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**Fusarium culmorum** affects expression of biofilm formation key genes in *Bacillus subtilis*

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**A B S T R A C T**

It is known that there is correlation between biofilm formation and antagonistic activities of *Bacillus subtilis* strains; but, the mechanism of this correlation is not clear. So, the effect of the plant pathogen (*Fusarium culmorum*) on the biofilm formation in a *B. subtilis* strain with high antagonistic and biofilm formation activities was studied. The expression of sinR and tasA genes involved in the biofilm formation was studied in both single culture of bacterium (*B*) and co-culture with *F. culmorum* (FB) using real-time PCR. The results revealed that the expression of the sinR gene in both B and FB conditions was continuously decreased during the biofilm formation period and, after 24 h (FB4 and FB4), it reached 1% and 0.3% at the planktonic phase (B1), respectively, whereas the expression of the tasA was continuously increased and was 5.27 and 30 times more than that at the planktonic phase (B1) after 24 h, respectively. So, the expression reduction rate for sinR (3 times) and the expression increasing rate for tasA (6 times) were significantly higher in FB conditions than the B ones. The relative expression of sinR in FB1 (planktonic phase), FB2 (8 h), FB3(12h), and FB4 (24 h) times was 0.65, 0.44, 0.35, and 0.29, whereas the tasA gene expression was 2.98, 3.44, 4.37, and 5.63-fold of the one at coordinate time points in B conditions, respectively. The significant expression reduction of sinR and increase of tasA confirmed that the presence of pathogen could stimulate biofilm formation in the antagonistic bacterium.

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**Introduction**

*Bacillus subtilis* is known as one of the most important antagonistic (biocontrol agent) and plant-growth promoting bacteria (PGPR) that is isolated from rhizosphere of different kinds of plants.\textsuperscript{1-4} *B. subtilis* strains have the potential to produce more than two dozens of different antimicrobial compounds and antibiotics with an amazing variety of structures\textsuperscript{5} and also are able to form multicellular structures or biofilm.\textsuperscript{6-8} Biofilm formation occurs in many bacterial species in response to diverse environmental conditions such as nutrient depletion and
drought, and it is mediated by many mechanical, biochemi-
cal, and genetic factors. Commonly, a mixture of polymeric
compounds (e.g. extracellular polysaccharides, proteins, and
DNA) and an aggregation of different microorganisms can be
found in biofilms. Ability to form biofilm is associated with
numerous benefits for its bacteria. For instance, antibiotics are
the most common tools to remove bacteria; but, they are not
efficient in the biofilm structure.

Biofilm formation depends on two matrix gene operons,
including yqxM (tapA-sipW-tasA genes) and epsA-O (15 genes)
which are directly controlled by a repressor SinR and are
responsible for the synthesis of amyloid-like fibers and an
exopolysaccharide as two major biofilm components. Derepression is triggered by sinR which is activated by
phosphorylated SpoOA (SpoOA–P) as a master and important
regulatory protein in biofilm formation process. The tasA gene in the operon yqxM is the major gene which encodes the
protein involved in antimicrobial activities, spore coat assem-
by, and germination. It is also found in the stationary phase,
sporulating cultures, and the biofilm matrix.

The environmental conditions and presence of other
organisms like plant pathogens, symbionts, commensal-
ism organisms, and plant hosts can affect biofilm for-
tation; therefore, different biofilm structures such as plaques,
slimes, pellicles, and colonies are seen under various condi-
tions. Previously, some researchers have shown that there is positive correlation between biofilm for-
tation as well as FGPR and antagonistic activities of B. subtilis strains. Bais et al. demonstrated that a B. subtilis strain
(ATCC 6051) was able to form biofilm-like structures on the
roots of Arabidopsis plants and protect Arabidopsis from in-
fec tions by Pseudomonas syringae. Chen et al. showed that
plant protection by antagonistic B. subtilis strains against Ralstonia solanacearum depended on widely conserved genes required
for biofilm formation, including regulatory genes and genes
for matrix production; so, they provided evidence suggesting
that matrix production is critical for bacterial colonization
on plant root surfaces.

