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# The Role of Wheat Germ Agglutinin in the Attachment of *Pseudomonas* sp. WS32 to Wheat Root

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**Wheat germ agglutinin (WGA), which is secreted on the surface of wheat root, has been defined as a protein that reversibly and non-enzymatically binds to specific carbohydrates. However, little attention has been paid to the function of WGA in the attachment of bacteria to their host plants. The aim of this study was to investigate the role of WGA in the attachment of *Pseudomonas* sp. WS32 to wheat roots. Wheat roots were initially treated with double-distilled water, WGA-H (WGA solution that was heated at 100°C for 15 min) and WGA, independently. Subsequently, the roots were co-incubated with cell solutions ($10^9$ cells/ml). A dilution plate method using a solid nutrient medium was employed to determine the adsorption of WS32 to wheat roots. WGA was labeled with fluorescein isothiocyanate and detected using the fluorescent *in situ* hybridization (FISH) technique. The number of adsorptive WS32 cells on wheat roots was significantly increased when the wheat roots were pretreated with WGA, compared with the control treatment ($p = 0.01$). However, WGA-H failed to increase the amount of bacterial cells that attached to the wheat roots because of the loss of its physiological activity. The FISH assay also revealed that more cells adhered to WGA-treated wheat roots than to control or WGA-H-treated roots. The results indicated that WGA can mediate *Pseudomonas* strain WS32's adherence to wheat seedling roots. The findings of this study provide a better understanding of the processes involved in plant-microbe interactions.**

***Keywords*:** wheat germ agglutinin, *Pseudomonas* sp., attachment, wheat roots, fluorescent *in situ* hybridization

## Introduction

Plant rhizospheres, which include the plant roots and the surrounding soil, are the sites of dynamic interactions between plant roots and soil microorganisms (Hartmann *et al*., 2009). Plant exudates, a variety of organic compounds including carbohydrates, carboxylic acids and inorganic ions, support the microorganisms. Lectins, one kind of exudate, are proteins that reversibly and non-enzymatically bind to specific carbohydrates (De Hoff *et al*., 2009). Numerous types of plant lectins have been studied, such as jack bean lectin (Inbar and Sachs, 1969), soybean lectin (Sela *et al*., 1970), and wheat germ agglutinin (WGA) (Burger and Goldberg, 1967). WGA, a glycoprotein, is secreted and exposed at the root surface (Mishkind *et al*., 1980). These root exudates play important roles in the regulation of the chemistry and biology of the root microenvironment (Antoun and Kloepper, 2001; Bais *et al*., 2004; Haichar *et al*., 2008; Hartmann *et al*., 2009).

Plant growth-promoting rhizobacteria (PGPR) are a group of soil microorganisms that can stimulate the growth of plants (Germida *et al*., 1998). Many species of bacteria, including *Pseudomonads*, have been reported to have beneficial traits (Compant *et al*., 2010). *Pseudomonas* is a large and common genus among plant root microbe communities. Recent research shows that *Pseudomonas* sp. can efficiently colonize their plant hosts and produce positive effects on plants by increasing dry weight or yield (Compant *et al*., 2010; Cummings and Orr, 2011). In fact, plants can also send beneficial bacteria adhering to their host roots into the rhizosphere by releasing soluble carbon compounds and specific stimulatory compounds (Hartmann *et al*., 2009). Here, we provide another explanation of how the genus *Pseudomonas* attaches to their host plants.

In our previous research, we found a specific affinity between WGA and wheat rhizobacteria (Zhang *et al*., 2012); however, the role of WGA remains unclear. The present study was undertaken to acquire a better understanding of the function of plant-derived WGA in the rhizosphere's colonization by a previously described PGPR, *Pseudomonas* sp. strain WS32. Fluorescence *in situ* hybridization (FISH) has been applied to detect microbes in various micro-environments because of its effectiveness and precision (Fernandez *et al*., 2008; Kenny *et al*., 2008; Wilén *et al*., 2008; Pilhofer *et al*., 2009). Thus, FISH was employed to detect the attachment of strain WS32 to wheat roots.

