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Performance of Transgenic *Chrysanthemum* Harbouring Wasabi Defensin Gene for White Rust Disease Resistance

Rinaldi Sjahril[1*], Irma Jamaluddin[2], Marhamah Nadir[3], Asman[4], Feranita Haring[1], Muhammad Riadi[5], Siti Halimah Larekeng[6], Totik Sri Mariani[7], Trisnawaty A.R[8], Nurhaya J. Panga[9], Astina Tambung[1], Dong Poh Chin[10], Masahiro Mii[10]

[1]*Laboratory of Plant Biosciences and Reproduction Biotechnology, Faculty of Agriculture, Universitas Hasanuddin Makassar, Indonesia*
[2]*Master Student, Agrotechnology, Faculty of Agriculture, Universitas Hasanuddin, Makassar, Indonesia*
[3]*Animal Feed Chemistry Laboratory, Faculty of Animal Husbandry, Universitas Hasanuddin, Makassar, Indonesia*
[4]*Plant Pest and Disease Laboratory, Department of Plant Pest and Disease Science, Faculty of Agriculture, Universitas Hasanuddin, Makassar, Indonesia*
[5]*Plant Breeding and Seed Sciences Laboratory, Department of Agronomy, Faculty of Agriculture, Universitas Hasanuddin, Makassar, Indonesia*
[6]*Biotechnology and Tree Breeding Laboratory, Department of Forestry, Faculty of Forestry, Universitas Hasanuddin, Makassar, Indonesia*
[7]*School of Life Science and Technology, Bandung Institute of Technology, Bandung, Indonesia*
[8]*Department of Agrotechnology, Faculty of Sciences and Technology, University of Muhammadiyah Sidenreng Rappang, Sidenreng Rappang, Indonesia*
[9]*Faculty of Agriculture, Musamus University, Merauke, Papua, Indonesia*
[10]*Graduate School of Horticulture, Chiba University, Matsudo, Chiba, Japan*

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ABSTRACT

This study was intended to obtain white rust (*Puccinia horiana*) disease resistance *Chrysanthemum* transformed with wasabi defensin gene through mediation of *Agrobacterium tumefaciens* from three explant sources, i.e., leaf, lateral shoot bud, and internode. Observations were made on transformation efficiency, PCR analysis, *in vitro* and *ex vitro* disease resistance tests. Results showed that efficiency of transgenic callus and shoot regeneration was found both highest from lateral shoot buds (57.5% and 50.0%, respectively). PCR analysis showed that three putative transgenic plantlets from lateral shoot buds and one from leaf explant were putative transgenic carrying the wasabi, *hpt*, and *nptII* genes. Rooting test showed that the highest number of rooted plants was found in treatment of hygromycin (Hg) 25 mg L^{-1} (81%) and lowest was in treatment combination of kanamycin (Km) 50 mg L^{-1} + Hg 25 mg L^{-1} (25%). *In vitro* disease resistance test with sorus inoculation of *P. horiana*, directly on the leaves, resulted in 20 resistant plants out of 30 putative transgenic plants (66.67%). *Ex vitro* testing on adult plants of the same samples in a confined closed greenhouse (CGH) resulted in average of 80% transgenic *Chrysanthemum* plants were resistant, whereas in control plants caused white rust disease symptom.

1. Introduction

Chrysanthemum is a type of cut flower that is familiar, not only in Indonesia but also in the world. The harvested area of *Chrysanthemum* in 2018 decreased by 4.56% from 1,163.55 hectares in 2017 to 1,110.52 hectares in 2018. Although the harvested area decreased, the production of *Chrysanthemum* plants actually increased by 1.56% and had the largest production compared to other types of cut flower ornamental plants with a production of 488.18 million stalks. The number of export destinations for this commodity has decreased. In 2018, Japan became the only export destination for Indonesian *Chrysanthemum*, whereas previously this commodity was able to penetrate the Kuwaiti market. Although the number of importing countries decreased, the volume of *Chrysanthemum* exports rose from 49.52 tons to 59.11 tons (Badan Pusat Statistik 2018).

The decline in the number of *Chrysanthemum* importing countries was caused by several obstacles,

* Corresponding Author
E-mail Address: rinaldi.sjahril@gmail.com

one of which was the quality of flowers produced by Indonesia that did not meet the quality standards set by the global market so that they could not compete with products from other countries. The decline in the quality of *Chrysanthemum* flowers is caused by pests and diseases such as white rust (*Puccinia horiana* P. Henn). According to Kristina (2018), white rust attack can reduce the vase-life of *Chrysanthemum* flowers to only 5 days, whereas vase life of healthy flowers can last up to 12 days at room temperature (27-29°C). Seedling health survey conducted in West Java showed that seedlings produced by farmers infected with white rust disease were approximately 28.5% (Suhardi 2009).

One potential alternative to control this disease is to develop disease-resistant crops through genetic engineering. The formation of disease-resistant plants can be done by inserting resistance genes introduced from other organisms into the plant genome which is called genetic transformation technology. Genetic transformation technique mediated by *A. tumefaciens* is becoming popular nowadays because of the ease with which this soil phytopathogen infects plant wounds and transmits T-DNA to host plant cells (Hwang *et al.* 2017). Several studies on genetic transformation of *Chrysanthemum* for pest and disease resistance were reported by Shinoyama *et al.* (2015) using the cry1Ab (mcbt) gene to produce *Chrysanthemum* plants resistant to *Helicoverpa armigera* insects. Gong (2018) reported resistance to *Botrytis cinerea* infection in *Chrysanthemum* transformation using *Arabidopsis thaliana* ESB1 Gene. Resistance to *Chrysanthemum* black spot disease (CBS) with genetic transformation of the CmWRKY33.1 gene was reported by Liu *et al.* (2020).

