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BIOTECHNOLOGY

Optimization of Protoplast Isolation Protocols from Callus Culture of *Anacardium occidentale* L.

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Abstract

Protoplast technology endeavors a unique single cell system that promotes several aspects of modern biotechnology. In the present study, an efficient protocol to isolate the protoplast from callus culture of a valuable plantation crop, *Anacardium occidentale* was developed. The phenolic compound released during digestion process caused protoplast injury resulted in low yielding. Further investigations revealed that pH 5.0 and osmotic concentration 0.5M mannitol were found suitable for maximum protoplasts yield. The results also indicated that the age of the callus tissues plays a key role in the yield of protoplasts in *A.occidentale*. During the study, youngcalli showed maximum protoplast yield when treated with cellulase (2.5 %), pectinase (2.5 %), hemicellulase (1.5%) and 20mM $CaCl_2$ in 0.5M mannitol. Meanwhile, an incubationperiod of 4hours with enzyme solution resulted in the maximum yield of protoplasts (4.71 ± 0.15)×10^3 protoplasts/g FW. In short, the results generated in the study can be used as a platform to work out the protoplast technology in *A.occidentale*.

Highlights

- Actively growing callus obtained from the leaf explants cultured on MS medium supplemented with 2,4-D and BAP have been used for protoplast studies.
- Young callus showed maximum protoplast yield when treated with cellulase (2.5 %), pectinase (2.5 %), hemicellulase (1.5%) and 20 mM $CaCl_2$ in 0.5M mannitol at pH5.0.

Keywords: *Anacardium occidentale*, Callus culture, Protoplast, Osmoticum

The cashew (*Anacardium occidentale* L.) is native to Brazil from where it spread widely in the tropical countries of Africa and India by the Portuguese explorers in the 16th century (Ohler 1967). It is one of the economically important woody plants. Two most important parts in commercial uses are cashew nut for diet and liquid from nut shell (CNSL). Anacardic acid, cardol and cardanol are the major components of CNSL.

Biological activities of the anacardic acid include molluscicidal activity (Kubo *et al.,* 1986), anti-tumor activity (Kubo *et al.,* 1993), antimicrobial activity (Himejima and Kubo 1991), antioxidant activity (Razali *et al.,* 2008), antimutagenic activity (Melo *et al.,* 2008) etc.

Today, India is one of the leading countries in commercial production, processing and exporting of raw cashew nut and cashew nut shell liquid (CNSL) in the world. This has resulted in the initiation of research and breeding programs, leads to the release of high-yielding elite varieties for commercial profiteering. Vegetative propagation by grafting creates large stocks of planting material necessary for commercial cashew plantations. This method is incapable of meeting the existing demand for planting material (Nambiar *et al.,* 1990).

Therefore, *in vitro* clonal propagation became one of the appropriate ways for generating more planting material. A complete regeneration system of cashew has been obtained mainly through organogenesis (Boggetti *et al.,* 1999).

In the present study, callus obtained from young leaves of cashew seedlings were used as the source tissue for the isolation of protoplasts from *A.occidentale*. Protoplast refers to all the components of a plant cell excluding cell wall, limited by its plasma membrane. The wall can be temporarily removed from plant cells using mechanical (Bhojwani and Rezdan 1983) or enzymatic (Bajaj 1989) means without significant loss of viability. They are unique, as they are single cell systems without cytoplasmic connections. Regeneration of plant from cultured protoplasts is one of the most important pre-requisites for genetic manipulation programmes (Patidar *et al.,* 2013). Thus, in order to completely explore the application of protoplast-based biotechnology, an effective protoplast isolation system is essential. This study aimed to determine hormonal concentration to induce callus from leaf explants, osmoticum, enzyme concentration and the incubation period for the maximum release of viable protoplasts.

Materials and Methods

Plant material

Mature cashew (*A. occidentale*) seeds were procured from the mother stock tree grown at the Kerala State Cashew Development Corporation, Kollam, Kerala, India. Microleaves which sprouted from cotyledonary node axils of decapitated seedlings germinated in plastic trays containing washed and sterilized sand as substrate in laboratory condition were used for the harvest of explants.

