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Effect of Organic Additives on Regeneration of Orchid Hybrid (*Dendrobium* 'Bertachong' X *Dendrobium* 'Blackspider X Sutiknoi')

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Abstract. *Dendrobium* is a genus of orchids that has been widely planted, both domestically and internationally. Orchid conventional breeding is generally done to produce new variants, but seeds from crosses are generally difficult to germinate. To solve this problem, tissue culture has long been used to germinate orchid seeds. This study aims to obtain a suitable medium composition for growing seeds from crosses of *Dendrobium* 'Bertachong' X *Dendrobium* 'Blackspider x Sutiknoi'. The seeds were grown on two different media types, MS (Murashige-Skoog) and VW (Vacin-Went), with the addition of 2% sucrose and with or without the addition of organic extracts (either mungbean sprouts extract or coconut water). Plantlets were sub-cultured every 4 weeks for 2 times on the same medium but with the addition of activated charcoal, and followed by a third subculture with the addition of 1 $mg.L^{-1}$ NAA (Naphthalene Acetic Acid) in each medium. The best growth shown by fast regeneration and good seedling quality (multiple buds and roots formed) was obtained on MS medium with the addition of 2% sucrose and 15% mungbean sprouts extract.

INTRODUCTION

Plants are potentially capable to propagate using their seeds (generative propagation), also propagating using leaf, stem, and root cutting in support environmental conditions [1]. Indonesia is one of the countries with the most orchid diversity. Orchids are ornamental plants that are extensively sold as cut flowers or potted flowers and very popular in the floriculture sector due to their lovely shapes and colors [2]. *Dendrobium* sp., an epiphytic sympodial orchid, is one of the most popular orchid species [3], which difficult to germinate in natural conditions and grow slowly [4]. This may give an impact to fulfill the market demand, particularly among orchid collectors. In order to solve these issues, plant tissue culture techniques or *in vitro* propagation might be used.

Seeds of orchids can be germinated to form protocorm like bodies (PLBs) and grow into seedlings using in vitro propagation procedures. By adding nutrients to the culture media, in vitro propagation can support in the rapid growth of *Dendrobium* sp. under sterile and regulated circumstances. *Dendrobium* sp. growth is influenced by a variety of factors, including genotype, explant type, and media composition [5]. The growth media, PGRs (Plant Growth Regulators), ambient conditions, and others are all important factors to consider when propagating in vitro.

There are several distinct types of media that can be used for in vitro plant propagation, each with its own macronutrient and micronutrient contents, such as MS, VW, NP (New Phalaenopsis), and KC (Knudson). Salt, vitamins, minerals, carbon sources, and growth regulators are commonly found in orchid propagation media [6] also in many other plant growth media. Growth regulators are frequently added to the culture media. Natural PGRs and chemical/artificial PGRs are the two types of plant growth regulators. Natural PGRs can be found in organic materials such as coconut water, mungbean sprout extract [7], apple extract, banana extract, potato extract, and many more, whereas chemical/artificial PGRs can be found in market such as 2,4-D (2,4-Dichlorophenoxyacetic acid), TDZ (Thidiazuron), BAP (Benzyl Amino Purin), IAA (Indole Acetic Acid), NAA, and others.The addition of organic compounds to the culture media can increased the number of orchids that germinated [8, 9, 10, 11].

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According to Lawalata *et al*. [12], coconut water contains auxin and cytokinin, which can help orchid explant growth in vitro.The use of mungbean sprout extract as a natural plant growth regulator may be owing to its high vitamin content compared to their seeds, where the use of 150 g/L mungbean sprout extract resulted in the maximum yield of moth orchid growth [14]. Ulfa [15] found 1.68 ppm auxin, 39.94 ppm gibberellin, and 96.26 ppm cytokinin in the mungbean sprout extract.

