Iran. J. Biotechnol. April 2021;19(2): e2762 DOI: 10.30498/IJB.2021.2762

Research Article

# Induction of Wheat Resistance to STB by the Endophytic Fungus *Serendipita indica* and *Pseudomonas protegens*

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**Background:** *Septoria tritici* blotch (STB) caused by fungus *Zymoseptoria tritici*, is one of the important wheat (*Triticum aestivum* L.) diseases difficult to control because of the lack of wheat resistant cultivars. The use of biological control agents is one possible way for triggering host plant resistance to biotic and abiotic stresses.

**Objective:** In this study, we examined the ability of *Serendipita indica* and *Pseudomonas protegens* CHA0-mCherry in inducing the local wheat cultivar Tajan resistance to STB.

**Materials and Methods:** The interaction between biological control agents and the roots of wheat was evaluated. The experiment was conducted in a completely randomized design by three replicates. Spore suspension was supplied at concentrations of $10^7$ and $10^9$ for *S. indica* and bacteria isolate (CHA0-mCherry) respectively. Five treatments were applied including *S. indica*, CHA0-mCherry, *S. indica* and CHA0-mCherry co-inoculation, positive and negative control. Twenty-one days after inoculation, the interaction between biological agents and plant roots were evaluated through morphological traits and qPCR. The plant resistance, disease severity, and the correlation between resistance and disease severity were assessed. Pycnidial variation and agronomic traits were also evaluated.

**Results:** Twenty-one days after inoculation, both biological agents clearly colonized all treated roots of all treatments except in control plants as demonstrated by qPCR analysis. Chlamydospores were observed in the *S. indica*-treated hosts with the CHA0-mCherry colonizing assessment showing $5\times10^9$ CFU $g^{-1}$ in the root. The asexual phase of the fungal pathogen, pycnidial diameter, was reduced in *S. indica* treated plants more considerably than in the other treatments. There was a positive correlation between resistance and disease severity mean when calculated by Pearson's correlation. There was a significant difference between the root length, fresh, and dry weight of root. Spore density was inversely correlated to resistance and disease severity, when compared with control, with CHA0-mCherry being the most effective in reducing the spore density. *S. indica* was the most effective in promoting root growth and stem biomass, when compared with control.

**Conclusions:** *Serendipita indica* and *Pseudomonas protegens* CHA0-mCherry colonies showed a potential biological control activity and efficiently enhanced the plant resistance to *Z. tritici* in the treated wheat roots. The microbial biological control agents are very practical in crop protection against plant disease and can be very useful in sustainable agriculture.

**Abbreviations:** PLSN: percentage of leave surface necrosis, DPI: day past inoculation, PLACL: percentage of leaf area covered by lesions, PPMLA: pycnidia per millimeter in leaf area.

*Keywords:* Biocontrol, Disease Severity, Endophyte, Interaction, Pycnidia, Septoria Blotch

## 1. Background

Wheat (*Triticum aestivum* L.) is one of the most important and strategic cereal crops in the world which is also affected by biotic and abiotic stress (1). The wheat cultivated area and yield in Iran was estimated about 5.843 million hectares and 11 million tons in 2018 (2). *Septoria tritici* leaf blotch (STB) is considered the most damaging wheat foliar disease affecting the wheat

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production causing global crop losses of approximately US $5 billion per year (3). *Z. tritici* is a filamentous fungus which exhibits a hemibiotrophic lifestyle. The disease was first reported from France in 1842 (4). In Iran, the first report of STB was observed in 1942 (5). While STB can reduce global wheat yields by about 30-53% (6), the damage of STB in some provinces of Iran such as Khuzestan and Golestan, were reported to be up to 40% (7). Tajan cultivar was resistant to STB which was introduced for the first time in the north of Iran in 1995. However, Tajan resistant cultivar becomes susceptible to STB due to the new pathogen strains that manage to overcome the host resistance (8). Due to the high costs of fungicide application and their negative impact on human health and the environment, the use of resistant cultivars is considered to be the best method in managing economically important plant diseases such as STB (9). Such resistance in plants can be achieved through induced systemic resistance which is occurs via stimulating beneficial root fungi and bacteria. This phenomenon is called "induced systemic resistance" (ISR) (10). In 1997, *Serendipita* (=*Piriformspora*) *indica*, an endophytic mycorrhiza fungus, was discovered in northwestern India which was able to protect plants against pathogens through the ISR mechanism (11). *S.indica* belongs to a new family Sebacinaceae and new order Sebacinales. Sebacinales are divided to two subgroups: Group A, Sebacinaceae and group B, for new family Serendipitaceae (12).*S.indica* colonizes the roots of a wide range of monocotyledonous and dicotyledonous plants such as wheat (13). *S. indica* significantly increased the growth parameters of the growing plant in the soil with a low organic matter content (14).

