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Research Article

Bacillus Subtilis RP24 a Potential Bioagent for Control of Mango Malformation

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Abstract

Floral malformation induced by *Fusarium mangiferae* is an intricate disease and major bottleneck hindering mango productivity in different countries. Our study re-confirmed the identity of pathogen *Fusarium mangiferae* (KF060921) in causing mango malformation disease under North Indian conditions. Various chemical measures employed to control mango malformation have shown limited success. Biological management of plant diseases by using antagonistic microorganisms is considered a viable alternative to chemical control. The present study was initiated with the hypothesis that the bio-control agents *Bacillus subtilis* RP24 (EF154418), a gram-positive bacterium and *Pseudomonas fluorescens* (MTCC 9858)*, a* gram-negative bacterium, can efficiently control *F. mangiferae* induced mango malformation. *In vitro* studies demonstrated the capability of *B. subtilis* RP24, and *P. fluorescens,* in reducing the proliferation of *F. mangiferae* Both *B. subtilis* RP24 and *P. fluorescens* inhibited the growth of *F. mangiferae* and suppressed the formation of macro conidia and micro conidia, its primary infective propagules. We further observed the expression of ethylene response factor (*ERF*) gene (450bp), using the semi quantitative reverse transcriptase (RT) PCR analysis, in the healthy buds, malformed multiple buds and the malformed multiple buds that were treated with *B. subtilis* RP24 but no ERF expression was detected in the single swollen malformed buds or the malformed multiple buds that were exposed to *P. fluorescens* (MTCC 9858). Further, the *in vivo* studies conducted over three consecutive years revealed the potential of *B. subtilis* RP24 alone in controlling mango malformation disease (82%) when compared with other treatments and control. Although there is the suggestion of antimicrobial activity by *B. subtilis* RP24, the *in-planta* control of *F. mangiferae* may primarily be associated with competitive exclusion. *B. subtilis* RP24 further reduced 18% of mango malformation that appeared on mango trees after foliar spray, through the expression of ERF transcription factors that triggered senescence and drying of malformed panicles. Hence manual de-blossoming of malformed panicles normally practiced can be avoided.

Keywords: *Mangifera Indica*; Floral Malformation; *Fusarium Mangiferae*; *Bacillus Subtilis*; *Pseudomonas Fluorescens;* RT-PCR Analysis; Ethylene Signaling; Senescence

Abbreviations

ACS:1-Minocyclopropane-1-Carboxilic Acid Synthase; ACO:1-Aminocyclopropane-1-Carboxilic Acid Oxidase; CTR: Constitutive Triple Response; EIN-2: Ethylene Insensitive2; EIN-3: Ethylene Insensitive3; EBF-1/2: EIN3-Binding F-Box Protein; ERF1/2: Ethylene Response Factor1/2; ETR: Ethylene Receptor; SAM:S-Adenosyl Methionine, MPK6: MPK6 - Mitogen-Activated Protein Kinase 6; SIMKK: Salt Stress-Induced MAPK Kinase

Introduction

Floral malformation is regarded as the most devastating and challenging disease of mango that is capable of causing severe reduction in fruit yield and financial losses to the mango industry globally. Its fast spread in mango growing areas is a major concern for mango producers and exporters [1]. Malformation was first reported in India in 1891 and subsequently in Brazil, South Africa, Mexico, Central America, Sudan, Pakistan, Egypt, Malaysia, Israel, Swaziland, and the United States [2]. The characteristic symptom of floral malformation is the thickening of panicles with clustering of flowers caused by a reduction in the length of the primary and secondary branches, thus, leading to witches' broom-like appearance [3,4]. The flowers in diseased inflorescence are either sterile or fruits abort shortly after setting, resulting in 30-90% lower fruit yield [5,6]. A large number of abiotic and biotic factors have been listed to cause malformation which includes nutritional deficiencies, viruses, fungis and mites [3-5]. A large number of *Fusarium* species viz., *F. proliferatum, F. sterilihyphosum, F. tupiense, F. mangiferae and F. mexicanum* have been reported to be associated with the disease. However recent studies support the involvement of only the *F. mangiferae* [3,4,6]. Studies using artificial inoculation with *F. mangiferae* isolates from mango transformed with the GUS reporter gene (ß-glucoronidase) indicated that the primary site of infection in the host is chiefly the growing buds. A specific PCR diagnosis indicated that the pathogen is neither seed borne, nor systemically translocated. The micro and macro conidia; infective propagules of *F. mangiferae* proliferate rapidly on the malformed inflorescences [7]. An airborne pathogen, *F. mangiferae* infects mango buds only when sufficient inoculum is available; and gradually spreads by using the mango bud mite *Aceria mangiferae* as the vector [6].

Management of the mango malformation disease through use of synthetic chemicals have met with limited success. Bio control measures have been in use for decades for disease control in fruit crops such as peach, grape and citrus [7]. Studies have suggested that microorganisms that can degrade chitin, a major constituent of the fungal cell walls, are crucial in the biological control of fungal pathogens [8]. Members of genus *Bacillus* are cited among the first successful examples of biological control agents for suppression of diseases caused by insects and other pathogens. A large number of commercially successful *B. subtilis* strains are now avai lable for control of the fungal diseases in several agricultural crops [9,10] and in aquaculture [11]. These agents may also be used as adjuvant, or in the spore form, as delivery system for development of new vaccines [12]. Plant growth promoting rhizobacteria (PGPR) such as the fluorescent *Pseudomonads* are known to promote plant growth, induce systemic resistance and help in reducing the severity of fungal, bacterial and nematode infection in horticultural, oilseed, cereal and other crops. Competition for nutrients and space [12], competitive exclusion of pathogens by rapid colonization in rhizosphere [13] and production of a variety of secondary metabolites such as siderophores, hydrogen cyanide and antibiotics [14], 2,4-diacetylphloroglucinol (DAPG), phenazines and surfactants [13] are the major mechanisms of disease suppression by *P. fluorescens*.

Identification of a potent antagonistic organism is the most important and the foremost requirement for an effective biological control strategy. *Bacillus subtilis* RP24 [National Center for Biotechnology information (NCBI) accession number: EF154418], and *Pseudomonas fluorescens* (MTCC 9858) selected for this study were identified based on our previous laboratory and field trials and from the available literature. The present study focused on elucidating the identity of the *Fusarium* species responsible for causing the malformation malady in the north India. Secondly, to study the antagonistic action of *B. subtilis* RP24, a Gram-positive bacterium and *P. fluorescence*, a Gram-negative bacterium for their ability to control IARI isolated pathogen *F. mangiferae* and mango malformation under *in vitro* and *in vivo* conditions. Our experiments conducted under filed conditions revealed rapid drying and senescence of malformed panicles after foliar spray with *B. subtilis* RP24.

Endogenous signals like the phyto-hormones and exogenous factors such as temperature, light, nutrients and pathogens regulate a chain of events that occur in a coordinated manner at the cellular and tissue level to induce flower senescence [15]. Ethylene is reported to influence and regulate genetic networks that ultimately determine the growth and senescence of different plant parts like leaves, flowers, and fruits. Ethylene may also induce, promote or inhibit senescence depending upon optimum or sub-optimum levels [16]. Different phytopathogens can autonomously produce ethylene *in vitro* and *in planta*. Studies have suggested that challenging plant tissues with pathogens triggers enhanced ethylene production, which is controlled by the rate-limiting steps involved in the biosynthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) in the presence of ACC synthase (ACS) and its subsequent conversion

to ethylene by ACC oxidase (ACO). Transcriptional regulation of *ACO* and *ACS* genes is the pivotal mechanism regulating the ethylene biosynthesis. By contrast, the ethylene response factors (ERFs) are the principal targets coordinating the stress-induced response that modulate ethylene signaling and biosynthesis. The examples of ethylene-producing pathogens include the fungus *Botrytis cinerea* and the bacterium *P. syringae* [17]. The implication of ethylene production by the pathogen and its significance in host-pathogen interaction is currently unclear and thus, warrants further investigation. Hence the present study was extended to determine the transcript expression of *ACS*, *ACO*, and *ERF* involved in ethylene biosynthesis with and without treatment of the biocontrol agents to decipher the relation between observed senescence in malformed panicles and ethylene production during host-pathogen-bio agent interactions and its significance in control of mango malformation. Our aim was to conclude whether biocontrol of mango malformation can be a reliable alternative approach over the chemical control measures.

Materials and Methods

In vitro study

Identification of casual organism

Ten trees of the mango variety Amrapali, with uniform age (18 years old), raised under similar soil and climatic conditions, were used as the experimental material. Morphologically different buds (Figure S1A and S1B) and healthy and malformed panicles that developed into healthy and malformed panicles (Figure 1a and 1b) were randomly collected (n 10/treatment) following visual observations and were cultured in potato dextrose agar medium. When mycelial growth and spores were observed, isolation was conducted through hyphal tipping and single spore isolation following the method suggested by Leslie and Summerell [18] and observations on fungal morphology were recorded. Molecular studies were performed following the extraction of total genomic DNA from the fresh fungal mycelia using a modified protocol of Leslie and Summerell [18]. The tef-1 region was amplified through polymerase chain reaction (PCR) by using primers, EF-1 and EF-2 [19] in a 3100-Avant genetic analyser. Nucleotide sequences were edited with the SEQMAN II v. 3.61 programs from the DNASTAR software package (LASERGEN). BLAST searches were performed with the tef-1 sequences to search highly identical sequences. A neighbor-joining tree was constructed (using MEGA 3.1) for phylogenetic analysis [20] and the sequence was submitted to the GenBank for obtaining the accession number. Two-year old mango plants of Amrapali variety (n 10) were maintained under controlled conditions of 27 ± 1 °C and 65% RH and 31 ± 1 °C and 65% RH in 2 different chambers of a phytotron. The sprouting buds were artificially inoculated with spores of fungal isolate in liquid suspension using cotton swab to test for Koch' postulates.

