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NARINGENIN ENHANCED EFFICIENCY OF GUS ACTIVITY IN *Passiflora mollissima* (H.B.K.) Bailey

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ABSTRACT

The flavonoid naringenin has been investigated as a possible *vir* gene inducer in *Agrobacterium*-mediated transformation in *Passiflora mollissima*, *P. giberti* and *Nicotiana tabacum* cv. Xanthi. The transformation efficiency percentage of explants showing blue GUS expression and the extent of staining following inoculation with *Agrobacterium tumefaciens* strains EHA 105 and 1065, carrying *gus* and *nptII* genes was enhanced with the supplementation of the co-cultivation medium with naringenin. Supplementation of medium with 100µM (strain EHA 105) and 300 µM (strain 1065) naringenin was most effective at enhancing mean (±s.e.m., n=3) GUS activity in leaf explants (20.3 ± 2.4%, strain EHA; 105; 6.0 ± 0.57%, strain 1065) and nodal segments (16.7 ± 2.4% strain EHA 105; 8.3 ± 0.57% strain 1065) of *P. mollissima*. In *P. giberti* and *N. tabacum* maximum GUS activity was obtained in leaf and root explants with 100µM naringenin for both strains analysed. Additionally, when naringenin was added to Luria Bertani (LB) medium, both bacterial growth via optical density and colony forming units were higher when compared to control. This is the first report of the use of naringenin to enhance gene transfer from *Agrobacterium* to plants. These findings suggest that naringenin can be used as an alternative to acetosyringone for *vir* gene induction in *Agrobacterium*. This approach may be especially useful in plants that are generally recalcitrant to *Agrobacterium*-mediated transformation.

Key words: naringenin, *Agrobacterium,* acetosyringone, *Passifloras*

RESUMEN

Se investigó al flavonoide naringenina como posible inductor de los genes *vir* en la transformación genética mediada por *Agrobacterium tumefaciens* en *Passiflora mollissima, P. giberti* y *Nicotiana tabacum* cv. Xanthi. El porcentaje de eficiencia en la transformación de los explantes se evidenció en la expresión del gen *gus*, posterior a la inoculación de los explantes con las cepas de *A. tumefaciens* EHA 105 y 1065. Estas cepas contenían los genes *gus* y *npt* II. Se observó un incremento en la expresión del gen *gus* al suplementar el medio de co-cultivo de los explantes con el flavonoide naringenina. En los explantes de *P. mollissima*, al suplementar el medio de cultivo con 100 µM (cepa EHA 105) y 300 µM (cepa 1065), naringenina fue más efectivo con un incremento medio (± s.e.m., n=3) de la actividad de GUS en explantes de hoja (20.3 ± 2.4%, cepa EHA; 105; 6.0 ± 0.57%, cepa 1065) y en segmentos nodales (16.7 ± 2.4% cepa EHA 105; 8.3 ± 0.57% cepa 1065). En *P. giberti* y *N. tabacum* la actividad máxima de GUS se obtuvo en explantes de hoja y raíz con 100 µM de

naringenina en ambas cepas analizadas. Cuando naringenina fue adicionada al medio de cultivo bacteriano Luria Bertani (LB) tanto la densidad óptica como el número de unidades formadoras de colonias fue mayor en relación al control. Esta es la primera vez en la cual se indica el empleo de naringenina como flavonoide que incrementa la transferencia de genes de *Agrobacterium* a las plantas. Los resultados sugieren que naringenin puede ser usado como alternativa frente a acetosiringona en la inducción de genes *vir* en *Agrobacterium* en experimentos de transformación.

