TOWARDS *SCLEROTINIA* RESISTANCE: SOMATIC HYBRIDIZATION BETWEEN WILD AND CULTIVATED SUNFLOWER

Dragana M. Vasic, Institute of Field and Vegetable Crops, 21000 Novi Sad, Maksima Gorkog 30, Serbia and Montenegro
E-mail: vasicd@ifvcns.ns.ac.yu

Ksenija J. Taski, National Laboratory for Seed Testing, 21000 Novi Sad, Maksima Gorkog 30, Serbia and Montenegro
E-mail: ksenijat@ifvcns.ns.ac.yu

Nevena M. Nagl and **Dragan M. Skoric,** Institute of Field and Vegetable Crops, 21000 Novi Sad, Maksima Gorkog 30, Serbia and Montenegro
E-mail: nagl@ifvcns.ns.ac.yu
E-mail: skoricd@ifvcns.ns.ac.yu

Abstract

The use of interesting characteristics from wild *Helianthus* species in sunflower breeding is limited by poor crossability or sterility of interspecific hybrids. Somatic hybridization by protoplast fusion is an efficient method for overcoming crossing barriers and increasing of the genetic variability of the plants. Protoplasts obtained from leaf mesophyll of *Sclerotinia sclerotiorum*-resistant accessions of *Helianthus maximiliani* and *Helianthus mollis* and etiolated hypocotyls of inbred lines PH-BC1-91A and Ha-74A were purified by floating on Ficoll. Prior to electrofusion wild species protoplasts were irradiated with UV light with the aim of inactivating a part of their genome. Fusion products were embedded in agarose droplets and subjected to different regeneration protocols. The best results were obtained with the protocol of Trabace et al. (1995). Developed microcalluses were released from the agarose and transferred to solid media. Shoot regeneration was achieved by culture of calluses on regeneration medium containing 2.2 mgL-1 BAP and 0.01 mgL-1 NAA (Krasnyanski et al., 1992). During the culture, calluses were treated with a high concentration of 2,4-D for a limited period. Studies at the molecular and morphological level showed that obtained plants were somatic hybrids.

Introduction

Cultivated sunflower (*Helianthus annuus* L.) is one of the most important oil crops in the world. White rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is the most important sunflower disease in regions with humid climate (Skoric and Rajcan, 1992). There is no efficient method of chemical control of this pathogen (Masirevic and Gulya, 1992), and no sources of resistance in cultivated sunflower have yet been found (Bazzalo et al., 1991).

Wild *Helianthus* species are regarded as important sources of disease resistance (Skoric and Rajcan, 1992) but their use in sunflower breeding is limited by poor crossability and sterility of interspecific hybrids (Atlagic et al., 1995). Somatic hybridization by protoplast fusion is an efficient method of overcoming crossing barriers and increasing the genetic variability of plants (Grosser et al., 1990).

In our work, we have used electrofusion of protoplasts in order to produce somatic hybrids between cultivated sunflower and its wild relatives *Helianthus mollis* Lam. and *Helianthus maximiliani* Schrader.

Materials and Methods

Hypocotyl protoplasts of the sunflower inbred line Ha-74A and leaf mesophyll protoplasts of *H. mollis*, accession 1298, were fused using three 30-μs electrical pulses, with the intensity of 1250 Vcm-1. The same was done with protoplasts of inbred line PH-BC1-91A and accession 1631 of *H. maximiliani*. Fusion products were cultured in agarose droplets according to the protocol of Trabace et al. (1995). Resulting microcalluses were transferred onto solid regeneration media as described by Henn et al. (1998).

DNA for RAPD analysis was isolated from leaves of both parents and their somatic hybrids, according to the protocol of Gentzbittel et al. (1994). RAPD analysis was done using five 10-base primers (Operon Technologies). PCR was carried out in a 25-μl reaction volume as described by Sossey-Alaoui et al. (1998).

Results and Discussion

Electrofusion is the most frequently used technique for generation of somatic hybrid plants between different species (Sihachakr et al., 1988; Guo and Deng, 1998; Kisaka et al., 1997). This method was also used for fusion of sunflower protoplasts, but we were able to find only reports describing regeneration of calluses whose hybrid nature was subsequently confirmed by isozyme (Barth et al., 1993) and RAPD analysis (Vasic et al., 2000). However, plant regeneration from these calluses was not reported. Sunflower somatic hybrid plants were only regenerated after fusion of protoplasts using PEG (Krasnyanski and Menczel, 1995; Trabace et al., 1996; Henn at al., 1998).