Microbial strains

Bacterial biocontrol agents, *P. fluorescens* (MTCC 9858) [21] and *B. subtilis* RP24(EF154418) [22], obtained from the NCIPM, New

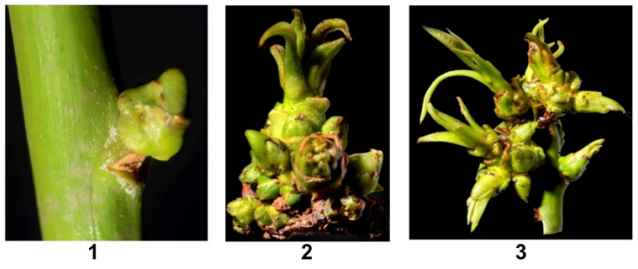


Figure S1A: Malformed bud samples selected for laboratory analysis**.**
(1) Single swollen malformed bud, (2) malformed multiple buds, (3) Malformed panicle development stage.

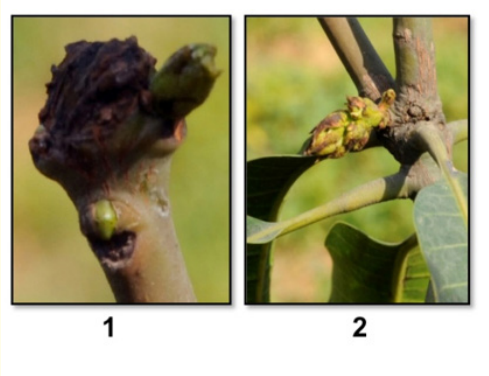


Figure S1B: Healthy bud samples selected for laboratory analysis.
(1) Single healthy bud (2) healthy panicle development stage (Usha., *et al*. 2019).

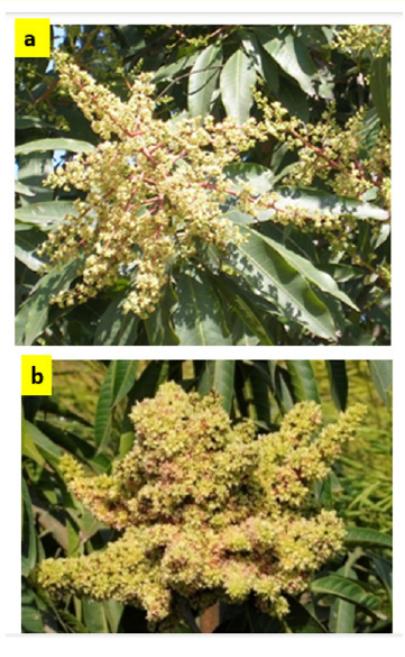


Figure 1: a) Healthy panicle, b) Malformed panicle as observed in third week of March (flowering season) in mango variety Amrapali.

Delhi and the Division of Microbiology, IARI, New Delhi, India, respectively, were preserved on nutrient agar slants overlayed with 30% glycerol. *F. mangiferae* (IARI isolate KF060921) [3,4], procured from the Division of Plant Pathology, IARI, New Delhi, was maintained on potato dextrose agar (PDA) slants under controlled temperature for further studies.

PDA, King's A medium, and nutrient agar medium of analytical reagent grade, obtained from HiMedia Laboratories, were used for culturing *F. mangiferae*, *P. fluorescens* and B. *subtilis* RP24, respectively. All laboratory experiments were replicated three times (n = 10 per treatment).

Dual culture technique

The sterilized melted potato dextrose agar (PDA) was poured aseptically in the pre-sterilized petri dishes (90-mm dia) and left to solidify. Each bacterial isolate was spotted on the plate (near the periphery) and 10 ul spore suspension of *F. mangiferae* was spotted on the opposite side of petri-plate. The bacterial isolates and pathogen *F. mangiferae* were placed ~60mm apart, opposite to each other [23]. The control plates had only the fungal isolate, without bacterial strains. The entire experiment was replicated 10 times with n = 3/treatment. The inoculated and control petri plates were incubated at 28°C ± 2°C for seven days following which the diameter, in terms of the vertical and horizontal dimension of the fungal spread on the plates was measured using a venire-caliper (Absolute Digimatic-Mitutoyo Corporation, Japan). The inhibitory effect of *B. subtilis* RP24 and *P. fluorescens*) on fungal growth was measured according to the following formula described by Kumar., *et al.* [24].

$$\text{Growth reduction (\%)} = \frac{\text{Radial diameter in control} - \text{Radial diameter in treatment}}{\text{Radial diameter in control}} \times 100$$

Scanning electron microscopy (SEM)

Healthy and malformed tissues were collected from *in vivo* in field conditions (Figure 1a and 1b) and used for SEM analysis. Pieces of the 4-mm area between the mycelia of *F. mangiferae*, and the zone of inhibition produced by *B. subtilis* RP24 (EF154418), (*In vitro* under laboratory conditions, (Figure 3a, circles) and *P. fluorescens* (MTCC 9858), (*In vitro* under laboratory conditions, Figure 4a, circles) were collected and used for SEM analysis. Similar samples were recovered from another petri dish where *F. mangiferae* was grown alone without the bacteria (control). The collected samples were fixed with glutaraldehyde 2.5% v/v in a 0.1M phosphate buffer (pH 7.2) for 24h at 4°C, followed by dehydration in series by using 10, 30, 50, 70, 96, and 100% ethanol. The samples were immersed in each alcoholic solution for 15 min and two changes in 100% acetone. Critical point drying was achieved with liquid CO_2 at its critical point, i.e., 31.5°C at 7.584MPa. The samples were then mounted onto aluminum stubs. Splutter coating was performed using a 35-nm-thick coat of palladium or gold. Finally, the samples were observed using Zeiss EVOMA10 scanning electron microscope.

Preparation of cultures for foliar application

Bacterial strains *B. subtilis* RP24 and *P. fluorescens,* were cultured in nutrient broth (50 mL) under aseptic condition and incubated for 48 h on a rotary shaker maintained at 180 rpm at 30°C [25] to get a final population of 3 × 10^7 CFU/mL Broth cultures were centrifuged to remove the broth. The bacteria were then resuspended in water (1:20) and were sprayed in three treatment combinations while maintaining a non-bacterial control treatment.

In vivo study

Fifteen-year-old trees of the mango variety Amrapali, a regular bearing, dwarf and highly susceptible to mango malformation, planted at a spacing of 0.45 m^2, having uniform growth, and receiving identical cultural practices, were selected in a randomized block design (RBD) for the study (n = 10/treatment). Foliar application of *B. subtilis* RP24 (T_1), *P. fluorescens* (T_2), and *B. subtilis* RP24 (EF154418), + *P. fluorescens*, (T_3) was performed in the last week of January (before flower bud sprouting), February (before flower bud sprouting), and February and March (before and after flower bud sprouting) (n = 10/treatment) by using a Knapsack Sprayer (ASPEE, Mumbai, India) at a rate of 5 L/tree (volume standardized for dwarf mango variety Amrapali in previous experiments). The experiment was repeated for three consecutive years. Trees with water spray were maintained as control (T4). The extent of malformation expressed as percentage was recorded in different treatments and compared with control. The data analyzed were not significantly different for the three experimental years and hence mean values were shown in tables.

Determination of ethylene concentration in the gas phase

The untreated healthy and malformed buds (control), the malformed buds treated with *B. subtilis* RP24 (EF154418), and the malformed buds treated with *P. fluorescens* (MTCC 9858), (n = 100 per treatment) were tightly covered with a polythene bag during the February-March flowering season and measurements were made after 2, 4, and 6 days of bagging. A syringe was used to draw the gas from the experimental bags encompassing different treatments and the gas was directly injected into the gas chromatograph equipment for analyzing ethylene [26].

Expression of ethylene and ERF transcripts

ERF expression and other ethylene responses were studied in mango variety Amrapali. Three independent biological replicates for each sample were collected from the malformed and the healthy tissues at different growth stages starting with the bud initiation to the panicle development stage [MB-1: single swollen malformed bud stage-1, MB-2: multiple malformed bud stage-2, MB-3: multiple malformed bud stage-3, HB-1: healthy bud stage 1and HB-2: healthy bud stage-2] (Figure S1A and S1B). The biological replicates were used for RNA extraction, cDNA library construction and sequencing. Sequencing was performed using 2x150PE chemistry on the Illumina NextSeq platform (Figure S6). The transcripts were assembled using Bridger with default parameters. All coding sequences (CDS) were predicted from the unigenes using Transdecoder, and the predicted CDS were annotated using BLASTX against NCBI nr database. For identification of differentially expressed ERF and ethylene transcripts in healthy and malformed tissues at different growth stages, the DESeq package was used. Differential gene expression analysis was performed for the commonly occurring coding sequences among the control and infected samples. Furthermore, to decipher the ethylene biosynthesis and signaling pathways across healthy and malformed tissues, the CDS were mapped to the reference canonical pathways in KEGG by using the KEGG automatic annotation server [27]. The analysis based on p value was performed using Graphpad. The transcriptomic data of healthy and malformed tissues at different growth stages were submitted to NCBI (accession number: SAMN05727981, SAMN05727982, SAMN05727983, SAMN05727984, and SAMN05727985).

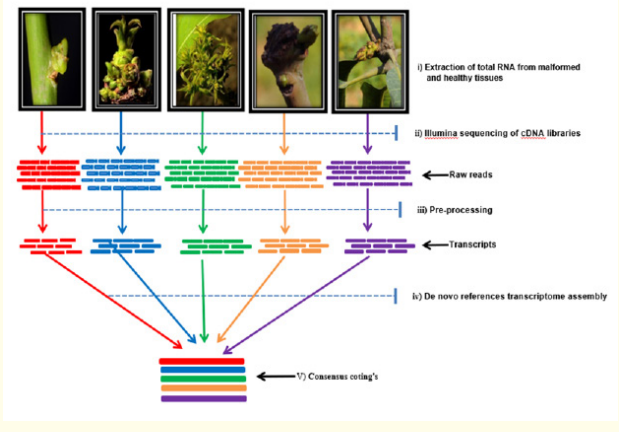


Figure S6: Workflow of transcritome sequencing in malformed and healthy bud tissues.

RNA isolation from mango buds

Three independent biological replicates for each sample namely healthy single buds (control), malformed single buds, malformed multiple buds (Figure S1A and S1B), malformed multiple buds treated with *B. subtilis* RP24and malformed multiple buds treated with *P. fluorescens* (MTCC 9858) were collected *in vivo* in field conditions, rapidly frozen in liquid nitrogen and preserved at −80°C. Total RNA was isolated using the Qiagen RNAase Easy kit (Qiagen, USA), according to the manufacturer's instructions. The isolated RNA was treated with DNase (Ambion DNA-free DNase kit) to contain DNA contamination. NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) was then used to determine the concentration and purity of the isolated RNA.