Palabras clave*:* naringenina, *Agrobacterium*, acetosiringona y *Pasifloras*

INTRODUCTION

The achievement of reliable transformation protocols in plant species depends on the establishment of an efficient regeneration procedure, high transformation frequency and optimum selection for regenerating transformed cells. This can be obtained by the interplay of a large number of factors including plant genotype, bacterial strain, the external conditions during pre-culture of Agrobacteria and plant material during co-cultivation (Gheysen *et al.*, 1998). One of the most common approaches to increase *Agrobacterium* transformation frequency is the use of certain metabolites (*e.g.* acetosyringone, α-hydroxyacetosyringone) during pre-culture of the Agrobacteria and/or co-cultivation which stimulate the virulence (*vir*) genes on the tumor-inducing (Ti) plasmid. As a consequence, several studies in woody fruit species have indicated the beneficial effect of adding acetosyringone during co-cultivation and/or *Agrobacterium* pre-culture (James *et al.*, 1993; Kaneyoshi *et al.*, 1994; Cervera *et al.*, 1998a). Although several other *vir* gene inducer compounds have also been identified and are commercially available, including quercetin (Zerback *et al.*, 1989), catechol (Bolton *et al.*, 1986), sinapinic acid (Melchers *et al.*, 1989), ferulic acid (Berthelot *et al.*, 1998) and vanillin (Stachel *et al.*, 1985), a limited number of *Agrobacterium*-mediated transformation protocols have used them to improve transformation. Therefore, the aim of this study was to examine whether flavonoids and acetosyringone (comparative metabolite) added to the co-cultivation medium were able to increase the transformation frequency in *P. mollissima*, *P. giberti* (a comparative species) and *Nicotiana tabacum* var. Xanthi (a model plant). The frequency of transformation was based on assessing the effect of these compounds on transient GUS activity using two *A. tumefaciens* strains (1065 and EHA 105). This hypothesis was based on findings obtained by Zerback *et al.* (1989), which established that flavonoid compounds are inducers of the *vir* region of the *A. tumefaciens* Ti plasmid and Messens *et al.* (1991), who found that the flavonoid naringenin was the most effective inducer of the expression of *nod* genes in *Azorhizobium*. Additionally, the growth of both strains following addition of naringenin and acetosyringone to Luria Bertani medium was evaluated in terms of optical density (OD) and colony-forming units (CFUs).

MATERIALS AND METHODS

Plant material

Leaf and root explants and nodal segments from 28-40 d-old *in vitro* cultured seedlings of *P. mollissima* and *P. giberti* were used in the experiments together with leaf explants of *Nicotiana tabacum* cv. Xanthi (21-28 d-old axenic

nodal segments derived from *in vitro* plants). To establish seedling/plants of *P. mollissima* and *P. giberti* seeds were soaked in sterile reverse-osmosis water for 2d then surface sterilised (2 min) by immersion in 70% (v/v) ethanol, followed by a second immersion in 30% (v/v) "Domestos" bleach solution (Lever Industrial Runcorn, UK) (60 min). Seeds were rinsed 7-10 times with sterile reverse-osmosis water and then placed into 9 cm Petri dishes (Bibby-Sterilin, Stowe, UK) (25 per dish) containing either Nitsch and Nitsch, (1969) (NN) based-*medium (P. mollissima*) or Murashige and Skoog (1962) (MS) based-medium (*P. giberti*) (25 ml per dish), all made semi-solid with 0.7% (w/v) agar (Sigma Chemical Co. Ltd., Poole, UK) and with no growth regulators, pH 5.7. The medium was sterilised by autoclaving. Dishes were sealed with Nescofilm (Bando Chemical Co., Kobe, Japan) and cultured under dark conditions (22 ± 2°C) until germination (45-60 d). The plantlets were then transferred to 175 ml capacity glass jars (Beatson Clark & Co. Ltd., Rotherham, UK.) (4 per jar) containing either 50 ml MS based-medium or NN based-medium as described above. Cultures were then kept under a 16 h photoperiod (47µE m^{-2} s^{-1}, Cool White fluorescent tubes, Thorn EMI Ltd.) at 22 ± 2°C for a period of 28 - 40 d.

P. giberti and *N. tabacum* were employed in this study for comparative purposes as both species (control species) have proven to be susceptible to *A. tumefaciens*, whilst this is the first study of *Agrobacterium* transformation in *P. mollissima*. In the current investigation two experiments involving the three species were performed. For simplicity, these experiments were designated as Experiment I (*Agrobacterium* strain 1065) and Experiment II (*Agrobacterium* strain EHA 105).

