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BROCCOLI: INSIGHTS OF SOME IN VITRO CELLS AND TISSUE CULTURE RESEARCH

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***Key words**: organogenesis, embryogenesis, haploid, microspores*

INTRODUCTION

The importance of broccoli culture rely on the fact that the plant is a relatively abundant source of vitamins, including provitamin A (primarily beta-carotene, a carotenoid), vitamin C (ascorbate), and vitamin E (tocopherol) (USDA Nutrient Database, 2011). It is also a source of phytochemicals that have been associated with health promotion. Phytochemical groups with reported health activity found in broccoli include glucosinolates, tocopherols, carotenoids, and flavonoids (Farnham et al., 2009). Several authors, reported that diets rich in broccoli reduce cancer incidence in humans. A strong case for a cause-effect association between consumption (dose) and reduction in disease risk exists for the glucosinolates (anti-cancer), tocopherols (cardiovascular) and carotenoids (particularly related to eye-health) (Higdon et al., 2007).

In this age of antibiotics, hormones, corticosteroids and other synthetic products it may seem strange to revert to therapies consisting solely of the use of plants, fruits, vegetables or cereals (Valnet, 1987). Broccoli and broccoli buds contain a phytochemical compound known as sulforaphan, which kills Helicobacter pylori bacteria. This bacterium is responsible for most stomach ulcers and cancers. Tests have shown that sulforaphan can destroy this bacterium, which is also resistant to antibiotic treatment. The sulfur substances that are contained by broccoli, as well as vitamins, play a particularly important role in stopping the chaotic proliferation of cancer cells, contributing to the prevention and even healing of certain types of cancer, such as: esophagus, larynx, stomach, prostate, colon and lungs. It is recommended to consume raw broccoli or juice as much as possible, especially since phytonutrients from cruciferous vegetables are very good protectors of soft tissues.

Broccoli is grown all over the globe, except for the humid tropical areas. China is the largest producer of cauliflower broccoli in the world with 8.5 million tons. In second place is India with 6.5 million tonnes, the production of the dosages, accounting for 80% of the world production.

Although the general tendency remains to cultivate an assortment of traditional vegetables, in recent years there is a beginning of diversification, the tendency being due, both to imports, to the emergence of categories of consumers that allow them to buy more varied and expensive vegetables, as well as to the presence of a large number of foreign nationals who work in Romania and have specific preferences, for example: Chinese cabbage, Brussels cabbage, brined salad, broccoli, asparagus and others. Although at first this assortment came mainly from import, a number of domestic producers have begun to cultivate such species, which they contract with the big chain stores or sell them on the free market, at acceptable price. In Romania, the high demand for broccoli registered by the big chains of stores, creates an opportunity for the local farmers in cultivating this species, especially since the supply of supermarkets with such assortments is mainly made of import. The culture of broccoli is sporadic, probably also because, in recent years, Romanians have discovered it in hypermarkets or have become more informed about it. The broccoli culture is not pretentious to the vegetation factors and each can have this wonderful vegetable in their own garden, with a minimum of effort, resembling, in terms of growing conditions, with cabbage and cauliflower.

All these considerations led to an increased interest in breeding this specie, and in the case of broccoli, research objectives focus on a number of important directions that need to be addressed. An integrated knowledge-based approach to crop improvement with associated cost and efficiency benefits, understanding, conserving, accessing, and harnessing genetic diversity; identifying and/or creating genotypes that adapt to climate change, markets, land use, and energy demands; obtaining improved sustainable production by exploiting the genome, including pest and disease control, water and nutrient efficiency, increasing marketable yields and harvest indexes, etc.

From the organic and conventional production point of view it is known that some organic farmers' desired traits were the same as those of conventional producers, such as drought tolerance, insect and disease resistance or high yield. Other characteristics

were thought to be more important to organic producers than to conventional growers: for instance, vigorous growth and ability to perform in soils with potentially low or fluctuating mineralization rates of nutrients, or the ability to cover the soil and withstand weed competition by having less erect architecture than displayed by modern broccoli hybrids.

Plants display a remarkable potential for cellular totipotency and it appears that any differentiated plant cell that retains its nucleus has the ability to revert to the embryogenic condition and regenerate entire plant (Zhao et. al., 2021). This is the main principle that led to a highly important tool for breeders in overcoming the needs that have to be solved through research.

