ISOLATION, CULTURE AND CALLUS FORMATION OF SOYBEAN PROTOPLAST

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Abstract

Protoplasts isolated enzymatically from cotyledons of immature pods can be rapidly obtained in great numbers. Protoplast regenerating a wall and sustaining division can be induced to form a callus. Protoplast division occurred after 2-3 days of culture and compact clusters of cells appeared in the second week of the culture. Transfers of these clusters to the liquid medium resulted in the formation of rapidly growing callus tissues, which turned green after exposure to light.

Isolation

One of the most significant developments in the field of plant tissue culture during recent years has been the isolation, culture and fusion of protoplasts. The techniques are especially important because of their far-reaching implications in studies of plant improvement by cell modification and somatic hybridization. Protoplasts also have the remarkable property of taking up small and large molecules, viruses, bacteria, chloroplasts, DNA and whole nuclei. This suggests a method by which "transformed" plants may be obtained (Bajaj, 1977). Protoplasts have been isolated from numerous different plant species. The recently developed technique of protoplast culture has led to the successful regeneration of whole plants in 30 species. In the last few years, plants have been regenerated from interspecific and intergeneric somatic hybrid cells. Somatic hybridization has become a useful tool for studying genetic engineering of plant and hopefully an experimental system for the uptake of extraneous genetic materials.

Soybean is one of the most important crops in the world market. Tissue culture of soybean has been established for a number of years. In 1975-76, we successfully induced plantlets from the callus culture of the soybean hypocotyl (Research Group of Soybean Tissue Culture, 1976). In 1979, we successfully induced a pollen plantlet from an anther (Jian *et al.*, 1980).

Protoplasts have been isolated from cultured root cells (Kao, 1970; Kao *et al.*, 1970; Kao *et al.*, 1971; Millev *et al.*, 1971) root (Xu Z.H., 1982), immature pod tissue (surface below the endocarp) (Zieg and Outka, 1980) and leaves (Schwenk, 1981) of soybean plants. To date pod and root protoplasts have produced callus in culture, but plant regeneration has not been obtained.

The objective is to enlarge the scope of soybean hybridization, to increase the range of variation and to create new cultivars or mutants with high photosynthetic rate, good quality, resistance and high yield by means of somatic hybridization through protoplast fusion and uptake of organelles and extraneous genetic material by protoplasts. In order to establish an experimental system for studying genetic engineering of soybean, first of all, the protoplasts must be obtained in large quantities, secondly, a protoplast may be induced to regenerate into an intact plant.

We have thus used various tissues of soybean, including leaf, stem, pith, root and pod as the materials for protoplast isolation. This report describes the method applied for protoplasts isolated enzymatically from cotyledons of immature pods and rapidly obtained in great numbers, and the conditions for sustained division to produce visible callus. There are no reports of isolation and cultivation in soybean cotyledon.

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Materials and methods

Sixty-nine varieties were used in an attempt to isolate protoplasts. Soybeans were grown under field conditions, immature pods (less than 1 cm x 4 cm) were surface-sterilized with alcohol, the epidermis and endocarp layer were removed, sterilized cotyledons were cut in vertical sections approximately 1-2 mm in width. These cotyledonary segments (per seed) were incubated in 3 ml of enzyme-protoplast medium mixture (1: 1), placed in a 60x50 mm dish to form a thin layer and the dish was incubated on a rotary shaker (60 rpm) in the dark at room temperature (25°C) for 24 hr. The enzyme solution containing 2% Onozuka R-10 cellulase, 2% Rhozyme hemicellulase, 1% Sigma pectinase, was dissolved in 10 ml of the following solution : 10 mg $CaCl_2$ $2H_2O$, 1 mg NaH_2PO_4 H_2O, 1.26 g glucose and 5.8 mg MES in 10 ml distilled water (pH 5.5). The medium used was Kao's plant protoplast medium (Kao, 1982).

After incubation, the enzyme-protoplast mixture was passed through a stainless steel filter 60 μm in pore size. The filtrate was then centrifuged (1000 rpm, 4-6 min.) to sediment the protoplasts, the supernatant was removed and the protoplasts were washed in 5 ml medium in which they were cultured. After the enzyme was washed away the protoplasts were resuspended in the protoplast medium in a 0.01-0.2 % suspension, 5-12 drops (50 μl) of the protoplast suspension were placed in a 60 x 50 mm Falcon petri dish, the dish was sealed with parafilm and incubated at 25°C in dim light in a plastic box. After protoplasts divided, fresh medium was added for dilution (the medium was made by mixing 1 part of a cell culture medium to 3 parts of the protoplast culture medium) (Kao, 1982).

Results

These experiments were repeated 24 times and 69 varieties were used in an attempt to isolate the protoplasts. Isolation of protoplasts was easier in 15 varieties than in the others. Protoplasts from *Glycine max* were usually isolated more rapidly than the protoplasts from *Glycine soja*. Protoplast release reflected clearly differences of the genotypes of *Glycine max* used. In some varieties protoplasts were easily isolated, unlike in others. Percentages of isolated protoplasts in most of the varieties exceeded 50%, and in some varieties production of 80% of protoplasts could be achieved. After the isolation, in viable protoplasts the cycle of differentiation inside the cell could be observed.

Generally speaking, young cotyledons from rapidly growing pods are the best source of protoplasts and enrichment of a mineral salt medium with proper amounts of organic acids, amino acids and vitamins usually resulted in a much rapid initiation of cell regeneration and division in protoplasts. A newly formed cell wall sometimes could be observed within 24 hr after isolation of the protoplast. First division of protoplast occurred after 2-3 days of culture, second division after 4-5 days, followed by the formation of 5 to 10 cell clusters within 2 weeks. Percentages of divided protoplasts in most of the varieties were more than 30-50%. After 5-8 days of culturing, fresh medium with a slightly lower osmolarity was added. At one month, when the protoplasts formed cells and divided several times, gradual dilution of the culture with fresh medium was necessary to sustain cell division. After dilution with fresh medium, the clusters were incubated in the incubator shaker (100 rpm), and they grew to form visible colonies up to 1-2 mm in size. They were transplanted onto solid medium with agar and subsequently they formed a callus 4-5 mm in diameter. Protoplast-derived tissues grew vigorously when maintained in Kao's medium and became green under diffuse light. Differentiated culture is continued.

References

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Discussion

Thulasidass, G. (India): Could you achieve any success in the transfer of economic characters like disease resistance from one variety to another through somatic hybridization ?
Answer: No success has been achieved yet.