Bacterial strains and plasmid

A. tumefaciens strains 1065 and EHA 105 were used to assess the effect of polycyclic compounds such as flavonoids (naringin, naringenin, hesperetin, daidzein, quercetin and myricetin; Sigma) and acetophenones (acetosyringone; Sigma) on transient *gus* gene expression in *P. mollissima*, *P. giberti* and *N. tabacum*. Strain 1065 was LBA4404 carrying pVDH65 [with the *gus* and *npt* II genes] and the super-virulent pTOK47 which possesses additional *vir* genes (*vir* B, C and G genes) (Curtis *et al.*, 1994). *A. tumefaciens*, EHA 105 contained the binary plasmid p35SGUSINT (Vancanneyt *et al.*, 1990) and was a disarmed derivative of *A. tumefaciens* A281 (Hood *et al.*, 1993). Two gene cassettes in the T-DNA, 35S-*uid*A (*gus*INT)-35S and NOS-*npt*II-NOS, served as a reporter and selectable marker genes respectively (Cervera *et al.*, 1998a). The *gus* gene in both strains, has the PIV2 intron of the gene *ST-L*1 from potato within its coding sequence preventing its expression in *Agrobacterium* (Vancanneyt *et al.*, 1990). Strain 1065 was provided by The Plant Science Division, University of Nottingham and strain EHA 105 was provided by Dr. Leandro Peña and Dra. Magdalena Cervera, Instituto Valenciano de Investigaciones Agrarias (IVIA) Valencia, Spain.

Storage, growth and maintenance of bacteria

Both *A. tumefaciens* strains 1065 and EHA 105 were maintained in 20% (v/v) glycerol at –70°C for long-term storage. Glycerol stocks were prepared according to the method proposed by Beckker *et al.* (1996). This method consisted of adding 750 ml aliquots of overnight bacterial culture to 750 µl LB medium (Sambrook *et al.*, 1989): glycerol mixture (6:4, v/v). Stocks were transferred to a 2.0 ml screw-

capped storage tube and used when required.

Bacterial cultures were initiated from –70°C glycerol stocks by streaking onto a 9 cm Petri dish containing LB semi-solid medium, supplemented with 100 mg ml^{-1} rinfampicin, 50 mg ml^{-1} kanamycin sulphate and 5 mg ml^{-1} tetracycline for strain 1065 and 25 mg ml^{-1} kanamycin and 25 mg ml^{-1} nalidixic acid for strain EHA 105. Cultures were incubated for 3-5 d (strain 1065) and 1-2 d (strain EHA 105) in the dark at 28 ± 1°C. Liquid cultures were initiated by transferring 100 µl loopfuls of bacteria from the semi-solid medium into 100 ml capacity Erlenmeyer flasks each containing 20 ml of liquid LB medium supplemented with antibiotics (40 mg ml^{-1} rinfampicin, 50 mg ml^{-1} kanamycin sulphate and 2 mg ml^{-1} tetracycline) for strain 1065 and the same antibiotic concentrations as used in the semi-solid medium for strain EHA 105. Subsequently, all cultures were incubated on a horizontal shaker (150 rpm) at 28 ± 1°C in the dark for 16-20 h. The growth of the bacterial cultures for both strains was monitored by their optical density (OD) using a Pye Unicam SP6-500 spectrophotometer at 600_{nm} against a blank of liquid LB medium containing the specified antibiotics.

Bacterial inoculation of the explants

Leaf and root explants and nodal segments of *P. mollissima* and *P. giberti*, and leaves of *N. tabacum*, were used as a source of tissues for inoculation with strains 1065 and EHA 105. The leaves were cut into segments (1 cm^2) and the mid-vein and outer borders were removed. The root explants were cut into 1.5 cm lengths whilst, the nodal segments were cut obliquely into 1 cm lengths. The wounded tissues were incubated (35 min) in 3 different dilutions [undiluted; 1:2; 1:5 (v/v)] in a mixture of overnight bacterial culture of *Agrobacterium* liquid medium (strains 1065 and EHA 105). Dilution of the overnight bacterial culture was with SRNN liquid medium (NN medium supplemented with BAP 3.0 mg l^{-1} + KIN 2.0 mg l^{-1}) for *P. mollissima*, SRMS (MS medium supplemented with BAP 1.0 mg l^{-1}+ KIN 0.5 mg l^{-1}) for *P. giberti* (Otoni, 1995) and SRMS1 [MS medium supplemented with indoleacetic acid (IAA) 0.1 mg l^{-1} + KIN 10 mg l^{-1}] (Horsch *et al.*, 1985) for *N. tabacum*. All inoculated explants were blotted free of excess bacteria with sterile paper and placed in 9 cm Petri dishes each containing 20 ml of semi-solid SRNN, SRMS or SRMS1 media (co-cultivation medium) as described above. The dishes were incubated in the dark for 7 d. Each experiment was repeated 3 times with each individual treatment replicated 3 times within an experiment. Explants were assessed for their GUS activity after 3, 5, 7 d. All bacterial cultures were used to inoculate the explants when the OD_{600} was 1.2-1.5. Explants not treated with *Agrobacterium* were employed as controls.

