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ORIGINAL RESEARCH

Effect of ethylene inhibitor, type of auxin, and type of sugar on anther culture of local East Java aromatic rice varieties

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Abstract

Breeding using the double haploid approach has been used in accelerating the rice breeding process. However, the main obstacle in the application of this technique in rice is the low level of callus formation and their regeneration into haploid plants. Based on these issues, this research is aimed at developing the best method for obtaining double haploid plants through anther culture using three kinds of aromatic rice of Indonesian local varieties. Anthers of Pendok, Genjah Arum, and Merah Wangi varieties were cultured in callus induction media which were either supplemented with different types of carbon sources, or auxins and ethylene inhibitors. However, the application of different sugar types did not significantly affect callus formation of Pendok and Genjah Arum. However, auxin treatment showed NAA to be more efficient for the callus induction of Pendok, while 2,4-D had no effect on callus induction in all tested varieties. The addition of ethylene inhibitor successfully induced callus formation of Merah Wangi varieties, while the rates of Pendok and Genjah Arum were not significantly affected. Callus of three plant lines were then regenerated into plantlet in regeneration media. Of the three callus varieties used, only Pendok could regenerate into plantlets and grow into adult haploid plants.

Keywords Aromatic rice · Anther culture optimization · Double haploid

Introduction

Aromatic rice is of high market value owing to its superior grain quality and pleasant aroma due to the synthesis of volatile 2-acetyl-1-pyrroline compounds. Fragrant accession has been identified in at least three of the distinct genetic subpopulations of rice, including *Group V* ("Basmati and "Sadri"), *indica* ("Jasmine" varieties), and *tropical japonica*. Indonesia has varieties of aromatic rice categorized as either local or breeding varieties. Several of them have been released by Indonesian Center for Rice Research (ICRR), including Sintanur, Celebes, Gilirang, Cimelati, and Batang Gadis (Tarigan et al. 2014). In addition to the above list, several local aromatic varieties such as Pandan Wangi, Rojo Lele, and Mentik Wangi are very popular within the community.

There are several local rice varieties categorized as aromatic, which are distributed in several regencies in East Java Province. Pendok is local rice originated from Tuban regency, and Genjah Arum is originated from Banyuwangi. However, both Pendok and Genjah Arum seeds contain awn, which are undesirable during milling process. Merah Wangi belongs to the brown rice type widely cultivated in Pasuruan regency. It produces aromatic fragrant during cultivation and when the grains are being steamed.

The double haploid technique is a useful tool for plant breeding due to its advantage to shorten the overall breeding period attributable to the ability to reach 100% homozygosity one generation after the induction of haploids with the

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small population size to obtain the desired genotype (Dunwell 2010; Dwivedi et al. 2015). Conventional rice breeding involves cross-breeding of selected parental lines, followed by mass selection for up to eight generations to produce new plant lines with high genetic stability. The production of new rice variety using double haploid is usually conducted using anther culture method. In China, anther culture techniques play a role in producing over 100 rice varieties, while several lines have been reported in India, Japan, South Korea, Hungary, and USA (Siddique 2015). However, in Indonesia there has been no report on the generation of new rice varieties through double haploid techniques.

The success of anther culture techniques in the production of double haploid plant depends on a number of endogenous and exogenous factors of the donor plant. The endogenous factor is tightly related to the physiological status, such as hormones and nutritional level within the cell, whereas the exogenous factors associated with the growing environmental conditions such as light intensity, photoperiodicity, nitrogen status, and temperature affect the quality of donor crops (Germanà 2011). It has been reported that rice plants grown in the fields brought about a higher number of double haploid plants than those grown in the greenhouse or pots (Mishra and Rao 2016). The growth phase of pollen has also been identified as a crucial aspect influencing the success of callus formation. Also, it was reported that anthers in early to mid-uninucleate developmental stage are suitable for callus induction in wheat (Tadesse et al. 2012). Moreover, cold pre-treatment by incubation of rice flowers at 4 °C for 3 to 7 days generated highest frequency of embryos and green plantlets (Khatun et al. 2012). The positive effects of this pre-treatment are the delay of pollen grain senescence, increase in the symmetric division of pollen grains, and induced metabolic change necessary for androgenesis (Kaushal 2014). Another very important factor contributing to the successful establishment of a double haploid plant is the composition of tissue culture media. The application of 40 g L^{-1} maltose as carbon source was reported to significantly increase the callus induction, compared to that of 40 g L^{-1} sucrose or 40 g L^{-1} mixture of sucrose and maltose using anther of four Iranian rice varieties. However, cultivar regeneration was not significantly affected (Bagheri et al. 2009). Rice androgenesis might also be increased by the addition of polyamides such as putrescine into the culture media, given that the application of 10^{-3} M putrescine was sufficient to inhibit ethylene biosynthesis as anther senescence delayed and decreased 1-aminocyclopropane-1-carboxylic acid (ACC) content in anther-derived calli (Dewi and Purwoko 2016).

