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Method Paper

A high-throughput screening assay to identify bacterial antagonists against *Fusarium verticillioides*

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A high-throughput antagonistic assay was developed to screen for bacterial isolates capable of controlling the maize fungal phytopathogen *Fusarium verticillioides*. This assay combines a straightforward methodology, in which the fungus is challenged with bacterial isolates in liquid medium, with a novel approach that uses the plant lectin wheat germ agglutinin (WGA) coupled to a fluorophore (Alexa-Fluor® 488) under the commercial name of WGA, Alexa Fluor® 488 conjugate. The assay is performed in a 96-well plate format, which reduces the required laboratory space and streamlines quantitation and automation of the process, making it fast and accurate. The basis of our assay is that fungal biomass can be assessed by WGA, Alexa Fluor® 488 conjugate staining, which recognizes the chitin in the fungal cell wall and thus permits the identification of potential antagonistic bacteria that inhibit fungal growth. This principle was validated by chitin-competition binding assays against WGA, Alexa Fluor® 488 conjugate; confocal laser microscopy confirmed that the fluorescent WGA, Alexa Fluor® 488 conjugate binds to the chitin of the fungal cell wall. The majority of bacterial isolates did not bind to the WGA, Alexa Fluor® 488 conjugate. Furthermore, including washing steps significantly reduced any bacterial staining to background levels, even in the rare cases where bacterial isolates were capable of binding to WGA. Confirmatory conventional agar plate antagonistic assays were also conducted to validate our technique. We are now successfully employing this large-scale antagonistic assay as a pre-screening step for potential fungal antagonists in extensive bacteria collections (on the order of thousands of isolates).

Additional supporting information may be found in the online version of this artilcle at the publisher's web-site

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Maize (*Zea mays* L.) is one of the four basic food staples that support the world's population [1]. Although developed countries mainly produce maize for animal feed and industrial uses [2], maize represents a major food source in Mexico [3]. The majority of the reported maize diseases which affect roots, stalks, ears, and kernels are caused by fungi [4]; this includes "stalk, root and ear rot", one of the most important diseases in countries which grow maize. In Mexico, the species that have been found to cause this disease are *F. verticillioides* [5] and *F. subglutinans* [6, 7]. Stalk, root and ear rot is the most severe agricultural disease in Mexico's central Highland Valley area, as it lowers both yield and grain quality [8].

Our approach to this problem is to find novel antagonistic bacteria against fungal phytopathogens. However, screening for individual isolates from large collections (containing hundreds or even thousands of

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isolates) can be challenging, as this generally involves time-consuming microbiological techniques and necessitates dedicated laboratory space. Another constraint is that antagonism assays traditionally require solid medium plates to grow both the bacterial antagonists and pathogens, for dual culture assays [9–11]. The objective of this study was to develop a high-throughput methodology to rapidly screen large numbers of bacterial isolates in the search for antagonists against *F. verticillioides* (*Fv*), the causative agent of the stalk, ear and root rot of corn [12, 13], which is a serious disease afflicting Mexico's maize production [14]. High-throughput screening using bioluminescent/fluorescent assays has mainly been developed and utilized for finding pharmacological targets for drug discovery [15–18]. To the best of our knowledge, our study is the first of its kind to apply high-throughput fluorescence assays in order to discover novel bacterial antagonists against fungal phytopathogens, although several reports have developed high-throughput screening using different approaches, for biocontrol purposes [19, 20].

Wheat germ agglutinin (WGA) is a well-characterized member of the chitin-binding class of lectins from the Poaceae family [21], which specifically binds to *N*-acetyl-D-glucosamine and *N*-acetyl-D-neuraminic (sialic) acid residues [22–24]. When conjugated to fluorescent molecules (such as Alexa-Fluor, tetramethylrhodamine or fluorescein conjugates, etc.), WGA can facilitate the localization of fungal tissue in complex samples, such as the arbuscular mycorrhizal fungi that colonize roots [25], and plant pathogenic fungi that infect plant tissues [26]. In our novel antagonism assay this fluorescent WGA, Alexa Fluor® 488 conjugate (Cat. No. W11261, Life Technologies, Eugene, OR, USA) eliminates any subjective measurements of fungal growth inhibition.

In our screening assay, fungal biomass is quantified as relative fluorescence units due to the specific binding of WGA, Alexa Fluor® 488 conjugate to different chitin residues of the fungal cell wall. To evaluate WGA, Alexa Fluor® 488 conjugate efficiency of binding to purified chitin, we prepared colloidal chitin (Cat C-7170, St. Louis, MO, USA) according to Shanmugaiah et al. [27]. Adding increasing amounts of chitin to 0.5 ml of phosphate buffer saline (1 × PBS; with a final concentration of 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, and pH 7.4) containing 0.5 μg of WGA, Alexa Fluor® 488 conjugate induced the precipitation of a chitin-WGA complex after overnight incubation at 4 °C. This complex was removed by centrifugation at 17,900 × *g*. Subsequently, fluorescence of the supernatant was evaluated with a DTX880 multimodal detector (Beckman Coulter, Brea, CA, USA) using the EX1 filter slide for excitation (485–535 nm wavelength) and the EMP1 filter slide for emission (465–625 nm wavelength). A blank tube containing 1× PBS and Alexa Fluor® 488 conjugate was used to set up a background control. This tube was also incubated overnight at 4 °C and washed three times with 1× PBS by centrifugation and then used for fluorescence counting. The remaining fluorescence from the supernatant corresponds to the amount of unbound WGA, Alexa Fluor® 488 conjugate (as compared to the original fluorescence level). Ten μg of colloidal chitin reduced the fluorescence of the supernatant by 80% (i.e. about 80% of the fluorescent WGA, Alexa Fluor® 488 conjugate was bound); amounts from 30 to 50 μg reduced it by more than 90% (Supporting Information Fig. S1). This result is consistent with the WGA, Alexa Fluor® 488 conjugate binding to chitin residues of the fungal cell wall.

