., 1979), N. sylvestris (Nagy and Malign, 1976), N. otophora (Banks and Evans, 1976) and N. plumbeiginifolia (Gill et al., 1978). Bourgin et al., (1979) reported isolation of mesophyll protoplasts from six species of Nicotiana.

Tobacco protoplasts were isolated from only mesophyll tissues where as cotyledon, hypocotyl and mesophyll tissues were tried in tomato and pepper from aseptically grown materials. Hypocotyl tissues yielded very few protoplasts. There are no reports of protoplast isolation from hypocotyl tissues in tomato and pepper as it was found to be very fragile, but in sunflower

hypocotyls were considered to be the best choice for of protoplast preparation and culture (Bohorava et al., 1986; Lenee and Chupeau, 1986)

Earlier reports indicate that cotyledons (Koblitz and Koblitz, 1982a,b, 1983; Saxena et al., 1981; Messerschmidt, 1974) and mesophyll tissues (Muhlbach, 1980; Gill et al., 1979, Cassells and Barlass, 1976, Patel et al., 1994) are commonly used explants although stem cortex (Gleddie et al., 1989), shoots (Diaz et al., 1988), fruit tissues (Gregary and Cocking, 1965; Raj and Herr, 1970), callus and cell cultures (Tan et al., 1987, Koblitz and Koblitz, 1982b) were utilized also for protoplast isolation.

Four different sets of enzymes were used for isolation. The crude liquid form and lyophyllised enzymes-cellulase and pectinase procured from Biocon, Bangalore, were found unsuitable for protoplast culture. Eventhough there was cell wall digestion observed, these induced lysis of the protoplasts simultinously. This observation was in agreement with the report of Vasil and Vasil(1980) that crude enzymes might contain contaminants like salts, ribonucleases, peroxidases, lypases and phenols which cause lysis of the isolated protoplasts. Tseng and Mount (1974) observed that purified protease and phospholipases from culture filtrate of Erwinia carotovara could lyse the

protoplasts of cucumber. Santos et al., (1980) reported that molecular sieving of crude enzymes using sephadex G-25 ensured maintenance of viability of protoplasts isolated from carrot suspension cultures.

When crude Novo enzymes were used for isolation, there was a good response even at lower concentrations and there was effective release of protoplasts at a faster rate. When protoplasts were exposed to enzymes for a longer time, it affected the viability. It might be due to the contaminants such as lipases, phenols and salts present in the crude enzymes as reported earlier (Vasil and Vasil, 1980). Irrespective of the source tissues the enzyme concentration required was 1.2% celluclast and 0.5%pectinex as compared to 2% cellulase and 0.5 % macerozyme of Onazuka type. Even the rate of digestion was faster compared to onazuka enzymes. Using these enzymes there was efficient release of protoplasts from tomato and pepper. Further, the whole cycle from protoplast isolation to plant regeneration was possible in tobacco cv. Thompson. This shows that the new set of enzymes tested from Novo industries can be effectively used in protoplast work.

By using Onazuka enzymes viz. cellulase at 2.0% and macerozyme at 0.5%, there was efficient release of protoplasts from cotyledonary and mesophyll tissues of tomato and Capsicum, and mesophyll tissue of tobacco. Since the enzymes were relatively pure there were no

deleterious effects observed on the viability when protoplasts were exposed to digestion environment for longer time. This was observed in N. rustica (Gill et al., 1979), N. Tabacum (Thanutony and Kitvijarn, 1985) and tomato (Gleddis et al., 1989).

There was a significant difference in rate of release and yield between the two set of enzymes. Eventhough the rate was better with Novo enzymes the yield was higher in Onazuka, the viability being the same in both the cases, in the three species tested.

Díaz et al., (1988) used 1.0% cellulase and 0.25% macerozyme and obtained a yield of 4×10^5 protoplasts g^{-1} FW in Capsicum annuum as compared to an yield 8×10^6 g^{-1} FW with the same enzymes-Cellulase at 2.0% and macerozyme at 0.5% protoplasts with the same enzymes after overnight digestion during the present study. This indicates that with increase in enzyme concentration the protoplast release was more effective.

