International Journal of Advance Research in Science and Engineering
Volume No.07, Special Issue No.04, March 2018
www.ijarse.com
IJARSE
ISSN: 2319-8354

Effect of various factors in protoplast isolation from temperate mulberry

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ABSTRACT:
In the present study, effect of various factors was studied for the isolation of protoplasts from the mesophyll cells of four superior temperate mulberry varieties viz. Morus albaChinese white, Morus alba var. Ichinose. Morus multicaulis var. Goshoerami and an interspecific hybrid i.e. PPR-1 (Pampore-1). Among all the factors, concentration and combination of enzymes used, concentration of osmoticum and duration of enzymatic treatments were found to be major factors effecting the isolation of viable protoplasts in maximum number. Among the different combinations of enzyme solutions tested, the maximum yield of viable protoplasts ($5.171\pm0.354x10^6$ g-1 fresh weight and $6.051\pm0.424x10^6$g-1 fresh weight) was obtained from the enzymatic combination of 2% Cellulase, 0.5% Macerozyme and 0.2% Pectinase for incubating the leaf peices for 8 Hrs and 10 Hrs duration with 80 RPM (Rotations Per Minute) on rotary shaker at 26° C from Goshoerami& PPR-1 varieties respectively. Similarly among the different concentrations of osmoticum (Mannitol) tested, 13% of Mannitol (CPW-13M) has given the maximum viable protoplasts of 72%, 77%, 88%, & 92% in Ichinose, Chinese white, Goshoerami and PPR-1 respectively. Viability of isolated protoplasts was carried out by using 0.2% Evans Blue stain.

Keywords:*Cellulase, Mesophyll cells, Protoplasts, Temperate mulberry, Viability.*

1. Introduction

In plants producing full hybrids is not easy because of sexual incompatibility barriers between desired individuals. This has often proved to be a serious threat in crop improvement programs through conventional breeding programmes.Whereas in somatic hybridization, protoplasts of any two mulberry varieties can be fused irrespective of their species type and breeding season, from the fused protoplasts a somatic hybrid can be raised due to their totipotency nature.

Protoplasts are the naked plant cells from which cell wall has been removed, but the plasma membrane is intact. Both from the isolated protoplasts and from their fusion product, a somatic hybrid, can be regenerated into whole plantlets through the regeneration studies.In somatic hybridization due to fusion of protoplasts derived from different mulberry varieties, there is high possiblity of genomic c ombinationswhich can lead to the production of somatic hybrids with more desirable charcaters [**1**]. It allows us to combine entire genomes from the sexually incompatible parents and expected to result in hybrids which are superior in characters [**2, 3**].

In recent days, as much success was reported in isolation of protoplasts from different plant species by enzymatic method [**4-9**], we have also carried the enzymatic method and identified the effect of various factors for the isolation of protoplasts from the mesophyll cells of four superior temperate mulberry variet ies: *Morus alba* var. Ichinose; *Morus alba* var. Chinese White, *Morus multicaulis* var. Goshoeramiand an interspecific hybrid i.e. PPR-1 (Pampore-1).

So, through this research paper we are reporting about theeffect of various factors onprotoplast isolation from the superior temperate mulberry varieties by enzymatic method.

2. Materials & Methods

2.1 Plant Material

Fully expanded le avesfrom the *in vitro* shootlets of Goshoerami, Ichinose, Chinese white and PPR-1 were used in this research study.

2.2 Enzymes:

Cellulase Onozuka R-10, Macerozyme R-10 and Pectinase enzymes were procured from Himedia and used in this study.

