Plant Protoplasts in Agricultural Research

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Under plasmolyzing conditions, cell walls can be removed from the cells of higher plants without disorganizing the remaining cellular structures. The resulting cell form, the naked protoplast, assumes spherical shape and remains viable when placed in suitable environments.

Although these facts have been known to botanists for a long time, it was since the introduction of an enzymic method for cell wall removal in 1960 that protoplast research made a striking progress[4]. Recent studies show that this wall less form of cells exhibits some unique properties which are of extreme interest for both fundamental and applied biology.

In 1968 we developed a method for isolating active protoplasts from the mesophyll tissue of tobacco[14]. In this method, leaf tissues are first macerated by pectinase to release single mesophyll cells. The cells thus isolated in suspension are then treated with cellulase which transforms the cells into protoplasts by dissolving the cellulosic walls (Fig. 1).

Tobacco mesophyll protoplasts are now used most extensively in protoplast research because they are readily isolated in very large numbers (up to 10^7 protoplasts from 1 g fresh leaf material) and show a wide range of activities.

In this article I shall discuss some of the uses of plant protoplasts in agricultural sciences. Although I shall deal specifically with tobacco mesophyll protoplasts with which we are working, the discussions should apply in principle to protoplasts from other tissues or from other plants.

Protoplasts as a new experimental material in plant virology

Plant viruses lack the ability to penetrate the rigid walls of plant cells. Inoculation with plant viruses requires, therefore, the presence

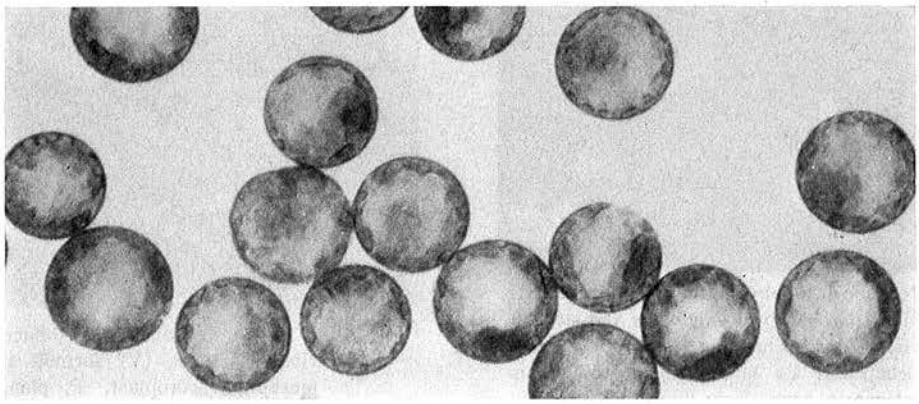

Fig. 1. Protoplasts of palisade parenchyma cells isolated from tobacco leaves. Structures peripherally arranged in protoplasts are chloroplasts. ×650

of wounds in the cell wall which are produced, for example, by rubbing the leaf surface with carborundum. This is one of the reasons why only an extremely small fraction of the cells in a leaf (usually less than one thousandth) are primary-infected.

Most of the leaf cells become infected later by cell-to-cell movement of virus but these secondary-infected cells are unsuitable for the analysis of infection events because the stage of infection differs from one cell to another.

The low frequency of primary infection and the asynchronous virus multiplication cause major technical difficulties in the plant virus studies using tissue systems.

We have shown that these technical difficulties are largely bypassed by the use of protoplasts, the isolated cells without walls. Protoplasts from tobacco leaves are infected by tobacco mosaic virus (TMV) at very high frequencies without mechanically wounding the cells. For inoculation, they are simply mixed for 10 minutes with TMV solution at 1 μg/ml under appropriate conditions[15].

The protoplasts thus inoculated are cultured in a synthetic liquid medium to allow the virus to multiply in protoplasts. Virus accumulated in the infected protoplasts can be visualized by staining with fluorescence-labelled antibody to virus, and we know by this method that up to 90% of protoplasts are infected [8] (Fig. 2).

