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Triple Layer Synthetic Seed Encapsulation as An Alternative to *In Vitro* Culture of Kribo Orchid (*Dendrobium spectabile*)

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ABSTRACT

Dendrobium spectabile is unique in that it has very diverse colors, shapes and sizes of flowers which make its market demand high but not commensurate with its production. The way to overcome this gap is with synthetic seed technology. The research design used was a completely randomized design with 2 factors, namely chitosan, which consisted of 5 levels, namely 0.05; 0.1; 0.15; 0.2% as well as control and betel leaf extract consisting of 4 levels, namely control; 1; 2; and 3%. Synthetic seeds are made with an Alginate concentration of 2.5%, starting with encapsulation and then providing chitosan soaking treatment and betel leaf extract according to the treatment. Observations were made 1 week after treatment and 3 weeks after treatment. The results show that the three-layer synthetic seeds have the characteristics of being white, non-wrinkling and dense. The lowest percentage of contamination was found in the treatment (0% chitosan + 3% betel leaf), (0.1% chitosan + 0% betel leaf), (0.1% chitosan + 1% betel leaf), (0.1% chitosan + 0.1% betel leaf). betel 3%), (chitosan 0.2% + betel leaf 0%), chitosan 0.2% + betel leaf 2%) of 25%. The cause of contamination is the fungus Rhizoctonia sp. and Aspergillus sp.. The highest shoot growth rate was found in the 0.15% chitosan + 1% betel leaf treatment. The highest number of roots was found in the treatments (0.15% chitosan + 0% betel leaf) and (0.2% chitosan + 2% betel leaf). The longest root growth was found in the treatment (0.15% chitosan + 0% betel leaf).

Keywords: Betel leaf; Chitosan; Contamination; Growth

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INTRODUCTION

Dendrobium sp. is one of the most dominant orchid genera in the Orchidaceae family and consists of more than 2,000 orchid species included in it (Martuti et al., 2021). *Dendrobium spectabile* (kribo orchid) is a very unique ornamental plant because it has a variety of flower colors, shapes and sizes (Sarsaiya et al. 2021). The uniqueness of the frizzy orchid means that market demand continues to increase but is not commensurate with the availability of national production. Production of friable orchids in Indonesia has actually decreased. According to data from the Central Statistics Agency (BPS), Indonesian production of kribo orchids in 2021 was 11,351,615 million stalks, then in 2022 it decreased to 6,793,967 million stalks, and in 2023 it decreased again to 2,522,933 million stalks. The way to overcome this problem is with synthetic seed technology.

Synthetic seeds are an encapsulation method consisting of a somatic embryo wrapped in endosperm and artificial seed coat. Synthetic seeds offer various very profitable advantages, including relatively affordable production costs, the ability to be produced on a large scale, ease of distribution, and resistance to drought and contamination. Synthetic seeds are given 3 layers consisting of alginate, chitosan and betel leaf extract. This combination of coating materials not only increases seed resistance to extreme environmental conditions, but can also increase plantlet growth and reduce contamination.

The first hydrogel coating in the form of alginate can provide maximum protection for explants produced in vitro. The second coating is chitosan which has a natural polycationic linear biopolymer structure of N-glucosamine which undergoes deacetylation and is obtained from chitin (Nawrotek et al., 2023). Chitosan is used in agriculture as a plant growth promoter (Bakhoum et al., 2022). Meanwhile, betel leaves as the third layer contain secondary metabolite compounds in the form of chavicol, cavibetol, eugenol, methyl eugenol, carvacrol, terpenes and sesquiterpenes, phenols, flavonoids, saponins, sugars and starch (Nasahi et al., 2023). The bioactive compounds contained in betel leaves are associated with antibacterial, antifungal and antioxidant properties (Tran et al., 2023). Previous research on synthetic seeds had shortcomings, namely that the seeds shriveled and there was quite a high level of contamination in synthetic seeds. The novelty of this research is the coating of betel leaf extract on synthetic seeds. Betel leaf extract can reduce the rate of contamination because it has antibacterial and antifungal properties.