For the *Fv*/bacteria antagonistic assays, we used a cryopreserved monoconidial *Fv* culture whose symptomatology, pathogenicity, and molecular identity were previously characterized by our group. The frozen stock (−70 °C) was used as a starter culture, and *Fv* was grown on potato dextrose agar (PDA; Cat. No. 213400, BD Difco, Franklin Lakes, NJ, USA) plates for seven to 10 days at 25 °C. Subsequently, a mycelium plug (0.5 cm in both diameter and height) was placed in a new PDA plate for 7 days. *Fv* mycelia-containing conidia were resuspended in sterile distilled water and counted (Neubauer chamber) to adjust the conidia concentration.

A dose–response experiment was conducted to determine the amount of WGA, Alexa Fluor® 488 conjugate necessary to measure fungal biomass in 96-well plates, in which each well contained 0.5 ml of potato dextrose (PD) broth (Cat. No. 254920 BD Difco) (Supporting Information Fig. S2). *Fv* inoculum (2×10^5 conidia ml^{-1}) was grown at 25 °C for 36 h, and the fungal biomass collected by centrifugation at 17,900 × *g*. Fluorescence associated to fungal biomass was quantitated after staining by overnight incubation at 4 °C with 1× PBS added with WGA, Alexa Fluor® 488 conjugate ranging from 0.2 to 2.5 μg ml^{-1}, and followed by washing three times with 1× PBS and centrifugation. A blank tube containing 1× PBS and Alexa Fluor® 488 conjugate was used to set up a background control. It was incubated overnight at 4 °C and also washed three times with 1× PBS by centrifugation and then used for fluorescence measurements. Five replicates were quantified per each dosage amount used. The fluorescence values for WGA, Alexa Fluor® 488 conjugate were similar when either a 2.5 or a 1 or 2 μg ml^{-1} doses were used. We selected 1 μg ml^{-1} of WGA, Alexa Fluor® 488 conjugate for further experiments, which is in accordance with the manufacturer's recommendations.

We next examined the influence of initial inoculum size on fungal biomass. Growth kinetics showed that the

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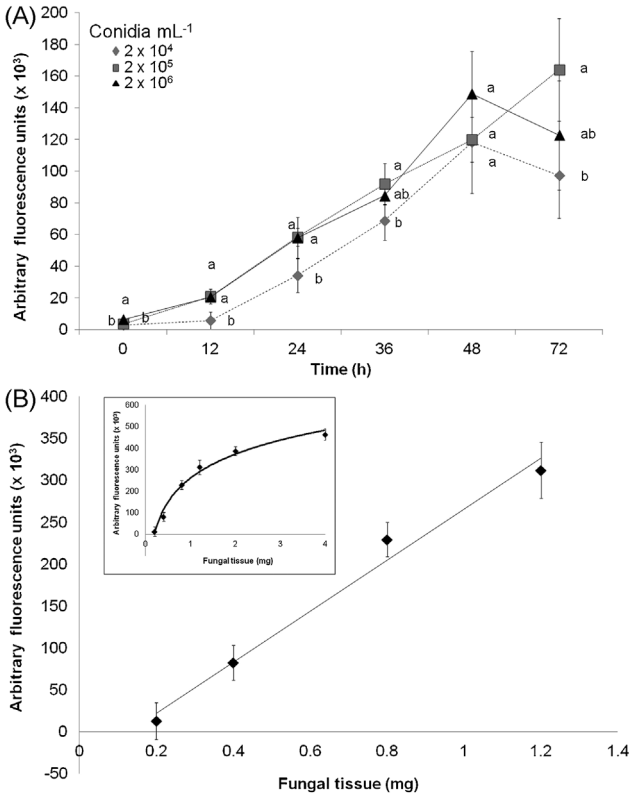

Figure 1. (A) Growth kinetics of *Fv* in PD broth using different conidia concentrations. For detection of fungal growth as relative fluorescence units, five replicates were used per point. Tissue was collected at different time points by centrifugation and transferred to a 96-well optical plate to be stained with 1 μg ml^{-1} of WGA, Alexa Fluor® 488 conjugate by overnight incubation at 4 °C. Fluorescence was counted after washing three times by centrifugation. Initial conidia concentrations are indicated by diamonds (2 × 10^4 conidia ml^{-1}), squares (2 × 10^5 conidia ml^{-1}) and triangles (2 × 10^6 conidia ml^{-1}). Different letters indicate significant differences ($p = 0.05$) between values measured at a given time point. (B) WGA, Alexa Fluor® 488 conjugate fluorescence counts correlate with fungal biomass. Increasing amounts of fungal biomass (dry tissue) were stained using 1 μg ml^{-1} WGA, Alexa Fluor® 488 conjugate. The correlation coefficient (R) was 0.9839. The inset shows that at higher amounts of fungal biomass WGA, Alexa Fluor® 488 conjugate fluorescence counts reach saturation. Each point represents the average of five replicates (diamonds) and bars indicate standard deviation.

