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Full Length Research Paper

Effect of *Agrobacterium* culture and inoculation density on transformation efficiency of a citrange (*Citrus reticulata x Poncirus trifoliata*)

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This study focused on the effect of two factors, initial culture density and subsequent inoculation density on the transformation efficiency of citrus rootstock US-812 (*Citrus reticulata* x *Poncirus trifoliata*) epicotyl explants. In the first experiment, *Agrobacterium* EHA105 containing pBINGUSint was grown in YEP medium to an OD_{600} of 1 and glycerol stocks made and stored at -80°C. The three factors incubation time (18 to 24 hours), glycerol stock dilution (1/10, 1/100, 1/1000), and temperature (26 or 28°C) were simultaneously varied and a response surface for OD_{600} was generated for culture density in baffled and unbaffled flasks. The resulting polynomial models for both flask types were highly significant with R^2 values of 0.98; this means that the polynomial model can be used to specify the stock dilution, temperature, and flask type to achieve a target density in a given amount of time (accurate from 18 to 24 h). In a second set of experiments, transformation efficiency of juvenile epicotyl explants was assessed from treatments in a 2x2 factorial arrangement of *Agrobacterium* culture OD_{600} (0.1 or 1) and inoculation density OD_{600} (0.1 or 1) using the GUS reporter gene. The polynomial model developed in the first experiment was used to determine the conditions to achieve each factorial treatment combination. No significant differences in main or interactive effects were detected.

Key words: Citrus, genetic transformation, rootstocks, *Agrobacterium*, GUS.

INTRODUCTION

Transgenic citrus is an important method for producing trees with traits such as, improved fruit production/quality and disease/pest resistance that may be difficult or impossible to attain via conventional methods. To incorporate transgenic production into a citrus breeding program requires the methodology for producing transgenics to be of sufficient efficiency and economy to produce requisite numbers of plants for laboratory, greenhouse, and field testing at realistic costs.

Agrobacterium-mediated transformation is the predominant method for producing transgenic citrus (Moore et al., 1992; Kaneyoshi et al., 1994; Peña et al., 1995; Yu et al., 2002; Dutt and Grosser, 2009; Costa et al., 2002).

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Given the large number of factors that potentially affect the efficiency of *Agrobacterium*-mediated transformation, we focused this study primarily on the effects of two factors, initial culture density and inoculation density on transformation efficiency. To illustrate this idea consider two cultures each grown to two different densities, and then brought to the same inoculation density – will each culture result in the same transformation efficiency? Inoculation density is commonly included in studies to improve transformation efficiency (Peña et al., 1995; Yu et al., 2002; Dutt and Grosser, 2009; Costa et al., 2002; Dutt and Grosser, 2009). Culture density has been shown to be potentially significant (De Clercq et al. 2002), but is not generally considered. Single factor experiments that vary only inoculation density, cannot determine the main effect of culture density or if it interacts with inoculation density. We tested this idea on US-812 (Bowman and Rouse, 2006), a citrus rootstock used in commercial ci-

trus production in Florida, USA.

MATERIALS AND METHODS

Seed germination

Fruit from 12-year old field-grown citrus rootstock US-812 (*Citrus reticulata* x *Poncirus trifoliata*) trees grown in Lake County, Florida, USA, were used as the source of seeds. Seeds were extracted in February, 2007 by manual extraction, treated with 1% (w/v) hydroxyquinoline sulphate, and stored in plastic bags at 4 °C. To disinfest the seeds the seed coats were first removed, the seeds were then incubated in 30% bleach (5.25% w/v sodium hypo-chlorite) for 30 min, followed by an 18 h presoak in water, and then the seeds were sown onto MT medium solidified with 8% (w/v) agar in Magenta GA-7 vessels (Magenta Corporation, Chicago, IL) at nine seeds per container. Seeds were germinated and grown in the dark at 27°C.

