AVOID CONTAMINATION IN SOYBEAN *(Glycine max, L. [Merrill])* MICROSPORES CULTURE

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Abstract

Microspore culture is done to obtain pure strains. The purpose of soybean microspore culture to obtain quality seeds. Two important step that must be done is isolation of microspores in starvation medium and subculture into embryogenesis medium. Many factors contributing to the contamination of soybean microspore culture. Contamination in the B medium temperature 34 ^{o}C is more common than 4 ^{o}C. Vulnerable to contamination because of embryogenesis medium rich in nutrients. Bacterial contamination can be caused by internal contaminants such as shape of the anther. Other internal contaminants that cause diseases such as fungi *Colletotrichum truncatum* and *Phakopsora pachyrhizi.* Antagonistic fungi which contaminate cultures that *Trichoderma spp., Alternaria spp., Fusarium spp*. Handling of contamination is done by selecting the appropriate methods in order to remain viable microspores. Sterilization soybean flower buds with 20% Tween for 10 minutes and then rinsed with distilled water. Moreover sterilization with 4% Hg Cl_2 and 10% NaOCl for 10 minutes, rinsed with distilled water times, followed by 96% alcohol for 1 minute, can press up to 70% contamination.

Keywords: soybean, microspore, contamination

1. Introductions

Microspore embryogenesis is the most commonly used method to produce doubled haploids [1] Androgenesis can result from the culture of intact anthers or the culture of mechanically isolated microspores [2]. To achieve best results, microspores in culture need to be relatively pure [3].

Contamination can be introduce in several ways. Initial contamination-due to incomplete sterilization of the explant. Latent contamination-usually resulting from endogenous bacteria present in the tissue explant that grow and multiply long after culture initiation. Culture shoud be checked 3-5 days after initiating or subculturing for contamination.

Bacteria are the most frequent contaminants. Fungi may enter culture on tissue explants or from airborne spores [4]. In many cases, the contaminating sources are not easily determined; however, the most common ones are associated with microorganisms of the environment and of the manipulating individual. A frequent form of penetration of spores and cells of microorganisms into the work environment is through air currents that are brought by the air conditioning equipment, remaining in the environment due to inadequate aseptis conditions [5]. Among the most common microorganisms, the fungi are frequently detected and are highly detrimental, as they grow right in the nutritional medium, competing with the plants for the nutrients of the medium, besides producing phytotoxic metabolites. Endophytic contamination, that is, microorganisms that colonize vegetable tissues internally, are also harmful for i*n vitro* plant cultures [6].

The establishment of an in vitro culture requires the removal of culturable fungal and bacterial contaminants. Chemical methods used include antibiotics and fungisides, alcohols, mercuric chloride, and oxidizing biocides such as halogen compounds (e.g. chlorine, bromine and iodine) and hidrogen peroxides.The methods used depends on the plant species, type of explant, phytoxicity, type of contaminant(s) and cost [7]. The research purpose to share an experience ovoid contamination in soybean microspore culture.

2. Materials And Methods

Plant material. Five cultivar of soybean flower buds: Argomulyo, Grobogan, Wilis, Anjasmoro and Black Malika. Length of bud selected 2,0 -3,5 mm. Seventy to eighty buds were washed with liquid detergent for about ten minutes then rinsing by acuadest three times and 70% alcohol

for 2 minutes. Soybean buds were opened with 2 spluit, take the anther, place them in a petridish. All of the anther were used in the laminar air flow cabinet to be sterilized using Hg Cl_2 1% for 10 min, rinse them with sterilized water three times, put 96% alcohol for one minutes, then threw it.

Every soybean flower bud had ten anthers. The diameter of five soybean cultivar was measured with *Optilab* Software. Sum of microspore every bud was counted under light microscope. The normality test and analysis of variance (Anova) were performed on the data.

