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EFFECT OF THE MAIN CYTOKININS ON ANDROGENESIS OF WHITE CABBAGE (*BRASSICA OLERACEA* L. var. *CAPITATA*) ANTHERS CULTIVATED "IN VITRO"

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ABSTRACT

The aim of the present research work was the screening of the effect of the main cytokinin (BAP, kinetin or zeatin) in different concentrations and combinations with the auxin NAA on androgenesis of white cabbage anthers cultivated *in vitro.* The results obtained are regarded as an intermediary stage for the development of a reproducible protocol for *in vitro* regeneration of plant from anther culture.
Thus, for the determination of the influence of plant growth regulators formula over the callus induction and plant regeneration from anthers cultivated *in vitro* in the present study the authors undergo a screening of the three most frequently utilized cytokinins (BAP, kinetin and zeatin) in different concentration and combination with the auxin NAA. The results obtained, indicated that the best morphogenetic reaction is obtained on variant with BAP as the main growth regulator.

Keywords: benzylaminopurine, kinetin, zeatin, haploid, morphogenesis

INTRODUCTION

The *Brassica* group is a large group of plants which contains many commercially important food and oilseed crop plants. The vegetable crops from this group, such as cabbage, cauliflower, broccoli and brussels sprouts belong to *B. oleracea* and they are essential for human nutrition due to their high content in vitamins and minerals being cultivated on large surfaces all over the world (Favela-González et al., 2020; Murat et al., 2018).
In *Brassica* spp. breeding programs, *in vitro* plant tissue cultures has been involved in a wide range of applications. Among these, haploid production through anther culture proved to be an important approach of tissue culture, during the last decades (Shariatpanahi and Ahmadi, 2016; Anshul et al., 2020). If traditionally, breeders can obtain homozygosity by self-pollination, in 8-10 years, with anther culture, homozygous plant can be produced within one year. Theanther culture is an alternative and efficient technique to conventional breeding methods by enabling production of haploid and di-haploid plants. This is the reason why the scientific communities show a huge interest in improvement the existing *in vitro* anther culture techniques in different *Brassica* species including *B. napus*, *B oleracea*, *B. campestris*, *B. juncea*, *B. carinata* and *B. nigra.* Due to the rather recalcitrant reaction of *Brassica* tissues to *in vitro* culture, the researchers are focused on the establishment of the most effective conditions that allow the development of embryos, shoots and finally plants from anthers culture in order to obtain microspore derived haploids (MDH). The regeneration of haploid plants depends on a wide range of factors, from which important are: genotype, plant growth regulators (PGR), culture media, physiological status of donor

plant, stage of pollen development, temperature and light (Fowler, 2000; Vishal, 2012; Zhang, 2003; Monisha and Saikat, 2020).
Plant biotechnology has been used for decades as a tool to improve the plant breeding programs. Among the many techniques employed, anther culture is designed for generating haploid plants which through different diploididization methods can be transformed in homozygous dihaploids, utilizable as parental lines for F1 hybrids. Plant breeders, traditionally, achieve homozygosity of the cross products through self-fertilization - which usually needs 8-10 year, by anther culture, homozygous plant can be produced within a year. Many factors influence the morphogenetic reaction of anthers cultivated on solid media *in vitro.* Among them, the most important are: the plant growth regulator added in culture media, genotype physiological status of donor plant, anther wall factor, stage of microspore development, and effect of temperature and light. All these inner and outer factors basically influence the future evolution *in vitro* of anthers either toward callus induction or plant regeneration directly from immature anther. The importance of plant growth regulators added to the *in vitro* culture medium for callus induction, organ formation and embryogenesis, has been demonstrated for a large number of plant species. Among the protocols in which cytokinins were used as PGR for induction of regeneration, the literature shows that N6-benzylaminopurine (BAP) was the most frequently employed (57%), followed by kinetin (37%), zeatin (3%) and thidiazuron (3%).

MATERIALS AND METHODS

Plant material
The biologic material was represented by unopened flower buds at 3.0 – 3.4 mm length, which contain anthers with microspores at late uninucleate or binucleate stage. The developmental stage of the microspores was established through observation under microscope according with 1% aceto-carmine method. The buds were collected from mother plants belonging to the variety – DL20 developed and maintained at Vegetable Research and Development Station Bacau. The mother plants were grown in controlled conditions in greenhouse, with proper regime of fertilisation, irrigation and phyto-sanitary control.
Sterilization
The excised buds were surface sterilized in 0.1% mercuric chloride (w/v) for 15 min, followed by rinsing in sterile distilled water for 3 to 4 times. After sterilization the buds were dissected under aseptic conditions, the anther filaments removed and the anthers were inoculated in sterile tubes, on NLN (Lichter, 1982) basal nutrient medium (macroelements, microelements and vitamins)containing 3% (w/v) sucrose,50µM $AgNO_3$ and supplemented with different quantities of cytokinin (BAP, Kinetin or zeatin) and auxin NAA – table 1.
The pH was adjusted to 5.8 prior to the addition of 0.8% agar and autoclaved at 121°C (1.06 kg/cm^2) for 25 min.
Culture techniques
The cultures were incubated at 33°C temperature for one week in complete dark, followed by their transfer in culture chambers with controlled light (16-h photoperiod, and 5000 lx light intensity), and temperature at (25^0C). Four to five weeks after inoculation the anthers were removed aseptically from the culture tubes and were transferred on freshly prepared sterilized medium with the same PGRs formula.

