**G & BM**
**Tome IV**
**Iaşi 2003**

# OBSERVATIONS ON *IN VITRO* BEHAVIOUR OF THE SPECIES *Brassica juncea* Czern.

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**Key words:** *Brasica juncea,* in vitro, micropropagation.

**Abstract:** This paper presents the morphogenetic reaction of different explants of *Brassica juncea Czern.* Species in " in vitro" cultures. The obtained results showed that the micropropagation of this plant could be recommended only for multiplying some valuable genotypes.

## INTRODUCTION

The species *Brassica juncea* Czern., synonym with *Sinapis juncea* L. (the violet-blue, or brown mustard), is a herbaceous annual plant, belonging to the family *Cruciferae*; it is spread in North-East Africa, East Asia and it is introduced into culture in many regions on the Earth. It is cultivated on large areas in the region of lower Volga River, Northern Caucasus, Ukraine, between Azov Sea and Black Sea, generally in those areas where its resistance to drying recommends it to be preferred instead of black mustard.

The seeds of brown mustard have a characteristic – piquant, hot, taste. The active substance in the seeds is the glycoside sinigrine which, under the influence of the specific enzyme (mirosine), is transformed into mustard oil (alyl - isosulfocyanate), kalium bisulfate and glucose. The glycoside occurs together with the enzyme within the seeds, but the enzyme is activated only by moistening the seed powder in warm water (up to $60^0$ C). The mustard oil is a light- yellow liquid, with an irritant odour and it represents 0.5 – 1.7 % of the dry weight. There are also greasy, non-siccative oil (35 – 40 %), proteins, mineral salts etc. in the seeds (GAMMERMAN, 1952; SHUPINSKAYA and KARPOVICH, 1963; PÂRVU, 1997).

The powder resulted from decorticated brown mustard seeds is used for preparing alimentary mustard and mustard paper (the paper is painted with rubber oil and spinkled on with mustard powder). After the extraction of the greasy oil from the seed mass, the resulted pomaces are used to prepare the mustard powder.

Mustard oil is toxic, it produces skin irritations, even vesicles and wounds. In alcoholic solutions (2 %), mustard oil is used for external treatments (as massages), based on its revulsive, anti-inflammatory and anti-rheumatic properties. The greasy oil from brown mustard is used, as other vegetable oils, in human nourishment.

## MATERIAL AND METHODS

The biological material (the seeds) used in this study was obtained from Bacău Vegetable Research Station, and the investigations dedicated to "in vitro" behaviour of the species *Brassica juncea* Czern. were carried out at the "Stejarul" Research Center Piatra Neamţ. For initiating the "in vitro" culture, the brown mustard seeds were sterilized for 15 minutes in 0.1 % mercury chloride solution; subsequently, they were

washed three times with sterile distilled water and inoculated on the ground nutritive medium Murashige-Skoog (1962). On this medium the seeds sprout rapidly and the germination capacity surpasses 95%.

For testing the morphogenetic reaction, there were used as explants three-day old germs, fragments of hypocotyls, tips of the small plants in cotyledonary stage, callus obtained by certain hormonal formulas.

Explants were cultivated in 100 ml Erlenmeyer flasks, on MS medium solidified with 8.5 g/l of agar, having succrose (25 g/l) as carbon source; the medium was supplemented with growth regulators in various concentrations and combinations (Table 1).

Table no. 1 Hormonal formulae used for "in vitro" culture of *Brassica juncea* Czern.

| No. | Hormonal formula | Growth regulators (mg/l) | | | | | |
|---|---|---|---|---|---|---|---|
| | | GA | BAP | IAA | IBA | NAA | 2.4 - D |
| 1 | $A_2$ | - | - | 2.0 | - | - | - |
| 2 | $B_{0.2}$ | - | 0.2 | - | - | - | - |
| 3 | $B_{1.0}$ | - | 1.0 | - | - | - | - |
| 4 | $BA_1$ | - | 1.0 | 0.5 | - | - | - |
| 5 | $BD_1$ | - | 1.0 | - | - | - | 0.5 |
| 6 | $BN_1$ | - | 1.0 | - | - | 0.5 | - |
| 7 | $D_2$ | - | - | - | - | - | 2.0 |
| 8 | GA | 0.5 | - | 0.5 | - | - | - |
| 9 | IBA | - | - | - | 1.0 | - | - |

## RESULTS AND DISCUSIONS

For the starting of "in vitro" cultures of *Brassica juncea* Czern., there were used small plants obtained from seeds aseptically germinated on hormone-free Murashige-Skoog medium, or on the same nutritive medium, supplemented with 0.2 and 1.0 mg/l benzyl-amino-purine (BAP). It was observed that the seeds germinate quickly and the plants present an intense growth on hormone-free MS medium. The introduction of BAP in the culture medium determined an inhibition of hypocotyls and stimulated strongly the rhizogenesis, consisting in the development of an abundant layer of absorbant hairs on the primary rootlet.

