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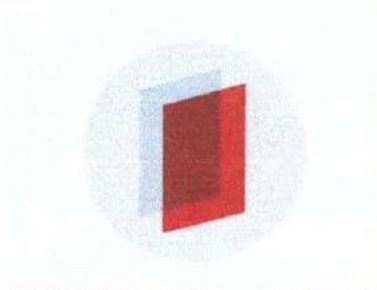
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Effect of selection agents to *Chrysanthemum (Chrysanthemum morifolium)* callus growth after *Agrobacterium*-mediated genetic transformation

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Abstract. Genetic transformation mediated by *Agrobacterium tumefaciens* requires an efficient selection method for successful progress of transformation. This study aims to determine the concentration and kind of antibiotics and selection agents used during transformation to formulate standard protocol of chrysanthemum in the process of propagating disease resistant Chrysanthemum mediated by *Agrobacterium tumefaciens* EHA105 (pEKB-WD). The experiments were performed by planting chrysanthemum explants leaf cutting (5 mm diameter on NAA medium 2 mg L⁻¹ BAP 2 mg L⁻¹) with addition of Kanamycin: 25, 50, 100, 150 and 200 (mg L⁻¹); Hygromycin: 5, 10, 25, 50 and 75 (mg L⁻¹); Paromomycin: 10, 25, 50, 75 and 100 (mg L⁻¹). Experiment was arranged in a Completely Randomized Design (CRD). Each treatment was repeated five times thus 75 bottles of culture were used, each bottle consists of 5 pieces of leaf cuttings, resulted in total of 375 pieces. The results showed that selection agent had a critical value for Hygromycin 25 mg L⁻¹ and Kanamycin 100 mg L⁻¹ which can make explant experienced necrosis better than Paromomycin. Paromomycin at 100 mg L⁻¹ was only able to kill explant's periphery. Remained callus stayed fresh more than 50% so that when used as the selection agent could produce more escape cell. The optimum transformation with concentration of 10% *Agrobacterium* (vol/vol) with 30 minutes co-cultivation can produce more efficient transformed callus. Considering the high price of Hygromycin, it was best to use Kanamycin as selective agents.

1. Introduction

Chrysanthemums occupy the largest floriculture commodity export to become a large foreign exchange producer as well but the development of chrysanthemums experiencing some obstacles and challenges. The most important obstacle is plant pests and diseases. Plants attacked by pests or diseases



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will decrease its quality, making it difficult to market. Plants that are free from pests and diseases are a key requirement to meet global export markets. One potential alternative is to develop resistant plants by utilizing biotechnology, especially in the field of genetic engineering. Genetic engineering technology allows genes from other organisms to be inserted into the genomes of target plants. Genetic engineering to produce disease-resistant chrysanthemums has been reported by [1]. Kim *et al.* [2] used the *chitinase* gene, but was limited to the resistance of pathogenic fungi. While resistance to viral disease was reported by Toguri *et al.* [3], who used the *pac1* gene (RNA double ribbon from the specific ribonuclease from *Schizosaccharomyces pombe*). Resistance to the fungal plaque *Alternaria* leaf spot (ALS) has also been reported by Xu *et al.* [4] using the Xpa1 hoGo gene from *Xanthomonas oryzae*. It has also been reported the resistance of Chrysanthemum plants to pests using the cry IAB gene from *Bacillus thuringiensis* [5]. So far there has been no report on the use of genes that can provide resistance to leaf rust disease in chrysanthemums caused by fungi.

