Euphytica 23 (1974) 633–649

POTENTIALS OF PROTOPLAST CULTURE WORK IN AGRICULTURE[1]

Y. P. S. BAJAJ[2]

Department of Horticulture, Agricultural University, P.O. Box 30, Wageningen, the Netherlands

Received 27 December 1973

SUMMARY

During a short span of the last four years there have been exciting developments in the field of protoplast culture and somatic hybridization. Enormous interest and enthusiasm was shown by plant scientists at the 'International Colloquium on Protoplasts and Fusion of Somatic Plant Cells', and 'Third International Symposium on Yeast Protoplasts' held in 1972 at Versailles (France) and Salamanca (Spain), respectively. At present, large quantities of protoplasts can be enzymatically isolated, and when cultured they regenerate a wall and divide to give rise to a clone of homogenous callus tissue. By manipulation of nutritional and physical conditions this tissue can be induced to differentiate into both haploid and diploid plants. The protoplasts can also be induced to fuse with one another, the fused product can be cultured, and made to regenerate into a somatic or 'parasexual hybrid'. The protoplasts take up micro- and macromolecules, viruses, bacteria, and genetic materials like DNA and nuclei, and serve for the transformation and cell modification studies. Isolated chloroplasts have also been transplanted into albino protoplasts and green plants obtained.

The protoplast culture work has unlimited potentialities for plant improvement. It is already being used as a novel research tool by the physiologists, virologists, molecular biologists, geneticists, and for morphogenetic studies. The work could have far-reaching implications in agricultural research as in future (1) the regeneration of somatic hybrids from fused protoplasts of sexually incompatible or distantly related plants, (2) the introduction of nitrogen-fixing bacteria and blue-green algae into non-legumes specially the cereals, (3) the induction of disease-resistance in a crop by the incorporation of a selective genome into the protoplast, (4) the induction and easy detection of mutations in haploid protoplasts, (5) the transplantation of 'foreign chloroplasts' into crop plants with inefficient photosynthetic system, (6) culture of protoplasts to raise clones for vegetative multiplication and also for genetic diversity and (7) insertion of a part or complete genome by transgenosis, cell modification, and nuclear transplantation could replace or at least help to modernize some of the conventional practices used for plant improvement. To get this gigantic task done a close collaboration between various disciplines of plant sciences is desired, and the resources pooled together to get the maximum benefits of the facilities and the talent available. A great deal of responsibility lies specially with the geneticists and plant breeders who will eventually deal with the ultimate product. These above mentioned possibilities are by no stretch of imagination meant to be any substitute for the present day plant breeding practices, but they are certainly new valuable tools which can be exploited.

INTRODUCTION

The importance of plant tissue culture in plant research has been realized for a long time, and it has been established beyond doubt that isolated cells if given proper environments have the capacity to unfold their morphogenetic potentialities. This has been repeatedly demonstrated (for literature see BAJAJ & BOPP, 1971), and now it is

[1] Publication 397 of the Department of Horticulture, Agricultural University, Wageningen, the Netherlands.

[2] Present address: Institut für Pflanzenphysiologie und Zellbiologie, Freie Universität Berlin, D-1 Berlin 33 (Dahlem), Germany.

possible to obtain a whole plant (BRAUN, 1959; VASIL & HILDEBRANDT, 1965), or even a tree (WINTON, 1970) from an isolated single cell and callus tissue under aseptic conditions. This work has now been extended to naked protoplasts removed from the confines of cell walls, and plants of tobacco (TAKEBE et al., 1971; NITSCH & OHYAMA, 1971; BAJAJ, 1972; CARLSON et al., 1972) carrot (GRAMBOV et al., 1972; KAMEYA & UCHIMIYA, 1972) and *Petunia* (DURAND et al., 1973; FREARSON et al., 1973) have been regenerated from them.

The isolation, culture and fusion of plant protoplasts have tremendous possibilities in agricultural research, specially because of their potential significance in somatic hybridization in sexually incompatible crosses, and also for plant modification studies. During the last four years this fascinating technique has increasingly attracted the attention of plant scientists for a variety of reasons (see COCKING, 1970, 1972; and Colloq. Internatl. CNRS, 1973), and is now being used as a tool for attacking various problems by physiologists, molecular biologists, and for morphogenetic studies. At this stage the message needs to be brought to the agriculturists regarding the potentialities and significance of this important discipline of research for plant improvement.

The technique though still in the formative stages has opened new avenues, and holds a great promise for the future, specially for genetic engineering. The researchers have already started to look upon these methods as a valuable tool, both for basic and applied work. The protoplasts have many advantages over the cells, and in this communication their use in plant research is outlined. The main purpose is to summarize some of the latest developments and point out their far-reaching implications in agricultural research, and also to provide technical details and a survey of literature for those who at present are not familiar with this fast-growing field of research. Before going into details of the work which has been done so far, it is pertinent to discuss the historical background regarding the development of the technique, facilities required for this work, and procedure for the isolation of protoplasts.

HISTORICAL BACKGROUND

KLERCKER (1892) for the first time used mechanical means to obtain protoplasts from water warrior (*Stratiotes aloides*). He first plasmolysed the cells and then cut the cell walls to liberate the protoplasts. Since then many workers (see COCKING, 1972) have tried to modify or develop techniques, but the main drawbacks were (*i*) the protoplast yield was low (*ii*) the methods were tedious and laborious, and (*iii*) these techniques could be used only for those cells in which protoplasts were ejected during plasmolysis. In 1960, Professor E. C. COCKING of the University of Nottingham, England, developed a very sophisticated and novel approach for the large scale production of higher plant protoplasts, and initiated with that a new field of research. He obtained protoplasts from root tips of tomato (*Lycopersicon esculentum*) seedlings by using *Myrothecium* enzyme-cellulase, which involved the digestion of cell walls. Enzymatic preparation of protoplasts has certain definite advantages (RUESINK, 1971) i.e. (*i*) large quantities of protoplasts can be isolated easily, (*ii*) there is less osmotic shrinkage, (*iii*) cells are not broken as is the case with mechanical methods. With the development of this technique it has become possible to isolate protoplasts in large quantities from almost any part of the plant. To date, protoplasts have been obtained from fruit tissues

(GREGORY & COCKING, 1965; RAJ & HERR, 1970), leaves (TAKEBE et al., 1968; POWER & COCKING, 1968, 1970; OTSUKI & TAKEBE, 1969; BOURGIN et al., 1972), roots (COCKING, 1960; BAWA & TORREY, 1971), root nodules (DAVEY et al., 1973), coleoptiles (RUESINK & THIMANN, 1965; HALL & COCKING, 1971), endosperm (MOTOYOSHI, 1972), aleurone layer (TAIZ & JONES, 1971), flower petals (POTRYKUS, 1971), potato tuber (LORENZINI, 1973), pollen mother cells, pollen tetrads, and pollen grains (BHOJWANI & COCKING, 1972; BAJAJ & COCKING, 1972, 1973; BAJAJ, 1974a,b), crown-gall tissue (SCOWCROFT et al., 1973), and callus tissue cultures (SCHENK & HILDEBRANDT, 1969; ERIKSSON & JONASSON, 1969; HELLMANN & REINERT, 1971; HOLDEN & HILDEBRANDT, 1972; BUTENKO & IVANTSOV, 1973; WALLIN & ERIKSSON, 1973).

Recently, HARADA (1973) combined the mechanical and enzymatic techniques to obtain protoplasts from the leaves of *Ipomea* and *Calystegia*. By this method tedious peeling of the epidermis is eliminated. His technique involved two steps, (*i*) mechanical isolation of free cells, and (*ii*) enzymatic transformation of these cells into protoplasts.