Callus induction

Young leaves of *A. occidentale* collected from decapitated seedlings served as the source of explants for the present study. Callus initiation was studied with 2,4-D and BAP alone and with various combinations in the MS (Murashige and Skoog 1962) medium. After inoculation, the cultures were preserved at a constant room temperature of 24 ± 2ºC and 55 ± 5% relative humidity in the culture room under dark conditions for 3 weeks before the first subculture. Subculturing was done frequently every four weeks until callus establishment.

Protoplast isolation

Callus obtained from the young leaves were used as the donor material for protoplast isolation. The sliced calli were treated with 20% (w/v) mannitol for 30 minutes to facilitate plasmolysis. The callus sections were washed twice in 0.6 M (11%) mannitol solution before incubation. About 1-2 gm of dark-grown calli were sliced to 1 mm thick and treated with enzyme mixtures (Eriksson 1985).

In Preliminary experiments, an effect of various concentrations and combinations of enzymes on protoplast yield was analyzed. Initially, Cellulase Onozuka R-10 (Merck, Germany), Pectinase (Sigma, USA) and Hemicellulase (Sigma, USA) were the major types of enzymes used in varying concentrations and combinations for testing the suitability for the isolation of protoplast. Later, enzyme mixtures consisting of various concentrations/combinations of enzymes along with 20 mM $CaCl_2$ and 0.6 M mannitol as osmoticum were treated. Unless otherwise mentioned, the pH of the enzyme mixture was adjusted to 5.5, and all the mixtures were filter sterilized with 0.45 µ Millipore membrane filter (Millipore High-Flow, Sartorius, Germany). Studies on the effects of various levels of pH and osmoticum were also carried out.

One to two grams of the callus tissue were treated with 20% mannitol for plasmolyzing the cells prior to enzyme treatment. Then they were treated with filter sterilized enzyme mixture (10 ml) and incubated on a rotary shaker (50 rpm) for up to 12 hours in the darkness at 25 ± 2°C. Samples were collected at regular intervals for microscopic examination and to optimize the time required for optimum cell wall digestion. Aliquots of protoplast suspensions collected at different intervals were collected in cavity slides and observed under the microscope.

Protoplast purification

After ascertaining the yield, the enzyme-protoplast mixture was sieved using a nylon mesh (75 microns) (Wilson Sieves, Nottingham, UK) and the filtrate was centrifuged for 5 minutes at 100 × gyration. Discarded the supernatant and the sediment

protoplasts together with undigested tissues were combined mildly with protoplast washing medium that consisted of full strength MS salts and 0.5 M of mannitol and centrifuged at 100 × gyration for 5 minutes. The pelleted protoplasts were then mixed with 3-4 ml protoplast washing medium and suspended over a density gradient solution containing 20% sucrose and centrifuged at 100 × gyration for 4-5 minutes. The protoplasts were collected at the interface of the two solutions. They were collected carefully using a pasture pipette and then washed twice in the culture medium as above. The protoplast yield was calculated with the help of a hemocytometer count under the microscope (Vancha *et al.,* 2004).

Determination of viable protoplast

Evan's blue staining was used to assess the viability of purified protoplasts (Huang *et al.,* 1986; Evans and Bravo 1983). Aliquots of protoplasts suspension mixed gently with 15 µl of 0.4% (w/v) Evan's blue dye (Sigma-Aldrich) and incubated for 10 minutes at room temperature. Living cells excludes Evan's blue whereas dead protoplasts are stained deep blue colour. The number of viable protoplasts was estimated using a hemocytometer and the percentage of protoplasts viability was calculated by dividing the total number of viable protoplasts by the total number of protoplasts count.

$$\% \text{ of protoplast viability} = \frac{\text{total no. of viable protoplast count} \times 100}{\text{total no. of protoplast count}}$$

Data analysis

Data were expressed as means standard deviation (SD) of three replicate determinations. All statistical analyses were carried out using an SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver. 20). To determine whether there were any differences among the means, one-way analysis (ANOVA) and the Duncan's New Multiple range test were applied to the result at 0.05 level of significance ($p < 0.05$).

