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R E V I E W:

In Vitro Selection and Somaclonal Variation for Biotic and Abiotic Stress Tolerance

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ABSTRACT

As an alternative technology, plant improvement through somaclonal variation is expected to support conventional breeding. New superior variants with a better performance and more attractive texture could be obtained through this method. To enhance genetic variation, both physical and chemical treatments such as gamma ray (Co^{60}) and Ethyl Methane Sulphonate (EMS) compound could be applied. In particular for vegetative propagated plants, in vitro induced mutation is the most effective method to improve variation. For obtaining the desired characteristic of plant, in vitro selection is the best method due to its capability to manipulate the variation to the expected result. Therefore, by applying the selection agent to the media, plant tolerance to both abiotic and biotic could be acquired. Generally, the tolerance at the callus level at the specific selection agent is positively correlated with the tolerance at the plant level. At this point, PEG (polyethylene glycol) and manitol is chemical compound useful for drought tolerance, fusaric or filtrate is for fusarium wilt, $A1Cl_3.6H_2O$ is for Al tolerance.

Key words: somaclonal variation, in vitro selection, induced mutation.

INTRODUCTION

Plant improvement through somaclonal variation and in vitro selection is some techniques of in vitro culture for obtaining plant genotype tolerance to the biotic or abiotic stress, such as drought, high salinity, Al stress, acid soil, and disease tolerance (Ahmed et al., 1996; Yusnita et al., 2005). In addition, the plants are expected to have some desired characters such as having bigger fruit size, more interesting flower texture, more delicious taste and higher production (Pedrieri, 2001; Ahloowalia and Maluszynski, 2001; Witjaksono, 2003). Ahloowalia (1986) states, that somaclonal variation would give advantages if it increases genetic variation, particularly the character which is not obtained at the mother plant. Because of its low genetic variation, particular plants which are only vegetative propagation or self-pollination, desired characters could be obtained through somaclonal variation.

In vitro selection is one of somaclonal variation method. Its effectiveness and efficiency are due to its ability of changing the plant to the desired character, either by applying a selection agent on the culture media or by giving particular condition to change the somaclone with the required character (Van den Bulk, 1991; Karp, 1995). Somaclonal variation occurs at the plant resulted from cell regeneration during in vitro culture period, generally are not originated from the axilar shoot or tip-shoot. Somaclonal variation are caused by several factors, such as: by genotype and polyploidy level, environment during the growing period, the applied growth regulator, culture period and the applied procedure (Maluszynski et al., 1995) and the presence of selection agent.

Somaclonal variation occurs among the population of plant resulted from in vitro culture. It is apparently caused by gene amplification, the alteration of a basic couple, transposing migration, methylation transform, chromosome instability, chromosome inversion, one spot mutation, translocation, ploidy change, restructuring or deletion (George and Sherington, 1984; Phillips et al., 1990; Dennis, 2004; Kumar and Marthur, 2004). The high range of mutation is called chimera, pleytrophy, genetic instability and epigenetic variation. Epigenetic variation is the one when the resulted change was so unstable that the resulted expression returns to its origin. The new mutant could be created through induced mutations by using chemical mutagen such as ethylene scimine (ES), diethyl sulphonate (DES), ethyl methane-sulphonate (EMS), and the azida group. Physical mutagen frequently used is x-rays, gamma-rays (Co^{60}), fast neutron (nf) and thermal neutron (Nth).

Through in vitro selection to numerous mutant or genetic material is possible (Maluszynski et al., 1995). Likewise, in vitro selection could also be conducted at the population such as cell and callus and shoot resulted from regeneration in the small area and in the controlled condition. Application of the selecting agent in the culture media, in vitro method is very advantageous. Only such a tolerant plant is capable to grow that to obtain the plant with the desired character does not take a long time (Biswas et al., 2002). The achievement of in vitro selection technique to obtain the tolerant plant requires the availability of: (i)

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high variation of cell, (ii) easy application of in vitro selection method, (iii) regeneration method of tolerant cell (Widoretno, 2003), and (iv) the desired character to be inherited (Yusnita, 2005).

IN VITRO SELECTION FOR *FUSARIUM* DISEASES TOLERANCE

The availability of the appropriate selection agent is the determining factor for the optimum result. Generally, the selection agent used is fungi culture filtrate or the familiar toxin such as oxalate acid and fusaric acid (Matsumoto et al*.,* 1995*).* Fusaric acid ($C_{10}H_{13}O_2N$) is a metabolite product from many strain of *Fusarium oxysporum* and applied as "selecting agent" for the cell culture and callus culture to inhibit the fungi germination. Based on the reality, toxin or filtrate can be used for selecting agent because there is a relationship between toxin tolerance and disease tolerance.

