STUDY ON PLANT REGENERATION AND FUNGAL DISEASE RESISTANT GENE TRANSFORMATION FOR VIETNAMESE GINGER (*Zingiber officinale* Rosc.) VIA *Agrobacterium* - MEDIATED METHOD

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SUMMARY

Ginger (*Zingiber officinale* Rosc.) has been used as valuable spice and medicine for a long time in South Asia. Ginger and other species in the *Zingiber* genus have known as a recalcitrant group for tissue culture and transformation. In this study, we assessed the regeneration media for ginger plant regeneration from *in vitro* leaf sheaths. Results showed that the MS medium containing 1 mg/L thidiazuron gave the highest efficiency with 68.33% in regeneration rate and 24.63 shoots forming per regenerated sample in average. These results were extremely useful for the application on genetic transformation in ginger which is susceptible to fungal diseases. A plant defensin gene (*Ca-AFP*) was transferred into *in vitro* leaf sheaths of ginger simultaneously with *β-glucuronidase* (*gus*) reporter gene and *hygromycin phosphotransferase* (*hpt*) selectable marker gene, by using the *Agrobacterium tumefaciens* strains EHA105. After 10 weeks in selective media containing 4 mg/L hygromycin, the expression of *gus* gene was recorded in some regenerating shoots by histochemical Gus assay. By using PCR detecting method, the positive bands of *Ca-AFP* and *hpt* transgenes were observed in four lines of transformed shoots that regenerated in hygromycin-selective media within 20 weeks. The results of this study show the potential to create a new ginger variety that could improve the resistance to soft rot disease caused by *Pythium aphanidermatum* fungus in the future.

Keywords: Agrobacterium tumefaciens, plant defensin gene, regeneration, *Zingiber officinale* Rosc.

INTRODUCTION

The genus *Zingiber* is distributed in tropical and subtropical Asia and Far East Asia and includes about 150 species. Ginger (*Zingiber officinale* Rosc.) is one of essential spice crops and herbs that have cultivated and used in South Asia for a long time (Ravindran, Babu, 2005). The ginger rhizome contains bioactive compounds such as gingerol, shogaol, zingiberene, α-terpineol and linalool (Ravindran, Babu, 2005; Zuraida *et al*., 2016). However, ginger is susceptible to fungi and bacteria, especially *Pythium aphanidermatum* causing soft rot disease and it is easy for the pathogen to be widely transmitted. Therefore, after being infected by pathogen, extreme yield losses can occur in ginger cultivation (Ravindran, Babu, 2005). Because of those problems, development and propagation of disease-free or disease-highly-resistant ginger varieties are required with effective methods. Genetic transformation is one of solutions that have been successful on some main crops (cotton, maize, and soybean) to enhance the resistance to insects or diseases (ISAAA, 2017).

For ginger, callus and embryogenic callus could be induced from *ex vitro* buds, young leaves and anthers. Type of auxin and their concentration supplied to media have the main role on induction of somatic embryogenesis. Many types of auxin have been reported for their efficiency, such as 2,4-D, dicamba or NAA (Kackar *et al*., 1993; Ravindran, Babu, 2005). Combination of 2,4-D and BA, or NAA and kinetin/BA could also induce embryogenic callus from shoots (Jamil *et al*., 2007; Suma *et al*., 2008). However, the number of such reports are limited and it is difficult to apply these results for genetic transformation because the limit of *ex vitro* explants (Suma *et al*., 2008). In previous study on Vietnamese ginger, the combination of BA and 2,4 D could induced embryogenic callus and somatic embryo from transverse thin cell layer of *ex vitro* rhizome sprouts (Tran *et al*., 2010). However, ginger rhizomes are usually collected at the end of cultivation seasons. Thus, the *in vitro* aerial stem (including shoot tips meristem) has been used for plant regeneration instead of *ex vitro* sprouts. The use of 2,4 D was inefficient when the regeneration rate was lower than 10%. On the other hand, thidiazuron (TDZ) is one of the most active cytokinin, can induce adventitious shoots more effective than purine-type cytokinin in many species such as carnation (Lu *et al*., 1991; Nugent *et al*., 1991), and rose (Lu, 1993). TDZ also stimulates endogenous purine cytokinin synthesis and inhibits their degradation (Capelle *et al*., 1983; Thomas, Katterman, 1986). Therefore, when TDZ was applied on media, regeneration rate from *in vitro* aerial stem was around 70% (Tran *et al*., 2019). This is the potential result which need to be studied more detail.