Histochemical detection of GUS activity associated with explants

The detection of gene activity was performed using the GUS assay (Jefferson *et al.*, 1987) in *P. mollissima*, *P. giberti* and *N. tabacum* explants after 3, 5 and 7 d of co-cultivation. A total of 15 explants (5 per period) from each treatment were randomly chosen. GUS histochemical assay buffer solution was prepared prior to use by dissolving 10mg X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, Gold Biotechnology Inc., St. Louis, USA) in 1 ml of EGME (ethylene glycolmonoethyl ether) and then adding 20 ml of 0.1M sodium phosphate (Na_2HPO_4, NaH_2PO_4) and 0.02% (w/v) sodium azide, pH 7.4

(Sambrook *et al.*, 1989). The explants were incubated in the GUS assay buffer overnight at 37°C in 96 well microtitre plates. After incubation, the GUS assay buffer was removed and replaced with ethanol 70% (v/v) for 4 d in order to remove the chlorophyll from the explants and to improve visualisation of the blue colour which resulted from the GUS activity in the explants. The intensity and localization of blue staining was quantified using a stereomicroscope. In order to minimise the possible inhibition of GUS activity from inhibitory compounds present in the explants, polyvinylpolypyrrolidone [(PVPP), 25 mg for each 100 mg of tissue] was added to the extract assay solution.

Procedure for dissolving flavonoids and acetosyringone and their addition to the co-cultivation medium

The different flavonoids used (naringin, naringenin, hesperetin, daidzein, quercetin and myricetin) were dissolved (10 mg) in reverse osmosis water (10 ml) at pH 9.8 to minimise their conversion to the chalcone form. The pH of the water was adjusted with 0.1M sodium hydroxide, filter sterilised and incorporated into autoclaved co-cultivation medium for each species, prior to cooling, giving a final pH of 6.8. For acetosyringone, 98.1 mg was dissolved in 1ml dimethyl sulfoxide (DMSO) and stored at –20ºC before use. The addition of acetosyringone to the co-cultivation medium was employed as described above for the flavonoids.

Determination of the effect of flavonoids and acetosyringone in the co-cultivation medium

Leaf and root explants and nodal segments of *P. mollissima*, *P. giberti* and *N. tabacum* were used in the experiments with flavonoid compounds, but leaf explants only for acetosyringone. The same *Agrobacterium* strains (1065 and EHA 105), bacterial dilution (undiluted, 1:2, 1:5 v/v) and co-cultivation medium (SRNN, SRMS, SRMS1) were employed as described earlier. However, in this case, explants cultivated with *Agrobacterium*, but without supplementation with flavonoid compounds or acetosyringone, were used as control. The co-cultivation medium was supplemented with 100, 200 and 300 µM of the different flavonoid compounds and 100 µM of acetosyringone. Each experiment was repeated 3 times with each individual treatment replicated 3 times within an experiment. The GUS assay was performed after 3, 5, 7 d.

Determination of the effects of naringenin and acetosyringone on bacterial growth

In order to determine the effects of naringenin and acetosyringone on bacterial growth, both *Agrobacterium* strains (1065 and EHA 105) were cultured in LB liquid medium supplemented with naringenin (100 µM) and acetosyringone (100 µM) for 24 h. The absorbance and the bacterial concentration of liquid cultures in both strains were estimated at 12, 16, 20, 24 h. The absorbance was monitored by using a spectrophotometer at $600_{nm.}$ The bacterial growth was estimated using a dilution series in which 1 ml of bacterial culture at each evaluation period was transferred to 9 ml liquid LB medium in a screw-capped tube to obtain a dilution of 10^{-1}. This procedure was repeated seven times until the original sample had been diluted by a factor of 10^{-7}. Subsequently, 0.05 ml of the final dilution was transferred to a 9 cm Petri dish containing 20 ml LB semi-solid medium supplemented with the appropriate antibiotic concentration for each strain. This was repeated with further 0.05 ml aliquots of bacteria to give 3 replicate plates. The bacterial droplet was

spread over the surface with a glass spreader. The estimation of bacterial concentration was calculated as colony-forming units (CFU) per ml (Jones *et al.*, 1998). The colony count per ml for each dilution was calculated by dividing the mean colony count per plate (C) by the volume (in ml) of liquid transferred to each plate (V) multiplied by the original dilution (M). For example, for a sample with a mean colony count of 5.5 colonies per plate for a volume of 0.05 ml at a dilution of 10^{-7}, the count would be: $(5.5/0.05) \times 10^{7} = 1.1 \times 10^{9}$ CFU ml^{-1}.

