INDUCTION OF SPOROPHYTIC DIVISION IN ORCHIDS MICROSPORES BY STRESS

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ABSTRACT

Orchid is one of the important ornamental plants in Indonesia this plant generally propagated by seed. Enhancing quality of this plant through breeding technology by various plant tissue culture methods and biotechnology, including doubled haploid technology are necessary. The most efficient method in creating doubled haploids plant is via microspore embryogenesis. We have develop new, innovative doubled haploid technology using the technique of isolated microspore culture. The goals are to obtain data on the male gametophyte development, viable embryogenic microspores, microspores derived embryos and double haploid plants of Orchid.

Development of male gametophytes were analysed by isolation of microspores and pollen at various stages and staining with DAPI. Isolated orchid buds of *Dendrobium* hybrid 1, *Vanda tricolor* and *Spathoglotis plicata* were subjected to cold temperatures ($4^{o}C$) for 7 days, microspores were then isolated by crushing the pollinia using glass rod and cultured them in embryogenesis A2, NP, MS and VW medium, viability of the microspores were determine by using Flourescein diacetate (FDA). Isolated Orchid pollinia were cultured in starvation medium B at various temperatures and duration of time to evaluate embryogenic response, isolated microspores then were cultured further in the basic embryogenesis medium and incubated at $25^{o}C$ in the darkness.

The result showed that floral characteristics for the late-uninucleate stage of the microspores were different for every orchid spesies. Ovulum lenght was used for Vanda, while in Dendrobium, Phalaenopsis, Arachnis, *Spathoglottis plicata* and Cattleya, varied length of flower bud was used. Isolated microspores of *Dendrobium* hybrid 1, *Vanda tricolor* and *Spathoglotis plicata* at 7th days of culture in different media formulation showing different respond of viability. Medium A2 keeping viability of *Dendrobium* hybrid 1 microspores better than any other medium, while in *Vanda tricolor* and *Spathoglotis plicata* embryogenesis NP medium was superior. Incubation of orchid pollinia at 4 and $25^{o}C$ were successfully maintain viability of the microspores during starvation periods but not able to block gametophytic development. In contrast starved pollinia at $33^{o}C$ were succesfully block gametophytic development, percentage of embryogenic microspores after starvation of isolated pollinia at 33°C for 4 days was superior compare to any other treatments. Symmetrical divisions and some multicellular structures were observed, which were clear indication for the sporophytic development of microspore-derived embryos, they had developed and after a few weeks they degenerated and died.

Keywords: flower bud-pollinia-microspore-stress-embriogenic-embryo-Orchid

INTRODUCTION

Orchid is one of the important ornamental plants in Indonesia this plant generally propagated by seed. Enhancing quality of this plant through breeding technology by various plant tissue culture methods and biotechnology, including doubled haploid technology are necessary.

The genetic diversity is inherited from generation to generation through pollen and ovule (male and female gamete cells). The nucleus of male gamete cell encountering and fused with the nucleus of egg cell resulted in zygote, then develop into a plant and in turn allowing a genetic inheritance in a continuous manner. Direct plant regeneration via microspore embryogenesis, and doubling of its chromosomes number, is more efficient in expressing

genetic diversity brought by haploid microspores because plant phenotype was not covered by the dominant effect (Morrison & Evans, 1988).

Quality improvement of orchid can be achieved by creating new hybrid, because of heterosis phenomena. The important tools that facilitate the production of F1 hybrids is a rapid way of producing homozygouse parent lines, complete homozygozity of parent lines ensures uniformity of the hybrid plants. Currently, the best way of making homozygous parent lines is through the regeneration of plants from haploid microspores or pollen, in which chromosome number is doubled (Touraev *et al*.,1996a).

The potential of immature pollen to deviate from its normal developmental pathway, giving rise to haploid plants via embryogenesis, is known since the original experiments of Guha and Maheshwari (1964, 1966) who observed the formation of embryos of pollen origin in anther cultures of *Datura innoxia*.

