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Response surface studies that elucidate the role of infiltration conditions on *Agrobacterium tumefaciens*-mediated transient transgene expression in harvested switchgrass (*Panicum virgatum*)

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ABSTRACT

Agrobacterium tumefaciens-mediated transient expression (agroinfiltration) experiments were performed in harvested switchgrass (*Panicum virgatum*) leaves to identify the effects of wounding by bead beating, surfactant concentration and vacuum application on *in planta* β-glucuronidase expression and leaf decay. Expression was scored based on a consistent pattern of visual observations of histochemical staining over the leaf surface as might be observed in stable gene expression in switchgrass leaves. Assays on extracts from leaves were also performed to measure expression levels; however, these assays showed low expression levels, which may have been due to low recombinant protein recovery and decomposition in the leaf. Bead beating was successful for wounding the plant surface, but did not improve the consistency of expression based on histochemical staining observations. Surfactant was necessary for improving contact between the leaf surface and *Agrobacterium* suspension and consistently improved expression when vacuum application level was low (25 kPa). Increasing vacuum application from 25 to 5 kPa improved expression only when surfactant concentration was low. When a suspension of *A. tumefaciens* containing 1000 ppm Break-Thru surfactant was added to harvested leaves and 25 kPa vacuum applied, a fairly uniform expression was visualized across the leaf surface within 2–3 days of incubation, suggesting that agroinfiltration is a rapid tool for examining expression of transgenes in switchgrass leaves.

1. Introduction

The resistance of plant material to enzymatic and acid hydrolysis is one of the most significant obstacles facing lignocellulose-based production of biochemicals and fuels [1]. As reviewed by Vogel and Jung [2], several studies have proposed and investigated genetic modification of herbaceous plants for improved bioconversion [2]. One area of recent interest is *in-planta* expression of cell wall-decomposing enzymes to facilitate lignocellulose pretreatment [3]. While procedures are well established for plant transformation, it may take several weeks to evaluate transgene expression in parts of the plants, such as leaves, that are harvested and converted to biochemicals and fuels. If multiple transgenes and plants are to be investigated the evaluation process could take months.

Transient transgene expression is a potential alternative to stable expression in transgenic plants for testing gene

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constructs and production of recombinant proteins. Agroinfiltration is one method of transient expression in which the bacterium *Agrobacterium tumefaciens*, modified to include a gene for the desired protein, is infused into the plant. The *A. tumefaciens* transfers the gene to nuclei of compatible plant cells where the transgene is transiently expressed without necessarily incorporating it into the plant's genome. Interesting features of agroinfiltration reported in expression studies using dicot plant species include (1) higher and more rapid expression of recombinant protein compared with stable expression in transgenic plants [4–7], (2) rapid co-expression of multiple genes by simultaneous agroinfiltration with multiple *A. tumefaciens* strains carrying genes encoding unique proteins [6,8–10] and (3) expression in tissues after harvest with incubation in complete darkness [11,12]. These features make agroinfiltration attractive for testing the expression of novel transgenes in plant biomass and mimicking expression in transgenic plants.

Very little research has been done on *Agrobacterium*-mediated transient expression in the leaves of monocot plant species. The goal of this research was to identify the role of agroinfiltration conditions on transient β-glucuronidase expression in harvested switchgrass leaves and to select conditions for uniform expression in leaves so as to mimic expression that might be achieved in the leaves of a transgenic plant. Switchgrass was selected because it is a potential biomass energy feedstock [13]. The reporter gene β-glucuronidase (GUS) was chosen as a model heterologous protein because its production has been studied extensively in plants, it is relatively stable, thereby reducing confounding effects of protein degradation *in planta*, and can be readily visualized by histochemical staining. Agroinfiltration conditions examined included mechanical wounding, vacuum application and surfactant concentration, and responses included expression uniformity and plant decay.

2. Methods

2.1. Switchgrass production and preparation

The switchgrass variety, *Panicum virgatum* L. cv. Alamo was used for the infiltration experiments. Switchgrass was cultivated in potting soil (pH 6.19) and maintained in UC Davis greenhouses with controlled fertigation and temperature. Leaves were harvested 10–36 d after planting. Upon harvest, leaves were cut into 2–4 cm pieces, rinsed with distilled water and placed on ice until infiltration. Leaves were infiltrated within 18 h of harvest.

