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Research Article
Micropropagation and Secondary Metabolites Content of White-Purple Varieties of *Orthosiphon aristatus* Blume Miq.

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Abstract

Background and Objective: The cat whiskers plant (*Orthosiphon aristatus* Blume Miq) is a plant that has been widely used as raw material for traditional medicine. The population of white-purple varieties of *O. aristatus* is decreasing efforts to maintain the white-purple *O. aristatus* need to be done keeping in mind its potential as raw material for traditional medicine. This study aims to determine the composition of a suitable medium in growing plantlet *O. aristatus* white-purple varieties and the content of its secondary metabolites. **Materials and Methods:** The internode explants were induced on MS medium added by various combinations of zeatin and 2,4-Dichlorophenoxyacetic acid (2,4-D). Root induction was carried out on shoots formed on MS medium with Indole-3-Butyric Acid (IBA). The acclimatization process was carried out using soil media. Determination of secondary metabolite levels was carried out on *O. aristatus* (*in vitro* culture) and wild-type plants aged ten months using high-performance liquid chromatography (HPLC). **Results:** MS+BAP 2ppm+NAA3 ppm media was the optimal medium for growing shoots in leaf explants. Media MS+zeatin 3 ppm+2,4-D 2 ppm produced good shoot growth on internode explants. The best root induction occurred in MS+IBA media of 0.75 ppm. The acclimatization process was successful on shoots originating from the internode, while those from leaf explants had not succeeded in growing and developing. **Conclusion:** The levels of rosmarinic acid and sinensetin in the white-purple variety *O. aristatus* (*in vitro* culture) were 1.08 and 1.62% w/w and higher than those of wild varieties.

Key words: *Orthosiphon aristatus*, white-purple variety, micropropagation, plant tissue culture, chemical composition, root induction

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

At this time, the use of traditional medicinal plants is increasingly in demand by the community. One class of traditional medicine that people in Indonesia widely use is *Jamu*. The *O. aristatus* plant has been used as a raw material for traditional medicine in several countries[1,2]. A plant that is often used in traditional herbal medicine raw materials is the *O. aristatus* (*Orthosiphon aristatus* Blume Miq). The pharmacological activity of cat whiskers (*O. aristatus*) has been widely reported, including diuretic[3], antioxidant[4], antidiabetic[5], antihypertensive[6], antimicrobial[7], anti-cancer[8], epilepsy treatment[9], antiviral[10]. Benefits in this traditional treatment are inseparable from the content of its main secondary metabolites, namely rosmarinic acid, sinensetin and eupatorin[11]. Based on *in silico* studies, rosmarinic acid can act as a COVID-19 inhibitor[12-14]. Sinensetin have potential as immunomodulators[15,16] and COVID-19 inhibitors (*in silico* study)[17].

Not many people know that there are three *O. aristatus*, namely white, White-purple and purple[18,19]. Batubara[20] latest report reveals that of the 15 genotypes of *O. aristatus* that grow in Indonesia, 14 are white varieties and one purple variety. This indicates that the population of the White-purple variety of *O. aristatus* is getting smaller. To increase raw materials for the traditional medicine of White-purple varieties of *O. aristatus* is necessary to propagate efforts using *in vitro* culture.

Several research reports on efforts to propagate *O. aristatus* using *in vitro* culture with growth regulators MS+BAP 1 ppm+IBA 6 ppm[21], MS+BAP 0.2 ppm[22], MS+BAP 1 ppm+NAA 0.2 ppm[23].

Previous reports did not mention the varieties of *O. aristatus* used. There were no reports on comparing secondary metabolites levels of the White-purple varieties of whiskers (*in vitro* culture) and wild types. This study's results could be the basis for the better quality of white-purple varieties of *O. aristatus* and to maintain the availability of raw materials for traditional medicine.

MATERIALS AND METHODS

Study area: The study was carried out at the School of Pharmacy Institut Teknologi Bandung, Indonesia from March, 2019-December, 2020.

Materials

Chemicals and reagents: Ethyl acetate, ethanol, acetone, acetonitrile HPLC grade and methanol HPLC grade were purchased from Merck (Jakarta, Indonesia). Rosmarinic acid and sinensetin were purchased from Sigma (St. Louis, MO, USA). Formic acid was purchased from Loba Chemie (Mumbai, India).

Instrumentation: HPLC (HP-Agilent 1100, USA), rotary evaporators (Heidolph, Germany), ovens (Memert, Germany), balance sheet (Mettler Toledo, Hong Kong), analytical scales (Shimadzu, Japan).

Collection of plants: White-purple varieties of *O. aristatus* were collected from the Manoko experimental garden, West Bandung, Indonesia. The plants were taxonomically identified at the School of Life Science and Technology, Institut Teknologi Bandung.

