SCREENING THE INFLUENCE OF TEMPERATURE AS STRESS FACTOR IN THE ORIENTATION OF MORPHOGENETIC REACTION OF *BRASSICA OLERACEA* ANTHERS CULTIVATED *IN VITRO*

STUDII PRIVIND INFLUENŢA TEMPERATURII CA FACTOR DE STRES DETERMINANT ÎN ORIENTAREA REACŢIEI MORFOGENETICE A ANTERELOR DE *BRASSICA OLERACEA* CULTIVATE *IN VITRO*

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Abstract. *Anther culture has become a powerful tool for the rapid production of haploid and inbred lines used for obtaining hybrid cultivars and it has reduced significantly the time required for breeding new cultivars by at least 2 to 3 years. Androgenesis results in homozygous progeny from a heterozygous parent in a single generation and provides excellent material for research, plant breeding and plant transformation. There are many factors that influence the effectiveness of anther culture and these factors may also interact. Some of major factors are genotype, donor plant growth conditions, anther pretreatment, time of their flowering, microsporogenesis phase, cultivation media composition, temperature shock and environment conditions. The aim of the present work was to assess the influence of temperature as stress factor in the orientation of morphogenetic reaction of Brassica oleracea anthers cultivated in vitro toward the obtaining of haploid plants.*

Key words: haploids, embryo, callus, cabbage, shoots

Rezumat*. Cultura de antere a devenit un instrument puternic de producere a plantelor haploide utilizabile pentru obţinerea de plante haploide, reducând semnificativ timpul necesar pentru crearea de noi cultivare. Androgeneza presupune realizarea unor plante homozigote pornind de la un părinte heterozigot, într-o singură generatie, oferind astfel un material excelent pentru cercetarea fundamentală dar şi pentru ameliorarea şi transformarea genetică a plantelor. Factorii care influenţează orientarea reacţiei morfogenetice a anterelor sunt multipli, iar eficacitatea androgenezei depinde şi de interacţiunea dintre aceşti factori. Principalii determinanţi sunt genotipul, condiţiile de creştere a plantelor donor, pre-tratamentul anterelor, timpul de înflorire, faza de microsporogeneză, compoziţia mediului de cultură, aplicarea unor şocuri termice şi condiţiile de creştere. Scopul prezentului studiu a fost determinarea influenţei temperaturii, ca factor de stress în orientarea reacţiei morfogenetice a anterelor de Brassica oleracea cultivate in vitro.*

Cuvinte cheie: haploizi, embrion, calus, varza, lăstari

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INTRODUCTION

The haploid plants derived from anther culture have been used to produce homozygous diploids, in many plant species due to the fact that using this technique we can accelerate breeding programs. For *Brassica* spp. haploid plants production through anther culture proved to be an important goal for tissue culture scientist all over the world. Traditionally, plant breeders usually achieve isogene lines, utilized as parent lines for F1 hybrid production by using the self-pollination, which is a highly time consuming process. Using tissue culture technique, more precisely anther culture, homozygous plant can be produced within a year as compared to the long inbreeding method, which might take 8-10 years. Therefore, *in vitro* techniques are considered to be alternative tools of conventional method of *Brassica* improvement.

The regeneration of haploid plants from anther culture depends on a wide range of factors, from which we underline: the genotype, culture media, physiological status of donor plant, anther wall factor, stage of pollen development, and effect of temperature and light.

In the last two decades, remarkable progress in anther culture technology has been made in all major *Brassica* species, but most of them are concentrated on oilseed rape (*Brassica napus*) (Palmer et al. 1996). Comprehensive utilization of this doubled-haploid production system has been involved in *Brassica* breeding programmes as well as in gene transfer, biochemical and physiological studies, and other manipulations (Palmer et al. 1996). However, all these applications largely depend upon efficient protocols that are specific for each species and sometime even for each genotype.

As mentioned before, there are various factors influencing anther culture success and among them is also temperature regarded as stress treatments (Dunwell, 1983). Among these stress factors in *Brassica* anther culture, many authors reported the importance of a short heat shock treatment that is basically required to stimulate anther development toward direct organogenesis and embryogenesis.

