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FACTORS AFFECTING EMBRYOGENESIS IN MICROSPORE CULTURES OF BROCCOLI (*Brassica oleracea* var. *italica*)

Badawi, M. A.*; E. E. Metwally **; Sahar S. Taha * and Marwa O. Arafeh*

*** Department of Vegetable Crops , Faculty of Agric., Cairo Univ. , Egypt.**

**** Department of Vegetable Crops , Faculty of Agric., Tanta Univ. , Egypt**

ABSTRACT

Microspore culture is a very important and useful tool in plant breeding for haploid production and has been developed for many years. Broccoli (*Brassica oleracea* var. italica) is an important vegetable crop. Conditions for reliable induction of embryogenesis from isolated microspores were studied in eight genotypes of broccoli (Hanin, Conde F_1, Baladi, Belstar F_1, Marathon F_1, Parthenon F_1, Naxos F_1 and Tiburom). The optimum timing for microspore culture was confirmed to be during the mid to late uninucleate stage. The highest yield was 237.33 embryo per dish from genotype Parthenon F_1. Embryo yields were significantly increased in broccoli genotypes by the incubation at 32.5 °C for 24 hours, than that incubated at 32.5 °C for 48 hours or 35.5 °C for 24 or 48 hours. The use of the 1/2 NLN-13 medium yielded greater number of embryos than the standard NLN-13 and B5 media. The magnitude of the response to the reduction of the concentration of major salts by half in the NLN medium varied with the different genotypes. Parthenon F_1 and Marathon F_1 presented a better response to the reduction of the concentration of major salts by half in NLN-13. Microspore culture density on embryo production was evaluated in selected genotypes. Microspore plating density was critical for efficient embryonic induction and development, with an optimal plating density of 4×10^4 microspore/ml. which obtained resulted in 237.33 embryo from Parthenon F_1 and 171.00 embryo from Marathon F_1. Activated charcoal (0.2 ml) added to the liquid NLN-13 medium, increased embryo yield significantly as compared to those cultures without activated charcoal.

INTRODUCTION

Brassica oleracea vegetables are an important and high diversified group of *Brassica* crops grown worldwide. Cauliflower (*B. oleracea* var. *botrytis*) and broccoli (*B. oleracea* var. *italica*) are the vegetable cole crops deserving the most breeding attention by seed companies. Microspore culture is an effective technology for : i) the production of homozygous parental lines (as an alternative to self-pollination) for the production of F_1 hybrids of modern cultivars of *B. oleracea* crops and ii) the increase in selection efficiency for desirable genetic recombinants. Successful microspore culture in different broccoli genotypes were described by Lichter (1989), Duijs *et al*., (1992) and Vicente and Dias (1996). A problem with the practical application of this technique is the very low embryo yield. Numerous factors are required for high levels of embryogenesis from *Brassica* microspores. These factors include culture media, growing conditions of donor plants, genotypes, microspore developmental stages, and the incubation of microspores at elevated temperature during the early stages of culture (Keller *et al*., 1987). Culture density is another factor that may influence microspore culture in *Brassica*. In this study, the effects of

genotype, culture medium, culture density, temperature treatment and activated charcoal on the efficiency of microspore embryogenesis in Broccoli *(Brassica. oleracea* var. italica) were examined.

MATERIALS AND METHODS

Plant material:

Eight commercial broccoli hybrids (Conde F_1, Belstar F_1 Marathon F_1, Parthenon F_1, Naxos F_1) and cultivars (Hanin, Baladi and Tiburom) were used in the present study.

Plant growth conditions:

Plants were grown in a greenhouse using plastic pots of 16 cm diameter, fertilized with fertilizers as necessary and watered as required. Then they were verbalized for eight weeks, in an incubator room at 4 ±1˚C with continuous light. After floral differentiation, and the start of generative development, all the plants were transferred to a growth chamber with a 16 h photoperiod and at temperature of 18˚C.

