CALLI FORMATION, LIMITED ORGANOGENESIS WITH HYPOCOTYL AND COTYLEDON PROTOPLASTS OF *HELIANTHUS ANNUUS*

Trendafil NEDEV and Günter HAHNE*

Institute of Genetics, Bulgarian Academy of Sciences, Sofia, (Bulgaria) *IBMP du CNRS, 12 rue du General Zimmer, 67084 Strasbourg, (France)

Abstract

The application of somatic hybridization of sunflower requires efficient methods of plant regeneration from isolated protoplasts. the objective of this research was to establishe a protocol for cultivation and subsequent regeneration with inbred lines of sunflower. Protoplasts isolated from tissues of seedlings germinated in special darklight photoperiod were cultivated in different modifications of LEENE and CHUPEAU (1986) medium using alginate films. protoplasts started to devide relatively late but small calli dveloped. Rapidly growing microcalli were transfered to agar solidified medium according FISCHER et al. (1992), containing 0.05 mg/l α-naphtylacetic acid and 0.2 mg/l benzylaminopurine. Few calli derived from hypocotyl protoplasts produced shoot-like structures. This aspect of regeneration remains the goal of further investigations.

Key words: *Helianthus*, alginate cultivated protoplasts, organogenesis, regeneration.

Introduction

Among the crop plants, sunflower is important as a source of vegetable oil. Efficient protoplast isolation and culture conditions (OCHATT and POWER, 1992) are powerful tools for modification of plant genome. This is based on totipotency which is unique characteristic of plant cell. Data on protoplasts regeneration from *Helianthus annuus* show recalcitrance and are rather limited (CHANABE et al., 1989). The procedure of protoplast immobilization has become increasingly popular in protoplast cultivation (DAMM and WILLMITZER, 1988).

In the present paper, we compare the regeneration capabilities of protoplasts isolated from four cultivated sunflower genotypes.

Material and methods

Helianthus annuus genotypes HA 300, 21,31,35 were obtained from CNRS collection \Dr. G. Hahne, Strasbourg, France\. Germination in vitro, protoplast isolation and culture were according FISCHER et al., 1992. The protoplast-containing alginate disks were transferred into liquid culturing solution consisting of LC medium with 3mg\l α-naptylacetic acid \NAA\ and 1mg\l 6-benzylaminopurine \BAP\; 660 mOsm; pH 5.6. The protoplasts have also been plated in LC medium with 1mg\L 2,4- dichlorphenoxiacetic acid \2,4-D\ and 1mg\l BAP. Cultures were kept in dark at 25°C. After 7-10 days first liquid medium was removed and alginate embedded protoplasts were transfered to LC medium with 0.1 mg\l NAA and 1 mg\l BAP in

which all other components are the same. Further, every next week the medium was replaced with LC of decreased osmolarity \1% sucrose; 550-600 mOsm.

The microcalli were transferred to agar-solidified regeneration medium containing several phytohormones (see Table 1) and osmotic preasure adjusted to 500 mOsm with manit. Ten days later microcalli were cultured on the same medium with pressure 300 mOsm.

Plating efficiency is expressed as percentage of protoplasts that form colonies convenient for the transfer to agar medium.

Results, discussion and conclusion

Low viscosity alginates \Sigma and Roth\ were used in different final concentrations \1.0 and 1.4%\ as supporting material for protoplast culturing. Thus Sigma and Roth alginates were compared. Roth alginate proved to be more convenient, so 1% solution of it was used in further experiments.

Plating densities ranging from 1.10^4 to 1.10^5 ppl\ml alginate were compared. Divisions could be initiated at all cell densities (Fig 1) and all tested genotypes. For the HA 300 line the maximum efficiency was observed at density 5.10^4 ppl\ml alginate, whereas line 31 showed possibility to form morphogenic type of colonies at 1.10^4 ppl\ml alginate but at so low protoplast density the grow was impeded. At 1.10^5 ppl\ml browning occurred. In our work hypocotyl protoplasts gave equal plating efficiencies as compared to cotyledon system, but only hypocotyl protoplasts from line 31 regenerated from 1.10^4 ppl\ml, compared to all other types and genotypes.