The selection of media and plant growth regulators, as well as the nutrient composition, also organic additives in growth media, are all key factors in determining the variance that occurs during in vitro propagation [16]. The use of different culture media showed different efficiency of orchid regeneration during in vitro propagation. According to Aktar*et al*. [17], utilizing half-strength MS medium with the addition of sabri banana resulted in the highest number of PLBs, fresh weight of PLBs, and number of shoots explant in *Dendrobium* sp. when compared to utilizing other media with the addition of sabri banana. Furthermore, in *Dendrobium* sp. cultivated on MS medium with 2,4-D, the production of PLBs and effective plantlet regeneration were added by Nasiruddin *et al.* [18]. According to Utami and Sucipto [4], VW media was recommended for in vitro germination and protocorm formation. *Phalaenopsis amboinensis* seedlings grew and developed roots optimally on VW media with the addition of 15% coconut water (v/v) and 10 g/ L banana homogenate. In vitro propagation of orchid needs specific media composition, so the aim of this research is determining the composition of media and appropriate organic extract for the fast-growing seedsfrom crosses of *Dendrobium 'Bertachong'* X *Dendrobium 'Blackspider x Sutiknoi'* hybrid orchids.

EXPERIMENTAL DETAILS

Plant Materials

Plant materials in this research were *Dendrobium* 'Bertachong' and *Dendrobium* 'Blackspider x Sutiknoi' obtained from Dede Orchid Nursery, Batu, Malang.

Surface Sterilization of Explants

Hybrid orchid pods from crosses of *Dendrobium* 'Bertachong' and *Dendrobium* 'Blackspider x Sutiknoi' with the complete cell wall were harvested, then washed under running tap water with detergent. After that, the pods were sterilized in LAF (Laminar Airflow) Cabinet by dipping in 96% ethanol and flamed. The cell walls of sterilized pods were split opened with sterile surgical blades and seeds of the hybrid orchid were cultured on all of treatment medium (Table 1). The cultures incubated at 26^{o}C under a photoperiod of 16 h light. Subculture was performed every 4 weeks with the addition of 2% charcoal at second subculture (8 weeks after seeds sowing) and addition of 1 mg.L^{-1} NAA at third subculture (12 weeks after seeds sowing) of all treatment medium.

Regenerating Cultures

The *Dendrobium* 'Bertachong' X *Dendrobium* 'Blackspider x Sutiknoi' (*Dendrobium* 'Bertachong' female) pods were then cultured in MS and VW media with the addition of coconut water and mungbean sprout extract as organic compound (Table 1).

The MS and VW media used in these experiments were full strength and half strength. Coconut water or mungbean sprout extract as organic compound of each medium according to treatment, and 2% sucrose was added to all medium.

Preparation of 15% Coconut Water and 15% Mungbean Sprout Extract

The 15% coconut water were prepared using young coconut. The coconut cracked opened and collect every 15 mL coconut water into plastic and kept frozen in a freezer at -4°C prior to use [19]. Then, 15 mL coconut water will be added into every 100 mL MS or VW media, so the final concentration of coconut water is 15%, whereas 15% mungbean sprout extract prepared by blender 15 gram mungbean sprout in 100 mL aquadest, then collect every 15 mL filtrate mungbean sprout as mungbean sprout extract and kept frozen in a freezer at -4°C prior to use. Then, 15 mL mungbean sprout will be added into every 100 mL MS or VW media, so the final concentration of mungbean sprout extract is 15%.

TABLE 1. Medium which contains organic extract for growth of seeds resulted from Crossing of *Dendrobium* 'Bertachong' X *Dendrobium* 'Blackspider x Sutiknoi'

Medium Code	Medium composition
A	1/2 MS + 15% coconut water + 2% sucrose
B	1/2 VW+ 15% coconut water + 2% sucrose
C	1/2 MS + 15% mungbean sprout extract + 2% sucrose
D	1/2 VW + 15% mungbean sprout extract + 2% sucrose
E	MS + 15% coconut water + 2% sucrose
F	VW+ 15% coconut water + 2% sucrose
G	MS + 15% mungbean sprout extract + 2% sucrose
H	VW + 15% mungbean sprout extract + 2% sucrose

Statistical Analysis

The experiment was designed in a completely randomized design with eight treatment (A-H) and tenth replication. Data was analysed using one way ANOVA (Analysis of Variance). Duncan's Multiple Range Test (DMRT) at 5% error level (α=0.05) was used in the case of significant difference was observed. Germination observed, percentage of normal dan uniform plantlets from each subculture stage (4, 8, and 12 weeks), percentage of plantlets with four leaves and many number of roots at the last subculture stage (16 weeks) were used as observed variables.

