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A scenario of suitable temperature and time for microspore culture in some species of *Brassica*

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Abstract. The genus *Brassica* comprises 37 different species. It is known for its outstanding agricultural and horticultural crops, including several weeds categorized as vegetables, oilseeds, spices, and fodder using buds, inflorescences, leaves, and roots. Due to its high nutritional and economic importance, the formation of haploid in different species from cultured and isolated microspores of this genus is considered a prime concern to meet emerging and expanding markets. We reviewed 38 research articles for microspore culture comprising 5 of *Brassica juncea*, 6 of *Brassica rapa*, 12 of *Brassica oleracea (*var. italic, *and* var. tronchuda), 12 of *Brassica napus* L., 2 of *Brassica carinata*, and 1 of *Brassica campestris.* The temperature used for microspore culture ranged from 15-33 °C, whereas the time taken ranged from 8 hours to 40 days. The observation concluded that the suitable temperature and time for microspores culture are 32 °C and 48 h, respectively.

Keywords: *Brassica species, microspore culture, embryogenesis, temperature, time.*

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1. Introduction

The first case of haploid regeneration plants from the *in vitro* cultured anthers was noted in *Daturainnoxia* (Guha and Maheshwari, 1964). Later, Bourgin and Nitsch (1967) achieved androgenic haploid from anthers cultured of *Nicotiana sylvestris* and *N. tabacum.* Many advanced approaches have been developed the isolated microspores in different species for haploid production (Ferrie and Caswell, 2011).

The genus *Brassica* belongs to Brassicaceae's family (Crucifer family) is the most economical genus and comprises 37 different species. The genus consists of 3 diploid species (*Brassica nigra, Brassica oleracea,* and *Brassica rapa*) and three amphidiploid species (*Brassica carinata, Brassica juncea*, and *Brassica napus*). The genus is categorized into forage, oilseed, condiment, and vegetable crops based on their uses. Among *Brasicca* crops, most of the vegetables and forms of good form choices are obtained from *B. oleracea* and *B. rapa* (Cartea et al., 2015).

The genus *Brassica* forms secondary defense metabolites known as glucosinolates (have an antibiotic-like effect) in the whole plant, and their seed oil contains a very long chain of fatty acids. *Brassica's species* are one of the most demanded few oilseeds adjusted to cool temperate agricultural zones and winter production, germinating and growing at low temperatures. There is an opportunity for industrial (non-edible) growth of *Brassica* oil due to the high demand for chemical feedstocks, renewable energy, industrial oils, and different vegetable oils. Different *Brasicca* derived products such as Canola (A modified fatty acid profiles include in canola), high erucic acid (22:1cisΔ13), rapeseed (HEAR), and super-high erotic acid rapeseed (SHEAR) cultivars can be developed and grown to expand markets. Industrial (non-edible) Brasicca oil is expected to meet double-digit annual growth (McVetty et al., 2016).

The embryogenesis process from microspore can be influenced by factors such as growing conditions of donor plant growth, genotype, microspore developmental stage, medium constituents, and culture conditions. The high-temperature treatment at 30-35 °C for 1-3 days of culture following the 25 °C significantly increased the embryogenesis in anther culture and microspore culture

of *Brassica* species (Constantine et al., 1996; Takahata, 1997). This technique is commonly used in microspore and another culture of *Brassica* species, including *B. rapa* (syn. *B. campestris*) (Zhang and Takahata, 1999). Here, we reviewed and enumerated articles to identify the more appropriate temperature and time for the microspore culture of some species of genus *Brassica* (Table1).

Table 1. Variation in temperature and time used for microspore culture in *Brassica spp.*

