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Anther culture of local upland rice varieties from East Kalimantan: effect of panicle cold pre-treatment and putrescine enriched medium

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Abstract. *Nurhasanah, Pratama AN, Sunaryo W. 2016. Anther culture of local upland rice varieties from East Kalimantan: effect of panicle cold pre-treatment and putrescine enriched medium. Biodiversitas 17: 148-153.* Anther culture has been evaluated as an effective method for homozygous plant production which is very important for the hybrid breeding program in rice. Low number of green plantlets is a main obstacle in the application of this technique. Culture conditions increasing anther culture efficiency can be applied to solve this problem especially for low regeneration frequency. This study aimed to investigate the effect of panicle cold pre-treatment and the addition of putrescine into culture medium to the anther culturability of local upland rice varieties originated from East Kalimantan. Two cold pre-treatment times, 8 and 10 days, at 4°C and anther culture medium with and without putrescine were examined in this research. The result showed that cold incubation time effect was a genotype-dependent in influencing the ability of rice microspores to develop into callus and green plantlet regeneration. The longer cold incubation time, 10 days, resulted in the highest number of callus, plantlet as well as green plantlets in *Serai Gunung* cultivar but the opposite result was found in *Geragai* cultivar. The effect of putrescine addition in the culture medium was also observed as a genotyped-dependent. It can either increase number of calli, plantlets and green plantlets or decrease them on a specific genotype.

Keywords: Anther culture, Panicle cold pre-treatment, Putrescine, East Kalimantan upland rice,
Abbreviations: IBA (Indole-3-butyric acid), MS (Murashige and Skoog medium), NAA (1-Naphthaleneacetic Acid)

INTRODUCTION

Hybrid rice technology is one of the breakthroughs to overcome increasing global demand for rice. Yield increasing of 15-20% was reported can be achieved through this technique (Virmani et al. 1997; Virmani and Kumar 2004). The development of hybrid varieties cannot be separated from the homozygous line production, which involves numerous cycles of selfing using conventional breeding method. In contrast to such technique, the application of anther culture technology can speed up the breeding process within one generation and significantly shorten the hybrid rice production.

Progress has been made to improve the in vitro androgenic response; nevertheless the application of this technique to rice breeding is still remain problem. Various factors influence culturability of anthers under in vitro condition (Silva 2010). Genotype, pre-culture condition and culture media affect the ability of microspore to form callus, and its regeneration to form shoots as well as green plantlets (Datta 2005; Hong and Rui-Zhen 2008; Silva 2010). High anther culture efficiency reflected by high number of green plantlet regeneration is actually the main objective of this method, and is mainly influenced by the genetics of the donor plant (Yan et al. 1996; Yamagishi et al. 1998). Some genotypes included into *indica* rice subspecies, which have early anther necrosis, poor callus proliferation, and high albino plant regeneration, are known as less responsive varieties to anther culture (Chen et al. 2005; Balachandran et al. 1999, Silva 2010). The poor response of these varieties could be increased by pre-treatment of panicle and modification of nutritional and other supplemental requirements in rice anther culture media.

Stress to heat or cold treatment of the anthers before culture highly influences the induction of microspores to initiate sporophytic pathway in many plant species (Datta 2005). Low temperature shock or cold stress of spikes is the most widely used pre-treatment to improve androgenic response in cereals including barley (Kruczkowska et al. 2002; Haque and Islam 2014), wheat (Slama Ayed et al. 2010), rye (Mikołajczyk et al. 2012) and rice (Caushal et al. 2014). Cold pre-treatment can increase the culturability of anthers by the lack of synchronous development of the tapetum and microspore symmetric division of first pollen mitosis (Nitsch 1974) and delay of pollen and anther wall senescence (Sunderland 1978). Cold pre-treatment effect on rice anther culturability has been examined at different temperature and incubation time. The variation of cold temperature and duration influences the number of embryo/callus formation including development of green plants, and the effects were reported as a genotype-dependent (Trejo-Tapia, et al. 2002; Datta 2005; Khatun et al. 2012).