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## Materials and Methods

### Strain and culture condition

PGPR Strain *Pseudomonas* sp. WS32 (NCBI GenBank, accession number JN210910) showed affinity to WGA in our previous research (Zhang *et al*., 2012). The physiological and biochemical characteristics of strain WS32 are listed in Table 1. Strain WS32 was stored in 40% glycerol at -20°C before being used. The cultures were cultivated in beef extract medium on a thermostated shaker at 150 rpm at 28°C for 10 h. The cells were washed twice by centrifugation at 5,000 rpm for 5 min with phosphate-buffered saline (PBS pH 7.2) (Yegorenkova *et al*., 2001).

### WGA purification and hemagglutination assay

WGA was purified by precipitation with ammonium sulfate and chitin affinity chromatography, and labeling with fluorescein isothiocyanate (FITC) was performed using the previously described method (Zhang *et al*., 2012). The WGA-FITC (2 mg/ml) was stored at -20°C. To detect the physiological activity of WGA, a hemagglutination assay was performed. A solution (20 µl) containing 2% rabbit red blood cells was placed in a rinsed glass beaker, and 10 ml of 0.5% glutaraldehyde solution was then added. The solution was fully mixed by slow rotation for 1 h on a magnetic agitator. Then, the rabbit blood cells were rinsed four times using PBS (0.01 mol/L). Finally, the cells were diluted to their original concentration in PBS and stored at 4°C. Three treatments were implemented: a control treatment (CK) that consisted of 25 µl prepared rabbit blood cell solution added to sterilized saline water, a test treatment with WGA in which 25 µl prepared rabbit blood cell solution was added to 25 µl WGA solution, and a WGA-H treatment in which 25 µl prepared rabbit blood cell solution was added to a 25-µl WGA solution that had been heated for 15 min at 100°C. For each treatment, 2 µl of the mixture was spread on glass slides, which were examined using a Leica DMLB fluorescence microscope (Germany).

### Sterilization and cultivation of wheat seeds

Wheat seeds (*Triticum aestivum* Wan 33) were surface sterilized as previously described (Yegorenkova *et al*., 2001). The seeds were placed in sterile Petri dishes, properly watered, and germinated in darkness for 2 d at 25°C. Healthy seeds with roots length of 1–2 cm were selected for further analysis.

### Plant inoculation and adsorption assay

Wheat seedling root segments 2 cm long were cut from the seedlings. Each segment was transferred aseptically to an individual test-tube containing 4.5 ml of PBS and inoculated with 0.5 ml of bacterial suspension at a specific optical density ($10^9$ cells/ml). Wheat roots were initially treated by double-distilled water, WGA-H (0.2 mg/ml) and WGA (0.2 mg/ml) for 1 h at 28°C, independently. Then, the roots were gently washed with sterilized water and co-incubated with the cell solution ($10^9$ cells/ml) for 2 h at 28°C. A dilution plate method using a solid nutrient medium was employed to determine the adsorption (log CFU/g fresh wheat seedling roots) of WS32 to wheat roots within 2 h as previously described (Yegorenkova *et al*., 2001). The experiments were repeated three times.

### Detection by FISH

Wheat root samples were prepared as described above and the amount of WGA and bacteria adhering to the roots was evaluated using two methods. In one method, the sample that was not co-incubated with the cell solution was treated by 0.2 mg/ml WGA-FITC to detect the distribution of WGA on the root's surface (WGA was detected with an FITC filter, maximum excitation = 490 nm and maximum emission = 520 nm, green fluorescence). In the other method, the sample that had been co-incubated with the cell solution was prepared for FISH according to the previous description (Watt *et al*., 2006). Two probes, EUB338 (5′-GCT GCCTCCCGTAGGAGT-3′) (Amann *et al*., 1990) labeled with Cy3 and PSE227 (5′-AATCCGACCTAGGCTCATC-3′) (Watt *et al*., 2006) labeled with FAM, which is specific for *Pseudomonas* sp., were used in this study. All the samples were examined using a Leica DMLB fluorescence microscope (Germany).