Meanwhile, ISR is one of the mechanisms used by plant growth promoting rhizobacteria, especially fluorescent pseudomonads (15). When a susceptible plant interacts with growth-promoting rhizobacteria (PGPR), this could enhance systemic resistance to subsequent biotic attacks. This systemic action can also occur in plant tissues distant from the initial infection site. It operates through activating some defense genes that were not active before. This inducing gene leads to accumulation and transport of defense compounds (16). *Pseudomonas protegens* strain CHA0, a member of Proteobacteria phylum and Pseudomonadaceae family (17), has been widely studied as a model strain for the biological control agent against several plant pathogens (18). Some reports have indicated that this strain can be used as plant resistance inducers against some plant pathogens (19). According to available literature, there is no report about the interaction of fungal endophyte *S. indica* and bacterium CHA0-mCherry against STB.

## 2. Objectives

The aims of the this study were to: 1) evaluate the interaction of two biological agents (*S. indica* & CHA0-mCherry) on cultivation of winter wheat, 2) induce resistance in susceptible Tajan cultivar to *Z. tritici* (20), and 3) compare the effect of the endophytic fungus and the bacterial strain on inducing resistance against STB and mitigating disease.

## 3. Materials and Methods

### *3.1. Cultivar and Strains*

*Z. tritici* was isolated from Golestan province wheat fields by Eyal *et al.*'s method (21). The isolates were identified by the morphological trait and fungus identification keys. For molecular identification, internal transcribed spacer (ITS) and large subunit (LSU) DNA regions were used. Most aggressive fungal ZTKJR isolates were isolated from Ramiyan County, registered by 3240C and MG969552 code in IRAN International Fungal Culture Collection and NCBI respectively, also explained in Ashrafi *et al.* (20). *S. indica* isolate was provided by Prof. Kogel, faculty member of the Phytopathology institute, University of Giessen, Germany, registered with the DSM11827 code in the cell culture and microorganism collections of the Brunswick, Germany. Strain CHA0-*mche* = mCherry-tagged; Gentamycin-resistant, was provided by Prof. Keel, faculty member of the university of Lausanne, Switzerland, registered with CCOS2 code in the collection of Swiss cultivation. In this study, Tajan sensitive bread wheat cultivar with Bow ″s″/Nkt ″s″ (CM67428-GM-LR-5M-3R-LB-Y) (www.spii.ir) pedigree was used.

### *3.2. Fungal Cultures and Plant Inoculation*

Tajan cultivar seeds were disinfected by applying 70 % ethanol for 1 min and then washed 5 min with sterile water, followed by 1.5% sodium hypochlorite for 1 min immediately. Then, the seeds were washed with sterile water for 1, 3, and 5 min respectively. The seeds were placed in 9 cm diameter petri dishes containing sterile filter paper (Whatman), after which 5 mL distilled water was added and incubated in darkness at 25 ˚C for 72 h. Germinated wheat seeds were inoculated with spore suspension of *Serendipita indica* ($5\times10^5$ spore /1 mL) and stored for 6 h at room temperature (11). In order to prepare the spore suspension, *S. indica* was cultured by transferring a plug of the young culture on complex medium (CM) in 25 ˚C and darkness for 21 days. CM contained Glucose 20g, Peptone 2g, Salt solution: ($NaNO_3$, Kcl, $MgSO_4.7H_2O$, $KH_2PO_4$) 50 mL,

Yeast extract 1g, plus micronutrient mineral solution Casemic acid 1g, agar 15g, and then dissolved in 1000 mL distilled water. Twenty-one days after inoculation, the Chlamydospore would be visible on the culture medium. Mycelia were harvested by scraping the surface of the culture medium by a scalpel and filtered by lace fabric. Spore cell suspension $5\times10^5$ was prepared by distilled water and 0.05% tween 20. The spore concentration was adjusted by a hemocytometer (11).

*3.3. Bacterial Culture*

Bacterial strain CHA0-mCherry was cultured in LB medium (LB consisted of: Tryptone peptone 10g, Yeast extract 5g, plus mineral elements in distilled water 1000 mL) for 36 h at room temperature on a shaker with 100 rpm/min. The cells were pelletized through centrifugation at 6000 rpm for 15 min. The supernatant was discarded and cells were washed with 0.15% NaCl and pelletized with the centrifuge again. Cell suspensions $5\times10^5$ were prepared by distilled water. Cell concentrations were measured by a spectrophotometer model SQ-2802 made by Unico, USA, at 600 nm and 0.7 absorption (adjusted about $10^9$ cells) (22).

*3.4. Treatment with Biological Inducers*

Soil was prepared by combining perlite, sand, and farm soil equally and then autoclaved at 120±1 ˚C for 60 min twice. Next, 1 kg soil per 15 cm diameter pots was used and the soil was irrigated with water about 12h before planting. Inoculation was performed by soaking germinated wheat seeds into *S. indica* spore cell suspensions (described as before 3.2), after which the solution was incubated at room temperature with shaking at 70 rpm overnight. Three treated wheat seeds were planted directly in pots (11). The inoculated seeds were grown on top of pot soil in greenhouse with 200 µL of $5\times10^5$ *P. protegens* CHA0-mCherry cells suspension added on top of each seed and then covered with soft soil (22). The pots were not irrigated until 72h. Next, they were irrigated by pouring adequate water in the plate under each pot every 48h. Three untreated wheat seeds were planted directly in pots as control.

