

A Model of *Agrobacterium tumefaciens* Vacuum Infiltration Into Harvested Leaf Tissue and Subsequent In Planta Transgene Transient Expression

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ABSTRACT: *Agrobacterium*-mediated gene transfer, or agroinfiltration, can be a highly efficient method for transforming and inducing transient transgene expression in plant tissue. The technique uses the innate DNA secretion pathway of *Agrobacterium tumefaciens* to vector a particular plasmid-encoded segment of DNA from the bacteria to plant cells. Vacuum is often applied to plant tissue submerged in a suspension of *A. tumefaciens* to improve agroinfiltration. However, the effects of vacuum application on agroinfiltration and in planta transient transgene expression have not been well quantified. Here we show that vacuum application and release act to drive *A. tumefaciens* suspension into the interior of leaf tissue. Moreover, the amount of suspension that enters leaves can be predicted based on the vacuum intensity and duration. Furthermore, we show that transient expression levels of an agroinfiltrated reporter gene vary in response to the amount of *A. tumefaciens* vacuum infiltrated into leaf tissue, suggesting that vacuum infiltration conditions can be tailored to achieve optimal transient transgene expression levels after agroinfiltration.

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Introduction

Agroinfiltration, wherein *Agrobacterium tumefaciens* is used as a vector for inserting transgenes into plant cells via its endogenous DNA secretion pathway, is an effective method for rapidly transforming and inducing transient transgene expression in many plant species. Bringing *A. tumefaciens* into contact with susceptible host plant cells is important for effective agroinfiltration. Vacuum infiltration, where plant

tissue is submerged in a liquid suspension of *A. tumefaciens* and subjected to decreased pressure followed by rapid repressurization, is a common method for introducing bacteria to the interior of the plant tissue (Bechtold and Pelletier, 1998; Bechtold et al., 1993; Tague and Mantis, 2006). In lettuce specifically, it has been shown that vacuum application substantially increases transient expression levels when compared to infiltrations at atmospheric pressure (Joh et al., 2005).

The application of vacuum may cause gases to evacuate from the leaf interior through stomata and possibly through wounding sites. As the vacuum is broken and pressure rapidly increases, cell suspension may be driven into the leaf to replace these gases. This phenomenon may expose *A. tumefaciens* to plant cells that are more susceptible to transformation than those present on the leaf epidermis. Studies have shown that the mesophyll cells of *A. thaliana* leaf explants, which reside in the tissue interior, are more readily transformed by *A. tumefaciens* compared to other cell types present in leaf tissue (Sangwan et al., 1992).

While vacuum infiltration is widely used, the process itself has not been modeled. Such a model may help predict vacuum conditions that lead to optimal transient expression. The objectives of this study were (i) to model the outflux of gases from leaf tissue during vacuum and the subsequent replacement of these gases by cell suspension after repressurization, and (ii) to correlate vacuum infiltration with transient expression levels of an agroinfiltrated reporter gene.

Materials and Methods

Cultivation of Lettuce and Preparation for Vacuum Infiltration

Romaine lettuce, *L. sactuca valmaine*, was grown in a greenhouse. Seeds were germinated on wetted paper towels

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over 3 days. Plants were watered twice daily to saturation with fertilizer water (1,250 ppm GrowMore 4-18-38 no boron, 1,250 ppm magnesium sulfate, 3,000 ppm calcium nitrate). Temperature varied between 21 and 38°C. The outermost leaves were harvested from plants 25 to 35 days after germination.

Lettuce leaves were rinsed, blotted dry, and cut into 2 cm × 3 cm rectangles, taking care to select tissue of uniform age, color, and texture, as described previously by Joh et al. (2005). Leaf sections were divided into aliquots of three to four sections and cut edges were sealed with paraffin wax. Leaf aliquots were placed into 50 mL tubes filled with 30 mL of water or cell suspension containing Break-Thru S-240 surfactant (Goldschmidt Chemical Company, Hopewell, VA). Wads of polyethylene mesh were inserted into the tubes to fully submerge the leaf sections. After vacuum was applied to the tubes, leaf sections were removed and blotted dry. Weight measurements on the leaf sections before and after vacuum were used to determine the amount of vacuum-infiltrated liquid. Leaves infiltrated with *A. tumefaciens* were sealed in Petri dishes lined with wetted filter paper and incubated in darkness for 3 days at 22°C post-vacuum.