### Statistical analyses

The results of the measurements were subjected to an ANOVA. The significance at the 1% level was tested by Fisher's least significant difference (LSD) using the Origin 8.0 software. Values are the means ± standard deviations of three replications. More than 15 microscopic fields were counted for a statistical evaluation.

## Results and Discussion

Bacterial attachment to plant roots is considered a very early

**Table 1. Physiological and biochemical characteristics of *Pseudomonas* sp. strain WS32**

| Tested experiments* | Strain WS32 | Tested experiments* | Strain WS32 |
|---|---|---|---|
| Colony Morphology | Regular, yellowish white | Hydrolyzed gelatin | + |
| Gram staining | $G^-$ | Production of $H_2S$ | – |
| Spore | – | Catalase | - |
| Flagellar | Round-forming | Oxidase | – |
| Starch | + | Methyl red Test | – |
| Fermentation Test [a] | Acid | Voges proskauer Test | – |

Notes: * +, positive; –, negative; [a] Utilization of glucose to produce acid

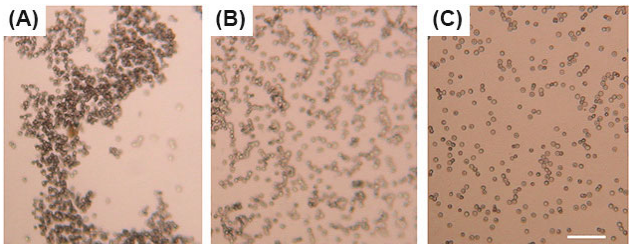


**Fig. 1. Visualization of the hemagglutination assay.** Rabbit blood cells were treated by sterilized saline water (CK), wheat germ agglutinin (WGA)-H, which was WGA solution (0.2 mg/ml) heated for 15 min at 100°C, and WGA (0.2 mg/ml), independently. The aggregation rate of CK, WGA-H, and WGA were scored as -, +, and ++++, respectively, according to the degree of rabbit red blood cells' distribution. Bar represents 50 μm.

step of plant root colonization by plant-associated microorganisms (Rodríguez-Navarro *et al.*, 2007). The capacity of the microbe to attach to plant cells is probably a key factor in determining its ability to be competitive in colonizing the host's roots. However, little attention has been paid to the plant lectins that are widely distributed on the root surface. Here, we examined the role of WGA in the attachment of *Pseudomonas* sp. WS32 to wheat plant roots. The physiological and biochemical characteristics of strain WS32 are listed in Table 1.

It is important to determine the physiological activity of WGA in the adsorption process. Here, we inactivated WGA by boiling. Then, the hemagglutination assay was used to determine the effectiveness of WGA. In this work, the aggregation rate of CK, WGA-H, and WGA were scored as -, + and ++++, respectively. As shown in Fig. 1, the purified WGA could agglutinate the rabbit blood cells (A). However, the WGA pretreated by heating for 15 min at 100°C showed an inability to agglutinate the rabbit blood cells (B) when compared with the control treatment (C).

To investigate the role of WGA in the attachment process, wheat roots were treated with double-distilled water, WGA-H and WGA, independently. Adsorption assays were performed at the same treatment time at a cell concentration of $10^9$ cells/ml. The total numbers of cells attached for CK, WGA-H, and WGA were 1.8±0.6, 2.0±0.5, and 5.5±1.1×$10^7$ cells/ml/g, respectively. Wheat roots pretreated with a 0.2 mg/ml WGA solution had a significantly (at the 1% level) higher strain WS32 adsorption number compared with the control treatment. However, when the WGA was heated for 15 min at 100°C, the number of bacteria adhering to the wheat roots decreased significantly (Fig. 2). Treating the roots with WGA is likely to increase the quantity of lectins on the root surface (Laus *et al.*, 2006). However, when the WGA was heat treated, the number of adhering cells was significantly reduced. This result suggested that when WGA lost its physiological activity, it could not mediate the bacterial adhesion to the wheat roots. It was previously determined that the maximum number of adhering cells is approximately 6.6 log N (number of cells adsorbed to the root per cm) in the attachment of *Azospirillum brasilense* to wheat roots (Yegorenkova *et al.*, 2001). In our study, we observed a slightly higher number of attached cells. This may be attributed to the different bacterial strains or wheat materials used in the experiments.

