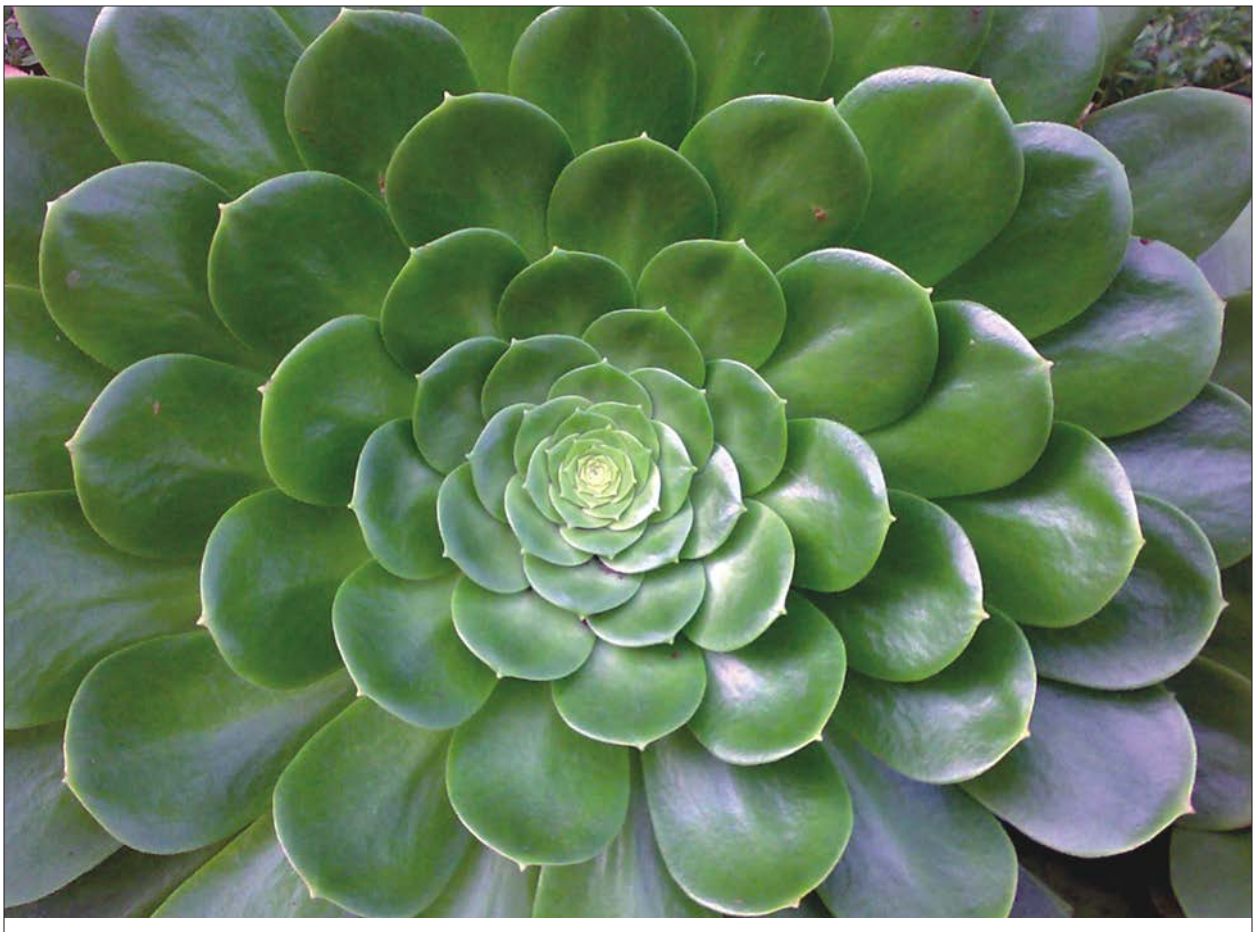

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Cysteine and Acetosyringone are the Two Important Parameters in *Agrobacterium*-mediated Transformation of Rose Hybrid (*Rosa hybrida* L.) cv. *Nikita*

