Effects of Essential Oil Made of Orange Peels on Fungal Contamination of Elephant Grass, Cassava Plant and Corn Kernel Explants on Tissue Culture Medium

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Abstract. Tissue culture technology is an alternative way that can be used to produce plant seeds that will be widely developed as feed for ruminants. However, this technique often has constraint in the form of fungal contamination which affect the growth of explants during culture. One way in preventing the fungal pollution is by immersing explants in an antifungal solution in the form of essential oil. The aim of this study was to obtain the optimum concentration of essential oils made from orange peels to inhibit fungal growth in explants of elephant grass, cassava plant and corn kernel. There are 6 concentrations of essential oils as the treatments, including 0%, 2%, 4%, 6%, 8% and 10%. Each treatment consisted of 4 replications and observations were carried out for 4 months period. Results obtained that concentration of essential oil as much as 10% was the best for preventing fungal growth followed by concentrations of 8%, 6%, 4% and 2%. However, explants of elephant grass and cassava plant can grow well at concentrations of 2% and 4%. It is concluded that essential oils with a minimum concentration of 2% can be used to prevent the growth of fungal contamination in explants of elephant grass and cassava plant.

1. Introduction

Animal feed, in general, is a mixture of two or more types of material. Feed ingredients for cattle or other ruminants consist mostly of forage and concentrate. Forage feed that is often given to cattle can be grass or legumes, while concentrate feed is a mixture of bran, grains, soybean cake and fish meal. According to Prabowo and Susanti [1] the ideal feeds comprise of balanced nutritional content and following the requirement of the animals.

Forage is a food source of life which is very important in the growth of livestock, therefore forage must be of good quality and easily digested by livestock. However, forage is only available in adequate quantities or even in abundance during rainy season. Rahardjo et al. [2] stated that the
availability of forage is a common problem experienced by farmers and animal owners, therefore most of them only rely on forages from fertile areas where grass grows.

Elephant grass, cassava and corn can be used as a source of forage for ruminants. Elephant grass (*Pennisetum purpureum*) is one of the forages favoured by livestock and this grass can thrive in the tropics. This grass can also grow well in the high and lowland areas. Elephant grass that is usually planted is in the form of stem cuttings that can grow quickly and can reach up to 2-4 meters in high [3]. According to Novianti *et al.* [4] forage of elephant grass has a large impact on feed efficiency because it is the most varied feed material in terms of digestibility and nutritional composition and is often given in greater proportions than other forages.

The use of cassava plants as an option for animal feed are also aimed to overcome the shortage of animal feed as the local feed ingredients. Cassava (*Manihot utilissima*) is a tropical plant that is easy to grow in all soil conditions and at harvest time its price is relatively cheaper. The protein content in cassava tubers is not too high, but cassava tubers are a potential source of feed energy. However, cassava leaves contain high levels of protein and can be used as feed ingredients for protein sources. Utilization of cassava plants and their waste as animal feed ingredients is often constrained by the presence of antinutrient compounds in the form of cyanide acid and linamarin [5]. These constraints can be overcome by physical, chemical or biological treatment as an effort to detoxify the antinutrient compound.

Cassava peels or cassava tuber skin can also be used as additional feed ingredients for ruminants. Cassava peels is a by-product of the cassava tuber processing industry such as cassava chips and tapioca flour. Cassava tuber skin is quite a lot in number, each kilogram of tubers produces 15-20% of the skin. Rukmana [6] sated that cassava peels are widely used at this time because it has a relatively good nutritional value as it contains crude protein as much as 8.11%, 15.2% crude fibre and 74.7% TDN.

Corn (*Zea mays*) is a plant commonly known as a raw material for concentrate. Corn used as raw material for animal feed is in the form of shelled corn. As published by ISB [7] that maize yields in the form of dried kernels that have been released and cleaned from the cob are generally white and yellow. Lately, it turns out that corn waste can still be utilized, namely 92.5% in the form of leaves, 5% stems and 2.5% cobs. Corn straw is agricultural waste that is widely available in rural areas and is almost evenly distributed in all dryland areas. Corn straw, when mixed with other feed ingredients that have complete nutrient content, will produce a rational and inexpensive feed composition. Corn straw is left over from the corn plant after the cob is harvested and can be given to cattle in both fresh and dry forms. Utilization of corn straw as animal feed has been given mainly to cattle, goats, and sheep [8].

