Effect of Medium pH and Light on Quinidine Production in *Cinchona calisaya* Wedd. Endophytic Fungi

*Cinchona calisaya* Wedd. Endatif Mantarında Kinidin Üretiminde Ortamın pH’sinin ve Işığın Etkisi

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**ABSTRACT**

Objectives: Quinidine has pharmaceutical importance as an antimalarial, antiarrhythmia, antimicrobial, anticancer, antioxidant, astringent, and bitter flavoring agent. Quinidine is in high demand, yet its production from the bark of the quina tree (*Cinchona calisaya*) is limited. Quinidine production from quina tree fungal endophytes, namely *Aspergillus sydowii*, *Diaporthe* sp., *Diaporthe lithicola*, *Fusarium oxysporum*, and *F. solani* is lower than the quinidine content of the tree bark. This study attempted to increase quinidine production from these fungi. This research aimed to determine the optimum culture conditions for quinidine production from endophytic fungi.

Materials and Methods: Quinidine was produced by *in vitro* culturing of the fungal endophytes in potato dextrose broth (PDB) medium under different culture conditions, i.e., a combination of an initial medium pH of 6.2 or 6.8, with or without light, in a static condition for 21 days of incubation at room temperature. Production under natural daylight in PDB medium without pH modification was used as the control. At the end of the incubation period, the mycelial mass was separated from the filtrate. The dried biomass and chloroform-extracted filtrate were weighed. Quinidine in the extract was analyzed qualitatively and quantitatively using high-performance liquid chromatograph.

Results: Quinidine production was affected by both light and the initial pH of the medium, depending on the fungal strain used. A significant increment in quinidine production, approximately 1.1-9.3-fold relative to its respective control was obtained from all fungi under their optimum conditions. Quinidine production in most of the fungi was significantly correlated with their biomass production but not with their extract production. Of those five fungi, *F. solani* that was cultured in PDB medium with an initial pH of 6.2 and incubated under continuous light produced the highest concentration of quinidine with low biomass.

Conclusion: The quinidine production of all fungal endophytes studied was affected by the culture conditions. *F. solani* is the most promising fungus for use as a quinidine production agent.

Key words: Fungal endophytes, *in vitro* culture, optimization, light, pH, quinidine

**ÖZ**

Amaç: Kinidin, antimalaryal, antiaritmik, antimikrobial, antiyansı, antioksidan, astrinjen ve acı tatlandırıcı olarak kullanıldığı için farmasötik öneme sahiptir. Kinidin yoğun talep görmesi rağmen, kinidin kaynağı olan quina ağacı (*Cinchona calisaya*) kabuğundan üretilmesi zorur veren ve endofitlerin birçoğundan üretilmesi zor veren etkisi, quina ağacı kabuğundaki kinidin içeriğini düşüktü. Bu çalışmadan endofit mantarların kinidin üretimini belirlemesi ve optimal kültür koşullarını belirlemesi amaçlanmıştır.

Gereç ve Yöntemler: Kinidin farkedik kültür koşullarında patates dekstroz et suyu (PDB) ortamında fungal endofitlerin *in vitro* kültürlenmesi yoluya üretilmiş, dışlamalı ortamlar olarak pH 6.2 veya 6.8’ın kombinasyonları ikişlik/dağık olarak kullanları statik koşullarda 21 gün süreyle inkübasyona tabi tutularak üretildiler. pH’yi değişirmeden PDB ortamında doğal gün işığı altında üretim kontrol olarak kullanılmıştır. İnkübasyon süresinin sonunda, misel külesi filtrtan ayrılmıştır. Kurutulmuş biyokütle ve kloroform ile ekstre edilmiş filtrat tartılmıştır. Ekstredeki kinidin, kalitif ve kantitif olarak yuksel basınıcı sivi kromatografisi kullanılarak analiz edilmiştir.

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INTRODUCTION

Quinoline is a major secondary metabolite produced in the bark and root of the quina tree (Cinchona, Rubiaceae). There are four members of quinoline, i.e., quinine, quinidine, cinchonine, and cinchonidine. These substances are commonly used as antimalarial drugs. Malaria is a very dangerous disease and a leading cause of death worldwide. Malaria is caused by four species of Plasmodium. The most serious form of malaria is caused by Plasmodium falciparum and P. vivax. The goal of the World Health Organization in 2016-2030 is to either reduce the incidence of malaria by at least 90% or eradicate the disease in all countries of the world.