Fusion products were embedded in agarose and subjected to different regeneration protocols. When the regeneration protocols of Krasnyanski et al. (1993) and Wingender et al. (1996) were used, no callus formation was observed. The best results were obtained by using the protocol of Trabace et al. (1995). During first week of culture in L4 medium symmetrical divisions of protoplasts were observed, and protoplasts developed into small, macroscopic colonies. Colonies kept for 3-4 weeks in the dark produced visible, white microcalluses. These observations are in accordance with the results of Guilley and Hahne (1989) and Trabace et al. (1995, 1996).

Developed microcalluses, transferred to solid KR-R-Ag medium, and exposed to the light, continued to grow. Green vigorously growing calluses were treated with 2,4-D. When calluses were transferred onto KR-R-Ag medium, shoots were observed. It seems that a high level of cytokinin subsequent with 2,4-D treatment is essential for regeneration of shoots. Krasnyanski and Menczel (1993) have reported that exposure of colonies to a high concentration of 2,4-D for a limited period induced subsequent development of somatic

embryos. 2,4-D treatment had a similar effect in our experiment, as it improved the organogenic response of protoplast-derived calluses.

Shoots were transferred onto MS medium (Murashige and Skoog, 1962), where some of them produced plants. Well-developed and normal plants were treated with IBA for root induction (Vasic et al., 2001), and transferred into sterile soil for acclimatization. Plants that flowered were pollinated with the B form of the recipient parent. As a result of pollination several seeds were obtained and for their rescue embryo culture is currently used.

The band patterns obtained by four primers showed bands characteristic for Ha-74A in its somatic hybrids with *H. mollis*. Only in band patterns obtained with primer C15 the presence of bands characteristic of *H. mollis* was observed, indicating the presence of a part of its genome in the hybrid plant (Figure 1). In hybrid PH-BC1-91A x *H. maximiliani* bands characteristic of the wild parent were found in the RAPD profile obtained with the primer E05.

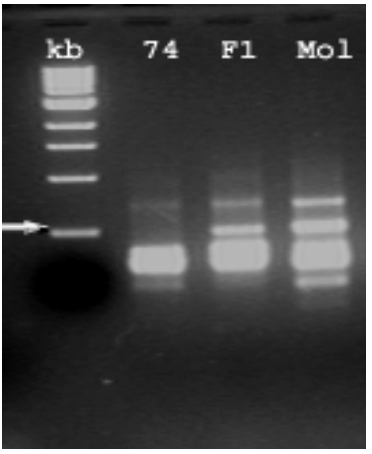
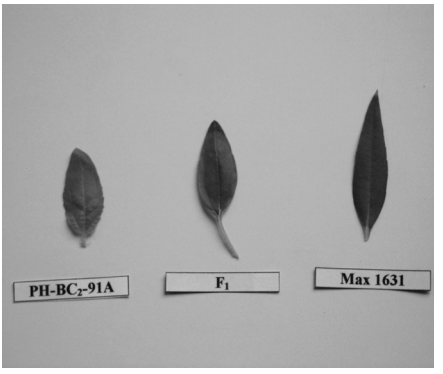


Figure 1. RAPD profiles of Ha-74A, *H. mollis* and their somatic hybrid obtained with primer C15.

Besides the RAPD analysis, morphological comparisons between parents and hybrids were done. Leaves of hybrid plants Ha-74A x *H. mollis* had a shape similar to that of the cultivated parent, but with smooth edges as in *H. mollis* (Figure 2). The same was observed in the hybrid between Ph-BC1-91A and *H. maximiliani* (Figure 2).

Figure 2. **Left:** Leaves of Ha-74A, *H. mollis* and their hybrid.



Right: Leaves of PH-BC1-91A, *H. maximiliani* and their hybrid.

Acknowledgements

This study was carried out within a project of the Ministry of Science, Technology and Development of the Republic of Serbia, Grant No. 5.02.0401.

References

Atlagic, J, Dozet, B, and Skoric, D. 1995. Meioses and pollen grain viability in *Helianthus mollis*, *H. salicifolius*, *H. maximiliani* and their F1 hybrids with cultivated sunflower. Euphytica. 81:259-263.

Barth, S, Voeste, D, Wingender, R, and Shanabl, H. 1993. Somatic hybrids of sunflower (*Helianthus annuus* L.) identified at the callus by isozyme analysis. Bot. Acta. 106:100-102.

Bazzalo, M.E, Dimarco, P, Martinez, F, and Daleo, G.R. 1991. Indictors of resistance of sunflower plant to basal rot (*Sclerotinia sclerotiorum*); Symptological, biochemical, anatomical and morphological characters of the host. Euphytica. 57:195-205.

