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ROLE OF YAMINES IN INHIBITION OF ETHYLENE BIOSYNTH AND THEIR EFFECTS ON RICE ANTHER CULTURE DEVELOPMENT

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ABSTRACT

The polyamines such as putrescine, spermidine, and spermine were reported to increase green plant regeneration in rice anther culture. Low response of anther culture of rice sub-species indica may be improved with the addition of putrescine in the culture media. Four experiments were conducted to study the role of polyamines in inhibition of ethylene biosynthesis and their effects on rice anther culture development. Anthers of two subspecies of rice, indica (IR64, Krowal, Jatiluhur) and japonica (Taipei 309) were cultured onto media supplemented with putrescine (N6P) and without putrescine (N6). Young panicles containing the anthers at mid-to-late nucleate microspores were cold pretreated at 5 + 2°C and incubated in the dark for 8 days before the anthers were cultured. Results showed that medium without putrescine produced an earlier senescence of indica rice anther than that of japonica. The addition of 10^{-3} M putrescine into the culture media inhibited ethylene biosynthesis as anther senescence delayed, increased the three polyamines contents, and decreased the ACC content as well as ACC oxydase activity in anther-derived calli. In the anther and anther-derived calli of subspecies indica, the total polyamines content was lower (10.14 nM g^{-1} anther and 8.48 nM g^{-1} calli) than that of subspecies japonica (12.61 nM g^{-1} anther and 10.16 nM g^{-1} calli), whereas the ethylene production was higher (32.31 nM g^{-1} anther and 2.48 nM g^{-1} calli) than the japonica (31.68 nM g^{-1} anther and 1.76 nM g^{-1} calli). This study suggests that application of 10^{-3} M putrescine in anther culture of rice subspecies indica improves androgenesis by inhibiting early senescence of cultured anthers and enhancing embryo or callus formation from microspores.

[***Keywords***: *Oryza sativa*, anther culture, ethylene, polyamines, senescence]

INTRODUCTION

Anther culture is useful for introgression of desirable traits, overcoming F_1 sterility, utilization of heterosis, and the production of spontaneous homozygous doubled haploid (DH) lines, which ease selection of phenotypes for quantitative characters (Bishnoi *et al*. 2000; Datta 2005). For effective utilization in rice breeding programs, the technique should allow production of large number of genetically stable spontaneous DH plants from a wide range of genotypes.

Rice variety development in Indonesia is generally derived from crosses between subspecies indica. Unfortunately, subspecies indica exhibits poorer androgenic response than that of japonica. Subspecies indica has been known as a recalcitrant genotype, with early anther necrosis, poor callus proliferation, and high albino plant regeneration (Chen *et al*. 1991; Dewi *et al*. 2006). On the other hand, japonica genotype is very responsive to anther culture or having high anther culturability compared to the indica (Bishnoi *et al*. 2000). The low androgenic response of rice subspecies indica in anther culture due to early necrosis or senescence of anthers may result from a higher rate of ethylene production by anthers compared to japonica.

In *in-vitro* culture, senescence has been associated with the accumulation of ethylene in the sealed culture vessel (Tiainen 1996). Ethylene generated by isolated anthers is likely to accumulate in the vessels used for anther culture, but reports of its effects on anther culture development are rare and the type of the effect appears to be species dependent. Ethylene may also inhibit plant development through cessation in cell division scientifically known as post-mitotic senescence. This inhibitory effect can be reversed by using anti-ethylene agents (Gan 2004).

During rice anther culture, microspores are initiated toward androgenesis. Early senescence of anther tissues will delay or halt dedifferentiation of microspores. This contributes to the decreased proportion of the viable anthers. The anthers, therefore, should be maintained viable long enough to ensure the anther cell wall, especially tapetum, supports microspore development. Study on anther culture of barley showed that early senescence in anthers inhibited the microspores to form embryoid (Cho and Kasha 1992).