To multiply the various sources of feed ingredients mentioned above requires various ways to meet the availability of forage. One of them is by using tissue culture technology. According to Husni and Kosmitian [9] opportunities for procurement of plant seedlings through tissue culture are very potential to meet the needs of seedlings to expand animal feed forage to increase livestock productions in Indonesia. Tissue culture methods for propagation of seeds have been carried out on plants of high economic value such as food crops, horticulture, plantations, and forestry. As found by Husni [10] that the principle of this method of propagation of tissue culture is an attempt to multiply a type of plant carried out in aseptic conditions on controlled growth media. Therefore, the tissue used as a source of explants for propagation must be aseptic, free of contaminants in the form of microorganisms or fungi.

The presence of fungal and microorganism contamination in tissues used as explants in tissue culture is often an obstacle and inhibitor in using tissue culture methods for plant propagation. One way to control it is by using anti-fungal ingredients, but anti-fungal ingredients with basic ingredients in the form of chemicals will harm health. It is necessary to try various other options, one of which is to use essential oils made from orange peels. The purpose of this study is to find an effective dose of antifungal material that will be used to sterilize explants from materials in the form of elephant grass, cassava plant and corn kernel which are inoculated in the medium for seeding.
2. Materials and methods
The study was conducted at the Cell and Tissue Biology Laboratory, Indonesian Center for Agriculture Biotechnology and Genetic Resources and at the Mycology Laboratory, Indonesian Research Center for Veterinary Research, in 2018. Plant materials used as the source of explants were young stems from elephant grass and cassava plants and corn kernels. In this study there were 6 concentrations of essential oils as treatment, including 0%, 2%, 4%, 6%, 8% and 10%. Each treatment consisted of 4 replications and observations were carried out for 4 consecutive months. The material used for sterilization was essential oils made from orange peels. While the growing media used was a mixture of Murashige & Skoog (MS) with sugar that is compacted using agar. The MS was made from macro and micro minerals, miyo-inositol, and vitamin.

2.1. Explant preparation
Explants of young stems of elephant grass and cassava plants were cut into pieces with a size of 1-2 cm consisting of one bud. The explants and corn kernels were washed using detergent, then rinsed with running water. The explants were then put into a sterile bottle (5 cm in diameter and 10 cm high), which contained 10 ml of 70% alcohol solution to soak the explants for 20 minutes. The immersion was carried out in a laminar airflow cabinet, the explants were then rinsed two times with sterile distilled water. Furthermore, the explants were transferred to other sterile bottles which contained essential oil solutions according to the treatment concentrations.

2.2. Essential oil solution preparation
The essential oils were made in a distillation kettle, the lid of the kettle was firstly opened and cleaned, then a porous plate was installed as a base for the orange peels. Then clean water was poured into the distillation kettle with a height of approximately 3-5 cm below the porous plate. After that, as much as 40 kg of dried orange peels were arranged properly on a porous plate. The orange peels were arranged so that they were not too dense to facilitate the passage of water vapor through the orange peels.

The distillation kettle was then tightly closed, and the connecting pipe was installed between the distillery boiler with the condenser and the condenser reservoir. Then the distillery was heated using a stove and cooling water flowing into the condenser. The distillation process was carried out for approximately 6 hours. When the water boils, the water vapor will pass through holes in the porous plate and crevices in the orange peels. Essential oils contained in the orange peels will be carried by hot steam to the condenser pipe and accommodated in the distillation valve faucet.

Oil and water will separate themselves according to the difference in specific gravity where the oil layer was on the top and the water layer was below. The distillation results were then passed through the distillation valve, the water layer was washed out and the oil layer was then collected. The essential oil obtained was then mixed with Sodium Anhydrous Sulfate, after which the filtrate was separated by filtering it using filter paper until essential oil obtained was free of water [11, 12]. The amount of essential oil needed was as much as 30 ml which was then stored in a tightly closed container. The yield of essential oils obtained from this process was approximately 0.32%.

2.3. Essential oil dilution
As much as 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, and 1.0 ml of essential oils were poured into separate test tubes containing respectively 9.8 ml, 9.6 ml, 9.4 ml, 9.2 ml and 9.0 ml of sterile distilled water. Then, the essential oil solutions were homogenized using a vortex mixer to obtain the dilution with concentrations of 2%, 4%, 6%, 8% and 10%.

2.4. Culture media preparation
Media solution that has been made according to the dose was mixed with 30 g/l sugar with a pH of 5.8. After stirring it until dissolved using a magnetic stirrer, the media was then heated on a hotplate at a temperature of 500 °C until it boils. Approximately 25 ml of the boiled media were poured into a
sterilized bottle in an oven at 150 °C for 2 hours. Then the bottle was covered with aluminum foil. Furthermore, it was sterilized in an autoclave at 121 °C with a pressure of 1.5 Atm for 15 minutes. All tools and materials used in the process of tissue culture preparation were also sterilized in the oven at a similar temperature, pressure and time.