RESULT AND DISCUSSION

Pods Resulted from the Crosses of Dendrobium 'Bertachong' X Dendrobium 'Blackspider x Sutiknoi'

The crosses of *Dendrobium* 'Bertachong X *Dendrobium* 'Blackspider x Sutiknoi' resulting in pods with were about 4.0 cm long, which were harvested after 4 months after crossing (Fig. 1).

FIGURE 1. The Pods as Result of Crossing of *Dendrobium* 'Bertachong X *Dendrobium* 'Blackspider x Sutiknoi'.

Effect of Medium and Organic Compound on the Growth of Embryo Resulted from Crosses of Dendrobium 'Bertachong X Dendrobium 'Blackspider x Sutiknoi'

Effect of Medium and organic compound on the growth of embryo resulted from crosses of *Dendrobium* 'Bertachong X *Dendrobium* 'Blackspider x Sutiknoi' to %Plantlet with normal and uniform growth and %Four-leafed Plantlet with many roots shown at Table 2 below:

TABLE 2. Effect of Medium and Organic Compound on the Growth of Embryo Resulted from Crosses of *Dendrobium* 'Bertachong X *Dendrobium* 'Blackspider x Sutiknoi'

Medium Code	Day to germinate	% Plantlet with normal and uniform growth			% Four-leafed Plantlet with many roots
		4 w	8 w (with 2% charcoal)	12 w (with 1 mg.L^{-1} NAA)	16 w
A	15	70±0.36^{ab}	70±0.28^{ab}	70±0.73^{a}	75±0.15^{a}
B	15	65±0.65^{a}	65±0.45^{a}	70±0.29^{a}	75±0.21^{a}
C	14	80±0.82^{c}	80±0.37^{c}	80±0.18^{c}	80±0.32^{b}
D	14	75±0.71^{bc}	75±0.58^{bc}	75±0.45^{b}	80±0.52^{b}
E	12	75±0.65^{bc}	75±0.24^{bc}	80±0.75^{c}	80±0.24^{b}
F	12	70±0.35^{ab}	70±0.46^{ab}	80±0.84^{c}	80±0.36^{b}
G	7	90±0.47^{d}	90±0.39^{d}	90±0.42^{d}	95±0.24^{d}
H	10	80±0.18^{c}	80±0.21^{c}	80±0.55^{c}	85±0.45^{c}

Values followed by the same letter in the same column were not significantly different ($p > 0.05$ by DMRT). w : week

Effect of Medium and Organic Compound to Germination Rate of Embryo Resulted from Crosses of Dendrobium 'Bertachong' X Dendrobium 'Blackspider x Sutiknoi'

Varied responses of *Dendrobium* 'Bertachong' X *Dendrobium* 'Blackspider x Sutiknoi' embryo cultured on different types of media (MS and VW medium), different strength of MS and VW medium (full strength and half strength), and different MS or VW medium supplemented with organic compound was observed at the Table 2. Based on Table 2, different type of medium could result in varied responses of embryo germination rates, in which MS media giving an equal to higher germination rate than VW media both in full strength or half strength (compared with same organic compound and strength medium in different type medium). The difference of the two types of media in this research were their compositions. MS medium is a medium with highly enriched with macroelements and microelements, also different vitamins [20], whereas VW medium is a medium contained macroelements and microelements, with lower concentration of vitamins compared to MS medium. Seed germination and seedling development of *Cymbidium aloifolium* (L.) Sw. was promoted by various medium [21].

Each type of medium, such as MS medium and VW medium result the higher germination rate of embryo at full strength medium than half strength medium, as shown at Table 2, which at MS medium both full strength and half strength, the germination started after 7, 12, 14, and 15 days after cultured, whereas at VW medium both full strength and half strength, the germination started after 10, 12, 14, and 15 days after cultured. According to research of Pradhan [21], showed the best composition medium for *Cymbidium aloifolium* (L.) Sw. was full MS medium supplemented with hormones and followed by hormone free full strength MS medium, ½ MS medium, and ¼ MS in which germination started after 10, 12, and 15 weeks of primary culture respectively.