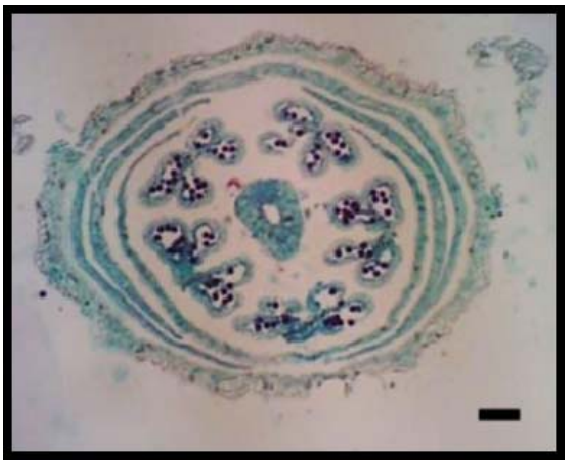
Soybean flower bud and anther treatments.

Freshly bud flower with microspores in the late uninucleate to pre-mitotic stage were washed in liquid deterjent, rinse three times, surface with an aerosol of 70% etanol, twice. For the anther treatment experiments, 30 or 36 harvested bud flower were out into a petridish. The sepala and petala were removed from anther. A small 3cm petridish containing 1,5 ml medium B was placed into a larger dish.

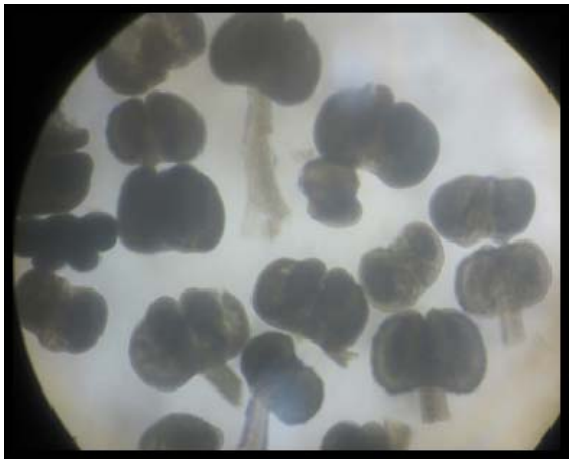
Anthers were isolated aseptically from freshly harvested bud flower, and all antrers of 10 bud flower were placed on the surface of 1,5 ml of starvation medium B containing 1,49 mg ml^{-1} KCl, 0,12 mg ml^{-1} mg SO_4 , 0,11 mg ml^{-1} $CaCl_2$, 0,14 mg ml^{-1} KH_2 PO_4, 54,7 mg ml^{-1} manitol, pH 7,0 [8]. The mix of microspores and 2 ml B medium in 2 microtubes were sentrifuged 1000 rpm for 10 minutes. Then, threw the B medium, and changed it with the 2 ml B medium till clearly and keep them in the incubator 34°C for 4 days.

Microspore isolation and culture.

Microspores were isolated, either from preculture anthers or from anthers excised from pretreated bud flower, by stirring the anthers in medium B with a magnetic stirer for 2-3 min at 1000 rpm. After three washes in medium B (centrifugation for 5 min at 100 g) the microspore suspension was diluted in 1 ml of B5-micro minerals and vitamins (5 mg l^{-1} $MnSO_4.7H_2O$, 1.0 mg l^{-1}Zn $SO_4.7H_2O$, 1.5 mg l^{-1} H_3BO_3, 0.375 mg l^{-1} KI, 0.00125 mg l^{-1}Cu $SO_4.5H_2O$, 0.0125 mg l^{-1} Na_2 $MoO_4.2H_2O$, 0.0125 mg l^{-1}Co $Cl_2.6H_2O$, 50 mg l^{-1} myo-inositol, 5.0 mg l^{-1} thiamine-HCl, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine-HCl [9], 32 g l^{-1} Fe-NaEDTA, 200 mg l^{-1} MES [2-(N-morph-olino) ethanesulfonic acid], 500 mg l^{-1} glutamine and 9.0% (w/v) maltose, pH 6.2. The condotion of culture was noted every two days for two weeks.