2.5. Explants sterilization
The next step was the sterilization of explants of elephant grass, cassava plants and corn kernels, that have been washed with running water, by immersing them in 70% alcohol solution for 20 minutes while on a shaker. They were then rinsed two times with a sterile distilled water and immersed in the solution of essential oils according to the concentration of the treatments (2%, 4%, 6%, 8%, and 10%) for 15 minutes. Finally, they were rinsed again twice with a sterile distilled water. The explants were now ready for culture.

2.6. Explants culture
The steps to culture the explants were as follows: Laminar airflow cabinet, tools and all materials used were first sterilized. Essential oils with concentrations of 2%, 4%, 6%, 8% and 10% are poured into sterile Petri dishes. A total of 5 explants of elephant grass soaked in the essential oils with a concentration of 2%, 4%, 6%, 8% and 10% for 5 minutes. The lid of the bottle containing the planting medium was opened and the mouth was burned using a Bunsen burner. Five explants of elephant grass that had been soaked in essential oils were planted into the culture bottle using tweezers, then the culture bottles were immediately closed. The same method was used for the cassava and corn explants. Each treatment was repeated 4 times and observation on the appearance of the fungus was carried out for 120 days. Identification of explant pollutants was carried out using the method developed by Thompson [13], Onions et al. [14], and Barnet and Hunter [15].

3. Results and discussion
It was found from this study that the concentration of essential oil as much as 10% was the best for preventing mold growth in the culture media followed by concentrations of 8%, 6%, 4% and 2%. In explants of elephant grass and cassava plant, the molds were only grown at concentrations of 2% and 4%, whereas in explants of corn kernels the fungi had grown on the 10th day of observation (Table 1). It should be considered that the possibility of fungal contamination originating from the place of origin of explants used as well as from the air surrounding the bottles used as seeding facilities.

**Table 1.** The duration (days) of resistance to fungal contamination in tissue culture media of various explants diluted in different concentrations using essential oils made of orange peels

| No. | Name of Explants     | Essential oil concentrations (%) | 0    | 2    | 4    | 6    | 8    | 10   |
|-----|-----------------------|----------------------------------|------|------|------|------|------|------|
| 1.  | Elephant grass        |                                  | 2-3  | 10-30| 30-45| 35-45| 120  | 120  |
| 2.  | Cassava plants        |                                  | 2-3  | 25-30| 80-90| 100-120| 120 | 120  |
| 3.  | Corn kernels          |                                  | 1-2  | 2-3  | 2-6  | 2-8  | 3-6  | 10-14|

Antifungal material possessed by the essential oils made of orange peels were analyzed using the Gas Chromatography-Mass Spectrophotometer (GC-MS) method. The identification results showed that the essential oils of orange peels contain 5 major compounds, namely limonene (26.04%), β-citral (10.40%), β-pinene (18.84%), α-Citral (13.09%), and β-phellandrene (6.29%) [16]. Based on these results, one of the dominant components of essential oils as antifungals was α-Citral which was included in the active allelopathic compound which has antifungal activity [17]. The α-Citral activity as an antifungal owned by essential oils of orange peels was caused by compounds contained in the essential oils which have the same mechanism as antifungal ofazole group which is an antifungal agent where the group of compounds will interact with C-14α demethylase (P-enzyme) 450 cytokines). The result of this interaction will inhibit the demethylation of lanosterol to ergosterol.
which is an important sterol for fungal membranes. This inhibitory process will interfere with the function of permeability in fungal cell membranes and it has a fungistatic characteristic [18, 19].

It was also obtained from the results of this study that the types of contaminated fungi that grow on all three types of explants both on agar media and the plants were the genera of *Aspergillus* sp., *Fusarium* sp. and *Penicillium* sp. (Figure 1). These results were in accordance with studies by Thompson [13], Onions *et al.* [14], and Ahmad [20] which stated that the 3 types of fungus are categorized as the contaminated fungi. The genus of *Trichoderma* sp. was also found as the contaminated fungi, this type of fungi is widely found in the soil. As the contamination fungus were generally grown on the explants indicated that the explant's place of origin has been contaminated with the fungus.

![Figure 1](image-url)  
*Figure 1.* Types of contaminated fungi grown on the agar media and explants diluted with essential oils made of orange peels: (A) *Aspergillus* sp., (B) *Fusarium* sp., (C) *Penicillium* sp., (D) *Trichoderma* sp. (using the Lactophenol cotton blue staining and with a magnification of 10 x 40).