Previously, we isolated and selected some native B. sub-
tilis strains which had high biofilm formation potential and
antagonistic capability against Fusarium culmorum, the causal
agent of foot and root rot on wheat. Finally, the strains
with high biofilm production and biocontrol potential were
selected. The B. subtilis strain Bs12 isolated from sugar beet
fields in Kermanshah region (Iran) showed high biofilm for-
tation, volatile production, protease activity, and 79.4% and
83% inhibitory effect against F. culmorum at laboratory and
greenhouse levels, respectively. It was shown that volatile
and protease production as well as biofilm formation by this
strain and also other selected strains had significantly positive
correlation with their antagonistic ability, which coordinated with the previous reports. The principal purpose of this investigation was to find a part of the mechanism for correlation between biofilm formation and antagonistic effect at molecular level; therefore, the effects of a plant pathogenic fungus (F. culmorum) on forming biofilm in B. sub-
tilis (Bs12) were evaluated. To do so, expression of the tasA and sinR genes in the strain Bs12 was investigated using
real-time PCR method both in the presence and absence of
F. culmorum.

Materials and methods

Microorganisms and culture conditions

The native B. subtilis strain Bs12 (GenBank accession num-
ber HQ234328) with high potential in biofilm formation and
antagonistic activity against F. culmorum was used. The bac-
terial strain was routinely grown on nutrient agar (NA) or
Luria-Bertani broth (LB) at 37 °C. For long maintenance, sterile 40% glycerol was used according to Weller and Cooks and,
then, transferred to –20 °C. The F. culmorum strain was kindly
provided by Plant Protection Research Institute of Iran (PPRI),
cultivated on potato dextrose agar (PDA) at 27 °C for routine
experiments, and transferred to 4 °C for long time mainte-
nance.

Primers designing

Two specific primers pairs, TasA-F (CAA GCC GTT CCA CTG
TGT AG)/TasA-R (AAC CGC TCC TGA ATA TGA TGG) and Sin-
R-F (AAA GGC TAC TCA CTA TCA GAA C/SinR-R (TCT AAT TGA
CCA TCG TAT TCG G), were designed using Oligo (National
Bioscience Inc., version 5) software for conducting the real-
time PCR experiments. These primers amplified 181bp and
188bp DNA fragments of tasA and sinR of B. subtilis, respec-
tively. The primer pairs, 16SrRNA-F (GTA ACC TGC CTG TAA
GAC TGG)/16SrRNA-R (CTG TAA GTG GTA GCC GAA GC), with
the PCR product length of 110bp were used as the internal
control. Primers were designed in order to have the length
of about 20–22 bases, G/C content between 40.9% and 55%,
and Tm of about 56–59 °C. Length of the PCR secondary structures and dimer formation was controlled using Oligo Analyzer 1.0.3
software. The primers were synthesized by MWG (Ebersberg,
Germany).

To evaluate the specificity of the primers, a PCR was per-
formed using genomic DNA of B. subtilis (Bs12) and F. culmorum. Genomic DNA of the bacterium and fungus was extracted
using GenElute™ Bacterial Genomic DNA Kit (Sigma–Aldrich,
Zwijndrecht, NL) and Core-one™ kit (CoreBio, USA), respec-
ively.

Co-culture of B. subtilis and F. culmorum

Bs12 cells were grown in biofilm growth medium (BGM) con-
taining an LB-based medium plus 0.15 M ammonium sulphate,
100 mM potassium phosphate, pH 7, 34 mM sodium citrate,
1 mM MgSO4, and 0.1% glucose, as described by Hamon and
Lazzarella. Sampling was performed under bacterial plank-
tonic and biofilm formation conditions, according to Stanley
et al. To obtain planktonic cells, the bacterial cells were
grown overnight in BGM medium at 37 °C with shaking at
200 rpm. Afterwards, the medium containing bacteria was
divided into two parts, one co-cultured with suspension con-
taining 106 spores per mL of F. culmorum and another without
any fungal treatment (as negative control). Both beakers were
put in the above growth conditions for three more hours
(OD600 = 2.5 for control). At this time, the bacterial medium
was diluted with an OD600 = 0.1 in fresh medium and, then, the
medium containing bacterium and fungus was diluted with
the same amount of fresh medium. An aliquot of contents of each beaker as planktonic cell population (6 mL) was harvested by centrifugation at 8000 rpm for 10 min for RNA isolation. In the second step, both beakers were incubated at 37 °C without shaking; these conditions were necessary to induce biofilm formation in bacteria. The next samples containing 6 mL taken from each beaker were harvested 8, 12, and 24 h after incubation by centrifugation at 8000 rpm for 10 min. All the samples were immediately put at −80 °C until RNA extraction. To normalize the experiments, 100 μL of the media containing microorganisms were cultured on NA and bacterial CFU was counted in each sample after 24 h at 37 °C. Before RNA isolation, normalization was performed by diluting the samples containing more bacterial cells as the final bacterial CFU was the same for all the treatments.