MATERIAL AND METHOD

Method, Time, and Place

This research used a Completely Randomized Design (CRD) with 2 factors, namely chitosan, which consisted of 5 levels, namely 0.05; 0.1; 0.15; 0.2% as well as control and betel leaf extract consisting of 4 levels, namely control; 1; 2; and 3%, resulting in 80 experimental units. The research was carried out offline in April-July 2024 at the Tissue Culture Laboratory, Faculty of Agriculture and Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret.

Tools and Material

The tools used include glassware, stereo microscope, spatula, dropper pipette, micropipette, hot plate stirrer, pH meter, oven, rotary evaporator, evaporation cup, water bath, Bunsen, Laminar Air Flow (LAF), culture bottles, scapel, paper filter, blender, tweezers, funnel, and millimeter block. Materials used are alginate, chitosan, betel leaves, 96% ethanol, distilled water, 70% alcohol, methylated spirits, 56 MSS (week after sowing) kribo orchid plantlets, CaCl2.2H2O, vermiculite, clorox, macronutrients, micronutrients, FeEDTA, vitamins , and sucrose.

Preparation of MS Media and 2.5% Sodium Alginate

Preparation of MS Media (Murashige and Skoog) was carried out by dissolving 500 ml of distilled water plus 20 ml/L of macronutrients; micronutrients 10 ml/L; vitamins 50 ml/L; Fe EDTA 50 ml/L; and sucrose 30 grams/L. The pH of the MS medium is then measured to be 6.2, if it is less than 6.2 then 1 N NaOH is added and if it is more 1 N HCl is added. The MS media was then incorporated into 12.5 grams of sodium alginate powder at a temperature of 45-50°C.

Preparation Betel Leaf Extract Solution

Betel leaves were cut and dried in an oven at 70°C for 2 days, then ground. 1.5 kg of betel leaf powder was soaked in 7 liters of 96% ethanol for 3 days and stirred once a day. The betel leaf supernatant is then filtered and the solute is taken in the form of the homogeneity of betel leaves and solvent. The solute is then evaporated using a rotary evaporator at a temperature of 70° until a semi-liquid concentration is formed. The semi-liquid concentrate was evaporated again using a water bath at a temperature of 85°C until a perfect paste concentration was formed. The paste was diluted to concentrations of 1%, 2%, and 3%. 1% paste is made by weighing 1 gram of paste dissolved in 1 liter of distilled water, then at concentrations of 2 and 3% it is made by replacing 2 grams and 3 grams of betel leaf paste.

Preparation Chitosan Solution and $CaCl_2.2H_2O$

Chitosan solution was made with a concentration of 0.05; 0.1; 0.15; and 0.2%. Konsentrations 0.05% chitosan solution was made by dissolving 0.125 mL of chitosan with distilled water until it reached a volume of 250 mL. Preparations chitosan concentration 0.1; 0.15, and 0.2% are the same as making 1% chitosan concentration, by changing the chitosan concentration to 0.25; 0.375; 0.5 mL. Control treatment using pure aqudest. A 100 mM $CaCl_2.H_2O$ solution was made by dissolving 1.1 grams of $CaCl_2.H_2O$ powder with 100 mL of distilled water.

Sterilization of Tools and Materials

Sterilization of tools and materials using an autoclave. The equipment is stored in an oven at 70℃, while the materials are stored in a storage room at 25.5℃ for 12-24 hours. Sterilization of synthetic seed seedling media in the form of vermiculite is carried out by soaking in a solution of Clorox and distilled water in a ratio of 3:1 for 6 hours. After that, the vermiculite was rinsed with sterile water 3 times. Vermiculite is placed in a culture bottle, tightly closed, and wet sterilized using an autoclave. Each culture bottle was filled with 10 grams of vermiculite.

Encapsulation and Treatment

Encapsulation was carried out by inserting each plantlet into a test tube. The reaction tube was then filled with MS + Alginate 2.5% until the plantlets were completely submerged ± 3-5 ml. After 3 minutes, add 100 mM CaCl2.2H2O solution and shake for 3 minutes until synthetic seeds form. The synthetic seeds were then treated with chitosan and betel leaf extract according to the scheme for 15 minutes. Synthetic seeds that have been treated are then placed in culture bottles that have been given sterile vermiculite seeding media.