Isolation of microspores:

For both isolation and culture of the microspores, a sterilized filter, Nitsch and Nitsch medium (1967), as modified by Lichter (1981) and 13% sucrose (NLN-13) was used. In the culture medium experiment, the NLN-13 medium was also used with concentration reduced by half ($_{1/2}$ NLN- 13). Flower buds were harvested from young inflorescences. Up to 50 buds cauld be used for one isolation procedure. The microspore stages were observed cytologically at the mid to late uninucleate stage. The buds were surface sterilized. Then each sample of buds were transferred into 50 ml beaker with 1.5 ml NLN-13 medium and squeezed gently with the plunger, microspore suspension was obtained by filtration through 45 *µ*m nylon mesh screens. This suspension was centrifuged 3 times at 900 rpm for 3 min and 20-30 ml of NLN-13 medium were added. (½NLN-13 Medium, B_5 Medium) then resuspended the total number of microspores with a haemocytometer to final density of (3, 4 and 5). 10^4 microspores /ml. 10 ml (9 ml medium + 1 ml medium with colchicine 0.01%) were incubated at 32.5˚C for 24h, then centrifuged for 3 min and10 ml medium were added and incubated at (32.5˚C, 35.5˚C for 24h or 48h) in the dark in a growth chamber at 25˚C, after one week early pro-embryos were observed. After 2-3 weeks the dishes were transferred to a gyratory shaker at 60 rpm at 24˚C in the dark for one week, then embryos were transferred to B_5 solid medium with 2% sucrose at 24˚C for 4 weeks to allow the embryos to develop into plantlets. An addition of a 0.2 ml per dish (60 mm. diameter) of activated charcoal to the culture dishes was performed according to Gland *et al*., (1988) and Dias (1999) procedures.

Data analysis:

Results were expressed as number of embryos per petri dish, In all experiments each treatment consisted of 8 dishes, a completely randomized design (CRD) was used with three replications, data were analyzed by Tukey's (HSD) test by Steel and Torrie (1960).

RESULTS AND DISCUSSION

1. Effect of genotypes on embryo induction and plantlets regeneration of broccoli:

The *B*. *oleracea* genotype is considered to be a key factor for obtaining microspore-derived embryos. There was a significant variation in the response of the eight different broccoli genotypes to microspore culture (Table1). Parthenon F_1 (237.33 embryo/dish). Marathon F_1 (171.00 embryo/dish) were the highest responsive accessions studied Hanin, Conde F_1 and Baladi, were the lowest responsive microspore culture genotypes (٤٦,٦٧, ١٨,٦٧ and ٣,٦٧ embryo/dish, respectivly) while Belstar F_1, Naxos F_1 and Tiburom showed no response. In total eight genotypes were tested, five produced embryos, but the yield of embryos was relatively low for most genotypes. *B*. *oleracea* is mostly self-incompatible and open-pollinated, and therefore genotypes are hetrogeneous to various degrees in the population. There is a plant to plant variation for microspore culture response within genotypes (Ferrie and Keller, 1995).

Table1. Effect of genotypes on embryo induction and plantlets regeneration of broccoli.

Genotypes	No. Embryos / dish	No. Plantlets/ dish
Conde F١	١٨,٦٧a	٠,٣٣a
Hanin	٤٦,٦٧a	١,٣٣a
Bellstare F١	٠,٠٠a	٠,٠٠a
Marathon F١	١٧١,٠٠b	٥٢,٦٧b
Parthenon F١	٢٣٧,٣٣c	٢٣,٣٣a
Naxos F١	٠,٠٠a	٠,٠٠a
Tiburom	٠,٠٠a	٠,٠٠a
Baladi	٣,٦٧a	٠,٠٠a

We compare means with a post-hoc test (Tukey's HSD test); Mean holding same letters are not significantly different on HSD at (0.05).

The present results confirmed the high embryogenic capacity of several broccoli genotypes: Parthenon F١ and Marathon F١ when compared to most of the *B. oleracea* genotypes, revealed a significant variability between genotype responses. Differences in responsiveness can be related to the different induction treatments used: 32.5°C for 24 hours by Takahata and Keller (1991) and the standard 30 °C for 48 hours (Duijs *et al*., 1992) Marathon F_1, was previously considered as unresponsive Pink (1999), but was highly responsive in the present study.

