Induction of Embryogenic Microspore in Oil Palm (*Elaeis guineensis* Jacq) by Starvation and Temperature Stress

Ari Indrianto[#], Totik Sri Mariani[*], Dini Astika Sari[#]

[#]Laboratory of Biotechnology, Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia

[*]School of Life Sciences and Technology, Bandung Institute of Technology, Ganesha 10, Bandung 40132, Indonesia
[*]*Email: totiksrimariani {at} yahoo.com*

ABSTRACT--- ***Gametophytic development (in vivo) of microspore was programmed to develop as pollen. By in vitro culture technique, normal development of microspores could be converted into embryogenic microspores. Developmental stages and stress were become the main roles of inducing the gametophytic pathway to embryo development. Embryogenic microspores then could be regenerated as haploid plants. Haploid culture technology can be used as a great solution to produce pure line parental with double haploid plants efficiently. The aim of this research was to determine the developmental stage of oil palm microspores and to induce normal development of oil palm microspores into embryogenic microspores by pre-treatment stress, i.e. carbohydrate starvation and temperature stress.***

Oil palm microspores were isolated from fresh spikelet (from the tip, middle and basal part vertically of spikes) and then were observed in the samples stained with DAPI (4,6-diamidino-2-phenylindole) under electron microscopes to determine nucleus position. Late uninucleate microspores were then cultured aseptically in the carbohydrate-starvation medium and each was incubated to 4ºC, 25ºC and 34ºC for 2, 4 and 6 days as treatments. Further treatment was sub-culturing of embryogenic microspores into A2 embryogenesis medium and then embryogenic microspores were incubated to25ºC. Observation of embryogenic microspores and symmetrical divisions were determined in the samples stained with DAPI (cytology observation) while viability observation was carried out in the samples stained using FDA (fluorescence diacetate). Percentage data of viable embryogenic-microspores with symmetrical division and multicelluler structures were collected as representative of quantitative data.

The results showed that basal part of spike contains the most mature stage of microspore development and Marihat was become the highest proportion contained late uninucleate stage of microspore with 71.26% at the tip position of spike and followed by 55.94% at the basal spike. That was characterized by brown to dark-brown color of morphological spike. Pre-treatment with 4º Ctowards spikeletwas able to retain the viability of microspore(59,7% from total population) until 6 days and decline until 35,9% at 12 days after treatment. Pre-treatment temperature and starvation stress were able to induce embryogenic microspore which is characterized by swollen cell with oval structure, cytoplasm of cell was fulfilled by smooth granules. Embryogenic microspore developed to symmetrical division (2,1%) and multinucleat structures (4 nuclei) in A2 embryogenesis medium. Symmetrical division and multinucleat structures were become thefirst step in producing haploid plants wherein subsequently continue to double chromosome technique for double haploid plants production (pure line).

Keywords--- starvation – cold shock – heat shock – microspores – embryogenic – oil palm

---

1. INTRODUCTION

Commercial hybrid of oil palm planting material known as Tenera (Dura x Pisifera) has an excellence productivity and high CPO content. Latif (1991) explained that Tenera is still not the pure hybrid yet hence heterocyst manifestation for each individual is never come identical, and as a resultthe potency of maximum production in the field is unprecedented. The effort of producing newly exeeded hybrids is now facing some constrains, other than require of accuracy and persistency, Morrison and Evans (1988) stated in the long term research study due to the requirement of passing throughseveral repetition of life cycles to gain representative data. Significant step could help hybrid production is by assembling the homozygot mother palm efficiently. Conventional breeding was applied by repeatedly back cross to produce homozygot mother palm (Morrison dan Evans, 1988). The best way to produce homozygot plants (pure line) is by regenerating oil palm planlet directly from haploid cells (microspore), subsequently continue with double-chromosome technique application to produce double haploid plants. Therefore, the ability of producing oil palm planlet directly from microspore cells is become innovation to solve the problems.

Microspore is a male gametophyte cell or an immature pollen. In the normal development (gametophytic, *in* vivo), microspore will be differentiated as mature pollen and produce 2 sperm cells, in which by *in vitro* culture technique can be converted into embryogenic microspore (sporophytic). Developmental stage of microspores and stress were become the main role in inducing embryogenic microspores. Embryogenic microspores may allow us to regenerate haploid plant (Touraev *et al*, 1997).

In oil palm, recent studies of producing haploid plant using microspore culture are not published yet beside the preliminary study of microspore regeneration process. Latif (1991) had identified developmental stages of oil palm microspore using conventional staining that was developed by Alexander (1969). Madon *et al* (2005) reported cytological analysis of microspore mother cell using fluorochromatic staining that was developed by Greilhuber and Temsch (2001).The aim of this research were to determine the developmental stages of oil palm microspore and to induce embryogenic microspore by carbohydrate starvation and temperature stress.

2. MATERIALS AND METHODS

2.1. Materials

Planting materials that been used for this research was *spathe* (male flower buds) from Dura, Pisifera and Tenera from IOPRI Medan (well-known as PPKS Medan) and var. Lonsum, var. Marihat and var. Socphin from estates of small holder oil palm plantation in Pangkalan Bun, Central Kalimantan. DAPI, FDA, nylon filter 40 µm, sterilized aquades, alcohol 70%, alcohol 96%, $HgCl_2$ (sublimat), label, parafilm, sterilized allumunium foil, disposable blue tips, maltose, mannitol, chemicals for B starvation medium(Kyo dan Harada, 1986), and A2 embryogenesis medium (Touraev, *et al* .,1996).

