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Research Article

Evaluation of fluorescence-activated cell sorting technology in agrobacterium biocontrol

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Abstract: Fluorescence-activated cell sorting (FACS) as a novel and sensitive technology was used to evaluate the biocontrol efficiency of *Bacillus subtilis* against *Agrobacterium tumefaciens,* a very destructive plant pathogen. The combination of two methods of culturing and cell sorting by FACS technology was used to distinguish a rapid and accurate method in monitoring the biocontrol effect of *Bacillus* (ATCC21332) on *Agrobacterium* (IBRC-M10701 and AGL1),. The culture method indicated that the *B. subtilis* could suppress *A. tumefaciens in vitro* and *in vivo*. We used a green fluorescent protein (GFP), reporter, to flow cytometric analysis using FACS. The mean of GFP expression levels was significantly reduced to 17.98, 16.48, and 11.27% in treatments 24, 48, and 72 h post-treatment; however, it was 31.57, 26.06, and 23.98% in the nontreated ones. The experiments demonstrated a positive biocontrol effect of *Bacillus* against *Agrobacterium*. Overall, our findings may provide a basis for improving the new rapid biocontrol agent detection method based on FACS.

Keywords: *Agrobacterium* spp., *Bacillus* spp., fluorescence-activated cell sorting (FACS)

Introduction

Fluorescence-activated cell sorting (FACS) technology is a type of flow cytometry that classifies and identifies cells according to specific light scattering and fluorescent properties. Fluorescent-based cell sorting and counting is a valuable scientific tool as it enables the rapid and accurate recording of fluorescent signals from each cell (Czechowska *et al.,* 2008). In situ detection and quantification of fluorescence from GFP-expressing reporter cells were mainly done by fluorescence microscopy combined with digital image analysis. However, this approach is laborious, and accurate reporter quantification is often hampered by the highly heterogeneous expression and distribution patterns occurring in bacterial populations colonizing natural environments. FACS-based flow cytometry is a powerful alternative to overcome some of these limitations because it allows an extensive assessment of population heterogeneity at the single-cell level by measuring the optical properties of tens of thousands of individual cells within a short time. Thus far, studies involving environmental bacteria have used flow cytometry mainly to enumerate GFP-tagged bacterial cells and determine their physiological state (Czechowska *et al.,* 2008). In 2010, Rochat *et al*. (2010) used this technology to investigate the biocontrol effect of *Pseudomonas* on the root of cereals. They simultaneously investigated the rate of

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colonization and production of antifungal compounds by *Pseudomonas fluorescens* CHA0 on wheat roots. Schmitd *et al.* (2006) used the above technology to count bacterial populations as a rapid and accurate method. They determined the populations of *Klebsiella oxytoca*, *Serratia marcescens,* and *Escherichia coli* using fluorescence dyes and the above technology.

Agrobacterium causes a tumorous disease in most plants called crown gall, a very destructive plant disease that reduces infected plants' yield by up to 40% (Schroth *et al.,* 1988). The disease has been reported in the Middle East, Japan, North and South America, South Africa, China, and several European countries (Burr *et al.,* 1998). The use of microbial antagonists to control plant diseases is a relatively new development and represents an important contemporary aspect of agricultural technology. The advantages of using biological control, compared to chemical control, would be (a) low cost and (b) minimal environmental impact since biological control agents have minimal implications in terms of an alteration of the general environment. A non-pathogenic strain of agrobacterium *A. radiobacter* (K84) has been reported as an agent for the biological control of crown gall disease (New and Kerr, 1972; Kerr, 1980). Multiple *Bacillus* spp strains have been exploited as biopesticides for plant disease control and stimulate plant defense responses (Fravel, 2005). In 1991, Hassanein *et al.* (1991) showed that *B. subtilis* suppresses *A. tumefaciens* in vitro and in vivo. The antagonistic mechanism of *B. subtilis* is by antibiotic production (Chang and Commedahl, 1968; Weinhold and Bowman, 1968), competition (Utkhede and Rahe, 1980), or both (Cubeta *et al.,* 1985). About 5-7% of the *B. subtilis* genome is dedicated to the antibiotic synthesis and can produce numerous antimicrobial compounds of different structures (Stein *et al.,* 2005). The cyclic lipopeptide surfactin might have an efficient role in triggering systemic resistance (Henry *et al.,* 2011). The increasing interest in surfactin is because of its amphiphilic character (Arima *et al.,* 1968). These compounds have potential applications in both medical and biotechnological fields (Asad *et al.,* 2010). This biosurfactant possesses antifungal and antibacterial activity (Kim *et al.,* 1998), resulting in its controlling effect by inducing apoptosis and cell cycle arrest (Cao *et al.,* 2011). *Bacillus subtilis* may be considered as an alternative to *A. radiobacter* for the biological control of crown gall disease because of the discovery of *A. tumefaciens* with resistance to *A. radiobacter*. The ability of *B. subtilis* to produce antibiotics (Waksman, 1969; Loeffler *et al.,* 1986), to form endospores that are tolerant to heat and desiccation, and to stimulate plant growth (Chang and Kommedahl, 1968; Broadbent *et al.,* 1971; Baker *et al.,* 1985) makes it particularly suitable as candidate bio-controlling agent without undesirable side effects on plant development.