FACILITIES REQUIRED

Since one of the main objectives is to obtain whole plants from the protoplasts via callus cultures, the work has to be carried out under sterile conditions. The protoplast isolation and culture work, contrary to the general impression, does not require many elaborate facilities other than the usual equipment needed for the tissue culture work (see WHITE, 1963; STREET, 1973). Small air-flow cabinets have proved to be more useful than the large inoculation rooms ordinarily used for the tissue culture work. These cabinets require very little space, and can be easily moved from one place to another. Most of the enzymes (Cellulase, Meicelase, Macerozyme, Pectinol, Rhozyme and Helicase) are nowadays commercially available. They can be easily sterilized through small Millipore filters, or if in large quantities through plastic Sartorious-Membrane filters. Furthermore a centrifuge, light microscope, haemocytometer, sterile plastic petri-dishes, disposable syringes, Pasteur pipettes, and general glasswares which are normally available in most plant physiology laboratories are sufficient to start the work.

ISOLATION OF PROTOPLASTS

As already mentioned the protoplasts can be isolated from almost any part of the plant. As a typical example the enzymatic method for the isolation of protoplasts from mesophyll cells of tobacco leaves is described here. The technique primarily involves four steps; (1) sterilization of leaves, (2) peeling off the epidermis, (3) enzymatic treatment and incubation, and finally (4) isolation and cleaning of the protoplasts.

First of all, fully expanded and healthy leaves (from about 2-month-old seedlings) are surface sterilized by dipping them in 70% alcohol for about a minute, and then immediately treated for 30 minutes with 2% solution of sodium hypochlorite, or 10% (v/v) solution of commercially available disinfectants like 'Bleach'. The leaves are then rinsed three times with sterile distilled water to remove traces of any sterilant that are left. Rest of the manipulations are carried out under sterile conditions.

With the help of a pair of very fine forceps the lower epidermis from the excised leaves is carefully peeled off (TAKEBE et al., 1968), and the stripped leaves are cut into small pieces (4 cm^2). The peeling is facilitated if after sterilization the leaves are allowed to become flaccid, or if the water supply to the plants is limited before the leaves are excised (TAKEBE et al., 1968). From the peeled segments the protoplasts can be isolated by two methods, (*i*) sequential method – first by using the pectinase enzyme (macerozyme) the cells are isolated, and then by treating the freshly isolated cells with cellulase the cell walls are digested and, the protoplasts are obtained (OTSUKI & TAKEBE, 1969), and (*ii*) the peeled leaf segments are directly incubated in a mixture of macerozyme and cellulase (POWER & COCKING, 1970. The second method which is obviously simple, is described here.

The peeled segments are placed with their lower surface downwards in a petri dish containing enzyme mixture. The enzyme mixture is prepared by dissolving 0.5% macerozyme (pectinase enzyme) and 2% cellulase (Onozuka P1,500 – obtained from *Trichoderma viride*) in 0.7 M sorbitol or mannitol solution prepared in distilled water. Addition of potassium dextran sulphate (0.3%) to the enzyme mixture is reported (TAKEBE et al., 1968) to enhance the cell separation. The pH of the mixture is adjusted to 5.4, and sterile-filtered through Sartorious-Membrane filters. About 10 ml of the enzyme mixture is used for 1 g of leaf material. The material is incubated at 25 °C for 4–6 hrs, and then gently teased to liberate more protoplasts. They are filtered through a fine wire gauze to remove leaf debris, transferred to 13 × 100 mm screw-capped tubes, and centrifuged at 100 *g* for 1 minute. The protoplasts are sedimented, and the supernatant containing enzyme mixture and debris is carefully removed. This process is repeated three times and the protoplasts are washed with 0.7 M sorbitol solution. For final cleaning, sorbitol is replaced by 20% sucrose solution, and centrifuged at 200 *g* for 1 minute. This time cleaned protoplasts float, while the debris settle down at the bottom. The floating protoplasts are gently pipetted out with Pasteur pipettes, and the sample bulked. A very small sample is taken to measure the density of protoplasts with a haemocytometer (Fuchs-Rosenthal). The protoplasts are finally resuspended in a known volume of liquid culture medium, and the volume so adjusted that final density after plating (procedure described later) is between 10^4 to 10^6 protoplasts per ml of the medium.

The method described for tobacco leaves can be easily modified to obtain protoplasts from various cereals (EVANS et al., 1972), *Asparagus* (MACKENZIE et al., 1973), legumes and cornflower (DAVEY & SHORT, 1973), barley (SCHASKOLSKAYA et al., 1973), *Petunia* (DURAND et al., 1973; FREARSON et al., 1973), rape and rye (WENZEL, 1973) and various other plants (OTSUKI & TAKEBE, 1969; KELLER et al., 1970; COCKING & EVANS, 1973).

Though the protoplasts can be isolated easily, their yield depends on a number of factors, the most important ones being the age and the physiological state of the plant. In addition, particular attention should be paid to the concentration and purity of the enzyme, their *p*H, period of incubation, and specially the plasmolyticum as they immensely effect the viability of the protoplasts and their subsequent growth and division in cultures.

The sequence of the isolation, fusion and culture of protoplasts, and their subsequent regeneration into plants is diagrammatically represented in Fig. 1.

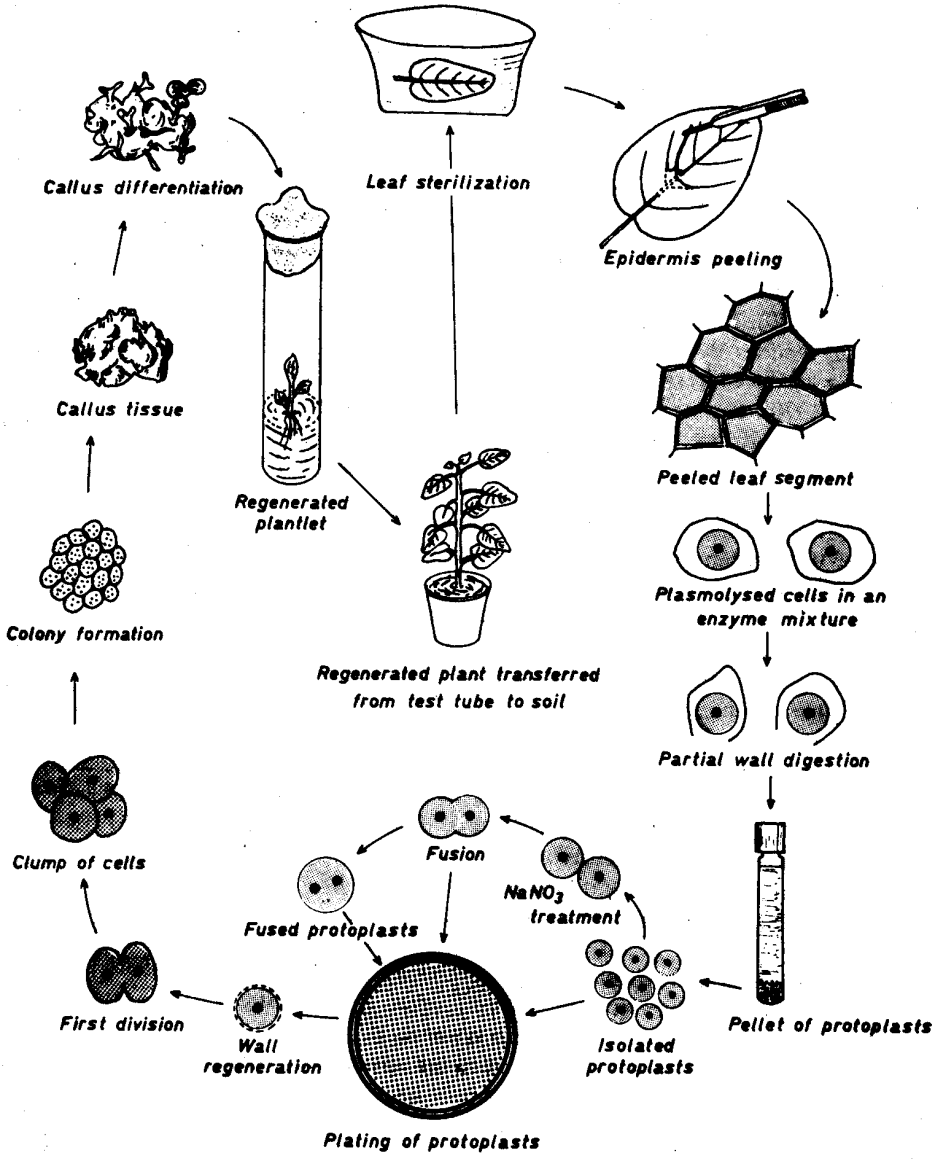

Fig. 1. Diagrammatic representation for the isolation, culture and fusion of leaf-cell protoplasts.