The optimum media composition for germination rate of embryo was full strength MS medium with 15% mungbean sprout extract and 2% sucrose. There were two types of organic compounds used in this research, such as coconut water and mungbean sprout extract. Organic compounds can be used to replace the role of synthetic hormones, because the application of synthetic hormones in tissue culture medium causing high production cost. The coconut water contain vitamin [22], such us vitamin B1, B2, B3, C, and others, also amino acids which can increase vitamin content in the tissue culture medium. Whereas enrichment with mungbean sprout extract, which contains essential amino acids and minerals, also affect the success of in vitro propagation. The germination of embryos on full strength MS medium with 15% mungbean sprout extract and 2% sucrose started at 7 days after cultured, whereas germination of embryo at same full strength MS medium, but with addition of 15% coconut water and 2% sucrose started at 12 days after planting. Similar results were observed using full strength VW medium,

which germination of embryos at full strength VW medium with 15% mungbean sprout extract and 2% sucrose were faster than germination of embryo at same VW medium with 15% coconut water and 2% sucrose. According to Table 2, germination of embryo was faster at MS medium or VW medium with addition of mungbean sprout extract than addition of coconut water (explanation above).

Effect of Medium and Organic Compound to Percentage of Normal and Uniform Plantlet

Percentage of plantlet which were normal and grow up uniformly was influenced by media composition, such as type of medium, level concentration of medium, and also organic compounds which is added into the medium. At second subculture (8 weeks after seeds sowing), 2% charcoal will be added. Charcoal act as anti-browning agent, that can cause death in explants. Similar results with the other observed variables, percentage of plantlet which normally and uniformly grow up was higher in MS medium than in VW medium, at both full strength and half strength. Plantlets which normally and uniformly grow up provide an indication of the suitable media composition for *Dendrobium* 'Bertachong' X *Dendrobium* 'Blackspider x Sutiknoi' hybrid orchid. The optimum treatment for this observed variable was full strength MS medium with 15% mungbean sprout extract and 2% sucrose, which resulted 90%, 90%, and 90% normal and uniform growth at 4, 8, and 12 weeks after incubated respectively (Table 2). The germination and development of *Dendrobium* 'Bertachong' X *Dendrobium* 'Blackspider x Sutiknoi' orchid seed on MS medium with 15% mungbean sprout extract and 2% sucrose can be seen at Fig. 2.

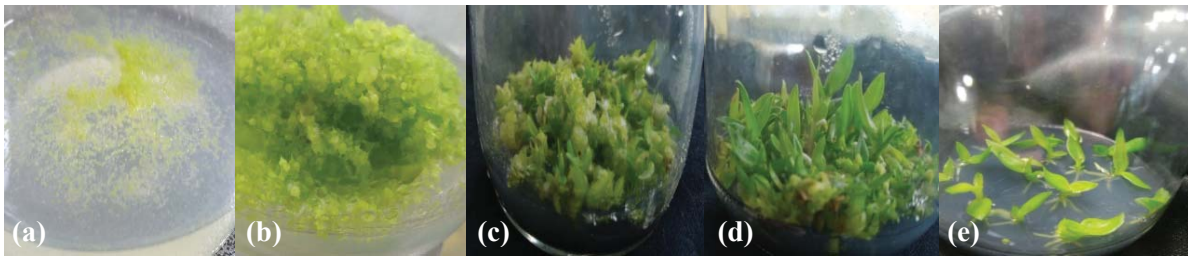

FIGURE 2. The germination and development of *Dendrobium*'Bertachong'X'Blackspider x Sutiknoi' orchid seed germination on MS + 15% mungbean sprout extract + 2% sucrose. (a) seed germination 14 days after sowing, (b) seedling growth 4 weeks after sowing, (c) seedling growth 8 weeks after sowing, (d) seedling growth 12 weeks after sowing, (e) plantlet 16 weeks after sowing.