In plant tissue culture, there exist two distinct mechanisms through which an explant can give rise to a whole new plant: organogenesis and somatic embryogenesis. Typically, in the case of organogenesis, the formation of shoots and roots occurs sequentially, guided by specific culture conditions, primarily influenced by the type and concentration of plant growth regulators within the culture medium. This developmental process is further distinguished by the establishment of vascular connections between the parent tissue and the regenerating segment (Terzi & Lo Schiavo, 1990).

Organogenesis

An indispensable tool for plant regeneration using tissue culture techniques and for plant transformation. Direct organogenesis can considered to be an important aspect when it comes to maintaining the genetic constitution of regenerated plantlets. Direct organogenesis in tissue culture in vitro revealed the integrity of regeneration protocol in many cases (Kazzaz and Taha 2002). Indirect organogenesis involves the regeneration of shoots from the in vitro cultured explants that evolved firstly in callus.

Brassica species have been widely exploited for tissue culture purposes. Regeneration protocols have been developed for most of the *Brassica* species. Organogenesis has been the widely used pathway for regeneration in *Brassica* crops compared to other means of regeneration. Regeneration of plants via organogenesis has been accomplished from various tissues such as cotyledons (Sharma et al., 1990; Hachey et al., 1991; Ono et al., 1994), hypocotyls (Yang et al., 1991), peduncle segments (Eapen and George, 1997), leaves (Radke et al., 1988), thin cell layers of epidermal and subepidermal cells (Klimaszewska and Keller, 1985), roots (Xu et al., 1982), and protoplasts (Glimelius, 1984; Spangenberg et al., 1986: Kik and Zaal, 1993; Hu et al., 1999). However, hypocotyl segments remain the most desirable explants for tissue culture and have been used for most *Brassica* species due to their regenerative capacity.

Robertson and Earle (1986) achieved successful plant regeneration from leaf protoplasts of *Brassica oleracea* L. var. *italica*. Leaf protoplasts were obtained from a hybrid cultivar through enzymatic treatment, which included the use of enzymes such as 2% cellulase (w/v), 1% Macerozyme (w/v), and 0.5% Driselase (w/v).

Somatic Embryogenesis

Somatic embryogenesis serves as an invaluable tool encompassing a broad spectrum of objectives, ranging from fundamental biochemical, physiological, and morphological investigations to the development of highly practical applications. This process can be defined as the transformation of haploid or diploid somatic cells into structures resembling zygotic embryos. These structures exhibit a bipolar nature and lack vascular connections with the parent tissue. Somatic embryogenesis unfolds through a well-ordered sequence of characteristic embryological stages without the fusion of gametes, as described by Williams and Maheswaran (1986), Emons (1994), and Raemakers et al. (1995). An intriguing feature of somatic embryos is their uninterrupted growth, which results from the absence of developmental arrest, as noted by Faure et al. (1998). Notably, both organogenesis and somatic embryogenesis have been observed to occur within the same explant, as reported by He et al. (1990). Furthermore, these processes can originate from specific tissue layers or cells within the explants, as demonstrated by Osternack et al. (1999).

Somatic embryogenesis development can be delineated into two primary phases. The first phase involves the transformation of differentiated somatic cells into embryogenic cells with the capacity for embryogenesis. These embryogenic cells then undergo proliferation. The second phase encompasses the expression of embryogenic competence by these cells, ultimately leading to their differentiation into somatic embryos. Notably, these two phases appear to operate independently of each other, each influenced by distinct factors.

Direct and indirect somatic embryogenesis have often been regarded as two ends of a spectrum (Williams & Maheswaran, 1986; Carman, 1990). Once the induction of embryogenically determined cells is achieved, there seem to be no fundamental distinctions between indirect and direct somatic embryogenesis (Williams & Maheswaran, 1986). Emons (1994) contended that in many systems classified as indirect embryogenesis, the embryogenic callus consists of young embryos (referred to as pre-embryogenic masses or (pre-globular embryos).

