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In-Vitro Growth of Transgenic Sengon Resulting From Co-Cultivation Using *Agrobacterium Tumefaciens* Bacteria

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Abstract
Sengon (*Paraserianthes falcataria* (L) Nielsen) is a forestry plant often developed for industrial purposes in Indonesia. Sengon is a fast-growing species and has a relatively short-cutting period. Sengon wood is widely used in the production of carpentry wood, plywood raw materials, and pulp. The purpose of this study was to optimize the transformation of the entC and pmsB genes into sengon plants using the embryo injury method through Agrobacterium tumefaciens as an intermediary and to determine the efficiency of the transformation using the injury to sengon embryos. This research was conducted from September 2022 to March 2023. The research was conducted at the Genetically Modified Organism (GMO) Tissue Culture Laboratory, Recombinant Protein (Microbe) Laboratory, and Greenhouse of the Genetic Engineering Center, National Research and Innovation Agency (BRIN) Cibinong, Bogor, Java West. The research procedure consisted of several steps, namely Preparation of MS Media, Preparation of LB Media, Preparation of Sengon Seed Infection Using Agrobacterium tumefaciens, Sterilization and Planting of Sengon Seeds, Acclimatization, and Molecular Analysis (including Sengon DNA isolation, PCR Amplification, and Electrophoresis). Transformation efficiency was calculated by the positive strain of hpt PCR bands compared to the number of transformed embryos and multiplied by 100%. In the results of the PCR analysis, it was found that one plant sample carried the hpt marker, which indicates the integration of the entC and pmsB genes. which when analyzed using transformation efficiency calculations, the data obtained for the entire transformation stage was 0.8%, where the success of the transformation obtained was still classified as low. Factors that cause the low success of transformation include the method of transformation, the growth phase of bacteria, genetic material, and culture aseptic conditions.

Keywords— entC, PCR, pmsB, Sengon, Transformation

Introduction

Sengon (*Paraserianthes falcataria* (L) Nielsen) is a forestry plant that is often developed for industrial purposes in Indonesia. Sengon plants are included in the fast-growing species and have a relatively short cutting period. Sengon wood is widely used to produce carpentry wood, plywood raw materials, and pulp. Sengon wood processing waste in the form of sawdust can be used for livestock purposes, and wood pellets can be made into raw material for processing briquettes and as a planting medium for mushroom cultivation. Another advantage of the sengon tree is that it can be used as a support plant to prevent erosion. This tree can protect the soil from the effects of rainwater and improve soil structure through spreading roots. It can be used as a shade tree, reforestation tree, and soil fertilizer. (Putri, et al., 2018 ; Istikorini, et al., 2020).

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Meanwhile, sengon roots have a symbiosis with rhizobium which can produce a nitrogen fixation process that plants can use (Suprapti, et al, 2014; Hermylina, et al, 2020).

The many advantages of the sengon plant have caused market demand for this type of wood to increase in the timber industry in Indonesia. This has also resulted in high interest in developing sengon plants on a wide scale. It causes the need for the availability of seeds in large quantities. Therefore, it is necessary to provide and propagate quality sengon seeds.

The challenge in providing large quantities of sengon seeds is a disturbance in the form of biotic stress, especially at the seedling stage. The condition of seedlings in the seedling phase is relatively susceptible to disease. This disturbance is one of the obstacles in the process of forest plant regeneration so that it can reduce the quantity and quality of seeds, for example, pathogens that cause damping off and rust on sengon (Hermylina et al., 2020).

Alternative that we can use is plant breeding based on genetic engineering can be done by developing plants resistant to biotic stress. Salicylic acid, which is naturally found in plants, is involved in overcoming pathogen attacks (Verberne et al., 2000). The pmsB and entC genes encode salicylic acid biosynthesis, which can increase disease resistance. Research that has been carried out by Verberne et al. (2000) in tobacco plants shows that the expression of genes that support increased resistance to various pathogens. Expression of salicylic acid (pmsB gene and entC gene) can increase plant resistance to pathogens but does not affect plant phenotype. The salicylic acid content in rice plants is positively correlated with rice resistance to Pyricularia grisea (Silverman et al., 1995). Martin et al (2010) once applied SA to elm trees infected with the fungus pathogen Dutch Elm Disease (DED). The results obtained by applying SA inhibited fungal growth on elm trees.