Statistical analysis

The experiments to analyse the effect of flavonoids and acetosyringone in the co-cultivation medium and on bacterial growth (liquid and semi-solid media) for *P. mollissima*, *P. giberti* and *N. tabacum* explants, using both strains (1065 and EHA 105), were performed at least 3 times (3 replications within each treatment for each experiment). Means and standard error (SE) were used throughout. The ANOVA test was performed using the statistical software Minitab Inc., version 12 (State College PA, USA).

RESULTS

GUS histochemical analysis

Evaluation of Agrobacterium strain 1065 (Experiment I)

Histochemical analysis failed to show GUS activity in both *P. mollissima* and *P. giberti* explants (leaf, root and nodal segments). In *N. tabacum*, GUS activity was observed, although at low levels in leaf explants for all bacterial dilutions evaluated. The most significant mean GUS activity obtained (% of explants showing blue staining) was 11.6 ± 3.6% when using a bacterial dilution of 1:2 (v/v). Interestingly, bacterial growth in the co-cultivation medium, at all bacterial dilutions evaluated [undiluted; 1:2; 1:5 (v/v)] was not seen in *P. mollissima*, *P. giberti* and *N. tabacum* explants.

Evaluation of Agrobacterium strain 1065 following supplementation of the co-cultivation medium with flavonoid and acetosyringone (Experiment I)

Among the different flavonoid compounds added to the co-cultivation medium, naringenin was the only one that resulted in GUS activity in *P. mollissima* and *P. giberti* explants when using *Agrobacterium* strain 1065. In *P. mollissima*, the maximum mean GUS activity for leaf explants (6.0 ± 0.57%) and nodal segments (8.3 ± 0.57%) was obtained with 300 µM naringenin in an undiluted bacterial culture. In *P. giberti* supplementation of the co-cultivation medium with 100 µM naringenin and undiluted bacterial culture was most effective in enhancing mean GUS activity in leaf explants (5.0 ± 2.0%), whilst in *N. tabacum*, the maximum mean GUS activity (25.0 ± 6.0%) was considerably higher (5-fold) when compared to control (11.6 ± 3.6%) at a bacterial dilution of 1:2 (v/v). Interestingly, GUS activity was not detected for both *P. mollissima* and *P. giberti* explants when acetosyringone was added to the co-cultivation medium.

Evaluation of Agrobacterium strain EHA 105 (Experiment II)

Enzymatic analysis revealed GUS activity in leaf and root explants and nodal segments in all 3 species used in this experiment. In *P. mollissima*, the maximum mean GUS activity obtained in leaf was 19.3 ± 2.8% and for roots was 14.0 ± 1.1% of explants at different bacterial dilutions [1:2 (v/v) for leaf; undiluted for root]. In *P. giberti* the most significant maximum mean GUS activity was in nodal segments (16.3 ± 1.8%) and leaf explants (14.0 ± 1.1%), with the same bacterial dilution of 1:2 (v/v). The *Agrobacterium* strain EHA

105 was highly effective in leaf explants of *N. tabacum* [bacterial dilution of 1:2 (v/v)], when compared to the other two species in this experiment, revealing a maximum mean GUS activity of 38.0 ± 2.0%.

Evaluation of Agrobacterium strain EHA 105 with supplementation of the co-cultivation medium with naringenin (Experiment II)

In contrast with Experiment I, explants co-cultivated with *Agrobacterium* strain EHA 105 supplemented with naringenin exhibited a wide range of detectable GUS activities in all 3 species analysed. In *P. mollissima* the 3 types of explants (leaf, root and nodal segments) evaluated showed a similar pattern of results as maximum mean GUS activity in leaf (20.3 ± 2.4%) and root (17.3 ± 3.9%) explants and nodal segments (16.7 ± 2.4%) was obtained when the co-cultivation medium was supplemented with 100 µM naringenin at a bacterial dilution of 1:2 (v/v). In *P. giberti* the addition of 100 µM naringenin in the co-cultivation medium and a 1:2 (v/v) bacterial dilution was also effective in leaf (22.6 ± 2.9%) and root explants (22.3 ± 4.3%). However, in *N. tabacum* there was no significant enhancement in maximum mean GUS activity (42.0 ± 6.0%) when compared to control (38.0 ± 2.0%).