An appropriate dose of essential oil will kill the contaminant fungi, but it will not kill the inoculated explants. An effective and the best doses of essential oils are those that have a wide concentration range between the dose of killing contaminated fungi and the non-lethal dose of the explants. However, these doses seem to be difficult to be achieved for the purpose of this study (Table 1). Besides that, on observations of more than 120 incubation days, almost all explants were died, so that these essential oils tended to be better used as fungicides on seed media than on plant explant fungicides.
4. Conclusion

Essential oils made from orange peels can be used as a fungicide in explant media with concentrations of up to 10%, but as a fungicide for the explants it can only be used at a maximal concentration of 4%.

References

[1] Prabowo A and Susanti A E 2013 Software-based beef cattle feed formulation to support beef and buffalo self-sufficiency program Proc. Nat. Seminar on Anim. Husbandry and Vet. Tech. p 180-186
[2] Rahardjo L, Subagiyono I, Chuzaiemi S and Nugroho A B 2011 Characterization of feed system in people’s dairy farming business during prolonged rainy season Proc. Nat. Seminar on Anim. Husbandry and Vet. Tech. p 717-724
[3] Reksohadiprodjo S 1985 Forage Production of Tropical Livestock Food. of the. (Yogyakarta: UGM Faculty of Economics Publishing Section)
[4] Novianti, Purwanto B P and Atabany A 2014 Milk production efficiency and productivity of Elephant grass (Pennisetum purpureum) on FH dairy cow with different cutting size J. Anim. Prod. and Tech.1 (2) 224-230
[5] Antari R and Umianti U 2009 Utilization of cassava plants and their wastes optimally as ruminant animal feeds Wartazoa 19(4) 192
[6] Rukmana R 1997 Cassava (Yogyakarta: Kanisius)
[7] ISB 1998 Corn Feed Raw Materials ISBN 01-4483-1998. (Jakarta: Indonesian Standard Bureau)
[8] Directorate General of Livestock and Health 2006 Plant wastes as ruminant feeds (Jakarta: Ministry of Agriculture)
[9] Husni A dan Kosmiatin M 2019 The potential use of SDGs and biotechnology to support sustainable agriculture Elephant grass and seed breeding opportunities through tissue culture to meet the needs of quality animal feed ed M Sabran, E G Lestari, D W Utami, R Purnamaningsih, Y Suryadi, M. Tasma, Susiptrayitno, and R A S Wibisono (Jakarta: IAARD Press) pp 231-248
[10] Husni A 2018 Quality seed production system for increasing plantation plant competitiveness Delivering sustainable agriculture: an Agenda for technologically Innovation and policy ed T Sudaryanto, I Inoumu, I Las, E Karnawati, B A Husin, and I W Rusastra (Jakarta: IAARD Press) Pp 291-321
[11] Guenther E 1987 Essential Oil Volume I Translated to Indonesian by Ketaren S (Jakarta: UI-Press Publisher)
[12] Wonorahardjo S 2013 Chemical Separation Methods (Jakarta: Akademia Permana) p 240
[13] Thompson J C 1969 Techniques for the Isolation of the Common Pathogenic Functions. II. Air Sampling. Dilution Plating and the Ringworm Fungi. Medium 2 110-120
[14] Onions, Allsopp and Eggins 1981 Smith’s Introduction to Industrial Mycology (London: Edward Arnold Ltd)
[15] Barnet A and Hunter B B 1998 Illustrated Marga of Imperfect Fungi (USA: Prentice Hall)
[16] Wibaldus, Jayuska A and Ardiningsih P 2016 Bioactive oil of Citrus aurantifolia against Leprosy (Coptotermes sp.) J. Chem. Equator 5 (1) 44-51
[17] Chaimovitsh D, Abu-Abied M, Belanov E, Rubin B, Dudai N and Sadot E 2010 The Plant J. 61(3) 399-408
[18] Richard A H and Pamela C C 2001 Pharmacological Illustrated Review (Jakarta: Widya Medika Muda)
[19] Shahzadi P, Muhammad A, Ferhat M F and Chaudhry M Y 2014 Synthesis of 3,7-dimethyl-2, 6- octadienal acetal from citral extracted from lemon grass (Cymbopogon citraes L) J. Antiviral and Antiretroviral 6(1) 28-31
[20] Ahmad R Z 2009 Contamination of molds on feed and its control J. Agric. Res. Dev. 28(1) 15-22