

Transformation efficiency in *Chrysanthemum* from various sources of explants

Abstract. Nowadays, *Chrysanthemum* is one of the most popular ornamental plants. However their production is constrained by problems with pests and diseases, mainly white rust disease (*Puccinia horiana* P. Henn). One potential alternative is the development of plants through genetic engineering, namely disease resistant transgenic plants. Problem that often occurs in the process is the inhibition of regeneration of calli from the transformation that makes it difficult for researchers to carry out DNA testing from leaves. Calli regeneration is regulated by several factors, such as the use of explant sources and media composition. This study aims to find best explant sources for transformation results into shoots, for DNA analysis. The study was carried out at the level of transformation with three types of explants namely leaves, lateral shoot buds, and internodes. Genetic transformation was carried out by two-stage co-cultivation method using *Agrobacterium tumefaciens* strain EHA105 which contained pEKB-WD binary vector T-DNA construct. Results showed that the most appropriate genetic transformation of explants originating from internodes was 69.33%, but the explants had the lowest regeneration efficiency (1.92%). The highest regeneration efficiency was obtained in explants originating from lateral shoot buds, which amounted to 77.78% with transformation efficiency 54.00%. Both lowest efficiencies were found in leaves (27.31%) for transformation efficiency and regeneration efficiency of 6.45%.

1. Introduction

Chrysanthemum production in Indonesia over the past three years from 2015-2017 continued to increase from the production of 442,998,194 stalks in 2015 which increased to 480,685,420 stalks in 2017 with a harvested area of 11,635,498 ha. The development of export and import of *Chrysanthemums* in Indonesia based on data from the Data Center and Information System of the Ministry of Agriculture shows that *Chrysanthemums* exported to Japan from January to September 2018 were very high. The export volume of *Chrysanthemums* had decreased in February to March and June but continued to increase until September reaching 9,626 kg from 4,263 in January. However,



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when compared to export data from 2014-2017, it declined in 2018 because this year the export destination country was only Japan, whereas in the previous year the export destination countries other than Japan were Kuwait, Singapore, Malaysia, Australia, and Canada. In addition, the volume of exports to Japan itself has decreased in 2018. While the volume of imports from China in 2018 it amounted to 9,941 kg which is quite large compared to the volume of imports from 2014-2017 of 1,571 kg (2017), 6,975 kg (2016), 5,250 kg (2015), & 240 kg (2014). Besides importing from China, are also from Colombia, Vietnam and Singapore [1].

The reduced export volume of *Chrysanthemums*, accompanied by an increase in import volume, may be due to a decrease in the quality of *Chrysanthemum*. This is caused by problems with *Chrysanthemum* pests and diseases. An important disease that commonly attacks *Chrysanthemum* plants is white rust (*Puccinia horiana* P. Henn). Plants that are attacked by pests or diseases will reduce their quality so that they do not meet the main requirements of the global export market.

Disease control carried out by *Chrysanthemum* farmers has only been limited to the use of fungicides which are usually not on target so that the disease was not completely controlled. The use of fungicides will cause negative impact such as of pollution on local environment, besides that also the production costs will also increase. Current global market demand tends to want residual-free agricultural products along with consumers who are more selective in buying so that other alternatives are needed to control rust that is more environmentally friendly and more economical in the long run.

One potential alternative is to develop resistant plants. Development of resistant plants can be done through plant breeding, but in *Chrysanthemum*, the limited germplasm that is resistant to this disease is a major obstacle. This can be overcome by utilizing biotechnology, especially in the field of genetic engineering.

Genetic engineering technology allows the insertion of genes from other organisms into the plant genome. The inserted gene can encode proteins with anti-microbial activity that allows plants to protect themselves from attacks of fungi or bacteria that cause disease. The insertion of this gene into the plant genome can produce plants that are free of the disease. Genetic engineering to produce disease-resistant *Chrysanthemums* have been reported by Takatsu [2]. Kim *et al.* [3] used the chitinase gene but was limited to the resistance of pathogenic fungi. Resistance to viral diseases has been reported by Toguri *et al.*, [4], who uses the *pac1* gene (double band RNA from specific ribonuclease from *Schizosaccharomyces pombe*). The resistance to the Alternaria leaf spot (ALS) fungus has also been reported by Xu, Chen and Chen [5] using the *hpaG* *Xoo* gene from *Xanthomonas oryzae*. In addition, the resistance of *Chrysanthemums* to pests has also been report by Sinoyama *et al.* [6] uses the cry1AB gene from *Bacillus thuringiensis*. So far there have been no reports that are known to use genes that can provide resistance to disease in *Chrysanthemums* caused by fungi and bacteria simultaneously.

