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ISOLATION AND YIELD OF PROTOPLASTS FROM DIFFERENT TISSUES OF *TRITICUM AESTIVUM* IN STERILE CULTURE

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Abstract

Protoplasts from leaves, roots excised from young seedlings and calli induced from roots of seeds have been isolated with a digestion mixture containing 2.5% Macerozyme, 2.5% cellulase Onozuka-R-10 and 1% hemicellulase in 0.7% mannitol at pH 5.6 after 6 h incubation. Young leaves yielded highest amount of protoplasts and appeared to be a good source of plant material.

Introduction

Since the enzymatic isolation of protoplasts from plant tissues (Cocking, 1960) there have been an increasing interest of plant scientists in the subject because of their great potential in genetic modification of plant cells and somatic hybridization (Dudits *et al.*, 1977; Evans *et al.*, 1980; Power *et al.*, 1976). The present communication reports method for the isolation of protoplasts from various tissues of *Triticum aestivum* cv. Chanab 70, a high yielding wheat variety under laboratory conditions.

Material and Method

Seeds of *T. aestivum* cv. Chanab-70 surface sterilized in 0.2% aqueous solution of mercuric chloride for 15 m, were washed in sterile distilled water and germinated on moist filter paper in dark at 30-33°C. Leaves from 5-6 day old seedlings were excised and used for isolation of single cells and protoplasts. In another set the germinated seedlings were transferred to pots and after one month mature leaves were excised. Similarly 1 cm. long distal parts of root tips from one week old seedlings were excised and used for the isolation of protoplasts.

For callus induction surface sterilized seeds were soaked in sterile water for 10-12 h. Germinated seeds were placed on complete medium of Murashige & Skoog (1962), containing 5 mg/l 2, 4-D, at pH 5.6. The cultures were incubated at 30°C in complete darkness. After two weeks calli showing active growth and more than 1 cm in diameter, were collected and used for the isolation of protoplasts. Mixed enzyme method was used for the isolation of protoplasts. Initially, the optimum concentration of Macerozgme for the isolation of single cells was determined which was then tested against a range of cellulase concentrations. "Macerozyme-R-10" (Kinki Yakult Mfg,

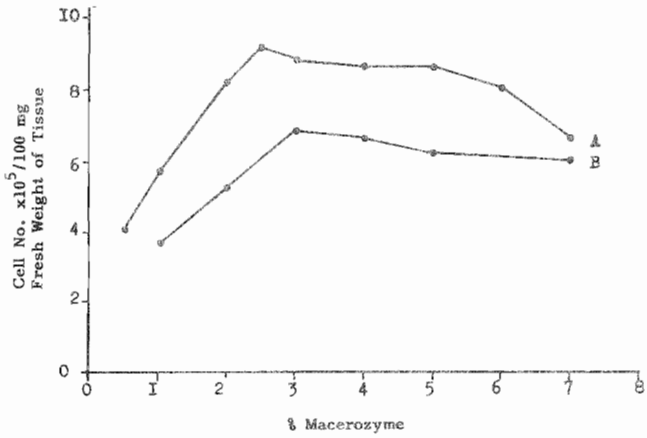

Fig. 1 Effect of Macerozyme concentration on the yield of single cells of wheat obtained from young leaves (A) and from one month old leaves (B). (Mannitol supplied at 0.6M; pH 5.4 after 6 hr. incubation).

Co. Ltd., Nishinomiya, Japan), "Cellulase Onozuka-R-10" (Kinki Yakult Mfg. Co. Ltd., Nishinomiya, Japan) and "Hemicellulase" (Sigma Chemical Co., St-Louis, Mo.) passed through 0.22 μm Millipore filters were used. Plant material in 100 mg quantities was used for each treatment. Enzyme-tissue mixture was incubated at 30^{O}C in dark and was frequently shaken to ensure mixing of the tissues with the enzyme solution. The digest containing single cells and protoplasts was repeatedly expelled through a hypodermic syringe, in order to dissociate the cell walls and finally passed through 100μm mesh and centrifuged at 100 g for 2-3 m., to remove cell wall debris. The pellet was washed three times with 0.6 M mannitol and resuspended in 20% sucrose solution in water. On centrifugation at 100 g protoplasts float on the surface whereas cells and cell debris settled to the bottom. Protoplasts were removed with the help of a fine pipette. The isolated fraction was diluted to a known volume with 0.7M mannitol and number of protoplasts recorded using a haemocytometer.