*3.5. Microscopic Examination of the Interaction between Root and Biological Agents*

In order to observe the interaction between *S. indica* and root, the following method (11) was used. Twenty-one days post-planting, inoculated roots of plants were removed from soil and washed gently with running water. Then, the roots were heated inside a solution of 10% KOH to become cleared off for 4 min and then washed twice with sterile water. The spore germination of *S. indica* on the roots was monitored by ink vinegar staining method (23). Pear shape spores in roots were observed by 40x lens of microscope model CX22 Olympus, Japan, **Figure 1**. For detecting *P. protegens*

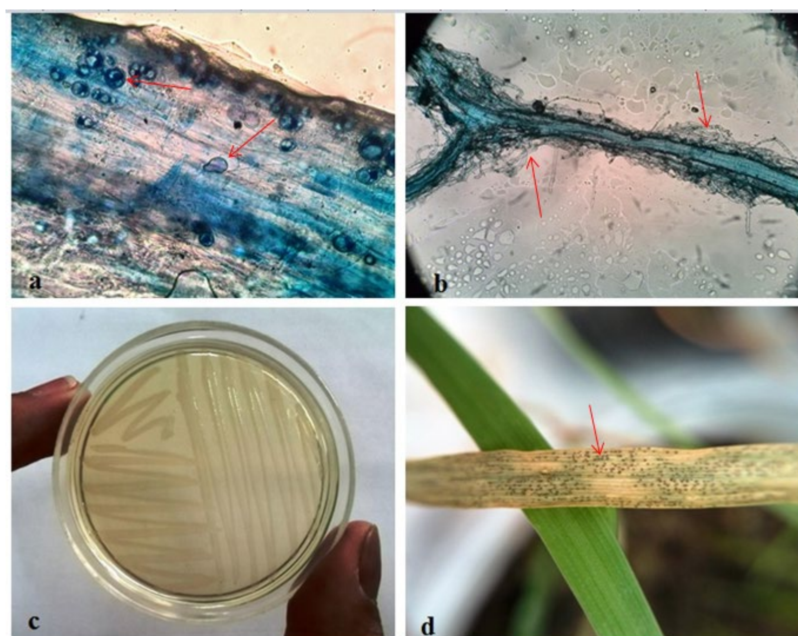


**Figure 1.** a: Colonization of *Serendipita indica* chlamydospore (stained small spores) into wheat root (Bar:15µm) indicated by red arrows. b: *S. indica* mycelium cluster on root (Bar:10mm) indicated by red arrows. c:Colony growth of *Pseudomonas protegens* (CHA0m-cherry) culture on NA medium after 24h at room temperature (Bar:8mm). d: Small pycnidium (black) indicated by red arrow on wheat leave (Bar:10mm).

(CHA0-mCherry) in roots, after harvesting roots and washing, crushed 1 gr dry tissues by sterile mortar and pestle in laminar flow hoods condition. The mixture was added to 99 mL sterile solution and agitated on a rotary shaker for 5 min (200 rpm). Subsequently, 10-fold serial dilutions were prepared as necessary in sterile distilled water, with 0.1mL of the diluted solution uniformly spread on a sterilized. Petri plates with Nutrient agar media (NA) containing 10 µg.$L^{-1}$ of gentamycin were added. The colony was counted after 24 h incubation at room temperature. Colony counts were expressed as colony-forming units per gram of root tissue mixture (CFU. $g^{-1}$) (24).

*3.6. Detection of Biological Agents in Roots*

In order to extract the *S. indica* and *P. protegens* (CHA0-mCherry), 21-day-old wheat seedlings were taken out with roots were removed and washed with running water. DNA was extracted by CTAB method (Murray and Thompson) (25). For detection of *S. indica*, two translation elongation factor *P. indica* Tef (accession no. AJ249911) Butehorn *et al.* (26) and Deshmukh *et al.* (27) for *S. indica* F-5' ACC GTC TTG GGG TTG TAT CC 3' and R-5' TCG TCG CTG TCA ACA AGA TG 3' were used. PCR program was as follows: 4 min at 94 ˚C and followed by 35 cycles for 30 s at 94 ˚C, 30 s in 54 ˚C, 45 s at72 ˚C and at the end 1 min at 72 ˚C. For detection of *P. protegens*, CHA0-mCherry two primers designed by von Felten *et al.* (28) for *P. protegens* CHA0, CHA0_1_for (AK-04) F-5'CGACA-CATGACCAACATCGTTCGA3' and CHA0_1_rev (AK-04) R-5'GGCTACAAGGTCATTACAAAATC-CAGTGAT 3' were used. PCR program was as follows: 4 min at 94 ˚C, followed by 35 cycles at 30 s at 94 ˚C, 30 s at 62 ˚C, 45 s at 72 ˚C and at the end 1 min at 72 ˚C. The PCR reaction consisted of (10 X (1 X) PCR buffer, 50 mM $MgCl_2$ (1.5 mM), 2 mM of each dNTP (0.2 mM), 10 pM of each primer (0.4 pM), 5 u.µ$L^{-1}$ Taq DNA polymerase 5 u.µ$L^{-1}$ (TA7505C Cinna Gen) (1 u), 100 ng DNA, dd$H_2O$. All PCR reactions were done using a thermocycler apparatus model CG1-96 Corbet Research, Australia. The products of the PCR reaction were then examined through electrophoresis using 1% agarose gel in 1 X TBE buffer, at 85 volts for 45 min.