Table 1. PGR combinations tested for their role on androgenetic potential of *Brassica*

Variant	BAP (µM)	KIN (µM)	ZEATIN (µM)	NAA (µM)
V_0	-	-	-	-
V_1	4.4	-	-	1.6
V_2	4.4	-	-	2.7
V_3	4.4	-	-	5.4
V_4	8.9	-	-	2.7
V_5	8.9	-	-	-
V_6	-	4.6	-	1.6
V_7	-	4.6	-	2.7
V_8	-	4.6	-	5.4
V_9	-	9.3	-	2.7
V_{10}	-	9.3	-	-
V_{11}	-	-	4.6	1.6
V_{12}	-	-	4.6	2.7
V_{13}	-	-	4.6	5.4
V_{14}	-	-	9.1	2.7
V_{15}	-	-	9.1	-

The sub-cultured culture tubes were then incubated at 25°C with 16 h photoperiod for 5-7 days. Repeated sub cultures were done at an interval of 30 days and incubated under the same temperature as mentioned previously. The culture vessels showing signs of contamination were discarded. Day to day observations were carried out to note the responses.

Rooting and acclimatization

After 3 to 4 weeks, when regenerated shoots reached a length of more than 4.0 cm, they were separated and transferred on NLN basal medium supplemented with 2.7 µM NAA for rooting. The rooted plantlets were transferred to the hydroponics conditions in bottles and hardened by maintaining a high humidity (90% RH) during first week. Then, by gradually decreasing the humidity, resulted a survival rate of over 95% of the plants.

Statistical analysis

Ten anthers per sterile tube, in three replications were inoculated for each variant. The percentage of anthers forming regenerative structures and the mean number of shoots per explant were recorded. The data were analyzed by ANOVA.

RESULTS AND DISCUSSIONS

Beside callusogenesis, the main morphogenetic responses of anthers on the 15 variants tested in the present study were organogenesis, and/or embryogenesis. Stereomicroscopic observations revealed that the regenerative structures got initiated after 14 - 20 days of cultures. The anthers started to grow in size and small protuberances emerged on the anther's surfaces that gradually evolved toward callus or direct regeneration of shoot and embryoids from anthers. In this stage the anthers acquired either directly or indirectly (which involved the callus phase) organogenic and embryogenic competence.

The poor regeneration response found on control variant (V0) lead us to the conclusion that the addition of PGRs in culture medium is essential for inducing morphogenetic competences in anthers' tissues (organogenesis, or embryogenesis) and afterwards plant regeneration. Among the PGRs tested, the best responses were found on variants with BAP.

The anthers cultivated on these media showed higher results both for indirect and direct organogenesis and embryogenesis- table 2. Similar results were obtained with culture media with zeatin, that induced either direct regeneration of somatic embryos and/or shoots from the tissues of the anthers, or indirect regeneration from calli with organogenic structures. The most effective medium variant was V4, containing 8.9 µM BAP and 2.7 µM NAA. On this variants 59,3% from reactive anther proved to have organogenic competences, 30,4% embryogenic competences, and only 10.2% differentiated calli. Due to the fact that is desirable to obtain the plants directly from the anthers, we considered this variant as being one of the most effective combinations of PGRs for plant regeneration.

Table 2. Effect of PGR on callus induction (callusogenesis), organogenesis, embryogenesis and plant regeneration in anther culture of *B. oleracea* (mean±SE)

Variant	organogenesis		embryogenesis		callusogenesis	
	%	Average no. of shoots/exp	%	Average no. of shoots/exp	%	Average no. of shoots/exp
V_0	15.9	4.69±0.18	19.1	0.41±0.46	65.0	5.62±0.24
V_1	35.9	11.52±0.32	28.3	1.70±0.38	35.7	14.17±0.41
V_2	38.6	14.78±0.36	22.1	2.02±0.28	39.2	18.03±0.29
V_3	18.7	16.66±0.52	29.6	2.13±0.52	51.6	19.80±0.64
V_4	59.3	21.33±2.21	30.4	3.18±0.60	10.2	25.51±0.98
V_5	33.7	16.09±0.48	21.6	1.42±0.29	44.5	19.79±0.14
V_6	18.5	7.33±0.35	7.8	0.38±0.34	72.8	8.20±0.31
V_7	10.3	7.28±0.28	0.4	0.62±0.12	89.1	8.51±0.45
V_8	26.7	6. 44±0.18	4.0	0.85±0.24	68.2	7.79±0.30
V_9	45.5	9.33±0.35	4.4	1.00±0.20	50.4	10.44±0.29
V_{10}	21.8	8.21±0.33	3.7	0.57±0.10	74.4	9.67±0.71
V_{11}	31.5	12.42±0.15	31.1	1.50±0.15	34.9	14.77±0.20
V_{12}	31.3	13.27±0.72	27.8	2.00±0.15	38.4	15.81±0.63
V_{13}	28.8	11.88±0.24	17.9	2.18±0.32	50.8	14.01±0.35
V_{14}	38.5	17.95±0.88	29.4	2.22±0.19	29.6	21.39±0.17
V_{15}	25.5	14.54±0.98	16.1	1.70±0.34	57.9	17.44±0.84