In our experiment, we tested the effects of auxin, carbon source, and ethylene inhibitor on anther culture of Pendok, Genjah Arum, and Merah Wangi varieties which originated from East Java province, Indonesia. The main object of our research is to analyze the anther culturability of the three varieties of aromatic rice under several treatments such as different types of auxin, carbon source, and addition of ethylene inhibitor. Our results showed that the supplementation of anther culture media using maltose results in the highest callus formation in Pendok variety. The utilization auxin in the form of 2,4-D resulted in the highest rate of callus formation in both Pendok and Genjah Arum varieties. Moreover, the application of ethylene inhibitor induces the callus formation on Merah Wangi variety, while Pendok and Genjah Arum did not exhibit substantial changes. In the plant regeneration step, we discovered that only callus of Pendok could regenerate into plantlet, whereas Genjah Arum and Merah Wangi did not develop any shoots after incubation in the regeneration medium.

Materials and methods

Experimental material

This research was conducted in January–September 2017 in the Division of Molecular biology and Biotechnology, Center for Development of Advanced Science and Technology (CDAST) University of Jember. Three local rice cultivars: Pendok, Genjah Arum, and Merah Wangi originated from three regencies in East Java Indonesia (Tuban, Banyuwangi, and Pasuruan, respectively) were used in this study. Based on their morphological characteristics, Pendok and Genjah Arum are classified as *javanica* varieties, while Merah Wangi is classified as *indica*. Seedlings of three rice varieties were grown in the irrigated rice field until the plant entering the late booting stage as indicated by the appearance of the swollen stem. The stems of the three rice varieties containing the developing panicles were then harvested for the materials of anther culture experiment in the laboratory.

Treatments

After harvesting, panicles were immediately tightly covered using plastic wrap and aluminum foil, after which were incubated at 4 °C for 10 days. The panicles were then sterilized by shaking gently for 3 min in 1% (v/v) of commercial bleach solution, and subsequently washed five times using sterilized double-distilled water. Anthers were then cultured in solidified N6 medium supplemented with 2 mg L^{-1} NAA or 2,4-D, 0.5 mg L^{-1} Kinetin, and were incubated in dark condition at 28 °C for callus induction. The effect of ethylene inhibitor on callus induction rate was evaluated by adding either putrescine or $AgNO_3$ to the callus induction medium for 1 mM and 10 mg L^{-1} final concentration, respectively. Afterwards, the formed calli

were transferred into solidified N6 medium which was supplemented by 2 mg L^{-1} Kinetin and 0.5 mg L^{-1} NAA, and then grown in a growth chamber with supplemental lighting (8-h dark/16-h light) at 28 °C. The effect of sugar type on callus induction and regeneration was evaluated by supplementing callus induction media and regeneration with 30 g L^{-1} of sucrose, 40 g L^{-1} of maltose, or a combination of glucose (20 g L^{-1}) and sucrose (30 g L^{-1}). The regenerated green plantlets were transferred onto solidified MS containing IAA (0.5 mg L^{-1}) and sucrose (30 g L^{-1}).