Sterilization of explants: Internodes and leaves washed with running water for 15 min, washed in 70% ethanol for 1 min and submerged in NaOCl solution (Bayclin®) for 5 min plus two drops of Tween 80. Explant washed with sterile water three times for 1 min each. The explants were then transferred to sterile petri dish and planted in solid media.

Shoot induction: Internode was cultured on basal MS medium containing vitamins and added with zeatin+2, 4-D growth regulator with a concentration variation ratio of 2:2, 3:2 and 4:2 ppm. Sterile leaf explants were grown on MS media with a combination of growth regulators BAP: NAA 2:1; 2:2 and 2:3 ppm. Each treatment consisted of five replications. The culture bottles were stored in the incubation room with a Philips 36 watt lamp illuminated for 8 hours darkness of 16 hours at a temperature of 19-20°C. Observations were made every two days to record the presence of shoots.

Prolonged and root induction of shoot: Shoots grown were subcultured on MS medium supplemented with IBA with various concentrations (0.5; 0.75 and 1 ppm) to elongate shoots and induce roots. The culture bottles were stored in the incubation room with a Philips 36 watt lamp at a temperature of 19-20°C. Observations were made every two days to record the presence of shoots.

Acclimatization: The plantlets resulting in the organogenesis were then acclimatized on the soil medium with husk charcoal in a ratio of 1:1. The white-purple variety *O. aristatus* plantlet in the bottle is removed and washed in running water with the aim that there is no gelatinous media involved, then planted on the prepared treatment media. Two months the plantlets were stored in a room at 25°C, after which they were planted in planting land. Watered the plantlets using water twice a day. Observations were made every other day and measurements were made once a month for nine months after planting on the media. Parameters observed and measured were the plant height.

Extraction of two varieties of *O. aristatus*: A total of 100 g of fresh plant *O. aristatus* was washed with running water and dried in an oven at 60°C. The dry material is then ground. Eighty gram of plant material powder was extracted separately by maceration using 500 mL of acetone, ethanol and ethyl acetate as a solvent. Extract was then concentrated using a rotary evaporator and thickened on a water bath.

Preparation of marker and sample solutions: Qualitative analysis of *in vitro* culture and wild type plants using HPLC was carried out by preparing standard and sample solutions. Rosmarinic acid and sinensetin were weighed as much as 1 mg and dissolved in 1 mL of HPLC grade methanol. The stock solution has been diluted into various concentrations of 60, 70, 80, 90 and 100 μg mL^{-1} with methanol. The sample solution is prepared by dissolving 15 mg of the extract in 1 mL of methanol. The sample solution is then filtered into a syringe filter.

High-performance liquid chromatography (HPLC) instrumentation and conditions: The HPLC used is a gradient method that uses the C18 column of the reversed phase. The column temperature is 37°C. The mobile phase consisted of 0.1% formic acid solution and acetonitrile with a gradient elution system with a ratio of 0.1% formic acid: acetonitrile at 0 (85:15), 1 (85:15), 12 (35:65), 15(85:15) and 18 min (85:15). The flow rate is 1 mL min^{-1}. The time of separation was 20 min. The test used to determine the levels of secondary metabolites *O. aristatus* corresponds to Saidan[24] with a change in the maximum wavelength used at 340.6 nm.

Data analysis: All test samples were prepared in three replications to determine the main secondary metabolite levels using HPLC. The data are expressed as a mean±SD. Data processing was carried out by one-way ANOVA, followed by a multiple-range test by Duncan using SPSS 22 software. The p-values<0.05 have been found statistically important.

RESULTS

The correctness of the plant identity used as the source of the explants is confirmed by determination. The determination results showed that the plants included the white-purple variety of *O. aristatus* with letter number 6115/I1.CO2.2/PL/2019.

Leaf explants and internodes sterilized with 70% ethanol for 1 min and submerged in NaOCl solution (Bayclin®) for 5 min plus two drops of Tween 80 were not contaminated during storage. Explants grown on MS medium added with a combination of zeatin and 2,4-D variations were able to grow white-purple varieties of *O. aristatus* with an average response rate of 93.3%. Variations of growth regulators zeatin 3 ppm and 2,4-D 2 ppm (Fig. 1a) can grow shoots faster, shoots higher and the number of leaves is more than shoots that grow on zeatin 2 ppm+2,4-D 2 ppm (Fig. 1b) and zeatin 4 ppm+2,4-D 2 ppm (Fig. 1c) (Table 1 and Fig. 1a-f). In MS medium, added with BAP and NAA growth regulators variations, leaf explants could develop into shoots with an average growth response rate of 100%. As Fig. 1d media BAP 2 ppm+NAA 1 ppm and BAP 2 ppm+NAA 2 ppm (Fig. 1e) produce low shoot and fewer leaves than media BAP 2 ppm+NAA 3 ppm (Fig. 1f) which produces higher shoots and more leaves (Table 1 and Fig. 1).

