Erratum

A simple culture method for *Brassica* hypocotyl protoplasts

Phan V. Chuong, K. P. Pauls, and W. D. Beversdorf

Department of Crop Science, University of Guelph, Guelph, Ontario, Canada

Plant Cell Reports (1985) 4:4–6

Increasing the variability of *Lycopersicon peruvianum* Mill. by protoplast fusion with *Petunia hybrida* L.

Z. Tabaeizadeh, C. Perennes, and C. Bergounioux

Laboratoire d'Amélioration des Plantes, Université de Paris Sud, F-91405 Orsay, France

Plant Cell Reports (1985) 4:7–11

Due to an unfortunate error, Fig. 1 of the article by P. V. Chuong et al. and Fig. 1 of the article by Z. Tabaeizadeh et al. were transposed. Consequently, the relevant pages are repeated here in their correct form. The page numbers correspond to those of the original pages in Volume 4, Number 1.

Further, in the title of the article by P. V. Chuong et al., “hypototyl” should have read “hypocotyl”.

RESULTS AND DISCUSSION

Shortly after plating, intact protoplasts were found floating on the surface of the medium but cell wall debris and lysed cells sank to the bottom of the culture dish (Fig. 1a). Cytoplasmic streaming could be observed at this time. Protoplasts gradually increased their volumes over the next few days. Irregularly shaped and swollen cells were often observed. The first division occurred 3-5 days after culture (Fig. 1b) and the division efficiency was around 20% after 6 days in culture. The protoplasts continued to divide (Fig. 1c) and formed clusters of cells at the surface of the medium (Fig. 1d). Microcolonies visible to an unaided eye (300-500 μm in diameter) developed from the protoplasts after 3-4 weeks in culture. The microcolonies also remained floating on the surface of the medium for this period of time. Usually 1,000 - 2,000 microcalli developed per plate inoculated with 2.5 ml media containing 2×10^5 protoplasts/ml (Fig. 1e). This number was markedly reduced to 100 - 300 microcalli per plate if the concentration of protoplasts originally plated was halved (i.e. $1 \times 10^5/\text{ml}$) but the microcalli were larger (approximately 1 - 1.5 mm in diameter) than those produced at the higher protoplast density. Increasing the Ficoll concentration from 5 g/l to 30 g/l helped the microcalli to remain on the surface of the medium but reduced the final yield of microcalli. Again, however, the reduction in number of microcalli was accompanied by an increase in size of the individuals.

After 4-6 weeks in culture the microcalli were transferred to MS regeneration medium. Approximately 30 - 80% of the microcalli transferred survived on the regeneration medium and the larger microcalli generally had the greatest chance of survival. After 3 - 4 weeks on the regeneration medium, shoots were produced by approximately 3 percent of the calli.

The protoplast culture technique described in this report employs relatively simple media and is easily performed. No handling of the plant material is required until the protoplasts have developed into microcalli and shoots are regenerated after a single transfer step. It compares favourably with other culture methods for *Brassica* protoplasts in terms of the division efficiencies which have previously been reported (Xu et al., 1982; Li and Kohlenbach, 1982; Glimelius, 1984). The unique feature of this procedure is the Ficoll and high sucrose concentrations used to float the protoplasts and the microcalli that develop from them on the surface of the medium. Calli that grew too large and thus sank to the bottom of the medium died if they were not transferred to regeneration medium shortly thereafter. This observation suggests that gas exchange may be an important factor controlling the division and development of *Brassica* protoplasts. Ficoll has previously been used in barley anther culture media to enhance the aeration and growth of pollen calli (Kao, 1981).

Fig. 1. Culture of hypocotyl-derived protoplasts from *B. napus* cv. Isuzu, bar = 100 μm . a) Protoplasts following isolation from hypocotyl tissue; b) First cell division; c) Second cell division, after 5 days in culture; d) Clusters of protoplast-derived cells floating on the surface of the medium, after 2 weeks in culture. Note the wrinkles of the thin film under the weight of the cell clusters; e) Microcalli on the surface of the medium, after 4 weeks in culture.

Another advantage accruing from the use of Ficoll and high sucrose in the media is that it separates, to a large extent, the live cells from the dead cells and debris. Thus the viable cells and microcolonies which develop from them may be removed from the deleterious effects of cell browning and particle release which often accompanies cell deterioration in *Brassica* protoplasts (Schenck and Hoffmann, 1979; Xu *et al.*, 1982). Microcolony browning was not a problem with the cultivar Isuzu but was observed in protoplast cultures of some other genotypes of *B. napus* and in *B. oleracea* (Chuong *et al.*, unpublished).