Chung et al., (1986) used high concentration of cellulase (3%) and macerozyme (2%) to isolate protoplast from mesophyll tissues of tomato as against 2.0% cellulase and 0.5% macerozyme used in this experiments and even lesser doses of 1.0% cellulase and 0.5% macerozymes (Tan et al., 1986; Shahin, 1985). Gill et al., (1979) and Schenk and Hilderbrandt (1969) reported

that cellulase alone can be used for protoplast isolation and pectinase is not required.

Enzymes other than Onazuka or Novo enzymes were also reportedly used for protoplast isolation. Niedz et al., (1988) used Meicelase-p (3.0%) and Macerase (0.1%) to isolate tomato mesophyll protoplasts. Koblitz and Koblitz (1982) used mescelase (0.2%), macerase(0.1%) and driselase (0.2%) to isolate protoplasts from tomato cortical tissues.

Nomura et al., (1982) and Zapata et al., (1977) isolated protoplasts from carrot tissues using a single Driselase which has both cellulytic and pectiolytic activity but Muhlbach (1980) maintained that driselase damages the protoplasts released.

While selecting appropriate enzyme combinations, it should be noted that not only the yield and rate of protoplasts released, but also the viability of protoplasts to be considered. In the present experiment the viability was not affected by use of the two sets of enzymes viz. Novo and Onazuka enzymes. Rethmeier et al., (1991) demonstrated that cellulaseR-10 is preferable to cellulysin which was found to reduced the viability of cultured tomato protoplasts.

There are reports (Hongwang et al.,1987) where they had shown that cellwall degrading enzymes inhibit nuclear division and not DNA synthesis in soybean.

Patnaik et al., (1982) also showed that cell wall degrading enzymes are toxic to varying degree and might affect physiology of cells. When crude enzymes were desalted using BioGel P₆ and tested for their activity and toxicity (Cassells and Barlass, 1976). Polygalactaronase fraction and cellulase fraction when used at the same concentration as crude enzyme resulted in reduction in protoplast yield but the viability at the same time was not effected.

Cassells and Barlass (1978) reported that cellulases were relatively less toxic and active in protoplast release for tomato explants. From these observations it was found that the yield and viability of protoplasts reflects on enzyme purity.

Gill et al., (1979) used only cellulase at 3% and obtained an yield of 2.2×10^6 protoplasts $g^{-1}FW$ as against 4×10^7 protoplast $g^{-1}FW$ in this experiment where both cellulase (2%) and macerozyme (0.5%) were used for tobacco mesophyll tissues. Gill et al., (1979) concludes that pectinase has no beneficial effect on protoplast release, but its beneficial effect was observed by efficient digestion in the present study.

There are reports to show that type and concentration of enzymes vary with species, variety and type of the explants used as observed in this experiment. Abdullah et al., (1986), showed that young

embryogenic cell cultures of rice require less quantity of enzymes as compared to old suspension cultures to release protoplasts. Pollen tetrads require a different set of enzymes like pectolyase, which is stronger than pectinase to release protoplasts (Vasil and vasil, 1980).

The other factors influencing the yield and viability of protoplasts are, the explant status, pretreatments, method of incubation and isolation procedure. In the present study, all the plants used were from axenic cultures, prior to isolation, plants were dark preincubated at 4⁰C for 18 hours, later cotyledon or mesophyll explants were cut into small strips and incubated in digestion mixtures in dark without shaking.

There are reports of dark pretreatment of stem segments (Gleddie et al., 1989), as well as leaves of tomato (Chaung et al., 1986; Patil et al., 1994) at 4⁰C for 16-18 hours prior to isolation. Patel et al., (1994) also has demonstrated that preincubation enhances the yield and viability of tomato mesophyll protoplasts. Cold conditioning of plant material enhanced the stability of Solanum and Lycopersicon protoplasts (Haberlach et al., 1985; Tan et al., 1987) by inducing the cells to adapt to stress conditions which are imposed during the rigorous protoplast isolation procedures.