Quinidine is actually three-fold more effective at treating malaria caused by P. falciparum than the other compounds mentioned. Further, it has the widest range of uses out of all members of the quinoline family. In addition to its application as an antimalarial drug, quinidine is also useful as an antimicrobial, anticancer, antioxidant, astringent, bitter flavoring, and antiarrhythmia agent. As an antimalarial drug, quinidine binds to free heme to prevent hemoglobin formation. The production of hemazoin in a heme detoxification system in the malaria parasite. Free heme is very toxic to the parasite, as it can induce oxidative stress, which leads to the parasite’s death. As an antibacterial, quinidine inhibits the internalization or invasion of bacteria. As an anticancer agent, quinidine can arrest MCF-7 human breast cancer cells in the G1 phase of the cell cycle and lead to apoptotic cell death. As an antibacterial, quinidine is classified as class Ia sodium channel blocker. Quinidine is very effective at treating atrial fibrillation and short QT syndrome. It can also be used as an anticonvulsant, an anticholinergic drug, and an antagonist of α-adrenergic and muscarinic drugs. Therefore, increased quinidine production is needed to fill the growing pharmaceutical demand.

Currently, quinidine availability is solely dependent on the bark and root of the quina tree. Indonesia has shown a drastic decline in the quina tree population and a consequent decline in quinidine production. Efforts have been made to increase the plant population and quinidine production, i.e., quina tree replanting, tree selection through hybridization and clonal propagation and quinidine production through cell culture. However, the quinidine supply is still limited. Therefore, it is necessary to explore other natural resources for quinidine production.

Endophytic fungi can produce secondary metabolites similar to those of their host plant. Endophytic fungi isolated from the Cinchona tree are potential sources of quinidine production.
Quinidine extraction and analysis

The mycelial mass was separated from the filtrate using filter paper and dried in an oven at 60°C for 24 hours or until a constant dry mass was reached. The biomass dry weight represented biomass production. The filtrate was extracted by adding chloroform (CHCl₃, ≥99.8%) at a 1:1 ratio. The mixture was homogenized and allowed to stand for 24 hours. The mixture was transferred to a separatory funnel and allowed to stand for a few seconds to form two layers. Afterward, the bottom layer was removed and evaporated using a rotary evaporator at 45°C and 60 rpm until a pellet was formed. The pellet was weighed and designated as the extract. Finally, the extract was stored at 4°C as the stock material for quinidine analysis.

Quinidine was analyzed using a Shimadzu Prominence 20AD high-performance liquid chromatograph (HPLC). The extract was first dissolved in 1 mL chloroform. About 20 µL of that solution was injected into the HPLC. The analytical conditions were set as follows: Thermo C-18 column (4.6x250 mm), methanol:acetonitrile (80:20) as the mobile phase, 1.0 mL sec⁻¹ flow rate, 40°C column temperature, and a detection wavelength of 210 nm. Quinidine was detected to have an Rt value of 9.9412±1.9 minutes (Figure 2F), and the unknown compound had a peak at ± Rt 5 minutes (Figure 2A-E). The quinidine standard concentration was highly correlated (R²: 1) with its area under the respective peak; therefore, the regression equation was y=mx + b, where y is the area under the standard quinidine peak, m is the regression coefficient, x is the quinidine concentration, and b is a constant.

Experimental design

A factorial design with a combination of two factors, i.e., light exposure (with or without light) and the initial pH of the medium (pH 6.2 and 6.8) was used in this research. The production condition in PDB medium without pH modification incubated at room temperature was used as a control. The treatments were tested on five C. calisaya endophytic fungi, i.e., A. sydowii, Diaporthe sp., D. lithicola, F. oxysporum, and F. solani. Each treatment was applied in triplicate to produce a total of 75 experimental units.

Statistical analysis

Data were analyzed statistically using analysis of variance followed by Duncan’s multiple range test (DMRT), both at the 5% level of significance. Correlation of quinidine production with biomass dry weight and with extract weight were calculated using Pearson’s correlation coefficient (r_p). The correlation was categorized into five groups, i.e., weakest (0.00-0.19), weak (0.20-0.39), moderate (0.40-0.69), strong (0.70-0.90), and strongest (0.90-1.00).28

RESULTS

Quinidine production

The HPLC chromatogram of all fungal extracts showed two peaks, indicating the presence of quinidine and an unknown substance (Figure 2A-E). Quinidine was detected to have an Rt similar to that of the quinidine standard (Rt 9.9412±1.9 minutes, Figure 2F), and the unknown compound had a peak at ± Rt 5 minutes (Figure 2A-E). The quinidine standard concentration was highly correlated (R²: 1) with its area under the respective peak; therefore, the regression equation y=123.915x+1442.8 was further used to calculate the quinidine concentration in the extract.