*3.7. Leaf Disease-Severity Evaluation*

Plant inoculation was performed during 30 days. In order to prepare *Z. tritici* spore, 250 mL of culture media containing yeast extract, malt extract, and sucrose (10 g per 1000 mL distilled water) was prepared in 500 mL flask and autoclaved for 20 min at 121 ˚C. Five agar plugs (about 0.5 cm) from 3-day-old *Z. tritici* culture on PDA were inoculated into the flask. Then, the cultures were incubated on a shaker at 120 rpm at 21 ˚C for 96 h. Afterwards, 0.5 mL per liter tween 20 was added and the yeast like spore concentration was adjust at $1\times10^7$ spore cells.$L^{-1}$ using a hemocytometer. Spore suspension was applied by spray inoculation onto plants surfaces. Saturation moisture was prepared for 72 h using nylon cover and treated plants were left in darkness condition for 12 h. After 72 h, up to 70% moisture was supplied (29).

The disease was evaluated through the method of Zhang *et al.*)29). Disease symptoms on wheat plants were quantified and leaf average in each replicate were separately evaluated at 21 days post-inoculation (DPI). Subsequently, disease development was calculated based on the Zhang *et al.* 1-9 scale (29). The plant response to the pathogen was quantified by measuring the percentage of symptoms of disease such as the amount of necrosis, chlorosis, and the density of pycnidia. The treated plant was classified into 4 categories on the basis of mean STB scores including resistant (R: average disease scores ranged from 1.0-4.9), moderately resistant (MR: average disease scores ranged from 5.0-6.9), moderately susceptible (MS: average disease scores ranged from 7.0-7.9), and susceptible (S: average disease scores ranged from 8.0-9.0) (29).

For calculating the disease severity of the *Z. tritici*, the percentage of leave surface necrosis (PLSN) was measured (29,). The correlation between resistance and disease severity data means was analyzed via Pearson correlation test**.** Also, for standard categorization of disease severity and treatment resistance, cluster analysis was calculated through Euclidean distance method by IBM SPSS Statistics v.19.

*3.8. Variation of Asexual Sporulation of Pathogen on Leaves*

Five leaves were randomly selected from each pot (3 replicates). The leaves were decolorized using the method of Chartrain *et al.* (30). All pycnidia evaluation was measured by 10 X magnification by microscope model CX22 Olympus, Japan lens, eye piece, and micrometry stage. To estimate the pycnidial density, the number of pycnidia (PYC) was visually estimated by 10 X magnification as the sum of pycnidia present on the adaxial and abaxial face of the inoculated leaf surface. The number of pycnidia produced on a lesion (PYCmax) was estimated as the maximal count of pycnidia (before the leaf became senescent). The pycnidial density ($PYC_{dens}$) was calculated as the surface density of pycnidia of the sporulation area (Lesion necrotic spot)

(PYCmax/SPOmax). To estimate the pycnidia diameter, the pycnidial diameter ($PYC_{diam}$) was calculated in millimeters for each sporulation area ($PYC_{diam}/SPO_{max}$). PYC= pycnidial density in sporulating area, SPO= the percentage of sporulating area (31).

*3.9 Growth Measurement*
The length, fresh/dry weight, the percentage of plant root and stem tissue water as well as total biomass for each treatment were estimated. The wheat plants were collected at the heading stage, the root systems were separated from shoot samples, the soil particles were gently removed and the roots were washed with running water. The root and stem length and fresh weight were measured. Plant parts were dried at 70 ˚C in an oven for 48 h to obtain the dry weight. The percentage of root and stem tissue water calculated using the equation: PTW= [(FW-DW) ×100]/FW, and the percentage of total biomass calculated using the equation: PB= (DW×100)/FW. FW is root and stem fresh weight; DW is root and stem dry weight (32).

*3.10. Statistical Analysis*
The statistical analysis was performed by the complementary randomized design, with 3 replicates. The five treatments included 1) wheat seeds treated by *S. indica*, 2) wheat seeds treated by bacteria (CHA0-mCherry), 3) wheat seeds in co-inoculation treatments by *S. indica*, and CHA0-mCherry, 4) wheat seeds without any treatment as negative control, and 5) wheat seeds without any biological treatments but inoculated with *Z. tritici* as positive control. The data were subjected to one-way analysis of variance (ANOVA), LSD, and Duncan tests (P=0.05) using SPSS version 19.0 statistical software.

## 4. Results

*4.1. Microscopic Examination of Root and its Interaction with Bacteria and Fungus*
Typical pear-shaped chlamydospores of *S. indica* was observed by root staining on Tajan cultivar root cortex after 21-day post treatment by *S. indica*, **Figure 1A and B,** while no spores were detected in the control plant. To confirm the CHA0-mCherry inoculation 21 days post-inoculation, endophytic bacteria were re-isolated from roots and were cultured on NA media, with the colonies of bacteria appearing on media after 24 h, **Figure 1C**. Nevertheless, the absence of bacteria in the control plant culture was observed. Colony number in the root suspension culture was measured by determining the number of colony forming units, at $5\times10^5$ CFU $g^{-1}$ concentration of bacterial cells (CHA0-mCherry).