There are reports which shows that just preplasmolysis of the tissues for 1-2hours may also improve the yield and viability. This has been reported in tomato (Niedz and Sink, 1988; Niedz et al., 1985 and Montagno et al., 1991) and Capsicum (Diaz et al., 1988). Gentle preplasmolysis reduces protoplast damage and spontaneous fusion during subsequent enzyme incubation and seals off plasmodesmata preventing the leakage of cell components, including ribonuclease which can trigger senescence and protoplast lysis (Galston, 1978). Some reports show that pre-treatment or preplasmolysis is not necessary but the source material should be grown in controlled conditions with sufficient light, nutrients, temperature and moisture. Even the in vitro grown cultures also yield good viable protoplassts. In vitro grown explants was used without pretreatments or preplasmolysis in tobacco (Sankara Rao and Gunasekari, 1991), N rustica (Gill et al., 1979) and tomato (Belline et al., 1990). Tan et al., (1985, 1987) reported that protoplasts isolated from the in vitro grown plants divided more rapidly and reached higher plating densities than field grown tomato plants.

Pretreatment of plant at 4⁰C and inclusion of kinetin in the digestion mixtures followed in the present study enhanced the frequency of cell division as also reported by Berry et al., (1982) in lettuce. Vasil (1976), Eriksson (1977). Bhojwani and Razdan

(1983) attributed importance to stringent control of the source material for efficient release of viable protoplasts.

The single step digestion procedure followed in this experiment has been reported earlier in Capsicum (Diaz et al., 1988) and tomato (Cassells and Barlass, 1976; Montagno et al., 1991 and Koblitz & Koblitz, 1982).

Some follow two step digestion procedure and reported enhanced yield and viability of protoplasts as observed by Gregory and Cocking, 1965 (locule tissues of tomato), Raj and Herr, 1970 (berries of Solanum) and Cassells and Barlass, 1976 (tomato mesophyll tissue).

Purification of protoplasts was done using the method followed by Sankara Rao and Gunasekari (1991), which involved washing with 9% mannitol and floating on 20% sucrose. This method was a modified method to that reported in tomato (Gleddie et al., 1989) and tobacco (Gill et al., 1979). There are reports where polyethylene glycol, dextran, sorbitol, percoll and ficoll are used for floatation (Vasil and Vasil, 1980). Even 13 % mannitol has been used in floatation of protoplasts in Nicotiana species (Bourgin et al., 1979). Koblitz and Koblitz (1982) directly cultured protoplasts without the step of floatation in tomato.

Generally fragile mesophyll tissues are kept for digestion in a stationary phase (Vasil and Vasil, 1980) as followed in this experiment but suspension cell cultures require a low speed agitation (Vasil and Vasil, 1980) to release the protoplasts.

The viability of protoplasts was estimated using Fluorescein diacetate (FDA) as shown by Sankara Rao and Gunasekari(1991) in tobacco and Patil et al., (1994) in tomato. The important factors which affect viability includes physiological state of explant, enzymatic toxicity, method of isolation and also the culture conditions.

There was a significant increase in the efficiency of protoplast isolation with increase in digestion temperature from 27⁰ to 30⁰C. This has been reported in N. rustica by Gill et al., during 1979. They observed that there was a considerable increase in yield when temperature was raised from 20 to 25⁰C and maintained at 30⁰ but protoplast yield reduced significantly at 35⁰C.