Polyamines, an important and interesting group of naturally occurring low molecular weight, polycationic, aliphatic nitrogenous compounds, present in all cells. They are found in plant cells at levels significantly higher than those of plant hormones. Their endogenous concentrations required for biological effects are in millimolar range (Bais and Ravishankar 2002). Polyamines include spermidine, spermine, and their obligate precursor putrescine known to play important roles in a wide range of plant physiological processes such as morphogenesis, flower differentiation and initiation, pollen viability, root growth, and biotic-abiotic stress responses (Bouchereau *et al*. 1999; Martin-Tanguy 2001). In biosynthetic pathway of polyamines and ethylene, it seems that both pathways use the same substrate, i.e. S-adenosyl-methionine (SAM). In biosynthesis pathway of polyamines, SAM is decarboxylated by SAM decarboxylase (SAMDC) and provides aminopropyl moiety that is used to convert putrescine to spermidine and spermine, respectively. SAM is also a precursor in ethylene biosynthesis, through the intermediate formation of 1-aminocyclopropane-1-carboxylic acid (ACC). Since ethylene tends to be a senescence inducer and polyamines tend to be senescence inhibitors (Tiainen 1996), the fate of SAM becomes very crucial. Increases in polyamines biosynthesis, particularly via SAMDC activity, therefore, are likely to decrease the rates of ethylene synthesis.

Cho and Kasha (1992) reported that in anther culture of barley in which anthers were high in the ACC content, application of putrescine delayed senescence of cultured anther and increased embryogenesis. Purwoko *et al*. (2001) found that polyamines increased green plant regeneration in rice anther culture of Taipei 309, a subspesies japonica. Further study confirmed that 10^{-3} M putrescine were the best concentration in regenerating green plantlet of both japonica and indica via anther culture (Dewi *et al*. 2004, 2006). The aims of this study were to investigate the role of polyamines in inhibition of ethylene biosynthesis and their effects on rice anther culture development.

MATERIALS AND METHODS

The study consisted of four experiments conducted in 2004 at Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Bogor. Anther culture works were conducted at the Molecular Biology Laboratory, while polyamine and ethylene analyses were carried out at the Plant Nutrition and Chemical Laboratory.

Experiment 1. The effect of putrescine on inhibition of anther senescence in anther culture of indica and japonica rice

Anthers were derived from Taipei 309 representing subspecies japonica, and Krowal, Jatiluhur and IR64 representing subspecies indica. The experiment was arranged in a factorial design with two factors, replicated five times. The first factor was subspecies of rice, i.e. indica and japonica, and the second factor was callus induction media. Callus induction media were N6 basal medium without and with addition of 10^{-3} M putrescine designated as N6 and N6P, respectively. Rice anther culture method followed the protocol of Dewi *et al.* (2004). Young panicles were cold pretreated at 5 + 2°C and incubated in the dark for 8 days. After cold pretreatment, spikelets were removed from the sheath leaf and surface sterilized with 20% commercial bleach. Spikelets were cut at the base with scissors so as to cut the anther filaments. Anthers containing mid-to-late uninucleate microspores were obtained from the central spikelets of the panicles. Each spikelet was picked up by the uncut end using a forcep and anthers were released on the callus induction medium by tapping the forcep on the rim of the Petri dish. The cultures were maintained in the dark at 25 + 2°C to induce callus formation from microspores. Observation was conducted on number of senescent anthers at 8 weeks after culture (WAC).

Experiment 2. The effect of putrescine on plant regeneration in anther culture of indica and japonica rice

Based on the first experiment, the explants used for culture initiation were anthers of Taipei 309 and Krowal representing subspecies japonica and indica, respectively. The experiment was arranged in a factorial design with two factors, replicated five times. The first factor was subspecies of rice, i.e. indica and japonica, and the second factor was callus induction media, i.e. N6 and N6P. Rice anther culture method followed the protocol of Dewi *et al.* (2004) as previously described in Experiment 1. In this experiment, the androgenic calli of 1-2 mm diameter were transferred onto MS regeneration media and incubated under light at 25 + 2°C to induce green plant regeneration. Observations were conducted on number of anthers inoculated, number of anthers forming calli, number of calli produced, number of calli

producing plant, number of green plantlet, and number of albino plantlet.

Experiment 3. Polyamines, ethylene, ACC, and ACC oxydase activity analyses in anthers of indica and japonica rice

The anthers containing microspores at uninucleate stage were derived from Taipei 309 and Krowal representing subspecies japonica and indica, respectively. Except for the fresh anthers, the cold pretreated anthers (at 5 + 2°C) were incubated in the dark for 8 days.

