Indian J. Genet., **63**(3): 243-246 (2003)

Protoplast isolation, culture and plant regeneration in Butterflypea [*Clitoria ternatea* (Linn.)]

Ravindra B. Malabadi

Division of Plant Tissue Culture, Department of Botany, Karnatak University, Dharwad 580 003

(Received: January 2003; Revised: July 2003; Accepted: August 2003)

Abstract

This study describes successful plant regeneration using protoplasts isolated from mesophyll cells of *Clitoria ternatea* (Linn.). Maximum protoplast yield (7.34×10^6 g/fresh wt of leaf tissue), and viability (86.3%) was recorded with an enzyme mixture containing 1.0% (w/v) Cellulase, 1.5% (w/v) Macerozyme and 0.3% (w/v) Pectolyase. An optimum density of 2.5×10^3 protoplasts/ml was required for the highest frequency (71.25 ± 6.85%) of division. Dividing protoplasts produced microcalli that proliferated on MS medium containing 9.29 pM Kinetin and produced shoot buds when MS medium incorporated with 100 mg 1-1 adenine sulphate. Elongated shoots were rooted on half strength MS basal medium without growth regulators. The well rooted shoots were transferred and acclimatized.

Key words : *Clitoria*, Plant regeneration, Protoplasts

Introduction

Plant protoplasts are the important ideal tools for genetic manipulations such as gene transfer, mutation breeding and somatic hybridization. Protoplast culture when coupled with an efficient protocol for gene delivery and plantlet regeneration serves as an excellent system for the recovery of transgenic plants [1. 2]. There are numerous reports on plant regeneration from protoplasts of several species [3, 4] including grain legumes [5, 6, 7] Plant regeneration through protoplast culture was reported in 320 higher plant species representing 146 genera and 49 plant families [8].

Clitoria ternatea (Linn.) (Family - Leguminosae) cv. white flowered variety commonly known as "Butterfly pea" is an important medicinal and a forage plant and also a good soil binder because of its twining stem and rhizomatous roots. The plant is considered to be a good brain tonic and is useful in throat and eye infections, skin diseases, urinary troubles, ulcer, antidotal and in improving memory and intelligence. The root has a sharp better taste, cooling, acrid, laxative, diuretic, cathartic and anthelmintic. Roots are emetic, used by the tribals to cause abortion and root paste is applied on the stomach of cattle for urinary and abdominal swellings. A phenol glycoside 3, 5, 7, 4 - tetra - hydroxyflavone - 3 rhamnoglycoside, an alkaloid called as Clitorin (MP-235°C) is extracted from roots [9]. Therefore, in the light of above mentioned important medicinal properties, an attempt has been made to regenerate plants via protoplasts. Till today nobody has reported protoplast derived plant regeneration in *Clitoria ternatea* (Linn.) This is the first successful report of plant regeneration using protoplasts in *Clitoria ternatea* (Linn.). Direct gene transfer via protoplasts will also be a future option for the introgession of useful genes particularly coding for key enzymes in alkaloid biosynthesis. This protocol is very simple, reproducible and may be suitable for producing transgenic plants.

Materials and methods

Seeds of white flowered variety of *Clitoria ternatea* (Linn.) were collected from the ripe pods of plants growing in Botanical Garden of Karnatak University, Dharwad. Seeds were surface sterilized with 70% ethanol for 3 min and 0.1% $HgCl_2$ for 2 min, rinsed three times with sterile double distilled water and germinated aseptically on MS basal medium [10] with $2gl^{-1}$ sucrose and 0.7% agar in 145 mm × 25 mm glass culture tubes containing 15 ml of the medium under cool white fluorescent light (100 µmol, m^{-2} s^{-1}) at 25 ± 2° C with a relative humidity of 55-60%.

For protoplast isolation, young leaves (1-2 cm) were harvested from 15 days old aseptically grown seedlings (1 g fresh wt) and chopped into small pieces (1 mm). The tissues were incubated in a 20 ml filter sterilized (Millipore, 0.45 µm pore size) protoplast isolation medium (PIM) (PIM - half strength MS minor salts, MS iron-EDTA, MS-vitamines, 5 mM $CaCl_2$. $2H_2O$, 5mM MES, 0.6 M mannitol, 0.3 M sucrose) containing 1 % (w/v) Cellulase Onozuka R-10 (Yakult Pharma Co. Ltd Japan), 1.5% (w/v) Macerozyme R-10 (Yakult Pharma Co. Ltd Japan), and 0.3% (w/v) Pectolyase Y-23 (Seishin Pharma Co. Ltd Japan). Protoplast isolation medium (PIM) was incorporated with different enzyme concentrations (A, B, C, D, E) (Table 1) for optimum protoplast yield. The mixture in 100 ml