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Abstract: An *Agrobacterium*-mediated transformation method for rose hybrid *Nikita* has been developed by using *Agrobacterium tumefaciens,* EHA 101 harboring the disarmed plasmid pIG 121-Hm with the eukaryotic *hpt11* gene, which confers resistance to the antibiotic hygromycin, as well as an intron containing β-glucuronidase (*gusA*) gene. In order to optimise the conditions for rose hybrid *Nikita* transformation, several factors known to influence *Agrobacterium*-mediated DNA transfer were examined using transient *gusA* gene expression. Nodal segment and leaf segment were examined. Two parameters, different cysteine concentrations (0, 50, 100, 150 and 200 mg L^{-1}) and acetosyringone concentrations (0, 50, 100, 150 and 200 μM) were evaluated to maximize transformation efficiency during co-cultivation in MS medium in both nodal and leaf segments. The inclusion of 100 mg L^{-1} cysteine and 50 μM acetosyringone in co-cultivation MS medium was proved to increased transient GUS expression in nodal segment and the inclusion of 100 mg L^{-1} cysteine and 0 μM acetosyringone in co-cultivation MS medium was proven increased transient GUS expression in leaf segment. Overall, the transient GUS expression level in nodal segment was found to higher than in leaf segment. The two optimum parameters concentrations will be applied subsequent transformation work in future with nodal segment as explants to obtained transgenic rose plantlets.

Key words: Rose, gene transfer, cysteine, acetosyringone

INTRODUCTION

Roses are one of the most important ornamental flowers. They offer a rainbow of colours, a variety of forms and fragrances. It is important for the improvement of commercial rose species to generate ornamental flowers with new and improved traits in floriculture industry. The genetic engineering of rose is enabled by *Agrobacterium*-mediated transformation method.

Agrobacterium-mediated genetic transformation is the technology used for the production of genetically modified transgenic plants and allows the transfer of foreign genes of interest into plants, together with the progress in gene identification, selection and isolation. It has enabled specific alterations of single traits such as plant morphology, new colours, altered flower form, flowers with better fragrance and longer vase life in already successful rose varieties (Casanova *et al*., 2005). Available methods for the transfer of genes could significantly shorten the breeding procedures, overcome some of the agronomic and environmental problems and has enabled a broadening of the available gene pool of a given species with genes from other species can be delivered to plant (Zuker *et al*., 1998).

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Reporter genes such as GUS gene and GFP gene, with selectable marker gene systems have been used in co-transformation experiments to confirm transgenic (Sreeramanan *et al*., 2006). In addition, they have been used to improve transformation systems and the efficiency of recovering transgenic plants by allowing the visual detection of transformed tissues.

There are few published reports on *Agrobacterium*-mediated transformation and biolistic transformation of roses. Li *et al*. (2002) reported on optimization of the *uidA* gene transfer into somatic embryos of rose via *Agrobacterium tumefaciens*. Li *et al*. (2003) also successful reported on an introduction of antimicrobial protein gene, *Ace-AMP1* into *Rosa hybrida* cv. Carefree Beauty via *Agrobacterium*-mediated transformation. Kim *et al*. (2004) reported on *Agrobacterium*-mediated transformation of embryogenic calluses of *Rosa hybrida* cultivar Tineke using the Green Fluorescent Protein (GFP) gene.

Cysteine is an inhibitor of polyphenol oxidases (PPOs), peroxidases (PODs) and enzymetic browning, either directly or in directly through the action of its thiol group (Olhoft *et al*., 2001). Generally, *Agrobacterium* infection caused tissue browning and necrotic on the explants. This is likely common defense responses of plant to wounding or pathogen infection. The defense mechanisms that firstly activated upon wounding or pathogen infection is oxidative burst (Olhoft *et al*., 2001). The oxidative burst involves the production of reactive oxygen species which are thought to activate programmed cell death and to generate a barrier of dead cells around the site of infection. The defense mechanisms are following by oxidation of phenolic via action of PPOs and POD, to the position of tannins and release of phytoalexins for further protection against pathogen infection. Tannins are the compound that results in the browning of wound tissues. As a result, by reducing wound- and pathogen-defense responses in plants, cysteine have the potential to increase the capacity of *Agrobacterium* to infect plant tissues and stably transfer its T-DNA as well as to increase the frequency of infected cells that remain viable and become transformed (Olhoft *et al*., 2001).