Incubation and Periodic Observations

Synthetic seeds that have been formed are then incubated in a room with a temperature of 25.5℃, with humidity of 53%, and a light intensity of 344 lux.24 hours^{-1}. Treatment is carried out by spraying 70% alcohol on the surface of the bottle to reduce the risk of contamination. Observations were carried out at the beginning of 1 week after treatment and at the end of 3 week after treatment.

Identify Microbial Contaminants

Identification was carried out by making macroscopic observations on the surface of synthetic seeds and microscopically using a stereo microscope with 100x magnification. Observations were made by observing all conditions of the synthetic seeds at the end of the observation. The results of the appearance of macroscopic and microscopic morphological characteristics were then identified based on similarities with literature sources.

RESULT AND DISCUSSION

Prosentage of Contamination

The percentage of contamination was calculated by dividing the total number of contaminated seeds by the total number of seeds for each treatment multiplied by 100%. Bacterial contamination is characterized by milky white clouds or slimy deposits, while fungal contamination is characterized by brown or blackish hyphae on the surface of synthetic beans (Li et al., 2022). Based on Figure 2, the highest percentage of contamination, namely 75%, was found in the K0D1 (0% chitosan + 1% betel leaf), K1D0 (0.05% chitosan + 0% betel leaf), K1D3 (0.05% chitosan + 0% betel leaf) treatment. betel 3%), while the lowest contamination, namely 25% was found in the treatment K0D3 (0% chitosan + 3% betel leaf), K2D0 (0.1% chitosan + 0% betel leaf), K2D1 (0.1% chitosan + betel leaf 1%), K2D3 (chitosan 0.1% betel leaf 3%), K4D0 (chitosan 0.2% + betel leaf 0%), and K4D2 (chitosan 0.2% + betel leaf

2%). These results were obtained due to the combination of chitosan and betel leaf extract in suppressing contamination. Chitosan has properties to strengthen plant resistance to disease (Samarah et al., 2020). Betel leaves also have bioactive compounds related to antibacterial, antifungal and antioxidant properties (Tran et al., 2023). According to Li et al. (2022), sources that can cause contamination in in vitro culture are improper sterilization, culture rooms, and transfer areas.

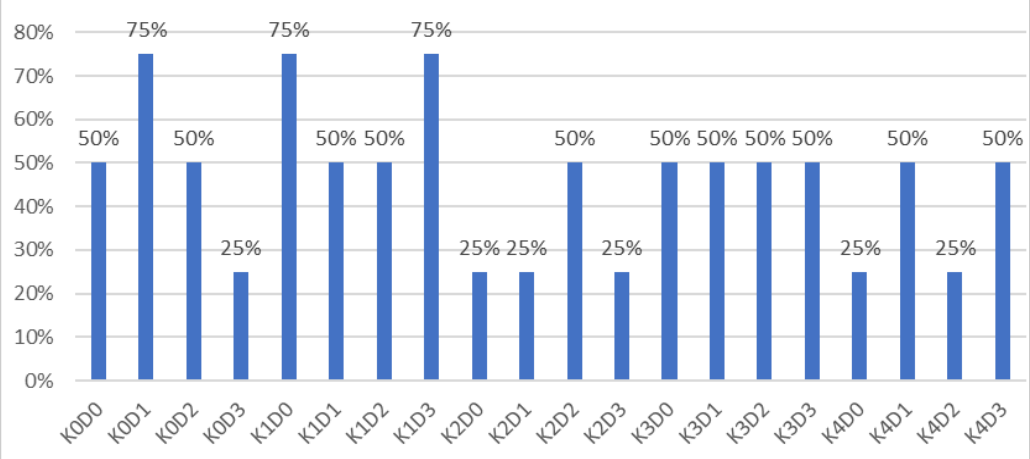

Figure 1. Prosentage Contamination

Identification of Microbes that Cause Contaminations

Identification of the microbes causing contamination is carried out by observation using a microscope. Identification is based on observing microbial morphology at 100x magnification. The identification results showed that the cause of the contamination was the fungus *Rhizoctonia* sp. and *Aspergillus* sp.