SA to control pathogenic fungi has never been applied before to in-vitro cultures of sengon plants. Warseno (2008) carried out gene transformation through Agrobacterium tumefaciens using callus. The highest transformation efficiency result obtained was 40%, with the PCR product showing six of the nine samples showing positive results, indicating that the gene was successfully integrated into the plant tissue.

SA acts as a resistance-inducing agent, a signal sender of responses to pathogen infection. The active SA compound is known to induce a plant resistance mechanism against pathogen attack, where when a pathogen infects the plant, the production of SA content increases. SA activates genes that control plant resistance to pathogen attacks (Martín et al., 2010; Leiwakabessy et al., 2018; Wijayanti et al., 2018; Lubis et al., 2020).

Transformation of A. tumefaciens is a genetic transfer method that is widely applied to plants. This method has several advantages, namely it has a smaller number of gene copies compared to using the shooting method, is stable, cheaper, and the process is more manageable (Nyaboga et al., 2014). It is hoped that these efforts will produce transgenic plants resistant to biotic criticism and thus produce quality sengon seeds. This method is carried out in vitro (tissue culture) so that it can produce large numbers of plants quickly and does not depend on the season. Therefore, it is necessary to introduce genes for resistance to pathogens through in-vitro tissue culture methods and genetic engineering in sengon so that it is resistant to biotic stress.

Literature Review

Sengon is one of the industrial wood plants that has a relatively fast growth period. The nature of sengon wood is quite dense, has straight grain, is a little rough but easy to work with. The color of the wood is yellow to ivory brown (Basyaruddin, et al, 2019; Putri, et al, 2018). Sawdust from sengon processing can also be used for livestock purposes or as an alternative growing medium in mushroom cultivation and can be used as raw material for wood pellets

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(Hermylina, et al, 2020). Apart from that, sengon wood waste can be made into raw material for briquette processing (Pujasakti, et al, 2018). The roots of sengon are also able to resist erosion, which is called sengonization. The Ministry of Environment and Forestry (KLHK) uses sengon around river flows to prevent erosion. The use of sengon is also mainly for its wood as an industrial material (Putri, et al., 2018).

Genetic transformation or transfer of genetic information can be carried out by utilizing the interaction of the Agrobacterium tumefaciens bacteria. Agrobacterium tumefaciens can naturally cause tumor disease in plants (crown gall disease) and is a gram-negative bacterium that lives freely in the soil and can infect plants that are injured. This bacteria lives at an optimum temperature of 28-30°C. These bacteria can enter plants through injured plant tissue. However, this bacterium has the ability to transfer the DNA contained in the Tumor inducing (Ti) plasmid into the plant nucleus (Aswan, 2015).

The initial interaction of bacterial infection with plant cells begins with the presence of metabolites (intermediaries) in injured plant cells. These metabolites can be sugar compounds, acids, or phenolic compounds. This compound will induce Agrabacterium tumefaciens to move towards the target cells. This is due to its ability to insert gene-carrying T-DNA from a plasmid into the plant cell genome. There is also induction of virulence factors (Vir) which regulate the process of cutting and transferring T-DNA to plant cells. Several metabolites released by plants can induce virulence factors, these metabolites are Acetosyringone, coniferyl alcohol, and ethyl pyruvate. In nature, the correlation between Agrobacterium tumefaciens bacteria is a natural phenomenon of spontaneous (naturally occurring) T-DNA transport (Budaya, et al, 2022; Handayani, 2013).

Research Method

Time and place

This research was carried out from September 2022 to March 2023. The research was carried out at the Genetically Modified Organism (GMO) Tissue Culture Laboratory, the Recombinant Protein (Microbe) Laboratory and the Genetic Engineering Center Greenhouse, National Research and Innovation Agency (BRIN) Cibinong, Bogor, Java West.

Tools and materials

The tools used include: Petri Dish, Culture Bottle, Tweezers, Spatula, Bunsen, Tip, Dropper Pipette, Autoclave, Incubator, Incubator Shaker, Öse Needle, Laminar Air Flow, Duran Schott, Ph Meter, Measuring Cup, Falcon, Aluminum Foil, heat-resistant plastic, Micropipette, Centrifudge, Heat Block.