There are several types of genes used in the transformation for pest and disease resistance, one of which is the wasabi defensin gene. Wasabi is a type of radish plant native to Japan, which can produce volatile allyl isothiocyanate (AITC) which can inhibit fungal infections (Atsumi and Saito 2015). Wasalexin and 6-methylsulfonylhexyl isothiocyanate (WjAMP-1) are secondary metabolites isolated from the wasabi plant which have antifungal and bacterial properties (Pedras *et al.* 1999; Saitoh *et al.* 2001; Kiba *et al.* 2003). The gene, named the wasabi defensin gene, is used in the transformation of rice plants for resistance to blast disease caused by fungi (Kanzaki *et al.* 2002). The gene encoding this antimicrobial protein is expressed in the transgenic plant *Nicotiana benthamiana* which inhibits the growth of fungi and bacteria (Saitoh *et al.* 2001). Wasabi defensin gene transformation provided resistance to Alternaria leaf spot and fusarium wilt disease in transgenic melon (Ntui *et al.* 2010) and tobacco (Ntui *et al.* 2011). Meanwhile, orchids can inhibit the growth of late blight caused by *Erwinia carotovora* (Sjahril *et al.* 2006).

Genetic transformation studies conducted on plants are generally constrained by the regeneration of plants from putative transgenic callus. This is likely caused by habituation of the callus due to repeated sub-cultures and the use of growth regulators and antibiotics for long term. Other factors that are thought to affect plant regeneration are the difference in explant sources such as leaves, lateral shoot buds and internodes as was reported by Jamaluddin *et al.* (2018). The success of the transformation is also influenced by age of explants, different ratios and concentrations of growth regulators and chemicals/antibiotics used as selective agents of transformed tissues (Bangash *et al.* 2013). Teixeira da Silva (2003) reported that the effect of antibiotic concentration used for genetic transformation on plant morphogenesis depends on explant size, source of explants, time of *A. tumefaciens* infection and selection pressure in genetic transformation. Nakano (2007) also reported that the optimal concentration of acetosyringone, used for enhancing *Agrobacterium* infection, depends on plant species, source of explants, duration of cultivation, and competency of the target tissue. This report substantiate previous study by Jamaluddin (2018) where their results showed that the efficiency of genetic transformation on callus occurred in internode (15.2%), lateral shoot buds (57.5%), and leaves (20.7%). The value of transformation efficiency (highest to lowest) on shoots regeneration was lateral shoot buds (50.0%), leaves (1.4%), and internode (0.3%). In this report, we show the persistence of the putative transgenic *Chrysanthemum* plants obtained are tested for white rust disease (*Puccinia horiana*) resistance.

2. Materials and Methods

2.1. Plant Materials and Culture Medium

Plant materials used were explants from plantlets of *Chrysanthemum* cv. Limeron (spray type with yellow flower), namely: leaf disks (5 mm),

internode cutting and lateral shoot bud cuttings. Culture medium consisted of inorganic and organic material components of MS medium (Murashige and Skoog 1962) supplemented with 0.1 mg L^{-1} 1-naphthalenacetic acid (NAA) (Sigma-Aldrich, Germany), 1.0 mg L^{-1} 6-benzylaminopurine (BAP) (Duchefa-Postbus, Netherland), 30 g L^{-1} sucrose, and solidified with 2.5 g L^{-1} gellan gum (Kanto Chemical Co., Inc. Chuo-ku, Tokyo, Japan). The pH of the media was adjusted in the range of 5.6-5.8 then autoclaved for 15 minutes at a pressure of 121°C. The tissue cultures were incubated at 25 °C under a photoperiod of 16 hours with cold white fluorescent light at 35 mol m^{-2} s^{-1}.

2.2. Plasmid Vector and Bacterial Strain

The *A. tumefaciens* strain used was EHA105 (pEKH-WD) harbouring wasabi defensin gene and hygromycin phosphotransferase (hpt) gene both driven by cauliflower mosaic virus 35S promoter (CaMV 35S-P), and the gene for neomycin phosphotransferase (*npt*II) driven by nopaline synthase promoter (nos-pro).

2.3. Transformation and Transgenic Plant Regeneration

Agrobacterium strain EHA105 (pEKH-WD) was cultured on solid LB medium with the addition of antibiotics 25 mg L^{-1} chloramphenicol (Affymetrix, Inc. Cleveland Ohio, USA), 50 mg L^{-1} kanamycin sulphate; (Wako Pure Chemical Industries, Osaka, Japan), and spectinomycin (50 mg L^{-1}). It was then incubated overnight using the same liquid LB medium supplemented with 200 µM acetosyringone (3',5'-Dimethoxy-4'-hydroxy-acetophenone; Sigma-Aldrich, St. Louis, MO, USA) on reciprocal shaker at 28°C for 12 hours at 50 rpm.