2.2. A. tumefaciens maintenance and cultivation

A. tumefaciens strain C58C1 containing plasmid pTFS40 (British Sugar, Norwich, UK) was used for infiltration and expression. This strain was chosen because it was found to be the most effective in over 6000 infiltration and transient expression experiments [5]. This strain contains an additional helper plasmid pCH32 [14] to enhance transient expression. The pTFS40 plasmid confers kanamycin and tetracycline antibiotic resistance and includes the p35S-*uidA*-intron gene coding for GUS. The presence of the intron prevents expression of the *uidA* gene in *A. tumefaciens*.

A. tumefaciens cultures were maintained in liquid YEP medium: 10 g L^{-1} yeast extract (Becton Dickinson, Franklin Lakes, NJ), 10 g L^{-1} peptone (Becton Dickinson) and 5 g L^{-1} sodium chloride supplemented with 50 mg L^{-1} kanamycin sulfate (Gibco, Grand Island, NY) and 5 mg L^{-1} tetracycline hydrochloride (Sigma, St. Louis, MO). Inoculum was prepared as a 15% glycerol stock culture at 1×10^9 colony-forming units (cfu)/mL YEP ($OD_{590} = 0.35$) and stored at −80 °C. Starting with a 2% inoculum, *A. tumefaciens* cultures were grown to log phase in 50-mL Erlenmeyer flasks at 28 °C and 180 rpm on a rotary shaker. When cultures were approximately $OD_{590} = 0.35$, the cultures were centrifuged at 4000*g* and 10 °C for 20 min. Pellets were resuspended in sterile distilled water to $OD_{590} = 0.4$ prior to infiltration.

2.3. Vacuum infiltration and incubation of leaf tissue

Experiments were designed as full factorial and response surface studies in which wounding, vacuum application and surfactant concentration were the independent variables, and expression and plant decay were the response variables. Procedures for vacuum infiltration and incubation were modified from those described elsewhere [11]. Plant material (20–25 leaves) was added to 50-mL tubes containing sterile distilled water. The concentrated aqueous *A. tumefaciens* suspension was added to reconstitute its original $OD_{590} = 0.35$ in a final volume of 20 mL. Leaf wounding was accomplished by bead beating; 0.5 mL zirconia/silica beads (0.5 mm diameter; BioSpec Products, Bartlesville, OK, USA) were added to the 50-mL tubes containing leaves and cell suspension and vortexed for 1 min. Unwounded treatments were vortexed without beads. After vortexing, Break-Thru S 240 (Goldschmidt Chemical, Hopewell, VA) non-ionic surfactant was added to a final concentration varying from 50 to 1000 ppm (vol vol^{-1}). The tubes were placed in a vacuum chamber on an orbital shaker set at 70 rpm, and vacuum, varying from 25 to 5 kPa applied for 20 min at room temperature. The vacuum was quickly released to facilitate infusion of the bacteria into the tissue. The infiltrated tissues were spread in a single layer in Petri dishes containing sterile filter paper (Whatman #1, Maidstone, UK) moistened with sterile water. The dishes were sealed with Parafilm and placed in an incubator maintained at 22 °C in complete darkness. Incubation time varied between 3 and 6 days. Leaves were examined for decay and GUS expression upon incubation.

2.4. Visualization of transgene expression in intact leaves

GUS expression was visualized in intact freshly incubated leaves using a histochemical assay [15]. For each assay, the leaves were placed in a 50-mL tube and covered with 10 mL of an aqueous solution containing 5% of 10 mg mL^{-1} X-Gluc (Bio-World, Dublin, OH) in dimethyl sulfoxide (Sigma); 100 mM sodium phosphate at pH 7.0; 10 mM EDTA (Bio-Rad, Hercules, CA); 0.5 mM potassium ferricyanide; 0.5 mM potassium ferrocyanide; and 0.006% Triton X-100 (Fisher, Fairlawn, NJ). Pressure was reduced to 25 kPa with shaking at 70 rpm for 20 min. The tubes were incubated at 37 °C and 150 rpm for 5 h.

Chlorophyll was removed by leaching in 70% denatured ethanol for a minimum of 24 h with moderate agitation. All leaves in each treatment were examined under a dissecting microscope at 10× and scored for the presence of indigo pigment resulting from the GUS reaction with X-Gluc. A score of "0" represented no expression, "1" represented very little expression, "2" moderate expression and "3" uniform expression over the leaf tissue (Fig. 1). Average scores were calculated for each treatment.