A. *tumefaciens* Strains and Cultivation

A. tumefaciens strain C58C1 was used for all agroinfiltration experiments (Wroblewski et al., 2005) and was cultivated using the methods of Joh et al. (2005). C58C1 employs a binary vector system (Hamilton, 1997) that includes the plasmid pTFS40 (British Sugar, Norwich, UK). The T-DNA present on pTFS40 contains an intronated *uidA* gene coding for β-glucuronidase (GUS) driven by the 35S promoter from cauliflower mosaic virus (Vancanneyt et al., 1990).

Leaf Protein Extraction and GUS Assay

After incubation, leaves were stored at -80°C for at least 24 h. Leaf extracts were obtained and GUS assayed using methods described by Joh et al. (2005) with the modification that extraction buffer was added to the leaves on a 2-to-1 volume per mass basis (e.g., 2 mL of extraction buffer to 1 g wet weight of leaves).

Development of Stomatal Gas Outflux and Bacterial Suspension Influx Models

The substomatal cavity is an open airspace proximal to the stomatal pore that acts as an entry point for all gases that diffuse into the intercellular airspaces of the mesophyll cells residing in the leaf interior. The system considered in this model, illustrated in Figure 1, includes the stomatal opening along with the proximal cavity and the air spaces associated with it, henceforth referred to as the substomatal cavity.

Assuming that only air occupies the substomatal cavity prior to vacuum application, that the molecular weight of the air is constant and that no mass enters the substomatal

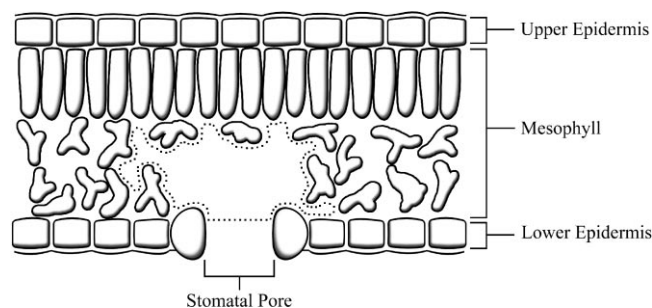


Figure 1. Leaf cross-section. The dotted line indicates the control volume used in developing the model, specifically the cavity proximal to the stomatal pore and associated airspaces.

cavity during vacuum application, the molar mass balance for the cavity is:

$$\frac{dn_{\text{cavity}}}{dt} = -\dot{n}_{\text{out}} \quad (1)$$

where n_{cavity} is the number of moles of air contained in the substomatal cavity and \dot{n}_{out} is the mole flow rate of gas out of the cavity. The molar value of air can be related to the pressure within the substomatal cavity through the ideal gas law. Assuming the cavity volume and temperature are constant (i.e., negligible cell structure changes and vacuum cooling effects over the span of vacuum application), the change in molecular mass is only a function of the change in pressure:

$$\frac{dn_{\text{cavity}}}{dt} = \frac{d}{dt} \left(\frac{P_{\text{cavity}} V_{\text{cavity}}}{RT} \right) = \left(\frac{V_{\text{cavity}}}{RT} \right) \frac{dP_{\text{cavity}}}{dt} \quad (2)$$

where P_{cavity} is the pressure within the substomatal cavity, V_{cavity} is the volume of the cavity, R is the universal gas constant, and T is the temperature of the gas in the cavity. The flow out of the substomatal cavity can be described in manner similar to Ohm's law (Upadhyaya et al., 1983), where flow is equal to the potential divided by resistance. In this case, the relationship is:

$$-\dot{n}_{\text{out}} = \frac{P_{\text{exterior}} - P_{\text{cavity}}}{R_{\text{res}}} \quad (3)$$

where P_{exterior} is the pressure external to the leaf tissue and R_{res} is the resistance to flow at the stomatal opening. It is assumed that any effects resulting from changes in gas density during vacuum are negligible.

The final mass balance for the gas in the cavity in terms of pressure is then:

$$\frac{dP_{\text{cavity}}}{dt} = \frac{RT}{V_{\text{cavity}} R_{\text{res}}} (P_{\text{exterior}} - P_{\text{cavity}}) \quad (4)$$

subject to the initial condition $P_{\text{exterior}} = P_{\text{cavity}} = P_0$ when $t = 0$, where P_0 is the initial pressure.

An empirical double decay function was used to represent vacuum chamber pressure over time during vacuum application, as this model accurately represented the pressure change dynamics of the particular vacuum system used in this research:

$$P_{\text{exterior}} = A e^{-k_1 t} + B e^{-k_2 t} \quad (5)$$

where A , B , k_1 , and k_2 are empirical constants.

