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REVIEW

Plant tissue culture of cat whiskers (*Orthosiphon aristatus Blume Miq*): A review of secondary metabolite production and micropropagation

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Abstract

Background: The cat whiskers plant (*Orthosiphon aristatus Blume Miq*) is widely used as a raw material in traditional medicine for one of its many properties, i.e. antiviral activity. Three varieties of cat whiskers grow in Indonesia, classified according to the colour of their flower: white, white-purple, and purple. The purple variety of cat whiskers is endangered, so it is necessary to propagate this plant. **Objectives:** This research aimed to obtain appropriate protocols for plant propagation and production of active compounds of cat whiskers by *in vitro* culture. **Methods:** Data were collected from various online journal sites such as PubMed, ResearchGate, and Scopus. This review discusses several aspects, including callus induction efforts, modification of cell suspension cultures, and efforts to propagate the cat whiskers plant by *in vitro* culture. **Results:** Callus induction of white cat whiskers with purple and purple hues could take place on Murashige and Skoog (MS) media added with growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) 0.4 mg/L. Suspension culture medium (MS+ 2,4-D 1 mg/L + NAA 1 mg/L) can increase cell biomass and rosmarinic acid levels. Media MS + 6-benzyl amino purine (BAP) 3 mg/L + 1-naphthaleneacetic acid (NAA) 2 mg/L can induce the growth of shoots of white cat whiskers with purple and purple patterns. Root induction of the two varieties of cat whiskers could take place on MS + Indole-3-butyric acid (IBA) 0.75 mg/L medium. **Conclusion:** Efforts to produce secondary metabolites and plant propagation of cat whiskers by *in vitro* culture have been successful. It is necessary to enhance the production and propagation using bioreactors to yield more active compounds and raw materials of the cat whiskers plant.

Introduction

Orthosiphon aristatus (Blume) Miq., better known as the cat whiskers, is a plant typical of the island of Java and popular in herbal medicine, especially in the Asian region. In some Southeast Asian countries, it is widely used in traditional medicine for treating urinary tract infections, urinary stones, and rheumatic gout (Awale *et al*., 2001). Based on the colour of their flower, cat whiskers plants are is divided into three varieties, namely white, white-purple, and purple (Faramayuda *et al*., 2020). The main active compounds in the cat whiskers plant are rosmarinic acid, sinensetin, and eupatorin (Guo *et al*., 2019). The levels and yields of the three principal secondary metabolites remain low (Cai *et al*., 2018). Several studies *in silico*, *in vitro*, and *in vivo* have demonstrated the potential antiviral activity of sinensetin compounds (Faramayuda *et al*., 2021b).

Based on the potential pharmacological activity of cat whiskers and the active compounds in these plants, it is necessary to multiply cat whiskers, especially the white-purple and purple varieties, and the production of the active compounds (rosmarinic acid, sinensetin, and eupatorin). One approach that can help achieve this goal is plant tissue culture. Efforts to propagate plants were carried out by *in vitro* culture (micropropagation), and the production of active

compounds started from callus induction, then continued at the stage of cell suspension culture, which was modified by the addition of elicitors and precursors.

Several studies have reported on media and growth regulators that can grow cat whiskers shoots. Nodal explants inoculated on MS + 2, 4-D 0.5 ppm medium were able to grow cat whiskers shoots (Elangomathavan *et al*., 2017). Young leaf explants inoculated on MS + BAP 2 ppm + 1-naphthaleneacetic acid (NAA) 3 ppm media were able to grow white-purple varieties of cat whiskers with many leaves. Root induction on cat whiskers shoots went well on MS + IBA 0.75 mg/L medium (Faramayuda *et al*., 2021c). In callus induction, it has also been reported that a suitable medium for growing white-purple and purple varieties of cat whiskers. Leaf explants inoculated on MS + 2,4-D 0.4 mg/L media were able to grow purple and white-purple varieties of cat whiskers callus and identified the presence of rosmarinic acid compounds (Faramayuda *et al*., 2020; Faramayuda *et al*., 2021a). The culture of cat whiskers cell suspension from MS + 2,4-D 1 ppm + NAA 1 ppm media can increase rosmarinic acid levels (Bordbar *et al*., 2015).