Genus / species	Temp. (°C)	Time (h, d)	References
Brassica juncea	32.5	7 d	Lionneton et al., 2001
Brassica rapa	33	24 h	Sato et al., 2001
*Brassica oleracea*var. *italica*	32.5	24 h	Carlos and Dias, 2001
Brassica oleracea var. tronchuda	32.5	24 h	Dias and Correia, 2002
Brassica napus L.	30	7 d	Zhou et al., 2002
Brassica napus L.	32	8 h	Seguí-Simarro et al., 2003
B. napus L.	30	7 d	Gu et al., 2004
B. rapa and *B. oleracea*	32	48 h	Gu et al., 2004
Brassica juncea	32.5 ± 1	10-15 d	Prem et al., 2005
Brassica juncea	32	72 h	Chanana et al., 2005
Brassica napus L.	30	7 d	Zhang et al., 2006
Brassica juncea	32	48 h	Agarwal et al., 2006
Brassica carinata	30	18 h	Abraha et al., 2007
Brassica carinata	30	14 d	Abraha et al., 2008
Brassica juncea	32.5 ± 1	10-11 d	Prem et al., 2008
Brassica campestris ssp	32	18 h	Wang et al., 2009
Brassica napus L.	30	48 h	Wan et al., 2011
Brassica oleracea	30.5	48 h	Winarto et al., 2011
Brassica napus L.	30	10 h	Yadollahi, et al., 2011
Brassica napus L.	30	14 d	Mohammadi et al., 2011
Brassica napus L.	32	48 h	Prem et al., 2012
Brassica napus L.	18	40 d	Prem et al., 2012
Brassica napus L.	32	16 h	El-Tantawy et al., 2013
Brassica rapa	32	48 h	Seo et al., 2014
Brassica rapa ssp. chinensis	25	48 h	Shumilina et al., 2015
Brassica napus L. euphytica	15	15 h	Javed et al., 2015
Brassica napus L.	30	14 d	Najafabadi et al., 2015
Brassica oleracea	32	48 h	Tuncer et al., 2016
Brassica rapa ssp. chinensis L.	33	24 h	Zhang et al., 2016
B. oleracea var. botrytis L.	30	24 h	Bhatia et al., 2016
Brassica rapa L. ssp. pekinensis	30	24 h	Lu et al., 2016
Brassica oleracea L. var. italica	32.5	24 h	Zeng et al., 2017
Brassica oleracea L. var. capitata	26 ± 1	16/8 d/n	Cilingir et al., 2017
*Brassica oleracea*var. capitata L.	30	48 h	Bhatia et al., 2018
B. oleracea L. convar. botrytis	32	48 h	Domblides et al., 2018
*Brassica oleracea*var. capitataL	30	48 h	Pilih et al., 2018
Brassica napus L.	32	48 h	Berenguer et al., 2019
Brassica oleracea L.	32	24 h	Zhang et al., 2020
Brassica napus L.	25	16 h	Su et al., 2020
Brassica rapa ssp. *Rapa* L.	32	48 h	Shumilina et al., 2020

Note. Tempt.-Temperature, d - day, h- hour, d/n- day/night

2. Scenario of microspore culture in *Brassica* sp.

Lionneton et al. (2001) developed a protocol of influential microspore culture based on sucrose concentration of culture media, which influenced embryo formation. The experiment was carried out and tested in 13 genotypes of *Brassica juncea*. From which, 12 genotypes produced embryos successfully, and only 7 of them germinated into plants. By the treatment of colchicines, doubled haploid plants were produced. The microspore cultures were incubated for seven days at 32.5 °C in the dark to induce embryogenesis.

Sato et al. (2001) examined the response of low-temperature pretreatment for buds and inflorescences on microspore culture to produce haploids of *Brassics rapa* (syn. *B. compestris*). Before isolation of microspores, flower buds or inflorescences were pretreated for 3, 7, 10, and 20 days (kept into a petri dish containing 5 ml of B5-13 medium) at 4 °C and found 10-day pretreatments of both were effective. More embryos were formed in buds than that of inflorescences after pretreatment for 7-20 days. The low temperature (4 °C) was influenced by decreasing the percentage of microspores at the unicellular stage of the late unicellular condition and increased bicellular stage percentage with two unequal nuclei sizes. Further, several binucleate stages of microspores containing the unequal size of nuclei were formed. The incubation of microspores was carried out at 33 °C for 24 days and then placed at 25 °C in the dark.

Carlos and Dias (2001) studied stable microspore embryogenesis conditions in ten genotypes of broccoli (*Brassica oleracea* var. Italic*)*. The numbers of embryos incubated at 32.5 °C for one day were increased in nearly all broccoli genotypes than the incubation period for two days at 30 °C. The treatments for 48 hours at 32.5 °C produced fewer microspores in broccoli than *B. Napus*. The produced embryo number is higher by the application of the ½ NLN-13 medium than the standard NLN-13. The higher concentration of some of the primary macronutrients in NLN-13, mainly NO_3, may inhibit embryo growth by decreasing its pH. Therefore, embryogenesis frequency can be increased by reducing major salts' concentration by half in the NLN-13 medium. The production of embryos increased in 7 out of 10 broccolis. The significant responses to the reduction of significant salts were obtained in SDB9, Shogun, Marathon, and Green Valiant, and this reduction in concentration did not affect embryo development in plants.

Dias and Correia (2002) carried out two experiments in tronchuda cabbages (*Brassica oleracea* var. tronchuda) to examine the role of medium renovation and incubation temperature for in vitro embryogenesis of microspore culture. The first experiment conducted to examine the effect of medium renovation for 0, 24, 48, 72, 96, and 120 h result in the decrease of embryo production with medium refreshing in tronchuda cabbages 'Couve Grelo, Penca da Povoa, and Couve Algarvia whereas the production of embryos increased only after 24 h in 'Penca de Mirandela.' The second experiment was carried out at 27.5 and 30 °C for two days and 32.5 and 35 °C for 1 and 2 days to examine the effect's incubation regime. In this experiment, the production of embryos was increased in 'Couve grelo', 'Penca de Povoa,' and 'Penca de Mirandela' at 30 °C for two days, whereas the production of embryos increased in 'Cauve Algarvia' at 32.5 °C for 24 h in compared to the 30 °C for 48 h.