Results and Discussion

Callus induction from leafexplants

Leaf segments (0.5-1.0 cm) were excised from micro leaves, which sprouted from cotyledonary node axils of decapitated seedlings and were cultured on MS medium containing different concentrations of 2,4-D and BAP alone and with different combinations. Maximum callus induction (46.55 ± 3.50 %) was observed on MS medium supplemented with 2,4-D (50 mg/l) with acombination of BAP (50 mg/l) (Table 1). Callus induced from leaf segments was initially light yellow and then turn to light to dark brown in colour and varied in texture from semi-friable to compact (Fig. 1).

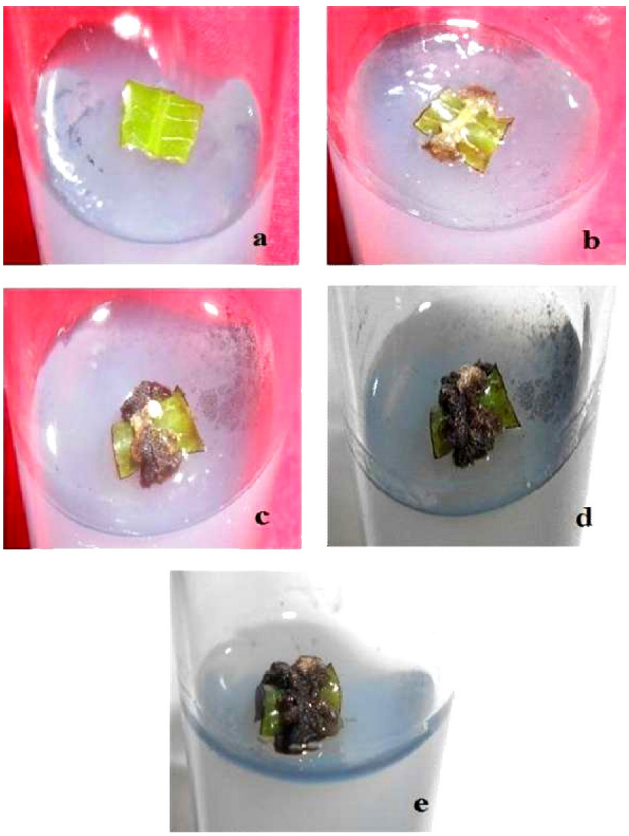

Fig. 1: Induction and proliferation of callus from leaf segment of *A.occidentale* on MS medium

a. Inoculated leaf explant; b. Callus initiated from the midrib region at the sixth day of culture; c. Callus after 1 weeks of culture; d. Callus after 2 weeks of culture; e. Callus after 3 weeks of culture.

Most of the leaf explants only grown bigger in size after 11 days. Callus initiation occurred from the midrib region of the explants during 1 weeks of culture in most of the leaf explants. The induction percentage, texture and colour of callus were different at various hormonal concentrations (Table 1). The concentration of 50 mg/l 2,4-D and 50 mg/l BAP was the first to induce callus production after 6 days with 46.55 ± 3.50 % and

Table 1: The callus induction from the leaf explants of *A.occcidentale* inoculated on the MS media supplemented with 2,4-D and BAP alone and with different combinations after 3 weeks of culture

Sl. No.	Growth hormones (mg/l)		Days to callus initiation	Callus score	Explant forming callus (%)	Callus texture	Callus colour
	2,4-D	BAP					
1	0	0	NC	NC	NC	NC	NC
2	10	—	NC	NC	NC	NC	NC
3	25	—	NC	NC	NC	NC	NC
4	50	—	NC	NC	NC	NC	NC
5	—	10	NC	NC	NC	NC	NC
6	—	25	NC	NC	NC	NC	NC
7	—	50	NC	NC	NC	NC	NC
8	25	10	NC	NC	NC	NC	NC
9	25	25	12	+	28.88±5.09b	semi friable	light brown
10	50	10	13	+	16.66±3.33a	compact	dark brown
11	50	25	7	+++	36.66±3.33c	compact	pale brown
12	50	50	6	++++	46.55±3.50d	semi friable	dark brown
13	75	75	NC	NC	NC	NC	NC