Efforts to develop the principal secondary metabolites in cat whiskers are directed at cell suspension cultures modified with the addition of elicitors and precursors. Further development to a larger scale (bioreactor) for the cat whiskers propagation, especially the purple and white-purple varieties, shoot propagation, and acclimatisation, were carried out until the *in vitro* cultured plants grew and were ready to be harvested. This review is expected to provide guidelines or protocols for producing secondary metabolites and the propagation of the cat whiskers plant by *in vitro* culture.

Distribution and diversity of Cat Whiskers

The cat whiskers plant originated from tropical Africa and then spread to Asia and Australia. It is called cat whiskers because the collection of long stamens protrudes from two different sides, similar to cat whiskers. Based on the colour of the flower, there are three varieties, namely purple flower with purple stems and crowns, purplish-white flower with purplish-green stems and purplish-white crowns, and white flower with green stems and white crowns (Faramayuda *et al.,* 2020; Faramayuda, *et al*., 2021f; Faramayuda, *et al*., 2022) (Figure 1).

(a) White-purple variety

(b) purple variety

Figure 1: Cat Whiskers Plant

Cat whiskers plant tissue culture

Cat whisker plant callus induction

Several studies have reported the success of cat whiskers callus induction, including leaf, petiole, and stem explants of cat whiskers inoculated on MS media with a combination of growth regulators 2,4-D and

NAA. The highest callus weight came from leaf explants. Root explants cultured on MS + 2,4-D + NAA media were not observed for callus formation. MS media added with the growth regulators 6-benzyl amino purine (BAP), kinetin, and picloram could not form callus properly. The choice of the type of explant used affected the callus induction of cat whiskers. Media MS + NAA 1 ppm + 2,4-D 1 ppm could induce cat whiskers callus well (2.86 g). Leaf explants grown on MS medium without NAA produced less friable callus (Bordbar *et al*., 2015; Wai-leng and Lai-keng, 2016).

Leaf stalk explants inoculated on MS+ NAA 1 ppm + 2,4-D 1.5 ppm could induce cat whiskers callus well. When inoculated on MS + NAA 3 ppm media, cat whiskers stem explants could produce callus with friable texture. In stem explants inoculated on media with 2,4-D added to culture media, the callus induced was not maximal, so the stems were not suitable for use as explants (Wai-leng & Lai-keng, 2016).

Leaf explants inoculated on MS media + kinetin 1 ppm could induce callus formation with a fresh weight of 0.12 g. The addition of the growth regulator Indole-3-Acetic Acid (IAA) to media containing kinetin could increase callus formation. Examples of the combination of kinetin and auxin that can increase the growth of cat whiskers callus are 0.5 ppm kinetin and 1 ppm IAA or 1 ppm kinetin and IAA 2 ppm. At the age of two months, the colour of the callus is green, and after four months, it becomes brown. Media MS + kinetin 2 ppm + IAA 1 ppm resulted in a green callus colour. MS medium + kinetin 0.5 ppm + IAA 2 ppm produced a cream colour callus, while MS + kinetin 0.5 ppm + IAA 1.5 ppm media produced a brown callus (Ali *et al*., 2017).

Callus from purple and white-purple varieties of cat whiskers leaf explants grew well on MS + 2,4-D 0.4, 1 and 2 ppm media in 14 days. The best medium for growing callus of the two varieties of cat whiskers was MS + 2,4-D 0.4 ppm, which produced white and friable callus. In identifying secondary metabolite content in the callus of the two varieties of cat whiskers using thin-layer chromatography, the presence of rosmarinic acid and sinensetin compounds was detected (Faramayuda *et al*., 2020). Quantitative analysis was carried out on the callus of the two varieties of cat whiskers derived from MS+2,4-D 0.4 ppm media using high-performance liquid chromatography (HPLC). Furthermore, white with purple hue by 1.28 and 2.22 % w/w rosmarinic acid levels in the callus of the white-purple variety was higher than that of the purple variety. Contrary to previous reports, the purple variety (wild type) had a higher secondary metabolite content than other varieties. One of the advantages of plant tissue culture is that it can produce and increase secondary metabolite contents (Faramayuda *et al*., 2021a).

Another report explained that purple and white-purple varieties of cat whiskers callus could grow on MS, Gamborg, N6, and SH media added with growth regulator 2,4-D 0.4 ppm. Based on the analysis of secondary metabolite content using TLC, the presence of sinensetin and rosmarinic acid compounds in the callus of two varieties of cat whiskers derived from the four primary media was identified. Sinensetin spot fluorescence was brighter in callus from N6 media. Rosmarinic acid in the callus of the purple variety looks lighter than the callus of the white-purple variety. Sinensetin fluorescence in the callus of white-purple cat whiskers was fainter than the wild type, but in rosmarinic acid, the fluorescence was brighter than that of the wild type (Faramayuda *et al*., 2021d).

