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MICROPROPAGATION OF *Helianthus maximiliani* (Schrader) BY SHOOT APEX CULTURE

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SUMMARY

H.maximiliani was micropropagated using culture of shoot apices on modified Murashige and Skoog medium (DV). Further propagation of *in vitro* grown plants was done by culture of their nodal segments and shoot tips on the same medium supplemented with phloridzin, silver nitrate and casein hydrolysate (DV'). Rooting was induced by dipping the explants into IBA solution prior culture. Viable protoplasts (90%) were isolated from leaf mesophyll. These protoplasts divided (18%) in culture in agarose droplets.

Key words: ***Helianthus maximiliani*, micropropagation, shoot apex culture, protoplasts**

INTRODUCTION

Sunflower is one of the world's most important oil crops. However, sunflower cultivation is largely hampered by its sensitivity to hard diseases among which white rot induced by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is of the most destructive. Up to date, there is no efficient chemical control of the fungus development nor high level of genetic resistance in the cultivated sunflower.

Wild sunflowers constitute an important source of resistance against several major sunflower diseases including *Sclerotinia*. Škorić and Rajčan (1992) studied 12 populations of eight wild sunflower species for their resistance to *Sclerotinia* and found that accession 1631 of *Helianthus maximiliani* possesses a high degree of resistance to white rot. This was recently confirmed by Henn *et al*. (1997) who demonstrated by a mycelium stem test that, among 8 wild species studied, *Helianthus maximiliani* has the highest resistance to *Sclerotinia sclerotiorum* infection.

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Several attempts have been made to cross these two species using conventional methods in order to transfer this trait to cultivated sunflower, but with limited success as the interspecific hybrids obtained showed complete sterility (D. Škorić, unpublished results).

Production of asymmetric somatic hybrids by fusion of protoplasts of these two species could be a means to overcome the problem of sexual incompatibility between them. In this paper we describe a reliable protocol for micropropagation of *H.maximiliani* by which a sufficient quantity of sterile donor plants for protoplast isolation could be produced.

MATERIALS AND METHODS

Plant material

Helianthus maximiliani, ecotype 1631, was obtained from the Institute of Field and Vegetable Crops, Novi Sad (Yugoslavia). The plants were propagated from rhizomes in the green house and the explants were taken from 50-60 cm tall plants (6-8 leaf stage).

Shoot tips culture

About 1 cm long shoot tips were sampled, sterilised by immersion in 3% calcium hypochlorite for twenty minutes and rinsed three times with sterile distilled water. Shoot apices 0.5 cm long were placed on a DV medium containing half strength macroelements, full strength microelements and vitamins of Murashige and Skoog (1962), 2 mM of ammonium ferric citrate, 10 g l^{-1} sucrose, 6 g l^{-1} agar, pH 5.7. After three weeks of culture shoots without root were transferred to the same medium but containing 2 mg l^{-1} silver nitrate, 50 mg l^{-1} phloridzin and 3 g l^{-1} casein hydrolysate (DV' medium).

Micropropagation of *in vitro* plantlets was obtained by dissection of plants about 10-12 cm tall into nodal segments and apical shoot tips. The leaves were cut off and the base of the explants dipped in 1 mg l^{-1} solution of IBA for 5 min. The explants were grown on DV' medium. The same protocol was repeated every two weeks.

During the whole experiment light intensity was 34 μEm^{-1}, photoperiod 16:8 (light:dark) and temperature 25°C.

Protoplast isolation and culture

Young leaves of *in vitro* cultivated shoots were cut into 2 mm wide strips. The leaf fragments were plasmolysed in 10 ml of a washing solution containing 16 g l^{-1} NaCl, 1.8 g l^{-1} $CaCl_2$ x $2H_2O$, 1 g l^{-1} glucose, 380 mg l^{-1} KCl, 700 mg l^{-1} MES and 80 mg l^{-1} BCP, pH 5.7. After 1.5 h, this medium was replaced by 9 ml of the same fresh medium and 1 ml of the enzyme mixture composed of 1% cellulase R10, 0.5%

macerozyme R10 (Onozuka, Yakult Housha Co. Ltd, Japan), 0.05% pectolyase (Seishin Pharmaceutical Co. Ltd, Japan) and 0.01% Driselase (Sigma, St Louis, USA). Cell wall digestion was carried out for 20 h in the dark at 26°C. After filtration through 100 μm sieve, protoplasts were purified by floating on 10% Ficoll in the washing medium and rinsed twice in the same medium.

Protoplasts were cultured in 250 μl agarose droplets in a density of 5 x 10^4 protoplasts per ml, four droplets per one 5 cm diameter Petri dish. The droplets were surrounded by 2.5 ml of V-KM medium (Binding and Nehls, 1977) with 0.5 mg l^{-1} each of NAA and BAP. Cultures were kept in the dark at 25°C. Every seven days, 1.8 ml of the medium was taken off and replaced with the same quantity of fresh medium.

RESULTS AND DISCUSSION

We have used several approaches in order to obtain a reliable protocol for *in vitro* micropropagation of *Helianthus maximiliani*. In the first experiments, the protocol proposed by Imhoff *et al*. (1996), for micropropagation from rhizomes of several wild species, was tested. The main problem encountered was the very low yield of sterile explants which did not allow further regeneration of sterile shoots. We tried to solve this problem by adding the antibiotics in the plant growth medium as suggested by the authors but even then, the yield of sterile explants was still quite low (16.1%). Furthermore the plants which regenerated from these explants were mainly deformed being very short with pale yellow leaves.