2.3 Isolation of Protoplasts:

Fully expanded leaves of 40- 50 days old were excised from the *in vitro* shootlets of Ichinose, Chinese white, Goshoerami and PPR-1 mulberry varieties with a sterile scalpel blade in asceptic conditions under laminar air flow cabinet and they were cut into small pieces of 1-2 mm in size. Leaf pieces of about 1 gram fresh weight were incubated in sterile conical flask (100 ml capacity) with 15 ml of filter sterilized enzyme solution (2% cellulase, 0.5% macerozyme and 0.2% pectinase) which were prepared in CPW-13M solution with 5.6 pH for 6-12 hrs duration on rotary shaker at 80 RPM in dark conditions at 26°C (Table-1 &Table-2). During the incubation period, pectin material present in the intracellular spaces get degraded by macerozyme and pectinase enzymesand cellolusic material of cellwalls by cellulase enzyme leading to release of protoplasts from the mesophyll cells.

2.4 Purification of Isolated Protoplasts:

The isolated protoplasts were initially filtered by using steel mesh with a pore size of 45µ. The obtained filterate was collected in a 20 ml sterile srew cap centrifuge tube and centrifuge at 1000 RPM for 5 minutes. After centrifugation, supernatant was discarded .Impurities like cell debris were removed from the pellet of protoplasts by carrying out centrifugation at 600 rpm for 5 Minutes after the addition of 10 ml of 20% sucrose solution (CPW-20S) (Table-3). After centrifugation, protoplasts w ere obtained as a distinct band, which were were collected into a seperate sterile screw cap centrifu ge tube with the help of a pasteur pipette. The collected protoplasts were suspended in 5ml of CPW-13 M solution to maintain the m in viabile state.

2.5 Yield and Viability of Isolated protoplasts:

Initially to determine the yield of pr otoplasts, 10 µl of suspended protoplasts soultion was taken on a clean glass slide and observed under the compound light microscope in different maginfiable lenses and results were expressed as number of protoplasts per gram fresh weight of leaf. For checking the viability and yield of viable protoplasts, to the suspended protoplasts solution (1 ml),20 µl of 0.2 % Evans blue (20 mg/10 ml sterile distilled water)solution was added and incubated for 2 minutes. T hen 10µlof 0.2% Evans Blue stain treated protoplast solution were placed on a haemocytometer slide and observed under compound light microscope and theyield results were expressed as number of protoplasts per gram fresh weight of leaf. Viability results were expressed as percentage which is determined by the number of viable (unstained) protoplasts to that of non-viable (stained) protoplasts.

2.6 Statistical Analysis:

The data obtained from this research study was the mean of 3 replications and the data was statistically analyzed by using SPSS Version 17 (SPSS Inc., Chicago, USA). The mean values were compared by Tukey's tests at the 5% level of significance. All means are represented with standard error.

3. Results & Discussion:

In this research study, we have identified the effect of various factors in isolation of protoplasts from the mesophyll cells of four superior mulberry varieties *viz. Morus alba* var.Chinese white, *Morus alba* var. Ichinose. *Morus multicaulis* var. Goshoeramiand an interspecific hybrid *i.e.* PPR-1 (Pampore-1).Severalauthorshavereported about the isolation of protoplasts from the mesophyll cells of tropical and sub-tropical mulberry[**10-19**],but there is scanty information on the isolation of protoplasts from the temperate mulberry varieties. And to the best of our knowledge this is the first report on protoplast isolation from temperate mulberry varieties.

Initially when different combinations of enzyme solutions tested, the maximum yield of viable protoplasts ($5.171\pm0.354x10^6$ g-1 fresh weight and $6.051\pm0.424 x10^6$g-1 fresh weight) was obtained from the combination of 2% Cellulase, 0.5% Macerozyme and 0.2% Pectinase for incubating the leaf peices for 8 Hrs in

Goshoerami and for 10 Hrs in PPR-1 respectively (**Fig.1**). Other two varieties (i.e. Chinese White and Ichinose)also yield the protoplast with the same set of factors and conditions as PPR-1 but the count of protoplasts in these two varieties is not comparable as that of PPR-1 (Table-4). Similar to our findings ,other researchers [**12, 16 &19**] also used 2% cellulase in isolation of maximum number of protoplasts from mesophyll cells of different tropical and sub-tropical mulberry varieties.