Plant defensins are small, cysteine-rich peptides, abundantly present in plant kingdom, usually are found in stomatal and peripheral cells. They play important roles on primary effect on the invading pathogens, especially against fungal pathogens (Khan *et al*., 2019). Three-dimensional pattern of plant defensins are conserved with

one alpha helix and three antiparallel beta sheets. These folding patterns are stabilized by four or five disulfide bridges from conserved amino acid residues of cysteine (Khan *et al*., 2019). *Ca-AFP* gene from chickpea (*Cicer arietinum*) can encode a plant defensin protein of 74 amino acids. The results from aligning Ca-AFP protein's sequence to those of other crops' defensin proteins showed that Ca-AFP contains eight common cysteine residues, which include four disulfide bridges (Kumar *et al*., 2019). Furthermore, it was demonstrated that Ca-AFP protein has the antifungal function to *Pythium aphanidermatum* and *Botrytis cinereal* (Islam *et al*., 2007).

On genetic transformation in plants, selecting agent plays an important role on the screening of stable transgenic explants, and eliminate chimeric status. Hygromycin B, an antibiotic, is often used as the selecting agent. This is the second most frequently used antibiotic for selection after kanamycin (Miki, McHugh, 2004). So far, there is only one report that uses hygromycin with 40 mg/L in concentration to screen the transformants in *Z. officinale* cultivated in India (Musfir Mehaboob *et al*., 2019).

In this study, we developed a regeneration protocol for *Z. officinale*, a Vietnamese variety, by using young *in vitro* leaf sheaths to create a stable source for genetic transformation of this species. Initially, *Ca-AFP* gene under the control of CaMV35S promoter was transferred into *Z. officinale* by *Agrobacterium tumefaciens* for an attempt to improve resistance to soft rot disease caused by *Pythium aphanidermatum* fungus.

MATERIALS AND METHODS

Plant materials

Sprouts forming in rhizome of cultivated ginger (*Zingiber officinale*) in the southern of Vietnam were collected and established *in vitro* as the description in our previous report (Tran *et al*., 2010). They were micro-propagated in solid Murashige and Skoog (MS) medium supplemented with 3 mg/L BA, and used as *in vitro* source. They were sub-cultured every two-month and maintained at 25 ± 2°C with photoperiod of 8 hours light and 16 hours dark (Tran *et al*., 2010; 2019).

Evaluation of shoot regeneration medium

Leaf sheaths were cut from 1-month-old *in vitro* explants with approximate 5 mm in length and we investigated the effect of TDZ on shoot regeneration from leaf sheaths. MS medium was supplemented with different concentrations of TDZ (0.5, 1.0, and 1.5 mg/L). We also examined the efficiency of shoot regeneration in the presence of both 0.5 mg/L IBA and TDZ (in the above range of concentration). Samples were transferred to fresh media every two-week, at 25 ± 2°C with photoperiod of 8 hours light and 16 hours dark.

Assessment of hygromycin concentrations transgenic shoot selection

In this study we assessed the effect of hygromycin on the regeneration of shoots from *in vitro* leaf sheaths of Vietnamese *Z. officinale* cultured in optimal regeneration media. Concentrations of hygromycin in this assessment were in range 0, 2, 4, and 6 mg/L, and samples were sub-cultured every two-week.