Semi quantitative real time PCR analysis

The cDNA was synthesized (Superscript III kit, Invitrogen, USA) using 1 µg of total RNA isolated from different experimental samples, according to the manufacturer's instructions. Forward and reverse primers of ACO, ACS, and ERF were designed using the Gene Fisher primer designing software (Table S1). These were degenerate primers, synthesized by aligning two different EST sequences for ACO (AJ297435, AM743172) and two different EST sequences for ACS (U22523, AF170705), where K represents the bases G and T, Y represents C and T, and N represents -any base.

*Primer ID	Primer Sequence (5'- 3')	Tm (°C)
ACO-F	AGGTKAGCNACTACCCACCATG	54.8
ACO-R	GCATANAGCTTCATGTAGTCTTC	51.7
ACCS-F	TGGAAGNAGCATATGAAAAAGCTC	52.3
ACCS-R	GAACYTNTCCACAAATTCATCATCATC	53.7
ERF-F	AGATGTTGCAACCAAAGAAGCCGCCAGAG	62.9
ERF-R	TAAGAGGTGATCACGATCAATATTCTGC	57.0

Table S1: List of primer sequences used for quantitative Reverse Transcriptase polymerase chain reaction (qRT-PCR).

*Primer sequences (5'-3') of 1-amino cyclopropane-1-carboxylate oxidase (ACO, Accession number AJ297435, AM743172), 1-amino cyclopropane-carboxylate synthase (ACCS, Accession number U22523, AF170705) and Ethylene-response Factor (ERF, Accession number EU513281) genes used for RT PCR analysis; K = G, T; Y = C, T; N = any base in the degenerate primers.

PCR amplification was performed on C1000 Touch Biorad Thermal Cycler, California, USA in a 25 µL reaction mixture, which comprised of 10 × PCR buffer (2.5 µL), 10 mM dNTPs (0.75 µL), forward and reverse primers (0.5 µL each) for the following genes viz., ACO, ACS or ERF (Sigma India, Bengaluru) and Taq DNA polymerase (0.30 µL; 3 U/µL; Agilent Technologies California, USA), and sterile water to make up the final volume. Following steps i.e., 55°C for 50 min,85°C for 5 min, initial denaturation at 95°C for 2 min, followed by 30 cycles at 95°C for 30 s, 56.5°C for 25 s, 72°C for 1 min and a final extension cycle at 72°C for 5 min were followed for the reverse transcription reaction which used 2µL of cDNA as the template in the reaction mixture. The specific amplification of the gene sequences in a reaction were identified on the agarose gel (1.2%) with bands having mass of ~ 220-278 (ACO), 287 (ACS), and 450 bp (ERF).

Statistical analysis

This study used completely randomized design for laboratory experiments with 10 replications (n = 3/treatment) and RBD for field experiments with three replications (n = 10/treatment). Statistical difference between the antagonists was determined using analysis of variance (ANOVA; General Linear Model, GLM). The significant statistical differences in radial diameter between the treatments were compared using one-way ANOVA at a significance level of 5%. All tests were applied using SPSS software version 23. The ERF gene concentration on RT-PCR was quantified by comparing it with the intensity of known concentrations on a molecular ladder (100bp plus, Thermo Fisher Scientific) and was normalized against the Actin gene.

Results

In vitro study

Identification of casual organism

The fungal mycelia isolated from mango malformed buds (Figure S1A) were identified as *F. mangiferae*, on the basis of its morphology and molecular characterization. Blast analysis revealed 100% sequence similarity of the Tef-1 region with that of the other *F. mangiferae* strains available on NCBI database. Further mango plants of variety Amrapali maintained in phytotron at temperature of (27^0C ± 1°C and 65% RH), developed into malformed panicles, when the sprouting buds were artificial inoculated with *F. mangiferae* confirming the causal agent.

Antagonism exhibited by biocontrol agents in dual culture

The radial diameter of *F. mangiferae* growth was 25.2 ± 2.1 mm after 168 h of incubation (control) and was significantly higher ($p < 0.05$) than the treatment given with *B. subtilis* RP24 or *P. fluorescens* (MTCC 9858), depicting that both the treatments have antagonistic effect on the radial growth of the fungus (Table 1, Figure S2). The percent inhibition of *F. mangiferae* was 17.46 with *B. subtilis* RP24 treatment and13.03 with *P. fluorescens* (MTCC 9858) treatment, however, both the bacterial bioagents showed no significant difference on the radial diameter of *F. mangiferae*.

SEM

SEM of malformed panicles collected from mango tree variety Amrapali (*in vivo* in field condition, Figure 2A-a), revealed fungal mycelia, macro conidia, and micro conidia. However, fungal mycelia, macro and micro conidia were absent in healthy panicles col-

S. No	Treatment	Mean (N=30/treatment)	LSD value at 5%
1	Radial Growth of *Fusarium mangiferae* in control plate (mm)	24.34[a] ±2.4	3.9
2	Radial Growth of *F. mangiferae* in treatment plate with *B. subtilis (*mm)	21.88[b]±1.5	2.8
3	Radial Growth of *F. mangiferae* in treatment plate with *P. fluorescens (*mm)	21.25[b]±1.6	2.4

Table 1: *In vitro* evaluation of bacterial bioagents against *Fusarium mangiferae*

* Values in each column having different letters are significantly different

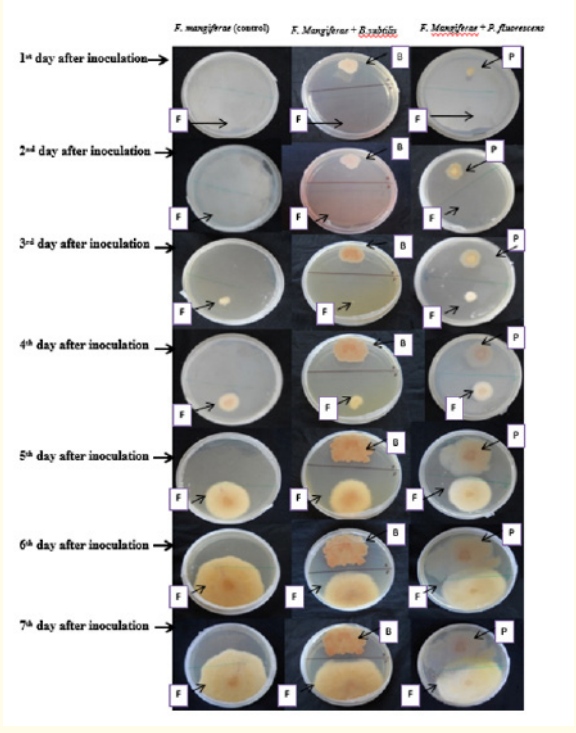


Figure S2: Radial growth of *Fusarium mangiferae* (KF060921) under dual culture technique using *F. mangiferae* (KF060921) control), *F.mangiferae* (KF060921) + *B. subtilis* RP 24 (EF154418) and *F. mangiferae* (KF060921) + *P.florescence (*MTCC 9858); F = *F.mangiferae*(KF060921), B = *B.subtilis* RP 24 (EF154418) P = *P.fluorescens*(MTCC9858).

lected from same trees which served as controls (*in vivo* in field condition, Figure 2A-b). Lysis and disruption of mycelia was absent in cultures of *F. mangiferae* alone grown under *in vitro* conditions (Figure 2B, control).

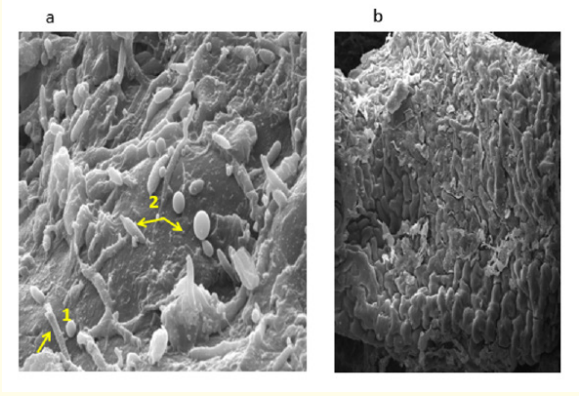


Figure 2A: Scanning electron micrograph of malformed and healthy tissues in mango variety Amrapali (in vivo) Control a) SEM of malformed tissue from mango tree variety Amrapali showing the presence of (1) fungal mycelia, (2) macro and micro conidia of Fusarium mangiferae (KF060921). Scale bar 2 µm.

b) SEM of healthy tissue from mango tree variety Amrapali with no fungal mycelia, macro and micro conidia (control). Scale bar 20 µm.

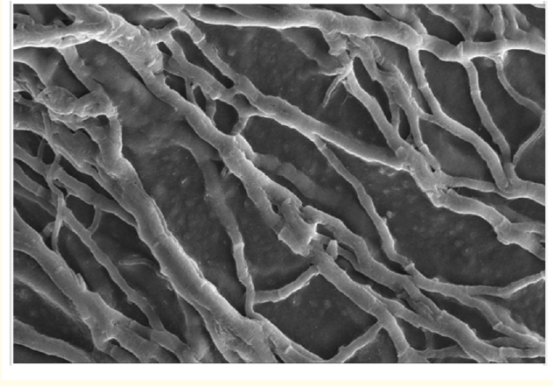


Figure 2B: Scanning electron micrograph of Fusarium mangiferae (KF060921) In vitro 168 h after inoculation (Control).
Scale bar 20 µm

SEM of the samples collected from the *in vitro* cultures using interacting sections of *F. mangiferae* +*B. subtilis* RP24 (Figure 3a, circle) and *F. mangiferae* +*P. fluorescens* (MTCC 9858) (Figure 4a, circle) indicated that both bacterial agents potentially controlled the growth of *F. mangiferae*, however the visible responses differed. In the samples collected from *in vitro* cultures of *F. mangiferae*, + *B. subtilis* RP24(Figure 3a, circle), numerous *B. subtilis* RP24cells, lysis and disruption of *F. mangiferae* mycelia were observed (Figure 3b and 3c), However, in samples collected from *in vitro* culture of *F. mangiferae* + *P. fluorescens* (MTCC 9858), although the fungal cell walls were intact, the cells were empty (Figure 4b and 4c).