Zhou et al. (2002) evaluated the role of immediate colchicines treatment on embryogenesis and doubling efficiency during microspore culture in two F_1 hybrids of *Brassica napus*. The colchicines treatment (at 50 and 500 mg/L) for 15 h produced a large number of healthy embryos. Thus, they formed embryos transferred to a tangible medium at the initial period of 2 °C for ten days germinated vigorously at 24 °C. The colchicine treatment at high concentration (1000 mg/L) showed positive effects less on embryogenesis, and efficiency is doubling. During the experiments, the cultures were in the dark incubated at 30 °C for seven days and then moved to 24 °C.

Seguí-Simarro et al. (2003) studied the behavior of two heat-shock proteins (Hsp70 and Hsp90) before and after embryogenesis induction. The stress treatment conducted for 8 h at 32 °C changed the gametophyte development of microspore. After division, some microspore developed haploid embryos, whereas the rest did not respond to induction regarding gametophytic development. The stress proteins are associating with the induction of microspore embryogenesis.

Gu et al. (2004) noted the response of cold pretreatment on microspore embryogenesis in spring and winter *Brassica napus* and *B. rapa* and *B. oleracea*. They obtained the result more boosted (by 1-7 fold) than standard microspore culture protocol in *B. napus*. It was found significantly less in *B. rapa* but harmful in *B. oleracea*. The appropriate period for cold pretreatment for the best result in microspore embryogenesis was 2-4 days that also induced the embryogenesis quality. The dishes were closed using the Parafilm double layer then incubated at 30 °C in the dark for seven days (*B. napus*) or 32 °C for 2-days (*B. rapa* and *B. oleracea*), and then moved to 25 °C still in the dark. Medium refreshing was managed after one day of induction following microspore isolation.

Prem et al. (2005) investigated the effect of different factors of exogenous (namely such as microspore density composition of media in culture and culture incubation conditions) and the endogenous factors (such as genotype, growth conditions, and microspore developmental stage) on microspore embryogenesis on donor plant in three varieties of Indian *B. juncea*. The microspores obtained from post-culture high-temperature incubation at 32.5 ± 1 °C for 10-15 d were most suitable for maximum microspore embryo production. In contrast, the highest density of microspores (78 embryos per Petri dish) was found from the late uninucleate microspores (contained in bud sizes 3.1-3.5 nm irrespective of genotype) cultured on NLN medium containing 13% sucrose and silver nitrate (10 µM) and incubated at 32.5 °C for 10-15 d.

Chanana et al. (2005) studied isolated microspore cultures in various varieties of *Brassica juncea* for morphogenesis. The maximum number of embryos was observed in the cultures of microspores from the plants maintained at low-temperature (10/5 °C) than the field has grown plants. The cultivar Rajat produced about 3500 embryos/100 buds. Around 52% of survival and 74% of fertile plants were produced from microspore embryos. The dishes of microspore suspension were incubated at 32 °C for 72 h and transferred to 25 ± 2 °C in the dark.

Zhang et al. (2006) examined the response of chilling, desiccation, and excision of cotyledons ion plant development from microspores-derived embryos in oilseed rape (*Brasicca napus*). Out of five media observations, the best response from all the genotypes found when the embryos were cultured in the half-strength of Murashige and Skoog medium with 2.0 mg/dm^3 benzyl amino purine. The frequency of embryo germination increased by 90%, and plantlet development increased by 58.46% after cold treatment for 3 or 5 days. The highest numbers of plantlets were obtained from the embryos smaller than 4.0 mm. The germination and development of plantlets were boosted in a successive subculture. About 99.78% of plantlets were formed in the genotype ZJU452 after the second subculture of embryos. The Petri dishes of microspores were incubated at 30 °C for seven days and later kept in the dark at 24 °C. All the treatments were repeated at least thrice in each experiment.

Agarwal et al. (2006) established a whole protocol to promote microspore embryogenesis in the *oleiferous* crop, *Brassica juncea* (Indian mustard). For the experiment, applied colchicines for increasing microspore embryogenesis and also to get embryos for the doubled haploid. High concentrated colchicine (>10 mg/L), for 24 h, showed convenience for direct connection of diploid embryos. Compared to colchicines, higher temperature treatment and an antiauxin PCIB (*p*-chlorophenoxyisobutyric acid) significantly increased the microspore embryogenesis rate. When the temperature increased from 32 °C to 35 °C, embryogenesis increased by 10-fold. The maximum rate of embryogenesis was achieved when PCIB was added at 35 °C in the culture after 24 h of culture initiation. 20 µM PCIB could embryogenesis enhance of microspore by 5-fold. It was added at various concentrations (0, 1, 10, 20, 30, 40, and 50 mg/L) to the Petri dishes containing microspore culture immediately after isolation and subjected to 32 °C for two days. After 6 and 24 h, colchicine was removed by centrifugation; microspores were washed two times with NLN-13 KI medium and again incubated at 32 °C in the dark. After high-temperature treatment, the Petri dishes were incubated at 25 ± 2 °C in the dark.