Root induction was carried out on shoots Fig. 1 b and f because they had a good growth profile. Shoots planted on MS medium added with IBA 0.5 media variation; 0.75 and 1 ppm can grow roots with an average growth response of 100%. The root growth medium added with 0.75 ppm IBA could grow roots faster with better shoot height and fewer leaves than IBA with concentrations of 0.5 and 1 ppm (Table 2; Fig. 2a-f).

For the shoots that have grown roots, Fig. 2b and e, the acclimatization process was carried out. Shoots derived from internode explants (Fig. 2b) from zeatin 3 ppm + 2.4 D 2 ppm can grow well until they are 10 months old. Plants grown from *in vitro* culture that were 2 months old were grown in the same conditions as the original plant (wild type). The white-purple variety of *O. aristatus in vitro* culture yield was greater than the wild type (Table 3; Fig. 3a-c). Shoots derived from leaf explants (2e) grown on 2 ppm+NAA 3 ppm BAP medium had not succeeded to grow during acclimatization process.

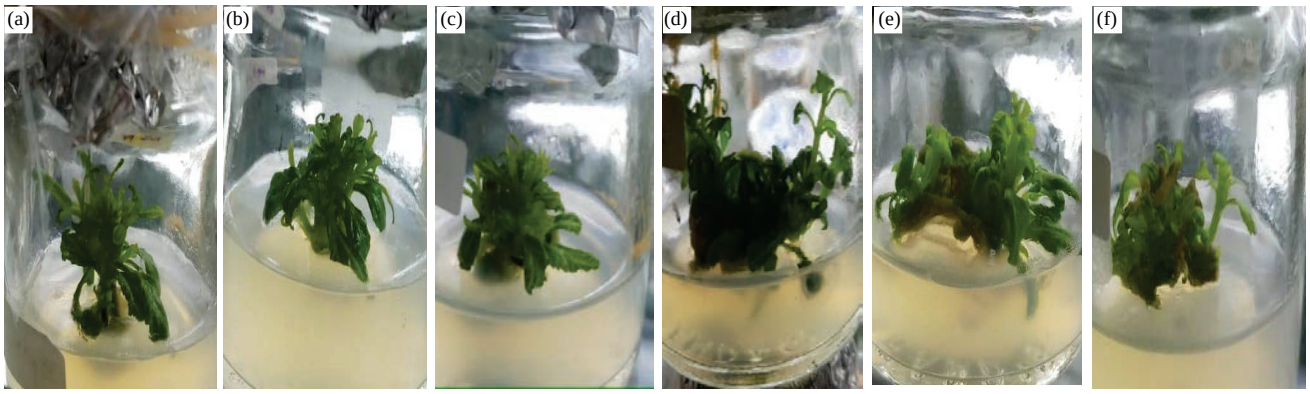

Fig. 1(a-f): Induction of white-purple varieties of *O. aristatus*

(a) MS+Zeatin 2 ppm+2,4-D 2 ppm, (b) MS+Zeatin 3 ppm+2,4-D 2 ppm, (c) MS+Zeatin 4 ppm+2,4-D 2 ppm, (d) MS+BAP 2 ppm+NAA 1 ppm, (e) MS+BAP 2 ppm+NAA 2 ppm and (f) MS+BAP 2 ppm+NAA 3 ppm