The materials used include: Aquadest, 70% Alcohol, 96% Alcohol, Sterile Water, Bayclean, Lurah Bertani (LB) Growing Media, MS Growing Media, Kinetin Growth Regulator, Liquid IK-III, Kanamycin, Acetosyringone , Higromycin, Cefotaxime.

Research procedure

The media used are sengon culture media and bacterial culture media. For sengon culture media, MS media is used, while LB (Luriah Bertani) media is used for bacterial culture media. There are two types of MS media used, namely MS + Acetosyringone media and MS + Hygromycin media. MS + Acetosyringone media functions to encourage the infection process of Agrobacterium tumefaciens so that in this study, this media was used as a culture medium for seeds and bacteria together. Meanwhile, MS + Hygromycin media was used as a selection medium for organism growth. The antibiotic Hygromycin functions to select and inhibit the growth of gram-positive and negative bacteria. The content of the media provided is:

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MS Media Creation

For 1000 mL of MS media, mix 100 mL of MS Macro I, 100 mL of MS Macro II, 1 mL of MS Micro, 10 mL of MS FeNaEDTA, 10 mL of Vitamin B5, 10 mL of Kinetin, 10 gr of Sucrose, 2.5 gr of Phytagel. Mix the ingredients in a 1000 mL Duran Schott bottle other than the phytagel. The ingredients are mixed with distilled water (the volume is equalized to the equivalent of 1000 mL). The pH of the media was measured until it reached pH 8.5. Added phytagel to the media. The media was autoclaved for 15 minutes at 121°C. The warm media was given 1 mL of the antibiotic Hygromycin /Acetosyringone .

LB Media Creation

To make 1000 mL of LB media, mix 10 grams of Bacto tryptone, 5 grams of NaCl and 5 grams of yeast extract (for solid LB media, add 15 grams of Bacto Agar), then add distilled water until it reaches 1000 mL, the media mixture is autoclaved for 15 minutes at a temperature of 121°C, 1 mL of the antibiotic kanamycin was added to the warm (but not yet hardened) medium.

Preparation of Sengon Seed Infection Using Agrobacterium tumefaciens

The infection method used is by injuring Sengon seeds using Agrobacterium tumefaciens bacteria. Agrobacterium tumefaciens bacteria resulting from the introduction of pmsB and entC genes were inoculated in 50 mL solid LB medium + 50 mL/g Kanamycin using the zig-zag spread plate method. The bacteria were then incubated at 28°C for three days.

The seed infection procedure is to prepare 10 mL of liquid IK-III media in a sterile petri dish. Then 100 µL of Acetosyringone was added. Bacteria that had been incubated for three days were taken using a spatula, placed in 10 mL IK-III medium + 100 µL Acetosyringone , and homogenized by stirring. The bacteria were left for ± 1 hour (to optimize bacterial fusion with IK-III media) before being infected with sengon seeds.

Sterilization and Planting of Sengon Seeds

Sengon seeds are first soaked for ± 4 hours in water so that the seeds become soft so that it is easy to infect the seeds. After that, surface sterilization was carried out by using 70% alcohol for 1 minute, Bayclean 100%, and distilled water each for 1 minute with 5 repetitions. The seeds are then dried on filter paper until completely dry.

Next, the seeds are infected by injuring (piercing) the sengon seeds 1-2 times using a sterile needle that has been dipped first in bacteria in MS + Acetosyringone media. The seeds that have been pierced are then dried again on filter paper before being planted on MS + Acetosyringone media. The seeds were then stored in the dark for three days.

After being cultured in the dark, the seeds were transferred into MS + Hygromycin media. The seeds are transferred to new media (MS + Hygromycin) every five to six days until roots and leaves form.

Results and Discussion

Results of Bacterial Inoculation

The recombinant DNA plasmid construct containing the pmsB and entC genes transformed into Agrobacterium tumefaciens can grow well on LB + Kanamycin media (figure 1). The obstacle to inoculating Agrobacterium tumefaciens bacteria obtained in the laboratory is contamination with other bacteria. This can be caused by careless inoculation work or less aseptic working conditions.