Abraha et al. (2007) investigated the embryogenic responsibility in six genotypes of *Brasicca carinata* to microspore culture. They modified the microspore culture protocol and produced a satisfactory amount of embryos. The highest numbers of embryos were obtained from 2.5 mm to 3.25 mm long buds in cultivar Dodalla. However, in other genotypes, favorable results were obtained only from 3-3.5 mm long buds when cultured at 30 °C. The frequency of microspore embryogenesis was also determined among genotypes. Microspore cultivation was more efficient in the liquid NLN medium with 0.05 mg/L colchicine for 18 h for embryo production than the control. Each microspore suspension was then placed to 9 cm diameter plastic Petri dishes and cultivated for 18 h in the thermostat at 30 °C in the dark.

Abraha et al. (2008) tested factors in microspores affecting embryogenesis culture of *Brassica carinata*. The response of three factors developmental as such stage of isolated microspores, level of the elevated temperature (30, 32 and 25 °C) and effect of density 70,000 to 150,000 microspores per 1 mL of the medium was examined and developed a reliable protocol for *Brassica carinata* microspore embryogenesis. The significant effects of all the examined factors were observed to form derived embryos in the other genotypes from genus *Brassica* from microspore. The values of the factors mentioned above determined in five various genotypes helped improve the protocol for sound and rapid production of homozygous plants of *Brassica carinata*. Several densities between 60,000 and 164,000 microspores per Petri dish were used. Each microspore suspension was then dropped to 9 cm plastic Petri dish and cultivated for two weeks in the thermostat at 30 °C in the dark or three days at 32 °C then after 2-3 weeks in 25 °C in the dark. After three weeks of cultivation, embryos were counted at torpedo and stage heart (about 2 mm in length), and the culture dish was placed on the shaker (70 rpm) under light at 22 °C until the embryos turned green.

Prem et al. (2008) investigated the activated charcoal (AC) effect for increased microspore embryo production and described the methodology for efficient diploid and regeneration to form doubled haploid (DH) *B. juncea* plants. The dependence genotype for microspore totipotency and an essential effect of genotype by bud size selection was determined. Microspore embryogenesis increased from 100 to 405 embryos per petri dish reciprocal to 2,700-10,935 embryos per 100 buds by applying activated charcoal in NLN containing medium 13% (w/v) sucrose and 10 µM $AgNo_3$. The embryos produced in the absence or presence of activated charcoal germinated embryo similarly for the air-dried. Microspore embryos incubated at 4 ± 1 °C for ten days in dark resulted in the conversion of 82.3%, and the majority of plants formed were haploid. The microspore-derived plants at 3-4 leaf stage after treatment with 0.34% colchicine's for 2-3 h produced 70% of survival and 75% of doubling frequencies of the chromosome. All the

doubled haploid plants were self-pollinated and grown under field conditions. After incubating the microspore cultures at 32.5 ± 1 °C in the dark for 10-11 days, heart-shaped embryos were observed. All the cultures were placed on a gyratory shaker. The temperature was 25 ± 1 °C in the dark conditions, with a 60 rpm rotator until the formation of embryos for 11-20 days.

Wang et al. (2009) studied the microspore embryogenesis in recalcitrant purple flowering stalk development (*Brassica campestris* ssp. *chinensis* var. *purpurea* Hort.) genotypes. The suitable condition for the microspore survival sustained induced embryogenesis and cell division was a short treatment of heat shock at 32 °C for 18 h. The suitable condition for the development of embryos was noticed in reduced macro salts concentration (1/2 NLN). Ten genotypes out of 12 responded to microspore culture in these suitable conditions, with the frequencies ranging from 2.7 to 70.5 embryoids per dish. Nevertheless, regenerated plants were obtained from 9 genotypes, and more than 75% of these regenerated plants were double haploid. This study has established an efficient protocol for microspore culture and provides an excellent possibility for breeding DH in the purple flowering stalk.

Wan et al. (2011) studied the microspore embryogenesis optimization and plant regeneration protocol *for Brasicca napus*. They evaluated the contribution of various factors such as inflorescence length, *in vitro* growth duration of donor plant, heat shock, activated charcoal, and sucrose concentrations on embryogenesis and shoot regeneration in two oilseed rape for that composition on nutrient media (*Brassica napus* L.) viz. ZS758 and ZS72. In both genotypes, the maximum numbers of embryos were formed from the NLN induction medium supplemented with 0.25 mg/mL activated charcoal and 130 g/L sucrose after heat shocked in darkness at 30 °C for two days. The embryos of 4 weeks of both genotypes produced the most considerable shoot regeneration. By applying colchicine treatment (150 mg/L for 30 h, 300 mg/L for 15 h), all the plants obtained from both genotypes doubled and transplanted to soil. The rate of plant survival was more than 95%, whereas doubling was 75%. The freshly isolated microspores were incubated in liquid NLN-13 medium in the dark at 30 °C for two days and 33 °C for five days separately at 25 °C in darkness for embryo differentiation microspore culture. Then microspore was transferred to an orbital shaker (60 rpm) and cultured at 25 °C in darkness cotyledons formation. The formation of healthy embryos from both genotypes ZS758 and ZS72 at 30 °C heat-shocked for two days was 81.5 and 74.6 embryos per bud. The embryo formation was deficient, i.e., 31.5 embryos per bud from ZS758 and 23.0 embryos from ZS72, when incubated at 33 °C for five days.