Effect of media (Herath et al. 2007) and its supplement compound, such as gelling agents (Lee and Lee 1995), carbon sources (Shahnewaz and Bari 2004; Hong and Rui-

zhen 2008), amino acids and plant growth regulators (Herath et al. 2008; Lal et al. 2014) in rice anther culture has been evaluated in previous studies. Putrescine, a plant growth regulator included into polyamines group, was reported to increase green plantlet regeneration in rice anther culture. Its addition in rice anther culture media might improve the culturability of less responsive varieties.

Less is known about anther culturability of East Kalimantan local upland rice cultivars, since it has not been optimally exploited in plant breeding program yet, especially for hybrid breeding technology. Some superior varieties such as Mayas Kuning and Buyung, were observed as less responsive varieties (Nurhasanah et al. 2015). Some efforts should be done to increase anther culturability of these varieties. The present study was conducted to evaluate the effect of cold pre-treatment incubation time and putrescine addition in culture media to the anther culturability of local upland rice varieties originated from East Kalimantan, Indonesia.

MATERIALS AND METHODS

Plant material

Four local upland rice cultivars, originated from Kutai Kartanegara District of East Kalimantan, Indonesia were used in this study i.e. Buyung, Geragai, Mayas Kuning and Serai Gunung. The four varieties are classified as *indica* varieties based on their morpohological characteristics. The mother plants were grown in green house as explant source plants.

Explant preparation and anther culture procedure

After harvested, panicles were wrapped using aluminum foil and incubated at ± 4 ºC for eight and ten days to evaluate the effect of cold pre-treatment time to rice anther culture. The general procedure for explant preparation and rice anther culture were conducted as explained in Nurhasanah et al. (2015). The effect of putrescine enriched medium to the anther culturability was evaluate by adding 0,1644 g L^{-1} putrescine to the callus induction medium (0,7% (w/v) agar solidified N6 medium supplemented with phytohormones, i.e. 0.5 mg L^{-1} Kinetin + 2 mg L^{-1} NAA and 60 g L^{-1} sucrose and incubated in dark condition (25 ± 2 ° C). Each petri dish is considered as one replication containing ±120 anthers from 20 spikelets.

Embryogenic calli were transferred in regeneration medium (0,7% (w/v) agar solidified N6 + 2 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA+ 40 g L^{-1} sucrose and placed in a light condition (25 ± 2 ° C). The regenerated green plantlets were transferred on root induction medium (0,7% (w/v) agar solidified MS + 0.5 mg L^{-1} IBA + 30 g L^{-1} sucrose.

Data analysis

Data were analysed using analysis of variance (ANOVA). Prior to variance analysis, data were transformed using $\sqrt{X+0.5}$. The differences of mean values were analized using Duncan Multiple Range Test (DMRT) at α 5%.

RESULTS AND DISCUSSION

Anther culturability of East Kalimantan local upland varieties was evaluated from its androgenic response. In this study, microspores could develop into callus, and regenerate into green plantlets. Subsequently, the green plantlets were acclimatized and grown in green house (Figure 1).

Effect of panicle cold pre-treatment duration

Different cold pre-treatment incubation time of panicles at ± 4 ºC prior to anther innoculation resulted in various anther culturability. Cold incubation of eight days gave a better result than in the ten days, in Geragai variety. In this variety, the incubation time of eight days produced almost two fold of callus and eight fold of green plantlet number than that of in the incubation period of ten days. Contrary to that, in Serai Gunung the longer incubation time of ten days resulted in the highest number of callus and green plantlet formation, as well as anther culture efficiency (Table 1). Cold pre-treatment duration for ten days increased the number of callus and green plantlet formation for about three and five times, respectively than that of the eight days.

A number of studies has been conducted to evaluate the cold pre-treatment effect in rice anther culture (Trejo-Tapia et al. 2002; Sen et al. 2011; Khatun et al. 2012; Rukmini et al. 2013). Cold shock pre-treatment was reported increased the development of callus and green plantlet regeneration from microspore. It could enhance the androgenic response, since it delays the mitotic stages thereby synchronizing the stage of all microspore during pre-treatment. According to Kiviharju and Pehu (1998), some of the positive effects of cold pretreatment on callus induction included delaying of anther wall senescence, increasing of symmetric division of pollen grains and releasing of substances necessary for androgenesis, mainly amino acids and shock-thermic proteins. The growth factors stimulating the embryogenesis of microspores can be optimally provided in an appropriate microenvironment during this process (Datta et al. 2005; Chen et al. 2005).