In spite of its critical role in other species, the cold pretreatment is less frequently used in *Brassica* species. Moreover, the significance of cold pretreatment in *Brassica* anther embryogenesis seems contradictory in previous studies. Some authors reported effective results from cold pretreatment of flower buds or inflorescences in *Brassica* sp. (Lichter 1982), while others underlined negative effects in *B. napus* (Dunwell et al. 1983) and *B. rapa* (Munshi 1996).

These contradictory results regarding the influence of cold pretreatment over the morphogenetic reaction of anther cultivated *in vitro* may lead to misunderstanding of its role and may conduct to an inappropriate application in *Brassica* anther culture.

Our study focused toward the evaluation of low temperature effect, regarded as stress pretreatment, applied to flower buds before inoculation to culture media combined with heat shock applied to anthers immediately after inoculation.

MATERIAL AND METHOD

The plant material utilized in this research is represented by two varieties BC 145 and BC 321 that belongs to Vegetable Research and Development Station

Bacau, Romania. Donor plants were grown in controlled conditions, in greenhouses, with a proper regime of watering, fertilization and pest control.

The explants, represented by buds measured 3.0 – 3.4 mm in length and a ratio of petal length to anther length (0.5–0.75) (Gu et al., 2004, cited by Wedzony, 2007). These buds contained anthers with microspores at late uninucleate to binucleate stage (observed using 1% aceto-carmine under microscope). Part of the selected buds with microspore were wrapped with polythene bag and kept at 4°C for 2 days (variant V1), 4 days (variant V2) and 7 days (variants V3). The control variant is represented by fresh buds with no temperature stress (V0). The abnormal anthers of the bud were discarded and those at the appropriate size and age were used in the culture.

The explants were washed thoroughly under running tap water for 30 min and treated with a surfactant, Tween 20 (10 drops per 100ml of sterilized distilled water). Later these explants were surface sterilized with 0.1% mercuric chloride (w/v) for 15 min and repeatedly washed using sterilized distilled water. Under aseptic conditions, anthers were removed from the sterilized buds using a fine Tweezers (forceps) and inoculated on sterile tubes with culture media containing MS macro and microsalts (Murashige Skoog, 1962) supplemented with vitamins MS, 1962 and the following hormonal formula - BA- 8.8 µM +2.7 µM NAA. The hormonal formula utilised was determined to be the most effective in different prior experiments.

The cultures were incubated at 33°C temperature for one week in complete dark. After that the cultures were transfer in culture chambers with controlled light, humidity and temperature control at 25^0C, a 16-h photoperiod, and 5000 lx light intensity. Fifty anthers of each genotype were inoculated into each treatment.

Four to five weeks after inoculation of anthers, they were removed aseptically from the culture tubes on a sterilized glass plate inside the laminar airflow cabinet and were placed again on freshly prepared sterilized medium containing appropriate hormonal supplements for plant regeneration. Sub culture was done in the MS media containing different combinations and concentrations of BA and NAA. The sub cultured culture tubes were again incubated at 25°C with 16 hrs photoperiod for 5-7 days. Repeated sub cultures were done at an interval of 15 days and incubated under the same temperature as mentioned previously. After shoot initiation, more light intensity was used for shoot elongation. The culture vessels showing signs of contamination were discarded. Day to day observation was carried out to note the responses.

In the course of the experiments the number of anthers producing callus and embryoids were recorded. The number of embryoids with cotyledons and roots developed in tubes were marked also.

The frequency of both type of reaction and the frequency of reacted anthers were calculated in percentage to the *in vitro* set initial explants. The frequency of the regenerated microplants was presented in percentage both to the total number of anthers and to the number of obtained direct embryoids from the respective genotype.

RESULTS AND DISCUSSIONS

Low temperature stress is one of the most important but least studied abiotic stresses affecting plant development in tissue culture. The reproductive stage is the most susceptible stage for temperature stress in most crops in which temperature response has been studied (Paulsen 1994; Angadi et al. 2000).

The results obtained in our experimentations and presented in the present paper sustain this theory. The application of low temperatures, regarded as stress factor,

before the inoculation of the anthers on the growth media had significant influence over the induction and orientation of the explant morphogenetic reaction.