The overwhelming advantage of FISH for plant rhizosphere ecosystem studies, compared with other microbiological methods, is the ability to identify, localize and quantify bacteria *in situ* (Watt *et al.*, 2006). First, to identify the distri-

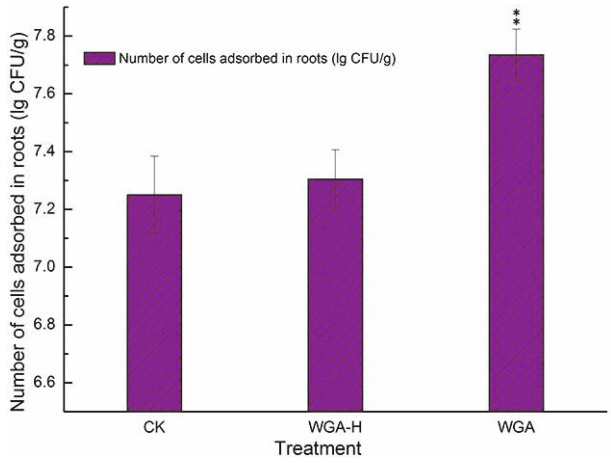


**Fig. 2. Adsorption of *Pseudomonas* sp. strain WS32 cells to wheat (*Triticum aestivum* Wan 33) roots.** Wheat roots pretreated with wheat germ agglutinin (WGA)-H, which was WGA solution (0.2 mg/ml) heated for 15 min at 100°C, and WGA solutions. CK indicates the sterilized water control treatment. Concentration of WGA solution was 0.2 mg/ml. Significant differences tested according to LSD at $^{**}p = 0.01$. Values are the means ± standard deviations.

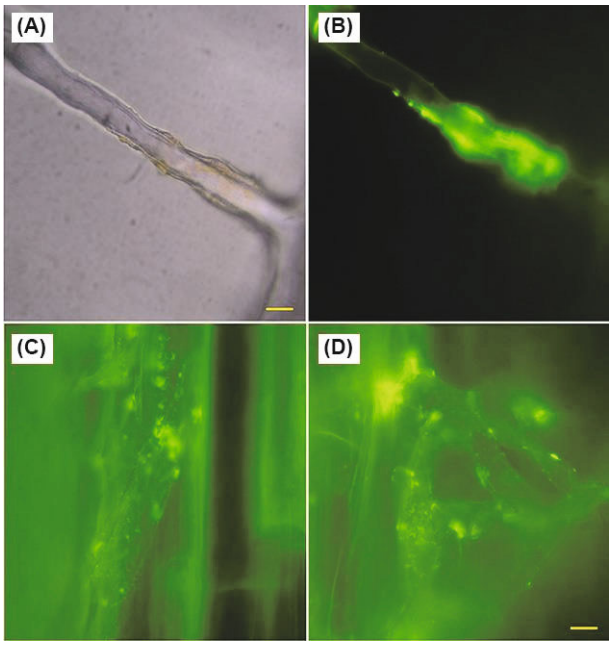


**Fig. 3. Photos of wheat (*Triticum aestivum* Wan 33) roots stained by wheat germ agglutinin (WGA) labeled with fluorescein isothiocyanate (FITC).** WGA was detected using a FITC filter (green fluorescence; Leica DMLB, Germany). (A) WGA located on the root hairs under fluorescence light. (B) the same location as in A. WGA was distributed on the root hairs (C and D) and the root elongation sites, as well as the axes of root surfaces. Bar represents 10 μm.