2.2. Equipments

Equipments that been occupied for this research were test tubes, test tube racks, sprayer, beaker glass, cell culture dish 35 mm, cell culture dish 100 mm, sterilized forceps, modified stirring rods, centrifuge tubes, centrifuge, magnetic stirrer,inverted fluorescent microscope, autoclave, analytical balance, pH meter, nylon filter 0,22 micrometer, hand sprayer, bunsen lamp, parafilm, allumunium foil, pinset, microtube1,5 ml, fixed micropippette 1 ml, incubator with temperature set : 4°C, 25°C, 34°C andLaminar Air Flow (LAF).

2.3. Methods

Determination of Microspore Developmental Stages

Spathe(male flower inflorescence) of oil palm with *spikelet*that contains anthera was prepared and late uninucleate microspores were isolated as explan. Determination of nucleus position of microspore was carried out using DAPI (4,6-diaminidino-2-phenylindole)staining under fluorescence microscope observation. Late uninucleate stage was identified by nucleus position in the edge, close to the cell wall. While to determine vacuola, microspore was observed without staining (Touraev dan Heberle-Bors, 1998).

Induction of embryogenic microspore by stress

Pre-treatment temperature of spike

*Spathe*was sprayed using alcohol 96% and was pared aseptically to isolate *spike*with the length of 13 – 15 cm (contains late uninucleate stage of microspores), and subsequently were sterilized by soaking into alcohol 96% for 3-5 sec. Each oftwo *spikes*then were inserted into one sterilized test tube contains 3 ml aquades. Tubes were placed in incubator with temperature set at 34; 25 dan 4°C for 6 dan 12 days.

Pre-treatment temperature and starvation of spikelet

Flower buds (*spikelet*) were isolated from spike and were sterilized usingHgCl2 0,1% for 10 menit. Sterillized *spikelet*s were thencultured in petridish (Ø 5 cm) contains 5 ml ofB starvation medium, which each of petridish contain 50 *spikelets*. Petridish was sealed using parafilm and was placed in incubator at the dark room at temperature 4; 25 and 34^{o}C for 2; 4; 6 and 8 days. Microspores were then isolated and cultured in petridish contain A2 embryogenesis medium. Culture was placed in incubator at temparature 25 ^{o}C (dark room).

Pre-treatment temperature and starvation of microspore

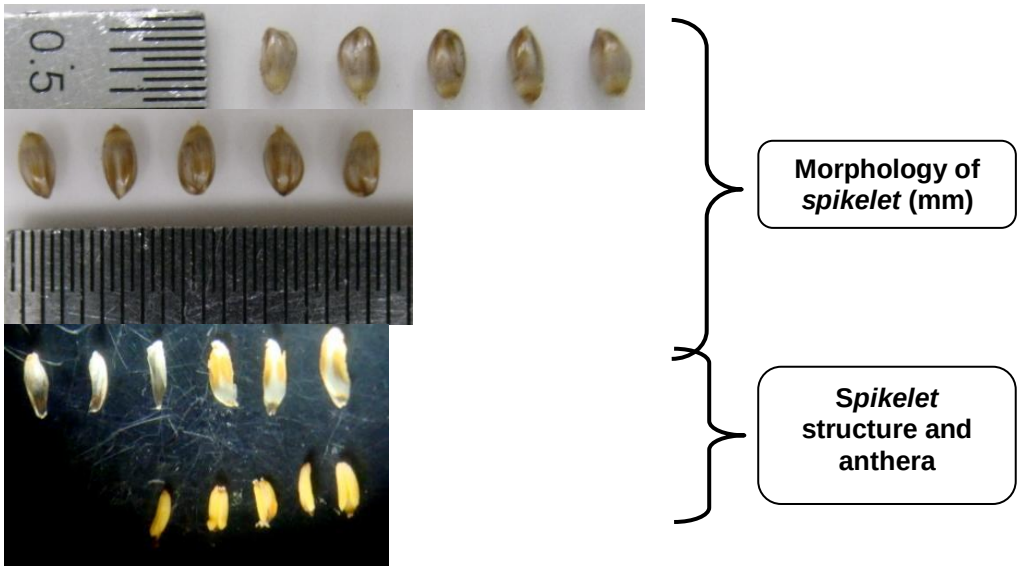
Microspores were isolated aseptically in B medium by grinding the spikelet using stirring rod. Microspore suspension was filtered using nylon filter 40µm then to wash by way of centrifugation at 1000 rpm for 10 minutes. Microspores were prepared to be cultured in petridish (Ø 5 cm) contains 5 ml of B starvation medium with the density 10^5 microspores/ml (density was measured by Haemocytometer). Petridish was sealed with parafilmand was incubated in the dark room with temperature set at: 4; 25 dan 34^{o}C for varied times 2; 4; 6 and 8 days. Micropores were then sub-cultured in A2 embryogenesis medium and incubated at 25 ^{o}C in the dark room.

3. RESULTS

Determination of Oil Palm Microspore Developmental Stages

Oil palm male inflorescence is composed by complex structure of *spikes* that contains *spikelet* structure for each. *Spikelet* is male flower unit which consists of 2 layers of carpium and is composed by 3 leaves for each carpium layer. Exocarpium as outer layer isnormally dark-yellowish color while endocarpium, is thinner and transparent-white in color. Inner end of carpium is consisted of 5-6 pairs of anthera with short filament structure (Pic 1.).

Developmental stage of microsopre is one of the most important key for inducing embryogenic microspre, that is to say related to cell cycle in which first stage of haploid mitosis *(late unicellular* atau *early bicellular*) was noted as the most critical stage for inducing embryogenic microspore. Refer to Indrianto *et al.* (1999) and Raina & Irfan (1998) over wheat and paddy, *late unicellular*stage until*premitosis*are the most optimum stage for microspore culture.