different conidia concentrations followed a similar trend (Fig. 1A). At 36 h of growth, the 2 × 10^5 and 2 × 10^6 conidia ml^{-1} concentrations showed similar growth patterns; by contrast, at 2 × 10^4 conidia ml^{-1}, *Fv* growth was significantly reduced as compared to the 2 × 10^5 conidia ml^{-1} condition. No significant differences were observed between different inoculum concentrations after 48 and 72 h of growth, and by 72 h a decrease in fluorescence was detected for both the 2 × 10^4 and 2 × 10^6 conidia ml^{-1} concentrations. This decrease may be due to excessive fungal growth that was not properly centrifuged, as well as manipulation of the fungal tissue. It is also possible that fungal material could change its cell wall structure during different growth stages, which could prevent WGA binding. Based on the obtained results, and to minimize changes to fungal cell wall structure that could affect fluorescence measurements (due to fungal tissue aging), we decided for the screening assay protocol to fix fungal growth at time 36 h and to add 2 × 10^5 ml^{-1} conidia as the initial inoculum.

Fungal growth kinetics are difficult to follow in a 96-well plate format, given the very small amount of fungal dry biomass that can be recovered from 0.5 ml of PD broth. For this reason, we only weighed fungal biomass at a late time point (72 h of growth), and could determine the maximal range of biomass growth (measured as dry biomass). Values for dry fungal tissues from different experiments ranged from 0.8 to 1.2 mg. To verify that fungal biomass can be accurately measured (at least up to 1.2 mg) using the WGA, Alexa Fluor® 488 conjugate, we grew the fungus for 72 h in 100 ml of PD broth, after which fungal tissue was collected and dried at 100 °C until constant weight was obtained (approximately, 12–16 h). Dry fungal tissue was ground to a fine powder using a porcelain mortar and pestle, and was similarly desiccated to a constant dry weight. A suspension of dry fungal biomass was prepared, ranging from 0.2 to 4 mg in 0.5 ml of PBS buffer. The fungal tissue was stained with WGA, Alexa Fluor® 488 conjugate (1 μg ml^{-1}) and quantitated as described above. A correlation between fungal biomass and fluorescence count was observed from 0.2 to 1.2 mg fungal dry weight, confirming that fluorescence count accurately reflects fungal biomass in this assay, when the selected 1 μg ml^{-1} concentration of WGA, Alexa Fluor® 488 conjugate is used (Fig. 1B). Fungal biomass higher than 1.2 mg could not be accurately measured, as saturation of the WGA, Alexa Fluor® 488 conjugate (reaching a plateau) in fluorescence counts was observed when we used up to 4 mg of fungal tissue (inset Fig. 1B). By performing the antagonistic *Fv*/bacteria assays at 36 h we assure that fluorescence will be accurately measured, and that fungal biomasses higher than 1.2 mg will not be reached in our assays, even in the remote case that some bacteria will cause growth promotion of the fungus.

The bacterial isolates in this liquid antagonistic assay belong to a cryopreserved (−70 °C) collection of 11,520 bacteria isolates from the maize rhizosphere (Scientific Collection CIIDIR-003), arranged in a 96-well plate format. The plate containing the stock isolates was handled in a laminar hood. Sterile 200 μl tips were carefully placed inside each well of the plate, and the tips

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were scraped against the frozen glycerol stocks to load inside a small amount of melted bacterial suspension. Subsequently, 500 μl of PD broth were aliquoted to each well of a 2 ml 96-well plate (Cat. No. 140504 Beckman Coulter) and inoculated with the tips containing the bacterial isolates. Finally, each well received 2×10^5 conidia ml^{-1}. The plates were covered with a sterile aluminum seal and incubated at 25 °C and 250 rpm for 36 h in an orbital shaker. After incubation, the 96-well plates were centrifuged at 6570 × *g* for 10 min in a Beckman J-30I centrifuge using a swinging-bucket rotor (JS 5.9, Beckman Coulter) and the supernatant was discarded. The mycelial pellet was resuspended and washed four times by centrifugation with 0.5 ml of 1× PBS solution. The pellet was then resuspended in 0.5 ml of 1× PBS, and 0.5 μg of WGA, Alexa Fluor® 488 conjugate was added to obtain a final concentration of 1 μg ml^{-1}. Following overnight incubation at 4 °C, the plate was centrifuged to remove the supernatant and the pellet was washed four times (in 1 ml 1× PBS) by centrifugation to eliminate residual WGA, Alexa Fluor® 488 conjugate and bacteria. After the final wash, the pellet was resuspended in 0.2 ml of 1× PBS and placed in an optical 96-well plate (Cat. No. 609844, Beckman Coulter). Subsequently, fluorescence of the fungal pellet was counted as described before. This protocol facilitates handling sets of two or four 96-well plates, depending on the type of centrifuge rotor available, to the point where four to eight 96-well plates can easily be processed in two 8-h work days by a single person. This workload can be improved when using robotic liquid-handling platforms under sterile conditions.