Plant specific phenolic compounds that induce the expression of *Agrobacterium vir* genes are important for the gene transfer. Acetosyringone is a phenolic compound produced during plant-cell wounding and induces the transcription of *Agrobacterium tumefaciens* virulence genes and hence to enhance transformation frequency by addition of this compound into bacteria culture medium or co-cultivation medium (Lee *et al*., 2006). Acetosyringone as a phenolic signal is transduced through a receptor *virA* protein in the inner membrane of the bacterial cell. The expression of these genes triggers the transfer of T-DNA from the Tumour inducing (Ti) plasmid to plant cells and its integration into plant genome (Sreeramanan *et al*., 2006). The objectives of this research project are to determine the two optimal parameters for transient *gusA* gene expression using different cysteine and acetosyringone concentrations.

MATERIALS AND METHODS

Plant Materials and Tissue Culture Procedure

Rose hybrid, *Nikita* plants were selected from the AIMST green house was used as plant material (Fig. 1). The nodal and leaf segments were washed with tap water and were stir in the teepol solution for half an hour. Then, about 1 or 2 drop of 1% Tween 20 solution was added and the explants were rinsed under running tap-water for 1 h. The shoot and leaf were surface-sterilized with 20% of 5.25% sodium hypochlorite for 10 min and then were soaked in 70% ethanol for 5 min with shaking. Then, they were rinsed 5 times with sterilized distilled water for 5 min. The shoot was cut into single-node segment approximately 2.0 cm in length and leaf was cut into approximately 1.3×1 cm^2. They were cultured on MS (Murashige-Skoog, 1962) medium supplemented with 2 mg L^{-1} of BAP for two days prior of transformation.

Fig. 1: Rose hybrid *Nikita*, The bar in the bottom of each image represents 2.5 mm

Agrobacterium Tumefaciens Strain and Plasmid

Agrobacterium tumefaciens strain, super-virulent strain EHA 101 (pIG 121-Hm) was used in this research project. *Agrobacterium tumefacien* strain EHA 101 contained the disarmed plasmid pIG 121-Hm with the eukaryotic *hpt 11* gene, which confers resistance to hygromycin, as well as an intron containing β-glucuronidase (*gusA*) gene (Fig. 2). *Agrobacterium* cultures were grown from single colonies in 25 mL of Luria-Bertani (LB) medium by incubation at 28°C and 120 rpm on shaker for 20 h to reach an optimal density of 0.7 units at 600 nm (OD_{600}). Appropriate antibiotic was included in the medium at the following concentration: kanamycin (50 mg L^{-1}). The schematic diagram of the plasmid pIG 121-Hm of *Agrobacterium* strain EHA 101 is given in Fig. 2.

Optimization of Parameters for Transient *gusA* Gene Expression

To assess factors affecting the transformation frequency, two different treatments were carried out. Each treatment included three replicates, each containing 10 single nodal segments and 10 single leaf segments. The two parameters included different cysteine concentrations (0, 50, 100, 150 and 200 mg L^{-1}) and acetosyringone concentration (0, 50, 100, 150 and 200 μM), which was used during co-cultivation with *Agrobacterium* in solid co-cultivation medium. All the parameters were evaluated and optimized based on percentage of GUS positive in nodal and leaf segments. The percentage of GUS positive calculated by the number of GUS positive explants per total number of explants used. The explants that have shown more than 20% of blue colouration are considered as GUS positive.

Agrobacterium-Mediated Transformation

Nodal and leaf segments were wounded on surface with a scalpel and immersed into a conical flask containing 25 mL of an overnight-grown *Agrobacterium* suspension for 30 min with shaking. Then, the explants were blotted dry with sterile filter paper and transferred to solid co-cultivation medium for 3 days at 25°C in the dark. Co-cultivation medium is a MS medium supplemented with 2 mg L^{-1} of BAP as well as containing 50 μM acetosyringone and 100 mg L^{-1} cysteine.