As demonstrated from previous reports (SCHMITZ AND SCHNABL, 1989) culture medium with 2,4-D has a beneficial effect to achieve cell divisions and subsequent regeneration at different plant cultures including sunflower protoplasts. Thus, we tested utilization of the hormone 2,4-D, but optimal results were not found. Remarkable differences between media with 2,4-D and NAA were observed. Compact yellowish calli were formed on BAP/NAA containing medium. Medium containing BAP/2,4-D was not suitable for callus formation, frequently calli turn to brown. The medium according to FISCHER et al., (1992) seemed to give the best results in obtaining a "shoot-like structures" at line 31 developed on some calli.

When calli were transferred to regeneration medium, high speed of grow occurred. Similar calli were obtained from genotypes 21 and 35, but regeneration appeared only in line 31.

Several reports have been published concerning the regeneration of *Helianthus annuus* from protoplasts during the last several years (FISCHER et al., KRASNYANSKI and MENCZEL, 1993). In all experiments regeneration was reached in two different ways - organogenesis and somatic embriogenesis at two different genotypes. Our work is based on immobilization of the protoplasts in alginate and variation of parameters to optimize microcolony formation and organogenesis.

In the present paper we show an evidence for morphogenesis from hypocotyl protoplasts isolated from absolutely new genotype - line 31.

Only alginate is suitable for cultivation, in our experiments. We support the view of Fischer and Hahhe, contradictory of that, agarose appears to be an essential gelling agent for Helianthus protoplast culture (BOHOROVA et al., 1986). For Helianthus annuus the protection of wall - less cells by alginate matrics represents a method by which decompartmentation and its accompanying processes may be delayed. Since the vacuoles are known to accumulate damaging enzymes and trash salts may be one of the possible biochemical explanations for the effect of alginate is that proteases released from dying cells diffuse very slowly trough the alginate, this way their degradetative effect on protoplasts being controlled (DAMM and WILLMITZER, 1988).

Cell divisions could be induced, fast growing colonies (Fig 2)and calli (Fig 3) were produced at all densities but latter structures stopped growing after two-three weeks. May be a second condition for obtaining of morphogenesis is the low plating density. Cultivation of about 1.10^4 densities proved to be very satisfactory due to the dilution of the phytotoxic factors and the pH of LC remained stable, because this decrease prevents acidification of the medium. Such culture densities are 5 time less than the normal optimal value for sunflower - 5.10^4 ppl\ml (CHANABE et al., 1989), which would result. in reducing of inhibitors of protoplast division.

The microcali arrising from alginate disks were transferred to a regeneration medium proposed by FISCHER et al., (1992). Morphogenesis started in ten days after transfer (Fig 4).

As for morphogenesis the induction was possible with NAA, but not with 2,4-D. NAA was as effective as BAP for the induction.

Recent studies have shown that treatment with 2,4-D of the culture medium can dramatically improve the frequency of initiation of shoot formation - 10 times or more, but it may inhibite organogenetic capabilities of the non-differentiated tissue too (BOHOROVA et al., 1986). The results obtained here, also, strongly suggested that NAA but not 2,4-D plays an important role in subsequent development of shoot formation. Therefore, we assume that NAA is an essential plant regulator for morphogenesis of cultured sunflower cells in our work though no direct evidence is available at present.

Our work indicates that some factors are important for regeneration from sunflower protoplasts:
- embedding in alginate and low starting plating densities
- using of modified LC medium with NAA and BAP, but not with 2,4-D
- choice of convenient genotype. In many cases, e.g. tomato, success was limited to specific genotype under special conditions. Investigations suggest that the regeneration capacity is a dominant trait, which can be used as a selectable marker in protoplast fusion experiments. A comparison of related genotypes reveals that *Helianthus annuus* regeneration potential *in vitro* is incompletely dominant and is under multigenetic control (PELLISSIER ET AL., 1990).