Pic 1. Morphology of *spikelet* (mm) Marihatand *spikelet*structure(male flower unit) with yellowish color of outer carpium; transparent-white color of inner carpium and 5 pairs of anthera with short filament structure.

Results of morphological and cytological observation showed that var. Marihat in which characterized by brown color of tip part of *spike* and progressively dark in color at the basal part of *spike* vertically, contained the highest percentage of late uninucleate stage of microspore in the number of 71.26 % with standard deviation equal to 0.33. (Table 1.). Other than late-uninucleatestage, var. Marihat also contained binucleate stage of microspore with 2 asymmetric nuclei. Binucleate stage with 2 asymmetric nuclei can be observed by DAPI staining under fluorescence microscopewhich indicate different intensity level of fluorescence.

Table 1. Developmental stage of microspore over 3 different varieties and showed different results based on *spikelet* position towards *spike* vertically, by DAPI staining

Varietas	Letak Spikelet	Stadium Perkembangan						Warna Bagian Spike
		MMC	Tetrad	Uninukleat awal	Uninukleat tengah	Uninukleat akhir	Binukleat awal	
Marihat	Ujung	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	9.77 ± 0.81	71.26 ± 0.33	18.97 ± 0.81	Coklat
	Tengah	0.00 ± 0.00	0.00 ± 0.00	56.62 ± 5.64	43.38 ± 5.6	0.00 ± 0.00	0.00 ± 0.00	Coklat tua
	Pangkal	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	44.06 ± 6	55.94 ± 6.02	0.00 ± 0.00	Coklat tua
Lonsum	Ujung	100 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	Kuning
	Tengah	11.95±4.04	89.05 ± 0.76	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	Kuning tua
	Pangkal	0.00 ± 0.00	15.85 ± 1.22	63.39 ± 0.77	20.76 ± 1.72	0.00 ± 0.00	0.00 ± 0.00	Coklat muda
Sophin	Ujung	100 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	Putih kekuningan
	Tengah	97.65 ± 0.83	2.35 ± 1.22	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	Kuning
	Pangkal	0.00 ± 0.00	0.00 ± 0.00	44.99 ± 0.23	55 ± 0.2	0.00 ± 0.00	0.00 ± 0.00	Kuning

Note : Percentage data was measured from 3 replication with ±300 microspores for each.

Table 1. showed us the results from determination of microspore developmental stage over 3 different varieties (var. Marihat, var. Lonsum and var. Socphin) based on *spikelet* position towards *spike* and *spikelet*'s color. There is significant correlation wherein the basal part of *spikelet* contains the most mature stage compare to middle and tip part. Basal part of *spikelet* was characterized by yellow-brown until dark brown in color. Color of *spike* also determined the

developmental stages of microspore contained in the *spikelet. Spike*with light yellow in color – yellow color were mostly consisted of *MMC* (*Microspore mother cell*) stage until early uninucleate stage. Cytologically, pollen mother cell was characterized by round shape and contains one nucleus located in the central position. Tetrad stage was characterized by 4 nuclei in the cell while early uninucleate was spherical shape of cell with central-located nucleus. Var. Lonsum (L) was characterized by yellowish*spike*at the basal part and light-yellow at the tip vertically, indicated the different developmental stage for each part. *Spikelet* located at the tip position contained*MMC* / *microspore mother cell* with one nuclues and roud shape of cell. *Spikelet* located at the middle part was mostly consisted of tetrad stage with stretch chromatin structures, at the 89.05 % with standar deviation equals to 0.76 (Table 1.). Basal partof *spikelet* contained mid-uninucleate stage of microspore which is characterized by spherical shape of microspore cell and central-located of nucleus. For var. Lonsum, maturity of microspore was indicated from basal to tip of *spike* vertically, while the tip part had the immature one as the light color of *spike.* Type of var. Lonsum inflorescence in this study may not be used as explan for induction of embryogenic microspore since the results of observation determined that developmental stage of this sample was too early and may unresponsive to be induced for embryogenic microspore.