Results

Effects of different types of auxins on callus formation

The effects of different types of auxins for anther-derived callus formation at 3-week incubation were examined using 2,4-D and NAA. We observed that anther of Pendok varieties was approximately 40% higher in callus induction media compared to Genjah Arum in callus induction media with NAA supplementation. However, the callus induction rate of Genjah Arum and Pendok was not different in callus induction media with 2,4-D supplementation. In contrast to Pendok and Genjah Arum, Merah Wangi variety did not show any callus formation in both auxin treatments. These results indicate that NAA is more effective for callus formation in Pendok, as 2,4-D did not affect callus induction rate in both Pendok and Genjah Arum. In addition, Merah Wangi was less responsive to the application of both NAA and 2,4-D at 2 mg L^{-1} concentration (Fig. 1).

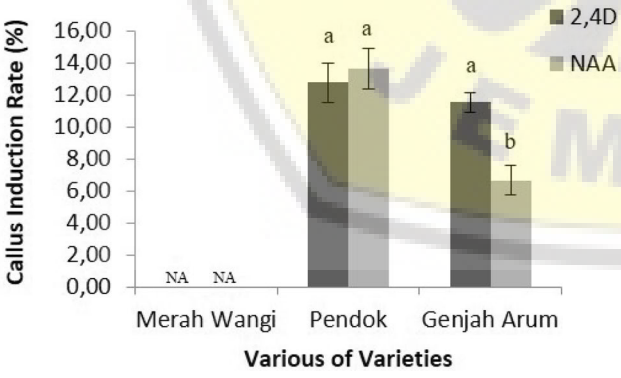

Fig. 1 Effect of different types of auxin on the formation of callus anther rice varieties Pendok, Genjah Arum, and Merah Wangi at 3 weeks of culture. Mean values of callus induction frequency marked with the same letters do not differ significantly ($P \leq 0.05$) in DMRT. Vertical bar represents ± SD ($n = 3$); NA, data are not available

Effects of different types of sugar on callus formation

Sugar is used as the primary energy source for the growth and development of explant in tissue culture. However, sucrose has been widely used as a source of carbon in anther culture, while maltose has been reported to result in superior androgenesis response in several species (Sen and Singh 2011). In this study, we compare and evaluate the effects of sucrose, maltose, and the mixture of sucrose and glucose for callus induction in Pendok, Genjah Arum, and Merah Wangi varieties. In our experimental condition, the application of maltose resulted in the highest rate of callus induction in Pendok, but Genjah Arum showed about 60% lower than Pendok in callus induction rate in maltose-supplemented media. Moreover, sucrose or combination of sucrose and glucose did not significantly affect the callus formation rate in Pendok dan Genjah Arum (Fig. 2). However, the application of the three types of sugar did not successfully induce callus formation on the anther of Merah Wangi variety. This result indicates that maltose is the best sugar form for callus induction, at least in Pendok variety.

Effect of ethylene inhibitor on callus formation

The effect of ethylene inhibitor on Pendok, Genjah Arum, and Merah Wangi anther culturability was evaluated in callus induction experiment by the addition of two ethylene-inhibiting agents (putrescine and $AgNO_3$). In the absence of these inhibitors, only Pendok and Genjah Arum showed callus formation at 3-week incubation period, which was not the case in Merah Wangi variety. Addition of 1 mM

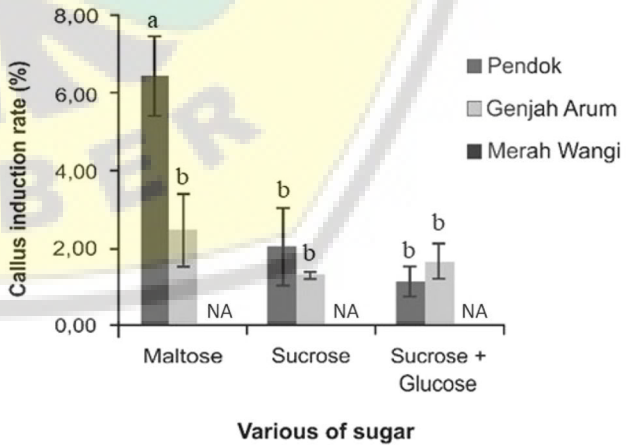

Fig. 2 Effect of different types of sugar on the formation of callus anther rice varieties Pendok, Genjah Arum, and Merah Wangi. Mean values of callus induction frequency marked with the same letters do not differ significantly ($P \leq 0.05$) in DMRT. Vertical bar represents ± SD ($n = 3$); NA, data are not available