In vivo Study

Effect of concentration of bioagent used on mango malformation

Preliminary studies conducted for three consecutive years showed promise in controlling mango malformation, when a final concentration of bio agents (3 × 10^8 CFU/ mL and 3 × 10^7 CFU/ mL) was used when compared with (3 × 10^6 CFU/mL) or control. No significant differences between the final concentration of bio agents (3 × 10^8 CFU/ mL and 3 × 10^7 CFU/mL) were observed on the extent of mango malformation (data not shown). Hence the data on effect of *B. subtilis* RP24, (T_1), *P. fluorescens* (T_2), and *B. subtilis* RP24 (EF154418), + *P. fluorescens*, (T_3) on mango malformation when a final concentration of (3 × 10^7 CFU/mL) was sprayed on mango trees is presented in the table 2.

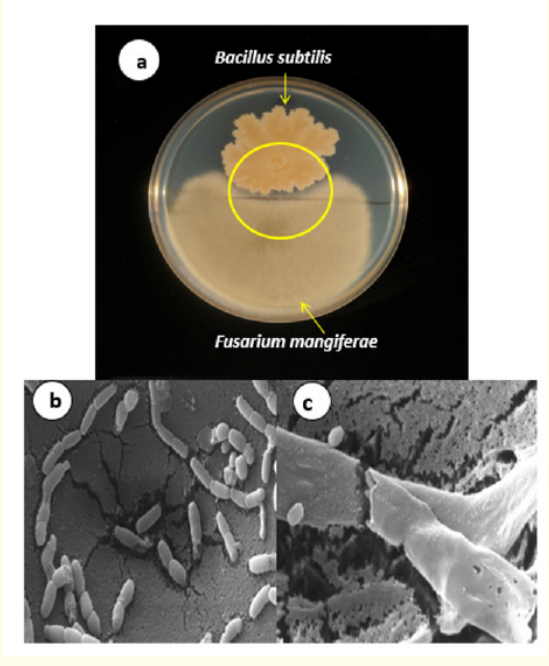


Figure 3: a) Inhibition in growth of Fusarium mangiferae (IARI isolate KF060921) using Bacillus subtilis RP-24 (EF154418),120 hours after inoculation. Circle showing interacting section used for SEM study. b) and c) SEM showing morphology of B. subtilis RP-24 (EF154418), and F. mangiferae (KF060921) 120 hours after inoculation (Scale bar 2 µm and Scale bar 1 µm).

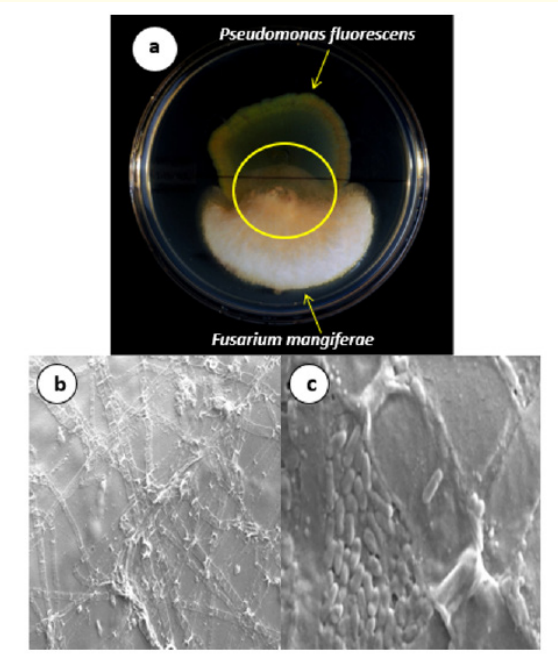


Figure 4: Interaction of radial growth of Pseudomonas fluorescens (MTCC 9858) and Fusarium mangiferae (KF060921); (b and c) SEM studies using interacting part of P. fluorescens (MTCC 9858) and F. mangiferae(KF060921).

Effect of bioagent on mango malformation

The foliar application of T_1, T_2, and T_3 significantly reduced the extent of mango malformation when compared with T_4. Studies on extent of mango malformation showed that the number of healthy panicles significantly ($p<0.05$) decreased in treatment T_2 (*P. Fluorescens* and T_3 (*B. subtilis* RP24, + *P. fluorescens* (MTCC 9858) compared to T_1 (*B. subtilis* RP24 and T_4 (Control) (Table 2).

S. No.	Treatment	No. of healthy panicles/ tree	No. of malformed panicles/ tree	Malformation (%)
T_1	*Bacillus subtilis* RP-24(NCBI accession number: EF154418)	325 ± 42	60 ± 15	18 ± 2.08
T_2	*Pseudomonas fluorescens* (MTCC 9858)	282 ± 29	168 ± 26	60 ± 5.57
T_3	*B. subtilis* RP-24 (NCBI accession number: EF154418) + *P. fluorescens* (MTCC 9858)	268 ± 11	136 ± 54	51 ± 15.1
T_4	Control	297 ± 11	184 ± 52	63 ± 11.24
LSD (P ≤ 0.05)		50.23	76.02	18.58

Table 2: Extent of malformation in mango variety Amrapali in different treatments. Applied during February + March.

**Values expressed are Mean ± SD.

***N = 30/treatment.

Effect of time of spray of bioagent on mango malformation

Data shown in tables 2, S2, and S3) revealed that the extent of mango malformation (18%) was significantly lower in Treatment T_1 when sprayed during (February + March) when compared with other treatments. The results showed that treatment with *B. subtilis* RP24 decreased the extent of malformation in mango variety Amrapali.

S. No.	Treatment	Malformation (%)
T_1	*Bacillus subtilis* RP-24 (EF154418)	27
T_2	*Pseudomonas fluorescens* (MTCC 9858),	45
T_3	*B. subtilis* + P. *fluorescens* (MTCC 9858),	48
T_4	Control (water spray)	64

Table S2: Extent of malformation in mango variety Amrapali after foliar spray with bioagents (3 × 10^7 CFU/mL) during February (before flower bud sprouting stage).

S. No.	Treatment	Malformation (%)
T_1	*Bacillus subtilis* RP-24 (EF154418)	26
T_2	*Pseudomonas fluorescens* (MTCC 9858),	51
T_3	*B. subtilis* + P. *fluorescens* (MTCC 9858),	46
T_4	Control (water spray)	68

Table S3: Extent of malformation in mango variety Amrapali after foliar spray with bioagents (3 × 10^7 CFU/mL) during March (After flower bud sprouting stage).

Effect of bio agent on drying of malformed panicles

Malformed panicles (18%) that developed on mango trees after foliar spray with T_1, completely dried and dropped within 4-7 days, depending on the environmental conditions (Figure 5), whereas the malformed panicles on the control trees (Figure S3 and S4) dried gradually and dropped with increase in temperature (> 40°C) over a period of 30-40 days. Approximately thirty-nine per cent of the dried malformed panicles continued to remain on the untreated control trees and later transformed into fresh vegetative or floral malformed panicles under favorable weather conditions in the months July-September when temperatures of 27±2^{0}C prevailed. Whereas in trees treated with T_1, all malformed panicles that developed (18%) dried and dropped after treatment. The malformed panicles in the trees treated with *P. fluorescens* (MTCC 9858) (T_2; Figure S5), did not exhibit any symptoms of drying as observed in the trees treated with T_1 or T_4 (Figure 5, S3, and S4).

Effect of bioagent on ethylene concentration in malformed panicles

Ethylene is known to regulate senescence in plants. Ethylene levels were therefore measured from different samples treated with bio agents to find a relation between ethylene levels and observed senescence. The extracted gas from healthy and malformed buds (Figure S1A and S1B), malformed multiple buds treated with *B. subtilis* RP24, and malformed multiple buds treated with *P. fluorescens* (MTCC 9858) had insufficient ethylene concentrations for quantification through gas chromatography. Hence, the study was extended to determine the expression levels of the genes involved in ethylene biosynthesis through RT-qPCR analysis.

Figure 5: Malformed panicles that dried and dropped 48 hours after foliar spray with *Bacillus subtilis* RP-24 (EF154418) in mango variety Amrapali.

Figure S4: Arrows showing Senescence and slow drying of malformed panicles in mango variety Amrapali (control trees) as observed in the month of April-May.

Figure S3: Extent of floral malformation and no fruit set in mango variety Amrapali as observed in third week of March (flowering season).

Figure S5: No symptoms of senescence and drying of malformed panicles observed after 168 hours after foliar spray with *Pseudomonas fluorescens* (MTCC 9858) in mango variety Amrapali.

Transcript accumulation of ethylene response genes in healthy and malformed buds

The CDS of healthy and malformed tissues at different growth stages when annotated with BLASTX against NCBI nr database revealed 100, 108, 99, 102, and 110 ethylene response genes (Table 3) as well as 8,10,14,17, and 11 ERF genes (Table 4) in MB-1, MB-2, MB-3, HB-1, and HB-2, respectively. The transcript accumulation of the ethylene response genes indicated 21 up regulated and 7 down regulated genes (Figure 6). According to the differentially expressed gene (DEG) analysis, the number of up regulated genes between HB-1 and MB-1; HB-1 and MB-2; HB-1 and MB-3 were 3, 5, and 2, respectively, whereas that of down regulated genes were 1, 2, and 2, respectively. Furthermore, the number of up regulated genes between HB-2 and MB-1, HB-2 and MB-2, and HB-2 and MB-3 were 7, 3, and 1, respectively, whereas those of down regulated genes were 0, 2, and 0, respectively (Tables 5 and 6, S4 and Figure 6). The number of ethylene biosynthesis genes (ACS and ACO) in both healthy and malformed bud developmental stages was similar. The ethylene signaling pathway genes, namely SIMKK, MPK6, CTR, EBF-1/2, EIN-2, and EIN-3, also exhibited a similar pattern in both healthy and malformed tissues at different developmental stages (Figure 7).

Samples	Stages	Number of ethylene genes
Malformed Buds	Single Swollen malformed bud (MB-1)	100
	Multiple malformed buds (MB-2)	108
	Multiple malformed buds (MB-3)	99
Healthy buds	Healthy bud stage (HB-1)	102
	Healthy panicle stage (HB-2)	110

Table 3: Number of ethylene genes observed in healthy and malformed tissues at different stages of bud development.

