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ISOLATION AND CULTURE OF NEEM (*AZADIRACHTA INDICA* A. JUSS.) CALLUS PROTOPLASTS[1]

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Abstract

Protoplasts were isolated from 3-week-old callus tissue of *Azadirachta indica* using a digestion solution of 2% Cellulase R-10 and 0.2% Macerozyme R-10. The optimum duration for enzyme treatment was 4 hr. During culture, protoplasts regenerated a cell wall and subsequently divided. Protoplasts cultured as thin liquid layers of the Murashige & Skoog's medium continued dividing for longer duration than those cultured in droplets.

Keywords : *Azadirachta indica*, callus, medicinal plant, protoplast culture, protoplast isolation, woody plant

Abbreviations : 2,4-D - 2,4-dichlorophenoxyacetic acid, Kn - Kinetin, MS - Murashige & Skoog's medium, pCPA - para-chlorophenoxyacetic acid, 2,4,5-T - 2,4,5-trichlorophenoxy-acetic acid

Introduction

Azadirachta indica A. Juss., a member of the family Meliaceae, is an evergreen tropical tree, popular as an avenue tree and for reclamation of poor soil and wastelands. It is of great commercial value because of its pesticidal, medicinal and pharmacological properties. Though many plants produce insecticidal and insect repellent agents, neem holds out maximum promise of providing highly effective, non-toxic and environment-friendly means of controlling or eliminating insect pests that cause losses in agricultural produce (Govindachari et al. 1992).

Despite its profound economic value, very little scientific work has been done on neem (Chaturvedi 2001). Improvement of neem by conventional breeding is difficult due to its highly heterozygous nature, long generation cycle, and recalcitrant nature of seeds.

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The application of protoplast technology for the improvement of woody plants offers new option to complement conventional breeding programs. The ability of isolated protoplasts to undergo fusion and take up macromolecules and cell organelles offers many possibilities in genetic engineering and crop improvement (Bhojwani et al. 1977). However, to fully explore the potentials for protoplast-technology on woody crops, efficient and reproducible methods for protoplast isolation and purification must first be established. In neem no work is reported in this regard.

In this paper, the isolation of protoplasts from callus tissue of *A. indica* and cultural conditions have been described whereby these protoplasts are made amenable to divide. The callus was a better source of protoplasts than the leaf mesophyll tissue.

Material and Methods

The anther calli, maintained by three weekly subcultures on Murashige & Skoog's (1962) medium — MS + pCPA(5μM), MS + 2,4-D(1μM), MS + 2,4-D(1μM) + KN(1μM), and MS + 2,4, 5-T(1μM), were used as the source of protoplasts. These callus lines were maintained for over 17 months during which they retained the potential for differentiation (Chaturvedi et al. 2003).

Callus was transferred directly to 55mm pre-sterilized Petriplates containing filter-sterilized enzyme solution (5ml per gram of tissue). The enzyme solution contained 2% cellulase R-10 (Yakult Honsha Co. Ltd., Japan), 0.2% Macerozyme R-10 (Yakult Honsha Co. Ltd., Japan) and 11% mannitol in CPW (cell-protoplast-washing medium; Frearson et al. 1973). pH of the enzyme solution was set at 5.6. Petridishes were sealed with parafilm and incubated in dark at 25°C for 4-12 hr.

After enzyme treatment, the incubation mixture was filtered through a nylon sieve (ca 45μm pore size). The filtrate was transferred to centrifuge tubes and spun at 100xg for 5 min. The supernatant was discarded and the pellet was resuspended in 1ml enzyme solution and layered over a 21% sucrose pad (in CPW) in another centrifuge tube. After centrifugation at 100xg for 10 min the purified protoplasts were collected from the interface of the sucrose pad and the enzyme solution. Prior to counting, the protoplasts were washed twice by repeated suspension and centrifugation in the culture medium at 100xg for 3 min.

Protoplasts were cultured (at a density of $1x10^4$/ml of medium) in liquid MS + Mannitol (11%) + 2,4-D (1μM) + Kn (1μM) or MS + Mannitol (11%) + pCPA (5μM). pH of the media was adjusted to 5.6. These were cultured as flattened drops (approx. 100μl) or as a thin layer in 35mm Petriplates. Pairs of culture plates were transferred to larger Petriplates. Before sealing the outer Petriplate, a few drops of SDW were placed along its inner margin to prevent desiccation of protoplast cultures. Cultures were then incubated at 25°C in continuous darkness. After 11 days of culture, 2 or 3 drops of mannitol-free fresh liquid medium were added to each culture plate to reduce osmolarity of the medium. This was repeated every 3 or 4 days for the next 2 weeks. After another week the dividing protoplasts were cultured in 35mm Petriplates as sitting drops or as thin layer over the semi-solid medium. All the cultures were maintained in dark at 25°C.