(A)



(B)

Figure 1. Anatomical stucture of soybean flower bud, bar =10 µm (A) variability of soybean anthers (B) bar = 100 µm

3. Results and Discussions

Table 1 . Diameter of soybean's anther, total of microspore and freuquency contamination

cultivars	diameter of anther mean±sd (µm)	total of micro-spores per bud (mean±sd)
Argomulyo	326.03±20.81^{b}	1644±173^{bc}
Grobogan	344.56±35.19^{b}	941±142^{a}
Wilis	284.98±18.16^{a}	1839±156^{c}
Anjasmoro	354.67±59.67^{b}	2003±216^{c}
Black Malika	278.00±17.51^{a}	1277±147^{ab}

Mean values followed by different upper-case letters different significantly by Duncan's multiple range test at P ≤0.05. sd = standard deviation

Contamination in microspores culture can be

Table 2. Contaminant in soybean microspore culture

Kind of contaminant	Culture condition	Note
Mold	Slime with several microspore	Non sterilized microspore isolation
Bacteria	Not clear medium, litle spots	Improper sterilization
Hyphae, mycelium fungi	Branched hyphae	Density the number of soybean planted.
	Phakopsora pachyrichi, Aspergilus niger, Colletotricum truncatum	Shading plant, less expose sunlight Antracnose disease.
	Trichoderma, Alternaria, Fusarium	Antagonist fungi, non disease for soybean plant, contaminant microspore culture
Tricomes and debris	Spots, lines of trichomes, part of sepals/petals	Surface of soybean flower buds, chush of sepals and petals

Table 3. Treatment avoid contamination in microspore culture

Explant source	Agent pre- sterilization	Treatment sterilization in laminar air flow	Culture result
soybean flower bud	Liquid detergent 10% five minutes, rinse water three times	1% $HgCl_2$ rinse steril water three times, 96% alcohol 1 minute	100% culture contamination in B medium: bacteria, fungi, debris, trichomes
	Liquid detergent 20% ten minutes, rinse water five times, alcohol 70% 2 minutes	4% $HgCl_2$ and 10% NaOCl rinse steril water five times, 96% alcohol 1 minute	30% culture contamination in B medium: fungi, debris, trichomes, slowly contamination in B5 medium, after 7 days
soybean anther	Liquid detergent 10% five minutes, rinse water three times	$HgCl_2$ 1% rinse steril water three times, 96% alcohol 1 minute	50% culture contamination bacteria, 50% microspore plasmolysis
	Liquid detergent210% ten minutes, rinse water five times, alcohol 70% 2 minutes	4% $HgCl_2$ and 10% NaOCl rinse steril water five times, 96% alcohol 1 minute	No contamination, empty anther, 70% microspore plasmolysis

influenced by many factors. The shape, size and type of anther and microspores can affect the contamination. Contamination often seen since starvation treatment in B medium until subculture to embryogenesis medium. Part of anther, sepals, petals of soybean flower bud can be contaminant agent. The soybean healty plant is needed for succesful microspore culture. Soybean floral buds possess morphological features that make it difficult to isolate viable male cells under aseptic conditions: trichomes covering the bud outer surfaces [10]. Contamination in the B medium temperature 34 ^{o}C is more common than 4 ^{o}C, because microorganism can't grow at cold temperature (data not showen).

Mean length of anthers in five cultivars various between 278.00±17.51 µm and 354.67±59.67 µm. The longest anther is Anjasmoro, althought Argomulyo and Grobogan are big too. Anjasmoro cultivar is the largest anther had the big number microspore per bud.

Anther shape and microspore pattern can complicated the process of sterilization. Responsive cultivars mean cultivars easy grown because of the contamination is rare. According to the data above, Anjasmoro soybean cultivars is the most responsive to microspore culture (Table 1). Anthers of the apple-shaped were notably shorter and smaller in size, than those of the suboblate group.

Shape and structure of soybean anther can be added contamination problem in microspore culture (Fig.1A, B). Diameter of soybean microspore is between 17.8 µm and 23.8 µm. Indonesian soybean microspore smaller than Brazilian soybean. The microspores of the soybean cultivars tested in Brazil study presented an average diameter of 25 µm [10].

4. Conclusion

Sterilization soybean flower buds with 20% Tween for 10 minutes and then rinsed with distilled water. Moreover sterilization with 4% $HgCl_2$ and 10% NaOCl for 10 minutes, rinsed with distilled water times, followed by 96% alcohol for 1 minute, can press up to 70% contamination.

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