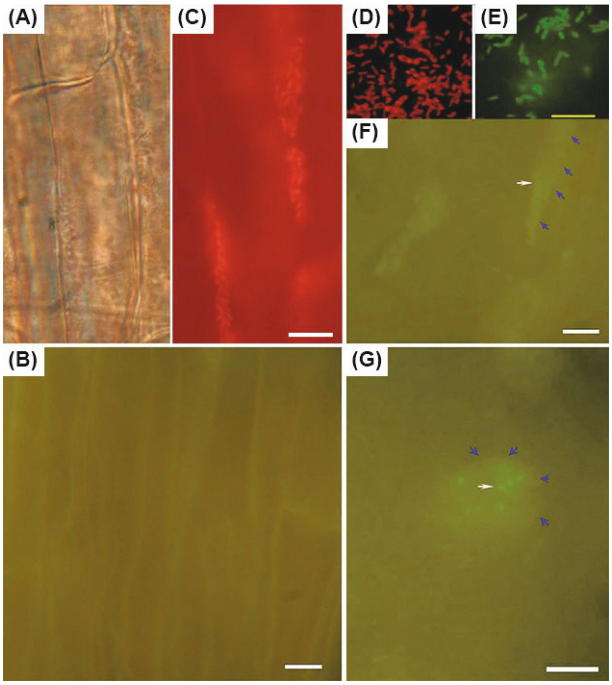


**Fig. 4. Fluorescence images of the *Pseudomonas* sp. strain WS32 detected by fluorescence *in situ* hybridization.** Image under optical view (A), control treatment without any cells (B). Probe EUB338 labeled with Cy3 (red) was used to detect the cells. Bacterial cells were distributed along the axis of the root's surface (C). To distinguish the bacteria from the wheat root, strain WS32 cells were detected by probe EUB338 (D) and species-specific probe PSE227 labeled with FAM (green) (E). Probe PSE227 showed that *Pseudomonas* cells attached to the wheat roots and were distributed at root elongation sites and the axes of root surfaces (F and G). White arrows point to the attached bacteria, blue arrows point to the root surface and root hair. Bar represents 10 μm.

bution of WGA on the root surface, wheat seedling roots were treated by WGA-FITC. The majority of WGAs were located on the root surface whereas WGA was undetectable at the root tip (Fig. 3). In this study, WGA attached itself more efficiently to the elongated and helical root surfaces, which is consistent with a previous report (Mishkind *et al.*, 1980). This may indicate that seedling roots in the elongation zone are in a more active physiological condition. In addition, two probes were used in FISH experiments, EUB338 labeled with Cy3 (red), which can be used for all bacteria and PSE227 labeled with FAM (green), which is specific for *Pseudomonas* sp. WS32. As shown in Fig. 4, under optical conditions, cells could not be distinguished from the root surface (Fig. 4A). The same results were obtained in the control treatment (Fig. 4B). To identify the bacteria from root surfaces, we detected the cells using each probe separately (Fig. 4D and 4E). The strain WS32 was well detected by the probe EUB338-Cy3, which showed bacterial cells distributed along with the axis of the root surface (Fig. 4C). The species-specific probe showed *Pseudomonas* cells attached to the wheat roots and distributed at root elongation sites and the axes of root surfaces (Fig. 4F and 4G). In addition, the *Pseudomonas* WS32 was found at varying distances from the root surfaces. Bacterial cells were not abundant on the root cap, but in the elongation zone and the region where root hairs cover the root surface. The higher number of bacteria on root hairs indicates that WGA can facilitate the adherence of strain WS32 to wheat roots.

The genus *Pseudomonas* has been extensively studied in PGPR research (Vessey, 2003; Wu *et al.*, 2008; Cummings and Orr, 2011). It is important to understand the processes through which this species can colonize their host plant's roots. However, many factors, such as bacterial chemotaxis, motility, and cellular hydrophobicity, are known to affect bacterial attachment (Rodríguez-Navarro *et al.*, 2007). In this study, we explored another explanation of the effective attachment of the genus *Pseudomonas* strain WS32 to plant roots. The results obtained here clearly showed that treating the roots with WGA can significantly increase the number of adsorbed *Pseudomonas* strain WS32 cells on the wheat root's surface.

In conclusion, when the wheat roots were treated with WGA, more cells were attached to the bulbs of the root hairs and in the elongation zone. The number of cells adhering to wheat roots was significantly increased, with the majority of the WGAs being located on the root hairs or along the axis of the root surface. We demonstrated the specific WGA-mediated binding of the *Pseudomonas* strain WS32 to wheat roots. The findings of this study provide a better understanding of the processes involved in plant-microbe interactions.

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