GUS Histochemical Assay

Nodal segments and leaf were assayed for GUS expression according to Jefferson *et al.* (1987) with 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc) staining solutions as substrate and incubated at 37°C over night before examination of GUS activity.On the following day, these explants were then washed several times with absolute ethanol to remove chlorophyll pigments.

Statistical Analysis

Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. All statistical analyses were performed at the level 5% using SPSS 10.0 (SPSS Inc. USA).

RESULTS AND DISCUSSION

Optimization of Parameters for Transient *gusA* Gene Expression

Efficiency of *Agrobacterium*-mediated transformation and delivery of T-DNA into plant cells is influenced by several physico-chemical and physiological conditions (Sanyal *et al*., 2005). The transfer of T-DNA into host plant genomes is known to be influenced by several factors including the type of explants, the *Agrobacterium tumefaciens* strain and vector combination and the conditions of infection and co-cultivation. There are more reports published on the effect of acetosyringone concentration on the transfer of T-DNA into plant host genome (Sreeramanan *et al*., 2006; Lee *et al*., 2006; Lievre *et al*., 2005; Shrawat *et al*., 2006) and few reports published on the effect of cysteine concentration on the transfer of T-DNA into plant host genome (Olhoft *et al*., 2001; Opabode, 2006). In this research project, transient expression of *gusA* (GUS) marker gene was used to assess and optimize *Agrobacterium*-mediated transformation of rose hybrid, *Nikita*. The effect of different cysteine concentrations (0, 50, 100, 150 and 200 mg L^{-1}) and different acetosyringone concentrations (0, 50, 100, 150 and 200 μM) on transient *gusA* gene expression in nodal segment and leaf segment of *Nikita* were investigated. In addition, the transient *gusA* expression level between nodal segment and leaf segment were compared. Other factors such as pre-cultured period for 2 days, wounding on the explants, *Agrobacterium* strain EHA 101 and co-cultivation period for 3 days were remain constant through out the experiment.

Pre-culture period is defined as the time between when the explants are first isolated and cultured and when the explants are inoculated with *Agrobacterium* (Shrawat *et al*., 2006). Pre-culturing explants prior to inoculation and co-cultivation with *Agrobacterium* has been shown to improve genetic transformation in banana (Sreeramanan *et al*., 2006) and barley (Shrawat *et al*., 2006). The co-cultivation period also is a crucial factor influencing *Agrobacterium*-mediated gene transfer. Co-cultivation for 3 days was the most effective for increasing the frequency of transient GUS expression in banana (Sreeramanan *et al*., 2006) and orchard glass (Lee *et al*., 2006). The purpose of wounding on the explants in this experiment is to provide *Agrobacterium* accessibility to transformation competent cells by allows the entry of the bacteria into plant cells and provides phenolic compounds.

Effect of Cysteine Treatment on Transient GUS Expression

The effect of cysteine on transient *gusA* gene expression in nodal segment and leaf segment were evaluated. Four concentrations of cysteine (50-200 mg L^{-1}) in solid co-cultivation medium were investigated. The percentage of GUS positive results increased with increasing cysteine concentrations and the highest expression was determined at 100 mg L^{-1} (Fig. 3). When more than 100 mg L^{-1} cysteine was used, the percentage of GUS positive in nodal were decreased and less GUS expression was observed in the nodal segment (Fig. 4). On the other hand, the percentage of GUS positive in leaf segment did not increased with the increased in cysteine concentration. The percentage of GUS positive in leaf segment is not significant between 0, 100 and 150 mg L^{-1} and the highest transient GUS expression was determined at 100 mg L^{-1} (Fig. 5 and 6). However, the GUS expression in the absence of cysteine was still detected in the both nodal and leaf segments. These results suggested that the addition of cysteine in co-cultivation medium not necessary for the transformation but it would enhance the transformation frequency for both nodal segment and leaf segment.