putrescine or 10 mg L^{-1} of $AgNO_3$ for callus induction had no effect on the callus induction rate of Pendok and Genjah Arum. However, both putrescine and $AgNO_3$ treatments resulted in callus formation in Merah Wangi even though the rate was lesser than that of Pendok and Genjah Arum varieties (Fig. 3). This result indicates that ethylene inhibitor for callus induction is effective for recalcitrant type, but not for the varieties with high ability to form callus.

Plantlet regeneration

Regeneration was carried out by transferring the anther-derived callus from three rice varieties onto solidified Murashige and Skoog (MS) medium which was then supplemented with 2 mg L^{-1} of kinetin and 0.5 mg L^{-1} of NAA to produce the haploid plantlets. The initial response for cytokinin treatment was shown by the formation of green spots in the callus of all three rice varieties. Only green spots of callus from Pendok successfully regenerated into plantlets at the subsequent culture period (Fig. 4). The green spots in Genjah Arum and Merah Wangi calluses which could not regenerate became shoot and turned brown over a longer culture period. The combination of sucrose and glucose sugar treatment gave the best results compared to the treatments of maltose and sucrose (Fig. 5).

The effect of ethylene inhibitor on the regeneration of haploid plantlet was examined by the addition of its agents such as putrescine or $AgNO_3$ in the regeneration media. Our result showed that the application of putrescine increases the percentage of callus regeneration of Pendok variety. The anther-derived callus of Pendok successfully produces viable and healthy plantlet in the regeneration media after several weeks of incubation. $AgNO_3$ application, on the other hand, had no effect on the callus becoming plantlet in the three tested varieties (Fig. 6). This result indicates that putrescine is more suitable for callus regeneration, at least in the Pendok variety.

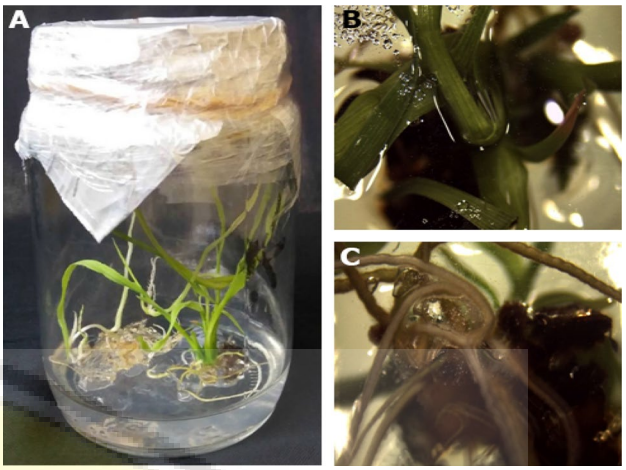

Fig. 4 Haploid rice plantlets. **a** The appearance of the plantlet in the regeneration media, **b** growth area of the stem, **c** root growth area

Discussion

In general, the success of anther culture is dependent on several factors such as physiological condition plant, genotype, conditions of donor, ages of anther, pre-culture treatment, presence of osmotic stress, gamma ray irradiation, and

