Tropical Agricultural Research Vol. 10 1998, 1-11

Isolation of Protoplasts from Leaf Tissue of Tea [*Camellia sinensis* (L.) O. Kuntz.] : Factors Affecting Protoplast Yield and Viability

M.T.K. Gunasekare and P.K. Evans[1]

Plant Breeding Division
Tea Research Institute
Talawakelle, Sri Lanka

ABSTRACT. *An isolation procedure that can release an adequate yield of viable protoplasts from leaf tissues of tea [Camellia sinensis (L.) O. Kuntz.] was established. Factors that affect protoplast yield and their viability were identified to improve the yield of intact protoplasts. To optimize the yield and viability of protoplasts, factors connected to explant and isolation medium were investigated. Among the parameters tested, the viability of the isolated protoplasts was affected only by the composition of the enzyme mixture. The osmoticum of the isolation mixture and the maturity of the leaf had no effect on the viability of protoplasts. On the other hand, all these factors affect the yield of protoplasts. Protoplasts cultured in MS based agarose medium were alive for 30 days but no cell proliferation was detected.*

INTRODUCTION

The utilisation of plant protoplasts in genetic modification techniques such as somatic hybridisation and genetic transformation has a great potential for crop improvement. This is particularly useful in a woody crop like tea since this crop requires long term breeding for the development of new genotypes with desirable characteristics. However, the primary limitation to the application of protoplasts in genetic manipulation is the inability, in a number of cases, to regenerate plants from protoplasts, especially in woody plant species. Protoplast technology in tea, has not reached the preparative stage yet. Therefore, to explore the potential application of protoplast based technology for tea an efficient method for isolation of viable protoplasts in quantity must first be established.

---

1 Department of Biology, School of Biological Sciences, University of Southampton, United Kingdom.

Many variables connected to the donor plant (Jenes *et al.*, 1994; Krasnyanski *et al.*, 1992) and isolation conditions (Mills and Hammerschlag, 1994; Ochatt, 1992; Park and Son, 1992) have been found to affect the yield and viability of protoplasts in studies carried out with other plant species. The experiments described in this paper were carried out to establish a method for isolating protoplasts from leaf tissue of tea by analysing some factors influencing the yield and viability of the isolated protoplasts. Preliminary studies have also been carried out in an attempt to culture these protoplasts.

MATERIALS AND METHODS

Plant material

Tea [*Camellia sinensis* (L.) O. Kuntz.] leaf tissue was used as a source of protoplast. Young leaves (2nd and 3rd leaf from the apex, unless otherwise mentioned) obtained from actively growing shoots of glasshouse grown seedlings were used. The donor plants were raised in pots and were maintained at 25°C with 16 h photoperiod using supplementary light when necessary.

Preparation of enzyme solution

Enzymes used in this study included Cellulase – Onozuka R–10 (Yakulta Honsha Co. Ltd., Minatouku, Japan); Macerozyme R–10 (Yakult Pharmaceutical Co. Ltd., Nishinomiya, Japan); Pectolayase and Driselase (Sigma Chemicals Co., U.K.). Enzymes at required concentrations (w/v) were dissolved in CPW (Cell and Protoplast Washing) solution (Power *et al.*, 1984) containing 11% (w/v) mannitol (CPW11M), unless otherwise mentioned. The enzyme solution was filter sterilised using Swinnex filter holders with Whatman membrane filters (pore size 0.2 μm, Cellulose nitrate).

Isolation of protoplasts

Leaves were surface sterilised in 7% (v/v) 'Domestos' (a commercial bleach containing 10.5% (v/v) Sodium hypochlorite; Unilever, U.K.) solution with one drop of Tween–20 added per 100 ml of solution. After 30 min leaves were washed 3–4 times in sterile distilled water. The mid–rib of the leaf was removed and fine cuts (< 1 mm) were made in the leaf lamina. Finely cut leaf tissue was plasmolysed in a Petri dish with the lower epidermis in