Results

Number of cells in 5-6 day old leaves was much greater as compared to one month old leaves (Fig. 1). Tissues of younger leaves required 2.5% Macerozyme for complete digestion of tissues into single cells as compared to 3% level for mature leaves. Out of different level of cellulases tested, in combination with 2.5% Macerozyme, 2.5% cellulase produced maximum number of protoplasts. Addition of a range of concentrations of hemicellulase to the mixture of enzymes upto 1% level increased the yield of protoplasts whereas above 1% level the yield gradually decreased (Fig. 2).

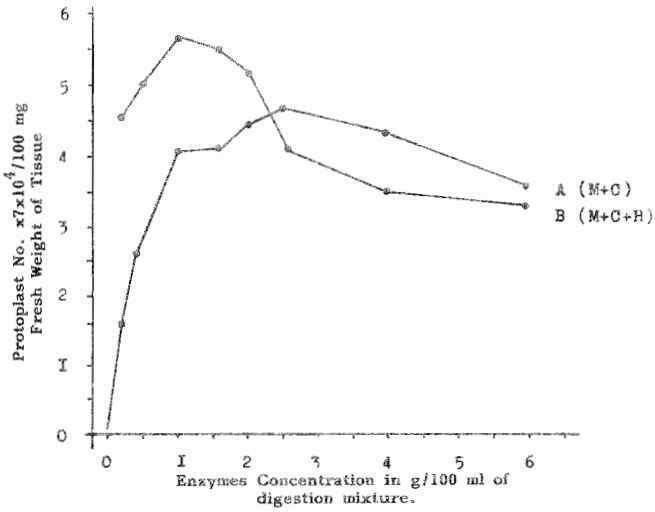

Fig. 2 Effect of cellulase Onozuka (A) and hemicellulase (B) on the frequency of protoplasts isolation from 5-6 days old leaves of wheat. (Mannitol supplied at 0.6M; pH 5.4 ofter 6 hr. treatment). Digestion mixture in A contained Macerozyme (M) at 2.5% in addition to a range of cellulase concentrations. Digestion mixture B contained a fixed level of Macerozyme (M, 2.5%) and cellulase Onozuka (C, 2.5%) along with a range of hemicellulase concentrations, tested.

The satisfactory concentrations of mannitol, as osmoticum, were spread over a wide range from 0.4-0.8 M. Although above 0.7M the decline in yield was not very rapid but the number of protoplasts was less when compared with 0.7M. Mannitol 0.7M was therefore selected for future experiments (Fig. 3). Other sugars and their derivatives

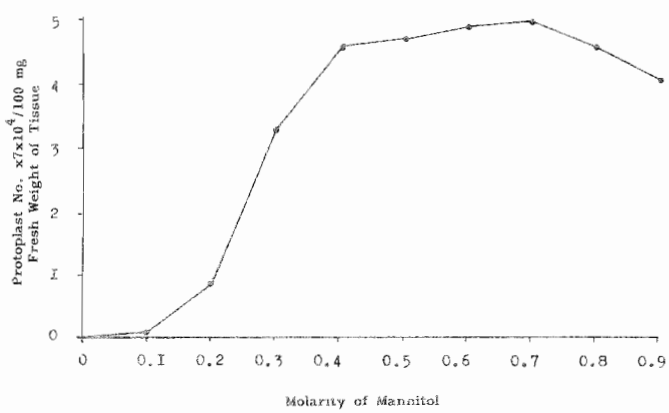

Fig. 3 Influence of mannitol concentrations on the yield of protoplasts from young wheat leaves. Digestion mixture contained 2.5% Macerozyme, 2.5% cellulase Onozuka, and 1% hemicellulase in the above mentioned range of mannitol concentrations at pH 5.4. Duration of treatment was 6 hr.