To verify whether the presence of bacteria could influence the WGA-based fungal staining assay, we first checked whether the WGA, Alexa Fluor® 488 conjugate could bind to the bacteria. In bacteria cultivated alone, we determined by confocal laser scanning using a white laser in a TCS SP5 X microscope (Leica) that WGA, Alexa Fluor® 488 conjugate binds to some isolates either at the extracellular matrix surrounding the cell wall (Supporting Information Fig. S3) or to their cell walls (Fig. 2A). It is possible that isolates showing fluorescence are gram-positive, since their bacterial cell wall has *N*-acetylglucosamine residues and they are recognized by WGA, Alexa Fluor® 488 conjugate [28]. In the case of gram-negative bacteria, WGA, Alexa Fluor® 488 conjugate should not be able to penetrate the outer membrane and thus could not attach to the peptidoglycan. Nevertheless, we verified that the final bacterial load is greatly reduced in our assay, thanks to the many washes included in the protocol. WGA, Alexa Fluor® 488 conjugate staining and washing of 96-well plates that contain the bacteria-only control revealed no significant fluorescence (Fig. 2E and I, see "bact" bar). These results suggest that bacteria (regardless of their ability to bind WGA) are effectively washed during the assay. Any bacteria remaining after washing will thus have a negligible effect on fluorescence counts, and should therefore not be considered in analyzing the results.

We also confirmed visually, by scanning confocal laser microscopy, that the fungal cell wall is stained with WGA, Alexa Fluor® 488 conjugate when the fungus is grown alone (Fig. 2B and F), and when it is grown in the presence of antagonistic bacteria (Fig. 2C and G). By adding excess chitin to *Fv* grown alone and allowing it to compete for the chitin residues of the fungal cell wall, we demonstrated that chitin will bind to the WGA, Alexa Fluor® 488 conjugate; this prevents the lectin moiety from binding to the fungal cell wall, and it is subsequently removed by washing (Fig. 2D and H). Excess chitin forms small precipitates that are fluorescent green and that are not easily removed by washing (see inset close-up of Fig. 2D), although no staining of the fungal hyphae was observed. This demonstrates that WGA, Alexa Fluor® 488 conjugate indeed acted directly in the assay by binding to the fungal cell wall. Visual observations were corroborated by fluorescence measurements, which indicated that the fungus was solely responsible for fluorescence counts in this procedure, and not the bacteria (Fig. 2I).

The decrease in fungal biomass caused by the antagonistic bacterial isolate was measured as a decrease in fluorescence as compared to the "fungus only" control. To evaluate this change, we calculated the percentage of fungal growth as follows: $F_{well} = F_f + F_b + F_{blank}$ where F_{well} equals to the fluorescence of the well which is the sum of the fluorescence of the fungus (F_f) plus the fluorescence of the remaining bacteria after washing (F_b) and the fluorescence of the blank (F_{blank}). Since F_b is negligible (Fig. 2), you can ignore it, and $F_{well} = F_f + F_{blank}$. Then, we get: $F_f = F_{well} - F_{blank}$. This calculation is true for all the wells (with or without bacteria), and the same F_{blank} value was substracted to all values. Please note that it should also be removed from the Control well (fungus only). To calculate the decrease in fungal biomass caused by the antagonistic bacterial isolate, the F_f value of the sample (F_{f_sample}) is compared to the F_f value of the control ($F_{f\_control}$), and is expressed as a percentage of $F_{f\_control}$. This gives: Fungal growth (% of control) (% FG) = $F_{f\_sample} \times 100/F_{f\_control}$.

The percentage of fungal growth inhibition (%FGI) was calculated by subtracting the percentage of fungal growth (%FG) from 100 (i.e. %FGI = 100 − %FG). The criterion used to select a good antagonist was arbitrarily