Bacterial strain, DNA construct and culture conditions

Agrobacterium tumefaciens strain EHA-105 strain (Hood et al., 1993) carrying the pBINGUSint binary plasmid (Vancanneyt et al., 1990) carrying the marker gene -glucuronidase (GUS) was used in all experiments. Bacterial stock cultures were made by selecting a single colony on a streaked YEP plate (10 g/L peptone, 5 g/L NaCl, 10 g/L yeast extract, 10 g/L agar, and pH 7), inoculating 50 mls of liquid YEP in a 125 ml flask with the colony, and culturing on a rotary shaker (225 rpm) at 27 °C until the culture achieved an OD_{600} of 1. *Agrobacterium* glycerol stocks were made using 8 parts culture + 2 parts 80% (v/v) glycerol, flash freezing in liquid nitrogen, and storing at -80 °C. *Agrobacterium* from glycerol stocks were used in all experiments.

Agrobacterium culture density curve and transformation experiments

One hundred microliters of *Agrobacterium* diluted glycerol stock were inoculated into 50 mls of YEP medium in a 125 ml flask, placed onto a rotary shaker at 225 rpm, and in the dark. The experiment was designed as a response surface with three numeric factors – incubation time, dilution, and temperature (note that due to logistical constraints temperature was treated as a categoric factor). The factor 'incubation time' ranged from 18 to 24 h and was the time when an OD_{600} reading was taken. The factor 'dilution' was the dilution of the glycerol stock using YEP medium and was 1/10, 1/100, or 1/1000; one hundred microliters of these dilutions were used to inoculate the culture flasks. The factor 'temperature' was either 26 or 28°C. Lastly, the culture density experiments were run in 125 ml unbaffled and baffled flasks.

Treatment combinations (experimental design points) were selected using modified D-optimal criteria to satisfy a quadratic polynomial; the treatment design points are listed in Table 1. The experiment included 9 model points, 10 lack-of-fit points, and 6 points to estimate pure error for 25 treatment design points. Each treatment design point was estimated from the average of three 125 ml flasks. Thus, the experiment, per flask type, utilized seventy-five flasks.

Transformation

One centimeter long US-812 epicotyl explants from 21 day old dark-grown seedlings were treated with *Agrobacterium* at a culture density of OD_{600} 0.1 or 1 and an inoculation density of OD_{600} 0.1 or 1 in a factorial arrangement. Each treatment combination was conducted three times and included 125 explants. Two responses were measured, 1) the number of explants that formed shoots and 2) the number of shooting explants that had GUS^+ shoots; explants with multiple GUS^+ shoots were counted as one transformation event. Transformation generally followed the protocol of Orbovi and Grosser (2006) and was conducted as follows:

i) Inoculate YEP media + 100mg/L kanamycin + 100 um acetosyringone with Agrobacterium glycerol stock culture. Grow overnight on a shaker at 225 rpm.
ii) Pellet cells and re-suspend in MSB3 (MS salts and vitamins, 3% (w/v) sucrose, 3 mg/L BA) shoot regeneration medium.
iii) Cut etiolated seedling epicotyls into 1 cm pieces and place in *Agrobacterium* solution for 20 min then blot explants on sterile filter paper.
iv) Plate explants onto CM1 (MS salts and vitamins, 3% (w/v) sucrose, 1 mg/L 2,4-D, 0.1 mg/L NAA, and 3 mg/L BA) co-culture medium. Place plates in the dark at 24°C for 3 days.
v) Triple rinse explants in sterile water and blot on sterile filter paper. Plate onto MSB3 + 100 mg/l kanamycin + 250 mg/l cefotaxime + 250 mg/l vancomycin.
vi) Place the plates in a dark growth chamber at 27 °C for two weeks and then move to 16/8 photoperiod (41-58 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 27 °C for shoot regeneration.

Explants were subcultured monthly for three months, and then regenerated shoots were assayed for GUS activity by histochemical staining (Jefferson, 1987).

Data analysis

For the *Agrobacterium* culture density experiment the software application Design-Expert® 7 (Stat-Ease, Inc, Minneapolis, MN) was used for experimental design construction, model evaluation, and analyses. Response surface methods (RSM) were used in this study for two reasons. First, RSM are more efficient than factorial designs for determining the effects of multiple quantitative factors. Second, RSM provides a highly graphical map generated from a polynomial model (contour plots in this paper) that intuitively summarizes the relationship between the factors varied and the responses measured. The result is a tool that researchers can effectively use without having to have a complete understanding of the sometimes complex mathematical underpinnings of the technique. Third, RSM is a tool to identify the combinations of multiply factors that result in the "best" (as defined by the application) response. The result is that RSM provides not only scientific information of the relationship between factors varied and responses measured, but specifies the factor levels to achieve an optimum response.