**Total RNA isolation and cDNA synthesis**

Total RNA was extracted from the harvested samples using Aurum Total RNA Mini Kit (Bio-Rad, USA) according to the manufacturer’s instructions. The concentration of RNA was quantified using a spectrophotometer (NanoDrop 1000 spectrophotometer-Thermo Scientific). PCR was performed using RNA (0.6 μg) as the template to ensure the absence of genomic DNA contamination in the RNA samples. The temperature profile for PCR consisted of a first denaturation step of 5 min at 94 °C, followed by 40 cycles of 94 °C/1 min for denaturation, 60 °C/1 min for annealing, and 72 °C/1 min for extension. A final extension was carried out at 72 °C/5 min.

Total RNA was transformed into cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, USA) following the manufacturer’s protocol. The temperature program for cDNA synthesis was 25 °C/5 min for the attachment of primers, 42 °C/45 min for cDNA synthesis, and 85 °C/5 min for enzyme inactivating.

**Real-time PCR**

Real-time PCR was carried out using iCyclerIQ real time PCR (Bio-Rad, USA) using IQ™ SYBR® Green Supermix Kit (Bio-Rad, USA) in 96-well plates. After the dilution of cDNA, 1 μL (20 ng) was added to 24 μL of PCR mixture (12.5 μL of IQ™ SYBR® Green Supermix, 1 μL of each primer at 10 pmol/μL and 9.5 μL of RNase-free water). Specific cDNAs were amplified by real-time PCR using the specific primers. The real-time PCR cycling conditions were designated as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 20 s, and the final extension was carried out at 72 °C for 5 min. Fluorescence measurements were recorded during each annealing step. To establish a melting curve and confirm the primers’ specificity, an additional step starting from 50 to 95 °C was performed. This step included ninety 10s cycles, in each of which there was temperature increase by 0.5 °C and, at the end of each 10s, the emitted fluorescence was recorded. The efficiencies of amplifications were determined by running a standard curve by the serial dilutions of cDNA. Efficiency can be calculated by the formula: \( E = [10^{(1/s - 1)} - 1] \times 100 \), where \( s \) is the slope of standard curve. For each measurement, a threshold cycle value \( (C_T) \) was determined. \( C_T \) is defined as the number of cycles required for the fluorescent signal to pass the threshold (i.e. exceeds the background level). Finally, the expression of genes was calculated by formula \( 2^{-\Delta \Delta C_T} \). The results were normalized using B. subtilis 16S rRNA gene as the internal gene. The ultrapure water was used instead of cDNA as a negative control and the gene expression levels were compared with the negative control.

**Statistical analysis**

Measures were taken for each condition by cDNA synthesized from RNA extracted from three independent cultures and performed in triplicate for each gene. Real-time PCR data analysis was performed using Bio-Rad software based on the threshold cycle (\( C_T \)). Analysis of variance, comparison of means, and score of treatment groups were obtained using SAS (version 9.1) and Duncan Multiple test (\( p < 0.01 \)).

**Results**

**Primer specificity and real-time PCR optimization**

To evaluate specificity of the designed primers, PCR was carried out using genomic DNA of B. subtilis (Bs12) and F. culorum. When bacterial genomic DNA was used as the template, TasA-F/TasA-R and SinR-F/SinR-R primers amplified 181 and 188 bp fragments, respectively. In addition, the PCR product of internal control primer was a 110 bp fragment. No PCR product was observed when the fungal genomic DNA or negative control was used. After sampling and RNA extraction, PCR was performed using the samples of RNA 16S rRNA-F/16S rRNA-R. No fragment was amplified in the samples. These results confirmed that there was no DNA contamination in the RNA samples. To determine the amplification efficiency, different serial dilutions of cDNA were used for each primer. For instance, five dilutions of cDNA from 1 to 0.0001 were used for 16SrRNA primers and, finally, cycle threshold, Tm, and standard curves were obtained. According to this experiment, the efficiency of 16S rRNA, TasA, and SinR primers was determined as 92.75%, 99.98%, and 96.78%, respectively.