Shoot apex culture

Starting from shoot apices, the application of published protocols (Bohorova *et al*., 1985; Punia and Bohorova, 1992) failed to give developing plantlets. The technique described in material and methods was the only one to allow shoot production from green house cultivated plants. In a typical experiment, 8% of apices gave rise to shoots (Figure 1).

When grown on DV medium, shoots showed signs of vitrification and developed very slowly. Adding of casein hydrolysate, silver nitrate and phloridzin to the culture medium (DV' medium), as suggested by Imhoff *et al*. (1996) who used these chemicals to prevent vitrification, helped us to overcome this problem. After transfer to DV' medium, rooted plants continued to develop normally and grew vigorously.

Further micropropagation of *H.maximiliani* from these primary shoots was very easy and enabled us to produce sufficient quantity of non-contaminated plant material suitable for protoplast isolation.

Dipping the shoots in IBA solution proved to be very efficient way for induction of rhizogenesis. The roots formed in all treated explants within five days after treatment. Burrus *et al*. (1991) and Wingender *et al*. (1996) also used dipping of the cut

shoots in high auxin solutions for generation of rooted shoots although they used NAA and IAA instead of IBA.

Figure 1a: Micropropagation of plants by culture of shoot apices

Figure 1b: Micropropagation of plants by culture of nodal segments

Protoplast isolation and culture

Protoplasts were isolated from leaves of two-weeks or one-month old plants obtained by micropropagation.

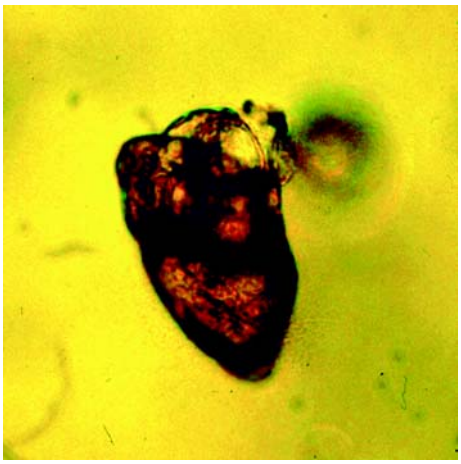

Figure 2: Embryo-like structure formed after division of H.maximiliani protoplasts

The yield of protoplasts depended of the age of donor plants. About 1.5 x 10^6 protoplasts per g of fresh weight were isolated from the leaves of two-weeks old plants while in one-month old plants the yield was much lower - 5 x 10^5 protoplasts per g of fresh weight.

The similar phenomenon was observed in *H.giganteus* (Krasnyanski *et al.*, 1992) where no protoplasts could be isolated from the plants older than 5 to 6 weeks due to incomplete digestion. However, the age of plants did not have an influence on viability and division frequency of protoplasts. In both cases viability of protoplasts after isolation was about 90% and percentage of divisions after 14 days of culture was about 18%.

CONCLUSIONS

The aim of this work was to develop the technique for mass micropropagation of the wild species *H.maximiliani*. *In vitro* cultivated plants are now available and viable protoplasts were produced from these plants. We plan now to fuse protoplasts of *H.maximiliani* with protoplasts of selected lines of *H.annuus* in order to introduce the resistance against *Sclerotinia sclerotiorum* of *H.maximiliani* into the cultivated sunflower. Techniques for protoplast fusion and protoplast regeneration have already been published (Chanabe and Alibert, 1990; Chanabe *et al*., 1991; Burrus *et al*., 1991) and trait transfer could be achieved following the strategy described by Kallerhoff and Alibert (1996).

Abbreviations *BAP* 6-benzylamino-purine, *BCP* Bromocresol purple, *IBA* indole-3-butyric acid, *MES* 2-(N-morpholino)ethanesulfonic acid, *MS* Murashige and Skoog medium, *NAA* 1-naphthalene acetic acid

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MICROPROPAGACION DE *Helianthus maximiliani* (Schrader) POR EL CULTIVO DE VAINAS DE LOS VASTAGOS

RESUMEN

La micropropagacion de *H.maximiliani* fue hecha por el cultivo de vainas le los vastagos en el medio modificado de Murashige y Skoog (DV). Las plantas cultivadas *in vitro* eran aun multiplicadas por el cultivo de segmentos nodales y vainas de los vartagos en el mismo medio al cual fueron adicionados floridzina, nitrato de plata y caseina (DV'). El arraigo fue inducido por la mojada de explantados en la solucion de IBA antes del cultivo. Los protoplastos vitales (90%) fueron aislados de los mesofilos de hojas. Despues de ser puestos en el cultivo en gotas de agarosis, los protoplastos se dividian (18%).

MICROPROPAGATION DE L'*Helianthus maximiliani* (Schrader) AU MOYEN DE LA CULTURE DE L'APEX DE LA POUSSE

RÉSUMÉ

Le *H.maximiliani* a été micropropagé au moyen de la culture de l'apex des pousses dans un médium modifié de Murashige et Skoog (DV). La propagation *in vitro* des plantes a été effectuée ultérieurement par la culture de leurs segments nodaux et des sommets des pousses dans le même médium auquel on avait ajouté du phloridzine, du nitrate d'argent et de la caséine (DV'). La formation de racines a été initiée par le trempage des explants dans une solution IBA avant la culture. Des protoplastes viables (90%) ont été isolées des feuilles mésophylles. Ces protoplastes se sont divisés (18%) dans une culture de gouttelettes d'agarose.