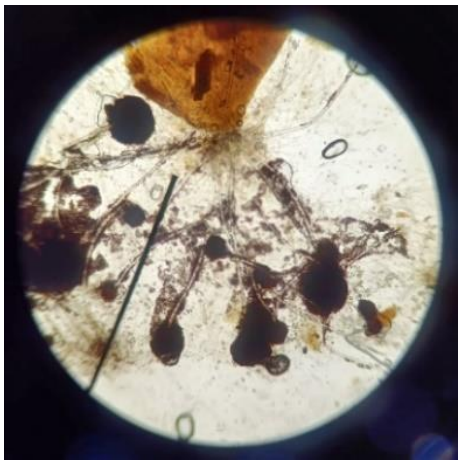

Figure 2. Microscopic appearance of the fungus *Rhizoctonia* sp.

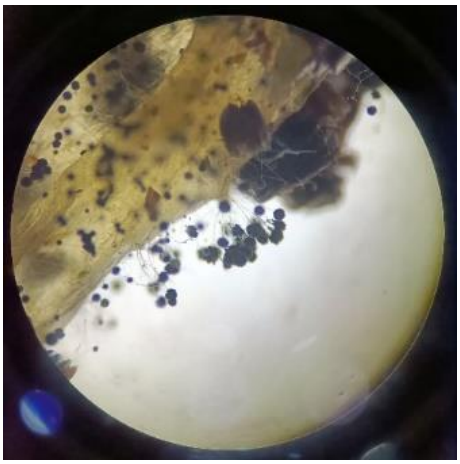

Figure 3. Microscopic appearance of the fungus *Aspergillus* sp.

Rhizoctonia sp. is a fungal pathogen commonly found in orchid tissue culture (Pereira et al., 2021). Rhizoctonia sp. has blackish brown hyphae with many branches, no septa, and a thread-like structure (Soelistijono et al., 2021). These hyphae will penetrate the orchid plant tissue and take up nutrients, thereby inhibiting the growth of roots, shoots and leaves (Sisti et al., 2023). This causes the plantlets to die, turn blackish brown, and rot.

Aspergillus sp. is a fungus that comes from the Ascomycota class. The characteristics of this fungus are that it has an oval, semi-spherical or round conidia structure (Saif et al., 2021). The presence of the fungus Aspergillus sp. can be detrimental to the growth of synthetic seeds because they emit dangerous mycotoxins. Aspergillus sp. capable of producing mycotoxins such as aflatoxins (AFTs), ochratoxin A (OTA), patulin (PAT), citrinin (CIT), aflatrem (AT), secalonic acids (SA), cyclopiazonic acid (CPA), terrein (TR), sterigmatocystin (ST), and gliotoxin (GT) (Navale et al., 2021). These mycotoxins can damage plant cells and inhibit the growth of synthetic seed roots.

Shoot Growth Rate

The shoot growth rate is calculated by dividing the shoot height at the end of the observation and the beginning of the observation. Based on Figure 4. The highest average shoot growth rate was in the K3D1 treatment (0.15% chitosan + 1% betel leaf extract) with a value of 3.12 mm. The K4D0 (0.2% chitosan) treatment gave the lowest shoot growth rate, namely 1 mm. According to Chakraborty et al. (2020), chitosan contains nutrients that can increase plant growth. According to El-Hady (2021), chitosan compounds can stimulate cell division and apical elongation through enzymatic activity. According to Bakashi et al. (2020), the natural ingredient chitosan can substitute natural stimulants at very low levels. Application of chitosan at appropriate concentrations can increase plant shoot growth (Kumaraswamy et al. 2021).