The initial switch from pollen development to embryogenic development is the main key to success in the generation of doubled haploid plants. Application of stress is an important factor controlling this switch in the developmental fate. Different stress conditions are effective in inducing the switch between the gametophytic and sporophytic generations in homosporus plants; similarly, microspore of flowering plants can be switched toward sporophytic development by a variety of shock treatments (Bell, 1992; Dickinson, 1994). Sucrose starvation, in particular, appears to be the major signal controlling the developmental fate of tobacco microspores, as it is the most effective treatment for embryogenic induction from different developmental stages, uninucleate microspores as well as mid-bicellular pollen. Starvation may also be a general trigger of pollen embryogenesis in higher plants, as it is effective not only in tobacco but also in other species such as barley (Hoekstra *et al.*, 1992), wheat (Indrianto *et al*, 1999); rice (Ogawa *et a*l, 1994).

Objective of this research is to develop new, innovative doubled haploid technology for Orchid using the technique of isolated microspore culture. The goals are to obtain data on the male gametophyte development, embryogenic microspores, microspores derived embryos of Orchid.

MATERIALS AND METHODS
Determination of Late-uninucleate microspore stage
Development of male gametophytes were analysed by isolation of microspores and pollen at various stages and staining with DAPI, to determine state of the nuclei. Data obtained were used to characterize developmental stages of male gametophyte of Orchid and identification of correlation between stage of male gametophyte and bud size, morphology or other simple markers.

Temperature stress on isolated Orchid buds
Cold shock pretreatments of buds were shown to be very effective to maintain viability of the microspores and to block gametophytic pathway and induce embryogenesis. Isolated orchid buds at different developmental stages were subjected to cold temperatures (4°C) for 7 days, microspores were then isolated by crushing the pollinia using glass rod and cultured them in embryogenesis A2, NP, MS and VW medium.

Starvation of isolated Orchid pollinia

Isolated Orchid pollinia at different stages of development were subjected to starvation in carbohydrate and nitrogen-free medium B, which was shown to trigger the formation of embryogenic microspores in the number of species, including tobacco (Touraev *et al*, 1996a), wheat (Indrianto, *et al*, 2001) and rice (Ogawa *et al*, 1994). Pollinia were cultured in medium B at various temperatures and duration of time to evaluate embryogenic response and simultaneously the block of gametophytic pathway, the microspores allowed to shed and isolated microspores were cultured further in the basic embryogenesis medium and incubated at 25°C in the darkness.

Regeneration of Orchid microspore-derived embryos.

It was shown that regeneration frequency is absolutely dependent on the quality of the embryos. Therefore, success of regeneration will be dependent on the optimization of culture conditions for initiation and further development of an embryo. Well developed Orchid microspore-derived embryo were then transferred to basic regeneration medium with a low concentration of carbohydrates on solid medium and incubated under light condition.

RESULTS AND DISCUSSION
Determination of Late-uninucleate microspore stage

Staging is a basic step to initiate microspore culture, since it allows to corelate the most suitable microspore stage for androgenesis induction with a flower bud trait measurement in each orchid species. The main objective is to obtain data on the male gametophyte development of orchids. Specific objective is the description of male development in relation to morphological characteristics of buds of various developmental stages. DAPI (Diamidino-phenyl-indole) is used to establish the state of microspore nucleus/nuclei at a given flower bud developmental stage ultimately allowing to find out the reaction of each microspore stage to the induction of androgenic haploidy through isolated microspore culture techniques.

Table 1. Floral characteristics for the Late-uninucleate stage of the microspores

Species	Morphological characteristic	Range (mm)
Dendrobium secundum	Flower bud length	11 – 14
Dendrobium hybrid I	Flower bud length	14 – 16
Dendrobium hybrid II	Flower bud length	13 – 15
Oncidium sweet sugar	Flower bud length	8.4 – 9.8
Spathoglottis plicata	Flower bud length	7 – 9
Vanda tricolor	Ovulum length	10 – 15
Vanda limbata	Flower bud length	5 - 8
Arachnis Megy Oei	Flower bud length	8 – 10
Cattleya	Flower bud length	36.3 – 38.2
Phalaenopsis amabilis	Flower bud length	10 – 12
Phalaenopsis belina	Flower bud length	10 – 12

A good correlation between the floral trait and the developmental stage was observed in Orchid. Table 1 shows the measurement range of Orchid flower buds corresponding to the late-uninucleate microspore stage (from young microspore to vacuolated microspore). Floral

characteristics for the late-uninucleate stage of the microspores are different for every orchid spesies. In Vanda, ovulum length is used for determining morphological characteristic of flower bud containing late-uninucleate microspores, while in Dendrobium, Phalaenopsis, Arachnis, *Spathoglottis plicata* and Cattleya, flower bud length is used.