NC = no callus formed; + = callus only formed at the edge of the explant; ++ = callus formed more at the edge; +++ = callus covered the surface; ++++ = callus covered more on the surface. For each treatment the means within the column by different letters are significantly different at P < 0.05. Each value is expressed as the means ±SD.

it was significantly different ($p < 0.05$) with all other hormonal concentrations. The results also displayed that auxin and cytokinin alone was not very effective for producing callus in MS medium. Besides that, 2,4-D when used in combination with BAP produced increased percentage of callus induction than when it used alone (Table 1). This concentration and combination for callus induction from leaves were not seemed to be reported earlier. These results were in agreement with an earlier study (Leva and Falcone1990) where callus was also initiated from leaf explants on MS and Schenk and Hildebrandt medium.

Protoplast isolation and purification

Results presented here indicated that the influence of enzymes and other factors are particular on isolation and purification of protoplasts. In the present study, actively growing callus tissues were used a source material for protoplast isolation. The callus raised *in vitro* was easily handled and could be digested to protoplasts with relatively less debris in enzyme mixture.

Effect of enzyme mixture

Treatment with 20% mannitol for 30 minutes was sufficient to plasmolyze the cells of *in vitro* tissues. A mixture of cellulase (2.5%) and pectinase (2.5%) in 0.6 M mannitol (pH 5.5) was identified as the basic enzyme mixture. The effect of hemicellulase was also analyzed where three concentrations of the enzyme (1%, 1.5% and 2%) were used along with the basic enzyme mixture. The yield of protoplasts from callus tissues varied at different concentration of hemicellulase. Hemicellulase at the concentration of 1%, 1.5% and 2% resulted the yield of $(1.42 \pm 0.18)\times10^3$ protoplasts/g FW, $(1.51 \pm 0.17)\times10^3$ protoplasts/g FW and $(1.37 \pm 0.20)\times10^3$ protoplasts/g FW respectively after 4 hours of incubation. Results also showed that hemicellulase at a concentration of 1.5% enhanced the yield to $(1.51 \pm 0.17)\times 10^3$ protoplasts/g FW after 4 hours of treatment (Fig. 2). The experiments also disclosed that 4 hours of incubation are best for cell wall digestion and protoplast yield from actively growing calli (Fig. 2). Relatively older callus tissues were less yielding in the enzyme mixture than the actively growing ones.

The protoplasts are not merely the representatives of thewall-less cell but serve as apotential tool for the study in specific problems in cell biology such as cell division and wall synthesis, membrane transport, cell physiology, genetic modification etc. (Bajaj 1989). Protoplasts also play an important role in the non-conventional breeding of certain crop

species. There has been no report on protoplast studies in this promising group of plants. Cellulase and pectinase were the extensively used hydrolytic enzymes for the isolation of protoplast. Pectinase chiefly used for the distribution of cell aggregates (Grezes *et al.,* 1994), although cellulase generally used to break the plant cell wall (Sondahl *et al.* 1980). The efficacy of isolation of protoplast mainly depends on the composition and concentration of the enzyme solution (Chabane *et al.,* 2007). Penetration of enzyme mixture into tissues was enhanced by slicing of the explants or by rupturing epidermal cover (Yasugi 1989).

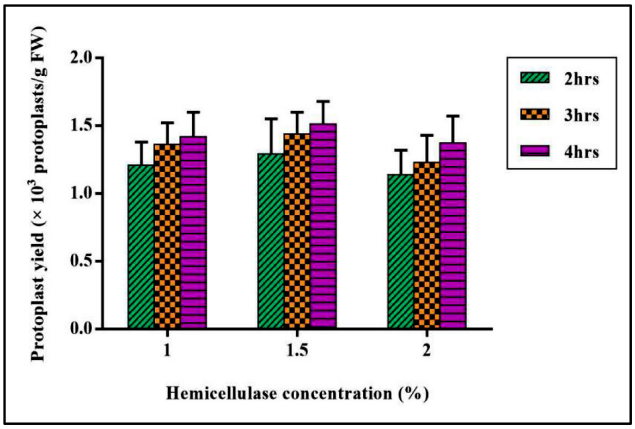

Fig. 2: Yield of protoplast from callus tissues of *A.occidentale* in presence of hemicellulase at different time periods

(Basic enzyme mixture: cellulase Onozuka R-10 (2.5%) and pectinase (2.5%) in 0.6 M mannitol at pH 5.5)(n=3, error bars represent standard deviation)

The time course dependent assay revealed that very few protoplasts were seen released during the initial hour of incubation. The isolation gradually increased with the progression of the incubation period. Freshly isolated protoplasts are spherical in shape as they were unrestricted by a cell wall. Protoplasts yielded from callus tissues were also broken or injured due to the release of phenolics during isolation.