One of the anti-microbial protein-coding genes that have been proven to be able to encode anti-fungal proteins and bacteria is the wasabi defensin gene. Wasabi is a Japanese radish plant that is used as a food ingredient and has anti bacterial substances. The results of secondary metabolites from wasabi namely *wasalexin* and *6-methylsulfonylhexyl isothiocyanate*, have been reported to have anti-fungal and anti-bacterial activities [7]. The anti-microbial protein-coding gene (WjAMP-1) has been isolated from wasabi [8], [9] and is called the wasabi defensin gene by Kanzaki *et al.*, [10]. The anti microbial protein encoded by this gene shows the expression and inhibiting effects of fungal and bacterial growth in transgenic *Nicotiana benthamiana* [11]. Furthermore, Kanzaki *et al.* [10] also reported that fungal growth causing blast disease in rice was inhibited by over expression of wasabi defensins. While orchids can inhibit the growth of late blight caused by *Erwinia carotovora* [12].

The insertion of the wasabi defensin gene has produced a hopeful *Chrysanthemum* calli that are thought to be transgenic resistant to white rust. The problem that is often found in the assembly of transgenic plants is the inhibition of the transformation of the calli to regenerate, making it difficult for researchers to analyze/examine DNA. In addition, there were very few calli that passed the selection in the previous study. This is probably caused by habituation of the calli due to multiple sub-cultures and the use of 2,4-D too long (6 months) [13]. The use of explants sources and media composition also

affects regeneration. The use of explant sources in previous studies is slow to regenerate, probably due to leaf explants not actively dividing. The leaves when they are old will fall apart from the explant sources of adventitious shoots, internodes, shoots or meristems which are still actively dividing. In addition, the inhibition of explant regeneration is caused by the effects of giving antibiotics.

2. Method

This research begins with preparation of tools and media making. Transformation begins with the multiplication of *Chrysanthemum* plants as material for explant sources for transformation. Preparation of various explant sources by initiating calli from 3 explant sources, namely leaves, internode, and adventitious shoots. After the calli initiation stage, then the co-cultivation and transformation are carried out, the Agrobacterium rinse / lethal stage, and the selection stage. Calli who successfully passed the antibiotic selection test was regenerated for molecular analysis.

2.1. Experimental apparatus

Materials needed are planting callus and plantlet *Chrysanthemum*, inorganic and organic material components for the manufacture of Murashige and Skoog (MS) media, Luria Bertani (LB) media, growth regulators α -Naphthaleneacetic Acid (NAA), Benzyl Amino Purine (BAP), antibiotics (kanamycin, spectinomycin, chloramphenicol, meropenem, acetosyringone), *Agrobacterium tumefaciens* strain EHA105 which contains constructs of *pEKB-WD T-DNA* region binary vector.