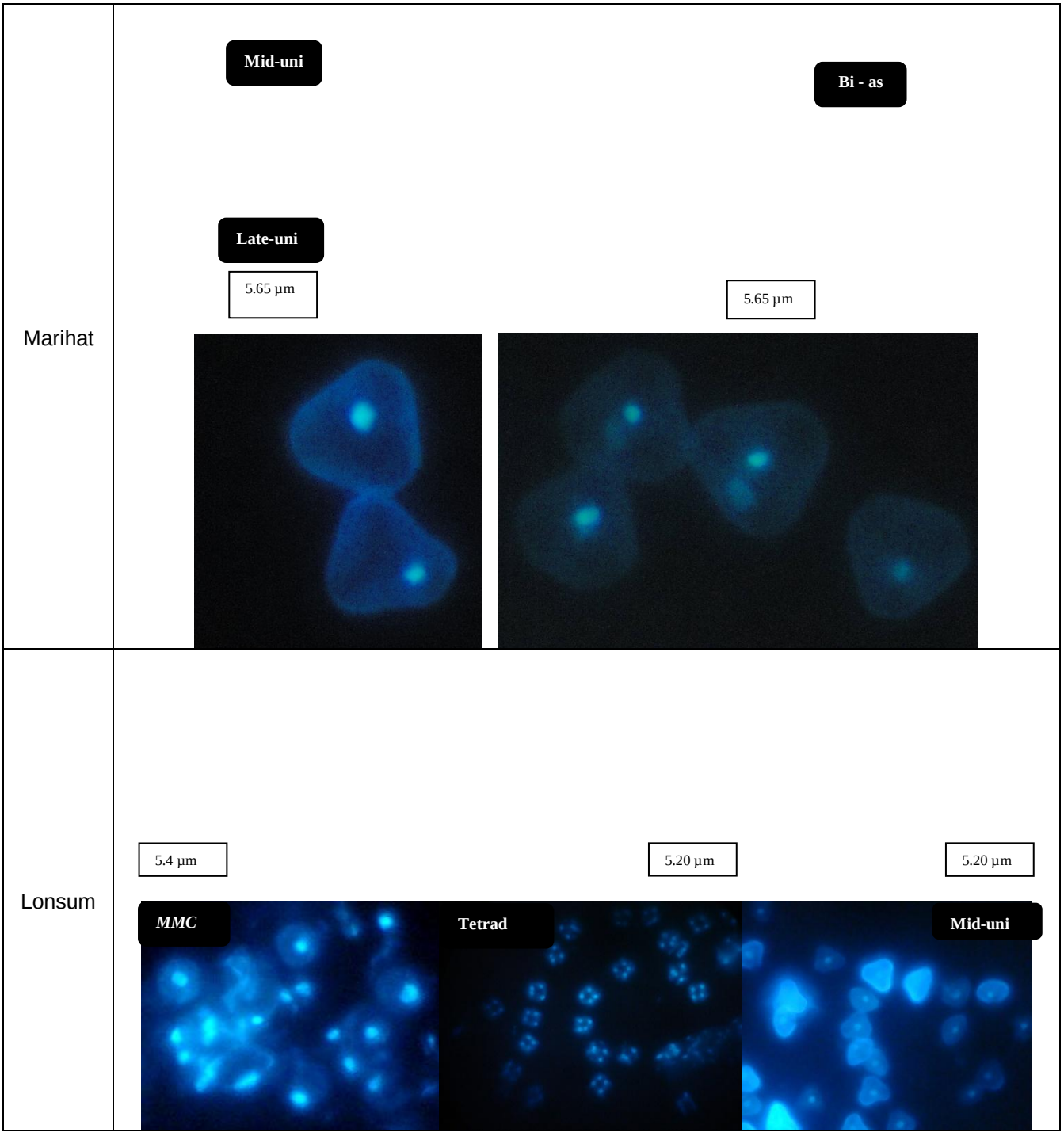
Variety of Socphin (S) indicated by light-yellow color of spike at the basal part and beige color for tip part of *spike.* This variety had the smaller dimension compare to two other varieties Marihat and Lonsum. At the basal part of *spike*, determination of developmental stage showed us early uninucleate stage, characterized by spherical shape of microspore cell and central-located of nucleus. While the tip and mid- part of *spike* contains *spikelet* withmicrospore mother cell (MMC) stage (Pic. 2). Data stated in Table 1. showed the percentage of microspore mother cell (MMC) was 100% at the tip part and 97.65 % at the mid-part over *spike* position. This informationindicated that var. Socphin may not be used as explan for induction of embryogenic microspore since the developmental stage of microspore was not sufficient and may contribute to difficulty level of microspore isolation due to smaller dimension of *spike.*

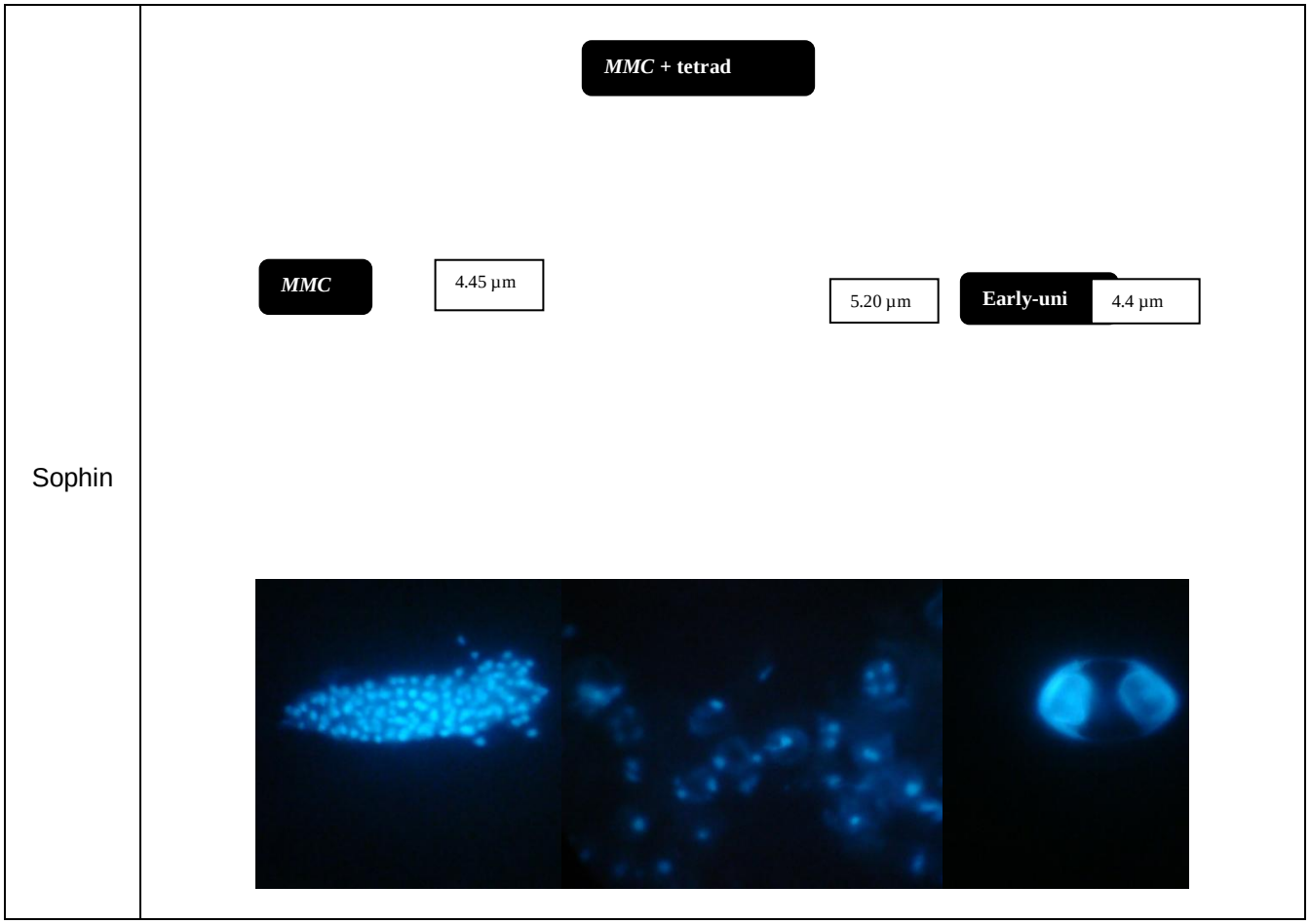
Viability of microspore plays an important role against induction of embryogenic since microspores that could survive during stress period may successfully convert its normal developmentinto sporophytic pathway (Indrianto *et al*., 1999). In this study, cold treatment as temperature stress (4°C) towards *spikelet* may defend the viability of microspore rather than normal temperature and high temperature (25°C and34°C).