Three different liquid culture media were tried. Tobacco protoplast was cultured in 8E medium (Niedz et al., 1985) as followed by Sankara Rao and Gunasekari (1991), and observed cell division, callus formation and finally organogenesis. But the response of tomato and pepper protoplasts to same 8E medium was not encouraging. The MS medium also did not favour the

culture. The TM medium (Shahin, 1985) with $1g^{-1}$ of low melting agarose and 1mM PVP was found the best. There are reports that MS medium is not good for initial protoplast culture as it contains higher amount of ammonical ion as demonstrated in potato (Shepard and totten, 1979), tomato (Tan et al., 1987) and Salpiglossis sinuata (Boyes et al., 1980). KM8 medium (Kao and Michayluk, 1975) has been reported to be beneficial for Capsicum protoplasts (Diaz et al., 1988). Montagno et al., 1991 used Lycopersicon culture medium (LCM) which is a combination of B5 macro and micro salts, and vitamins from Kao and Michaylak, (1975) for different tomato cultivars. Koblitz and Koblitz (1983) used KP2 medium to culture tomato protoplasts.

Most of the recent reports show that TM medium directly or with slight modification are used in tomato (Patil et al., 1994; Rajeshwariand Ramaswamy, 1994) and Capsicum (Diaz et al., 1988)

The important ingredient of the culture medium is found to be inositol, replacement of ammonical to nitrate nitrogen, MES buffer, antioxidants (ascorbic acid) and polyphenol adsorbers [polyvinylpyrrolidone (PVP)] to avoid browning and maintain viability of protoplasts. Browning has been observed in tomato

(Morgan and Cocking, 1982; Niedz et al., 1985) and Capsicum (Diaz et al., 1988; Donato et al., 1989) protoplast culture.

The effect of ammonical ion on viability has been shown in Asteraceae plants (Okamura et al., 1984), Potato (Shepard and Totten, 1979) and Salpiglossis sinuata (Boyes et al., 1980). MS medium did not favour initial culture and hence TM medium was used in this study.

There are reports of production of ethylene by protoplasts and injured cells in cultures. This effects the viability of protoplasts as reported in tomato (Cassells et al., 1980, 1986). Phenols are also secreted by cells during growth in cultures which when oxidised lead to inhibition of growth. Hence the antioxidant (ascorbic acid) and the polyphenol adsorber (PVP) were incorporated into the medium, in the present study. These were earlier used by (Shahin, 1985; O'Connell and Hanson, 1985) in tomato and Capsicum (Saxena et al., 1981; Donato et al., 1989) to prevent oxidation. protoplast medium. Niedz et al., 1985, showed that browning can be reduced to a greater extent by frequently changing the culture medium and dark culturing of protoplasts initially. The antioxidants/polyphenol adsorbers need not be used in this case.

In the present study liquid media were used for protoplast culture initially. Protoplasts at $2-4 \times 10^4$ protoplasts ml^{-1} was plated in liquid 8E/TM medium in petridishes and dark incubated at $25 \pm 2^\circ\text{C}$. Similar method of culture of protoplast was employed for tobacco (Sankara Rao and Prakash, 1995), tomato (Muhlbach, 1980; Shahin, 1985; Tan et al., 1987; Niedz and Sink, 1988; Montagno et al., 1991; Patil et al., 1994) and Capsicum (Diaz et al., 1988; Saxena et al., 1981). The advantages of this technique is that replenishing/ changing of culture media is easier. The solid agar medium has also been followed by some workers where the protoplasts are mixed in warm agar medium and allowed to solidify in petridishes. This was reported in tobacco (Nagata and Takebe, 1971; Gill et al., 1979), tomato (Morgan and Cocking, 1982; Koblitz and Koblitz, 1982a,b, 1983). The advantage of solid medium over liquid culture is that the protoplasts are held in a fixed position which is essential for efficient cell wall synthesis but the disadvantage being accumulation of phenols which may cause browning (Vasil, 1980).

Saxena et al., (1981) and Diaz et al., (1988) used agarose bead method of protoplast culture in Capsicum. This method helps to overcome some of the disadvantages observed in previous methods. Since the protoplasts are embedded and immobilised in agarose beads, and liquid cultures are used to provide nutrients which can be

diluted/changed with time. By this method it was observed the cell wall synthesis was uniform and browning was avoided.