Effect of Media and Organic Compound to Percentage of Four-leafed Plantlet with Many Roots Resulted from Crosses of Dendrobium 'Bertachong' X Dendrobium 'Blackspider x Sutiknoi'

Effect of media and organic compounds to percentage of four-leafed plantlet with many numbers of roots also observed. The use of MS medium resulted higher percentage of four-leafed plantlet with many roots than VW medium, both full strength and half strength. When compared the use of organic compounds mungbean sprout extract and coconut water with the same type and strength of medium, the result shown that mungbean sprout extract would produce a higher percentage of four-leafed plantlet with many roots than use coconut water as organic compound (Table 2). Mungbean sprout extract contains essential amino acids, such as tryptophan which is the most important organic substance in auxin biosynthesis, especially in the biosynthesis process of IAA (Indole Acetic Acid), in which tryptophan act as precursor [13]. Auxin can promote root growth, so the plantlet would have many roots. According to research of Amilah dan Astuti [14], addition of beansprout extract in the media stimulate the root growth of moon orchid (*Phalaenopsis amabilis* L.) when compared to control. The tissue differentiation for formation of shoots was promoted by cytokinins, which will form a leaf.

Cytokinins and auxin in coconut water both can provide interaction effects on tissue differentiation, while cytokinin at relatively high levels will promote the formation of stems or shoots [24], whereas if auxin at relatively at high level will promote root formation.The optimum medium composition for the highest percentage of four-leafed plantlet with many roots was half strength MS medium with 15% mungbean sprout extract and 2% sucrose, with percentage at 16 weeks was 95% (Table 2). High number of roots at plantlet also can caused by addition of 1 $mg.L^{-1}$ NAA started at third subculture (12 weeks after seeds sowing) into all medium treatments.

Effect of Medium and Organic Compound to Number of Leaves and Roots Resulted from Crossing of Dendrobium 'Bertachong' X Dendrobium 'Blackspider x Sutiknoi'

Effect of Medium and organic compound on the growth of embryo resulted from crosses of *Dendrobium* 'Bertachong X *Dendrobium* 'Blackspider x Sutiknoi' to number of leaves and roots shown at Table 3.

TABLE 3. Effect of medium and organic compound on 20th weeks after planting

Medium Code	No of Leaves	No of Roots
A	3.2±0.027[a]	4.5±0.025[a]
B	3.4±0.064[a]	5.2±0.099[b]
C	3.5±0.055[a]	5.5±0.092[b]
D	3.5±0.058[a]	5.9±0.081[c]
E	5.2±0.079[b]	5.2±0.085[b]
F	5.1±0.052[b]	5.8±0.085[bc]
G	5.9±0.025[c]	7.1±0.085[d]
H	5.5±0.013[b]	6.9±0.059[d]

Values followed by the same letter in the same column were not significantly different ($p > 0.05$ by DMRT). All treatment medium contained 1 $mg.L^{-1}$ NAA

Effect of Medium and Organic Compound to Number of Leaves Resulted from Crossing of Dendrobium 'Bertachong' X Dendrobium 'Blackspider x Sutiknoi'

Effect of medium and organic compounds to number of leaves also observed. The use of half strength MS medium results less number of leaves than half strength VW medium, but the use of full strength MS medium result higher number of leaves than full strength VW medium. When compared the use of organic compounds mungbean sprout extract and coconut water with the same type and strength of medium, the result shown that mungbean sprout extract would produce a higher number of leaves than use coconut water as organic compound (Table 3). Coconut water contain high endogenous cytokines which can induce shoot, also amino acids, organic acids, purines, sugars, alcohol, vitamins, minerals, nucleic acids, and growth regulators, can induce callus and morphogenesis process [24]. So, number of leaves of *Dendrobium 'Bertachong' X 'Blackspider x Sutiknoi'* with organic compound coconut water less than use mungbean sprout extract but not significantly.

