INTEGRATED MANAGEMENT FOR CONTROL OF VERTICILLIUM WILT OF COTTON

A Thesis

Submitted in partial fulfillment of the requirements for the award of the degree of

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IN
(BOTANY)

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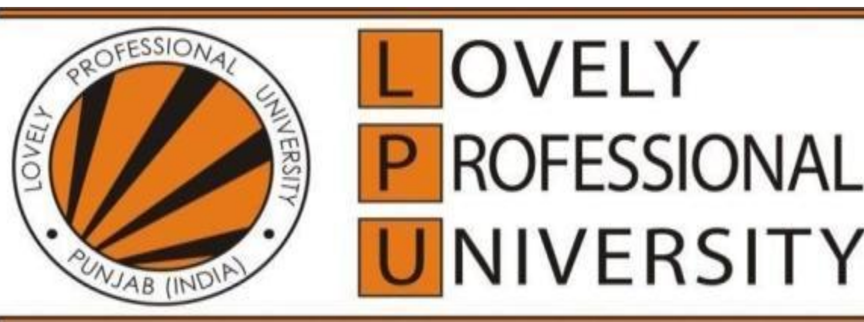
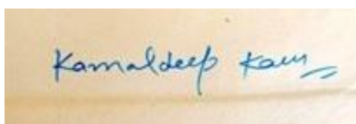


DECLARATION

I hereby declare that the thesis entitled**, "Integrated management for control of Verticillium wilt of cotton"** submitted for Ph.D. Botany Degree to School of Bioengineering and Biosciences, Lovely Professional University is entirely original work and all ideas and references have been duly acknowledged. The research work has not been formed the basis for award of any other degree.



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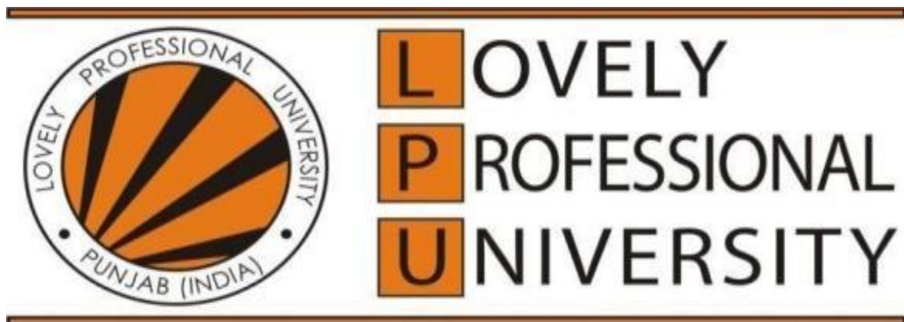

CERTIFICATE

This is to certify that **Ms. Kamaldeep Kaur** has completed the Ph.D. Botany titled**, "Integrated management for control of Verticillium wilt of cotton"** under my guidance and supervision. To the best of my knowledge, the present work is the result of his original investigation and study. No part of this thesis has ever been submitted for any other degree or diploma. The thesis is fit for the submission for the partial fulfillment of the condition for the award of degree of Ph.D. in Botany.

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ABSTRACT

Cotton is a crop of fibre, oil and protein and an economically important crop grown throughout the world. Cotton is the major vital kharif crop of Punjab, India but cotton yield starts reducing annually due to attack of soil borne pathogenic fungus *Verticillium dahliae* causing *Verticillium* wilt disease in cotton. Various biological agents viz. endophytic bacteria, fungal strains and actinomycetes have been used for control of witing in cotton. Major focus of this research is to isolate antagonistic fungi from different organic manures and endophytic bacteria from healthy cotton plant parts; followed by *in vitro* antagonistic activity and biosurfactant based studies with enzymatic screening for evaluation of potential biocontrol agents to suppress *Verticillium* wilt of cotton under both poly house and natural field conditions in Bathinda, Punjab, India. Bathinda, major cotton producing district of Punjab is selected for this study. Pathogenic fungus has been isolated from collected samples growing under natural field conditions of Bathinda, Punjab, India. A total of 12 antagonistic fungal strains have been isolated from different organic manures and 7 endophytic bacterial strains have been isolated from collected plant parts. viz. root, stem and leaf from different sites of Bathinda, Punjab, India. Biocontrol potential of antagonistic isolates was assessed on following criteria: (1) *in vitro* inhibition of *Verticillium dahliae* by Dual culture assay, (2) activities of cell wall degrading enzymes including protease, cellulase, and chitinase. (3) *in vivo* field trials under poly house and natural field conditions. Preliminary screening of all antagonistic strains has been done for checking their antagonistic potential *V. dahliae* by Dual culture assay in which all strains have shown different antagonism towards *Verticillium dahliae*. Prevalence rate of *Vertcillium dahliae* out of 160 collected samples, from 20 different sites of Bathinda, Punjab, India is 73%. Most significant percentage inhibition of radial growth (PIRG) has been calculated (73.46%) in *Aspergillus fumigatus* followed by *T. harzianum* (72.91%), *Aspergillus niger* (63.20%), *Aspergillus nidulans* (61.12%), *Gliocladium sp.* (61.81%), *Aspergillus terreus (59.90%), Fusarium solani* (47.22%) and *Cladosporium sphaerospermum* (6.95%) towards *V. dahliae* during Dual culture assay. Additional screening has been done by in vitro hydrolysis enzymatic activity in which maximum cellulase activity was recorded in *Aspergillus terreus* (11.5 mm) followed by *Gliocladium sp.* (10.83mm)., *Aspergillus niger* (10.04mm), *Aspergillus fumigatus* (9.83mm),

Trichoderma harzianum (8.5mm), *Aspergillus nidulans* (7.5mm) and *Fusarium solani* (5.0mm) around the well. *Aspergillus fumigatus* has shown most significant growth while *Cladosporium sphaerospermum* (0mm) was not able to produce inhibition zone. clear inhibition zones during protease enzyme activity, maximum inhibition zone has been recorded in *A. terreus* (10.7mm) followed by *Aspergillus niger* (9.5mm), *A. fumigatus* (9.33mm), *Gliocladium sp.* (9.06mm), *Aspergillus nidulans* (8.1mm), *Trichoderma harzianum* (6.7mm), *Fusarium solani* (6.46mm) and *Cladosporium sphaerospermum* (3.56mm) clear zones respectively around the well. During chitinase activity maximum hydrolysis zone has been recorded in *Aspergillus niger* (9.66mm) followed *by Aspergillus terreus* (9.5mm), *Aspergillus nidulans* (8.5mm), *Aspergillus fumigatus*(8.43mm), *Gliocladium sp.*(8.43mm), *Trichoderma harzianum* (8.1mm), *Fusarium solani* (6.6mm) and *Cladosporium sphaerospermum* (3.2mm) around the well. PIRG of all bacterial strains in which maximum percentage of inhibition growth has been shown in *Pseudomonas aeruginosa* (VTS DB-V1/NI /S/S1/B2) with 61.12% followed by *Lysinibacillus macroides* (VTS DB-V1/NI /S/S1/B1), *Bacillus subtilis* (VTS DB-V1/NI /R/S1/B2), VG DB-V1/NI /L/S1/B2, VTS DB-V1/NI /L/S1/B2, VG DB-V2/NI /L/S1/B1 and VG DB-V1/NI /S/S1/B1 with 59.2%, 57.64%, 56.25%, 48.62%, 45.2% and 40.29% respectively during Dual culture Assay. Inhibition zones produced by various endophytic bacterial strains at 5th day of incubation, in which *Pseudomonas aeruginosa* (VTS DB-V1/NI /S/S1/B2) has shown maximum growth of inhibition zone (8.56mm) followed by *Lysinibacillus macroides* (VTSDB-V1/N1/S/S1/B1)(7.3mm), VTSDBV1/N1/L/S1/B2(6.8mm), *Bacillus subtilis* (VTSDBV1/N1/R/S1/B2) (5.23mm), VG DB-V2/N1/L/S1/B2(5.2mm), VGDB-V2/N1/L/S1/B2(5.0mm) and VGDB-V1/N1/S/S1/B1(4.5mm) during cellulase activity. *Pseudomonas aeruginosa* (VTS DB-V1/NI /S/S1/B2) has shown most significant growth followed by *Lysinibacillus macroides.* Maximum inhibition zone has been recorded in *Pseudomonas aeruginosa* (VTS DB-V1/NI /S/S1/B2) (8.70mm) followed by *Lysinibacillus macroides* (VTS DB-V1/NI /S/S1/B1) (7.32mm), *Bacillus subtilis* (VTS DB-V1/NI /R/S1/B2) (6.66mm) , VG DB-V1/NI /L/S1/B2 (5.5mm), VG DB-V2/NI /L/S1/B1 (4.2mm), VG DB-V1/NI /S/S1/B1(3.9mm) and VTS DB-V1/NI /L/S1/B2 (3.2mm) during protease activity. Maximum growth of purple coloured inhibition zone in *Pseudomonas aeruginosa* (VTS DB-V1/NI /S/S1/B2) (7.4mm) followed by *Lysinibacillus macroides* (VTS DB-V1/NI /S/S1/B1) (7.1mm), *Bacillus subtilis* (VTS DB-V1/NI /R/S1/B2) (6.3mm), VG DB-V1/NI /L/S1/B2

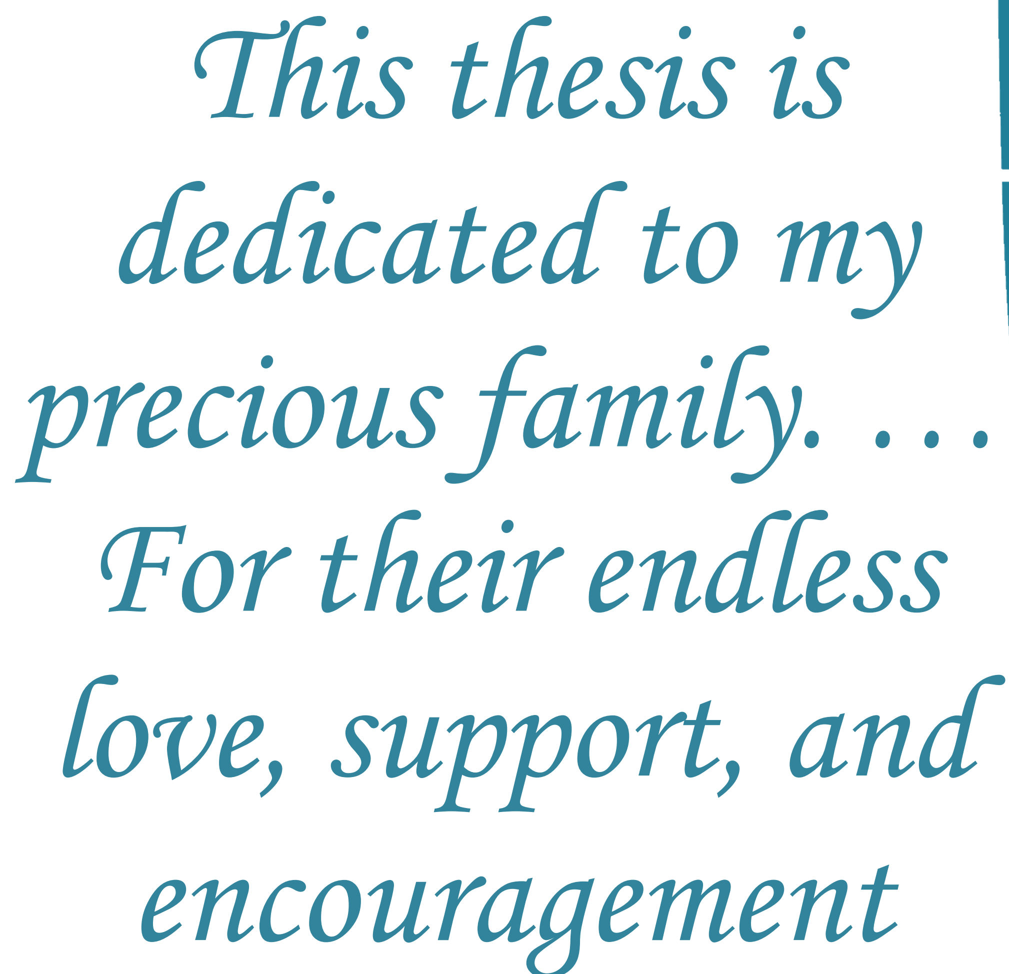
(6.1mm), VTS DB-V1/NI /L/S1/B2(5.7mm), VG DB-V2/NI /L/S1/B1(4.5mm) and VG DB-V1/NI /S/S1/B1(3.9mm) during chitinase activity. *in vitro* antifungal activity of both biosurfactant Fengycin and Iturin against *V. dahliae* in which Fengycin at concentration of 5ppm and 10 ppm have shown 2.3±0.1ab mm and 3.2± 0.05b mm inhibition zones respectively and Iturin at same concentrations have shown 2.9±0.15b mm, 3.8±0.2ab mm inhibition zones respectively as compared to control plate. Inhibition zones have been recorded at 10, 50 and 100ppm. Inhibition zone of 7.6±0.8mm and 12±0.2mm has been recorded after 5 days of incubation at 27^0C around the well at 50ppm and 100ppm concentration respectively when compared to control. Various active compounds such as 3-Glc-campesterol, Momordol , Dihydroisocucurbitacin B-25-acetyl, 2-O-β-D- glucopyranose- (22-27)-hexanocucurbitacin I, 2-O-β-D-glucopyranosyl-cucurbitacin L, 2-O-β-D-glucopyranosyl-cucurbitacin E, Arvenin I, 6'-acetyl-2-O-β-D- glucopyranosyl-cucurbitacin E, 2,3-Dihydrowithaferin A3β-O-sulfate, 23, 24-dihydrowithagenin, Dihydrowithanolide, Dihydrowithferin A, Petunidin 3-O-rhamnoside, Adenylosuccinate, Lysoplasmenylcholine, GDP-L-galactose, Verbascoside, Deoxycholic acid 3- glucuronide, 3 β -Hydroxydeoxo dihydrodeoxygedunin, Hydrocortisone cypionate, Dihydro deoxy streptomycin, Withanolide S, Solasonine, β -2 solamargine, Germanicol cinnamate, Pubesenolide and Cucurbitacin E, 2-O-β-D-glucopyranosyl-cucurbitacin I, 25-p-coumaroyl-3'-acetyl-2-O-β-D glucopyranosyl-cucurbitacin I, Petunidin 3-O-rhamnoside, Aswagandhanolide, Sitoindoside X, 4-Deoxywithaperuvin and Solanesol have been identified by LCMS of biochemical formulation.

in vivo study of selected potential biocontrol agents has been done to evaluate these potential antagonistic strains against Verticillium dahliae. In field experiment, biocontrol protection has been calculated in which, *Aspergillus terreus has shown* (74.5%) followed by *Trichoderma harzianum* (72.3%), *Aspergillus fumigatus* (68.8%), *A. niger* (66.2%), *A. nidulans* (61.2%) as compared to control 1 (43.2), (41.3), (36.7), (41.3), (33.8) and control 2 (26.5), (24.2), (39.5), (22.6) and (39.5) respectively. Most significant biocontrol efficacy has been recorded in *Pseudomonas aeruginosa* (73.6%) followed by *Lysinibacillus macroides* (68.5%) and *Bacillus subtilis* (62.5) as compared to control plant with (40.3), (36.7) and (39.2) biocontrol protection percent, respectively in monoculture experiment. Control plants (non-

bacterized) have shown wilting or death (81%) after 135 days of sowing and treated plants have shown no disease symptoms (0% healthy) at 0-4 rating scale and 71.3% biocontrol protection percent during co- culture experiment. During natural field conditions with biochemical formulation, the percentage of biocontrol protection for *Pseudomonas aeruginosa* (76.6%) followed by *Lysinibacillus macroides* (69.5%) and *Bacillus subtilis* (58.3%) as compared to control plants (35.5%), (39.7%) and (36.6%) respectively. Most significant biocontrol efficacy in *Trichoderma harzianum* (79.5%) followed by *Aspergillus terreus* (78.3%), *A. fumigatus* (72.8%), *A. niger (69.2%)* and *A. nidulans* (68.2%) as compared to control 1 (35.6%), (33.6%), (39.7%), (31.5%) and (32.8%) and control 2 plants (24.2%), (26.5%), (39.5%), (22.6%) and (38.5%) respectively. During natural field condition trials, 78.2 percent significant biocontrol efficacy has been recorded in test plants as compared to control plants during co culture experiment with biochemical formulation. During poly house experiment, biocontrol protection percent is (69.6%) in *Pseudomonas aeruginosa* followed by *Lysinibacillus macroides* (65.2%) *Bacillus subtilis* (62.5%) as compared to control with (36.4%), (29.7%) and (32.2%) respectively in monoculture experiment. During pot trials, most significant biocontrol efficacy has been recorded in *Trichoderma harzianum* (70.3%) followed by *Aspergillus terreus* (66.5%), *A. niger* (64.2%), *A. fumigatus* (61.5%) and *A. nidulans* (59.2%) as compared to control 1 plants with (38.1), (36.2), (38.4), (42.7) and (35.8) and control 2 plants (22.1), (28.8), (22.6), (39.5) and (38.5) respectively. Control 2 plants have shown wilting or death (83%) after 135 days of sowing during pot trials and 69.5% biocontrol protection percent during co-culture experiment.

During pot trials with biochemical formulation, most significant biocontrol efficacy has been recorded in *Trichoderma harzianum* (73.2%) followed by *Aspergillus terreus* (68.5%), *A. fumigatus* (66.2%), *A. niger* (61.3%) and *A. nidulans* (57.2%) as compared to control 1 (36.5), (33.2), (37.5), (39.5) and (34.6) and control 2 plants (25.1), (26.6), (33.5), (20.4) and (36.5) respectively in monoculture experiment and biocontrol protection in *Pseudomonas aeruginosa* (70.6%) followed by *Lysinibacillus macroides* (68.6%) *Bacillus subtilis* (64.5%) as compared to control plant with (37.5%), (36.5%) and (39.2%) respectively has been calculated. 72.5% significant biocontrol efficacy has been recorded in test plants as compared to control plants during co-culture experiment in pot trials. Antagonistic fungal strains that showed

positive biocontrol efficacy in the greenhouse and field experiments were analysed for their potential effects on growth on cotton plant. Antagonistic strain of *Aspergillus terreus* has shown most positive antagonistic activity during *in vitro* screening as well as bio control efficacy and helps in growth promotion of cotton plants. Endophytic bacterial strains that showed positive biocontrol efficacy in the greenhouse and field experiments were analysed for their potential effects on growth on cotton plants. Yield and growth of seed bacterized plants with *Pseudomonas aeruginosa* under natural field conditions was more than *Lysinibacillus macroides* followed by *Bacillus subtilis* when compared with control (non-bacterized). Maximum root length was recorded in treated plant than control plants which shows the symptoms of wilting after first blooming stage. Evaluation of antagonistic fungal strains, endophytic bacterial strains under poly house and natural conditions field trials of Bathinda, Punjab in combination with soil solarization and biochemical formulation have shown positive biocontrol efficacy against wilt disease and effects on growth of plants and increases yield of cotton than control plant**.**


This thesis is
dedicated to my
precious family. …
For their endless
love, support, and
encouragement

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Kamaldeep Kaur

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CHAPTER- I

INTRODUCTION

Cotton is a crop of fibre, oil and protein and an economically important crop grown throughout the world (Zhang et al., 2018). Cotton is cultivated all over the world from the prehistoric era. More than 80 countries of tropical and sub-tropical regions in world cultivate cotton (Romo et al., 2021). Cotton belongs to order Malvales, family Malvaceae and genus *Gossypium*n (Shim et al., 2018). The major cotton producing countries are USA, India, Pakistan, Turkey, Argentina and Egypt (Li et al., 2012). *Gossypium arboreum, G. herbaceum, G. hirsutum* and *G. barbadense* are the four main species of cotton. *Gossypium arboreum, G. herbaceum,* are diploid (2n=26) in nature and native to old world (Karena et al., 2021). There are four growth stages of cotton plant:

1. **Germination of seed and seedling establishment**
2. **Leaf area and development of canopy**
3. **Flowering and development of cotton bolls**
4. **Maturation of cotton plant**

1.1 Morphology of cotton plant: Different species of cotton plants shows different growth habits. The root can go below three meters in soil. Tap root system in cotton plays a function to absorb and transport water and uptake of nutrients from the earth to all plant parts. There is an erect, indeterminate, and monopodial stem of cotton. The main stem of the cotton plant is having many nodes, and they have the capability of producing a branch (Newman., 2002). Monopodium and sympodium branches are present in cotton. Monopodial branches (vegetative) are those branches which do not bear fruits directly on it. Sympodial branches are those branches which bear fruit directly, therefore known as fruiting branches. Sympodial branches enter the fruiting phase of growth. There are two types of leaves in cotton plant: (1) leaves originate from cotyledons, (2) true leaves (Patki and Sable., 2016). Phyllotaxy (arrangement of leaves on the main stem) is mostly spiral in form and is in the ratio of 1:1 clockwise or anti clockwise arrangement of leaves in cotton plants. Colour of cotton leaves are with different shades of green or dark green and red colour in cotton plants. Hairy leaves are present on leaves which provide protection against different insects. Leaves are also indicating that nutrients are present in very low amount. Non infected leaves

have the large amount of nitrogen from all upper parts of the cotton plant. Flower of cotton plant is complete; bracteoles are in the surrounding, calyx, corolla, and well-developed androecium and gynoecium. Fertilized flower becomes an open boll after 50 days of germination followed by additional 30 days for development of bolls into full size. Seeds also have grown to full size after the same time. Very low temperature and frost conditions are responsible for dryness in green bolls. The time of bolls increases with a decrease in temperature. Seed of cotton plant is a well-developed ovule that gives rise into mature seed or not to a full-grown seed. The average growth in seed is of 10 mm x 6 mm. The depth for plantation of cotton seed is 3-4 cm. The germination process begins only when moisture is absorbed into the cotton seed. Cotton fibres are epidermal hairs known as trichomes that are arising from the protoderm of the ovule. Formation of fibres is initiated after 15-20 days, and deposition of the cellulosic secondary cell wall begins (Lang et al., 2011).

1.2 History of cotton cultivation in India

Cotton fibre were discovered more than 4000 years ago in Coastal Peru and at Mohanjodaro in the Indus Valley. In the middle of 18th century only *ideogram arboreum* & *herbaceum* varieties of cotton were grown in different regions of the country. American Cotton (variety Combodian) was introduced in India was in 1904-05. The ICCC established Cotton Technological Research Laboratory (now CIRCOT) in 1924 to undertake study on Cotton fibre. Major loss has been reported in India during partition in 1947 with the transfer of large areas of irrigated Cotton to Pakistan. Therefore, the production in India touched low of 23 lakh bales in 1948. Due to concentrated efforts made by the State Government under the aegis of ICCC the area under cotton increased to 78 lakh ha with increased production of 53 lakh bales by 1966-67 (Dr. N.B. Singh, 2009, Revolution in Indian Cotton, 14, Directorate of Cotton Development Ministry of Agriculture, Department of Agriculture & Cooperation Government of India).

1.3 Cotton production in India: Punjab, Haryana, Rajasthan, Uttar Pradesh, Madhya Pradesh, Gujarat, Andhra Pradesh, Karnataka and Tamilnadu are nine major cotton cultivated states in India. These nine states contribute 95 percent of cotton production in India. These nine states are reported as major cotton cultivated states (Uppal et al., 2008). According to the Ministry of Agriculture and Farmers Welfare 322.7 lakh bales cotton has been cultivated during 2019-2020. In Punjab, Bathinda, Faridkot,

Firozepur, Sangrur are major cotton producing districts and Ludhiana, Mukatsar, Barnala and Fazilka are the minor cotton producers (**Figure 1.1**).

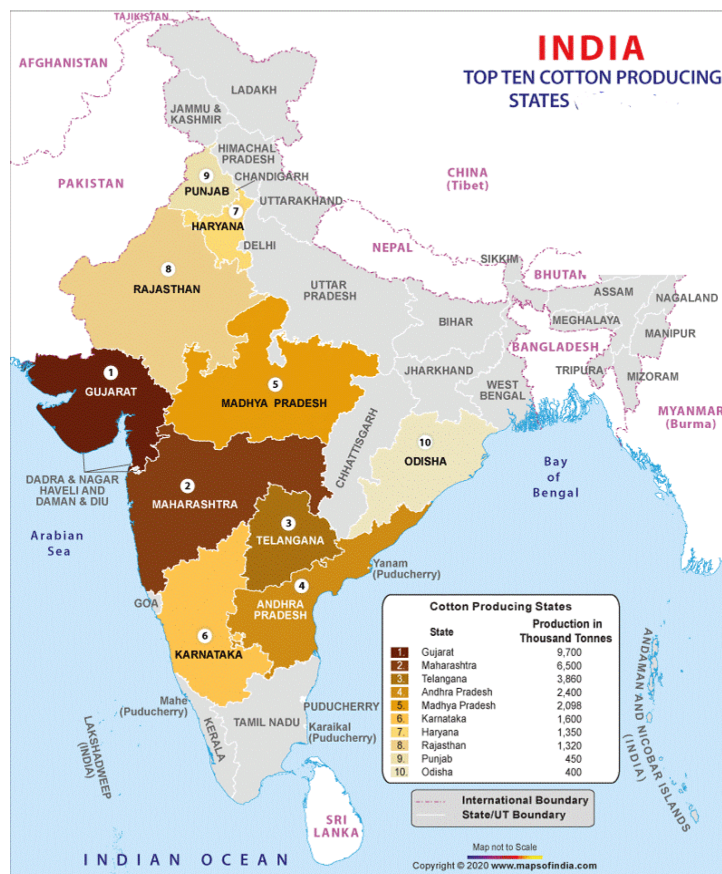

Figure 1.1- Cotton production map of India

1.4 Cotton cultivation in Punjab: Cotton is a vital Kharif crop of the Punjab State. April to May is the sowing period and harvesting period starts from December to January in Punjab. Sowing temperature is 25-35^{0}C and harvesting temperature is 15^{0}C to 25^{0}C is required daily for germination of cotton plant and 21^{0}C is required for proper crop growth. 27^{0}C to 32^{0}C day temperature is required during fruiting phase and cool nights are also needed during fruiting phase in cotton. Cotton picking period is starts from mid-September to November. Cotton can be successfully grown on all soils without water logging, sandy or saline types. At different intervals of time cotton needs four to six irrigations depending upon the rainfall in season. The cotton crop does not require irrigation during the flowering and fruiting stages. From healthy cotton fields, uninfected cotton bolls should be separately picked, stored and ginned. The purified seed should be labelled and kept in a clean and moisture less place. Before the use of cotton seeds its genetic purity and germination should be tested. The

cotton grows infinitely because of its feature. Various plant disease and insect pests can also be harmful for cotton plants. In the world, annual loss in cotton cultivation due to *Verticillium* wilt were reported as 1.5 million bales (Celik et al., 2017). In Punjab, nowadays 4.5 lakh hectare areas come under cultivation. If no prevention and control measures has been taken, attack of insects and pests can result into huge loss (Mohan et al., 2014).

1.5 Diseases reported in cotton plant: Many organisms like fungi, nematodes and bacteria which germinate on and within tissues of plants are infection causing agent in cotton plants. Various diseases reported in cotton plants are Root rot of cotton, Angular leaf spot, Seed and seedling diseases such as *Fusarium* wilt of cotton and *Verticillium* wilt of cotton especially in India (Hagan et al., 2017). Diseased plants show the symptoms of stunting in height of cotton plants, poor colour development, reduced vigour and yields of cotton and sometimes causes death in plant. Seedling diseases are responsible for moderate loss in yield of crop but soil, cultural and environmental impacts are also taking part for delaying of the seed development. Primary agents that because diseases are named as *Pythium sp., Rhizoctonia solani*, *Phomaexigua* (*Ascochyta*), and *Fusarium sp.,* and *Verticillium* species. (Huang et al., 2016). Damaged seedlings are characterized by yellowish, irregular, slower growing, and rarely die in a short lifespan. Checking of infected seedlings might show dark spots on the shoots and roots. *Pythium spp. Fusarium spp*. is usually attacked from the seeds and attacked below-ground parts of young seedlings *Pythium sp.* causes seedling disease in cotton and known as water moulds, because it produces spores which are moving actively in soil water (Zhu et al., 2013). Boll rot is mostly occurring only when attack of insects done on cotton plant or excessively wet conditions are there. Boll rot at commencing stage of infection reveals small brown lesions that generally spread until when the whole boll becomes blackened and dried (Nargale et al., 2020). *Xanthomonas compestris* is responsible for bacterial or angular leaf spot of cotton disease. The infection comes on several parts of cotton plant, in seedling and in mature plant stages (Wang et al., 2009). Bolls, bracts, flowers, leaves, petioles, and shoots possess symptoms. Water-soaked spots produces on leaves which then convert reddish into black forming angular leaf spots, are covered by veins and vein lets. Blighted patches are formed by coalescing of spots were appear before. *Fusarium* wilt of cotton disease is the resultant of pathogen named *Fusarium oxysporum* and

diseased plants shows the symptoms of withering of leaves by virtue of loss of turgidity or stiffness then ultimately leaves turns into brown and drooping starts which finally drop off. Whitefly is also a dominant factor to harm the cotton plants. Cotton Leaf curl disease is a viral disease caused by attacking *Bemisia tabaci* (whitefly) (Khan et al., 2015).

1.6 *Verticillium* wilt of cotton: *Verticillium* wilt, most devastating disease of cotton occurred by soil borne fungus known as *Verticillium dahliae* (Zhang et al., 2019). Defoliating and non- defoliating pathotype of *V. dahliae* has been reported firstly in Turkey. *V. dahliae* has wide host range of more than 400 plant species. *Verticillium* wilt has reported first time in Manisa province of Turkey in 1941 (Pullman and Devay.,1981). *Verticillium* fungus is present in the soil and infects plants through the roots, often entering through wounds. The fungus survives in the soil as a thread-like body called a mycelium and as microscopic, dark, resistant structures called microsclerotia. These microsclerotia can survive in soil or dead plant material for up to ten years. Therefore, it is virtually impossible to eradicate the fungus from the soil (Duang et al., 2013). *Vertcillium dahliae* have long lifespan and long viability of the resting structures, fungicides are not effective to control the effect of pathogen in diseased plants. *Verticillium dahliae* remains in viable conditions for long time in the soil as microsclerotia. Single microsclerotium is enough for start-up of infection in cotton plants. Dissemination of *V. dahliae* takes place at long distance with seed infected by mycelia or with infected seed with microsclerotia. Maximum growth of the fungi enhances the chances to increase of the disease. The roots act as an agency to commence fungal infection which invades trough the vascular system causes a systemic infection (Khadi., 2010). Significant loss in cotton yield has been reported throughout the world due to attack of this pathogenic fungus. Fiber quality has also been affected due to attack of *V. dahliae*. Significant loss in cotton lint with 32% has been reported in diseased cotton plants (Ayele et al., 2020).

1.6.1 Symptoms of *Verticillium* wilt in cotton: Infected leaves reveal wilting and becomes yellow between veins before showing necrosis. Vascular discolouration is light to dark brown which is more in the shoot and branches in the plants showing symptoms of wilting. Diseased plants can die after defoliation or remain defoliated. Symptoms of the cotton plants caused by *Verticillium* wilt generally appear at the first flowering stage. Abscising or malformation of younger bolls occurs in infected plants.

Infected leaves start appearing in darker green colour as compared to the healthy plant and become somewhat cracked among the veins (Presley., 1953). When plants infected with defoliating strain of fungus that may lose their leaves **(Figure 1.2)**. Foliar symptoms may be confused with those of cotton root if plants wilt and die but plants infected with *Verticillium dahliae* only have no rotten roots (Vandenkoornhuyse et al., 2015). The significant losses were occurring by this disease throughout the 1970's and 1980's (Mansoori et al., 2013). The partially resistant varieties which are used now a days are Paymaster HS-26, in the 1990's is believed to have disrupted soil populations of *Verticillium dahliae.*

Figure 1.2: Cotton plant showing symptoms of *Verticillium* wilt

1.7 Life cycle of *Verticillium dahliae*

Three major phases of life cycle of *V. dahliae* are as follow:

- Dormant phase
- Parasitic phase
- Saprotrophic phase

V. dahliae remains dormant during dormant phase refers to the extended phase. During this phase round, thick walled microsclerotia made of melanin have been formed that helps to survive in soil during unfavourable conditions for a long time (Daayf., 2015). Microsclerotia starts growing during favourable conditions.

Microsclerotia of *V. dahliae* have been germinate in the rhizosphere of cotton plant in the presence of root exudates. Fungi starts colonization in plant cortex from one plant to other and infects tips of cotton plant (Klimes et al., 2015). After colonization in plant cortex, conidia have been formed in vascular system of plant and allowed to transfer to other parts of cotton plant through the sap stream. *V. dahliae* produces pectinolytic enzymes causing damage to the xylem and vessels of plant. A glue-like substance produced by fungus results into discolouration of vessels and filled with hyphae of pathogenic fungus. Toxins produced by *V. dahliae* also affects respiration and photosynthesis results into wilting of cotton plant during parasitic phase/stage. During saprophytic stage, microsclerotia starts increasing particularly in the roots and shoot. Fungus grow and reproduce continuously in cortex and petioles of plant during this stage **(Figure 1.3)**. Once the complete death of plant occurs, microsclerotia remains in the soil and continue life cycle by stimulation of root exudates (Klosterman et al., 2009).

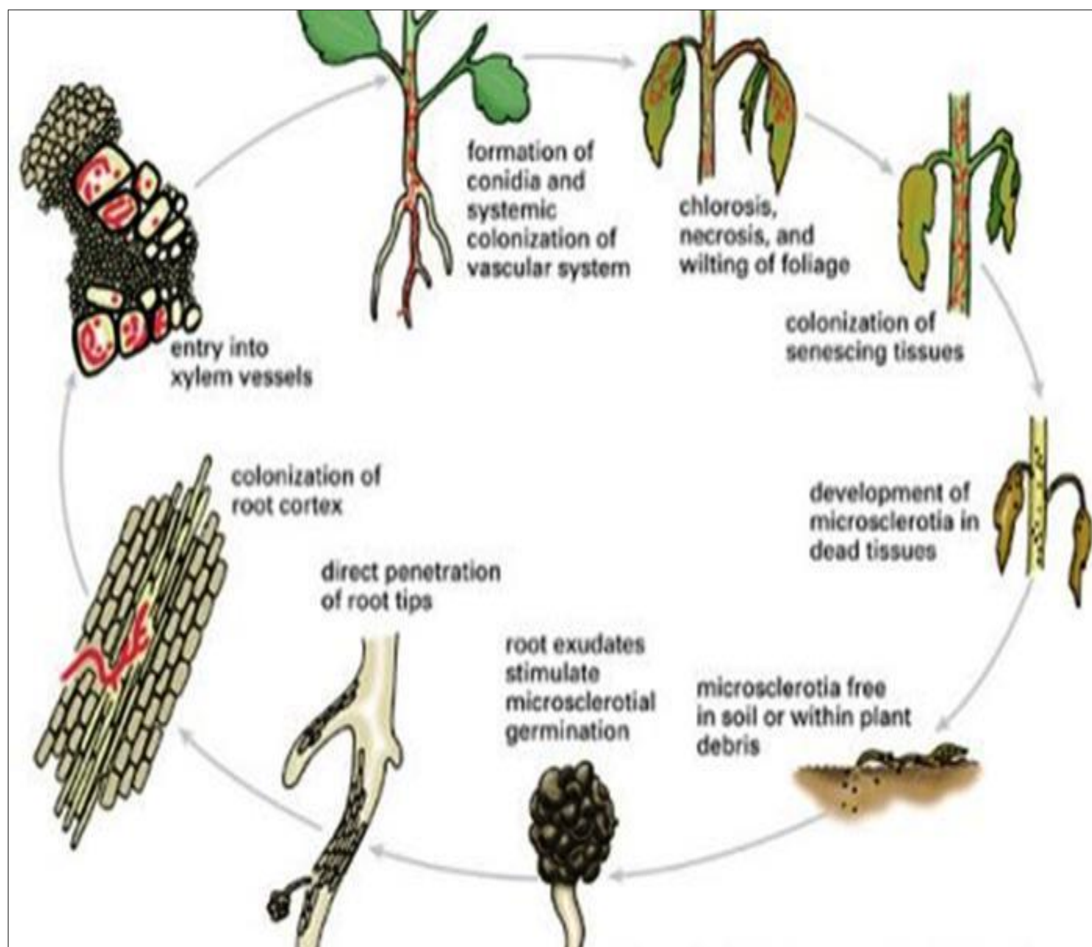

Figure 1.3- Life cycle of *Verticillium dahliae*

1.8 Methods for control of *Verticillium* wilt of cotton

Various techniques have been utilized for the control of *Verticillium* wilt of cotton. The fixation or thickness of inoculum in soil is a central point in picking the board methodologies for *Verticillium* wilt. If the density of microsclerotia of *V. dahliae* is low the regular rotation with no susceptible crops such as crops of grass family has been used for control of wilt. Tolerant varieties of cotton help to reduce loss in yield, but they are not helpful to stop increasing level of inoculum. Rotation of cotton crop with other crops is necessary for many years to control wilt disease and soil solarization is also an important method for control of wilt disease. Use of resistant varieties is another important method. Crop rotation with wheat, sorghum, corn, barley, or rice also help to reduce microsclerotia of *V. dahliae*. If first irrigation is delayed and disease pressure is more in soil (more than 10 microsclerotia per gram of soil), but temperature is cool it leads to reduce the effect of pathogen.