Erlenmeyer flasks was incubated with constant agitation of 50 rpm, in the dark for 15 hrs at 27±2°C to liberate protoplasts. The pH of the protoplast isolation medium (PIM) was adjusted to 5.8 before filter sterilization. After incubation for 15 hrs, the tissues were separated into small cell clumps with a pipette. The enzyme-protoplast mixture was then gently passed through nylon sieves (60 and 30 µm pore size successively) under aseptic conditions to remove large debris and undigested cells. The filtered protoplasts were collected by centrifugation at 100 × g for 5 min, and the supernatant was discarded. The pellet was resuspended in protoplast - isolation - medium (PIM) (4 ml) and centrifuged at 100 × g for 5 min. This step was repeated four times to remove all the enzyme. The washed pellet was resuspended in 2 ml PIM, layered on the top of approximately 3 ml of sacrose pad (25% sucrose) and centrifuged for 5 min at 100 × g to collect the purified protoplasts. Finally, the isolated protoplasts were collected with a Pasteur pipette and again suspended in PIM (10-15 ml) [10]. The protoplast yield was determined by counting the number of protoplasts per milliliter using a haemocytometer and the culture density of protoplasts was adjusted before plating. The viability of protoplasts was assessed using the fluorescein-di-acetate (FDA) staining method [11], and observed under a fluorescent microscope. Cell - wall digestion and resynthesis was confirmed by staining with 0.01% Calcofluor white ST dye [12].

The protoplasts were further subcultured in a liquid protoplast culture medium (PCM) (PCM - half strength MS - minor salts, MS - iron EDTA, MS - vitamines, 0.1 M sucrose, 0.2 M mannitol, 5 mM $CaCl_2$ $2H_2O$, 4.54 µM 2, 4-D, 9.29 µM kinetin (KN) in 50 ml Erlenmeyer flasks (Each containing 10 ml of protoplast culture medium (PCM). All the cultures were initially kept in the dark at 25±2°C with gentle shaking (50 rpm) on a rotatory shaker incubated for 6 hrs / day and then transferred to static condition in the culture room at the same temperature in the darkness. Cultures were also observed for changes in the protoplasts with respect to shape, size and division. The division frequency was calculated after a week as the percentage of dividing protoplasts.

After two weeks, 200 µl of the suspension containing protoplast derived microcalli was plated on a thin layer of Shoot Induction Medium (SIM) (13) which is MS basal medium supplemented with $7gl^{-1}$ agar, $20gl^{-1}$ sucrose, 9.29 µM kinetin and 100 mgl^{-1} adenine sulphate (AS) in a glass culture tubes and kept in dark at 25±2°C. After 2 weeks, the callus was again subcultured on the same medium for further proliferation, and cultures were maintained for 2 weeks on 16 hour photoperiod under cool fluorescent light 100 µ mol, m^{-2} s^{-1} with a relative humidity of 55-60%. Shoots regenerated from callus were rooted on half strength MS basal medium without growth regulators. The well rooted shoots were transferred and acclimatized. Data presented in the tables were analyzed statistically by using ANOVA .

Results and discussion

The enzyme solution containing a combination of 1% (w/v) Cellulase, 1.5% (w/v) Macerozyme and 0.3% (w/v) Pectolyase Y-23 was effective for digesting cell-walls and releasing mesophyll protoplasts. Release of protoplasts started within 5 hrs of enzymatic digestion, but 15 hrs incubation at 27±2°C in the dark was necessary for the complete digestion of cell-walls. The spherical shape of the released protoplasts and the absence of fluorescence after staining with calcofluor white ST dye confirmed complete cell-wall digestion. Isolated protoplasts were densely cytoplasmic showing small starch grains. Two different types of protoplasts small (about 25-30 µm) and slightly large (35-50 µm) were observed (Fig. 1A,B). Freshly isolated protoplasts were spherical in shape but slowly turned oval during the successive period of incubation followed by culture. Fluorescein diacetate staining revealed that 90% of the isolated protoplasts were presumed viable as indicated by yellow green fluorescence. Protoplasts were cultured initially at a density of 10^5 protoplasts / ml. Protoplast isolation medium (PIM) with different combinations of enzyme concentrations (Table 1) have profound effect on the yield, viability and division frequency of isolated

Table 1. Influence of enzyme concentration on yield, viability and division frequency of protoplasts (data's represents mean ± SE of three independent experiments)

Code	Enzyme Solution Concentration (% (w/v))			Yield/g[a] leaf segment (X 10^6)	Viability[b] (%)	Division[C] frequency (%) after 3 weeks
	Cellulase	Macerozyme	Pectolyase			
A	1.0	1.5	0.3	7.34 ± 0.32	86.30 ± 3.80	69.82 ± 5.96
B	1.2	1.7	0.4	4.85 ± 0.36	64.42 ± 1.50	71.25 ± 6.85
C	1.5	1.8	0.5	3.31 ± 0.40	65.92 ± 1.85	65.32 ± 4.86
D	1.5	2.0	0.5	1.68 ± 1.06	64.81 ± 1.08	66.34 ± 5.60
E	1.5	2.3	0.5	1.25 ± 1.02	59.31 ± 2.06	60.28 ± 5.92