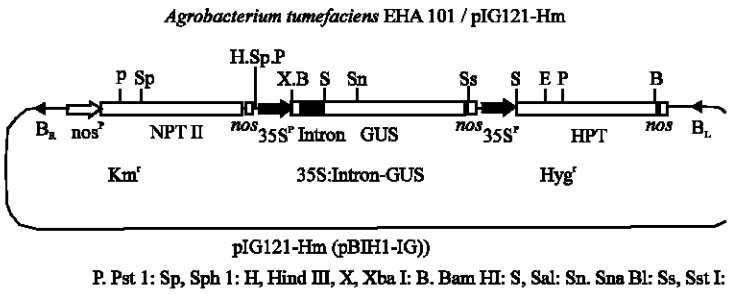

Fig. 2: The plasmid pIG 121-Hm. Abbreviations: B_R, border right; B_L, border left; $35S^P$, 35S promoter; NPT11, neomycin phosphotransferase; HPT, hygromycin phosphotransferase; GUS, β-glucuronidase; nos, E, *Eco*R1; H, *Hind*III; B, *BamH*I; X, *Xba*1; P, *Pst*1; Sp, *Sph*1; S, *Sal*1; Sn, *Sna* B1; Ss, *Sst*1

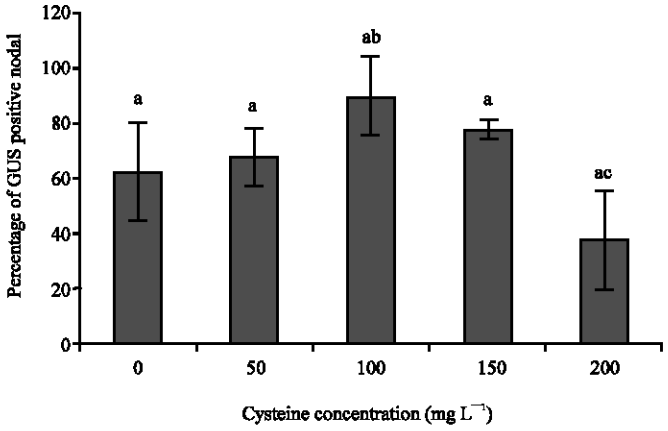

Fig. 3: Influence of cysteine concentrations (mg L^{-1}) on transient GUS expression in nodal segment. Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letter(s) indicate values are significantly different ($p<0.05$)

The results obtained from this experiment shown that the transformation frequency of *Nikita* rose was optimal at 100 mg L^{-1} cysteine and shown reduced as the level of cysteine beyond 100 mg L^{-1}. However, there is a published report demonstrated the enhancement of *Agrobacterium*-mediated transformation at even much higher level of cysteine concentration. Olhoft and Somers (2001) have successful to increased *Agrobacterium* infection from 37 to 91% of explants in the cotyledonary-node region by amending the solid co-cultivation medium with L-cysteine, which resulted in a fivefold increase in stable T-DNA transfer in newly developed shoot primordial in producing transgenic soybeans [*Glycine max* (L.) Merrill]. The addition of cysteine to the solid co-cultivation medium increased the average frequency of explants containing a GUS positive focus at the cot-node from 37% in explants cultured on medium containing no cysteine to 91% in those cultured on medium containing between 600 and 1,000 mg L^{-1} of cysteine (Olhoft and Somers, 2001). Besides, Zeng *et al*. (2003) have reported on the optimized both *Agrobacterium* infection and glufosinate selection in the presence of L-cysteine for Expressed Sequence Tag (EST) and functional genomics analyses (Williams 82) in modern genetic analysis and manipulation of soybean (*Glycine max*).