PROTOPLAST CULTURE AND REGENERATION OF PLANTS

Protoplasts can be grown and induced to divide both in liquid as well as in the agar media. In the liquid media, however, there is limited growth, and the cell aggregates have to be transferred eventually to an agar-solidified nutrient medium for further growth and morphogenesis. Plating of the protoplasts has an advantage in that the whole sequence of division, growth and development of a plant from a single protoplast can be easily observed, and the plating efficiency (% age of protoplasts forming colonies) estimated. Plating is a very efficient technique to bypass sex and raising single cell clones, the significance of which has been emphasized in detail by STEWARD et al. (1970). It has proved to be very valuable specially for vegetative multiplication of sugar cane (MARETZKI & NICKELL, 1973). At present, fields of sugar cane are already being produced in Hawaii from single cell clones, and new genomes selected (HEINZ & MEE, 1970). Plating could also be utilized for evaluating the effect of various phytohormones, drugs and herbicides (BOULWARE & CAMPER, 1972). The technique of plating was first employed by BERGMANN (1960) to grow isolated callus cells of tobacco and beans, and has since been modified (NAGATA & TAKEBE, 1971) for the culture of isolated protoplasts. Two ml aliquots of protoplasts (density approximately 4×10^5) suspended in the liquid culture medium are poured into Falcon plastic petri-dishes, and mixed gently with an equal volume of the same medium containing 1.2% agar. At the time of mixing, particular care should be taken so that the temperature of the medium is not higher than 45 °C. Although the petri-dishes are tight lided, however, to prevent desiccation, it is advisable to seal them with Parafilm M. The dishes are then kept in an inverted position at 28 °C under continuous illumination from fluorescent tubes at 2300 lux. Light intensity and protoplast density are critical for plating efficiency.

The isolated protoplasts in culture regenerate new walls around themselves (POJNAR et al., 1967), which can be demonstrated by staining the reconstituted protoplasts for five minutes with a flourescent brightner Calcofluor White (0.1%). Each isolated protoplast normally regenerates its own separate wall, but if a large number of protoplasts are in contact with one another then common walls are regenerated, and a cell aggregate or a so called 'tissue' is formed (BAJAJ, 1974a). Detailed ultrastructural, freeze-etching and carbon shadowed replica studies by WILLISON (1973) throw considerable light on the nature of wall; however, for studying the biochemistry of wall formation, protoplasts are a superb system.

MURASHIGE & SKOOG's (1962) medium modified by NAGATA & TAKEBE (1971) – hereafter referred to as TMS-medium- has proved to be sufficient to support the growth of protoplasts. This medium contains major and minor elements, vitamins, myo-inositol, Fe-EDTA, sugar and growth regulators (for detailed composition see NAGATA & TAKEBE, 1971). To prevent bursting of protoplasts an extra amount of osmoticum is added.

The protoplasts divide only once or twice (ERIKSSON & JONASSON, 1969), continue to divide to form an aggregate of cells (KAO et al., 1970), or a mass of callus tissue. Small cell aggregates and colonies are difficult to pick up, and sometimes are damaged by rough handling with the forceps. A better practice is to cut small pieces of agar (containing colonies of cells), and transfer them onto the top of an other medium. The

growth of colonies can be enhanced if they are transferred to a mannitol-free medium.

Tobacco leaf protoplasts are a unique material for regeneration and morphogenetic studies. The protoplasts when plated on TMS-medium show an increase in size, and regenerate a wall within two days to form a cell, at this stage the cell wall can be easily observed by plasmolysis. The reconstituted protoplasts or the newly formed cells start to divide in 3–4 days, and form an aggregate of cells in two weeks. With repeated divisions the number of chloroplasts per cell decrease, and the cells become more vacuolated. Pale green visible colonies (0.3–1 mm diam.) are formed in 3–4 weeks. The nature of further growth is manipulated by the auxin/cytokinin ratio in the medium. When 4–6 week old colonies are transferred to tobacco callus differentiation medium containing cytokinin (2 mg/l) and indoleacetic acid (4 mg/l), within next 3–4 weeks shoots are differentiated from callus tissue. To induce root formation, these shoots are transplanted on WHITE's basal medium (WHITE, 1963). So, in short it takes about 10 weeks from the time the protoplasts are plated to the regeneration of plantlets. These plants can subsequently be transferred to pots. This remarkable feat in addition to tobacco (NAGATA & TAKEBE, 1971; TAKEBE et al., 1971; NITSCH & OHYAMA, 1971; BAJAJ, 1972; CARLSON et al., 1972), has also been achieved with carrot (GRAMBOV et al., 1972; KAMEYA & UCHIMIYA, 1972) and *Petunia* (DURAND et al., 1973; FREARSON et al., 1973). To extend this work to other plants would be more a matter of refining the technique and finding suitable media and conditions.

PROTOPLAST FUSION AND SOMATIC HYBRIDIZATION

A plant breeder usually obtains hybrids by using various conventional techniques, but the difficulty arises with the sexually incompatible crosses; in such cases protoplast fusion and somatic hybridization could be exploited. In animal cells, somatic hybridization was achieved as early as 1960 (BARSKI et al.), and later a man-mouse hybrid cell was obtained (see EPHRUSSI & WEISS, 1965; HARRIS, 1970). However, unfortunately, or rather fortunately a hybrid cell never differentiated into a 'Man-Mouse Hybrid', which would obviously have been a freak. On the other hand a plant cell is totipotent, and it can be induced to regenerate into a whole plant. It is this inborne nature of the plant cell or the manifestation of totipotency which forms the basis for the formation of somatic hybrids, and for the possible combination of genotypes that are not closely related.

POWER, CUMMINS & COCKING (1970), and POWER & COCKING (1971) for the first time successfully demonstrated the intra and inter-specific fusion between protoplasts obtained from root tips of maize and oat seedlings, and outlined the technique and conditions for fusion of protoplasts. This technique in short involves the following procedure. Isolated protoplasts are suspended in an aggregation mixture (5.5% sodium nitrate in 10% sucrose solution), kept in a water-bath at 35 °C for 5 minutes, and then centrifuged for 5 minutes at 200 *g* to obtain a dense pellet. The tube with a pellet of protoplasts is once again transferred to a water-bath (30 °C) for 30 minutes – during which most of the protoplasts undergo fusion. The aggregation mixture is very gently (without disturbing the pellet) replaced by TMS-medium supplemented with extra 0.1% $NaNO_3$. The protoplasts are left undisturbed in this medium till they float, after which they are washed a couple of times with liquid TMS-medium, and plated.