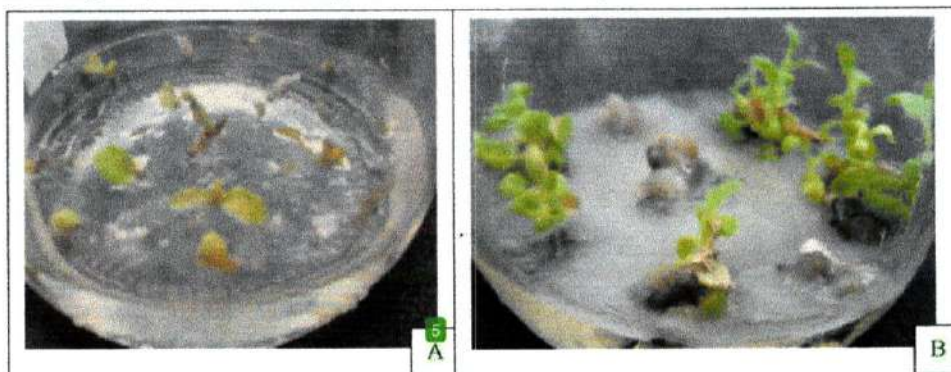
2.2. Data Analysis

In this experiment, research was carried out at the level of transformation with various explant sources, namely leaves, adventitious shoots, and internodes. Observations will be carried out on explant sources which provide faster germination rates and which sources provide the highest transformation effectiveness. The transformation method used was the method obtained in the previous study, namely the concentration of the mixture of cocultivation solution from Murashige and Skoog (MS) media with *Agrobacterium tumefaciens* which had been grown for one night in Luria Bertani (LB) media was 1:10 with long co-cultivation during 30 minutes [13].

3. Result and Discussion

3.1. The development of explants was transformed with wasabi defensin gene (*pEKB-WD*) from various explant sources.

Figure 2 shows the development of the explants resulting from the transformation with wasabi defensin gene (*pEKB-WD*) from explant, internode, and leaf explants sources.



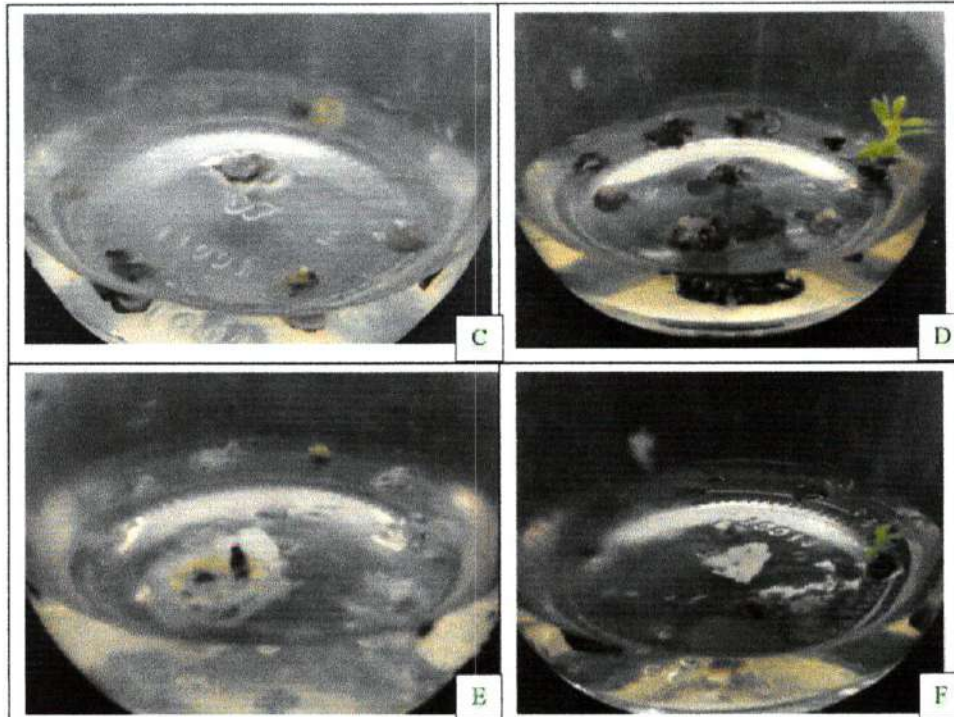


Figure 1. Development of explants resulting from transformation. (A and B) development of explants from lateral shoots buds, (C and D) explant development from leaf sources, (E and F) explant development from internode sources