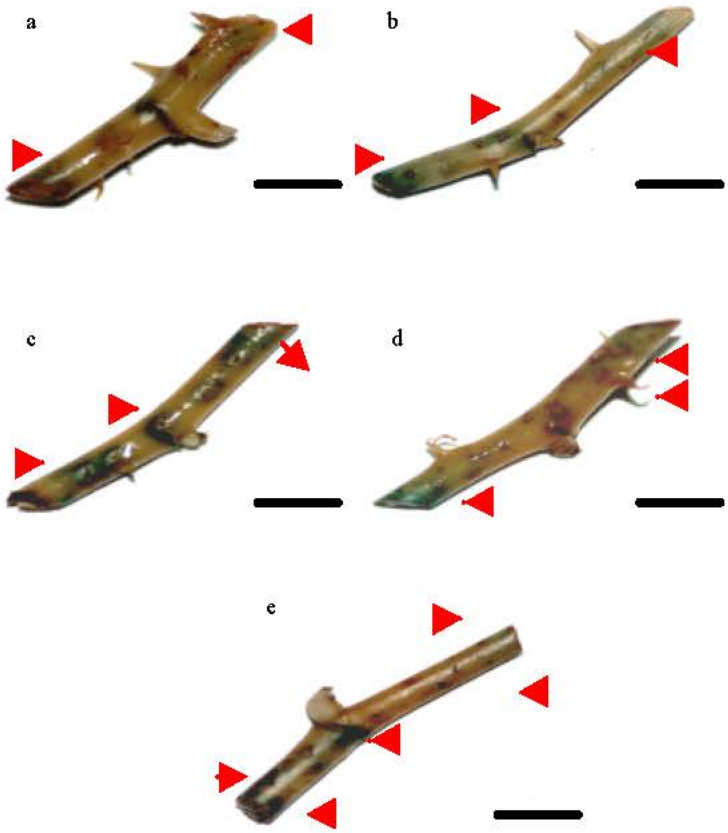

Fig. 4: Comparison of transient *gusA* gene expression in single nodal segments with different concentrations of cysteine (mg L^{-1}). (A) 0 mg L^{-1}, (B) 50 mg L^{-1}, (C) 100 mg L^{-1}, (D) 150 mg L^{-1} and (E) 200 mg L^{-1}. The bar in the bottom of each image represents 2.5 mm. The arrows in the image note the blue colouration

In addition, Enríquez-Obregón *et al.* (1999) have evaluated the effect of cysteine and silver nitrate with known antioxidant activity on the viability of stem sections taken from *in vitro* rice plantlets and on their interaction with *Agrobacterium tumefaciens* (AT 2260) containing a shuttle vector bearing the *gus* and *bar* gene. Olhoft *et al.* (2001) reported that *Agrobacterium*-mediated transformation of soybean cells and the production of fertile transgenic soybean *Glycine max* (L.) Merrill plants using the cotyledonary-node method were improved by amending the solid co-cultivation medium with L-cysteine. The frequency of transformed cells was increased only when L-cysteine was present during co-cultivation of *Agrobacterium* and cot-node explants (Olhoft *et al.*, 2001). In addition, Opabode *et al.* (2006) reported that inclusion of thiol compounds, L-cysteine, dithiothreitol and sodium thiosulphate in co-cultivation medium increased transformation efficiency as high as 16.4% in soyabean plant.

Effect of Acetosyringone Treatment on Transient GUS Expression

The effect of acetosyringone on transient *gusA* gene expression in nodal segment and leaf segment were evaluated. Four concentrations of acetosyringone (50-200 μM) in solid co-cultivation medium were investigated. The percentage of GUS positive nodal was highest at 50 μM of acetosyringone (Fig. 6). The frequency of nodal segment showed GUS expression was higher at 50 μM compared to the frequency of nodal segment showed GUS expression was higher at 50 μM. This result suggests

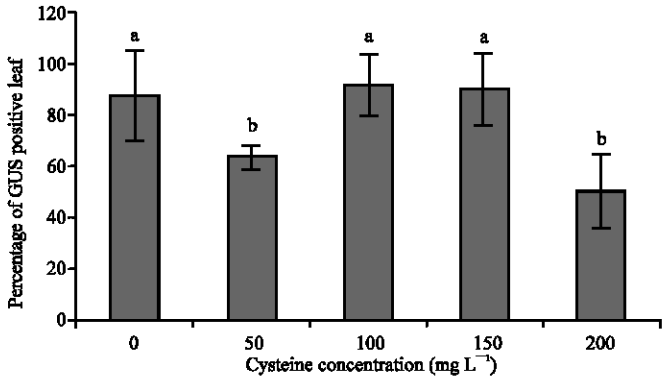

Fig. 5: Influence of cysteine concentrations (mg L^{-1}) on transient GUS expression in leaf segment. Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letter(s) indicate values are significantly different ($p<0.05$)