As the intactness of plasmembrane and viability of isolated protoplasts depends on the appropriate concentration of osmoticum used, we have tested several concentrations of mannitol as osmoticum in our study. Among the different concentrations tested, 13% of Mannitol (CPW-13M) has given the maximum viable protoplasts of 72%, 77%, 88%, & 92% in Ichinose, Chinese white, Goshoerami and PPR-1 respectively. Viability of isolated protoplasts was ascertained by using 0.2% Evans Blue stain, after staining the viable protoplasts have excluded the stain and remained unstained, where as the non-viable or dead protoplasts has retained the stain and stained blue in colour (**Fig-2A& Fig-2D**).

Enzymatic incubation for more than 10 hrs has resulted in the increase of yield of protoplasts from all the varieties but the viability has reduced.Similar to our findings, more than9 hours of enzymatictreatment duration has decreased the viability of isolated protoplasts in three cultivars of mulberry *i.e.* K2, S13 and S36[**20**].

4. Figures and Tables

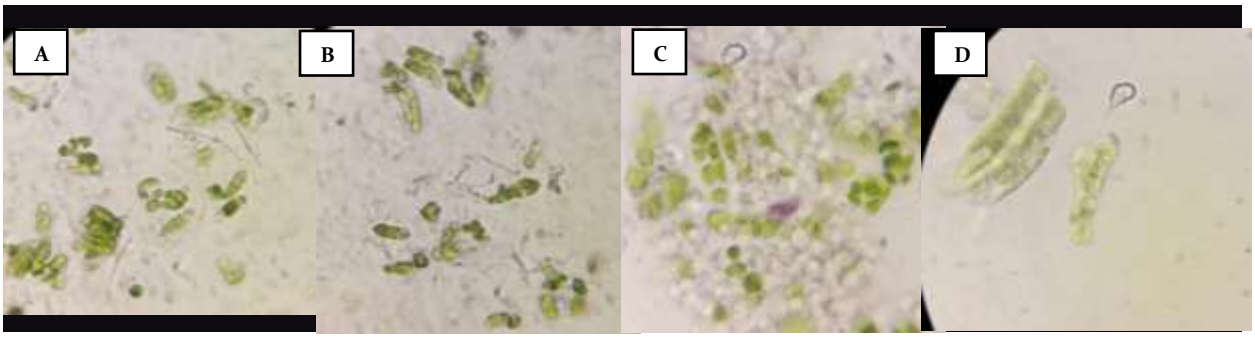

Fig.1:Microscopic views (under 40 x objective lenses) of isolated protoplasts from mesophyll cells of temperate mulberry varieties *viz.* A) PPR-1 B) Chinese White C) Ichinose D) Goshoerami by using enzymatic combination of 2% cellulase, 0.5% macerozyme and 0.2% pectinase

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www.ijarse.com
IJARSE
ISSN: 2319-8354

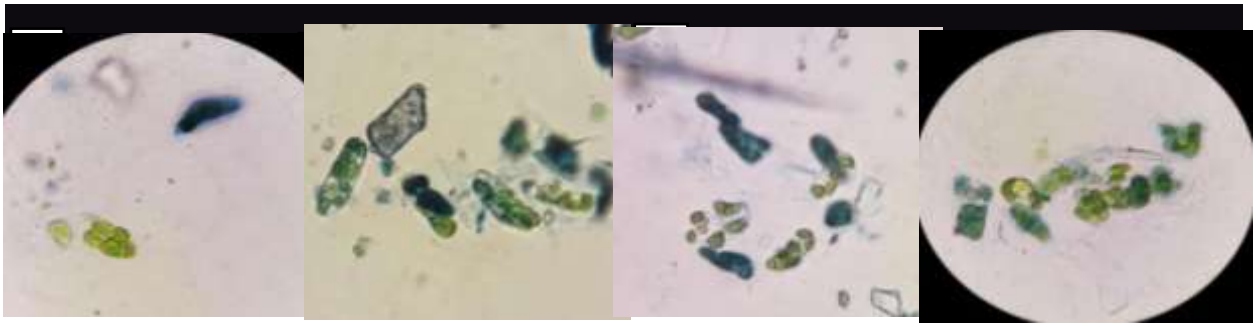

Fig.2:- Microscopic views of evaluation of viability of isolated protoplasts with evan's blue (0.2%) under 40 x objective lenses