The size increase of the microcalli produced at lower protoplast plating densities suggests that nutrients might be limiting in the medium at the higher protoplast densities. This was tested by media replenishment. Enhanced growth of the microcalli in cultures treated in this way was observed and is consistent with the proposal that nutrients become limiting but may also indicate that inhibitory by-products accumulate in the medium during the development of the microcalli. High cell densities are however a requirement for efficient culture of the protoplasts since 1/10 of the number of microcolonies were formed after halving the concentration of protoplasts placed into culture. A minimum cell density for survival of protoplasts in culture is a frequently observed phenomenon (Nagata and Takebe, 1971; Schenck and Robbelen, 1982) and may be due to a conditioning effect of the protoplasts on the culture medium.

The frequency of plant regeneration from calli in this experiment (3%) was lower than that reported by Xu *et al.* (1982) and Glimelius (1984), who observed 19% and 70% respectively. This low frequency of plant regeneration with calli from the cultivar Isuzu may be due to a poor regeneration ability of the genotype used and/or to some inadequacy of the regeneration medium. A search for genotypes with high regeneration ability and an appropriate regeneration medium is underway.

The hypocotyl protoplasts used in this study have a dense light brown cytoplasm and are relatively stable cells in culture. We have used these cells for fusion studies with *Brassica* leaf mesophyll protoplasts (Pauls and Chuong, 1984). Heterokaryons are easily identified after fusion because the light brown cytoplasm of a hypocotyl protoplast and the green chloroplasts of a mesophyll protoplast are combined in one cell. It was found that the protoplast culture medium described in the present report does not support the growth of the leaf mesophyll protoplasts. (Leaf protoplasts were isolated in the same manner as the hypocotyl protoplasts from young leaves of plants raised in a growth room, after sterilization of the leaves with 10% bleach for 10 min followed by several rinses with sterilized water, except that the enzymatic digestion was performed under static conditions in an enzyme solution containing 1.5% w/v cellulase Onozuka R-10 from Yakult Pharmaceutical Industry Co., Nishinomy, Japan 0.3% Macerase from Calbiochem-Behring Corp., La-Jolla,

USA, plus CPW salts and 10% Mannitol, pH 5.7). Thus the culture method could be used to select against unfused leaf protoplasts after fusion experiments with *Brassica* leaf and hypocotyl protoplasts. Finally it should be noted that protoplasts from all six *Brassica* species including the 3 primary diploids and 3 amphidiploids of (U, 1935) triangle developed microcalli in the protoplast culture medium (Chuong *et al.*, in preparation). This suggests that the technique may be useful in interspecific or even intergeneric protoplast fusion studies in *Cruciferae*.

ACKNOWLEDGEMENTS

This research was supported by the Natural Sciences and Engineering Research Council of Canada, the Ontario Ministry of Agriculture and Food and the Canola Council of Canada.

REFERENCES

- Adams TL, Townsend JA (1983) *Plant Cell Reports* 2:165-168
- Bidney DL, Shepard JF, Kaleikan E (1983) *Protoplasma* 117:89-92
- Glimelius K (1984) *Physiol Plant* 61:38-44
- Kartha KK, Michayuk MR, Kao KN, Gamburg OL (1974) *Plant Sci Lett* 3:265-271.
- Kao KN (1981) *Z Pflanzenphysiol.* 103:437-443
- Koop H, Weber G, Schweiger H (1983) *Z Pflanzenphysiol.* 112:21-34
- Horsch RB, Jones GE (1980) *In Vitro* 16:103-108
- Li L, Kohlenbach HW (1982) *Plant Cell Reports* 1:209-211
- Lichter R (1981) *Z Pflanzenphysiol.* 103:229-237
- Nagata T, Takebe I (1971) *Planta* 99:12-20
- Pauls KP, Chuong PV (1984) *First Plant Genet Eng Workshop. IAPTC. Canada Section*; p. 36
- Schenck HR, Hoffmann F (1979) *Z Pflanzenzuchtg* 82:354-360
- Schenck HR, Robbelen G (1982) *Z Pflanzenzuchtg* 89:278-288
- U N (1935) *Jap. J. Bot.* 7:389-452
- Xu ZH, Davey MR, Cocking EC (1981) *Z Pflanzenphysiol* 104:289-298
- Xu ZH, Davey MR, Cocking EC (1982) *Plant Sci Lett* 24:117-121

Fig. 1

(a) and (b) Mitotic metaphase of *L. peruvianum* and *P. hybrida*, respectively.

(c) Mitotic metaphase of plant R_3 .

Chromosomes the dimension of *L. peruvianum* and also larger chromosomes the size of *P. hybrida* (see arrow) are present.

(d) Anaphase of the meiosis of plant R_3 . Two lagging chromosomes are observed at the equatorial plane.

Fig. 2

(a) and (b) Leaf morphology of *L. peruvianum* seed grown plant and plant regenerated from protoplast culture, respectively.

(c) *P. hybrida* (seed grown plant).

(d) and (e) R_3 plants. (f) and (g) R_6 plant.