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Figure 1. Agrobacterium tumefaciens bacteria ready to be infected..

Results of In-vitro Growth Observation of Transgenic Sengon

Good in-vitro seed culture resulting from co-cultivation with Agrobacterium tumefaciens bacteria is characterized by seeds that have bloomed and have not bacterial overgrowth. In-vitro culture of transgenic sengon seeds on good and fresh selection media is characterized by seeds that germinate and are green in color, do not experience browning, and do not experience bacterial overgrowth. Seeds grown beyond 5 cm are transferred to MS + Hygromycin media in culture bottles.

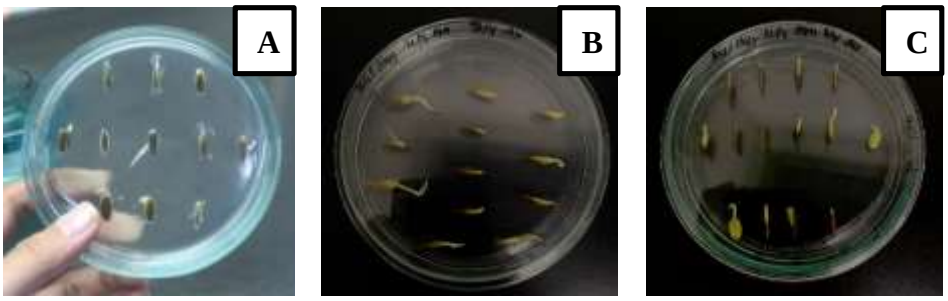

Figure 2. (A) Sengon seeds resulting from infection in petri dish. Sengon aged three days on MS + Asetoryrigone co-cultivation media. (B) and (C) Six day old Sengon on MS+Hygromycin selection media.

Sengon plants resulting from infection require 3-4 weeks of growth before they can be acclimatized. Sengon on petri media that were long enough were transferred to culture bottles so that their growth was not hampered (Figure 3 (C)).

(A) (B)

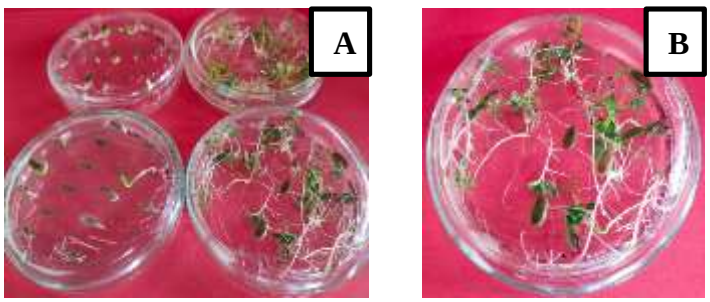

Figure 3. (A) Control sengon plants. (B) Transgenic putative sengon.

Some cultured seeds can initially grow well. However, after being subcultured several times, some seeds survive, and others cannot. The obstacle found in the laboratory was seed contamination

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with Agrobacterium tumefaciens bacteria resulting from infection, which had overgrown, thus disrupting or inhibiting the seed germination process and causing failure to grow in sengon seeds. Bacterial overgrowth can occur in the co-cultivation process and in the regeneration process. Apart from that, bacteria that overgrow also contaminate the growth media and change the color of the media.

Improvements to the obstacles faced require a long time to obtain improvements in the infection methods used in cases of bacterial overgrowth. Seeds or plantlets that experience overgrow and are subcultured after first being sterilized using cefotaxime can experience overgrow again.

Figure 4. Sengon seeds experiencing bacterial overgrow.

Conclusion

In-vitro growth of Transgenic Sengon resulting from co-cultivation using Agrobacterium Tumefaciens bacteria successfully carried out using the wound method. Factors influence growth success include the transformation method, bacterial growth phase, and aseptic culture conditions.

Suggestion

The success of gene introduction and its stability in the genome of transgenic sengon plants to determine that carry the pmsB and entC genes, the molecular analysis should be carried out for gene integration tests or gene expression tests on the transformed plants. This study can be continued to acclimatization stage.

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