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PRELIMINARY STUDIES ON THE INFLUENCE OF EXOGENOUS HORMONAL FACTORS ON THE „IN VITRO" ORGANOGENESIS OF *BRASSICA OLERACEA* L. VAR. *CAPITATA*

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***Key words*:** *PGR, zeatine, BAP, cabbage, vitro*

INTRODUCTION

Brasica oleracea contains many important vegetable crops, such as cabbage, cauliflower, broccoli and brussels sprouts. In specialized literature, there are numerous reports regarding plant regeneration from explants in several *Brassica* species including *B. napus* (Glimelius, 1984; Zhao et al., 1995), *B. oleracea* (Jourdan and Earle, 1989; Hansen and Earle, 1994), *B. campestris* (Zhao et al., 1994), *B. juncea* (Kirti and Chopra, 1990; Bonfils et al., 1992), *B. carinata* (Jaiswal et al., 1990) and *B. nigra* (Narasimhulu et al., 1993).

However, it is well known that the ability to regenerate shoots is highly dependent on the genotype (Zhao et al., 1995).

Another important factor is the type and combination of growth factors. The importance of the culture medium, especially of the levels of cytokinins and auxins, in callus induction, organ formation and multiplication, has been demonstrated for a large number of plant species. Due to the importance played by the PGRs in the orientation of the morphogenetic responses of the explant, this aspect has been revised by many authors, in different species. In 2004, Gaj presented statistics about the number of species that respond to and about the protocols that use different PGR groups and combinations to induce regeneration.

In his work, he stated that, among auxins, the most frequently used was 2,4-D (49%) followed by naphthalene acetic acid (NAA, 27%), IAA (6%), indole-3-butyric acid (6%), Picloram (5%) and Dicamba (5%).Among the protocols in which cytokinins were used as PGR for induction of regeneration, Raemakers et al. (1995) mentioned that N6-benzylaminopurine (BAP) was the most frequently employed (57%), followed by kinetin (37%), zeatin (3%) and thidiazuron (3%).

MATERIAL AND METHODS

The experiments were carried out in the Laboratory of Tissue Culture at Vegetable Research and Development Station Bacau, Romania. The biological material is represented by seeds belonging to two lines IS 21 and IS 57.
The seeds were surface sterilized with 0.1% (wt/vol) mercuric chloride ($HgCl_2$) for 7 minutes, followed by repeated washing with sterile distilled water.

Seeds were germinated in the dark in full strength of basal medium Murashige and Skoog, 1962, supplemented with 3% sucrose and 0.8% agar. Hypocotyls from five days seedlings were used as a source of explants for the experiments.

Under aseptic conditions, explants were inoculated on basal MS (Murashige and Skoog, 1962) medium containing 3% (w/v) sucrose, supplemented with different concentrations and combinations of 6-benzylaminopurine (BA) indole-3-acetic acid (IAA) or naphtyl acetic acid (NAA) and zeatin for direct plant regeneration – table 1.

Table 1. Components of different nutrient media for shoot induction at white cabbage

Components	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8
Macro elements	MS	MS	MS	MS	MS	MS	MS	MS
Micro elements	MS	MS	MS	MS	MS	MS	MS	MS
Vitamins	MS	MS	MS	MS	MS	MS	MS	MS
zeatin*	9.1	9.1	9.1	13.6	-	-	-	-
BAP*	-	-	-	-	8.8	8.8	8.8	13.3
NAA*	2.6	-	-	-	2.6	-	-	-
IAA*	-	2.8	-	-	-	2.8	-	-
Sucrose**	30	30	30	30	30	30	30	30
Agar**	8	8	8	8	8	8	8	8
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8

* expressed in µM, ** expressed in g/l

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.

Cultures were then incubated at 26±1°C, a 16-h photoperiod, and 5000 lx light intensity.

The elongated shoots were cut out and transferred into rooting medium that contained auxins in different concentrations (NAA and IAA) – table 2.

Table 2. Components of different nutrient media for root induction at white cabbage

Components	V1	V2	V3	V4	V5
Macro and micronutrients	Murashige and Skoog, 1962				
Vitamins	Murashige and Skoog, 1962				
NAA*	4.8	3.2	1.6	-	-
IAA*				5.1	3.4

* expressed in µM

The rooted plantlets were washed and transferred to the hydroponics conditions in bottles. Different variants were tested: simple water, addition of Previcur (a substance used to control fungus) in concentration of 0.15% and directly to potting mixture in plastic pots. The pots were covered with clear bags to provide 100% relative humidity. They were placed in an acclimatization room under a 16/8 h photoperiod at 20 - 23°C. The acclimatized plants were planted in a potting mixture of sterilized sand + vermiculite (1:1 ratio) in plastic cups, hardened in a mist chamber (80% relative humidity) for 2 weeks before transfer to green house. The plants were allowed to grow to maturity. They normally passed vegetative phase.For each genotype, 10 hypocotyls were cultured and the experiment was repeated 3 times. The percentage of buds forming regeneration structures and the mean number of shoots per explant was recorded. The data were analyzed by ANOVA (analysis of variance). The means were compared using the Duncan multiple comparison test at $P < 0.05$. In tables the mean values and standard error for each genotype were shown.