contact with the CPW11M for 1 h. After plasmolysis, the solution was replaced with filter–sterilised enzyme solution which also contained appropriate concentration of mannitol in CPW (pH = 5.8). Approximately 50–60 mg of leaf tissue was digested in 3 ml of enzyme mixture in a sealed, 60×15 mm size, Petri dish. Incubation of leaf tissue was carried out overnight at room temperature in the dark. The undigested leaf tissue was removed the following day and the crude suspension of protoplasts was filtered through a 39 μm nylon mesh sieve. The filtrate was transferred to screw–capped, sterile centrifuge tubes and diluted with CPW11M solution. Protoplasts were pelleted by spinning at 100×g for 5 min in a swing–rotor centrifuge. Then the pellet was re-suspended in CPW containing 21% (w/v) sucrose and 5% (w/v) Ficoll (Ficoll 70, Pharmacia, Sweden) and was spun at 100×g for 5 min to recover intact protoplasts from debris. Finally, protoplasts were re-suspended in a known volume of culture medium for quantification and/or culture.

Effect of enzyme composition

Protoplast yield and viability were compared in various enzyme mixtures as shown in Table 1, containing different types and concentrations of enzymes dissolved in CPW11M.

Effect of osmolarity

The enzyme mixture containing 1% (w/v) Cellulase and 0.05% (w/v) Pectolyase was prepared in a series of mannitol concentrations ranging from 5–13% (w/v) (5, 7, 9, 11 and 13%). Protoplast yield and viability at various osmoticum were compared.

Effect of leaf age

Preliminary studies showed that the maturity stage of the leaf is crucial to obtain high yield of quality protoplasts. Therefore, the leaves of 1st to 4th positions from the apex of the shoot were used to assess whether there is any effect of the leaf position on protoplast yield and viability. The same enzyme mixture used in the previous experiment was used with CPW11M.

Quantitative estimation of protoplast

Protoplast counts were made using a double chamber heamocytometer (modified Fuchs Rosenthal, Weber Scientific Ltd., England). Counts were made in five squares of each chamber per sample. Five such samples were counted for every treatment in a given replicate. Each experiment consisted of 3 replicates and experiment was repeated twice. Data were presented as means ± standard error (S.E.). For quantitative estimation of viability, protoplasts were stained with FDA (Fluorescein diacetate) according to Widholm (1972). The confirmation of removal and reformation of cell wall was done by using 0.02% (w/v) Tinapol solution (Power *et al.*, 1984) . The samples were observed under the U.V. microscope (Olympus) equipped with a mercury vapour lamp, excitation filter BG12 and suppression filter K510.

Protoplast culture

After washing the protoplast suspension, the pellet was collected and re-suspended in a known volume of culture medium. The number of protoplasts per ml was adjusted according to Power *et al.* (1984). Protoplasts were cultured at a density of 4×105 protoplasts ml^{-1} of medium consisting of MS (Murashige and Skoog) basal medium supplemented with 0.2 mg l^{-1} NAA (∝-napthaleneacetic acid), 1 mg l^{-1} 2,4–D (2,4-dichlorophenoxyacetic acid), 0.2 mg l^{-1} Zeatin, 3% (w/v) sucrose, 8% (w/v) mannitol (pH=5.8). Culture medium was solidified with 0.6% (w/v) agarose (Type VII, low gelling T^{0}, Sigma, USA). Protoplasts were cultured as thin layers in 50×8 mm sterile plastic Petri dishes with tight lids (Falcon 1006, Becton Dickinson Labware, New Jersey). Cultures were incubated in the dark at 25±2°C.

RESULTS AND DISCUSSION

Effect of enzyme composition

All the tested enzyme mixtures gave a yield of protoplasts in the range of 1.0–4.6×10^{6} g^{-1} fresh wt. of tissue with viability ranging from 51–79% (Table 1). The enzyme mixture containing 1% Cellulase and 0.05% Pectolyase (EN2) was the best mixture in terms of both yield (4.14×10^{6} g^{-1} fresh wt.) and viability (79%) of protoplasts. Although addition of Driselase at 1% to the same enzyme mixture (EN5) increased yield (4.62×10^{6} g^{-1} fresh wt.), viability (51%) was significantly reduced. The reduction of viability as

a result of addition of Driselase may partly be due to the presence of toxic or other unsuitable compounds in Driselase. Increase in the total concentration of enzyme (cellulase) in the isolation mixture by the addition of 1% Driselase may also be attributable to the reduction in protoplast viability. This higher concentration of enzyme may not be suitable for the viability of protoplasts if incubated for as long as 16 h. Mills and Hammerschlag (1994) found that incorporation of Driselase caused a reduction in viability in peach protoplasts from 89% (without Driselase) to 51% at 0.5% concentration with 15–16 h incubation.