In this study, we applied a combination of culture method and cell sorting by FACS that could be a model system to assess the viability of *Agrobacterium- Bacillus* cell interactions. Hence, for this study, *B. subtilis* ATCC21332 with surfactin secretion ability was used to determine if this strain has the biocontrol effect against *Agrobacterium*. We successfully quantified variations in the expression of GFP-based reporter fusions to the Agrobacterium gene in response to the biocontrol agent. Therefore, the study showed that FACS technology is a powerful tool for studies on the activity of beneficial bacteria, and it can be a new rapid biocontrol agent detection method.

Materials and Methods

Bacteria, plant, and infection

The bacteria used in this study were *A. tumefaciens* strain IBRC-M10701, purchased from the Iranian Biological Research Center, and *B. subtilis* ATCC21332, kindly provided by M. A. Marahiel (Department of Chemistry, Biochemistry, Philipps-University Marburg, Hans-Meerwein-Strasse, D-35032 Marburg, Germany). 40 ml of Nutrient Broth (NB) and Luria Broth (LB) media were inoculated with the strains ATCC21332 and IBRCM10701, respectively, and subsequently incubated in an incubator shaker at 180 rpm at 28 ºC for 24 h. The main vein of four-week-old tobacco leaves

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(*Nicotiana tabacum* L. var Xanthi) was scratched and treated with 20 µl of strain ATCC21332 with OD600 = 0.5 in water. The plants were inoculated again with strain IBRC-M10701 with the same above concentration, three days after pre-treatment with *B. subtilis*. Plants were kept in a growth chamber at 26 ± 2 ºC and 70% RH for 72 h. Three plants were used for each treatment, and water treatment as a control.

Agrobacteria population

Agrobacteria population *in vivo*

The agrobacterium population of treated plants was determined 24, 48, and 72 hours post-inoculation. After surface sterilization of the leaves with 70% ethanol in distilled water, the samples were cut into sterile Petri dishes in small portions, added to one ml of Luria–Bertani (LB) medium, and incubated at room temperature for 10 min. Serial dilution of bacteria was prepared and immediately cultured on LB agar plates containing Kanamycin antibiotic (*Agrobacterium*) and Nutrient Agar (*Bacillus*), then stored at 28 °C for 1-2 days. The bacterial population (CFU / ml) was then evaluated (Pruss *et al.,* 2008).

Agrobacteria population *in vitro*

A. tumefaciens IBRC-M10701 and *B. subtilis* ATCC21332 were inoculated in LB and NB medium, respectively, and incubated at 28 °C on a rotary shaker (180 rpm) for 24 h. Cell concentration was measured by optical density at 600 nm. Bacterial strains were separately washed with distilled water (5000 rpm, 10 min) and co-incubated with 1:1 portion (OD 600 = 0.5) in LB medium. Serial dilution was prepared (10^{-5}) and incubated on LB medium at 28 °C (Overnight). Living colonies were counted after 24 h (Pruss *et al.,* 2008).