2.5. *Protein extraction and GUS assay*

Proteins were extracted from leaves using a buffer consisting of 50 mM sodium phosphate at pH 7.0 and 40 mM dithiothreitol (Bio-Rad). Frozen leaf tissue and ice-cold buffer at a 1:2 ratio (wt vol^{-1}) were ground for 10 min in a chilled mortar and pestle. The homogenate was centrifuged for 20 min at 19,000*g* at 10 °C, the supernatant removed and centrifuged for an additional 20 min. Extracts were stored at −80 °C prior to analysis.

To measure GUS activity in plant extracts, 50 μL of extract was added to 500 μL of buffer containing 50 mM sodium phosphate at pH 7.0, 10 mM dithiothreitol (Bio-Rad), 1 mM EDTA, 0.1% sodium lauryl sarcosyl (Sigma), 0.1% Triton X-100 and 1 mM *p*-nitrophenyl glucuronide (PNPG, Sigma) at 37 °C. At timed intervals, 100 μL was withdrawn and the enzymatic reaction terminated in 800 μL of 0.2 M sodium carbonate. The optical density of terminated samples was measured at 405 nm on a microplate reader (Molecular Devices Vmax, Sunnyvale, CA). One unit of activity (U) is defined as 1 nmole PNPG converted to *p*-nitrophenol per minute at 37 °C.