The analysis of polyamines (putrescine, spermidine, and spermine) was based on Smith and Davies (1985) using freeze-dried samples and an HPLC technique. Solvent system used for HPLC technique was water and methanol (35:65) conducted as isocratic elution system at 70% methanol with the flow rate of 1 ml/minute through reversed-phase Bondapak column C18 4.6 x 250 mm, and polyamines were detected at 254 nm with a UV-detector.

Ethylene was analyzed using Gas Chromatography (GC) Hitachi type 263-70 with Flame Ionization Detector (FID). Spiral column 2000 mm x 4 mm made of 80-100 mesh activated aluminum was used for ethylene analysis. The carrier was N2 with gas flow rate of 50 ml/minute. Initial temperature was 100°C, injection temperature was 120°C, column temperature was 180°C, and detector temperature was 220°C.

ACC content was quantified based on Lizada and Yang (1979) using GC Hitachi type 263-70 with FID. Column and GC condition were the same as those for ethylene analysis. ACC oxydase activity can be identified by the amount of ethylene produced when ACC was added to samples. Observation was conducted on polyamine content, ethylene content, ACC content, and ACC oxydase activity of fresh and cold pretreated anthers.

Experiment 4. Polyamines, ACC, and ACC oxydase activity analyses in anther-derived calli of indica dan japonica rice

Calli (size 2 + 0.5 mm) were derived from anther culture of Taipei 309 representing japonica and Krowal representing indica rice**.** Rice anther culture method followed the protocol of Dewi *et al.* (2004) as previously described in Experiment 1. The analyses of polyamines, ACC, and ACC oxydase activity were the same as previously described in Experiment 3. Observation was conducted on polyamine content, ACC content, and ACC oxydase activity of anther-derived calli.

RESULTS AND DISCUSSION

The Effect of Putrescine on Inhibition of Anther Senescence in Anther Culture of Indica and Japonica Rice

The result indicated that there was interaction between media and rice genotypes on number and percentage of senescent anthers at 8 WAC (Table 1). In media without putrescine (N6), the onset of senescence began at 3 days after anther was cultured (DAC) for subspecies indica, while for japonica occurred at 7 DAC. Generally, the first embryoid or callus formation occurred at 3-8 WAC (Sasmita *et al*. 2001). According to Cho and Kasha (1992), prolonged viability of cultured anthers was important to ensure tapetum to support microspore development into embryoid or calli. In this research at 8 WAC, the highest number of senescent anthers were achieved by IR64 (indica) in N6 medium without putrescine, while the lowest were achieved by Taipei 309 (japonica) in N6 medium supplemented with 10^{-3} M putrescine (Table 1).

Percentage of senescent anthers in callus induction medium N6 ranged between 25.5-62.0% for indica and 6.1% for japonica, while in N6P medium the senescent anthers ranged between 18.2-57.0% for indica and 1.4% for japonica (Table 1). Therefore, the addition of 10^{-3} M putrescine in N6P medium decreased senescent anthers in subspecies indica by 5.0, 12.3, and 7.3% for IR64, Jatiluhur, and Krowal, respect-

Table 1. Interaction between media and genotypes on senescent anthers in anther culture of indica and japonica rice at 8 weeks after culture.

Treatment		Number of senescent anthers
Media	Genotypes	
N6	Subspesies indica	
	IR64	96.0a (62.0)
	Jatiluhur	66.0b (45.0)
	Krowal	39.5c (25.5)
	Subspesies japonica	
	Taipei 309	11.5e (6.1)
N6P	Subspesies indica	
	IR64	86.0ab (57.0)
	Jatiluhur	47.0c (32.7)
	Krowal	27.5d (18.2)
	Subspesies japonica	
	Taipei 309	2.5f (1.4)

N6 = media without putrescine, N6P = N6 + 10^{-3} M putrescine. The different letters following the values in the same column mean a significant different according to DMRT at $P < 0.05$. Number in parentheses indicated percentage of senescent anthers.

ively. In subspecies japonica represented by Taipei 309, however, the decrease was only 4.7%.