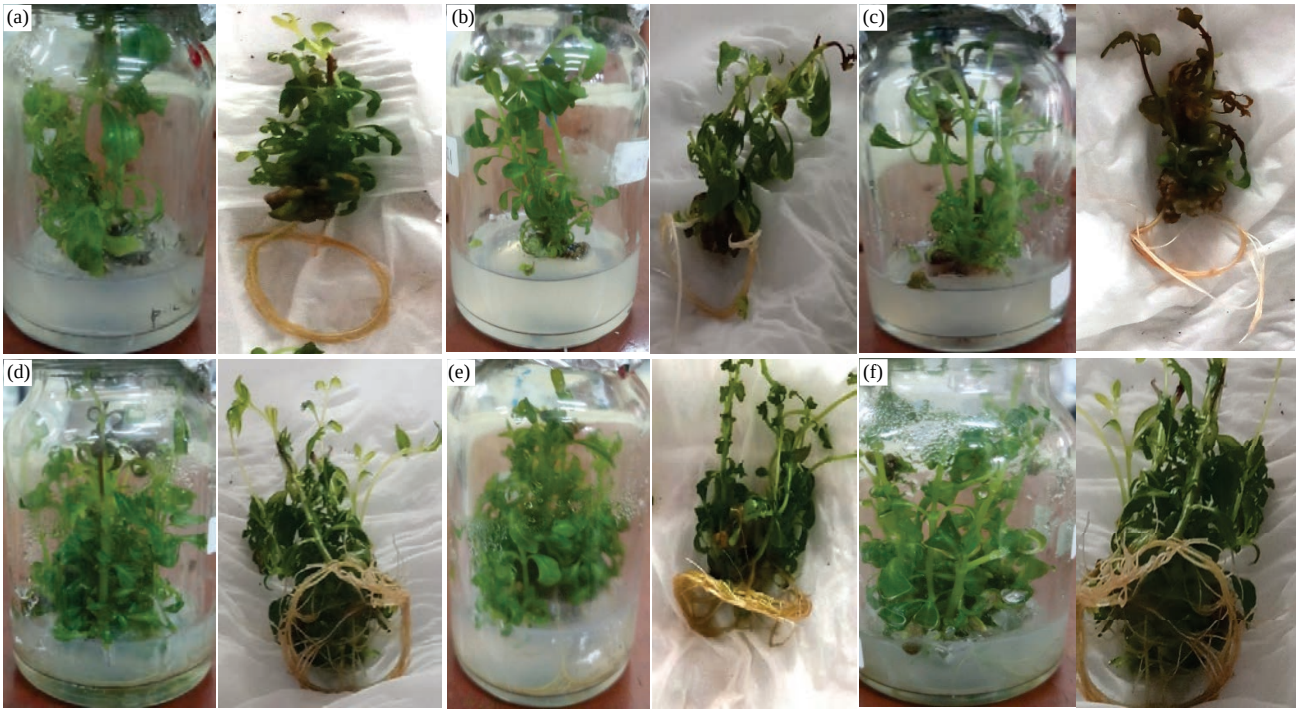

Fig. 2(a-f): Root induction of white-purple varieties of *O. aristatus*

(a) 1b+MS+IBA 0.5 ppm, (b) 1b+MS+IBA 0.75 ppm, (c)1b+MS+IBA 1 ppm, (d)1f+MS+IBA 0.5 ppm, (e)1f+MS+IBA 0.75 ppm and (f)1f+MS+IBA 1 ppm

Table 1: Induction of white-purple varieties of *O. aristatus* shoots in a variety of growing media

Growth regulator	Time to develop n = 5 x±SD	Shoot height (cm) n = 5 x±SD (day 30)	Number of leaves (strands) n = 5 x±SD (day 30)	Response to shoot growth (%)
MS+Zeatin 2 ppm+2,4 D 2 ppm (1a)	16±0.47^{ab}	4.60±0.12^{a}	13±0.82^{a}	100
MS+Zeatin 3 ppm+2,4 D 2 ppm (1b)	14±0.81^{a}	4.86±0.16^{a}	14±0.47^{a}	100
MS+Zeatin 4 ppm+2,4 D 2 ppm (1c)	17±0.47^{b}	4.76 ±0.12^{a}	12±0.47^{a}	80
MS+BAP 2 ppm+NAA 1 ppm (1d)	14±0.47^{a}	2.76±0.12^{b}	20±0.47^{b}	100
MS+BAP 2 ppm+NAA 2 ppm (1e)	16±0.47^{ab}	3.01±0.20^{b}	22±0.82^{bc}	100
MS+BAP 2 ppm+NAA 3 ppm (1f)	15±0.47^{ab}	3.30±0.29^{c}	24±0.82^{c}	100

Mean value of the different superscript letters shows significant differences ($p<0.05$)

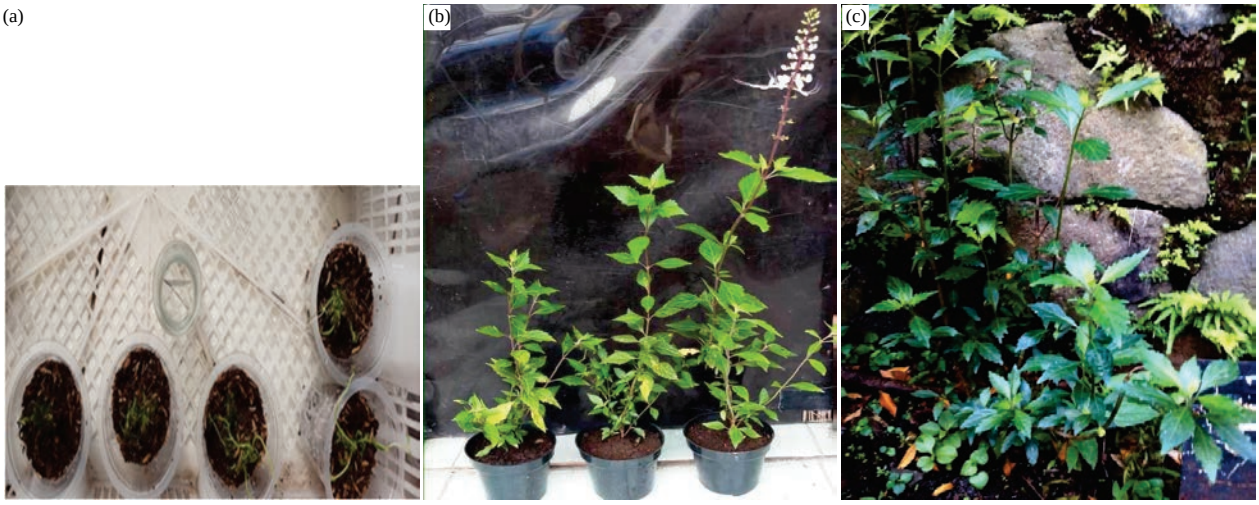

Fig. 3(a-c): Acclimatization process of White-purple varieties of *O. aristatus* (R_2)
(a) Process of transferring plantlets to acclimatization media, (b) 5 months of *O. aristatus* (*in vitro* culture) and (c) 10 months of *O. stamineus* (*in vitro* culture)