1.9 Biological control of *Verticillium* wilt of cotton: Various biological agents viz. endophytic bacteria, fungal strains and actinomycetes have been used for control of witing in cotton (Eljounaidi et al., 2016). Endophytic bacterial strains could be separated from upper disinfested plant tissue or extracted from inside the plant, and which does not damage the plant (Hallmann et al., 1997. Endophytic bacterial strains viz. *Enterobacter genus* (Li et al., 2012, *Bacillus megaterium* (Safiyazov et al., 1995; McInroy and Kloepper., 1995), *Acinetobacter baumannii* and *Arthrobacter spp.* (McInroy and Kloepper., 1995), *Bacillus pumilus* (Compant et al., 2005), *Bacillus subtilis* (Guo et al., 2010), *Pseudomonas* fluorescent (Erdogana and Benlioglu., 2010) have been used as biocontrol agents against wilting disease. *Trichoderma harzianum* and *Trichoderma viride* are biocontrol fungal strains reported against wilt of cotton. Compost manures have stimulated proliferation of antagonistic strains in rhizospheric soil and also suppress the effect of soil borne pathogenic fungus (De Brito Alvarez et al.,1995; Termorshuizen et al., 2006).

1.10 Biosurfactants: Biosurfactants are morphologically unmatched molecules which are surface stimulated compounds generated by variety of microorganisms. They are classified by their microbial origin and chemical nature. They are also amphipathic in nature and belong to a structural group of surface-active molecules. Chemical structure of biosurfactants is comprising with a hydrophilic portion, an acid, anions, peptides, cations, monosaccharides, disaccharides or polysaccharides and a

hydrophobic part of unsaturated or saturated hydrocarbon series or fatty acids (Oluwaseun et al., 2017). Microbial biosurfactants can be classified in two major categories that are low-molecular-weight compounds known as biosurfactants, comprising lipopeptide, glycolipids, proteins and polymers of high-molecular-weight called polysaccharides, lipo-polysaccharides proteins, or lipoproteins (Yin et al., 2009). They are surface active compounds and classified into low molecular and high molecular weight compounds. Biosurfactants have been produced from very cheap and raw material that are very easily available and in large amount (Mohan et al., 2006). Biosurfactants have been used in different fields such as agriculture, cosmetics, and industries. Most of the synthetic surfactants shown negative impact on the environment and causing toxic effects due to long term availability in the environment (Makkar et al., 2003). Biosurfactants can be produced from very cheap raw materials which are available in large quantities. Microorganism viz. *Pseudomonas aeruginosa, Burkholderia glumae*, *Ustilago maydis, Bacillus subtilis, Pseudomonas fluorescens* and *Candida lipolytica* been used for production of biosurfactants. Biosurfactants are very helpful in field of agriculture by way of remediation of hydrocarbon, inorganic compounds, and remediation of inorganic compound such as heavy metals. Rhamnolipids isolated from *Pseudomonas* strains have potential application in phytopathogen elimination; and increases the constitution of soil by removing the heavy metals from contaminated soil (Debode et al., 2007).

1.11 Bioformulation as biocontrol agent:

Biochemical formulations are biologically active products containing one or more beneficial microbial strains in an easy-to-use and economical carrier material with the addition of several chemicals, promoting plant growth. It continues to stimulate research and development in many areas. Increasing soil fertility, promoting plant growth and controlling phytopathogens are the goals of the biopharmaceutical industry, which leads to the development of an environmentally friendly environment. Rhizobacteria have immeasurable uses in sustainable agriculture as environmentally friendly biofertilizers and pesticides. Intensive commercial agriculture involves overuse of fertilizers and pesticides. There is concern that continuous use of fertilizers and pesticides will gradually reduce soil fertility (M et al., 2017). Reduction of disease or disease inoculums caused by various phytopathogens viz. bacterial and fungal strains, viruses in its dormant stage or in active stage has been done by using

different biocontrol agents to reduce the effect of harmful chemicals on environment. In the past few years so many experiments have been initiated to produce sustainable broad-spectrum formulations (Mitchell et al., 1991; Whitbread et al., 2003). Study has revealed that continuous use of bioformulations has also been increased the concentrations of nitrogen, organic matters, and important nutrients (Huang et al. 2010). Microbial bioformulations are more effective than man-made chemicals because only one microbe present in formulations helps to reduce the effect of pathogen with direct interaction with phytopathogen and many other mechanisms take part in growth of plants and in suppression of plant diseases. (Mendes et al., 2011).

Types- Mainly two types of bioformulations viz. solids and liquids are available (Burges and Jones.,1998). There are many other types of bioformulations available in the market throughout the world. **Fig 1.4** describes different types of solid formulations and types of encapsulations (Larena et al., 2003; Abadias et al., 2005; Guijarro et al., 2007a). Bioformulations have been produced by the addition of different wetting agents, binder and dispersant etc. (Tadros., 2005; Brar et al., 2006; Knowles., 2008).

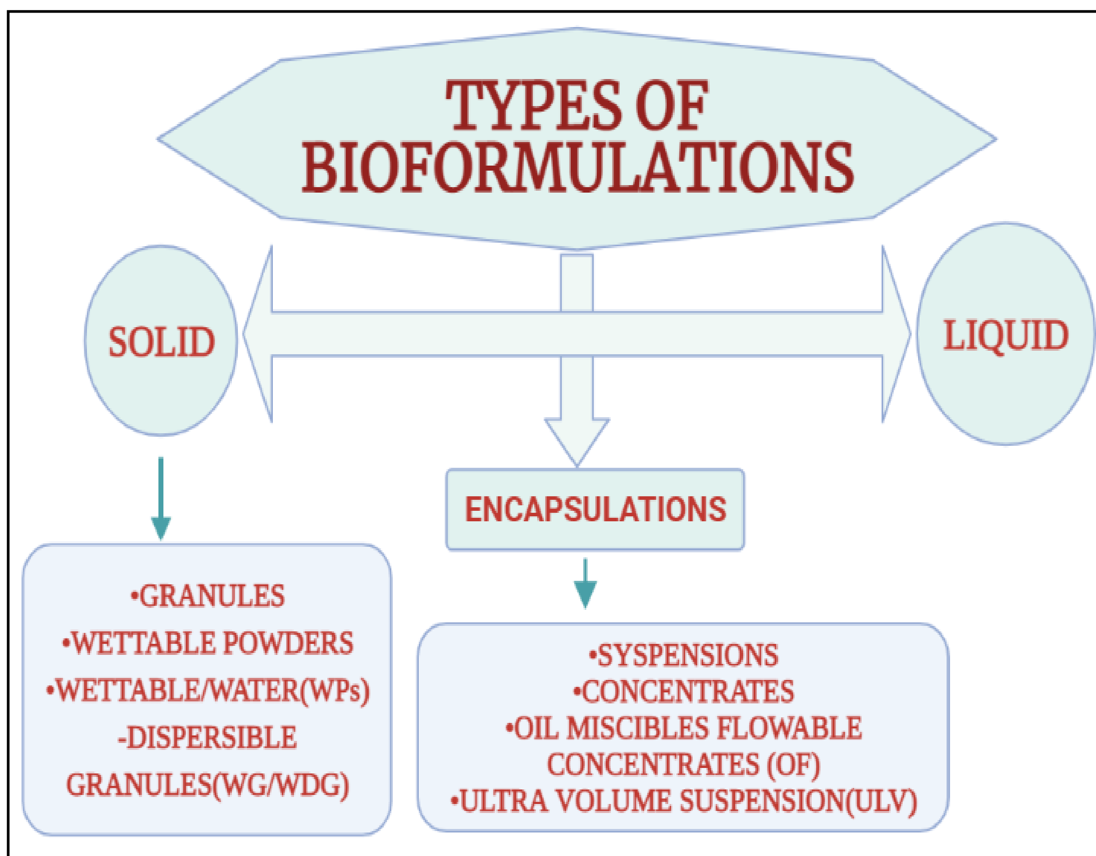

Figure 1.4: Types of bioformulations

Microbial bioformulation or plant based bioformulations helps to control wilt of cotton. Bioformulations has been mass multiplied and standardized in the form of biopesticides and bio fungicides. In Assam Agricultural University, Jorhat, Assam, India at Department of Plant Pathology, some PGR based bioformulations have been developed named as Bioveer, Biozium, Biozin PTB and some talc based biopesticides in combination with microbial strains viz. *Trichoderma harzianum Pseudomonas aeruginosa* and *Bacillus brevis*. These microbial based biocontrol formulations have shown positive effect in control of wilt diseases of chilli, tomato and reduce wilt diseases in of pepper, French bean and damping off cabbage. Similarly, bioformulation named as BioPf-2 produced with *T. harzianum* and *P. fluorescence* helps to increased concentration of nutrients in, plant growth development and reduce disease caused by soil borne pathogens (Kumar et al., 2017).

Therefore, such research is needed to isolate antagonistic fungi from different organic manures and endophytic bacteria from healthy cotton plant parts; followed by *in vitro* antagonistic activity and biosurfactant based studies with enzymatic screening for evaluation of potential biocontrol agents to suppress *Verticillium* wilt of cotton under both poly house and natural field conditions. Looking forward at the fact that Bathinda major cotton producing district of Punjab hence selected for present studies and loss in cotton yield has been reported due to attack of soil borne pathogenic fungus *V. dahliae* in Bathinda, Punjab, India. Therefore, the research will focus on isolating, screening and identification of both pathogenic and antagonistic biocontrol strains.*in vivo* field trials under natural field conditions and poly house will be done for evaluation of both endophytic bacterial strains and antagonistic fungal strains along with biochemical formulation for control of *Verticillium* wilt of cotton. A combinatorial approach with resistant variety, antagonistic fungal strains, endophytic bacterial strains used for seed bacterization and use of biochemical formulation will be controlled *Verticillium* wilt of cotton.

CHAPTER -II

REVIEW OF LITERATURE

Cotton is an economically important crop grown all over the world. Cotton belongs to order Malvales, family Malvaceae and genus *Gossypium. Gossypium arboreum, G. herbaceum, G. hirsutum* and *G. barbadense* are the four main species of cotton. *Gossypium arboreum, G. herbaceum,* are diploid (2n=26) in nature and native to old world. The global production of cotton was 123 million bales (480 lb each) during 2017-18, and 2.27 per cent to 121.30 million bales increased production was reported during 2019-20 **(Figure 2.1)**. Cotton production increased majorly in India and US but decreased in Pakistan and Australia. While increasing the harvested area of cotton., India becomes the world's largest cotton producer country and India has remained highest producer from past 5 years with increase of 14% over previous years and total production of 29.5 million bales during 2019-20. Punjab, Haryana, Rajasthan, Uttar Pradesh, Madhya Pradesh, Gujarat, Andhra Pradesh, Karnataka and Tamilnadu are nine major cotton cultivated states, in India. 95 percent of cotton production takes place only in these nine states of India. These nine states are reported as major cotton cultivated states in previous years (Uppal et al*.,* 2008). According to the Ministry of Agriculture and Farmers Welfare 32.3 million bales cotton has been cultivated during 2019-2020 **(Figure 2.2)**. *Verticillium* wilt is among the vital diseases of cotton which causes grave money losses in lots of crops. It is almost impossible to control this disease chemically, and soil solarization technique or fumigation with broad-spectrum chemicals is not very economical methods in bigger cotton growing areas; alternatively, method of biological control could be environmentally helpful for the control of this harmful pathogenic soil borne fungus. Some biological mediums such as endophytic bacterial strains, fungal strains, actinomycetes and biosurfactants have studied to control *Verticillium* wilt in cotton.

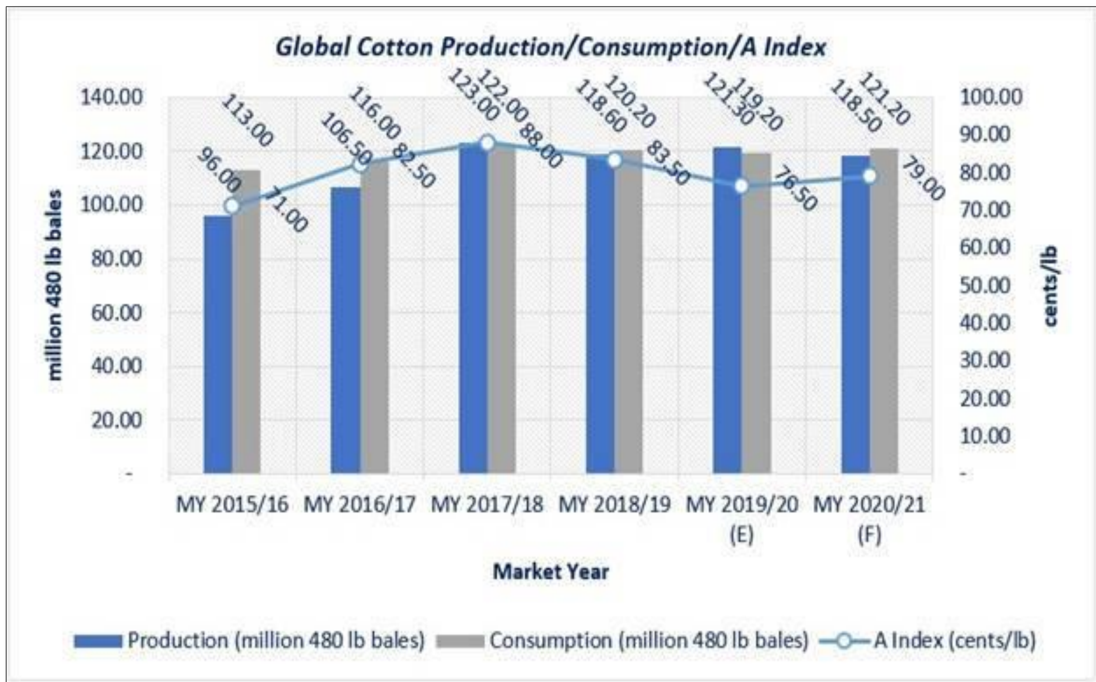

Figure 2.1: Cotton production throughout world

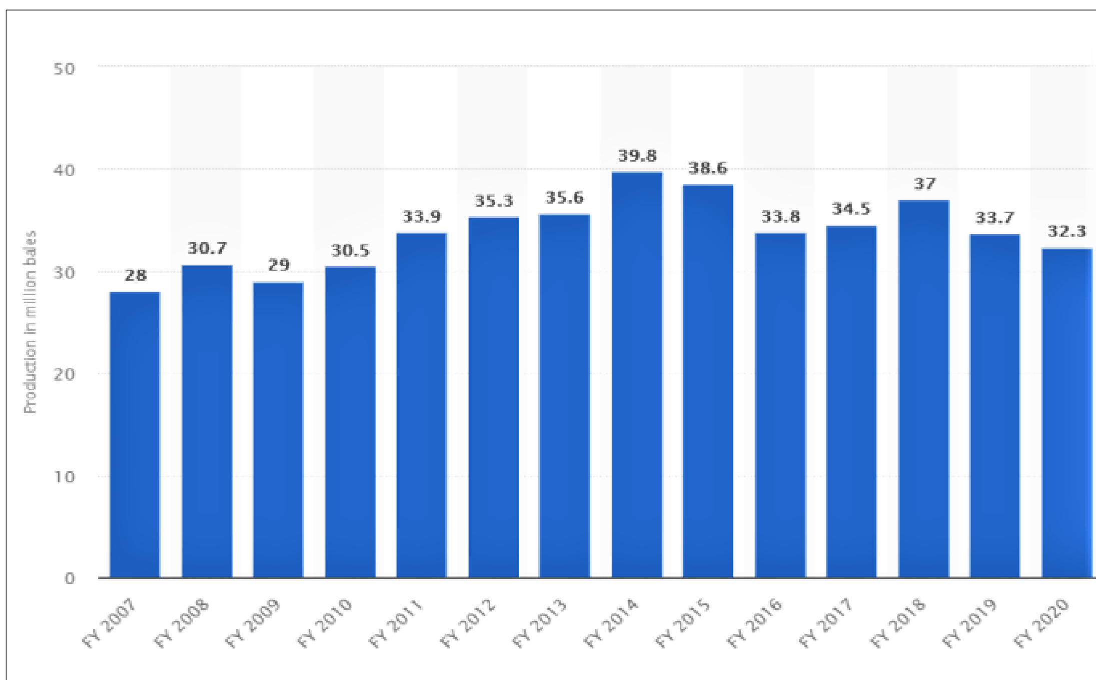

Figure 2.2: Production of raw cotton in India from financial year 2007 to 2019, with an estimate for 2020*(in million bales)*

2.2 Reported cotton diseases throughout world

There are different types of cotton diseases occurs by attack of different insects, pests, nematodes and fungi like Root rot of cotton, Angular leaf spot, Seed and seedling

diseases such as *Fusarium* wilt of cotton and *Verticillium* wilt of cotton specially in India (Heather M. Kelly., 2016).

Seedling diseases are responsible for moderate loss in yield of crop is of five percent annually & maximum infection hazards in cotton plants only because fungus borne from soil but, cultural and environmental impacts are also taking part for delaying of the seed development and formation of seedlings is most common problem in different cotton growing regions. Infection of seedlings occurs more favourably within moisturized cool and wet conditions. Environmental factors which also plays a major role for the formation of seedling diseases. Different types of fungi cause seedlings diseases in cotton are named as *Pythium* spp., *Rhizoctonia solani*, *Phomaexigua* (*Ascochyta*), and *Fusarium sp.* and *Verticillium* species. (Huang et al., 2016).

Damaged seedlings are characterized by yellowish, irregular, slower growing, and rarely die in a short lifespan. Checking of infected seedlings might show dark spots on the shoots and roots. *Pythium sp.* and *Fusarium sp*. is usually attacked from the seeds and attacked below-ground parts of young seedlings. Different fungal sp. of the genus *Pythium* may produce seedling disease in cotton and in many other crops. *Pythium sp.* is generally known as water moulds because it produces spores which are moving actively in soil water (Zhu et al., 2013). Several *Fusarium sp.* are mostly reported on infected seedlings of cotton. There is another disease of cotton out of so many diseases known as boll rot of cotton. Boll rot is mostly occurring only when attack of insects done on cotton plant or excessively wet conditions are there. Boll rot at stage of infection reveals small brown lesions that generally spread until when the whole boll becomes blackened and dried (Barber and Cotty., 1986). In leaf spot of cotton, the size of leaf gets reduced and is generally getting small, brown, and round lesions which enlarge by approximately half inch. Then old lesions rarely grow into grey centres that can fall out. Root rot of cotton is another crucial cotton disease. Wilting of entire plants takes place when the first visible symptom appears on plant and every leaf from top to bottom shows drooping down in infected cotton plants. The abrupt wilting is another major characteristic feature of root rot of cotton. In the onset of infection or disease, it reported on few plants in the field which shows wilting thereafter some time get converted into large or small round shaped diseased patches. Disease appears much early and manifestation on top of ground parts in the form of

wilting is a very late symptom. *Xanthomonas compestris* is responsible for bacterial or angular leaf spot of cotton disease. The infection comes on several parts of cotton plant, in seedling and in mature plant stages (Wang et al., 2009). Bolls, bracts, flowers, leaves, petioles, and shoots may all possess symptoms. Water-soaked spots produces on leaves which then convert reddish into black forming angular leaf spots, are covered by veins and vein lets. In Boll rot of cotton disease, the bolls become able to soak water and spots form and changes to dark black and sunken irregular spots forms when disease starts on bolls. The infection spreads very smoothly into the entire bolls and shedding of bolls occurs. In case of black arm disease of cotton, dark brown to black lesions visible on shoot and can cause a unique dry black twig features therefore this disease named as "black arm disease". *Fusarium* wilt of cotton disease is the resultant of pathogen named *Fusarium oxysporum* and diseased plants shows the symptoms of withering of leaves by virtue of loss of turgidity or stiffness then ultimately leaves turns into brown and drooping starts which finally drop off. Diseased plants also possess dried shoots and twigs. Whitefly is also a dominant factor to harm the cotton plants. Cotton Leaf curl disease is a viral disease of cotton. Cotton leaf curl virus disease (CLCuD) is a disease which is transmitted to cotton plants by the attack of whitefly *Bemisia tabaci*. If any other host plant species of the whitefly, infected by the leaf curl virus can also serve as the source of inoculums for the insect to acquire and transmit the virus to cotton (Khan et al., 2015).

2.3 *Verticillium* wilt of cotton

Significant loss in yield and plant biomass has been recorded due to attack of most devastating soil borne pathogen *Verticillium dahliae* (Ma et al., 2002). *Verticillium dahliae* remains in viable conditions for long time in the soil as microsclerotia. A single microsclerotium is sufficient to start infection in a cotton plant. Roots act as an agency to commence fungal infection which invades through the vascular system causes a systemic infection (Hampton et al., 1990; Liu et al., 2021). Vascular discolouration is light to dark brown which is prominent in the shoot and branches at onset of infections in cotton plants. Diseased plants can be dying after defoliation or remain defoliated. Symptoms of the cotton plants caused by *Verticillium* wilt generally appear at the first flowering stage (Wei et al., 2021).

2.4 Biological strains used by the previous researchers for control of Verticillium wilt of cotton

2.4.1 Biological control of *Verticillium* wilt of cotton by bacterial strains

Endophytic bacterial strains are the ones which could be separated from upper non-infected tissues of plants or isolated from inside the plant, and does not damage the plant (Hallmann et al., 1997). Endophytic bacteria can be administered for cotton seeds to protect *Verticillium* wilt also to keep a tab on the possible way of actions as growth promoting bacteria too. Different types of bacterial strains have been used for control of wilt disease **(Table 2.1)**.

According to Çubukcu and Benlioglu., 2015 roots of different cotton varieties and weeds from fields of cotton in Aydin province were used for isolation of endophytic bacterial isolates. Out of 158 isolates only 12 isolates had shown significant *in vitro* inhibition zones against *Verticillium dahliae*. A total of twenty-two weeds and twenty-three root samples of cotton were collected in two-year period used for isolation of 158 isolates out of them 12 isolates only showed significant suppression of wilt disease. Both C5 (*G. barbadense* (Giza 45)) and E2 (*G. hirsutum* (DPL882)) endophytes are gram positive also producing endospore. Tow strains F5 (*G. hirsutum* (Stonville 825)) and F2 (G. *hirsutum* (Stonville 825)) produced siderophore and did not induce hypersensitive reactions. E21, C5 and F5 bacterial endophytes caused significant inhibition in *Verticillium* wilt.

Yang et al., 2013 have studied that *Verticillium* wilt is most devastating disease of cotton. Three antagonistic bacterial strains of *Bacillus subtilis* YUPP-2, *Paenibacillus xylanilyticus* YUPP-1, and *Paenibacillus polymyxa* YUPP-8 have been obtained from boll-setting stage, germination stage of cotton plants and squaring stage respectively. These bacterial strains have been evaluated in different growth stages of cotton against wilt disease of cotton. Colonization of these three bacterial strains has shown the maximum growth in the germination stage, stage at squaring and boll-setting stage, discretely. In 2010- 2011, field experiments had been conducted in which treatment with combined bacterial strains were more effective than individual application. This study illustrated that use of endophytic bacterial strains with combined application can achieve good control has the capability to control *Verticillium* wilt of cotton.

Mansoori et al., 2013 have studied the possibility of different types of bacterial strains as positive antagonists against *Verticillium* wilt of cotton. *Pseudomonas fluorescens* and *Bacillus spp*. were isolated from different plant roots as well as rhizospheric soils in the cotton fields of Iranian. Suspension of eight bacterial strains of both *Bacillus sp.* and *Pseudomonas flourescens* were prepared and the concentration of each strain was 108 cfu/ml. Each bacterial suspension was used for coating on 10 cotton seeds. Most of the isolates were shown positive antagonism against wilt disease when compared to the untreated cotton seeds (control) after 90 days of sowing. Isolates *Pseudomonas fluorescens* (B5), *Pseudomonas fluorescens* (B6)*, B. subtilis* (B2), *P. fluorescens* (B7*),* and *Bacillus coagulans* (B3) were the most effective, respectively against wilt disease. *Pseudomonas fluorescens* isolates were more effective than *Bacillus* bacterial strains. This study revealed that bacterial strains shown best positive antagonism against wilt disease hence good source of biocontrol.

Li et al., 2012 have studied that *Verticillium* wilt of cotton is caused by a defoliating pathogen *V. dahliae*. Thirty-nine antagonistic endophytic bacterial strains were evaluated against *Verticillium* wilt of cotton under controlled conditions. Experiments under controlled conditions has described that HA02 (endophytic bacterium) has shown positive antagonism against *V. dahliae*. HA02- gfp gene tagged was inoculated in cotton seedlings and populations of HA02-gfp were higher in the root than in the stem. Results of cotton plants grown under controlled conditions as well as green house has shown that out of 39 DAEB isolates only 1 isolate was best biocontrol potential against *V. dahliae*. HA02-gfp gene was colonized in roots of cotton plants.

According to Erdogan and Benlioglu., 2009 cotton and weed rhizosphere were used for isolation of 59 fluorescent Pseudomonas spp. Out of 59, only 4 strains were selected according to criteria followed by them (1) *in vitro* constraint of *V. dahliae* (2) Reduction in wilt disease by using bacterized seeds with stem-injection method of *V. dahliae* and (3) under greenhouse conditions (seedling vigor test). *Convolvulus arvensis, Xanthium strumarium, Portulaca sp.* and *Gossypium hirsutum* were used for isolation of 4 *Pseudomonas sp*. and very well-known biocontrol agent *Serratia polymuthica* were evaluated against *Verticillium* wilt disease. This study revealed that seed treated with both *Pseudomonas sp.* and *Serratia plymuthica* were effective to control *Verticillium* wilt of cotton.

L. Soesanto., 2000 has evaluated the *in vitro* effect of *P. fluorescens* against *Verticillium dahliae*. *Pseudomonas fluorescens* (P60) was reduced the mycelial growth of 20 isolates of *Verticillium dahliae by* 31-64%. Inhibition of *V. dahliae* was slightly less and ranged from 29-52% at 1/5 strength of PDB. 717.96 isolate of *V. dahliae* did not produce microsclerotia on PDA at 1/5 strength of PDB agar as compared to other isolates which produced more microsclerotia at same concentration. *P. fluorescens* (P60) was reduced the formation of microsclerotia of *V. dahliae* when compared with control plates; showed no mycelial growth. In this study, P60 was chosen because of its *in vitro* inhibition of formation of microsclerotia of *V. dahliae* as compared to other strains.

Table 2.1- Biocontrol Efficacy of different Endophytic bacterial strains against *V. dahliae*

S. No.	Name of Endophytic Bacterial Strains	Mode of Isolation	Method of Treatment	Results Obtained After treatment	References
1	*G. barbadense* (Giza 45) *G. hirsutum* (DPL882) *G. hirsutum* (Stonville 825)	Roots and Weeds of different cotton varieties	*in vitro* inhibition against *V. dahliae*	**Endospore produced by gram +ve strains against *V. dahliae*** helps to reduce the effect of wilt disease.	Çubukcu and Benlioglu., 2015
2	*Bacillus subtilis* YUPP-2 *Paenibacillus xylanilyticus* YUPP-1 *Paenibacillus polymyxa* YUPP-8	Different Growth Stages of Cotton	*in vivo* evaluation in both poly house and natural fields	**Infection rate in Poly House-** 6.7% (YUPP-8), 6.7% (YUPP-1), 13.3% (YUPP-2)	Yang et al., 2013
3	*Pseudomonas fluorescens* *Bacillus spp*.	Roots and Rhizospheric soil of Cotton	Seed Bacterization	**Disease % in** (B5)- 19% (most effective), (B6)- 28%	Mansoori et al.,2013

4	(*Gossypium barbadense*) Haidao20 (HA02), HA03 Changkan (*G. hirsutum L.*) (CK06)	Roots of Healthy Cotton Plants	*in vivo* evaluation in both poly house and fields	**Protection Rate in Poly House** - 72.4% (HA02), 51.4% (HA03), 70.3%(CK06) **Protection rate in Field Trials-** 45.8% (HA02), 48.3% (CK06)	Li et al., 2012
5	*Pseudomonas spp. Serratia polymuthica*	*Convolvulus arvensis* (FP35), *Xanthium strumarium* (FP22), *Portulaca sp.* (FP23) *and Gossypium hirsutum* (FP23	Seed Bacterization	**Reduction in Wilt Disease in Field Trials-** 39.2% to 50.9% (FP22 and FP35) 22.1% to 36.8% (FP23 and FP30)	Erdogan and Benlioglu., 2009
6	*Pseudomonas fluorescens* (P60)	Wheat growing fields	*in vitro* evaluation against *V. dahliae*	**Reduction in mycelial growth of *V. dahliae*-** 31-64%	L. Soesanto., 2000

2.4.2 Fungal strains for control of wilt disease

Various fungal strains viz. *Acremonium sp*, *Penicillium simplicissimum*, *Talaromyces flavus*, *Penicillium simplicissimum, Leptosphaeria sp.* etc. have been used for control of *Verticillium* wilt of cotton **(Table 2.2)**.

According to Yuan et al., 2017, *Verticillium* wilt of cotton is the most harmful soil-borne disease caused by *V. dahliae* leads to decrease in cotton production. CEF193 (*Acremonium sp.*), CEF-818 (*Penicillium simplicissimum*), CEF-642 (*Talaromyces flavus*) and CEF-714 (*Leptosphaeria sp.)* were separated from healthy roots of different cotton plants. These isolates had been assessed against wilt disease of cotton caused by defoliating strain of *Verticillium dahliae* Vd080. Evaluation of all the strains against *V. dahliae* has been done in green house conditions. Experiments conducted in green house conditions have revealed that disease severity index and

disease incidence was controlled significantly varying from 26 percent (CEF-642) to 67 percent (CEF-818) after 25 days of injection. Results of this study has been described that seed treated with fungal strains CEF-818 and CET-714 had shown best antagonism against *V. dahliae* when evaluated as biocontrol potential against wilt disease of cotton under controlled conditions and helped in enhancement of cotton yield.

Six hundred and forty-two endophytic fungal strains have been obtained from stem, leaf tissues and roots of *G. hirsutum*. According to resistivity shown in wilt disease, cotton plants were ranked and sampled accordingly. Most of endophytic fungal strains have been isolated from leaves (391) as compared to root sections (140) and stem (111) of cotton plants. There was no significant difference has been shown in endophytic bacterial strains in resistant varieties of cotton. Endophytic fungus isolated from stem tissues (0.702) has shown more evenness and uniformity as compared to other tissues. *in vitro* antagonistic activity of 80 endophytic fungal strains has been evaluated against *Verticillium dahliae*. Out of 80 endophytic strains only seven strains have shown most positive antagonism against pathogenic fungus and rate of inhibition was less than 75% among these 7 fungal strains. These endophytic fungal strains have been investigated for antifungal activity along with non- volatile and volatile metabolites. Non-volatile compounds released by *Talaromyces flavus, Penicillium simplicissimum, Leptosphaeria sp.* and *Fusarium solani* helped to control the growth *V. dahliae*. This study revealed that interactions between cotton and endophytic bacterial strains may provide the better screening platform for control of wilt disease of cotton (Fang- Li et al., 2014).

Naraghi et al., 2012 have studied the effect of antagonistic fungus *Talaromyces flavus* on cotton plant. Five *Talaromyces flavus* fungal isolates were selected, shown the highest antagonistic activity against *V. dahliae*. These five isolates were used under controlled conditions. A split-plot trial was developed with four replications in randomized complete block design for evaluation of *T. flavus* on cotton plants. Soil was treated with *T. flavus* and seed treatment has been done along with combination of both treatments. Disease severity index was assessed by crown length, height of plant, length of roots, fresh weight of plant, and dry weight of plant. In seed treated plants most positive results have been shown. In crown length treatment, *T. flavus* was not shown any significant results. This study has been revealed that antagonistic fungus *T.*

flavus acts as both antagonistic fungus against *V. dahliae* as well as promotes growth of cotton plants.

Zheng et al., 2011 have isolated a total of three hundred and seventy-three fungus strains from the rhizospheric, bulk soil and endorhiza of cotton plants sown under field conditions. Antagonistic activities of all these fungal strains have been tested against *V. dahliae*. Out of 373 only 105 fungal strains had shown inhibition zones towards *Verticillium dahliae Kleb.,* hence selected as best antagonists against *V. dahliae*. Evaluations of these 105 antagonistic strains have been done to check its biocontrol potential. *in vitro* screening system has been developed against *V. dahliae* along with different activity of hydrolytic fungal cell wall lysis enzymes named as cellulase, protease and chitinase. Thirty-three antagonists were evaluated for their biocontrol potential on cotton plants under controlled conditions.

Kobra et al., 2009 was investigated the effect of development of 3 *Glomus species* namely *G. versiforme*, *G. etunicatum* and *G. intraradices* on wilt disease of cotton. The effect of ABM fungus on soil borne pathogen of cotton plant has been studied. This study revealed arbuscular mycorrhizal decreased the risk of wilt disease in cotton by colonization of ABM in cotton plants when compared to non-ABM. Chlorophyll content was lower in diseased cotton plants. Content of sugar and protein was increased by all these three Glomus *sp.* in shoot and root of cotton plants. Proline concentration has been increased in shoot as well as roots of pathogen affected cotton as compared to healthy plants (Kobra et al., 2011). Higher concentration of proline in infected plants as sensor of tension had shown that *Verticillium* wilt of cotton leads to death of plant and reduces yield too. This study described the significant effect of arbuscular mycorrhizal fungal strains on soil borne pathogen *V. dahliae.*

Linda E. Hanson., 2000 has developed a seed treatment method for control of *Verticillium* wilt of cotton. *Trichoderma virens* has been used for evaluation of biocontrol potential against *V. dahliae*. *T. virens* was grown on PDA plates and dried. Afterwards seeds of cotton has been treated with dried *T. virens* and sown in fields. When cotton plants reached at six true leaf stages, *Verticillium dahliae* has been inoculated in stem by technique known as “Stem Puncture”. Symptoms of wilt disease have been assessed after 10 days of inoculation and height of plant was also measured. Seed treated with *T. virens* persuade resistance in cotton plants but extracts of

terpenoid phytoalexins was not different in infected plants as compared to healthy cotton plants. Treatment with G-4 strains of *T. virens* helped to increase length of cotton when compared to untreated plants. This study has revealed, *T. virens* act as a biocontrol agent against *Verticillium* wilt of cotton and increase yield of cotton too.

