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Submission date: 09-Feb-2022 11:28AM (UTC+0700)
Submission ID: 1758259528
File name: 11-AIP_2019_-ARTIKEL.pdf (896.87K)
Word count: 2492
Character count: 13154

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Cite as: AIP Conference Proceedings **2120**, 030028 (2019); https://doi.org/10.1063/1.5115632
Published Online: 03 July 2019

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The Production of Cinnamic Acid Secondary Metabolites through in Vitro Culture of Callus *Camellia sinensis L* with the Elicitor of Cobalt Metal Ions

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Abstract. Cinnamic acid is one of the secondary metabolites found in *Camellia sinensis* L plants which can be bioactive. These Cinnamic acid bioactives are useful as anti-cancer, antioxidants, antibacterial, corrosive and as an inducer to increase secondary metabolites in biotechnology's field. The problem of cinnamic acid production from plants constrained by climate change, weather and narrow land conditions with uncontrolled population explosions. The purpose of this study was to obtain cinnamic acid's method production with *in vitro culture* techniques. The methods used to achieve these objectives: 1) planting shoots of *Camellia sinensis* L leaf explants, 2) increasing cinnamic acid in callus culture, 3) Qualitative observation of callus growth. 4) Quantitative observation of cinnamic acid content in callus. The results of the study were shaped with increased wet weight callus containing cinnamic acid. The significant finding in this study is that with the addition of cobalt metal ion elicitors, the secondary metabolite of cinnamic acid was obtained by 11.9%. This study implies that the use of the *in vitro culture* technique of *Camellia sinensis L* in a relatively short time can produce secondary metabolites of cinnamic acid which increase with the cobalt metal ions elicitor.

INTRODUCTION

Cinnamic acid is one of the secondary metabolites found in various plants and is found in several fruits. In *Camellia sinensis* L cinnamic acid is a secondary metabolite formed through the phenylpropanoid pathway [1]. This cinnamic acid has bioactive properties in various fields; some of them can be as allelochemical/ inhibits root growth in undesirable plants in agriculture [2]. Besides, it can be an anti-oxidant, anti-cancer and anti-inflammatory which can be applied to the medical field [3]. In the area of food and beverages, it can reduce damage and extend the service life in the food industry [4]. In the cosmetics industry, cinnamic acid can be a candidate drug that is useful for whitening or brightening the skin [5]. The acquisition of cinnamic acid bioactive from plants is constrained by climate change, weather and land conditions that are increasingly narrow due to uncontrolled population explosions. To reduce these constraints, the production of secondary metabolites of cinnamic acid needs to be carried out through in vitro culture techniques. The advantage of using in vitro culture techniques is that it can be done on a narrow field, or a laboratory scale. The other benefits do not depend on climate change, no need to wait for plants to harvest, biomass can be harvested with a relatively short time between 4-8 weeks with in vitro techniques especially with suspense culture techniques [6]. Besides, having the advantage, in vitro culture has its deficiency too, that is the

International Conference on Biology and Applied Science (ICOBAS)
AIP Conf. Proc. 2120, 030028-1–030028-5; https://doi.org/10.1063/1.5115632
Published by AIP Publishing. 978-0-7354-1860-8/$30.00

biomass sometimes obtained still under the biomass harvested from plants collected from the land. To overcome this deficiency, a technique with media optimization, growth-regulating substances and the addition of precursors and elicitors is carried out. This study aimed to obtain a method of the production of secondary metabolites of cinnamic acid in vitro by modifying the medium and elicitor of cobalt metal ion as the inducer.

EXPERIMENTAL DETAILS

Plant Materials and Explant Source

Camellia sinensis L plant material is obtained from the garden, then maintained in polybags to adapt to the weather environment of greenhouses by providing complex fertilizer nitrogen-phosphorus and potassium. The subsequent plant material as explant source material is that leaf picked from the position of the first leaf to the third leaf. Before planting the explants of the leaf shoots, the aseptic procedure is applied. The aseptic procedures applied are 1) washed in tap water that flows for twenty minutes. 2) soaked in a solution containing two percent anti-bacterial and anti-fungal substances for fifteen minutes then drained. 3) leaves that have been drained, soaked in a solution containing three percent vitamin C. 4) then soaking the leaves in a solution containing five percent sodium hypochlorite [7]. for ten minutes on a laminar air flow cabinet. 5) after being soaked with sodium hypochlorite, then rinsed with distilled water which was repeated three times. 6) Sterile *Camellia sinensis* L leaf shoots are ready to be cut by one centimeter [8] to be planted in Murashige and Skoog media enriched with growth regulators.

Planting shoots of *Camellia sinensis L* Leaf Explants

The pieces of *Camellia sinensis L* leaf shoots as explants were planted in Murashige and Skoog (MS) media enriched with 2.4 dichlorophenoxyacetic acid (2,4-D) growth regulators of one ppm [9]. The piece of leaf shoots on this MS medium then was incubated in the incubation room with irradiation for six weeks to obtain callus biomass. Callus biomass is further enhanced by elicitor.