Winarto et al. (2011) established a protocol on isolated microspore culture for *Brasicca oleracea* of Indonesian origin (cv. 'Kemeh'). During the study, different factors such as the developmental, microspore vitality, bud size selection, stage of microspores genotype selection and temperature treatment, and the effect of embryogenesis frequency were taken under consideration. Due to low microspore viability, many embryos were formed with high microspore density, i.e., 15×10^4 cells/ml. The higher numbers of embryos were obtained using 4.5-4.6 mm long flower buds as explants at 30.5 °C for 48h and then transfer to 25 °C continuously until the embryos formed. Altogether, were obtained 295 embryones from 189 buds, 30% of which were abnormal; 165 healthy embryos were successfully acclimatized to the soil.

Yadollahi et al. (2011) studied the response of carbon sources, polyethylene glycol (PEG), and abscisic acid (ABA) on a secondary embryo (SE) induction and maturation in *Brasicca napus*. Microspore-derived embryos (MDE) of cultivars Global, PF_{704}, and Option. The highest percentage of secondary embryogenesis was obtained by the application of 0.3 M (300 $mOsml^{-1}$) glucose and 0.2 M (200 mOsm/L) sorbitol in SE induction medium (for cultivars Global and PF_{704}) and sorbitol at 0.2 and 0.3 M (200 and 300 mOsm/L, for cultivar Option. In the three studied cultivars, the mean number of SE/MDE reduced by applying ABA in SE induction medium, whereas the percentage of mature SEs in each cultivar increased by 40-80 µM ABA in SE induction medium. The joint application of PEG with or without ABA also reduced the mean number of SE/MDE. However, a significant enhancement of the percentages of mature SEs for the three cultivars occurred. All the donor plants were grown under sodium lamps in a growth chamber with a 16 h/day photoperiod (300 $IE/m^2/s$) under 15/10 °C day/night temperatures. The microspores were 30.0 ± 0.5 °C incubated in the dark for ten days.

Mohammadi et al. (2011) established a valuable method following colchicine treatment of microspore-derived embryos of oilseed rape (*Brassicca napus* L.) to produce double haploid plants. The colchicines treatment of cotyledonary haploid embryos was carried out with one of four concentrations (125, 250, 500, and 1000 mg/L); for one of three treatments periods (12, 24, and 36 h) at one of the two temperatures (8 and 25 °C) and compared to control embryos (without colchicines treatment). After this treatment, the seed recovery, number of chromosomes, leaf stomatal density and size, stomatal density, and size and size of a pollen grain from regenerated plants were observed. The double haploid plants were developed from colchicine treatments, but double haploid plants did not form from control embryos. The formation of double haploid plants was recorded about 64.29% from 250 mg/L colchicine treatment and 66.66% from 500 mg/L colchicine treatment for 36 h, at 8 °C. Only nine plants were obtained from 500 mg/L colchicine treatment for 36 h, and no plants were obtained from the higher colchicine treatment (1,000 mg/L). With

the help of these results, it was concluded that colchicine treatment of embryos derived from microspores could induce doubled haploid lines of oilseed rape. The culture's density was determined (40,000 microspores/ml), and 8 mL of microspore suspension dispensed into each petri dish. Cultures were incubated at 30 °C for 14 days in darkness and then transferred to 25 °C in the dark on a shaker (40 rpm).

Prem et al. (2012) developed under low temperature (18 °C) in *Brassica napus* as a new microspore embryogenesis system. All the cultures were placed after isolation to three conditions of temperature in the dark at 32.0 ± 1 °C, 18 ± 1 °C and 25 ± 1 °C (control). The process was observed every two days under stereomicroscope till the formation of heart-shaped embryos. The cultures maintained by 25 ± 1 °C did not result in any embryo induction (observations up to 40 d), and microspore embryogenesis was observed from Petri dishes cultured at 32.0 ± 1 °C and 18 ± 1 °C. The maximum number of embryos was recorded from 32.0 ± 1 °C (1281.1 ± 98.9 embryos/ Petri dish), but the size of embryos was more minor obtained from Petri dishes cultured to 32 °C compared to those induced at 18 °C. The similar types of mature microspore embryos were obtained from at 18 °C and 32 °C after 30-40 days of culture. This experiment concluded that low temperature applied for an extended period induces the embryogenesis process originating asymmetric cell identities, early polarity establishment, and the formation of suspensor-like structures, mimicking zygotic embryogenesis.

El-Tantawy et al. (2013) studied distribution patterns and the profile of arabinogalactan protein during microspore embryogenesis and pollen development in *Brasicca napus*. *Brassica napus* cv. Topas donor plants were grown at 15 °C days, 16 h photoperiod, and 10 °C night. The microspore culture and embryogenesis induction were carried out at 32 °C for 48 h. All the samples collected a different times, and anthers from flower buds from the development of pollen different were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.3, overnight at 4 °C.