In the present study liquid culture along with 1 gL^{-1} low melting agarose was used for protoplast culture. Agarose slightly changes the viscosity of the culture medium by which uniform cell wall formation is facilitated and budding of protoplasts avoided. Cell wall formation was observed within two days irrespective of the hormonal concentration. The freshly formed cell wall is seen as a ring around the plasmolysed protoplasts. This method was for the first time reported to study the cell wall synthesis. This method of plasmolysis to monitor cell wall synthesis is quite easy and could be observed by a simple microscope as compared to fluorescence microscope when calcofluor white was used. In the earlier reports, either calcofluor white (Galbraith, 1981; Muhlbach, 1980 and Patil et al., 1994) or tinopol B.O.P.T (Bilkey and Cocking, 1982) were used to ascertain cell wall synthesis. Shahin (1985) reported that tomato protoplasts form cell wall but do not divide in absence of hormones. Nicotiana, Petunia, Datura and Brassica protoplasts form cell wall within 30-36 hours (Nagata and Takebe, 1970) but cereal and legumes take 3-4 days for wall regeneration (Giles, 1972).

In tobacco and tomato the first division was observed by 4th day and by 7th day in Capsicum. Similar

results has been reported in tobacco (Nagata and takebe, 1971),, tomato (Shahin, 1985) and Capsicum (Diaz et al., 1988; Donato et al., 1989).

Plating density also effects the cell division. The plating denisty was 2×10^4 protoplasts ml^{-1} of culture medium as followed by Sankara Rao and Gunasekari (1991). This was also reported by Kao and Michayluk(1975), and Shepard and Totten (1975), as larger protoplast populations act as detoxifing mechanism against deleterious substances in the immediate environment.

Tan et al., (1987) reported that an initial protoplast density of $0.5-2.0 \times 10^5$ protoplasts ml^{-1} was required to stimulate growth. Lower than 10^3 protoplasts ml^{-1} , the protoplasts did not divide and degenerated. Eventhough higher plating density (1×10^6 protoplasts ml^{-1}) favoured initial cell division later caused aggregation of protoplasts and leads to browning of colonies. Kao and Michayluk (1975) observed that based on the plating density, the nutritional requirements of the protoplasts varied in Vicia hajastana. When <10 protoplasts ml^{-1} was plated, it required various vitamins and coconut milk to sustain growth but at >250 protoplasts ml^{-1} plating density, simple B5 medium was sufficient for division. Gleba (1978) found that culturing of protoplasts at high density for three days

and lowering of the culture densities as hanging drop cultures increased the plating efficiency.

Cell wall synthesis is not only controlled by genetic factors but also by culture conditions. The cell wall synthesis is affected by higher sucrose (0.3M) and sorbitol (0.5M) concentration in the protoplast culture medium, as reported by Shepard and Totten (1977).

Plating efficiency was recorded on 15th day in tobacco and tomato and 20th day in pepper. The plating efficiency in tobacco was 75-80% as also observed by Sankara Rao and Gunasekari (1991). There are reports of very low plating efficiency of 6-40% in N. rustica (Gill et al., 1979). Similar variation from 0-60% has been observed by Bourgin et al., (1979), in different species of Nicotina viz. N. glauca and N. glutinosa (50%), N. plumbaginifolia (60%) and N. acuminata, N. alata, N. paniculata (as low as 0-5%).

In tomato cotyledonary protoplast cultures, the plating efficiency was 25%. Shahin (1985) also observed a similar PE of 16% in cultivar UC-8L of tomato. Tan et al., (1985) observed that PE varied with cultivar and showed low PE in Abunda (5%) and as high as 35% in Money maker. Niedz et al., 1985, reported that the PE varied from 0.1% in LA 1622 line to 87% in LA 1268. Similar results were observed by Montagno et al., 1991 and Patil et al., (1994).

Muhlbach (1980) observed that plating efficiency is the combined effect of species and media used. Among species L. peruvianum has higher PE of 22% than L. esculentum with 11%. With change in the medium the PE varied from 4-11% in L. esculentum and 1-22% in L. peruvianum.