*4.2. PCR Detection of Biological Inducers in the Root*
Translation elongation factor (Tef) gene was evaluated by specific Tef primer, a 160 bp band for *S. indica*-treated plants and in the root co-inoculated by *S. indica*,

**Figure 2.** PCR detection of *Serendipita indica* in DNA of wheat roots on 1% agarose gel with the designed specific Tef primer pair gene; amplify a fragment of 110 bp.
Notes: “L” denotes a 100 bp pluse; ladder A: S.i: DNA extracted from pure culture of *S. indica* (positive control); Wh-Co: DNA from root of non-inoculated wheat (negative control); S.i-Treat: DNA from root of inoculated plants by *S. indica*; S.i+P.p: DNA from root of inoculated plants by *S. indica* and *Pseudomonas protegens*, B: P.p: DNA extracted from pure culture of *P. protegens* (positive control); Wh-Co: DNA from root of non-inoculated wheat (negative control); P.p-Treat: DNA from root of inoculated plants by *P. protegens*; P.p+S.i: DNA from root of inoculated plants by *P. protegens* and *S. indica*, Co: technical control.

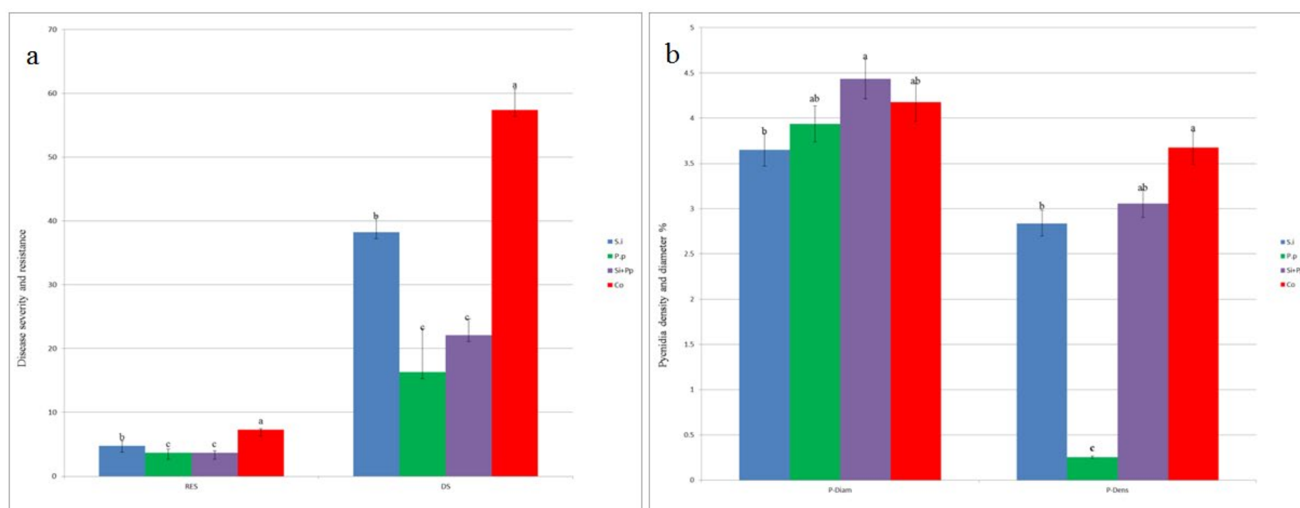


**Figure 3.** Effect of *Serendipita indica* (S.i) and *Pseudomonas protegens* (P.p) on disease severity (Necrosis %) and resistance (a) and pycnidia density (number per millimeter leaf surface) and pycnidia diameter (b) of wheat crops infected by *Zymoseptoria tritici* when compared statistically by LSD test (P : 0.05), Bar indicated by Standard Deviation. A: RES:Resistance, DS :Disease Severity (Necrosis %), B: P-Diam :Pycnidia Diameter, P-Dens:Pycnidia Density, Co: Control, plants without biological agent.
Note: treatment with lesser amount is effective than other treatments.

**Table 1.** Evaluation of disease severity, sporulation Pycnidia, and reaction of Tajan cultivar susceptibility to *Z. tritici* based on the 1-9 scale method (Zhang *et al.* 2001).

| Treatments | Scale Range | Reaction | Disease Severity (Leave pycnidia %) | Pycnidia Diameter | Pycnidia Density (n/mm) |
|---|---|---|---|---|---|
| *S. indica* | 4.78 | R | 38.22 | 3.649 | 2.837 |
| *P. protegens* | 3.67 | R | 16.33 | 3.938 | 0.255 |
| *S. indica* + *P. protegens* | 3.67 | R | 22.11 | 4.435 | 3.057 |
| Control | 7.27 | MS | 57.39 | 4.178 | 3.673 |

n/mm: Number of pycnidia in leaf area (millimeter). R: Resistant, MS: Moderately susceptible, Control: Positive control, plants without biological agent.

while CHA0-mCherry bacterial strains were observed on 1% agarose gel, **Figure 2A**. The results revealed that the amplification of strain CHA0-mCherry with AK-04 primers for in the wheat root treated by CHA0-mCherry and root co-inoculated by *S. indica*, and CHA0-mCherry bacterial strain produced a fragment of approximately 110 bp on 1% agarose gel, **Figure 2B**. We did not observe any fragment using universal fungal and bacterial primers for wheat root samples grown.