Seo et al. (2014) examined the microspore efficiency of embryogenesis in *Brassica rapa* using various genotypes and cultural conditions. Out of 50, only 30 accessions of *Brasicca rapa* induced embryos from microspores. The highest number of embryos was obtained from IT135449 of turnip type (1.194 per bud), but three accessions of season (winter oil) type did not produce an embryo. During the embryogenesis period, the best stock effect was also noticed with four accessions (IT135449; Turnip type, IT199710; Chinese cabbage type, IT212886, Pak choi type, IT218043; summer oil type). The highest number of embryos was obtained from pre-cultured microspores at 32 °C for two days in IT135449, IT199710, and IT212886. High embryogenesis was noticed in IT218043 after three days of heat shock treatment.

Shumilina et al. (2015) studied the content on microspore-derived embryogenesis in Chinese cabbage (*Brasicca rapa* ssp. chinensis) for the genotype effect and medium culture. The mass of embryos and plants was collected from microspores isolated from flower buds of 2-2.9 mm in length and cultured in liquid NLN 13 with 13% sucrose (w/v) supplemented 24-epibrassinolide and 1% of activated charcoal. The 1/2 Murashige-Skoog culture medium with 2% sucrose (w/v), 0.1 mg/L benzylaminopurine, and 3 g/L Phytagel were applied for embryo culture that activated the formation of secondary embryos resulting in the formation of doubled haploid plants of the large number. After isolation and washing, microspores from 10 buds were kept in a Petri dish (6 cm in diameter) with 5 mL medium mentioned above and incubated at 32 °C in the dark for 48 h. Further incubation was at 25 °C in the dark until embryos were formed.

Javed et al. (2015) studied QTL (quantitative trait loci), finding influencing seed oil content, fatty acid profile, and days to flowering in *Brassica napus*. For the experiment, two spring canola varieties, Polo and Topas were selected to develop a mapping population to detect QTL, influencing seed oil content, fatty acid profile, and flowering time. The DH lines were produced from Polo, and Topas has grown in a growth chamber at 15 °C for 15 h day length. In this study, QTL identification results were obtained from a saturated linkage map derived from SSR markers: relatively large population and reduced marker interval 3.77 cM for linkage with map QTL detection. Stable QTL for oil content, fatty acid profile, and flowering time have been identified and compared with published results. In this study, QTL explaining significant phenotypic variations in oil content (27%), linolenic acid content (35%), and flowering time (43%) have been reported.

Najafabadi et al. (2015) assessed the responses of heat shock and 2,4-D treatment on morphological and physiological characteristics of microspores and microspore-derived doubled haploid (DH) plants in *Brasicca napus*. Isolated microspores were cultured either in NLN-13 medium and incubated at 30 °C for 14 d or treated with 2,4-D (35 mg/L) for 30 min to induce embryogenesis. The embryos derived from microspores were transferred into B5 medium for the formation of plantlets. For the determination of chlorophyll-a (Chla), chlorophyll b (Chlb), and carotenoids contents, spectrophotometric readings at 490, 663, and 645 nm were carried out. The extraction of proteins from microspores and leaves was conducted with the help of TRIzol and cetyl-three-ethyl-ammonium bromide (CTAB). The total protein content of treated microspores was reduced by using stressors, but the

protein content and concentration of Chla and Chlb of the DH plants were increased by heat shock treatment. The carotenoids were not affected by applied stressors. The 2,4-D treatment induced longer and broader stomata, and the size of pollen grains were decreased by heat shock and 2,4-D treatment.

Tuncer et al. (2016) studied the effect of heat shock treatment on the embryogenesis of microspores in *Brassica oleracea* species. During this study, the effect of heat shock treatment at 32 °C and 35 °C for two days was applied in six genotypes of Turkish white head cabbage (Yalova-1, Ercis, 177C, 177T, 531C, 538C), three genotypes of Turkish kale (Balkaya, Yanmaz, Karadere 077) and five commercial F_1 ornamental kale hybrids (Red Piegon, Victoria Piegon, Red Chidori, white Kamome, and Pink Kamome). The maximum number of embryos was obtained from cv. Yalova-1 (9.92 embryones per petri dish) and Pink Kamome F_1 (11.13 embryo per petri dish) at 32 °C and cv. Karadere 077 (5.63 embryones per petri dish) at 35 °C.

Zhang et al. (2016**)** investigated histone deacetylase inhibitors' response on microspore embryogenesis and plant regeneration in Pakchoi (*Brassica rapa* ssp. chinensis). The study was especially carried out to improve the microspore embryogenesis rate and improve direct plant regeneration protocol. For the study, F_2 plants of Pakchoi (421, 424, and 426) were used as donor plants for microspore culture. Verbalization of seed was carried out in an ultra-low temperature freezer at -80 °C for 21 days after seed germination. The incubation of microspore culture was conducted at 33 °C for 1 d and then transferred to 25 °C in the dark. After the embryos' visibility, the culture dishes were placed in a rotary shaker in the dark at 50 rpm, 25 °C.