In one of the experiments it was tested the morphogenetic reaction of hypocotyl fragments on the medium MS supplemented with several hormonal formulae. Contrary to expectations based on the results obtained on other plant species (GHIORGHIȚĂ et al., 1990, 1997), the weakest reaction of this type of explants was recorded on the MS medium supplemented with 2.0 mg/l 2.4-D. The fragments of hypocotyl grew in thickness, generating a compact callus, cream-coloured or light-green at the ends; the proliferation speed of callus cells is rather low (Tab. 2). Callus and root formation processes were absent on this hormonal formula.

The best morphogenetic reaction showed the hypocotyl fragments grown on nutritive medium supplemented by 1 mg/l BAP and 0.5 mg/l IAA. Under these culture conditions the explants generated callus on all their surface; this was compact and cream-coloured along the hypocotyl fragments and light-green and round-shaped at the ends of hypocotyl fragments, giving to these formations the general shape of dumb bells. Sporadically, some of the appeared calluses generated small, isolated, sprouts or fine bunches of roots.

On the MS medium supplemented with 1 mg/l BAP and 0.5 mg/l NAA ($BN_1$), hypocotyl fragments had a decreased callus formation reaction as compared with $BA_1$, in this case callus formation being more active only on several parts of the explant. This callus presented a solid consistence and was cream-coloured. $BN_1$ favours root formation, most of the explants presenting bunches of white, thin, small roots on certain parts (especially at the ends).

The hypocotyl fragments introduced on MS medium supplemented with 2 mg/l IAA formed a quite friable callus, cream-coloured, with reduced proliferation. IAA induced, on the other hand, an intense root generation. The supplementation of the nutritive medium with 2,4-D influenced unfavorable both callus generation processes and organogenesis processes. The association of 2,4-D with BAP ($BD_1$) stimulated the formation of a solid-consistence callus, green-coloured and presenting a high speed cell proliferation.

In an another experiment we investigated the morphogenetic reaction of the shoot tips in cotiledonary stage, under different hormonal formulae. On the medium supplemented with 2,4-D, the explants remained in cotiledonary stage. At the contact of the stem with the nutritive medium a small, compact, cream-coloured callus appeared. In spite of the increasing of the cotyledons, $D_2$ medium inhibited the organogenesis.

On the medium $BD_1$, at the contact on the stem with the nutritive medium a well represented, round-shaped, compact, white-greenish callus was generated. This medium formula determined shoot development and the appearence of the true leaves, but the process of caulogenesis is still weakly represented. The leaves became white-yellowish and they dried in time.

On the $BA_1$ nutritive medium the shoots continue their growth and developing processes, their strength being lower to that recorded at plants grown on hormone-free MS medium. The size of the shoots, at the same age, is smaller than on MS, $B_{10}$ and $BN_1$. The phenomenon of multiple shooting was rarely observed. At the contact of the explant with the nutritive medium appears a well developed, cream-colored, compact callus (Tab. 2).

The nutritive medium supplemented with 1 mg/l BAP ($B_{10}$) provided a good growth and development of the shoots, which present a vigorous aspect. The same age shoots are however smaller than that grown on hormone-free MS medium. In this variant too, at the contact of the stem with the nutritive medium was generated a rich callus, with a high cell proliferation capacity, cream-colored and of solid consistence. Occasionally, $B_{10}$ medium determines multiple shooting and root generation.

Table 2 : The morphogenetic reaction in vitro cultures of *Brassica juncea* Czern.