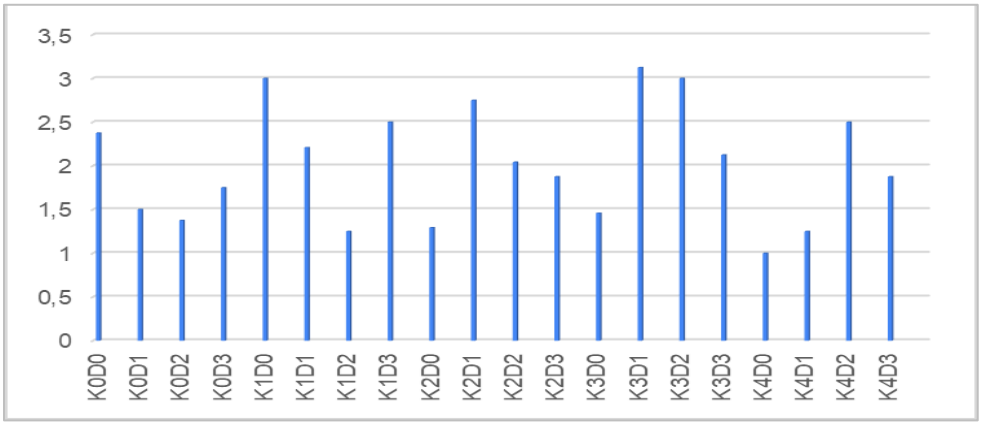

Figure 4. Shoot Growth Rate

Number and Length of Root

The increase in the number of roots is calculated based on the root length at the end of the observation and the root length at the beginning of the observation. Based on Figure 5, there are 8 treatments that can support root growth in synthetic seeds, namely K2D0, K2D2, K3D0, K3D1, K3D2, K3D3, K4D0, and K4D2. The K2D0 treatment (0.1% chitosan + 0% betel leaf) contained 1 root. The K2D2 treatment (0.1% chitosan + 2% betel leaf) contained 2 roots. In the K3D0 treatment (0.15% chitosan + 0% betel leaf), there were 3 roots. The K3D1 treatment (0.15% chitosan + 1% betel leaf) contained 1 root. The K3D2 treatment (0.15% chitosan + 2% betel leaf) contained 1 root. The K3D3 treatment (0.15% chitosan + 3% betel leaf) contained 1 root. The K4D0 treatment (0.2% chitosan + 0% betel leaf) contained 1 root. In the K4D2 treatment (0.2% chitosan + 2% betel leaf), there were 3 roots.