Samples	Stages	Number of ERF genes
Malformed samples	Single Swollen malformed bud stage (MB-1)	8
	Multiple malformed bud stage (MB-2)	10
	Malformed panicle stage (MB-3)	14
Healthy samples	Healthy bud stage (HB-1)	17
	Healthy panicle stage (HB-2)	11

Table 4: Number of ERF genes in healthy and malformed tissues at different stages of bud development.

Samples	Up-regulated genes		Down-regulated genes	
	Number	Unigenes Id	Number	Unigenes Id
HB-1$_{VS}$ MB-1	3	CDS_27786_Unigene_31862 CDS_23917_Unigene_25761 CDS_9019_Unigene_9606	1	CDS_871_Unigene_1047
HB-1$_{VS}$MB-2	5	CDS_27786_Unigene_31862 CDS_31121_Unigene_41064 CDS_33158_Unigene_49268 CDS_2319_Unigene_2671 CDS_23917_Unigene_25761	2	CDS_8152_Unigene_8726 CDS_24282_Unigene_26283

HB-1_{VS} MB-3	2	CDS_7119_Unigene_7684 CDS_31121_Unigene_41064	2	CDS_27057_Unigene_30512 CDS_28472_Unigene_33385
HB-2_{VS} MB-1	7	CDS_28719_Unigene_33993 CDS_25082_Unigene_27276 CDS_28026_Unigene_32402 CDS_30172_Unigene_37899 CDS_17459_Unigene_18145 CDS_22986_Unigene_24437 CDS_22320_Unigene_23606	0	
HB-2_{VS}MB-2	3	CDS_2728_Unigene_3080 CDS_25082_Unigene_27276 CDS_22320_Unigene_23606	2	CDS_24405_Unigene_26321 CDS_17969_Unigene_18666
HB-2_{VS}MB-3	1	CDS_25415_Unigene_27785	0	
Total	21		7	

Table 5: Number and Unigenes sequence identity of differentially expressed ethylene genes in six possible experimental comparison combinations between the healthy and the malformed tissues.

** MB-1: Single Swollen malformed bud stage-1, MB-2: Multiple malformed bud stage-2, MB-3: Multiple malformed bud stage-3; HB-1: Healthy bud stage 1 and HB-2: Healthy bud stage-2.

Genes	Malformed stages			Healthy stages	
	MB-1	MB-2	MB-3	HB-1	HB-2
ETR	4	4	4	3	5
CTR	3	2	4	3	3
SIMKK	-	-	-	-	-
MPK6	1	3	2	1	2
EIN-2	2	3	2	4	3
EIN-3	5	4	4	5	4
EBF-1/2	3	3	3	3	1
ERF-1	1	4	4	4	4
ERF-3	2	4	2	4	1

Table 6: Expression of ethylene plant hormone signaling pathway genes in healthy and malformed buds at different growth stages (P-value < 0.005).

Abbreviation: ETR: ethylene receptor1; CTR: constitutive triple response1; SIMKK: SIMK Kinase; MPK6: Mitogen-activated protein kinase-6; EIN-2: Ehylene insensitive-2; EIN-3: Ethylene insensitive-3: EBF-1/2: EIN3 binding F-box protein; ERF-1: Ethylene Response Factor1; ERF-3: Ethylene Response Factor 3.

UP-REGULATED GENES								
HB-1 vs MB-1	**Id**	**Hit description**	**Base Mean**	**Base Mean (HB-1)**	**Base Mean (MB-1)**	**Fold Change**	**pval**	**padj**
	XP_006478313	PREDICTED: ethylene-responsive transcription factor ERF023-like [Citrus sinensis]	1109.195774	91.85155248	2126.539996	23.15192219	0.000558484	0.10896
	XP_006421847	hypothetical protein CICLE_v10005869mg [Citrus clementina] >gi|568874437|ref|XP_006490322.1| PREDICTED: ethylene-responsive transcription factor ERF026-like [Citrus sinensis] >gi|557523720|gb|ESR35087.1| hypothetical protein CICLE_v10005869mg [Citrus clementina] >gi|641841190|gb|KDO60104.1| hypothetical protein CISIN_1g028004mg [Citrus sinensis]	1019.981922	231.4369826	1808.526862	7.814338235	0.016863504	0.543848
	XP_006490015	PREDICTED: ethylene-responsive transcription factor ERF105-like [Citrus sinensis] >gi|641816156|gb|KDO38348.1| hypothetical protein CISIN_1g026385mg [Citrus sinensis]	2722.022247	684.9088204	4759.135673	6.948568234	0.027286263	0.65839

HB-1 vs MB-2	Id	Hit description	Base Mean	Base Mean (HB-1)	Base Mean (MB-2)	Fold Change	pval	padj
	XP_006478313	PREDICTED: ethylene-responsive transcription factor ERF023-like [Citrus sinensis]	1129.76132	109.4527195	2150.069921	19.64382366	0.00114095	0.17607
	XP_006464539	PREDICTED: ethylene-responsive transcription factor ABR1-like isoform X1 [Citrus sinensis]	180.8751628	27.57863798	334.1716877	12.11704827	0.030586817	0.690035
	XP_004141277	PREDICTED: ethylene-responsive transcription factor ERF011-like [Cucumis sativus] >gi|700200068|gb|KGN55226.1| hypothetical protein Csa_4G641590 [Cucumis sativus]	251.8690054	43.09162185	460.646389	10.68992925	0.031685864	0.695016
	AAX68525	putative ethylene responsive element binding protein 2 [Gossypium hirsutum]	29389.69438	5206.329752	53573.05901	10.28998576	0.02555775	0.656298
	XP_006421847	hypothetical protein CICLE_v10005869mg [Citrus clementina] >gi|568874437|ref|XP_006490322.1| PREDICTED: ethylene-responsive transcription factor ERF026-like [Citrus sinensis] >gi|557523720|gb|ESR35087.1| hypothetical protein CICLE_v10005869mg [Citrus clementina] >gi|641841190|gb|KDO60104.1| hypothetical protein CISIN_1g028004mg [Citrus sinensis]	1435.709276	275.7863798	2595.632172	9.411748953	0.009736982	0.438648

HB-1 vs MB-3	Id	Hit description	Base Mean	Base Mean (HB-1)	Base Mean (MB-3)	Fold Change	pval	padj
	XP_007016682	Ethylene insensitive 3 family protein [Theobroma cacao] >gi|508787045|gb|EOY34301.1| Ethylene insensitive 3 family protein [Theobroma cacao]	853.8448902	108.74349	1598.94629	14.70383459	0.00068617	0.066427
	XP_006464539	PREDICTED: ethylene-responsive transcription factor ABR1-like isoform X1 [Citrus sinensis]	165.0776406	27.83833345	302.3169478	10.85973585	0.018610714	0.450002
HB-2 vs MB-1	Id	Hit description	Base Mean	Base Mean (HB-2)	Base Mean (MB-1)	Fold Change	pval	padj
	AIT39449	ethylene receptor 1 [Mangifera indica]	2331.13146	180.3404697	4481.92245	24.85256059	0.000933959	0.100531
	XP_006478313	PREDICTED: ethylene-responsive transcription factor ERF023-like [Citrus sinensis]	1006.218598	170.3215548	1842.115641	10.81551682	0.006959685	0.307942
	XP_007035093	Ethylene-responsive transcription factor 1B [Theobroma cacao] >gi|508714122|gb|EOY06019.1| Ethylene-responsive transcription factor 1B [Theobroma cacao]	545.1042201	106.8684265	983.3400138	9.201408179	0.018125018	0.487741
	XP_006472752	PREDICTED: ethylene-responsive transcription factor ERF014-like [Citrus sinensis]	367.9096073	73.47204323	662.3471713	9.014955107	0.030708763	0.585704
	XP_007009698	Ethylene-responsive transcription factor RAP2-7, putative isoform 1 [Theobroma cacao] >gi|508726611|gb|EOY18508.1| Ethylene-responsive transcription factor RAP2-7, putative isoform 1 [Theobroma cacao]	391.7199916	85.16077738	698.2792059	8.199540063	0.034865415	0.615641
	XP_006490015	PREDICTED: ethylene-responsive transcription factor ERF105-like [Citrus sinensis] >gi|641816156|gb|KDO38348.1| hypothetical protein CISIN_1g026385mg [Citrus sinensis]	2394.429975	666.2578465	4122.602104	6.187697639	0.04164304	0.653441

	XP_006421847	hypothetical protein CICLE_v10005869mg [Citrus clementina] >gi|568874437|ref|XP_006490322.1| PREDICTED: ethylene-responsive transcription factor ERF026-like [Citrus sinensis] >gi|557523720|gb|ESR35087.1| hypothetical protein CICLE_v10005869mg [Citrus clementina] >gi|641841190|gb|KDO60104.1| hypothetical protein CISIN_1g028004mg [Citrus sinensis]	931.5148053	296.3929017	1566.636709	5.285675535	0.05013591	0.706421
HB-2 vs MB-2	Id	Hit description	Base Mean	Base Mean (HB-2)	Base Mean (MB-2)	Fold Change	pval	padj
	AAX68525	putative ethylene responsive element binding protein 2 [Gossypium hirsutum]	25257.73091	3462.880332	47052.58149	13.58770069	0.03877131	0.625474
	XP_006478313	PREDICTED: ethylene-responsive transcription factor ERF023-like [Citrus sinensis]	1044.279357	200.1778373	1888.380878	9.433516235	0.008988481	0.311334
	XP_006421847	hypothetical protein CICLE_v10005869mg [Citrus clementina] >gi|568874437|ref|XP_006490322.1| PREDICTED: ethylene-responsive transcription factor ERF026-like [Citrus sinensis] >gi|557523720|gb|ESR35087.1| hypothetical protein CICLE_v10005869mg [Citrus clementina] >gi|641841190|gb|KDO60104.1| hypothetical protein CISIN_1g028004mg [Citrus sinensis]	1314.030799	348.3486874	2279.712911	6.5443419	0.024319157	0.50614
HB-2 vs MB-3	Id	Hit description	Base Mean	Base Mean (HB-2)	Base Mean (MB-3)	Fold Change	pval	padj
	XP_007016682	Ethylene insensitive 3 family protein [Theobroma cacao] >gi|508787045|gb|EOY34301.1| Ethylene insensitive 3 family protein [Theobroma cacao]	742.5949455	100.4522409	1384.73765	13.78503494	0.000661247	0.056179