Pre-treatment temperature of spikelet

Morphologically, *spikelet* of Dura that were treated by temperature stress at 34; 25 and 4°C for 6 and 12 days indicated the noticeable difference. *Spikelet* with 4°C of pre-treatment stress is hardly found any changesand looks fresh for appearance. For*spikelet* that were treated at temperature 25°C, some of the samples looks dark-brown in color and about to rot or decay while *spikelet* with high-temperature pre-treatment 34°C cause the *spikelet stalk* become soft in texture, dark brown in color and rot of *spikelet.*

Variety	Developmental stage of microspore over 3 different varieties using DAPI staining


Marihat
Mid-uni
Bi - as
Late-uni
5.65 µm
5.65 µm
Lonsum
5.4 µm
5.20 µm
5.20 µm
MMC
Tetrad
Mid-uni



Pic 2. Developmental stage of microspore over var. M; var. L; and var. S : Microspore at microspore mother cellstage(MMC); tetrad stage; early uninucleate (early-uni); mid uninucleate (mid-uni); late uninucleate (late-uni); and binucleate asymmetric (bi-as) using DAPI staining.

Viable microspores were isolated from pre-treated *spikelet* and for rot *spikelet* were removed from test unit. Cytological observation of microspores had collected from Dura *spikelet* (pre-treated with 4°C) showedthat 59,7% from total population of microsore were still viable until 6 days after treatement and decline until 35,9% at 12 days after treatment (tabel 2).

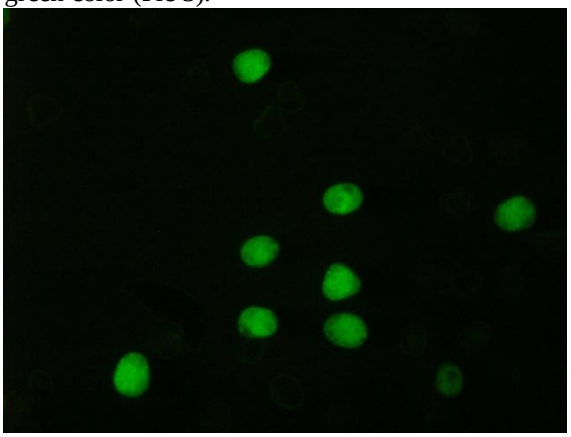
Table 2. Effect of temperature and length of incubation days over *spikelet* of Dura towards microspore viabillity.

Length of incubation time (days)	**Temperature of incubator**		
	4°C	**25°C**	**34°C**
6	59,56 ± 2,99	21,74 ± 1,46	0,8 ± 0,7
12	35,99 ± 2,9	9,85 ± 0,91	0,00 ± 0,00

Note : Microspores were isolated from randomly taken *spikelet* of *spike*part, 10 s*pikelet* for each treatment. Mean of percentage and standar deviation were measured from ±300 microspores with 5 replication.

The result of this study is in accordance with previous research study that had carried out by Sunderland (1978) in which described about the principal effect of cold pre-treatment against the survival rate of microspore viability. Cold effect may convert the composition of plasma membrane by synthesis mechanism and formation of unsaturated-fatty acid thus affect the fluidity of plasma membrane (Lyons, 1973). This effects of fluidity then lead the plasma membrane to be

more adaptive against stress, this is showed by FDA staining of microspore from cold pre-treated*spikelet*. Viable microspores were indicated by its ability to break the fluorescein diacetate into fluorescein by esterase enzyme activity in cytoplasm, so that fluoresces in yellow-green color (Pic 3).

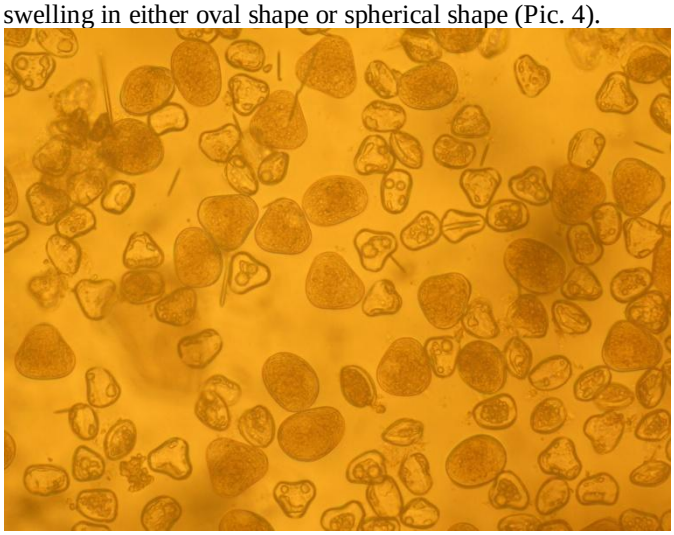


Pic 3. Viable oil palm microspore will be indicated by yellow-green fluorescence color using FDA staining under fluorescence microscope observation.