Bhatia et al. (2016) described an isolated microspore culture protocol for a cauliflower (*Brasicca oleracea* var. botrytis) that evolved under Indian conditions. During the study, the different factors such as the size of the bud, developmental stage of the microspores, culture density, and heat shock treatments were taken under consideration for adequate microspore embryogenesis in four different genotypes representing four different maturity groups. The most suitable density for microspore embryogenesis was 6-8 × 10^4 cells per ml. The microspores were incubated at 30/32.5 °C for 24 h followed by maintenance at 25 °C in different maturity groups of Indian cauliflower. Based on cytometry examination of 646 microspores-derived plants, more than 60% of plants were spontaneous diploids, and less than 15% were haploids.

Lu et al. (2016) evaluated the application of ethyl methanesulfonate (EMS) for mutagenesis in Chinese cabbage. For the experiment, about 30 to 35 buds of five Chinese cabbage genotypes were soaked in solution were different concentrations of WMS (0.03, 0.05, 0.1, and 0.2%) at room temperature to induce mutagenesis. All the buds were treated with each concentration of EMS for 5, 10, or 15 min. Microspores were collected by filtration and washed by centrifugation at 1000 r/min two times for 3 to 4 min each and were suspended at a density of 1 × 10^5 /mL in NLN 13 medium containing active carbon at a concentration of 0.05 to 0.01 mg/mL. The suspension was transferred into Petri dishes of 60 mm diameter for culturing (3 mL/dish). All the cultures were incubated at 30 °C for 34 h in the dark and transferred in the dark at 25 °C.

Zeng et al. (2017) studied to set up an efficient technique allowing broccoli DH plants' formation at a high rate. For this experiment, three cultivars of broccoli ('B415', 'B429', and 'B844') were used. Ascorbate (ASC) or glutathione (GSH) was dissolved in distilled water at room temperature in the dark, and pH was maintained to 6.0 at -20 °C. ASC or GSH will be used to the culture media at a specific concentration (1, 5, 10, 20, 50 or 100 mg/L). 2 mL of liquid culture medium with various ASC or GSH concentrations was placed in 60 × 15 mm Petri dish and incubated at 32.5 °C for 24 h and then placed to 25 °C in the dark. Just after the stress treatment, the number of dead cells reaches about 80% after two days. The mortality rate decreased by adding 10 mg/L ASC and 20 mg/L GSH and producing many embryos.

Cilingir et al. (2017) examined the effects of medium, genotype, and sucrose concentration to determine the frequency of anther culture for future breeding efforts. For this experiment, three red cabbage cultivars (Zencibas, Integro F_1, and Cabellero F_1) were used for anthers culture. Flower buds (3-4 mm in length and 1-2 mm in width) were immersed in 70% ethanol for 1 min and 10% commercial bleach solution for 15 min. The anthers were cultured in jars containing 25 mL of induction medium. All the jars were sealed with the help of stretch film and incubated at 26 ± 1 °C in a growth chamber illuminated with white inflorescent 32 W lamps (3000 Lux) under 16/8 h (day/night) photoperiod. Altogether, ten jars with five anthers for each replicate were cultured for each medium and genotype. The sucrose concentration and genotype were highly responsible for androgenesis for the culture media. The genotype 'Zencibas' produced embryogenesis and plantlet initiation, whereas 'Integro F_1 and Cabellero F_1 did not show any androgenic response.

Bhatia et al. (2018) studied the response of factors for efficient embryogenesis in the microspore in field-grown cabbage without a controlled growing condition. During the study, the role of different factors such as developmental stages of bud, culture density, heat shock, and flow cytometry was investigated. Four genotypes (Golden Acre, KTCBH-2085, KTCBH-6621, and KTCBH-6045) were selected for the experiment. Maximum viability was found in bud size of 4-4.5 mm (from genotypes, Golden Acre and

KTCBH-2085) and 4.5-5.0 mm (from KTCBH-6621 and KTCBH-6045). The most suitable temperature shock treatments were 30 °C for 48 h + 25 °C (for Golden Acre and KTCBH-2085) and 32.5 °C for 24 h + 25 °C (for KTCBH-6621 and KTCBH-6045). The bud size and microspore developmental stage were the most critical among various factors for microspore embryogenesis in cabbage. The interaction among genotypes, bud size, culture density, and heat treatment factors also played a significant role in white cabbage's microspore embryogenesis. Out of 221 plants, 29.0% were haploids, 10.9% were tetraploids, and 16.3% were mixoploids and aneuploids.

Domblides et al. (2018) produced doubled haploid plants of broccoli (*B. oleracea* L. convar. Botrytis) from macrospore culture. The highest numbers of embryos were obtained from 4-5 mm long buds. The suitable temperature for microspore treatment was 32 °C for two days. The embryoids were obtained from Arcadia F_1, Everest, Green Valiant, Marathon F_1, and Furio. The most considerable number of embryoids (140 embryoids per Petri dish) was found from Green Valiant, and the minimum number of embryoids (up to 3 embryoids per Petri dish) found from Furio. The growth of embryoids takes place either directly into normal embryoid or suspensor-like structures. The embryoid with a suspensor grows more slowly than the normal one.