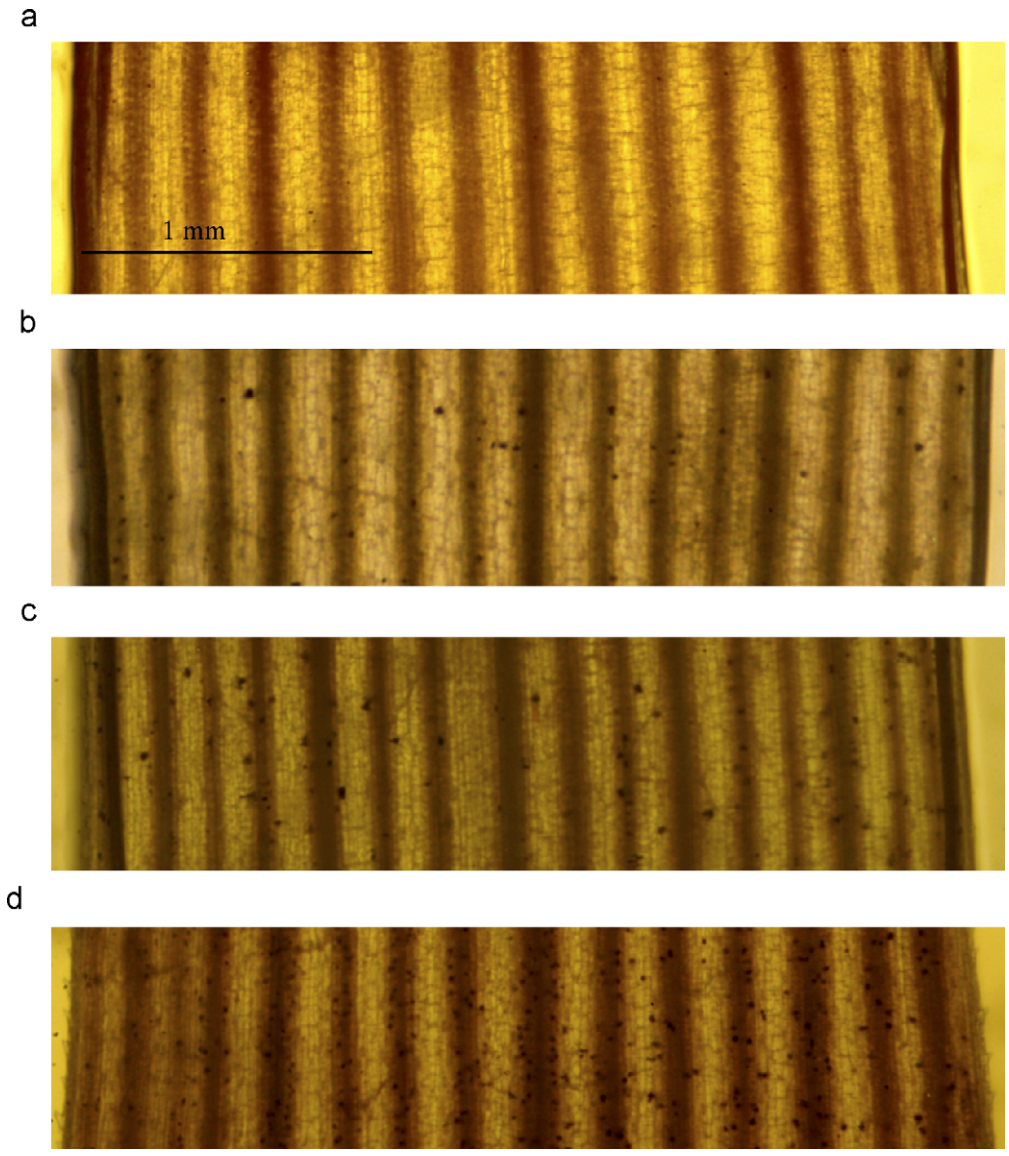

Fig. 1 – Visualization scores for representative switchgrass leaves infiltrated with C58C1 and incubated for 3 days where (a) expression score = 0, (b) expression score = 1, (c) expression score = 2 and (d) expression score = 3.

2.6. Data analysis

Results from full factorial and response surface studies were analyzed using JMP IN v5.1 (SAS Institute, Cary, NC) to determine whether bead beating, surfactant concentration and vacuum application had significant effects on expression and leaf decay. Expression scores from the full factorial study were examined using the following model:

$$E\{Y\} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3, \quad (1)$$

where $E\{Y\}$ is the expected value of the response variable, β_0 is the model intercept, X_1 is the coded level (−1, 1) for surfactant concentration, X_2 is the coded level (−1,1) for vacuum application, X_3 represents the level of bead beating (L1, L2), β_1 is the parameter estimate for surfactant concentration, β_2 is the parameter estimate for vacuum application, β_3 is the parameter estimate for bead beating, β_{12} is the parameter estimate for the interaction between surfactant concentration and vacuum application, β_{13} is the parameter estimate for the interaction between surfactant concentration and bead beating, and β_{23} is the parameter estimate for the interaction between vacuum application and bead beating. Expression scores from the response surface study were examined using

$$E\{Y\} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2, \quad (2)$$

where β_{11} is the parameter estimate representing second-order effects of surfactant concentration and β_{22} is the parameter estimate representing second-order effects of vacuum application.

Reduced models were determined for expression score for Eq. (1) and expression score and decay for Eq. (2). Reduced models were found using a mixed stepwise regression platform in JMP-IN, which computes parameter estimates that are the same as those estimated by other least-squares methods, but it facilitates searching and selecting among many models. JMP-IN was also used to calculate significance probabilities (termed *p*-values here) for each parameter estimate. Since there was a hierarchy of terms in the models, JMP-IN's "combine" rule was used, which groups a parameter estimate (e.g. β_{12}) with its precedent estimates (e.g. β_1 and β_2) and calculates the group's significance probability for entry as a joint *F*-test. The significance probability for a parameter to enter and leave the model was set to 0.25.

3. Results

The first experiment examined the influence of vacuum application level, surfactant concentration and wounding on GUS expression measured using the histochemical assay. Levels for each independent variable and expression scores are listed in Table 1. Moderate expression was observed in all treatments, indicating that conditions were favorable for infiltrating *A. tumefaciens* into leaves, transfer of the transgene to the nuclei of plant cells and transgene expression *in planta*. Parameter estimates from mixed stepwise regression of expression scores using Eq. (1) are listed in Table 2. The interaction between vacuum and surfactant was slightly significant ($p = 0.064$); at low vacuum application (25 kPa) increasing surfactant concentration improved expression, while at high vacuum application (5 kPa) increasing surfactant concentration had little effect on expression. Wounding by bead beating did not have a significant effect on expression. Wounding resulted in patches of expression where significant abrasions had been made on the leaves, but these abrasions did not improve the uniformity of expression over the leaf. For this reason, wounding by bead beating was not considered in future infiltrations.

The second experiment was designed as a response surface study and further examined the effect of surfactant concentration and vacuum level on expression. A longer incubation time was also investigated to determine whether expression increased and leaf decay occurred with extended incubation time. Treatment surfactant concentrations and vacuum levels and corresponding expression scores and leaf decay after incubation are presented in Table 3. Parameter estimates from mixed stepwise regression of expression scores using Eq. (2) are listed in Tables 4 and 5. For leaves examined 3 days after infiltration, increasing vacuum level significantly reduced expression (Table 4). There was a slightly significant curvature indicating a maximum in expression with respect to vacuum application (Fig. 2). The interaction between vacuum level and surfactant concentration was also slightly significant; at low vacuum levels (25 kPa), increasing surfactant concentration had a small effect on expression, while at