Vacuole of the late-uninucleate microspores are not clearly seen, DAPI stained of these microspores showed that position of the nucleus was in the periphery close to the microspore wall. Microspores gametophytically programmed to differentiate into mature pollen, the first nuclear mitosis division is asymmetric result in small generative cell inside the cytoplasm of vegetative cell, in this binucleate pollen both vegetative and generative nucleus have different fate of development, vegetative nucleus is not develop further (remain in G1 of the cell cycle) while generative nucleus will divide symmetrically to produce sperm cells (McCormic, 1993). The most responsive stage of pollen development to induce embryogenesis is late-uninucleate to pre-mitosis. Table 1 showed that the flower bud length for Dendrobium containing microspores at the late-uninucleate is ranging between 11 to 16 mm.

Temperature stress on isolated Orchid buds

Cold shock pretreatments of buds were shown to be very effective to maintain viability of the microspores. Viability of the microspore means that it have not plasmolysed, the cytoplasm still intact and able to grow, germinate or develop. Isolated orchid buds at different developmental stages were subjected to cold temperatures (4°C) for 7 days, microspore were then isolated by crushing pollinia using glass rod and cultured in embryogenesis medium. Maintenace of the viability of isolated microspores during the first days of *in vitro* culture is absolutely essential for microspore embryogenesis. To find optimal media formulations for maintaining the viability of isolated Orchid microspores, some basic medium formulation of orchid were tested namely NP, MS, VW and A2.

According to Duncan and Heberle, (1976) cold pre-treatment slows down degradation processes in the anther tissues thus protecting microspores from toxic compounds released in the decaying anthers . Therefore Cold shock pretreatments of buds were shown to be very effective to maintain viability of the microspores. According to Dafni and Firmage (2000) viability of the microspore means that it had the capacity to live, grow, germinate or develop. Maintenance of the viability of isolated microspores during the first days of *in vitro* culture is absolutely essential for microspore embryogenesis. Isolated microspores of *Dendrobium* hybrid 1, *Vanda tricolor* and *Spathoglotis plicata* at 7th days of culture in different media formulation showing different respond of viability. Viability of the microspores were determine by using Flourescein diacetate (FDA), this test have two aspects of viability that were reaction of esterase activity present in the cytoplasm and an intactness of cell membrane. Table 2 showed that embryogenesis medium A2 keeping viability of *Dendrobium* hybrid 1 microspores better than any other medium, while in *Vanda tricolor* and *Spathoglotis plicata* embryogenesis NP medium was superior. Experiments by using fresh flower bud, without any cold pretreatment, was not able to maintain viability of isolated microspores more than 4 days, almost all isolated microspores plasmolysed and finally die (data not shown).

Table 2.   Percentage of viable isolated microspores of *Dendrobium* hybrid 1, *Vanda tricolor* and *Spathoglotis plicata* at 7th days of culture in different media formulation, percentage is counted from at least 300 microspores per culture dish.

Orchid	Embryogenesis Media			
	NP	MS	VW	A2
Dendrobium hybrid 1	17.55 ± 1.77	21.22 ± 3.92	10.98 ± 2.31	22.62 ± 2.12
Vanda tricolor	25.48 ± 1.45	16.83 ± 1.58	13.49 ± 1.43	22.19 ± 1.68
Spathoglottis plicata	26.46 ± 0.99	16.2 ± 1.29	13.32 ± 1.6	22.76 ± 0.83

Starvation of isolated Orchid pollinia

Isolated Orchid pollinia of *Dendrobium* hybrid 1, *Vanda tricolor* and *Spathoglottis plicata* were subjected to starvation in carbohydrate and nitrogen-free medium B, which was shown to trigger the formation of embryogenic microspores in wheat and tobacco (Indrianto, *et al,* 1999; Touraev *et al,* 1997). Pollinia were cultured in starvation medium B and incubated at various temperatures and duration of time to evaluate embryogenic response and simultaneously the block of gametophytic pathway. The microspores allowed shedding and isolated microspores were cultured further in the basic embryogenesis A2 medium and incubated at 25°C in the darkness. Incubation of orchid pollinia at 4 and 25 °C were successfully maintain viability of the microspores during starvation periods but not able to block gametophytic development as indicated by germinating pollen. In contrast starved pollinia of *Dendrobium* hybrid 1, *Vanda tricolor* and *Spathoglottis plicata* at 33 °C were succesfully block gametophytic development, pollinia spontaneously shed their embryogenic microspores.