Table 1. Effect of digestion enzyme mixture on protoplast yield and viability.

Composition of enzyme [% (w/v)]	Code	Protoplast yield ($\times 10^6$ g^{-1} fresh wt.) Mean ± S.E.	Viability (%)
Cell. (2) Pec. (0.5)	EN1	1.06 ± 0.12	75.2
Cell. (1) Pec. (0.05)	EN2	4.14 ± 0.20	79.0
Cell. (1) Mac. (0.05)	EN3	2.42 ± 0.18	72.5
Cell. (1) Mac. (0.1) Pec. (0.05)	EN4	3.05 ± 0.15	67.4
Cell. (1) Dri. (1) Pec. (0.05)	EN5	4.62 ± 0.16	51.0

Cell. - Cellulase R–10 Pec. - Pectolyase
Mac. - Macerozyme R–10 Dri. - Driselase
Data presented as mean ± standard error. Mean values of 2 experiments, each with 3 replicates for each treatment.

Protoplast yield seemed to decrease as the concentration of total enzyme in the mixture increased. The lowest yield (1.06×10^6 g^{-1} fresh wt.) was recorded with the 2% Cellulase and 0.5% Pectolyase (EN1), which was the most concentrated enzyme mixture. The reduction in protoplast yield in tissues incubated in mixtures with higher enzyme concentration may be due to inappropriate incubation time. The incubation time in the enzyme mixture is also important in determining optimum yield of viable protoplasts (Smith

and McCown, 1983; Russell and McCown, 1986). Therefore, it would be interesting to examine the influence of both the incubation time and the concentration of enzyme mixture in relation to protoplast yield and viability.

When 1% Cellulase and 0.05% Pectolyase (EN2) was used, the yield of protoplasts was 4.14×10^6 g^{-1} fresh wt. However, when Pectolyase was replaced with the same concentration of Macerozyme (EN3) the protoplast yield decreased to 2.42×10^6, which was about a 50% reduction (Table 1). This shows that, as a pectinase source, Macerozyme may not be suitable compared to Pectolyase. The properties of commercial preparations vary depending on their origin and may, therefore, differ in the degree of cell wall digesting activities. Macerozyme is derived from *Rhizopus spp.* whereas *Aspergillus japonicus* is the source for Pectolyase. Grezes *et al.* (1994) found that the use of Cellulase from *Aspergillus niger* instead of Cellulase R10, which is from *Trichoderma viride*, decreased the protoplast yield of *Coffea arabica* cell suspensions by a factor of ten. Thus, the present results are in agreement with those of Grezes *et al.* (1994) suggesting that the yield of protoplasts may be dependent on the type or the origin of the enzyme source.

Effect of osmolarity

The highest yield and viability of isolated protoplasts was found when 11% (w/v) mannitol was used. Though there was a variation in yield with different mannitol concentrations tested, viability did not show any great variation and was in the range of 74–77% (Figure 1). Protoplast yield was optimum in 11% (w/v) mannitol and decreased when lower or higher concentrations were used. The degree of plasmolysis in cells of tissues exposed to 11% mannitol may be optimum. At lower or higher concentrations a large proportion of the cells may not reach the appropriate level of plasmolysis and are thus damaged during protoplast isolation.