Statistically, different incubation times of eight and ten days had no significant effect to the number of callus, plantlet and green plantlet formation in this study (Table 2), since the incubation period tested in this study was not too different. However, it was observed that the effect of incubation time very depended on the genotype. The same cold incubation duration can either increase or decrease the rate of callus, plantlet and green plantlet production in different genotypes (Figure 2). Serai Gunung significantly produced the highest number of callus, plantlet as well as green plantlet regeneration than Geragai in incubation period of ten days (Table 3, Figure 1). It showed a genotype-dependent effect, verified by a significant interaction of genotype and cold incubation time in variance analysis results, especially for plantlet and green plantlet formation (Table 2).

Table 1. Effect of panicle cold pre-treatment duration to the anther culturability of East Kalimantan upland rice variety

Variety	Cold preatment	Callus+		Plantlet#		Green plantlet*		Anther culture eficiency**
		Σ	%	Σ	%	Σ	%	%
Geragai	8 days	65	5.42	49	75.38	23	46.94	1.92
	10 days	37	3.08	9	24.32	3	33.33	0.25
Serai Gunung	8 days	83	6.92	52	62.65	8	15.38	0.67
	10 days	220	18.33	128	58.18	41	32.03	3.42

Note: +Percentage of callus = (Σ of callus /Σ of anther innoculated (1200)) x100%. #Percentage of plantlet = (Σ of plantlet/Σ of callus) x100%. *Pecentage of green plantlet = (Σ of green plantlet/Σ of total plantlet) x100%. **Percentage of anther culture efficiency = (Σ of green plantlet /Σ of anther innoculated) x 100%

Table 2. Variance analysis of the effect of genotype and panicle cold pre-treatment duration to the number of callus, plantlet and green plantlet

Source of variation	Number of callus	Number of plantlet	Number of green plantlet
Genotypes (G)	*	*	ns
Cold pre-treatment (C)	ns	ns	ns
G x C	ns	*	**

*Significant at p=0.05; **Significant at p=0.01; nsnot-significant

Table 3. Effect of panicle cold pre-treatment duration to the mean value of callus, plantlet and green plantlet formation

Genotype	Cold pre-treatment duration				Mean	
	8 days		10 days			
Callus						
Geragai	6.50	a A	3.70	a A	5.10	A
Serai Gunung	8.30	a A	22.00	a B	15.15	B
Mean	7.40	a	12.85	a		
Plantlet						
Geragai	4.90	b A	0.90	a A	2.90	A
Serai Gunung	5.20	a A	12.80	a B	9.00	B
Mean	5.05	a	6.85	a		
Green plantlet						
Geragai	2.3	b A	0.3	a A	1.30	A
Serai Gunung	0.8	a A	4.1	b B	2.45	A
Mean	1.55	a	2.20	a		

Note: Data presented as mean value from ten replications; Different lowercase and capital letters show significant differences in the same row and column respectively, according to Duncan Multiple Range Test (DMRT) at α= 0.05

Table 4. Variance analysis of the effect of genotype and putrescine to the number of callus, plantlet and green plantlet

Source of variation	Number of callus	Number of plantlet	Number of green plantlet
Genotypes (G)	*	ns	ns
Putrescine (P)	ns	ns	ns
G x P	ns	ns	*

*Significant at p=0.05; **Significant at p=0.01; nsnot-significant

Table 5. Effect of putrescine addition in callus induction medium to the mean value of callus, plantlet and green plantlet formation

Genotype	Callus induction medium		Mean
	Without putrescine	With putrescine	
Callus			
Buyung	3.50 a A	2.00 a A	2.25 A
Geragai	7.50 b AB	3.17 a AB	5.34 AB
Mayas Kuning	2.17 a A	14.83 a B	8.50 AB
Serai Gunung	12.67 a B	7.33 a AB	10.00 B
Mean	6.46 a	6.83 a	
Plantlet			
Buyung	3.17 b A	2.00 a A	2.58 A
Geragai	5.67 b AB	2.00 a A	3.84 AB
Mayas Kuning	1.50 a A	7.67 a A	4.59 AB
Serai Gunung	8.00 a B	5.83 a A	6.92 B
Mean	4.58 a	4.38 a	
Green plantlet			
Buyung	0.17 a A	0.50 a A	0.33 A
Geragai	2.33 b B	0.50 a A	1.42 B
Mayas Kuning	0.17 a A	1.50 a A	0.83 AB
Serai Gunung	1.17 a AB	0.83 a A	1.00 AB
Mean	0.96 a	0.83 a	

Note: Remarks are the same as Table 3.