Table 2: Root induction white-purple varieties of *O. aristatus* shoots in a variety of growing media

Growth regulator	Root growth time n = 3 x±SD	Shoot height (cm) n = 3 x±SD (day 30)	Number of leaves (strands) n = 3 x±SD (day 30)	Response to root growth (%)
S2+MS+IBA 0.5 ppm (2a)	16±0.33^{ab}	6.36±0.33^{a}	26±0.82^{a}	100
S2+MS+IBA 0.75 ppm (2b)	14±0.80^{a}	6.80±0.08^{b}	29±0.47^{b}	100
S2+MS+IBA 1 ppm (2c)	17±0.04^{b}	6.36±0.04^{a}	20±0.82^{c}	100
S6+MS+IBA 0.5 ppm (2d)	14±0.47^{a}	6.40±0.08^{a}	38±0.82^{d}	100
S6+MS+IBA 0.75 ppm (2e)	10±0.47^{c}	6.70±0.08^{b}	42±0.82^{e}	100
S6 +MS+IBA 1 ppm (2f)	14±0.47^{a}	6.30±0.12^{a}	35±0.82^{f}	100

Mean value of the different superscript letters shows significant differences (p<0.05)

Table 3: Observations of the growth of white-purple variety *O. aristatus* (*in vitro* culture and wild type)

Samples	Plant height (cm) n = 3 x±SD				
	2 month	4 month	6 month	8 month	10 month
White-purple variety *O. stamineus* (*in vitro* culture)	25.76±0.44^{a}	38.30±0.45^{a}	51.43±0.15^{a}	65.96±1.10^{a}	79.63±1.40^{a}
White-purple variety *O. stamineus* (wild type)	21.47±0.47^{b}	35.20±0.37^{b}	42.13±0.96^{b}	58.50±1.20^{b}	71.30±1.16^{b}

Mean value of the different superscript letters shows significant differences (p<0.05)

Table 4: Levels of rosmarinic acid, sinensetin from acetone, ethyl acetate and ethanol extracts of white-purple variety of *O. aristatus* (*in vitro* culture and wild type)

Samples	Rosmarinic acid (% w/w) x±SD (n = 3)	Sinensetin (% w/w) x±SD (n = 3)
White-Purple variety acetone extract *in vitro* culture (PI 1)	0.77±0.01^{ad}	1.10±0.02^{a}
White-Purple variety ethyl acetate extract *in vitro* culture (PI 2)	1.08±0.06^{b}	1.62±0.07^{b}
White-Purple variety ethanol extract *in vitro* culture (PI 3)	0.78±0.02^{a}	0.73±0.03^{c}
White-Purple variety acetone extract wild type (PW 1)	0.30±0.01^{c}	0.46±0.08^{cd}
White-Purple variety ethyl acetate extract wild type (PW 2)	0.31±0.08^{c}	0.35±0.07^{d}
White-Purple variety ethanol extract wild type (PW 3)	0.48±0.01^{dc}	0.64±0.02^{c}

Mean value of the different superscript letters shows significant differences (p<0.05)

The results of the qualitative analysis of acetone, ethyl acetate and ethanol extracts of *O. aristatus* from *in vitro* culture that was ten months old showed the presence of rosmarinic acid and sinensetin compounds because the three extracts appeared peaks with the same retention time as rosmarinic acid and sinensetin standards (Fig. 4, 5a-c and 6a-c).

Quantitative analysis of rosmarinic acid and sinensetin levels of acetone, ethyl acetate and ethanol extracts of White-purple varieties of *O. aristatus* were higher than wild-type extracts. *O. aristatus* ethyl acetate extract (*in vitro* culture) produced the highest rosmarinic acid and sinensetin levels than other extracts with a value of 1.08 and 1.62% w/w. (Table 4).