Table 2.2: Biocontrol Efficacy of different Fungal strains used against *V. dahliae*

S. No.	Name of Fungal Strains	Mode of Isolation	Method of Treatment	Results Obtained After treatment	References
1	CEF193 (*Acremonium sp.*), CEF-818 (*Penicillium simplicissimum*), CEF-642 (*Talaromyces flavus*) and CEF-714 (*Leptosphaeria sp.)*	Roots of different cotton plants	*in vivo* evaluation in Green house Experiments (Seed Bacterization)	**Disease Severity Index**- 69.5% (CEF193), 69.2% (CEF-818), 54.6% (CEF-642) and (CEF-714) 45.7%	Yuan et al., 2017
2	*Alternaria sp.* *Acremonium sp.* *Penicillium sp.*	Stem, leaf tissues and roots of cotton plants	*in vitro* evaluation against *V. dahliae*	Exhibition of Mycelial Growth- *Alternaria sp.* (7.9%) *Acremonium* (6.6%) *Penicillium* (4.8%)	Fang- Li et al., 2014
3	*Talaromyces flavus*	Selection from Fungal Collection	Seed Bacterization	TF-Co-M 23(*Talaromyces flavus)* most effective to increase height, fresh weight, length of roots and plant dry weight also by 2.26-, 1.23- 1.80- and 1.19-fold.	Naraghi et al., 2012

4	*Phomopsis sp.*, *Fusarium oxysporum* *Nectria haematococca*	Rhizospheric, Bulk soil and Endorhiza of cotton plants	*in vivo* evaluation in Green house	**Biocontrol Efficacy**- 63–69.78% in all 3 species.	Zheng et al., 2011
5	*G. versiforme*, *G. etunicatum* *G. intraradices*	Culture supplied by Agrology Department of Tabriz University	*in vivo* evaluation against wilt disease of cotton	Sugar and Protein content was increased by 3 *Glomus sp.* and Proline concentration was also increased in effected cotton plants	Kobra et al., 2009
6	*Trichoderma virens*	Inoculum prepared in lab produced Gliovirin	Seed Treatment for Green house trials	*T. virens* induced growth in cotton and reduced the symptoms of disease	Linda E. Hanson., 2000

2.4.3 Biological Control of *Verticillium* Wilt of Cotton by Actinomycetes

Actinomycetes are group of gram-positive filamentous fungi, which make up a significant proportion of soil microbial biomass and plays a major role in rhizospheric ecosystems (Sardi et al., 1992). Actinomycetes are also produced antibiotics, some of which are very effective against soil-borne pathogenic fungal strains (Barakate et al., 2002; ElMehalawy et al., 2004; Jain and Jain, 2007). Some actinomycetes may also promote plant growth (El Tarabily et al., 2009).

According to Xue et al., 2013, *V. dahliae* is the most serious causative agent of soil bore disease known as *Verticillium* wilt. Rhizosphere soil of different crops has been used for isolation of seven hundred and twelve actinomycetes and evaluated as biocontrol potential. Degradation of cell wall of fungus has been followed by various enzymes known as cellulase, protease b-1, 3-glucosidase and chitinase. Results obtained from microscopy analyzed those antagonistic isolates have been curved around *V. dahliae* and responsible for lysis. This study has described a significant correlation between antagonistic strains and *V. dahliae.in vitro* screening of 4 strains of *Streptomyces* had made indole acetic acid (IAA) and siderophores) responsible for enhancement in growth of cotton plants under controlled/ greenhouse conditions.

Results obtained from this study have revealed that selected species of *Streptomyces* acts as a positive biocontrol agent against *Verticillium* wilt of cotton.

Aghighi et al., 2004 have studied that antifungal activity of various actinomycetes against *Verticillium dahliae Kleb.* Now days, use of harmful chemicals has been increased and responsible for harmful effects on environment. *Streptomyces spp.* (soil actinomycetes*)* was increased fertility of soil and has antagonistic activity against *V. dahliae.* For the evaluation of 110 isolates of actinomycetes, a screening system has been developed in which only 14 isolates had shown positive antagonism against pathogenic fungus. Most positive antifungal activity has shown by different strains viz. *Streptomyces* sp., *Streptomyces plicatus* strain 101 and *Frankia* sp. strain 103.

2.5 Biosurfactants and it's used in Agriculture

Biosurfactants are morphologically unmatched molecules which are surface stimulated compounds generated by variety of microorganisms. They are divided by their microbial origin and chemical nature. They are also amphipathic in nature and belong to a structural group of surface-active molecules. The chemical skeleton comprises of a hydrophilic portion, an acid, anions, peptides, cations, monosaccharides, disaccharides or polysaccharides and a hydrophobic part of unsaturated or saturated hydrocarbon series or fatty acids (AC et al., 2017). **Table 2.3** describes the microorganism producing microbial biosurfactants. Microbial strains viz. *Pseudomonas, Bacillus* and *Candida* have been confirmed for large-scale production of biosurfactants (Gaur et al., 2021). Rhamnolipids most important biosurfactant has also been produced by *Pseudomonas aeruginosa* and *Burkholderia thailandensis* via a 10-L bioreactor. Biosurfactants have been isolated from different sources such as rhizospheric soil, petroleum soil and endophytic bacterial strains (Rodriguez et al., 2021) **(Table 2.4).**

Table 2.3: Microorganism producing microbial biosurfactants

Name of Microorganisms	Group of biosurfactants	Class of biosurfactants
Pseudomonas sp., Pseudomonas aeruginosa, Burkholderia glumae, Burkholderia thailandensis Burkholderia plantarii,	Glycolipids	Rhamnoloipids

Rhodococcus erythropolis, Nocardia erythropolis, Mycobacterium sp., Arthobacter sp	Glycolipids	Trehalose lipids
Torulopsis apicola, Torulopsis bombicola, Torulopsis petrophilum	Glycolipids	Sophorolipids
Ustilago maydis, Ustilago zeae,	Glycolipids	Cellobiolipids
Bacillus subtilis, Bacillus licheniformis	Lipopeptides and lipoproteins	Surfactin/ iturin/fengycin
Bacillus licheniformis	Lipopeptides and lipoproteins	Lichenysin
Pseudomonas fluorescens	Lipopeptides and lipoproteins	Viscosin
Serratia marcescens	Lipopeptides and lipoproteins	Serrawettin
Bacillus subtilis	Lipopeptides and lipoproteins	Subtilism
Bacillus polymyxa	Lipopeptides and lipoproteins	Polymixin
Acinetobacter sp., Rhodococcus erythropolis Mycococcus sp.	Fatty acids/neutral lipids/ Phospholipids	Phosphatidylethanolamine
Penicillium spiculisporum	Fatty acids/neutral lipids/ Phospholipids	Spiculisporic acid
Acinetobacter calcoaceticus	Polymeric surfactants	Emulsan
Acinetobacter radioresistens	Polymeric surfactants	Alasan
Acinetobacter calcoaceticus A2	Polymeric surfactants	Biodispersan
Acinebacter calcoaceticus	Polymeric surfactants	Polysaccharide protein Complex
Candida lipolytica	Polymeric surfactants	Liposan
Saccharomyces cerevisiae	Polymeric surfactants	Mannoprotein
Pseudomonas aeruginosa	Polymeric surfactants	Protein PA
Acinetobacter calcoaceticus, Pseudomonas marginalis	Particulate Biosurfactants	Vesicles
Cyanobacteria	Polymeric surfactants	Whole microbial cells

Table 2.4 Source of isolation of different biosurfactants

Source of Isolation	Name of microorganisms producing biosurfactants
Native flora of apple Contaminated soil with petroleum	*Pseudomonas aeruginosa*
Land farming soil	*Bacillus megaterium* *Bacillus cereus* *B. thuringiensis*
Soil from farming area	*Stenotrophomonas maltophilia*
Petroleum-contaminated soil	*Psuedomonas nitroreducens*
Petroleum-contaminated soil	*Acinetobacter sp.*
Crude oil-contaminated soil	*Staphylococcus sp*
Crude oil-contaminated soil	*Pseudomonas sp*
Petrochemical waste-contaminated soil	*Pseudomonas aeruginosa Bacillus cereus*
Endosulfan sprayed cashew plantation soil containing hydrophobic substances	*Bacillus subtilis*
Hydrocarbon-contaminated soil	*Serratia marcescens*
Heavy crude oil-contaminated soil	*Pseudomonas sp.* *Enterobacter cloacae*
Soil contaminated with crude oil	*Streptomyces rochei*
Rhizosphere of fique	*Pseudomonas fluorescens*
Petroleum-contaminated soil	*Pseudomonas aeruginosa*
Antarctic soil	*Rhodococcus fascaians*
Soil	*Bacillus subtilis*
Endophytic bacteria from maize	*Bacillus mojavensis*
Phenanthrene-contaminated soil microcosm	*Sphingomonas paucimobilis*
Agriculture soil	*Pseudomonas sp.*

Oil-contaminated soil	*Pseudomonas sp*
Rhizosphere of black pepper	*Pseudomonas putida*
Oil-contaminated soil	*Pseudomonas aeruginosa*
Fuel oil-contaminated soil	*Burkholderia cenocepacia*
Soil	*Rhodococcus wratislaviensis*
Contaminated soil	*Nocardia otitidiscaviarium*
Diesel-contaminated soil	*Pseudomonas aeuroginosa*
Ornithogenic soil of Antarctica	*Pantoea sp.*
Petroleum oil-contaminated soil	*Pseudomonas aeruginosa Bacillus subtilis*
Rhizosphere of white and red cocoyam plants	*Pseudomonas sp.*
Soil	*Pseudomonas chlororaphis*
Long Beach soil	*Acinetobacter junii*
Sugar beet rhizosphere	*Pseudomonas fluorescens*
Hydrocarbon/metal-contaminated soil	*Flavobacterium sp.*
Soil	*Bacillus sp*
Petroleum-contaminated soil	*Pseudomonas fluorescens*

2.6 Biocontrol of various diseases by biosurfactants

Pseudomonas and *Bacillus* are the isolates of rhizosphere produced by biosurfactants. They show their biological activity as to control soft rot disease caused by *Pectobacterium* and *Dickeya sp.* Best well known biosurfactants used as biological agents are rhamnolipids and cyclic lipopeptides and Rhamnolipids inhibits zoospore formation of disease-causing microorganisms in plants and helps to become plant resistant from different economically used pesticides. *Pseudomonas* is antagonistic to aphid of green peach. *Pseudomonas putida* is a plant growth-promoter which produces microbial biosurfactants. Such biosurfactants helps in breakdown of zoospores of the *Phytophthora capsica*, causing disease damping-off in cucumber plants. *Bacillus* strain produced biosurfactants that retards the growth of disease-causing fungi such as *Fusarium sp.*, and *Aspergillus sp.* Isoform of surfactin produced

by *Brevibacillus brevis* strain HOB1. It is antagonistic to bacterial and fungal disease-causing pathogens according to Haddad· Fluorescent pseudomonad's produces biosurfactant and they are helpful to minimize fungal pathogens growth viz. *Pythium ultimum* causes diseases such as damping off and root rot of plants*, Fusarium oxysporum* causative agent for wilting in crops and *Phytophthora cryptogea* is a causative agent of rotting of flowers and fruits. *Pseudomonas sp*. plays a crucial role in biological control of *Verticillium* wilt of potatoes. Mature microsclerotia are enough to cause disease in potatoes according to Debode et al*.,* 2007. *Pseudomonas sp.* retards the *Rhizoctonia solani* growth that is a causative agent of plant diseases and *Phythium ultimum* a causative agent of root rot and damping off the plants with the help of double function performing compounds such as tensin, viscosin and viscosinamid. *Bacillus subtilis* strain is isolated from soil produces biosurfactants helps to control anthracnose on papaya leaves (Singh et al*.,* 2021). *Pseudomonas aeruginosa* inhibited by production of biosurfactants through *Staphylococcus sp*., derived from fossil oil-impure soil (Eddouaouda et al*.,* 2012).

Plant pathogens viz. *Pythium* and *Phytophtora spp*. (Debode et al., 2007) has been controlled by *Pseudomonas* CMR12 while producing cyclic lipopeptide and produces rhamnolipids were derived from *P. aeruginosa* PNA1. Biosurfactants obtained from *Pseudomonas fluorescens* have also shown antifungal activities (Nielsen and Sørensen, 2003, Saikia et al.,2012). For e.g., *Pseudomonads fluroscens* was efficient enough to control fungal growth of many pathogenic strains viz. *Phytophthora cryptogea* (causative agent of rotting of fruits and flowers) *Pythium ultimum* (damping off and root rot of plants) and *Fusarium oxysporum* (wilting of crops). *in vitro* viability of *Verticillium sp.* has also been reduced by biosurfactants isolated from *Pseudomonas sp.* (Debode et al*.,* 2007). Biosurfactant produced from *Bacillus subtilis* (isolate of soil) has shown inhibition in pathogenic growth of *Colletotrichum gloeosporioides* (Kim et al*.,* 2010) causes anthracnose on papaya leaves.

2.7 Integrated approach for control of *Verticillium* wilt disease

Integrated management includes control of wilt disease by using different methods viz. crop rotation, pre- and post-plant culturing practices, using resistant varieties, soil solarization, seed bacterization and soil fumigation. It also includes growth and development of cotton plant and relationship of disease symptoms with fiber and yield

quality and integration of the components of the crop management system (El-zik and K.M., 1985).

According to the study of Jason E. Woodward, Extension Plant Pathologist, Texas Agri Life Extension Service Terry A. Wheeler, Research Plant Pathologist Texas Agri LIFE Research control of wilt disease is not possible with help of single management strategy therefore different control methods have been used such as chemical control, fumigation, and some other cultural practices. Management of wilt disease is also done by using resistant varieties or tolerant cultivars in some cotton growing areas. Some other methods such as proper irrigation, crop rotation and seeding rate can also help to reduce wilt disease symptoms or population of *V. dahliae* present in soil. Various other practices will remain the focus of future on Southern High Plain for control of wilt disease.

Loss in cotton production has been occurred in China due to both *Fusarium* and *Verticillium* wilt of cotton. According to survey in 1982 almost 16% loss in yield has been reported in moderate high and highly infested cotton fields with estimated loss of about 100000 tons of lint cotton. In this study integrated management have been developed for control of wilt disease. *F. oxysporum* f. sp. *vasinfectum* and *Verticillium dahliae* virulent strains have been identified in different cotton growing regions. Since 1951, 47 *Fusarium*-wilt-resistant and *Verticillium*-tolerant cultivars have been grown in heavy infected fields and about 50% of the potential loss in cotton yield has been saved annually. An organic solvent infusion (OSI) technique, with fungicides such as carbendazim or ethylethene thiosulphate has been developed in which these fungicides help to produced dormant seeds from internal soil-borne pathogens. A combinatorial treatment of systemic fungicides such as carbendazim and quintozene provides a good integrated management control by forming a coated seed and helps to control diseases caused with soil insects, aphids and thrips at the seedling stage (Rekanovic et al., 2007).

2.8 Biochemical/ bioformulation-

Biochemical formulations contain one or more beneficial microbial strains in an easy-to-use and economical carrier material with the addition of several chemicals, best as a biologically active product that promotes plant growth. Microbial-based formulations, also known as bioforms, can involve a single microbial formulation

product with direct interaction with pathogens including mechanisms for controlling disease and helps to promote plant growth. Therefore, it is more important than synthetic chemicals (Mendes et al. 2011). About 890 synthetic chemicals are approved as pesticides throughout the world, but a total of 20,700 marketed products are available approximately out of which organophosphorus insecticides are the biggest group of insecticides available in market (Stenersen., 2004).

2.9 Bioformulations available in market: Met 52 bioinsecticide is available in market that contains spores of the soil fungus *Metarhizium anisopliae*. Soil-borne and foliar diseases have been controlled with the help of bactericide/biofungicide available in market named as Taegro (Mehnaz., 2016). Trichoder Max is the most common fungicide available contains spores of *T.asperellum* , helps to control various fungal diseases. Similarly, Broca (*Hedypates betulinus*) infestations in Erva-mateplantation has been controlled with Bio Max containing spores of *Beauveria bassiana* (Mehnaz., 2016). Some biopesticides have been developed by using consortia of microbial strains viz. such as *B. thuringiensis, M. anisopliae, B. bassiana* and *Verticillium lecanii* Biollium under the brand name as Biosona, and Biometa **(Table 2.5)**.

Table 2.5 - WP bioformulations available in the market throughout the world

Name of product	Category of bioformulation	Target organism	Carrier material	Application method	Name of country	References
Gypchek	Mycoinsecticides	Gypsy moth (Lymantria dispar) Multicapsid nuclear polyhedrosis virus (LdMNPV)	Virus infected gypsy moth larvae processed to produce a finely ground powder	Applied using aerial or ground application equipment	USA	Reardon and Podgwaite (1992)
Boverin, Naturalis, Boverosil, Trichobass-P, and L BioGuard Rich Mycotrol ES® Mycotrol O® Spain	Mycoinsecticides	*Beauveria bassiana*	Talc or other inerts such as perlite, kaolin, bentonite, starch	Used to protect agricultural, fruit, decorative, and flowering plants from beetle and moths	Russia, India, South Africa, Former USSR,	De Faria and Wraight (2007
Bio-fungus	Fungicide	*Trichoderma spp.*	Barley kernels or clay granules	Fungi causing wilt, take-all, root rot, and wood decay	Denmark	Monte (2001)
Trichoject, Trichoseal, Trichoprotection ®	Fungicide	*Trichoderma harzianum and Trichoderma viride*	Autoclaved corn or rice seed	*Chondrostereum purpureum* and other soil and foliar pathogens	New Zealand	Burges and Jones (1998)
Trichodex	Fungicide	*T. harzianum*	Autoclaved corn or rice seed	*Chondrostereum purpureum* and other soil and foliar pathogens	Israel	Monte 2001

Binab®	Fungicide	*T. harzianum*	Autoclaved corn or rice seed	Control *Botrytis, Fusarium, Gaeumannomyces, Pythium, Rhizoctonia, Sclerotinia, Sclerotium, Verticillium*, and wood-rot fungi	Denmark, USA, Israel	Monte 2001
Binab T		*T. harzianum and T. polysporum*	Autoclaved corn or rice seed	Control *Botrytis, Fusarium, Gaeumannomyces, Pythium, Rhizoctonia, Sclerotinia, Sclerotium, Verticillium*, and wood-rot fungi	Sweden	Monte 2001
Aspire®	Fungicide	*Candida oleophila*	Skimmed milk	Postharvest decay of citrus fruits, apples, and pears caused by *Penicillium sp*. and *Botrytis sp*.	USA	Droby et al. (1998)
Yield Plus	Fungicide	*Cryptococcus albidus*	Skimmed milk	Postharvest decay of citrus fruits, apples, and pears caused by *Penicillium sp*. and *Botrytis sp*.	South Arica	http://www. anchor.co. za/

Soridex®	Fungicide	*Pseudozyma flocculosa*	Skimmed milk	Powdery mildew on greenhouse roses and cucumbers	Canada	Punja and Utkhede (2003)
Di TeraTM	Nematicide	Killed *Myrothecium verrucaria*	Wheat bran, talc powder, silica	Plant parasitic nematode specially root-knot nematodes (*Meloidogyna Incognita*)	USA	Warrior et al. (1999)
BlightBan® A506 A506	Bactericide	*Pseudomonas fluorescens*	Talc and peat	Postharvest decay in almond, apple, cherry, wettable powder	Canada	Stockwell and Stack (2007)
Bio-Save 10 LP, Bio-Save 100, and Bio-Save 1000. ESC-100	Fungicide	*Pseudomonas syringae strain*	Talc and peat	Postharvest decay in citrus and pome fruit	USA	Stockwell and Stack (2007)

CHAPTER-III

RESEARCH OBJECTIVES

1. Isolation of pathogenic and non-pathogenic strains along with *in vitro* screening of antagonistic potential against *V. dahliae*.
2. Identification of microbial strains and it's hydrolytic enzymatic activities of microbial strains.
3. Antifungal activities of Biosurfactant and Biochemical formulation against *Verticillium dahliae*.
4. Field trials studies for lab to land utilization.

CHAPTER-IV

HYPOTHESIS

Cotton is the major vital kharif crop of Punjab, India but cotton yield starts reducing annually due to attack of soil borne pathogenic fungus *Verticillium dahliae* causing *Verticillium* wilt disease in cotton. The control of wilt disease cannot be achieved by single management technique, an integrated management plan is required. Therefore, the research will be focused upon isolating, screening and identification of both pathogenic and antagonistic biocontrol strains under *in vivo* field trials in natural field conditions and poly house along with biochemical formulation for control of *Verticillium* wilt of cotton. A combinatorial approach with resistant variety, antagonistic fungal strains, endophytic bacterial strains used in seed bacterization and use of biochemical formulation will control *Verticillium* wilt of cotton.

CHAPTER-V

RESEARCH METHODOLOGY

5.1 Research Methodology

5.2 Site selection and Sample collection-

Bathinda is the major cotton producer division of Punjab, hence selected for present studies (**Figure 5.1**). Samples have been collected from 20 different sites from different villages of Bathinda region. Soil samples were collected from below mentioned villages.

Figure 5.1: Map showing sample collection site in Punjab, India

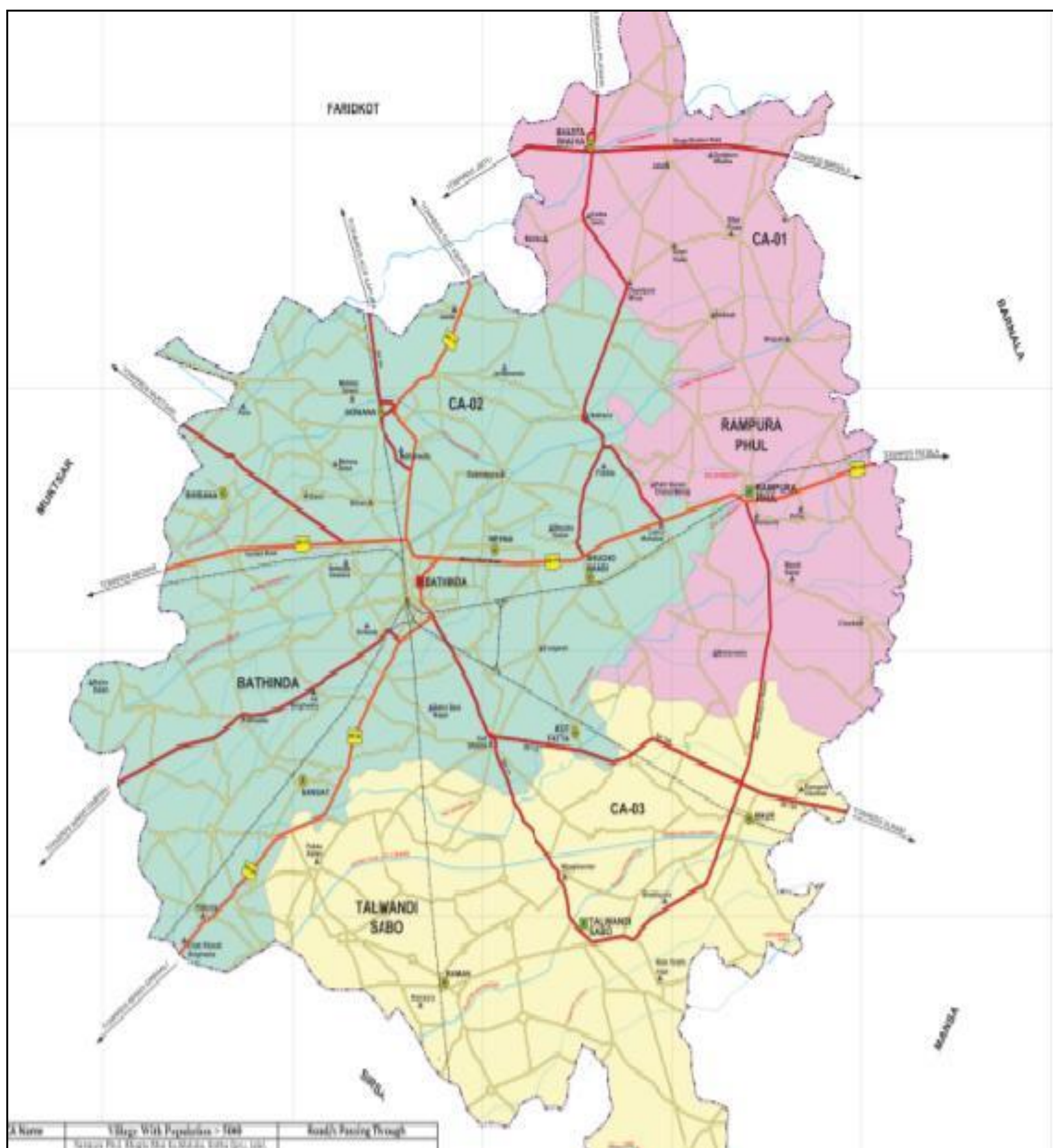

Figure 5.2: Map showing District of Bathinda, Punjab, India

Bathinda district of Punjab is one of the major cotton cultivated area hence selected for the research purpose (**Figure 5.2**). Bathinda is in Malwa region of northwest India and most important district of Punjab for cotton production. The location of Bathinda is between 30.20° North latitude and 74.95°East longitude. The District is situated about a height of 201 m from the mean sea level. 20 villages / town have been selected for study related to the cotton disease. In almost all the fields under cotton cultivation is affected with the diseases. Farmers from these villages have been regularly consulted for the fungal attack. Infected plant materials viz. infected leaves, cotton bolls and infected stems of cotton plants has been collected for characterization of fungal strains. Two sites per village have been selected for collection of non-

infested and infested cotton samples. Infected plant and soil samples have been collected from such sites. Field of cotton crop has been segregated into four plots, and every segregated plot of field will be of 250 m^2. Soil samples and plants have been collected in replication of five per sample from the different layers of each segregated plot. Replication of soil sample from plots has been come from the layers of soil in each segregated field that are 0–15 cm in depth within 1 m^2 area (Oros sichler et al., 2006). The cotton plants and soil were characterized as:

(A) Healthy cotton plants in non-infested fields (non - infected)
(B) Healthy plants in infested fields (infected)
(C) Unhealthy plants in infested fields (infected)
(D) Rhizosphere soil of healthy plants in non-infested fields (non-infected)
(E) Rhizosphere soil of healthy plants in infested fields (infected)
(F) Rhizosphere soil of unhealthy plants in infested fields (infected)
(G) Bulk soil of non-infested fields (non- infected)

Total 160 samples have been collected from both healthy as well as unhealthy fields of Bathinda, Punjab.

Sample collection of organic manures

A total of 8 types of organic manures viz. Poultry Manure, Farmyard Manure, Bio Manure (Organic manure), Vermicomposting, Compost Manure, Green Manure, Oilseed Manure and Animal Manure were collected from different sites of Punjab, India. Sample (10gm each) has been collected by randomized method. Poultry manure, Farmyard manure, Organic manure, Compost manure, Green manure and Animal manures have been collected in replicates of two from each site (total10gm of each organic manure sample) and Vermicompost, Oilseed manures have been purchased from Phillaur, Distt. Ludhiana, Punjab, India, Jagraon, Distt. Ludhiana, Punjab, India respectively.

Sample collection for isolation of antagonistic fungus from rhizospheric soil

Rhizospheric soil of healthy cotton plants from previously cotton growing fields has been collected from five different sites of Bathinda; Punjab followed by complete randomized block design. Soil samples have been collected in replication of five per sample from the different layers of each segregated plot. Replication of soil sample

from plots has been come from the layers of soil in each segregated field that are 0–15 cm in depth within 1 m^2 area (Oros-schiler et al., 2006).

Sample collection for isolation of endophytic bacterial strains

Different sites of Bathinda, Punjab has been selected for collection of healthy cotton plant parts. Cotton plant parts such as healthy leaf, stem and roots have been collected from previous cotton growing fields.

5.3 Isolation of Pathogenic fungal strains from Soil and plant Samples

Isolation of pathogenic fungus has been done from collected plant and soil samples which show symptoms of disease from infected fields as well as samples from healthy cotton fields (Zheng et al., 2011). Roots of collected plant samples from both sites have been washed under running water, surface sterilization with 70% ethanol and cut into small pieces with sterile knives. PDA plates were prepared, and sterile samples been inoculated for 6 days at 25^0C.

Fungal strains obtained from rhizospheric as well as bulk soil samples of both sites been sieved with 2mm mesh and mixed thoroughly. Soil samples were transferred into sterile Erlenmeyer flask with sterile distilled water (1:9) and put-on rotatory shaker for 30 min at 150 rpm. Supernatant from all soil samples were serially diluted and 2ml of each sample were transferred into PDA plates (Zheng et al.,2011). Plates were incubated at 25^0C for 6 days. Fungal strains obtained from both plant and soil samples have been purified and stored until further use.

5.4 Isolation of Antagonistic Fungal Strains from Organic Manures

Poultry Manure, Farmyard Manure, Bio Manure, Vermicompost, Compost Manure, Green Manure, Oilseed Manure and Animal Manure were collected from different sites of Punjab, India. These manures have been used for the isolation of antagonistic fungal strains. Isolation is performed as per method of Serial dilution method described by Aneja (2011). Antagonistic fungus has also been isolated from all manure samples by repeating the same process. After isolation, purified colonies of different fungal strains have been preserved at 4^0 C for further studies.

5.5 Screening the Rhizospheric soil for isolating fungi antagonistic to *Verticillium dahliae*

Antagonistic fungal strains have been isolated from rhizospheric soil of cotton growing areas in Bathinda region (Oros-schiler et al., 2006). Isolation of fungus has been done by Serial dilution method **(Figure 5.3)** (Aneja., 2011). PDA medium was prepared and 1ml of soil sample was poured into the petri plates. Afterwards, medium was poured into plates. Plates were incubated for 5-6 days at 28^0 C. Different types of antagonistic fungus has been separated and purified. Antagonistic fungus has been preserved at 4^0C for further use.

5.6 Isolation of endophytic bacterial strains from healthy plant parts

Endophytic bacterial strains have been isolated from healthy cotton plant parts viz. roots, stems and leaves. Endophytic bacterial strains have been isolated on Nutrient Agar medium (NAM) by streaking method. Different bacterial strains were purified by streaking using NA plates. Isolation protocol was performed with slight modifications for endophytic bacteria. 3-cm-long segments from middle vascular bundle was cut into pieces and soaked in sterile water overnight to free the endophytic bacteria. Inoculations consist of 1ml of the aliquot on Nutrient agar medium plates separately for roots, leaves and stem segments. These plates were then incubated at 37^0 C. Observations were taken after 48 to 72 hrs. Bacterial isolates were picked from plates and purified by streaking techniques and incubated at 37^0C. The isolation process repeated till pure cultures were obtained for further experimentations (Zheng et al., 2011).

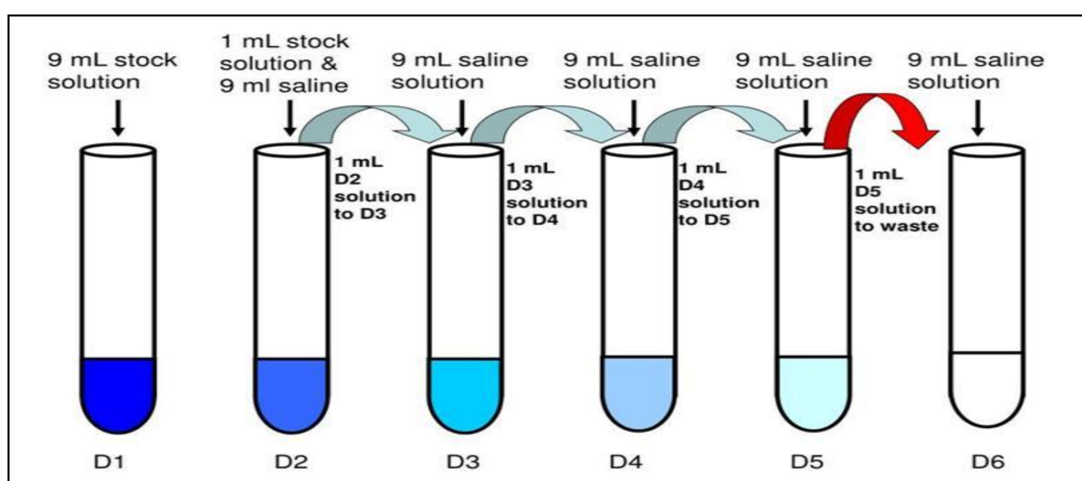

Figure 5.3: Method of Serial dilution followed for isolation of fungal strains from rhizospheric and bulk soil samples

5.7 *in vitro* Screening of Antagonistic fungal strains against *Verticillium dahliae*

Dual Culture Assay: Preliminary *in vitro* screening of antagonistic fungal strains obtained from rhizospheric soil of healthy cotton fields and organic manures against *Verticillium dahliae* has been done by Dual Culture Assay in which PDA plates were prepared **(Figure 5.4)**. In each pate *Verticillium dahliae* was inoculated at 2cm apart from side of petri pate and antagonistic strain opposite to *V. dahliae* and incubate for 144 hours at 26^0 C. For control, only *Verticillium dahliae* was inoculated. After incubation at 26^0 C for 6 days, each plate was observed, and growths of both strains have been calculated. Antagonistic activities were tested 6 days after incubation by measuring the radius of the *V. dahliae* colony in the direction of the antagonist colony (R2) and the radius of the *V. dahliae* only in the control plate (R1). Growth is measured (in mm) for *Verticillium dahliae* in control. This experiment was performed triplicates. PIRG has been calculated by using two readings according to the formula of Skidmore and Dickinson (1976).

Formula used for calculation of Percentage of Growth Inhibition was:

PGI= R1-T1/R1*100

PGI=Percentage of Growth Inhibition

R1= Growth of *Verticillium dahliae* in control

T1= Growth of *Verticillium dahliae* in Test

5.8 *in vitro* screening of endophytic bacterial strains against *V. dahliae* by Dual Culture Assay

in vitro antagonistic activity of bacterial isolates against *Verticillium dahliae* has been done by dual culture method in which PDA plates were prepared. In each plate, *Verticillium dahliae* was inoculated at 2cm apart from side of petri pate and incubate for 48 hours at 26^0 C. After 48 hours, bacterial isolates from NA medium were inoculated at other half of the plate by zigzag streaking. *Verticillium dahliae* was only inoculated for control. After incubation at 28 ± 1^0 C for 6 days, each plate was observed. After six days measure the growth of both endophytic bacterial strains and *Verticillium dahliae* for calculation of percentage of growth inhibition. Measure the growth (in mm) of *Verticillium dahliae* in control also. This experiment has been done in triplicates (Skidmore and Dickinson 1976) with slight modifications.

Formula used for calculation of Percentage of Growth Inhibition was:

PGI= R1-T1/R1*100

PGI=Percentage of Growth Inhibition, R1= Growth of Fungus in control, T1= Growth of Bacterial Culture in Test

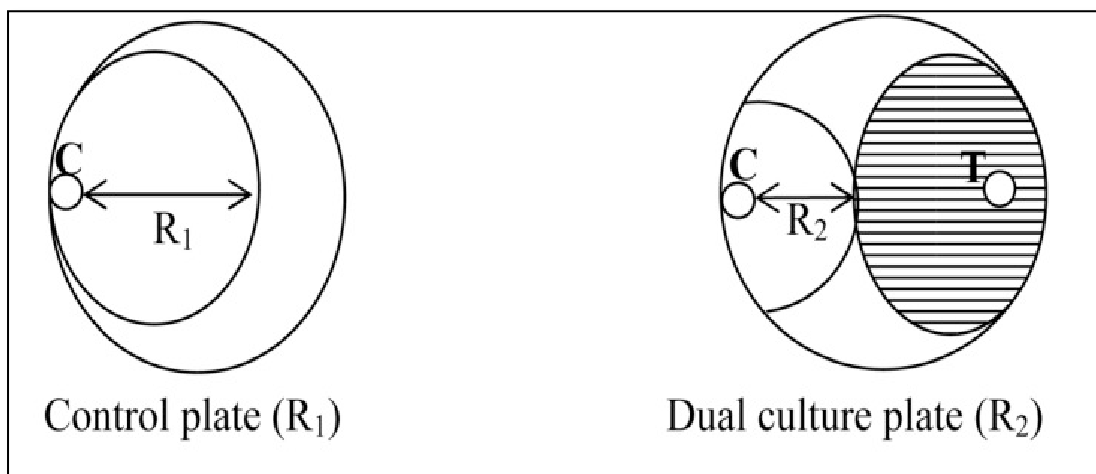

Figure 5.4: Dual culture Assay followed for preliminary *in vitro* screening of antagonistic fungal strains and endophytic bacterial strains against *Verticillium dahliae*

5.9 Additional screening of antagonistic microbial strains by evaluating hydrolytic enzymatic activity

5.9.1 *in vitro* Cellulase, Protease, Chitinase Activity of Antagonistic Fungal Strains

5.9.1.1 Preparation of Crude Enzyme from Antagonistic fungal Strains

Additional screening of antagonistic isolates isolated from collected samples has been done by studied *in vitro* activities of hydrolytic enzymes viz. chitinase, protease and cellulase by well diffusion method. A loopful culture of antagonistic fungal strains was inoculated into a Czapek Mineral Salt Broth Medium. Fungal cultures were incubated for 96 h at 26^0C. The crude enzymes were obtained by centrifugation at 10000 rpm, at 4^0C for 10 min. Both protease and chitinase crude enzymes from antagonistic microbial strains were obtained by inoculating the antagonistic strains on Casein Agar Medium and Chitinase Basal Medium respectively with slight modifications (Cherkupally et al., 2017). The resultant supernatant of all antagonists in cellulase, protease as well as chitinase activity have been separated and stored in 1ml vials at 4^0C until further screening.

