Plant regeneration system in protoplast culture of *Gentiana* genus

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Introduction

Long term studies on morphogenetical potential of various species of *Gentiana* genus revealed high morphogenic potential of their different tissues and organs. Obtained long-term cell suspensions characterised by embryogenic capability appeared background for changing the level of experiments: from tissue to cell. Protoplast - plant cell without cell wall - in sense of structure, organization and function reveals as the final subject of experiments concerning plant regeneration from single cell.

The aim of the presentation was to work out plant regeneration system initiated from protoplasts and completed by the culture of *Gentiana kurroo* Royle plantlets.

Material and Methods

Protoplasts were isolated from one year old cell suspension originated from cotyledons and hypocotyl embryogenic callus cultured on MS medium supplemented with A) 0.5mg/l 2,4-D + 1.0 mg/l kinetin and B) 1.0 mg/l dicamba + 0.5 BAP + 0.1 mg/l NAA + 80.0 mg/l AS. Before protoplast isolation cell suspensions were divided on the fractions according to the size of cell aggregates: >500µm, 500-300µm and 300-150µm. For protoplast isolation 1 gram of suspension was treated with 10ml of the enzyme mixture: Hemicellulase 0.25%, Pectolyase Y23 0.04%, Driselase 0.5%, Macerozyme R10 1.5%, Cellulase RS 1.5%. For protoplast culture three types of culture were employed: liquid medium or thin agarose (Sea Plaque) layer and "bead culture" (Tab.1). Optimalization of osmoticum required the use of the following concentrations of mannitol: 3%, 6% and 9%. Additionally, 11% and 13% mannitol was tested for the creation of system of direct protoplast development in somatic embryo. Protoplasts were implanted with density 2 x 10^5/ml. After 10 days of culture plating efficiency (PE) was scored. The osmotic pressure of the medium was decreased weekly. Cultures of beats and thin layers were subcultured with the help of induction medium (IM): MS + 0.5mg/l 2,4-D + 1.0mg/l kinetyny + 8g/l agar for callus proliferation and regeneration medium (RM): MS + 0.5mg/l GA_3 + 1.0mg/l kinetin + 80 mg/l AS + 8g/l agar. Embyos were transferred to 1/2MS medium for conversion.

Tab. 1. Media used for protoplast cultures of *G. kurroo*

Name of medium	Medium compositions
MS 2,4-D	MS + 0.5mg/l 2,4-D + 1.0mg/l kinetin + 30g/l glucose + mannitol, pH=5,8
MS DIC	MS + 1.0mg/l dicamba + 2.0mg/l BAP + 80mg/l AS + 0.1mg/l NAA + 30g/l glucose + mannitol, pH=5,8
KM 2,4-D	KM + 0.5mg/l 2,4-D + 1.0mg/l kinetin + 30g/l glucose + mannitol, pH=5,8
KM DIC	KM + 1.0mg/l dicamba + 2.0mg/l BAP + 80mg/l AS + 0.1mg/l NAA + 30g/l glucose + mannitol, pH=5,8
MS (-NH_4NO_3) 2,4-D	MS (-NH_4NO_3) + 0.5mg/l 2,4-D + 1.0mg/l kinetin + 30g/l glucose + 3g/l glutamine + mannitol, pH=5,8
MS (-NH_4NO_3) DIC	MS (-NH_4NO_3) + 1.0mg/l dicamba + 2.0mg/l BAP + 80mg/l AS + 0.1mg/l NAA + 30g/l glucose + 3g/l glutamine + mannitol, pH=5,8

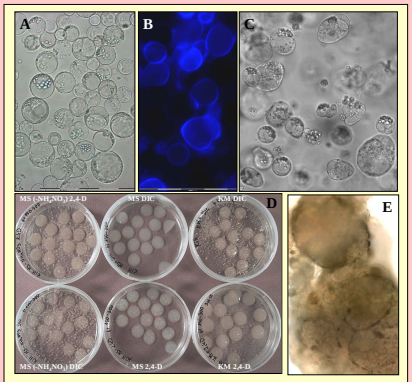


Fig. 1. A) freshly isolated protoplasts, B) cell wall reconstruction after 24 hours, C) initial cell division after 10 days, D) cultures maintained on various media, E) globular embryos on KM DIC medium.