DOWN-REGU-LATED GENES								
HB-1 vs MB-1	Id	Hit description	Base Mean	Base Mean (HB-1)	Base Mean (MB-1)	Fold Change	pval	padj
	XP_006428165	hypothetical protein CICLE_v10025961mg [Citrus clementina] >gi|568819346|ref|XP_006464216.1| PREDICTED: ethylene-responsive transcription factor RAP2-4-like [Citrus sinensis] >gi|557530155|gb|ESR41405.1| hypothetical protein CICLE_v10025961mg [Citrus clementina]	2538.999839	4469.626727	608.3729506	0.136112698	0.022552049	0.609888
HB-1 vs MB-2	Id	Hit description	Base Mean	Base Mean (HB-1)	Base Mean (MB-2)	Fold Change	pval	padj
	XP_006449387	hypothetical protein CICLE_v10016982mg [Citrus clementina] >gi|568826791|ref|XP_006467752.1| PREDICTED: ethylene-responsive transcription factor 12-like [Citrus sinensis] >gi|220029657|gb|ACL78786.1| putative ethylene responsive element binding protein 1 [Citrus unshiu] >gi|557551998|gb|ESR62627.1| hypothetical protein CICLE_v10016982mg [Citrus clementina] >gi|641858970|gb|KDO77660.1| hypothetical protein CISIN_1g030994mg [Citrus sinensis]	804.3628084	1492.693781	116.031836	0.077733181	0.005630872	0.341645
	XP_007014251	AP2-like ethylene-responsive transcription factor AIL5, putative [Theobroma cacao] >gi|508784614|gb|EOY31870.1| AP2-like ethylene-responsive transcription factor AIL5, putative [Theobroma cacao]	212.7422527	392.9955913	32.48891408	0.08266992	0.028594283	0.670783

HB-1 vs MB-3	Id	Hit description	Base Mean	Base Mean (HB-1)	Base Mean (MB-3)	Fold Change	pval	padj
	XP_006447343	hypothetical protein CICLE_v10015144mg [Citrus clementina] >gi|568877222|ref|XP_006491642.1| PREDICTED: ethylene-responsive transcription factor ERF053-like [Citrus sinensis] >gi|557549954|gb|ESR60583.1| hypothetical protein CICLE_v10015144mg [Citrus clementina]	211.9553178	387.1268246	36.78381114	0.095017469	0.018193626	0.449818
	XP_010663806	PREDICTED: ethylene-responsive transcription factor ERF114, partial [Vitis vinifera]	227.4119886	405.3957309	49.42824621	0.121925917	0.030625893	0.572321
HB-2 vs MB-2	Id	Hit description	Base Mean	Base Mean (HB-2)	Base Mean (MB-2)	Fold Change	pval	padj
	XP_007014251	AP2-like ethylene-responsive transcription factor AIL5, putative [Theobroma cacao] >gi|508784614|gb|EOY31870.1| AP2-like ethylene-responsive transcription factor AIL5, putative [Theobroma cacao]	187.4603935	346.3861596	28.5346274	0.082378082	0.031200319	0.570775
	XP_006466424	PREDICTED: AP2-like ethylene-responsive transcription factor ANT-like [Citrus sinensis] >gi|641860173|gb|KDO78862.1| hypothetical protein CISIN_1g005737mg [Citrus sinensis]	274.2466616	496.5195375	51.97378562	0.104676215	0.03615063	0.606336

Table S4: Differetially expressed ethylene genes in healthy and malformed tissues in mango cultivar Amrapali.

Note: MB-1: Malformed Bud-1; MB-2: Malformed Bud-2; MB-3: Malformed Bud-3; HB-1: Healthy Bud-1 & HB-2: Healthy Bud-2.

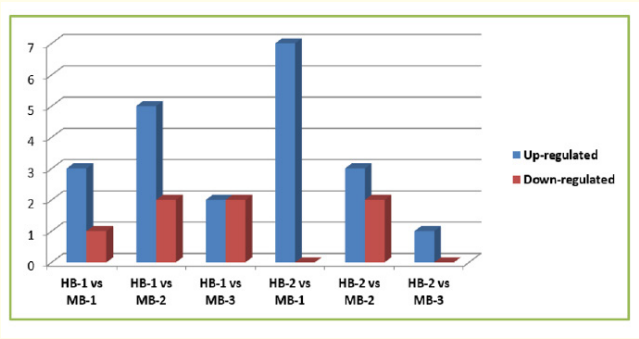
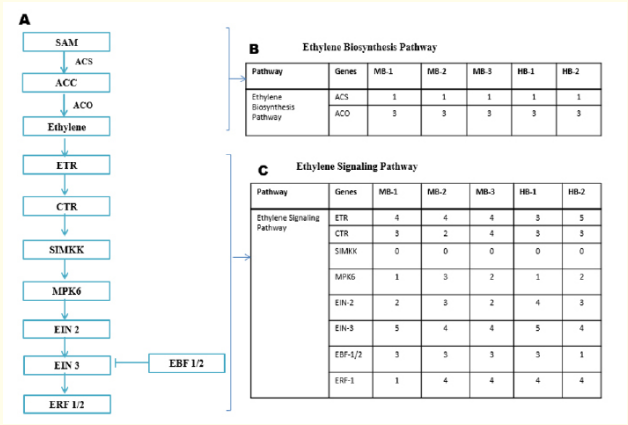


Figure 6: Differentially expressed ethylene genes between the healthy and the malformed tissues in mango variety Amrapali. MB-1: Single Swollen malformed bud stage-1, MB-2: Multiple malformed bud stage-2, MB-3: Multiple malformed bud stage-3. HB-1: Healthy bud stage 1 and HB-2: Healthy bud stage-2.



Pathway	Genes	MB-1	MB-2	MB-3	HB-1	HB-2
Ethylene Biosynthesis Pathway	ACS	1	1	1	1	1
	ACO	3	3	3	3	3

Pathway	Genes	MB-1	MB-2	MB-3	HB-1	HB-2
Ethylene Signaling Pathway	ETR	4	4	4	3	3
	CTR	3	2	4	3	3
	SIMKK	0	0	0	0	0
	MPK6	1	3	2	1	2
	EIN-2	2	3	2	4	3
	EIN-3	5	4	4	5	4
	EBF-1/2	3	3	3	3	1
	ERF-1	1	4	4	4	4

Figure 7: A) Ethylene biosynthesis and signaling pathway genes. B) Number of genes observed in ethylene biosynthesis pathway and C) Signaling pathways in transcriptomic analysis of healthy and malformed tissues.

RT-qPCR analysis

The quality of the extracted RNA, assessed using the ratio of absorbance at 260/280nm and 260/230nm, was in the range (> 1.52 to 2.44) for all five samples (Figure 8a). The concentrations of total RNA were 692.0, 378.6, 365.6, 117.3, and 291.1ng/µL for the healthy buds, malformed single buds, malformed multiple buds, malformed multiple buds treated with *B. subtilis* RP24and malformed multiple buds treated with *P. fluorescens* (MTCC 9858) respectively. The transcript expression of Actin, a housekeeping gene, was used as control (Figure 8b).

The RNA isolates were further used for analyzing the expression of ethylene genes such as ACO, ACS and ERF following the optimization of the semi quantitative RT-PCR conditions. The annealing reaction at 56.5°C yielded a sufficient amplification product of only ERF. The primers used for ACO and ACS failed to produce the expected amplification. ERF yielded PCR amplicons of the expected size of 450bp in samples collected 48 h after inoculation (Figure 8c). Despite varying the annealing temperature by ±3°C, ACO and ACS demonstrated no amplification. ERF was expressed in healthy buds, malformed multiple buds, and malformed multiple buds treated with *B. subtilis* RP24 but not in the swollen malformed single buds and malformed multiple buds treated with *P. fluorescens* (MTCC 9858) (Figure 8c). Furthermore, the relative expression of ERF normalized against the housekeeping gene revealed similar expression in healthy and malformed buds (Figure 8c). ERF (ng/µl) showed similar expression in healthy and malformed buds (Figure 8d). Our results suggest that the treatment with *P. fluorescens* (MTCC 9858) reduced the expression of ERF, which is primarily responsible for ethylene signaling and activation of ethylene target genes.

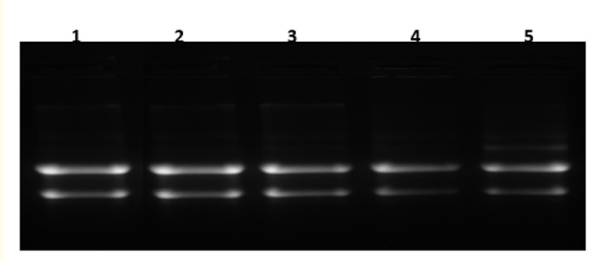


Figure 8a: Total RNA separated on agarose gel.

Figure 8b: Actin gene expression (350bp) in mango variety Amrapali.
Lane 1-Healthy single bud.
Lane 2-Malformed single bud.
Lane 3-Malformed Multiple bud.
Lane 4-Malformed multiple bud treated with Bacillus subtilis RP 24 (EF154418).
Lane 5-Malformed multiple bud treated with Pseudomonas fluorescens (MTCC 9858).
Actin gene expression was used as an endogenous control for normalizing the ERF expression.