5.9.2 Semi Quantitative Assay of Hydrolytic Enzymes

5.9.2.1 *in vitro* screening of Cellulase Enzyme by Well Diffusion Method

Cellulase Assay has been done by preparing Czapek Mineral Salt Agar Medium (KH_2PO_4/1.00 g, $MgSO_4.7H_2O$/0.50 g, $NaNO_3$/2.00 g, KCl/0.50 g, Peptone/2.00 g, and Agar/15.00g, Distilled water 1000ml comprising Carboxy Methyl Cellulose (CMC) 5.00g) petri plates. The plates were allowed to solidify, and well (4mm) diameter was punched in center. The crude enzyme extracted from antagonistic fungal were added into the holes and distilled water was used as a control. The plates were incubated for 5 days at 26^0 C. The plates were flooded with Congo Red Aqueous Solution for 15 min. and washed with NaCl for 1.5 min. Production of cellulase was observed and calculated by formation of clear inhibition zones around the well (Vijayaraghavan et al., 2011).

5.9.2.2 *in vitro* screening of Protease Enzyme by Well Diffusion Method

in vitro screening of protease enzyme extracted from antagonistic microbial strains have been done on Casein Agar medium (Beef extract/1.50g, Peptic digest of animal tissue/5.00g, Sodium chloride/5.00g, Yeast extract/1.50g, Agar/15.00g, Casein/10.00g and Distilled water 1000ml) (Vijayaraghavan and Samuel., 2013). The medium was aseptically transferred into petri plates and allowed to solidify for 30 min. Well (4mm) was punctured in Center, crude culture supernatant from antagonistic fungal strains was loaded into well, incubate for 5 days at 26^0C and flooded the plates with Bromo Cresol Green dye. Formation of clear zones of inhibition around the well determinates the proteolytic activity while the rest of plates shows blue colour. Proteolytic activity was determined by calculating the width of clear zones carefully.

5.9.2.3 *in vitro* screening of Chitinase Enzyme by Well Diffusion Method

The Chitinase Detection Medium comprising basal chitin medium (Colloidal chitin/4.5g, $MgSO_4.7H_2O$/0.30g, $NH4SO_4$/3.00g, KH_2PO_4/2.00g, Citric acid monohydrate/1.00 g, Agar/15g, Bromo cresol purple/0.15g and Tween-80/0.15g and Distilled water 1000ml) was used for determination of chitinase activity. Afterwards medium was poured into petri plates and solidify. Crude enzymes from all antagonistic fungal were poured into holes punched in center. Incubate petri plates for 5 days at 26^0 C for fungal strains and at 37^0 C for bacterial strains. Plates were flooded with Bromo Cresol Green dye. Activity of chitinase enzyme was observed and

calculated by the formation of inhibition zones around the well. All activities were carried out in triplicates and distilled water was used as a control.

5.9.3 Preparation of crude Cellulase, Protease and Chitinase from Endophytic bacteria

Endophytic bacterial strains have been grown on liquid Czapek-Mineral salt Medium in conical flask for 5 days at 26^0 C, liquid Casein Medium in conical flask for 5 days at 26^0 was used for protease and endophytic bacterial strains have been grown on Chitinase Detection Medium in conical flask for production of chitinase enzyme. Filter the medium through filter paper. The sample was centrifuged for 10 min. at 10000 ppm at 4^0 C. The resultant supernatant was separated and stored in 1ml sterilized vials for further screening (Vijayaraghavan et al., 2011).

5.9.3.1 Determination of Cellulase Activity by Well Diffusion Method

Czapek- Mineral Salt Agar medium was poured in petri plates. Plates were solidified and hole was punched in center. A crude culture supernatant from endophytic bacterial strains was loaded into holes. Plates were incubated for 3-5 days at 26^0 C. The plates were flooded with aqueous Congo red solution for 15 mints. Then, plates were flooded with NaCl (1M) for 1 min. Inhibition zones around the well has been recorded. Diameter of colonies was measured and recorded (Vijayaraghavan et al., 2011).

5.9.3.2 Determination of Protease Activity by Well Diffusion Method

Casein Agar medium was poured in petri plates. Plates were solidified and hole was punched in center. A crude culture supernatant from endophytic bacterial strains was loaded into holes and distilled water as control. Plates were incubated for 3-5 days at 26^0 C. The plates were flooded with Bromo Cresol Green Dye. Diameter of clear inhibition zones around the well was measured and recorded (Vijayaraghavan et al., 2011).

5.9.3.3 Determination of Chitinase Activity by Well Diffusion Method

Chitinase Agar medium was prepared, and pH of medium was adjusted to 4.7 then autoclaved. Agar medium was poured into plates and allow to be solidifying. Hole was punched in center. A crude culture supernatant of endophytic bacterial strains was loaded into holes. After incubation, plates were flooded with Bromo Cresol Green Dye. Diameter of purple coloured zone formation around the well was

measured and recorded. This experiment was done in triplicates wherein distilled water used as a control in all above activities (Vijayaraghavan et al., 2011).

5.10 Identification of Microbial Strains.

Identification of selected antagonistic microbial strains has been done by examined characteristics features of colony and airy hyphae, morphology of resting spores, conidiophores (Xue et al., 2013 and Gilman 2002). These fungal cultures were identified based on morphological characteristics using taxonomic guides and standard procedures with the commonly used lacto phenol cotton stain. Fungal strains have also been sent to ITCC Delhi for identification. Bacterial culture was further identified based on 16s rRNA sequencing and sent to Barcode & Biosciences, Bangalore, India for identification.

5.11 *in vitro* biosurfactant activity against *Verticillium dahliae* by Well diffusion method

Biosurfactants., Fengycin and Iturin have been used at different concentrations viz. 1ppm, 5ppm and 10ppm for *in vitro* activity against *V. dahliae* by Well diffusion method in which PDA plates were prepared and hole (4mm) has been punctured in center. Afterwards, pathogenic fungal strain has been inoculated around the well and biosurfactant compound at different concentrations were poured into well (Debode et al., 2007). Distilled water is used as a control. Plates were incubating for 5 days at 26^0 C. Inhibition zone around the well was measured (mm) in each plate after 5 days.

5.12 Selection of plants for biochemical formulation: As per the literature survey, a biochemical formulation has been developed by using different plant parts of various medicinal plants along with antagonistic fungus and endophytic bacteria showing antifungal activity against pathogenic fungal diseases (Haas and Defago., 2005).

5.12.1 Preparation of phytochemical formulation

Plant parts of *Ricinus communis, Tinospora cardifolia, Withania somnifera, Datura stramonium, Clotropis procera, Azadiaracta indiaca, Citrullus colocynthis, Solanum xanthocarpum,* and Copper sulphate has been used for preparation of formulation (**Table 5.4**). Selected plant parts were cut into small pieces with sterile knife and soaked into cow's urine with fermented acidophilus milk for 30 days in a large bucket. After 30 days, extract has been sieved through muslin cloth. Plant extract is

used in ratio of 1:9 (biochemical formulation: water) in the form of spray mixed with $3.4x10^5$ conidia/ml of antagonistic fungal strains/endophytic bacterial strains.

Table 5.4: Various medicinal plants along with its parts and chemical used for preparation of biochemical formulation

S. No.	Name of Plant/ Chemical/ Liquid solvent used	Plant parts used	Weight/Volume used
1	Cow urine	-----	1000ml
2	Fermented acidophilus milk (4-5 weeks old)	------	500ml
3	Jaggery (for stickness)	-----	500gm
4	*Ricinus communis*	Leaves, Fruit	500gm
5	*Tinospora cardifolia*	Leaves, Stem	500gm
6	*Withania somnifera*	Leaves	500gm
7	*Datura stramonium*	Leaves, Fruit	500gm
8	*Clotropis procera*	Leaves	500gm
9	*Azadiaracta indiaca*	Leaves	500gm
10	*Citrullus colocynthis*	Leaves, Fruits	500gm
11	*Solanum xanthocarpum*	Leaves, Fruits	500gm
12	Copper sulphate	-----	5gm

5.12.2 Analysis of biochemical formulation:

Liquid chromatograph Mass Spectrometry technique has been used for the analysis of biochemical formulation. 10ml of biochemical formulation has been transferred into petri plate and put into hot air oven at 40^0C for overnight. Next day biochemical formulation has been prepared into powdered form using sterilized pestle mortar. 1mg of biochemical formulation has been sent to Central Drug Research Institute, Lucknow, India for LCMS.

5.13 *in vitro* antifungal activity of biochemical formulation against *Verticillium dahliae*: Antifungal activity of bioformulation has been checked at 10ppm, 50ppm and 100ppm concentration by Well diffusion method (Saha et al., 2008 and M.L Suleiman., 2011). PDA plates were prepared, and well (4mm) has been punctured in center. Afterwards, pathogenic fungal strain has been inoculated around the well and

biochemical formulation at different concentration was poured into well (Debode et al., 2007). Distilled water is used as a control. Plates were incubating for 5 days at 26^0 C. Experiment has been done in triplicates. Inhibition zone around the well was measured (mm) in each plate after 5 days.

5.14 Field trials studies for lab to land utilization.

5.14.1 Cultures Practices prior to seed sowing under natural field conditions

5.14.1.1 Preparatory tillage

The field was prepared by ploughing before sowing of seeds, with tractor drawn plough followed by two cross harrowing. Dry weeds and stubble were removed by deep ploughing. The field was again ploughing by repeated harrowing with a blade harrow and finally leveled with wooden leveler (PrEm et al., 2016). Then field has been prepared according to the experimental field layout according to the plan of layout represented below **(Table 5.5**).

5.14.1.2 Soil solarization

Soil solarization technique has been applied before sowing of seeds in prepared experimental field. Four equal ridges have been prepared with wooden leveler and properly covered with polythene sheets. The selected plot area for cultivation is 35x10 feet with four equal ridges at distance of 2 feet in each row. In the first step, deep ploughing of field has been done and debris was removed from selected area of field. Soil was covered with clear/transparent polythene films. Each row was separately covered with plastic sheets for 30-40 days (Gill et al., 2009).

5.14.1.3 Sowing, seed rate and spacing:

The experiment was conducted for 6 months duration from May to November 2019. Seed rate of 7kg/hectare was used for experiment. Treated seeds of Rasi 134 Bt were sown by hand dibbling methods in lines at depth of 5cm and each seed has been sown at distance of 1.5 feet.

5.14.1.4 Soil Sterilization for Poly house Experiments

Evaluation of potential antagonistic microbial strains against *Verticillium dahliae* has been performed in poly house experiments. Soil, sand, peat and vermiculite will be used in ratio of 1:1:1:1 respectively for each pot. Soil was collected from previously cotton growing fields in polythene bags. Soil was air dried in hot air oven and sieved

to remove the debris. Soil and sand were sterilized separately in autoclave for 30 minutes at least 15 psi. (Wolf and Skipper., 1994).

5.15 Seed bacterization for Poly house and Field trials

Bacterization of delinted seeds of *Verticillium dahliae* sensitive local variety Rasi 134 Bt has been done for both field and pot experiments with slight modifications (Göre et al., 2008). Cotton seeds are first treated with 1% NaOCl for 1 min for surface sterilization followed by three times washing in sterile phosphate buffer having concentration 0.05 M (pH 7.4) and antagonistic bacteria (Bölek et al., 2005). Nutrient Broth medium has used for antagonistic bacterial growth for 24 hours at 37^0C. Afterwards, centrifugation has been done at 5000g for 5 min at 4^0C, pellets have been collected and suspended in sterile phosphate buffer with 1.5% CMC solution. For control seeds have been treated with 1.5% CMC without bacteria and soaking of seeds with antagonistic bacterial suspension of 100mg with 1ml of 1.5% CMC. The mean concentration of bacterial strains was 7.1^{107}–1.0^{108} cfu/seed for test in both polyhouse and field trials. After soaking all the seeds were kept separately in sterile condition in laminar flow for 12 hours (Bölek et al., 2005).

5.16 Biological control of *Verticillium* wilt by using endophytic bacterial strains in Poly house and Natural Field conditions (for monoculture experiment)

Verticillium dahliae sensitive local variety has been used for evaluation of endophytic bacterial strains against *V. dahliae* under both natural and controlled conditions. A mixture of autoclaved soil-sand-peat-vermiculite in an equal ratio has been transferred into plastic pots for sowing of both bacterized and control seeds (Naraghi et al., 2010). Seeds treated with each endophytic bacterial strain (for monoculture experiments) has been sown into pots for poly house experiments and in natural field also.10 days old plates of *V. dahliae* have used for inoculation after sowing of bacterized seeds., diluted into $3x10^6$ conidia/ml. Drops of conidial suspension (5-l0 drops) has been inoculated into stem of cotton plant through 22-gauge needle by puncturing the first internode of stem at six true leaf stage (Hanson., 2000). Cotton plants were fertilized with 16-8-24 N-P-K liquid fertilizer. Experiment has been repeated in triplicates for each bacterial strain. Non-bacterized plants used as control been inoculated with sterile distilled water only. Disease severity of each plant has been checked after fifteen days of inoculation from sowing to harvesting period. At 0-to-4 rating scale 0 = (healthy plant), 1 = 1–33% (Acropetal Chlorosis), 2 = 34–66%

(Necrosis), 3 = 67–97% (Wilt or Defoliation) 4 = (Dead plant), disease severity index was assessed for both control and test plants (Bejarano-Alcazar et al., 1995).

Formula used for calculation of Biocontrol protection % (Li et al., 2012) and disease index (Zhu et al., 2013) is mentioned below:

Disease Incidence (%) = $[(n_1+n_2+n_3+n_4)/n] \times 100$.,
Disease Severity Index (%) = $[(0n_0+1n_1 + 2n_2 + 3n_3 + 4n_4)/4n \times 100$;
Biocontrol Protection% = 100- Vascular discoloration (treatment)/Vascular discoloration (control) x 100

5.17 Biological control of *Verticillium* wilt by using fungal strains in Poly house and Natural Field conditions (monoculture experiment)

Verticillium dahliae susceptible variety (Rasi 134Bt) has been used for evaluation of antagonistic fungal strains against *V. dahliae* under controlled poly house and natural field (Village Akalian Kalan, Bathinda, Punjab, India). A mixture of autoclaved soil-sand-peat-vermiculite mixed with each antagonistic fungal strain (1×10^8 conidia/ml) has been transferred into plastic pots and then seeds has been sown (Naraghi et al., 2010). After sowing of seeds, 10 days old plates of *Verticillium dahliae* have used for inoculation., diluted with 3 $\times10^6$ conidia/ml with distilled water. Drops of conidial suspension (5-l0 drops) have been inoculated into stem of cotton plant through 22-gauge needle by puncturing the first internode of stem at six true leaf stage (Hanson, 2000). Cotton plants were fertilized with 16-8-24N-P-K liquid fertilizer. Control 1 plants have been inoculated with sterile distilled water only and control 2 plant has been inoculated with *V. dahliae*. Disease severity of each plant has been checked after fifteen days of inoculation from sowing to harvesting period (15 to 180 days). At 0-to-4 rating scale 0 = (healthy plant), 1 = 1–33% (Acropetal Chlorosis), 2 = 34–66% (Necrosis), 3 = 67–97% (Wilt or Defoliation) 4 = (Dead plant), disease severity index was assessed for both control and test plants (Bejarano-Alcazar et al., 1995).

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Disease Incidence (%) = $[(n_1+n_2+n_3+n_4)/n] \times 100$.,
Disease Severity Index (%) = $[(0n_0+1n_1 + 2n_2 + 3n_3 + 4n_4)/4n \times 100$;

Biocontrol Protection% = 100- Vascular discoloration (treatment)/Vascular discoloration (control) x 100

5.18 Biological control of *Verticillium* wilt by using fungal strains in Poly house and Natural Field conditions (co- culture experiment)

Verticillium dahliae sensitive local variety has been used for checking the effect of both endophytic bacterial strains and antagonistic fungal strains against *V. dahliae* under both natural and controlled conditions. Seeds treated with combination of all bacterial strains have been used for this experiment. After sowing of seeds mixture of all fungal cultures (1×108 conidia/ml) each mixed autoclaved soil-sand-peat-vermiculite in an equal ratio has been transferred into plastic pots for sowing of both bacterized and control seeds. After sowing of bacterized seeds and non-bacterized seeds (control) same procedure has been followed as discussed above for both poly house and natural field conditions. Experiment has been repeated in triplicates for assessment of disease severity index and biocontrol protection from 15 to 180 days of sowing.

5.19 Biological control of *Verticillium* wilt in Poly house and Natural Field conditions along with biochemical formulation (monoculture and co-culture experiment)

Verticillium dahliae sensitive local variety (Rasi Bt 134) has been used for checking the effect of biochemical formulation along with each endophytic bacterial strain and each antagonistic fungal strain against *V. dahliae* under both natural and controlled conditions. Bacterized seeds and non-bacterized seeds (control) has been sown in an equal mixture of autoclaved soil-sand-peat- vermiculite into plastic pots.10 days old plates of *Verticillium dahliae* have used for inoculation. diluted into 3 x10^6 conidia/ml and distilled water in control plants. Drops of conidial suspension (5-l0 drops) have been inoculated into stem of cotton plant through 22-gauge needle by puncturing the first internode of stem at six true leaf stage (Hanson., 2000). After 60 days of sowing, biochemical formulation in ratio of 1:10 (biochemical formulation: water) was used in a spray after every fortnight in bacterized seeds and only distilled water used for control plants. Biochemical formulation mixed with each antagonistic fungal culture (1×108 conidia/ml) has been used in spray form after every fortnight for monoculture experiment. Seeds treated with combination of all bacterial strains have also been sown and mixture of all antagonistic fungal strains (1×108 conidia/ml) each mixed

with biochemical formulation during co-culture experiment. Disease severity of each plant has been checked after fifteen days of inoculation from sowing to harvesting period. At 0-to-4 rating scale 0 = (healthy plant), 1 = 1–33% (Acropetal Chlorosis), 2 = 34–66% (Necrosis), 3 = 67–97% (Wilt or Defoliation) 4 = (Dead plant), disease severity index was assessed for both control and test plants (Bejarano-Alcazar et al., 1995).

Formula used for calculation of Bio control protection % (Li et al., 2012) and disease index (Zhu et al., 2013) is mentioned below:

Disease Incidence (%) = $[(n_1+n_2+n_3+n_4)/n] \times 100$.,
Disease Severity Index (%) = $[(0n_0+1n_1 + 2n_2 + 3n_3 + 4n_4)/4n \times 100$;
Biocontrol Protection% = 100- Vascular discoloration (treatment)/Vascular discoloration (control) x 100

Table 5.5: Experimental field layout (PF- Pathogenic Fungus, EB- Endophytic Bacteria, AF- Antagonistic Fungus, BF- Biochemical Formulation)

FIELD TRIALS			
ROW 1 (18 PLANTS/ROW)	ROW 2 (17 PLANTS /ROW)	ROW 3 (17 PLANTS /ROW)	ROW 4 (18 PLANTS /ROW)
F1			
C1 (CONTROL 1 FUNGUS 1) UN INOCULATED WITH P.F (NON-BACTERIZED)	T1 B2 (SEED BACTERIZATION WITH B2 + INOCULATED WITH P.F)	T2 B2 (SEED BACTERIZATION WITH B2 + INOCULATED WITH P.F)	T3 B. F+ CO CULTURE (INCOLUATED WITH P.FI+ B.F+ 3 E.B + 5 A.F)
C2 ((CONTROL 2 FUNGUS 1) (INOCULATED WITH P.F (NON-BACTERIZED)	C B1 (CONTROL BACTERIA 2 INOCULATED WITH P.F) NON BACTERIZED	T3 B2 (SEED BACTERIZATION WITH B3 + INOCULATED WITH P.F)	T2 B.F+ CO CULTURE (INCOLUATED WITH P.FI+ B.F+ 3 E.B + 5 A.F)
T1F1(TEST 1FUNGUS 1) PF + F1 (NON-BACTERIZED)	**B2**	**B3**	T1 B.F+ CO CULTURE (INCOLUATED WITH P.FI+ B.F+ 3 E.B + 5 A.F)
T2F1 (TEST 2 FUNGUS 1) PF + F1 (NON-BACTERIZED)	T3 B1 (SEED BACTERIZATION WITH B1 + INOCULATED WITH P.F)	C B3(CONTROL BACTERIA 3 INOCULATED WITH P.F) NON BACTERIZED	**BIOFORMULATION+ CO CULTURE**
T3F1 (TEST 3 FUNGUS 1) PF + F1 (NON-BACTERIZED)	T2 B1 (SEED BACTERIZATION WITH B1 + INOCULATED WITH P.F)	T1 B3 (SEED BACTERIZATION WITH B3 + INOCULATED WITH P.F)	T3 B.F+ B2 (INCOLUATED WITH P.F+ B.F+ E.B)

F2	T1 B1 (SEED BACTERIZATION WITH B1 + INOCULATED WITH P.F)	T2 B3 (SEED BACTERIZATION WITH B3 + INOCULATED WITH P.F)	T2 B.F+ B2 (INCOLUATED WITH P.F+ B.F+ E.B)
			T1 B.F+ B2 (INCOLUATED WITH P.F+ B.F+ E.B)
C1 (CONTROL 1 FUNGUS 2) UN INOCULATED WITH P.F (NON BACTERIZED)	C B1 (CONTROL BACTERIA 1 INOCULATED WITH P.F) NON BACTERIZED	T3 B3 (SEED BACTERIZATION WITH B3 + INOCULATED WITH VERTI)	**BIOFORMULATION+ B3**
C2 ((CONTROL 2 FUNGUS 2) (INOCULATED WITH P.F (NON BACTERIZED)	**B1**	**BIOFORMULATION WITH ANTA FUNGUS**	T3 B.F+ B2(INCOLUATED WITH P.F+ B.F+ E.B)
T1F2(TEST 1FUNGUS 2) PF + AF (NON BACTERIZED)	T3 CO CULTURE (INOCULATED WITH P.F + 5 A.F (F1,F2,F3,F4,F5) + 3 E.B (B1, B2,B3)	**BIOFORMULATION+ F1**	T2 B.F+ B2(INCOLUATED WITH P.F+ B.F+ E.B)
		CONTROL OF BIOFORMULATION (INOCULATED WITH P.F) (NON BACTERIZED)	T1 B.F+ B2(INCOLUATED WITH P.F+ B.F+ E.B)
T2F2 (TEST 2 FUNGUS 2) PF + AF (NON BACTERIZED)	T2 CO CULTURE (INOCULATED WITH P.F + 5 A.F (F1,F2,F3,F4,F5) + 3 E.B BACTERIA (B1, B2,B3)	T1 B.F+ F1(INCOLUATED WITH P.F+ B.F + A.F)	**BIOFORMULATION+ B2**

T2F3 (TEST 3 FUNGUS 2) PF + AF (NON BACTERIZED)	T1 CO CULTURE (INOCULATED WITH P.F + 5 AF (F1,F2,F3,F4,F5) + 3 E.B BACTERIA (B1, B2,B3)	T2 B. F+ F1(INCOLUATED WITH P. F+ B. F+ A.F)	T3 B. F+ B1(INCOLUATED WITH P.F+ B.F+ E.B)
F3	CONTROL OF CO CULTURE (NON BACTERIZED) (INOCULATED WITH PF)	T3 B. F+ F1(INCOLUATED WITH P. F+ B.F + A.F)	T2 B. F+ B1(INCOLUATED WITH P. F+ B.F+ E.B)
C1 (CONTROL 1 FUNGUS 3) UN INOCULATED WITH PF (NON BACTERIZED)	**CO CULTURES**	**BIOFORMULATION+ F2**	T1 B.F+ B1(INCOLUATED WITH P.F+ B.F+ E.B)
		T1 B. F+ F2(INCOLUATED WITH P. F+ B. F+ A.F)	CONTROL OF BIOFORMULATION (INOCULATED WITH P.F)
C2 ((CONTROL 2 FUNGUS 3) (INOCULATED WITH PF (NON BACTERIZED)	T3F5(TEST 1FUNGUS 5) PF + AF (NON BACTERIZED)	T2 B. F+ F2(INCOLUATED WITH P.F + B.F + A.F)	**BIOFORMULATION+ B1**
T1F3(TEST 1FUNGUS 3) P. F+ A.F (NON BACTERIZED)	T2F5(TEST 1FUNGUS 5) P.F+ A.F (NON BACTERIZED)	T3 B. F+ F2(INCOLUATED WITH P.F+ B.F+ A.F)	**BIOFORMULATION WITH ANTA BACTERIA**
T2F3 (TEST 2 FUNGUS 3) P.F+ A.F (NON BACTERIZED)	T2F5(TEST 1FUNGUS 5) P.F + A.F (NON BACTERIZED)	**BIOFORMULATION+ F3**	T3 B.F+ F5 (INCOLUATED WITH P.F+ B.F+ A.F)

T3F3 (TEST 3 FUNGUS 3) P.F + A.F (NON BACTERIZED)	C2 ((CONTROL 2 FUNGUS 5) (INOCULATED WITH P.F (NON BACTERIZED)	T1 B.F+ F3(INCOLUATED WITH P.F+ B.F+ A.F)	T2 B.F+ F5 (INCOLUATED WITH P.F+ B.F+ A.F)
F4	C1 (CONTROL 1 FUNGUS 5) UN INOCULATED WITH P.F (NON BACTERIZED)	T2 B.F+ F3(INCOLUATED WITH P.F+ B.F+ A.F)	T1 B.F+ F5 (INCOLUATED WITH P.F+ B.F+ A.F)
C1 (CONTROL 1 FUNGUS 4) UN INOCULATED WITH P.F (NON BACTERIZED)	**F5**	T3 B.F+ F3 (INCOLUATED WITH P.F+ B.F+ A.F)	**BIOFORMULATION+ F5**
C2 (CONTROL 2 FUNGUS 4) (INOCULATED WITH P.F (NON BACTERIZED)	T3 F4 (TEST 1FUNGUS 4) P. F+ A.F (NON BACTERIZED)	**BIOFORMULATION+ F4**	T3 B.F+ F4((INCOLUATED WITH P.F+ B.F+ A.F)
T1F4 (TEST 1FUNGUS 4) P.F + A.F (NON-BACTERIZED)	T2 F4 ((TEST 1FUNGUS 4) P.F + A.F (NON BACTERIZED)	T1 B.F+ F4((INCOLUATED WITH P.F+ B.F+ A.F)	T2 B.F+ F4((INCOLUATED WITH P.F+ B.F+ A.F)

CHAPTER-VI

RESULTS AND DISCUSSION

6.1 Introduction

Cotton is an economically important crop grown throughout the world. Cotton plants are being affected by the most devastating soil borne pathogen *Verticillium dahliae (*Dervis and Bicici., 2005). The disease was first discovered in the Manisa province of Turkey in 1941 (Iyriboz., 1941). Different strategies have been used by various researchers such as usage of fungicides, chemical fumigation but more a more intensified, powerful and effective strategy need to be developed for control of wilt disease (Usami et al., 2017). Biological control is the most effective and sustainable method to reduce the effect of *Verticillium dahliae* on cotton and safe, environment friendly and convenient approach to be used (Deketelaere et al., 2017; Fravel., 2005; Li et al., 2012). Lipopeptide biosurfactants also helps to control different fungal diseases (AC et al., 2017). Bioformulation developed from different medicinal plants along with antagonistic strains and chemicals has been used against *V. dahliae* is also effective approach to control wilt disease. The primary aim of this research is to develop a strategy to control *Verticillium* wilt of cotton by evaluating the antagonistic fungal strains as potential biocontrol agents along with biochemical formulation. The objectives of the present study are to isolate antagonistic fungi from different organic manures and endophytic bacteria from healthy cotton plant parts; followed by *in vitro* antagonistic activity and biosurfactant based studies with enzymatic screening for evaluation of potential biocontrol agents to suppress *Verticillium* wilt of cotton under both poly house and natural field conditions. Efficacy of biochemical formulation with antagonistic fungal strains and endophytic bacterial strains has also been checked under both polyhouse and natural field conditions.

6.2 Site selection and sample collection

In the current study, Bathinda district of Punjab, India was selected. Bathinda is located in the Malwa region of northwestern India. The Bathinda district is one of the most important districts in Punjab. Bathinda is located between latitude 30.20 ° north and longitude 74.95° east. The district is about 201 meters above sea level. Bathinda is located in the long Indo-Ganges plain and is known for its very high yields due to its alluvial soil. The 20 villages from Bathinda, which the samples were taken are listed in **(Table No. 6.1).** Both the infected and unaffected fields were divided into

four equal compartments, with an area of approximately 250 m2. Plant and soil samples were taken from the core of each plot. Rhizosphere soil samples were taken from soil layers 0-15 cm deep in a 1 m2 area (Orosschiler et al., 2006).

A total of 160 samples have been collected from both healthy as well as unhealthy fields of Bathinda, Punjab for isolation of pathogenic fungal strains. **Table 6.2** describes various sites of Punjab, India from where samples of organic manures have been collected. **Table 6.3** describes sample collection sites of Bathinda, Punjab along with their co- ordinates and date of collection from five different sites for isolation of endophytic bacterial strains from healthy cotton plant parts. **Table 6.4** describes the sample collection sites of Bathinda, Punjab, India for isolation of antagonistic fungus from rhizospheric soil of healthy cotton plants.

Table 6.1 - List of Selected Sites for Sample Collection (Cotton Plant Parts)

S.No.	Name of Selected Sites	Co-ordinates	Samples	Size (I, N.I) in Inches /Weight (R.S, B.S) in Gram of Collected Samples	Date of Collection
1	Vill. Bir talab Basti no.2 Distt. Bathinda	Lat:30.18821, Long:74.89384	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.1,4.0), (5,5)	10th August,2017
2	Vill. Bir talab Basti no.3 Distt. Bathinda	Lat:30.18821, Long:74.89384	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.0,5.1), (5,5)	10th August,2017
3	Vill. Naruana Distt. Bathinda	Lat:30.16381, Long:74.89603	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(2.9,4.7) (5,5)	10th August,2017
4	Vill. Nwan pind (Bir behman) Distt. Bathinda	Lat:30.19948, Long:74.86387	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.2, 4.6), (5,5)	11th August,2017

5	Vill. Multania Distt. Bathinda	Lat:30.17857, Long:74.85637	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.5, 5.0), (5,5)	11th August,2017
6	Vill. Talwandi sabo Distt. Bathinda	Lat:29.98741, Long:75.08706	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(2.5, 4.8), (5,5)	12th August,2017
7	Vill. Laleana Distt. Bathinda	Lat:29.99913, Long:75.02617	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.5,5.0), (5,5)	12th August,2017
8	Vill. Jajjal Distt. Bathinda	Lat:29.96789, Long:75.03288	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.0, 4.0), (5,5)	12th August,2017
9	Vill. Malkana distt. Bathinda	Lat:29.93726, Long:75.03312	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.7,4.8), (5,5)	13th August,2017
10	Vill. Akalia Kalan, distt. Bathinda	Lat:30.37241, Long:74.88678	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.5,5.2), (5,5)	13th August,2017
11	Vill. Bhaagi vander distt. Bathinda	Lat:30.02592, Long:75.06842	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.0,5.0), (5,5)	14th August,2017
12	Vill. Nat bager distt. Bathinda	Lat:30.03091, Long:75.0705	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(2.4,4.8), (5,5)	14th August,2017
13	Vill. Sivian distt. Bathinda	Lat:30.26073, Long:74.89968	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.1,4.5), (5,5)	16th August,2017
14	Vill. Gill Patti distt. Bathinda	Lat:30.27663, Long:74.93914	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(2.5,5.0), (5,5)	16th August,2017

15	Vill. Deon distt. Bathinda	Lat:30.22934, Long:74.94158	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.2,5.1), (5,5)	17th August,2017
16	Vill. Mehma sarja distt. Bathinda	Lat:30.30971, Long:74.8434	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.0,4.2), (5,5)	17th August,2017
17	Vill. Rampura Phul distt. Bathinda	Lat:30.27509, Long:75.24211	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(2.8,4.2), (5,5)	19th August,2017
18	Vill. Bhucho mandi distt. Bathinda	Lat:30.21322, Long:75.09032	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(2.5,4.0), (5,5)	19th August,2017
19	Vill. Goniana mandi distt. Bathinda	Lat:30.31913, Long:74.91464	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.3,4.6), (5,5)	19th August,2017
20	Vill. Nehian wala distt. Bathinda	Lat:30.30637, Long:7491065	Roots, Rhizospheric Soil, Bulk Soil (I & N.I)	(3.7,4.9), (5,5)	20th August,2017

Table 1: indicates- **I-** Infected, **N.I-** Non- Infected, **R.S-** Rhizospheric Soil, **B.S-** Bulk Soil

Table 6.2 Sample collection of organic manures from Various Sites of Punjab

S.No.	Name of Organic Manure	Name of Sites	Co-ordinates	Weight of Collected Samples	Date of Collection
1	Bio Manure	Goniana Mandi, Distt. Bathinda, Punjab, India	Lat:30.31913, Long:74.91464	10gm	12th January,2018
2	Poultry Manure	Sewe Wala, Distt. Faridkot, Punjab, India	Lat:30.41683, Long:74.88895	10gm	10th January,2018

3	Vermicompost	Phillaur, Distt. Ludhiana, Punjab, India	Lat:31.02101, Long:75.78579	10gm	17th January,2018
4	Oil Seed Manure	Jagraon, Distt. Ludhiana, Punjab, India	Lat:30.78812, Long:75.47016	10gm	20th January,2018
5	Green Manure	Chandbhan, Distt. Faridkot, Punjab, India	Lat:30.38515, Long:74.89975	10gm	10th January,2018
6	Compost Manure	Aklia Kalan, Distt. Bathinda, Punjab, India	Lat:30.37201, Long:74.88589	10gm	11th January,2018
7	Animal Manure	Chandbhan, Distt. Faridkot, Punjab, India	Lat:30.38381, Long:74.89895	10gm	10th January,2018
8	Farmyard Manure	Dabrikhana, Distt. Faridkot, Punjab, India	Lat:30.40857, Long:74.94564	10gm	17th January,2018

Table 6.3 Sample collection for endophytic bacterial strains

S. No.	Name of Selected Sites	Site condition	Co-ordinates	Samples	Size (in inches) of Collected Samples	Date of Collection
1	Vill. Goniana mandi distt. Bathinda	Non-infected	Lat:30.31974, Long:74.91464	Leaves, Stem and Roots (N.I)	Stem 6, Root 5.8 (cm)	19th August,2017

2	Vill. Goniana mandi distt. Bathinda	Non-infected	Lat:30.31913, Long:74.91464	Leaves, Stem and Roots (N.I)	Stem 6.2, Root 5 (cm)	19th August,2017
3	Vill. Talwandi sabo Distt. Bathinda	Non-infected	Lat:29.98741, Long:75.08706	Leaves, Stem and Roots (N.I)	Stem 5.1, Root 5.2 (cm)	12th August,2017
4	Vill. Talwandi sabo Distt. Bathinda	Non-infected	Lat:29.98774, Long:75.09102	Leaves, Stem and Roots (N.I)	Stem 5.3, Root 5.8 (cm)	12th August,2017

Table 6.4: Sample collection for isolation of antagonistic fungus from rhizospheric soil

S. No.	Name of Selected Sites	Site condition	Co-ordinates	Source of Collection	Weight of Sample Collected	Date of Collection
1	Vill. Goniana Mandi, Distt. Bathinda	Non-infected Field	Lat:30.31913, Long:74.91464	Rhizosphere Soil from roots of healthy plant	10 gm	10th August 2019
2	Vill. Rampura Phul Distt. Bathinda	Non-infected Field	Lat:30.27509, Long:75.24211	Same as above	10 gm	11th August 2019
3	Vill. Akalia Kalan, distt. Bathinda	Non-infected Field	Lat:30.37241, Long:74.88678	Same as above	10 gm	12th August 2019
4	Vill. Talwandi sabo Distt. Bathinda	Non-infected Field	Lat:29.98741, Long:75.08706	Same as above	10 gm	12th August 2019
5	Vill. Gill Patti distt. Bathinda	Non-infected Field	Lat:30.27663, Long:74.93914	Same as above	10 gm	13th August 2019

Figure 6.1 - Map showing District of Bathinda, Punjab, India

Figure 6.2.- Pictorial view of sampling site (a) non infected (healthy field) (b) infected (unhealthy field) village in Bathinda District, Punjab

Figure 6.3: Pictorial view of rhizospheric soil from (a) infected cotton plant (b) healthy cotton plant from Bathinda district Punjab, India

Figure 6.4: Pictorial view of bulk soil sample collection from Bathinda district Punjab, India

6.3 Isolation of pathogenic fungal strains from collected plant/soil samples

Verticillium dahliae, most devastating pathogenic fungus of cotton has been isolated from infected cotton plant parts and rhizospheric and bulk soil. The pathogenic fungal strain isolated from infected samples shows whitish mycelium in initial incubation period on PDA plates. Colony on maturation (7 days) start changing colour to dark brown **(Figure 6.5)**. *V. dahliae* has also been isolated from rhizospheric and bulk soil samples collected from infected cotton fields **(Figure 6.6)**. Purification of fungi has been performed by using PDA plates **(Figure 6.7). Table 6.5** depicts prevalence rate of *V. dahliae* isolated from collected samples from different sites. The samples codes from the sites are A, B, C, D, E, F, G and H. The sample code description has been

mentioned at the bottom of the table. Based on the description from the code of the sample, it has been presumed that sample code B, C, E, F, H has very high probability of getting pathogenic strains of *V. dahliae.* Description from sample code A, D, G is presumed to have very less or no probability for the presence of pathogenic *V. dahliae*. The results depicted in **Table 6.5** clearly confirms the presumptions made and establishes the presence of *V. dahliae* from the infected samples and fields. Infected and non-infected samples such as cotton plant parts, rhizospheric soil and bulk soil have been collected from both infected and non-infected sites. *V. dahliae* has been isolated from all collected samples from all sites. The results from the total (8) collected samples from both infected and non-infected sites, maximum prevalence rate of *V. dahliae* has been reported in three sites Vill. Bir talab Basti no.3 Distt. Bathinda, Vill. Naruana distt. Bathinda, Vill. Jajjal distt. Bathinda is 62% followed by 50% in ten sites named as Vill. Bir Talab Basti no.2 distt. Bathinda, Vill. Multanian distt. Bathinda, Vill. Talwandi sabo distt. Bathinda, Vill. Laleana distt. Bathinda, Vill. Akalia Kalan, distt. Bathinda, Vill. Nat bager distt. Bathinda, Vill. Gill patti distt. Bathinda, Vill. Bhucho mandi distt. Bathinda, Vill. Goniana mandi distt. Bathinda, 37% in six sites viz. Vill. Nwan pind (Bir behman) distt. Bathinda, Vill. Bhaagi vander distt. Bathinda, Vill. Sivian distt. Bathinda, Vill. Deon distt. Bathinda, Vill. Rampura phul distt. Bathinda, Vill. Nehian wala distt. Bathinda and minimum rate of prevalence (12%) of *V. dahliae* has been observed in Vill. Malkana distt. Bathinda. Out of 8 collected samples, prevalence rate of *V. dahliae* has also been calculated from 5 infected samples only. Highest prevalence rate of *V. dahliae* (100%) has been observed in three sites such as Vill. Bir talab Basti no.3 distt. Bathinda, Vill. Naruana distt. Bathinda and Vill. Jajjal distt. Bathinda is 100% followed by 80% in ten sites, Vill. Bir Talab Basti no.2 distt. Bathinda, Vill. Multanian distt. Bathinda, Vill. Talwandi sabo distt. Bathinda, Vill. Laleana distt. Bathinda, Vill. Akalia Kalan, distt. Bathinda, Vill. Nat bager distt. Bathinda, Vill. Gill patti distt. Bathinda, Vill. Bhucho mandi distt. Bathinda, Vill. Goniana mandi distt. Bathinda followed by 60% prevalence rate in six sites viz. Vill. Nwan pind (Bir behman) distt. Bathinda, Vill. Bhaagi vander distt. Bathinda, Vill. Sivian distt. Bathinda, Vill. Deon distt. Bathinda, Vill. Rampura phul distt. Bathinda, Vill. Nehian wala distt. Bathinda and least prevalence rate of *V. dahliae* (20%) has been observed in Vill. Malkana distt. Bathinda. Prevalence rate of *Vertcillium dahliae* out of 160 collected samples, from 20 different sites of Bathinda, Punjab, India is 73%.