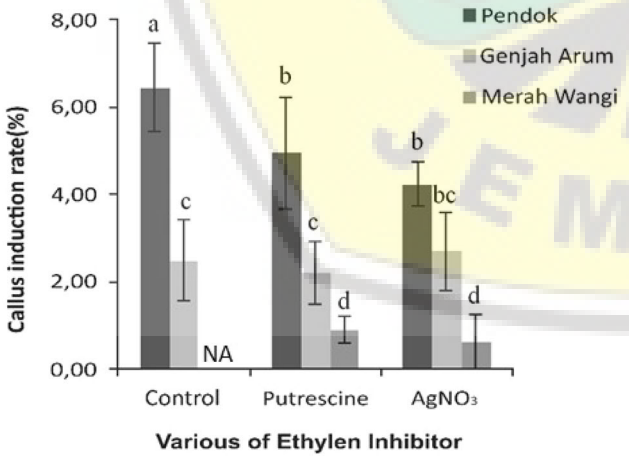

Fig. 3 Effect of ethylene inhibitor on the formation of callus anther rice varieties Pendok, Genjah Arum, and Merah Wangi. Mean values of callus induction frequency marked with the same letters do not differ significantly ($P \leq 0.05$) in DMRT. Vertical bars represent ± SD ($n = 3$); NA, data are not available

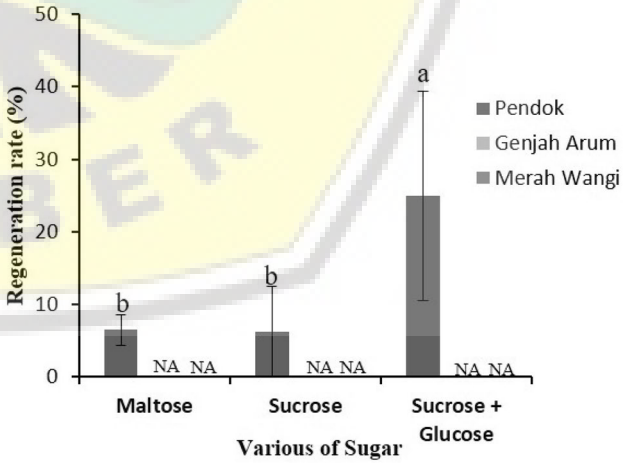

Fig. 5 Effect of different types of sugar on the regeneration haploid plantlet from callus varieties Pendok, Genjah Arum, and Merah Wangi. Mean values of regeneration frequency marked with the same letters do not differ significantly ($P \leq 0.05$) in DMRT. Vertical bars represent ± SD ($n = 3$); NA, data are not available

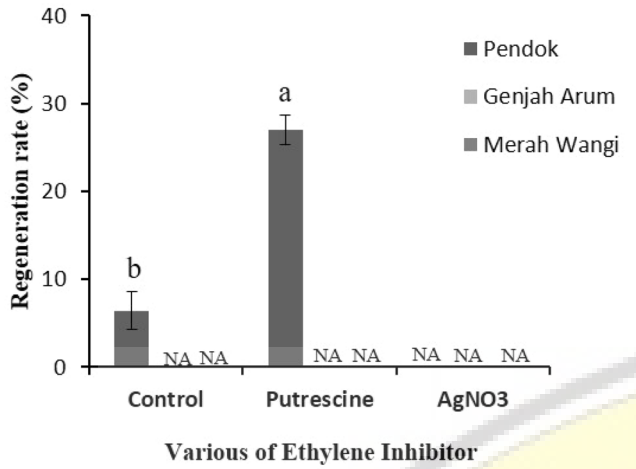

Fig. 6 Effect of ethylene inhibitor on the regeneration haploid plantlet from callus varieties Pendok, Genjah Arum, and Merah Wangi. Mean values of regeneration frequency marked with the same letters do not differ significantly ($P \leq 0.05$) in DMRT. Vertical bars represent ± SD ($n = 3$); NA, data are not available

hormone composition. In the case of hormone composition, our result showed that the application of two different forms of auxins in callus induction media (NAA and 2,4-D) has successfully induced callus formation from anther of Pendok and Genjah Arum variety, while Merah Wangi variety did not result in anther formation. NAA brought about higher callus formation in Pendok, but a different preference was shown by Genjah Arum that callus induction was higher in media with 2,4-D supplementation. It has been reported that the applications of 2 mg L^{-1} NAA to the callus induction media resulted in higher callus formation than 2,4-D at 2 and 2.5 mg L^{-1} in the anther culture of superior IR-69690 rice varieties (Islam et al. 2004). The other studies reported that the application of 2,4-D at 2 mg L^{-1} in anther culture of japonica rice varieties of Azucena resulted in higher level of callus development than that of various concentrations of NAA (Sharma et al. 2017). These results indicate that both NAA and 2,4-D as auxin sources can be used for callus induction. Apparently, efficiency of NAA or 2,4-D in callus induction tightly correlates with rice genotype.