Detailed descriptions of the statistical methods used to analyze the response surface data can be found in (Niedz and Evens, 2007; Evens et al., 2008). Briefly, all possible models from the mean to cubic polynomial were calculated. Initial model selection was based on a battery of model adequacy tests (Anderson and Whitcomb, 2005). Normality and constant variance were deter-mined graphically via normal probability plots of residuals; Box-Cox plots were used to identify, if required, the necessity and type of data transformation (Box and Cox, 1964). Overly influential data points were identified with DFFITS and DFBETAS plots (Belsley et al., 1980). Potential outlier points were checked with externally studentized "outlier-t" (Weisberg, 1986) and Cook's Distance (Cook and Weisberg, 1982) graphical plots. R^2, adjusted-R^2 (R^2_{adj}), and predicted-R^2 (R^2_{pred}), were estimated for each selected model (Myers and Montgomery, 2002).

For the transformation experiment, the number of explants that had shoots and the number of shooting explants that were GUS^+ were converted to percentages to adjust for differences in explant

Table 1. Treatment points and data for the OD_{600} values in unbaffled and baffled flasks. Experiment is a three-factor response surface. Each treatment design point represents the mean of three duplicate flasks.

Treatment	Factors			unbaffled flask	baffled flask
design points	Incubation time (h)	Dilution	Temperature (ºC)	OD_{600}	OD_{600}
1	18.0	0.100	26	2.38	3.11
2	24.0	0.001	28	2.32	2.76
3	18.0	0.100	26	2.24	3.17
4	21.0	0.100	26	2.84	3.84
5	24.0	0.010	26	2.82	3.43
6	18.0	0.001	26	0.20	0.18
7	18.0	0.001	28	0.54	0.54
8	21.0	0.100	28	3.02	4.69
9	18.0	0.001	28	0.52	0.68
10	24.0	0.100	26	3.20	4.34
11	24.0	0.001	26	1.69	1.70
12	18.0	0.001	26	0.12	0.24
13	24.0	0.100	28	3.38	4.53
14	24.0	0.001	28	2.21	2.93
15	24.0	0.010	28	3.06	3.75
16	21.0	0.001	26	0.61	0.59
17	18.0	0.100	28	2.56	3.51
18	18.0	0.100	28	2.36	3.69
19	18.0	0.010	28	2.09	2.26
20	19.5	0.010	26	1.96	2.12
21	21.0	0.010	28	2.66	3.10
22	18.0	0.010	26	1.38	1.44
23	22.5	0.010	26	2.65	3.02
24	19.5	0.010	28	2.39	2.71
25	22.5	0.010	26	2.23	2.98

number. The data were analyzed by ANOVA as a two-factor factorial with two levels.

RESULTS

Prior to designing these experiments, a culture density curve was constructed for EHA-105 strain carrying the pBINGUSint binary plasmid and is presented in Figure 1. A culture density of OD_{600} of 1 occurred at the approximate midpoint of the log phase of growth. Thus, the glycerol stock cultures were made from cultures grown to this density.

Agrobacterium culture density curve

The OD_{600} values for *Agrobacterium* culture density ranged from 0.121 – 3.376 for unbaffled flasks and 0.181 – 4.693 for baffled flasks (Table 1). For OD_{600}_unbaffled, the best fitting polynomial was a reduced quadratic model obtained by backward elimination; for OD_{600}_baffled the best fitting polynomial was a 2-factor interaction model. Summaries of the ANOVA, lack-of-fit test and three R^2 statistics, and coded regression coefficients for OD_{600}_unbaffled and OD_{600}_unbaffled are presented in Tables 2 and 3, respectively.