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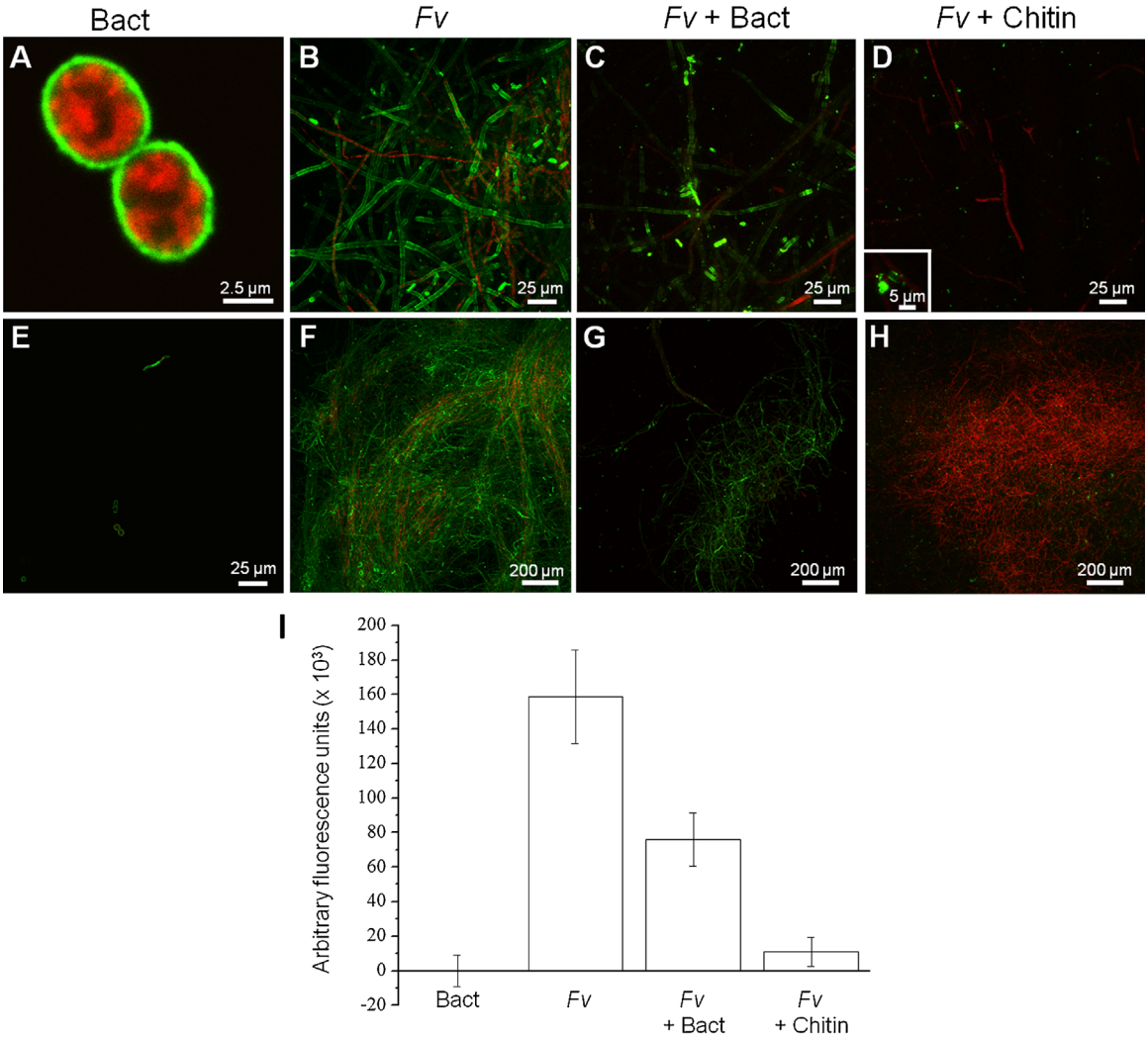

Figure 2. Dual WGA, Alexa Fluor® 488 conjugate and propidium iodide staining revealed that lectin binds to the cell walls of the fungus and bacteria. Merged images are shown in panels (A–H). A Leica TCS SP5 X confocal laser scanning microscope was used to obtain the two merged acquisition channels using 497 and 489 nm excitation laser (white laser), and emission ranges of 502–548 and 598–706 nm for WGA, Alexa Fluor® 488 conjugate (green fluorescence) and for propidium iodide (red fluorescence), respectively. (A) Close-up of bacterial cells showing WGA binding to the cell walls. (E) Last wash following WGA, Alexa-Fluor® 488 conjugate staining from a well of bacteria-only control, illustrating that the remaining bacterial cells are greatly reduced in number. (B and F) *Fv* fungal mycelium grown alone and stained with WGA, Alexa Fluor® 488 conjugate showing green fluorescence in their cell walls. (C and G) *Fv* fungal mycelium grown with bacteria in an antagonistic assay and stained with WGA, Alexa Fluor® 488 conjugate showing green fluorescence in their cell walls. (D and H) *Fv* fungal mycelium stained with WGA, Alexa Fluor® 488 conjugate in the presence of excess colloidal chitin (50 µg) reveals that competition with chitin prevents lectin from binding to the fungal cell wall, as indicated by the absence of green fluorescence. The inset in (D) indicates that the green spots observed in this image are possibly chitin precipitates bound to WGA, Alexa Fluor® 488 conjugate. (I) Quantitation of fluorescence after the series of washes demonstrates that any remaining bacteria are not responsible for fluorescence in the antagonistic assay, and that the green fluorescent stain is due to the binding of WGA, Alexa Fluor® 488 conjugate to *N*-acetyl glucosamine residues. Bact stands for bacteria; *Fv* indicates *F. verticillioides* grown alone. *Fv* + Bact is the antagonistic assay in which *Fv* and bacteria are incubated together. Under this condition, the bacteria have an antifungal activity; similar results are obtained with bacteria that do not inhibit fungal growth only that fluorescence counts will change. *Fv* + Chitin refers to a binding assay between chitin and WGA, followed by the staining of the fungus grown alone (set up as a lectin–chitin binding control). The same antagonistic bacterium was used for panels A, C, E, G, and I.

set to >60% growth inhibition. We did not perform growth kinetics for each isolate, as this is typically an unmanageable task in such a massive screen. In the present work, we did perform bacterial growth kinetics for several isolates (data not shown) and observed that they reached the stationary phase by 36 h. The conditions used in this assay, such as PD broth selection, are biased to favor fungal growth. Based on the assumption that this would enable finding bacterial isolates that are antagonists against *Fv*, we performed a

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(A)

	1	2	3	4	5	6	7	8	9	10	11	12
A	74	77	70	65	32	80	17	85	24	19	78	71
B	62	84	79	13	73	63	82	12	54	3	57	21
C	72	63	26	-19	88	51	78	62	11	31	-34	50
D	87	23	-13	84	81	44	21	31	27	16	82	23
E	51	24	-20	-23	55	3	-5	7	25	68	-7	-28
F	52	42	77	90	12	37	17	69	63	56	-47	56
G	39	62	51	65	68	7	61	49	34	99	51	50
H	46	51	50	46	46	-1	32	-66	12	71	65	74