*4.3. Disease Development*

The results of analysis of variance showed that the mean of plant resistance data was statistically significant at 1% level. Treatment mean comparisons for plants resistant to *Z. tritici* by LSD's test comparing the average of treatment plants resistance to *Z. tritici* by LSD test revealed that their means fell into three different groups, **Figure 3A**. The CHA0-treated plants showed the highest levels of resistance to *Z. tritici* (29), and the mean fell into the third group (C). The co-inoculation treatment had a second mean resistance, **Table 1**. No significant differences were observed between the mean of this treatment and bacterial treatment, and it fell into group C, **Figure 3A**. The *S. indica* treatment had a higher resistance grade than another treatment and fell into group B **Figure 3A**. All the three treatment were grouped as resistant (R) according to the Zhang *et al.* (29), **Table 1**. The control plants' treatment presented a significantly higher number of scales compared to the other three treatments and they were categorized in the moderately sensitive (MS) class (group A) according to Zhang *et al.* (29), **Table 1, Figure 3A**.
For evaluating the disease severity in this study, the mean percentage of PLSN was calculated (5, 7). We quantified the diseased plant to compare the treated and non-treated plants based on the symptoms on

wheat plants including the amount of leaf necrosis, leaf chlorosis, and the density of pycnidia as described by Zhang *et al.* (29). The analysis of variance indicated that the treatment means had statistically significant differences at 1% level. The LSD test showed three different groups for these treatment means, **Figure 3A**. The CHA0-treatment and co-inoculation treatment scored in the minimum mean and they fell into the group C by LSD test. These treated plants showed a lower percentage of leaf area covered by lesions (PLACL) and a significantly lower number of pycnidia. The *S. indica* treatment had greater damage than another treatment and fell into group B. The mean necrosis of control plants was 57.39% of leaf surface and was categorized in group A.

Regarding the correlations between disease severity and resistance, the Pearson correlation coefficient was 0.934 and it stood significantly at 0.01, **Figure 4** (the correlation coefficient is near +1). These results demonstrated that there is a strong positive correlation between these two data groups.

The cluster analysis of disease severity showed there were 2 groups for all 4 treatments. Control and *S. indica* fell into group 1; CHA0-mCherry and co-inoculation were placed in group 2. In this classification, the rates of damage in control and *S. indica* were very close to each other and they were categorized in the same group. Thus, CHA0-mCherry and co-inoculation treatments were placed in group 2.

### *4.4. Variation of Asexual Sporulation of Pathogen on Leaves*

Analysis of variance of pycnidia density means showed that there were significant differences between the treatments at 1%. The mean value of all pycnidial density treatments was recorded to range from 0.255 pycnidia per millimeter in leaf area (PPMLA) for CHA0-mCherry treatment to 3.673 PPMLA for control, **Table 1**. The difference between *S. indica* and co-inoculation treatments means was 0.22 PPMLA. The difference between higher and lower range was 3.418 PPMLA. Thus, it can be concluded that the biocontrol agents were effective in reducing the number of pycnidia in each necrotic spot. Additionally, CHA0-mCherry was more effective than three other treatments in lowering the pycnidial density. The LSD test showed three groups for means of treatments, **Figure 3**. B. CHA0-mCherry treatment had the least average and fell into group C. *S. indica* belonged to group B and co-inoculation treatment was related to intermediate group AB. The highest mean of pycnidial density was related to the control (group A).

The results of means pycnidial diameter showed that, the analysis of variance was not statistically significant. Meanwhile, in this section, the least mean diameter was related to the fungus *S. indica* with 3.649 PPMLA, **Table 1**.

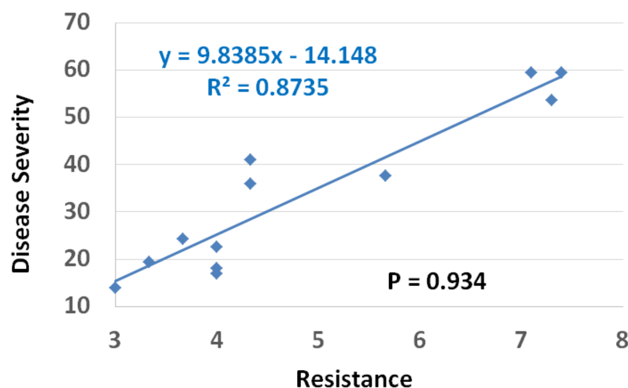


**Figure 4.** Correlations between disease severity (leaf surface necrosis %) and resistance of Tajan cultivar treatment by biocontrol agents by Pearson correlation test.