Table 6.5: Prevalence rate of *Verticillium dahliae* from sites of Bathinda, Punjab

S. No.	Name of Villages	Number of fungal strains isolated sample(code) from collected fields								occurrence of *Verticillium dahliae* from Of Sample (8) fields	No of infected sample (8) fields	No of non-infected sample (8) fields	Total number of fungal strains isolated from sample (8) fields	Total No. of *V. dahliae in* sample (8) fields	Prevalence rate of *V. dahliae* in sample (8) fields	Prevalence rate of *V. dahliae* infected sample (5) fields	Prevalence rate of *V. dahliae* in infected sample fields in 20 villages
		A (NI)	B (I)	C (I)	D (NI)	E (I)	F (I)	G (NI)	H (I)								
1	**Vill. Bir Talab Basti no.2 distt. Bathinda**	1	2	1	5	1	1	1	1	B, C, E, H	5	3	10	4	50%	80%	
2	**Vill. Bir talab Basti no.3 distt. Bathinda**	2	1	1	1	1	1	1	1	B, C, E, F, H	5	3	9	5	62%	100%	
3	**Vill. Naruana distt. Bathinda**	1	1	2	1	1	1	1	1	B, C, F, H	5	3	9	4	62%	100%	73%
4	**Vill. Nwan pind (Bir behman) distt. Bathinda**	1	1	1	1	3	1	1	2	B, C, E	5	3	11	3	37%	60%	
5	**Vill. Multanian**	1	1	1	1	1	2	1	2	B, C, E, H	5	3	10	4	50%	80%	

	distt. Bathinda																
6	**Vill. Talwandi sabo distt. Bathinda**	1	2	1	1	2	1	1	1	B, E, F, H	5	3	10	4	50%	80%	
7	**Vill. Laleana distt. Bathinda**	1	1	1	1	1	1	1	2	C, E, F, H	5	3	9	4	50%	80%	
8	**Vill. Jajjal distt. Bathinda**	1	1	2	1	1	1	1	2	B, C, E, F, H	5	3	10	5	62%	100%	
9	**Vill. Malkana distt. Bathinda**	1	1	1	1	1	2	1	1	B	5	3	9	1	12%	20%	
10	**Vill. Akalia Kalan, distt. Bathinda**	1	1	1	2	1	1	1	1	B, C, F, H	5	3	9	4	50%	80%	
11	**Vill. Bhaagi vander distt. Bathinda**	1	1	1	1	1	1	1	1	B, C, E	5	3	8	3	37%	60%	
12	**Vill. Nat bager distt. Bathinda**	1	1	1	1	1	1	1	1	B, C, E, H	5	3	8	4	50%	80%	
13	**Vill. Sivian distt. Bathinda**	1	2	1	1	1	1	1	1	B, C, E	5	3	9	3	37%	60%	

14	**Vill. Gill patti distt. Bathinda**	1	1	1	1	1	1	1	2	B, E, F, H	5	3	9	4	50%	80%	
15	**Vill. Deon distt. Bathinda**	1	1	1	1	1	1	1	1	C, E, H	5	3	8	3	37%	60%	
16	**Vill. Mehma sarja distt. Bathinda**	1	1	1	1	2	1	1	1	B, E, F, H	5	3	9	4	50%	80%	
17	**Vill. Rampura phul distt. Bathinda**	1	1	1	1	1	1	1	1	B, C, E	5	3	8	3	37%	60%	
18	**Vill. Bhucho mandi distt. Bathinda**	1	1	1	1	1	1	1	2	B, E, F, H	5	3	9	4	50%	80%	
19	**Vill. Goniana mandi distt. Bathinda**	1	1	1	1	1	2	1	1	B, C, E, F	5	3	9	4	50%	80%	
20	**Vill. Nehian wala distt. Bathinda**	1	2	1	1	1	1	1	1	B, C, E, H	5	3	9	3	37%	60%	

Sample fields (code) indicates: N.I (Non- infected) and I (Infected)

Formulas used for percentage of *V. dahliae* from collected samples-=

Prevalence rate of *V. dahliae* from total 8 sample fields= Total number of fungal strains isolated from 8 sample fields- No of *V. dahliae* isolated from 8 sample fields/100

Prevalence rate of *V. dahliae* from total 8 sample fields= Total number of fungal strains isolated from 5 infected sample fields- No of *V. dahliae* isolated from 5 infected sample fields /100

6.4 Isolation of antagonistic fungal strains from organic manures

In this present investigation, isolation of antagonistic fungal strains was performed by using PDA plates from different types of organic manures by Serial dilution method. A total of 12 antagonistic fungal strains have been isolated from different organic manures (Bio manure, Animal manure, Vermicompost, Poultry manure, Green manure, Oil seed manure, Farmyard and Compost manure) using PDA medium **(Figure 6.9)**. Maximum number of fungal cultures (3) have been isolated from Animal manure followed by two each from Bio manure and compost manure and single fungus has been isolated from Oil seed, Poultry and Vermicompost manure **(Table 6.6)**. Similar study has been done by (Oka and Yermiyahu., 2002; Bailey and Lazarovits., 2003) in which antagonistic strains have been isolated form compost manure and lead to the development of an effective management against various disease caused by nematode, fungi and bacterial strains. Study of Cotxarrera et al., 2002; Kavroulakis et al., 2005, have confirmed that field compost is full of diversity with different microbes, and helps to suppress various disease more consistently than compost produced in an enclosed area (Kuter et al., 1983).

Table 6.6 Isolation of antagonistic fungal strains from organic manures

Type of manure	Organism code	Colony characteristics	Number of fungus isolated
Oil seed manure (OSM)	OSM/Y/F1	Yellowish White (white in center and yellow at Periphery)	1
BIO MANURE (BM)	BM/W/F1	White colour (parallel growth covering full Petri plate)	2
	BM/DG/ F2	Dull green in colour, parallel growth and covered full petri plate	
ANIMAL MANURE	AM/BG/F1	Black green colour (parallel growth covering full Petri plate)	3

(AM)	Am/B/F2	Black in colour (Parallel growth, covering full petri plate)	
	AM/GG/F3	Greyish Green in colour, Parallel growth, covering full petri plate)	
COMPOST MANURE(CM)	CM/DG/F1	Dark green colour (perpendicular growth covering full Petri plate)	2
	CM/B/F2	Brown in colour (parallel growth in center)	
Poultry Manure (PM)	PM/G/F1	Green in colour, parallel growth covering full petri plate	1
Vermicompost	VC/LB/F1	Light Brown in colour, parallel growth, covering full Petri plate	1

6.5 Isolation of antagonistic fungal strains from rhizospheric soil

Isolation of antagonistic fungal strains was carried out from rhizospheric soil of healthy cotton growing areas in Bathinda region by using PDA medium. Different types of antagonistic fungus have been isolated and purified. A total of 3 antagonistic fungal strains have been isolated from rhizospheric soil of healthy cotton plants. Different strains isolated from rhizospheric soil have been purified by using PDA plates and stored for further use.

6.6 Isolation of endophytic bacterial strains from healthy plant parts

Endophytic bacterial strains have been isolated from healthy cotton plant parts viz. roots, stems and leaves. A total of 7 endophytic bacterial strains have been isolated on Nutrient Agar medium (NAM) by streaking method. Different bacterial strains were purified by streaking using NA plates **(Figure 6.10)**. Total 7 endophytic bacterial strains have been isolated from collected plant parts. viz. VTS DB-V1/NI /L/S1/B2 from healthy stem of cotton plant, VG DB-V1/NI /L/S1/B2 has been isolated from healthy stem of cotton, VG DB-V2/NI /L/S1/B1 from healthy leaves of cotton plant,

VG DB-V1/NI /S/S1/B1 has been isolated from healthy stem of cotton plant, VTS DB-V1/NI /S/S1/B2 from healthy stem of cotton plant, VTS DB-V1/NI /R/S1/B2 was isolated from healthy cotton root and VTS DB-V1/NI /S/S1/B1 has been isolated from healthy stem of cotton plant collected from different sites of Bathinda, Punjab, India. Similar study has been done by Mcinroy and Kloeeper., 1995, in which isolation of endophytic bacterial strains from cotton roots and stem tissues has been reported. These 7 isolates were further screened through antagonistic test and positive bacterial strains were identified through 16s rRNA sequencing. 39 endophytic bacterial strains have been isolated and tested for their antagonism against *Verticillium* wilt of cotton (Li et al., 2012).

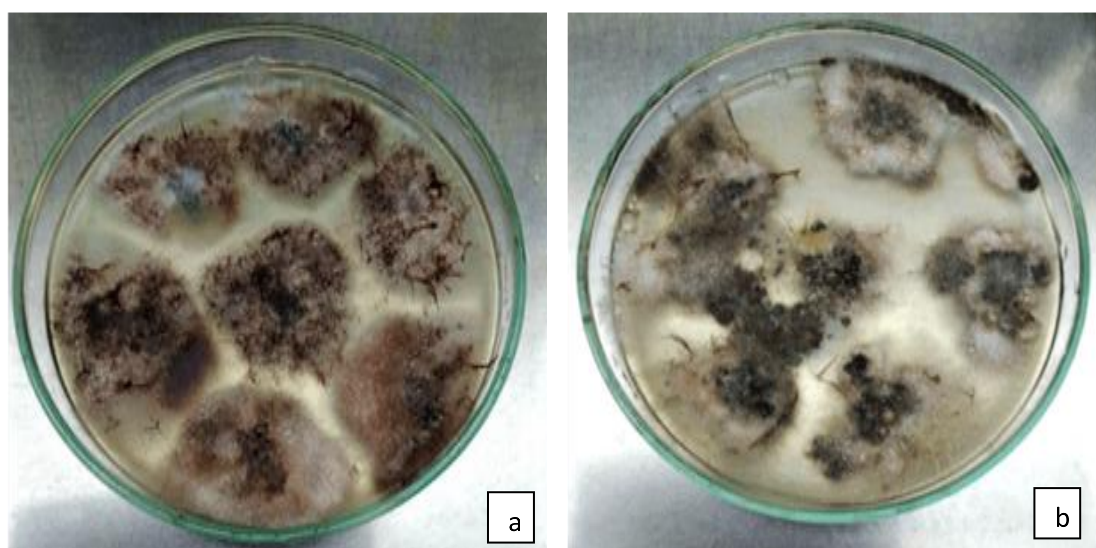


Figure 6.5: (a, b) Pathogenic fungal strains isolated from infected cotton leaves, roots

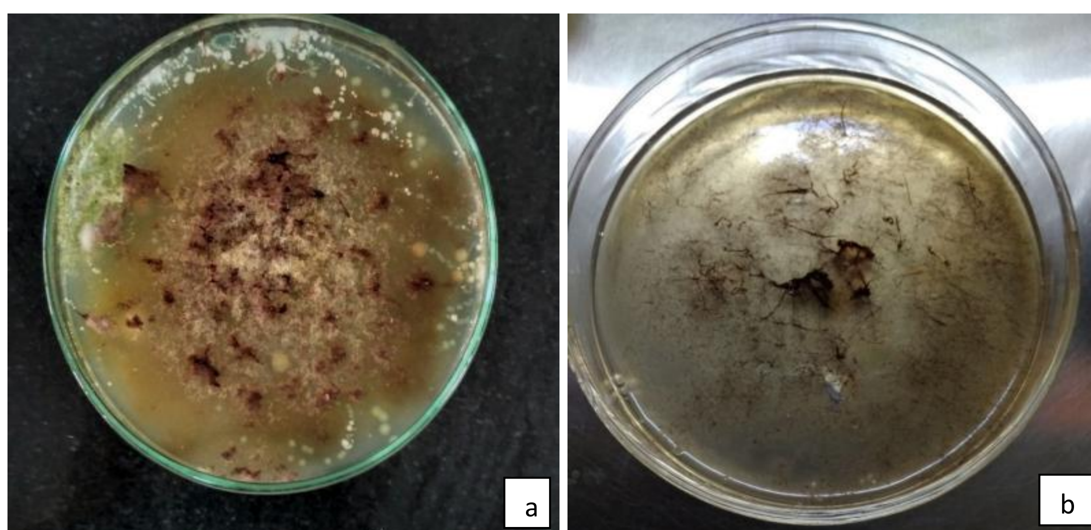


Figure 6.6: (a) Pathogenic fungal strains isolated from rhizospheric soil of infected cotton field (b) Pathogenic fungal strain isolated from bulk soil of infected cotton field

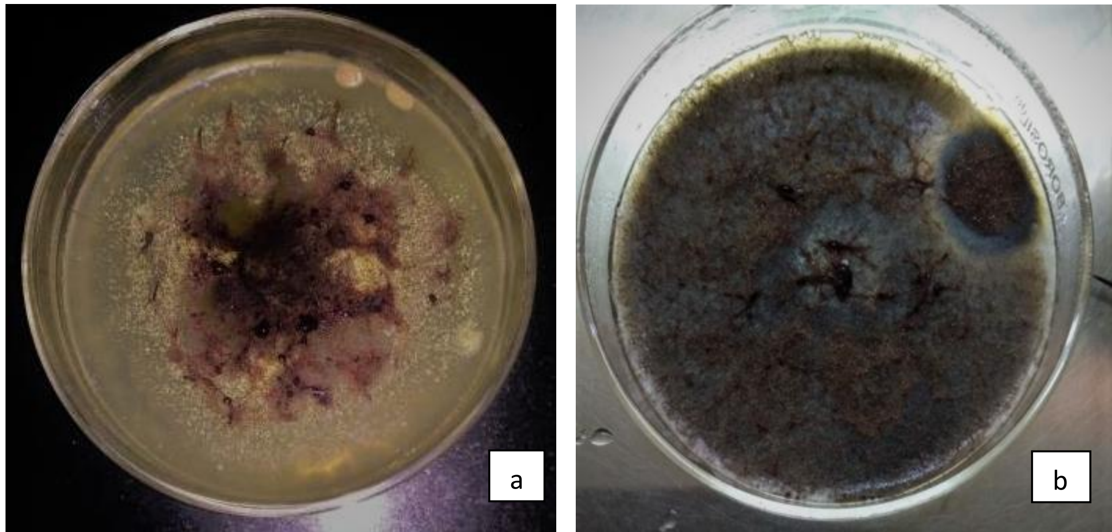


Figure 6.7: Isolation of *Vertcillium dahliae* in PDA plates- (a) Colony of *V. dahliae* (7 days) (b) Colony of *V. dahliae* after 14 days of inoculation

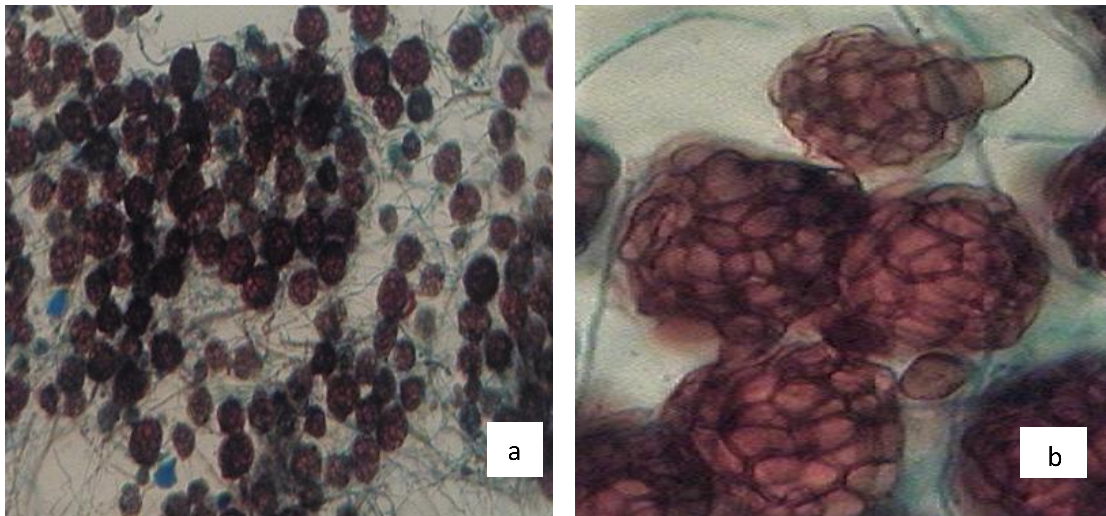
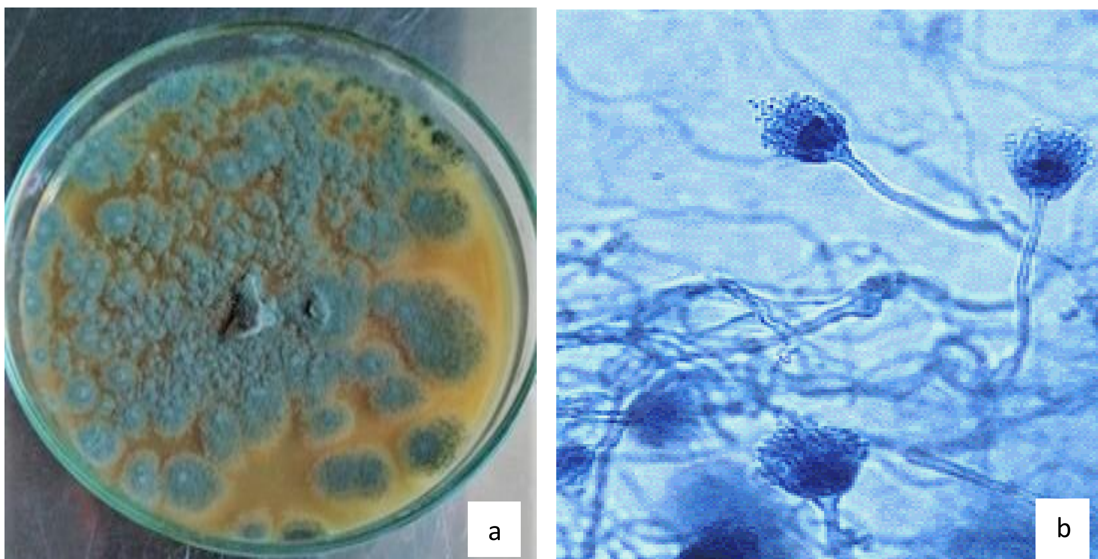
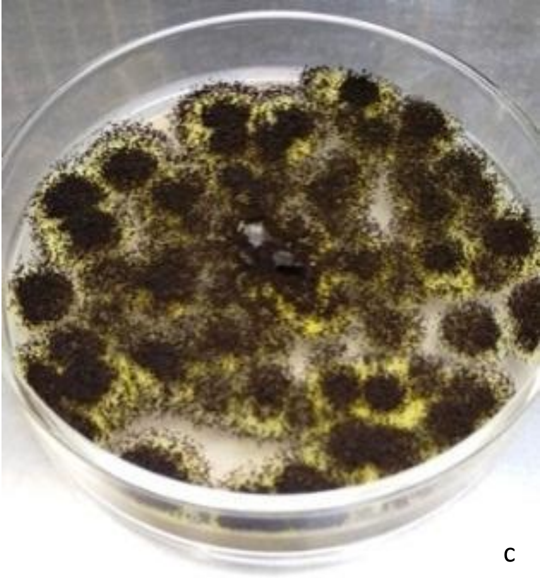


Figure 6.8: (a) Microsclerotia of *V. dahliae* under light microscope (10x) (b) Microsclerotia of *V. dahliae* under light microscope (40x)

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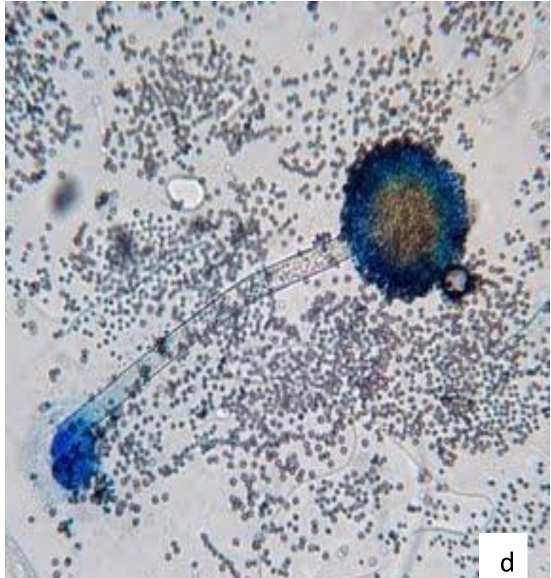
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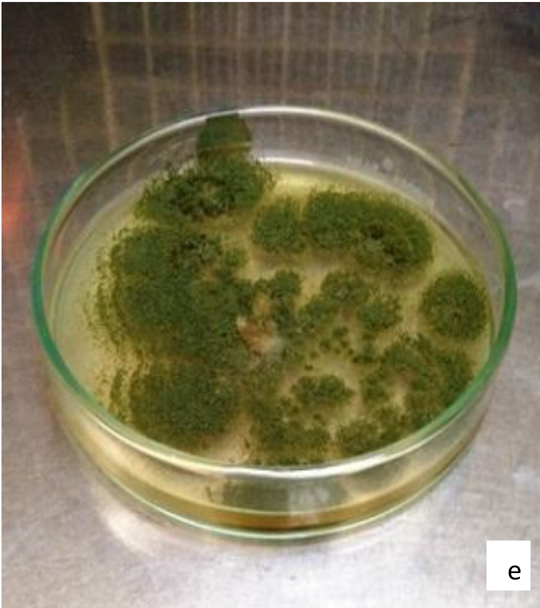
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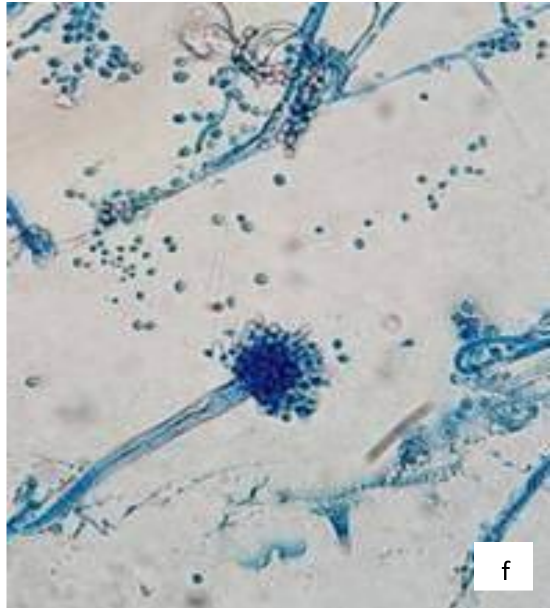
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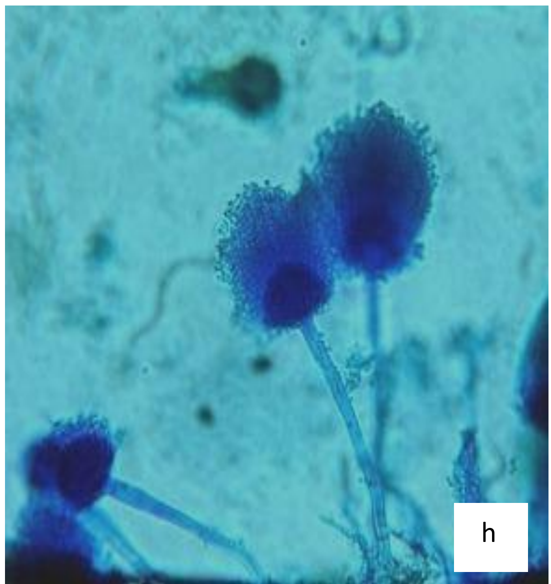
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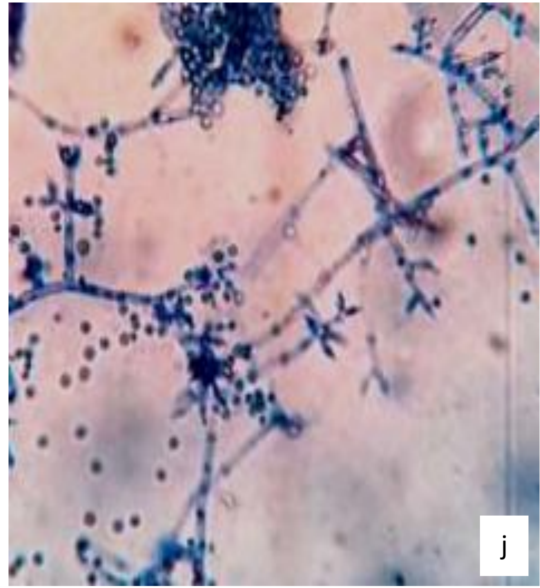
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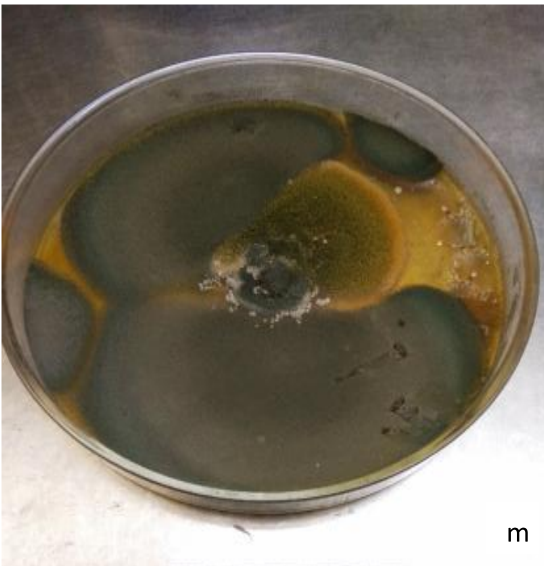
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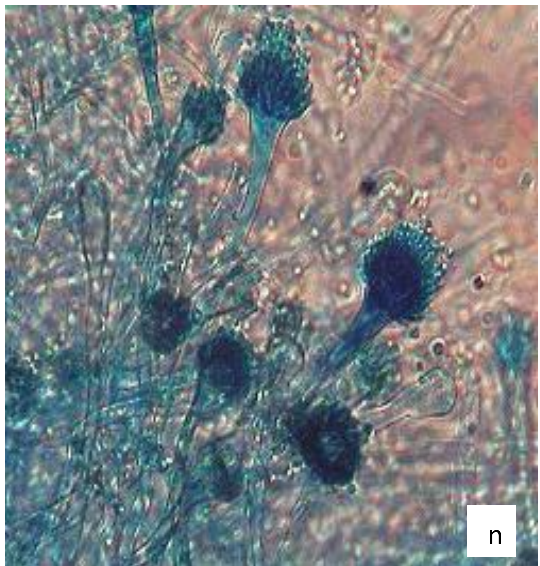
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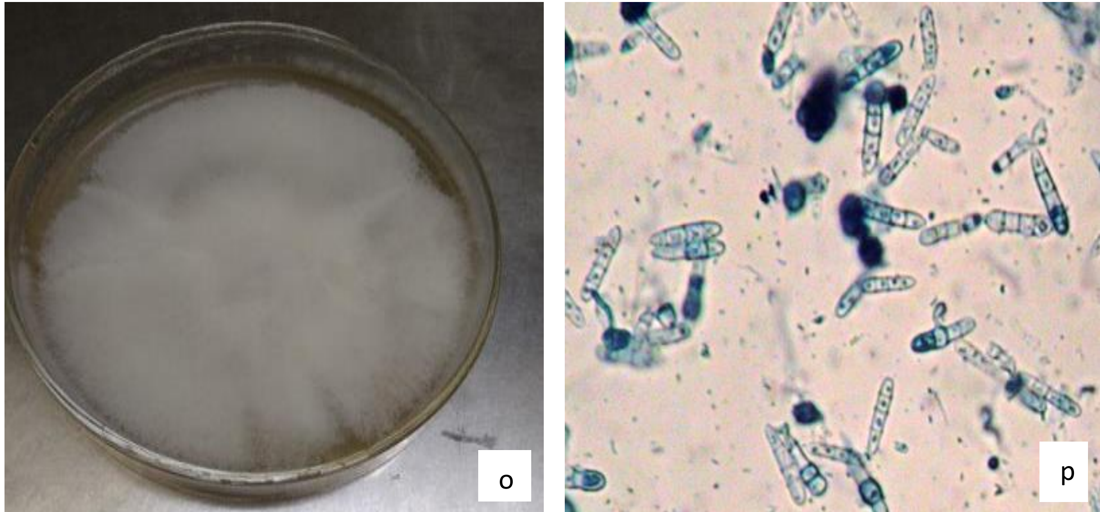
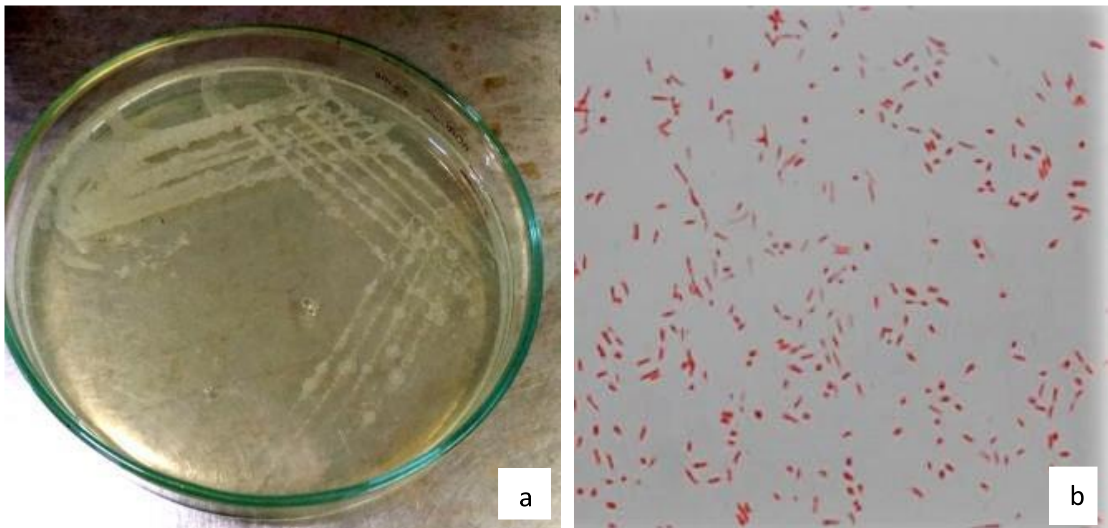
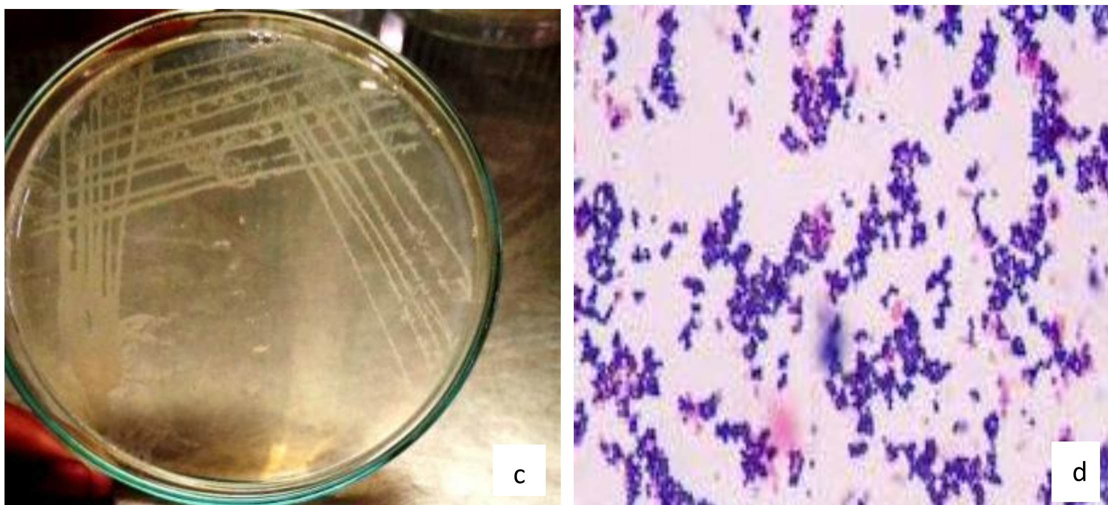