Following treatment with $NaNO_3$, POTRYKUS (1971) reported interspecific fusion of the flower petal protoplasts of *Torenia fournieri* and *T. bailloni*; and by electron microscopy WITHERS & COCKING (1972) followed the sequence of both induced and spontaneous fusion in tobacco leaf protoplasts. KAMEYA & TAKAHASHI (1972) studied the effect of various inorganic salts on interspecific fusion between the root protoplasts of *Brassica chinensis* and leaf protoplasts of *B. oleracea*, and concluded that (i) the fusion effect was due to sodium ions and not to nitrate, (ii) sodium was more effective than potassium, and no fusion was observed with calcium ions. The next logical step was to combine the process of isolation, fusion and culture. GILES (1972) had limited success and obtained an interspecific aggregate cell from soybean callus and *Digitaria* mesophyll protoplasts, which was capable of regenerating a cell wall. CARLSON, SMITH & DEARING (1972) from the Brookhaven National Laboratory, USA went a great step ahead and achieved the desired goal in somatic hybridization studies by demonstrating the formation of an interspecific somatic cell hybrid or a 'parasexual hybrid' between *Nicotiana glauca* and *Nicotiana langsdorfii*. This elegant work has greatly enhanced the confidence of plant breeders in protoplast work. It has also sparked the research activities in many laboratories, and has given a powerful impetus to the study of somatic cell hybridization. This work has for the first time practically demonstrated what was thought to be only a theoretical academic excercise (see BURNET, 1971).

MARX (1973) mentions that three results are possible with the mammalian cell hybridization: (i) extinction, in which a phenotype present in one parent cell but not the other is no longer expressed, (ii) continued expression of the phenotype in the hybrid, and (iii) activation of a phenotype that was expressed in neither parent. In 1967 WEISS & GREEN reported that during proliferation of a man-mouse somatic cell hybrid there is a complete elimination of human chromosomes. KAO & PUCK (1970) observed almost the same with a man-chinese hamster hybrid cell. It is a very interesting situation for the geneticists as a hybrid clone is available which has all the chromosomes of mouse with a few or non from man. Advantage can be taken for selective gene insertion into plants if a similar situation occurs in plant somatic cell hybrids.

GILES (1973) studied the genetic complementation by fusing various types of protoplasts of iojab and the white deficient mutant of *Zea mays*. This work is a sophisticated attempt to demonstrate complementation as this plant material is from known stocks, and from species about which sufficient genetic information is already available. His results are encouraging since the genetic complementation is one of the prerequisites for somatic hybridization. GILES stressed that 'a more immediate, and potentially important, application for protoplast fusion could be a new tool in the dissection of gene expression and controlling-gene structural-gene interactions'.

Though somatic hybridization can be achieved, still one of the main difficulties which faces the workers at present is the criteria by which one can establish the true identity of a somatic hybrid. The phenotypic characters in tissue cultures could sometimes be inconclusive, or even misleading. Various biochemical and genetical markers like isoenzyme zymograms, nucleic acid patterns, drug resistance, karyotype, chromosome number, Giemsa banding, and selective nutrient media could be employed. The process for a thorough screening and rigorous selection has to be devised, as the frequency of successful culture of the fused protoplasts is extremely low. As we are

dealing with a single cell system, the knowledge from the animal-cell fusion system and the microbial genetics could also be utilized.

HAPLOID PROTOPLASTS AND MUTATIONS

Haploid plants are of great importance in plant breeding and genetics, and their significance has been fairly emphasized (KIMBER & RILEY, 1963; NICKELL & TORREY, 1969; MELCHERS & LABIB, 1970; SUNDERLAND, 1971; NITSCH, 1972). They are desired mainly for two reasons, (i) for obtaining homozygous plants, and (ii) for easy detection of mutants. They can be experimentally induced by anther/pollen culture (GUHA & MAHESHWARI, 1964). By this technique callus and/or their regeneration has been obtained in a variety of crop plants i.e. barley, rice, wheat, potato, tomato, and the subject recently reviewed (BAJAJ, 1974b). These plants can be easily diploidized (KOCHHAR et al., 1971) to form homozygous plants. The success in culturing anthers has, however, been limited because of a number of reasons: (i) nutritional requirements are not fully understood, (ii) environmental factors under which the plants are grown considerably influence the success in culture, (iii) the stage of development of the anther is critical, (iv) obscure effect of anther wall on the development of pollen embryoid is not known, (v) the frequency of haploid tissue/plant formation is rather low – in some cases even less than 1%. Although anther culture has accelerated the interest in haploid production, it has one main pitfall, i.e. the plants not only originate from pollen grains (haploid) but also from various other parts of the anther, with the result that a mixed population of plants of various levels of ploidy is obtained. This drawback can be eliminated by the culture of isolated pollen grains, their protoplasts and also by haploid leaf protoplasts. Pollen grains are the naturally occurring haploids if conditions conducive to their growth could be provided. It is presumed that removal of the wall might facilitate the growth and division in naked pollen cells. For instance mesophyll cells of tobacco occasionally divide and regenerate, but if the mesophyll cell-wall is digested by the enzyme, the naked cells or the protoplasts readily grow, divide and regenerate. Pollen protoplast culture is by no means intended to be a substitute for the anther or the pollen culture but certainly an additional technique which could be taken into consideration, specially in cases where other possibilities for the induction of haploids fail. The initial studies on the isolation and culture of pollen tetrads, and pollen protoplasts have been encouraging (BHOJWANI & COCKING, 1972; BAJAJ & COCKING, 1972, 1973; BAJAJ, 1974a,b). The pollen tetrads surrounded by the thick callose wall are treated with millipore-filtered commercially available 0.75% enzyme Helicase prepared in 10% sucrose solution. This enzyme complex obtained from the snail (*Helix pomatia*) gut is rich in β-1:3 and β-1:4 glucanase activity and is capable of digesting the callosic wall material. Within 45–60 min the tetrad protoplasts are completely released from their callose walls. The protoplasts are spherical and densely cytoplasmic with a prominent nucleus. In microcultures some of the protoplasts regenerate a wall, and occasionally divide, or show elongation and binucleated condition (BAJAJ, 1974a).

Like the diploid tobacco leaf protoplasts, the haploid leaf protoplasts also grow and differentiate into haploid plants (NITSCH & OHYAMA, 1971; BAJAJ, 1972). In

addition, haploid protoplasts have also been isolated from the leaves of *Petunia*, rye and rape (WENZEL, 1973). The haploid cell cultures are mostly unstable and have a tendency to undergo endomitosis and as a result a mixed population of cells is obtained. In this connection it is pertinent to point out that Parafluorophenylalanine – an amino acid analogue has been reported to have an inhibitory effect on the diploid cells, and causes preferential growth of haploid fungi, and haploid tobacco tissue cultures (GUPTA & CARLSON, 1972).

One of the main objectives of the culture of haploid cell/protoplasts is to induce mutants by subjecting them to various mutagens. By this means mutant tobacco cell lines have been obtained by treating them with streptomycin (BINDING et al., 1970; MALIGA et al., 1973a) and, 5-bromodeoxyuridine (CARLSON, 1970; MALIGA et al., 1973b), DNA base analogues (LESCURE, 1973). Recently CARLSON (1973b) obtained methionine sulfoximine resistant calluses from haploid tobacco protoplasts. The calluses that retained resistance on subculturing were diploidized, and regenerated into whole plants. This imaginative work could have practical applications in plant pathology as the toxin produced by bacterial pathogen (*Pseudomonas tabaci*) that causes the wildfire disease of tobacco is an analogue of methionine.

UPTAKE STUDIES AND CELL MODIFICATION

Plant protoplasts have the remarkable property to take up particles and macro-molecules, like polystrene latex particles, ferritin, proteins, DNA, viruses, bacteria, or even whole chloroplasts and nuclei can be transplanted. MAYO & COCKING (1969) reported that the uptake is by pinocytosis, and demonstrated this by differential staining effects of phosphotungstic acid on the plasmalemma. This pinocytic property of protoplasts can be utilized for the cell modification studies; keeping this in perspective let us look at the various aspects of uptake.