Effect of mannitol

All studies on the effect of enzymes on protoplast isolation were initially carried out using 0.6 M mannitol. But the protoplasts were marginally plasmolyzed as evidenced by microscopic examination after 3-4 hours of incubation. Later, different concentrations of mannitol (0.5, 0.6 and 0.7 M) were tried. Mannitol at a concentration of 0.5 M was found more effective for protoplast yield than the other concentration (Fig. 3). The enzyme mixture with this concentration of the osmoticum yielded (2.6 ± 0.21)× 10^3 protoplasts/g FW callus tissue and it was significantly different with mannitol at the concentration of 0.4 M [(2.1 ± 0.17)× 10^3 protoplasts/g FW] and 0.6 M [(1.85 ± 0.20)× 10^3 protoplasts/g FW] respectively (Fig. 3).

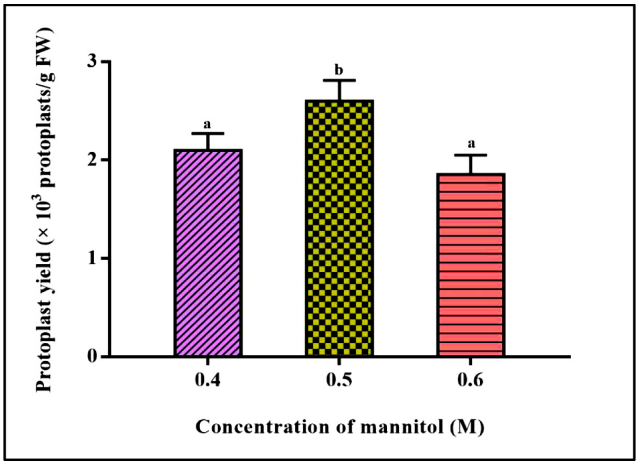

Fig. 3: Effect of mannitol on protoplast isolation from callus tissues of *A.occidentale*

(Basic enzyme mixture: cellulase Onozuka R-10 (2.5%), pectinase (2.5%) and hemicellulase (1.5%) at pH 5.5) (Values with different letters are significantly different by Duncan's multiple range test ($p < 0.05$), (n=3, error bars represent standard deviation)

Generally, the mannitol acted as flotation agent and sole osmotic sustainer for the effective isolation of viable protoplast (Jullien *et al.,* 1998). The freshly isolated protoplasts were prone to breakage when mannitol solution was not added to the washing medium. Without osmoticum like mannitol, the water moleculesenter into the protoplasts and resulted in the cell to rupture (Karp 2005). Usually, presence of 0.3 - 0.6 M mannitol in the enzyme mixture was reported suitable for optimum isolation of protoplasts (Bajaj 1989). In the present study 0.5M mannitol was found theoptimum for the isolation of protoplasts from callus tissues.

Effect of pH

All the above-mentioned experiments were carried out at the pH of 5.5. Effect of different pH levels (4.0, 4.5, 5.0 and 5.5) on protoplast isolation was also studied. A pH of 5 was found to be an ideal condition for enhanced isolation. Based on these experiments, an improved yield of (4.71 ± 0.15)×

10^3 protoplasts/g FW was obtained when calli were incubated in an enzyme mixture containing cellulase (2.5%), pectinase (2.5%), and hemicellulase (1.5%) in 0.5 M mannitol at pH-5 and it was significantly different with pH-4 [(2.32 ± 0.16)× 10^3 protoplasts/g FW], pH-4.5 [(3.12 ± 0.16)× 10^3 protoplasts/g FW] and pH-5.5 [(3.48 ± 0.21)× 10^3 protoplasts/g FW] respectively (Fig. 4). Protoplasts isolated from callus tissues consisted of small aggregates of protoplast and individual protoplast of spherical in shape and often contained less number of chloroplasts (Fig.5). They were very brittle and longer incubation period resulted in the rupturing of protoplast (Fig. 6).