GFP tagging of *A. tumefaciens* and co-incubation

To tag *A. tumefaciens* strain AGL1 with GFP, the construct of GFP vector (GFP-Constitutive bacteria Promoter BBa_J23100) was introduced into *Agrobacterium* AGL1 (Lazo *et al.,* 1991) by electroporation (Mersereau *et al.,* 1990). AGL1 (GFP) and *B. subtilis* ATCC 21332 inoculums were grown overnight on a shaker (180 rpm) at 28 °C in LB medium supplemented with 50 mg/l spectinomycin and NB medium, respectively. After 24 h bacterial strains were separately washed with distilled water (4000 rpm, 2 min) and co-incubated with 1:1 ratio (OD 600 = 0.4) in LB medium. Fluorescence excitement was measured after 24, 48, and 72 h co-incubation using FACS (Becton Dickinson, San Jose, Calif.).

Preparation of AGL1 cells for FACS sorting

The AGL1 cells expressing GFP were grown at 28 °C for 24 h in an LB liquid medium containing Spectinomycin (one colony inoculated into 3 ml medium containing 50 mg/ml of Spectinomycin). Wild-type AGL1 cells were incubated for 24 h in an LB-only medium. After incubation, AGL1 and GFP-expressing AGL1 cells were resuspended into PBS (ionic strength = 0.021) three times. Immediately before sorting, the cells were resuspended again into phosphate buffer (4.3 mM $Na_2HPO_4 \times 7H_2O$, 1.4 mM KH_2PO_4) containing 10^{-5} M, SDS and diluted to a concentration of 10^8 cells/ml. Fluorescence was excited by the 488 nm Blue Laser, Sapphire 20 mW, and the emitted fluorescence was detected by using the 530/30 nm filter. The Becton-Dickinson FACSDiva software (version 8.0.1) was used for data analysis.

Statistical analysis

All data were analyzed using SPSS ver 16.0 (SPSS, Chicago, IL, USA). Tukey's test and independent samples t-test were used to show statistical differences between treatment methods and controls. $p \leq 0.05$ was considered significantly different. Data are shown as mean ± standard deviation (SD).

Results

Pathogenicity and biocontrol tests in tobacco

This test was performed on 4-week-old tobacco plants according to the modified Hassanein (1991) method on stems and leaves with *Agrobacterium* and *Bacillus* bacteria. After 4 to 6 weeks of inoculation,

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symptoms including galls on stems, chlorotic leaves, and slow growth of treated plants were considered as positive. The results showed that stem gall, chlorination of leaves, and decrease in leaf surface growth rate in plants initially treated with *Bacillus* were relatively lower than ones treated with *Agrobacterium* individually.

The population of Agrobacteria in vivo

After serial dilution and suspension culture on NA and LBA media containing the relevant antibiotic on the first, third, sixth, and 30th days after treatment, the *Agrobacterium* population was counted using the related formula (Fig. 1). The results showed that the *Agrobacterium* population's reduction rate in the *Bacillus*-*Agrobacterium* combination treatment was 1.13 compared to the individual *Agrobacterium* treatment.

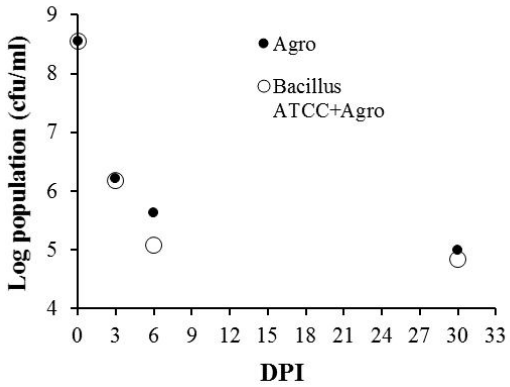

Figure 1 Measurement of *Agrobacterium* population at 1, 3, 6, and 30 days post-inoculation (DPI).