Figure 6.9: Colony of antagonistic strains on PDA after 7 days of incubation at 28±2^{0}C and microscopic structure stained with lactophenol cotton blue (40X) (a, b) *Aspergillus fumigatus*, (c, d) *Aspergillus niger*, (e, f) *Aspergillus nidulans* (g, h) *Aspergillus terreus* (i, j) *Trichoderma harzianum* (k, l) *Gliocladium sp*., (m, n) *Cladosporium sphaerospermum* (o, p) *Fusarium solani*





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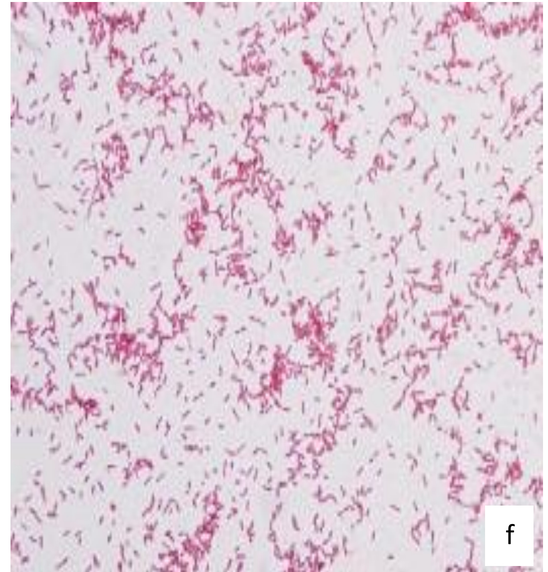
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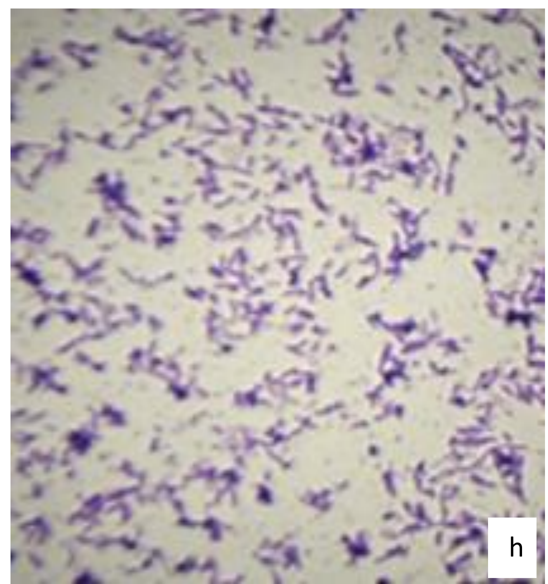
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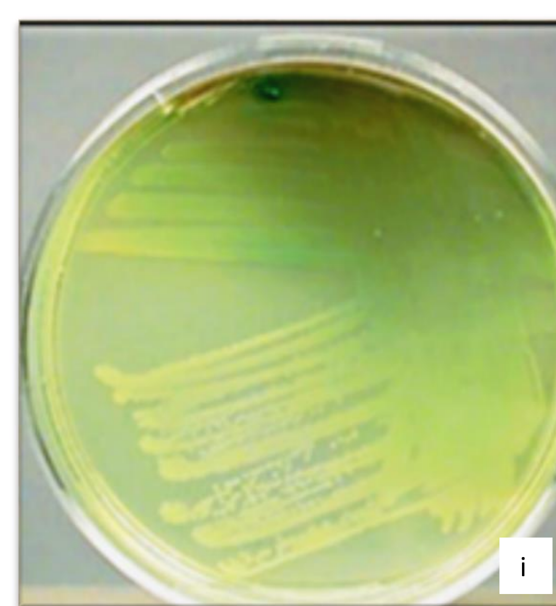
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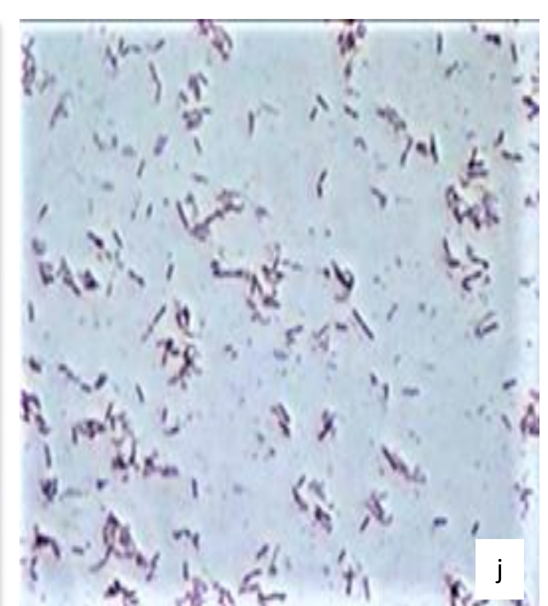
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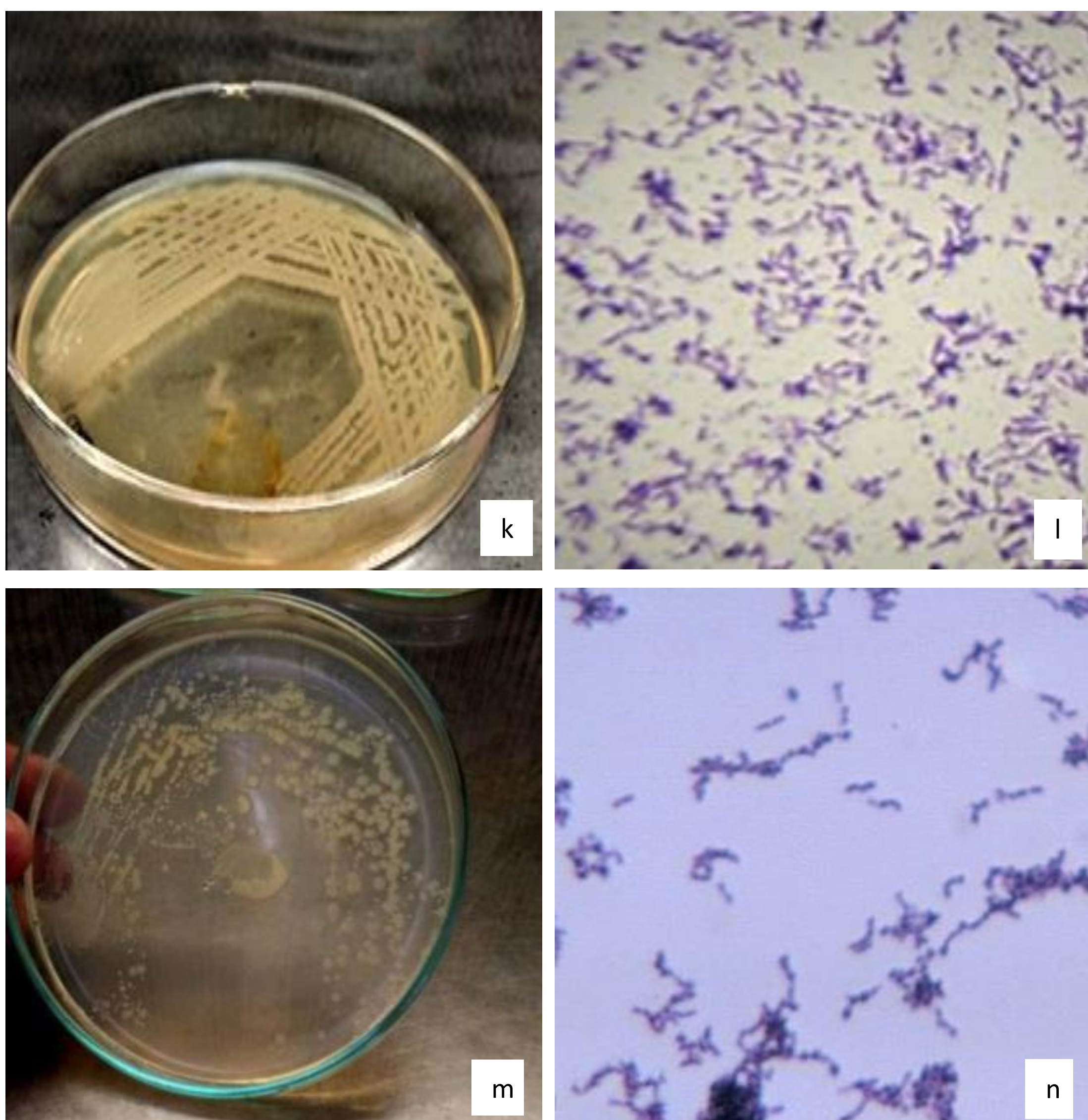


Figure 6.10- Colonies of endophytic bacterial strains along with gram staining under light microscope (a, b)- VTS DB-V1/NI /L/S1/B2 **(c, d)** VG DB-V1/NI /L/S1/B2 **(e, f)** VG DB-V2/NI /L/S1/B1 **(g, h)** VG DB-V1/NI /S/S1/B1 **(i,j)** VTS DB-V1/NI /S/S1/B2 **(k,l)**VTS DB-V1/NI /R/S1/B2 **(m,n)** VTS DB-V1/NI /S/S1/B1

6.7 Identification of microbial strains

Verticillium dahliae has been isolated from infected cotton plant parts, rhizospheric and bulk soil of cotton fields. *V. dahliae* has been preliminary identified by their morphological colony characters, air hyphae and structure of microsclerotia (Figure 6.8). Conidiophores are erect, hyaline, verticillately branched and septate. Phialides frequently arise directly from hyphae. Microsclerotia of *V. dahliae* is dark brown to black in colour, thick walled and abundant with almost swollen globular cells (Inderbitzin et al., 2011). However, morphological and microscopic examination may not give actual species for a particular organism. Based on this, molecular 18S rRNA

gene identification by PCR was conducted by outsourcing through Science Farmers, Bangalore India by DNA extraction method. Integrated Transcribed Spacer (ITS1 5´ -GTAGTCATATGCTTGTCTC-3´) and (ITS4 5´ -CTTCCGTCAATTCCTTTAAG-3´) primers were amplified by 18S rRNA gene region, sequenced and analyzed for similarity among species in NCBI data base. Figure 6.12 shows the phylogenic tree based on ITS region gene sequences showing relationship between fungal strain *Verticillium dahliae* and the closest species of *Verticillium zaregamsianum* based on the neighbor-joining analysis.

A total of 9 antagonistic fungal strains have been isolated from different organic manures and identified by studying their resting spore size, structure, hyphae under light microscope by making their LCB mounts. 2 strains of *Aspergillus* viz. *A. niger, A. nidulans*, were identified under light microscope (Gilman., 2002). *Aspergillus niger* has smooth conidia and conidiophores. The conidiophores are having septate and hyaline hyphae and becomes dark at the apex and terminating into a globose vesicle. *A. nidulans*, have septate and hyaline hyphae. Conidiophores are brown, short and smooth-walled, conidia are globose and rough. *Cladosporium sphaerospermum* has thick walls, septate and olive brown color. Fungal colonies have a velvety texture and are flat. The conidia are branched, septal, dark and tree-like. The *Trichoderma harzianum* has branched conidia that cluster in a bundle. They have wide, straight / flexible branches. The conidiophores are located at the tip of the hyaline hyphae and *Gliocladium sp*. were identified by examining their pore size, structure and hyphae, and were identified under a light microscope.

Fusarium solani, Aspergillus fumigatus and *Aspergillus terreus* were identified by Indian Type Culture Collection (ITCC), Division of Plant Pathology, Indian Agriculture Research Institute, New Delhi. Colonies of antagonistic fungal strains have been purified by using PDA plates and microscopic structure has been studied under light microscope **(Figure 6.9)**. 3 antagonistic fungal strains have been isolated from rhizospheric soil of healthy cotton plants and they are identified as *Aspergillus niger*, *A. nidulans* and *Fusarium solani.*

A total of 7, endophytic bacterial strains have been isolated and purified **(Figure 6.10)** out of which 3 strains have shown most positive antagonistic activity during *in vitro* screening hence used for *in vivo* trials. Bacterial strains were identified as a first

through microscopic and morphological examination. However, morphological and microscopic examination may not give actual species for a particular organism. Based on this, molecular 16S rRNA gene identification by PCR was conducted by outsourcing through Barcode Bioscience Bangalore India by DNA extraction method (**Figure 6.11**). Integrated Transcribed Spacer (ITS1 5´ -GTAGTCATATGCTTGTCTC-3´) and (ITS4 5´ -CTTCCGTCAATTCCTTTAAG-3´) primers were amplified by 16S rRNA gene region, sequenced and analyzed for similarity among species in NCBI data base. 3 endophytic bacterial strains have been identified through 16s rRNA and named as VTS DB-V1/NI/S/S1/B2 (*Pseudomonas aeruginosa)*, VTS DB-V1/NI /R/S1/B2 (*Bacillus subtilis*)**,** VTS DB-V1/NI /S/S1/B1(*Lysinibacillus macroides*).

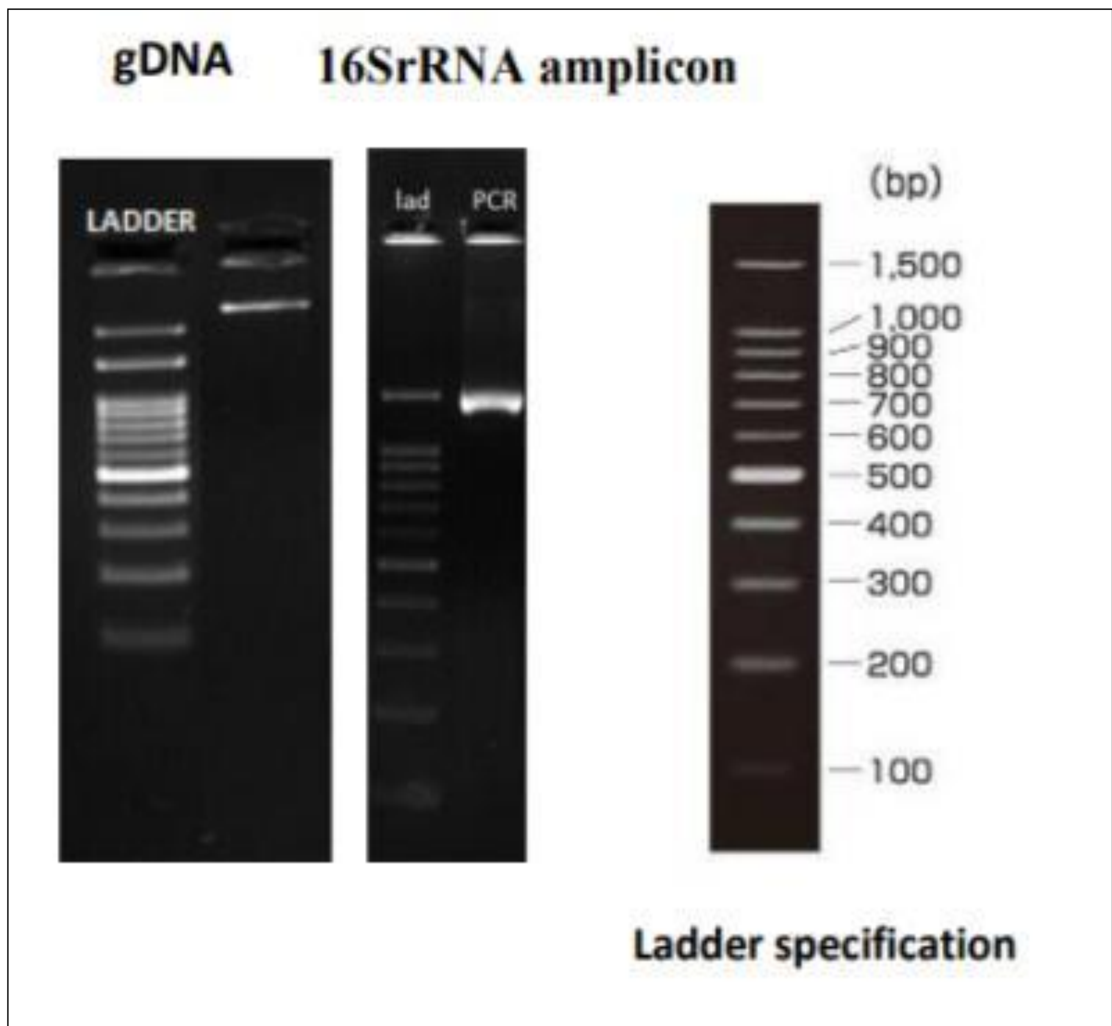


Figure 6.11: gDNA and 16S Amplicon QC data

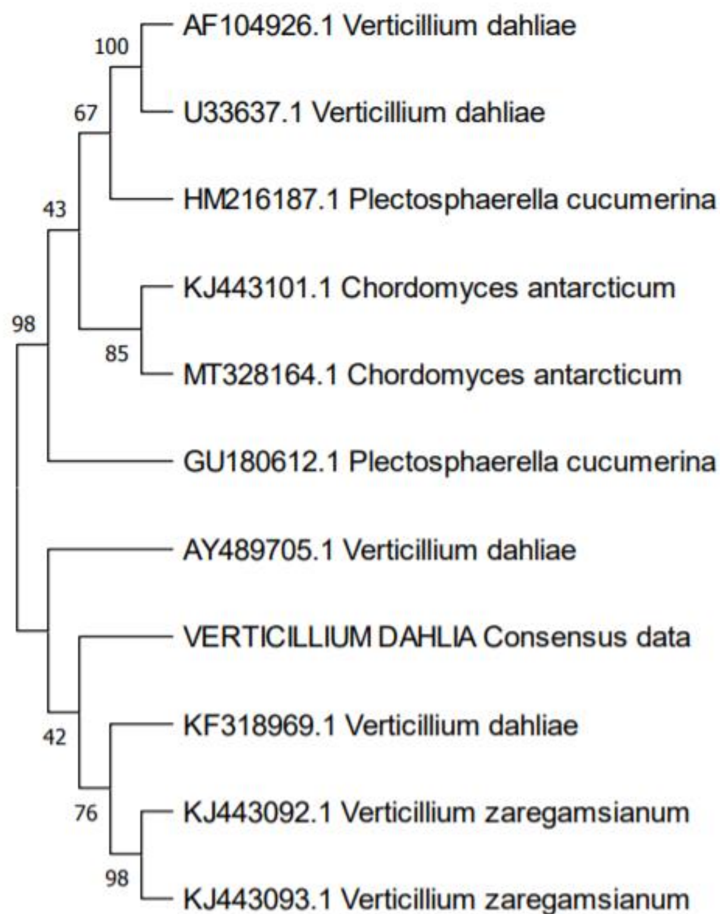


Figure 6.12: Phylogenic tree based on ITS region gene sequences showing relationship between fungal strain *Verticillium dahliae* and the closest species of *Verticillium zaregamsianum* based on the neighbor-joining analysis

6.8 Selection of Antagonistic Fungal Strains as Potential Biocontrol Agent

6.8.1 Preliminary screening of antagonistic fungal strains by Dual Culture Assay

Preliminary screening of antagonistic fungal strains against *V. dahliae* was performed by Dual culture assay. The results for the growth of *V. dahliae* and different antagonistic fungal strains in control and test plates during Dual culture assay has been shown in **Figure 6.13.** Different antagonistic strains have shown *in vitro* antagonistic activity towards *V. dahliae*. Experiment has been performed in triplicates. The result has been recorded from 0 to 144 hours of incubation. The result depicted in **Figure 6.14** shows the growth of *V. dahliae* in control and growth of both *V. dahliae* and *A. nidulans* in test plate from 0 hours to 144 hours of incubation in which *V. dahliae* has shown 48mm of growth as control plate and 16mm during test

plate *V. dahliae* along with 42mm growth of *A. nidulans* at 144 hours of incubation. Percentage inhibition of radial growth (PIRG) is 61.12% exhibited by *A. nidulans*. The result for *in vitro* screening of *T. harzianum* against *V. dahliae* has shown in **Figure 6.15** in which growth *V. dahliae* in control and growth of both *V. dahliae* and *T. harzianum* in test plate from 0 to 144 hours of incubation has been measured. *V. dahliae* has shown 48mm growth as control plate and in test plate (12mm) growth of *V. dahliae* along with 46mm growth of *T. harzianum* has been calculated at 144 hours of incubation. In *in vitro screening*., PIRG exhibited by *T. harzianum* against *V. dahliae* is 72.91%. The results in **Figure 6.16** depicts the growth of *V. dahliae* and *A. niger* during *in vitro* screening., growth of *V. dahliae* in control and growth of both *V. dahliae* and *A. niger* in test plate from 0 to 144 hours of incubation has been measured., in which *V. dahliae* has shown 52mm of growth in control plate and during test plate *V. dahliae* has shown 17.5mm along with 43mm growth of *A. niger* at 144 hours of incubation. *A. niger* has shown 63.20% inhibition activity against *V. dahliae*. The results in Figure **6.17** shows the growth of *A. terreus* against *V. dahliae* in which growth *V. dahliae* in control and growth of both *V. dahliae* and *A. terreus* in test plate from 0 to 144 hours of incubation has been calculated. *V. dahliae* has shown 54mm growth in control plate and in 12mm growth of *V. dahliae* in test plate along with 47mm growth of *A. terreus* at 144 hours of incubation has been recorded against *V. dahliae*. PIRG of *A. terreus* against *V. dahliae* is 59.90% during *in vitro* screening. The results in **Figure 6.18** depicts the growth of *Gliocladium sp*., against *V. dahliae* in which *V. dahliae* has shown 52mm growth in control plate and *Gliocladium sp*., against *V. dahliae* has shown 48mm and 17mm growth respectively in test plate at 144 hours of incubation. PIRG of *Gliocladium sp*. during *in vitro* screening is 61.81%. The results in **Figure 6.19** depicts the growth of *Cladosporium sp*., against *V. dahliae* in which *V. dahliae* has shown 50mm growth in control plate and *Cladosporium sp*., against *V. dahliae* has shown 32mm and 27.5mm growth respectively in test plate from 0 hours incubation to 144 hours of incubation. PIRG of *Cladosporium sp*. against *V. dahliae* is 6.95% during *in vitro* screening**.** The results in **Figure 6.20** depicts the growth of *Fusarium solani* against *V. dahliae* in which growth *V. dahliae* in control and growth of both *V. dahliae* and *Fusarium solani* in test plate from 0 to 144 hours of incubation has been calculated. *V. dahliae* has shown 52mm growth in control plate and in test plate 22mm growth of *V. dahliae* along with 36mm growth of *F. solani* at 144 hours of incubation has been recorded against *V.*

dahliae. In total., PIRG of *Fusarium solani* is 47.22%. The result in **Figure 6.21** depicts the growth of *A. fumigatus* against *V. dahliae* in which *V. dahliae* has shown 54mm growth in control plate and *A. fumigatus* against *V. dahliae* has shown 46mm and 12mm growth respectively in test plate at 144 hours of incubation. During *in vitro* screening, antagonistic activity of *A. fumigatus* is 73.46% against *V. dahliae*. The results in **Table 6.7** clearly shown that most significant percentage inhibition of radial growth (PIRG) (73.46%) in *Aspergillus fumigatus* followed by *T. harzianum* (72.91%), *Aspergillus niger* (63.20%), *Aspergillus nidulans* (61.12%), *Gliocladium sp.* (61.81%), *Aspergillus terreus (59.90%), Fusarium solani* (47.22%) and *Cladosporium sphaerospermum* (6.95%) towards *V. dahliae* during Dual culture assay. Out of all antagonistic strains *Aspergillus fumigatus* has shown most positive antagonism against *V. dahliae* during preliminary screening and *Cladosporium sphaerospermum* shown least percentage inhibition against of radial growth. Dual culture method is the perfect choice for initial *in vitro* screening., it helps to eliminate host plant pathogen and it finds the direct antagonism by antibiosis (Khan., 2013). Similar work has been done by Naraghi et al., 2012 in which *T. flavus* has been used for the control of *Verticillium* wilt in cotton and also in tomato, potato and eggplant. According to the study of Ibara Medina et al., 2010 in which *Trichoderma sp.* has shown positive antagonistic activity and helps to inhibit growth of pathogenic strain. Antibiotic compounds and lytic enzymes are released, which can lead to hyphal attachment and direct penetration into the host during the interaction of hyphal and fungal mechanisms. In fungal mycoparasitism, the fungus acquires its nutrients directly or indirectly by invading another fungus (Gao et al., 2010). Physical, chemical or nutritional conditions are the main factors that determined the ability of a fungal strain to parasite another fungus (Benítez et al., 2004). Statistical analysis of **Figure 6.14** depicts no significant difference between *V. dahliae* and *Aspergillus nidulans* at 24, 48 and 72 hours of incubation and denoted by same alphabets. *V.dahliae* and *Aspergillus nidulans* shows different activity at 96,120,144 hours of incubation hence denoted by different alphabets. Statistical analysis of **Figure 6.15** depicts a least significant difference (LSD) has shown in *Trichoderma harzianum* at (48, 72 96,120,144 hours) denoted by different alphabets between compared means in columns and no significant difference between compared means was observed in *Trichoderma harzianum* at (0, 24, hours of incubation) and *V. dahliae* at (0, 24, 48, 72, 96,120 incubation hours) denoted by same alphabets. Statistical analysis of

Figure 6.16 shows a least significant difference (LSD) in *Aspergillus niger* at (48,72 96,120,144 hours of incubation) denoted by different alphabets between compared means in columns and no significant difference between compared means was observed in *Aspergillus niger* at *(*0,24 hours) and *V. dahliae* during 144 hours of incubation hence denoted by same alphabets. **Figure 6.17** shows a least significant difference (LSD) in *A. terreus* (48, 72 96,120,144 hours of incubation) denoted by different alphabets between compared means in columns and no significant difference between compared means was observed in. *Aspergillus terreus* at *(*0, 24 hours of incubation) and *V. dahliae* at (0, 24 incubation hours) denoted by same alphabets. Statistical analysis of Gliocladium *sp.* at (96,120,144 hours of incubation) denoted by different alphabets between compared means in columns and no significant difference between compared means was observed in *Gliocladium sp.* at (0,24,48,72 hours of incubation) and *V. dahliae* at (9,120 incubation hours) denoted by same alphabets. A lack of LSD shows that *V. dahliae* as well as *Gliocladium sp.* shows the same activity at different hours of incubation but LSD between columns shows the different activity of *V. dahliae* and *Gliocladium sp.* **(Figure 6.18)**. **Figure 6.19** describes no significant difference between *Cladosporium sphaerospermum* at 0, 24,48 and 144 hours of incubation, denoted by same alphabets but shows significant difference during remaining incubation hours (different alphabets). *V.dahliae* shows no difference at (24,72,96,120 hours of Incubation) and shows different activity at 0,48,144 hours of incubation hence denoted by different alphabets. Statistical analysis of **Figure 6.20** describes a least significant difference (LSD) has shown in *Fusarium solani at* (48, 72 96,120,144 hours) denoted by different alphabets between compared means in columns and no significant difference between compared means was observed in *Fusarium solani* at *(*0, 24, hours of incubation) and *V. dahliae* at (0, 24, 48, 72, 96,120 incubation hours) denoted by same alphabets. Statistical analysis of **Figure 6.21** describes a least significant difference (LSD) has shown in *Aspergillus fumigatus at* (48, 72 96,120,144 hours) denoted by different alphabets between compared means in columns and no significant difference between compared means was observed in *A. fumigatus* at *(*0, 24, hours of incubation) and *V. dahliae* at (0, 24, 48, 72, 96,120 incubation hours) denoted by same alphabets. A lack of LSD shows that *V. dahliae* as well as antagonistic strains shows the same activity at different hours of incubation but least significant difference between compared means in columns shows the different activity of *V. dahliae* and antagonistic strains.

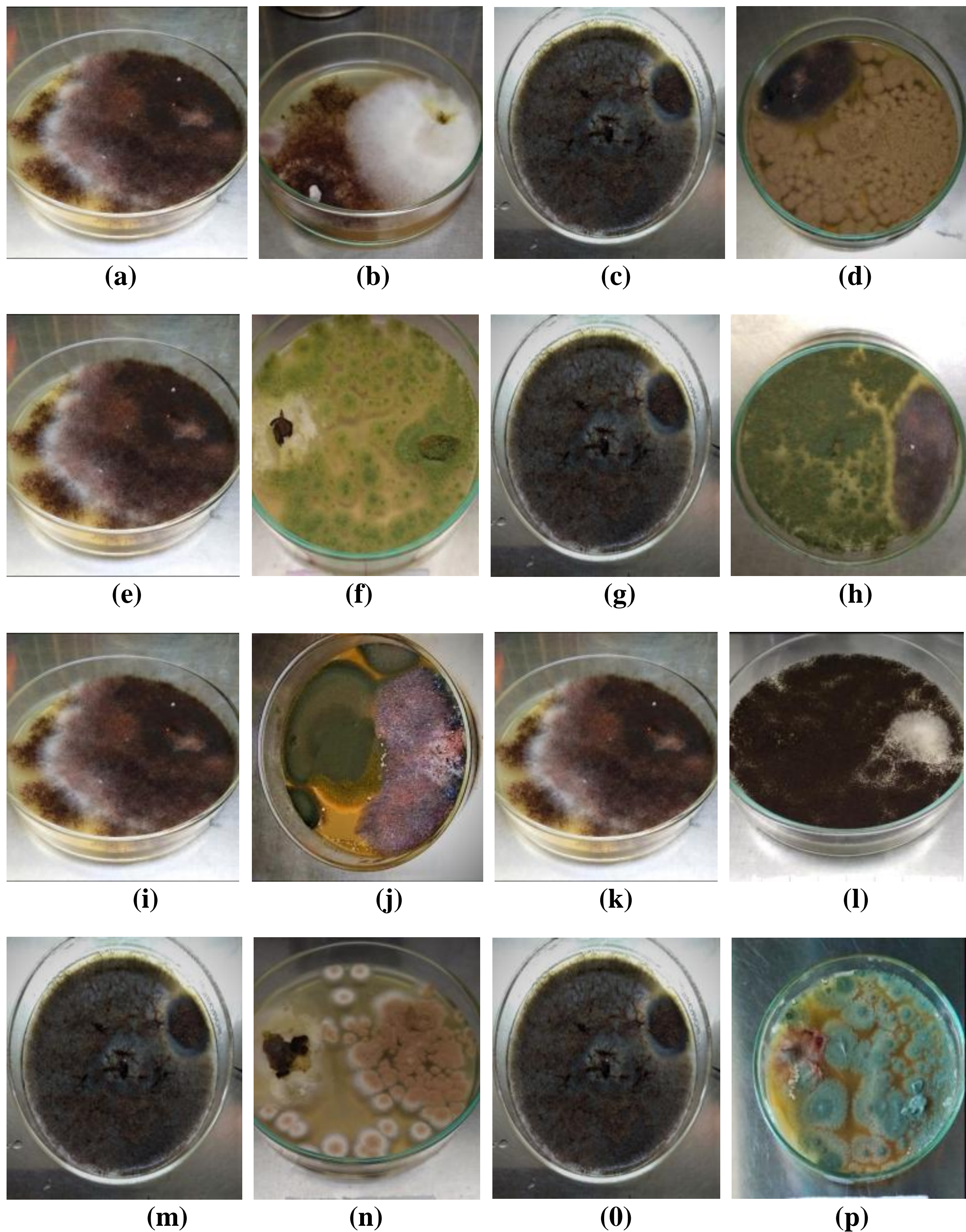


Figure 6.13: ***in vitro*** **antagonism of antagonistic fungal strains towards** ***Verticillium dahliae*** **during "Dual Culture Assay" along with control plates (a) Growth of** ***V. dahliae*** **in control plate (b) Growth of** ***Fusarium solani*** **against** ***V. dahliae*** **in test plate (c) Growth of** ***V. dahliae*** **in control plate (d) Growth of** ***Gliocladium sp.*** **against** ***V. dahliae*** **in test plate (e) Growth of** ***V. dahliae*** **in control plate (f) Growth of** ***A. nidulans*** **against** ***V. dahliae*** **in test plate (g) Growth of** ***V. dahliae*** **in control plate (h) Growth of** ***T. harzianum*** **against** ***V. dahliae*** **in test plate (i) Growth of** ***V. dahliae*** **in control plate (j) Growth of** ***Cladosporium sphaerospermum*** **against** ***V. dahliae*** **in test plate (k) Growth of** ***V. dahliae*** **in control plate (l) Growth of** ***A. niger*** **against** ***V. dahliae*** **in test plate (m) Growth of** ***V. dahliae*** **in control plate (n) Growth of** ***A. fumigatus*** **against** ***V. dahliae*** **in test plate (0) Growth of** ***V. dahliae*** **in control plate (p) Growth of** ***A. terreus*** **against** ***V. dahliae*** **in test plate**

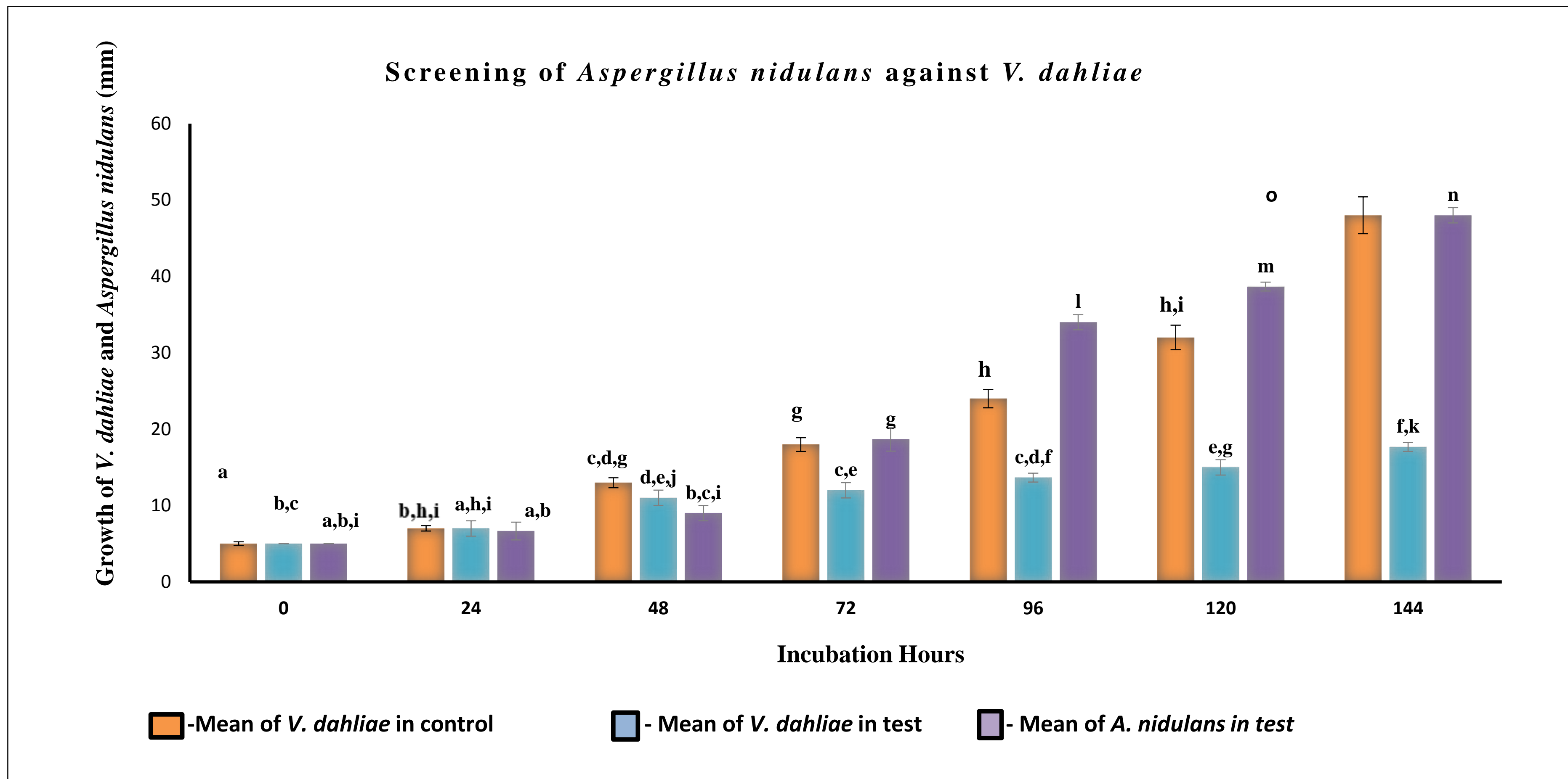


Figure 6.14: *in vitro* **screening of** *Aspergillus nidulans* **against** *Vertcillium dahliae* **in Dual Culture** *Assay* **after every 24 hours of incubation (Standard error bars are indicated. Means are Mean with different letters denote a significant difference between compared producers; same letters in columns denote a lack of significant differences between compared means (ANOVA. p≥0.05))**