The amount of quinidine varied depending on the fungal strain used and the incubation conditions. A. sydowii, F. oxysporum, and F. solani produced the maximum concentration of quinidine under continuous light incubation with an initial medium pH of 6.2. In contrast, the maximum quinidine production of Diaporthe sp. and D. lithicola were obtained under dark incubation with an initial medium pH of 6.8. Under optimum conditions, all fungi (A. sydowii, Diaporthe sp., D. lithicola, F. oxysporum, and F. solani) produced quinidine at their highest concentrations, i.e., 29.026 (Figure 3A), 8.913 (Figure 3B), 11.148 (Figure 3C), 23.967 (Figure 3D), and 65.177 µg (Figure 3E), respectively, in 200 mL PDB medium after 21 days of incubation.

The quinidine production of all fungi increased significantly (p<0.05) under optimum conditions. A. sydowii (Figure 3A), Diaporthe sp. (Figure 3B), D. lithicola (Figure 3C), F. oxysporum (Figure 3D), and F. solani (Figure 3E) increased
2.7-, 1.6-, 1.1-, 9.3-, and 6.0-fold, respectively, compared with their corresponding controls. The DMRT test (p<0.05) indicated that quinidine production in *F. solani* and *Diaporthe* sp. increased significantly under the combined conditions of light and initial medium pH, whereas the other strains were affected only by either light or the initial pH of the medium. Quinidine production in *F. solani* increased significantly with continuous light and an initial medium pH of 6.2, whereas that of *Diaporthe* sp. increased with dark incubation and an initial medium pH of 6.8. Unlike *F. solani* and *Diaporthe* sp., quinidine production in *F. oxysporum* increased under continuous light regardless of the pH, and that of *A. sydowii* and *D. lithicola* was only affected by the initial pH of the medium.

**Biomass and extract production and the final pH of the culture medium**

The optimum condition for biomass production of each fungus varied and was different from those for quinidine production except for that of *F. oxysporum*. This fungus produced both the maximum quinidine concentration and biomass dry weight (0.247 g) under continuous light and an initial medium pH of 6.2 (Figure 4A). Like *F. oxysporum*, *A. sydowii* grew better (0.387 g) under continuous light but at a different initial medium pH (pH 6.8) (Figure 4B). In contrast, that of *D. lithicola* (0.307 g) occurred under the dark condition with an initial medium pH of 6.2 (Figure 4C). The highest biomass production of all fungi occurred in modified-pH medium, and the highest biomass production of *Diaporthe* sp. (Figure 4D) and *F. solani* (Figure 4E) were obtained under the control conditions, i.e., 0.351 g and 0.470 g, respectively.

The optimum conditions for extract (pellet) production were also different from those for quinidine production, except in *Diaporthe* sp. and *D. lithicola*. Similar to their quinidine production, *Diaporthe* sp. (Figure 5A) and *D. lithicola* (Figure 5B) produced the highest extract dry weight, i.e., 0.765 g and 0.115 g, respectively, under darkness and at an initial medium pH of 6.8. Under these conditions, *F. solani* (Figure 5C) also produced the highest extract dry weight (0.102 g), whereas that of *A. sydowii* (Figure 5D) and *F. oxysporum* (Figure 5E) were obtained under control conditions, i.e., 0.164 g and 0.122 g, respectively. The extract production of each fungus did not reflect the quinidine production. Of the fungi studied, *Diaporthe* sp. produced the highest extract dry weight, but its quinidine production was the lowest (Table 1). Based on extract production, the quinidine content in the extract of *F. solani* was the highest (1551.83 µg/g extract), followed by that of *A. sydowii*, *F. oxysporum*, *D. lithicola*, and *Diaporthe* sp., i.e., 518.32, 230.45, 96.94, and 11.65 µg/g extract, respectively.
Correlation of quinidine with biomass and extract production

Quinidine production can be either negatively or positively correlated with the corresponding biomass production (Table 2). The correlation can also either be significant or insignificant (p<0.1). Of the fungi studied, only *F. solani* and *F. oxysporum* showed a significant correlation. In *F. solani*, quinidine production was strongly negatively ($r_p$: -0.862) correlated with biomass production. In contrast, there was a strong positive correlation between quinidine and biomass production in *F. oxysporum* ($r_p$: 0.835). In addition, *D. lithicola* showed a negative correlation ($r_p$: -0.595, moderate), albeit at a lower level of significance (p<0.2).