The subsequent developmental trajectory hinges on the duration of the inductive stimulus application. A relatively brief period results in a direct process, while a protracted duration leads to an indirect one. Conversely, in other investigations, direct embryogenesis has been employed to describe

the generation of an embryo from a single cell, omitting an intermediate callus stage. This is despite the embryo originating from the dedifferentiation of a previously specialized cell within the explant.

Though somatic embryogenesis has been used in transformation and regeneration systems in many plant species, *Brassica* crops seem to be lagging in this direction: probably because of the tractability and advanced state of organogenesis techniques in *Brassica.* Microspores or anthers have been somatic embryogenesis explants of choice in most *Brassica* species (Lichter, 1989; Sato et al., 1989; Aslam et al., 1990; Baillie et al., 1992). There have been a few reports using other explants, however. Somatic embryos have been obtained from hypocotyls (Kohlenbach et al.. 1982), protoplast-derived colonies (Kranz, 1988), and immature cotyledons (Turgut et al., 1998) in *B. napus.* Somatic embryogenesis has been successfully triggered in Chinese cabbage from cotyledonary explants (Choi et al., 1996). In cauliflower, somatic embryos were achieved by Deane et al. (1997) and Leroy et al. (2000) through the use of hypocotyl explants. Furthermore, somatic embryogenesis has been documented in rapid-cycling *B. napus*, utilizing hypocotyl explants on a basal medium following the Murashige and Skoog (1962) formulation, with an adjusted pH range of 3.5 to 5 (Koh and Loh, 2000).

As for organogenesis, genotype is a very important factor in the embryogenic frequency of most *Brassica* species. For instance, among three distinct Chinese cabbage cultivars examined, only one (Top Salad*) demonstrated the ability to produce somatic embryos (Choi et al., 1996). The influence of genotype has also been highlighted as a crucial factor in species like *B. rapa* (Baillie et al., 1992), *B. carinata* (Barro and Martin, 1999), and *B. napus* (Chuong et al., 1988), where it significantly affects the propensity for embryogenesis.

Somaclonal variation

Genetic variation is very important in crop improvement and forms the basis of development of new varieties.

Somaclonal variation is an important resource in plant breeding, where the variability observed in tissue culture-regenerated plants from somatic cells can be harnessed to develop crops with unique and novel traits. By applying selection pressure during tissue culture it is also possible to develop somaclones resistant to bio tic and abiotic stresses (Jain, 2001).

Somaclonal variation has been linked to alterations in chromosome number and structure, point mutations, and DNA methylation (Brown et al., 1993). Instances of somaclonal variation have been documented in cauliflower plants propagated from adventitious root meristems (Grout and Crisp, 1980) and doubled haploids of *Brassica napus* derived from anther culture (Wenzel et al., 1977; Hoffmann et al., 1982). In anther culture- derived plants of *B. juncea* var. Rai-5, variation in agronomic characters, oil content and fatty acid composition were observed (George and Rao, 1983). Yellow-seeded variants were observed in the progeny of the plants regenerated from cot vie do nary explants of *Brassica juncea* cv. TM-4 (George et al., 1987). Somaclonal variants in the R1 generation were selected from Indian mustard *(B. juncea* cv. Prakash) plants developed via shoots induced from cotyledonary callus (Jain et al., 1989). These Indian mustard plants displayed large variation in all the characters evaluated.

Some of the plants also showed significantly higher yield and other improved agriculturally important characteristics compared with controls. Somaclonal variation has led to the selection of a dwarf mutant and true breeding lines in the R2 generation. Somaclones of *B. juncea* producing high yields and resistant to shattering have been selected and commercially released (Kaliyar and Chopra, 1995). Selection pressure *in vitro* yielded salt-tolerant somaclones of *B. juncea* (Jain et al., 1990; Kirti et al., 1991).

Anther/Microspore Culture and Doubled Haploids

One of the most intriguing advancements in biotechnology has centered around the creation of haploid and doubled haploid plants. In Brassica species, these haploid and doubled haploid plants have been successfully generated through techniques like anther culture or the isolation of microspores. This breakthrough offers an efficient means to rapidly produce homozygous lines, particularly beneficial for generating hybrid seeds. This technology holds immense value in the breeding of self-incompatible, outcrossing lines, which are prevalent in vegetable Brassica.