(a) DNA uptake. The discovery that *Diplococcus pneumoniae* transforms itself by taking up exogenous DNA into its genomes (OSWALD et al., 1944) has made genetic engineering a routine task in bacteria (COSLOY & OISHI, 1973). New genes can be synthesized, and DNA-RNA hybrids can be obtained (MILO & SRIVASTAVA, 1969). The question arises whether one can do the same with the higher plant cells. Certainly, the information which has accumulated during the last few years (LEDOUX, 1965; LEDOUX et al., 1971; HESS, 1970, 1972) is very promising. DNA can now be incorporated into the higher plant cells/protoplasts (OHYAMA et al., 1972; HEYN & SCHILPEROORT, 1973; HOFFMANN & HESS, 1973). Recently the elegant experiments of DOY, GRESSHOFF & ROLFE (1972, 1973a,b) from the Australian National University have attracted much attention. These workers claim to have successfully 'transformed' haploid tomato cell cultures by phage lambda (bacterial virus). The fact that these higher plant cells or the transformants can grow on a medium containing galactose and lactose as the exclusive source of carbon is believed to be a sufficient proof to demonstrate that phage DNA is functioning. DOY and his coworkers however, prefer to use the term 'Transgenosis' for this type of transformation in higher plants.

For transgenosis much more outcome would be expected if the protoplasts instead of callus cultures are utilized. The protoplasts: (i) are much more uniform, homoge-

nous and synchronous as compared to callus tissues, (ii) have a uniform level of ploidy, (iii) would have higher uptake as they are devoid of walls, and there is more pinocytic activity. COCKING (1973) emphasized that using isolated protoplasts 'is likely to reduce the chances of degradation of input DNA, if bare DNA is supplied, because of the absence of cell wall'. He further stressed that 'transformation even in bacteria is a rare event and perhaps we should experiment with protoplasts to see if these naked cells offer any means of increasing the frequency and, therefore, more ready unambigous characterization of any transformation that may occur in the eukaryotic higher plant'. In connection with the DNA uptake, it is pertinent to point out that transfer of DNA from a disease-resistant cell into a susceptible cell/protoplast would be a major step in plant breeding and pathology.

(b) Virus uptake. In the past, studies on the pathology, host-parasite relationship, and the mechanism of infection by the viruses have been hampered because of a number of reasons. Though callus tissue cultures were used for infection of tobacco mosaic virus (CHANDRA & HILDEBRANDT, 1965; KASSANIS, 1967; MOTOYOSHI & OSHIMA, 1968), and with its RNA (MURAKISHI, 1968), the process was not synchronous, and because of the presence of a hard cell-wall the extent of infection was largely restricted to cells with broken or damaged walls. However, when the wall is enzymatically digested, the protoplasts can be infected synchronously.

In 1966, COCKING reported that tomato fruit protoplasts take up tobacco mosaic virus (TMV) particles by pinocytosis, and electron microscopically demonstrated the initial stages of infection. This work has since been followed by other workers (AOKI & TAKEBE, 1969; COCKING & POJNAR, 1969; HIBI & YORA, 1972; OTSUKI et al., 1972; BURGESS et al., 1973; COUTTS, 1973) and extended to mesophyll protoplasts. These initial studies have shown that TMV/RNA is taken up by pinocytosis through plasmalemma, and is greatly affected by poly-L-ornithine. The virus enters the cytoplasm of the protoplast, and multiplies. The intracellular amount of virus is almost the same as formed in the cells of infected growing plants. Infection is synchronous and 60–80% have been seen to be infected, and about the same amount of virus is produced in 2–3 days in protoplasts as in 10 days in a similar number of cells of a growing plant (COUTTS, 1973). The virus aggregates are often found near the nuclear membrane 48 hours after incubation, and in areas of virus multiplication there is an accumulation of large amounts of ribosomal material.

For future work protoplasts offer a model system for the study of (i) mechanism of infection, and (ii) nucleo-protein replication.

(c) Bacterial uptake and nitrogen fixation. Nitrogen fixing bacteria *Rhizobium japonicum* can be introduced into the pea leaf protoplasts during enzymatic digestion of the cell wall (DAVEY & COCKING, 1972). This uptake occurs by invaginations of the plasmalemma during plasmolysis. Such investigations could produce results in new endosymbiotic relationships. Recently, DAVEY et al. (1973) have also successfully isolated legume root-nodule protoplasts containing packets of bacteria, and the possibility of fusing these protoplasts with the non-legume protoplasts is being worked out. Introduction of free nitrogen fixing bacteria like *Azotobacter*, and blue-green algae into non-legumes are the future possibilities. COCKING (1973) comments that 'The agricul-

tural and social implications of this sort of modification of non-legume food plants such as the cereals would be profound – the need for plant biologists to work actively in this field is pressing'.

(d) Chloroplast transplantation. In 1969 NASS reported the incorporation of spinach chloroplasts into the cytoplasm of isolated animal cell cultures. These green cells divided normally and the chloroplasts retained their structural integrity. Following this, GILES & SARAFIS (1971) implanted *Nitella* chloroplasts into hen's eggs and demonstrated that these chloroplasts survive, remain metabolically active, maintain their morphological integrity, and divide repeatedly. This report on the successful in vitro culture of an organelle demonstrates evidence for the autonomy of the chloroplasts. Recently CARLSON (1973a) incorporated the nonmutant tobacco chloroplasts into the cytoplasm of an albino protoplasts. As the albino trait is transmitted cytoplasmically, it is a mutant of the chloroplast-DNA and not of a nuclear gene. The externally supplied 'foreign chloroplasts' escaped destruction in pinocytotic vacuoles and entered the cytoplasm; and whole tobacco plants were later regenerated from albino protoplasts containing 'foreign chloroplasts'.

POTRYKUS (1973) is also trying to duplicate the same with albino *Petunia* protoplasts, and the details of his further work are awaited. These remarkable manipulations of transplantation of chloroplasts from one plant to another when extended specially to those plants with a defective or inefficient photosynthetic system could have far-reaching significance in plant husbandry. As CARLSON put it 'Perhaps chloroplast transfer will provide a method for plant breeders to incorporate highly efficient chloroplasts into important crop species'.

(e) Transplantation of nuclei. It has been seen that organelles and other particles of rather small size such as DNA molecules, viruses, and bacteria can be taken up by the isolated protoplasts. It is amazing indeed to note, that the organelles of such a large size as the nuclei can also be introduced through the plasmalemma into the protoplasts without causing it to burst, as recently reported by POTRYKUS & HOFFMANN (1973). These workers successfully achieved both the intra and interspecific transplantation of isolated nuclei from *Petunia hybrida* into the protoplasts of *Zea mays*, *Petunia hybrida* and *Nicotiana glauca*. The fate of these implanted nuclei is still to be studied, and it is not known whether they are functional as such or are degraded during cultures. It certainly opens new vistas for the cytogeneticists to study nuclear-cytoplasmic interactions, more so if fertile plants could be regenerated from such protoplasts.

We know the fact that (i) isolated protoplasts take up genetic material, and (ii) whole plants can be regenerated from the protoplasts. If these two steps are combined, it would not be too much to speculate that in the very near future one should be able to engineer the genetic make up at a single cell/protoplast level, and synthesize a plant which would be a reality for plant breeders.