(B)

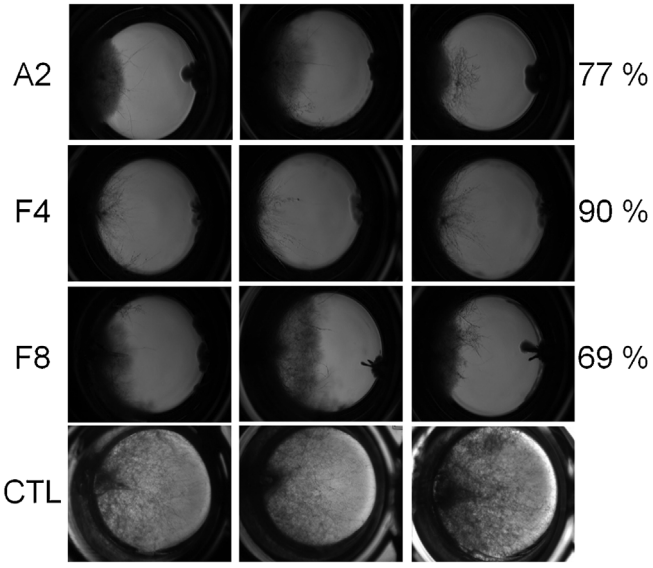

Figure 3. Bacterial antagonism assay against *Fv* in 96-well plates. (A) Percentage of growth inhibition caused by 96 bacterial isolates against *Fv* in PD broth (liquid antagonism assay). Negative values denote promotion instead of inhibition of fungal growth by those specific bacterial isolates. The gray boxes indicate isolates showing higher than 60% fungal growth inhibition that were selected for further confirmation as potential antagonists, while white boxes indicate $\leq$60% growth inhibition. (B) 96-well plate antagonism assays against *Fv* in PDA solid medium for three selected isolates. Isolates were chosen on the basis of their fungal growth inhibition in liquid medium assays (indicated as a percentage to the right of their respective photographs), and correspond to A2, F4, and F8 from panel (A). Photographs corresponding to only one well from different 96-well plates are displayed, with results shown in triplicate. *Fv* conidia were placed on the left side of each well and the bacterial isolates were placed on the right side of each well, except for the fungus-only control (CTL). Plates were evaluated after 48 h of incubation at 25 °C, at which point the fungal hyphae reached the right edge of the CTL well.

test with 96 isolates (Fig. 3A). One plate was randomly selected from the Scientific Collection CIIDIR-003 and tested as previously described for the liquid antagonism assay. Thirty-six isolates exhibiting >60% *Fv* growth inhibition were identified (Fig. 3A), and subsequently their potential antagonistic activity was tested in a 96-well solid medium antagonistic assay. For this assay, isolates were defrosted and grown in 2 mL deep-well 96-well plates in 0.5 ml of LB broth (Cat. L3022 Sigma, St. Louis, MO, USA) overnight at 25 °C and 200 rpm. Bacterial pellets were then picked using a multichannel micropipette (eight channels). In order to inoculate the bacteria on 96-well plates filled with 200 μl of PDA solid medium, the pellet was touched with a 10 μl tip to deposit the bacteria on the right side of each well. After bacterial inoculation of each well of the plate, 2 μl of water containing 1×10^4 *Fv* conidia were deposited in the left side of the well. In this assay, *Fv* was challenged with the bacterial isolates for 2 days in PDA medium cultured at 25 °C in 96-well agar plates (Fig. 3B),

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performed in triplicate. All thirty-six isolates exhibiting >60% *Fv* growth inhibition were subsequently tested in this assay, as well as a confirmatory assay using a conventional agar plate antagonist assay (data not shown). Three out of the thirty-six isolates were confirmed as growth inhibitors of *Fv* (Fig. 3B). This diminished number of isolates is consistent with the view that the liquid medium assay is a preliminary screen, and follow up confirmation with other assays such as dual culture analysis is just one step in the selection procedure for antagonists of a fungal pathogen. In our experience with extensive collections of rhizospheric organisms, *in vitro* plate screening for antagonists can diminish the number of potential antagonists to <10% of the original isolate number [29]. Since dual culture analysis is normally performed in 50–100 mm diameter Petri plates, we conducted confirmatory experiments that gave similar results for fungal growth inhibition (see Supporting Information Fig. S4). The isolate in Supporting Information Fig. S4B corresponds to isolate F8 from Fig. 3A exhibiting 69% growth inhibition in the 96-well plate assay. In contrast to the 96-well plate system, using a 50 mm diameter Petri plate resulted in a lower percentage of *Fv* growth inhibition (39%). In general, this was observed with all other isolates tested by the conventional Petri plate assay. This trend can be explained by considering that this type of dual culture assay allows screening for fungal growth inhibition mechanisms where diffusion of secreted inhibitory compounds is involved. It is possible that the closer proximity between the two organisms in the 96-well plates (only 8 mm in diameter) allows for a more rapid diffusion of these substances and for faster growth inhibition responses than in the larger volume Petri plates.

Antagonistic bacteria secrete diverse compounds that can act upon fungal pathogens, and as such, various antagonistic mechanisms can be detected in the liquid assay. These compounds can affect either spore germination [30] or elongation and growth of hyphae; in *Bacillus* spp. these effects are due to the action of enzymes such as chitinases [31, 32], or antibiotics such as kanosamine and zwittermicin A [33, 34]. In the case of this particular assay where the fungus and bacteria are in direct contact, another plausible antagonistic mechanism could involve mycoparasitism [35]. *In vitro* screening only allows selection, which must be confirmed *in planta*, where the tripartite interaction occurs between the antagonistic bacteria, the fungus and the plant root [36].