Agrobacterium strain, T-DNA construct and transformation

Agrobacterium tumefaciens strain EHA105 harbors a T-DNA construct shown in Figure 1. *A. tumefaciens* strain was cultured in liquid LB medium supplemented with kanamycin and rifampicin by incubating at 28°C, and shaking at 200 rpm overnight. Before the infection, cultured *A. tumefaciens* was centrifuged at 3500 rpm for 10 minutes. Then, the pellet was resuspended in liquid ½ MS medium supplemented with 200 µM acetosyringone, and the optical density (OD_{600}) was in the range of 0.8–1.0. The leaf sheath segments were soaked in *A. tumefaciens* solution for 20 minutes. After that, the excessive bacteria in infected samples was absorbed by aseptic filter papers. Then, infected samples were co-cultured in basal MS medium supplemented with 200 µM acetosyringone in the dark at 28°C for 3 days.

Figure 1. Schematic diagram of the T-DNA region

hpt, *hygromycin phosphotransferase* gene; *Ca-AFP*, *anti-fungal protein* gene derived from *C. arietinum*; Gus, *β-glucuronidase* gene; p35S, CaMV 35S promoter; t35S, CaMV 35S terminator; tNOS, nopaline synthase terminator. LB and RB, left and right border.

Selection of transgenic shoots

After co-culture period and washing re-grew *A. tumefaciens*, samples were placed on shoot-regeneration medium supplemented with 4 mg/L hygromycin to select the transformed line and antibiotic (cefotaxime and augmentin) to eliminate *A. tumefaciens*. The sub-cultivation was performed in selective medium every two-week until getting the regenerating shoots.

Histochemical Gus assay

In order to evaluate the expression of *gus* gene in infected ginger leaf sheaths, histochemical Gus assay was performed as the Jefferson's protocol (Jefferson, 1987). X-Gluc was dissolved in 100 mM sodium phosphate buffer (pH 7.0) containing 10 mM EDTA, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, and 0.1% (v/v) Triton™ X-100 to reach the 1 mM concentration. Samples were incubated on X-Gluc solution at 37° C overnight. Then, samples were soaked in 70% ethanol for 4 hours to remove chlorophyll (Jefferson, 1987).

PCR analysis

Leaf blades of hygromycin-resistant shoots regenerating in selective medium were collected and genomic DNA were extracted by using DNeasy® Plant Mini Kit (QIAGEN, Germany). PCR amplification was carried out with Swift™ Maxi Thermal Cycler (ESCO, Singapore) according to the following step-cycle program: (1) pre-incubation at 95° C for 5 minutes, follow by 30 cycles of denature at 95° C for 60 seconds, and annealing at 55° C for 60 seconds and extension at 72° C for 2 minutes each cycle for *hpt* gene; and (2) pre-incubation at 95° C for 5 minutes, follow by 35 cycles of denature at 95° C for 90 seconds, and annealing at 65° C for 90 seconds and extension at 72° C for 2 minutes each cycle for *Ca-AFP* gene. The primer pairs for PCR were the following; 5'- AGCTGCGCCGATGGTTTCTACAA -3' and 5'- ATCGCCTCGCTCCAGTCAATG -3' for *hpt* gene and 5'- CGCGGATCCATGGCGAGGTGTGAGAATTTGGCT -3' and 5'- TGCTCTAGAATGGCGAGGTGTGAGAATTTGGCT -3' for *Ca-AFP* gene (El-Siddig *et al*., 2009; Nguyen *et al*., 2012).

RESULTS AND DISCUSSION

Regeneration of shoots from leaf sheaths

The initial shoots were induced in the cutting side of leaf sheaths after 4 weeks in regeneration media. Shoots developed into clusters alternating by porous callus (Figure 2b). After 8 weeks, by the magnifying glass, it is clear to see that each bud cluster was covered by a layer, and contained shoots inside (Figure 2c and 2d). By the 12th week, the shoots grew large, turned green, and were around 3 mm in height (Figure 2e). Then, regenerated shoots were transferred to media without TDZ for plant development and root formation (Figure 2f). Those shoots started to form roots after two weeks in medium containing 3 mg/L BA and we could get *in vitro* ginger plants with roots growing well after about nine weeks (Figure 2g).