One of the anti-microbial protein coding genes that has been proven to encode antifungal proteins and bacteria is the wasabi defensin gene. Wasabi is a Japanese horseradish plant used as foodstuff and has anti-bacterial substance. Secondary metabolites from wasabi such as wasalexin and 6-methylsulfonylhexyl isothiocyanate have been reported to have anti-fungal and anti-bacterial activity [6]. The antibacterial protein coding gene (WjAMP-1) has been isolated from wasabi [7, 8] and is called the wasabi defensin gene by Kanzaki *et al.* [9]. Anti-microbial proteins encoded by this gene exhibit expression and inhibitory effects on the growth of fungi and bacteria in transgenic *Nicotiana benthamiana* [7]. Furthermore, Kanzaki *et al.* [9] also reported that the growth of fungi causing blast disease in rice was inhibited by over expression of the wasabi defensin gene. While on orchids can inhibit the growth of leaf blight caused by *Erwinia carborovora* [10].

Transformation with *Agrobacterium* mediation involves the use of soil bacteria known as *Agrobacterium tumefaciens* which have the ability to infect plant cells with a piece of DNA. There are several requirements that need to be considered in making genetic transformation such as efficient selection method [11]. Selection of transformant cells is a key factor in the success of methods developed for genetic transformation. The aim of selection for separating transformed and non-transformed cells, in vitro selection was performed using antibiotics as a selection agent [12].

Antibiotics as a selection agent work by inhibiting protein synthesis is disrupting the function of ribosomal subunits that reversibly inhibit protein synthesis by inhibiting the translocation of the tRNA-peptide complex from the location of the amino acid to the peptide site, consequently the polypeptide chain cannot be prolonged, but the resistance of the antibiotic will have no effect against cells that have a marker-resistant gene. Antibiotic selection agencies have different lethal doses of each type to be used as concentration selectors, as well as for each plant having different tolerance limits for each type of antibiotic. Pre-transformation selection can facilitate the transformation, by knowing the lethal dose of antibiotic selection agency so that the selection process becomes efficient. For that we need a series of studies to obtain the concentration and type of antibiotic selection agency that is effectively used in selecting callus transformation of wasabi defensin gene with mediation *Agrobacterium tumefaciens*.

2. Research Methods

2.1. Place and Time

The research was conducted at the Laboratory of Plant Biosciences and Reproduction Biotechnology, Department of Agronomy, Faculty of Agriculture, Hasanuddin University, Makassar. The study took place from April to November 2017.

2.2. Materials

Materials needed were plantlets of Chrysanthemum var. Pasopati, inorganic and organic chemical material components for Murashige and Skoog (MS) media, Luria Bertani (LB) media, growth regulators α -Naphthalenacetic Acid (NAA), Benzyl Amino Purin (BAP), and several kind of antibiotics (kanamycin, hygromycin, paromomycin, spectinomycin, chloramphenicol, meropenem).

2.3. Implementation of Research

This experiment was carried out by testing the concentration and kind of antibiotic selection agency on chrysanthemum leaf explants to determine lethal doses of various types and concentrations of antibiotics used. The media used were MS medium with incorporation of NAA (2 mg L^{-1}) BAP (2 mg L^{-1}), placed in Erlenmeyer flask then sterilized. Addition of antibiotics is done in the laminar air flow after the medium has a temperature of 40°C then poured into culture bottles. The concentration of antibiotic selection agent that is examined were: with no antibiotics; with Kanamycin (25 mg L^{-1} , 50 mg L^{-1} , 100 mg L^{-1} , 150 mg L^{-1} , and 200 mg L^{-1}); Hygromycin (5 mg L^{-1} , 10 mg L^{-1} , 25 mg L^{-1} , 50 mg L^{-1} , and 75 mg L^{-1}); Paromomycin (10 mg L^{-1} , 25 mg L^{-1} , 50 mg L^{-1} , 75 mg L^{-1} , and 100 mg L^{-1}).