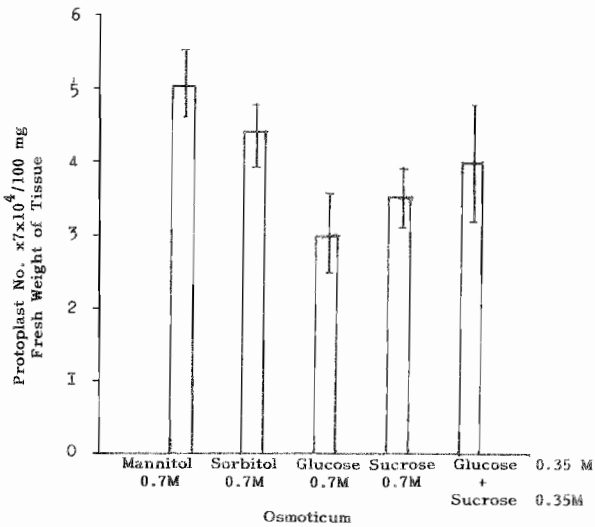

Fig. 4 Comparison of different osmotic agents on the yield of wheat protoplasts. The incubation mixture and conditions of incubation were same as in Fig. 3.

used as osmotic agent did not give yield higher than mannitol. Yield of protoplast considerably increased when glucose was used with sucrose at 0.35M, (Fig. 4). Mannitol was therefore consistently used as the osmotic agent, since none of the treatment showed an improvement in yield. Highest yield of protoplasts was obtained when pH of the digestion

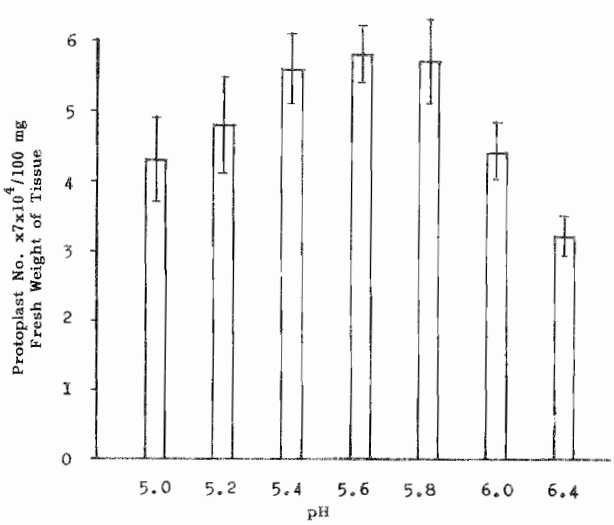

Fig. 5 Effect of pH on the yield of protoplasts from wheat leaves. Composition of digestion mixture and other conditions of incubation were same as in Fig. 3. Mannitol concentration was maintained at 0.7M.

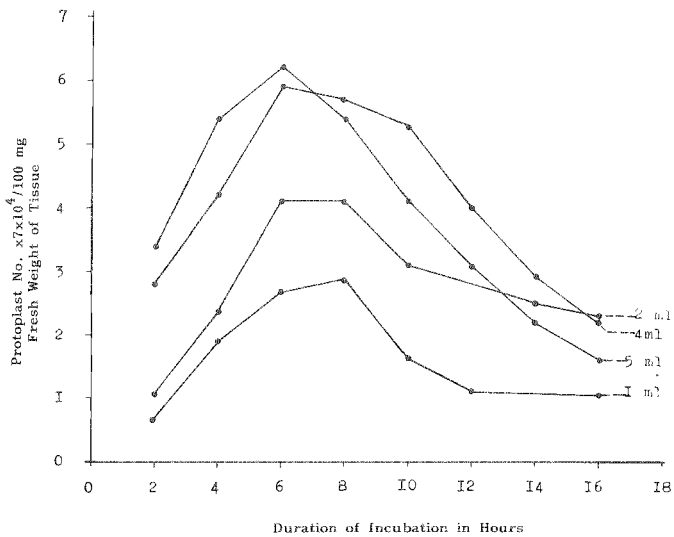

Fig. 6 Effect of the duration of incubation and the volume of cell wall digestion mixture on the isolation of protoplasts from leaves of wheat. Composition of digestion mixture and other conditions of incubation were same as in Fig. 3. Mannitol was supplied at 0.7M, at pH 5.6.