The population of Agrobacteria *in vitro*

To investigate the population of treated *Agrobacterium* in media, we counted the living colonies 24 h after co-incubation according to the mentioned protocol. Our data indicated a 92.5% reduction in the number of *Agrobacterium* cells compared to nontreated ones with *Bacillus*. The results showed a significant biocontrol effectiveness of *B. subtilis* ATCC21332 on *A. tumefaciens* IBRC-M10701. Experiments were repeated at least three times (Fig. 2).

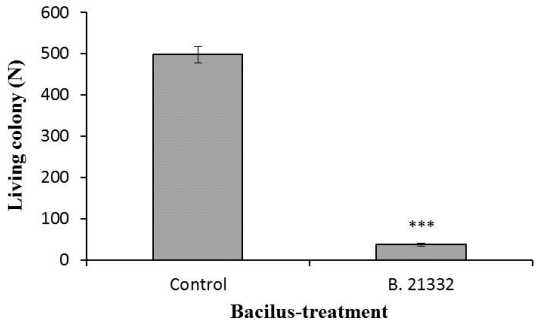

Figure 2 Number of agrobacterial living colonies counted after 24 h. Nontreated *Agrobacterium* (control) was compared with *Bacillus* ATCC21332 treated one. *** indicate significant differences at $p < 0.001$ using independent samples t-test.

FACS analysis

We have developed an experimental approach that allowed us to monitor the effect of *Bacillus* (ATCC21332) biocontrol on *Agrobacterium* using an AGL1 strain *Agrobacterium*-containing GFP vector (Constitutive bacteria Promoter BBa_J23100-GFP) and FACSbased flow cytometry. For this purpose, we tagged *A. tumefaciens* AGL1 by inserting a vector containing the GFP gene. After co-incubation of these two bacteria in equal proportions for OD600, 24, 48, and 72 h after co-incubation in LB medium, fluorescently labeled expression *Agrobacterium* light was evaluated by FACS. An example of the FACS analysis is shown in Figure 3. As a primary step, a zone P1 encompassing particles of bacterial cells was defined on the forward scatter (FSC-A)/side scatter (SSC-A) density plots (Fig. 3). The P1-gated particles include the bacterial inoculants. Treated AGL1 cells were recorded for the approximately 63,000 to 96,000 particles (events) gated in zone P1 defined on the FSC-H/SSC-H density plot (Fig. 3). In the example, the mean green fluorescence value (geometric mean) per P1gated particle is 17.98 and reflects the high expression levels average of GFP expression after 24h co-incubation with Bacillus (Table 1). The poor expression level in the treated AGL1 cells was 72h after coincubation with the values of 11.27 (Table 1). In contrast, most of the nontreated AGL1cells expressed high GFP expression levels compared with the treated ones (Table 1).

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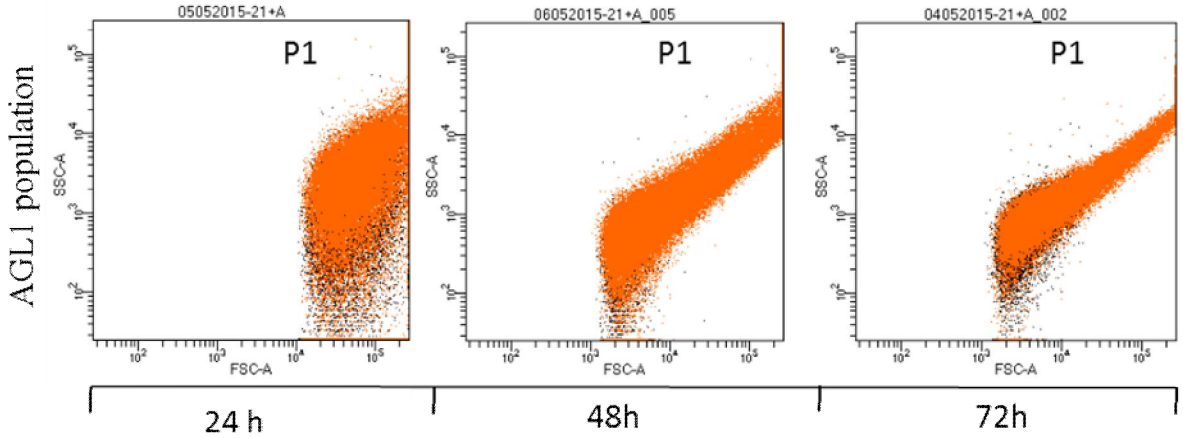

Figure 3 FACS analysis of the expression of GFP reporter in AGL1 during 24, 48, and 72 h post-treatment with *Bacillus subtilis* ATCC21332. Events detected by the forward scatter (FSC-A) and side scatter (SSC-A) detector, respectively. Data shown in histograms are gated with P1 defined on the FSC-A/SSC-A density plot.

