GLOBAL JOURNAL OF PURE AND APPLIED SCIENCES VOL. 6 NO. 1 JANUARY 2000: 37-40
 ISSN 1118-0579

EFFECT OF SORBITOL PRETREATMENT ON YIELD AND VIABILITY OF PROTOPLASTS ISOLATED FROM ETIOLATED SHOOTS OF THREE CULTIVARS OF *SORGHUM BICOLOR*

E. O. UMOH and A. E. NKANG

(Received 26 February 1999; Revision accepted 7 August 1999)

ABSTRACT

This study aimed at improving *Sorghum* protoplasts isolation procedures and maximizing yield using etiolated shoots of three cultivars (MH5I, CH9 and CVX) for *Sorghum bicolor* (L) Moench. Hypocotyl sections were pretreated in sorbitol for varying periods of time and protoplast isolated in medium (pH 5.7) containing 3% cellulase (ONOZUKA RS.), 0.5% macerozyme (R-10) and 0.1% pectolyase (Y-23) in protoplast wash solution. Pretreatment time of 3.5 hours in sorbitol was found to be optimal for protoplast yield and viability. Optimal protoplast yields of 9.22 x 10^5, 8.10 x 10^5 and 6.80 x 10^5 were obtained per gram fresh weight of hypocotyl tissue in cultivars MH5I, CH9 and CVX respectively, at 2.5hour digestion time. This low enzyme digestion time decreased the toxic effects of enzyme preparations and consequently enhanced viability up to 75%. Results show that etiolated seedlings can provide a rapid and high-yielding source of viable *Sorghum* protoplasts. Efforts should, therefore, be geared towards improving the plating efficiency and regeneration capacity of protoplasts.

Keywords: *Sorghum bicolor*, protoplasts, etiolated shoot, sorbitol, yield, viability.

INTRODUCTION:

Plant protoplasts have become indispensabl materials in several crop-breeding programmes as they are used for mutant selection, somatic hybridization, genetic transformation and plant regeneration (Galun, 1981). Such cellular genetic manipulations offer new possibilities for developing new varieties of *Sorghum*, an important crop used as food, fibre, fodder and fuel in the tropical and subtropical regions. Most of the processes require efficient methods for protoplasts isolation, culture and plant regeneration. Although several reports on *Sorghum* plant regeneration from cell calli have been described (Sharma *et. al.*, 1989; Cai and Butler, 1990), little has been reported on isolation, culture and regeneration from protoplasts (Chin and Scott, 1979; Wei and Xu, 1990).

The few recorded (Chourey and Sharpe, 1985; Murphy and Cocking, 1988; Wei and Xu, 1989, 1990; Guo and Liang, 1991; Hagio *et. al.*, 1991) successes in *Sorghum* protoplasts isolation and culture have been achieved through the use of cell suspension cultures obtained from calli of different ex-plant sources, mostly immature embryos. The process of obtaining protoplast from cell suspension cultures takes at least 3 months and requires 14-16 hours exposure to enzyme mixture (Wei and Xu, 1990). The use of etiolated tissue offers several advantages over the cell suspension culture method.

These include easy manipulation, rapid growth and rapid isolation.

No record is available on the etiolated tissue method in *Sorghum* protoplasts isolation. Also, data on protoplast yield and viability in *Sorghum* are scarce in literature. This work was, therefore, carried out to determine the effect of sorbitol pretreatment on protoplast yield and viability in etiolated shoots of some *Sorghum bicolor* cultivars.

MATERIALS AND METHODS

(a) Sources of *Sorghum bicolor* cultivars.

Three cultivars of *Sorghum bicolor* (CH9, MH51 and CVX) were used in this study. The first two cultivars (CH9 and MH51), which are commercial hybrids, were supplied by seed production unit of Spic Science Foundation, Madras, India. The third cultivar (CVX) was supplied by Swaminathan Research Foundation, Madras, India.

(b) Germination.