Evaluation of Agrobacterium strain EHA 105 following addition of acetosyringone to the co-cultivation medium (Experiment II)

The results provide evidence that acetosyringone (100 µM) enhanced mean GUS activity in leaf explants of *P. mollissima* (22.0 ± 3.5%), *P. giberti* (54.0 ± 5.7%) and *N. tabacum* (42.0 ± 6.0%) at a bacterial dilution of 1:2 (v/v). Interestingly, the results were significantly higher for *Agrobacterium* strain EHA 105 (Experiment II) when compared to strain 1065 (Experiment I).

Bacterial Growth

Determination of bacterial growth in LB liquid medium supplemented with naringenin and acetosyringone

As shown in Fig 1A, 2A when quantifying bacterial growth via optical density an increased trend was observed for both *Agrobacterium* strains 1065 and EHA 105, in LB liquid medium. Maximum bacterial growth for control (1.29 ± 0.3%, 1065); (2.42 ± 0.4%, EHA 105), naringenin (1.64 ± 0.5%, 1065); (2.91 ± 0.6%, EHA 105) and acetosyringone (0.84 ± 0.4%, 1065); (2.22 ± 0.4%, EHA 105) was obtained at 24h. Interestingly, the highest values observed for both strains was with the addition of naringenin at all 4 time intervals evaluated when compared to acetosyringone and control. In addition, *Agrobacterium* strain EHA 105 displayed higher values at all time intervals analysed when compared to strain 1065.

Determination of bacterial growth in semi-solid LB medium supplemented with naringenin and acetosyringone

In Fig 1B, 2B bacterial growth (colony-forming units) in semi-solid medium for both *Agrobacterium* strains 1065 and EHA 105 displayed a similar pattern, as in both cases, a maximum level of bacterial growth was achieved with a subsequent decline however, the peak point was at different times for each compound analysed. For example, with the addition of naringenin in the co-cultivation medium, maximum bacterial growth for both strains (3.30 x 10^{11}, 1065; 4.01 x 10^{11}, EHA 105) was at 12 h, and for acetosyringone 20 h for strain 1065 (8.59 x 10^{10}) and 16 h for strain EHA 105 (2.32 x 10^{11}). In this case, although there was a similar trend for the compounds analysed, the addition of naringenin for both strains still displayed higher values (colony-forming units) when compared to control and acetosyringone.

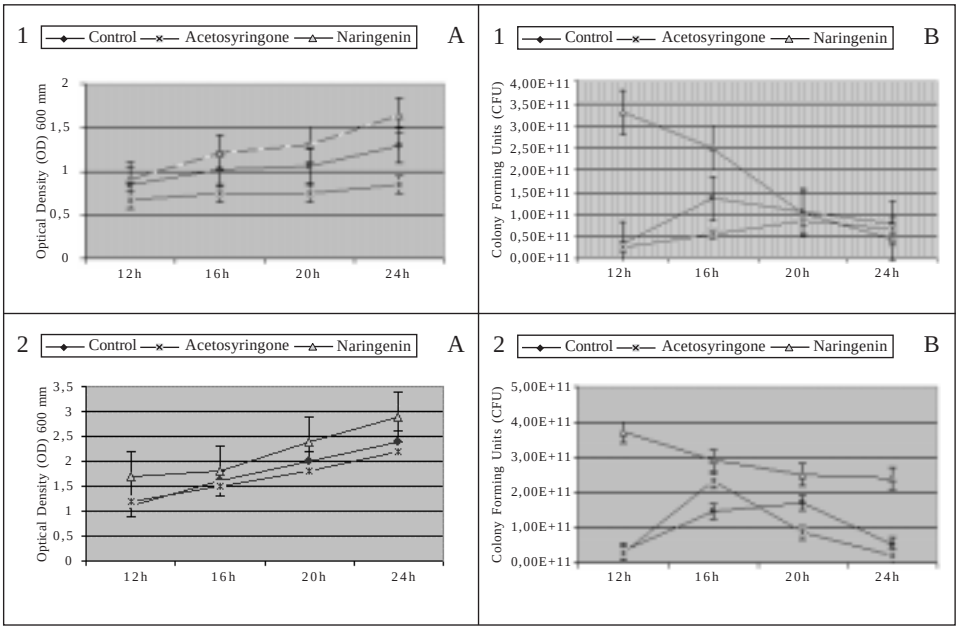

FIGURA 1. Effect of naringenin and acetosyringone on growth of Agrobacterium strains 1065 (1) and EHA 105 (2)