2– Effect of temperature treatment on embryo induction and Plantlets regeneration of broccoli.

Microspore embryogenesis in *B. oleracea* was routinely initiated by high temperature stress treatment of 32.5°C for 24 hours. For most of the *B. oleracea* varieties, this incubation temperature seems to be good. In the present experiment, out of the eight broccoli genotypes studied, two genotypes (Parthenon F_1 , Marathon F_1) produced more embryos when incubated at 32.5°C for 24 hours (237.33, 171 embryos/ dish) than when

incubated at 3◦.5◦C (Table 2). Embryo yields at 32.5◦C for 24 hours was always significantly higher than at 32.5◦C for 48 hours. Parthenon F_1 produced 109.67 embryo/dish when incubated at 35.5◦C for 24 hour.

Table 2. Effect of temperature treatment on embryo induction and Plantlets regeneration of broccoli.

Genotypes	Temperature C°		No. Embryos / dish	No. Plantlets/ dish
Conde F_1	32.5	24 h	18.67 ab	0.33 a
		48 h	0.0 a	0.0 a
	35.5	24 h	1.0 a	0.33 a
		48 h	0.0a	0.0 a
Hanin	32.5	24 h	46.67 bc	1.33 a
		48 h	1.0a	0.0 a
	35.5	24 h	14.33 ab	0.33 a
		48 h	0.67 a	0.0 a
Bellstare F_1	32.5	24 h	0.0 a	0.0 a
		48 h	0.33 a	0.0 a
	35.5	24 h	0.0 a	0.0 a
		48 h	0.0 a	0.0 a
Marathon F_1	32.5	24 h	171 e	52.67 d
		48 h	3.00	0.0 a
	35.5	24 h	79.67 cd	18.0 bc
		48 h	9.33 ab	0.0 a
Parthenon F_1	32.5	24 h	237.33 f	23.33 c
		48 h	18.0 ab	1.0 a
	35.5	24 h	109.67 d	8.0 ab
		48 h	2.33 a	0.0 a
Naxos F_1	32.5	24 h	0.0 a	0.0 a
		48 h	0.0 a	0.0 a
	35.5	24 h	0.0 a	0.0 a
		48 h	0.0 a	0.0 a
Tiburom	32.5	24 h	0.0 a	0.0 a
		48 h	0.0 a	0.0 a
	35.5	24 h	2.33 a	0.33 a
		48 h	0.0 a	0.0 a
Baladi	32.5	24 h	0.0 a	0.0 a
		48 h	3.67 a	0.0 a
	35.5	24 h	0.0 a	0.0a
		48 h	0.0 a	0.0 a

We compare means with a post-hoc test (Tukey's HSD test); Means holding same letters are not significantly different on HSD at (0.05).

Elevated culture temperature, as a pre-treatment for microspore embryogenesis is required for different *Brassica* species. The standard treatment of 48 hours at 30 ◦C used by Duijs *et al.,* (1992) in six different cultivar groups of *B. oleracea* was shown to be less effective than the treatment of 32.5◦C for 24 hours. This is in agreement with the results of Takahata and Keller (1991) and Halkjaer and Ringgaard (1997) which suggested 32.5◦C for 24 hours as an incubation temperatures for broccoli microspores. Embryo yield was significantly reduced when the incubation temperature of 35.5◦C was longer than 24 hours. Takahata and Keller (1991) also found that treatments at 35.5◦C for 48 hours or longer produced less embryos than the optimal treatment of 32.5◦C for 24 hours; this suggested

that *B. oleracea* microspores are more sensitive to high temperatures than those of *B. napus* in order to enter into embryogenic development, where as the low temperature conditions; were considered as a nonembryogenic culture conditions; moreover it inhibits microspore embryogenesis in broccoli. Custer *et al*. (1994) in *B. napus* cv. Topas, reported a quite high number of embryos were produced at 25 °C. This means that it is important to study the induction temperature for the different *Brassica* microspore culture systems.