Numerous methodologies for microspore and anther culture have been devised and refined for various Brassica species (Palmer et al., 1996b). Somatic embryos and plantlets were first successfully produced in B. napus back in 1977 by Keller and Armstrong (1977), and this technology has since found various applications. However, it is worth noting that most genotypes exhibit more favorable responses to isolated microspore culture, which tends to yield a higher number of embryos compared to anther culture (Cao et al., 1995). Both microspore and anther culture techniques (see Figure 1) are currently employed as valuable breeding tools aimed at enhancing vegetable Brassica varieties, such as B. oleracea and pakchoi (B. rapa ssp. chinensis) (Cao et al., 1994).

Microspore culture is used as an alternative to conventional breeding and is used in as an effective alternative technique for the production of doubled-haploid (DH) broccoli lines that can also be parental plants for the production of hybrids (Abercrombie et al. 2005).

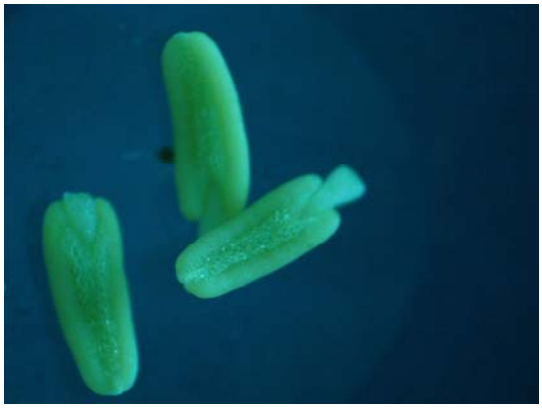

Fig. 1. Excised anthers (Foto stereomicroscope)

Double haploid line development is an important tool for plant breeding process due to the fact that it saves a lot of labour and time. Successful microspore technique of culture of different broccoli genotypes has been described by Takahata and Keller (1991), Duijs et al. (1992) and Dias (2001). DH parental lines have been developed using microspore culture (Wang et al. 1999) and introduced into breeding schemes (Hale et al. 2007). Still, there are limitations to the implementation of the microspore culture technique. The main problem is that embryo yield in many broccoli genotypes is still very low (Dias 2001), and certain genotypes are unable to undergo embryogenesis. In some experiments 10 genotypes were tested and most of them produced less than seven embryos per dish (Dias et al.,2001).

Research continue to be done in order to adapt and improve existing protocols to enhance use of this technique for large-scale DH line screening. Takahata and Keller (1991) and Halkjaer and Ringgaard (1997) suggested heat shock pretreatments for broccoli microspores at 32.5^0C and 35^0C, respectively for 1 day in both cases. Pink (1999) reported that reducing the concentration of macronutrients by half in NLN medium (½NLN-13) resulted in a significant increase in microspore responsiveness. While, Dias (2001) reported that embryo yields were significantly increased with nearly all broccoli genotypes by heat shock at 32.5^0C for 1 day. There are several advantages and applications of microspore culture. Microspore culture can yield up to ten times more embryos than anther culture (Siebel and Pauls, 1989). This also reduces the number of plants that need to be screened, and because recessive traits are not masked, smaller populations are needed. In plant breeding, microspore culture produces true breeding lines which can be used for studies of inheritance patterns (Verma et al., 2023). The time about 3 or 4 years of which a new cultivar is released is also decreased which frees up economic resources to be focused on other aspects of the breeding program (Sharma et al., 2011). Selection is more efficient because no masking by dominant genes occurs and recessive traits are easily identified.

Other applications include mutagenesis, transformations, and biochemical studies. Mutagenesis has led to the development of B. napus lines with a thinner seed coat, higher oil and protein content, and low fibre content as well as other developments (Yang et al., 2010). Mutation techniques have also improved yield and resistance to diseases and pests in B. carinata. Gene linkage and interactions have been studied in B. campestris through the use of haploid plants (Gambhir et al., 2017). Genetic engineering has been used with microspore culture in Agrobacterium transformations (Singh et al., 2022) and with the use of haploid technologies novel cultivars can be developed with a wide range of adaptations allowing crops to be farmed on marginal lands (Rani et al., 2013; Sharma et al, 2014). Embryos have can be used in B. napus to investigate biochemical pathways and screen end products (Ravanfar et al., 2009).