Table 6. Effect of putrescine encriched media to the anther culturability of East Kalimantan upland rice variety

Variety	Cold preatment	Callus+		Plantlet#		Green plantlet*		Anther culture eficiency**
		Σ	%	Σ	%	Σ	%	%
Buyung	No	21	2.92	17	80.95	1	5.88	0.14
	Yes	12	1.67	12	100.00	3	25.00	0.42
Geragai	No	45	6.25	34	75.56	14	45.16	1.94
	Yes	19	2.64	12	63.16	3	25.00	0.42
Mayas Kuning	No	13	1.81	9	69.23	1	11.11	0.14
	Yes	89	12.36	46	51.69	9	19.57	1.25
Serai Gunung	No	76	10.56	48	63.16	7	14.58	0.97
	Yes	44	6.11	35	79.55	5	4.29	0.69

Note: Remarks are the same as Table 1.

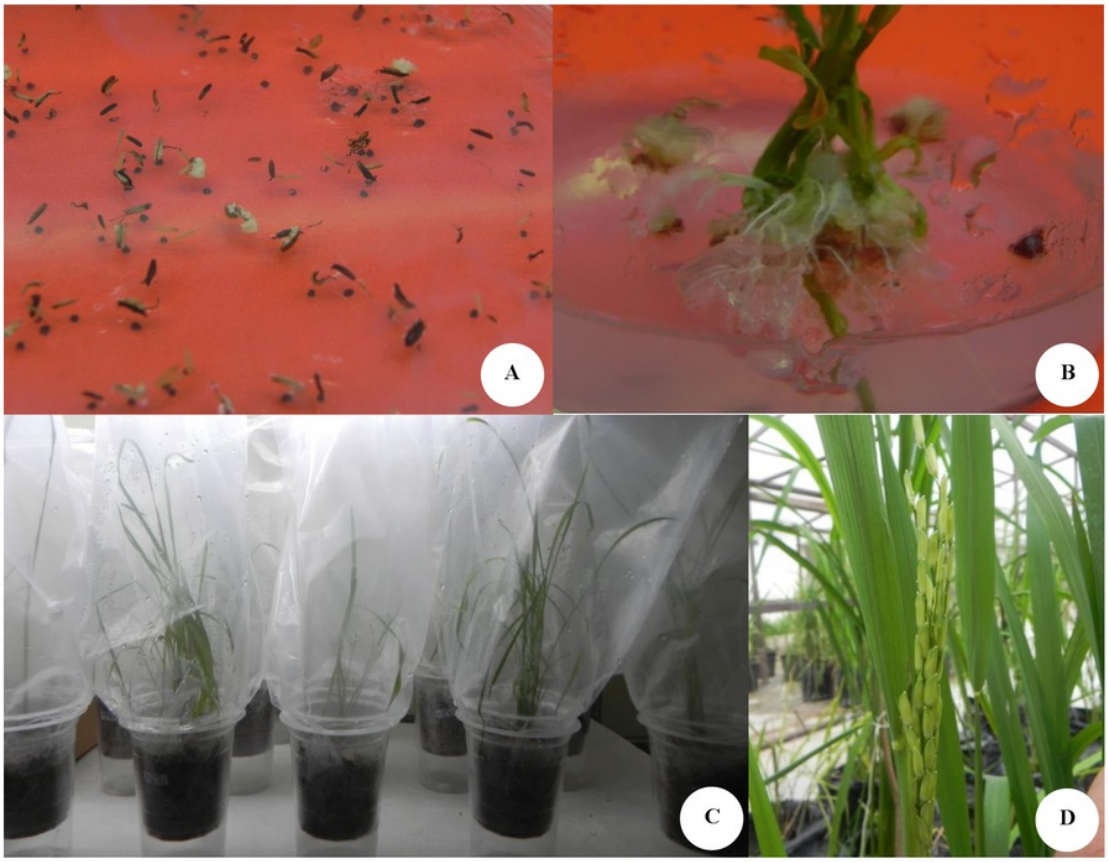

Figure 1. Anther culture of East Kalimantan upland rice varieties, A. Anther forming callus; B. Green plantlet; C. Acclimatization step; D. Fertile plants producing grains