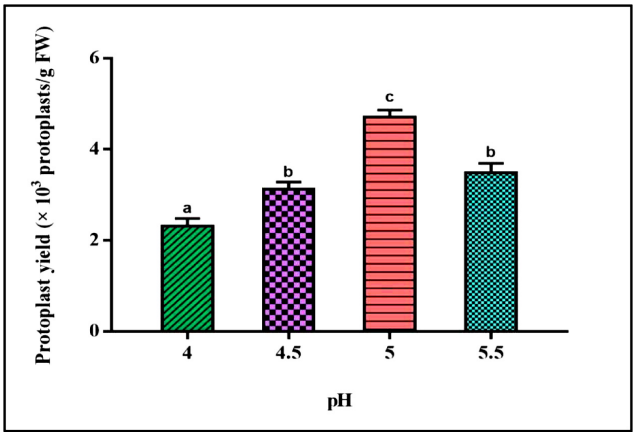

Fig. 4: Effect of pH on protoplast isolation from callus tissues of *A.occidentale*

(Basic enzyme mixture: cellulase Onozuka R-10 (2.5%), pectinase (2.5%) and hemicellulase (1.5%) in 0.5M mannitol) (Values with different letters are significantly different by Duncan's multiple range test (p < 0.05), (n=3, error bars represent standard deviation)

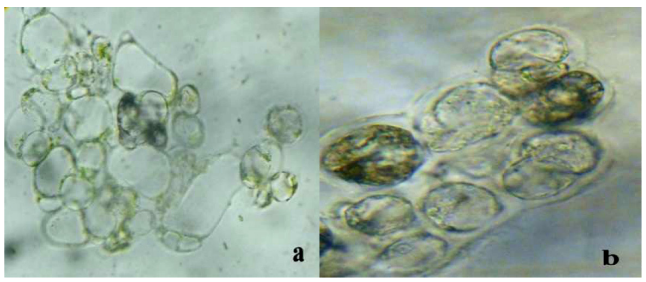

Fig. 5: Protoplast aggregates in a microscopic field from *A.occidentale* callus (a&b) - 40X

pH of the enzyme mixture has to be adjusted to optimize the digestion of cell wall because hydrolytic enzymes are active in certain acidic pH. In *A.occidentale* maximum protoplast yield was recorded when the enzyme mixture was adjusted to pH 5.0. The level of pH requirement varied significantly from species to species, usually ranging from 5.0-6.2. In *Phaseolus*, a higher pH of 6.0-7.0 was found beneficial for releasing most protoplasts from mesophyll tissues (Pelcher *et al.*, 1974), whereas a lower pH 5.6 has been reported for the optimum yield from *in vitro* derived leaves of different poplar hybrids (Park and Son 1992). In soybean, thebetter release of mesophyll protoplasts was observed when pH was adjusted to 5.8 (Schwenk *et al.*, 1981).

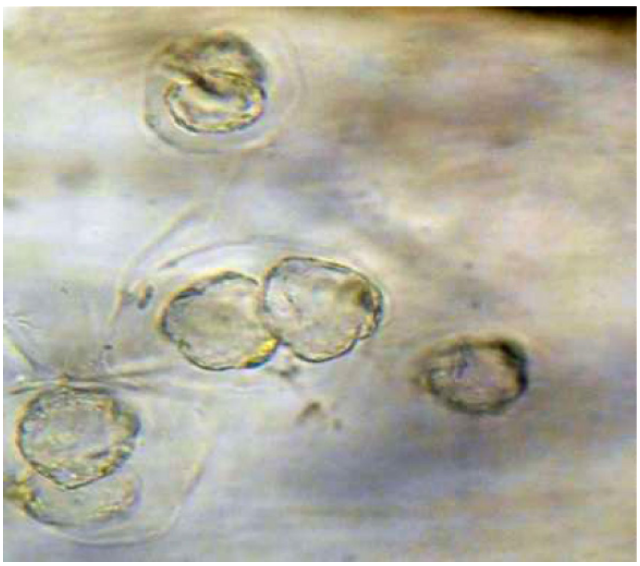

Fig. 6: Rupturing of protoplasts in a microscopic field from *A.occidentale* callus - 40X

Viability of protoplast

The viability of protoplasts was determined using Evan's blue. In the present study, the viable protoplasts were non-stained (Fig. 7) because in the living protoplast the dye is reduced to a colourless form.