Using the LaPlace transform, Equation (4) can be solved to yield $P_{\text{cavity}}(t)$:

$$P_{\text{cavity}}(t) = \left(P_0 - \frac{CA}{C - k_1} - \frac{CB}{C - k_2} \right) e^{-Ct} + \frac{CA}{C - k_1} e^{-k_1 t} + \frac{CB}{C - k_2} e^{-k_2 t}, \quad C = \frac{RT}{V_{\text{cavity}} R_{\text{res}}} \quad (6)$$

Given that the volume of the substomatal cavity is constant, and assuming gases that come out of solution within the leaf tissue are negligible, Boyle's Law can be used to relate gas pressure and volume within the substomatal cavity during and after vacuum application

$$V_{\text{gas,post-vacuum}} = V_{\text{cavity}} \frac{P_{\text{cavity,tf}}}{P_0} \quad (7)$$

where $V_{\text{gas,post-vacuum}}$ is the volume of gas within the cavity after vacuum has been broken and the cavity has reached atmospheric pressure and $P_{\text{cavity,tf}}$ is the final pressure of the gas within the cavity at the moment prior to breaking vacuum as determined by Equation (6).

Assuming that all gases evacuated from the substomatal cavity during vacuum are replaced with cell suspension upon re-pressurization, the volume of cell suspension infiltrated into the substomatal cavity can be expressed as:

$$V_{\text{suspension}} = V_{\text{cavity}} - V_{\text{gas,post-vacuum}} \quad (8)$$

where $V_{\text{suspension}}$ is the volume of cell suspension present within the substomatal cavity after vacuum has been released and re-pressurization has fully occurred.

The volume of infiltrated cell suspension is expressed in terms of the volume of the substomatal cavity and the fractional pressure change during vacuum application by replacing $V_{\text{gas,post-vacuum}}$ in Equation (8) with Equation (7) and then simplifying:

$$V_{\text{suspension}} = \left(1 - \frac{P_{\text{cavity,tf}}}{P_0} \right) V_{\text{cavity}} \quad (9)$$

Determination of Stomatal Resistance to Gas Flow

The resistance to gas flow through stomata, R_{res} , was determined by nonlinear regression of experimental data.

Matlab v 7.2 (The Mathworks, Natick, MA) was used to compare an experimental data set of P_{cavity} values at various time points to a function describing the external pressure change in the form of Equation (5). The program then used Matlab's nonlinear fit function to estimate R_{res} in Equation (6) given the P_{cavity} and P_{exterior} data. The temperature, T , was set constant at room temperature, 295.15 K, and any vacuum cooling effects were assumed to be negligible. The initial pressure, P_0 , was taken to be atmospheric pressure, 101 kPa.

Leaf samples were vacuum infiltrated with water for a given span of time, rapidly repressurized and then weighed to measure infiltrated water mass. These same samples were subsequently infiltrated at 5 kPa for 5 min and then weighed for infiltrated water mass once more, allowing for an approximation of V_{cavity} to be made based on the maximum amount of water that can be infiltrated. Vacuum infiltrations were performed at three target pressures: 45, 25, and 5 kPa, using surfactant concentrations of 20, 10, and 10 ppm, respectively. $P_{\text{cavity}}(t)$ values were calculated by inserting $V_{\text{suspension}}$ versus t and V_{cavity} data into Equation (9).

The parameter values describing the change in external pressure, A , B , k_1 , and k_2 in Equation (5), were estimated using Matlab's nonlinear fit function from a data set of pressure measurements over time obtained by running a vacuum pump attached to an empty vacuum chamber until steady state was reached at the target vacuum level (data not shown). The parameter values are given in Table I.

Effect of Infiltrated Suspension Volume on GUS Expression

Vacuum intensities were varied to control the amount of bacterial suspension infiltrated into leaf tissue to test the effect of infiltration volume on GUS transient expression. The bacterial density within the infiltrated suspension was held constant by using a bacterial suspension of $\text{OD}_{590} = 0.30$ across all treatments. All infiltrations used 20 ppm surfactant, a vacuum time of 7.5 min, and an agitation rate of 100 rpm during vacuum application.

Effect of Infiltrated Suspensions With Varying Bacterial Densities on GUS Expression

The bacterial concentration of *A. tumefaciens* suspensions was varied while the volume of suspension infiltrated into leaf discs was set constant in order to examine the effect of bacterial density within the leaf on GUS expression levels.