Genotype-dependent effect of cold pre-treatment in rice anther culture, showing genotypic differences for anther culturability at different temperature and duration was also observed in previous studies (Trejo-Tapia, et al. 2002; Datta 2005; Khatun et al. 2012). The optimum temperature and incubation time of cold pre-treatment varied in different genotypes (Genovessi and Magill 1979; Rukmini et al. 2013; Kaushal et al. 2014). Therefore, optimation research to find the optimal temperature and incubation period for particular genotype is very important to increase rice anther culturability.

The presence of putrescine in anther culture medium

The effect of putrescine in anther culture medium did not significantly influence callus, plantlet and green plantlet formation based on analysis of variance (Table 4). Nevertheless, the effect of putrescine varied on different genotypes. The mean value of each genotype was significantly different in the presence and absence of putrescine based on DMRT test (Table 5).

In this study, the presence of putrescine in callus induction medium enhanced anther culturability of Mayas Kuning evaluated from the number of calli, plantlets and green plantlets (Table 6). Mayas Kuning, which was not responsive to anther culture according to previous study (Nurhasanah et al. 2015), showed a better response in putrescine enriched medium.

Putrescine is a naturally occurring low molecular weight polycation, that is obligate requirement for cell growth and sustenance. It has been implicated in many important cellular processes such as cell division, protein synthesis, DNA replication, and response to abiotic stress (Kakkar and Sawhney 2002). Putrescine can improve androgenesis and enhance embryo or callus formation from microspores by inhibiting early senescence of cultured anthers. Putrescine inhibites ethylene biosynthesis, a senescence inducer, because they compete for the same substarate S-adenosyl-methionine (SAM) in their biosynthetic pathway Dewi et al. (2008).

Putrescine has been reported to increase green plantlet regeneration in rice anther culture (Sasmita 2007). In this current study, the total number of green plantlet could be elevated from one to nine in Mayas Kuning. Interestingly, putrescine enriched medium increased the number of green

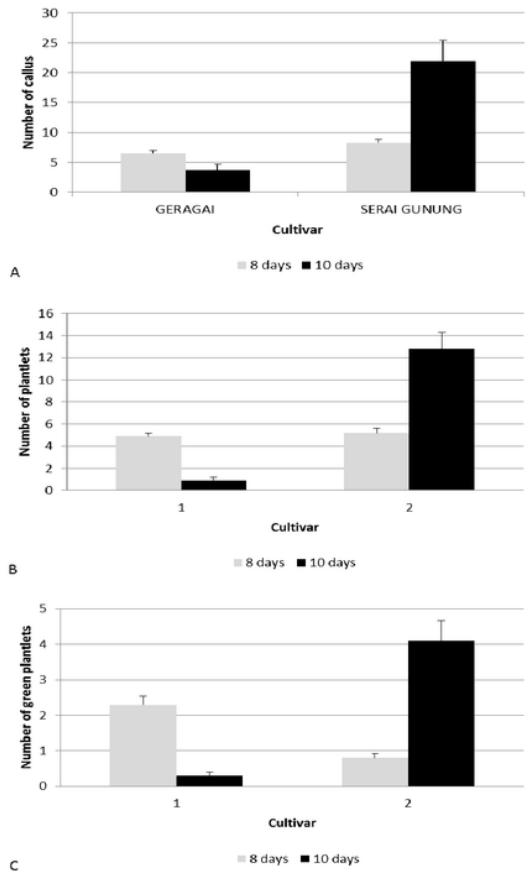

Figure 2. Effect of different cold incubation time of panicles to anther culture of East Kalimantan local upland rice varieties A. Number of callus; B. Number of plantlet; C. Number of green plantlet formation. (Data presented as mean value of original data from ten replications and the standard deviations).