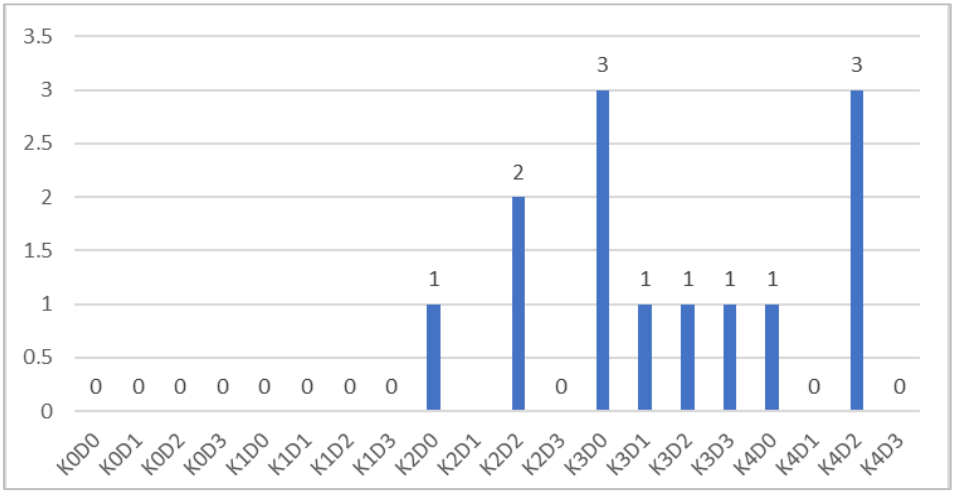

Figure 5. Number of Root

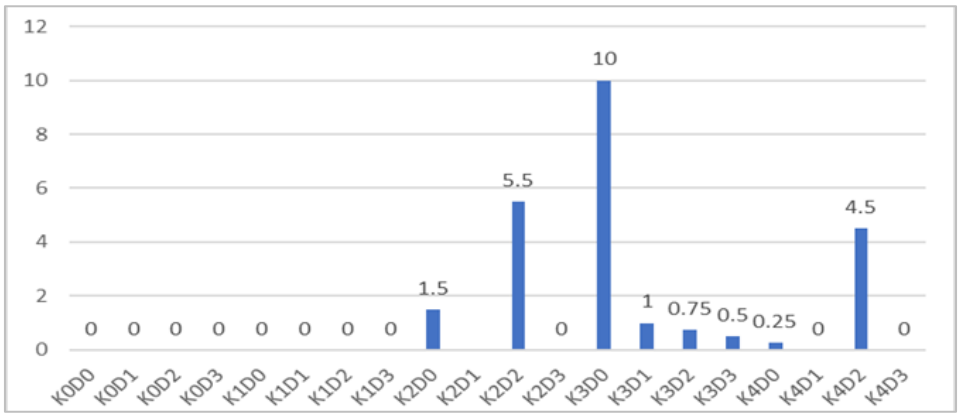

Figure 6. Length of Root

Root length is calculated based on the length at the end of the observation minus the root length at the beginning of the observation. Each treatment has varying root lengths. The K2D0 treatment (0.1% chitosan + 0% betel leaf) had an average of 1.5 mm. The K2D2 treatment (0.1% chitosan + 2% betel leaf) had an average of 5.5 mm. The K3D0 treatment (0.15% chitosan + 0% betel leaf) had an average of 10 mm. The K3D1 treatment (0.15% chitosan + 1% betel leaf) had an average of 1 mm. The K3D2 treatment (0.15% chitosan

+ 2% betel leaf) had an average of 0.75 mm. The K3D3 treatment (0.15% chitosan + 3% betel leaf) had an average of 0.5 mm. The K4D0 treatment (0.2% chitosan + 0% betel leaf) had an average of 0.25 mm. The K4D2 treatment (0.2% chitosan + 2% betel leaf) had an average of 4.5 mm.

Based on Figure 5, it is known that the highest number of roots was produced by the K3D0 and K4D2 treatments with a total of 3 roots. Meanwhile, the longest average root growth (Figure 6) was in the K3D0 treatment with a value of 10 mm. Factors that influence root growth and length are the combination of MS media and chitosan. This is because MS is a basic medium that contains inorganic salts, nitrogen, macronutrients and micronutrients so that it is able to support plant cell growth (Pesternak and Steinmacher, 2024). Meanwhile, chitosan is a substitute material that contains macro and micro nutrients as well as growth regulators (Stasińska-Jakubas and Hawrylak-Nowak, 2022). Plant cells will absorb nutrients from MS and chitosan, resulting in cell division at the tip of the root meristem. This division stimulates the enlargement and elongation of synthetic seed roots (Wasiati et al. 2021). Chitosan can form films and membranes well (Suarez-Fernandez et al., 2020). This is able to maintain the condition of the alginate in synthetic seeds and can also support the growth and development of synthetic seeds.

CONCLUSION

Triple layer synthetic seeds have the characteristics of being white, non-wrinkling and dense. The lowest percentage of contamination was found in the treatment (0% chitosan + 3% betel leaf), (0.1% chitosan + 0% betel leaf), (0.1% chitosan + 1% betel leaf), (0.1% chitosan + 0.1% betel leaf). betel 3%), (chitosan 0.2% + betel leaf 0%), chitosan 0.2% + betel leaf 2%) of 25%. The cause of contamination is the fungus *Rhizoctonia* sp. and *Aspergillus* sp. The highest shoot growth rate was found in the 0.15% chitosan + 1% betel leaf treatment. The highest number of roots was found in the treatments (0.15% chitosan + 0% betel leaf) and (0.2% chitosan + 2% betel leaf). The longest root growth was found in the treatment (0.15% chitosan + 0% betel leaf).

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