(h) and (i) Plant R_7 in vivo and in vitro.

In addition, at the meiosis of the R_3 plant, two chromosomes appeared in the equatorial plane (Fig 1d).

Meiosis permits the elimination of supernumerary or different chromosomes. Since we observed two different morphological types of chromosomes in R_3 Plant mitosis, it seems that at meiosis chromosomes are eliminated.

Analysis of the Expression of Genetic Information Morphological Observations

It is necessary to point out that since no two species of the genera Lycopersicon and Petunia have ever been crossed sexually, it is very difficult to predict the morphological form of such a hybrid plant.

Among the plants regenerated after fusion, the leaf morphologies of the R_3 and R_6 plants were intermediate between the two parents. The leaflets were round and fewer than those of L. peruvianum parent (Fig. 2). The flower of the R_3 and R_6 plants resembled those of L. peruvianum, but were male sterile. Study of the progeny of these plants (using L. peruvianum as the male parent) will permit us to verify whether this sterility is nuclear or cytoplasmic. Plant R_7 , had round and nondentate leaflets (Fig. 2h,i). This plant showed poor growth and failed to flower.

Another morphological criterion which was studied in regenerated plants was the form of leaf hairs. Preliminary observations of the fully grown parental plants revealed simple hairs on L. peruvianum leaves and glandular hairs on P. hybrida leaves. The *in vitro* control plants had the same type of hairs as the seed grown parental type. Among the plants regenerated after heterofusion experiments, both types of hairs were observed on the R_6 plant *in vitro*. When this plant was transferred into greenhouse, the first leaves still showed both types of hairs, but when they were well developed only simple hairs were observed. Closer examination of seedgrown parental plants revealed that L. peruvianum cotyledons have glandular hairs, the first leaves have glandular and simple hairs, and only later leaves of fully grown plants have exclusively simple hairs. In P. hybrida, at all stages of development, the leaves have glandular hairs.

As the plant R_6 has intermediate leaf morphology, two interpretations are possible: either it could be the result of hybridization followed by elimination of Petunia chromosomes, or the *in vitro* culture could have induced a longer juvenile stage.

Analysis of RUBPCase

Although the stability of this enzyme already has been reported by Chen et al. (1976), we verified this fact in our experimental parental plants. The same electrophoretic pattern of RUBPCase was found in the seedlings and the flowering plants (Fig. 3a1-a4). The electrophoretic pattern of L. peruvianum plants regenerated from protoplasts and from autofusion was similar to that of the seed grown plants, indicating that the RUBPCase was stable during plant development as well as *in vitro*.

Among the regenerated plants, large subunit of R_7 has a more basic isoelectric point than either parent (Fig 3a5). In most previous analyses of RUBPCase in somatic hybrid plants, authors have

Fig. 3

Electrophoregram of RUBPCase for parent plants and some of the regenerated plants. SS: Small subunit; LS: Large subunit. (a1) and (a2) L. peruvianum flowering and seedling plants, respectively. (a3) and (a4) P. hybrida flowering and seedling plants, respectively. (a5) plant R_7 . (b1) L. peruvianum seed grown plant. (b2) L. peruvianum plant regenerated from protoplast culture. (b3) R_3 plant. (b4) R_6 plant.

found the large subunit of only one of the parents (Chen et al. 1977; Aviv et al. 1980). There are only two reports of the presence of the two parental types of the large subunit in interspecific somatic hybrids of Nicotiana (Glimelius et al. 1981; Kung et al. 1975). Since the RUBPCase was analysed in the control *in vitro* plants, and two independent electrofocusing experiments for plant R_7 were carried out, the possibility that the new form of the large subunit is an artifact due to *in vitro* culture or to experimental conditions can be excluded. Therefore, the new form of the large subunit in plant R_7 could be interpreted in two ways: (i) Expression of Lycopersicon genetic information coded by Chloroplast DNA might be modified by the presence of information originating from Petunia. (ii) Recombination of chloroplast DNA could have occurred. This latter hypothesis also has been suggested by Conde (1980) who observed a new pattern of chloroplast DNA in somatic hybrids of Nicotiana glauca + N. langsdorffii.

The RUBPCase electrophoretic pattern of the other presumptive regenerated hybrid plants analysed (including R_3 and R_6) was the same as of L. peruvianum. These plants therefore either lacked the genetic information of P. hybrida relative to RUBPCase, or the gene was transferred but not expressed.

In conclusion, after somatic fusion experiments of L. peruvianum and P. hybrida, one of the regenerated plants (R_3) possessed the whole chromosome number of the first parent (L. peruvianum) with some additional chromosomes similar to those of the second parent (P. hybrida), and was male sterile. Until now unambiguous expression of Petunia characters has not been observed in this plant. Another plant (R_7) presented a new form of the large subunit of RUBPCase which could mean that the direct expression of the parental characters in somatic hybrids is not systematic.