Cat's whisker plant cell suspension culture

Cat whiskers cell suspension culture derived from liquid media MS + NAA 1 ppm + 2,4-D 1 ppm had a good growth profile. Callus weighing 0.75 g was inoculated in a 20 mL liquid medium to produce the best dry and wet weights. Cat whiskers cell suspension culture from MS + 2,4-D 1 ppm + NAA 1 ppm + sucrose 30 g/L media resulted in high wet cell weight. The addition of the higher amount of sucrose up to 45 g/L reduced the wet weight of the suspension culture. The production of rosmarinic acid compounds was also higher in the media with 30 g/L sucrose added. Media conditioned at pH 5.65 produced the highest levels of rosmarinic acid, while at an alkaline pH, the levels of rosmarinic acid compounds were low. The irradiation conditions in the incubation room of the cat whiskers suspension culture must be considered. The dim lighting conditions can increase the levels of rosmarinic acid in the cell suspension culture of cat whiskers. Incubation of cell suspension culture at 26^{o}C affected increasing levels of rosmarinic acid and was not significantly different when cells were placed at 29^{o}C (Lim *et al*., 2013; Bordbar *et al*., 2015; Wai-leng and Lai-keng, 2016).

Cat whiskers plant micropropagation

The purple variety cat whiskers leaf explant inoculated on MS + BAP 2 ppm + NAA 1 ppm media; MS + BAP 2 ppm + NAA 2 ppm; MS + BAP 2 ppm + NAA 3 ppm could grow cat whiskers shoots well within one month. Media MS + BAP 2 ppm + NAA 3 ppm produced more leaves, but the shoots formed were not too high. Root induction took place well on MS + IBA 0.75 ppm media, and an increase in shoot height and number of leaves was also observed (Faramayuda *et al*., 2021b).

Internode explants were inoculated on MS media + zeatin 2 ppm + 2,4-D 2 ppm; MS + zeatin 3 ppm + 2,4-D 2 ppm; MS + zeatin 4 ppm + 2,4-D 2 ppm could grow shoots of white cat whiskers with purple pattern well.

The best medium for growing shoots was MS + zeatin 3 ppm + 2,4-D 2 ppm. Root induction on shoots of a white-purple variety of cat whiskers occurred well on MS + IBA 0.5 ppm medium; MS + IBA 0.75 ppm; MS + IBA 1 ppm. However, the best medium for growing roots was MS + IBA 0.75 ppm because it succeeded in making higher shoots and more leaves. Acclimatisation of shoots that have grown roots takes place well on sand and husk media. Previously, the plantlets were incubated at 25^{o}C for two weeks for the hardening process and then placed in the planting area. The white-purple variety cat whiskers plantlets could grow up to 10 months of age. Comparative analysis of secondary metabolites levels of the white-purple variety of the plant cultured *in vitro* with the wild type was performed using HPLC. The results showed that the secondary metabolites rosmarinic acid, sinensetin, and eupatorin in the cat whiskers plant *in vitro* culture were higher than the wild type. These results can be used as a protocol for the propagation of cat whiskers, especially the white-purple variety, which has better quality than the wild type. The plant tissue culture method allows an increase in secondary metabolite levels after modifying growth regulators, which will affect the enzymes that play a role in the biosynthesis of secondary metabolites (Faramayuda *et al*., 2021e; Faramayuda, *et al*., 2021g).

Tables I and II summarise media and growth regulators that are good for callus induction, cell suspension culture, and shoots of cat whiskers.

Table I: Media and plant growth regulators (PGR) in callus induction and cat whiskers shoots