The shoot's regeneration data were collected at 15th weeks in all treatments. The highest regeneration rate (68.33%) was obtained in medium containing 1 mg/LTDZ. In addition, this treatment also produced highest shoot number per sample (24.63 shoots) (Table 1). The combination between TDZ and 0.5 mg/L IBA gave the lower efficiency in both shoot regeneration and average number of shoot when compared to those of treatments without IBA (Table 1). These results were different to those of Lincy (2009). They could induce the direct regeneration of shoots from aerial stems of Jamaican ginger variety with 100% in efficiency, when using 1 mg/L TDZ and 0.5 mg/L IBA (Lincy *et al*., 2009). However, according to Aasim (2009), when using TDZ alone, the regeneration rate and the number of regenerated shoots were high, but the height of those shoots were limited by the inhibition effect of TDZ. The presence of IBA could increase the height of the regenerated shoot, but reduced the regeneration rate as well as the number of regenerated shoots (Aasim *et al*., 2010). In this study, there were the same issues, and to solve this disadvantage of TDZ, all regenerated shoots were moved to other media without TDZ to develop height of shoots and induce roots. In another report, the combination of TDZ in range of 0.1-1.0 mg/L with 2 mg/L 2,4-D were applied on leaf sheaths of 3-month-old *in vitro* explants of Indian ginger cv "Maran". The results indicated that it could induce embryogenic callus with efficiency in the range of 47.5-82.03% (Musfir Mehaboob *et al*., 2019).

In current time, there are several reports on shoot regeneration of *Z. officinale* from different materials such as young leaves, shoot tips and sprouts from rhizome using both organogenesis and somatic embryogenesis systems. However, the protocols and efficiencies are quite different, and highly depend on genetic resource, as subspecies or varieties (Ali *et al*., 2016; Ibrahim *et al*., 2015; Lincy *et al*., 2009; Mehaboob *et al*., 2019; Sathyagowri *et al*., 2011; Solanki *et al*., 2014; Suma *et al*., 2008; Tran *et al*., 2010). This is the first report of shoot regeneration from *in vitro* leaf sheath (*in vitro* explant) of Vietnamese *Z. officinale*. It has potential to provide the stable source, and effective method to the application on genetic transformation in Vietnamese *Z. officinale*.

Table 1. Effect of TDZ and IBA on shoot regeneration from leaf sheaths

IBA (mg/L)	TDZ (mg/L)	Shoot regeneration rate (%)	Average number of shoot/ regenerated sample
0	0.5	33.89 ± 2.00 ^{c}	14.31 ± 0.30 ^{d}
0	1.0	68.33 ± 0.96 ^{a}	24.63 ± 0.17 ^{a}
0	1.5	63.89 ± 1.47 ^{a}	20.18 ± 0.22 ^{b}
0.5	0.5	13.33 ± 1.67 ^{d}	9.65 ± 0.30 ^{e}
0.5	1.0	37.78 ± 3.09 ^{bc}	17.29 ± 0.24 ^{c}
0.5	1.5	46.11 ± 1.11 ^{b}	19.55 ± 0.13 ^{b}

Values were expressed as the mean ± SE (standard error), taking 30 samples in each treatment with three replications. All data were taken after 15 weeks after starting induction in regeneration media. With each treatment, the different letters showed the significant differences on values among treatments by the Tukey-HSD test (α = 0.05).