ACKNOWLEDGMENTS

This article is the outcome of my discussions with Professor E. C. COCKING (University of Nottingham, England), to whom I am greatly indebted for his kind help and

counsel during my stay (1972-1973) in his laboratory. Grateful acknowledgment is also made to the Agricultural University of Wageningen, the Netherlands, for the financial support and to Professor S. J. WELLENSIEK for critical appraisal of the manuscript.

REFERENCES

AOKI, S. & I. TAKEBE, 1969. Infection of tobacco mesophyll protoplasts by tobacco mosaic virus ribonucleic acid. Virology 39:439–448.

BAJAJ, Y. P. S., 1972. Protoplast culture and regeneration of haploid tobacco plants. Amer. J. Bot. 59(6):647

BAJAJ, Y. P. S., 1974a. Isolation and culture studies on pollen tetrad and pollen mother-cell protoplasts. Plant Sci. Letters 3: 93–99.

BAJAJ, Y. P. S., 1974b. Protoplast culture and production of haploids. In: B. M. Johri commemoration volume. University of Delhi.

BAJAJ, Y. P. S. & M. BOPP, 1971. Gewebekulturen in der Angewandten Botanik (Plant tissue culture in applied botany). Angew. Bot. 45:115–151.

BAJAJ, Y. P. S. & E. C. COCKING, 1972. The isolation and culture of microspore tetrads and microspore mother protoplasts of some angiosperms. P. 79. Proc. 3rd int. Symp. Yeast Protoplasts, Salamanca (Spain).

BAJAJ, Y. P. S. & E. C. COCKING, 1973. The isolation, culture and fusion of pollen protoplasts. In Vitro 8(5):420.

BARSKI, G., S. SORIEUL & F. CORNEFERT, 1960. Production dans des cultures in vitro de deux souches cellulaires en association de cellules de caractère 'hybride'. C.R. Acad. Sci. (Paris) 251: 1825–1833.

BAWA, S. B. & J. G. TORREY, 1971. 'Budding' and nuclear division in cultured protoplasts of corn, *Convolvulus* and onion. Bot. Gaz. 132:240–245.

BERGMANN, L., 1960. Growth and division of single cells of higher plants in vitro. J. gen. Physiol. 43: 841–851.

BHOJWANI, S. S. & E. C. COCKING, 1972. Isolation of protoplasts from pollen tetrads. Nature New Biol. 239:29–30.

BINDING, H., K. BINDING & J. STRAUB, 1970. Selektion in Gewebekulturen mit haploiden Zellen. Naturwiss. 57:138–139.

BOULWARE, M. A. & N. D. CAMPER, 1972. Effects of selected herbicides on plant protoplasts. Physiologia Pl. 26:313–317.

BOURGIN, J. P., Y. CHUPEAU & G. MOREL, 1972. Obtention et culture in vitro de protoplastes multinucléés de mésophylle de Tabac (*Nicotiana tabacum* L.) C. R. Acad. Sci. Ser. D. 274(26):3545–3548.

BRAUN, A. C., 1959. A demonstration of the recovery of the crown-gall tumor cell with the use of complex tumors of single-cell origin. Proc. nat. Acad. Sci. (U.S.) 45:932–938.

BURGESS, J., F. MOTOYOSHI & E. N. FLEMING, 1973. Effect of poly-L. ornithine on isolated tobacco mesophyll protoplasts: evidence against stimulated pinocytosis. Planta 111:199–208.

BURNET, M., 1971. Genes, dreams and realities. Aylesbury, Medical and Technical Publishing Co.

BUTENKO, R. G. & A. I. IVANTSOV, 1973. The isolation of protoplasts from different strains of carrot and tobacco tissue cultures. P. 79–84. In: Colloq. int. CNRS. Protoplastes et fusion de cellules somatiques végétales (Versailles). Editions de l'Institut National de la Recherche Agronomique, Paris.

CARLSON, P. S., 1970. Induction and isolation of auxotrophic mutants in somatic cultures of *Nicotiana tabacum*. Science 168:487–489.

CARLSON, P. S., 1973a. The use of protoplasts for genetic research. Proc. nat. Acad. Sci. (U.S.) 70: 598–602.

CARLSON, P. S., 1973b. Methionine sulfoximine-resistant mutants of tobacco. Science 180:1366.

CARLSON, P. S., H. H. SMITH & R. D. DEARING, 1972. Parasexual interspecific plant hybridization. Proc. nat. Acad. Sci. (U.S.) 69:2292–2294.

CHANDRA, N. & A. C. HILDEBRANDT, 1965. Tobacco mosaic virus inclusion bodies in tobacco tissue cultures. Nature (Lond.) 206:325–326.

COCKING, E. C., 1960. A method for the isolation of plant protoplasts and vacuoles. Nature (Lond.) 187:962–963.

COCKING, E. C., 1966. An electron microscopic study of the initial stages of infection of isolated tomato fruit protoplasts by tobacco mosaic virus. Planta 68:206–214.

COCKING, E. C., 1970. Virus uptake, cell wall regeneration, and virus multiplication in isolated plant protoplasts. Int. Rev. Cytology 28:89–124.

COCKING, E. C., 1972. Plant cell protoplasts - Isolation and development. An. Rev. Pl. Physiol. 23:29–50.

COCKING, E. C., 1973. Plant cell modification: problems and perspectives. P. 327–341. In: Colloq. int. CNRS. Protoplastes et fusion de cellules somatiques végétales (Versailles). Edition de l'Institut National de la Recherche Agronomique, Paris.

COCKING, E. C. & P. K. EVANS, 1973. The isolation of protoplasts. In: H. E. STREET (Ed.), Plant tissue and cell culture. P. 100–120. Botanical Monographs. Blackwell Sci. Publ. Oxford.

COCKING, E. C. & E. POJNAR, 1969. An electron microscopic study of the infection of isolated tomato fruit protoplasts by tobacco mosaic virus. J. Gen. Virol. 4:305–312.

COLLOQ. INT. CNRS. (Versailles, 1972). Protoplastes et fusion de cellules somatiques végétales. Edition de l'Institut National de la Recherche Agronomique, Paris. 1973.

COSLOY, S. D. & M. OISHI, 1973. Genetic transformation in *E. coli* K 12. Proc. nat. Acad. Sci. (U.S.) 90:84–87.

COUTTS, R. H. S., 1973. Viruses in isolated protoplasts, a potential model for studying nucleoprotein replication. P. 353–365. In: Colloq. int. CNRS. Protoplastes et fusion de cellules somatiques végétales (Versailles). Edition de l'Institut National de la Recherche Agronomique, Paris.

DAVEY, M. R. & E. C. COCKING, 1972. Uptake of bacteria by isolated higher plant protoplasts. Nature (Lond.) 239:455–456.

DAVEY, M. R., E. C. COCKING & E. BUSH, 1973. Isolation of legume root nodule protoplasts. Nature (Lond.) 244:460–461.

DAVEY, M. R. & K. C. SHORT, 1973. The isolation, culture and fusion of legume and cornflower leaf protoplasts. P. 437–453. In: Colloq. int. CNRS. Protoplastes et fusion de cellules somatiques végétales (Versailles). Edition de l'Institut National de la Recherche Agronomique, Paris.

DOY, C. H., P. M. GRESSHOFF & B. G. ROLFE, 1972. Phage mediated transfer and subsequent expression of the galactose operon from *E. coli* in cells of haploid tomato callus. Proc. Austr. Biochem. Soc. 5:3.

DOY, C. H., P. M. GRESSHOFF & B. G. ROLFE, 1973a. Biological and molecular evidence for the transgenosis of genes from bacteria to plant cells. Proc. nat. Acad. Sci. (U.S.) 70:723–726.

DOY, C. H., R. M. GRESSHOFF & B. G. ROLFE, 1973b. Time course of phenotypic expression of *E. coli* gene Z following transgenosis in haploid *Lycopersicon esculentum* cells. Nature New Biol.244:90–91.