Transformation was done by co-cultivation of leaf pieces, internodes, and lateral shoot buds in overnight-cultured *Agrobacterium* solution, that had been diluted to 10% (vol/vol) with liquid MS medium, for 30 minutes (Sjahril *et al.* 2018). The explants were then filtered with nylon mesh (42 µm) and rinsed with sugar-free liquid MS medium, dried on sterile tissue paper and placed on the surface of the solid MS medium added with 200 µM acetosyringone for co-cultivation in culture bottles for 3 days in dark conditions at 25°C.

Bacterial elimination of explants was carried out by washing with a solution of 10 mg L^{-1} Meropenem® for 10 minutes and subsequently the explants were inoculated onto the fresh medium containing 5 mg L^{-1} Meropenem®. The selection agents used were kanamycin (50 mg L^{-1}) for callus selection and during plantlet stage according to the results obtained in the previous study (Sjahril and Mii 2006; Sjahril *et al.* 2018). Cultures were incubated under TL-28W (Himawari®) lighting at 25°C. Explants were subculture onto the same fresh medium every 2 weeks until callus appeared. Explants that remained green on kanamycin-containing medium to produce callus and shoots were considered to be resistant. Hygromycin (hygromycin B; MP Biomedical, Illkirch, France) at 25 mg L^{-1} was used to confirm the putative transgenic shoots by observing root growth as indicator.

2.4. Plant Test on Rooting and Disease Resistance of Putative Transgenic Plantlets

To confirm the transgenic nature, a total of 64 putative transgenic plantlets regenerated from all the explant sources on 50 mg L^{-1} kanamycin-containing medium was subjected to the test for rooting ability on medium containing hygromycin and that on *in vitro* white rust disease resistance test.

2.4.1. Rooting Test on Hygromycin-containing Medium

Putative transgenic plantlets resulting from transformation were grown on MS hormone-free medium with the addition of 50 mg L^{-1} kanamycin for multiplications. Shoots with 5 leaves were then cut and grown on MS medium with 50 mg L^{-1} kanamycin and 25 mg L^{-1} hygromycin singly and in combination. Observations were made two weeks after planting by confirming whether rooting occurs on the plantlets grown on each of the test media.

2.4.2. *In Vitro* White Rust Disease Resistance Test

Resistance test for white rust disease is carried out on putative transgenic *Chrysanthemum* as a result of the transformation. During *in vitro* resistance testing, plantlets were infected with the sorus of *Puccinia horiana*. This experiment was carried out by testing two inoculation methods on putative and non-transgenic *Chrysanthemum* plants as a control.

The first method (M1) was done aseptically by directly inoculating on the leaves in a culture bottle, while the second method (M2) was done by attaching

already infected leaves from *ex vitro* to the underneath of culture bottle cap without inoculating the sorus onto test plants *in vitro*. Each treatment was repeated three times. Plants that showed the signs of infection and later died were regarded as non-transgenic.

2.4.3. *Ex Vitro* White Rust Disease Resistance Test

Chrysanthemum plants suspected of being transgenic were propagated and acclimatized for further planting in confined closed greenhouse. The experiment was designed in a randomized block design consisting of 3 replications. The test was carried out based on the method (Hansen *et al.* 2005), in which the outside of the experimental plot was planted with non-transgenic clones as infector rows or as sources of inoculum. Tests were carried out by inoculating fungal and bacterial diseases against transgenic and non-transgenic *Chrysanthemum* cultivars. Symptoms of attack were observed when the plants were 30 days after planting and the attack level was scored. The results of the observations will show that *Chrysanthemum* cultivars are capable of producing resistance to white rust disease.

2.5. Data Analysis

Data obtained for rooting test to *Chrysanthemum* were subjected to the analysis of variance (ANOVA). The least significant differences (LSD) test was performed to identify significant differences among the treatments, with significance level of $p<0.01$.

3. Results

3.1. Efficiency of Transformation and Regeneration

Table 1 shows the results on the efficiencies of callus and shoots regeneration from three different sources of explants after infection with *A. tumefaciens* strain EHA105 (pEKH-WD). Among the three explants, lateral bud origin explants gave the highest efficiency of callus formation (57.5%) and shoot regeneration (50%) on kanamycin-containing medium. In contrast, internode origin explants formed 52 kanamycin-resistant calli from 342 explants (15.2%) on kanamycin-selection medium, on which only 1 callus regenerated shoot (0.3% efficiency). In leaf origin explants, 61 out of 295 explants produced kanamycin-resistant calli (20.7%), from which only 4 calli regenerated shoots (1.4% efficiency).

Four weeks after infection with *A. tumefaciens* strain EHA105 (pEKH-WD), some of the explants from 3 different sources produced green callus on a kanamycin-containing medium, while most explants turned brown with necrosis (Figure 1A). These calli survived to grow and some shoots emerged from between the calli four month after culture (Figure 1B).