Mesophyll protoplasts of Capsicum annuum cv. California wonder showed a plating efficiency of 20% after 20 days. Díaz et al., (1988), reported that shoot protoplasts of Capsicum annuum showed a wide range of PE based on the species and cultivars. Capsicum annuum cv. nigrum showed 0% and cv. Americanum-26%, and C. chinense has a PE of 24%. This indicates that the PE is the combined effect of genotype and medium used.

Capsicum cotyledonary protoplasts formed cell wall by 2nd day and by 4-5 days assumed a dumbbell shape and appeared as if prepared for division. However, no cytokinesis was observed even after 15-20 days and later slowly degenerated. Messerschmidt (1974) observed similar phenomenon in Pharbitis nil cotyledonary protoplasts. Saxena et al., (1981) and Donato et al., (1989) reportedly used cotyledonary tissues of Capsicum annuum cv. California wonder for protoplast culture and regenerated plants.

The other conditions which favour cell division are temperature, light, pH and carbon source in the

medium. In the present study the pH of the culture medium was adjusted to 5.8 before autoclaving and protoplasts cultured at 25 ± 2 °C and in dark for 10-15 days based on the species.

A pH range of 5.5 to 5.8 is recommended as reported earlier (Gamborg et al., 1985; Patil et al., 1994; Koblitz and Koblitz, 1987). Temperature range of $22-28$ °C are recommended. Zapata et al., (1977) observed that from $25-29$ °C the protoplasts showed normal growth but at 31 °C the cell division was inhibited.

Nagata and Takebe (1971) were the first to assign a role for light in division of protoplasts. In the present study, tobacco protoplasts were cultured using 2,4-D, NAA, GA and BA in requisite amounts as followed by Sankara Rao and Gunashekari (1991). Cotyledonary and mesophyll tissues of tomato and pepper were cultured in various combinations and found that NAA, 2,4-D and BA were essential but kinetin had inhibitory effect on initial protoplast division. Eriksson (1977) observed that $1-3 \text{ mgL}^{-1}$ of NAA/2,4-D along with $0.1-1.0 \text{ mgL}^{-1}$ of BAP/ Zeatin are beneficial for cell division. O'Connell and Hanson (1985) observed that tomato protoplasts divided in the presence of 2,4-D (0.6 mgL^{-1}) and BA (0.4 mgL^{-1}). Tan et al., (1987) used NAA (1mg), 2,4-D (0.2mg) and BAP (0.5mg) to initiate cell division in Solanum species and L. pennelli.

Niedz and Sink, (1988) and Tan et al., (1987) reported that 2,4-D at a range of $0.1-1.0 \text{ mg}^{-1}$ is essential for protoplast division. Uchimiya and Murashige (1976) and Grawbow et al., (1972) reported that cytokinins are not essential for cell division in tobacco and carrot protoplasts.

Seven days after culture, CM-1 without hormones was added to the petridishes and this was continued every three days till 20th day. After 20-25 days it was transferred to CM-II for microcalli development and then to CM-III for macrocalli formation. This was later transferred to CM-IV for organogenesis and CM-V for rooting.

The advantage of regular dilution with culture medium has been reported in tobacco (Gill et al., 1979; Sankara Rao and Gunashekari, 1991), tomato (Koblitz and Koblitz, 1982a,b, 1983; Shahin, 1985, Patil et al., 1994) and pepper (Saxena et al., 1981; Diaz et al, 1988, Donato et al., 1989).

Tan et al., (1987) reported that if auxin concentration is not reduced after 7-10 days the cells turned brown. The advantage of adding fresh medium is to reduce the deleterious effects of auxin and phenols in the medium.

Once microcalli are formed osmolarity of the culture medium is gradually reduced. It has been

reported that gradual reduction in osmotic strength enhances cell division (Tan et al., 1987; Koblitz and Koblitz, 1982). Shahin (1985) observed that cell division will cease if there is no reduction in mannitol concentration in the culture medium.

Based on the stage of growth of protoplasts hormonal concentration varies. After initial culture, 2,4-D was entirely removed and replaced by 2.0 mgL^{-1} NAA. This has also been recommended by Tan et al., (1987), Gleddie et al., (1989) in tomato to sustain growth and retain morphogenetic capacity.