2.4. Methods and Analysis

Testing the concentration and type of antibiotic selection agency on Chrysanthemum leaf explants were to determine lethal doses of various types and concentrations of antibiotics used. Testing was done by planting leaf disc slices on media in previous experiment that is NAA (2 mg L^{-1}) and BAP 2 mg L^{-1} with addition of various antibiotic selection agents. The experiments were prepared in a Completely Randomized Design with Kanamycin treatment (Km): 25, 50, 100, 150 and 200 (mg L^{-1}); Hygromycin (Hyg): 5, 10, 25, 50 and 75 (mg L^{-1}); Paromomycin (Par): 10, 25, 50, 75 and 100 (mg L^{-1}). Each treatment was replicated 5 times so that there were 75 bottles of culture, each bottle consisted of 5 leaves of whole leaves 375 ring pieces of leaves. Data were analysed using statistic to determine the best treatment further tests. Data were performed by bar chart.

3. Result and Discussion

3.1. Result

3.1.1. Percentage (%) of Explants Life on Determining Concentration and Kind of Antibiotic Selection.

Figure 1 shows the highest percentage of live explant on control which is not different from K1, K2, K3, H1, H2, P1, P2, P3 and P4 that is 100%.

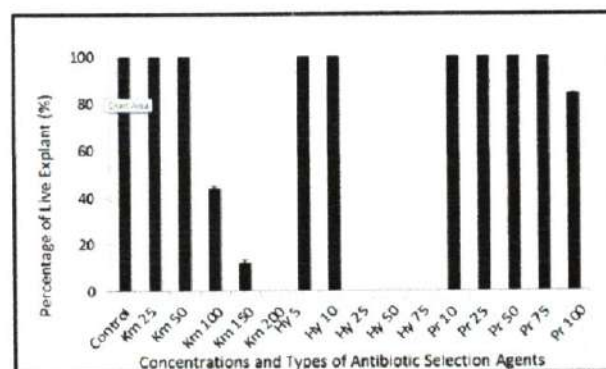


Figure 1. Average live of explant percentage of experimental results of determination of concentrations and types of antibiotics

3.1.2. Percentage (%) Smooth Explants on Determination of Concentrations and Types of Antibiotics Selection Agents. Figure 2 shows the results of the highest percentage of the highest controlled explant with K1, H1, H2, P1, P2 and P3 that is 100%.

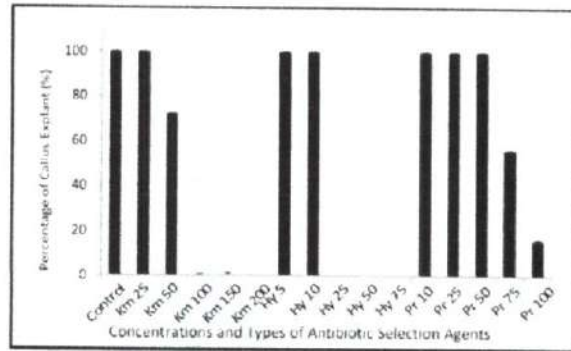


Figure 2. Average percentage of blunt explant results of experiments determining the concentration and type of antibiotics

3.1.3. *Weight explants callus on Determining Concentration and type Antibiotic Selection Agent (g).*

Figure 3 shows the highest yield of callus on control (1.022 g) and in the treatment of paromomycin antibiotic gave the highest callus result of 0.174 g.

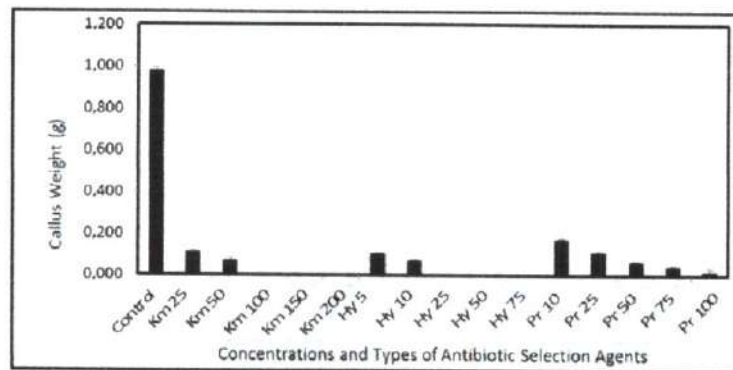


Figure 3. Graph of average percentage of blunt explant results of experiments determining the concentration and type of antibiotics