In this study subspecies indica which was classified as recalcitrant genotypes seemed more responsive to putrescine treatment than japonica. However, in both media, N6P and N6, number and percentage of senescent anthers were still higher in indica than that of japonica (Table 1).

Observation on anthers in N6 and N6P media showed that the addition of 10^{-3} M putrescine in N6 medium delayed the onset of senescence to become 5-7 DAC in indica and 13 DAC in japonica. Addition of 10^{-3} M putrescine into callus induction media maintained and prolonged viability of anther cell wall, i.e. tapetum, which was essential for androgenesis to occur. Tapetum, a thin layer of active cells around anther wall secreted proteins and β-1,3- glucanase, synthesized sporopolenin and orbicule precursor, and maintained locule viability where young pollen develop (Dickinson 1992). Early degeneration in anther tissue will inhibit androgenesis by causing the failure of proliferated microspores to regenerate into plantlet (Cho and Kasha 1992). Proliferated microspore still attach to anther cell wall through suspensor-like structure to take up nutrient excreted from tapetum (Chen 1983).

The study indicated that faster senescence of indica rice anthers might be the main reason of the low anther culture ability in indica compared to that of japonica. Naturally, pollen *in-vivo* contains ethylene precursor ACC, which causes high cell death in anthers due to accumulation of ethylene in culture vessel (Tiainen 1996). The source of ethylene production in anther culture has not yet been understood, but may be regulated by the endogenous ACC because of anther sensitivity to stress such as dehydration, wounding, or media composition (Cho dan Kasha 1992).

The Effect of Putrescine on Plant Regeneration in Anther Culture of Indica and Japonica Rice

Microspore or pollen development during the period before first mitosis is generally unstable. Cultured pollen in that stage of development can be changed its course from gametophytic to sporophytic and develops into embryoid, the calli (Dickinson 1992). The result indicated that addition of 10^{-3} M putrescine increased the number and percentage of anthers forming calli, number and percentage of calli produced, number of calli producing plant, number of green plantlet, and number of total plantlet of indica and japonica rice (Table 2 and 3).

The increase in green plants was also followed by the increase in albino plants produced in indica (Krowal), but both growth parameters decreased in japonica (Taipei 309). These results supported the previous anther culture studies on indica, japonica, and javanica (Datta 2005). In media without putrescine, Krowal failed to regenerate green plants but produced small number of albino plants (Table 3). George and Sherrington (1984) suggested that accumulation of ethylene in culture vessel might inhibit chlorophyll synthesis and chloroplast development which could be the cause of albino plant formation.

Increase in green plantlet occurred in media supplemented with putrescine from 0% to 52.60% in Krowal, while in Taipei 309 the increase was more than 4 folds from 20.30% to 94.20% (Table 3). These results confirmed our previous study (Experiment 1) indicating that the application of 10^{-3} M putrescine delayed senescence of cultured anthers and thus increased androgenesis.

The efficiency of putrescine application to green plant regeneration was indicated by the ratio of green plant to calli producing plant (GP/CPP) and percent-

Table 2. Interaction between media and genotypes on callus induction and regeneration in anther culture of indica and japonica rice.

Treatment		Number of AFC[1]	Number of CP[2]	Number of CPP[3]	Number of TP (GP+AP)
Media	Genotypes				
N6	Krowal (indica)	2.4d (1.83)	6.8d (5.14)	0.4d (5.88)	1.6d
	Taipei 309 (japonica)	4.8c (3.62)	11.2c (8.20)	2.0c (17.86)	13.8c
N6P	Krowal (indica)	15.0b (7.18)	33.4b (24.30)	5.6b (16.77)	34.6b
	Taipei 309 (japonica)	22.6a (9.23)	66.4a (43.59)	18.4a (27.71)	58.4a

N6 = media without putrescine, N6P = N6 + 10^{-3} M putrescine, AFC = anther forming calli, CP = calli produced, CPP = calli producing plant, GP = green plants, AP = albino plants, TP = total plantlet. The different letters following the values in the same column mean a significant different according to DMRT at $P < 0.05$. Number in parentheses indicated percentage of AFC, CP, and CPP.

Table 3. Interaction between media and genotype on plant regeneration efficiency in anther culture of indica dan japonica rice.