Table 3.   Percentage of embryogenic microspores after starvation of isolated pollinia at different temperature incubation

Temperature	Starvation period		
	2 days	**4 days**	**6 days**
Spathoglottis plicata			
4 °C	-	-	-
25 °C	1.28 ± 0.5	0.82 ± 0.18	0.57 ± 0.23
33 °C	2.47 ±0.47	5.18 ±0.75	0.89 ± 0.72
Vanda tricolor			
4 °C	-	-	-
25 °C	5.39 ± 0.53	3.56 ± 0.7	1.57 ± 0.49
33 °C	13.86 ± 1.25	21.25 ± 1.62	8.15 ± 1.28
Dendrobium hybrid1			
4 °C	-	-	-
25 °C	20.05 ± 1.64	10.03 ± 1.17	6.7 ± 1.52
33 °C	15.23 ± 2.89	49.16 ± 0.89	27.72 ± 0.63

Population of shed microspores contain big enlarge embryogenic microspores and small plasmolysed microspores. Enlarge microspore have thinner wall layer compare to normal gametophytic microspores. Cytoplasm of enlarged microspores, from pollinia pretreated at 33°C were clear without starch grains indicate that amylogenesis which is normally occur in normal gametophytic development of the microspores as reserve materials, had blocked during temperature stress of the pollinia. The nucleus of big enlarge embryogenic microspores

have condenced chromatin, some nucleus of embryogenic microspores had divide symmetrically (Figure 1).

Figure 1. Embryogenic microspores of *Dendrobium* hybrid 1 after starvation of isolated pollinia at 33 °C for 4 days.

Symmetrical division of embryogenic microspores was the indication of blocking normal gametophytic development of the microspores, leading to formation of multicellular structure (proembryo). Table 3 showed that percentage of embryogenic microspores of *Dendrobium* hybrid 1, *Vanda tricolor* and *Spathoglottis plicata* after starvation of isolated pollinia at 33°C for 4 days was superior compare to any other treatments

Regeneration of Orchid microspore-derived embryos

Orchid pollinia isolated from flower buds of *Dendrobium* hybrid 1 and *Spathoglottis plicata* were cultured in starvation medium B and incubated at 33°C for 4 days. Microspores then were isolated and cultured in embryogenesis medium A2 incubated at 25 °C in the darkness. In the first week of culture isolated microspores were able to keeping their viability in embryogenesis A2 medium. Symmetrical divisions and some multicellular structures were observed (Figure 2), which are clear indication for the sporophytic development of microspore-derived embryos, they had developed and after a few weeks they degenerated and died.
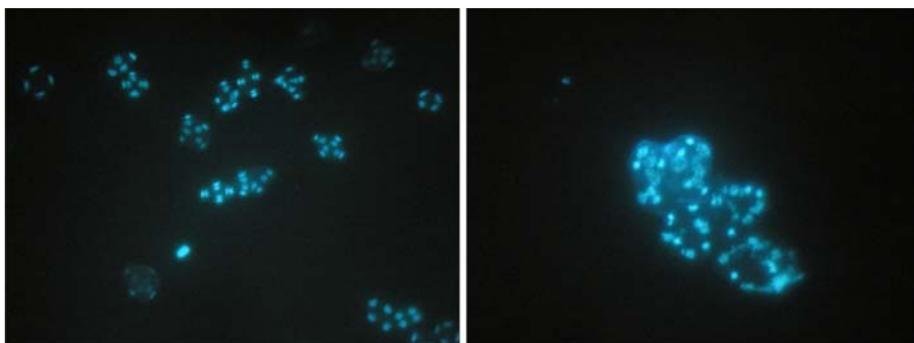

Figure 2. Symmetrical divisions and multicellular structures of embryogenic microspores of *Dendrobium* hybrid 1 in embryogenesis medium A2

ACKNOWLEDGEMENT

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