**Evaluating effect of pathogen presence on sinR expression**

Relative expression levels of sinR gene in the strain were calculated in the absence (B) and presence of the fungus (FB) from three independent cultures in triplicate. The results indicated that the maximum expression of sinR was observed when the bacterial cells were in the planktonic phase in the absence of F. culorum (B1) (Fig. 1(a)). By entering the biofilm formation phase, the expression of the gene was critically decreased, which was continued over the time from B1 to B4 (24 h after entering the biofilm formation period). The maximum reduction rate of the gene expression was observed 8 h after starting the biofilm formation compared with planktonic phase (about 80% reductions) and the minimum expression was observed 24 h after starting biofilm formation (B4) which was about 1% of the expression level in planktonic phase (Fig. 1(a)). When the bacterial cells were co-cultured with F. culorum, the sinR expression reduction trend was critically increased. Similar to the experiments in which plant pathogen was absent, the
maximum expression in the co-culture system occurred when the bacterial cells were in planktonic phase (FB1). It was continuously reduced 8 (FB2), 12 (FB3), and 24 (FB4) h after entering the biofilm formation phase and reached 3% of the expression level in planktonic phase (FB1) (Fig. 1(b)). The maximum reduction rate of the gene expression was observed 8 h after starting the biofilm formation (about 64% reductions). Comparison of the results of both treatments (B and FB) showed that the relative expression of sinR in FB condition was significantly lower than that of B condition in the same growth conditions and time points (Fig. 2(a)). The relative expression for FB1, FB2, FB3, and FB4 was 0.65, 0.44, 0.35, and 0.29 compared with B1, B2, B3, and B4, respectively, and by increasing the time during the biofilm formation period, the reduction rate of the sinR gene expression in FB condition was continuously increased compared with that of B condition in the same growth conditions and time points (Fig. 2(a)). The relative expression of the sinR gene during the planktonic and biofilm formation period in B and FB conditions compared with the planktonic phase of B condition is shown in Fig. 3(a). The maximum and minimum expressions were observed in the planktonic phase of B condition (100%) and 24 h after starting the biofilm formation in FB treatment (0.3%), respectively (Fig. 3(a)).

**Evaluating effect of the pathogen presence on expression of tasA gene**

The results of quantitative PCR showed that the expression of tasA gene continuously increased from planktonic to the final biofilm formation phases in B condition (B1–B4). The maximum gene expression was observed when the bacterial cells were at 24 h after starting the biofilm formation period in the absence of F. culmorum (B4), which was 5.27 times more than that in the planktonic phase (B1) (Fig. 4(a)). By entering the biofilm formation phase, the gene expression was critically increased, which was continued over the time from B1 to B4 phases. The maximum increasing rate of the gene expression
was observed at 24 h after starting the biofilm formation in which the expression of the gene was increased up to 315% compared with the phase B3, whereas the minimum increasing rate was observed at 8 h after starting biofilm formation (B2) in which the expression of the gene was increased by about 18% compared with the previous phase (B1) (Fig. 4(a)).

When the bacterial cells were co-cultured with *F. culmorum*, the *tasA* expression was significantly and continuously increased, which was critically increased during different phases from FB1 to FB4 (Fig. 4(b)). This increasing rate was significantly more than that of the treatments containing only bacterial cells. The levels of expression of the *tasA* gene 8, 12, and 24 h after entering biofilm formation phase were 1.59, 2.99, and 3.26 times more than those of the planktonic phase (FB1) (Fig. 4(b)).

The expression of the *tasA* gene in FB1 to FB4 phases was 2.98, 3.44, 4.37, and 5.63-fold of the one at similar time points in B (B1 to B4) conditions, respectively (Fig. 2(b)). These results suggested that the pathogenic fungus stimulated the expression of *tasA* gene. Fig. 3(b) shows the relative expression of the *tasA* gene during the planktonic and biofilm formation periods in treatments B and FB compared with the planktonic phase of treatment B (B1). The maximum expression was observed 24 h after starting the biofilm formation in treatment FB, which was 30 times more than that of the planktonic phase of treatment B (3000% increase) (Fig. 3(b)).