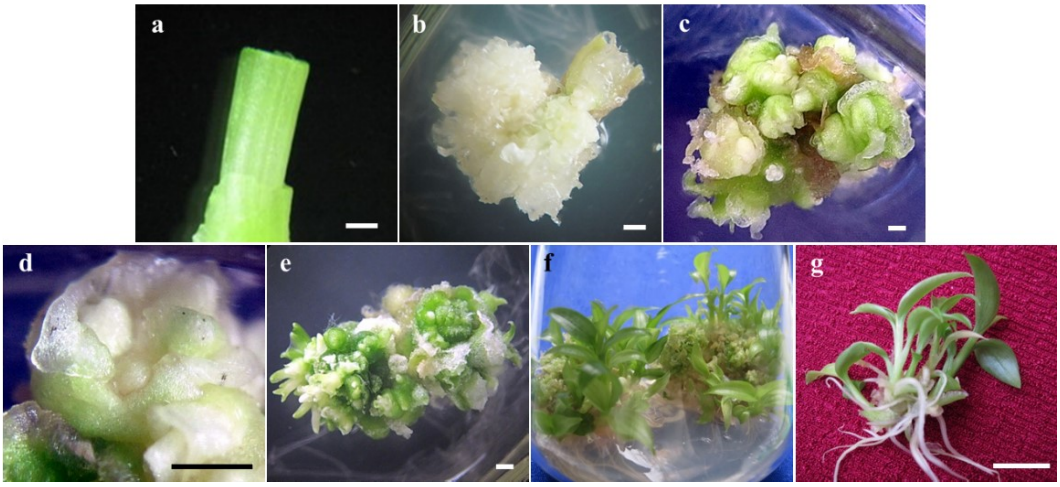

Figure 2. Regeneration of shoots from leaf sheaths

(a) Leaf sheaths are cut from 1-month-old in vitro explants. (b) 4 weeks after induction (WAI), small shoots are created in regeneration media. (c) 8 WAI, buds carry several small shoots in regeneration media. (d) Small shoots in closed-up view; (e) 12 WAI, shoot clusters develop in regeneration media. (f) Multi-shoots form roots in micro-propagation media. Scale bar: 1 mm. (g) in vitro ginger plants. Scale bar: 1 cm.

Effect of hygromycin on leaf sheaths

In this study, after 4 weeks in treatment of 6 mg/L hygromycin, no sample could survive. And after 6 weeks, no shoot was induced in treatment of 4 mg/L hygromycin. Consequently, this assessment was stopped and data were collected in all treatments after 6 weeks of screening. The results indicated that in the control media (without hygromycin) 66.67% of leaf sheaths formed heathy shoots. However, this value dropped down to 10%, when hygromycin concentration increased to 2 mg/L (Table 2). Moreover, results from other experiment showed that treatment of 4 mg/L hygromycin could inhibit the growth of more than 70% of 1 cm-shoots after eight weeks (detail data not shown). Therefore, 4 mg/L was chosen as the concentration of hygromycin to screen transgenic shoot regenerating in infected leaf sheaths. In other reports, hygromycin concentration used to select the transformants were 15 mg/L with maize callus (Walters *et al*., 1992), 20 mg/L with *Arabidopsis* (Ziemienowicz, 2001), and more than 50 mg/L with some grasses and monocotyledonous plants (Miki, McHugh, 2004). However, in this study, the suitable concentration is only 4 mg/L, much lower than those of mentioned reports.

Table 2. Effect of hygromycin on shoot regeneration from leaf sheaths

Hygromycin (mg/L)	Rate of samples forming shoots (%)	Note
0	66.67 ± 1.67	Heathy shoots
2	10.00 ± 2.89	Porous calli were formed in most of samples
4	0	Porous calli were formed in some samples
6	0	All samples died after 4 weeks

Values were expressed as the mean ± SE (standard error), taking 20 samples in each treatment with three replications. All data were taken after 6 weeks from starting point.

Selection of transformed shoots, *Gus* expression and PCR detection

After 3 days of co-culture period, infected samples were sub-cultured in regeneration media supplemented with 4 mg/L hygromycin every two-month until forming resistant shoots. In this study, the hygromycin-resistant shoot clusters were obtained after 10 weeks of infection (Figure 3a). Nine shoot clusters were got (from more than 1000 infected leaf sheaths) and each of them were cut a small part to perform histochemical Gus assay. The results showed that the blue color of X-Gluc stain was observed in the base of shoot cluster, but not in the upper part (Figure 3b and 3c). All hygromycin-resistant shoot clusters were maintained in regeneration media with 4 mg/L hygromycin until forming full shoots. After that 10 weeks, the Gus assay was performed with four lines of grew resistant-shoots which were cut in half vertically. The blue color of X-Gluc stain was really light and only observed in the base of shoot, but not in the leaf blades (Figure 3d).