Application of fusaric acid or filtrate for in vitro selection has frequently been applied for tomato, banana (Mariska et al*.,* 2005), and abaca (Damayanti, 2002; Sukmadjaja et al*.,* 2003). The result of the experiment showed that the new regenerate resulted from toxin tolerant cell or tissue is also tolerant to disease. Accordingly, Jin et al. (1993) obtained filtrate culture from *Fusarium solani*, which has pathogenic characteristic, inhibit the callus culture growth of soybean that was susceptible to *death syndrome disease*.

Fusaric acid produced by *Fusarium oxysporum* is a toxically organic component functioning among others: to inhibit cytocinine oxidation, to restrict respiration process at mitochondria, as well as to ATP at the membrane plasma and polyvinyl oxidation activity. In short, it inhibits growth and regeneration (Sukmadjaja et al*.,* 2003). According to Mariska et al. (2005) the fusaric acid application at a rate of 45 mg/l on the green and yellow "Ambon" banana (*Musa paradisiaca Sp.)* was capable of inhibiting the controlled propagation for 80%. Meanwhile, shoot radiated at the dosage of 5,7.5 and 10 gray inhibit 60, 60 and 26%. Using *Fusarium oxysporum* conidia in rice culture of 5 g/5 kg soil and 2.5g/5 kg soil, the resistance test conducted at the green house, showed that several number of plants remained green of being tolerance to the disease because of radiation with the dosage of 7,5 gray, without selection and selection using 30 ppm fusaric acid, and 10 gray without selection and selection using 40% fusaric acid. The highest percentage of the tolerant plant was produced by the irradiation treatment with fusaric acid selection.

Greenhouse test of the abaca somaclone by Sukmadjaja et al. (2003) showed a correlation between pathogenic fusarium and fusaric acid tolerance. After inoculation with conidia, the highest percentage of survived plant was obtained from plant selected with fusaric acid 15-30 ppm followed by filtrate 50% cross selection, as much as 75% either from the application of 10^4 or 10^5. Those plants are creating from the irradiated explants with the dosage of 1 Krad. The use of 15-30 fusaric acid at the plant without radiation produced 25% tolerant plants (Table 1). For this reason, induced mutation treatment tends to produce mutant with increasing disease tolerance. The physical mutagen treatment with gamma rays combined with in vitro selection will increase the disease tolerance plant.

Somaclonal variation and in vitro selection applied to peanut has produced somaclone R1 and R2 which are resistant to stem-rot disease caused by *Sclerotium*. Induced with *S. rolfsii* in the plastic house, several plant has more full pods and disease resistant than their mother plant (Yusnita, 2005). Another example of selecting agent is DON (dioxynivalenol), toxin produced by pathogenic fungi to produce wheat which is tolerant to scab disease (Yang et al*.,* 1998) and fungi culture filtrate containing toxin obtained from mango which is tolerant to anthracnose disease (*Collectrotrichum glocorporioides* Pens) (Jayasandar et al*.,* 2000). Several plants resulted from somaclonal variation and in vitro *selection* is presented at Table 2.

IN VITRO SELECTION FOR AL TOLERANCE

In vitro selection for acid soil and Al toxic tolerance could be applied with $AlCl_3.6H_2O$ as the selection component on the low acid media as much as pH 4 (Short et al*.,* 1987). Those method of application has resulted several Al tolerance plants such as in rice (Van Sint Jan et al*.,*1997), soybean (Mariska, 2003), and tobacco (Yamamoto et al*.,* 1994). Van Sint Jan (1997) shows that one of three Al tolerance was obtained from unselected callus using Al, while the others were selected with 250 and 1000 µmol from the total Al. The Al tolerant plants could not be determined from selection result because of the somaclonal variation.

Hutami et al*.,* 2001 attained the somatic embryo structure of soybean which is capable of proliferation after selection at the media containing Al and low pH, mainly which was originated from radiation at 4 gray in Wilis and Sindoro. Stress condition caused by Al results in the decreasing capability of growth and the development of somatic cell. It happened in the tobacco (Yamamoto et al*.,* 1994), and rice (Purnamaningsih et al., 2001; Edi, 2004).

From in vitro soybean selection using Al and gamma ray, several acid tolerant somaclones are obtained. Until the 5^{th} generation (G5), soybean lines with 60-65 pods is acquired, of which the stems are higher than their control plants (Sindoro). In this research, it is shown that there is a positive correlation between the mass of somatic cell tolerant to Al and low pH and their tolerance to acid field (Mariska, 2003). Al toxicity to selection media could be emerged by modifying macro nutrient MS, i.e. by increasing NH_4NO_3, $CaCl_2$ ($2H_2O$) and decreasing KH_2PO_4 and the application of Fe which was not chelated by EDTA (Purnamaningsih et al., 2001).