1A, 2A: mean values of optical density (n=3) at different intervals, 1B, 2B: mean values of the number of colonies (n=3) calculated at different intervals
Control: LB liquid medium without naringenin or acetosyringone, Acetosyringone (100 µl) supplemented in LB liquid medium. Naringenin (100 µl) LB liquid medium.

DISCUSSION

In a number of plants, such as woody species, it has not been straightforward to establish an efficient *Agrobacterium*-mediated transformation system (Cervera *et al.*, 1998b), perhaps because they fail to produce *vir* inducing compounds or they secrete anti-bacterial metabolites that inhibit *vir* induction. This difficulty was observed in *P. mollissima* using one of the strains (1065) evaluated in the current study. In an attempt to overcome this restraint, supplementation of the medium with flavonoids was an alternative approach to the traditional use of acetosyringone (Stachel and Zambryski, 1986), although it is well establish that several factors play an important role in *Agrobacterium*-mediated transformation.

The data obtained in the current study supported those expectations, in particular, that phenolic compounds were able to increase transient GUS expression in *P. mollissima* (target species), *P. giberti* (comparative species), and *N. tabacum* (model plant) explants, with both *Agrobacterium* strains, 1065 and EHA 105. However, among the flavonoids evaluated (naringin, naringenin, hesperetin, daidzein, quercetin and myricetin), naringenin was the only one to show a positive effect in terms of stimulating GUS activity. Interestingly, it was observed that *P. mollissima* explants incubated with strain

1065 only displayed GUS activity after the flavonoid naringenin was added to the co-cultivation medium. However, explants inoculated with strain EHA 105 behaved differently because *gus* gene expression was observed without supplementation of naringenin in the co-cultivation medium, although when added it further increased GUS activity. A possible explanation of the differences in GUS expression of the *P. mollissima* explants when using the two *Agrobacterium* strains (1065, EHA 105) could be the natural variability associated with three genetic entities: the plant genome that is responsible for the synthesis of the molecular signal; the bacterial chromosome; and the Ti plasmid coding for v*irA* and v*irG*; which require some form of compatibility (co-ordination) between them (Bénlanger *et al.,* 1996). This may not have occurred with *P. mollissma* explants when inoculated with *Agrobacterium* strain 1065.

This is the first report of the proven beneficial effects of naringenin in the co-cultivation medium, in stimulating *Agrobacterium*-mediated transformation of *P. mollissima*. The effectiveness of naringenin in stimulating *Agrobacterium* infection was at 100 and 300µM, similar to concentrations generally used in the most commonly employed virulence-stimulating metabolite (acetosyringone) in *Agrobacterium*-plant transformation systems. This was not, however, in agreement with the findings of Zerback *et al*. (1989), who showed that the concentrations of all flavonoids (*e.g*. quercetin) had to be 100 times higher than the phenolic acetosyringone in order to induce virulence in *Agrobacterium tumefaciens*.

In the current study the assessment of the effect of naringenin on bacterial suspension for both strains 1065 and EHA 105, showed an increase in bacterial cell growth (colony-forming units). These findings were consistent with previous observations of the growth-stimulating effect of flavonoids in certain Rhizobia species (Hartwig *et al*., 1991). Recently, O'Callaghan (1999) also found that naringenin-treated wheat stimulated the growth of secondary lateral roots. Although the mechanism(s) responsible for the stimulation of bacterial and/or plant cell growth by naringenin is yet unknown several probable explanations could be considered. For example, when flavonoids are added to the bacterial suspension the amount of extracellular proteins produced may increase therefore, inducing higher activity of bacterial cell growth (Lin *et al*., 1999). In the case of plant cell growth, it may be that naringenin does not influence bacterial cell growth directly; plants might have modified the exogenous flavonoid, thus stimulating bacterial colonization (O'Callagahan, 1999).

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