Explant	Target	Medium and PGR	Reference
Leaf	Callus	MS + 2,4-D 1 ppm + NAA 1 ppm	(Wai-leng and Lai-keng, 2004)
Leaf	Callus	MS + 2,4-D 1 ppm + NAA 1 ppm	(Bordbar *et al*., 2015)
Nodal	Shoots	MS + 2, 4-D 0.5 ppm	(Elangomathavan *et al*., 2017)
Nodal	Shoots	MS + NAA 1.0 ppm	(Elangomathavan *et al*., 2017)
Nodal	Shoots	IBA MS 2.0 ppm	(Elangomathavan *et al*., 2017)
Shoots	Root	¼ MS + 2, 4-D (0.5 ppm, 1.0 ppm, and 2.0 ppm)	(Elangomathavan *et al*., 2017)
Shoots	Root	¼ MS + IBA (0.5 ppm, 1.0 ppm, and 2.0 ppm)	(Elangomathavan *et al*., 2017)
Shoots	Root	¼ MS + NAA (0.5 ppm, 1.0 ppm, and 2.0 ppm)	(Elangomathavan *et al*., 2017)
Leaf	Green callus	MS + kinetin 1 ppm + IAA 1 ppm	(Ali *et al*., 2017)
Leaf	Cream colored callus	MS + kinetin 1.5 ppm + IAA 1.5 ppm	(Ali *et al*., 2017)
Leaf	Callus brown	MS + Kinetin 2 ppm	(Ali *et al*., 2017)
Nodal	Planlet	MS + BA 2 ppm	(Lim *et al*., 2013)
Seed	Adventist shoots	Gamborg (B5) + sucrose 3 % w/v + BAP 20 ηM	(Zarnadze *et al*., 2018)
Seed	Adventist shoots	Gamborg (B5) + sucrose 3% w/wv + BAP 15 ηM + NAA 3 ηM	(Zarnadze *et al*., 2018)
Shoots	Root	MS + IBA 1.5 ppm + 1GA3 1.5 ppm	(Elangovan *et al*., 2016)
Nodal	Shoots	MS + IBA 1 ppm	(Sheena and Jothi, 2015)
Shoots	Root	MS + IBA 4 ppm	(Sheena and Jothi, 2015)
Leaf	Callus	MS + 2, 4-D 2 ppm	(Sheena and Jothi, 2015)
Leaf	Root	MS + IAA 3 ppm + Sucrose 3%	(Ling *et al*., 2009)
Leaf	Callus	MS + 2, 4-D 0.4 ppm	(Faramayuda *et al*., 2020)
Leaf	Shoots	MS + BAP 2 ppm + NAA 3 ppm	(Faramayuda *et al*., 2021c)
Internodes	Shoots	MS + zeatin 3 ppm + 2,4-D 2 ppm	(Faramayuda *et al*., 2021e)

Table II: Culture media of cat whiskers cell suspension with the addition of elicitor

Sample	Medium	Elisitor	Results	Library
Callus	Liquid proliferation cell medium (MS + 2,4-D 1 ppm and NAA 1 ppm)	30 g / L sucrose	Increase cell biomass and rosmarinic acid levels	(Bordbar *et al*., 2015)
Callus	Liquid medium (MS + 2,4-D 1 ppm and NAA 1 ppm)	NaCl (1-3 g / L)	Not Increase right levels of total phenolic	(Lim *et al*., 2013)
Callus	Liquid medium	45 g / L sucrose	Increase levels of total phenolic	(Lim *et al*., 2013)
Callus	Liquid medium	Casein hydrolysate (0.3–3.0 g / L)	Not Increase levels of total phenolic	(Lim *et al*., 2013)
Callus	Liquid medium	yeast extract (0.25–0.75 g / L)	Not Increase levels of total phenolic	(Lim *et al*., 2013)
Callus	Liquid medium	1.5 g / L Chitosan	Increase levels of total phenolic	(Lim *et al*., 2013)

Internode	4.4 g MS + sucrose 3% b / v + gel 2.2% b / v + 0.1 myo-inositol	Yeast extract 5.0 mg/L	Shoot induction and increase phenolic levels	(Razali *et al*., 2017)

Conclusion

The cat whiskers plant has an excellent potential to be used as raw material in traditional medicine. The compounds thought to play a role in the pharmacological activity of the cat whiskers plant are sinensetin, eupatorin, and rosmarinic acid. Plant tissue culture of the cat whiskers plant is beneficial for the propagation process, especially the endangered purple and white-purple varieties. Callus induction and suspension culture can increase the principal secondary metabolites of the cat whiskers plant. MS media added with growth regulators zeatin 3 ppm and 2,4-D 2 ppm could induce the growth of cat whiskers shoots well. Shoots that grew on 0.75 ppm IBA media could induce root growth on shoots, and when acclimatised, plantlets could grow up to 10 months of age. Media MS + 2,4-D 0.4 ppm can induce the growth of cat whiskers callus well. This review can be the basis for developing micropropagation protocols to produce active compounds of the cat whiskers plant *in vitro*.

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Conflict of interests

The authors declare no conflict of interest.

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