In vitro selection in rice varieties (*Rojolele* and *T309*) using 100, 200, 300, 400 and 500 ppm Al, showed that the higher Al concentration given to the media, the less callus could regenerate. Equally, no callus regenerated at Al 500 ppm. Based on the nutrient test and using acid soil in the green house (Edi, 2004), shoot resulted from callus tolerant to selection media showed the tolerance characteristic to Al (Purnamaningsih et al., 2001). As indicated by Taylor (1995) somatic embryo cells remained developed in the Al and low pH media because of the tolerance characteristic at the cell level. It is similar to Rath's research (1996) that only the tolerant cells survive in the media containing selection component. From several researches above, it could be concluded that cell selection is potential for producing new genotype adapted to the environmental stress (Adkin et al*.,* 1995). In vitro selection for drought tolerance

In vitro selection for drought tolerance commonly uses PEG as selection component. This method has been applied to several plants (Bouslama and Scapaugh, 1994). Petcova et al. (1995) and Lestari et al. (2005) found a positive correlation between the capability of germinating seed at the media containing PEG and the growth of the

Table 1. Percentage of survived plant resulted from in vitro selection with *F oxysporum* conidia suspension in the green house after 30 days (Sukmadjaya et al., 2002).

Dosage of Radiation (Krad)	Early selection	Cross selection	Percentage of living plant		
			Conidia concentration per ml		
			10^3	10^4	10^5
0	Without fusaric acid selection (ppm) Filtrate (%)	Filtrate (%)	0	0	0
	0-0	50	0	0	0
	15-10	50	25	25	25
	30-45	50	-	-	-
1	Without fusaric acid selection (ppm) Filtrate (%)	Filtrate (%)	0	0	0
			45	50	40
	0-0	50	70	60	60
	15-10	50	75	75	75
	30-45	50	60	40	30

Table 2. Several disease tolerant plants resulted from somaclonal and in vitro selection (Yusnita, 2005).

Plant	In vitro culture system for	Selecting agent	Resistant to
Potato	Callus culture	Fungi Filtrate culture	*Phytophtora*
Tomato	Callus from leaf explants	-	*Fusarium oxysporum* f.sp. *infestan lycopersici* ras 2
Papaya	Shoot culture	-	*Phytoptora palmivora*
Soybean	Embryonic culture	-	*Fusarium oxysporum* f.sp *cubense* ras 4
Banana	Multiple bud clumps	Fusaric acid	*Fusarium* sp. ras I
Mango	Somatic embryo culture	Fungi Filtrate culture	*Coleetotricum gloesporoides*
Strawberry	Morphogenetic callus		*Rhizoctonia fragariae* and *Botrytis cemerea*
Apple	Shoot culture	Fungi Filtrate culture	*Phytophtora cactorum*
Wheat	Morphogenic callus	Fungi Filtrate culture	*Fusarium graminearum* and *Fusarium culmorum*

Table 3. Shows PEG, root penetration, proline content tests and grain production after drought stress (Lestari, 2005)

Treatment (radiation and selection)	Genotype	20% PEG examination	Root penetration test	Proline nmol/g	Weight of grain/panicle	Grain/panicle%	Decreasing (%) filled grain	Notes
Control	IR 64	NS	NE	17.07	TP	TP	100	NT
0 gray (20% PEG)	IR64-1	G	P	169.6	8.90	62	44	NT
“	IR64-2	G	P	140.1	5.51	51	52	NT
5 gray (0%PEG)	IR64-17	G	P	202.40	0	0	100	NT
“	IR64-3	G	P	176.78	23.53	49	17	Tolerant
“	IR64-3.1	NS	NE	NE	NE	NE	NE	NT
“	IR64-3.2	G	P	204.45	26.0	58	26	Tolerant
“	iR64-3.3	NS	NE	NE	NE	NE	NE	NT
“	IR64-4.1	G	P	150.0	18.97	55	31	Tolerant
“	IR64-4.2	G	P	267.2	20.0	53	29	Tolerant
“	IR64-5.1	G	NP	NE	NE	NE	NE	NT
“	IR64-5.2	NS	NE	NE	NE	NE	NE	NT
“	IR64-5.3	G	NP	NE	NE	NE	NE	NT
“	IR64-5.4	NS	NP	NE	NE	NE	NE	NT
“	IR64-7.1	G	P	180.18	18.08	52	25	Tolerant
“	IR64-7.2	G	P	147.69	19.43	56	30	Tolerant
“	IR64-8.2	NS	NP	NE	NE	NT	NT	NT
“	IR64-8.3	G	NP	NE	NE	NT	NT	NT
“	IR64-11	G	P	160.35	12.9	49	36	Tolerant
“	IR64-11.1	G	P	156	20.65	61	37	Tolerant
7 gray ()% PEG)	IR64-18	NS	NP	NE	NE	NT	NT	NT
“	IR64-19	G	NP	NE	NE	NT	NT	NT
“	IR64-22	G	NP	NE	NE	NT	NT	NT
“	IR64-23	G	P	113.6	NE	NE	100	NT