Aggregates of large numbers of virus particles are found in the protoplasts by electron microscopy[8] (Fig. 3). The course of virus multiplication can be followed by assaying the infectivity in protoplasts at intervals after inoculation. As shown in Fig. 4, TMV multiplication starts 6 hours after inoculation

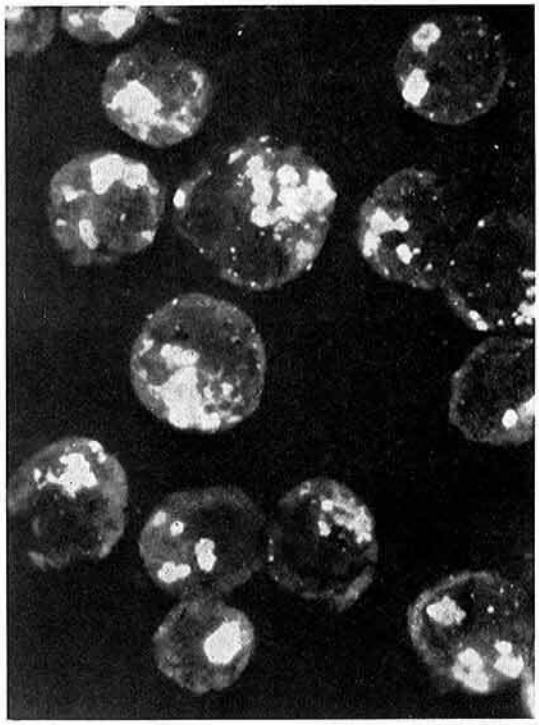

Fig. 2. Tobacco mesophyll protoplasts inoculated with tobacco mosaic virus and stained with fluorescence-labelled antibody to virus after 24 hours of culture. Virus antigen in protoplasts fluoresces brightly in yellow under fluorescence microscope. ×400

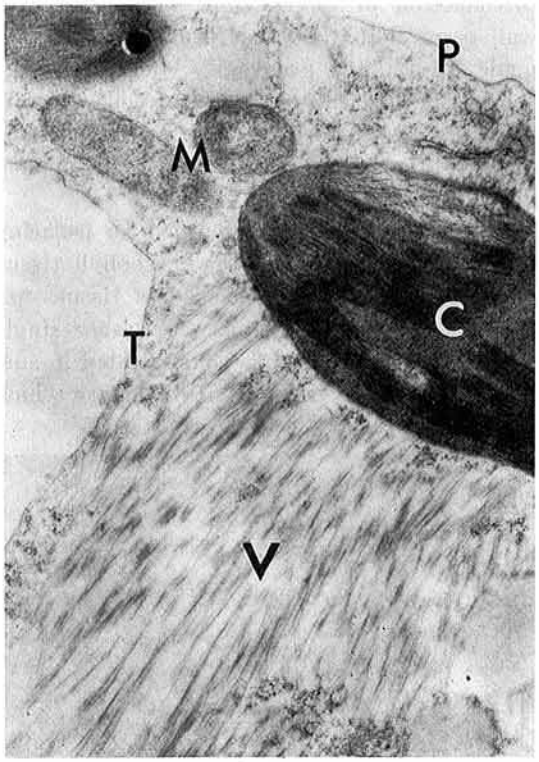

Fig. 3. A large aggregate of tobacco mosaic virus particles (V) formed in tobacco mesophyll protoplast. P, plasmalemma; T, tonoplast; C, chloroplast; M, mitochondria. ×17,000

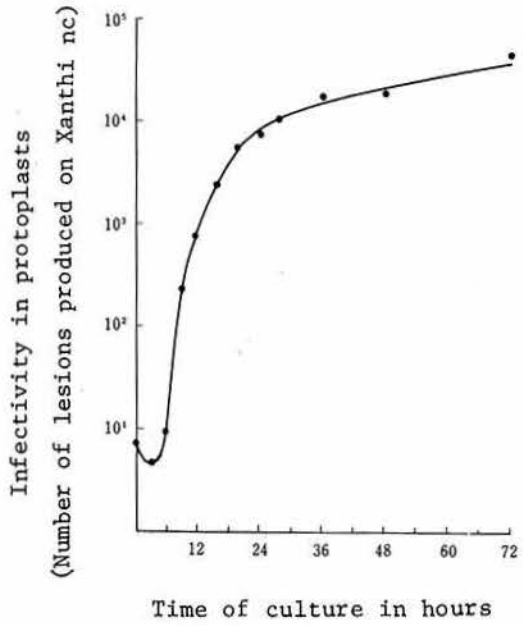

Fig. 4. Growth curve of tobacco mosaic virus in tobacco mesophyll protoplasts

and proceeds very rapidly during the first 24 hours. The infectivity assay also enables us to estimate that more than 10^6 TMV particles are produced per infected protoplast.