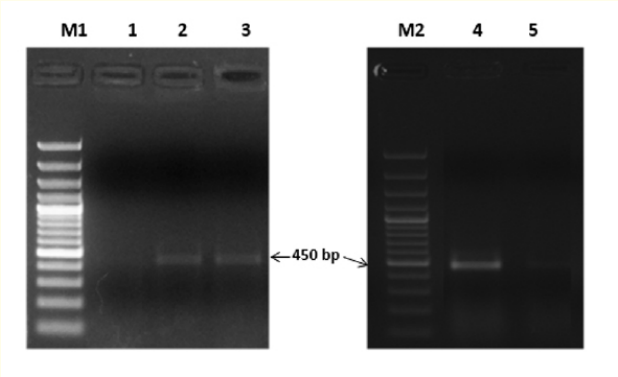


Figure 8c: ERF gene expression in mango variety Amrapali.
Lane-1: Malformed multiple buds treated with Pseudomonas fluorescens (MTCC 9858) 48 hours after inoculation.
Lane-2: Malformed multiple buds treated with Bacillus subtilis (EF154418) 48 hours after inoculation.
Lane-3: Healthy single bud control (without any treatment).
Lane-4: Malformed multiple buds' control (without any treatment);
Lane-5: Malformed single bud control (without any treatment)

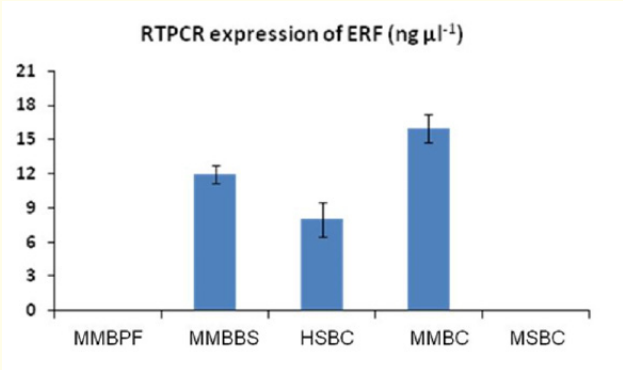


Figure 8d: RT-qPCR expression of ERF gene (Acc. No. EU513281) in healthy and malformed buds of mango variety Amrapali.
Lane-1: MMBPF - Malformed multiple buds treated with *Pseudomonas fluorescens* (MTCC 9858) 48 hours after inoculation.
Lane-2: MMBBS - Malformed multiple buds treated with *Bacillus subtilis* (EF154418) 48 hours after inoculation.
Lane-3: HSBC -Healthy single bud (without any treatment) control
Lane-4: MMBC - Malformed multiple buds (without any treatment) control
Lane-5: MSBC - Malformed single bud (without any treatment) control
Histogram represents mean (n = 3, ± SE) relative ERF level (ng/µl) in comparison to concentration of the 100bp molecular ladder.

Discussion

Management of the mango malformation disease through sanitation, pruning of the diseased panicles, application of synthetic fungicides, acaricides, hormones etc. have met with limited success. Due to widespread use of chemicals to control mango malformation and pesticide resistance has been reported in *Fusarium* [28,29] presents risk to ecology, environment and to the safety of personnel engaged in orchard management. Therefore, the discovery of most potent bio agent as alternative pathogen control method has become a priority.

The gram-positive *Bacillus spp.* and gram-negative *Pseudomonas* spp. are effective antagonists against *Fusarium* spp. The current study was therefore initiated with the hypothesis that use

of biocontrol microorganisms will lower the incidence of mango malformation. For this study the selection of *B. subtilis* RP24 and *P. fluorescens* (MTCC 9858) was determined from results obtained in previous experiments, relevance and availability.

In vitro studies and SEM analysis revealed the potential of *B. subtilis* RP24 and *P. fluorescens* (MTCC 9858) in reducing the radial growth of *F. mangiferae*, a causative agent of mango malformation disease. SEM analysis of the samples collected from *in vitro* dual cultures of *F. mangiferae*, + *B. subtilis* RP24 revealed the lysis and disruption of *F. mangiferae*, mycelia and absence of macro conidia and micro conidia when compared with control. And SEM samples treated with *F. mangiferae*, + *P. fluorescens* (MTCC 9858) revealed bacterial cells collating near the intact cell walls of *F. mangiferae*; however, the fungal cells were empty. Our study revealed that both *B. subtilis* RP24 and *P. fluorescens* (MTCC 9858) either completely inhibited *F. mangiferae* or reduced its growth rate since neither macro conidia nor micro conidia, the primary infective propagules of the pathogen were present. Studies have indicated that microbial antagonists of plant pathogens may have more than one mechanism of restricting the development of a target pest and that the inhibition of hyphal growth plays a determining role in inhibiting the development of *Fusarium* pathogens in the presence of the *Bacillus* strains [30]. *B. subtilis* also secrete cell wall-degrading enzymes, acetyl glucosaminidase and glucanase [31], which inhibit pathogens spore germination, disrupt germ tube growth, and interfere with the pathogen-plant attachment [32]. Biocontrol strain *B. subtilis* used in the present study RP24 has been reported to exhibit antagonism against a wide range of fungal pathogens and exhibited production of volatile organic compounds, lytic enzymes and lipopeptide antibiotic iturin *in vitro* [33, 43]. Studies have suggested that competition for niche and nutrients suppressed the growth of soil borne plant pathogens in the presence of *Pseudomonas* spp. [30]. A similar mechanism seems to govern the interaction between the biocontrol agent *P fluorescens* (MTCC 9858) to restrict the growth of the pathogenic fungal hypha.

Foliar application of the bio control agents at a final concentration of (3×10^7 CFU/mL) once before flowering in February and then again at flower bud initiation in March on the mango variety Amrapali showed a significant reduction (58.33%) in occurrence of malformed panicles in trees treated with *B. subtilis* RP24 (T1) than the control (T_4). 41.67% of malformed panicles that developed after foliar spray with T_1 completely dried and dropped within 4-7 days, depending on the environmental conditions. In the control trees (T_4), the malformed panicles dried slowly and dropped at higher temperature > 40 °C over a period of 30-40 days. The bio control properties of *Bacillus* spp. include the production of ammonia, antibiotics, chitinase, hydrogen cyanide, siderophores, and various hydrolytic enzymes such as lipase and glucanase and volatile compounds [34-36]. *Bacillus subtilis* is reported to synthesize different antibacterial compounds including different types of lipopeptides like surfactin, potent biosurfactants*: B. subtilis* is reported to form adherent biofilm on inert surfaces under the control of various transcription factors [37,38]. Earlier studies made in our laboratory [43] successfully extracted and partially characterized the antifungal metabolites of *B. subtilis* RP24 (EF154418), by thin layer chromatography (TLC) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Their studies revealed the presence of temperature and pH-stable cyclic lipopeptides of iturin group of peptide antibiotics [43]. Although there is the suggestion of antimicrobial activity, the in-planta control of *F. mangiferae* by *B. subtilis* RP24 may primarily be associated with competitive exclusion as described for other *Bacillus* species [39,40].

Major success in fungal disease control viz., *Pythium* root and seed rot across crops, *Fusarium* wilt in tomato and cotton, *Verticillium* wilt of potato, rhizoctonia stem and root rot of peanut, banana wilt caused by *Fusarium oxysporum*, bean disease caused by *Sclerotium rolfsii, Rhizoctonia solani* root infection in tomato and late blight of tomato has been achieved through the use of specific strains of *Pseudomonas*. Although *P. fluorescens* (MTCC 9858) showed potential to reduce the pathogen *F. mangiferae* under *in vitro* conditions, it failed to suppress the mango malformation disease when used as foliar spray on mango trees. Treatment with *P. fluorescens* (T_2) neither restricted the occurrence of floral malformation nor caused any visible drying symptoms in the malformed panicles. This could be due to the reason that *P. fluorescens* is adapted to survival in soil and colonization of plant roots and its application as foliar spray on mango trees was found ineffective to control an airborne pathogen, *F. mangiferae* induced mango malformation disease. However, *B. subtilis* RP24 efficiently controlled mango malformation disease. This may be because of their inherent ability to produce resistant endospores as well as their tolerance to extreme temperature and pH and resistance to pesticides, fertilizers, and production of antifungal comounds [43,41].

In the current study, foliar application of the biocontrol agents differentially regulated the onset of senescence and drying of malformed panicles. Ethylene has been implicated with several regulatory functions including flower senescence in plants. Senescing flowers showed climacteric rise of endogenous ethylene and an increased ethylene triggered a chain of events leading to the death of some of the floral organs [42]. According to Fick's law, the endogenous ethylene concentration of a fruit increases in league with the level of ethylene gas applied [47]. However, in the present experiment, concentration of ethylene in the gas samples drawn using a syringe from the polythene bag were lower than the detection limit of gas chromatography. This could be due to a possible leakage of gas caused by strong winds under open field conditions. Regulated functionality of the transcription factors during the floral senescence mediates the age-dependent expression of several senescence-related genes and those involved in ethylene biosynthesis and signaling, either directly or indirectly [43]. Hence, this study further extended to elucidate ethylene biosynthesis and it's signaling networks. We showed that the number of genes associated with the ethylene biosynthesis (*ACS, ACO, ERF*) and ethylene signaling pathway genes (*SIMKK, MPK6, CTR, EBF-1/2, EIN-2,* and *EIN-3*) did not differ in the healthy and the malformed buds at different stages of development indicating that ethylene is not the primary cause of mango malformation, as mooted by Ansari., *et al.* [44]. The current studies showed that the bacterial bio control agents that differed in their ability to control mango malformation also differed in their expression for the ethylene biosynthetic genes. ERF was expressed in healthy buds (control), malformed single buds, and malformed multiple buds treated with *B. subtilis* RP24 but not in malformed multiple buds treated with *P. fluorescens*. The transcription of ethylene-regulated genes is mediated by Ethylene Response Factors (ERFs) [47]. ERFs are the downstream components of ethylene signaling, known to modulate the activity of ethylene-responsive genes and have a pivotal role in mediating ethylene responses. Our results suggest that treatment with *P. fluorescens* reduced the expression of ERF, the primary ethylene response gene responsible for ethylene signaling and ethylene target gene activation. This minimized ethylene signaling and sensitivity could be the reason for inability of the bio agent *P. fluorescens* to cause drying and dropping of malformed tissues under field condition. Furthermore, we identified the CDS of the genes related to ethylene response that were differentially regulated in healthy and malformed tissues.

The significant reduction in occurrence of malformation incidence under T_1 when compared with other experimental treatments indicates that the use of gram- positive bacteria, *B. subtilis* RP24 has immense disease management advantage over the use of gram-negative bacteria *P. fluorescens*. However, safety aspects related to application of *B. subtilis* RP24 as a bio-control agent for the control of malformation disease in mango cannot be ignored. *B. subtilis* has been recognized as a nonpathogenic bacterium and has been granted the "generally regarded as safe" status by the US Food and Drug Administration. The spores of *B. subtilis* are resistant to electromagnetic radiation, ultraviolet rays, heat and some chemical agents, thus, these spores can be exploited as potential bio-control agents against plant pathogenic fungi [45,46]. Our study indicates that *B. subtilis* RP24 interferes with the complex regulatory pathways, within the plant-pathogen continuum, to restrict the development of a target pathogen *F. mangiferae* and consequently restricted the incidence of mango malformation by 58.33% *in vivo*. Foliar spray with *B. subtilis* RP 24 will allow optimal long-term maintenance of low levels of *F.mangiferae* infection by decreasing the reoccurrence of *F. mangiferae* loads on mango buds, and will prevent contamination of other pathogens by removing biofilms [62].Thus, *B. subtilis* RP 24 can help to overcome the limited action of chemical methods being followed to control mango malformation.