Table 1 – Full factorial experimental settings and corresponding expression scores for GUS expression in agroinfiltrated switchgrass leaves

Vacuum (−1 = 25 kPa, 1 = 5 kPa)	Surfactant (−1 = 50 ppm, 1 = 500 ppm)	Bead beating (L1 = yes, L2 = no)	Expression score (3 days post-infiltration)
−1	−1	L2	1.6
−1	−1	L1	1.2
−1	1	L2	2.0
−1	1	L1	1.9
1	−1	L2	2.2
1	−1	L1	2.0
1	1	L2	2.0
1	1	L1	1.1

Table 2 – Reduced model parameter estimates for full factorial experiment (Table 1)

Term	Parameter	Parameter estimate	*p*-Value
Intercept	β_0	1.73	0.0003
Surfactant	β_1	0.003	0.975
Vacuum	β_2	0.091	0.389
Wounding (L1)	β_3	0.203	0.110
Vacuum × surfactant	β_{12}	−0.259	0.064

The model R^2 was 0.83.

Table 3 – Response surface settings and results for GUS expression and leaf decay in agroinfiltrated switchgrass leaves 3 days and 6 days post-infiltration (dpi)

Vacuum (−1 = 25 kPa, 1 = 5 kPa)	Surfactant (−1 = 100 ppm, 1 = 1000 ppm)	Expression score		Leaf decay (%) (6 dpi)
		3 dpi	6 dpi	
−1	−1	1.7	1.2	5
−1	0	2.2	2.0	60
−1	1	2.0	2.1	65
0	−1	1.7	1.6	15
0	0	2.1	1.6	30
0	0	1.7	1.5	35
0	0	1.8	1.6	20
0	0	1.8	1.4	35
0	0	1.8	1.8	50
0	1	2.0	1.6	25
1	−1	1.5	2.2	10
1	0	1.3	1.8	50
1	1	1.1	1.5	15

Table 4 – Reduced model parameter estimates for fit of expression scores collected 3 days after infiltration for response surface experiment (Table 3)

Term	Parameter	Parameter estimate	*p*-Value
Intercept	β_0	1.800	<0.0001
Surfactant	β_1	0.033	0.654
Vacuum	β_2	−0.325	0.002
Vacuum × surfactant	β_{12}	−0.188	0.065
Vacuum × vacuum	β_{22}	−0.192	0.085

The model R^2 was 0.79.

Table 5 – Reduced model parameter estimates for fit of expression scores collected 6 days after infiltration for response surface experiment (Table 3)

Term	Parameter	Parameter estimate	*p*-Value
Intercept	β_0	1.56	<0.0001
Surfactant	β_1	0.033	0.584
Vacuum	β_2	0.017	0.783
Vacuum × surfactant	β_{12}	−0.400	0.0005
Vacuum × vacuum	β_{22}	0.202	0.035

The model R^2 was 0.83.

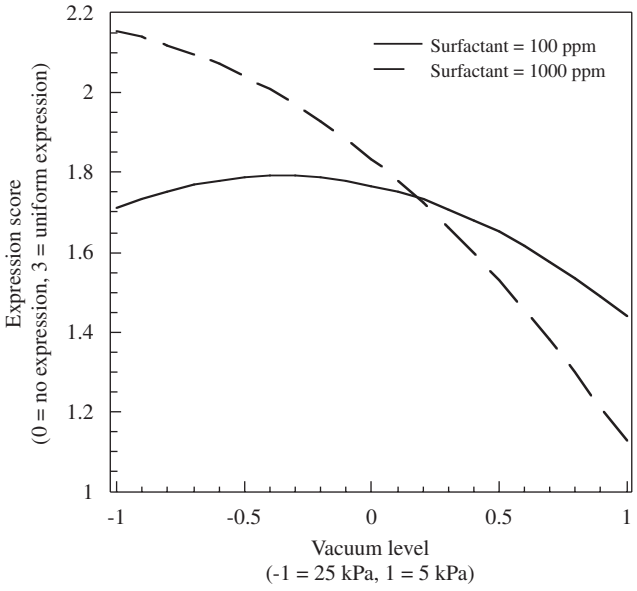

Fig. 2 – Expected expression score estimated using a reduced form of Eq. (2) and parameter estimates from response surface experiment after 3 days of incubation (Table 4). Lines represent expression under constant surfactant concentrations.