In addition to the type of auxin, various types of sugar were used as carbon sources of anther culture. Our result showed that the application of maltose produces higher callus formation than sucrose or mixture of glucose and sucrose in the Genjah Arum or Pendok. The different types of sugar did not result in the positive effect on callus formation in Merah Wangi variety, given that callus formation was absent in all types of callus induction media (Fig. 7). Maltose has been reported to be the carbon source that gives better effects compared to the other types of sugar in the stimulation of callus development. Moreover, it is also known for its slow hydrolysis and its ability to maintain the osmotic stability of culture media.

The recalcitrant-type Merah Wangi showed a difficulty in callus formation, and this might be consequently due to high ethylene activity during callus induction period. Putrescine and $AgNO_3$ have been known for their ethylene inhibitory activity, and we, therefore, investigate the effect of these two ethylene inhibitors on anther culture.

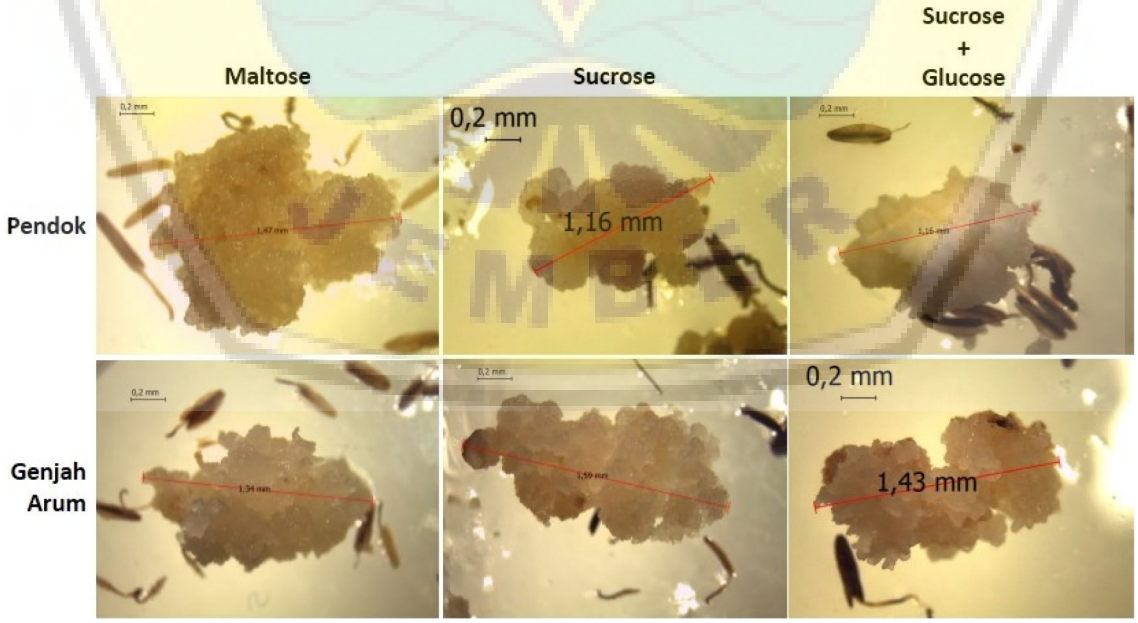

Fig. 7 Condition of anther callus of varieties of Pendok and Genjah Arum in media of callus induction containing maltose, sucrose, and combination sucrose + glucose