Induction of embryogenic mcrospore by temperature stress and starvation

Microspores were isolated from pre-treated of cold temperature *spikelet* (6 days incubation) and then were cultured in B starvation medium,subsequently incubated at temperature 4; 25 and 34°C, for the length of the days 2; 4 and 6 days. Embryogenic microspores were observed using inverted-microscope.

Touraev *et al*. (1997) reported embryogenic microspore was characterized morphologically by bigger size of cell due to swelled-cell and reorganization of cytoplasm structure that is indicated by disappearance of amilum granules, nucleus is moving in centripetal and formation of *cytoplasm bridge*that connects between perinuclear cytoplasm and subcortical cytoplasm. *Cytoplasm bridge* causes entirely microspore looks as *star-like* structure. This structure was not appeared under microscope observation of Dura microspore since the cytoplasm was entirely fullfilled by smooth granules and microspore was swelling in either oval shape or spherical shape (Pic. 4).



Pic 4. Swelling microspore after pre-treatment temperature and starvation stress. Cytoplasm looked dark in color and is fullfilled by smooth granules.

Observation towards swelled-microspore due to stress pre-treatment of temperature and starvation showed us that percentage of swelled-microspore due to cold pre-treatment was increase as the length of duration (days of incubation) (Table. 3). This is may caused by better fluidity effect of plasma membran (Lyons, 1973).

Heat temperature of pre-treatment was set at 34°C and showed us high percentage of contamination by bacteria starting from day-4 after treatment thus data could not be recorded (Table 3). The availability of smooth granules in cytoplasm from swelled-microspore is further need to be confirmed whether the amilum fullfilled the cytoplasm or others. Research study had been carried out by Sangwan dan Sangwan-Noreel (1987) in the development of plastid of microspore from both of androgenic and non-androgenic plants showed that androgenic plants was identified by the availability of proplastid while amiloplast was defined for recalcitrant microspores. Observation over Dura‘s

microsporeshowed that amilogenesis has been started as microsporogenesis process so that it is lead to the identification of Dura as recalcitrant microspore.

Tabel 3. Percentage of embryogenic microspore after temperature stress pre-treatment and starvation (B medium).

Length of duration of starvation (days)	**Temperature of incubation**		
	4°C	**25°C**	**34°C**
A2(0) control	15,53 ± 1,5	22,7 ± 2,6	11,11 ± 0,9
B (2)	19,74 ± 1,13	47,8 ± 3,7	27,84 ± 2,06
B (4)	23,2 ± 1,7	37,75 ± 1,43	0,00 ± 0,00
B (6)	27,9 ± 1,5	27,9 ± 2,63	0,00 ± 0,00

Note : Mean and standar deviation were measured from min. 300 microspores with 4 replication

Development of embryogenic microspore in A2 embryogenesis medium

Embryogenic microspores were then subcultured in A2 embryogenesis medium and were incubated at room temperature25°C for 21 days. Monitoring was carried out over microspore cultur to observe the development of nucleus of embryogenic microspores. Cytological observation using fluorescent microscope over microspore's nucleus with DAPI staining, showed us population of microspore with varied number of nuclei. Uninucleate microspore with 1 nucleus, symmetrical binucleate with 2 nuclei which has identical intensity of fluorescence, asymmetrical binucleate with 2 different intensity of fluorescence of nucleus, trinucleate with 3 nuclei and multinucleate with 4 or more nuclei (Pic. 5).

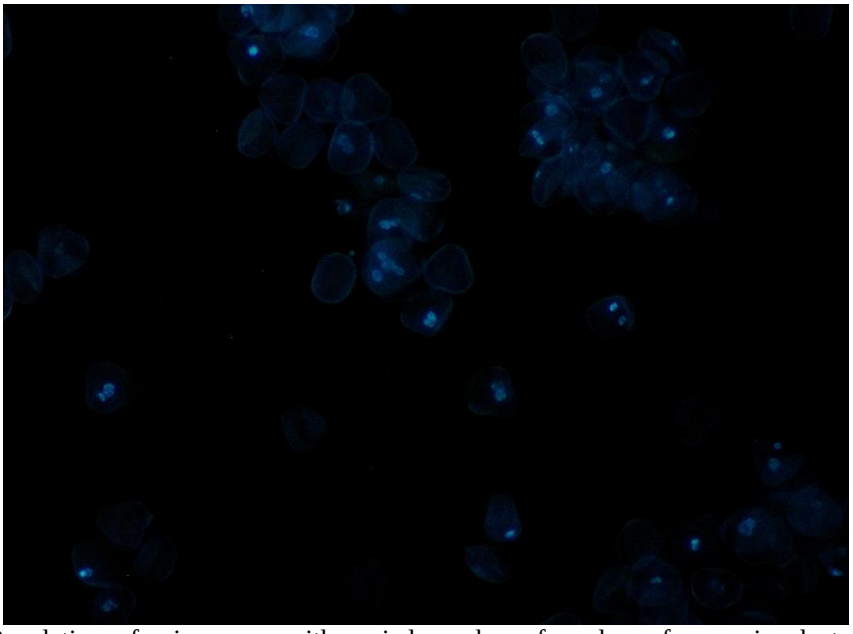
In this research, embryogenic microspores that has been induced by cold-temperature at 4°C,then subcultured in A2 embryogenesis medium and incubated at room temperature, showed us any alteration of normal development from gametophytic into sporopyhtic pathway. This alteration was identified by increasing of percentage of symmetrical division of nuclei from 0,57% at 2 days after treatment into 2,28% at 6 days after treatment(Table4).