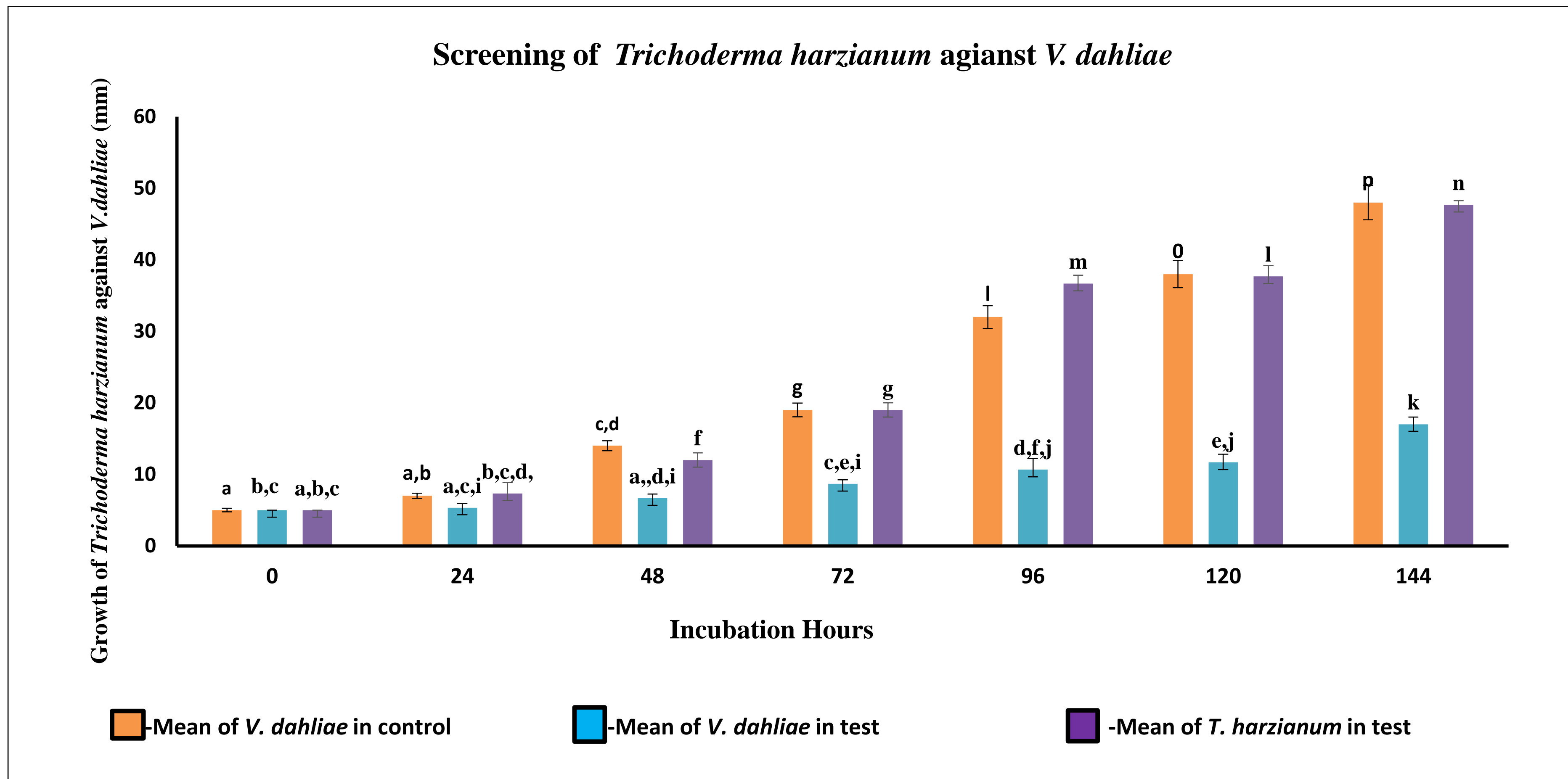


Figure 6.15: ***in vitro*** **screening of** ***Trichoderma harzianum*** **against** ***Vertcillium dahliae*** **in Dual Culture Assay after every 24 hours of incubation (Standard error bars are indicated. Mean with different letters denote a significant difference between compared producers; same letters in columns denote a lack of significant differences between compared means (ANOVA. p≥0.05))**

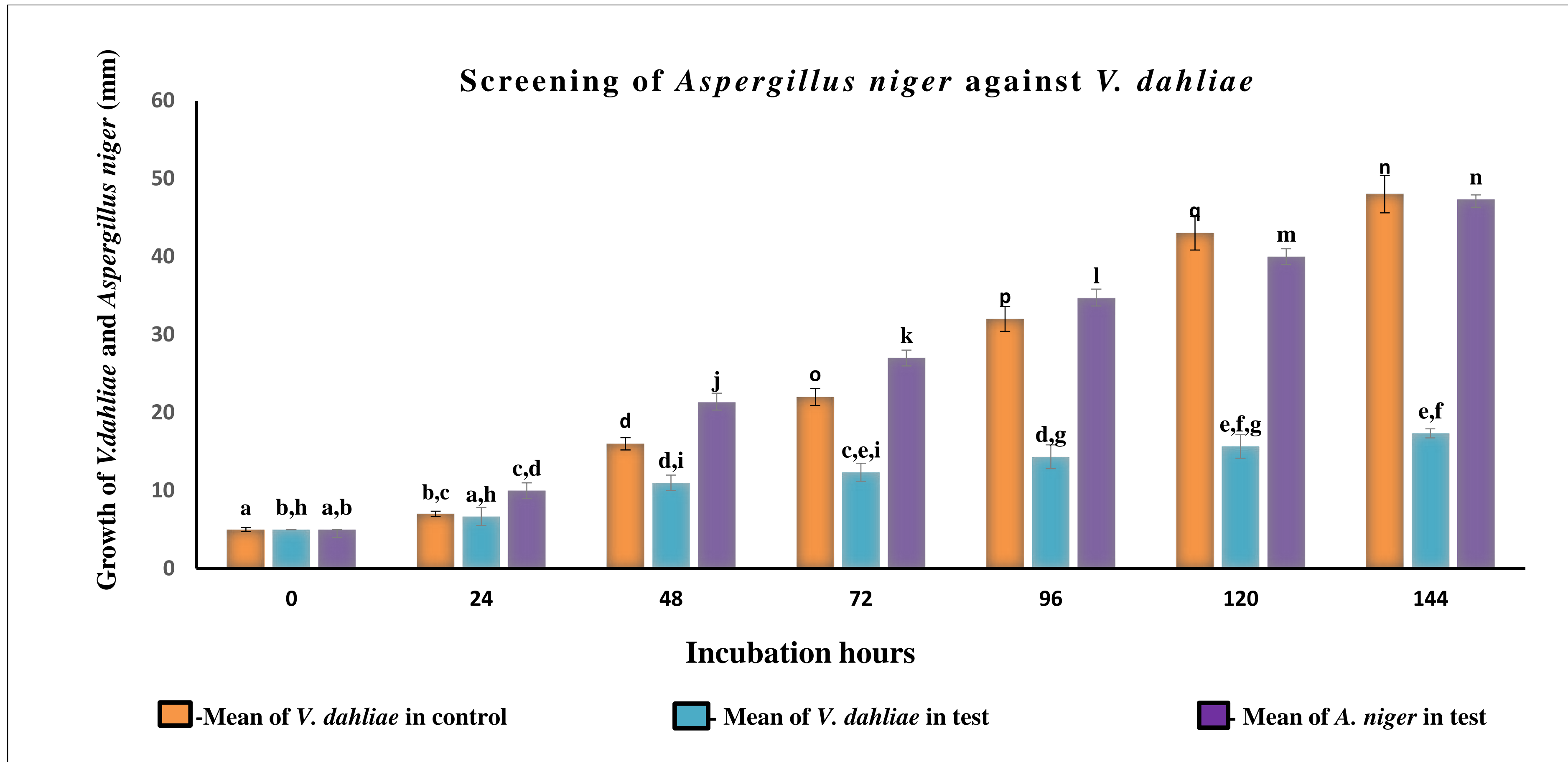


Figure 6.16: ***in vitro*** **screening of** ***Aspergillus niger*** **against** ***Vertcillium dahliae*** **in Dual Culture Assay after every 24 hours of incubation (Standard error bars are indicated. Mean with different letters denote a significant difference between compared producers; same letters in columns denote a lack of significant differences between compared means (ANOVA. p≥0.05))**

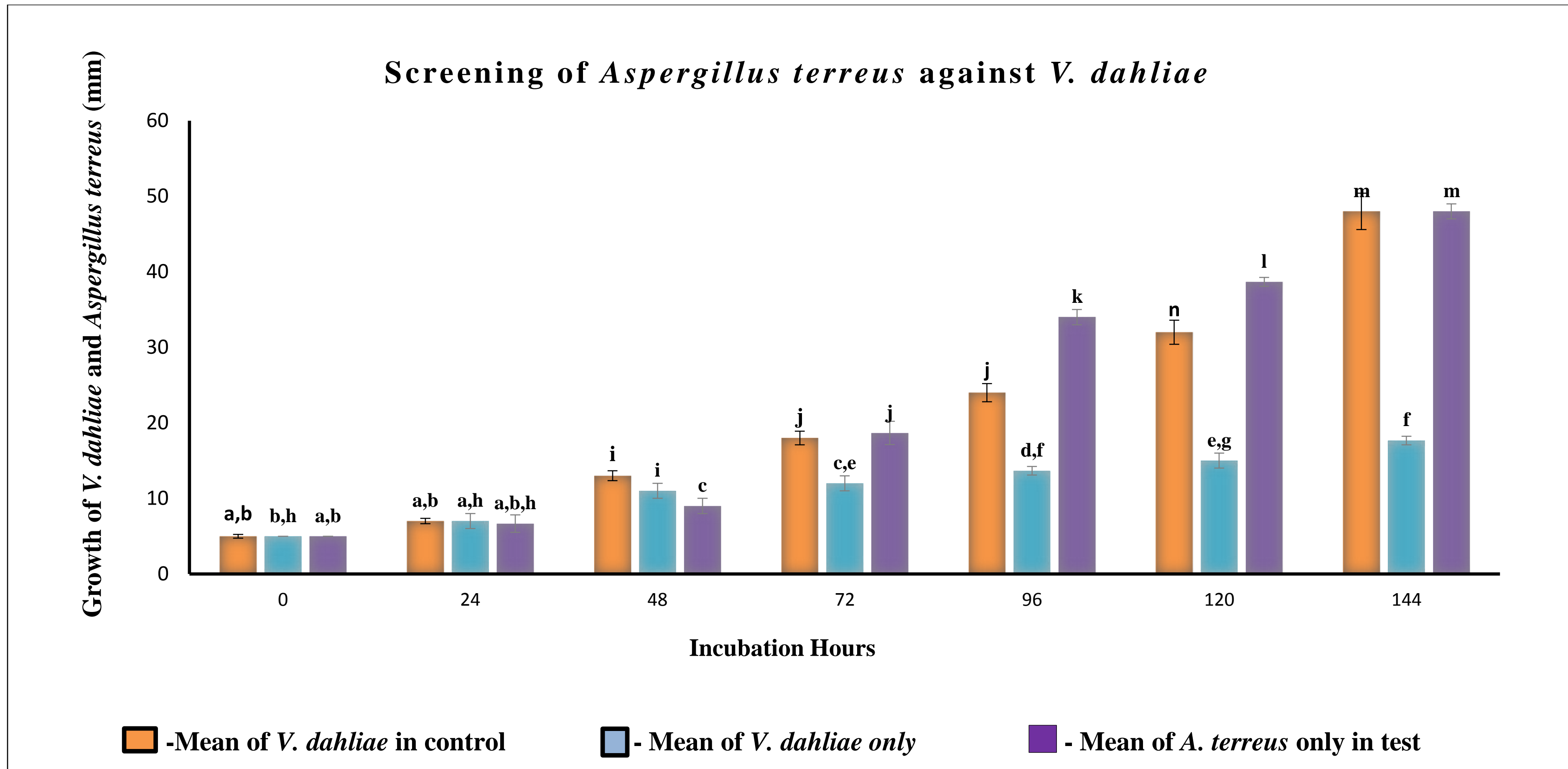


Figure 6.17: ***in vitro*** **screening of *Aspergillus terreus* against *Vertcillium dahliae* in Dual Culture Assay after every 24 hours of incubation (Standard error bars are indicated. Mean with different letters denote a significant difference between compared producers; same letters in columns denote a lack of significant differences between compared means (ANOVA. p≥0.05))**

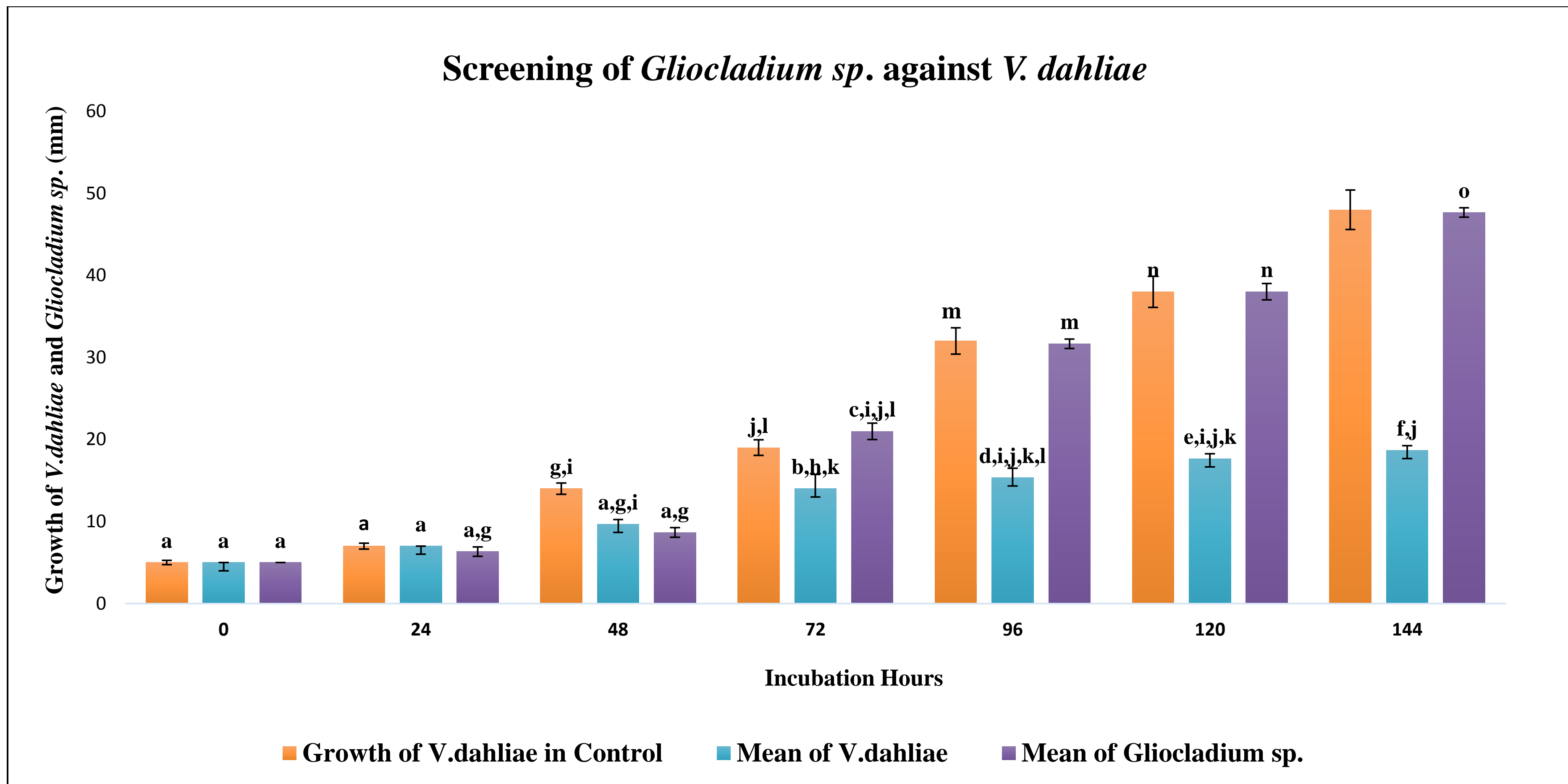


Figure 6.18: ***in vitro*** **screening of** ***Gliocladium sp.*** **against** ***Vertcillium dahliae*** **in Dual Culture Assay after every 24 hours of incubation (Standard error bars are indicated. Mean with different lowercase denote a significant difference between compared producers; same letters in columns denote a lack of significant differences between compared means (ANOVA. p≥0.05))**

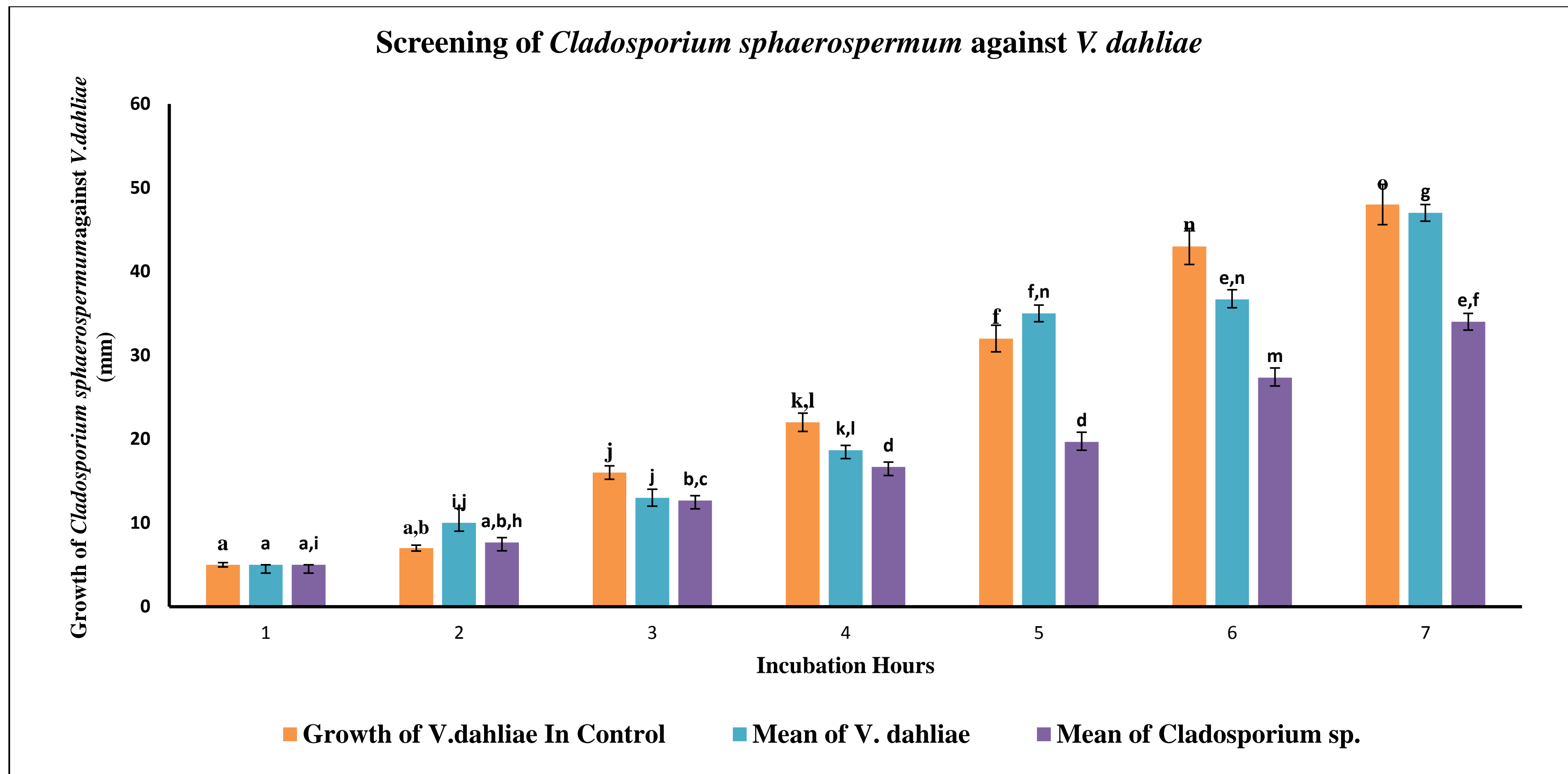


Figure 6.19– *in vitro* **screening of** *Cladosporium sphaerospermum* **against** *Vertcillium dahliae* **in Dual Culture Assay after every 24 hours of incubation (Standard error bars are indicated. Mean with different letters denote a significant difference between compared producers; same letters in columns denote a lack of significant differences between compared means (ANOVA. p≥0.05)**

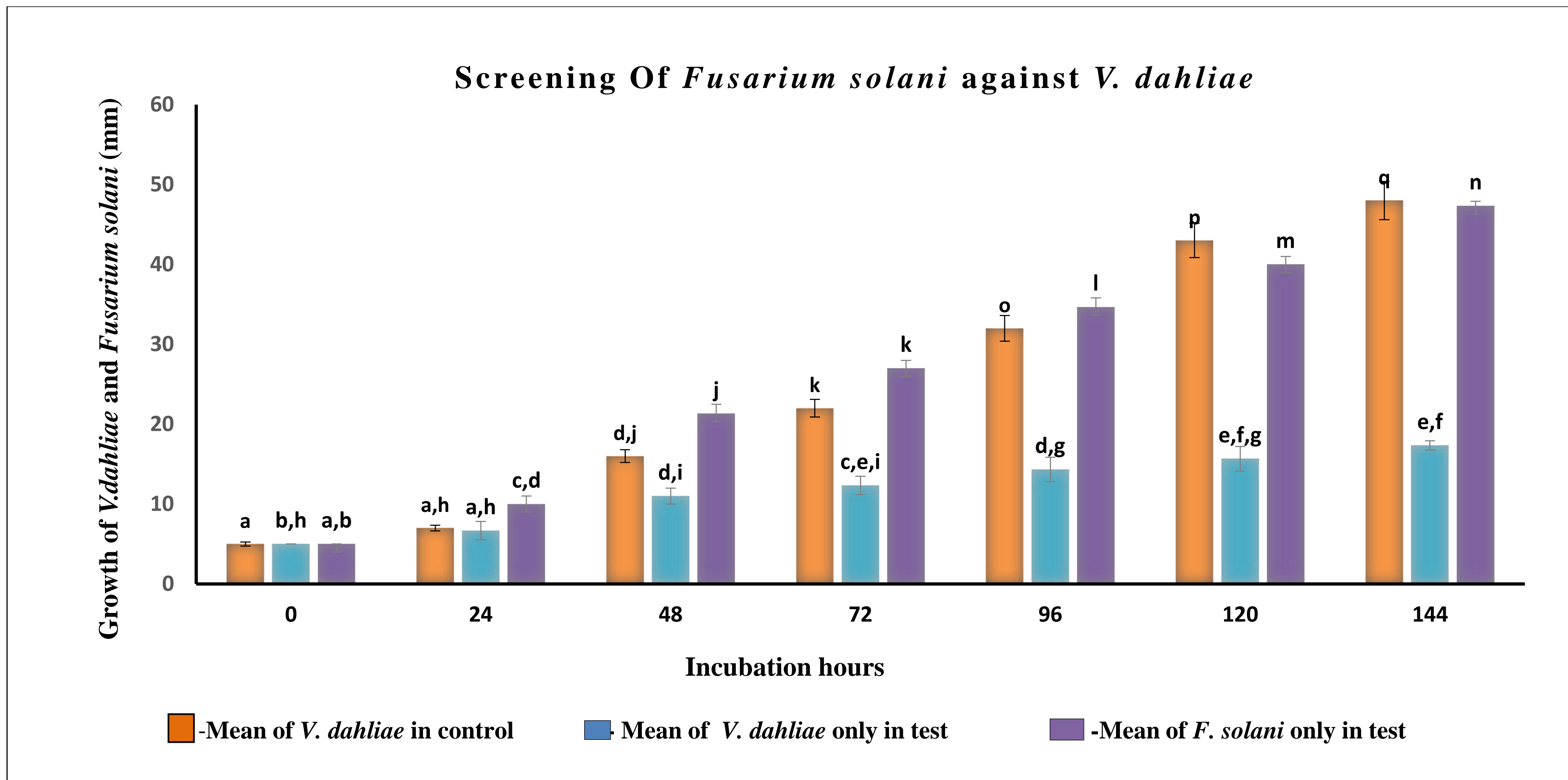


Figure 6.20 – *in vitro* screening of *Fusarium solani* against *Vertcillium dahliae* in Dual Culture Assay after every 24 hours of incubation (Standard error bars are indicated. Mean with different letters denote a significant difference between compared producers; same letters in columns denote a lack of significant differences between compared means (ANOVA. p≥0.05))

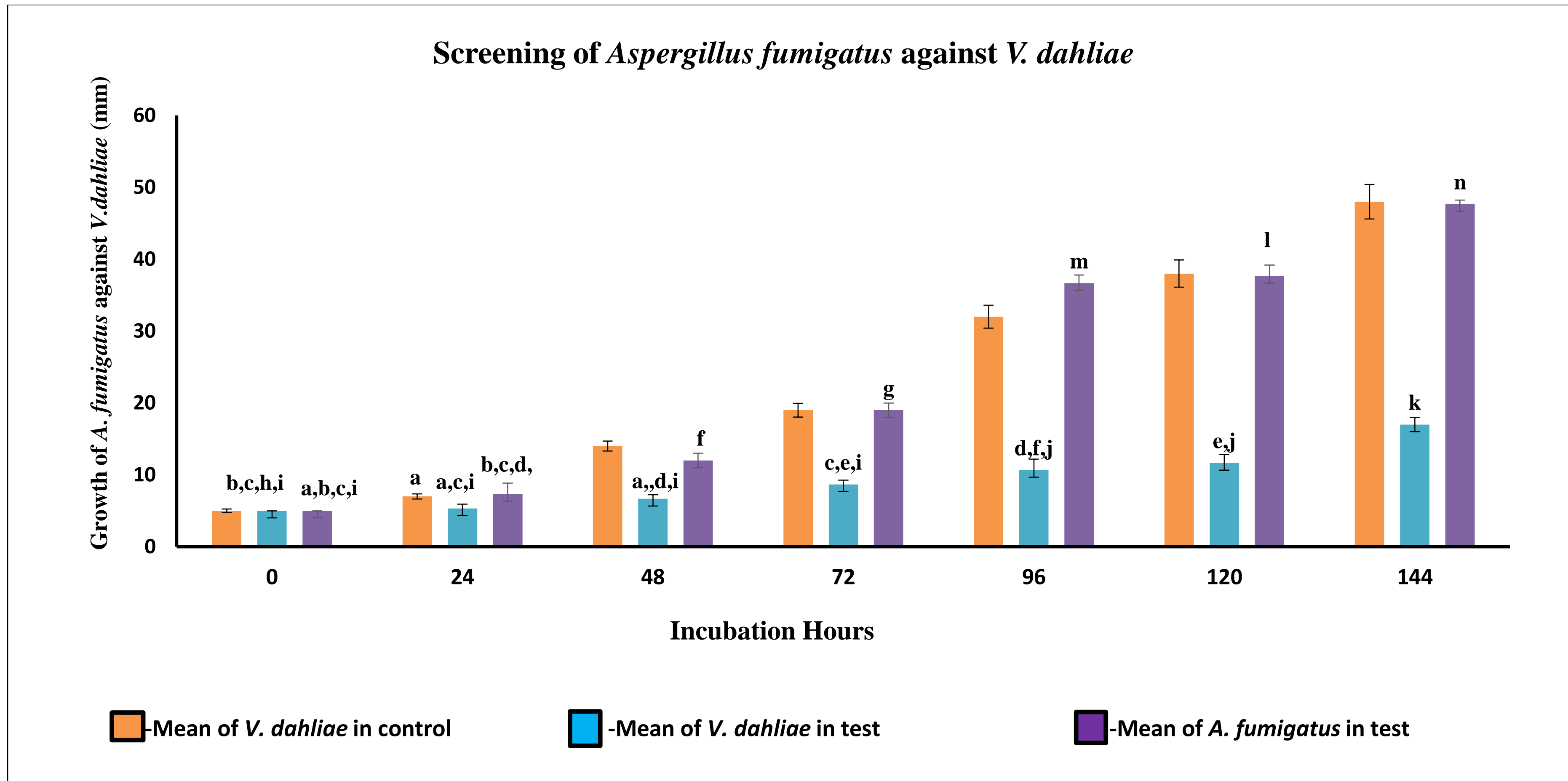


Figure 6.21: ***in vitro*** **screening of *Aspergillus fumigatus* against *Vertcillium dahliae* in Dual Culture *Assay* after every 24 hours of incubation (Standard error bars are indicated. Means are Mean with different letters denote a significant difference between compared producers; same letters in columns denote a lack of significant differences between compared means (ANOVA. p≥0.05))**

Table 6.7: Percentage inhibition of radial growth (PIRG) during Dual Culture Assay of different antagonists

S. No	Name of Antagonistic fungal strains	Source of isolation	PIRG
1	***Aspergillus niger***	Animal manure	63.20%
2	***A. terreus***	Bio manure	59.90%
3	***A. fumigatus***	Animal manure	73.46%
4	***A nidulans***	Compost Manure	61.12%
5	***Trichoderma harzianum***	Poultry Manure	72.91%
6	***Fusarium solani***	Bio manure	47.22%
7	***Cladosporium sphaerospermum***	Animal manure	6.95%
8	***Gliocladium sp.***	Vermicompost	61.81%

6.8.2 *in vitro* activities of Cellulase, Protease, Chitinase for selected antagonistic fungal strains

6,8.2.1Semi Quantitative Assay of Cellulase Enzyme

Production of fungal cell wall-degrading enzymes was studied as enzymes play important mechanism of fungal inhibition. Cellulase enzyme activity of antagonistic strains has performed by growing on liquid Czapek Mineral Salt Agar Medium. All the selected fungal strains have shown positive cellulase activity by producing yellow opaque inhibition zone around the well as compared to control **(Figure 6.22)**. The results were recorded on 5th days of incubation. The maximum cellulase activity was recorded in *Aspergillus terreus* (11.5 mm) followed by *Gliocladium sp.* (10.83mm)., *Aspergillus niger* (10.04mm), *Aspergillus fumigatus* (9.83mm), *Trichoderma harzianum* (8.5mm), *Aspergillus nidulans* (7.5mm) and *Fusarium solani* (5.0mm) around the well. *Aspergillus fumigatus* has shown most significant growth while *Cladosporium sphaerospermum* (0mm) was not able to produce inhibition zone. This result is in accordance with earlier reports of Kamala and Indira.,2011, wherein clear inhibition zone around the well was reported, after adding Congo red solutions. Enzymatic degradation of cell wall of pathogenic fungal strains has been done due to the secretion of two enzymes viz. endocellulases and exocellulase. These two

enzymes play an important role in cell wall degradation of pathogenic fungus (Kamala and Indira., 2014). The results in **Table 6.8** shows zone of hydrolysis recorded during cellulase assay of antagonistic fungus as compared to control. Statistical analysis of **Table 6.8** has shown no least significant difference (LSD) in *Aspergillus terreus, Trichoderma harzianum, Aspergillus niger*, *Aspergillus fumigatus, Gliocladium sp., Aspergillus nidulans* and *Fusarium solani* hence denoted by same alphabets between compared means in columns and has significant difference between compared means was observed in *Cladosporium sphaerospermum* hence denoted by different alphabet.

6.8.2.2 Semi Quantitative Assay of Protease Enzyme

in vitro screening of protease enzyme for selected antagonistic fungal strains have been performed by using Casein Agar medium. Antagonistic fungal strains have shown clear inhibition zones during protease activity around the well as compared to control plates **(Figure 6.23)**. The results have been shown in **Table 6.8** and shows the clear inhibition zones during protease enzyme activity, maximum inhibition zone has been recorded in *A. terreus* (10.7mm) followed by *Aspergillus niger* (9.5mm), *A. fumigatus* (9.33mm), *Gliocladium sp.* (9.06mm), *Aspergillus nidulans* (8.1mm), *Trichoderma harzianum* (6.7mm), *Fusarium solani* (6.46mm) and *Cladosporium sphaerospermum* (3.56mm) clear zones respectively around the well. Among 8 strains, *Aspergillus terreus* has shown the most positive result and *Cladosporium sphaerospermum* with least significant result in quantitative assay of protease enzyme. Similar studies have been reported by (Howell., 2003) in which *T. harzianum* (antagonistic strain) has plays an important role in biocontrol of fungal strains and shows positive results during protease activity. Kapat et al.,1998 has described that protease enzyme produced by pathogenic fungus has been inactivated by biocontrol strains. Lysis of cell wall has been occurred by catalyzing the breakage of peptide bonds in proteins (Mata et al., 2001). **Table 6.8** shows no significant difference in *Aspergillus terreus, Aspergillus nidulans, Aspergillus niger, Aspergillus fumigatus* and *Gliocladium sp*., denoted by same alphabets between compared means in columns and *Trichoderma harzianum, Cladosporium sphaerospermum* and *Fusarium solani* has shown significant difference between compared means hence denoted by different alphabet.

6.8.2.3 Semi Quantitative Assay of Chitinase Enzyme

Chitinase enzyme activity of antagonistic strains have been performed by using Chitin Detection Medium comprising basal chitin medium. **Figure 6.24** depicts the purple-coloured inhibition zone around the well during chitinase activity in test plates as compared to control. Experiment was carried out in triplicates and growth of all purple-coloured zones was measured. The results in **Table 6.7** shows the hydrolysis zones during chitinase activity in which maximum hydrolysis zone has been recorded in *Aspergillus niger* (9.66mm) followed *by Aspergillus terreus* (9.5mm), *Aspergillus nidulans* (8.5mm), *Aspergillus fumigatus*(8.43mm), *Gliocladium sp.*(8.43mm), *Trichoderma harzianum* (8.1mm), *Fusarium solani* (6.6mm) and *Cladosporium sphaerospermum* (3.2mm) around the well. Statistical analysis of **Table 6.7** has shown no significant difference (LSD) in *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus terreus*, *Trichoderma harzianum*, *Aspergillus fumigatus* and *Gliocladium sp.* hence denoted by same alphabets between compared means in columns (ANOVA $p>0.05$) and a significant difference was observed in *Cladosporium sphaerospermum* hence denoted by different alphabet. However, the result was in similar with work of Benitez et al., 2004 that antagonistic fungal strains produce chitinase enzyme for breakdown of chitin and other compounds. Hence, the quantity of chitinase enzyme produced, relates with the extent of antagonism shown by biocontrol agents against pathogenic strain. Result of Benitez et al., 2004 have described those strains of *Trichoderma* have produced chitinase enzyme, plays a major role for control of various pathogens and act as biocontrol agent. Enzymes produced by biocontrol agents helps to degrade cell wall of pathogenic strain because the cell wall is made up of chitin, proteins and glucans (Benitez et al., 2004).

