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RESEARCH ARTICLE

Effect of enzymes on isolation of protoplast of *Catharanthus roseus*

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SUMMARY

In present investigation the protoplast was isolated from surface sterilized 3-4 weeks grown fully expanded leaves of Catharanthus *roseus.* Different enzymes were successfully used for protoplast isolation. The effect of pH, temperature and incubation period found out for the release of protoplast. The viability of protoplast was checked by haemocytometer which was increased by culturing in white media in suspension culture with proper combination of auxin and cytokinins.

Key Words : Protoplast, Osmoticum treatment, Macerozyme, Purification, Plating, Viability

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Isolated protoplasts have been successfully used in studies on cell physiology and engineering. The yield of protoplast depends on several factors, such as, physiological state of experimental material, purity and composition of enzyme, choice of osmotic solution and hydrogen ion concentration (Bhojwani and Razdan, 1996). The cell wall is consists of cellulose, hemicellulose and pectic substances which can be digested by proper enzymes to release the protoplasts (Cocking, 1960). Since the technique of tissue culture has become important tool for studying the wide range of basic and applied problems in plant improvement. As the genetic engineering is one of the important method in the field of agriculture through tissue culture(Power *et al.*,1986) . A survey of literature indicates that tissue culture studies during last decade have certainly provided a useful tool for the induction of genetic variability and combinations, which do not exist in nature.

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Protoplast cloning is an innovative and novel experimental approach to solve the wide range of problems with the potential and possibilities of somatic hybridization by cell fusion, regeneration and modifies the plant genome involving a new crop species. Cocking (1960) first used enzyme to isolate and to release protoplast from tomato root tip using enzyme from celluloytic fungus - *Myrothecium verrucaria.* Later Takebe *et al.* (1968) successfully applied the commercially available macerozyme and cellulase to isolate the large number of protoplast.

MATERIAL AND METHODS

The young and fully expanded 3-4 weeks old leaves of *Capsicum annum* (Linn*)* was taken as explant, which was treated with 70 per cent ethanol and mercuric chloride (0.1%). The sterilized explant was then kept in sterilized cellular protoplast washing medium (CPW) with 13 per cent mannitol (Gamborg, 1977). The macerozyme was produced according to Chauhan and Wadegaonkar (1999). During macerozyme treatment to the surface sterilized leaves, the epidermis was carefully peeled off and minced by sterile scalpel and then incubated at 26 ± 2^0 C for 16-18 hrs in dark. The pure protoplasts formed a band near the lower minscus of sucrose gradient solution, which was sucked out with a sterile pipette (Fig. A and B).

The culturing of protoplast (Fig.C) was done on NT

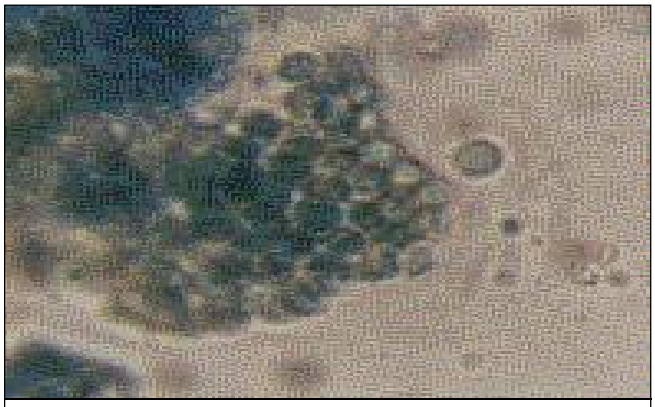

Fig. A: Cell wall degradation due to macerozyme activity

medium (Takebe and Nagata, 1971) with 2,4-D-1mg/l , NAA-1mg/l, BAP-0.5mg/l and 9.1 per cent mannitol, pH- 5.6 - 5.8 and was sterilized at 121^{0}C at 15 lbs for 15 minutes. In disposable Petriplates 1ml of protoplast culture was added with micro pipette to this 10 ml of NT media was poured at lowered temperature. After solidification the Petriplates were packed with parafilm and kept at 26 ± 2 ^{0}C in incubator for 6-8 days. The haemocytometer slide was used to check the viability.