Tabel 4. Development of nuclei with DAPI staining observation after 21 days incubation in A2 embryogenesis medium (room temperature; 25^{o}C).

Length of duration (days)	Temperature (^{o}C)	Uninucleate	Binucleate		Trinucleate	Multinucleate
			Symmetrical	Asymmetrical		
2	4	8,2 ± 0,8	0,57 ± 0,4	5,9 ± 0,7	0,00 ± 0,00	0,00 ± 0,00
	25	11,2 ± 0,4	0,4 ± 0,1	8 ± 0,57	0,22 ± 0,3	0,00 ± 0,00
	34	9,6 ± 0,29	2,1 ± 0,4	1,21± 0,42	0,1 ± 0,14	0,4 ± 0,1
4	4	6,08 ± 0,82	1,04 ± 0,2	5,7 ± 0,9	0,1 ± 0,1	0,3 ± 0,2
	25	9,2 ± 0,7	1,4 ± 1,05	8,8 ± 0,7	0,79 ± 0,15	0,00 ± 0,00
	34	0,00 ± 0 00	0,00 ± 0 00	0,00 ± 0 00	0,00 ± 0 00	0,00 ± 0 00
6	4	3,4 ± 0,7	2,28 ± 0,4	4,76 ± 0,3	0,58 ± 0,2	1 ± 0,2
	25	4,4 ± 0,4	0,4 ± 0,14	11,7 ± 0,9	1,1 ± 0,1	0,00 ± 0,00
	34	0,00 ± 0 00	0,00 ± 0 00	0,00 ± 0 00	0,00 ± 0 00	0,00 ± 0 00

Note : Microspores were pre-treated by starvation (B medium) at 4^{o}C; 25^{o}C and 34^{o}C for 2; 4; and 6 days, then subcultured in A2 embryogenesis medium. Mean and standar deviation were measured from min. 300 microspores, with 3 replication under fluorescence microscope observation.

Multicellular structure of microspore (proembrio, Pic. 5)that has been developed lead us to the theory that temperature and length of duration of starvation as stress pre-treatment were become the main role in inducing embryogenic microspore of Dura.



Pic 5. Population of microspore with varied number of nucleus, from uninucleate, symmetrical binucleate, asymmetrical binucleate, trinucleate and multinucleate (4 nuclei). Embryogenic microspore was previously induced by stress of cold temperature and starvation for 6 days.

4. CONCLUSION

1. Basal part of *spikelet* contains the most mature of microspore developmental stage and var. Marihat has the highest percentage of late uninucleate microspore population at the tip and basal part with the number 71.26% and 55.94% respectively, which was identified by morphological characteristic of brown until dark-brown in *spikelet* color.
2. Microspore isolated from pre-treated *spikelet* by cold temperature 4°C could retain the viability (59,7% from total population) until 6 days of incubation and decline to 35,9% at 12th days of incubation.
3. After starvation and temperature stress of pre-treatment (34°C), microspore was induced to embryogenic microspore andidentified by swelled-cell with ovale shape as well as its cytoplasm was fullfilled by smooth granules.
4. Embryogenic microspores developed and symmetrically divided (2,1%) then formed multinucleate stucture (4 nuclei, 0,4%) in A2 embryogenesis medium.

5. REFERENCES

1. Alexander, M. P., 1969.Differential staining of aborted and nonaborted pollen. *Stain Technol.* 14(3): 117-122.
2. Benito Moreno RM, Macke F, Alwen A, Heberle-Bors E, 1988. In situ seed production after pollination with in vitro matured, isolated pollen. *Planta* 176: 145-148.
3. Binarova P, Hause G, Cenklova V, Cordewener JHG, van Lookern Campagne MM, 1997. A short severe heat shock is required to induce embryogenesis in late bicellular pollen of *Brassica napus* , *Sex Plant Reprod* 10: 200-208.
4. Cho, M. S. and F. J. Zapata, 1988. Callus formation and plant regeneration in isolated pollen culture of rice (*Oryza sativa* cv. Taipei 309). *Plant Sci*. 58: 239-244.
5. Cordewener, J.H.G., Busink, R., Trass, J. A., Custer J. B. M., Dons, H. J. M., Van Lookern Campagne, M. M., 1994. Induction of microspore embryogenesis in *Brassica napus* is accompanied by specific changes in protein synthesis. *Planta* 195: 50-56.
6. Custers JBM, Cordewener JHG, Noellen Y, Dons JJM, van Lookern Campagne MM, 1994. Tempereture controls both gametophytic and sporophytic development on microspore cultures of *Brassica napus*. *Plant Cell Rep.* 13: 267-271.
7. Greilhuber, E and Temsch, E., 2001.Feulgen densitometry: Some observations relevant to best practice in quantitative nuclear DNA content determination. *Acta Bot of Croat*, 60(2): 285-298.
8. Heberle-Bors E, 1989. Isolated pollen culture in tobacco: Plant reproductive development in a nutshell. *Sex Plant Reprod* 2: 1-10.
9. Heberle-Bors E, 1999. Microspore culture, totipotency, and double haploids in plant breeding, In vitro Cell Dev. *Biol-Plant* 35: 157-181.