Table 1 Expression of the GFP cell tag of *Agrobacterium tumefaciens* AGL1 during 24, 48, and 72 h post-treatment with *Bacillus subtilis* ATCC21332 strain.

Post-treated hours	Relative GFP fluorescence[1, 2]			
	AGL1 (Non-treated)		AGL1 (Treated)	
	No. of cells[3]	Expression (%)[4]	No. of cells[3]	Expression (%)[4]
24 h	92.26b	31.57a	62.79c	17.98a
48 h	96.93a	26.04b	95.72a	16.48a
72 h	92.59b	23.98c	90.01b	11.27b

[1] Values represent the means calculated from pooled data from two independent repetitions of the same experimental setup, with three replicates.
[2] Per treatment in each experiment. Values in the same column followed by different letters are significantly different according to Tukey's test ($P \leq 0.05$).
[3] Number of GFP-tagged cells per ml of medium ($\times 10^9$) = number of events gated with P1, corresponding to *Agrobacterium* cells expressing the GFP tag.
[4] Percentage of GFP-tagged *Agrobacterium* cells (P1-gated events).

The results showed that the decrease in GFP signal in *Agrobacterium* nontreated with *Bacillus* during 24, 48, and 72 hours after culture was 31.57, 26.06, and 23.98%, respectively. These values in *Agrobacterium* treated with *Bacillus* ATCC21332 strain were 17.98%, 16.48%, and 11.27%, respectively (Fig. 4). The *Bacillus* biocontrol effect on *Agrobacterium* by measuring fluorescent light expression showed a significant decrease in the mean fluorescent cells counted by FACS in *Agrobacterium* treated with *Bacillus* 24, 48, and 72 h post-treatment compared to the nontreated *Agrobacterium*, indicating a positive biocontrol effect of *Bacillus* against *Agrobacterium*. Experiments were repeated at least three times.

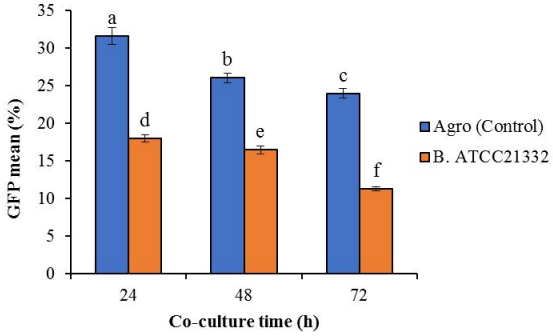

Figure 4 Comparison of average GFP produced in *Agrobacterium* and *Agrobacterium* treated with *Bacillus* ATCC21332. Different letters indicate a significant difference between averages (Tukey's test, $P \leq 0.05$).

Discussion

The recent development of monitoring tools based on autofluorescent proteins has dramatically facilitated the study of bacterial behavior and function on biocontrol. Several fluorescent dyes are available for assessing the viability of bacterial cells at a single-cell level

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without the use of cultivation methods that can be applied in epifluorescence microscopy or flow cytometry (Alvarez-Barrientos *et al.,* 2000; Berney *et al.,* 2008). In the present study, we provide a relatively robust methodology that combines the culture method and GFP reporter with FACS for studying *Agrobacterium*-*Bacillus* biocontrol interaction at the single-cell level. The technique provides several advantages. First, many cells can be easily monitored within a short period (de Werra *et al.,* 2008). Second, detection sensitivity is significantly improved compared with culture media (Schmidt *et al.,* 2006). Bacterial detection systems could be divided into culture methods like BacT/ALERT or Pall eBDS and rapid detection systems like ScansystemTM, FACS, or NAT. BacT/ALERT, Pall eBDS (Schmidt *et al.,* 2006).