There has been a great focus on developing techniques to identify antagonists that inhibit plant pathogens, in an effort to control disease in crops of interest such as corn [32, 37, 38]. The present methodology was designed to explore the antagonistic ability of a collection of 11,520 native bacterial isolates from the rhizosphere of cultivated maize in Sinaloa, Mexico (unpublished work). Our protocol is ideal for performing an antagonism bioassay with large numbers of specimens, and its advantages include less required laboratory/incubator space coupled with a shorter assay period, as well as the possibility of automation using liquid handling robotic platforms. We have demonstrated that this assay is suitable for screening large collections of bacteria in search of potential antagonists against fungal plant pathogens, as well as other types of fungal pathogens. It is always advisable to perform a small test of the liquid *Fv*/bacteria antagonistic assay in duplicates or triplicates to corroborate repetitiveness of the assay before starting a massive screening procedure. Nevertheless, performing this assay in duplicates or triplicates has to be evaluated by the researchers considering the size of the screening, time and/or budget constraints. In our experience, a combination of the massive liquid assay with confirmatory tests would work the best. Our high-throughput screening assay, in combination with dual culture analysis and other screening procedures (including *in planta* confirmatory bioassays), has allowed our group to select potential bacterial antagonists against the stalk, root and ear rot disease in maize. Preliminary results for these antagonists in field trials show promising results for the biological control of *F. verticillioides* in maize (manuscript in preparation).

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References

[1] Anderson, P.K., Cunningham, A.A., Patel N.G., Morales, F.J. et al., 2004. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. Trends Ecol. Evol., **19**, 535–544.

[2] Farnham, D.E., Benson, G.O., Pearce, R.B., 2003. Corn perspective and culture, in: White, J.P., Jonson, A.L. (Eds.), Corn Chemistry and Technology, 2nd edn., American Association of Cereal Chemists Inc., St. Paul, MN, 1–34.

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[3] Figueroa, J., de D., 1999. La tortilla vitaminada. Avance Perspectiva, **18**, 149–158.

[4] White, G.D., (Ed.), 2000. Compendium of Corn Diseases, APS Press, St. Paul MN.

[5] Perez-Brito, D., Jeffers, D., Gonzales-de-Leon, D., Khairallah, M. et al., 2001. QTL mapping of *Fusarium moniliforme* ear rot resistance in highland maize. Mexico Agrociencia, **35**, 181–196.

[6] Desjardins, A.E., Plattner, R.D., Gordon, T.R., 2000. *Gibberella fujikuroi* mating population A and *Fusarium subglutinans* from teosinte species and maize from Mexico and Central America. Mycol. Res., **104**, 865–872.

[7] Steenkamp, E.T., Coutinho, T.A., Desjardins, A.E., Wingfield, B.D. et al., 2001. *Gibberella fujikuroi* mating population E is associated with maize and teosinte. Mol. Plant Pathol., **2**, 215–221.

[8] Romero Cova, S., Félix Gastélum, R., 1981. Etiología de la germinación prematura del maíz en Huamantla,Tlaxcala. Agrociencia, **43**, 81–87.

[9] Lim, H.S., Kim, Y.S., Kim, S.D., 1991. *Pseudomonas stutzeri* YPL-1 genetic transformation and antifungal mechanism against *Fusarium solani*, an agent of plant root rot. Appl. Environ. Microbiol., **57**, 510–516.

[10] Montealegre, J.R., Reyes, R., Pérez, L.M., Herrera, R. et al., 2003. Selection of bioantagonistic bacteria to be used in biological control of *Rhizoctonia solani* in tomato. Electron. J. Biotechnol., **6**, 115–127.

[11] Romero, D., Pérez-García, A., Rivera, M.E., Cazorla, F.M. et al., 2004. Isolation and evaluation of antagonistic bacteria towards the cucurbit powdery mildew fungus *Podosphaera fusca*. Appl. Microbiol. Biotechnol., **64**, 263–269.

[12] Wang, B., Jeffers, S.N., 2000. *Fusarium* root and crown rot: a disease of container-grown hosts. Plant Dis., **84**, 980–988.

[13] Yang, D., Wang, B., Wang, J., Chen, Y. et al., 2009. Activity and efficacy of *Bacillus subtilis* strain NJ-18 against rice sheath blight and *Sclerotinia* stem rot of rape. Biol. Control, **51**, 61–65.

[14] Hernández, D.S., Reyes, L.A., García, O.G.J., Mayek, P.N. et al., 2007. Incidencia de hongos potencialmente toxígenos de maíz (*Zea mays L.*) almacenado y cultivado en el norte de Tamaulipas, México. Rev. Mex. Fitopat., **25**, 127–133.

[15] Fan, F., Wood, K.V., 2007. Bioluminescent assays for high-throughput screening. Assay Drug Dev. Technol., **5**, 127–136.

[16] Leveridge, M.V., Bardera, A.I., LaMarr, W., Billinton, A. et al., 2012. Lead discovery for microsomal prostaglandin E synthase using a combination of high-throughput fluorescent-based assays and rapidfire mass spectrometry. J. Biomol. Screen. **17**, 614–650.