3- Effect of genotype-media interaction on embryo induction and plantlets regeneration of broccoli.

Embryo yields were increased by reducing the concentration of major salts by half NLN-13 medium (Table 3). The reduction of major salts concentration in the NLN-13 medium never have any detrimental effect on the embryo yield. Therefore, reducing concentration of major salts at half NLN-13 medium seems to increase embryogenesis frequency in broccoli microspore culture. The magnitude of the response to the reduction of major salts in the medium varied with the different broccoli genotypes. The best results were obtained with the highly responsive genotypes 'Parthenon F_1', 'Marathon F_1' and 'Hanin' with significant increases in embryo yield (203.00, 171.00, 46.67 embryo/dish, respectively) (Table 2). Moderate differences were observed in the development of embryos into plantlets between the responsive genotypes. No differences were shown between the two other studied media (NLN-13, B5).

Table 3. Effect of genotype-media interaction on embryo induction and plantlets regeneration of broccoli.

Genotypes	Medium	No. Embryos / dish	No. Plantlets/ dish
Conde F_1	NLN-13	18.67 ab	0.33 a
	½NLN-13	1.00 a	0.33 a
	B_5	0.33 a	0.00 a
Hanin	NLN-13	3.33 ab	0.67 a
	½NLN-13	46.67 cd	1.33 a
	B_5	0.00 a	0.00 a
Bellstare F_1	NLN-13	0.33 a	0.00 a
	½NLN-13	0.00 a	0.00 a
	B_5	0.00 a	0.00 a
Marathon F_1	NLN-13	26.33 bc	14.00 ab
	½NLN-13	171.00 e	52.67 c
	B_5	2.00 a	0.33 a
Parthenon F_1	NLN-13	69.00 d	29.67 bc
	½NLN-13	203.00 f	23.33 ab
	B_5	2.67 ab	0.00 a
Naxos F_1	NLN-13	0.00 a	0.00 a
	½NLN-13	0.00 a	0.00 a
	B_5	1.67 a	0.67 a
Tiburom	NLN-13	0.00 a	0.00 a
	½NLN-13	1.67 a	0.33 a
	B_5	0.33 a	0.00 a
Baladi	NLN-13	3.67 ab	0.00 a
	½NLN-13	0.00 a	0.00 a
	B_5	0.00 a	0.00 a

We compare means with a post-hoc test (Tukey's HSD test); Means holding same letters are not significantly different on HSD at (0.05).

The nutritional requirements for induction and production of embryos vary widely from species to species. For microspore culture of *B. oleracea*, most researchers have used the standard NLN-13 culture media. In the

present study, 1/2 NLN-13 medium proved to be significantly better than NLN-13 medium for most of the broccoli genotypes studied. The explanation for the promotion of embryogenesis in all the genotypes tested, due to the reduction of major salts by half in the NLN-13 medium, needs to be clarified. Recently, Pink (1999) also reported a significant increase in embryo yield in an F_1 from a broccoli × cabbage cross by reducing the concentration of macronutrients by half in NLN medium. Sato *et al*., (1989), in *B. campestris* ssp. obtained similar results.

4- Effect of genotype-microspores density on embryo induction and plantlets regeneration of broccoli.

To investigate whether microspore plating density affected the efficiency of embryogenesis, isolated microspores transferred to NLN-13 medium at the densities of 3, 4 and 5 × 10^4 per/ml and embryonic induction and development were investigated after 4 weeks of culture. Results obtained from cultures with different densities are shown in Table 4. The best embryo yield **(**237.33, 171.00 embryos / dish) was achieved in cultures from Parthenon F_1, Marathon F_1, respectively with the density 4 × 10^4 microspores per 1 ml of cultivation medium. Higher and lower densities (3 or 5 × 10^4 microspores per 1 ml of cultivation medium, decreased the number of derived embryos per Petri dish. Culture density has significant effect on embryogenesis, and thus the capability of regeneration into whole plants (Huang *et al*., 1990 and Kott *et al.,* 1988). In the present experiments, the best embryo yield was achieved with the density of 4 × 10^4 microspore/ ml of cultivation medium. Barro and Martin (1999) reported optimal density in *Brassica carinata* 10 × 10^4 – 15 × 10^4 microspore /ml. Lichter (1982) and Zhou *et al*., (2002) reported the optimal density for *Brassica napus* 2 × 10^4 per ml. Higher and lower density caused remarkable decrease in number of derived embryos per Petri dish and in the case of high density often protracted development and incomplete embryos. This issue could be the cause of nutrient competition among developing embryos and/or some toxic substances when older microspores are presented in the cultivation medium.