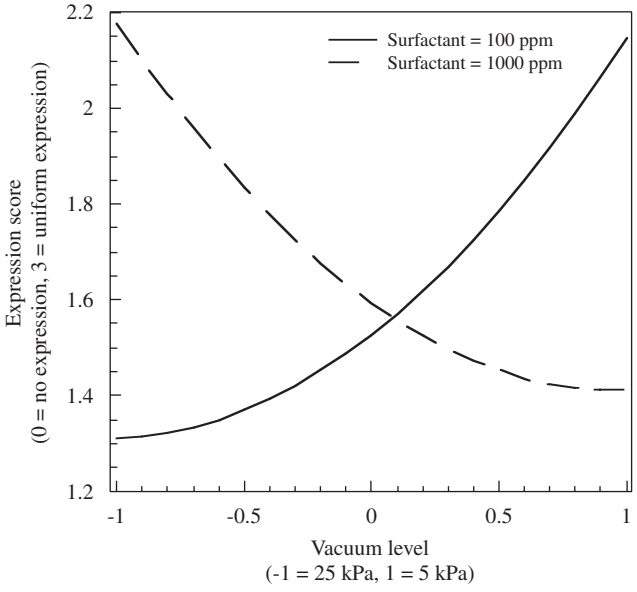

Fig. 3 – Expected expression score estimated using a reduced form of Eq. (2) and parameter estimates from response surface experiment after 6 days of incubation (Table 5). Lines represent expression under constant surfactant concentrations.

high vacuum levels (5 kPa), increasing surfactant concentration reduced expression (Fig. 2). For leaves examined 6 days after infiltration, the interaction between vacuum level and surfactant concentration was highly significant (Table 5). At low surfactant concentration increasing vacuum application improved expression; however, at high surfactant concentration increasing vacuum application reduced expression (Fig. 3). There was also a significant curvature in expression due to vacuum level indicating a minimum in expression. When replicate treatments infiltrated using 550 ppm surfactant and 15 kPa vacuum were compared ($n = 5$), there was a significant drop ($p = 0.02$) in expression with incubation time; mean expression scores for 3- and 6-day incubations were 1.81 and 1.55, respectively.

Leaf decay upon incubation was examined to determine whether any of the treatments resulted in deterioration of the leaves. Decay was not detected in any of the treatments after 3 days of incubation; however, decay was observed in all treatments after 6 days of incubation. Selected decayed and healthy leaves are presented in Fig. 4. Decay data were analyzed by mixed stepwise regression using Eq. (2) and parameter estimates are listed in Table 6. Leaf decay increased significantly with increasing surfactant concentration. There was also a maximum in leaf decay with respect to surfactant concentration. The interaction between surfactant concentration and vacuum level was significant: when surfactant concentration was low, increasing vacuum level resulted in leaf decay; however, when surfactant concentration was high, increasing vacuum significantly reduced decay (Fig. 5). When leaf decay was regressed against expression scores measured 6 days after infiltration, a slightly significant increase in decay was observed with increasing expression score ($p = 0.09$). If one outlier observed at 5 kPa and 100 ppm surfactant is excluded from the regression, the relationship between decay and expression becomes very significant ($p = 0.0008$).

A third switchgrass agroinfiltration was done to determine expression level measured on leaf extracts when agroinfiltration used vacuum at 25 kPa and surfactant at 1000 ppm. In samples incubated 2–3 days after infiltration, expression levels measured on plant extracts were 0.23 ± 0.06 U (g fresh weight)$^{-1}$ ($n = 3$). GUS activity was not detected in extracts from non-infiltrated switchgrass controls.

4. Discussion

Three variables hypothesized to be important to uniform transient expression in agroinfiltrated switchgrass were examined in this study: wounding by bead beating, surfactant concentration and vacuum application. While patches of expression were observed in abraded areas associated with bead beating, beating leaves did not result in a significant improvement in uniform expression over the leaf. It is possible that more excessive beating could have resulted in additional abrasion and higher expression, but also could have resulted in rapid decay of the leaf. One report in the literature observed improved transient expression in switchgrass leaves upon wounding using carborundum and subsequent co-cultivation with *Agrobacterium* [16]. However, the referenced study used a *uidA* gene encoding GUS that was not

Table 6 – Reduced model parameter estimates for fit of leaf decay observations collected 6 days after infiltration for response surface experiment (Table 3)

Term	Parameter	Parameter estimate	*p*-Value
Intercept	β_0	36.38	0.0001
Surfactant	β_1	12.50	0.030
Vacuum	β_2	−9.17	0.087
Vacuum × surfactant	β_{12}	−13.75	0.045
Surfactant × surfactant	β_{11}	−22.33	0.014
Vacuum × vacuum	β_{22}	12.67	0.105

The model R^2 was 0.80.