### *4.5. Measurement of Agronomic Traits*

Analysis of variance of root length showed statistically significant differences at 0.05 levels among treatments. LSD test showed that, CHA0-mCherry had the highest mean and it fell into group A. *S. indica* was related to group B. The co-inoculation treatment belonged to group BC. Finally, negative and positive controls were related to group C, **Figure 5A**.

Analysis of variance of stem length data means revealed that there were not statistically significant differences at 0.05 levels. As indicated in **Figure 5A**, the means of treatments were very close to each other. LSD test made 1 group for stem length data mean. However, the mean of CHA0-mCherry was higher than that of other treatments and the lowest mean belonged to positive control **Figure 5A**.

Analysis of variance of root weight data means confirmed that there were statistically significant differences between fresh and dry root weights and fell into two groups by LSD test at 0.05 level, **Figure 5B**. Bacterial strain CHA0-mCherry showed the highest mean and it was related to group A. The mean comparison of root dry weight data fell into three groups, **Figure 5B**. The highest mean belonged to bacteria and was assigned into group A. The lowest average belonged to the positive control and it was related to group C. *S. indica* average belonged to group B.

Means comparison of fresh and dry weight of the stem revealed that there were statistically significant

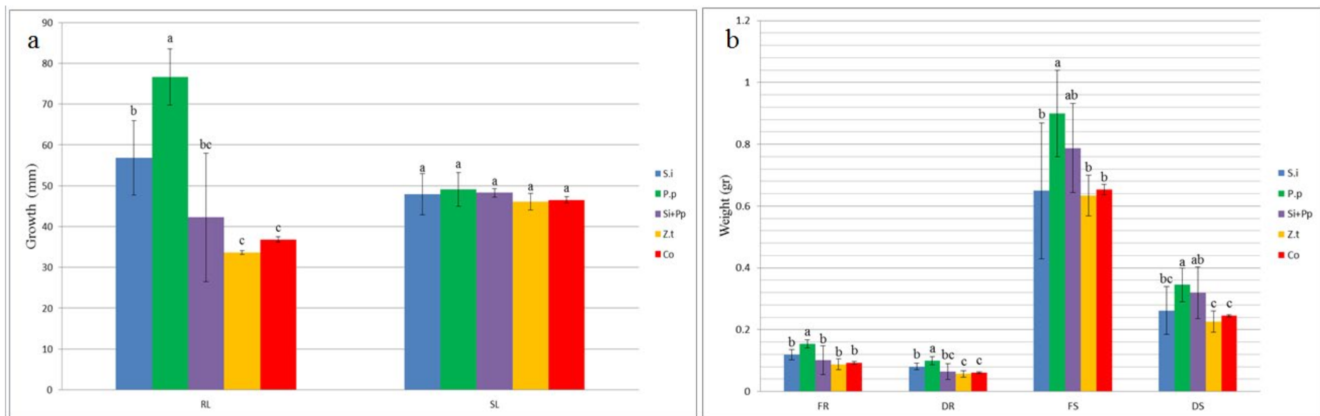


**Figure 5.** Effect of *Serendipita indica* (S.i) and *Pseudomonas protegens*(P.p) on growth (a) and growth parameters (b) of wheat plants infected by *Z. tritici* (Z.t) at 21˚C in green house when compared statistically by LSD test (P : 0.05), Bar indicated by Standard Deviation. Means root and shoot length per plant, A: RL:Root Length, SL:Stem Length, B: DR: Root dried weight, FR: Root fresh weight, DS:Stem dried weight, FS:Stem fresh weight, Co: Control, plants without biological agent.

differences at 0.05 level and fell in two groups. In fresh stem weight data, CHA0-mCherry belonged to group A. The means of co-inoculation was related to intermediate group AB. Negative control, *S. indica*, and positive control had the lowest means and they fell in the same group B. The mean stem dry weight fell in three groups and co-inoculation was related to intermediate group AB. *S. indica* mean belonged to intermediate group BC. Positive and negative controls belonged to group C.

Analyses of variance of stem and root water as well as total biomass showed that there were not significant differences at P=0.05 level. The total biomass of root and root water treatments were placed in one group by LSD test. The positive control had a higher mean stem water percentage and fell in group A. *S. indica* had the minimum mean and fell in group B. All other treatments belonged to intermediate group AB. The average of negative control, CHA0-mCherry and co-inoculation were higher respectively between treatments. Means comparison of stem total biomass showed that *S. indica* had the highest mean between other treatments and it fell in group A. Co-inoculation, CHA0-mCherry, and negative control had a higher stem biomass mean respectively.

## 5. Discussion

This study indicated that root endophytic fungus *S. indica* and bacterium *P. protegens* CHA0-mCherry are potent biocontrol agents playing pivotal roles in improving plant growth and enhancing plant resistance against *Z. tritici*, which has not been reported before. In this study, an attempt was made to compare the ability of both biocontrol agents in inducing resistance in susceptible Tajan wheat cultivar against STB. The results showed that both biocontrol agents made effective interaction with plant roots as confirmed by qPCR. It is evident that *S. indica* developed early in the beginning of interaction with plant root in the first three days and colonized the root cortex between 7 to 14 days post-sowing. The chlamydospores were also visible within the root cortex cells of 14-to 28-day old plants and indicated recently by Moharam *et al.* (33). Colonization of CHA0-mCherry was studied by Keel *et al.* (34).