5- Effect of activated charcoal on embryo induction and plantlets regeneration of broccoli.

Embryo yields were significantly increased by the addition of 0.2 ml. of activated charcoal (AC) to the microspore culture media (Table 5). In no instance did the addition of AC have any detrimental effect on embryo yield. Therefore AC at a concentration of 2% seems to act as a promoter of embryogenesis in the microspore culture of different B. oleracea genotypes. The magnitude of the response to the addition of AC varied with the different genotypes. The best results were obtained with Parthenon F_1 and Marathon F_1 with increases in embryo yield of 237.33, 171.00 embryo/dish, respectively which responded positively to the addition of AC. A qualitative improvement of the subsequent development of embryos in plants was also observed due to better embryos in presence of AC. These observations are in accordance with those of Gland *et al*., (1988) in *B. napus*. The response to the addition of AC also seems to be dependent on the growth stage of microspores.

Table 4. Effect of genotype-microspores density on embryo induction and plantlets regeneration of broccoli.

Genotypes	Density	No. Embryos / dish	No. Plantlets/ dish
Conde F_1	3×10^4	0.00 a	0.00 a
	4×10^4	18.67 a	0.33 a
	5×10^4	1.67 a	0.33 a
Hanin	3×10^4	5.67 a	0.67 a
	4×10^4	46.67 a	1.33 a
	5×10^4	18.67 a	1.33 a
Bellstare F_1	3×10^4	0.00 a	0.00 a
	4×10^4	0.33 a	0.00 a
	5×10^4	0.00 a	0.00 a
Marathon F_1	3×10^4	35.00 a	10.33 ab
	4×10^4	171.00 c	52.67 c
	5×10^4	42.33 a	9.33 ab
Parthenon F_1	3×10^4	102.67 b	8.33 ab
	4×10^4	237.33 d	23.33 b
	5×10^4	124.33 bc	10.67 ab
Naxos F_1	3×10^4	0.00 a	0.00 a
	4×10^4	0.00 a	0.00 a
	5×10^4	0.00 a	0.00 a
Tiburom	3×10^4	0.00 a	0.00 a
	4×10^4	0.00 a	0.00 a
	5×10^4	0.00 a	0.00 a
Baladi	3×10^4	0.00 a	0.00 a
	4×10^4	3.67 a	0.67 a
	5×10^4	1.67 a	0.33 a

We compare means with a post-hoc test (Tukey's HSD test); Means holding same letters are not significantly different on HSD at (0.05).

Addition of AC has promoted embryogenesis in microspore culture of different *B. oleracea*, The explanation for the promotion of embryogenesis by AC is not yet well clarified. AC possesses strong adsorptive properties and is usually used in chemistry to absorb both gases and dissolved solids. In different, *Brassicaceae* species, Lichter (1989) pointed out that addition of AC in an isolated microspore culture might remove toxic substances from the media. Gland *et al*., (1988) suggested also that AC acts by removing toxic substances released by non-reactive microspores thus allowing a larger number of embryogenic cells to develop. This explanation was also given by Kott *et al*., (1988), AC is capable of trapping gases and so may inactivate ethylene, or other gases, released from cultured tissues (Johansson *et al*., 1982). Dias and Martins (1998) observed that the inclusion of the ethylene antagonist silver nitrate in the medium increased embryo yields significantly in different morphotypes of *B. oleracea* as kales, kailan, cauliflowers, cabbages, tronchudas and broccolis. AC could absorb some phenols commonly produced by wounded tissues (Fridborg *et al*., 1978 and Weatherhead *et al*., 1979). However in *Brassica* microspore cultures, only low levels of phenolic compounds are expected in the microspore suspension since microspore cultures are almost devoid of somatic cell fragments. Although the release of some phenolic compounds of somatic cell fragments to the media can be a reality.