Effect of leaf stage

No notable difference in protoplast viability was shown among the leaves from different positions and this was in the range of 73–76% (Figure 2). However, protoplast yield increased with maturity up to the 2nd leaf from the apex and then decreased in the leaves below. However, leaves taken from 2nd and 3rd leaf positions gave the best yield. On the other hand, a low yield was recorded for leaves from both the 1st and 4th leaf positions, the latter giving the lowest yield among the leaf positions tested (Figure 2). The

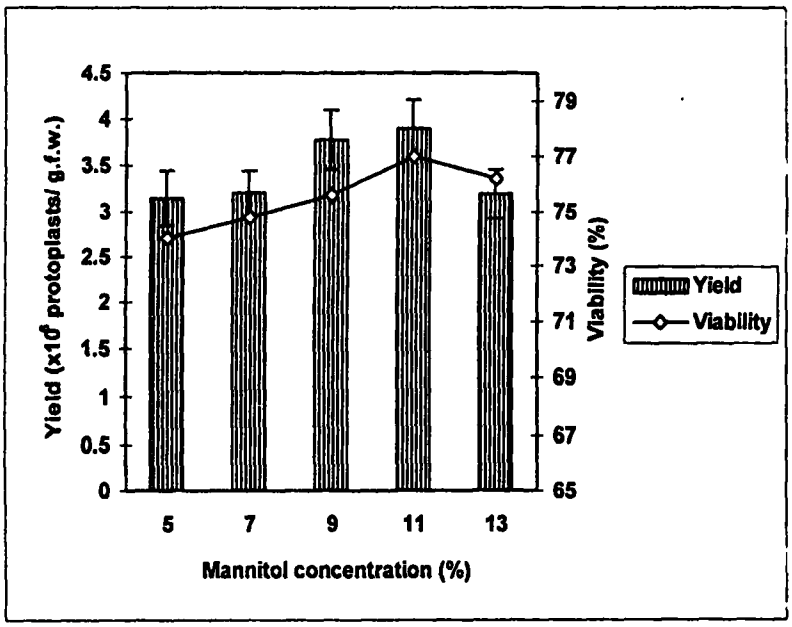

Figure 1. Effect of different osmoticum (mannitol levels) on protoplast yield and viability.
[Note: Data represent mean values of two experiments with 3 replicates. In each replicate, 5 sample counts were made. Bar indicates S.E. of the mean]

composition of the cell wall matrix varies considerably in different stages of wall deposition (Brett and Waldron, 1996). Younger leaves, which show a fast growth rate, may contain cells with a relatively thin cell wall. These cells could be damaged easily during the digestion and may cause a change of pH in the isolation solution thus reducing the effectiveness of the digestion process. Hence, the effectiveness of the enzyme digestion may be dependent upon both the isolation conditions and cell wall composition.

Quite often young leaves have been found more satisfactory than mature leaves for high protoplast yield (Revilla *et al.*, 1987; Mills and Hammerschlag, 1994). However, in the present study, the youngest leaf (1st leaf) gave a lower yield than the 2nd and 3rd leaf. This may be partly due to unsuitable isolation conditions (condition of the isolation mixture and or incubation time) with the cells of tissues at a very young stage. Therefore, for optimum yields different isolation conditions may be required for tissues having different degrees of maturity.