Seeds of the three cultivars were surface-sterilized by soaking in 2% sodium hypochlorite for 10 minutes. Thereafter, they were rinsed four times with sterile distilled water and further surface-sterilized by soaking in 0.1% $HgCl_2$ solution for 7 minutes. The double sterilization technique used was to ensure that the seeds were completely sterile before germination. The seeds were again rinsed four times with sterile distilled water and were germinated in sterile magenta boxes at 25°C in a dark cupboard.

(c) Protoplast Isolation and Purification

Ten-day old etiolated seedlings were used for protoplast extraction. Hypocotyl sections measuring 3cm in length were cut twice longitudinally and

E. O. UMOH, Department of Biological Sciences, University of Calabar, P.M.B 1115, Calabar - Nigeria
A. E. NKANG, Department of Biological Sciences, University of Calabar, P.M.B 1115, Calabar - Nigeria

soaked in 600mM sorbitol for varying periods of time (0,1, 3.5, 5 and 7 hours) prior to enzymic treatment. The protoplast isolation medium (Frearson *et. al.*, 1973) contained 3% cellulase (Onozuka RS), 0.5% macerozyme (R-10) and 0.1% pectolyase (Y-23) dissolved in protoplast wash solution. The pre-treated hypocotyl sections were incubated in the enzyme mixture (pH 5.7) at 27°C for varying periods of time (0,1, 2.5, 3.5 and 5 hours) in complete darkness but interrupted by gentle manual agitation at 20 mins intervals. Protoplasts were separated from undigested tissue by sieving through 30µm steel mesh and purified by sedimentation method recommended by Millam *et. al.*, 1991. Complete digestion of cell wall was confirmed in the purified protoplasts using calcoflour white (Nagata and Takebe, 1970).

(c) Protoplast Yield and Viability Test

Protoplasts were counted using a Fush-Rosenthal haemocytometer and yield determined per gram fresh weight of hypocotyl tissue. Ten counts were made per replicate. The counts were subjected to analyses of variance tests and their means separated using the Duncan's multiple range tests. Viability of protoplasts was determined using the fluorescein diacetate (FDA) technique (Widholm, 1972; Bengochea and Dodds, 1986).

RESULTS AND DISCUSSIONS

Protoplasts were readily isolated from etiolated seedlings of the three *Sorghum* cultivars (Plate 1). The optimal pre-treatment time, which gave the highest yield and viability values in 600 mM sorbitol was 3.5 hours in all cultivars. Pre-treating the tissues for longer periods was detrimental to both yield and viability of protoplasts (Table 1, Fig. 1). In general, cultivar MH51 gave the highest proportion of viable protoplasts while cultivar X gave the least. The unpretreated tissues (control) gave yields which were significantly lower than ($P<0.01$) yields from pre-treated tissues for each cultivar (Table 1).

Protoplast viability for the unpretreated tissue did not differ significantly ($P>0.05$) from those pretreated for 1 hour in sorbitol but differed significantly ($P<0.01$) from those pretreated for longer periods. This was observed in all the cultivars which further consolidates reports from previous workers that pre-treating the tissues in sorbitol before enzyme digestion enhances protoplast viability and yield (Nishimura *et. al.*, 1984; Wright, 1985; Theodoropolous and Roubelakis-Angelakis, 1990).

Protoplast release started within 2 hours of enzymic digestion but the optimal incubation time in the enzyme mixture was 2.5 hours (Fig. 2). Incubating beyond this period resulted in a considerable reduction in yield due to a high proportion of the protoplasts becoming lysed. Protoplast yield at optimal conditions (2.5 hours digestion in enzyme mixture following a 3.5 hour pre-treatment in sorbitol) was in the range of 6.8-9.22 x 10^5 per gram fresh weight of hypocotyl tissue (Table 1). The purified isolate did not show fluorescence when stained with calcoflour white indicating that cell wall digestion was complete. Cultivar MH51 gave the highest protoplast yield ($P<0.05$) and the best viability while the unimproved cultivar (X) gave the lowest yields.