| Variant | Used explant | Hormonal formula | **Morphogenetic reaction and proliferation speed** |
|---|---|---|---|
| 1 | Hypocotyl fragments | $BA_1$ | Cream-colored callus(+++), irregular, light-green, compact callus(+++), round shaped at the ends of the fragment, sporadically shoots (+) and roots (+) |
| 2 | Hypocotyl fragments | $BN_1$ | Reaction similar to that on $BA_1$; compact, cream colored callus(+++) along the explant, compact callus(++), light-green and rounded at the ends of the explant, root generation (+++), caulogenesis absent |
| 3 | Hypocotyl fragments | $BD_1$ | White-greenish, compact, calus (++), rugged at only one of the ends of hypocotyle fragment; caulogenesis and root generation – absent |
| 4 | Hypocotyl fragments | $D_2$ | Compact callus(+), cream-greenish at the ends of the fragment |
| 5 | Shoot tips in the cotyledonary stage | $B_{10}$ | Compact, cream-coloured callus (++) at the contact of the stem with the nutritive medium, vigorous shoots (++), sporadically roots (+), sporadically multiple shooting (+) |
| 6 | Shoot tips in the cotyledonary stage | $BA_1$ | Compact, cream-coloured callus(++) at the contact of the stem with the nutritive medium, sprouts (++), sporadically multiple shooting |
| 7 | Shoot tips in the cotyledonary stage | $BN_1$ | Compact, cream-coloured callus(++), shoots (+++),roots(+++) as bunches, presenting a lot of absorbant hairs |
| 8 | Shoot tips in the cotyledonary stage | $D_2$ | Very weak morphogenetic reaction; compact, cream-coloured callus(+) at the contact with the nutritive medium; the small plants remain in the cotyledonary stage and degenerate in time |
| 9 | Shoot tips in the cotyledonary stage | GA | Compact, cream-coloured callus (++) at the contact with the nutritive medium, sporadically roots(+),small sprouts(+) are generated sporadically at the basis of the stem |
| 10 | Leaves | $D_2$ | Compact, cream-brown-coloured callus(+), friable |
| 11 | Stem callus obtained on $B_{10}$, $BA_1$, $BN_1$ | $B_{02}$,$B_{10}$, GA | The callus doesn't proliferate, and after about six weeks degenerates, colour changing to brownish one |
| 12 | Stem callus obtained on $B_{10}$, $BA_1$, $BN_1$ | $A_2$ | The callus proliferate slightly (++) and keeps its consistence and colour (cream or light-green); several fragments of callus generate on their surface bunches of absorbant hairs (++); burgeons of sprouts (+) appear sporadically in the zones with light-green callus |
| 13 | Stem callus obtained on $B_{10}$, $BA_1$, $BN_1$ | $BA_1$ | The callus cultivated subsequently on $BA_1$ keeps its initial characteristics, proliferates best (+++); light-green callus generates sporadically small shoots (+) forming a rosette |
| 14 | Stem callus obtained on $B_{10}$, $BA_1$, $BN_1$ | $BN_1$ | The callus proliferate weakly (+) but is viable even after seven weeks after the transfer; it keeps its initial characteristics |
| 15 | Three-day old germs | $BA_1$,$BN_1$ | Two weeks after the inoculation the first two true leaves appear; the growth of the stem and especially of the root – strongly inhibited; the root – as a tap root (0.5 cm long), rounded by a lot of absorbant hairs |
| 16 | Three-day old germs | $BD_1$, $D_2$ | Root growth is entire inhibited; the stem and cotyledons growth is also strongly inhibited |
| 17 | Three-day old germs | $A_2$ | Stem growth and elongation is less inhibited; root growth is strongly inhibited (0.5-1 cm), being entirely covered by thin absorbant hairs |
| 18 | Three-day old germs | GA,IBA | Stem growth and elongation – similar to that on the control medium (MS), the first true leaf appears; the growth of the root is inhibited, the root is covered with bunches of absorbant hairs (especially on IAB) |

+ : low proliferation capacity; ++ : good proliferation capacity; +++ : very good proliferation capacity

Shoot tips in the cotiledonary stage grew about normally on the medium $BN_1$ too (Tab. 2). The size of the shoots is inferior to that of MS medium, and the shoots are less vigorous as that grown on $B_{10}$. At the contact of the stem with the nutritive media also appears a compact, cream-coloured callus. This medium favors root generation. Both normal roots and groups ofroots, forming short and dense bunches of roots are observed.

The introduction of IBA in the culture medium permitted the normal development of the shoots, their leaves being wider, dark-green and with goffered aspect. IBA stimulates theforming of normal roots and obtaining of neoplantlets.

On MS, hormone-free medium, the caulogenesis develops normally, the leaves are greater but more thin than on the media $B_{10}$, $BA_1$ and $BN_1$. The process of root forming runs more slowly compared to the variant on IBA medium. Generally, this process lasts for 3 weeks – one month.

On all the tested culture media the shoot tips in cotyledonary stage also generated neoplantlets. The highest frequency was recorded on the media supplemented with IBA, and, secondly, on MS. It is to be specified that, in spite of the favorable effects of $BN_1$ on root generating, it also determines callus formation and on this basis it may not be recommended for the stimulation of rooting of sprouts. All the media which induced caulogenesis also determined the generation of shoots forming a rosette.