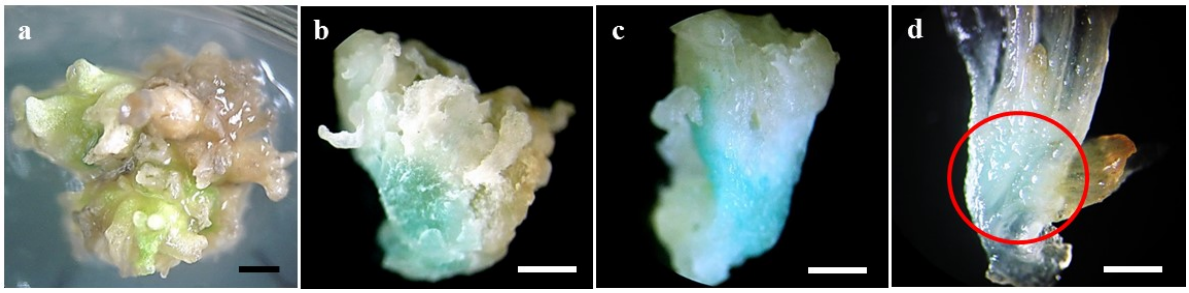

Figure 3. *Gus* expression in hygromycin-resistant shoots

(a) Shoots clusters regenerate in selective media supplementing 4 mg/L hygromycin, 10 weeks after infection. (b) and (c) Expression of Gus in resistant shoot cluster, 10 weeks after infection. (d) Expression of Gus in resistant shoot (inside the red circle), 20 weeks after infection. Scale bar: 2 mm.

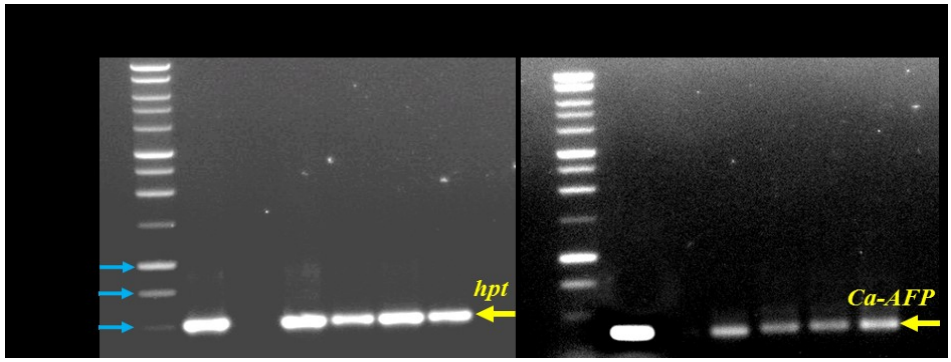

Figure 4. PCR analysis of genomic DNA from *in vitro* leaf blades of the transformed and non-transformed *Z. officinale*

(a) Amplification of the selectable marker gene (hpt) (508 bp). (b) Amplification of the target gene (Ca-AFP) (300 bp). Lane M: DNA ladder (1 kb), 1: positive control (plasmid DNA), 2: non-transformed explant, 3-6: independent transformed ginger shoots.