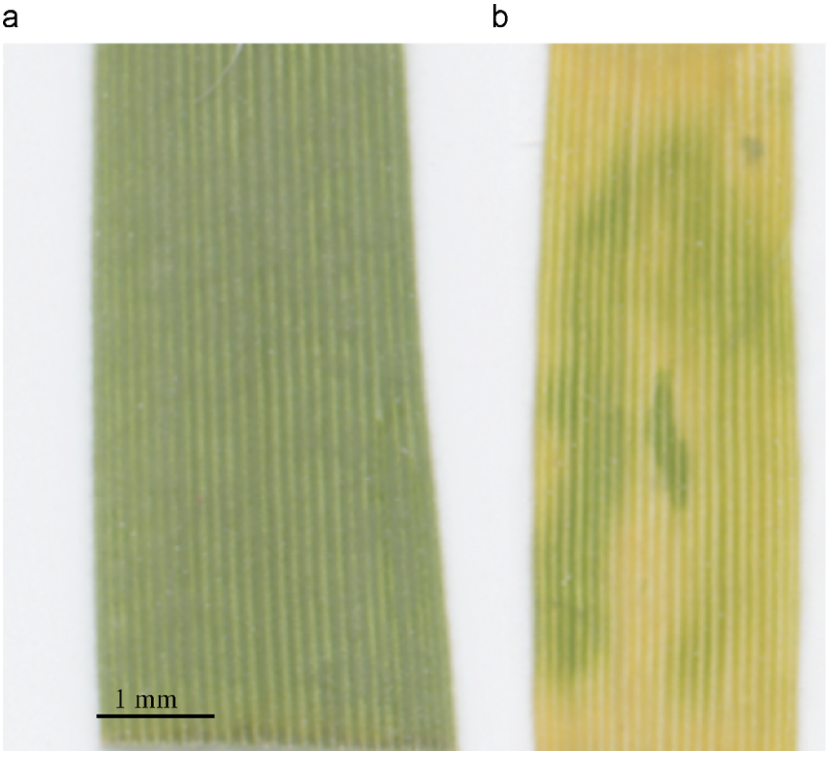

Fig. 4 – Decay of leaves 6 days post-infiltration where (a) represents a leaf with no decay and (b) represents a decayed leaf.

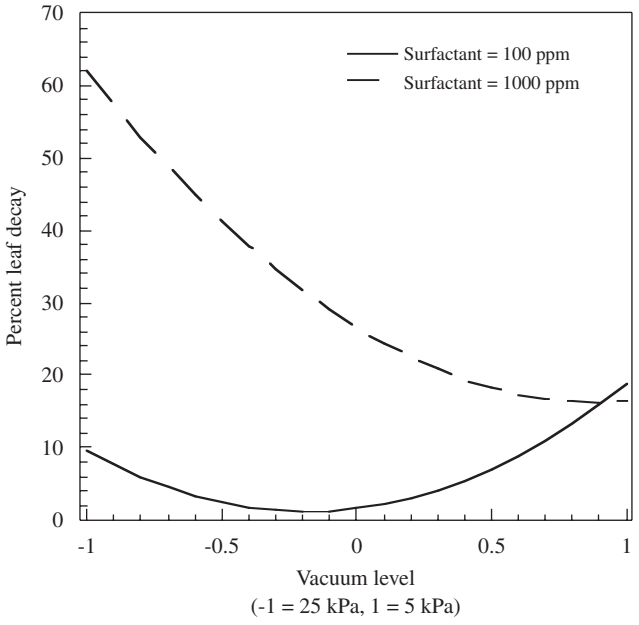

Fig. 5 – Expected percent leaf decay estimated using a reduced form of Eq. (2) and parameter estimates from response surface experiment after 6 days of incubation (Table 6). Lines represent decay under constant surfactant concentrations.

interrupted by an intron so it is possible that GUS was expressed by both the plant and *Agrobacterium*.

Surfactant and vacuum play several possible roles in the infiltration process. Surfactants lower the surface tension between the cell suspension and leaf surface, but may also wound the leaf by permeabilizing the cuticle and solubilizing plasma membranes as has been shown in herbicide formulation and delivery studies [17,18]. Such phenomena might allow better access and infection of plant cells by *A. tumefaciens*. Vacuum application likely evacuates plant stomata cavities, leaving sites for bacterial entry upon release of vacuum. The rapid increase in pressure associated with vacuum release may also damage the plant tissue and provide additional entry sites for bacteria. While wounding could result in the production of compounds that induce *vir* genes and improve expression [19], the presence of the helper plasmid pCH32 [14] in C58C1 makes such induction unnecessary. In other studies with C58C1, the addition of the *vir* gene-inducing compound acetosyringone during cultivation and infiltration had no effect on transient GUS expression in lettuce [20].