10. Henry, Y. and J., De Buyser, 1990. Wheat anther culture: Agronomic performance of doubled haploid lines and the release of variety "Florin", in: Biotechnology in Agriculture and Forestry: Wheat, Bajaj, Y. P. S (ed). *Springer Verlag*, pp: 285-352.
11. Hoekstra, S., Zijderveld, M. N van, Heidekamp F, Mark F van der, 1993. Microspore culture of *Hordeum vulgare* L.: the influence of density and osmolality. *Plant Cell Rep.* 12: 661-665.
12. Illic-Grubor K, Attree SM, Fowke LC, 1998. Induction of microspore-derived embryos of *Brassica napus* L with polyethylene glycol (PEG) as osmoticum in a low sucrose medium. *Plant Cell Rep* 17: 329-333.
13. Indrianto A, Heberle-Bors E, Touraev A, 1999. Assessment of various stresses and carbohydrates for their effect on the induction of embryogenesis in isolated wheat microspores. *Plant Sci* 143: 71-79.
14. Indrianto A, Barinova I, Touraev A and Heberle-Bors E, 2001. Tracking individual wheat microspores in vitro: identification of embryogenic microspores and body axis formation in the embryo. *Planta* 212(2): 163-174.
15. Indrianto A, 2003. Cytological and Ultrastructural features of initiation of wheat microspore embryogenesis. *BIOLOGI, Vol. 3*. No. 2: 65 – 79.
16. Indrianto A, Semiarti E dan Surifah, 2004. Produksi Galur Murni melalui Induksi Embriogenesis mikrospora Cabai Merah Basar dengan Stres. *ZURIAT Vol. 15*, No. 2: 133 – 139.
17. Jähne, A. and H.,Lörz, 1995. Cereal microspore culture. *Plant Sci* 109: 1-12.
18. Kyo M dan Harada H, 1986. Control of the developmental pathway of tobacco pollen in vitro. *Planta* 168: 427-432.
19. Latif S.,1991. Identifikasi mikrospora kelapa sawit (*Elaeis quinaensis* Jacq) untuk kultur haploid. *Bul. Perkeb*, 22(4), 231 – 238.
20. Madon,M., Heslop-Harrison,J.S., Schwarzacher, T., Mohd Rafdi, M. H and Clyde, M. M., 2005. Short communication: Cytological analysis of oil palm pollen mother cells (PMCs). *Journal of Oil Palm Res.* Vol. 17: 176-180.
21. Morrison, R. A. and D. A. Evans, 1988. Haploid plants from tissue culture : New Plant varieties in a shortened time frame. *Biotechnol.* 6: 684-690.
22. Nitsch, C., Andersen, S., Godard, M., Neuffer, M.G., dan W.F., Sheridan, 1986. Production of haploid plants of Zea mays and Penisetum through androgenesis, In: Crops I (Biotechnology and Agriculture 2), Bajaj, Y.P.S. (ed). *Springer Verlag*, pp: 168-180.
23. Ogawa T, Fukuoka H, Ohkawa Y, 1994. Induction of cell division of isolated pollen grains by sugar starvation in Rice. *Breed Sci* 44:75-77.
24. Raghavan V. 1997. *Molecular Embrylogi of Flowering Plants.* Cambridge University Press, Cambridge.
25. Raina SK and Irfan ST, 1998. High frequency embryogenesis and plantlet regeneration from isolated microspores of indica rice. *Plant Cell Rep* 17: 957-962.
26. Simmonds, D. H., 1994. Mechanism of induction of microspore embryogenesis in *Brassica napus*: Significance of the preprophase band of microtubules in the first sporophytic division, *In: Biomechanics of active movement and division of cells (NATO ASI series), Akkas, N. (ed)*. Springer-Verlag, Berlin, pp: 569-574.
27. Stauffer C, Benito Moreno RM, Heberle-Bors E, 1991. Seed set after pollination with in vitro matured, isolated pollen of *Triticum aestivum. L. Theor Appl Genet* 81: 576-580.
28. Stoeger, E., Benito-Moreno, R. M., Ylstra, B. Vicente, O. and E., Heberle-Bors, 1992. Comparison of different techniques for gene transfer into mature and immature tobacco pollen. *Transgenic Res.* 1: 71-78.
29. Touchet (de) B., Duval, Y. and C., Pannerier, 1991. Plant regeneration from embryogenic suspension cultures of oil palm (*Elaeis guineensis* Jacq). *Plant Cell* Rep. 10: 529-532.
30. Touraev A, Indrianto A, Wratschko I, Vicente O, Heberle-Bors E, 1996. Efficient microspore embryogenesis in wheat (*Triticum aestivum* L.) induced by starvation at high temperature. *Sex Plant Reprod* 9: 209-215.
31. Touraev A, Vicente O, Heberle-Bors E, 1997. Induction of microspore embryogenesis by stress. *Trends in Plant Sci* 2: 297-302.
32. Touraev A and Heberle-Bors E, 1998. Microspore embryogenesis and in vitro pollen maturation in tobacco. *In: Methods in Molecular Biology: Plant Cell Culture protocols RD. Hall (ed).* Humana Press Inc. Totowa, pp: 281-291.
33. Zarsky, V., Garrido, D., Rihova, L., Tupy, J., Vicente, O. and E. Heberle-Bors,1992. Derepression of the cell cycle by starvation is involved in the induction of Tobacco pollen embryogenesis. *Sex. Plant. Repr*. 5: 189-194.
34. Zhao JP, Simmond DH, Newcomb W, 1996. Induction of embryogenesis with colchicine instead of heat in microspores of *Brassica napus* L cv. Topas. *Planta* 198: 433-439.