To our knowledge, this is the first study detecting the biocontrol effect of *Bacillus* on the *Agrobacterium* population using FCAS. Also, our work adds a further example to the rare studies that combined sophisticated methods and green fluorescent proteins for monitoring bacterial populations by flow cytometry (Hakkila *et al.,* 2003; Sörensen *et al.,* 2003; Schmidt *et al.,* 2006; Rochat *et al.,* 2010). Another apparent advantage of the GFP tag in FACS analysis is that it allows the quantification of *Agrobacterium* populations without classical culturing methods.

Population levels recorded by FACS were more accurate than those determined by CFU counts (Table 1 and Fig. 2). This method is following previous studies in which numbers of bacterial cells from root samples able to grow on media were markedly lower than those recorded by FACS (Unge *et al.,* 1999; de Werra *et al.,* 2008; Gamalero *et al.,* 2004; Rochat *et al.,* 2010), highlighting the limits of CFU counting methods. Schmitd *et al.* (2006) used the above technology to count bacterial populations (*Klebsiella oxytoca*, *S. marcescens,* and *Escherichia coli*) as a rapid and accurate method. Our data showed a significant reduction in the mean of GFP expression levels counted by FACS in *Agrobacterium* treated with *Bacillus* compared with the nontreated ones during the co-incubation times (Fig. 4), indicating the positive biocontrol effect of *Bacillus* against *Agrobacterium*. We proved this effect by classical methods following previous studies that *B. subtilis* could suppress *A. tumefaciens* in vitro (Hassanein *et al.,* 1991). The GFP reporter FACS approach can be easily adapted for studying other biocontrol bacteria.

Overall, the FACS-based technique exposed here constitutes a new and sensitive tool for detecting viability and biocontrol effect by using detailed single-cell information about the bacterial population. A significant advantage of the presented approach is that it allows an overview of choosing the correct beneficial bacteria against the plant pathogens rapidly and accurately.

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Conflict of interests

The authors have no conflict of interest to declare.

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چکیده: مرتب‌سازی سلولی فعال‌شده با فلورسنس (FACS) یک فناوری حساس، دقیق و جدید می‌باشد. از این فناوری برای ارزیابی کارایی بیوکنترلی *Bacillus subtilis* علیه *Agrobacterium tumefaciens* که یک بیمارگر گیاهی بسیار مخرب است، استفاده شد. به‌منظور تشخیص سریع و دقیق اثر کنترلی باسیلوس (ATCC21332) علیه آگروباکتریوم (IBRC-M10701 و AGL1)، دو روش کشت محیطی و مرتب‌سازی سلولی با استفاده از فناوری FACS ترکیب گردید. نتایج حاصل از روش کشت محیطی نشان داد که *B. subtilis* قادر به کنترل *A. tumefaciens* در شرایط *in vitro* و *in vivo* است. هم‌چنین از گزارشگر GFP برای تجزیه‌وتحلیل فلوسیتومتری با استفاده از FACS استفاده شد. نتایج آنالیزهای FACS بیانگر کاهش قابل توجه میزان بیان GFP بیان شده در Agrobacterium پس از تیمار با باسیلوس طی ۲۴، ۴۸ و ۷۲ ساعت به‌ترتیب ۱۷/۹۸، ۱۶/۴۸ و ۱۱/۲۷ درصد) در مقایسه با کنترل (تیمار نشده) (به‌ترتیب ۳۱/۵۷، ۲۶/۰۶ و ۲۳/۹۸ درصد) بود. مقایسه نتایج دو روش بیانگر اثر مثبت بیوکنترلی باسیلوس علیه آگروباکتریوم بود. به‌طور کلی، یافته‌های این پژوهش می‌تواند زمینه استفاده از تکنیک سریع و دقیق FACS را در شناسایی عوامل بیوکنترلی فراهم کند.

واژگان کلیدی: آگروباکتریوم، باسیلوس، فناوری مرتب‌سازی سلولی فعال شده با فلورسنس

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