Yields obtained in the present study were similar to those obtained from suspension cultures of other *Sorghum* cultivars reported by Wei and Xu (1990). Also, since etiolated seedlings synthesize cell wall with less pectate components (Bengochea and Dodds, 1986; Tan *et. al.*, 1987; Bellini *et. al.*, 1990) enzyme treatment time was considerably lowered (2.5 hours) which in turn decreased the toxic effects of enzyme preparations thereby facilitating the production of viable protoplasts. Previous workers have reported 14-16hours as optimal enzyme treatment time for protoplast isolation when suspension cells of some *Sorghum* cultivars were employed (Wei and Xu 1990).

Table 1: Protoplast yield (X ± S.E) in three *Sorghum* cultivars following different periods of pre-treatment in sorbitol and 2.5 hrs digestion in enzyme mixture

Cultivar	Pre-treatment Period (hrs)	Yield (per gm fresh weight)
MH51	0	701,000*c± 48.9
	1	740,000b± 52.2
	3.5	922,000a ± 64.7
	5	890,000a ± 43.2
	7	820.000b± 39.0
CH9	0	580,000e ± 32.8
	1	640,000d ± 47.2
	3.5	810,000b ± 84.3
	5	780,000b ± 64.2
	7	720,000c ± 54.9
CVX	0	490,000f ± 39.8
	1	540,000ef ±54.7
	3.5	680,000d ± 62.1
	5	580,000e ± 43.2
	7	590,000e ± 44.0

* *Figures followed by the same case letter are not significantly different from each other (P>0.05)*

Plasmolysing cells with alcoholic sugars (sorbitol or mannitol) or carbohydrates (sucrose and glucose) before enzyme treatment enhances the stability of

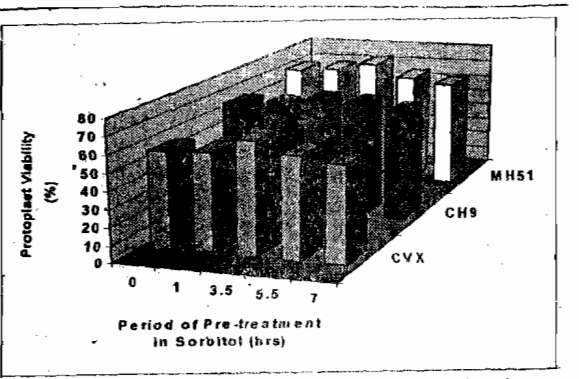

Fig. 1: Viability of protoplasts in three cultivars of *Sorghum bicolor* under different pre-treatment periods

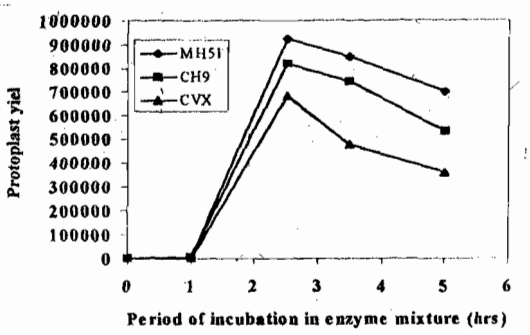

Fig 2: Effect of incubation time in enzyme mixture on protoplast yield

the protoplasts (Eriksson, 1985). It also improves protoplast yield (Nishimura *et. al.*, 1984; Wright, 1985; Theodoropolous and Roubelakis-Angelakis, 1990). The results of this study show that 3.5 hours of 600mM sorbitol pre-treatment time was optimal for yield and viability of protoplasts. Keeping the tissues for longer periods in sorbitol adversely affected viability (Fig. 1) and yield (Table 1). The exact pretreatment time, however, depended on the thickness of the materials as well as the conditions under which they were raised. The latter determines the amount of cutin deposits on the cell walls. For example, whole vines of *Vitis vinifera* raised in the green house required 24 hours of pretreatment for optimal yield (Wright, 1985) while those grown in vitro required 72 hours for a significant increase in yield (Theodoropolous and Roubelakis-Angelakis, 1990). Both groups of investigators used similar pre-treatment media.

In summary, the results of the present study showed that etiolated seedlings can provide a rapid and high-yielding source of viable *Sorghum* protoplasts.