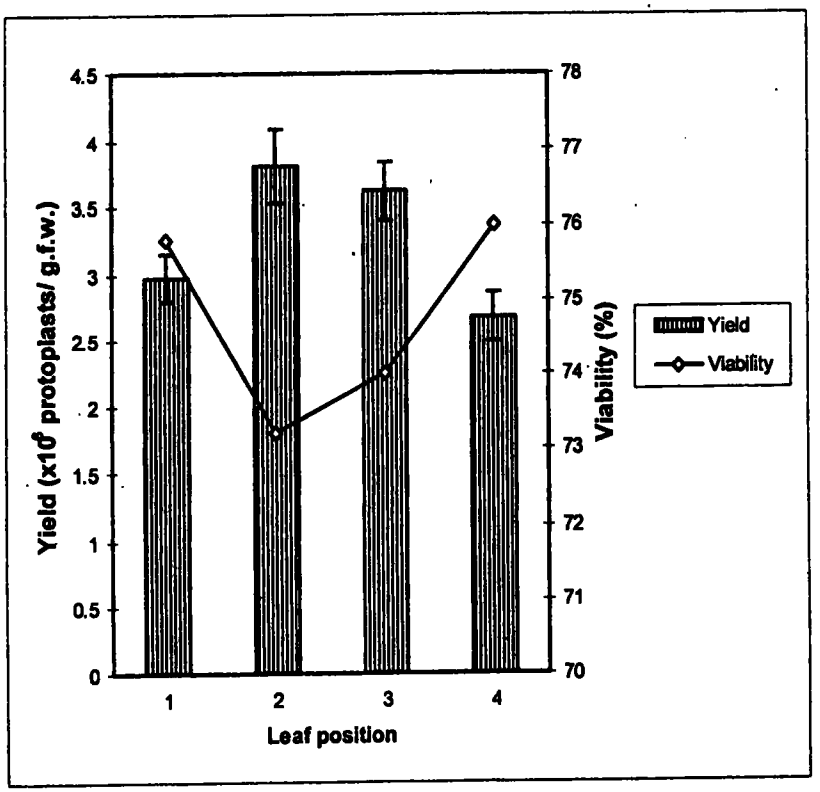

Figure 2. **Effect of leaf maturity on protoplast yield and viability.**
[Note: X – axis represents the position of the leaf on the shoot from the apex. Data are based on mean values of 2 experiments, each consisting of 3 replicates. In each replicate 5 sample counts were made. Bar indicates S.E. of the mean.]

Photomicrographs of freshly isolated protoplasts and protoplasts stained with FDA to assess the viability, are shown in Figure 3.

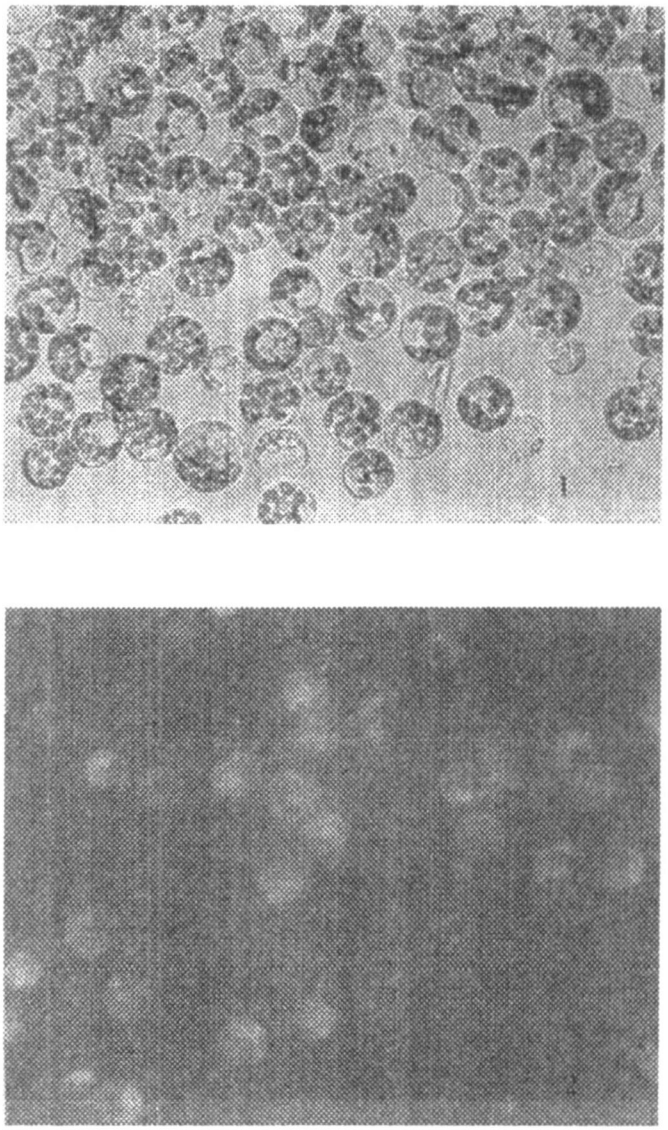

Figure 3. **(1) Freshly isolated protoplasts from leaf tissue of tea (× 410)**
(2) Freshly isolated protoplasts stained with FDA (× 410).