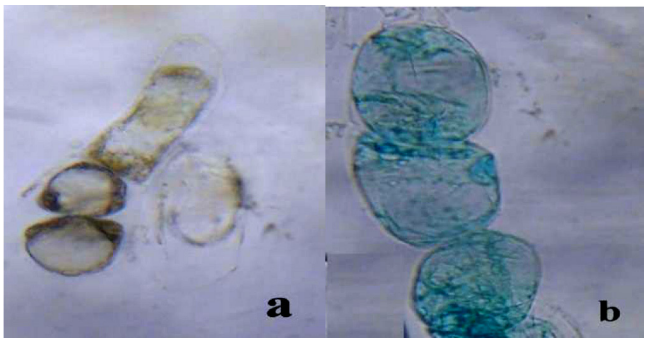

Fig. 7: Freshly isolated protoplasts from *A.occidentale* callus stained with Evans blue- 40X

(a) Non- stained protoplasts (viable) (b) Stained protoplasts (non-viable)

The incubation time of plant tissues in an enzyme solution also influenced the protoplast viability.

Consequently, optimum incubation time has to be decided because inadequate incubation length resulted in poor protoplast yield. As shown in Fig. 2, the protoplast yielded was gradually increased from 2 hrs to 4 hrs. The yield of protoplasts reached the maximum of (4.71 ± 0.15) × 10^3 protoplasts/g FW when the incubation time was 4 hrs. The number of viable protoplasts that obtained from 4 hrs incubation time was at the maximum ((4.71 ± 0.15)× 10^3 protoplasts/g FW) with viability percentage close to 69.5% in the enzyme mixture of cellulase Onozuka R-10 (2.5%), pectinase (2.5%) and hemicellulase (1.5%). They were very fragile and most of them got broken and did not survive in the initial 12 hrs of incubation.

It was reported that the duration of plant tissues in an enzyme solution also greatly influenced the viability of protoplast and nature of plasmalemma, subsequently affected the wall biosynthesis, and hence the division process (Kaur *et al*., 2006). It has been reported that cell suspension cultures are suitable for protoplast preparation of several species such as *Allium cepa* (Karim and Adachi 1997), *Lycopersicon chilense* (Latif *et al.,* 1993) etc. Isolation of protoplasts was found to be maximum from actively growing callus and cell suspensions of*Pinus lambertiana* compared to young sprouts initiated from mature explants (Gupta and Durzan 1986).

Conclusion

In the present study, callus development was noticed on the cut margins and midrib regions of the leaf lamina on full-strength MS supplemented with appropriate concentration of BAP and 2,4-D. The developed calli were light to dark brown in colour and slow growing. Maximum callus induction (46.55 ± 3.50%) was observed on MS medium supplemented with 2,4-D (50 mg/l) with acombination of BAP (50 mg/l) in the dark. Protoplasts could be isolated substantially from callus tissues. Thin sections of callus were found comparatively desirable for the release of up to (4.71 ± 0.15) × 10^3 protoplasts/g FW. During the study, maximum protoplast yield was achieved when calli were treated with cellulase (2.5 %), pectinase (2.5%), hemicellulase (1.5%) and 20 mM $CaCl_2$ in 0.5M mannitol. The investigations revealed that the pH of the enzyme mixture was one of the important factors for cell wall digestion and yield in *A. occidentale*. In *A.occidentale* pH 5.0 was found theoptimum for maximum yield of protoplasts. Osmotic regulation during the isolation of protoplasts was also standardized and 0.5M mannitol was found theoptimum for the isolation of protoplasts from callus tissues. It was also evident from the experiment that 4hrs of incubation was needed for optimum wall digestion under the conditions tested.

Acknowledgements

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