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Protoplast culture and transformation studies of Triticale (x Triticosecale Wittmack).

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1. INTRODUCTION

Plant regeneration from protoplasts offers a unique and essential technology for genetic engineering studies of higher plants. The major cereal crops however are still considered to be difficult for protoplast culture. There are very few reports on plant regeneration from protoplasts of the Gramineae. Fertile plants have been regenerated from protoplasts of Oryza sativa L. (Fujimura. et al.,1985, Yamada et al., 1986, Abdullah et al.,1986) sterile plants of Zea mays L. (Rhodes et al., 1987) and albino plantlets of Hordeum vulgare L.(Lührs and Lörz 1988) for more details see reviews (Vasil, 1987; Lörz et al., 1988).

Stable integration of defined genes in cereal species has been achieved by transformation of protoplasts with naked DNA in the presence of polyethylene glycol (Potrykus et al., 1985, Lörz et al., 1985, Uchimija et al., 1986) by electroporation (Fromm et al., 1986) or by in situ injection of plasmid DNA into floral tillers of Secale cereale L. (De la Pena et al., 1987).

Non-stable transformation so-called transient gene expression, in the Gramineae family has been reported in tissue culture derived protoplasts of Zea mays L. (Fromm et al.,1985),Triticum monococcum L., Sorghum bicolor L. and leaf derived protoplasts of Oryza sativa L.(Ou-Lee et al. 1986). More recently PEG-facilitated plasmid DNA uptake has been shown in dividing and non-dividing protoplast culture systems of Zea mays L., Hordeum vulgare L., Oryza sativa L. and Secale cereale L. (Junker et al.,1987).

In this report we present a protoplasts culture system of Triticosecale used for transformation studies with different plasmid constructions containing a selectable chimeric gene coding for kanamycin resistance.

2. PROCEDURE

2.1 Materials and Methods : Establishing cell suspension cultures from embryogenic callus of Triticosecale has been described previously (Stolarz and Lörz, 1986). In order to select fast growing cell suspension sublines macroplates (Greiner No 657102) with 6 units were used as culture vessels. The cell suspensions were maintained by subculturing 0.2 ml aliquots to 2 ml of fresh medium (Kao$_0$1977) into each unit at 4 days intervals and cultured at 26^{0}C in light on a gyratory shaker at 120 rpm. After 10-15 subcultures each subline used for further experiments was characterized by growth curves (fresh and dry weight). Samples for measurements have been collected at daily intervals in 6 replication. Protoplasts

were isolated from cell suspension cultures 2-10 days after subculture. The cell aggregates from a complete macroplate were collected and fresh weight was determined. 0.5 g. of the cells was mixed with 10 ml of an enzyme mixture consisting of 2% Cellulase RS Onozuka, 1% Macerozyme R 10 and 0.05% Pectolyase Y23 with osmolarity adjusted to 780 mOsm/kg H_2O with glucose. The cells were incubated on a gyratory shaker at 50 rpm for 2-2.5 h at 27°C in dark. Protoplasts were then passed through a series of sieves of 250,100,50 and 25 µm diameter mesh size to remove undigested cell clusters and cell debris. The protopsts were washed twice by centrifugation in seawater. Isolated protoplasts we resuspended in culture medium and plated at densities of 1-5 x 10^5 protoplasts/ml in 2 ml or 6 ml of liquid medium in Petri dishes (5 or 10 cm diameter) or directly embedded in agarose. The medium used for protoplast culture was identical to the suspension medium but with different concentrations of 2,4-D (0.5-8 mg/l). Plating efficiency was evaluted after 2 weeks. For transformation experiments 4 different plasmid constructions containing the NPT II gene have been used namely pLGVneo1103 and pLGVneo2103 (Hain et al., 1985), pRT99 and pRT100neo (Töpfer, 1987). Protoplast transformation was performed according to the method described by Krens et al. (1982). After treatment with DNA the protoplasts were washed and resuspended in culture medium at density 2.5 x 10^5 protoplasts/ml. The NPT II assay followed the procedure described by Reiss et al. (1984).

3. RESULTS

3.1 PROTOPLASTS ISOLATION AND CULTURE :Protoplasts of the triticale cell suspension (Fig.1) can be isolated with different efficiency depending from the time after subculture and osmolarity of the enzyme solution used for protoplast isolation.