$F_{a,b,c}$ value is significant at 1 % level.

protoplasts. The highest protoplast yield (7.34×10^6 g / fresh wt of leaf tissue), viability (86.3%) and 69.82% of division frequency recorded with enzyme solution (A) (Table 1) was noticed when protoplasts isolated with enzyme solution (A) (Table 1; Fig. 1C). On the other hand lowest protoplast yield (1.25×10^6/g fresh wt of leaf tissue), viability (59.31%) and division frequency (60.28%) was noticed with enzyme solution - E (Table 1). Our study revealed that, protoplast yield, viability and division frequency goes on decreasing with increase in the concentration of macerozyne and pectolyase. Therefore, protoplasts isolated with enzyme solution (A) was found to be optimum concentration of enzyme mixture in case of *Clitoria ternatea* (Linn.).

Dhir *et al.* [14] reported maximum protoplast yield from young petioles in sweet potato (*Ipomoea batatas* (L.) with an enzyme mixture of 1% (w/v) cellulose, 1.5% (w/v) macerozyme and 0.3% (w/v) pectolyase which also confirmed our present findings. In case of mango (*Mangifera indica* L.), the protoplast yield was drastically decreased with higher concentration of hemicellulases and pectinases [15].

Isolated protoplasts were subcultured in protoplast culture medium (PIM) for further growth and development. Protoplast derived cells were actively dividing within 2 weeks and developed microcalli, which were visible to the naked eye as shining glossy minute granules in the liquid medium (Fig. 1D). The growth of protoplasts in terms of division was greatly influenced by osmoticum and the presence of growth regulators such as 2, 4-D as well as kinetin. The lower concentration of mannitol from 0.6 to 0.2 M and sucrose from 0.3 to 0.1 M influenced cell-division processes in case of *Clitoria ternatea*. Within 2 weeks of plating on protoplast culture medium (PCM), solidified with 7 gl^{-1} agar and 20 gl^{-1}. Sucrose, the size of the microcalli increased to approximately 1-2 mm. Their proliferation was greatly influenced by the presence of growth regulators such as 9.29 μM kinetin in the PCM. The callus was initially watery and developed perfect shape and size within two weeks (Fig. 1E). The incorporation of 100 mg/l^{-1} adenine sulphate in the PCM resulted in the formation of small green bud like structures (Fig. 1E). Adenine in the form of adenine sulphate can stimulate cell-growth and greatly enhance shoot formation (Fig. 1F). It provides an available source of organic nitrogen to the cell and can generally be taken up more rapidly than inorganic nitrogen. Further development of shoot buds was noticed only when buds were separated from clumps and cultured on the same fresh medium (PCM). Shoots were rooted on half-strength PCM without growth regulators. The plants derived from protoplasts survived the transfer to soil and showed continued growth.

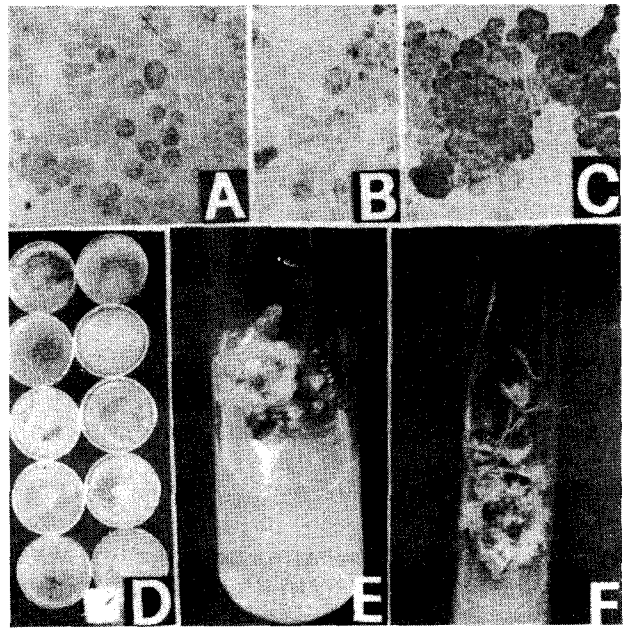

Fig. 1. Regeneration of plant by using protoplasts isolated from mesophyll cells. A, B - Freshly isolated protoplasts. C - Successive cell division given rise to colony formation. D - Visible protoplast derived microcalli appearing 2 to 3 weeks after culture initiation. E - Growth of protoplasts derived calli showing initiation of green shoot buds. F - Luxurient growth of green healthy shoots.