Table 1. Efficiency of callus and shoot regeneration from three explant sources after co-cultivation on hormone-free medium containing 50 mg L^{-1} kanamycin

Explant sources	No. of explants	No. of explants with Km resistant callus	Km resistant callus (%)	No. of Km resistant callus with shoot regeneration	Km resistant Callus with Shoot Regeneration (%)
Lateral bud	42	23	57.5	21	50.0
Internode	342	52	15.2	1	0.3
Leaf	295	61	20.7	4	1.4

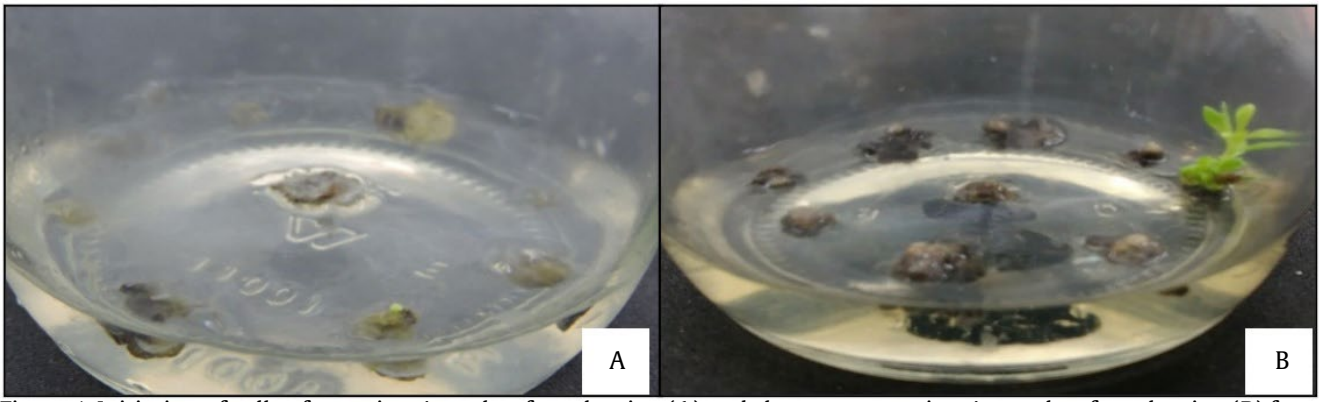


Figure 1. Initiation of callus formation 4 weeks after planting (A) and shoot regeneration 4 months after planting (B) from leaf explant on kanamycin-containing selection medium, after infection with *A. tumefaciens* harbouring wasabi defensin gene (pEKH-WD)

3.2. Analysis of pEKH-WD Gene Integrity in Putative Transgenic *Chrysanthemum* Plants

PCR was performed using a pair of primers from wasabi defensin gene, *hpt*, *npt*II and DNA from 13 putative transgenic *Chrysanthemum* plantlets, non-transgenic *Chrysanthemum* plantlets as control and pEKH-WD plasmid DNA as a positive control. The PCR results are presented in Figure 2.

3.3. Evaluation of Rooting Ability and Disease Resistance in Putative Transformants

Putative transgenic *Chrysanthemum* plantlets obtained on Km-selection medium were further tested for their rooting ability on media with kanamycin and hygromycin antibiotics (rooting test) and by infection with the sorus of *Puccinia horiana* (white rust resistance test). The results of the rooting test are presented in Table 2, Figures 3 and 4. Whereas the *in vitro* results of the testing of the disease are presented in Table 3, Figures 5 and 6; and the *ex vitro* results of the testing of the disease are presented in Figures 7 and 8.

3.3.1. Rooting Test

The results of the F-LSD test on the average number of rooted putative transgenic plants (%) on various *in vitro* antibiotic testing media at 8 WAP (week after plant) showed that the highest number of rooted plantlets in the test of hygromycin 25 mg L^{-1} (81%), did not differ in the test of kanamycin 50 mg L^{-1} (69%), and significantly different in the test of the combination of the two antibiotics Hg25 + Km 50 (25%). Antibiotic testing based on explant sources obtained results, transgenic putative from internode explant sources (33.0%), leaf explant sources (55.3%), and lateral bud explant sources (63.7%). The data can be seen in Table 2 and Figure 3. Rooting response of the putative transgenic shoots was observed two weeks after planting on medium containing antibiotics. The shoots grew and developed well as plantlets and show good growth of rooting in antibiotic test media, while non-transgenic plants as a comparison grew

Table 2. F-LSD test results mean the number of rooted transgenic putative plants (%) on various *in vitro* antibiotic testing media, 8 weeks after planting

Trangenic plant	Antibiotic medium			Average	
	Km + Hg	Km	Hg	Sample	Explant source
In K1.3	0	50	50	33	33.0
Lf K1.1	0	63	100	54	
Lf K1.2	0	63	100	54	55.3
Lf K1.3	25	50	100	58	
Lb K1	75	88	100	88	
Lb K2	25	75	50	50	
Lb K3	25	75	50	50	63.7
Lb K4	25	63	75	54	
Lb K5	25	75	83	61	
Lb K7	50	88	100	79	
Average	25[b]	69[a]	81[a]		

Hg = hygromycin, Km = kanamycin, In = transgenic from internode explant, Lf = transgenic from leaf explant, and Lb = transgenic from lateral bud explan. The numbers followed by unequal letters in the same row (a and b) mean that they are significantly different in the BNTα = 0.01 test with the comparison value of NP BNT 0.21