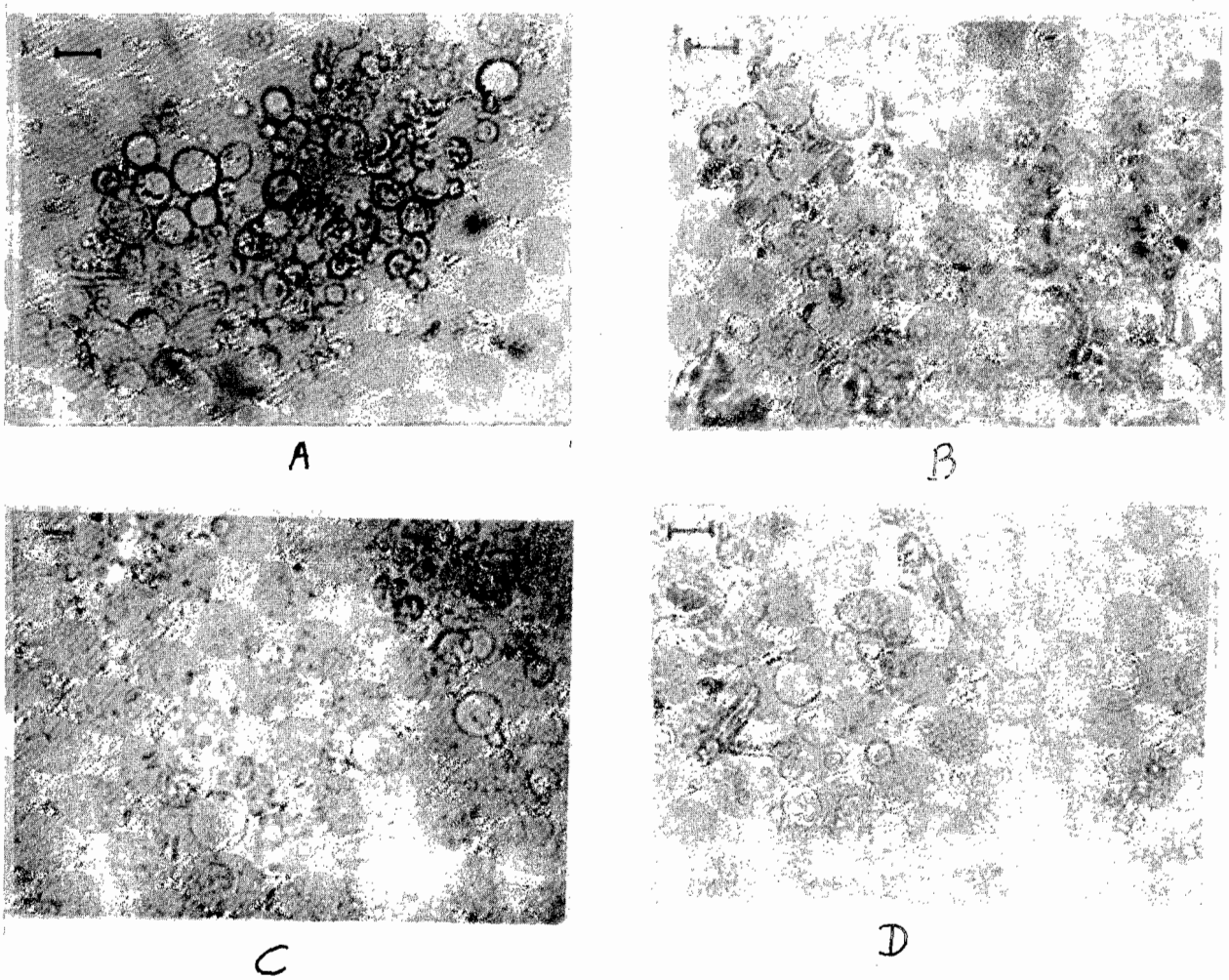

Plate 1: Freshly isolated protoplasts of *Sorghum bicolor* following pretreatment in sorbitol and digestion in enzyme mixture. (Scale bar = 40 µ)

A - MH51
B - CH9
C - CVX
D - Protoplasts from untreated tissues (control)

ACKNOWLEDGEMENT

This research was supported by a grant to E. O. Umoh from the Third World Academy of Sciences (TWAS) Italy. The authors wish to thank Raw Materials Research and Development Council (RMRDC) of Nigeria and COSTED, Madras, India, for some financial support, and Spic Science Foundation, Madras, for providing some materials and facilities.