Pilih et al. (2018) studied the effect of activated charcoal (AC) generate into the induction media on the generation of embryos and their subsequent development into plantlets. Thirty flower buds having late uninucleate microspores were selected for the experiment. The microspores were collected by gently crushing the buds in 2 mL of filter-sterilized hormone-free NLN 13 medium with13% sucrose. According to the experimental scheme, the adjusted microspore suspension was distributed into 50 mL sterile test tubes (half of them containing 0.02% AC, according to the experimental scheme) and incubated in the dark at 30 °C for 48 h. Following this stress treatment, the microspore suspensions were centrifuged at 190 g for four min., the supernatant was removed and replaced with fresh NLN-13 medium before distribution in 50-mm Petri dishes. The dishes were incubated in a growth chamber at 25 °C for ten days in the dark and then transferred to an orbital shaker, operating at 40 rpm. A total of 20,032 embryos were formed.

Berenguer et al. (2019) analyzed the cell death levels in microspore cultures of *Brassica napus* during the first stages after embryogenesis induction and involvement of caspase 3-like activity in cell death. For the experiment, *Brassica napus* (rapeseed) cv, 'Topas' line DH407 were selected as donor plants. Vacuolated microspores from flower buds were isolated for microspore culture and placed to stress treatment at 32 °C for 48 h to induce embryogenesis. After heat treatment, a high level of cell death was noticed in isolated microspore cultures. Cell death activities were reduced with a specific inhibitor of caspase 3-like activity, increasing embryogenesis induction efficiency.

Zhang et al. (2020) conducted the combined system of a molecular marker to breed *Brasicca napus* restores lines in the research. Genic male sterile hybrid F_1 generation HY15A x HF06 was selected for the experiment. Buds about 2-4 mm long were immersed in 70% alcohol for 30 s and sterilized with 0.1% mercuric chloride for 10 min. The buds rinsed thrice with sterile water, placed into a sterile glass containing 3 ml microspore isolation solution of B5 medium at pH 6. After homogenization, microspore suspension was centrifuged at 1,000 rpm for 5 min. Finally, isolated microspores were suspended on the NLN 13 culture medium. The suspension was centrifuged at 32 °C in the dark for two days after culture. The mature embryos were inoculated at 25 °C for a 16 h photoperiod in B5 solid medium in light condition.

Su et al. (2020) studied to identify differentially expressed proteins after 32 °C for 24 h and 25 °C for 24 h in isolated, cultured microspores of two cabbage accessions (Zhonggan 628 and 87-534). The 32 °C treatments produced 19.7 embryos/bud in Zhonggan 628, whereas at 25 °C, treatments did not produce any embryo. In the case of 87-534 type, no embryos were formed at both the treatments. The total number of identified differentially expressed proteins (DEPs) in Zhonggan 628 were 363 and 282 in 87-534. Only in Zhonggan 628, 97 DEPs were identified but not in 87-534 after 32 °C heat shock treatment.

Shumilina et al. (2020) studied genotype effect and culture conditions on embryogenesis of microspore and regeneration of plants in cultivars of turnip (*Brasicca rapa* sp. Rapa). During low temperature, treatment inflorescences were placed into a plastic Petri dish with 5 mL of sterile and transferred at 6 °C for two days after disinfected flower buds were crushed in ½ NLN 13 modified media with 135 sucrose (w/v) without potato extract and plant growth regulators at pH 5.8. Afterward, the microspore suspension was placed in 60 mm Petri dishes (5 mL /petri dish) at 32 °C for 48 h in the dark. Then continuous incubation was followed on a gyratory shaker at 60 rpm till the initiation of the embryo.

3. Future prospectus on microspore culture

The value of haploid and doubled haploid plant products from the microspore technique is an advanced and reliable technique. The recently applied technique will be modified for the economic and time point of view. The above observation of different experiments conducted shows that many researchers found better production for embryo development and economic efficiency. The relative observation of the past 20 years has the significant evolution on the microspore-derived plants for the haploid and the doubled of haploid (DH) plants for the future demand to manage and conserve the valuable for economic and conservation point of view essential plants. As here, *Brassica* is an example of the suitable treatment and the time for culture. The observation of different systems will be applied at 32 °C as better and caring about the sample collection, the 4.5-5 mm bud. The future will be evaluated from the application of different modern genetic technology.

4. Conclusion

In *Brassica* species, microspore embryogenesis is considered a unique technique under different workers' various specific stress treatments. We reviewed research articles to identify the suitable temperature and time for microspore embryogenesis in *Brassica* species. We reviewed 38 experimental works for microspore culture comprising 5 of *Brassica juncea*, 6 of *Brassica rapa*, 12 of *Brassica oleracea (*var. *italic, and* var. tronchuda), *12 of Brassica napus*., 2 of *Brassica carinata,* and 1 of *Brassica campestris.* The temperature used for microspore culture ranged from 15-33 °C, whereas the time taken ranged from 8 h to 40 days. The above observation concludes that suitable temperature and time for microspores culture are 32 °C and 48 h, respectively.

Conflicts of interest. There are no conflicts of interest.

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