In contrast, quinidine production in *A. sydowii* and *Diaporthe* sp. was not correlated with biomass production. The correlation coefficients ($r_p$) for the latter two fungi were 0.002 (weakest) and 0.199 (weak), respectively.

Quinidine and extract production also showed a similar pattern of correlation. However, most of the fungi (*A. sydowii*, *D. lithicola*) showed very weak or no correlation with biomass production.

**Figure 3.** Quinidine production by *Cinchona calisaya* endophytic fungi in 200 mL potato dextrose broth. Bar data followed by the same letter not significantly different in Duncan’s multiple range test at the 5% level of significance

**Figure 4.** Biomass production by *Cinchona calisaya* endophytic fungi in four treatments in 200 mL potato dextrose broth. A vertical bar above the data block indicates the standard error
Diaporthe sp., F. oxysporum, and F. solani showed insignificant correlations, except in the case of D. lithicola, where the correlation was strongly positive and significant ($r_p$: 0.708, p<0.2).

**DISCUSSION**

Only two of the fungal strains used in this study had ever been investigated for quinoline production. In that previous study, Diaporthe sp. and D. lithicola were grown under similar conditions to this study, and those fungi were reported to produce quinine. In contrast, quinine was not detected in the current study. Further, only quinidine was detected. All these quinolines (quinine, quinidine, cinchonine, and cinchonidine) are synthesized from the same structure; therefore, the possibility exists of structural changes among these compounds.

Quinidine was the only quinoline compound produced in this study, as quinidine is probably structurally more stable than quinine.

The quinidine biosynthetic pathway in C. calisaya endophytic fungi has not yet been clarified. In Cinchona, the quinoline biosynthetic pathway is hypothetically synthesized from tryptophan and geraniol to form two intermediates, strictosidine and cinchonaminic, prior to the formation of cinchonidinone. Cinchonidinone becomes a basal substance for the synthesis of the four quina alkaloids. C-8 epimerization of cinchonidinone via enol will form cinchoninone. Both cinchonidinone and cinchoninone are catalyzed by nicotinamide adenine dinucleotide phosphate oxidoreductase to form cinchonidine and cinchonine, respectively. Moreover, cinchonidinone may form quinonine by the hydroxylation and methylation, as do cinchoninone to form quinidinone. Quinonine is reduced to quinine, while quinidinone is reduced to quinidine. Quinidine can also be synthesized.
from quinine by oxidation, epimerization, and reduction steps. Cinchonidine and quinine can be differentiated by their R groups, a hydrogen in cinchonidine, and a methoxy in quinine. Quinine is diastereoisomer of quinidine, as are cinchonine and cinchonidine.

In this study, the medium became more acidic at the end of the incubation period. This means that the pH of the medium was not buffered. It is expected that a medium with an uncontrolled pH will create conditions conducive to greater quinidine production. With a dynamic pH level that decreases from pH 6.5 to pH 3.8 during incubation, swainsonina alkaloid production was induced to a higher level than at a constant pH (pH 6.5). A preliminary study showed that no quinidine was detected in hyphal cells. The acidity of the medium might influence the permeability of the cell wall, thereby affecting nutrient uptake from the environment into cells, and simultaneously allows the quinidine produced in the cell to be excreted into the medium. This simplifies the harvesting of quinidine. In addition, the pH may also affect the expression of genes associated with the synthesis of secondary metabolites and enzyme activity.

Not only pH but also light exposure affects quinidine production. This is in accordance with studies on other secondary metabolites. Secondary metabolite production of Penicillium isariiforme was inhibited by light, in contrast to that of A. flavus and A. ochraceus, which were induced by light. F. verticilloides, Isoria farinosa, and Emericella nidulans produced maximum secondary metabolites with dark incubation and an initial synthetic medium pH of 5.6. F. graminearum produced a higher quantity of trichotheccenes when it was grown under dark conditions rather than under light exposure. Fusarium spp. from Taxus wallichiana bark produced maximum secondary metabolites under optimum conditions, which were medium pH 6 and daylight incubation, whereas A. terreus from seaweed Codium decorticatum produced maximum antibacterial compounds at an initial medium pH of 5.5 and with daylight incubation. In addition, blue light inhibited mycotoxin production in A. flavus, A. parasiticus, and Alternaria alternata. Green light stimulated citrinin production by Monascus ruber.