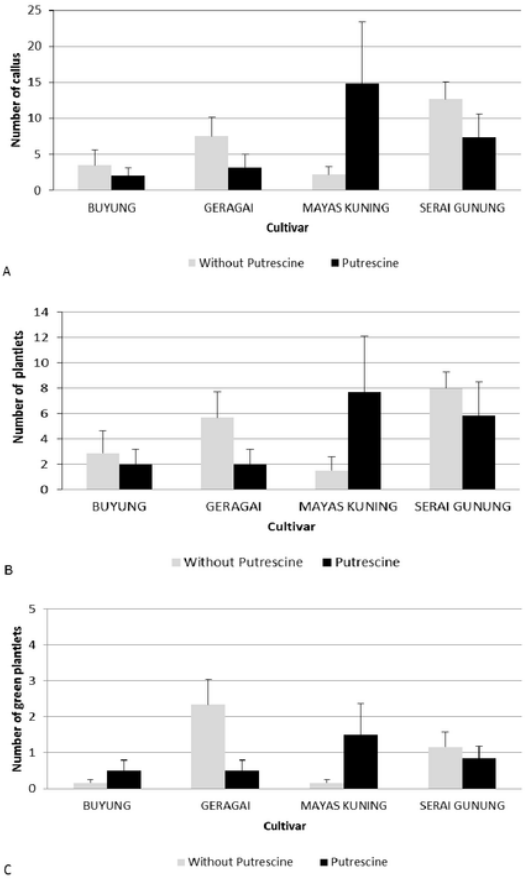

Figure 3. Effect of putrescine in callus induction medium to anther culture of East Kalimantan local upland rice varieties A. Number of callus; B. Number of plantlet; C. Number of green plantlet (Data presented as mean value of original data from six replications and the standard deviations).

plantlets in Buyung cultivar, although the number of callus and plantlet was lower than that of in the medium without putrescine. Therefore, the presence of putrescine in callus induction medium increased anther culture efficiency in Mayas Kuning and Buyung (Table 6).

The occurrence of a large proportion of albinos in anther culture limits the application of this technique to rice breeding program. The accumulation of ethylene in culture vessel might inhibit chlorophyll synthesis and chloroplast development that lead to albino plant formation (George and Sherrington 1984). A higher rate of ethylene production by anthers in *indica* compared to *japonica* types is also suggested as a reason for the poor response of these genotypes to anther culture (Dewi et al. 2008). Therefore, the use of anti-ethylene agent such as putrescine might prevent it and increase the green plantlet regeneration, especially in the improvement of anther culture in a recalcitrant genotype such as in subspecies indica which has early anther necrosis, poor callus proliferation, and high albino plant regeneration (Chen et al. 2005).

The effect of genotype x medium interaction, related to the presence or absence of putrescine, was observed in this research. The effect of putrescine was specific for each genotype, in which it can either increase the callus, plantlet and green plantlet regeneration, as found in Mayas Kuning and Buyung varieties, or decrease it on other genotypes (Table 6, Figure 3). The addition of putrescine in anther culture medium significantly reduced the ability of anther to form calli and plantlets as well as green plantlets regeneration in Geragai variety (Table 5). The presence of 0,1644 g L^{-1} of putrescine in callus induction medium decreased the number of callus for almost a half in Serai Gunung and two-thirds in Geragai. Furthermore, a drastic reduction of about 85% of green plantlets was exhibited by Geragai variety (Table 6).

A genotype x medium interaction was also observed by Talebi et al. (2007). A specific media requirement was needed for anther culture by different genotypes. In their study, high frequency of callus induction of a specific medium for a variety has low calussing frequency for another variety and vice versa, showing a genotype-dependent effect. The genotype-dependant effect of putrescine enriched medium in improving androgenesis was also reported in another study. Dewi et al. (2007) observed lower number of calli, callus forming shoots, and green plantlets in the presence than that of in the absence of putrescine in N6 callus induction medium in the F1 genotype from the cross of Taipei 309 x Asemandi. On the other hand, the contrary effect was showed by the F1 of the reciprocal cross.

The genotype-dependent effect of putrescine indicates that a general concentration of putrescine cannot be applied for all genotypes. Redha and Suleman et al. (2011), that studied the effect of exogenous application of polyamine on wheat anther culture, observed that the formation of embrio-like stucture and green plantlet varied significantly among genotypes depend on the duration of pre-treatment of anthers using polyamines. These results appear to underscore that each genotype requires a certain concentration of putrescine to optimally improve its androgenic response and increase anther culture efficiency.

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