A) Viable (unstained) and Non-viable (stained) protoplasts of PPR-1

B) Viable (unstained) and Non-viable (stained) protoplasts of Chinese White

C) Viable (unstained) and Non-viable (stained) protoplasts of Ichinose

D) Viable (unstained) and Non-viable (stained) protoplasts of Goshoerami

Table-1: Composition of CPW-13M solution

Sl.No	Chemical	Quantity (mg/L)
1	KH_2PO_4	28.00
2	KNO_3	108.00
3	NH_4NO_3	50.00
3	$CaCl_2.2H_2O$	1500.00
4	$MgSO_4.7H_2O$	240.00
5	KI	0.14
6	$CuSO_4.5H_2O$	0.025
7	D-Mannitol	130000
8	Sterile D.W	1000 ml
	pH	5.6

Table-2: Composition of Standardized Enzyme Solution

Sl.No	Enzyme	Quantity (Grams/100ml)
1	Cellulase Onozuka R-10	2.00
2	Macerozyme R-10	0.50
3	Pectinase	0.20
3	CPW-13M	100 ml
	pH	5.6

Table-3: Composition of CPW-20S solution

Sl.No	Chemical	Quantity (mg/L)
1	KH_2PO_4	28.00
2	KNO_3	108.00
3	NH_4NO_3	50.00
3	$CaCl_2.2H_2O$	1500.00
4	$MgSO_4.7H_2O$	240.00
5	KI	0.14
6	$CuSO_4.5H_2O$	0.025
7	Sucrose	200000 (200 Grams)
8	Sterile D.W	1000 ml
	pH	5.6

Table-4: Isolation of protoplasts from three superior mulberry varieties with 2% cellulase, 0.5% macerozyme and 0.2% pectinase

Sl.No	Mulberry Variety	D-Mannitol (%)	Incubation Time (Hrs)	Total Yield ofProtoplasts (× 10^6 g^{-1} Fresh Weight)	Viable yieldof Protoplasts (× 10^6 g^{-1} Fresh Weight)	Viability (%)
01	Ichinose	12	06	1.032±0.112[a]	0.612±0.024[a]	59.30±1.46[a]
			08	1.134±0.343[a]	0.704±0.041[a]	62.08±1.28[b]
			10	2.212±0.154[b]	1.230±0.212[b]	55.61±1.89[a]
		13	06	1.462±0.243[a]	0.744±0.054[a]	50.88±1.19[a]
			08	**2.546±0.312[b]**	**1.843±0.146[b]**	**72.39±2.11[c]**
			10	1.890±0.234[a]	1.045±0.117[b]	55.29±2.15[a]
		14	06	1.573±0.156[a]	1.004±0.222[b]	63.82±1.20[b]
			08	2.465±0.268[b]	1.720±0.240[b]	69.77±1.21[b]
			10	2.664±0.342[b]	1.584±0.326[b]	59.45±1.45[a]
02	Chinese White	12	06	1.824±0.282[a]	0.982±0.045[a]	53.94±1.38[a]
			08	1.877±0.214[a]	1.399±0.124[b]	74.57±1.44[c]
			10	1.645±0.188[a]	1.004±0.182[b]	61.03±1.65[b]
		13	06	1.254±0.213[a]	0.852±0.124[a]	67.94±1.73[b]
			08	**2.123±0.342[b]**	**1.644±0.321[b]**	**77.46±1.22[c]**
			10	2.415±0.270[b]	1.626±0.344[b]	67.32±1.92[b]
		14	06	1.845±0.208[a]	1.204±0.206[b]	65.25±1.52[b]
			08	2.812±0.324[b]	1.934±0.328[b]	68.77±1.42[b]
			10	2.923±0.423[b]	2.006±0.214[c]	68.62±1.79[b]
03	PPR-1	12	06	1.946±0.170[a]	1.420±0.204[b]	72.97±1.95[c]
			08	2.982±0.248[b]	2.224±0.362[c]	74.58±1.04[c]
			10	3.385±0.426[c]	2.795±0.188[c]	82.58±1.53[d]
		13	06	2.167±0.322[a]	1.662±0.145[b]	76.69±1.94[c]
			08	4.148±0.184[d]	3.024±0.266[d]	72.90±1.26[c]
			10	**6.574±0.508[e]**	**6.051±0.424[e]**	**92.04±1.04[d]**
		14	06	2.515±0.312[b]	1.845±0.226[b]	73.35±1.92[c]
			08	3.265±0.384[c]	2.544±0.214[c]	77.91±1.75[c]
			10	5.864±0.422[c]	4.811±0.326[d]	82.05±1.39[d]
04	Goshoerami	12	06	2.041±0.278[c]	1.645±0.332[b]	80.15±1.93[f]
			08	4.462±0.120[f]	3.775±0.409[d]	84.62±2.44[f]
			10	4.534±0.156[f]	2.741±0.201[c]	60.47±1.32[d]
		13	06	2.864±0.218[d]	2.381±0.123[c]	83.16±2.02[f]
			08	**5.867±0.237[g]**	**5.171±0.354[f]**	**88.14±1.38[f]**
			10	5.963±0.125[g]	3.246±0.312[d]	54.45±2.35[b]
		14	06	2.837±0.336[d]	2.188±0.261[c]	77.13±1.81[e]
			08	4.082±0.165[f]	3.257±0.108[d]	79.81±1.42[e]
			10	4.097±0.110[f]	1.926±0.021[b]	47.03±1.91[b]