A Box-Cox power transform plot analysis indicated that data transformation was not required for either the OD_{600}_unbaffled or the OD_{600}_baffled data. The residual and model diagnostics were within acceptable limits (Anderson and Whitcomb, 2005). The lack-of-fit test was not significant ($p = 0.5854$) for OD_{600}_unbaffled, but it was significant ($p = 0.0055$) for OD_{600}_baffled. R^2, R^2_{adj} and R^2_{pred} statistics ranged from 0.97 – 0.98 for OD_{600}_unbaffled and 0.96 – 0.98 for OD_{600}_baffled. The overall models for both responses were highly significant ($p < 0.0001$), indica-ting incubation time, inoculation dilution, and temperature significantly affected OD_{600}. The ANOVA for OD_{600}_unbaffled contained six significant terms; four of the terms, incubation time, dilution, temperature, and temperature^2 had highly significant p-values (that is, < 0.0001; Table 2). The ANOVA for OD_{600}_baffled contained four significant terms; three of the terms, incubation time, dilution, and temperature had highly significant p-values (that is, < 0.0001; Table 3). Examination of the F-values indicates that dilution has the single largest influence on OD_{600}. Contour plots of final OD_{600} for unbaffled/26 °C, unbaffled/28°C,

Table 2. ANOVA, regression coefficients, and summary statistics for culture density (that is, OD_{600}) of *Agrobacterium* EHA105-P20 in unbaffled flasks.

Source	F-value	p-values	Regression coefficients[c]
Model	186.37	< 0.0001	
Incubation Time	336.30	< 0.0001	+ 0.62
Dilution	585.50	< 0.0001	- 0.87
Temperature	50.30	< 0.0001	+ 0.20
Hours x Dilution	19.36	0.0003	+ 0.18
Dilution x Temperature	7.36	0.0142	+ 0.095
Temperature^2	43.82	< 0.0001	- 0.39
Lack of Fit	p = 0.5854		
R^2	0.98		
R^2 adj	0.98		
R^2 pred	0.97		
Model type	reduced quadratic[b]		
Transformation[a]	None		

[a] Determined by a Box Cox plot analysis.
[b] Model reduction by backward elimination.
[c] Presented in coded form. Coding normalizes the factor ranges by placing their low and high range value between -1 and +1 and can thus be directly compared. Final equation in coded terms: OD_{600} in unbaffled flasks = 2.38 + 0.62 * hours - 0.87 * dilution + 0.20 * temperature + 0.18 * hours * dilution + 0.095 * dilution * temperature - 0.39 * dilution^2.