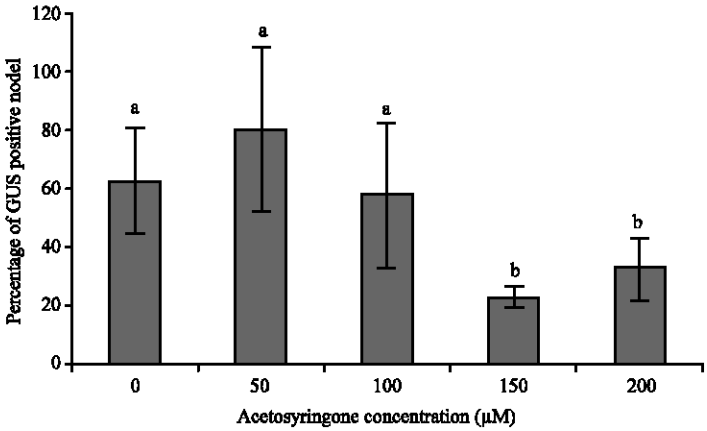

Fig. 6: Influence of acetosyringone concentrations (µM) on transient GUS expression in nodal segment. Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)

that inclusion of acetosyringone in co-cultivation medium may increase the transformation frequency. However, when more than 50 µM acetosyringone was used, the percentage of GUS positive nodal decreased with the increasing acetosyringone concentrations. The lowest percentage of positive was detected at 150 µM. This may because of high concentration of acetosyringone is toxic due to the harmful effect of supra-optimal concentration of acetosyringone and absolute ethanol solvent used in this experiment.

On the other hand, the percentage of GUS positive leaf was highest in the absence of acetosyringone (0 µM) and the percentage of GUS positive leaf decreased as the concentration of acetosyringone increased (Fig. 7). This may because of the leaf segment is more fragile compared to nodal segment and more susceptible to the toxic effect of acetosyringone at high concentration. In addition, when more than 100 µM acetosyringone was used, necrotic zones on the leaf surface was

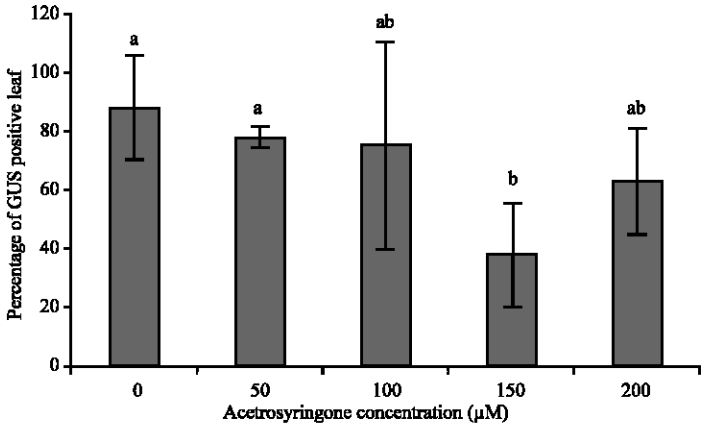

Fig. 7: Influence of acetosyringone concentrations (µM) on transient GUS expression in leaf segment. Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letter(s) indicate values are significantly different ($p<0.05$)

observed. Transient GUS expression was observed in the absence of acetosyringone for both nodal segment and leaf segment indicating that acetosyringone is not necessarily essential for *Agrobacterium*-mediated transformation of rose.

The results from this experiment shown the frequency of GUS expression in nodal segment and leaf segment are optimal at level below 50 µM acetosyringone. However, most published reports demonstrated that the frequency of GUS expression and transformation were enhanced at higher level of acetosyringone. Sreeramanan *et al*. (2006) reported that the single bud of banana cultivar, Rastali (AAB) shown the highest level of transient GUS expression at 100 µM acetosyringone and the transient GUS expression was decreased as the concentration of acetosyringone increased. Lee *et al*. (2006) reported that seed-derived calli of orchardgrass plants respond positively to the presence of 200 µM acetosyringone in both the inoculation and co-cultivation media. As well as, Wu *et al*. (2003) demonstrated the addition of 200 µM acetosyringone in the co-cultivation medium was statistically significant increased on the number of GUS foci per explant at the 95% level in Cadenza and Florida varieties of wheat. Besides, Sanyal *et al*. (2005) demonstrated that level of acetosyringone beyond 200 µM significantly affected the regeneration frequency and size of GUS foci whereas complete withdrawal of acetosyringone reflected significant decrease in the frequency of explants expressing GUS and mean number of GUS spots per explant in all the genotypes of chickpea. In addition, Lievre *et al*. (2005) demonstrate that the transformation of *R. graveolens* L. was improved with 14.2% of the initial explants produced kanamycin resistant plants when 250 µM of acetosyringone was added to the co-culture medium.