Effect of Medium and Organic Compound to Number of Roots Resulted from Crossing of Dendrobium 'Bertachong' X Dendrobium 'Blackspider x Sutiknoi'

Effect of medium and organic compounds to number of roots also observed. The use of half strength MS medium results less number of roots than half strength VW medium, but the use of full strength MS medium result higher number of leaves than full strength VW medium. When compared the use of organic compounds mungbean sprout extract and coconut water with the same type and strength of medium, the result shown that mungbean sprout extract would produce a higher number of leaves than use coconut water as organic compound (Table 3). That can be understood, because coconut water contains a cytokine which has role to stimulate shoots not for improve the roots [25], whereas mungbean sprout extract contains essential amino acids, such as tryptophan which is the most important organic substance in auxin biosynthesis, especially biosynthesis process of IAA (Indole Acetic Acid). Auxin can promote root growth, so the plantlet would have many roots. Visually, roots morphology of *Dendrobium* 'Bertachong' X *Dendrobium* 'Blackspider x Sutiknoi' with organic compound coconut water usually shorter and less. This happen because high content of cytokines on coconut water.

Selected plantlets resulted from crossing of Dendrobium 'Bertachong' X Dendrobium 'Blackspider x Sutiknoi' for acclimatization stage

After the germination of embryo resulted from crosses of *Dendrobium* 'Bertachong' X *Dendrobium* 'Blackspider x Sutiknoi', several plantlets were selected as plantlets which ready for acclimatization at the end of

the 20th weeks, with the criteria have many long roots and at least four leaves. The plantlets which ready for acclimatization can be seen below at Fig. 3:

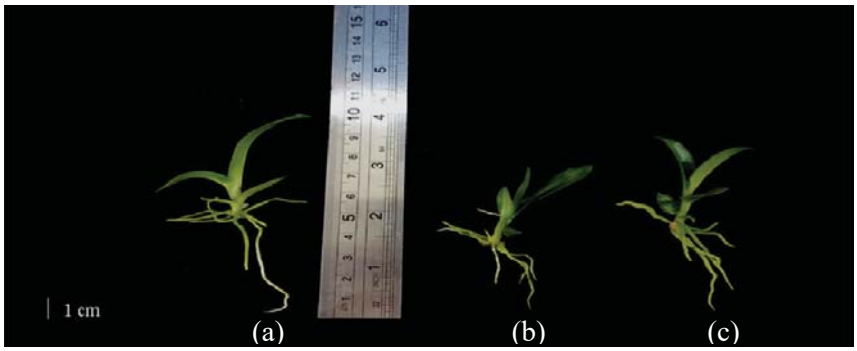

FIGURE 3. Plantlets ready for acclimatization stage at the end of the 24th weeks. (a) MS medium + 15% mungbean sprout extract + 2% Charcoal + 1 mg.L^{-1} NAA + 2% sucrose. (b) MS medium + 15% coconut water + 2% Charcoal + 1 mg.L^{-1} NAA+ 2% sucrose. (c) VW medium + 15% mungbean sprout extract + 2% Charcoal + 1 mg.L^{-1} NAA+ 2% sucrose.

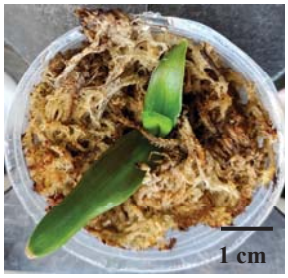

FIGURE 4. Acclimatization stage of plantlet with sphagnum moss on flexible cup

According to Fig. 3, plantlets which were cultured on MS medium with 15% mungbean sprout extract, 1 mg.L^{-1} NAA, and 2% sucrose had the most number and the longest roots (Table 3), followed by plantlets which were cultured at VW medium with 15% mungbean sprout extract, 1 mg.L^{-1} NAA, and 2% sucrose, and then plantlets which cultured at MS medium with 15% coconut water, 1 mg.L^{-1} NAA, and 2% sucrose (Table 3). Acclimatization stage of cultured plants is a critical period for adaptation to ex vitro condition, so acclimatization stage needs special attention [26]. For adaptation to ex vitro conditions, selected plantlets must have sufficient number and length roots, so they can survive well at ex vitro condition. Factor that can affect acclimatization process are plantlet age, culture medium, microorganism, nutrients, microenvironments likely humidity, light intensity, temperature [27], and acclimatization method [28]. Another advantage from economic side of using mungbean sprout, because mungbean sprout is cheaper than coconut water. The selected plantlets had been planted with sphagnum moss on flexible cup for acclimatization stage until the plantlets grow up (Fig. 4).