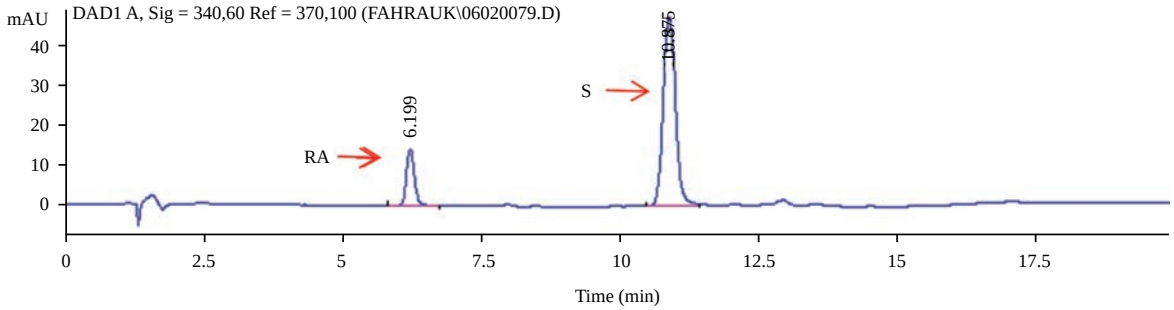

Fig. 4: Chromatogram of a standard mixture of rosmarinic acid (RA) and sinensetin (S) at 340.60 nm

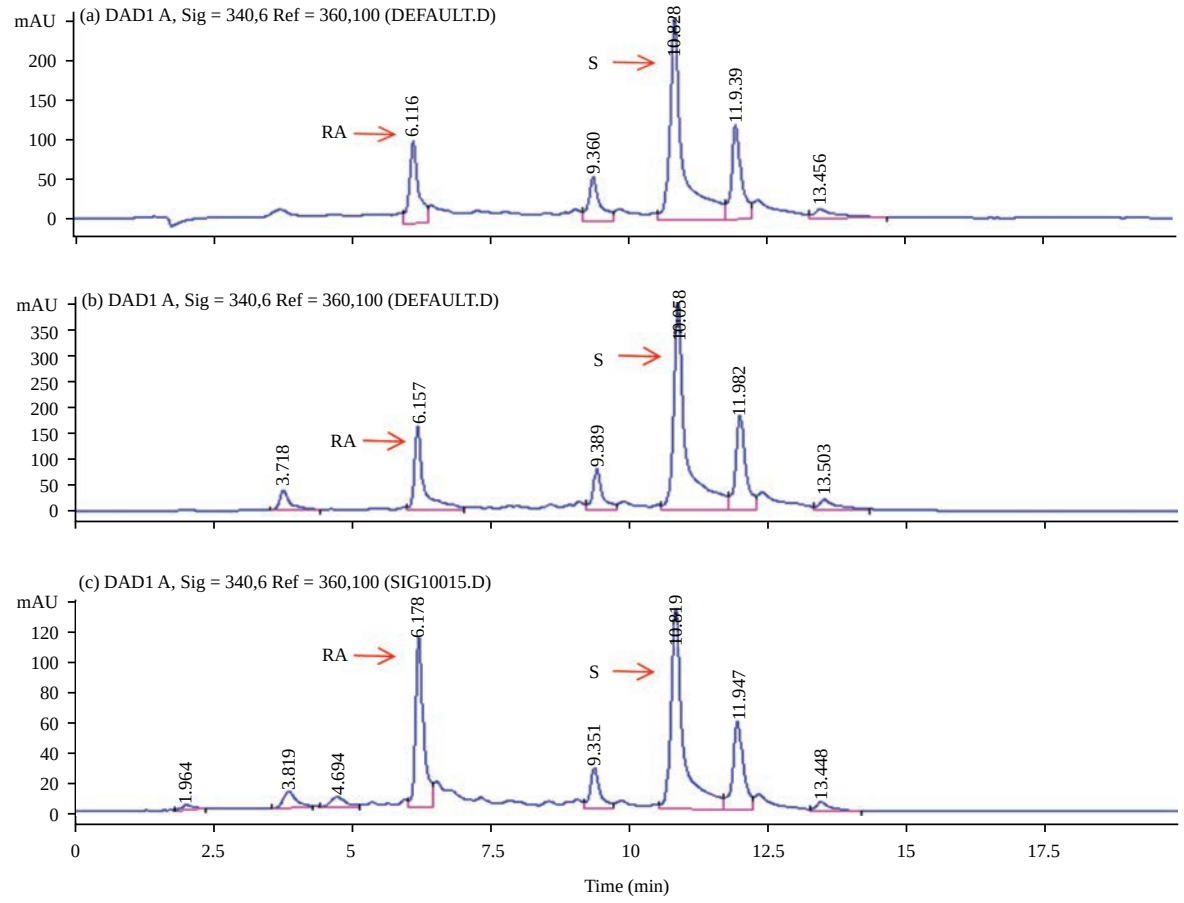

Fig. 5(a-c): Chromatogram of extract of white-purple variety at 340.60 nm (*in vitro* culture)
(a) Acetone extract, (b) Ethyl acetate extract and (c) Ethanol extract

DISCUSSION

Shoots derived from internode explants and grown on a combination medium of growth regulators zeatin and 2,4 D gave a good growth profile of white-purple varieties of *O. aristatus*. In-plant tissue culture, 2,4-Dichlorophenoxyacetic acid (2,4-D) is a synthetic auxin that acts as a growth regulator. Zeatin belongs to the class of cytokinins[25]. Zeatin affects shoot regeneration at the multiplication stage[26]. A higher concentration of cytokinins than auxins can induce shoots[27,28]. In this study, shoots formed from zeatin concentrations higher than 2,4-D produced an excellent growth profile. Another study report states that MS+Zeatin 0.5 ppm+2,4-D 0.5 ppm media can grow *Linum usitatissimum* L. shoots.