Increasing Cinnamic Acid in Callus Using Cobalt Metal Ion Elicitor

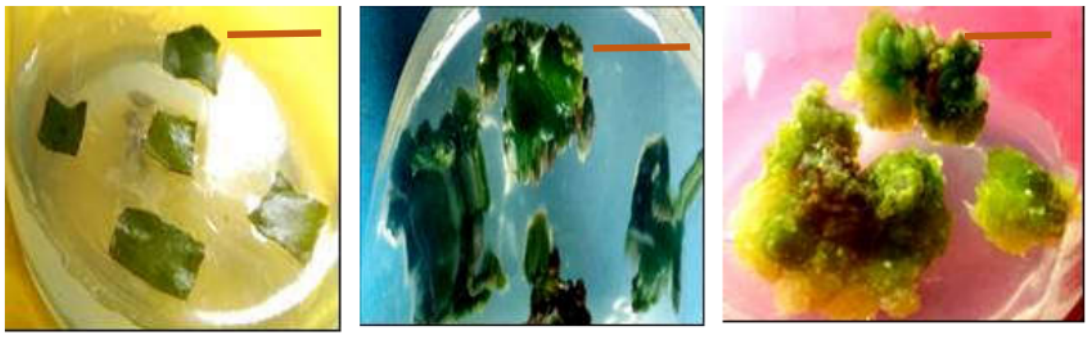
The increase in callus biomass was carried out by subculture by planting 10mg of callus on MS media enriched with 2,4-D growth regulator and with the addition of elicitor. Addition of Cobalt (II) chloride ionic elicitor [10] with different concentration variations, respectively: 0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2 ppm [11]. Media that has been added to elicitor is incubated for one month then evaluated both qualitatively and quantitatively.

Qualitative-Quantitative Observation of Cinnamic Acid Content in Callus

Qualitative evaluation by observing callus shape using a trinocular microscope while quantitative evaluation by weighing callus wet weight. Besides observing callus wet weight, a cinnamic acid content test was carried out, firstly by isolating using High-performance liquid chromatography (HPLC) [12].

RESULT AND DISCUSSION

Six weeks after planting, changes in the shape of the explants began to appear as callus with changes in morphology in Fig. 1. The appearance of morphological changes from leaf explants forming successive calluses (Fig. 1): A. leaf explant pieces, B. curvature swelling of explant, C. callus formation from explant edges, then all explants forming a callus. This morphological change is caused by the presence of nutrients and the role of growth regulators that can differentiate cells and cells elongation. This study is relevant to [13] with the addition of a growth regulator, amounting to 0.5 ppm in young leaves of the *Clinacanthus nutans* plants, which can produce callus with a wet weight of one hundred percent. Besides producing combined callus between 2,4-D 8 ppm and 6 benzylaminopurine (BAP) 8 ppm in Pelargonium x domestic plants, it can also form embryos [14]. A similar study in the *Trachyspermum Ammi* plants, a combination of 2,4-D two ppm and BAP 0.25 ppm was able to induce callus and shoots [15].



(a) (b) (c)

FIGURE 1. Changes in the morphology of explants forming a callus, 5 mm bars

The callus that formed by the addition of elicitor of cobalt (II) chloride metal ion with various concentrations: 0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2 ppm wet weight can be observed in Fig. 2.

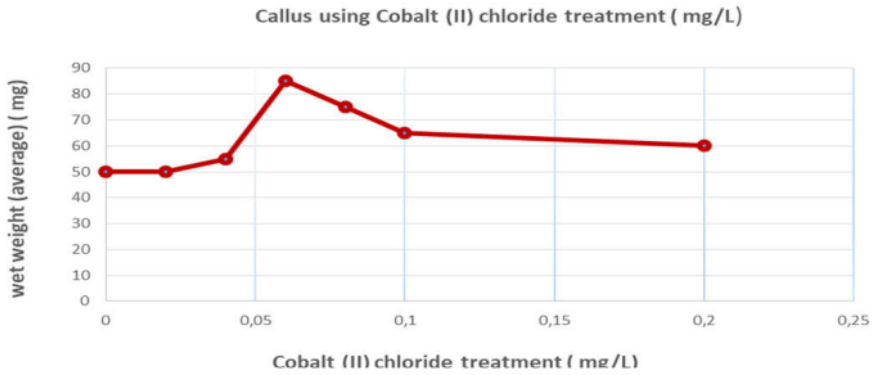


FIGURE 2. Callus with a treatment of cobalt (II) chloride metal ion

The use of Cobalt (II) chloride metal ions at a concentration of 0.06 ppm has the highest wet weight of about 85 mg (Fig. 2). The highest wet weight is caused by the presence of microelements of Cobalt (II) chloride metal ion which functions as energy for callus cell proliferation. But the use of these metal ions needs to be optimized to determine the optimum requirements of each plant. From the data in Fig. 2, there appears to be a decrease in wet weight when the concentration level of Cobalt (II) chloride is increased. It can be concluded that for elicitation, optimization needs to be done first to get the optimum elicitor level. The use of Cobalt (II) chloride metal ions in orchid plants can also increase the biomass at the level of 0.06 ppm [16]. The use of elicitor to increase the biomass, this is relevant to the research [17] that the use of elicitor dipotassium hydrogen orthophosphate at a concentration of 20 ppm can increase the biomass production. The presence of cinnamic acid in in vitro culture is the same as the presence of cinnamic acid in plants [18] because cinnamic acid acts as an intermediate in the biosynthesis of secondary metabolites [19]. Subsequent research on the use of Cobalt (II) chloride metal ions in *beta vulgaris* plants can produce secondary metabolites betalain [20].