Table 5. Effect of activated charcoal on embryo induction and plantlets regeneration of broccoli.

Genotypes	Activated charcoal 0.2ml	No. Embryos / dish	No. Plantlets/ dish
Conde F_1	Without AC	10.00 a	0.67 a
	With AC	18.67 a	0.33 a
Hanin	Without AC	28.67 a	1.00 a
	With AC	46.67 a	1.33 a
Bellstare F_1	Without AC	0.00 a	0.00 a
	With AC	0.33 a	0.00 a
Marathon F_1	Without AC	108.33 b	26.00 b
	With AC	171.00 c	52.67 c
Parthenon F_1	Without AC	131.67 bc	9.67 ab
	With AC	237.33 d	23.33 b
Naxos F_1	Without AC	0.00 a	0.00 a
	With AC	0.00 a	0.00 a
Tiburom	Without AC	0.00 a	0.00 a
	With AC	0.00 a	0.00 a
Baladi	Without AC	2.67 a	2.00 a
	With AC	3.67 a	0.67 a

In summary, our successful generation of embryos and plants from isolated broccoli microspores is mainly attributed to five key factors: (1) genotypes, (2) culture in 1/2 NLNS-13 medium supplemented with sucrose 130 g/l as carbon source, (3) an optimization of microspore plating density at 4×10^4 microspore/ml, (4) high temperature stress treatment of 32.5°C for 24 hours and (5) activated charcoal at a concentration of 2% seems to act as a promoter of embryogenesis .This information provides the basic framework for further improvements, and for future large-scale generation of embryos and plants from isolated microspores of broccoli. This microspore culture system, when optimized, may be used for genetic transformation, mutant selection for dominant and recessive traits, molecular studies on embryogenesis in addition to homozygous doubled haploid plants.

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العوامل المؤثرة على تكوين الأجنة عند زراعة حبوب اللقاح في البروكولي

محمد عبد المجيد بدوي *، المهدي ابراهيم متولي ، سحر سميح طه* و مروة عمر عرفة***

*** قسم الخضر – كلية الزراعة – جامعة القاهرة- مصر.**

**** قسم الخضر – كلية الزراعة – جامعة كفر الشيخ – مصر.**

تستخدم تقنية زراعة حبوب اللقاح لانتاج نباتات أحادية متضاعفة كوسيلة مهمة و فعالة في مجال تربية النبات، يعتبر البروكولي من محاصيل الخضر الهامة . تم دراسة العوامل المؤثرة على تكوين أجنة أحادية من زراعة حبوب اللقاح و امكانية انتاج نباتات احادية متضاعفة في ثمانية أصناف من البروكولي (Hanin, Conde F_1, Baladi , Belstar F_1 , Marathon F_1, Parthenon F_1 , Naxos F_1 and Tiburom)

أفضل مرحلة لاستخلاص حبوب اللقاح كانت في مرحلة حبة اللقاح وحيدة النواة . أفضل النتائج كانت ٢٣٧,٣٣ جنين من كل طبق من الصنف Parthenon F_1 كما زاد محصول الاجنة معنويا عند التحضين على حرارة ٣٢,٥ م° لمدة ٢٤ ساعة بالمقارنة بالتحضين على حرارة ٣٢,٥ م لمدة ٤٨ ساعة أو ٣٥,٥ م لمدة ٢٤ ساعة . كما تبين ان بيئة NLN-13 1/2 و كثافة حبوب اللقاح 4×10^4 microspore/ml مع استخدام الفحم النشط في البيئة كانت الافضل من حيث عدد الاجنة المتكونة بالمقارنة مع NLN-13 او B_5 حيث ابدى كل من Marathon F_1, Parthenon F_1 أفضل النتائج من حيث عدد الاجنة و عدد النباتات الناتجة.