REFERENCES

Bellini C; Chupeau, M; Gervais, M; Vastra, E and Chupeau, Y. 1990. Importance of myoinositol, calcium and ammonium for the viability and division of tomato *Lycopersicon esculentum* protoplasts. Plants cell tissue and Organ culture 23: 27 – 37.

Bengochea, T, and Dodds, J. H. 1986. Plant Protoplasts: A Biotechnological Tool for Plant improvement (1st Edition), Chapman and Hall, London 90 p

Cai T, and Butler, L. 1990. Plant Regeneration from embryogenic callus initiated from immature inflorescence of several high-tannin *Sorghum*. Plant cell tissue and organ culture 20: 101-110.

Chin, T. C and Scot, K. 1979. A large-scale procedure for isolation of cereal mesophyll protoplasts. Annals of Botany 43: 23-32.

Chourey, P.S. and Sharpe, D. 1985. Callus formation from protoplasts of *Sorghum* cell suspension cultures. Plants Science 39: 171 – 175

Eriksson, T. R. 1985. Protoplast Isolation and Culture in Plant Protoplasts, Fowke, L,C. and Constable, F. (eds) CRC Press, Boca Raton pp 1-10.

Frearson, E. M., Power T, B and Cocking, E 1973. The Isolation Culture and Regeneration of *Petunia* Leaf Protoplasts. Developmental Biology. 33: 130-137.

Galun, E 1981. Plant protoplasts as physiological tools. . Annual Review of Plant Physiology 32: 237-266.

Guo, J and Liang, G. 1991 Isolation Culture and regeneration of *Sorghum* Protoplasts. *Sorghum* Newsletter 32: 5.

Hagio, T., Blowers, A. D and Earle, E. D 1991. Stable transformation of *Sorghum* cell cultures after bombardment with DNA-coated micro-projectile. Plant Cell Reports 10(5): 260-264.

Millam, S., Burns, A. T. H. and Hocking, T 1991. A comparative assessment of purification techniques for mesophyll protoplasts of *Brassica napus* L. Plant cell Tissue and Organ Culture 24: 43-48.

Murphy, U. R. and Cocking E. C 1988. Somatic hybridization attempts between *Sorghum bicolor* (L) Moench and *Oryza sativa* (L). Current Science 57: 668–670.

Nagata,T. and Takebe, T. 1970. Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta 92: 301-308.

Nishimura, M., Hara-Nishimura, T. and Robinson, S. 1984. Isolation of metabolically competent protoplast from grapevine. Plant Science letters 37: 171-175

Sharma, V.; Kothari, S. L and Chandra, N 1989. In vitro regeneration, field transfer of plantlets and growth to maturity of plants of *Sorghum bicolor* (L). Moench. Current Science 58(10): 586-588.

Tan. M., Rietveld F., Van Marrewi, G. F. and Kool, A. J. 1987. Regeneration of leaf mesophyll protoplasts of tomato cultivars (*L. esculentum*): factors important for efficient protoplast culture and regeneration. Plant cell Reports 6: 172-175.

Theodoropolous, P. A. and Roubelakis-Angelakis, K. A. 1990. Progress in Leaf protoplast isolation and culture from virus-free axenic shoot cultures of *Vitis vinifera* L. Plant cell tissue and organ culture 20: 15-23.

Wei, Z. and Xu, Z. 1989. Plantlet regeneration from *Sorghum* protoplast cultures. Plant physiology communications. 6:45-46.

Wei, Z. and Xu, Z. 1990. Regeneration of fertile plants from embryogenic suspension culture of protoplasts of *Sorghum vulgare*. Plant cell reports 9: 51-53.

Widholm, J. M. 1972. The use of fluorescein diacetate and Phenosafranine for determining viability of cultured plant cells. Stain Technology 47(4): 189 –194.

Wright, D. C. 1985. Factors affecting isolation of protoplasts from leaves of grape (*Vitis vinifera*). Plants cell Tissue and organ culture 4: 95-100.