During these investigation program the morphogenetic reaction of brown mustard germs on various hormonal formulae was also tested. Compared to the germs inoculated on MS nutritive medium (considered as control) after two weeks from the beginning of the experiment, it was stated that the seeds grown on hormonal supplements showed higher or lower disturbances in root and stem development. On media GA and IBA these disturbances are the most reduced. The length of hypocotyls reaches within this time interval about 5-6 cm, the roots are well developed and show similar sizes; on the roots there are bunches of absorbant hairs, especially on IBA. The media $BA_1$ and $BN_1$ inhibited obviously the growth of hypocotyls which reach 1.5 – 2 cm, compared to the 5-6 cm determined in the control (MS), but caused the appearance ofthe first two true leaves. The stem becomes thicker at the limit of the root, and the root development is strongly inhibited (it reaches only 0.5 cm), seeming to a pivote surrounded by a dense layer of absorbant hairs. A similar reaction showed the germs inoculated on nutritive media supplemented with BAP (0.2 and 1.0 mg/l), with the difference that the root becomes thicker and reaches superior sizes (1.5 – 2.5 cm). On $A_2$ medium stem growth intensity ranges between that recorded on the media supplemented with IBA or GA and that supplemented with BAP (about 4 cm high); the small plants remain yet in cotyledonary stage, and root growth is also strongly inhibited (0.5 – 1 cm length), being covered on all the surface by short absorbant hairs. The strongest inhibition of growth was recorded on the media containing 2,4-D. The hypocotyls become thicker and reach 1 cm length, and root growth is suppressed.

In order to accomodate the obtained neoplantlets to the various hormonal formulas a hydroponic system was used, the process running in the laboratory room used for "in vitro" cultures. Accomodation process of brown mustard regenerants lasts about 12 days and no significant losses were recorded. In this stage of the research program it may be considered that the micropropagation is not recommended in this species. We can

affirm that generative reproduction is easy and doesn't arise difficulties, and "in vitro" multiplication is a expensive method and doesn't show a high efficiency in this species. As an allogamous plant, the micropropagation in brown mustard may be useful for the conserving of bioproductive characteristics of certain valuable genotypes.

During this research program it was studied also the capacity of keeping proliferation speed and even its stimulation by subsequent cultivation of the calluses obtained on the same or on the other hormonal formulae. It was stated that callusal tissue transferred from $BA_1$ medium (on which it was generated) to one of the media $B_{02}$ or $B_{10}$ did not proliferate and degenerated in time, acquiring a brownish colour. The callus transferred from $B_{10}$ on $BN_1$ proliferates easily and keeps its viability even after seven weeks. The best results as concerning callus proliferation are offered by $BA_1$ nutritive medium and by the transfer of the callus from this medium to $A_2$ medium. The other tested media ($B_{02}$, $B_{10}$, GA, $D_2$, $BN_1$ etc) provides a weak or insignificant proliferation of callusal tissue. Also, the organogenetic processes through the transfer of the callus onto various hormonal formulas are weakly developed. In this case the callus would contain active substances of economic interest, the amount of biomass offered by callus cultures seems to present no perspective, at least on the media tested in this study.

## CONCLUSIONS

The investigations carried out in order to know the morphogenetic reaction on "in vitro" cultivation of the species *Brassica juncea* Czern. evinced the following aspects:

1. The starting of "in vitro" culture of *Brassica juncea* Czern. may be easily realized using explants originating from plantlets obtained from seeds germinated aseptically on hormone-free nutritive medium Murashige-Skoog (1962).
2. For callus obtaining there may be recommended as explants fragments of hypocotyl, and as nutritive media – that supplemented with IAA, or with BAP + IAA. As reported to auxins, the brown mustard show a clear positive reaction for IAA, and a negative one for 2.4-D.
3. Subsequent cultivation of the callus on various hormonal formulae didn't determine the intensification of its proliferation capacity. Indirect organogenesis, via callus, is weakly representative within this species.
4. Caulogenesis could be provided by the culture of shoot tips in cotiledonary stage on MS nutritive medium supplemented with BAP, BAP + IAA, BAP + NAA, IBA. At the contact with the nutritive medium, at the shoot basis a callus layer more or less developed and, sporadically, roots appear on this media . The phenomenon of multiple shooting is also rare.
5. For rooting of rosette-shaped shoots, obtained on various hormonal formulae, the hormone-free nutritive medium MS is recommended, or the same medium supplemented with 0.5 mg/l IBA; the process efficiency is about 75 % and lasts for three weeks – one month from the inoculation.
6. The accomodation of the neoplantlets obtained "in vitro" to the "ex vitro" conditions is performed easily under hydroponic system, during a period of

time of 10 – 12 days, that implies certain losses(about 10 – 20 %). The regenerants adapted to septic conditions resist well to the transplantation in soil if water supply provided is suitable.

7. Considering that the brown mustard has no difficulties in generative reproduction, the micropropagation in this allogamous species could be recommended only for the multiplication of some valuable genotypes.

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