**Discussion**

We characterized 30 Iranian native *B. subtilis* strains isolated from the rhizosphere of various hosts in different regions of Iran. The results of laboratory and greenhouse experiments showed that volatile and protease production as well as biofilm formation by some strains had significantly positive correlation with their antagonistic ability and, finally, the most powerful antagonist strains with high biofilm production were selected. Strain Bs12 isolated from sugar beet fields in Kerman-shah region showed 79.4% and 83% inhibitory effect against *F. culmorum* at laboratory and greenhouse levels, respectively, and high biofilm formation, volatile production, and protease activity; therefore, it was selected for the present study.27
Previously, it has been shown that many different factors such as different fungal compounds (fungal culture supernatant), pH, temperature, nutrient compounds, \textsuperscript{21} indole, \textsuperscript{13} complex polysaccharides, \textsuperscript{22} and oxygen rate affect the biofilm formation. So, to explore the detailed mechanisms of different factors on biofilm formation in \textit{B. subtilis}, it is necessary to perform detailed studies covering all biotic and abiotic environmental factors. Different genetic pathways that are induced by environmental signals are involved in the interaction of cells and abiotic surfaces. These factors can be changed in the amount or type of nutrient content, osmotic factor, pH, temperature, iron, oxidative stress, and substrate type. \textsuperscript{33,34} Stanley and Lazazzera\textsuperscript{35} showed that environmental signals and regulatory proteins affect the initial steps of bacterial biofilm formation and the nature of mature biofilm structure. So, surface attachment and biofilm formation on different biotic and abiotic substrates are influenced by nature and various environmental stimulations. \textsuperscript{24} The presence of other organisms, such as pathogens, is known as one of the factors affecting the biofilm formation and structure in \textit{B. subtilis}; but, the mechanism is not well known. \textsuperscript{1,25,26} So, the principal purpose of this investigation was to find a part of the mechanism for correlation between biofilm formation and antagonistic effect at molecular level; therefore, the effects of a plant pathogenic fungus (\textit{F. culmorum}) on forming biofilm in \textit{B. subtilis} (Bs12) were evaluated. To do so, real-time PCR as a sensitive and quantitative technique was used to measure the expression profiles of two important genes (\textit{sinR} and \textit{tasA}) involved in the biofilm formation process of the bacteria in the presence and absence of \textit{F. culmorum}, the causal agent of wheat common root rot. \textit{B. subtilis} is commonly isolated from rhizosphere of different plants, shows antagonistic activities against plant pathogens, and may be used as plant-growth promoting bacteria. \textsuperscript{1,3,4} Various microorganisms can be found together in the rhizosphere. The presence and production of metabolites by other microorganisms can be very effective for biofilm formation in target bacteria. Our results indicated that the expression of \textit{sinR} was significantly reduced in the presence of the pathogenic fungus. Expression of this gene was at a high level in the planktonic phase of bacterial growth; but, it decreased upon entering the production of biofilm, as was expected. Several previous studies have demonstrated that \textit{sinR} as one of the most important regulatory genes has a direct negative control on biofilm formation of \textit{B. subtilis}. \textsuperscript{17,20,21} Leiman et al.\textsuperscript{16} showed that point mutations in the \textit{sinR} gene resulted in a significant increase in biofilm formation in \textit{B. subtilis} and confirmed that it is a key matrix regulatory gene for biofilm formation. Previously, this subject has been also confirmed by other researchers. \textsuperscript{37,38} Synthesis of main components of biofilm matrix, such as extracellular polysaccharides and proteins, is mediated by two operons of 15-gene \textit{eps} and three-gene \textit{yqxm}, respectively. \textsuperscript{16} Both of these operons are under direct negative control of the \textit{sinR} gene. Indeed, this repressor protein binds to multiple sites within the promoter region for the mentioned operons, thereby repressing its transcription. When this negative regulator is active, expression of these 18 important genes will be suppressed. So, \textit{sinR} gene is known as a master negative regulator in the biofilm formation process of \textit{B. subtilis}. \textsuperscript{17} Transcription of the \textit{sinR} gene is controlled by another gene called \textit{sinI}. When bacteria are in biofilm formation conditions, such as environmental stress, shortage of some nutrient sources, etc., transcriptional factor \textit{SpoA} becomes phosphorylated and activates expression of \textit{sinI}. Activated \textit{sinI} can be prevented from \textit{sinR} expression. Thus, \textit{eps} and \textit{yqxm} operons are activated and, consequently, the genes involved in biofilm formation are expressed. \textsuperscript{20} In the present study, it was observed that the expression of \textit{sinR} was decreased in the biofilm compared with the phase of planktonic cells in both treatments. On the other hand, expression of \textit{sinR} in free-swimming cells was higher than that of the sessile ones in the presence or absence of \textit{F. culmorum}. The expression of \textit{sinR} in FB condition was less at each time point compared with B condition. In planktonic cells, the gene expression level was decreased in the co-culture system (FB1) compared with single culture of bacterium (B1). This diminution rate was repeated at each time point of biofilm formation phase, namely FB2, FB3, and FB4 samples compared with B2, B3, and B4 samples, respectively. These results suggested that the presence of fungus in Bs12 growth medium caused a significant reduction of \textit{sinR} gene expression in both planktonic and sessile cells.