In the absence of surfactant, switchgrass leaves appeared to repel the cell suspension. The surfactant Break-Thru, a wetting agent used in agricultural chemical tank mixes, was examined for improving contact of the leaf surface with the *Agrobacterium* suspension. An interaction between surfactant concentration and vacuum level was consistently detected among independent experiments. In general, when vacuum application was low (25 kPa), increasing surfactant concentration improved expression. However, when vacuum application was high (5 kPa), increasing surfactant concentration reduced expression. The interaction became more pronounced and significant with increasing incubation time. This suggests that additional surfactant may have been required to break the surface tension between the plant and cell suspension interface for cell infusion at low vacuum levels. Alternatively, if surfactant and vacuum wound the plant, assisting with cell infection, higher surfactant concentrations might be required to balance the low frequency of wounding at lower vacuum levels. The combination of high surfactant (1000 ppm) and high vacuum (5 kPa) resulted in relatively low short-term and long-term expression of GUS, indicating that the combination was too severe for infiltration. For vacuum infiltration of lettuce at 25 kPa, addition of Break-Thru at levels used in tank mixes (1000 ppm) caused physical deterioration of the leaves. GUS expression also decreased from approximately 8000 U g dw^{-1} at 100 ppm Break-Thru to 150 U g dw^{-1} at 1000 ppm Break-Thru. At levels of 100 ppm or less, Break-Thru did not have a consistent effect on GUS expression in agroinfiltrated lettuce. Leaf decay measured after extended switchgrass incubation time did increase with increasing surfactant concentration. In contrast to reports with lettuce, expression in switchgrass appeared to increase with leaf decay and decay was significantly correlated with expression.

Vacuum application was examined for agoinfiltration of switchgrass because it was required for high transient transgene expression in lettuce [11]. Increasing vacuum application reduced short-term (3 day) expression in switchgrass at both low and high surfactant concentrations, but had a positive effect on long-term (6-day) expression at low surfactant concentrations. Plant decay decreased with increasing vacuum application. The lack of an effect of vacuum on expression at long incubation times and the absence of plant decay at high vacuum levels suggests that if the leaves were wounded by excessive vacuum application they likely recovered from any associated trauma. While vacuum infiltration was required for lettuce, we have observed that excessive vacuum reduces and delays transgene expression (unpublished data). We believe that this is due in part to plant cell damage and flooding of the stomata cavities reducing plant cell respiration. While switchgrass is less fragile and likely more resilient to the infiltration environment compared with lettuce, excessive vacuum appears to have a negative effect on transient expression in switchgrass.

Despite uniform expression of GUS over the leaf, expression levels measured by extracting GUS from leaves and performing assays on the extract indicated very low concentrations of GUS. Concentrations were several orders of magnitude lower than those reported in other transient expression systems. While expression in switchgrass was likely lower than these other systems, other explanations for the low concentration of GUS in extracts include poor extraction efficiency and low stability of GUS in switchgrass extracts. Switchgrass leaves were very difficult to grind and for this reason extracted pulps may have retained a significant amount of the expressed protein. Joh and co-workers showed that under certain conditions GUS was very unstable in plant extracts [12]. While the buffer used here for extracting GUS from switchgrass worked well for stabilizing GUS in lettuce extracts, it may not have been appropriate for switchgrass extracts. Further research is needed in this area if protein extracts are

to be used to quantify *in planta* protein expression in switchgrass.

5. Conclusions

Transient GUS expression in harvested switchgrass leaves was accomplished by agroinfiltration. Surfactant was necessary for improving contact of the leaf surface with cell suspension and consistently improved expression when vacuum application levels were low (25 kPa). At high vacuum levels (5 kPa) high surfactant concentration reduced short-term and long-term expression. When a cell suspension of *A. tumefaciens* containing 1000 ppm Break-Thru surfactant was added to harvested leaves and 25 kPa vacuum applied for 20 min, fairly uniform expression was visualized across the leaf surface within 2–3 days of incubation, suggesting that this is a viable, rapid tool for examining expression of transgenes in switchgrass leaves.

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