Since the protoplasts are in contact with inoculum virus only for a short time, the infection, and hence the subsequent virus multiplication should be synchronous.

The synchronous infection at very high frequencies is characteristic for protoplasts and makes this system far more suitable than conventional tissue materials for studying the process of plant virus multiplication.

We have been able to show with this system that, in addition to virus coat protein, a protein with high molecular weight is synthesized in the cells upon infection by TMV[13].

Experiments with protoplasts from local lesion hosts of TMV showed that the protoplasts, in contrast to the cells in leaves, do not respond to TMV infection by necrotic reaction[9], suggesting that cell necrosis is a result of interaction between cells in tissue.

The facile infection of protoplasts is not restricted to TMV. We have shown that tobacco mesophyll protoplasts are infected as well by cucumber mosaic virus[10] and potato virus X[11] as by TMV. The list of viruses which can be studied using protoplasts is expanding rapidly, and it is likely that protoplast infection will become one of the standard techniques in plant virology.

Protoplasts and plant breeding

Many problems have been encountered in the use of plant tissue cultures for crop improvement, and among them is the difficulty in obtaining single cells and in culturing them as single cell clones. By dissolving the walls which connect cells to each other, it is now possible to isolate a large number of single cells as protoplasts. Protoplasts are thus a potential source of large numbers of single cell clones and this is one of the most important features of protoplasts when their use for plant breeding is considered.

In addition, the lack of rigid walls confers some unique properties on protoplasts which may have great consequences for the attempts to modify genetic constitution of plants. The infection of protoplasts by viruses, or with their nucleic acid[1], indicates that these wall-less cells are capable of taking up nucleic acid molecules or nucleoprotein particles, and suggests a way to introduce foreign genes into plant cells.

With even more straightforward consequence for plant breeding is the finding that protoplasts can be induced to fuse with each other to form multinucleates[12].

Since protoplast fusion is possible between species or genera[12,18], it is envisioned that hybrids may be produced through protoplast fusion between such plants which cannot be crossed by sexual means. If such a possibility can be realized, it will obviously have an enormous impact on plant breeding.

One of the prerequisites for the use of protoplasts in plant breeding is the technique by which protoplasts can be cultured to regenerate whole plants. We have been able to show that this is possible with tobacco mesophyll protoplasts[16].

When these protoplasts are cultured in a

simple medium containing inorganic salts, vitamins, an auxin and a cytokinin, they rapidly form new surface walls and initiate cell division[5].

In an improved medium, the cells derived from protoplasts grow actively to form callus-like aggregates of large numbers of daughter cells[6]. It is also possible to culture the protoplasts in an agar-containing medium, and under optimal conditions up to 70% of plated protoplasts form colonies of 1 mm diameter in 6 weeks[6] (Fig. 5).

These colonies can be subcultured as callus on the same medium or they are transplanted to a differentiation medium to induce shoot formation.

On further transfer to a medium containing no hormone, these shoots produce roots and develop into plantlets (Fig. 6).

The plantlets thus derived *in vitro* from single mesophyll protoplasts grow normally on soil, flower and give seeds (Fig. 7). When appropriate precautions are taken during *in vitro* culture, the plants regenerated from protoplasts are morphologically identical to the parent plants and have the regular chromosome number[17].