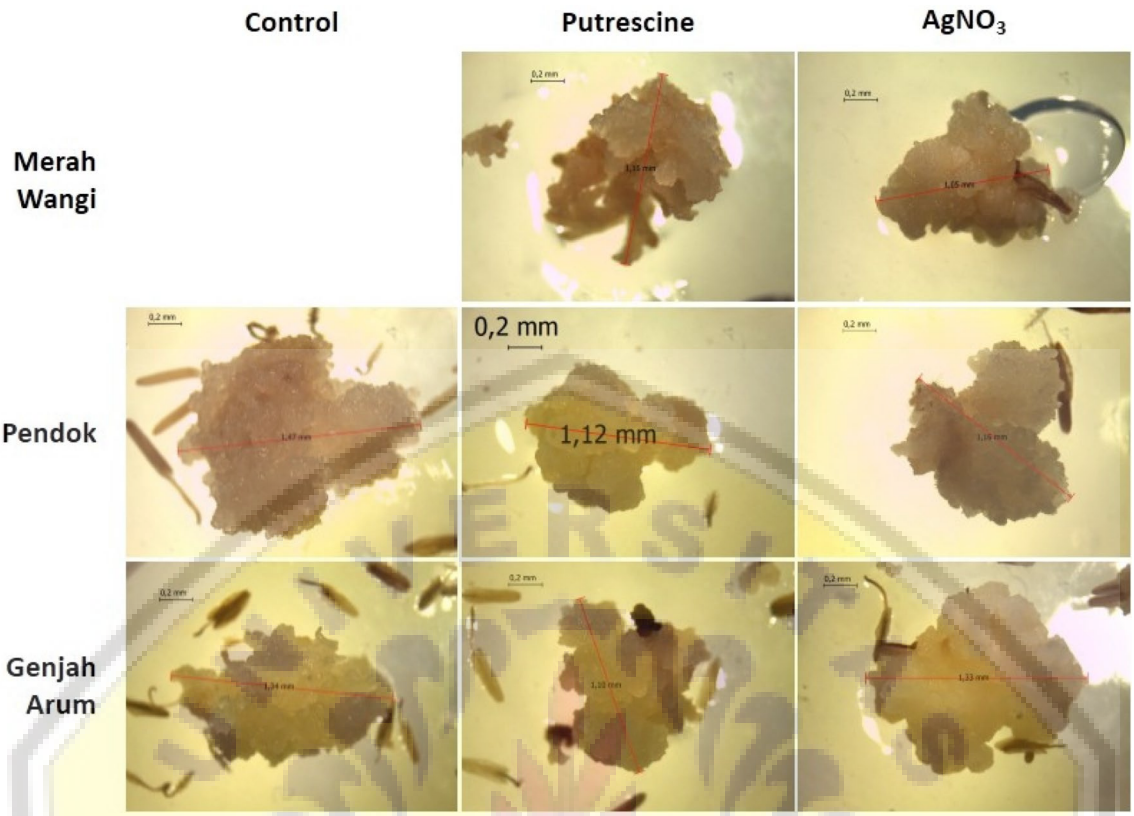

Fig. 8 Condition of anther rice callus of Pendok, Genjah Arum, and Merah Wangi varieties in callus induction media containing two types of ethylene inhibitor

Our results showed that the application of these inhibitors resulted in callus development in Merah Wangi variety, although to a lesser extent. However, no significant effect was observed in the callus development of Genjah Arum and Pendok (Fig. 8). Dewi and Purwoko (2016) reported that the application of 10^{-3} M putrescine to the callus induction media inhibits anther senescence and induces the regeneration plantlet. Moreover, Kaushal et al. (2014) reported that $AgNO_3$-supplemented media result in an identical effect as putrescine on callus formation and plantlet regeneration. The ethylene inhibitory activities of putrescine and $AgNO_3$ have been reported to prevent the biosynthesis of ethylene. The biosynthesis of ethylene initiates the activation of ACC synthase enzyme (ACS) to catalyze the conversion of *S*-adenosyl-L-methionine (SAM) to 1-aminocylopropane-1-carboxylic-acid (ACC), which is the precursor for ethylene synthesis. With the presence of ethylene inhibitor, SAM could not be processed into ethylene, but was used for the morphogenetic responses, and in stimulating the development of root, callus, apical, and the regeneration process.