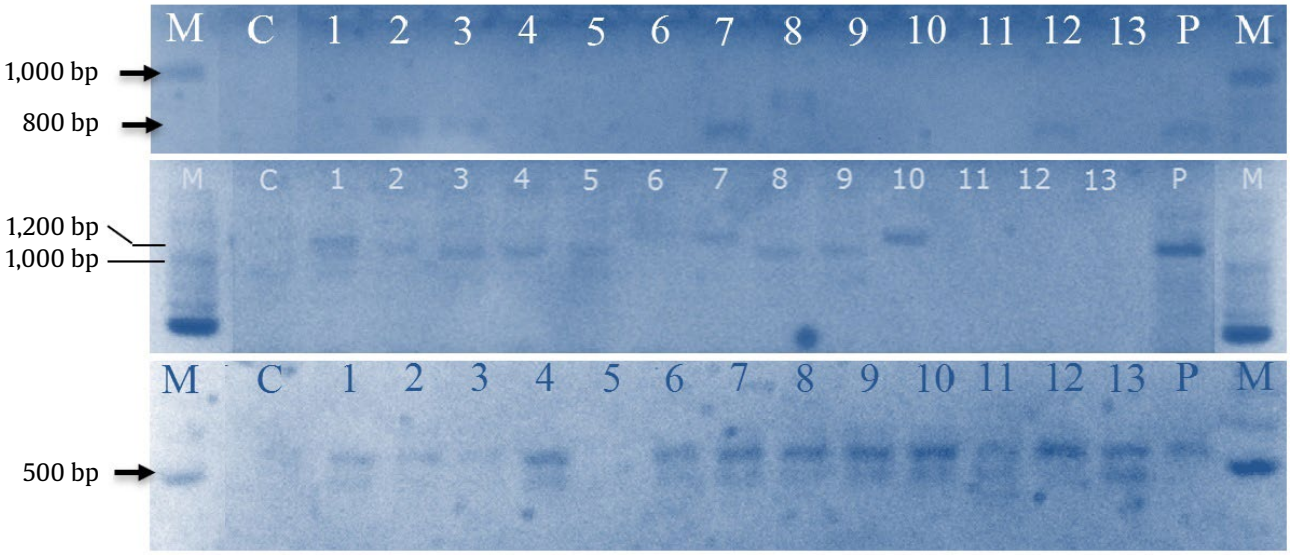


Figure 2. PCR amplification of *npt*II (800 bp, top), *hpt* (1,200 bp, middle), and wasabi defensin genes (500 bp, bottom), on several putative transgenic *Chrysanthemum* plantlet samples. M = marker, P = positive control (pEKH-WD plasmid DNA), C = control (non-transgenic plant), 1-13: putative transgenic plantlets regenerated from lateral shoot bud explants (lanes 1-7), leaf explants (lanes 8-9) and internode explants (lanes 10-13)

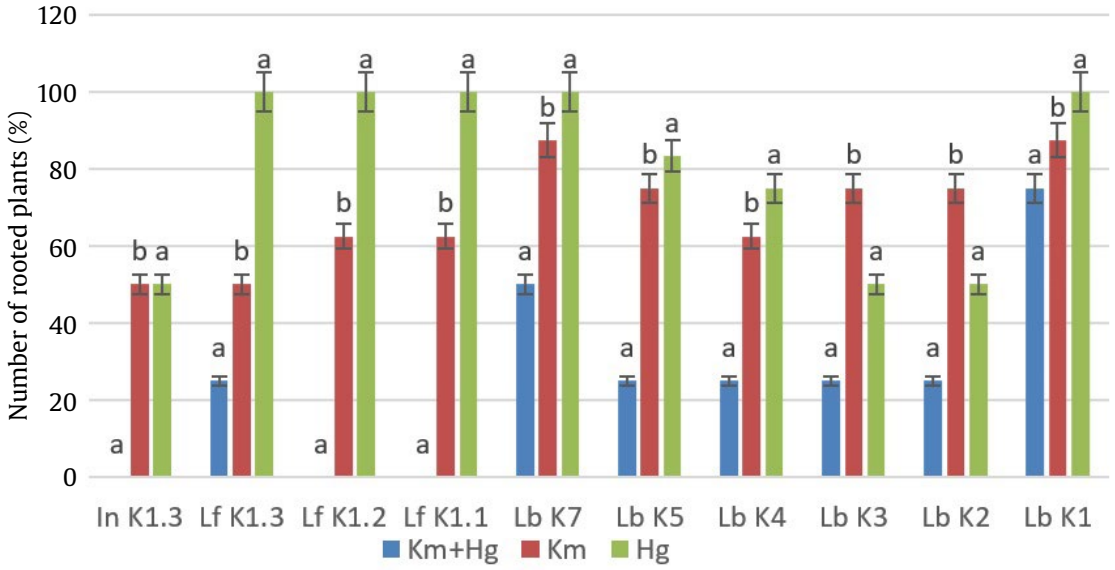


Figure 3. Rooting tests on putative transgenic *Chrysanthemum* shoots grown on MS medium containing 25 mg L^{-1} hygromycin (Hg), 50 mg L^{-1} kanamycin (Km). In = transgenic plantlet from internode explant, Lf = transgenic plantlet from leaf explant, and Lb = transgenic planlet from lateral bud explant. Numbers followed by different letters in same row (a and b) mean that they are significantly different in the LSDα = 0.01 test with the comparison value of LSD 0.21

Table 3. Infection rates of white rust inoculation in putative transgenic *Chrysanthemum* plantlets grown *in vitro* three weeks after inoculation