DURAND, J., I. POTRYKUS & G. DONN, 1973. Plantes issues de protoplastes de *Pétunia*. Z. Pflanzenphysiol. 69:26–34.

EPHRUSSI, B. & M. C. WEISS, 1965. Interspecific hybridization of somatic cells. Proc. nat. Acad. Sci. (U.S.) 53:1040–1042.

ERIKSSON, T. & K. JONASSON, 1969. Nuclear division in isolated protoplasts from cells of higher plants grown in vitro. Planta 89:85–89.

EVANS, P. K., A. G. KEATES & E. C. COCKING, 1972. Isolation of protoplasts from cereal leaves. Planta 104:178–181.

FREARSON, E. M., J. B. POWER & E. C. COCKING, 1973. The isolation, culture and regeneration of *Petunia* leaf protoplasts. Develop. Biol. 33:130–137.

GILES, K. L., 1972. An interspecific aggregate cell capable of cell wall regeneration. Pl. Cell Physiol. 13:207–210.

GILES, K. L., 1973. Attempts to demonstrate genetic complementation by the technique of protoplast fusion P. 485–495. In: Colloq. int. CNRS. Protoplastes et fusion de cellules somatiques végétales (Versailles). Edition de l'Institut National de la Recherche Agronomique, Paris.

GILES, K. L. & V. SARAFIS, 1971. On the survival and reproduction of chloroplasts outside the cell. Cytobios 4:61–74.

GRAMBOV, H. J., K. N. KAO, R. A. MILLER & O. L. GAMBORG, 1972. Cell division and plant development from protoplasts of carrot cell suspension cultures. Planta 103:348–355.

GREGORY, D. W. & E. C. COCKING, 1965. The large-scale isolation of protoplasts from immature tomato fruits. J. Cell Biol. 24:143–146.

GUHA, S. & S. C. MAHESHWARI, 1964. In vitro production of embryos from anthers of *Datura*. Nature (Lond.) 204:497.

GUPTA, N. & P. S. CARLSON, 1972. Preferential growth of haploid plant cells in vitro. Nature New Biol. 239:86.

HALL, M. D. & E. C. COCKING, 1971. The bursting response of isolated *Avena* coleoptile protoplasts to indole-3-ylacetic acid. Biochem. J. 124:33.

HARADA, H., 1973. A new method for obtaining protoplasts from mesophyll cells. Z. Pflanzenphysiol. 69:77–80.

HARRIS, H., 1970. Cell fusion. Harvard Univ. Press, Cambridge (Mass.).

HEINZ, D. J. & G. W. P. MEE, 1970. Colchicine-induced polyploids from cell suspension cultures of sugarcane. Crop Sci. 10:696–699.

HELLMANN, S. & J. REINERT, 1971. Protoplasten aus Zellkulturen von *Daucus carota*. Protoplasma 72:479–484.

HESS, D., 1970. Molekulare Genetik bei höheren Pflanzen. Ber. dt. bot. Ges. 83:279–300.

HESS, D., 1972. Transformation an höheren Organismen. Naturwiss. 59:348–355.

HIBI, T. & K. YORA, 1972. Electron microscopy of tobacco mosaic virus infection in tobacco mesophyll protoplasts. Ann. Phytopathl. Soc. Japan 38:350–356.

HEYN, R. F. & R. A. SCHILPEROORT, 1973. The use of protoplasts to follow the fate of *Agrobacterium tumefaciens* DNA on incubation with tobacco cells. P. 385–395. In: Colloq. int. CNRS. Protoplastes et fusion de cellules somatiques végétales (Versailles). Edition de l'Institut National de la Recherche Agronomique, Paris.

HOFFMANN, F. & D. HESS, 1973. Die Aufnahme radioaktiv markierter DNS in isolierten Protoplasten von *Petunia hybrida*. Z. Pflanzenphysiol. 69:81–83.

HOLDEN, D. J. & A. C. HILDEBRANDT, 1972. Behaviour of plant cell protoplasts in microculture. Phyton 29:47–54.

KAMEYA, T. & N. TAKAHASHI, 1972. The effects of inorganic ions on fusion of protoplasts from roots and leaves of *Brassica* species. Jap. J. Genet. 47:215–218.

KAMEYA, T. & H. UCHIMIYA, 1972. Embryoids derived from isolated protoplasts of carrot. Planta 103:356–360.

KAO, F. T. & T. T. PUCK, 1970. Genetics of somatic mammalian cells: linkage studies with human-chinese hamster cells. J. Cell Physiol. 80:119–128.

KAO, K. N., W. A. KELLER & R. A. MILLER, 1970. Cell division in newly formed cells from protoplasts of soybean. Exp. Cell Res. 62:338–340.

KASSANIS, B., 1967. Plant tissue culture. In: K. MARAMOROSCH and H. KOPROWSKI (Ed.), Methods in Virology. Vol. 1, p. 537–566. Academic Press, N.Y.

KELLER, W. A., B. HARVEY, O. L. GAMBORG, R. A. MILLER & D. E. EVELEIGH, 1970. Plant protoplasts for use in somatic cell hybridization. Nature (Lond.) 226:280–282.

KIMBER, G. & R. RILEY, 1963. Haploid angiosperms. Bot. Rev. 29:480–531.

KLERCKER, J. AF., 1892. Eine Methode zur Isolierung lebender Protoplasten. Svenka. Vet-Akad. Forh. Stockholm 9:463–471.

KOCHHAR, T., P. SABHARWAL & J. ENGELBERG, 1971. Production of homozygous diploid plants by tissue culture technique. J. Hered. 62:59–61.

LEDOUX, L., 1965. Uptake of DNA by living cells. In: Progress in nucleic acid research. Vol. 4, p. 231–267. Acad. Press N.Y.

LEDOUX, L., R. HUART & M. JACOBS, 1971. Fate of exogenous DNA in *Arabidopsis thaliana*. P. 159–172. In: L. LEDOUX (Ed.), Informative molecules in biological systems. Proc. int. Symp. Mol., Belgium 1970, North Holland Publ. Co. Amsterdam.

LESCURE, A. M., 1973. Selection of markers of resistance to base-analogues in somatic cell cultures of *Nicotiana tabacum*. Plant Sci. Letters 1:375–383.

LORENZINI, M., 1973. Obtention de protoplastes de tubercule de pomme de terre. C.R. Acad. Sci. (Paris) Ser. D. 276:1839–1842.

MACKENZIE, I. A., D. BUI-DANG-HA & M. R. DAVEY, 1973. Some aspects of the isolation, fine structure and growth of protoplasts from *Asparagus officinalis* L. P. 291–299. In: Colloq. int. CNRS. Protoplastes et fusion de cellules somatiques végétales (Versailles). Edition de l'Institut National de la Recherche Agronomique, Paris.