In tomato cotyledonary protoplasts, eventhough cell wall formation and first division occurred as reported, subsequent division was irregular and it formed are irregular-shaped cell clump. After microcalli formation, there was no further growth and the cells degenerated. Similar observations have been reported by Gleddie et al., (1989) in stem cortex protoplasts of Lycopersicon species, but the protoplasts from intergeneric crosses yielded good protoplasts and also showed sustained growth and regenerated into plants.

This indicates that protoplast growth is mainly controlled by genetic factor of plants. But there are reports where cotyledon and mesophyll protoplasts from L. esculentum have regenerated into plants (Morgan and

Cocking, 1982; Koblitz and Koblitz, 1982a,b; 1983; Niedz, 1985; Tan et al., 1987; Shahin et al., 1985).

In the present study mesophyll protoplasts of tobacco and pepper reached the stage of callus formation and organogenesis. The tobacco callus when exposed to CM-IV-1 culture medium (Table-11) formed shoots. The shoots elongated sufficiently by 25 matured by 25 days which were then transferred to CM-V medium for rooting. It is significant that the protoplasts isolated by both Novo and Onazuka enzymes in the present study, maintained viability and regeneration.

The Pepper calli produced 2-5 shoots in CM-IV-3 culture medium (Table-11). The microshoots were successfully rooted. There are reports of complete regeneration from Capsicum (Saxena et al., 1981; Diaz et al., 1988), but Donata et al., (1989) could achieve only callus formation with no organogenesis using cotyledonary protoplasts of C. annum.

Nagata and Takebe (1971) for the first time regenerated whole plants from mesophyll protoplasts of N. tabacum. Similarly, Vasil and Vasil (1974) obtained shoots from protoplasts calli when exposed to 4 mgL^{-1} IAA and 2.56 mgL^{-1} Kinetin. Similar reports are reported in N. tabacum (Chupeau et al., 1973; Ohya and Nitsch, 1972; Bourgin et al., 1979). Plant regeneration from protoplasts via organogenesis has also been reported in

tomato which was initially thought to be recalcitrant species (Vasil and Vasil, 1980)

Shahin (1985) using 14 diverse cultivars of tomato was able to regenerate plants from protoplasts. Tan et al., 1987 reported that the shooting frequency varied among the tomato cultivars viz. Moneymaker (30%), Bellina (22%), Sonatine (9%) and Abunda (4%) Similar observation has been reported by Koblitz and Koblitz (1982b), where L. esculentum cv. Nadja was efficiently regenerated into whole plants from mesophyll protoplasts.

Patil et al., (1994) observed regeneration from three Indian cultures of L. esculentum with shoot regeneration of 71%. O'Connell and Hanson (1985) reported that regeneration character can be transferred from L. pennelli to L. esculentum which has a low regeneration capacity by somatic hybridization of protoplasts from the two species.

Morgan and Cocking (1982) reported regeneration from only one out of nine cultivars tested. Zapata et al., (1981) reported callus formation and no organogenesis from L. hirsutum f glabratum. Gleddie et al., (1989) reported that protoplasts from intergeneric hybrids regenerated whereas regeneration from either parent failed. Zapata et al., (1977) showed that protoplasts from L. peruvianum was able to regenerate shoots whereas L. esculentum did not.

The protoplasts from three species viz. L. esculentum, L. pimpilellofolium and L. hirsutum did not differentiate into shoots (Zapata et al., 1981). There are reports where there are varied levels of regeneration based on the crop species and cultivars (Maheshwari et al., 1986; Vasil and Vasil, 1980) used.