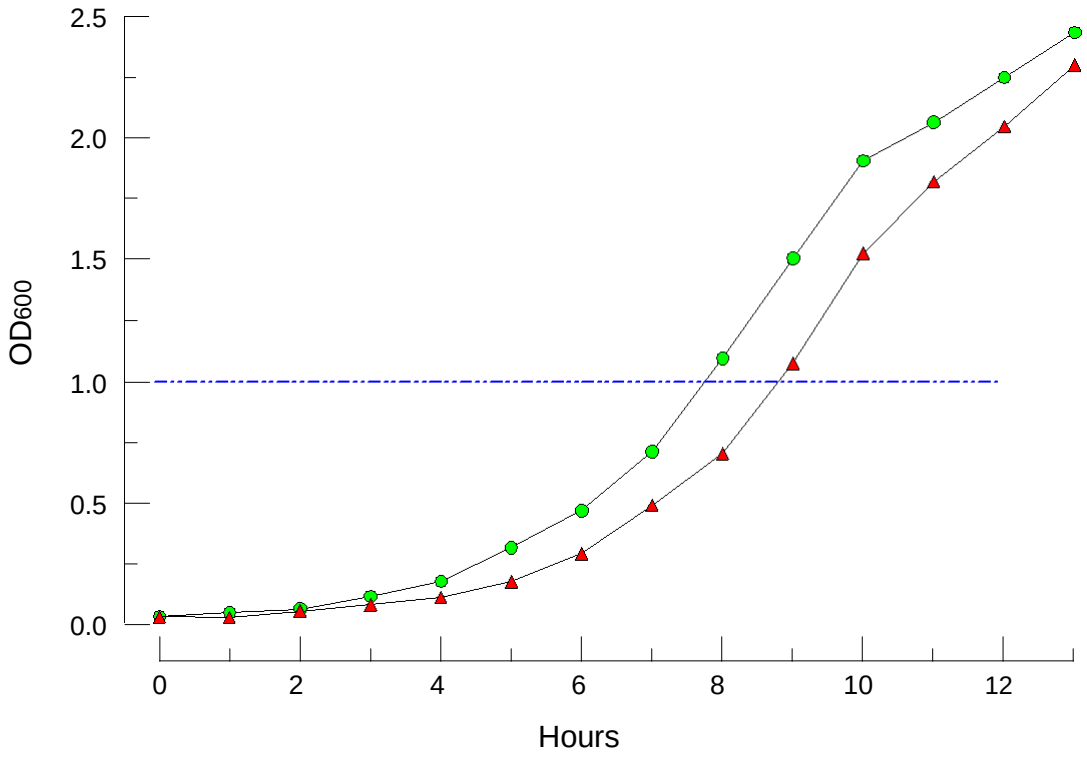

Figure 1. OD_{600} curve over incubation time. OD_{600} values were measured for *Agrobacterium* strain EHA105 carrying binary plasmid pBINGUSint grown over thirteen hours. Two starter cultures were grown to OD_{600} of 1.26 ( ) and 0.79 ( ), then 1 ml of each of these cultures was added to 50 mls of YEP media, and OD_{600} readings taken over thirteen hours. The OD_{600} of 1 was approximately at the midpoint of both curves and was the basis for selecting this density for the glycerol stocks.

Table 3. ANOVA, regression coefficients, and summary statistics for culture density (that is, OD_{600}) of *Agrobacterium* EHA105-P20 in baffled flasks.

Source	F -value	p-values	Regression coefficients[c]
Model	202.60	< 0.0001	
Incubation Time	214.03	< 0.0001	+ 0.81
Dilution	519.04	< 0.0001	- 1.34
Temperature	42.29	< 0.0001	+ 0.30
Hours x Dilution	8.68	0.0003	+ 0.19
Lack of Fit	p = 0.0055		
R^2	0.98		
R^2 adj	0.97		
R^2 pred	0.96		
Model type	reduced 2FI[b]		
Transformation[a]	None		

[a] Determined by a Box Cox plot analysis.
[b] Model reduction by backward elimination.
[c] Presented in coded form. Coding normalizes the factor ranges by placing their low and high range value between -1 and +1 and can thus be directly compared. Final equation in coded terms: OD_{600} in baffled flasks = 2.71 + 0.81 * hours -1.34 * dilution + 0.30 * temperature + 0.19 * hours * dilution.

Table 4. Final equations for *Agrobacterium* culture density models.

OD_{600} in unbaffled flasks at 26^{o} C (actual terms) =	-0.3368 + 0.1472* incubation time - 1.4168* dilution + 0.0587* incubation time * dilution - 0.3883 * $dilution^2$
OD_{600} in unbaffled flasks at 28^{o} C (actual terms) =	-0.1294 + 0.1472* incubation time - 1.2267* dilution + 0.0587* incubation time * dilution - 0.3883 * $dilution^2$
OD_{600} in baffled flasks at 26^{o} C (actual terms) =	-0.5269 + 0.2040* incubation time -2.6972* dilution + 0.0645* incubation time * dilution
OD_{600} in baffled flasks at 28^{o} C (actual terms) =	+0.0728 + 0.2040* incubation time - 2.6972* dilution + 0.0645* incubation time * dilution

baffled/26°C, and baffled/28°C are shown in Figure 2; the polynomial equations in actual terms that were used for the ANOVA and in the construction of the contour plots are listed in Table 4.

The contour plots in Figure 2 show the relationship between glycerol stock dilution, incubation time, temperature, and type of flask. The relationship is most easily seen using the colors in the contour plots. The culture density increases as the colors move from blue to red. For example, any region in any of the four plots that is blue has a low culture density; and for red, the greatest culture density. In addition, colors are comparable among the four plots. If a density corresponding to the yellow zone is required, then the contours graphically show under what conditions and how long it will take to achieve that particular density. The yellow zone in the four plots can be examined to determine what combinations will work.

*Agrobacterium-m*ediated transformation

An explant was scored as GUS^+ if it had at least one shoot that stained blue. Figure 3 shows the three types of responses observed – GUS^- shoots, GUS^- and GUS^+ shoots, and GUS^+ shoots (Figure 3). Explants that were GUS^+ commonly had both GUS^- and GUS^+ shoots. The ANOVA for the 2-factor factorial was not significant for either response – percentage explants that formed shoots (p = 0.8531) or the percentage shooting explants with GUS^+ shoots (p = 0.9075). For percentage explants that formed shoots, a log transformation of the data was conducted as suggested by a Box- Cox power transform plot analysis; percentage shooting explants with GUS^+ shoots required no data transformation. Residual and model diagnostics were within acceptable limits. A summary of the transformation data is presented in Table 5.