Notes: * = drought-stress tolerance indication, NS: no sprouting, NE = not examined, NP = not penetrating, NT = no tolerant, ND = no producing. G= germination, P = penetrating.

plants under stress condition. It is similar to Dragisga et al. (1996) who obtained the similar result that PEG could be applied after treating an osmotic stress on the in vitro selection.

Short et al. (1987) stated that in the in vitro culture, PEG is capable to induce water stress and positively correlated with that in the field or the green house (Damii and Hughes, 1997). PEG could be applied for stimulating drought because it could inhibit water in such a way that no water is provided for somatic cell, except for the callus/somatic cell which has particular mechanism for absorbing water. The research of Adkin et al. (1995) showed similar result. Only the tolerant callus which bears PEG media selection could increase its tolerance against drought stress. From the experiment result, the second generation is tested against the plant of callus origin selected with PEG media. Result shows that the dry-weight of the plant is higher than that of the mother plant.

Selective media applied with PEG could inhibit the growth and development of the soybean explants and could decrease the number of the formed somatic embryo (Widoretno et al., 2003; Husni et al., 2005). The increasing amount of PEG added to the selective media bring the deterioration influence of PEG to somatic/callus embryo (Widoretno et al., 2003). The result shows that the treatment with 15% PEG will cause 90% tolerant soybean genotype (explants *B3731*) remained survived. The percentage is lower, 54% and 30% respectively, when it is applied to the moderate genotype soybean (*Tidar* variant) and the sensitive soybean genotype (*MSC8606*).

In vitro selection for obtaining the tolerance to the drought stress has been conducted to green grams mungbean (*Vigna radiata* L.) (Gulati and Jaiwal, 1993), rice (O*ryza sativa* L.) (Adkins et al., 1995; Biswas et al., 2002; Lestari, 2005, 2006), barley (S*orghum bicolor* L.) (Duncan et al., 1995), potato (Prakash et al., 1994) and soybean (Widoretno, 2003; Husni et al., 2005). Husni et al., 2005 obtained three drought tolerant soybean somaclones originated from somatic embryo which had been selected with 20% PEG (BM 6000). During penetration test of the root, those somaclone have faster capability of penetrating paraffin layers than that of the previous variants *Tanggamus* and *Nanti*.

Selection to the callus of several rice variety conducted by Prakash et al., 1994 using 5, 10, 15 mg/l of PEG (BM 6000) successfully obtained drought tolerant plants. Five plants from three varieties increased their tolerance to drought. PEG as selection component significantly inhibit callus growth and therefore it could inhibit seed germination with drought intolerance. In vitro selection to the rice of Gajahmungkur, Towuti and IR64 varieties conducted by Lestari (2005; 2006) using PEG 20% produced some tolerant plants after listing for their tolerance in green house, i.e. five plants from Gajahmungkur, 9 from Towuti and 8 from IR64. Those plants could produce more full grains per panicle than that of the mother plants.

Germination test for the seed from in vitro selection shows that the seed from IR64 (mother plant) did not germinate at the 20% PEG solution (BM 6000); however, the seed from other somaclone (17 somaclone) germinated at the 20% PEG solution (Lestari, 2005). The seed from the germinated somaclone eventually penetrated paraffin layers at the base of the vase (Table 3). From the test, 12 somaclone could rapidly penetrate paraffin layers, in addition to the thicker and longer size of root (Lestari et al., 2005). Proline analysis to the plant showed that the leaf of the control plants (IR 64) has very low proline content, as much as 17,07 nmol/g, while the leaves from various somaclone produce higher proline, 113.6-287.2 nmol/g (Lestari, 2004).

CONCLUSION

Induced mutation could increase genetic variation to the plant which eventually could give the advantage for providing breeders with genetic material for plant selection. In vitro culture and irradiation effectively produce new somaclones with desired characteristics. Through in vitro selection, by giving particular emphasize on the media, certain expected traits could be produced.

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