SUMMARY

VI. SUMMARY

The study entitled "PROTOPLAST ISOLATION AND REGENERATION STUDIES IN SELECTED SOLANACEOUS CROPS" was taken up with the broad objectives of

- 1) Identification and preparation of explant suited for enzymatic digestion of cellwall
- 2) Standardization of incubation medium for enzyme digestion of cellwall and optimisation of conditions for release of greater number of protoplasts, and
- 3) Testing viability and callus forming capacity of the protoplasts by culturing in vitro wherein the media constituents and the environmental conditions are standardised.
- 4) Testing the totipotency of the protoplast derived callus

The experimental crops chosen were Nicotiana tabacum cv. Thompson, Lycopersicon esculentum cv. Arka vikas & Arka saurabh, and Capsicum annum cv. California wonder & Arka lohit. The work was mostly carried out with plants raised under aseptic conditions.

Initially to identify the explants most suitable for isolation of culturable protoplasts, preparations were made from cotyledon, hypocotyl and mesophyll tissues. The criteria used for selecting the explants were the quality and the yield of protoplast. Based on the empirical results, cotyledon and mesophyll tissues were finally selected for further experiments.

The osmolarity, various ingredients and pH of the incubation mixture were identified and their effect on the yield and viability of protoplasts were

systematically and critically examined. Mannitol was chosen as the osmoticum and osmolarity ranging from 300 to 700 mosm was tested for efficient protoplast release and osmolarity most suited for all species studied was found to be 580mosm (0.55M mannitol). The CPW salts used as other additive in the digestion mixture have also contributed to the final osmolarity used.

CPW salts with ascorbic acid as anti-oxidant was found to improve the viability of protoplast at later stage. The pH of digestion mixture used was 5.8.

The efficacy of the enzymes from four sources namely ; Liquid and lyophilised powder of cellulase and pectinase from Biocon, Liquid enzymes of celluclast and pectinex from Novo industries and Cellulase and macerozyme from Onozuka company, were evaluated for protoplast isolation. Enzymes from Biocon, Bangalore, was found unsuitable for protoplast isolation. Onozuka enzymes yielded moderate yield with good viability at lower temperature, while Novo enzymes yielded protoplasts at faster rate with moderate viability even at elevated temperature. Both these enzyme sources were used for obtaining protoplast for culture in this experimental study. In order to optimise the yields of protoplasts, the donor plants

were pre-conditioned, this was besides the appropriate enzyme ratio and incubation temperature carefully determined for the purpose. The strategy for preconditioning of the donor plants was to subject them to continuous light or dark periods prior to isolation and the yield evaluated. Keeping donor plants in darkness for 18 hours was most suitable for explanting.

An array of protoplasts culture media were systematically tried for protoplast culture for the initial period, for culturing protoplast derived small cell clumps, microcalli, macrocalli, callus proliferation and regeneration. The media tried were Shahins TM media, Niedz's 8E media and MS media. Further, the different additives needed at different stages of protoplast culture were also carefully determined. Growth regulator combination, ratio and levels appropriate for different stages was worked out.

The protoplasts were initially cultured for 10-15 days in dark and later exposed to cool white light of fluorescent lamp. Initial protoplast culture was maintained in liquid medium and later as microcalli formed, they were plated on to solid medium in petri dishes.

Mesophyll protoplasts of tobacco were shown to regenerate into healthy plants with a plating efficiency of 75-80%. In tomato, though the cotyledonary

protoplasts divided and formed small cell clumps of 10-15 cells, further development was not achieved. The mesophyll protoplasts of pepper developed into plants with a plating efficiency of 20-25%.

It can be concluded that Novo enzymes can be used in protoplast isolation, with a step of purification of enzymes, in all the species. There is not much variation in osmotic requirements in all the species tested. Since a single TM-medium (Shahin, 1985) was effectively used for protoplast culture in this study, this medium can be used to culture somatic hybrids during intergeneric and interspecific protoplast fusion.

Tomato mesophyll protoplasts were found to be fragile and lysed during the rigorous protoplast purification process itself.

During the present study, regeneration of plants were achieved from mesophyll protoplasts of tobacco and pepper, but only 10-15 celled clumps from tomato cotyledonary protoplasts. The earlier reports was from shoot tissues (Diaz et al., 1988) and cotyledons (Saxena et al., 1981, Donato et al., 1989).

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VII. REFERENCES:

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