Once haploid plants are produced, doubled haploids can be obtained by either colchicine application on haploid plants or by the process of spontaneous doubling of chromosomes. In *Brassica* species, spontaneous doubling depends on genotype, microspore stage, and culture conditions. Low-temperature treatment has especially facilitated the production of doubled haploids (Chen and Beversdorf, 1992). *B.juncea* doubled haploids have been produced by culturing microspores and treating the haploid plants with colchicine (Lionneton et al., 2001). Spontaneous doubled haploids have been reported in *B. napus* cv. Topas (XuHan el al., 1999), and doubled haploids from colchicine applications have been reported by Murphy and Searth (1998). In *B. napus* (Zhao et al., 1996), colchicine simultaneously induced embryogenesis in microspores and doubled the ploidy level. Doubled haploids obtained using microspores are preferred over the anther culture doubled haploids especially in the vegetable *(B. oleracea)* species, since ploidy levels are mixed in anther culture-derived doubled haploids as compared with the microspore-derived doubled haploids, which were found to lie mostly diploid (Wang et al., 1999). Because the most popular broccoli *(B. oleracea* var. *italica)* cultivars and breeding lines used in breeding programs are F1 hybrids, microspore-derived doubled haploids are helpful in crop improvement. Recently, breeders have used doubled haploids to produce homozygous lines in relatively short periods.

Working protocol for the cultivation of microspores on liquid media in vitro

Equipment and consumables needed for microspore culture:

- sterile containers for buds;
- 1200 ml sterile distilled water;
- 4 sterile 400 ml bottles;

- sterilisation solution: mercuric chloride,Tween;
- scissors sterile tweezers;
- 1 bottle of 250 ml medium sterile;
- sterile Pasteur pipette;
- sterile 50 ml containers;
- piston sering 50 ml;
- filtration device with 45 µm nylon filter;
- 11 or 12 ml sterile centrifuge tubes;
- sterile micropipe;
- Petri dishes, 45x20 mm and Petri dishes, 90x20 mm;
- growth chambers for donor plants;
- laminar airflow;
- centrifuge;
- incubators;
- inverted microscope;
- pH meter.

Growing conditions of donor plants

Mother plants are grown in plastic pots of 20 cm diameter in greenhouses until the 10-leaf stage. At this stage, the plants are vernalized for 90 days under conditions of a 16-hour photoperiod with photosynthetic active radiation of about 60 µmol m^{-2} s^{-1} , after vernalization they are placed under temperature conditions of 15^0 C during the light period and 10^0 C during the dark period.

Plant fertilization is done with liquid fertilizer (N:P:K - 20:10:20). Spraying with pesticides or fungicides should be avoided as it affects microspore reactivity, by taking preventive measures: reducing humidity in the growth chamber, removing dead leaves quickly, etc.

Excitation of flower buds

Flower buds are excised with a portion of stem to avoid oxidation and placed in Petri dishes on moistened filter paper in the refrigerator. Operations from bud harvesting to microspore isolation must be carried out quickly to avoid spoilage. The size of the flower buds is 3,1-3,5mm – fig. 2.

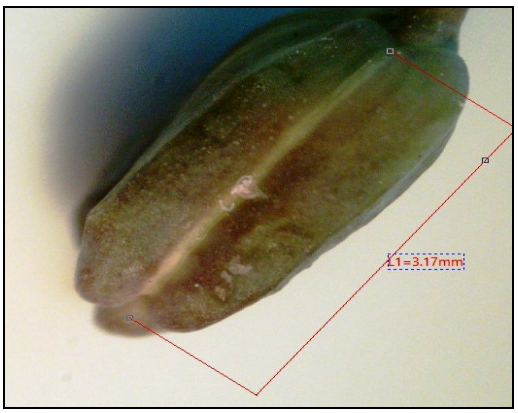

Fig. 2. Buds that contain mainly microspores in the uninucleate stage

Pre-treatment application

- Place buds in petri dishes on dampened filter paper in the refrigerator at $+4^0$ C for 1 day.