Table I. Parameter values in the double decay model describing the pressure external to the leaves within the vacuum chamber at time t (Eq. 5).

| Pressure (kPa) | A | B | k_1 | k_2 |
|----------------|------|------|-------|-------|
| 45 | 49.3 | 51.1 | 0.073 | 0.001 |
| 25 | 40.2 | 60.1 | 0.005 | 0.080 |
| 5 | 45.6 | 55.2 | 0.021 | 0.132 |

Suspensions of *A. tumefaciens* at varying absorbance intensities were infiltrated into leaf discs under uniform vacuum conditions (25 kPa for 7.5 min while shaking at 100 rpm) to achieve relatively constant infiltration volumes across all treatments. The number of bacteria infiltrated per unit leaf mass was based on the volume of suspension infiltrated into leaves and the cell density of the suspension.

Results and Discussion

Solution and Validation of the Stomatal Gas Outflux Model

Selected plots comparing the external pressure to both the theoretical and empirical substomatal cavity pressures are shown in Figure 2. The theoretical solutions utilized values of R_{res} estimated from fitting the data to Equation (6). For applied pressures of 45, 25, and 5 kPa, mean R_{res} estimates were 36.8 ± 1.46 (SEM), 34.9 ± 2.92 , and 24.6 ± 2.38 $\text{GJ s mol}^{-1} \text{m}^{-3}$ ($n=4$). Mean V_{cavity} values were not significantly different across treatments. Variability in the estimated value of R_{res} can stem from inaccuracies in the estimation of V_{cavity} and variation in the P_{cavity} data. Changes in the estimated value of V_{cavity} lead to proportional changes in the fitted value of R_{res} . The width of the confidence interval for the estimated value of R_{res} is dependent upon how the P_{cavity} data deviate from the model's prediction. Figure 2 shows the sensitivity of the model's output to changes in the fitted value of R_{res} . The upper and lower bounds of the 95% confidence interval for the value of R_{res} were inserted into the model to generate additional P_{cavity} versus time plots. The model output is most sensitive to error in R_{res} during the initial period in which the rate of P_{cavity} change decreases. The model becomes less sensitive to R_{res} error as P_{cavity} approaches steady-state. The solution shows considerable lag between the substomatal cavity and the vacuum chamber once vacuum is initiated and the pressure begins to change rapidly. However, as the external pressure approaches steady state and the rate of pressure change decreases, the discrepancy between the cavity and external pressures decreases as well. By 3 min of vacuum application, the pressure within the substomatal cavity has reached equilibrium with the environment, suggesting the outflow of gas from the cavity has ceased.

Effects of Infiltrated Cell Suspension Volume on Transient Expression

Transient expression levels were examined in response to varying infiltrated quantities of *A. tumefaciens* cell suspension. As seen previously, the mass of infiltrated cell suspension increased with vacuum intensity (Fig. 3a). The linear trend has $R^2 = 0.98$ and a P -value of <0.0001 . Greater volumes of infiltrated suspension led to increased transient