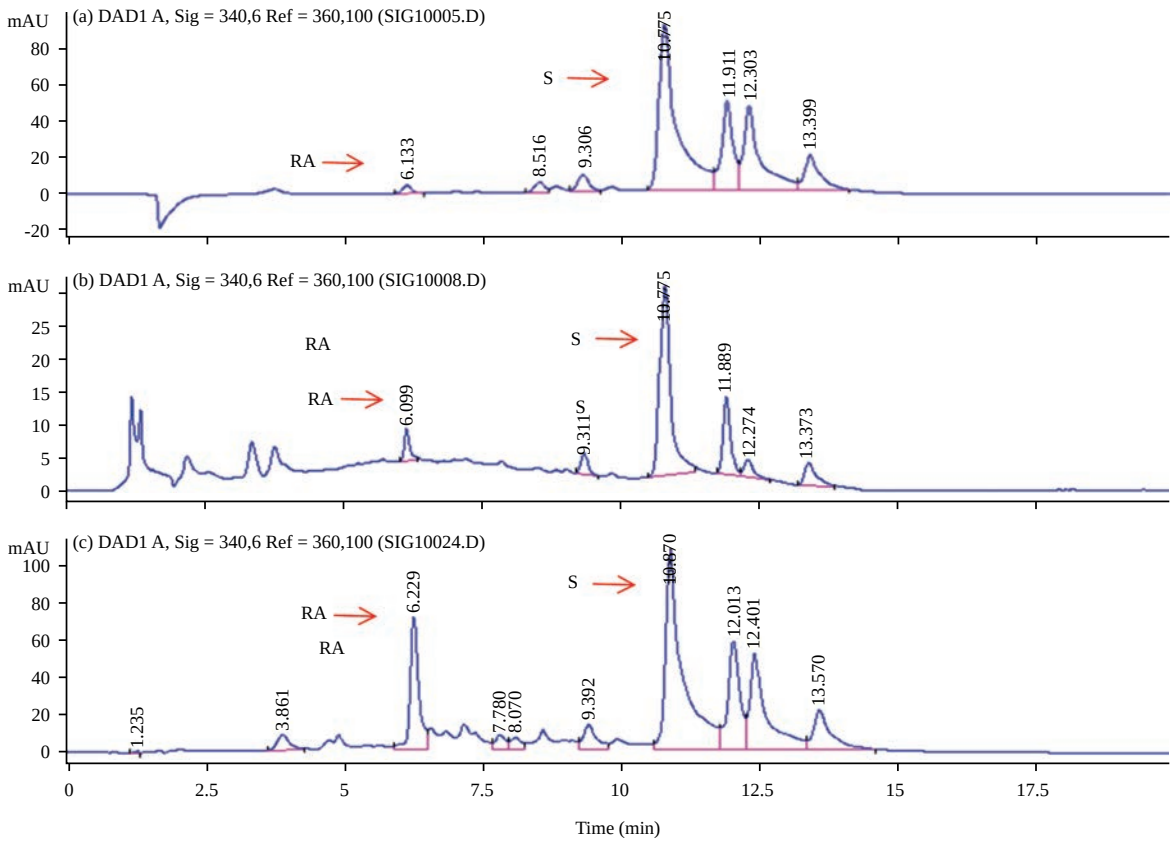

Fig. 6(a-c): Chromatogram of extract of white-purple variety at 340.60 nm (wild type)
(a) Acetone extract, (b) Ethyl acetate extract and (c) Ethanol extract

BAP growth regulators play a role in the development of axillary shoots and shoots in explants[29]. Naphthaleneacetic Acid (NAA) is an auxin hormone that functions to induce cell elongation and rooting initiation[30]. The shoots produced from leaf explants grown on media containing BAP and NAA looked shorter than shoots grown on zeatin and 2,4-D media, but more leaves were formed. On the other hand, if BAP concentration is higher than NAA, it will trigger more shoot growth[31] On the S6 medium, where the NAA concentration was greater than BAP, the roots formed were seen to be more. The effect of adding BAP on the number of shoot is proportional to the effect of BAP on the increase in the number of leaves. Several studies also reported the effect of the combination of BAP and NAA media on shoot growth, including MS media added with 1 ppm NAA and 0 ppm BAP to grow roots well[31]. Growth media with higher BAP concentrations than NAA can grow grape shoots[32,33], orchids[34]. *Lilium longiflorum* Thunb leaf explants added with NAA 1 ppm+BAP 1 ppm were able to grow shoots maximally with a growth percentage of 100%[30].