Transformation of three explant sources of chrysanthemum with *Agrobacterium tumefaciens* strain EHA105 containing T-DNA binary region pEKB-WD constructs produced several shoots from the successful calli which were called transgenic putative shoots. *Chrysanthemum* explants that have escaped and can live on *kanamycin* containing media show that pEKB-WD-EHA105 T-DNA is integrated so that it can detoxify *Kanamycin* which expresses *Kanamycin sulphate*. To turn off bacteria, *Meropenem* and *Cefotaxime acid* are used. In this study, the use of *Cefotaxime* was more effective at killing bacteria than *Meropenem*, but in terms of cost it was less effective because of its much more use. The antibiotic selection agent used is *Kanamycin* which contains 100 mg L^{-1} for callus selection and continues to be reduced to 30 mg L^{-1} at the regeneration stage. The development of explant chrysanthemum results can be seen in Figure 1. Development of explants from lateral shoots buds sources (Figure 1A and 1B), explant development from leaf sources (Figure 1C and 1D), and explant development from internode sources (Figures 1E and 1F). The green calli show the transformer calli while the nontransformed will experience necrotic, browning and then die. Dynamic antibiotics are used to interrupt transformants or non-contrast in explants. One explant resistance to kanamycin was characterized by the growth of explants on media selection [14]. Non-transgenic explants that do not contain a marker gene can cause the necrotic calli to die. This cell death occurs because of the presence of explant poisoning by antibiotics [15].

3.2. Efficiency of transformation and regeneration of *Chrysanthemum* plants with wasabi defensin gene (pEKB-WD).

Table 1 shows the efficiency and regeneration of *Chrysanthemum* plants with wasabi defensin gene (pEKB-WD) from 9 times transformation with total explant sources of 342 internodes and sources of leaf explants 313 as much as 75 and 227 forming the calli. While the number of calli was resistant / passed antibiotic selection as much as 52 and 62. Resistant calli which succeeded in regenerating only 1 shoot from explant internode sources and as many as 4 shoots from leaf explant sources.

Table 1. Efficiency and regeneration of *Chrysanthemum* plants with wasabi defensin gene (pEKB-WD)

	Explant source	Number of explants	Number of explants forming calli	Number of calli resistant	Number of calli regenerates	Transformation efficiency	Regeneration efficiency
Total	LSB	50	50	27	21	54,00%	77,78%
	IN	342	75	52	1	69,33%	1,92%
	LF	313	227	62	4	27,31%	6,45%
Average	LSB	5,56	5,56	3,00	2,33	54,00%	77,78%
	IN	42,75	9,38	6,50	0,13	69,33%	1,92%
	LF	34,78	25,22	6,89	0,44	27,31%	6,45%

LS= lateral shoots buds, IN= Internode, LF= Leaf.

Transformation of *Chrysanthemum* plants using various explant sources, namely adventitious shoots, internodes, and leaves, from 9 transformations resulted from a total of 27 transformed 50 adventitious shoots that were transformed and 21 explants which were resistant to transformation efficiency of 54% and regeneration efficiency. 77.78%, the highest compared to other explant sources. As many as 342 explant sources of explants internodes were 75 resistant and only 1 calli succeeded in regenerating so that the transformation efficiency was 69.33%, the highest compared to other explant sources and regeneration efficiency of 1.92%, lower compared to other explant sources. Explant sources of leaves as many as 313 total transformed resistant resistants were 62 calli and those that succeeded in regenerating were shoots of 4 calli so that the transformation efficiency was 27.31%, lower than other explant sources and regeneration efficiency of 6.45%. The highest regeneration efficiency in the source of adventitious shoot explants is caused by explant sources from adventitious shoots including the fastest and most actively dividing compared to others so that the possibility of adventitious shoots is transformed escape but will be proven through DNA analysis tests. The young part of the plant such as buds has cells that are still actively dividing so that the growth rate is faster. This is in accordance with the results of research by Dewi [16] explants originating from shoots have a better ability in the formation of callus, green spots and shoots. Adventitious shoots include meristematic tissue consisting of cells that are actively dividing and the presence of hormones released by plants on these tissues stimulates growth for regeneration. This is in accordance with Baidowi's study [17], the percentage of epicotile callus is higher than that of hypocotyl and cotyledons, presumably because epicotiles are in the apex region which is the site of auxin synthesis, which encourages higher cell division than hypocotyl and cotyledons.