[17] Severson, W.E., Shindo, N., Sosa, M., Fletcher, T., III et al., 2007. Development and validation of a high-throughput screen for inhibitors of SARS CoV and its application in screening of a 100,000-compound library. J. Biomol. Screen., **12**, 33–40.

[18] Wei, D., Bu, Z., Yu, A., Li, F., 2011. Identification of a lead small-molecule inhibitor of anthrax lethal toxin by using fluorescence-based high-throughput screening. BMB Rep., **44**, 811–815.

[19] Janisiewicz, W.J., Buyer, J.S., 2010. Culturable bacterial microflora associated with nectarine fruit and their potential for control of brown rot. Can. J. Microbiol., **56**, 480–486.

[20] Wang, H., Li, W., Chen, Q., Huang, Y. et al., 2012. A rapid microbioassay for discovery of antagonistic bacteria for *Phytophthora parasitica* var. *nicotianae*. Biol. Control., **102**, 267–271.

[21] Goldstein, I.J., Hayes, C.E., 1978. The lectins: carbohydrate-binding proteins of plants. Adv. Carbohydr. Chem. Biochem., **35**, 127–340.

[22] Peumans, W.J., Stinissen H.M., Carlier, A.R., 1982. Isolation and partial characterization of wheat-germ-agglutinin-like lectins from rye (*Secale cereale*) and barley (*Hordeum vulgare*) embryos. Biochem. J., **203**, 239–243.

[23] Van Damme, J.M., Peumans, W.J., Pusztai, A., Bardocz, S. (Eds.), 1998. Handbook of Plant Lectins: Properties and Biomedical Applications, John Wiley and Sons, West Sussex, England.

[24] Pellegrina, C.D., Rizzi, C., Mosconi, S., Zoccatelli, G. et al., 2005. Plant lectins as carriers for oral drugs: is wheat germ agglutinin a suitable candidate? Toxicol. Appl. Pharmacol., **207**, 170–178.

[25] Javot, H., Penmetsa, R.V., Terzaghi, N., Cook, D.R. et al., 2007. A *Medicago truncatula* phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. Proc. Natl. Acad. Sci. USA, **104**, 1720–1725.

[26] Galindo-Flores, H., Martínez-Álvarez, J.C., Nava-Pérez, E., García-Estrada, R.S. et al., 2005. A saprotrophic fungal isolate with homology to members of the *Chaetomiaceae* behaves as an antagonist of phytopathogenic fungi *in vitro*. Rev. Mex. Fitopatol., **23**, 130–139.

[27] Shanmugaiah, V., Mathivanan, N., Balasubramanian, N., Manoharan, P.T., 2008. Optimization of cultural conditions for production of chitinase by *Bacillus laterosporous* MML2270 isolated from rice rhizosphere soil. Afr. J. Biotechnol., **7**, 2562–2568.

[28] Sizemore, R.K., Caldwell, J.J., Kendrick A.S., 1990. Alternate gram staining technique using a fluorescent lectin. Appl. Environ. Microbiol., **57**, 2245–2247.

[29] Cordero-Ramírez, J.D., López-Rivera, R., Figueroa-Lopez, A. M., Mancera-López, M.E. et al., 2012. Native soil bacteria isolates in Mexico exhibit a promising antagonistic effect against *Fusarium oxysporum* f. sp.*radicis-lycopersici*. J. Basic Microbiol., DOI 10.1002/jobm.201200128

[30] Rosales, A.M., Mew, T.W., 1997. Suppression of *Fusarium moniliforme* in rice by rice associated antagonistic bacteria. Plant Dis., **81**, 49–52.

[31] Kishore, G.K., Pande, S., Podile, A.R., 2005. Biological control of late leaf spot of peanut (*Arachis hypogaea*) with chitinolytic bacteria. Phytopathology, **95**, 1157–1165.

[32] Chang, W.T., Hsieh, C.H., Hsieh, H.S., 2009. Conversion of crude chitosan to an anti-fungal protease by *Bacillus cereus*. World J. Microbiol. Biotechnol., **25**, 375–382.

[33] He, H., Silo-Suh, L.A., Handelsman, J., Clardy, J., 1994. Zwittermicin A, an antifungal and plant protection

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agent from *Bacillus cereus*. Tetrahedron Lett., **35**, 2499–2502.

[34] Silo-Suh, L.A., Lethbridge, B.J., Raffel, S.J., He, H. et al., 1994. Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. Appl. Environ. Microbiol., **60**, 2023–2030.

[35] Baker, R., 1987. Mycoparasitism: ecology and physiology. Can. J. Plant Pathol., **9**, 370–379.

[36] Kamilova, F., Leveau, J.H., Lugtenberg, B., 2007. *Collimonas fungivorans*, an unpredicted *in vitro* but efficient *in vivo* biocontrol agent for the suppression of tomato foot and root rot. Environ. Microbiol., **9**, 1597–1603.

[37] Cavaglieri, L., Passone, A., Etcheverry, M., 2004. Screening procedures for selecting rhizobacteria with biocontrol effects upon *Fusarium verticillioides* growth and fumonisin B1 production. Res. Microbiol., **155**, 747–754.

[38] Cavaglieri, L., Andres, L., Ibanez, M., Etcheverry, M., 2005. Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides in vitro* and at the maize root level. Res. Microbiol., **156**, 748–754.

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