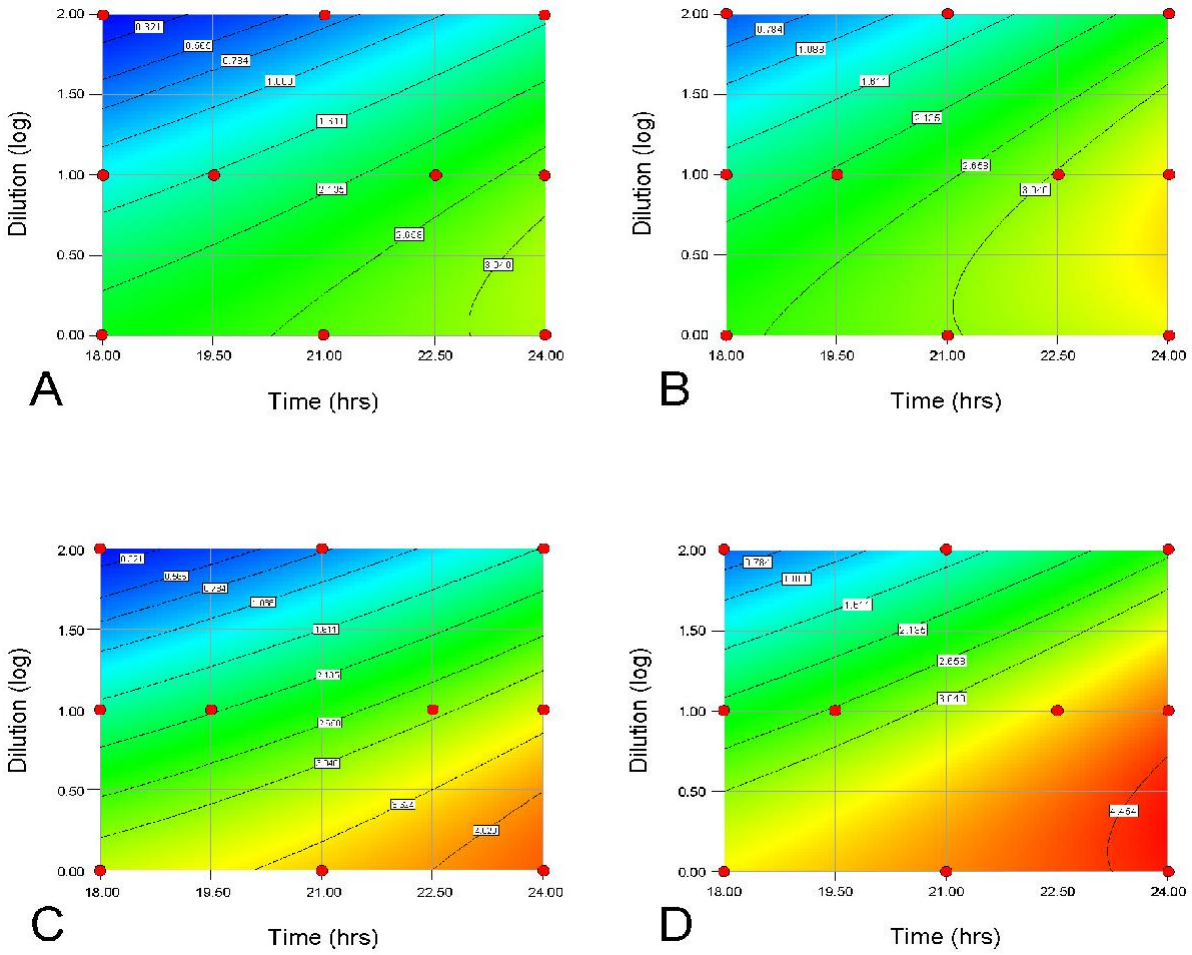

Figure 2. OD_{600} time x dilution contour plots for *Agrobacterium* culture density in unbaffled and baffled flasks. A) unbaffled flask at 26C; B) unbaffled flask at 28C; C) baffled flask at 26C; D) baffled flask at 28C. Dilution was in logs but coded as 0 = 1/10, 1 = 1/100, and 2 = 1/1000.