Treatment		Plantlets		Anther culture efficiency	
Media	Genotypes	GP	AP	Ratio GP/CPP (%)	GP/AI (%)
N6	Krowal (indica)	0.0d (0.00)	1.6d (100.00)	0.0c	0.0d
	Taipei 309 (japonica)	2.8c (20.30)	11.0b (79.70)	2.1b	2.5c
N6P	Krowal (indica)	18.2b (52.60)	16.4a (47.40)	3.3a	12.7b
	Taipei 309 (japonica)	55.0a (94.20)	3.4c (5.80)	3.3a	36.1a

N6 = media without putrescine, N6P = N6 + 10^{-3} M putrescine. GP = green plants, AP = albino plants; CPP: calli producing plant; AI = anther inoculated. The different letters following the values in the same column mean a significant different according to DMRT at $P < 0.05$. Number in between parentheses indicated percentage of GP and AP.

age of green plant production to number of anther inoculated (GP/AI). In indica (Krowal), GP/CP ratio increased from 0 to 3.3 plants, and GP/AI ratio increased from 0% to 12.70%. In japonica (Taipei 309), GP/CPP ratio increased from 2.1 to 3.3 plants and GP/AI ratio increased from 2.50% to 36.10 % (Table 3).

Although results of this study confirmed previous research in the application of polyamines for increasing green plant regeneration, the application of putrescine, however, did not eliminate albino regeneration. It seems that formation of albino plants in rice anther culture cannot be nullified. Therefore, delayed senescence of anthers by putrescine will enhance callus and embryo formation and support embryo development towards green plant regeneration.

Polyamines, Ethylene, ACC, and ACC Oxydase Activity Analyses in Anthers of Indica and Japonica Rice

Polyamine content in rice anthers of indica (Krowal) and japonica (Taipei 309) previously cold pretreated by incubating the panicles at 5 + 2°C in the dark are presented in Figure 1. Anthers of subspecies indica contained lower endogenous polyamines than that of japonica, while endogenous putrescine content was higher than spermidine and spermine.

The three polyamines, putrescine, spermidine and spermine were found readily in fresh anther before incubation (Fig. 1). Polyamines are needed for maturation and germination of pollen that led to the formation of pollen tube in nature (Chibi *et al*. 1994; Tiainen 1996). Santos *et al*. (1995) also reported that polyamine contents were higher in *in-vivo* pollen (microsporogenesis pathway) than *in-vitro* pollen (androgenesis pathway).

All three polyamines and total polyamine contents decreased following incubation of anthers at 5°C for 8 days (Fig. 1). The change in polyamine content indicated the possibility of polyamines used during the incubation period. Polyamines are needed at the onset of androgenesis as indicated in anther culture of maize. It was shown that polyamines were metabolized during cell division (Santos *et al*. 1995). Other reports stated that active polyamine metabolism during development of both somatic and zygotic embryos of *Picea abies* suggested that polyamines regulate embryo development, with an as yet unknown mode of action (Santanen 2000).

Ethylene production, ACC content, and ACC oxydase activity in rice anthers are presented in Table 4. Ethylene content in fresh anthers (0 DAI) was higher than that after incubation (8 DAI). The decrease in ACC content and ACC oxydase activity of anthers after incubation indicated that cold pretreatment at 5°C for 8 days delayed ethylene biosynthesis. Cold pretreatment is necessary to reduce ethylene production in rice anthers as reported by Zapata *et al*. (1983). In our experiment, anthers without cold pretreatment in room temperature began to wither at 2 DAI.

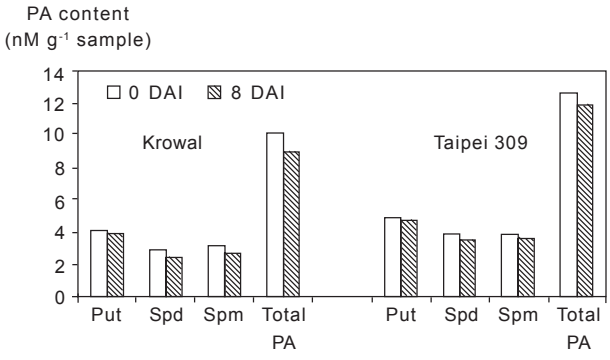

Fig. 1. Polyamine (PA) content in rice anther of Krowal (indica) and Taipei 309 (japonica) at 0 and 8 days after incubation (DAI); Put = putrescine, Spd = spermidine, Spm = spermine.