Treatment		Samples inoculated	Infected	Uninfected	Infection rate (%)
M1	T	30	10	20	33.33
	Nt	30	26	4	86.67
M2	T	14	-	14	0
	Nt	14	-	14	0

Remark: M1 = Streak/touch method, M2 = leave with disease placed in culture bottle with tested plantlets, T = transgenic, Nt = non-transgenic

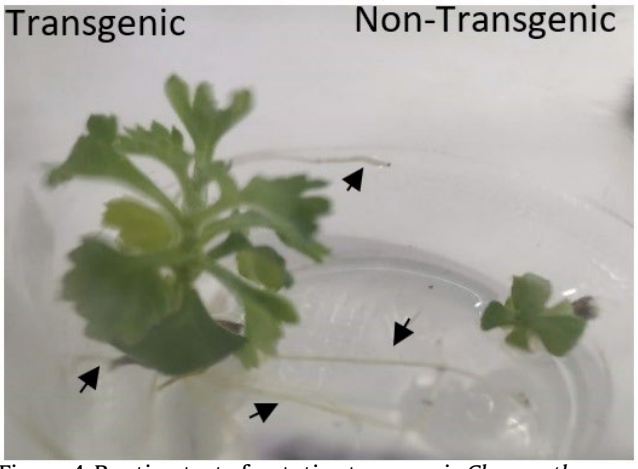


Figure 4. Rooting test of putative transgenic *Chrysanthemum* plant samples on 25 mg L^{-1} hygromycin and 50 mg L^{-1} kanamycin incorporated medium, two weeks after planting. Arrows point at growing roots from resistant plant

stunted, not rooted, and some leaves begin to turn brown and show signs of death (Figure 4).

3.3.2. White Rust Disease Resistance Test *In Vitro*

The results of the white rust disease resistance test against putative transgenic *Chrysanthemum in vitro* at 3 weeks shown in Table 3 and Figure 5 and also in Figure 6 (3 months old plantlet).

3.3.3. White Rust Disease Resistance Test *Ex Vitro*

The results of the white rust disease resistance test against putative transgenic *Chrysanthemum ex vitro* at the age of 8 and 12 weeks after planting showed an average of 80% (data not shown) resistance to white rust disease. Observations at the 12th week showed

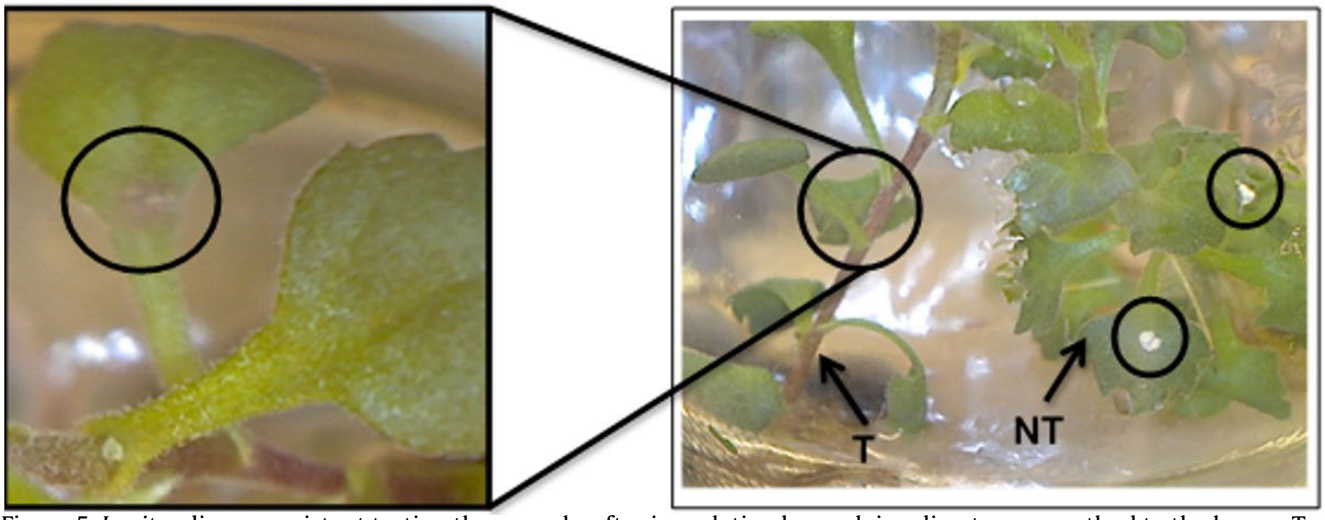
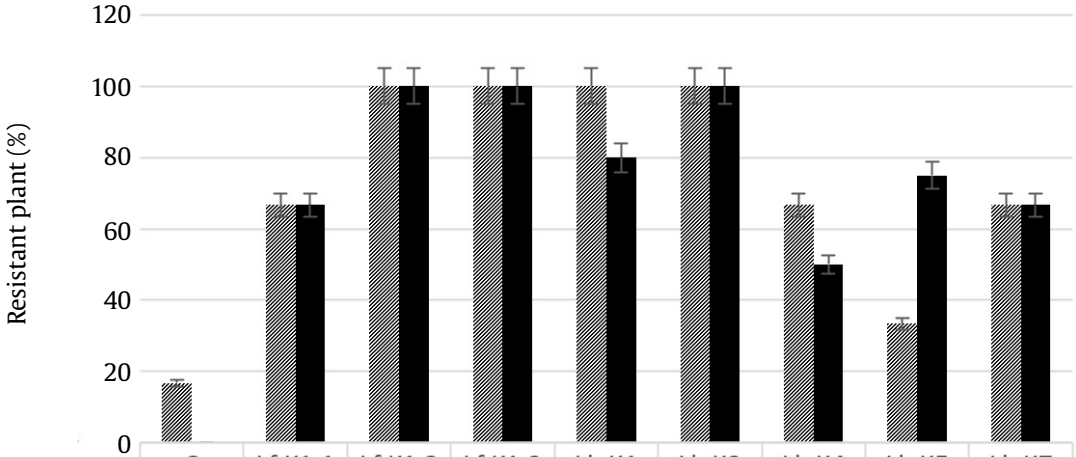