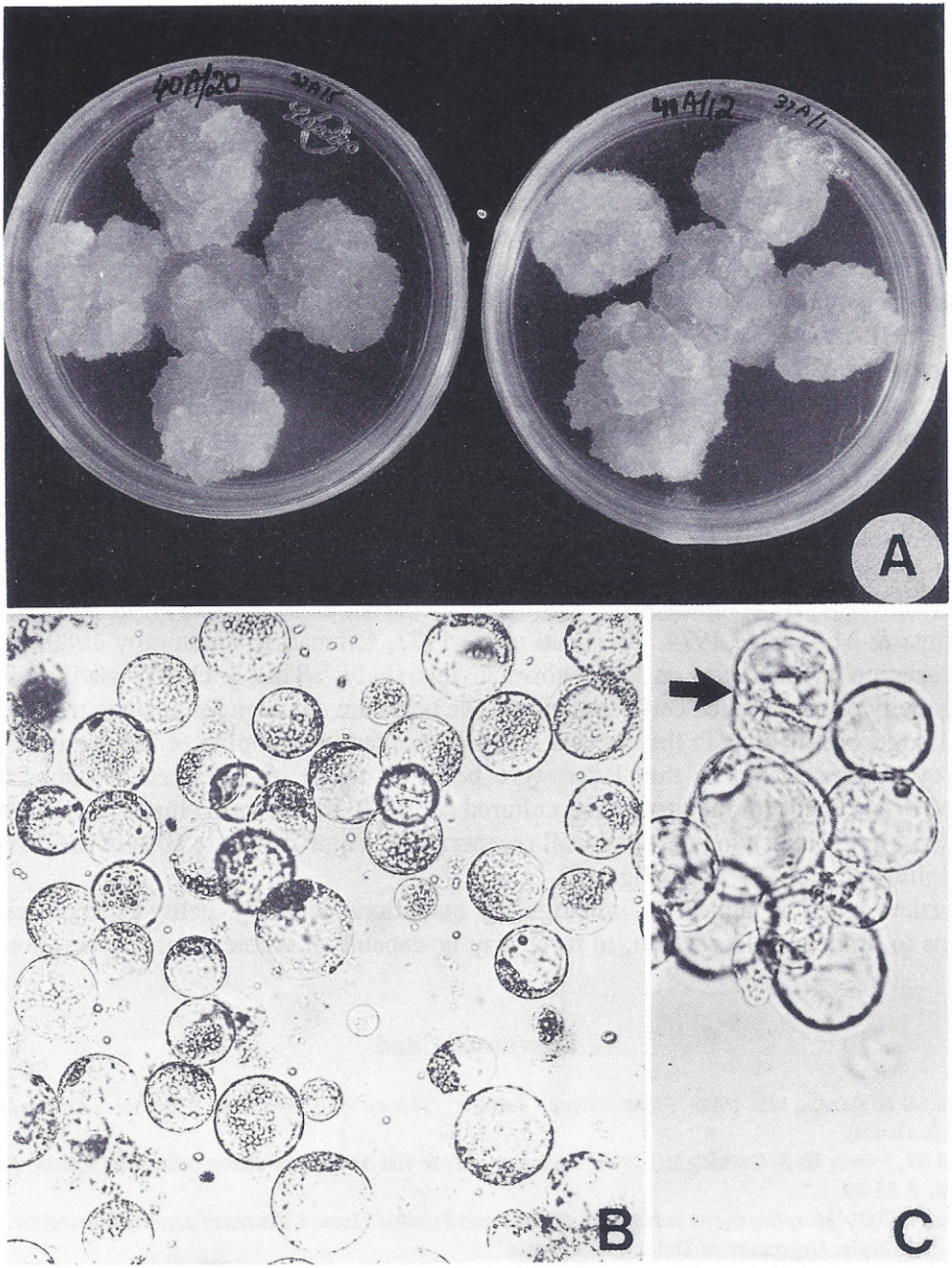

Fig. 1A-C — A. Three-week-old proliferating granular, friable, light brown anther callus on MS+pCPA (5μM). x 1.1. **B.** Freshly isolated protoplasts from calli as in **A**, showing considerable variation in size. x 470. **C.** Eleven-day-old culture of protoplast in liquid MS medium supplemented with pCPA (5μM). The protoplasts have regenerated walls as indicated by the change in their shape. One of the protoplasts has also divided (arrow). x 400.

Observations and Discussion

Leaves (from in vitro grown shoots or adult trees of neem) were first used, in the present study, as a possible source of protoplasts. However, this gave limited success owing to the difficulty in removing the lower epidermis and the low frequency of protoplasts released from leaf fragments. Although it is often desirable to use mesophyll protoplasts in somatic hybridization studies since leaf tissue is a readily accessible source of genetically uniform cells, in many plant species, including neem, leaf tissues do not yield large number of protoplasts capable of division. An alternative source is the cultured cell material where protoplasts can show greater potential to divide (Bhojwani & Razdan 1996). Therefore, anther callus, cultured on MS+pCPA(5μM), MS+2,4-D(1μM), MS+2,4-D(1μM) + Kn(1μM) and MS+2,4,5-T (1μM) was used for protoplast isolation. The soft and friable callus, obtained after four subcultures, was found to yield more protoplasts than earlier subcultures. Maximum yield ($5.8x10^6$ protoplasts/g fresh wt) of viable protoplasts was obtained from granular, friable, light brown callus grown on MS+pCPA(5μM) (Fig. 1A). Besides cycles of subcultures, the age of the callus also appeared to influence protoplast production. Calli were used from 2 -, 3 - and 5-week-old subcultures. The calli from 3-week-old subcultures consistently gave maximum yield of protoplasts. The protoplasts were characteristically spherical but varied considerably in size (Fig. 1B). As compared to mesophyll protoplasts, those isolated from cultured tissues (Uchimiya & Murashige 1974, Bhojwani et al. 1977, Othman & Paranjothy 1980) exhibit a great variation in their size as also shown in this study. This is chiefly attributed to the heterogeneity of the cultured cell population. The optimum duration for enzyme treatment was 4 hr. Longer incubations in the enzyme solution resulted in clumping of the protoplasts.

Protoplasts cultured as thin layer were better in terms of sustained divisions than as flattened drops. Most of the protoplasts cultured on MS + pCPA(5μM) liquid medium became oval after 4 days, indicating new cell wall regeneration. Approximately 10% of the protoplasts entered division after 11 days (Fig. 1C).

Further work is aimed at investigating conditions whereby cells undergo sustained divisions to produce callus which, in turn, may be capable of whole plant regeneration.

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