The regeneration of buds and shoots in protoplast derived calli from sunflower could not give clues to find a mechanism of shoot regeneration. Part of our findings are not in agree with published results referring to sunflower protoplasts. Those facts make the generalization of our specific results difficult. So the control

mechanisms of organogenesis can not be entirely clarified. An alternative approach would be the work on *Helianthus annuus* screening for genotypes which may show a wide genetic variability and high regeneration capacities.

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Table 1. Media composition for plant regeneration

Media type	Basic media	Supplements \mg/l\					
		Sucrose	Vitamins	Myoinositol	NAA	BAP	GA
RS - 1	MS	20 000	SH	1 000	1	1	0.1
RS - 2	MS	20 000	SH	1 000	0.05	0.2	-
RS - 3	MS	30 000	MS	1 000	0.01	0.2	0.1
RS - 4	MS	20 000	SH	1 000	0.1	0.2	0.1

MS - see MURASHIGE and SKOOG (1962); SH - see SCHENK and HILDERBRANDT (1972)

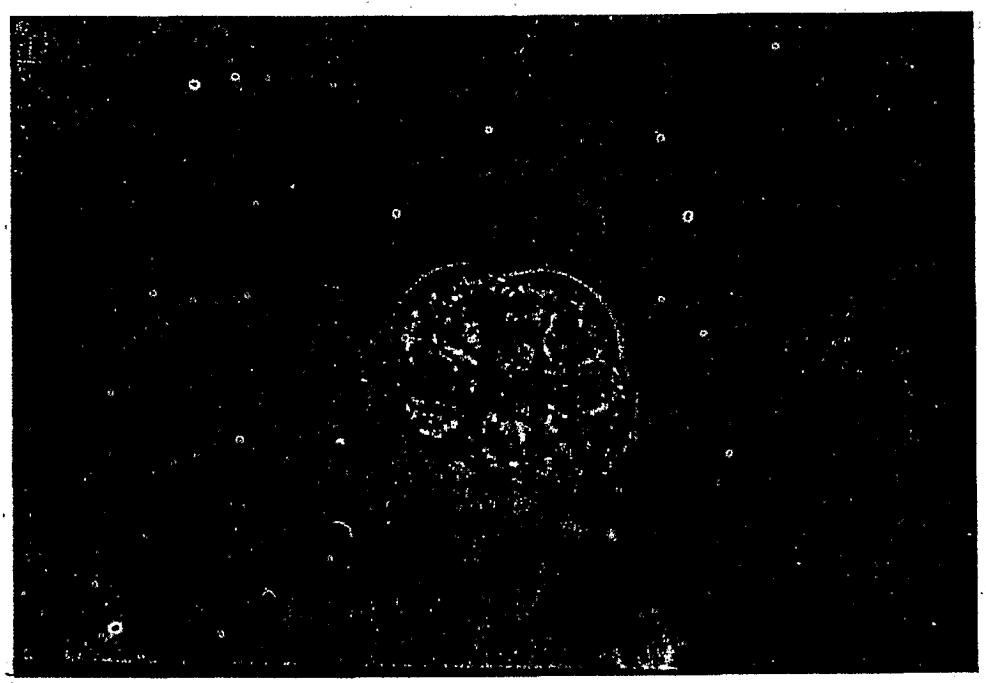

Fig.1 Hypocotyl protoplast development after 8 days, x 102

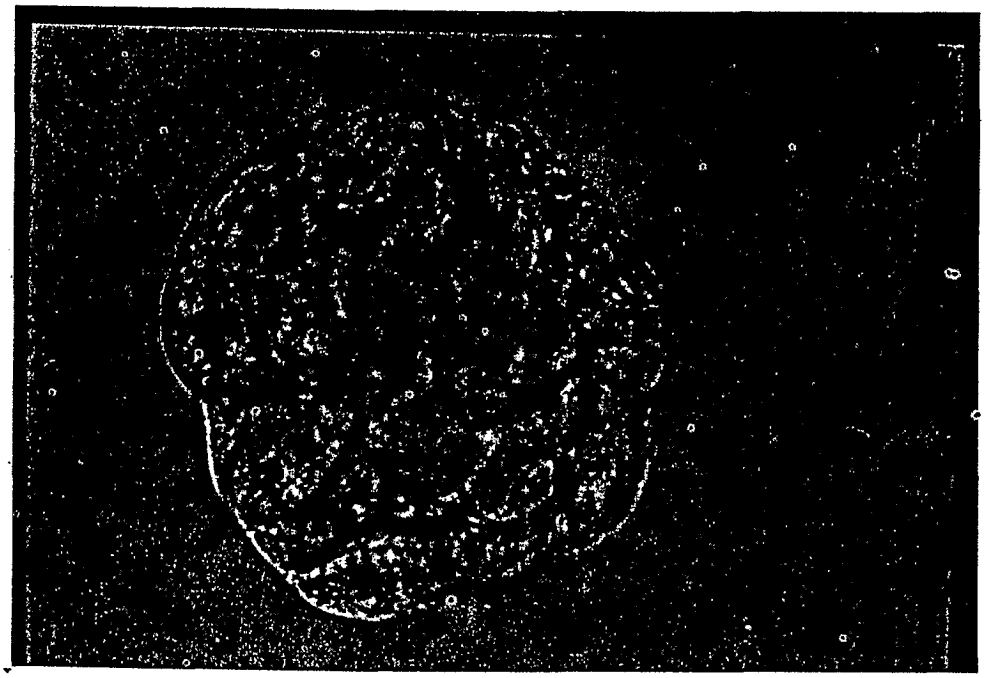

Fig.2 Colonies after 20 days, x 102

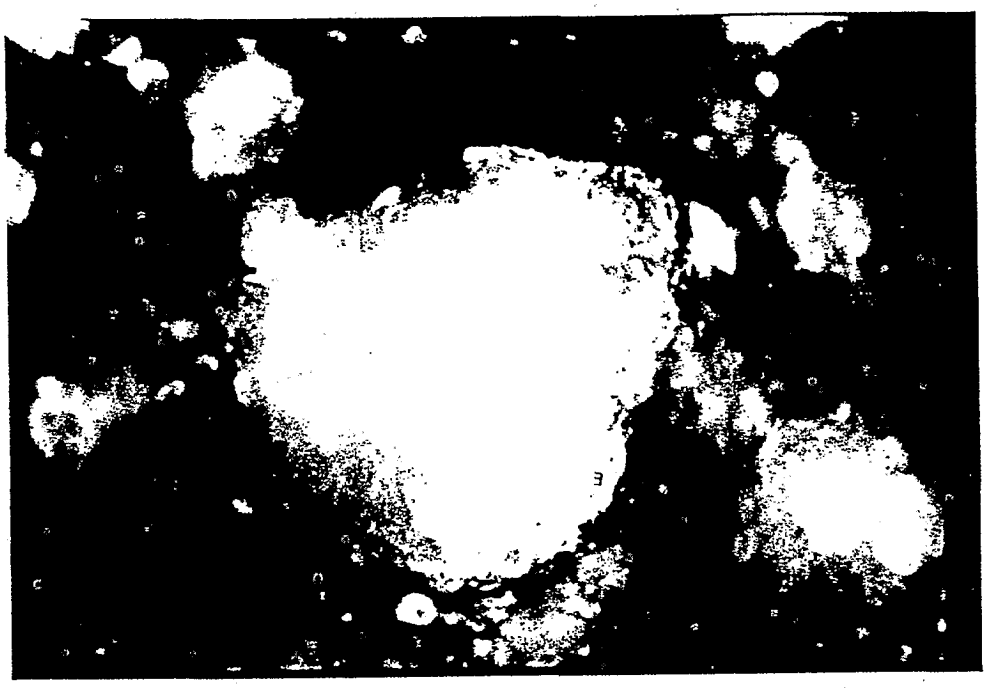

Fig.3 Growing callus at alginate disk 60 days old, x40

Fig.4 "Shoot-like structure" on the callus, x 16