The highest transformation efficiency was obtained at the explant source from the internode, from the total explants 342 as many as 75 formed calli and as many as 52 resisted so that the transformation efficiency was 69.33%. This is because the tissue on the internode is thicker than the leaves, so it lasts longer on the media selection than the explant source from the leaves before finally blackening and dying (not regenerating). The results of the Teixeira & Fukai [18] study of the transformation of *D. indicum* and *D. zawadskii* using internal explant sources obtained transformation efficiency ranging

from 3-27%. Annadana *et al.* [19] transformation efficiency of 74-76%. Tosca *et al.* [20] 7.8%. Shirasawa *et al.* [21] <38%. Takatsu *et al.* [22] 0.8%. Takatsu [23] <2.5%. Yepes *et al.* [24] 8-73%. Fukai *et al.* [25] 6-16%, and Lowe *et al.* [26] 0-36%. Compared with previous studies, the efficiency of transformation in *Chrysanthemums* with explant sources from internodes is still higher, but low compared to Annadana *et al.* [19] with a transformation efficiency of 74-76% with the use of kanamycin 25 antibiotics and Yepes *et al.* [24] with a transformation efficiency of 8-73% with the use of kanamycin antibiotic selection. Although the transformation is efficient at the explants source of the highest internodes, the regeneration efficiency is lower than a total of 52 resistant calli, which only regenerate so that the regeneration efficiency is only 1.92%. This is probably due to the use of kanamycin antibiotics that are too high, which is 30-100 ppm so that the calli has a regenerating effect. In addition, the low ability to regenerate may be due to the source of the explants internodes older than the meristematic tissue so that organogenesis occurs more to the root growth rather than the formation of shoots. This is in accordance with the study of Teixeira da Silva [25] from explant internal sources producing more roots than shoots, organogenesis which formed 0.01% embryo, 100% calli, 100% root and 2.4-3.2% shoots.


The lowest transformation efficiency was obtained from explant sources from leaves, from total explants 313 as many as 227 forming calli and those who managed to escape / resistant to antibiotics were only 62 calli, so the transformation efficiency was 27.31%. Low transformation efficiency is probably due to the source of leaf explants used being too young and thin so that they are very resistant to antibiotics. Other causes are because the leaves are too young, so that when the leaf/perforation leaves too quickly wilt so that it affects the formation of the calli. Calli formed from a few explant sources leaves, of a total of 342 explants, only 75 succeeded in forming the calli. This is in line with the study of Teixeira da Silva [18] that the transformation of *Chrysanthemums* with *Agrobacterium* infection obtained by explants with the age of older leaves gave the highest transformation efficiency (100%) compared to medium age and young age of 0% in 'Shuhou no cultivar -chikara'. Whereas in the 'Lineker' cultivar, obtained explants with older leaf ages provide the highest transformation efficiency (86%) compared to medium age and young age of 14% respectively. Transformation efficiency is high when compared to previous studies, including Toguri *et al.* [4] with a transformation efficiency of 0.4-2%; Kudo *et al.* [26] 2.8-14.5%; Jeong *et al.* [27] 2.5-3.3%; Ishida *et al.* [28] 20%; Shinoyama *et al.* [29], [30] 3.4%; Sherman *et al.* [31] 10%; Boase *et al.* [32] 1.3-2.1%; Dolgov *et al.* [33] 0-4%; Dolgov [34] 4%; Benetka and Pavingerova [35] 13-27%; Urban *et al.* [36] 4-7%; Courtney-Gutterson *et al.* [37] 2.3-3.6%; De Joung *et al.* [38] 0-16%; and Courtney-Gutterson *et al.* [39] 8%. However, it is relatively low when compared with Young *et al.* [40] transformation efficiency of 77-99% with the use of antibiotics kanamycin 25 ppm; Sherman *et al.* [31] 50-96% kanamycin antibiotics, hygromycin, geneticin 7.5-15; Kim J *et al.* [32] 65-99% km 20; Boase *et al.* [33] ~ 100% Km 25; Pavingerova *et al.* [41] 36-85% km 50-100; and Van Wordragen *et al.* (1992) 100% km 10-25. The possibility of using antibiotic selection for each explant source is different and different from the lethal dose of antibiotics for the initial selection of calli and the final selection at the regeneration stage so further research is needed for lethal doses of different explant sources and lethal doses at the callus selection and regeneration stages. In addition, it is also necessary to note the best composition of cytokine and auxin hormones for regenerating various explant sources.

4. Conclusion

In the case of *Chrysanthemum* in our study, the best source of explant for optimum transformation efficiency was from lateral shoot buds (54%) since they have better shoot regeneration efficiency (77.78%).

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