RESULTS AND DISCUSSIONS

Shoot buds got initiated on the initial explants after 6 days of culture. Only 0.04% of the axillary buds didn't have any positive development, the noted reaction being toward partial or total necrosis of the tissues. The higher frequency (97.5%) formation of maximum number of shoots was observed in 9.1 µM zeatin in combination with 2.8 µM IAA (variant V2). Initially 1 or 2 buds developed, later up to 16 shoots of above 4 cm length were formed in node in only two weeks. BAP in combination with IAA was less effective than zeatine as it induced only up to an average value of 74,37% (IS 21), respectively 82.05 (IS 57) formation of 5 - 6 shoots. Preliminary studies proved that cabbage explants culture in MS medium individually supplemented with both BAP and zeatin showed remarkable response. The literature presents data regarding the implication of 6-benzylaminopurine in the process of direct somatic embryogenesis and organogenesis, as responsible for preservation of the mitotic stimulus in the cells of the immature zygotic embryo (Pretova et al. 2000). The results obtained in our experiment proved the fact that among the tested cytokinins, 9.1 µM zeatin responds well compare to BAP in medium for shoot proliferation (see Table 3).

Table 3. Effects of different concentrations of BAP, zeatin alone and in combination with IAA or NAA in MS medium for multiple shoot induction from axillary buds of *B. oleracea* L. after 60 days (3 subcultures) – means ±SE

	% of explant showing response		Average no. of shoots		Average length of shoots	
	IS 21	IS 57	IS 21	IS 57	IS 21	IS 57
V_1	95.2	96.3	34.3±1.5	30.5±1.8	4.4±0.46	3.85±0.24
V_2	97.5	100	43.9±2.9	38.4±1.3	3.7±0.38	4.00±0.20
V_3	96.4	98.1	42.0±0.5	36.2±0.4	3.5±0.28	3.57±0.10
V_4	90.9	95.4	37.9±0.5	34.2±0.3	4.1±0.52	3.50±0.15
V_5	72.0	81.3	18.0±0.5	16.4±0.3	3.1±0.60	3.00±0.15
V_6	75.5	85.0	11.0±0.4	14.3±0.2	3.4±0.29	4.18±0.32
V_7	74.7	79.3	10.8±1.6	13.4±0.4	3.3±0.34	4.22±0.19
V_8	75.3	82.6	12.8±0.6	17.8±0.2	3.6±0.12	3.70±0.34

In order to evaluate the synergistic effect of zeatin with IAA and NAA for direct plant regeneration, IAA combinations responded well compare to NAA.

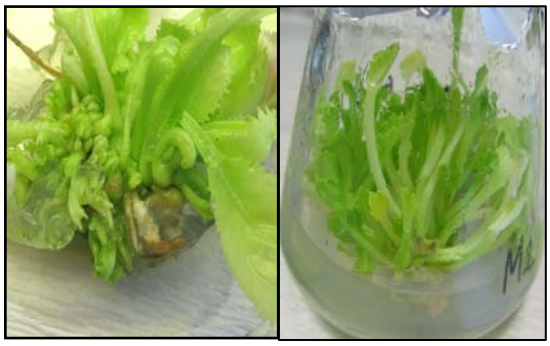

Fig. 1 a - b. Development of new shoots

The maximum induction of multiple shoots (43.9±2.9 – IS 21 and 38.4±1.3 – IS 57) was achieved from medium supplemented with 9.1 µM zeatin and 2.8 µM IAA, with an average shoot length of 3.7 cm (Figures 1a-b). The maximum number of shoots is tightened with the

obtaining of shoots with strong roots that could be directly transferred to hydroponic conditions.

If, in the combination zeatin with auxins, IAA is replaced by NAA – variant V1, the obtained results have lower values and this is due to the vitrification of the shoots and of course such plants gradually perish. The regenerative potential of this cultures are still high the poor results being related with this phenomena that is directly reflected in the number of shoots/explant. After 3 to 4 weeks, when regenerated shoots reached a length of more than 4.0 cm, they were separated and transferred on MS basal medium with different concentration of NAA and IAA.