However, there is a published report demonstrated on the acetosyringone did not help in increasing transformation efficiency. Mondal *et al*. (2001) reported on the use of the phenolic inducer, acetosyringone, did not enhance the efficiency of transformation in production of transgenic tea *Camellia sinensis* (L.) via *Agrobacterium*-mediated genetic transformation of somatic embryos. The inability of acetosyringone to improve upon the transformation efficiency could be due to the inherent prevalence of high amounts of phenolics in woody plant tissues such as tea (Mondal *et al*., 2001).

Types of Explants

Two types of explants which are nodal segment and leaf segment were used for transformation in this experiment. GUS expression level in nodal segment is higher than in leaf segment even though

the percentage of GUS positive in leaf might be higher than the percentage of GUS positive in nodal segment. In addition, the blue colour density on nodal segment is higher than leaf segment. Furthermore, there is more necrotic area around the wounding area in leaf segment compared to nodal segment especially the effect of acetosyringone in leaf segment shown higher level of necrotic areas. This result suggested that the leaf segment is more fragile compared to nodal segment. As a result, it is suggested that nodal segment is more stable as an explant for *Agrobacterium*-mediated transformation of rose hybrid *Nikita*. In addition, shoot formation in nodal segment is faster compared to the callus formation in leaf segment. Each auxiliary node or bud was capable of reproducing the main shoot having the natural branching system with unlimited proliferation potential (Rout *et al*., 1999). Hence, the nodal segment was chosen for the subsequent transformation experiment in this research project.

Tissue culture or micropropagation of roses has been reported by various researchers using cultures of auxiliary bud of nodal segment. Jabbarzadeh *et al*. (2005) reported that using single node segment as explants to evaluate the factors affecting tissue culture of Damask rose (*Rosa damascena* Mill.). In addition, Saxena *et al*. (2000) reported on the used of nodal segment as explants to establish an efficient *in vitro* procedure for micropropagation and regeneration of somaclones of rose scented *Pelargonium*. However, most researchers reported on the use of somatic embryo and callus as explants for *Agrobacterium*-mediated transformation. Li *et al*. (2003) and Kim *et al*. (2004) use primary embryogenic calli as explants for *Agrobacterium*-mediated transformation of *Rosa hybrida* cv. Carefree Beauty and *Rosa hybrida* cultivar Tineke, respectively. There are also researchers that used leaf segments as explants for transformation. Aida *et al*. (1996) reported on a method for *Agrobacterium*-mediated transformation of *Kalanchor hlossjtldiana* Poelln using leaf segment. Kishimoto *et al*. (2002) reported on a method for *Agrobacterium*-mediated transformation of *Elatior begonia* using young leaf disc.

CONCLUSIONS

An efficient system for the successful *Agrobacterium*-mediated transformation of rose hybrid, *Nikita* was developed by optimizing two main parameters using different concentrations of cysteine (0, 50, 100, 150 and 200 mg L^{-1}) and acetosyringone (0, 50, 100, 150 and 200 μM) based on transient GUS expression. This research provides the possibility for using *Agrobacterium*-mediated transformation method to deliver genes of potential horticultural value such as genes that regulate flower colour, shelf life and genes that confer insects resistant and disease resistant. The transient GUS expression assay was found to be an easy and reliable way of establishing optimal conditions for transformation. The optimized conditions investigated in this experiment include co-cultivation period for 3 days in the presence of 100 mg L^{-1} cysteine and 50 μM acetosyringone for nodal segment and co-cultivation period for 3 days in the presence of 100 mg L^{-1} cysteine and 0 μM acetosyringone for leaf segment.

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