In the case of \textit{tasA} gene, opposite results were obtained. In the planktonic phase, expression of the gene was at the lowest level. By entering the biofilm production phase, the expression of the \textit{tasA} gene was increased and, at final time point of biofilm formation (B4), it reached the highest levels of 5.28-fold. Previously, some other researchers have reported these results and shown significant increase in the expression of \textit{tasA} gene during biofilm formation. \textsuperscript{20,39,40} This increasing rate was observed in bacterial growth phases in both single and co-culture conditions; but, the increasing rate in FB was significantly more than that of treatments B. Branda et al.\textsuperscript{16} showed that \textit{TasA} is a major protein in biofilm extracellular matrix and the absence of this protein results in a residual matrix. \textit{TasA} has been detected in stationary phase and sporulating cultures. It appears that \textit{TasA} has several other functions; for instance, it acts as a broad-spectrum antibiotic factor and seems to have roles in spore coat assembly and germination. \textsuperscript{40,41} Evaluation of the relative expression of \textit{tasA} gene showed that the expression in FB treatments was significantly increased compared with B treatments at each time point. Similar results were observed in planktonic and all biofilm formation levels. Based on these data, expression of \textit{tasA} gene increased in the presence of pathogenic fungus. Many transcriptional factors in physiological activities of bacteria are regulated by environmental stress; for instance, biofilm formation by \textit{B. subtilis} stimulated in non-optimal growth conditions. As \textit{F. culmorum} is a pathogenic fungus, its presence in growth medium of the antagonist bacteria has provided a non-optimal condition; consequently, induced and enhanced biofilm formation down-regulate \textit{sinR} and up-regulating \textit{tasA} genes.

In addition to the mentioned key genes involved in biofilm formation in \textit{B. subtilis}, it has been recently demonstrated that other different genes and factors affect biofilm formation. For instance, it has been shown that the genes encoding antimicrobial proteins, such as surfactin and bacillomycin which are involved in the antagonistic activities of bacterium against plant pathogens, significantly and positively affect biofilm formation. \textsuperscript{25,26} Gerwig et al.\textsuperscript{42} confirmed that the protein
tyrosine kinases EpsB and PtkA differentially affected biofilm formation in B. subtilis.

Also, it has been shown that the RapP-PhrP Quorum-sensing system of B. subtilis affects biofilm formation through multiple targets due to an atypical signal-insensitive allele of RapP.\textsuperscript{43} Recently, complete genome of some biofilm-forming B. subtilis strains with antagonistic activities has been sequenced. Also, more detailed information about the correlation of antagonistic activities and biofilm formation is expected to be explored.\textsuperscript{44}

In conclusion, according to our results, for the first time, it was shown that the major genes, including sinR and tasA involved in biofilm formation in B. subtilis, were significantly affected by the interaction of bacteria and fungus. Also, the presence of F. culmorum stimulated biofilm formation in B. subtilis. These findings confirmed that the presence of other organisms, such as plant pathogens in the environment of the bacterium, stimulated biofilm formation. The present study could be the first step to determine the mechanism of relationship between antagonistic activities and a biofilm formation. But, to characterize the detailed mechanisms, it is necessary to perform more detailed studies in this field.

Conflicts of interest

The authors declare no conflicts of interest.

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