Table 4. Effects of different concentration of auxins NAA and IAA alone on root formation at cabbage *in vitro* regenerated shoots (mean +SE)

	% rooted shoots		Average no. of roots/shoot		Average length of roots	
	IS21	IS57	IS21	IS57	IS21	IS57
V_1	95.3	96.4	9.66±1.2	11.40±1.3	2.00±0.2	2.70±0.2
V_2	99.6	98.9	7.42±0.1	8.60±0.3	4.50±0.2	3.80±0.3
V_3	91.2	90.3	6.05±0.1	5.90±0.2	2.80±0.4	3.42±0.4
V_4	93.2	95.5	6.57±0.3	4.63±0.2	3.85±0.3	2.95±0.2
V_5	96.8	97.1	7.59±0.2	6.85±0.2	3.10±0.2	4.00±0.8

As it is illustrated in the table 4, both the NAA and IAA allows the development of roots, the best results being obtained on variant V2, characterized through the presence of NAA in a quantity of 3.2 μM. The increase in the quantity of NAA (variant 1 – 4.8 μM) is not reflected in the increase of rooted shoots, while the decrease at a level of 1.6 (variant V3) is directly related with the decrease of number of rooted shoots. In what concern the addition of IAA the values obtained are almost similar with NAA, the best results being obtained on the variant V5 with a lower concentration of IAA.The rooted plantlets were transferred to the hydroponics conditions in bottles.

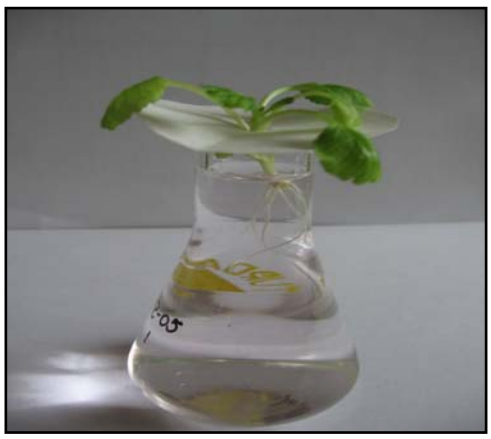

Fig. 2. Plants at acclimation stage

Before their transplantation the root system of the plantlets were continuously washed with tap water. Then the plantlets were transferred to different hydroponic variants: simple tap water, addition of Previcur (an accredited substance utilized for fungus control on vegetable plants) in concentration of 0,15% and directly to potting mixture. About 98.7 % of the plants established under *ex vitro* conditions when they were initially transferred on the variant with Previcur. It was also observed that 83% of the plants survived if they were directly transferred to potting mixture in plastic cups.

Fig. 3. Plants in greenhouse conditions

The plants were then transferred to greenhouses and allowed to grow to maturity. They normally passed vegetative and generative phase.

CONCLUSIONS

The researches finalize through the obtaining of viable plants through direct organogenesis and embryogenesis. In the experimental condition tested in the present study, the axillary buds used for the initiation of the regeneration processes allowed the multiplication of a large number of plants that could be used as parental lines for the obtaining of F_1 hybrids. The capacity of regeneration is strongly depending on the type and quantity of exogenous hormones.

The best morphogenetic reaction was obtained on the variants that contained as growth regulators zeatin and IAA. These hormones allowed at both genotypes the development of meristematic centers that rapidly evolved in shoots. Optimum values for shoot induction, were obtained on MS medium, supplemented with 9.1 μM zeatin and 2.8 μM IAA. One hundred percent of explants cultured on this medium turned green and showed a good differentiation.

The transfer on new medium with the same composition allows both the development of the existing shoots and the initiation of new ones – in a bigger percent when comparing with the other variants, 43.9±2.9 (IS21), respectively 38.4±1.3 (IS57), comparing with 10.8±1.6, respectively 13.4±0.4, on the variants with BAP. For the development of rooting system of regenerated shoots, the best results were obtained on variant V2, characterized through the presence of NAA in a quantity of 3.2 µM. The rooted plants were gradually adapted to normal *ex vitro* conditions.

About 98.7 % of the plants established under *ex vitro* conditions when they were initially transferred on the variant with Previcur in a concentration of 0.15%. The plants transferred to greenhouses normally passed the vegetative and generative phase.

ABSTRACT

The implication of tissue cultures „in vitro" in the conservation of valuable genotypes of *Brassica oleracea* has a tremendous importance for the breeding activity of this species, due to its increased multiplication outturn, aspect associated with also with the assurance of a high degree of uniformity and conformity of the obtained descendencies.

Comparing with the classical methods, the tissue culture "in vitro" presents the following advantages: the process is not limited by the seasonal changes imposed by the succession of seasons, the control of growth regulators is assured, it prevents the manifestation of negative influences determined by the expression of genetic variability in descendencies, the genetic conservation of selected plants is assured for a longer period of time.

An extremely important stage for the establishment of an efficient multiplication method "in vitro" is the determination of optimum conditions for the induction, support and development of regenerative processes of cultivated explants.

The factors that influence the capacity of regeneration of plants "in vitro" are numerous, but among them, the most important are the exogenous growth regulators.

Thus, for the determination of the best hormonal combinations, in the present study the authors tested 8 medium variants, that differ both from the point of view of quantity of hormons and the combinations between them.

The results obtained, indicated that the best morphogenetic reaction is obtained on variant with Zeatine as the main growth regulator, the results on this variant being superior comparing with the mediums with BAP.

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