These experiments show that it is now possible to produce vast numbers of clonal

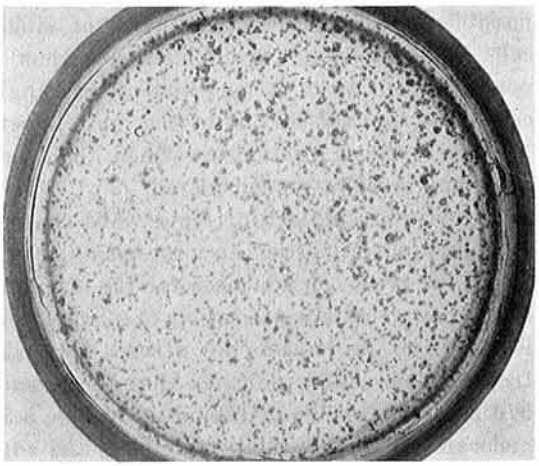

Fig. 5. Colonies formed by tobacco mesophyll protoplasts in an agar plate

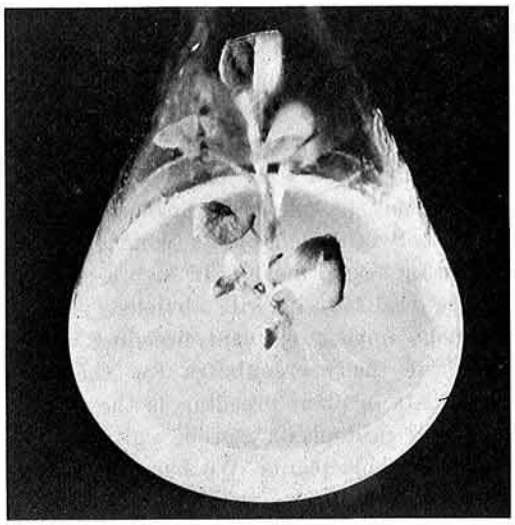

Fig. 6. A tobacco plantlet derived *in vitro* from a single mesophyll protoplast

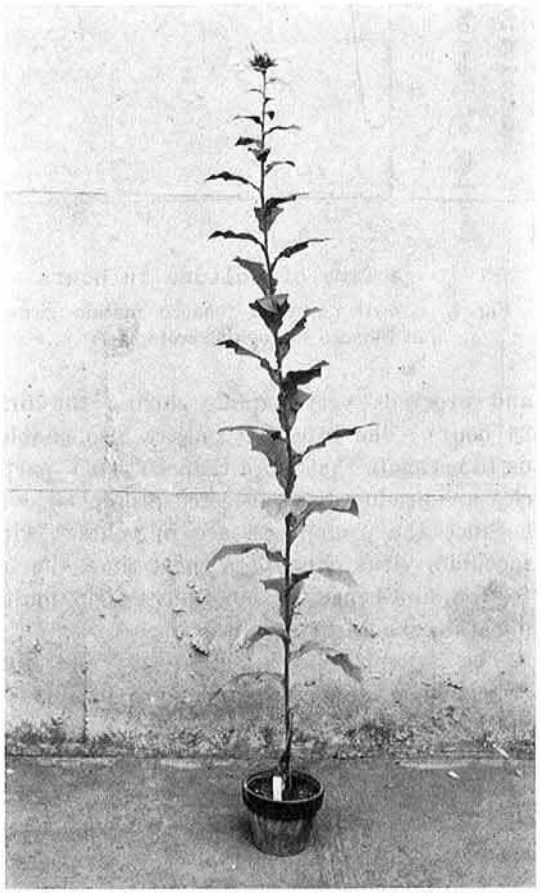

Fig. 7. A flowering tobacco plant (Xanthi) regenerated from a single mesophyll protoplast

cell lines or plants by plating isolated protoplasts. The same technique applied to protoplasts from haploid plants[7),17)] provides the possibility to manipulate somatic plant cells like unicellular microorganisms. For example, it should be possible to treat haploid protoplasts with a chemical or a physical mutagen and to select mutant clones on agar plates. This should enormously improve the efficiency for producing mutant plants.

Successful plating of protoplasts and subsequent regeneration into plants will also encourage any attempt to utilize for plant breeding the ability of protoplasts to take up genetic materials or to undergo interspecific fusion.

Protoplasts are an artificial form of cells which never exists in natural environments. Their unusual properties make it possible to mix genomes in hithertofore unfeasible combinations. It should be reasonable to assume that the chance of genetic variation in the existing plants will be greatly increased by the use of protoplasts. Although attempts to explore this possibility were initiated only very recently, the success reported in some model experiments[2),3)] appear to suggest that the use of protoplasts for plant breeding is promising.

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