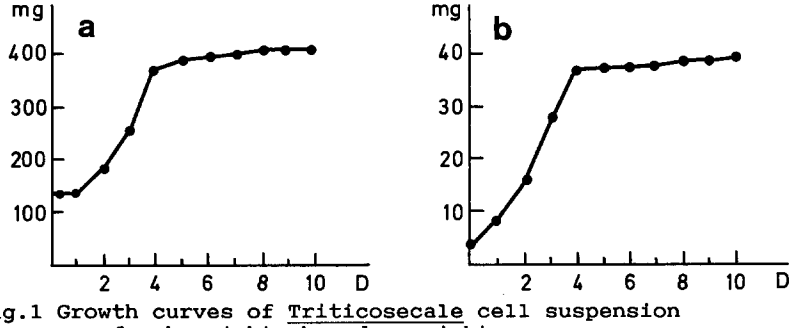


Fig.1 Growth curves of Triticosecale cell suspension
a - fresh weight, b - dry weight

Protoplast isolation at day 2 or 3 after subculture yields a low number of protoplasts (1-2x10^6) and these are capable to form colonies with efficiencis of 3-6%. The highest yield of 7x10^6-3x10^7 protoplasts per gram of fresh weight could be obtained from cell suspensions at day 7 or 8 after subculture.

In that case, plating efficiency evaluated after 2 weeks in liquid medium was high and ranged from 36% to 54%. The highest plating efficiency (60%) has been achieved from protoplasts isolated at day 10 after subculture. First divisions occurred after 3-5 days in protoplast cultures isolated 7 or 8 days after subculture. In protoplast cultures isolated after 2-3 and 10 days first divisions were observed only after 8 days or later. During the course of the experiments we found that embedding protoplasts in agarose redused plating efficiency significantly. Variation of the concentration of 2,4-D in the culture medium between 2-6mg/l had no effect on plating efficiency and time of the first divisions. Further experiments are aiming towords plant regeneration from protoplasts.

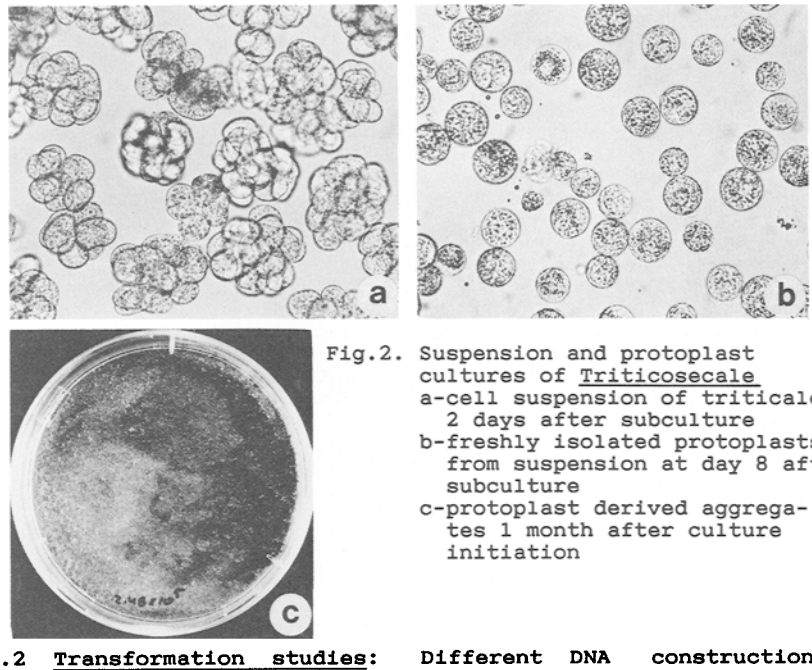
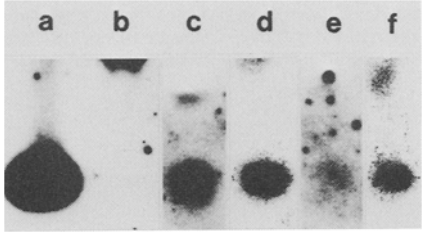


Fig.2. Suspension and protoplast cultures of Triticosecale
a-cell suspension of triticale 2 days after subculture
b-freshly isolated protoplasts from suspension at day 8 after subculture
c-protoplast derived aggregates 1 month after culture initiation

3.2 Transformation studies: Different DNA constructions carrying the NPT II gene as a marker gene have been used to transform suspension protoplasts of triticale. Positive results in transient gene expresion have been obtained by using the plasmid pRT99 and pLGV1103. The activity of the gene product neomycin phosphotransferase has been measured in the extract of treated protoplasts from day 2 to day 10 after transformation. The suspension protoplasts of triticale were treated with identical amounts of plasmid DNA (40µg). Intensity of expresion of the NPT II gene seems to be influenced by the promoter (p35S or pNOS) fused to the marker gene NPT II (Fig.3). Meanwhile protoplasts treated with

plasmid DNA have been transferred to selection medium containing 100mg/l geneticin. Further experiment are needed to optimize DNA uptake and the selection scheme.



a-transformed tobacco (positive control)
b-triticale protoplasts without DNA
c-10^6 protoplasts treated with pLGVneo1103 assayed after 4 days
d-10^6 protoplasts treated with pLGVneo1103 assayed after 10 days
e-10^6 protoplasts treated with pLGVneo2103 assayed after 4 days
f-10^6 protoplasts treated with pRT99assayed after 2 days

Fig.3.Transient expresion of the NPT II gene constructions in triticale suspension protoplasts.

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