These findings are in accordance with our previous results of senescence in anthers during cultured and regeneration of plant from rice anther-derived calli (Experiments 1 and 2). Subspecies indica appeared more responsive to the application of putrescine in decreasing the number and percentage of senescent anthers, however, the number and percentage of senescent anthers in indica were still higher and significantly different to that of japonica (Table 1). This was because of the nature of ethylene and ACC contents as well as ACC oxydase activity in subspecies indica (Krowal) which was higher than that of japonica (Taipei 309) (Table 4). Therefore, the number of calli formed and their regeneration into green plants were higher in japonica than that of indica (Tables 2 and 3).

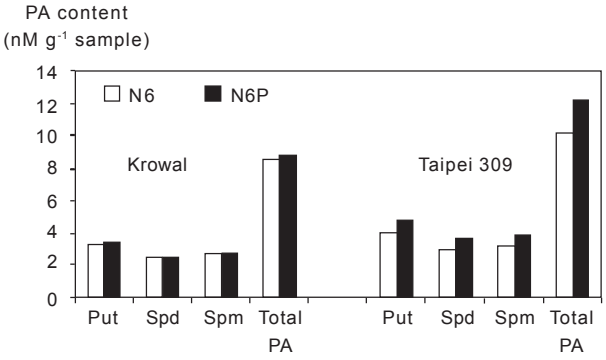

Fig. 2. Polyamine (PA) contents in anther-derived calli of indica (Krowal) and japonica rice (Taipei 309); N6 = media without putrescine, N6P = N6 + 10^{-3} M putrescine, Put = putrescine, Spd = spermidine, Spm = spermine.

Polyamines, ACC, and ACC oxydase Activity Analyses in Anther-Derived Calli of Indica and Japonica Rice

Polyamine content in anther-derived calli from anther culture of indica (Krowal) and japonica (Taipei 309) is presented in Figure 2. As shown in anthers, putrescine content in calli was higher than spermidine and spermine contents. Polyamine content in anther-derived calli in indica (Krowal) was lower than that of japonica (Taipei 309). These findings are in agreement with those observed in anthers of both rice genotypes (Fig. 1). Addition of 10^{-3} M putrescine increased the three polyamine contents, putrescine, spermidine and spermine in calli for both indica and japonica (Fig. 2). Putrescine content increased by 0.15 nM (from 3.28 to 3.43 nM) in Krowal-derived calli, while in Taipei 309-derived calli the increase was 0.70 nM (from 4.03 to 4.73 nM).

Total polyamines in anther culture-derived calli of both indica and japonica increased by 0.25 nM (from 8.48 to 8.73 nM) in indica (Krowal) and by 2.05 nM (from 10.16 to 12.21 nM) in japonica (Taipei 309) after the application of 10^{-3} M putrescine. In higher plant, rapid division, enlargement, extension, and proliferation of cells occur due to the application of auxin together with embryogenesis process related to polyamine content (Apelbaum 1990). In case of androgenesis induction, cell division, and embryogenesis, the increase in endogenous polyamines were observed in maize anthers (Santos *et al*. 1995). In japonica rice, putrescine uptake and its metabolism were higher than that of indica (Fig. 2). As a result, androgenesis in japonica (Taipei 309) should also be higher than that of indica (Krowal) (Table 3).

On the contrary, ACC content and ACC oxydase activity appeared to decrease following putrescine increase in anther-derived calli (Table 5). It seemed that ethylene biosynthesis was reduced due to diversion of SAM to polyamine biosynthesis pathway. Previous experiments in tomato pericarp disc culture (Mencarelli *et al*. 1990) and tomato shelf life storage (Saftner and Mehta 1990) showed that polyamine applications inhibit ethylene biosynthesis through inhibition of ACC formation and its conversion to ethylene. Roustan *et al*. (1993) reported that in carrot and sunflower tissue cultures, the inhibition of polyamine biosynthesis was caused by the decrease in activity of key enzyme (i.e. SAMDC) that decreased plant regeneration.