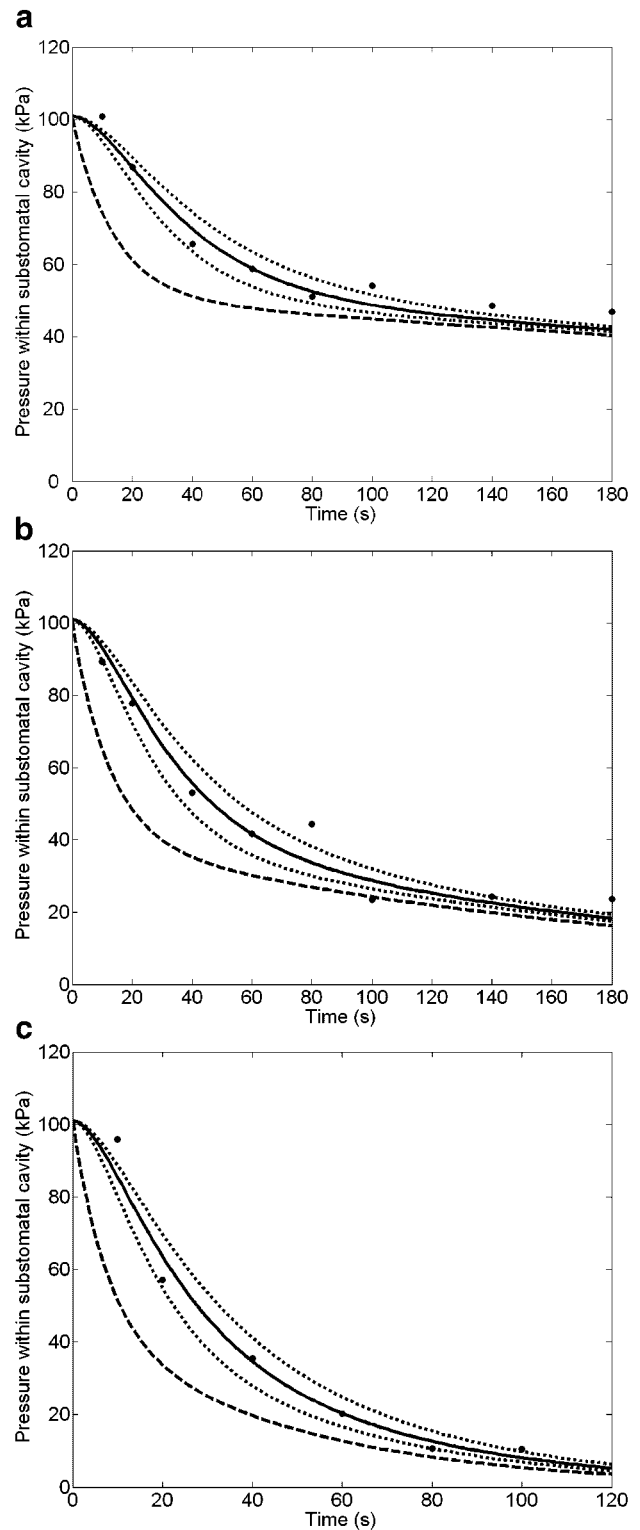


Figure 2. External and substomatal cavity pressure as a function of time during vacuum application for (a) 45 kPa, (b) 25 kPa, and (c) 5 kPa final pressures. Dashed lines represent the external pressure. Solid lines represent the theoretical substomatal cavity pressure. Circles represent empirical measurements of substomatal cavity pressure. Dotted lines show the sensitivity of the model's output to changes in R_{res} . The dotted lines are the predicted P_{cavity} profiles when the upper and lower bounds of the 95% confidence interval for the value of R_{res} are inserted into the model.