Conclusion

Based on our results, we conclude that the application of maltose as the carbon source results in the highest rate of callus formation in Pendok, whereas the addition of sucrose or mixture of sucrose and glucose did not improve the callus formation in all tested rice varieties. For the induction of callus, auxin in the form of NAA is more effective for Pendok variety, while 2,4-D is more effective for Genjah Arum. The addition of ethylene inhibitor only brings effect on callus formation in Merah Wangi variety, while Pendok and Genjah Arum showed no difference in their callus formation rate. Moreover, callus of Pendok has the highest plantlet regeneration rate among the three used rice varieties.

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Author contributions DPA: performed the tissue culture experiment, collected the data, and data analysis. FVP: performed tissue culture experiment and collected the data. BS: data analysis. SA: data analysis. WIDF: design the analysis, data analysis, and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

Novelty statement Pendok, Genjah Arum, and Merah are aromatic rice varieties originated from East Java, Indonesia. We subjected these three rice varieties to anther culture optimization using culture media supplemented with either of ethylene inhibitor, auxin, and sugar. Among the three varieties, we found that Pendok showed the highest rate in callus formation and plant regeneration, whereas Merah Wangi showed the lack of response to those two parameters. Furthermore, maltose is the best sugar for callus induction in Pendok, and 2,4-D is the suitable auxin for callus induction. Finally, we found that ethylene inhibitor was only effective for callus induction rate of recalcitrant-type Merah Wangi.

References

Bagheri N, Babaeian-Jelodar N, Ghanbari A (2009) Evaluation of effective factors in anther-culture of Iranian Rice (*Oryza sativa* L.) cultivars. Biharean Biol 3:119–124

Dewi IS, Purwoko BS (2016) Role of polyamines in inhibition of ethylene biosynthesis and their effects on rice anther-culture development. Indo J Agric Sci 9:60–67

Dunwell JM (2010) Haploids in flowering plants: origins and exploitation. Plant Biotechnol J 8:377–424

Dwivedi SL, Britt AB, Tripathi L, Sharma S, Upadhyaya HD, Ortiz R (2015) Haploids: constraints and opportunities in plant breeding. Biotechnol Adv 33:812–829

Germanà MA (2011) Anther-culture for haploid and doubled haploid production. plant cell. Tissue Organ Cult (PCTOC) 104:283–300

Islam MM, Adhikary SK, Gain P, Rahman MM, Siddique NA (2004) Effect of plant growth regulators on callus induction and plant regeneration in anther-culture of rice. Pak J Biol Sci 7:331–334

Kaushal L (2014) Effect of culture media on improving anther-culture response of rice (*Oryza sativa* L.). Int J Agric Innov Res 1:1473–2319

Kaushal L, Sharma R, Balachandran SM, Uluganathan K, Shenoy V (2014) Effect of cold pretreatment on improving anther-culture response of rice (*Oryza sativa* L.). Exp Biol Agric Sci 2:234–242

Khatun R, Islam SMS, Ara I, Tuteja N, Bari MA (2012) Effect of cold pretreatment and different media in improving anther-culture response in rice (*Oryza sativa* L.) in Bangladesh. Ind J Biotechnol 11:458–463

Mishra R, Rao GJN (2016) In-vitro androgenesis in rice: advantages, constraints and future prospects. Rice Sci 23:57–68

Sen C, Singh RP (2011) Anther-culture response in Boro rice hybrids. Asian J Biotechnol 3:470–477

Sharma A, Lal D, Sutradhar M (2017) Effect of growth regulators on callus morphology of rice anther-culture. J Appl Biol Biotechnol 5:068–071

Siddique R (2015) Impact of different media and genotypes an improving anther-culture response in rice (*Oryza sativa*) in Bangladesh. Eur Sci J 11:164–169

Tadesse W, Inagaki M, Tawkaz S, Baum M, van Ginkel M (2012) Recent advances and application of doubled haploids in wheat breeding. Afr J Biotechnol 11:15484–15492

Tarigan E, Jumali J, Kusbiantoro B (2014) Karakteristik flavor beras varietas padi aromatik dari ketinggian lokasi yang berbeda. Jurnal Penelitian Pertanian Tanaman Pangan 33:27–35