The reaction of the anthers at both genotypes utilized in the present paper ranged between direct embryogenesis and organogenesis and induction of callusogenesis. Regarding the callusogenesis, the temperature influenced the type and consistence of callus. We identified three callus morphotypes that were normally developed at the end of each subculture period: a friable white callus with no buds – fig. 1, a green-yellowish callus with no or scarce buds, and a hard green callus – fig. 2 covered with patches of buds and leafy structures that eventually developed into elongated shoots. White friable calluses were identified mainly on the variant V1 and V0, and even if subcultured on new fresh media it did not show reversion to green callus. The hard green calluses with buds were found to be developed from the anthers that were treated with a shock temperature of $+4^0$C for 4 days. While at variant V3 the callus structures were more yellowish colored than the previous one and proved to have a low capacity to develop shoots.

Fig. 1 - Friable white callus with no buds developed from anthers with no temperature stress (V0 - control)

Both genotypes of *Brassica* utilized in our experiments are responsive to cultivation in vitro. The anthers after incubation at 33^0, in dark conditions started to increase in size – fig. 3, becoming globular-shaped. On their surfaces started to appear small meristematic centers that evolved in fully developed plants or in plantlets without a root system. After four to five weeks from the inoculation times, the cultures were transferred on freshly prepared sterilized medium containing MS media with BA and NAA.

Fig. 2 – Hard green callus generated from anthers that were treated with a shock temperature of $+4^0$C for 4 days – variant V2.

On these media, incubated at 25°C with 16 hours photoperiod, the cultures continue to develop as more shoots arise at the base of the first appeared plantlet. The best morphogenetic reaction quantified as the percentage of reactive anthers comparing with the total number of explants placed in culture, as well as regenerated plants.

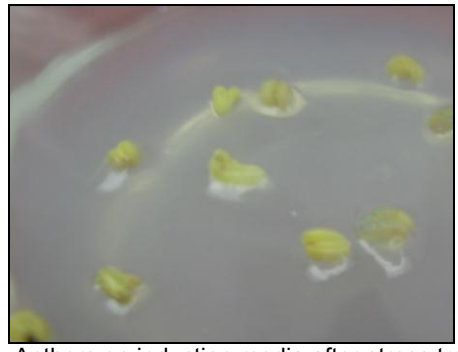

Fig. 3 – Anthers on induction media after stress treatment

The interpretation of results obtained after the development of the experiment underline the fact that anthers respond well to a short stress treatment at $+4^0$C for 4 days.

Fig. 5 – Small meristematic clusters formed at the base of the initial plantlet

This temperature pre-treatment of flower buds increased frequencies of organogenesis upto 34.17% and 43.32% at genotype BC 145 and genotype BC 321 respectively in MS-medium supplemented with BA- 8.8 μM +2.7 μM NAA.

After the first plantlets appeared, they were removed aseptically from the culture tubes on a sterilized glass plate inside the laminar airflow cabinet and were placed again on freshly prepared sterilized medium containing the same hormonal supplements. Gradually, at the basis of each new plantlet, both on the surface and inside the medium started to appear small meristematic centers - fig. 4 that evolved in embryoids and fully formed plants. Rooted plants were hardened by maintaining a high humidity (90% RH) during first week of hardening, which resulted in more than 80% survival of plantlets.

CONCLUSIONS

1. The purpose of the study was the accomplishment of a screening regarding the influence of temperature utilised as stress factor in the orientation of morphogenetic reaction of *Brassica oleracea* anthers cultivated *in vitro*.

2. The results obtained showed that the application of low temperatures $+4^0$C, before the inoculation of anthers on growth media had significant influence over the induction and orientation of the explant morphogenetic reaction.

3. The reaction of the anthers at both genotypes utilized in the present paper ranged between direct embryogenesis and organogenesis and induction of callusogenesis.

4. From the three variant tested, the variant V2, meaning $+4^0$C for 4 days increased frequencies of organogenesis upto 34.17% and 43.32% at genotype BC 145 and genotype BC 321 respectively in MS-medium supplemented with BA-8.8 μM +2.7 μM NAA.

5. The development of a standardized *in vitro* haploid production protocol for Brassica spp. will help in the production of homozygous inbred lines for use in development of synthetics or hybrid varities.

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