mixture was set at 5.6. However, pH below 5.2 or above 6.0 were less effective as they yielded less number of protoplasts (Fig. 5).

An incubation period for complete digestion of 100 mg of tissue for maximum yield of protoplasts was 6 h when the volume of digestion mixture was 2 ml or more. A decrease in the volume of digestion mixture resulted in an increase in the duration of incubation, affecting maximum yield of protoplasts (Fig. 6). Longer duration of incubation was deleterious for the survival of the protoplasts as their number declined rapidly. Rate of bursting of protoplasts was faster in the treatments containing larger volume of digestion mixture as compared to 1-2 ml of the incubation mixture as is evident from the rate of fall in yield during incubation extending from 6-16 h.

Young leaves were the best source for the isolation of protoplasts since number of protoplasts produced by excised roots was less than half of the number produced by young leaves. However, callus tissues gave slightly better yield than excised roots. Supplementing the digestion mixture with the M.S. salts (Murashinge & Skoog, 1962) reduced the yield (Fig. 7).

Discussion

Small blade of cereal leaves does not constitute a suitable plant material for peeling or stripping of epiderims, which is a common practice in the isolation of proto-

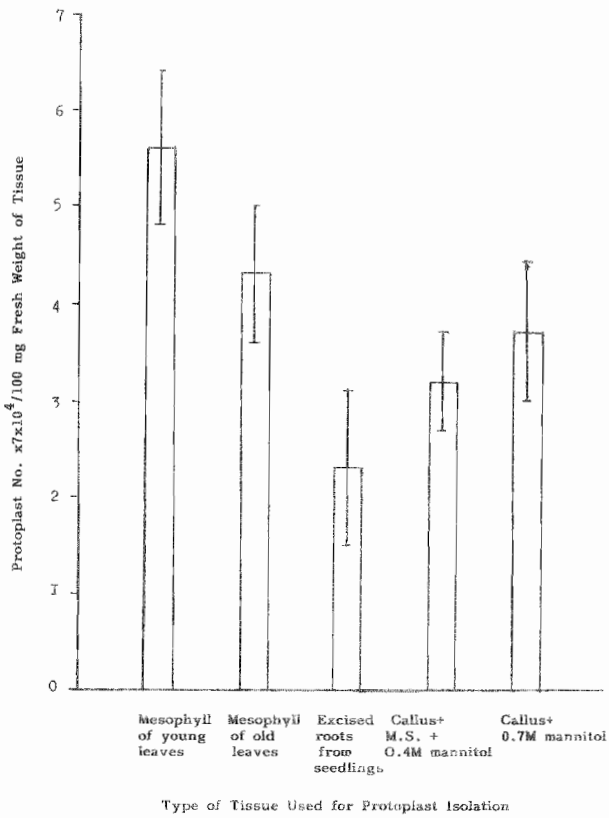

Fig. 7 Comparison of the type of tissue used for the isolation of protoplasts and its effect on the yield. Composition of the isolation medium and conditions of incubation were same as in Fig. 5. Volume of the digestion medium was 4 ml. with pH 5.6.

plasts from leaf tissues (Ruesink & Thimann, 1965; Bawa & Torrey, 1971; Hsiang hui, 1978; Hsiang-hui *et al,* 1978; Chin-sheng, 1978). The method of cutting leaves into small pieces and incubating them in the digestion mixture for longer periods with intermittent shaking proved satisfactory as it yielded large quantities of protoplasts.