Several studies on the induction of *O. aristatus* have been reported, including *O. aristatus* nodal explants that can grow into shoots on MS+benzyl adenine 6.7 mM[35], MS+2,4-D 0.5 ppm[36], MS+BAP 1.00 ppm[37]. *Orthosiphon aristatus* shoots can grow on MS+BAP 2 ppm[38], MS+BAP 1 ppm[21] and MS+BAP 0.2 ppm[22]. This study provides new information regarding the combination of zeatin+2,4-D and BAP and NAA media that can grow white-purple variety *O. aristatus*, which researchers have never previously reported.

Root induction on *O. aristatus* shoots went well on 0.75 ppm IBA media. IBA has a role in root development, including regulating the apical root meristem's size, root hair lengthening, lateral root development and adventitious root formation[39].

In the acclimatization of shoots from 2,4-D 2 ppm+3 ppm zeatin, they managed to grow until the age of 10 months. Shoots that have been transferred to soil growing media and husks are not directly conditioned on planting land and but are stored in a room with a 36-watt lamp illuminated for 8 hrs and darkness of 16 hrs at a temperature of 25°C for two

months. Plantlets take time to adapt to high radiation, autotrophic conditions and low air humidity after *ex vitro* transfer. Several weeks of shade growth and a gradual decrease in air humidity are typically needed before developing a robust plant[40].

In the observation of shoot development for ten months, the plant height resulted from *in vitro* culture was higher than that of the wild type. Plants cultured *in vitro* can produce plantlets with different physiology, morphology and anatomy from plants that grow naturally[41]. Shoots from BAP and NAA media have not succeeded in growing. The main problem in micropropagation is the high rate of water loss from plantlet shoots. Although the water potential in soil or sand is higher than the media, plantlets can quickly wither. The cause is an unlimited rate of transpiration due to inhibition of the development of the epicuticular wax, cuticle and stomata[40]. Plants from *in vitro* culture of white-purple varieties of *O. aristatus* have rhombic leaves with white-purple stalks and stamens. This result is consistent with those reported previously[18,42,19].

Quantitative analysis of acetone, ethyl acetate and ethanol extracts from white-purple varieties of *O. aristatus* (*in vitro* culture) showed an increase in rosmarinic acid levels sinensetin compared to wild types. The selection of the three extracts is based on their different levels of polarity. The levels of rosmarinic acid and sinensetin in the ethyl acetate extract of White-purple *O. aristatus* (*in vitro* culture) were 1.08 and 1.62% w/w. Research on rosmarinic acid and sinensetin levels from *O. aristatus* has been reported by Guo *et al.*[11] that the levels of rosmarinic acid and sinensetin in methanol: water (50:50) extract of *O. aristatus* were 2.83 mg g^{-1} (0.283% w/w) and 0.057 mg g^{-1} (0.0057% w/w)[11]. The levels of rosmarinic acid and sinensetin in the ethanol extract of *O. aristatus* were 19.861 (1.98% w/w) and 2.719 mg g^{-1} (0.27% w/w). The level of sinensetin in the purple variety of *O. aristatus* grows in Indonesia is 1.82 mg g^{-1} (0.182% w/w)[43]. Acetone extract: water (70:30) *O. aristatus* contain sinensetin 0.27% w/w[44]. The rosmarinic acid levels of the ethyl acetate extract of *O. aristatus in vitro* culture (PI 2) were higher than those reported by Guo *et al.*[11] However, it is still lower than reported by Cai *et al.*[43]. Sinensetin levels in PI 2 were higher than those reported by previous research. Previously reported[19,42] a qualitative and quantitative analysis of the chemical content of the acetone callus extract of the white-purple variety of *O. aristatus*, showing the presence of rosmarinic acid and sinensetin with rosmarinic acid levels of 2.22% w/w[42].

Cytokinin hormones play a role in regulating gene expression that can increase plant defence against environmental influences from pathogens[45]. Research can be expanded to a larger scale to produce raw materials for the white-purple varieties of *O. aristatus* that are of good quality and standard. This research further develops what has been done previously[19,42,46], where an effort to propagate the white-purple variety of *O. aristatus* with modified *in vitro* culture was carried out.

CONCLUSION

The acclimatization process of white-purple varieties of *O. aristatus* was successfully carried out using shoots from the growing medium 2,4-D 2 ppm+zeatin 3 ppm. There was an increase in rosmarinic acid and sinensetin level in the *O. aristatus* which were *in vitro*.

SIGNIFICANCE STATEMENT

This study found a micropropagation protocol for the white-purple variety *O. aristatus*, which had higher secondary metabolites than wild type. This study will help researchers uncover critical areas of the secondary metabolite content profile of white-purple varieties of *O. aristatus* (*in vitro* culture) that many researchers have not explored. Thus, new information about the composition of the media and the quality and quantity of secondary metabolites can be identified.

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