Qualitative results of cinnamic acid observed with trinocular microscope [21] obtained the shape and color of callus cells compared to standard cell forms and the color of cinnamic acid cells that have similarities. In a microscope, the standard color of cinnamic acid is the same as the cell color of callus containing cinnamic acid (Fig. 3).

Cinnamic acid has the shape of a crystal, which is yellowish white. In Fig. 3, the shape and color of the cell with the arrow sign that has similarities are also yellowish white. Besides using a microscope, the qualitative test results were carried out also by observing the chromatogram of the cinnamic acid standard retention time that compared with the chromatogram of sample retention time from the biomass of callus are shown in Fig. 4.

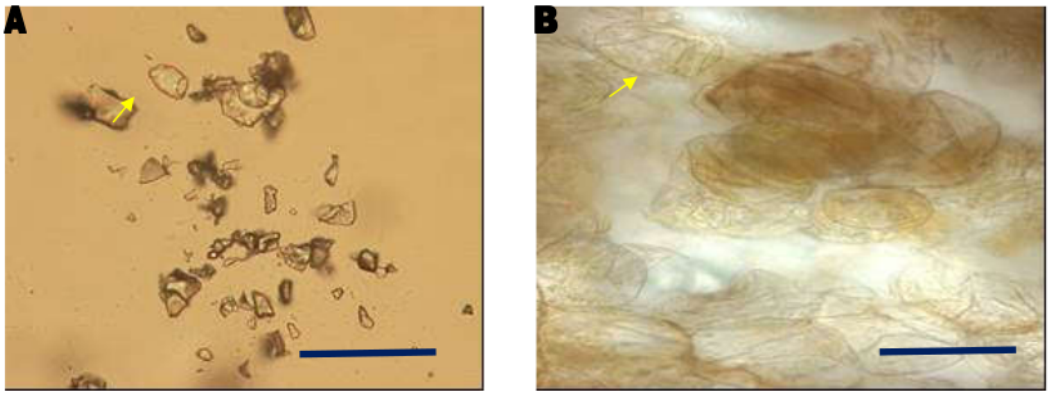


FIGURE 3. Cinnamic acid standard cells (A), cinnamic acid callus cells (B), 5 mm bars

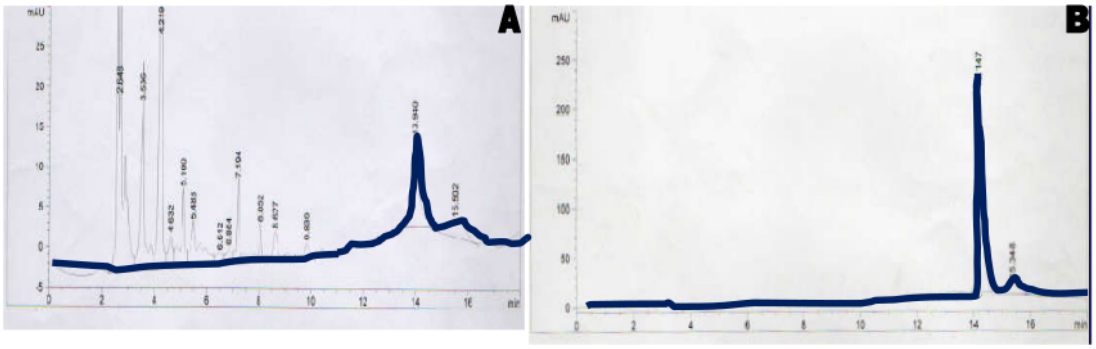


FIGURE 4. Chromatogram: (A) cinnamic acid standard, (B) cinnamic acid callus

Chromatogram was obtained with a standard retention time of 14, whereas callus retention time was approximately 14 min (Fig. 4). Then it can be said that callus contains cinnamic acid. The quantitative test results with partially modified High-performance liquid chromatography [22] were obtained from comparing the standard area with the sample area, obtained a cinnamic acid level of 11.9 %. This study is relevant to number patent of POO201709375 [23].

SUMMARY

Cinnamic acid secondary metabolites are obtained from the land, but can also be produced through the in vitro culture technique of the *Camellia sinensis* L plants by optimizing the addition of cobalt metal ions. The use of cobalt metal ion elicitor (ii) chloride at a concentration of 0.06 ppm can form the highest secondary metabolite of cinnamic acid at 85 milligrams

ACKNOWLEDGEMENT

The author would like to acknowledge the ministry of research, technology, and higher education of the Republic of Indonesia, which has funded the research through competition grants 2017-2019.

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