Gentzbittel, L, Zhang, G, Vear, F, Griveau, Y, and Nicolas, P. 1994. RFLP studies of genetic relationships among inbred lines of cultivated sunflower (*Helianthus annuus* L.): evidence for distinct restorer and maintainer germplasm pools. Theor. Appl. Genet. 89:419-425.

Grosser, J.W, Gmitter, F.G, Tusa, N, and Chandler, J.L. 1990. Somatic hybrid plants from sexually incompatible woody species: *Citrus reticulata* and *Citrus gilletiana*. Plant Cell Rep. 8:656-659.

Guilley, E, and Hahne, G. 1989. Callus formation from isolated sunflower (*Helianthus annuus*) mesophyll protoplast. Plant Cell Rep. 8:226-229.

Guo, W.W, and Deng, X.X. 1998. Somatic hybrids plantlets regeneration between *Citrus* and its wild relative *Marraya paniculata* via protoplast electrofusion. Plant Cell Rep. 18:297-300.

Henn, HJ, Wingender, R, and Schnabl, H. 1998. Regeneration of fertile interspecific hybrids from protoplast fusions between *Helianthus annuus* L. and wild *Helianthus* species. Plant Cell Rep. 18:220-224.

Kisaka, H, Kisaka, M, Kanno, A, and Kameya, T. 1997. Production and analysis of plants that are somatic hybrids of barley (*Hordeum vulgare* L) and carrot (*Daucus carota* L.). Theor. Appl. Genet. 94:221-226.

Krasnyanski, S, and Menczel, L. 1995. Production of fertile somatic hybrid plants of sunflower and *Helianthus giganteus* L. by protoplast fusion. Plant Cell Rep. 14:232-235.

Krasnyanski, S, Polgar, Z, Nemeth, G, and Menczel, L. 1992. Plant regeneration from callus and protoplast cultures of *Helianthus giganteus* L. Plant Cell Rep. 11:7-10.

Krasnyanski, S, Polgar, Z, Nemeth, G, and Menczel, L. 1993. Somatic embryogenesis and plant regeneration from hypocotyls protoplast of sunflower (*Helianthus annuus* L.). Plant Cell Rep. 12:260-263.

Masirevic, S, and Gulya, T.J. 1992. *Sclerotinia* and *Phomopsis:* two devastating sunflower pathogens. Field Crops Research. 30:271-300.

Murashige, T, and Skoog, F. 1962 A revised medium for growth and bioassays with tobacco tissue cultures. Physiol Plant. 15:473-497.

Sihachakr, D, Haicour, H, Serrafi, A, Barrientos, E, Herbreteau, C, Ducreux, G, Rossignol, B., and Souvannavong, V. 1988. Electrofusion for the production of somatic hybrid plants of *Solanum melangea* L. and *Solanum khasinum* C.B. Clark Plant Science. 57(3):215-223.

Skoric, D, and Rajcan, I. 1992. Breeding for *Sclerotinia* resistance in sunflower. *In*: Proc. 13th Int. Sunflower Conf. Pisa, Italy. p. 1257-1262.

Sossey-Alaoui, K, Serieys, H, Tersac, M, Lambert, P, Schilling, E, Griveau, Y, Kaan, F, and Berville, A. 1998. Evidence for several genomes in *Helianthus*. Theor. Appl. Genet. 97:422-430.

Trabace, T, Fiore M.C, D'Ambrosio, C, Vanadia, S, and Sunseri, F. 1996. Sunflower cytoplasmic hybrids relevant by PCR assay using male sterility as selectable marker. J. Genet. Breed. 50:29-34.

Trabace, T, Vischi, M, Fiore, M.C, Sunseri, F, Vanadia, S, Marchetti, A. and Olivieri, A.M. 1995. Plant regeneration from hypocotyl protoplast in sunflower (*Helianthus annuus* L) J. Genet. Breed. 49:51-54.

Vasic, D, Alibert, G, Berville, A, and Skoric, D. 2000. RAPD analysis of sunflower somatic hybrid calli. *In*: Proc15th Int. Sunflower Conference, Toulouse, France, p. L42-L45.

Vasic, D, Alibert, G, and Skoric, D. 2001. Protocol for efficient repetitive and secondary somatic embryogenesis in the *Helianthus maximiliani* (Schrader). Plant Cell. Rep. 20:121-125.

Wingender, R, Henn, H.J, Barth, S, Voeste, D, Machlab, H, and Schnabl, H. 1996. A regeneration protocol for sunflower (*Helianthus annuus L.*) protoplasts. Plant Cell. Rep. 15:742- 745.