Sterilisation of buds

The sterilization operation is performed as follows:

- the buds are washed in a continuous stream of tap water;
- soak quickly (about 1 minute) in 96ºC ethyl alcohol;
- Sterilisation is carried out in 0.1% mercuric chloride (HgCl2) to which 2-3 drops of Tween 20 (per 1 litre solution) are added for 15 minutes, during which time continuous and vigorous stirring is carried out;
- rinse with sterile distilled water, 3 successive times.

In the case of microspore cultures, the temperature at which the operations are carried out is very important, both the sterilizing agent and the distilled water must have a temperature of 4^0C.

Preparation of stock solutions and culture media

A number of rules must be taken into account when preparing stock solutions and culture environments:

- to facilitate the operations of preparing culture media, stock solutions can be made with a concentration of 1000x or 100x, solutions that are stored in the freezer until use;
- each component of the environment is added according to the environmental guidelines to avoid precipitation;
- also, each component dissolves completely before the next one is added to the list;
- start with a smaller quantity than required, bringing the final volume after the addition of all the components of the medium (except agar in the case of solid media);
- after sterilization , the liquid medium is left overnight at room temperature to detect possible infections;
- The liquid medium is stored in the refrigerator, while the solid medium is stored at room temperature.

Preparation of the NLN culture media (Lichter, 1982)

- stock solutions aredissolved in distilled water;
- add 13% maltose;
- adjust the pH to 5.8 by adding 0.5N HCl or 0.5N KOH;
- The medium is sterilized by filtration or autoclaving at 15 psi, 121^0 C, for 20 minutes.

Stock solutions for the NLN culture media:

Micro NLN (1000 x) - 100 ml

- 41,5 mg KI
- 500 mg H3 BO3
- 1250 mg MnSO4 x 4 H_2O
- 500 mg ZnSO4 x 7 H_2O
- 12.5 mg Na2 MoO4 x 2 H_2O
- 1.25 mg Cu2 SO4 x 5 H_2O
- 1.25 mg CoCl2 x 6 H_2O

Dissolve each microelement completely before adding the next, bring the volume to 100 ml freeze in 10 ml vials.

Vitamin NLN (1000 x) - 100 ml

- 25 mg Tiamin HCl
- 250 mg Nicotinic acid
- 25 mg Pyridoxine HCl
- 100 mg glicine
- 2.5 mg Biotin
- 25 mg folic acid

Dissolve each vitamin completely before adding the next, bring the volume to 100 ml freeze in 10 ml vials.

For 1l final medium:

- 62,5 mg KNO3
- 62.5 mg Mg SO4 x 7 H O2
- 62.5 mg KH2 PO4
- 250 mg Ca (NO3) 2 x 4 H O2
- 20 mg Fe EDTA
- 400 mg L-glutamine
- 15 mg Glutathione
- 50 mg myo-inositol
- 50 mg L serin
- 10 ml NLN vitamins (from 1000x stock solution)
- 10 ml NLN microelements (from 1000x stock solution)

Maceration

Sterile buds are transferred to 50 ml vials with 5 ml medium. Microspores are released into the medium by easy crushing of the buds with the plunger of a 20 or 50 ml syringe – fig.3. The operation must be carried out carefully so as not to damage the microspores, which would result in a much reduced number of somatic embryos.

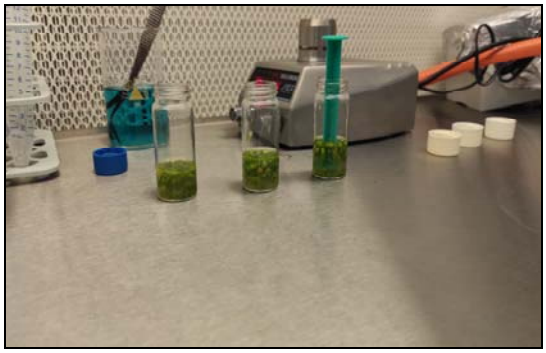

Fig. 3. Releasing the microspores

Filtering

The culture medium containing isolated microspores is taken up with a sterile Pasteur pipette and filtered through a 45 µm nylon filter into a graded test tube.Filter with 20 ml of medium. This operation results in a volume of 40 ml of medium with filtrate that can be divided into centrifuge tubes.