Light is a crucial signal for every living cell. It seems that not only secondary metabolite production but also regulation of gene transcription, enzyme activation, nutrient uptake, reproduction, morphogenesis, cell wall components, and metabolism of lipids, nucleic acids, and amino acids involves metabolic pathways. Blue light stimulates sexual reproduction in Phycomyces blakesleeanus. Mycelia of Fujikuroi gibberella are orange and produce maximum carotenoids when incubated under light conditions, yet the mycelia become white and produce low amounts of carotenoids when incubated under dark conditions. Glucose absorption by A. ornatu decreased significantly when it was incubated under light conditions. In this case, light may stimulate biosynthesis of an inhibitor that blocks glucose absorption into fungal cells.

In vitro growth of a microorganism is often correlated with its secondary metabolite production. The correlation can be either negative or positive, as shown in this study. When biomass production was negatively correlated with quinidine production, primary metabolism was more supported than secondary metabolism. Otherwise, primary metabolism is inhibited and shifted to secondary metabolism. When the correlation is positive, the carbon source might be used for both primary and secondary metabolism.

During in vitro production, endophytic fungi produced not only beneficial secondary metabolites but may also produce toxins. F. oxysporum f. sp. lycopersici has been reported to produce fusaric acid after 3 days of incubation; maximum production was reached after 10 days of incubation in potato sucrose broth. F. oxysporum could also produce fumonisin in glucose yeast asparagine malic acid medium at 25°C for a 2-week incubation period. Fumonisin has high solubility in water, methanol, and acetonitrile, while quinidine is very soluble in chloroform solvents, moderately soluble in alcohol and benzene, and slightly soluble in water solvents.

Another toxin, such as cyclosporine had been reported to be produced by F. solani f. sp. radicicola in modified liquid culture medium at 25°C for 7 days. F. solani M11 produces T-2 toxin, trichothecene, and solaniol in Czapek dox peptone medium at 25°C for 12 days. A. sydowii produced sydonic acid in liquid medium with an initial pH 7.2 at 28°C for 3 days, while Diaporthe produced phomopsin A in Czapek Dox broth at 24°C for 28 days. Regardless of toxin solubility, these fungi may produce an unwanted substance, consideration should be taken when in vitro production of quinidine using these fungi is brought into practice.

Quinidine is a high-value substance. In this study, quinidine production by F. solani, F. oxysporum, and A. sydowii was higher than that of Diaporthe sp. and D. litchicola. Further, the amount of quinidine produced by Diaporthe sp. (8.913 µg) and D. litchicola (11.148 µg) in 200 mL PDB medium after 21 days of incubation was much higher than by D. phaseolorum (3.5 µg/L) and Schizopyllum sp. (0.01 µg/L) on synthetic medium pH 6.2 after 4 days of incubation. This indicates that all the fungi studied are prospective agents for quinidine production.

The quinidine production from five C. calisaya endophytic fungi was still lower than that from C. calisaya bark extraction (2.240 µg/g). However, compared with quinidine production in C. ledergeriana cell culture (349.38 µg/g), in vitro quinidine production from A. sydowii and F. solani was higher. In contrast, quinidine production from these fungi was lower than in the cell culture (8.078 µg/g) modified by adding 5 ppm of paclobutrazol and incubating it under 10 µmol/m²/sec light intensity. Nonetheless, quinidine production from endophytic fungi is more beneficial than that from plants, considering that its production requires a shorter time. An attempt to scale up quinidine production should, therefore, be pursued using F. solani, which is capable of producing the highest amount of quinidine with low biomass production using PDB medium with an initial pH of 6.2 and incubation under light.
CONCLUSION
In quinidine production by A. sydowii, Diaporthe sp., D. lithicola, F. oxysporum, and F. solani, fungal endophytes from C. calisaya, was affected by light and the initial pH of the medium. Quinidine production by A. sydowii, F. oxysporum, and F. solani were higher with incubation under continuous light and an initial medium pH of 6.2, while Diaporthe sp. and D. lithicola were higher with incubation under continuous darkness and an initial medium pH of 6.8. *In vitro* culture of F. solani in PDB medium with an initial pH 6.2 and with incubation under continuous light produced the highest concentration of quinidine with low biomass and thus could potentially be used for quinidine production. Five endophytic fungi from C. calisaya, mainly F. solani, are natural resources that could be exploited commercially for quinidine production. Production under optimum light and initial medium pH were considered as economically and environmentally friendly conditions. However, the stability of this quinidine production under optimum conditions should be further investigated to find models for production on an industrial scale.

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