Figure 5. *In vitro* disease resistant testing three weeks after inoculation by applying direct sorus method to the leaves. T = transgenic plants showed dead and black sorus, NT = non-transgenic plants showed live and white sorus

Figure 6. *In vitro* disease testing by applying a method of applying direct sorus to the leaves, observed 3 months after inoculation. T = transgenic plants. NT = non-transgenic plants



	C	Lf K1.1	Lf K1.2	Lf K1.3	Lb K1	Lb K3	Lb K4	Lb K5	Lb K7
8 WAP	17%	67%	100%	100%	100%	100%	67%	33%	67%
12 WAP	0%	67%	100%	100%	80%	100%	50%	75%	67%

Figure 7. *Ex vitro* white rust disease resistance test in the confined closed greenhouse at 8 and 12 weeks after planting. C = control/non-transgenic, Lf K1.1-Lb K7 = transgenic plant

Figure 8. *Ex vitro* white rust disease resistance test in confined closed greenhouse at 8 weeks after sorus inoculation. (A) control/non-transgenic, (B) and (C) = transgenic plant, (B) shows the whole plant and (C) shows the lower leaf of plant (B) where the sorus inoculation were applied

samples from leaves of plants K1.2, K1.3, K3 lateral buds originated were 100% resistant, whereas resistance of K1 and K4 lateral bud originated sample plants decreased. While in K1.1 leaf and K7 lateral buds originated samples remained the same. However, in plants K5 lateral bud originated sample, the resistance increased from 33% to 75%, while all leaves from control plants were infected. The data is presented in Figures 7 and 8.

4. Discussion

The transformation of *Chrysanthemum* plants with wasabi defensin gene for white rust disease resistance in this study has succeeded in obtaining putative white rust resistant transgenic *Chrysanthemums* from three sources of explants (leaves, lateral bud shoots, and internodes) that have been tested for antibiotic resistance, DNA analysis, and tested for disease resistance on a laboratory-scale and confined closed greenhouse scale. The efficiency of transformation and regeneration is a determining factor and is a necessary parameter to determine the success rate of genetic transformation in plants through *Agrobacterium*-mediated methods. In this study, the value of transformation efficiency was obtained by comparing the number of explants that survived on the selection medium with the number of explants co-cultivated. While the regeneration efficiency value was obtained by comparing the number of explants that we're able to regenerate to form new shoots with the number of explants that we're able to survive on the selection medium.

The results of our study show that among the 3 explants, lateral bud origin explants gave the highest efficiency of callus formation (57.5%) and shoot regeneration (50%) in a kanamycin-containing medium. Since lateral shoot bud was thought to contain actively dividing cells to form shoots compared to others, the calli formed on kanamycin-containing medium might have high shoot regeneration ability (Table 1). On the other hand, internode explants gave the lowest efficiency of resistant callus and shoot regeneration in kanamycin-selection media.

Explant sources from internodes formed more calli compared to explant sources from leaves, which formed more somatic embryogenesis. This caused the low shoot regeneration efficiency from internodes explant sources. This result is supported by a study Sedaghati, Haddad and Bandehpour (2019) which compared stem and leaf explants, stem explants failed to form somatic embryogenesis and formed more callus. Callus formation, somatic embryogenesis and shoot regeneration of various types of explants depend on the type of tissue, age, interactions between metabolites and levels of endogenous hormones (Kumari *et al.* 2017; Jin *et al.* 2021).

The results of PCR analysis using a pair of wasabi gene primers (Figure 2) showed that all of the plantlets except for lane 5 obtained from 3 different explants showed a positive amplification of 500 bp wasabi defensin gene fragments. The *hpt* gene amplification by PCR shows that *Chrysanthemum* DNA plantlets from lateral shoot bud explant sources (lanes 1-7),

leaf explant sources (lanes 8 and 9), and internode explant source (lane 10) shows positive amplification around 1,200 bp *hpt* gene fragments. Whereas sample numbers 11-13 from internode explant sources were not amplified. The results obtained from PCR analysis using *nptII* primers show that samples at lanes 2, 3, and 7 from lateral shoot bud explant sources, sample lane 8 from leaf explant sources, and sample at lane 12 from amplified internode explant sources with fragment sizes around 800 bp. While samples at lanes 1, 4, 5, and 6 from leaf explant sources, sample number 9 from leaf explant sources, and sample at lanes 10, 11, and 13 from internode explant sources are not amplified.