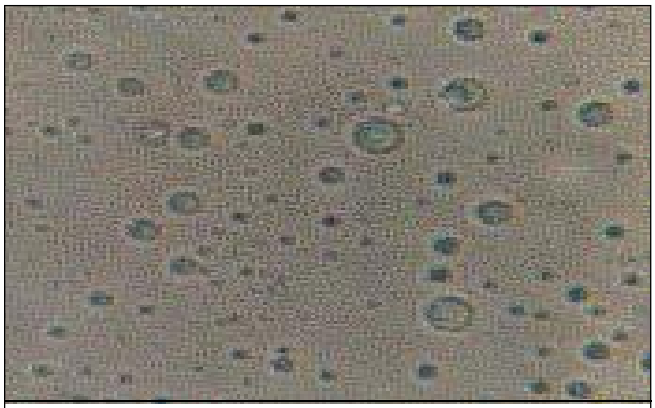

Fig. B: Isolated protoplasts from *Catharanthus roseus*

Fig. C: Cultured protoplast on NT media

The effect of different pH *viz.*, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 was found out to optimize the release of protoplasts. Similarly the effect of temperature *viz.*, 20 ^{0} C, 25 ^{0}C, 28 ^{0}C, 32 ^{0}C and 37^{0}C was screened at optimum pH for the yield of protoplasts. The effect of incubation period *viz.*, 5, 7, 9, 12 and 15 hours to the protoplast optimized system were screened to get higher density of protoplasts.

RESULTS AND DISCUSSION

The viable protoplasts were released by macerozyme produced by *A.niger* (Chauhan and Wadegaonkar, 1999) in 13 hrs incubation with cellular protoplast washing medium (CPW) and 10 per cent mannitol. The viable protoplast observed was green in colour, where as dead cells exhibited blue colouration by trypan blue.

Table 1 : Effect of pH on protoplast isolation from *Catharanthus roseus*

Sr. No	pH	No. of viable protoplasts
1.	4.5	1.440 x 10 ^{7}
2.	5.0	2.928 x 10 ^{7}
3.	5.5	4.028 x 10 ^{7}
4.	6.0	4.368 x 10 ^{7}
5.	6.5	2.240 x 10 ^{7}
6.	7.0	3.132 x 10 ^{7}

Table 2. : Effect of temperature on protoplast isolation from *Catharanthus roseus*

Sr.No	Temperature	No. of viable protoplasts
1.	20 ^{o}C.	0.940 x 10 ^{7}
2.	25 ^{o}C.	3.628 x 10 ^{7}
3.	28 ^{o}C.	4.708 x 10 ^{7}
4.	32 ^{o}C.	4.068 x 10 ^{7}
5.	37 ^{o}C.	2.240 x 10 ^{7}

Table 3 : Effect of incubation time on protoplast release

Sr.No.	Incubation hour	No. of viable protoplasts
1.	5	1.213×10^7
2.	7	4.254×10^7
3.	9	4.734×10^7
4.	13	5.234×10^7
5.	15	3.350×10^7

Table 4 : Viability of protoplast in suspension culture

Sr. No.	Incubation time (min)	No. of viable protoplasts
1.	0	2.0×10^4
2.	10	4.3×10^4
3.	20	5.8×10^4
4.	30	7.5×10^4

The protoplast culture was done on NT medium on disposable Petriplates containing 1ml protoplast. Out of seven replica three Petriplates showed colony growth after 3-4 days when incubated at $26 \pm 2\ ^0$C. The cultured protoplasts showed wall forming colonies after 5-6 days indicated by creamish colouration.

The harvested protoplast shown increase in number of viable count when cultured on a white medium containing 2,4-D –1mg/l, NAA- 1mg/l and BAP- 0.5 mg/l.

The different pH of macerozyme was set to analyze the optimized pH for release of protoplast. It was found out that pH 6.0 of the system enhances the release of protoplasts *i.e.* 4.368×10^7 (Table 1). The system with pH 6.0 with different temperature was set to analyze the effect of different temperature on release of protoplasts shows the maximum suitable temperature was 28^0 C which shows 4.708×10^7 number of released protoplasts (Table 2). The pH 6.0 with temperature $28\ ^0$C the system was incubated for different time intervals, which shows that 13 hours incubation of systems enhanced the releases of protoplasts to 5.234×10^7 (Table 3).

The viability of released protoplasts from above system with white media at suspension culture containing 4mg/lit. 2,4 D and 1mg/lit kinetin was set up. This system showed the increase in viability after 30 minutes (Table 4).

Conclusion :

The conclusion from the above experiments can be drawn as macerozyme isolated from *A. niger* can be successfully used for protoplast isolation from *Capsicum annum* (Linn) leaves. The optimization of protoplasts release system at pH 6.0, 28^0C and incubating for 13 hrs in dark can enhance the release of protoplast as alone system used. The culturing of protoplast can be done on NT media with concentration of 2, 4-D-1mg/l, NAA-1mg/l and BAP-0.5 mg/l and low conc. of mannitol and the viability can be increased by using white media in suspension culture.

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