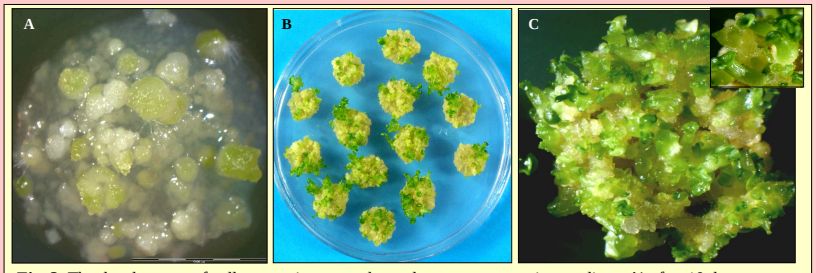
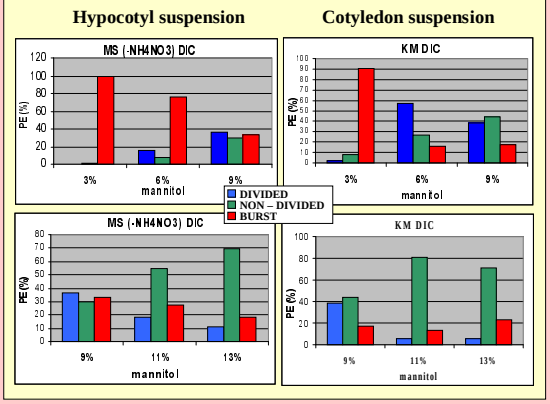


Fig. 2. The development of cell suspension protoplast culture on regeneration medium: A) after 10 days, B) after 4 weeks, C) after 6 weeks.

Results

The yield of protoplast isolation was dependent on the physiological condition of cell suspension and was scored 3.6 - 8.22 x 10^6 /ml and 6.0-7.2 x 10^6 /ml for hypocotyl and cotyledon derived cell suspension, respectively. After 24 hrs of culture cell wall formation with the help of calcofluor was recognised. And after next 24 hrs the initial division of protoplasts sporadically occurred when cultures were maintained in agarose medium (Fig.1). In liquid medium only not numerous protoplasts started to divide with at least 2 weeks of delay. The highest plating efficiency was obtained when protoplast were cultured in the presence of KM and MS (-NH_4NO_3) medium. There was not significant differences between two studied PGR combinations and fractions of cell suspension. Plating efficiency of cell suspension protoplasts was significantly important for organ origin of cell suspension but inconsiderable different when agarose method of culture was considered (Fig.3). Lower concentration of mannitol in culture media speeded up the development of the culture by two weeks.

Fig. 4. The effect of various mannitol concentrations on plating efficiency of various suspension of *G. kurroo* protoplasts after 10 days of culture for the best media.



It was observed that 6% of mannitol appeared to be enough sufficient concentration for protoplast growth stimulation of protoplasts originated from cotyledon derived suspension (Fig.4). Protoplast cultures originated from hypocotyl suspension required 9% mannitol. The highest mannitol concentrations did not support the better response than lower one. After six weeks of culture protocalli and embyoids in agarose media were observed (Fig.2), however, the somatic embryo formation sporadically occurred. After next three weeks protocalli were subcultured on RM and IM medium. Because of very fragile callus tissue, cultures were transferred together with agarose. After next two weeks somatic embyos were recognised, which usually developed in plantlets. Somatic embryos were regenerated only from cultures originated from KM and MS (-NH_4NO_3) media (Fig.5). Obtained plantlets were maintained on 1/2 MS basal medium and used for flow cytometry and molecular analysis.

Fig. 3. The plating efficiency of suspension protoplast in correlation to: source of suspension, type of cultur, fraction and culture media used.

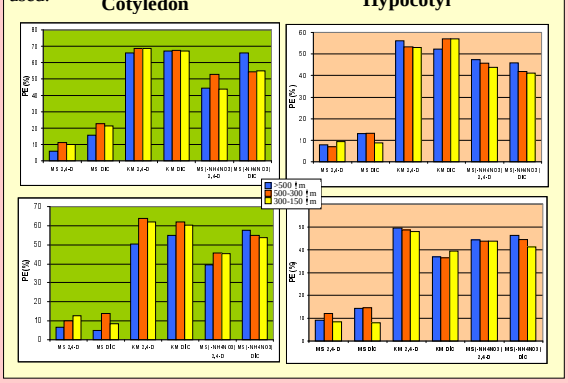
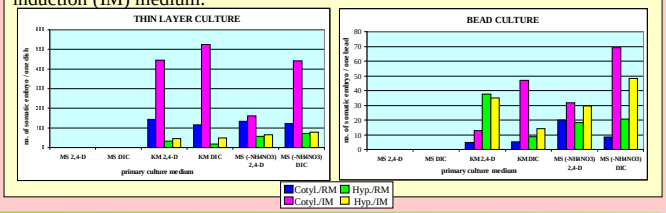


Fig. 5. The effect of type of protoplast culture and primary medium on somatic embryo production by protocalli depending on regeneration (RM) and induction (IM) medium.



Conclusions

1) Effective system of plant regeneration from protoplasts originated from various suspension cultures was developed,

2) The highest studied mannitol concentrations did not stimulate the growth and development of protoplast cultures and did not help to create system of direct embrygenic development of individual protoplast in somatic embryo,

3) The results suggest that age and physiological status of suspension could be responsible for gen expresions which are connected with direct embryogenesis likely to zygot divisions.

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