The results obtained are samples number 2, 3, and 7 from the treatment of lateral shoot bud explant sources, sample number 8 from the treatment of leaf explant sources contained all three marker genes. Samples containing wasabi and *hpt* genes were sample numbers 1, 4, and 6 from the treatment of lateral shoot bud explant sources, sample number 9 from the treatment of leaf explant sources, and sample number 10 from internode treatment. Samples containing wasabi and *npt*II genes were only sample number 12 from the treatment of internode explant sources. Samples containing only wasabi genes were sample numbers 11 and 13 from the treatment of internode explant sources. Sample number 5 from the treatment of lateral shoot bud source explants carries only the *hpt* gene.

Rooting test showed the highest average number of rooted plants was at hygromycin 25 mg L^{-1} (81%) and was not much different from kanamycin 50 mg L^{-1} (69%), and the lowest was at combination Hg25 + Km 50 (25%). Rooting tests of the non-transgenic shoot on hygromycin media (25 mg L^{-1}) and kanamycin media (50 mg L^{-1}) cause *Chrysanthemum* plantlet growth to be stunted. Shoots begin to die showing changes in color from green to yellowish-green and subse quently turned brown, dry and blacked. Hygromycin inhibits metabolic processes by binding to the 80S ribosome so that mRNA translation errors occur (Bashir *et al.* 2004). The wasabi defensin gene contains kanamycin resistance (*npt*II) gene and hygromycin resistance (*hpt*) gene in the T-DNA region, so that transgenic plants carrying the gene can survive and rooted in media containing hygromycin antibiotics with a lethal limit of 25 mg L^{-1} and kanamycin antibiotics with a lethal limit of 100 mg L^{-1} in *Chrysanthemum* (Sjahril *et al.* 2018).

Hygromycin has been used as a selective agent for transgenic and non-transgenic plants in the genetic transformation studies of various plant species. For example, Harwood *et al.* (2009) reported transformation in wheat using the pBract 204 gene contains the *hpt* gene which provides resistance to the hygromycin antibiotic with a lethal dose of 50 mg L^{-1}. Transgenic wheat plantlets formed roots in a medium containing hygromycin while non-transgenic plants did not survive. Harwood (2014) also reported barley transformations using the pBract hygromycin resistance gene. Transgenic plants are characterized by plantlets with a hygromycin resistance of 50 mg L^{-1} and quickly form strong roots. The hygromycin resistance gene is the best selection system for wheat plants. In the present study, only about a half of the plantlets, selected as the putative transformants due to their resistance against 50 mg L^{-1} kanamycin after infection with *A. tumefaciens* strain EHA105 (pEKH-WD) and subsequent plant regeneration process, showed resistance to 25 mg L^{-1} hygromycin. Therefore, it is possible that they are non-transgenic escapes.

Table 3 shows the infection rates of white rust disease after inoculation to kanamycin-resistant putative transgenic *Chrysanthemum* plantlets. Inoculation was carried out by two methods in aseptic conditions. The first method is direct inoculation and the second method is to attach infected leaves from *ex vitro* to underside of the bottle cap. After three weeks of inoculations, the highest infection rate of 86,67% was obtained in method 1 in non-transgenic (M1Nt) plants, whereas in the first method on putative transgenic plants, the infection rate was 33,33%. In the second method, there were no attacks of the fungus either on non-transgenic plants (0%) or transgenic plants (0%).

Testing resistance to disease using the first method in the form of direct scratching into the leaves of plants. Observation three weeks after inoculation (Figure 5) shows that the sorus attached to transgenic plants turned brown and then black (shown in dark circles). Whereas the sorus affixed to non-transgenic plants grew and turned white in color. The observation three months after inoculation (Figure 6) shows that non-transgenic plants decayed, turned brown and eventually died, while putative transgenic plants continued to grow and turn green. Then after 3 months of testing, the putative transgenic plants remained alive and the non-transgenic plants were seen dead (Figure 6).

These results suggest that direct smearing (Method #1) was more effective to be used compared to the method of attaching infected leaves to the culture bottle cap (Method #2). Based on observations, sorus-containing leaves affixed to the culture bottle caps dry out and then die before infecting the test plants underneath.

The white rust inoculation test in the confined closed greenhouse showed that the putative transgenic *Chrysanthemum* carrying the wasabi defensin gene gave an average resistance of 77% compared to non-transgenic controls and the remaining 33% were attacked but not as severely as the control. Two samples experienced a decrease in resistance in the 12th week of observation and 1 sample showed an increase in disease resistance, from 33% in the 8th week to 75% in the 12th week of observation, and showed brownish and dead sorus. This proves that we have produced transgenic plants using the wasabi defensin gene insertion method through the intermediary of *A. tumefaciens*, although there are still some samples that are suspected to have escaped.

In conclusion, the highest efficiency of putative genetic transformation was obtained from lateral shoot bud, which amounted to 50%. Since most of the putative transgenic plants obtained in the present study showed rooting on hygromycin-containing medium and resistance against infection with *Puccinia horiana*, it is highly possible that large portion of putative transgenic plants are real transgenic plants conferring the resistance against white rust disease. Further studies on molecular analyses of three marker genes (wasabi, *hpt*, and *npt*II) in the putative transgenic shoots obtained in the present study are now in progress.

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