Conclusion

With a rapid development of resistance in disease causing pathogens against the disease controlling chemicals, there is a need to explore, develop, validate and exploit potent and sustainable alternatives to chemicals for the control of floral malformation in mango. The results indicate that *B. subtilis* RP24 (EF154418), a gram-positive bacterium, has immense potential to control *F. mangiferae* induced mango malformation, primarily through competitive exclusion and paves way for developing a novel and cost-effective strategy for the control of mango malformation. Since *F. mangiferae* infects mango buds through conidia that are disseminated by air; foliar spray with *B. subtilis* RP24 once before flowering in February and then again at flower bud initiation in March is recommended to reduce the extent of mango malformation under north Indian conditions. The present study revealed that the protection of buds from fungal infection is necessary to control the malformation disease when inocula persist. Hence, *B. subtilis* RP24

can be an effective bio-control agent to control the floral malformation malady in mango in an ecofriendly and sustainable manner.

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Declarations

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Conflicts of Interest/Competing Interests

The authors foresee and have no conflict of Interest or competing interest.

Ethics Approval

The study did not involve use of animals for research and high ethical standards were followed during conduct of the study and until its reporting.

Consent to Participate

All authors had consented to participate in this multidisciplinary research.

Consent for Publication

The corresponding author has consent of all participating authors to submit the research for publication.

Bibliography

1. Schoeman MH., *et al.* "Integrated control of mango blossom malformation in South Africa". *South African Journal of Plant and Soil* 36 (2018) 1-6.
2. Usha K., *et al.* "Hormonal profiling of the Fusarium mangiferae infected mango buds in relation to mango malformation". *Scientia Horticulturae* 254 (2019): 148-154.
3. Yadav A., *et al.* "Correction to: Transcriptome analysis of flowering genes in mango (*Mangifera indica* L.) in relation to floral malformation". *Journal of Plant Biochemistry and Biotechnology* 29 (2020): 571-572.
4. Usha K., *et al.* "Antifungal activity of *Datura stramonium*, *Calotropis gigantea* and *Azadirachta indica* against *Fusarium mangiferae* and floral malformation in mango". *European Journal of Plant Pathology* 124 (2009): 637-657.
5. Usha K., *et al.* "Scanning electron microscopic studies on floral malformation in mango". *Scientia Horticulturae* 71 (1997): 127-130.
6. Calvo-Garrido C., *et al.* "Survival of the biological control agent Candida's sake CPA-1 on grapes under the influence of abiotic factors". *Journal of Applied Microbiology* 117 (2014): 800-811.
7. Abdullah MT., *et al.* "Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary with *Trichoderma harzianum* and *Bacillus amyloliquefaciens"*. *Crop Protection* 27 (2008): 1354-1359.
8. Zhao Y., *et al.* "Antagonistic Action of Bacillus *subtilis* Strain SG6 on *Fusarium graminearum"*. *PLoS One* 9 (2014): e92486.
9. Adam M., *et al.* "Bacterial antagonists of fungal pathogens also control root-knot nematodes by induced systemic resistance of tomato plants". *PLoS One* 9 (2014): e90402.
10. Ntushelo K., *et al.* "The Mode of Action of *Bacillus* Species against *Fusarium graminearum*, Tools for Investigation, and Future Prospects". *Toxins* 11.10 (2019): 606.
11. Bakker PAHM., *et al.* "Induced systemic resistance by fluorescent *Pseudomonas* spp". *Phytopathology* 97 (2007): 239-243.
12. Haas D and Defago G. "Biological control of soil-borne pathogens by fluorescent pseudomonads". *Nature Reviews Microbiology* 3 (2005): 307-319.
13. O'Sullivan DJ and O'Gara F. "Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens". *Microbiological Reviews* 56 (1992): 662-676.
14. Iqbal N., *et al.* "Ethylene role in plant growth, development and senescence: Interaction with other phytohormones". *Frontiers in Plant Science* 8 (2017): 475.
15. Pierik R., *et al.* "The Janus face of ethylene: growth inhibition and stimulation". *Trends in Plant Science* 11 (2006): 176-183.
16. Weingart H and Voelksch B. "Ethylene production by *Pseudomonas syringae* pathovars *in vitro* and in planta". *Applied and Environmental Microbiology 63* (1997): 156-161.

17. Leslie JF and Summerell BA. "The Fusarium laboratory manual". Oxford, UK (2006): 259-269.

18. Skovgaard K., *et al.* "Evolution of *Fusarium oxysporum* f. sp. *vasinfectum* races inferred from multigene genealogies". *Phytopathology* 91 (2001): 1231-1237.

19. Kumar S., *et al.* "MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment". *Briefings in Bioinformatics* 5 (2004): 50-163.

20. Iqbal ZM., *et al.* "RAPD analysis of *Fusarium* isolates causing mango malformation disease in Pakistan". *World Journal of Microbiology and Biotechnology* 22 (2006): 1161-1167.

21. Grover M., *et al.* "Molecular and biochemical approaches for characterization of antifungal trait of a potent biocontrol agent *Bacillus subtilis* RP24". *Current Microbiology* 60 (2010): 99-106.

22. Nawrot CK. "The Use of Interactions in Dual Cultures in vitro to evaluate the Pathogenicity of Fungi and Susceptibility of Host Plant Genotypes". *Environmental Biotechnology* (2013): 287-301.

23. Kumar P., *et al.* "Biocontrol potential of *Trichoderma* species against mango malformation pathogens". *Archives of Phytopathology and Plant Protection* 45 (2012): 1237-1245.

24. Huang JY., *et al.* "Infection with pathogens transmitted commonly through food and the effect of increasing use of culture-independent diagnostic tests on surveillance-foodborne diseases active surveillance network, 10 U.S. Sites, 2012-2015". *Morbidity and Mortality Weekly Report* 65 (2016): 368-371.

25. Elmo M., *et al.* "A method for determining the concentration of ethylene in the gas phase of vegetative plant tissues". *Plant Physiology* 46 (1970): 352-354.

26. Kanehisa M and Goto S. "KEGG: kyoto encyclopedia of genes and genomes". *Nucleic Acids Research* 28 (2000): 27-30.

27. McDonnell G and Russell AD. "Antiseptics and disinfectants: activity, action, and resistance". *Clinical Microbiology Reviews* 12 (1999): 147-179.

28. Frabetti A., *et al.* "Experimental evaluation of the efficacy of sanitation procedures in operating rooms". *American Journal of Infection Control* 37 (2009): 658-664.

29. Baysal OC., *et al.* "An inhibitory effect of a new Bacillus subtilis strain (EU07) against *Fusarium oxysporum* f. sp. radicis-lycopersici". *Physiological and Molecular Plant Pathology* 73 (2008): 25-32.

30. Manjula K and Podile AR. "Production of fungal cell wall degrading enzymes by a biocontrol strain of *Bacillus subtilis* AF1". *Indian Journal of Experimental Biology* 43 (2005): 892-896.

31. Piggot PJ and Hilbert DW. "Sporulation of *Bacillus subtilis"*. *Current Opinion in Microbiology* 7 (2004): 579-586.

32. Grover M., *et al.* "Comparison between *Bacillus subtilis* RP24 and its antibiotic-defective mutants". *World Journal Microbiology Biotechnology* 25.8 (2009): 1329-1335.

33. Huang CJ., *et al.* "Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus*". *Journal of Biochemistry and Molecular Biology Research* 38 (2018): 82-88.

34. Trivedi P., *et al.* "*In vitro* evaluation of antagonistic properties of *Pseudomonas corrugate"*. *Microbiological Research* 163 (2008): 329-336.

35. Srividya S., *et al.* "Multifarious antagonistic potentials of rhizosphere associated bacterial isolates against soil borne diseases of Tomato". *Asian Journal of Plant Science and Research* 2 (2012): 180-186.

36. Hamon MA and Beth AL. "The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis"*. *Molecular Microbiology* 42 (2001): 1199-1209.

37. Stanley NR., *et al.* "Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays". *Journal of Bacteriology* 185 (2003): 1951-1957.

38. Vandini A., *et al.* "Hard surface biocontrol in hospitals using microbial-based cleaning products". *PLoS One* 9 (2014): e108598.

39. Sparado D and Droby S. "Development of biocontrol products for post-harvest diseases of fruit: the importance of elucidating the mechanisms of action of yeast antagonists". *Trends in Food Science and Technology* 47 (2016): 39-49.

40. Khan N., *et al.* "Antifungal Activity of *Bacillus* Species Against *Fusarium* and Analysis of the Potential Mechanisms Used in Biocontrol". *Frontiers in Microbiology* 9 (2018): 2363.

41. Iordachescu M and Verlinden S. "Transcriptional regulation of three EIN3-like genes of carnation (*Dianthus caryophyllus* L. cv. Improved White Sim) during flower development and upon wounding, pollination, and ethylene exposure". *Journal of Experimental Botany* 56 (2005): 2011-2018.

42. Olsen A., *et al.* "Ethylene resistance in flowering ornamental plants-improvements and future perspectives". *Horticulture Research* 2 (2015): 15038.

43. Ansari MW., *et al.* "First evidence of ethylene production by *Fusarium mangiferae* associated with mango malformation". *Plant Signaling and Behavior* 8 (2013): e22673.

44. Thakore Y. "The biopesticide market for global agricultural use". *Industrial Biotechnology* 2 (2006): 194-208.

45. Zhao J., *et al.* "Fungal pretreatment of unsterilized yard trimmings for enhanced methane production by solid-state anaerobic digestion". *Bioresource Technology* 158 (2014): 248-252.

46. Liu W., *et al.* "An ethylene response factor (MxERF4) functions as a repressor of Fe acquisition in *Malus xiaojinensis*". *Scientific Reports* 8 (2018): 1068.