Microscopic analyses of *S. indica* and CHA0-mCherry co-inoculation treated plants showed that both biological agents colonized the root cortex of Tajan cultivar. The results clearly showed that in co-inoculation treatments, both of the biological control agents interact with plant roots in soil. The *S. indica* interaction necessary condition observed in this research, it is better to supply 27 ˚C within the first 7 days post-inoculation (33). The optimal temperatures for survival of bacterium CHA0-mCherry were about 15-25 ˚C (24). It may complicate the application of biological agents together in co-inoculation treatment. However, the results of this study showed that, CHA0-mCherry could survive as well at 27 ˚C for about 7 days. Several studies have revealed that *S. indica* and bacterial strain CHA0-mCherry have the capability to stimulate resistance in wheat plants when infected with powdery mildew and rust leaf (10, 19, 22). *S. indica* is considered as a model endophytic fungus in crop protection (14).

Bacterial strain CHA0-mCherry has been found in suppressive soil as a soil-borne pathogen (35).

Nevertheless, in this study our assessment of these two biological agents together against airborne *Z. tritici* in Tajan wheat cultivar showed that, the resistance arises in all treatments and biocontrol agents could reduce disease development in the treated plants, **Figure 3A**. The minimum average belonged to CHA0-mCherry and co-inoculation treatment. We observed that CHA0-mCherry was very effective compared with *S. indica* in protecting treated plants against STB. In general, the treatment of the Tajan cultivar by *S. indica*, co-inoculation and *P. protegens* induced resistance against *Z. tritici*. The results showed a significant difference between mean resistances in control treatments. The result of this research showed that, the mean of control treatment was different in *S. indica* treatment with 2.49 grades, in *P. protegens* with 3.6 grades, and in co-inoculation treatments. *P. protegens* and co-inoculation treatments had the same resistance grade and their resistance mean (3.6) was equal, **Table 1**. On the other hand, the difference between co-inoculation treatments and *S. indica* treatment was 1.1 resistance grades. *S. indica* was present in these two treatments but they were different 1.1 grades in the mean resistance. With these interpretations, it can be concluded that in co-inoculation treatments, resistance arises for CHA0-mCherry inducing. This result showed, bacterial biocontrol agent was more effective than *S. indica*. The laboratory culture test of CHA0-mCherry and microscopic analysis of *S. indica* that was confirmed by qPCR, **Figure 2Aand B,** showed that both of these two biocontrol agents are able to colonize wheat plant roots. Thus, from this data we can conclude that *P. protegens* CHA0-mCherry is more effective than *S. indica*.

Disease severity data were scored from 16.33 to 57.39 (PLSN) with their differences being equal to 41.06. As expected, we found that there is an opposite relationship between disease severity and disease resistance. In addition, the control treatment showed the highest disease severity and was the most sensitive. However, there was 16.11% (PLSN) difference between co-inoculation and *S. indica*, and they did not fall in the same group. *S. indica* showed more severe disease than co-inoculation where the difference between these two treatments was 5.78% (PLSN). Thus, the group of co-inoculation treatment was the same as CHA0-mCherry treatment (group C) and *S. indica* belonged to group B, **Figure 3A**.

The mean pycnidia density was calculated for all treatments (2, 5, 20). This result confirms that the bacterial strain is a more effective resistance inducer than *S. indica*. However, the results of pycnidial diameter analysis showed that *S. indica* is more effective than CHA0-mCherry in reducing the pycnidia diameter, **Figure 3B**. According to Poppe *et al.* (36), when biological agents interact with the host plant, *Z. tritici* pycnidial is affected by this interaction. So, disease symptoms, spore production, and diameter are reduced.

## 6. Conclusions

Data evaluation and statistical analysis of pycnidial dimensions in this research indicated that the pycnidial diameter is a more effective factor to enhance resistance in the Tajan wheat cultivar. The amount of spore per pycnidia could be reduced using these two biocontrol agents and it could also be useful for reducing the inoculum pressure on wheat farms and epidemic condition. These biocontrol agents and other wheat cultivars should be investigated in subsequent studies. To sum up, it can be concluded that, *S. indica* is a potential biological agent in elevating the total biomass of Tajan cultivar attacked by *Z. tritici* and it is necessary to perform such experiments by this endophytic fungus in field trials in the future.

## Acknowledgments

We wish to express our gratitude to Professor C. Keel faculty member of the University of Lausanne, Switzerland, his kind attitude for his advices and sending an isolate of specific modified bacterial *Pseudomonas protegens* strain registered with CCOS2 code (CHA0-mCherry) from the Swiss culture collection without any hesitate. We are also especially grateful to Prof. Dr. G. Defago for her advices. We are also especially grateful to Dr. A. Ali-Arab and Dr. A. Najafifar for their advices in statistical analysis. We wish to thank Dr. Sareh Hatamzadeh for her advic in molecular analysis.

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