3.2. *Discussion*

Lethal testing of doses of various types of antibiotics selection agent was done for subsequent use in the selection medium of callus / plant transformant and nontransformant where the results obtained that the higher concentration of antibiotic in explant without carrier resistance gene will inhibit cell division in leaf explant even will cause death on explant but each plant has different tolerance limits to each type of antibiotic, seen in the results of experiments determining the concentration and type of antibiotic selection agent on the percentage of explants that grew in concentration is still within the limits of chrysantherin tolerance that control without antibiotics can grow 100% in the administration of kanamycin concentrations of 25 mg L⁻¹ and 50 mg L⁻¹ can still grow up to 100%, the administration of hygromycin at concentrations of 5 mg L⁻¹ and 10 mg L⁻¹ still grow 100% and paromomycin administration all given concentrations are able to grow up to 100% except at concentrations of 100 mg L⁻¹ growth decreased 16%.

For each concentration given to each type of antibiotic effect on the growth of explant, as well as the percentage of explant smooth concentration that is still within the limits tolerant explant can remain to

grow and smooth but when the concentration of the stress on explant will affect the process of growth and development explant, ranging from explant will survive but not smooth to cause death. In general, antibiotics have lethal doses which may inhibit plant growth or delay, according to Anggraito [13], antibiotic poisoning causes explants to respond more to stress than to form buds.

The result of dead explant percentage shows lethal dose, where kanamycin at concentration of 100 mg L⁻¹ explant yellowish green and not smooth finally experience nekrotik or die, explant color change is sign of cell death, for hygromycin that start from 25 mg L⁻¹ causing death on explant in a fast time while for paromomycin the highest concentration of 100 mg L⁻¹ is not yet in lethal doses because explant still able to live up to 84%, explant very little (16%) only explant brown (dead). Explant experiencing stress will affect the explant not able to form callus even death, although the time required each concentration and type of antibiotic is different.

In high concentrations of hygromycin, the growth of explant is inhibited to cause death from excessive stress, besides it can also inhibit the transformant cells, so the growth or regeneration takes a long time. Hygromycin inhibits metabolic processes by binding to the 80S ribosome resulting in translational error of mRNA [14]. The concentration of high concentrations of hygromycin will begin with the explant from color change from green to yellowish green and then brownish browning.

Kanamycin at concentrations of 100 mg L⁻¹ causes the plants to turn yellowish green and calluses are not formed, these concentrations have explant signs that they will inhibit the formation of new cells or callus until finally explants die. In contrast to hygromycin, kanamycin takes longer and high concentrations can cause toxicity to chrysanthemum explants. Kanamycin is a selection agency commonly used to select whether the transformed gene is successful.

Giving antibiotics paromomycin in this experiment is not toxic seen from the results the percentage of explants callus or not experience stress and callus produced by the weight of callus was highest after the control is Par 10: 0174 g, while the highest concentration of paromomycin of 100 mg L⁻¹ remains formed a little callus and stresses experienced by explant only visible on the outskirts of explants brown this is what caused very rarely using paromomycin as the selection agent because it requires higher concentrations to be used as the selection agent. Among the three antibiotic agents who attempted hygromycin have toxic effects that high compared to kanamycin and paromomycin for hygromycin inhibit the binding 80S ribosomal subunit so that there was an error of translation, to kanamycin 40S subunit and paromomycin 16S subunit, thus seen from the number of ribosomal subunits bound hygromycin may hamper explant mensitesis more protein causes the metabolism process stalled so explant die.

4. Conclusion

Selection of antibiotic agents that have a critical value for Hygromycin was 25 mg L⁻¹ and Kanamycin was 100 mg L⁻¹, which can make the Chrysanthemum explant experienced necrosis. Paromomycin at 100 mg L⁻¹ was only able to damage the explant periphery so that when used as the selection agent could produce more escapes.

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