CONCLUSION

The fastest embryo germination rate (parameter: day to germinate) and growth young plantlet (parameters: %plantlet with normal and uniform growth, %four-leafed plantlet with many roots, number of leaves, and number of roots) which resulted from crossing of *Dendrobium* 'Bertachong' X *D.* 'Blackspider x Sutiknoi' at composition medium MS medium with addition of 15% mungbean sprout extract and 2% sucrose.

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Global Platform on Biodiversity and Biotechnology

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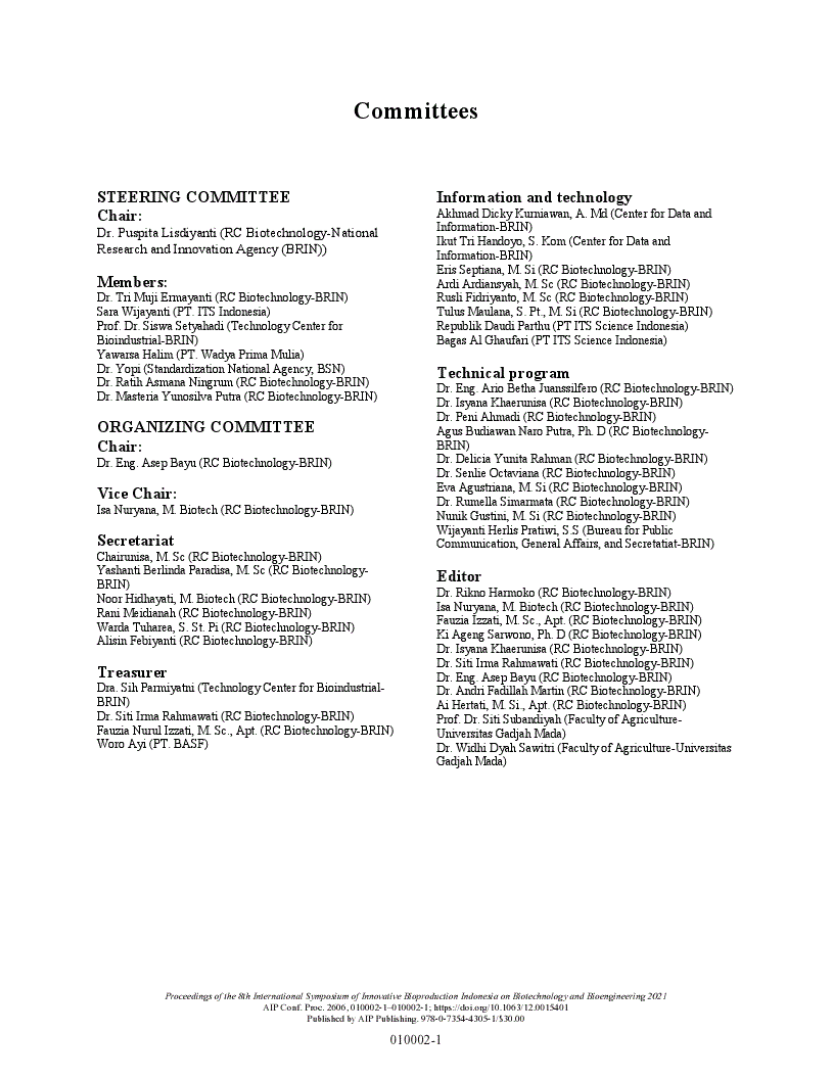

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Application of mulch and soil microbes to increase growth and yield of chili pepper

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Expression analysis of candidate structural genes involved in starch biosynthetic pathway of Indonesian cassava (*Manihot esculenta*) storage roots

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Identification of *Typhonium flagelliforme* and its closely related species in Java Island using morphology and SRAP markers

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The polymorphisms determination of the FecG/PstI and FecX/HinfI genes in Indonesian backcross sheep (75% Merino ×25% Garut)

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Isolation and identification of endophytic bacteria in lempuyang wangi (*Zingiber zerumbeth* var. *aromaticum Val*.) from Enggano Island, Bengkulu Province

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Senlie Octaviana; Tjandrawati Mozef; Joachim Wink

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