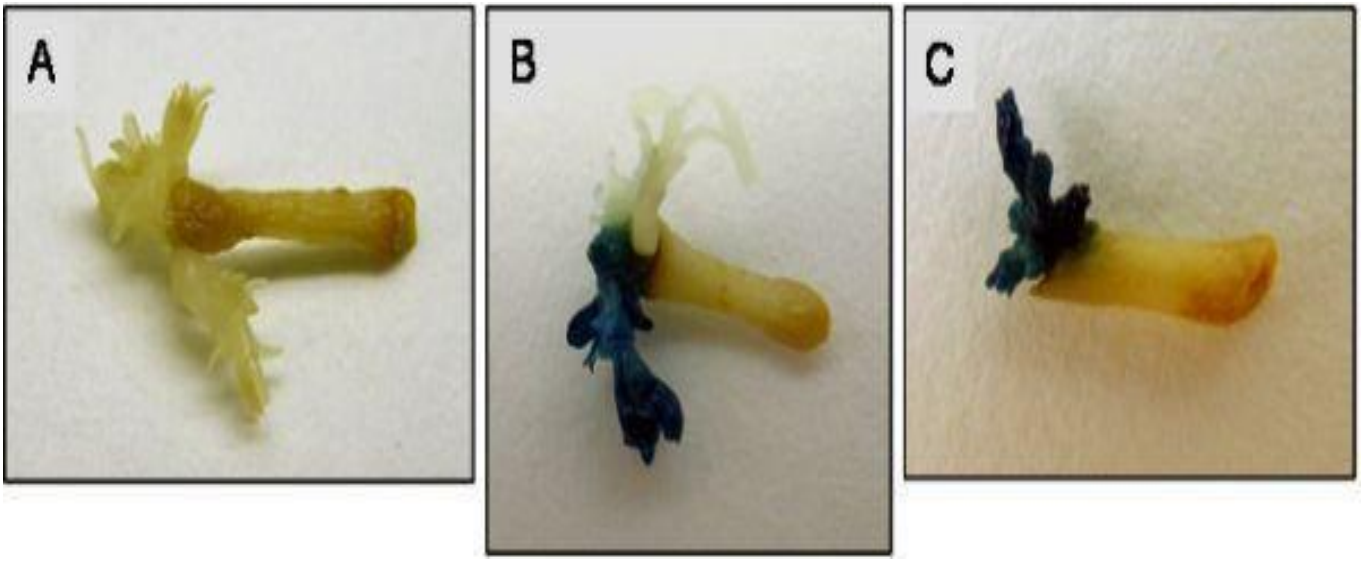

Figure 3. Shoot transformation types observed. A) GUS^- shoots only; B) both GUS^+ and GUS^- shoots; C) GUS^+ shoots only.

Table 5. The effect of culture and inoculation densities on transformation efficiency of the citrange rootstock US-812.

Culture density OD_{600}	Inoculation density OD_{600}	Number of explants[a]	Number of explants with shoots (%)[b]	Number of explants with GUS^+ shoots (%)[b]
0.1	0.1	335	58 (17.3 %)	33 (9.9 %)
0.1	1	351	41 (11.7%)	22 (6.3 %)
1	0.1	376	54 (14.4 %)	39 (10.4 %)
1	1	334	39 (11.7 %)	28 (8.4 %)

[a] Total number of explants from three replications.
[b] Average number and percentage from three replications.

DISCUSSION

The effects of *Agrobacterium* culture density and inoculation density on the transformation of the citrus rootstock variety US812 were determined. Culture density was used as a convenient empirical proxy of the growth phase of the bacteria and would be the method typically used in laboratories using *Agrobacterium* for genetic transformation. It was unknown to what extent culture density would affect or interact with inoculation density. The basic approach was to develop a "growth" model, using culture density, for *Agrobacterium*. The growth model provided the information required to grow the *Agrobacterium* to the two specific growth densities in a specified amount of time. This facilitated conduct of the transformation experiment where it was necessary to have two *Agrobacterium* cultures ready at the same time but at two different densities.