Cellulose has been used either alone (Grambow *et al,* 1972; Eriksson & Jonasson, 1969; Motoyoshi, 1971; Bawa & Torrey, 1971) or in combination with other enzymes (Scott *et al,* 1978, Cassell & Barlass, 1978) in such studies. In the present work mixed enzyme method was used. Addition of hemicellulase to the enzyme mixture increased the yield. Cereal leaves are known to contain large amount of hemicellulose in their cell walls (Buchala & Wilkie, 1974) hence the increase in yield resulting from incorporation of hemicellulase may be attributed to the breakdown of hemicellulose, becoming

unaffected by cellulase and pectinase treatment. In barley, a ten fold increase in the yield of protoplasts after the addition of 1% hemicellulase has been reported by Scott *et al,* (1978), however, in the present work it amounts to approximately 11.5%.

Glucose or sucrose either used alone or in combination proved to be a less effective osmoticum as compared to sorbitol or mannitol. A detrimental effect of sucrose, glucose and fructose on the survival of protoplasts in barley has been reported by Scott *et al* (1978). They observed a bursting of 80% of the initial population, incubated in the presence of three sugars, whereas sorbital gave the highest yield. However, in corn Bawa & Torrey (1971) observed maximum release, survival and nuclear division of protoplast in the presence 0.3M mannitol containing 1% socrose. There are reports of the use of two sugar derivatives viz., mannitol and sorbitol either singly (Chin-sheng, 1978; Hsiang-hui, 1978; Hsiang-hui *et al,* 1978; Scott *et al,* 1978 Ruesink & Thimann, 1965) or in combination (Bawa & Torrey, 1971; Kao *et al,* 1973 and Kao, 1978). However, mannitol has widely been used, as osmotic agent. In the present study with *T. aestivum* cv. Chanab-70 gave maximum yield. Since the culture of isolated single cells and protoplasts of cereals has not met with great success, therefore, none of the sugars or their derivatives can be considered as most suitable.

Commercially available preparations of enzymes contain a certain amount of closely related enzymes as contaminants. Cellulase preparations obtained from *Trichoderma viride* and from abscission zone of *Acer pseudoplatanus* have been shown to give two peaks of activity with respect to pH, indicating the presence of two isozymes (Linkins & Lewis, 1974; Sheldrake, 1970). Uchimiya & Murashige (1974) also observed two optima of yield with respect to pH during isolation of protoplasts from cultured cells of tobacco which have been suggested to be due to two isozymes present in the preparation of cellulase used. In the present work maximum yield of protoplasts over a wide range of pH ranging from 5.4-5.8 might be a manifestation of the activity of individual enzymes of the digestion mixture or isozymes of any one or more of the enzymes used during investigation (Fig. 5).

Volume of digestion mixture and period of incubation in the enzyme mixture proved to be two important parameters in determining the maximum yield of protoplasts (Fig. 6). The tissues required at least 6 h of incubation in the enzyme mixture provided the volume of digestion mixture was more than 2ml. In the presence of sufficiently high concentration of enzymes peeling of the epidermis, absence of vacuum infiltration of the tissues with the enzyme mixture at zero time and lack of continuous shaking appear to be the factors requiring such prolonged incubation period during isolation.

Out of two physiological stages: younger (5-6 day old) leaves always yielded more protoplasts as compared to the old ones. This observation is in line with the view that

young tissues or organs constitute a more suitable plant material for the isolation of protoplasts as compared to the old ones (Uchimiya & Murashige, 1974, Wen-an, 1978). Excised roots from seedlings gave the lowest yield of protoplasts. Roots lack large intercellular spaces and a large proportion of cells is vacuolated parenchyma. A slow rate of penetration of enzyme mixture in the tissues or perhaps the low rate of survival might be the possible reasons for such low yield. Protoplasts released from large vacuolated cells stand a lesser chance of long survival as they are more prone to bursting than the ones from small, non-vacuolated cells. Calli induced from roots of the germinating seeds though released an appreciable amount of protoplasts but their number was less than the leaf tissues. Whether the calli were not at the appropriate metabolic state to release large quantities of protoplasts needs experimentation.

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