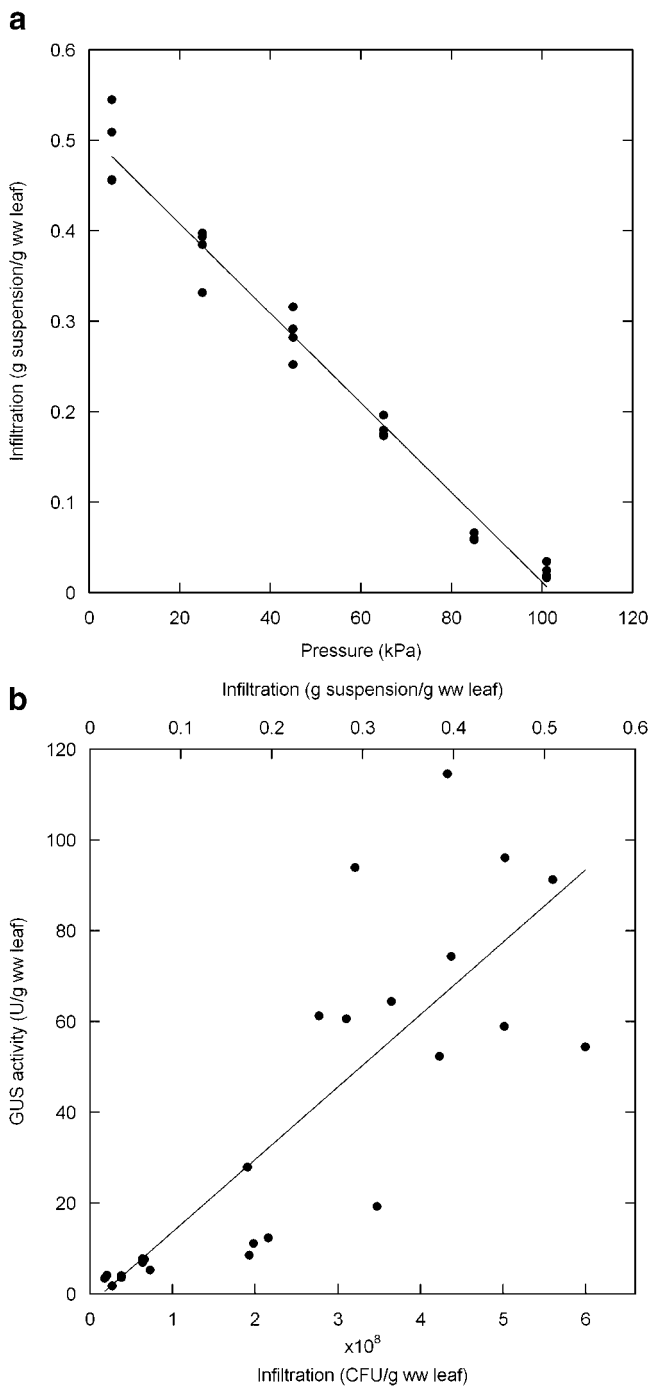


Figure 3. a: Infiltrated suspension versus pressure during vacuum application. b: GUS expression versus infiltrated suspension volume.

GUS expression, as indicated by GUS activity in the agroinfiltrated leaves (Fig. 3b). The linear trend has $R^2 = 0.70$ and a P -value of <0.0001 . These data may be explained by the fact that lower vacuum levels lead to smaller quantities of infiltrated suspension, resulting in fewer bacteria coming into contact with competent plant cells

in the leaf interior. Alternately, higher vacuum levels drive greater amounts of cell suspension into leaf tissues, maximizing the penetration of bacterial suspension into leaves and promoting contact between *A. tumefaciens* and plant cells. The increased contact with plant cells on the leaf interior may lead to more plant cells being transformed and higher overall transient expression levels of transgenes within leaf tissue.

Bacterial Density Effects on GUS Transient Expression

Figure 4 shows the expression trend for varying amounts of infiltrated bacteria. The fitted linear model shown in Figure 4 exhibits a significant negative slope ($P < 0.0001$). There was no correlation between the volume of suspension infiltrated and GUS expression levels, which rules out any infiltration volume effects in the expression data. Interestingly, GUS activity within the transformed tissue decreased as more bacteria were infiltrated into the leaves. The greatest GUS activity was observed when suspension containing 2.7×10^8 CFU/mL was used. This suggests that simply inserting more copies of GUS-encoding T-DNA into plant cells does not equate to increased transient expression. It has been documented previously that an inverse relationship between transgene copy number and expression level exists as a result of transcriptional gene silencing in plants (Vaucheret and Fagard, 2001). Additionally, certain plants are known to have a threshold for *A. tumefaciens* density beyond which cell viability (Cheng et al., 1997; Wroblewski

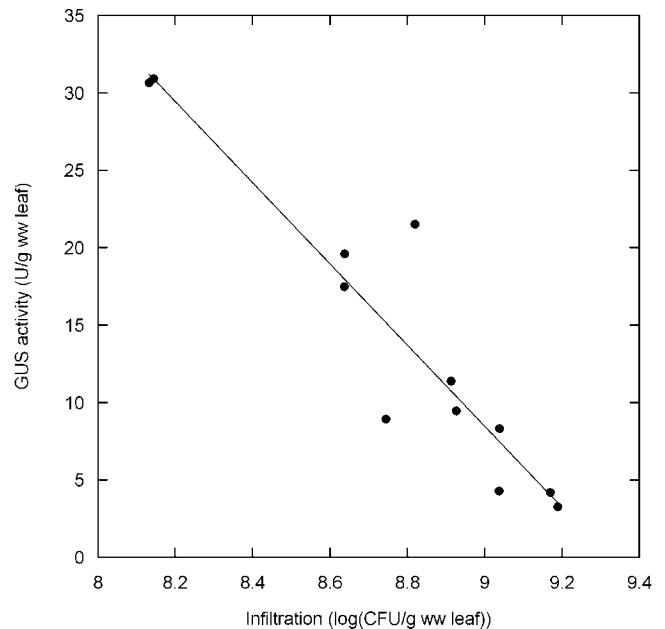


Figure 4. GUS transient expression level versus the logarithm of infiltrated *Agrobacterium*. The infiltrated volume was between 0.51 and 0.62 g suspension/g leaf.

et al., 2005) and transient expression (Amoah et al., 2001) decrease, although the mechanisms behind these phenomena remain unclear.

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