Protoplast culture

After 2– 3 days culture, re-synthesis of cell wall started and protoplasts changed in shape. Although protoplasts remained alive for 25–30 days in culture, cell division did not occur. The culture conditions used in this study (in terms of growth regulator composition, osmoticum, protoplast plating density and/or method of culture) may not be optimum for sustained growth of cultured protoplasts.

CONCLUSIONS

Among the parameters tested, the viability of the protoplasts isolated from leaf tissues of tea was affected only by the composition of the enzyme mixture *i.e.* both the concentration and the type of enzymes used. However, protoplast viability was not affected by the osmoticum of the isolation mixture or by the maturity of the leaf. On the other hand, all these factors, *viz.* composition of the enzyme mixture, osmoticum of the isolation mixture and the leaf age, affected the yield of protoplasts. The osmoticum, composition of enzyme digestion mixture and the maturity of the leaf required for optimum protoplast yield and viability are 11% (w/v) mannitol, 1% Cellulase + 0.05% Pectolyase and, tissues obtained from 2nd leaf from the apex, respectively. Under these conditions, a yield of $3–4\times10^6$ protoplasts per g fresh weight of leaf tissue can be obtained with the viability ranging from 73–79%.

Although sustained division of protoplasts was not observed under the present culture conditions, competence of such protoplasts is indicated by long–term survivability in culture. However, as a first step towards the application of protoplast based genetic manipulation techniques for tea, an isolation procedure that can release adequate yields of viable protoplasts were established in the present study. Further experiments are in progress to improve culture conditions necessary for continued growth of protoplasts to form proto–colonies and to regenerate plants.

ACKNOWLEDGEMENTS

The financial support received from the Tea Research Institute of Sri Lanka to carry out this work at the University of Southampton, U.K., is gratefully acknowledged.

REFERENCES

Brett, C. and Waldron, K. (1996). Physiology and Biochemistry of Plant Cell Walls. 2nd Edition. Chapman and Hall, London.

Grezes, J., Thomas, D. and Thomasset, B. (1994). Factors influencing protoplast isolation from *Coffea arabica* cells. Plant Cell, Tiss. Org. Cult. 36: 91-97.

Jenes, B., Puolimatta, M., Bittencourt, P. and Pulli, S. (1994). Time–saving method for protoplast isolation, transformation and transient gene expression assay in Barley. Agric. Sci. in Finland. 3: 199-205.

Krasnyanski, S., Polgar, Z., Nemeth, G. and Menczel, L. (1992). Plant regeneration from callus and protoplast cultures of *Helianthus giganteus* L.. Plant Cell Rep. 11: 7-10.

Mills, D. and Hammerschlag, F.A. (1994). Isolation of cells and protoplasts from leaves of *in vitro* propagated peach plants. Plant Cell, Tiss. Org. Cult. 36: 99-105.

Ochatt, S.J. (1992). The development of protoplast–to–tree system for *Prunus cerasifera* and *P. spinosa*. Plant Sci. 81: 253-259.

Park, Y.G. and Son, S.H. (1992). *In vitro* shoot regeneration from leaf mesophyll protoplasts of hybrid poplar (*Populus nigra* × *P. maximowiczii*). Plant Cell Rep. 11: 2-6.

Power, J.B., Chapman, J.V. and Wilson, D. (1984). Laboratory Manual: Plant Tissue Culture. Plant Genetic Manipulation Group, Dept. of Botany, University of Nottingham.

Revilla, M.A., Ochatt, S.J., Doughty, S. and Power, J.B. (1987). A general strategy for the isolation of mesophyll protoplasts from deciduous fruit and nut tree species. Plant Sci. 50: 133-137.

Russell, J.A. and McCown, B.H. (1986). Culture and regeneration of *Populus* leaf protoplasts isolated from non–seedling tissue. Plant Sci. 46: 133-142.

Smith, M.A.L. and McCown, B.H. (1983). A comparison of source tissue for protoplast isolation from three woody plant species. Plant Sci. Lett. 28: 149-156.

Widholm, J.M. (1972). The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol. 47: 189-194.