MALIGA, P., A. SZ. BREZNOVITS & L. MÁRTON, 1973a. Streptomycin-resistant plants from callus culture of haploid tabacco. Nature New Biol. 244:29.
MALIGA, P., L. MÁRTON & A. SZ. BREZNOVITS, 1973b. 5-bromodeoxyuridine-resistant cell lines from haploid tobacco. Plant Sci. Letters 1:119–121.
MARETZKI, A. & L. G. NICKELL, 1973. Formation of protoplasts from sugarcane cell suspensions and the regeneration of cell cultures from protoplasts. P. 51–63. In: Colloq. int. CNRS. Protoplastes et fusion de cellules somatiques végétales (Versailles). Edition de l'Institut National de la Agronomique, Paris.
MAYO, M. A. & E. C. COCKING, 1969. Detection of pinocytic activity using selective staining with phosphotungstic acid. Protoplasma 68:231–236.
MARX, J. L., 1973. Somatic cell hybrids: impact on mammalian genetics. Science 179:785–787.
MELCHERS, G. & G. LABIB, 1970. Die Bedeutung haploider höherer Pflanzen für Pflanzenphysiologie und Pflanzenzüchtung. Die durch Antherenkultur erzeugten Haploiden, ein neuer Durchbruch für die Pflanzenzüchtung. Ber. dt. Bot. Ges. 83:129–150.
MILO, G. E. & B. I. S. SRIVASTAVA, 1969. RNA-DNA hybridization studies with the crown gall bacteria and the tobacco tumor tissue. Biochem. Biophys. Res. Commun. 34:196.
MOTOYOSHI, F., 1972. Protoplast isolation from callus cells of maize endosperm. Expl Cell Res. 68: 452–456.
MOTOYOSHI, F., & N. OSHIMA, 1968. Multiplication of tobacco mosaic virus in suspension culture of tobacco cells. Jap. J. Microbiol. 12:317–320.
MURAKISHI, H. H., 1968. Infection of tomato callus cells in suspension with TMV-RNA. Phytopathology 58:993–996.
MURASHIGE, T. & F. SKOOG, 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plantarum, 15:473–497.
NAGATA, T. & I. TAKEBE, 1970. Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta 92:301–308.
NAGATA, T. & I. TAKEBE, 1971. Plating of isolated tobacco mesophyll protoplasts on agar medium. Planta 99:12–20.
NASS, M. M. K., 1969. Uptake of isolated chloroplasts by mammalian cells. Science 165:1128–1131.
NICKELL, L. G. & J. G. TORREY, 1969. Crop improvement through plant cell and tissue culture. Science 166:1068–1070.
NITSCH, J. P., 1972. Haploid plants from pollen. Z. PflZücht. 67:3–18.
NITSCH, J. P. & K. OHYAMA, 1971. Obtention de plantes à partir de protoplastes haploides cultivés in vitro. C.R. Acad Sci. (Paris) Ser. D. 273:801–804.
OHYAMA, K., O. L. GAMBORG & R. A. MILLER, 1972. Uptake of exogenous DNA by plant protoplasts. Can. J. Bot. 50:2077–2080.
OSWALD, T., M. D. AVERY, C. M. MACLEOD & M. MCÇARTY, 1944. Studies on the chemical nature of the substance inducing transformation of *Pneumococcal* types. Induction of transformation by a desoxyribonucleic acid fraction isolated from *Pneumococcus* types III. J. exp. Med. 79:137–158.
OTSUKI, Y. & I. TAKEBE, 1969. Isolation of intact mesophyll cells and their protoplasts from higher plants. Pl. Cell Physiol. 10:917–921.
OTSUKI, Y., I. TAKEBE, Y. HONDA & C. MATSUI, 1972. Ultrastructure of infection of tobacco mesophyll protoplasts by tobacco mosaic virus. Virology 49:188–194.
POJNAR, E., J. H. M. WILLISON & E. C. COCKING, 1967. Cell wall regeneration by isolated tomato fruit protoplasts. Protoplasma 64:460–475.
POTRYKUS, I., 1971. Intra and interspecific fusion of protoplasts from petals of *Torenia bailloni* and *Torenia fournieri*. Nature New Biol. 231:57–58.
POTRYKUS, I., 1973. Transplantation of chloroplasts into protoplasts of *Petunia*. Z. Pflanzenphysiol. 70:364–366.
POTRYKUS, I. & F. HOFFMANN, 1973. Transplantation of nuclei into protoplasts of higher plants. Z. Pflanzenphysiol. 69:287–289.
POWER, J. B. & E. C. COCKING, 1968. A simple method for the isolation of very large numbers of leaf protoplasts by using mixtures of cellulase and pectinase. Biochem. J. 3(5) 33 p.
POWER, J. B. & E. C. COCKING, 1970. Isolation of leaf protoplasts: macromolecule uptake and growth substance response. J. exp. Bot. 21:64–70.
POWER, J. B. & E. C. COCKING, 1971. Fusion of plant protoplasts. Sci. Progr. Oxford. 59:181–198.

POWER, J. B., S. E. CUMMINS & E. C. COCKING, 1970. Fusion of isolated plant protoplasts. Nature (Lond.) 225:1016–1018.

RAJ, B. & J. M. HERR, Jr., 1970. The isolation of protoplasts from placental cells of *Solanum nigrum* L. Protoplasma 69:291–300.

RUESINK, A. W., 1971. Protoplasts of plant cells. In: A. SAN PIETRO (Ed.), Methods in enzymology. 23A:197–209.

RUESINK, A. W. & K. V. THIMANN, 1965. Protoplasts from the *Avena* coleoptile. Proc nat. Acad. Sci. (U.S.) 54:56–64.

SCHASKOLSKAYA, N. D., G. N. SACHAROVSKAYA & E. V. SACHAROVA, 1973. The optimal conditions for isolation and incubation of barley mesophyll protoplasts. P. 93–98. In: Colloq. int. CNRS. Protoplastes et fusion de cellules somatiques végétales (Versailles). Edition de l'Institut National de la Recherche Agronomique, Paris.

SCHENK, R. U. & A. C. HILDEBRANDT, 1969. Production of protoplasts from plant cells in liquid culture using purified commercial cellulases. Crop Sci. 9:629–631.

SCOWCROFT, W. R., M. R. DAVEY & J. B. POWER, 1973. Crown gall protoplasts-isolation, culture and ultrastructure. Plant Sci. Letters 1:451–456.

STEWARD, F. C., P. V. AMMIRATO & M. O. MAPES, 1970. Growth and development of totipotent cells; some problems, procedures, and perspectives. Ann. Bot. 34:761–787.

STREET, H. E., 1973. Plant tissue and cell culture. Botanical Monograph, Vol. 11. Blackwell Sci. Publ. Oxford.

SUNDERLAND, N., 1971. Anther culture: a progress report. Sci. Progr. Oxford 59:527–549.

TAIZ, L. & R. L. JONES, 1971. The isolation of barley-aleurone protoplasts. Planta 101:95–100.

TABEKE, I., G. LABIB & G. MELCHERS, 1971. Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Narturwiss. 58:318–320.

TABEKE, I., Y. OTSUKI & S. AOKI, 1968. Isolation of mesophyll cells in intact and active state. Pl. Cell Physiol. 9:115–124.

VASIL, V. & A. C. HILDEBRANDT, 1965. Differentiation of tobacco plants from single, isolated cells in microcultures. Science 150:889–892.

WALLIN, A. & T. ERIKSSON, 1973. Protoplast cultures from cell suspensions of *Daucus carota*. Physiol. Plantarum 28:33–39.

WENZEL, G., 1973. Isolation of leaf protoplasts from haploid plants of *Petunia*, rape and rye. Z. PflZücht. 69:58–61.

WEISS, M. C. & H. GREEN, 1967.Human-mouse hybrid cell lines containing partial complements of human chromosomes and functioning human genes. Proc. natn. Acad. Sci. (U.S.) 58:1104–1111.

WHITE, P. R., 1963. The cultivation of animal and plant cells. The Roland Press Co. N.Y.

WILLISON, J. H. M., 1973. Fine structural changes occuring during the culture of isolated tomato fruit protoplasts. P. 215–241. In: Colloq. int. CNRS. Protoplastes et fusion de cellules somatiques végétales (Versailles). Edition de l'Institut National de la Recherche Agronomique, Paris.

WINTON, L. L., 1970. Shoot and tree production from aspen tissue cultures. Amer. J. Bot. 57:904–909.

WITHERS, L. & E. C. COCKING, 1972. Fine structural studies on spontaneous and induced fusion of higher plant protoplasts. J. Cell Sci. 11:59–75.