Ethylene precursor, ACC, in anther-derived calli of indica was higher than that of japonica (Table 5). In relation to plant regeneration, it was shown that in media without putrescine (N6), plant regeneration of

Table 4. Ethylene production, ACC content, and ACC oxydase activity in anthers of indica and japonica rice.

Incubation period (d)	Genotypes	ACC content (nM g^{-1} sample)	ACC oxydase activity (nl ethylene g^{-1} sample h^{-1})	Ethylene production (nl g^{-1} sample h^{-1})
0	Krowal (indica)	32.31	132.00	465.87
8	Krowal (indica)	21.99	124.00	384.60
0	Taipei 309 (japonica)	31.68	115.50	438.87
8	Taipei 309 (japonica)	28.55	88.30	375.33

Table 5. ACC content and ACC oxydase activity in anther-derived calli of indica dan japonica rice.

Media	Genotypes	ACC content (nM g^{-1} sample)	ACC oxydase activity (nl ethylene g^{-1} sample h^{-1})
N6	Krowal	2.48	13.20
	Taipei 309	1.76	8.92
N6P	Krowal	2.09	11.73
	Taipei 309	1.39	8.56

N6 = media without putrescine, N6P = N6 + 10^{-3} M putrescine.

indica was lower than that of japonica (Table 3). The response of indica to certain level of ethylene was higher than that of japonica. Cho dan Kasha (1992) who studied the relationship between senescence and androgenesis in anther culture of barley reported that there was an optimum level of ethylene, which did not affect embryogenesis while embryogenesis itself depended on barley genotypes. In barley anthers with low ACC content, the addition of ethephon or ACC increased embryogenesis. On the contrary, in barley anthers with high ACC content, androgenesis was increased due to the application of polyamine, i.e. putrescine.

In the case of ACC oxydase, the addition of 10^{-3} M putrescine into media caused the decrease of its activity in anther-derived calli (Table 5). The decrease in ACC oxydase activity was indicated by the decrease in ethylene formation rate by 1.47 nl ethylene g^{-1} sample h^{-1} (from 13.20 to 11.73 nl ethylene g^{-1} sample h^{-1}) in indica (Krowal), and by 0.36 nl ethylene g^{-1} sample h^{-1} (from 8.92 to 8.56 nl ethylene g^{-1} sample h^{-1}) in japonica (Taipei 309). Therefore, the improvement observed in androgenesis in both subspecies were also due to the decrease in ACC content and ACC oxydase activity in callus during regeneration period.

The Impact of Putrescine Application on Anther Culture Media of Subspecies Indica

Although number of green plant regeneration was higher in japonica, represented by Taipei 309, the effect of putrescine application on green plant regeneration was higher in indica, represented by Krowal. The addition of 10^{-3} M putrescine in Krowal showed an infinite increase in ratio of green plant to calli forming plant (GP/CPP), from 0 to 3.3 plants, and in percentage of green plant to anther inoculated (GP/AI) from 0% to 12.70%, while in Taipei 309 ratio of GP/CPP increased from 2.1 to 3.3 plants and percentage of GP/AI increased from 2.50% to 36.10% (Table 3). Zhuo *et al*. (1996) reported that the highest regeneration frequency obtained from anther culture of rice subspecies indica was 3% and about 8-10% in japonica by using combination of auxin and cytokinine alone. This study suggests that the application of 10^{-3} M putrescine in anther culture of indica rice will improve androgenesis by inhibiting early senescence of cultured anther and enhancing embryo or callus formation from microspores that will lead to green plant regeneration needed for double haploid plant development to be used for breeding purposes.

CONCLUSION

The low response of anthers to produce calli in rice anther culture of subspecies indica due to early senescence of anthers may result from a high rate of ethylene production by anthers when compared to that of the responsive subspecies japonica. Polyamine contents, i.e. putrescine, spermidine, spermine were lower, but ethylene production, ACC contents, and ACC oxydase activity were higher in anthers and anther-derived calli from subspecies indica than that of japonica. The findings explain why the green plant regeneration in anther culture of indica rice was lower than that of japonica. Therefore, application of 10^{-3} M putrescine in anther culture of indica rice is necessary to increase green plant regeneration needed for double haploid plant development to be used for breeding purposes.

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