Pre-calibrated *Agrobacterium* glycerol stock cultures were used because of the variability in growth observed in our previous experiences with single colony selection from *Agrobacterium* stock plates. We think that the use of pre-calibrated *Agrobacterium* glycerol stocks contri-buted to the highly accurate and uniform ability of the growth models to predict OD_{600} 18-24 h after inoculation.

The growth model was developed from three factors – incubation time, dilution, and temperature. The type of flask (baffled or unbaffled) was treated separately, though it could have been included as a categoric factor. Baffled and unbaffled flasks were included with the idea that growth would be significantly different in the two flasks, probably due to aeration differences, and that the researcher could use this effect for even greater control in growing *Agrobacterium* to a specified target density in a certain amount of time. Incubation time ranged from 18-24 and were selected based on two criteria, 1) *Agrobacterium* grows relatively slowly (vs. *Escherichia coli*) and overnight culture is typically required, and 2) to have the *Agrobacterium* ready by mid to late morning, a time convenient for transformation experiments as it allows for setup and explant preparation. For example, if explants are prepared and ready for inoculation by 10:00 am, then by varying the temperature, dilution, and/or type of flask (unbaffled or baffled) the *Agrobacterium* cultures can be initiated anytime from 10:00 am to 4:00 pm the preceding day to achieve a specific, final culture density. The temperatures, 26 and 28°C, were selected for practical reasons; 26°C was the temperature of our large culture room and allowed use of open tabletop rotary shakers, whereas 28°C is the standard temperature used to grow *Agrobacterium*. The dilution factor covered three orders of magnitude from 1/10 to 1/1000 and had the single largest main effect on final OD_{600}. It should be noted that the relatively small effect of temperature observed in these studies is primarily due to the narrow temperature range examined. It is quite likely that a broader temperature range would have increased the relative contribution of its effect on OD_{600}.

Because the growth model was developed with one *Agrobacterium* strain/binary plasmid combination, there is no reason to expect it to predict the growth of other strain/binary combinations with the same accuracy – that is, the term coefficients would vary. However, we think that the conclusions from the experiments presented here would remain unchanged and that running these factors over the same ranges on a different strain/binary would have similar effects – that is, the relative relationships of the term coefficients would be unchanged and the factor dilution would still have the largest effect.

We did not detect any significant main or interactive effects on transformation efficiency by Agrobacterium culture density and inoculation density. Four related studies in citrus provide additional information to help interpret our results. Using the rootstock 'Carrizo' citrange, Peña et al (1995) ran a 2x2 factorial experiment that included Agrobacterium inoculation density (107 or 108 cells ml-1) and two types of coculture; culture density was fixed. They observed a strong interaction between inoculation density and type of coculture. Yu et al (2002) ran a single factor experiment that varied inoculation density (OD600 varied from 0 to 0.6) and determined th effect on transformation frequency of 'Carrizo' citrange and 'Xuegan' sweet orange. The culture density was variable but was less than 0.6. Transformation efficiency was inversely related to OD600 for both genotypes and was significantly improved at OD600 levels < 0.2. Costa et al. (2002) ran a single factor experiment that varied inoculation density (2, 3, 4, and 5x108 cfu ml-1) and exa-

mined its effect on transformation frequency of 'Duncan' grapefruit. No significant differences were detected. Dutt and Grosser (2009) ran a single factor experiment that varied inoculation density (OD_{600} 0.15, 0.3, and 0.6) and its effect on transformation frequency of four citrus genotypes – 'Carrizo' citrange, 'Duncan' grapefruit, 'Hamlin' sweet orange, and 'Mexican' lime. Genotypic differences were observed where higher OD_{600} resulted in higher transformation efficiencies for 'Carrizo' and the reverse for the other three genotypes. They also observed a higher transformation frequency when the *Agrobacterium* were subcultured 3 h prior to transformation, indicating a possible growth phase effect.

Taken together, result differences can be attributed to differences in genotypic responses, interacting genotype x cultural factors, variations in uncontrolled factors (e. g, culture density), and differences in factor ranges. We have demonstrated that *Agrobacterium* culture density is easily controlled and highly predictable. By controlling this factor, future experimentation will benefit and a clearer understanding of the role of *Agrobacterium* growth phases and densities will result.

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