To identify the presence of transgenes in *Z. officinale* regenerating shoot lines, molecular analysis was performed by PCR method. First, to confirm the absence of *A. tumefaciens* on hygromycin-resistant shoot clusters, leaf blades were cut and shaken in 1.5 mL tubes (containing 1 mL LB) with 200 rpm at 37°C for one hour. After that, leaves were discarded and 1 mL solution was centrifuged at 3500 rpm for 10 minutes to get pellets. Then, pellets were resuspended in 100 µL LB and spread on LB plates. These plates were incubated at 28°C for 48 hours. No colony appearing on these plates indicated that *A. tumefaciens* did not remain on resistant shoot clusters. Next, *in vitro* leaf blades from each shoot clusters were collected and extracted genomic DNA. PCR analysis was carried out by using two specific primer pairs of selectable maker gene (*hpt*) and target gene (*Ca-AFP*). The specific DNA fragments were amplified, with 508 bp and 300 bp for *hpt* and *Ca-AFP*, respectively. The obtained results after electrophoresis indicated that there are four transgenic lines harboring both *hpt* and *Ca-AFP* genes, with the respective bands in lanes 3 - 6 in Figure 4a, and 4b.

In previous study, transgenic ginger was transformed with reporter gene (*gus*), or selectable marker gene (*hpt*, *nptII*) in order to study the genetic transformation in *Z. officinale* (Musfir Mehaboob *et al*., 2019; Suma *et al*., 2008). It is obvious that transformation in ginger has great potential. In this study, we showed initial result of transformation of a plant defensin gene (*Ca-AFP*)-target gene-into ginger leaf sheaths to create transgenic plants.

CONCLUSION

This is the first time that shoots regenerated from *in vitro* leaf sheaths of Vietnamese ginger by using TDZ. This material was applied on genetic transformation of *Z. officinale* with *Ca-AFP*, a defensin gene with antifungal

function. The initially obtaining transformed ginger shoot lines give the potential to develop a new ginger variety that could improve the resistance to soft rot caused by *Pythium aphanidermatum* fungus in the future.

Acknowledgements: *This research was supported by the fund from Institute of Tropical Biology, Vietnam Academy of Science and Technology.*

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NGHIÊN CỨU TÁI SINH TỪ NGUỒN MẪU *IN VITRO* VÀ BƯỚC ĐẦU BIẾN NẠP GEN KHÁNG NẤM VÀO CÂY GỪNG (*Zingiber officinale* Rosc.) BẰNG VI KHUẨN *Agrobacterium*

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TÓM TẮT

Các nghiên cứu trước đây cho thấy, cây gừng (*Zingiber officinale* Rosc.), cũng như các loài khác trong chi *Zingiber,* rất khó tái sinh chồi từ nguồn mẫu *in vitro*. Trong nghiên cứu này, chúng tôi khảo sát môi trường tái sinh cho bẹ lá gừng (leaf sheath) *in vitro*. Kết quả cho thấy môi trường MS chứa 1 mg/L TDZ cho hiệu quả cao nhất với 68,33% mẫu tái sinh và trung bình 24,63 chồi hình thành trên mỗi mẫu tái sinh. Sau đó, kết quả này được áp dụng cho quy trình biến nạp gen ở cây gừng. Gen kháng nấm *Ca-AFP* đã được chuyển vào bẹ lá gừng *in vitro* đồng thời với gen *β- glucuronidase* (*gus*) và gen *hygromycin phosphotransferase* (*hpt*), bằng cách sử dụng vi khuẩn *Agrobacterium tumefaciens*. Kết quả ban đầu cho thấy, sau 10 tuần trong môi trường chọn lọc có chứa 4 mg/L hygromycin, sự biểu hiện của gen gus đã được ghi nhận ở một số chồi tái sinh. Sau 20 tuần chọn lọc trên môi trường có chứa 4 mg/L hygromycin, chúng tôi đã nhận được bốn dòng chồi tái sinh mang gen *Ca-AFP* và *hpt* thông qua phương pháp PCR. Kết quả của nghiên cứu này cho thấy tiềm năng tạo ra một giống gừng mới có thể cải thiện khả năng kháng bệnh thối mềm do nấm *Pythium aphanidermatum* trong tương lai.

Keywords: Agrobacterium tumefaciens, gen kháng nấm, tái sinh, *Zingiber officinale* Rosc.

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