Centrifugation

Centrifuge the tubes at 190 g for 3 minutes and remove the green supernatant. Add 10 ml of fresh medium and centrifuge again, repeating the operation three times.

Concentration adjustment

After the last centrifugation, the supernatant is decanted and the suspension is re-suspended in new medium.

The optimum culture density is 40,000-50,000 microspores per ml of medium – fig. 4. The microspore suspension is placed in Petri dishes (5 ml/Petri dishes) and sealed with parafilm.

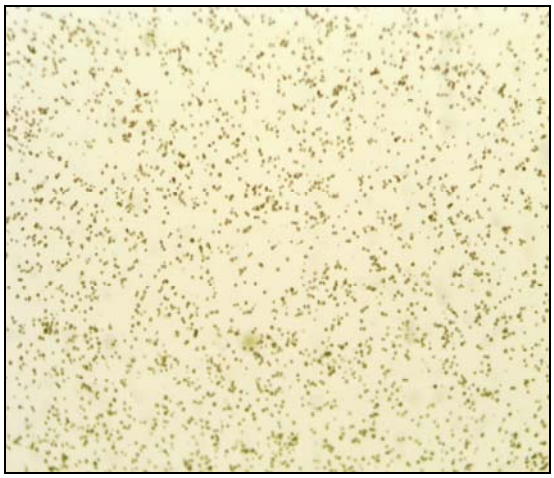

Fig. 4. Freshly isolated microspores (foto inverted microscope 10x)

Incubation of cultures

Petri dishes are placed in the incubator, in the dark for thermal achievement, at 33^0 C for 48 hours. After the thermal incubation period the cultures are transferred to culture chambers at 24^0 C in the dark.

Transfer of microspores on fresh culture media

- using sterile Pasteur pipettes, collect microspore suspension from Petri dishes into Falcon flasks;

- To ensure collection of all microspores – fig. 5, Petri dishes are filled with 1 ml of medium, which is added to the initial microspore suspension;

- adjust the medium volume up to 20 ml culture medium;

- centrifuge at 190 g for 3 minutes;
- the supernatant is removed;
- Resuspend the culture in 5 ml of fresh medium in Petri dishes;
- incubate the cultures at 24^0 C in the dark for 3 weeks;
- observations on the morphogenetic reaction of microspores are made daily, using an inverted microscope;
- cultures which show traces of infection or are unreactive are noted for statistical processing and removed from the culture.

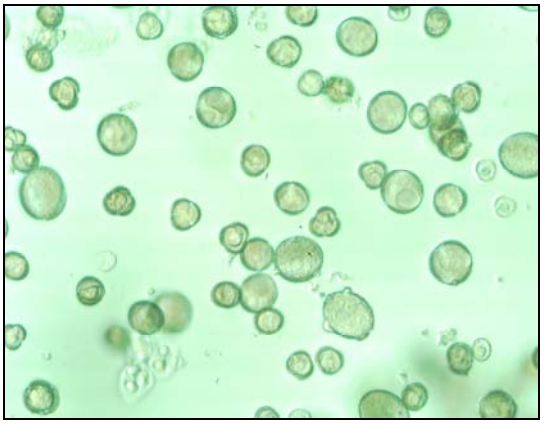

Fig. 5. Heterogenous population of brocolii microspores (foto inverted microscope 40x)