Represented Data is the mean and standard error of three replication. Mean±standard errors followed by same letter is not significantly different at P=0.05 according to SPSS Version 17 (SPSS Inc., Chicago, USA) and means were compared using Tukey's tests at the 5% level of significance

Conclusion:

Overall by considering all the factors the combination of enzyme solution (2% Cellulase, 0.5% Macerozyme and 0.2% Pectinase), duration of enzyme treatment (8-10 Hrs) and osmoticum concentration (13% Mannitol) were found to be the major factors influencing the isolation of protoplasts in maximum number from the mesophyll cells of temperate mulberry varieties. The developed protocol with standardized parameters will be utilized in isolation of protoplasts from temperate mulberry, which can be used as raw materials for somatic hybridization studies and improvement of mulberry through genetic engineering approaches.

5. Acknowledgements

Authors thank Central Silk Board, Ministry of Textiles, Government of India and Director, Central Sericultural Research & Training Institute, Pampore (J&K), India for providing financial support under the project Code:3571. Authors also thankCORD, University of Kashmir for providing all the necessary facilities in carrying out this research work.

References

[1]P Narayana, S.P. Chakraborti, M.K. Raghunath, and K. Sengupta K, A new method to improve mulberry, Indian silk,1992, 19-20.

[2]G Melchers, Potatoes for combined somatic and sexual breeding methods; plants from protoplasts and fusion of protoplasts of potato and tomato. In: Production of natural compounds by cell culture methods. A. W. Alfermann and E. Reinhard, eds. Proc. of an Internat.Symp.on plant cell culture. Ges. f. Strahlen- und Umweltforschung, 1978, 306–311.

[3]G Melchers, and G. Labib, Somatic hybridisation of plants by fusion of protoplasts. I. Selection of light resistant hybrids of "haploid" light sensitive varieties of tobacco,Molec. gen. Genet, 1974, 135, 277–294.

[4]A Wallin, and M. Welander, Improved yield of apple leaf protoplasts from in vitro cultured shoots by using very young leaves and adding L-methionine to the shoot medium, Plant Cell Tissue Organ, 1985, 5(1), 69–72. doi: 10.1007/BF00033571.