The effect of PGRs on the number of anthers that generated callus

Still, one of the most frequent regeneration responses was callus formation. On all media, including control medium, the anthers generated small calli, mainly on the filament side of the anthers in two three weeks after inoculation. Transferred calli on fresh media with the same composition of PGRs developed shoots and somatic embryos. Better results were noticed on media with BAP and zeatin as cytokinin. The stereomicroscopic observations underlined two types of callus: embryogenic and nonembryogenic. The embryogenic callus, with a relatively undifferentiated structure had isodiametric cells and areas with somatic embryos and shoots in different areas and in different stages of development, while the nonembryogenic callus had large, anisodiametric cells, with tracheides but no regenerative areas. The type and concentration of PGRs determined also differences in the colour of callus. Thus, on media with BAP and zeatin the predominant colour of callus varied between white green to dark green, while on media with kinetin the calluses were mainly white or white-brownish. The results obtained revealed that the consistence of callus obtained on media with kinetin was friable, non regenerative, the rate of regenerated shoots per callus being relatively low when compared with BAP or zeatin containing media.

Plant regeneration started two weeks after the transfer of calli to fresh media. Some of them regenerated both green and albino plantlets. Plant regeneration was greatly affected by the type of cytokinin added to medium. Thus, on media with kinetin the regeneration were significantly reduced, while BAP promoted the development of shoots from callus. The shoots, aseptically removed from tubes and placed on new fresh media allowed the continuation of regeneration processes through the apparition of adventitious shoots at the base of the newly formed ones.

The effect of PGRs on the number of anthers that generated shoots and embryos

The frequency of adventitious shoot regeneration was highly influenced by growth regulator concentration and combination. During the first week after inoculation, the first visible change in anthers was their slight enlargement in size. Adventitious shoots developed especially on the filament side of anthers and initially appeared as small multiple outgrowths. Regeneration percentage was affected by the addition of naphthalene acetic acid (NAA) with higher regeneration at higher concentrations (Table 2). Among the different combinations of plant growth regulators tested, NLN medium supplemented with BAP 8.9 µM and NAA 2.7 µM gave the best results, with higher frequency of direct shoot regeneration (59,3%) and number of shoots per explant (21.33 ± 2.21).

The embryos formation was also higher on this variant, 3.1±0.60 embryos per anther being the highest value obtained. At lower concentrations of BAP (4.4 µM) the frequency of embryo and shoot regeneration and the number of shoots per explant decreased.

The addition of zeatin in culture media also proved to be effective in Brassica anthers cultivated *in* vitroThe combination of zeatin with NAA was also beneficial for shoots and embryos development the values obtained on these variants being higher when compared with zeatin alone.

The kinetin proved to be less effective for inducing the shoot and embryo formation. On the variants with kinetin both, the frequency of shoot and embryo regeneration, and the number of embryos and shoots per anther were lower.

The induction of callus was the main morphogenetic reaction as 50.79% from total reactive anthers, gained callusogenetic competence. Direct organogenesis was observed at 30.03% of anthers, where small meristematic centers started to appear directly on the surface of globular-shaped anthers. Only 18.35% from total reactive anthers generated proembryo and embryo directly from anther's tissue.

After the first plantlets appeared, they were removed aseptically from the culture tubes and transferred on freshly prepared sterilized medium containing the same combination of PGRs. Gradually, at the basis of each new plantlet, both on the surface and inside the medium started to appear small meristematic centers that evolved in shoots. The transfer of the shoots on NLN basal medium supplemented with 2.7 µM NAA allowed root formation. 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.

While Górecka et al. (1997)reported a better response on B5-2 medium, containing sucrose (20 g/l) and kinetin (20 mg/l), in the experimental conditions of our study the ability of anthers to gain organogenic and embryogenic competence was higher on NLN medium supplemented with BAP 8.9 µM and NAA 2.7 µM .

CONCLUSIONS

- The results highlighted in the present study show that the morphogenetic reaction of anthers is highly linked to the addition of PGRs in culture media, the type of PGRs and their quantities, as it directly influence the ability of anthers to gain organogenic and embryogenic competence. Thus, on Brassica anthers the most effective PGRs combination,

among the ones tested by us, was BAP 8.9 µM with NAA 2.7 µM with the highest frequency of direct shoot regeneration and number of shoots per explant.

- Shoot development and rooting were successful on NLN basal medium supplemented with 2.7 µM NAA, and acclimatized plants were transferred to grow to maturity in the greenhouse.

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