Embryo transfer to solid culture media

- as the embryos develop (about 3 weeks), they are taken from the culture under sterile conditions to the laminar airflow niche and placed on sterile, solid culture media;
- the solid culture medium used for embryo transfer and sub-culturing is B5 medium (Gamborg et al., 1968);
- the working protocol for the production of the culture medium is the same as for the liquid medium, with a number of modifications. Thus, for making one litre of final medium, the working procedure is as follows:
- 500 ml of sterile double-distilled water is added to a 1 litre flask;
- the following quantities of macroelements, microelements and vitamins are weighed and added:
- 2500 mg KNO3
- 500 mg MgSO4 x 7H2O
- 130,5 mg NaH2PO4
- 113,2 mg CaCl2
- 5 ml NaFe EDTA (from 200x stock solution)
- 10 ml B5 micro (from 1000x stock solution)
- 10 ml vitamins (from 1000x stock solution).
- supplement with 3% sucrose;
- add hormones -, the best hormone combination to support organogenesis and embryogenesis processes in the Brassica genotypes tested is BAP- 8.9 µM and NAA 2.7 µM;
- brought to the final volume of 1 l;
- adjust pH to 5.8 by gradually adding small amounts of 1M NaOH;
- to solidify the medium add 0.8% Difco agar;
- sterilisation of the culture media is carried out by autoclaving at 121ºC (1.06 kg/cm2) for 25 minutes.
- to ensure the development of the embryos, the culture flasks are incubated in climatic chambers under photoperiod conditions of 16 hours light, at a temperature of 240C and a light intensity of 5000 lux.
- After embryo development, the seedlings thus obtained either undergo a new culture cycle by transfer to fresh media to induce somatic organogenesis and obtain new propagules, or are placed in hydroponic conditions for acclimatisation.

Acclimatisation of plantlets

- For acclimatisation, the plants are removed from the pot with tweezers, taking great care not to damage the root tips and apical growth tips;
- After they have been removed from the flasks, the culture medium remaining on the root rinses with water in a plastic dish;
- The plants are placed in 50 ml Erlenmeyer flasks with filter paper tips in a hydroponic system;
- The hydroponic solution contains PREVICUR at a concentration of 0.15% and is intended to revigorate the plant and support it in the acclimatisation process, while reducing the possibility of pathogens developing in the solution;
- During the acclimatization period, plants placed in pots with hydroponic solution are placed on a tray at a distance of 5 cm between pots and covered with a transparent lid, the plants a covered for 3-4 days, after which they are uncovered every day graduated;
- the lid is only completely closed when the plants are no longer wilting (about 14 - 15 days after the passage in septic conditions), at which point they are left uncovered.

Passing seedlings on soil substrate

- after a period of 14 to 15 days the plants are transferred to natural mineral growing medium in nutritious pots made of PVC, measuring 6x6x6 cm;
- the nutrient substrate consists of perlite-30%, peat-30%, peat -20%, celery soil-10% and sand-10%;
- after 21-28 days, the plants are fully adapted to septic environmental conditions and can be transferred to greenhouses or field conditions.

CONCLUSIONS

Broccoli, a valuable Brassica species, has seen limited cultivation under in vitro conditions. While some experiments have demonstrated successful in

vitro culture of Brassica species using hypocotyl segments, root segments, primary leaf discs, cotyledons, and anthers, further research is needed to achieve similar success as seen with other cole crop vegetables.

Microspore culture is used as an alternative to conventional breeding and is employed as an effective alternative technique for the production of doubled-haploid (DH) broccoli lines that can also be parental plants for the production of hybrids. Double haploid line development is an important tool for plant breeding process due to the fact that it saves a lot of labour and time.

Research continue to be done in order to adapt and improve existing protocols to enhance the use of this technique for large-scale DH line obtaining.

ABSTRACT

Cole crop vegetables (*Brassica oleracea* L.) are cool-season crops that include broccoli (*B. oleracea* Italica), cauliflower (*B. oleracea* Botrytis), and cabbage (*B*. oleracea Capitata).

Brassica oleracea, convar. *botrytis*, var Italica, Fam. Brassicaceae (Cruciferae), is grown for its dark green inflorescences. Its English name derives from the Italian “brocco” and the Latin “bracchium”, which means arm or branch (Boswell, cited by Singh et al., 2004).

Biotechnology is part of the broader field of genetic mapping, analysis and research called genomics.

In contrast to earlier methods of plant breeding, the new techniques allowed a much wider set of traits to be introduced into plants, in a much shorter period of time. These included resistance to herbicides, pest resistance, cold and drought tolerance, tolerance to salt in soils, enhanced nutrition and vitamin content and many other traits.

Brassica biotechnology includes organo-genesis, somatic embryogenesis, microspore culture and doubled haploids, somatic cell fusion, molecular markers for genetic fidelity of *in vitro*-grown plants, marker-assisted selection, and transformation.

In the present mini-review we outline the main achievements presented by the literature on the main and more often employed techniques used as tools for amplifying classic breeding results.

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