[5]P Wang, W. Lv, X. Hu, J. Chen, andJ. Shi, Isolation and Cultivation of Protoplasts of Liriodendron tulipifera,Acta Bot Boreal-Occident Sin, 2013, 33(2), 254–60.

[6]F Jiang, J. Zhu, and H. Liu, Protoplasts: a useful research system for plant cell biology, especially dedifferentiation. Protoplasma, 250(6), 1231-1238.

[7]A Nicolia, E. Proux-Wéra, I. Åhman, N. Onkokesung, M. Andersson, E. Andreasson, and L. Zhu, Targeted gene mutation in tetraploid potato through transient TALEN expression in protoplasts. Journal of Biotechnology, 2015, 204, 17-24.

[8]Y Guohui, C. Qiang, X. Zheni, X. Bin, H. Bingru,and Z. Bingyu, An efcient protocol for perennial ryegrass mesophyll protoplast isolation and transformation, and its application on interaction study between LpNOL and LpNYC1, Plant Methods, 2017, 13, 46 DOI 10.1186/s13007-017-0196-0

[9]R Siti Aminah, D. Noer Hartini, A. Asmah, O. Mohamad, A.Maheran, and N. Mohd Roslan,Protoplast Isolation from *Hibiscus sabdariffa* L,Advanced Science Letters, 2017, 23(2), 1333-1336. DOI: https://doi.org/10.1166/asl.2017.8362

[10]PKTewary, HY.Mohan Ram, andNS RangaSwamy, Isolation and culture of mulberry (*Morus alba* L.) protoplasts from callus tissues, Indian Journal of Plant *Physiology*, 1995, 38,114-117 .

[11]PK Chand, Y. Sahoo, SK.Patnaik, and SNPatnaik,Evaluation of Procedures for Inter-Specific ProtoplastFusion in Mulberry, *Indian Journal of Sericulture*. 1996, 35, 46-49.

[12]AM Hassanein, and DM.Soltan, *S.nigrum*is a model systemic plant tissue and protoplast cultures, Bio Plant, 2000, 43, 501-509.

[13]U Pavan, AV. Rao, V. Yashodhara, N. Rama Swamy,and A. Sadanandam, A simple protocol for rapid and efficient isolation of protoplast from callus cultures of mulberry (*Morusindica*L.) cv. S13. Sericologia.2010[a], 40, 647–651.

[14]U Pavan U, AV. Rao, V. Yashodhara, N. Ramaswamy,and A. Sadanandam, Evaluation of specific parameters in the isolation of protoplasts from mesophyll cells of three mulberry cultivars, Sericologia, 2000[b], 40(3), 469-474.

[15]U Pavan, R.Venugopal, K. Kiranmayee, T. Jaya Sree,and A. Sadanandam A, Plant regeneration of mulberry (*Morus indica*) from mesophyll-derived protoplasts. Plant Cell Tissue and Organ Culture, 2005, 82,289–293.

[16]P Salak, C. Rommanee, P. Ploenchit, and T. Takeshi, Plant Regeneration from Protoplasts of Paper Mulberry, Thai Variety. Pro of Mulberry and Handmade paper for Rural Development, 19-24 Mar 2001, Rama Gardens Hotel, Bangkok.

[17]FY Tohjima, H. Amanouchi, A. Koyama, and H. Machii. Effects of various Enzymes on the efficiency of protoplast isolation from mulberry mesophylls.Journal of Sericultural Sciences, 1996, 65, 485-489.

[18]R Chakravarthy, M. Ashish, and M. Raja, Protoplast information system in *Morus sps*: An interface for research data on mesophyll protoplasts isolated from mulberry cultivars S34, S54, M5 and mysore-local. In.J. Sft Com &Bioi, 2011, 2, 21-30.

[19]A Kapur, S. Bhatnagar, and P. Khurana, Isolation and culture of leaf protoplasts of Indian mulberry (*Morus indica*var. K-2). Sericologia, 2003, 43(1), 95-107.

[20]U Pavan, Mulberry improvements via plastid transformation and tissue culture engineering. P S Behav, 2010, 5(7), 785-87.