Maximum protoplast yield of 7.34×10^6 g/fresh wt of leaf tissue was observed with mesophyll cells. When cultured at plating density of 6×10^3 to 10×10^3 ml, protoplasts failed to regenerate cell walls and division was never noted. A plating density as low as 4×10^3 ml was necessary to obtain a few divisions (Table 2). The plating density of 2.5×10^3 ml was found to be optimum and was used routinely. The plating density higher than 2.5×10^3 ml reduced the cell divisions and very few protoplasts developed cell wall. On the other hand lower plating density below 2.5×10^3 resulted in the higher protoplast division. In the present study the plating density of 2.5×10^3 ml yielded higher percentage (82%) of shoot regeneration as compared to other plating densities (Table 2).

Table 2. The effect of protoplast plating density on division frequency and shoot regeneration (data represents mean ± SE of three independent experiments)

Protoplast plating density protoplast $(ml)^{-1}$	Cell division frequency (%)	Shoot regeneration (%)
10×10^3	0	0
6×10^3	0	0
4×10^3	22.00 ± 1.87	05 ± 0.84
2.5×10^3	71.25 ± 6.85	82 ± 7.56
1.25×10^3	54.40 ± 3.97	64 ± 2.90

In conclusion, the present study deals with successful isolation and regeneration of plants using protoplasts. Protoplast derived plants exhibit normal development and 100% survival. This protocol is very simple and reproducible. Exploitation of this technology will permit this important forage and medicinal legume to be incorporated into genetic improvement programmes involving somatic hybridization and transformation by direct DNA uptake which rely upon the use of isolated totipotent protoplasts.

Acknowledgement

I am grateful to the Head, Department of Botany, Karnatak University, Dharwad, India for providing the necessary facilities for this work.

References

1. **Dhir S. K., Dhir Savka M. A., Belanger F., Kriz A. L., Farrand S. K. and Widholm J. M.** 1992. Regeneration of transgenic soyabean (*Glycine max*) plants from electroporated protoplasts. Plant Physiol., **99**: 81-88.
2. **Shimamoto K., Terada R., Izawa T. and Fujimoto H.** 1989. Fertile transgenic rice plants regenerated from transformed protoplasts. Nature, **338**: 274-276.
3. **Binding H.** 1986. Regeneration from protoplasts. *In*: Vasil I.K. (ed). Plant regeneration and genetic variability. Cell culture and somatic cell genetics of plants, Vol. 3, Academic Press, New York, PP 259-274.
4. **Puite K. J.** 1992. Progress in plant protoplasts research. Physiol Plant, **85**: 403-410.
5. **Sawhney R. K., Shekhawat N. S., Gaiston A. W. and Kaur S. R.** 1985. Polyamine levels as related to growth, differentiation and senescence in protoplast derived cultures of *Vigna aconitifolia* and *Avena sativa*. Plant Growth Regulation, **3**: 329-337.
6. **Tian D. and Rose R. J.** 1999. Asymmetric somatic hybridization between the annual legumes *Medicago Truncatula* and *Medicago scutellata*. Plant Cell Rep., **18**: 989-996.
7. **Tegeder M., Kohn H., Nibbe M., Schieder O. and Pickardt T.** 1996. Plant regeneration from protoplasts of *Vicia narbonensis* via somatic embryogenesis and shoot organogenesis. Plant Cell Rep., **16**: 22-25.
8. **Rosest S. and Gillissel L. J. W.** 1989. Plant regeneration from plant protoplasts literature review. Acta Bot. Neerl., **38**: 1-23.
9. **Kulkarni C., Paftasheffi J. R. and Amruthraj G.** 1988. Effect of alcoholic extract of *Clitoria ternatea* on central nervous system in rodents. Indian J. Exp Biol., **26**: 957-960.
10. **Murashige T. and Skoog F.** 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant, **15**: 473-497.
11. **Widholm J. M.** 1972. The use of fluoresein-di-acetate and phenosafranine for determining viability of cultured plant cells. Stain Technol., **47**: 189-194.
12. **Nagata T. and Takebe I.** 1970. Cell-wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta, **92**: 301-308.
13. **Malabadi R. B. and Nataraja K.** 2001. Shoot regeneration in leaf explants of *Clitoria ternatea* (Linn.) cultured *in vitro*. Phytomorphology, **51**: 169-171.
14. **Dhir S. K., Dhir S. and Widholm J. M.** 1991. Plantlet regeneration from immature cotyledon protoplasts of soyabean (*Glycine max* (L.)). Plant Cell Rep., **10**: 39-43.
15. **Ara H., Jaiswal U. and Jaiswal V. S.** 2000. Plant regeneration from protoplasts of mango (*Mangifera indica* L.) through somatic embryogenesis. Plant Cell Rep., **19**: 622-627.