Table 6.8: Hydrolysis Zone formation during *in vitro* hydrolytic enzymatic activities (Standard error bars are indicated. Mean with different letters denote a significant difference between compared producers; same letters in columns denote a lack of significant differences between compared means (ANOVA. $p \geq 0.05$))

		Hydrolytic enzymatic activity of antagonistic strains(mm)		
Isolate	**Origin**	**Cellulase activity**	**Protease activity**	**Chitinase activity**
Aspergillus niger	Animal manure	10.4±0.5bcd	9.5±0.5ce	9.6± 0.2ac
Aspergillus nidulans	Compost	7.5±0.5de	8.01±0.3ace	8.5±0.3bcd
Aspergillus terreus	Bio manure	11.5±0.1abd	10.7±0.9ab	9.5±0.8abd
Trichoderma harzianum	Poultry manure	8.5±0.5bcd	6.46±0.2f	8.1±0.2ab
Aspergillus fumigatus	Animal manure	9.83±0.5abc	9.33±0.2bd	8.5±0.5cde
Cladosporium sphaerospermum	Animal manure	0±0.0 f	3.5±0.5g	3.2±h
Gliocladium sp.	Vermicompost	10.83±0.3cd	9.06±0.3bce	8.43±cef
Fusarium solani	Bio manure	5.0 ±0.1de	6.7±0.2f	6.66±g

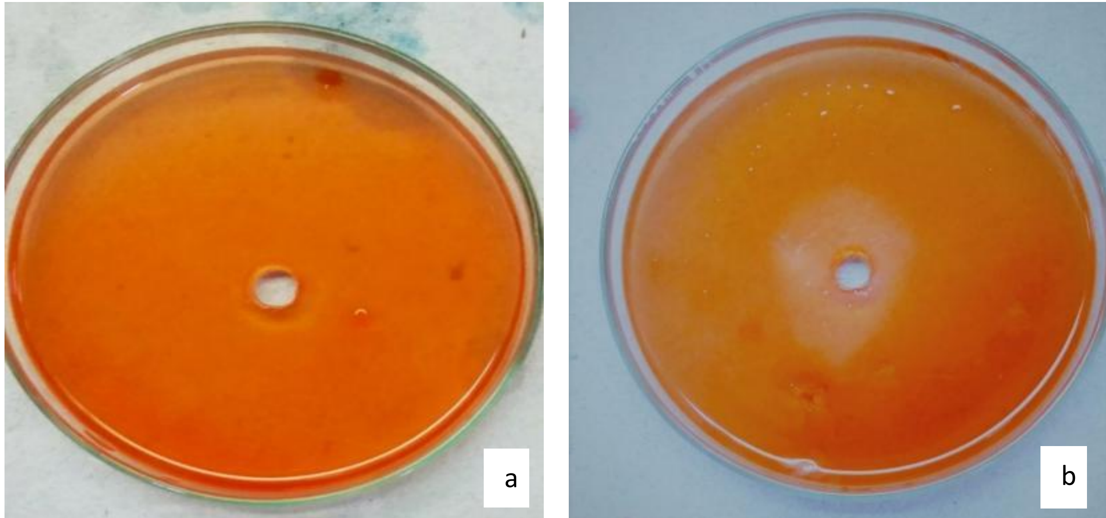


Figure 6.22: Cellulase Activity of Antagonistic fungal strain (a) Control (b) Test plate (yellow-opaque inhibition zone around well)

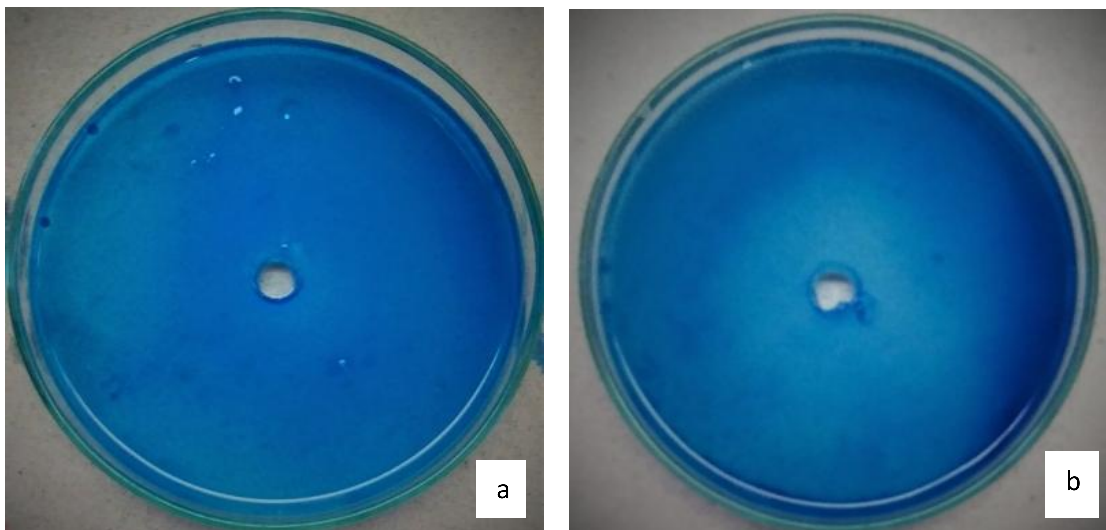


Figure 6.23: Protease Activity of Antagonistic fungal strain (a) Control (b) Test plate (clear inhibition zone around well)

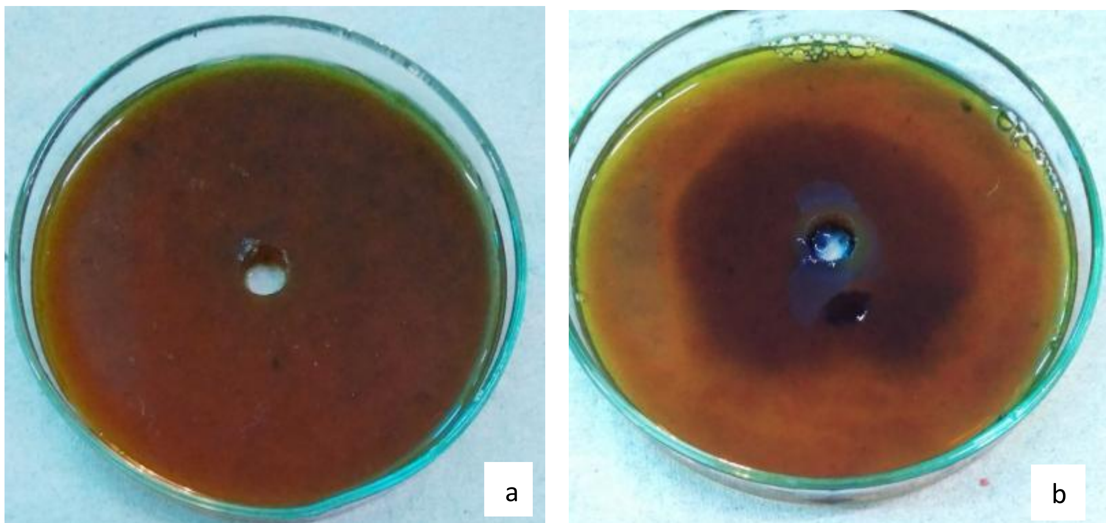


Figure 6.24: Chitinase Activity of Antagonistic fungal strain (a) Control (b) Test plate (purple coloured inhibition zone around well)

6.9 Selection of endophytic bacterial strains as potential biocontrol agents

6.9.1 Preliminary screening of endophytic bacterial strains by Dual Culture Assay

During this study, endophytic bacterial strains have been isolated and *in vitro* screening has been done for checking its antagonism against *V. dahliae*. Dual culture method is the preliminary step to check the antagonistic activity because it eliminates the growth of host pathogen and most likely by direct antagonism against pathogenic strain (Khan, 2013). Preliminary screening of all strains has been done through Dual culture assay for the selection of best positive endophytic bacterial strains for *in vivo* evaluation. The results in **Figure 6.25** depicts the growth of *V. dahliae* as control and growth of both *V. dahliae* and endophytic bacterial strains in test plates during *in vitro* screening towards *V. dahliae.* Different endophytic bacterial strains have shown *in vitro* antagonistic activity towards *V. dahliae*. Experiment has been performed in triplicates. The result has been recorded from 0 to 144 hours of incubation. The result depicted in **Figure 6.26** shows the growth of *V. dahliae* in control and growth of both *V. dahliae* and VTS DB-V1/NI /L/S1/B2 in test plate from 0 hours to 144 hours of incubation in which *V. dahliae* has shown 48mm of growth as control plate and 14mm during test plate in *V. dahliae* along with 38mm growth of VTS DB-V1/NI /L/S1/B2 at 144 hours of incubation. Percentage inhibition of radial growth (PIRG) is 48.62% exhibited by VTS DB-V1/NI /L/S1/B2. The result depicted in **Figure 6.27** shows the growth of *V. dahliae* in control and growth of both *V. dahliae* and VG DB-V1/NI /L/S1/B2 in test plate from 0 hours to 144 hours of incubation in which *V. dahliae* has shown 52 mm of growth as control plate and 12 mm during test plate in *V. dahliae* along with 40 mm growth of VTS DB-V1/NI /L/S1/B2 at 144 hours of incubation. Percentage inhibition of radial growth (PIRG) is 56.25% exhibited by VG DB-V1/NI /L/S1/B2**.** The result for *in vitro* screening of *T. harzianum* against *V. dahliae* has shown in **Figure 6.28** in which growth *V. dahliae* in control and growth of both *V. dahliae* and VG DB-V2/NI /L/S1/B1 in test plate from 0 to 144 hours of incubation has been measured. *V. dahliae* has shown 52mm growth as control plate and in test plate (19mm) growth of *V. dahliae* along with 48mm growth of VG DB-V2/NI /L/S1/B1 has been calculated at 144 hours of incubation. In *in vitro screening*., PIRG exhibited by VG DB-V2/NI /L/S1/B1 against *V. dahliae* is 45.2%. The result depicted in **Figure 6.29** shows the growth of *V. dahliae* in control and growth of both *V. dahliae* and VG DB-V2/NI /L/S1/B1in test plate from 0 hours to 144 hours of

incubation in which *V. dahliae* has shown 50mm of growth as control plate and 23mm during test plate in *V. dahliae* along with 30mm growth of VG DB-V2/NI /L/S1/B1 at 144 hours of incubation. Percentage inhibition of radial growth (PIRG) is 40.29% exhibited by VG DB-V2/NI /L/S1/B1. The result depicted in **Figure 6.30** shows the growth of *V. dahliae* in control and growth of both *V. dahliae* and *Bacillus subtilis* (VTS DB-V1/NI /R/S1/B2) in test plate from 0 to 144 hours of incubation in which *V. dahliae* has shown 52mm of growth as control plate and 18mm during test plate in *V. dahliae* along with 36mm growth of *Bacillus subtilis* (VTS DB-V1/NI /R/S1/B2) at 144 hours of incubation. Percentage inhibition of radial growth (PIRG) is 57.64% exhibited by *Bacillus subtilis* (VTS DB-V1/NI /R/S1/B2). The result depicted in **Figure 6.31** shows the growth of *V. dahliae* in control and growth of both *V. dahliae* and *Pseudomonas aeruginosa* (VTS DB-V1/NI /S/S1/B2) in test plate from 0 to 144 hours of incubation in which *V. dahliae* has shown 52mm of growth as control plate and 16mm during test plate in *V. dahliae* along with 39mm growth of *Pseudomonas aeruginosa* (VTS DB-V1/NI /S/S1/B2) at 144 hours of incubation. Percentage inhibition of radial growth (PIRG) is 61.12% exhibited by *Pseudomonas aeruginosa* (VTS DB-V1/NI /S/S1/B2)**.** The result depicted in **Figure 6.32** shows the growth of *V. dahliae* in control and growth of both *V. dahliae* and *Lysinibacillus macroides* (VTS DB-V1/NI /S/S1/B1) in test plate from 0 to144 hours of incubation in which *V. dahliae* has shown 52mm of growth as control plate and 23mm during test plate in *V. dahliae* along with 30mm growth of *Lysinibacillus macroides* (V TS DB-V1/NI /S/S1/B1) at 144 hours of incubation. Percentage inhibition of radial growth (PIRG) is 59.2% exhibited by *Lysinibacillus macroides* (V TS DB-V1/NI /S/S1/B1)**.** The results in **Table 6.9** describes the PIRG of all bacterial strains in which maximum percentage of inhibition growth has been shown in *Pseudomonas aeruginosa* (VTS DB-V1/NI /S/S1/B2) with 61.12% followed by *Lysinibacillus macroides* (VTS DB-V1/NI /S/S1/B1), *Bacillus subtilis* (VTS DB-V1/NI /R/S1/B2), VG DB-V1/NI /L/S1/B2, VTS DB-V1/NI /L/S1/B2, VG DB-V2/NI /L/S1/B1 and VG DB-V1/NI /S/S1/B1 with 59.2%, 57.64%, 56.25%, 48.62%, 45.2% and 40.29% respectively during Dual culture Assay. Similar work has been done by Swadling and Jeffries 1996, in which clear inhibition zone has been produced due to the production of either toxic metabolites, antibiotics or siderophores by endophytic bacterial strains. According to the study of Laitila et al., 2002; Cavaglieri et al., 2004., bacterial antagonistic strains help to suppress the activity of various microbial strains. Out of 7, 3 strains such as

Pseudomonas aeruginosa, Bacillus subtilis and *Lysinibacillus macroides* have shown most positive *in vitro* percent inhibition of radial growth during Dual Culture Assay against *V. dahliae* hence evaluated as potential biocontrol agents during both natural and controlled conditions. According to the study of Weller.,1988; Sùrensen.,1997, root associated endophytic bacterial strains plays an important role for control of different soil borne pathogenic strains.

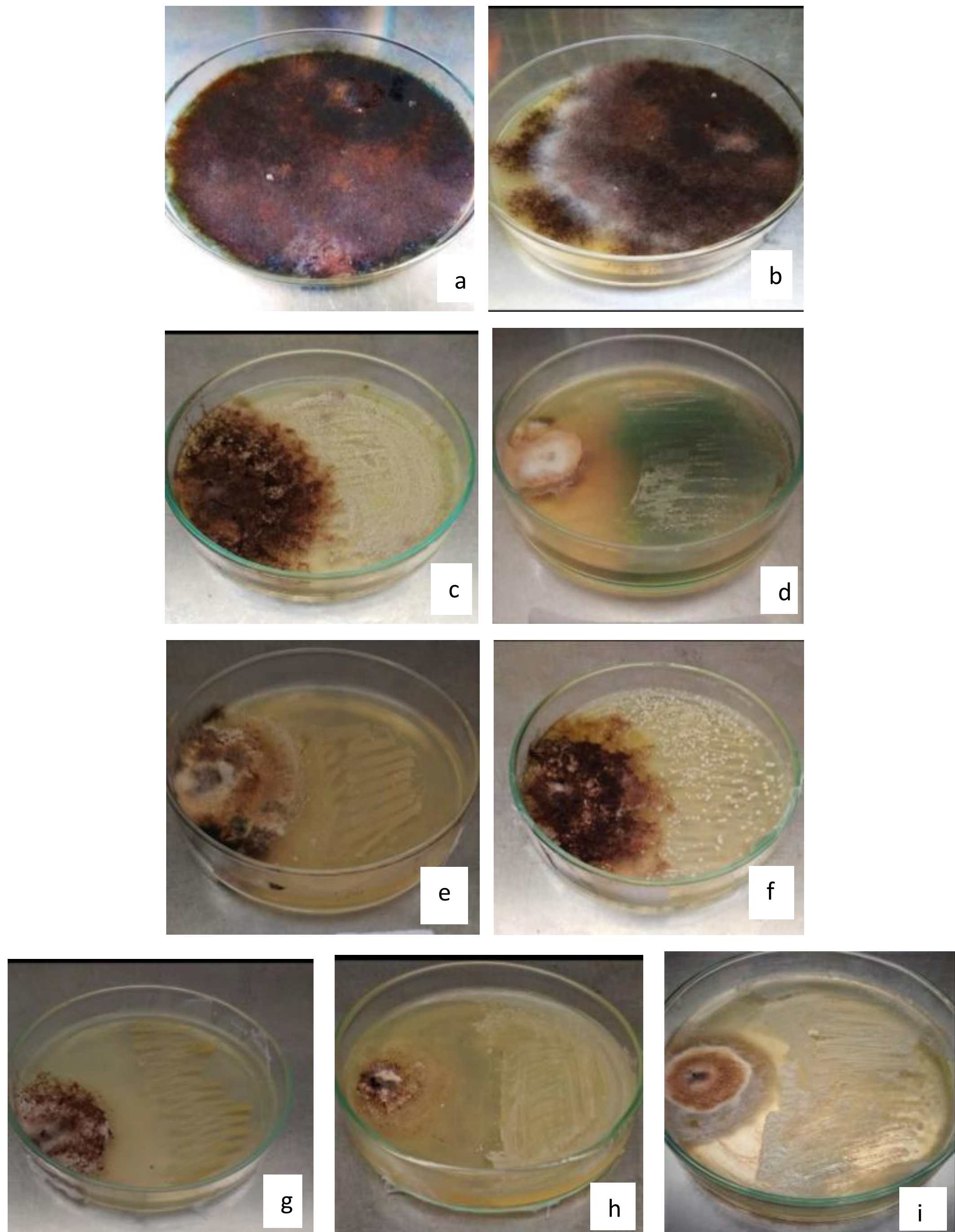


Figure 6.25: Figure 6.11: *in vitro* antagonism of endophytic bacterial strains towards *Verticillium dahliae* during "Dual Culture Assay" along with control plates (a, b) Growth of *V. dahliae* in control plates (c) Growth of VTS DB-V1/NI

/L/S1/B2 against *V. dahliae* (d) Growth of VTS DB-V1/NI /S/S1/B2 against *V. dahliae* (e) Growth of VG DB-V1/NI /L/S1/B2 against *V. dahliae* (f) Growth of VG DB-V1/NI /S/S1/B1 against *V. dahliae* (g) Growth of VG DB-V2/NI /L/S1/B1 against *V. dahliae* (h) Growth of VTS DB-V1/NI /R/S1/B2 against *V. dahliae* (i) Growth of VTS DB-V1/NI /S/S1/B1 against *V. dahliae*

Table 6.9: Percentage inhibition of radial growth (PIRG) during Dual Culture Assay of different endophytic bacterial strains isolated from healthy cotton plant parts

S. No	Endophytic bacterial strains (code)	Mode of isolation	PIRG
1	**VTS DB-V1/NI /L/S1/B2**	Healthy stem of cotton plant	48.62%
2	**VG DB-V1/NI /L/S1/B2**	Healthy stem of cotton plant	56.25%
3	**VG DB-V2/NI /L/S1/B1**	Healthy leaves of cotton plant	45.2%
4	**VG DB-V1/NI /S/S1/B1**	Healthy stem of cotton plant	40.29%
5	**VTS DB-V1/NI /S/S1/B2 (*Pseudomonas aeruginosa*)**	Healthy stem of cotton plant	61.12%
6	**VTS DB-V1/NI /R/S1/B2 (*Bacillus subtilis*)**	Healthy cotton root	57.64%
7	**VTS DB-V1/NI /S/S1/B1** (***Lysinibacillus macroides***)	Healthy stem of cotton plant	59.2%

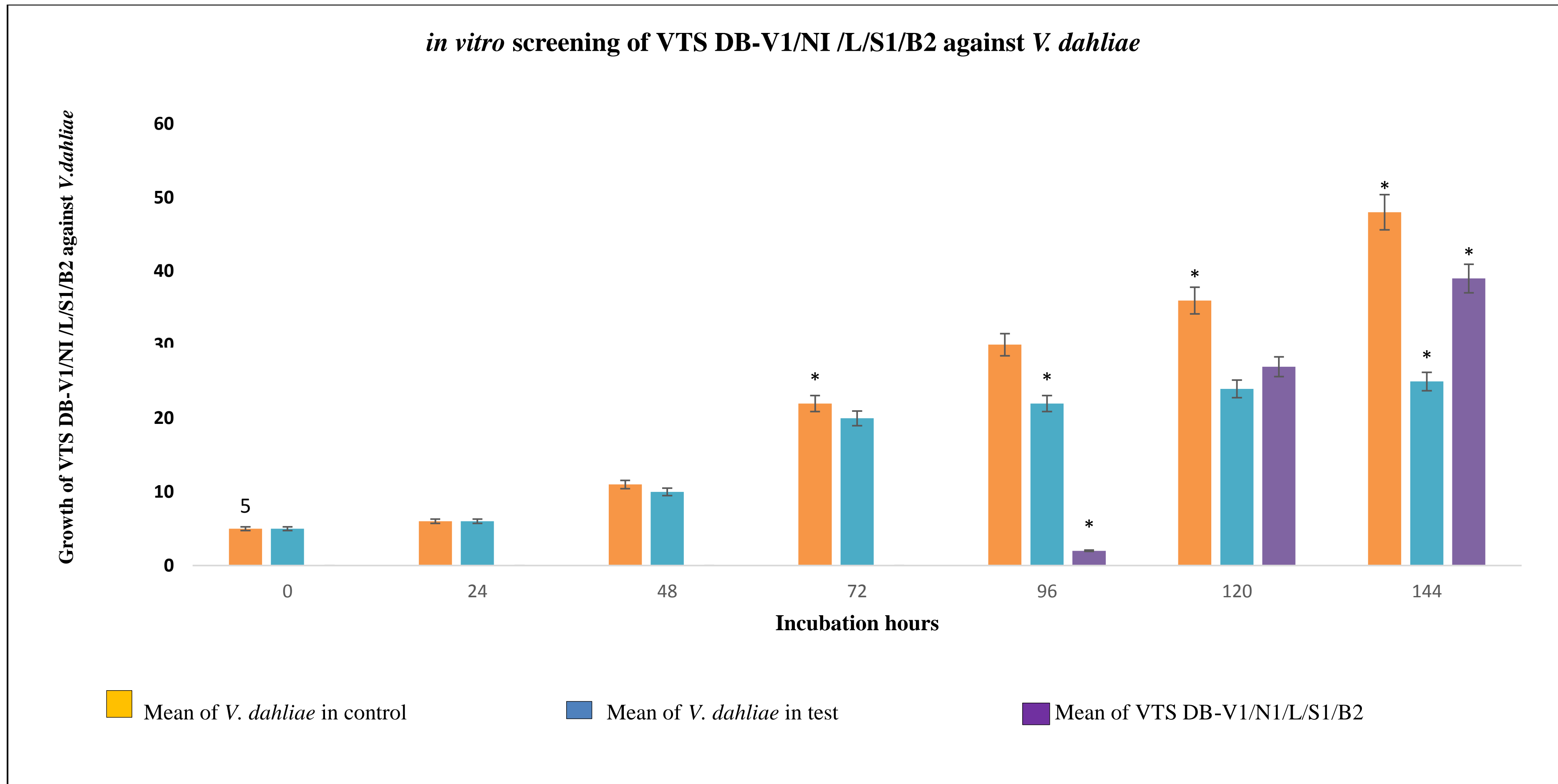


Figure 6.26: *in vitro* screening of VTS DB-V1/NI /L/S1/B2 against *Vertcillium dahliae* after every 24 hours of incubation (Standard error bars are indicated. Mean with star denote a significant difference between compared producers; without star denote a lack of significant differences between compared means (ANOVA. p≥0.05))

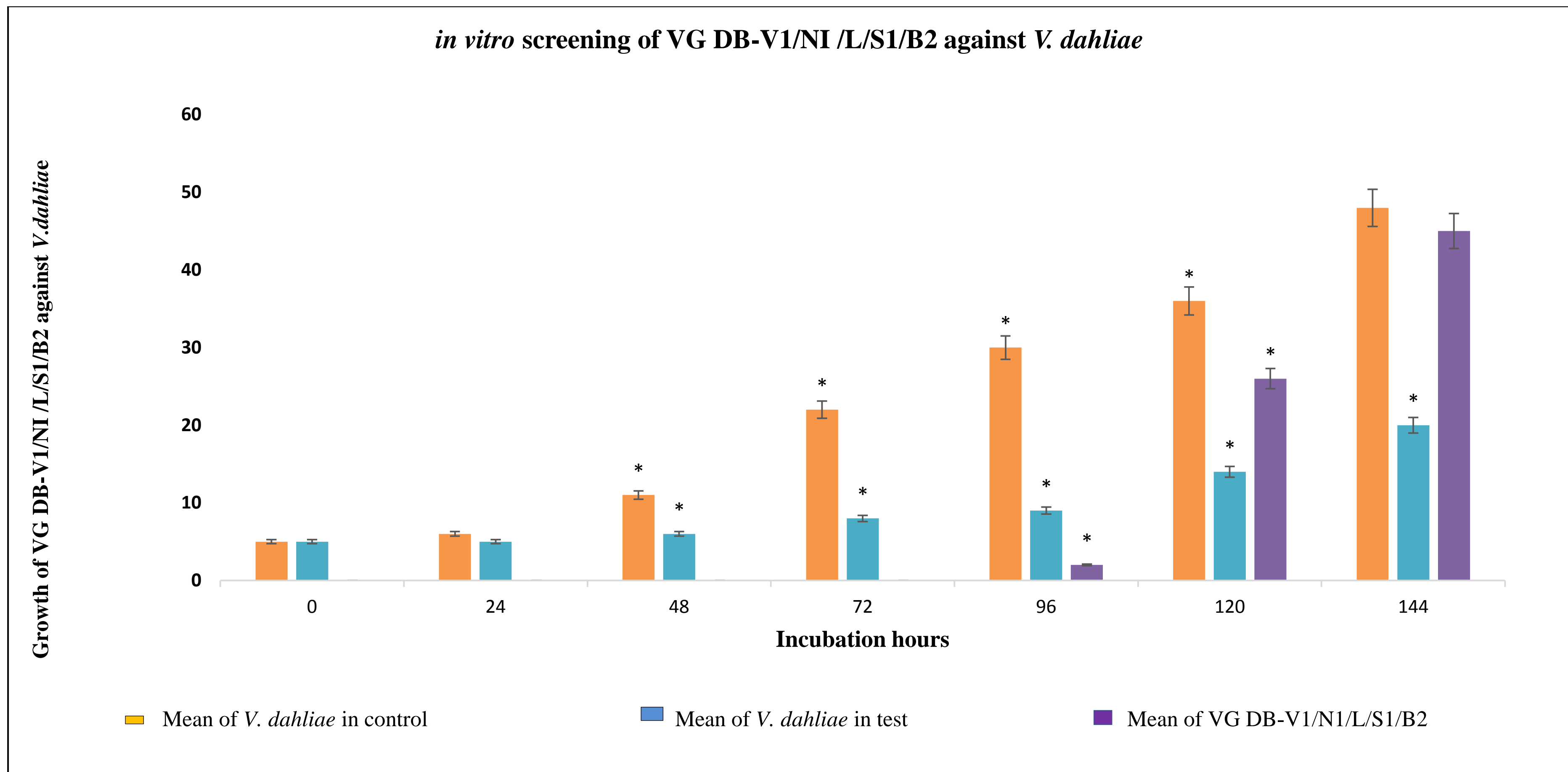


Figure 6.27: *in vitro* screening of VG DB-V1/NI /L/S1/B2 against *Vertcillium dahliae* after every 24 hours of incubation (Standard error bars are indicated. Mean with star denote a significant difference between compared producers; without star denote a lack of significant differences between compared means (ANOVA. p≥0.05))

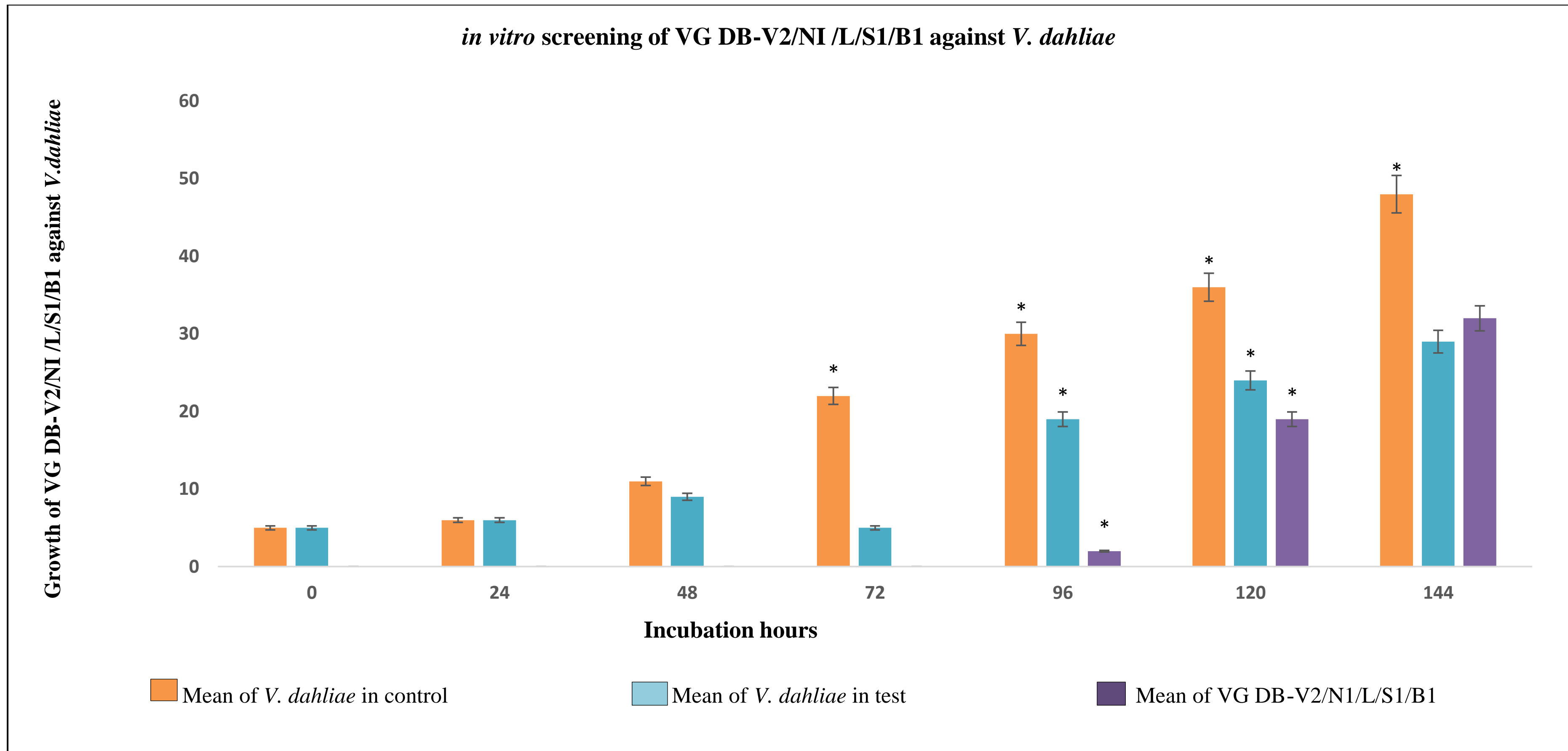


Figure 6.28: ***in vitro*** **screening of VG DB-V2/NI /L/S1/B1 against** ***Vertcillium dahliae*** **after every 24 hours of incubation (Standard error bars are indicated. Mean with star denote a significant difference between compared producers; without star denote a lack of significant differences between compared means (ANOVA. p≥0.05))**

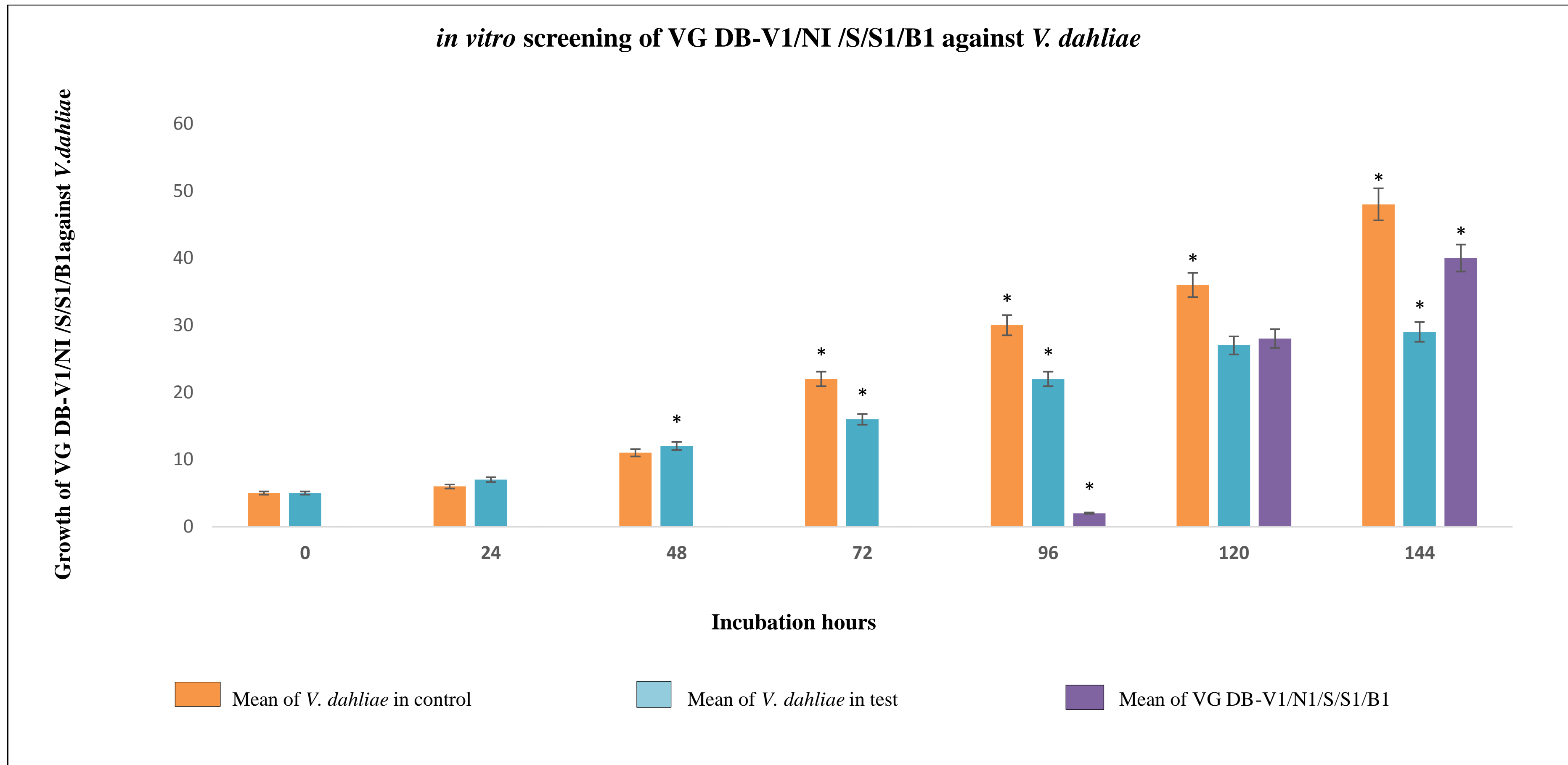


Figure 6.29: ***in vitro*** **screening of VG DB-V1/NI /L/S1/B1 against** ***Vertcillium dahliae*** **after every 24 hours of incubation (Standard error bars are indicated. Mean with star denote a significant difference between compared producers; without star denote a lack of significant differences between compared means (ANOVA. p≥0.05))**

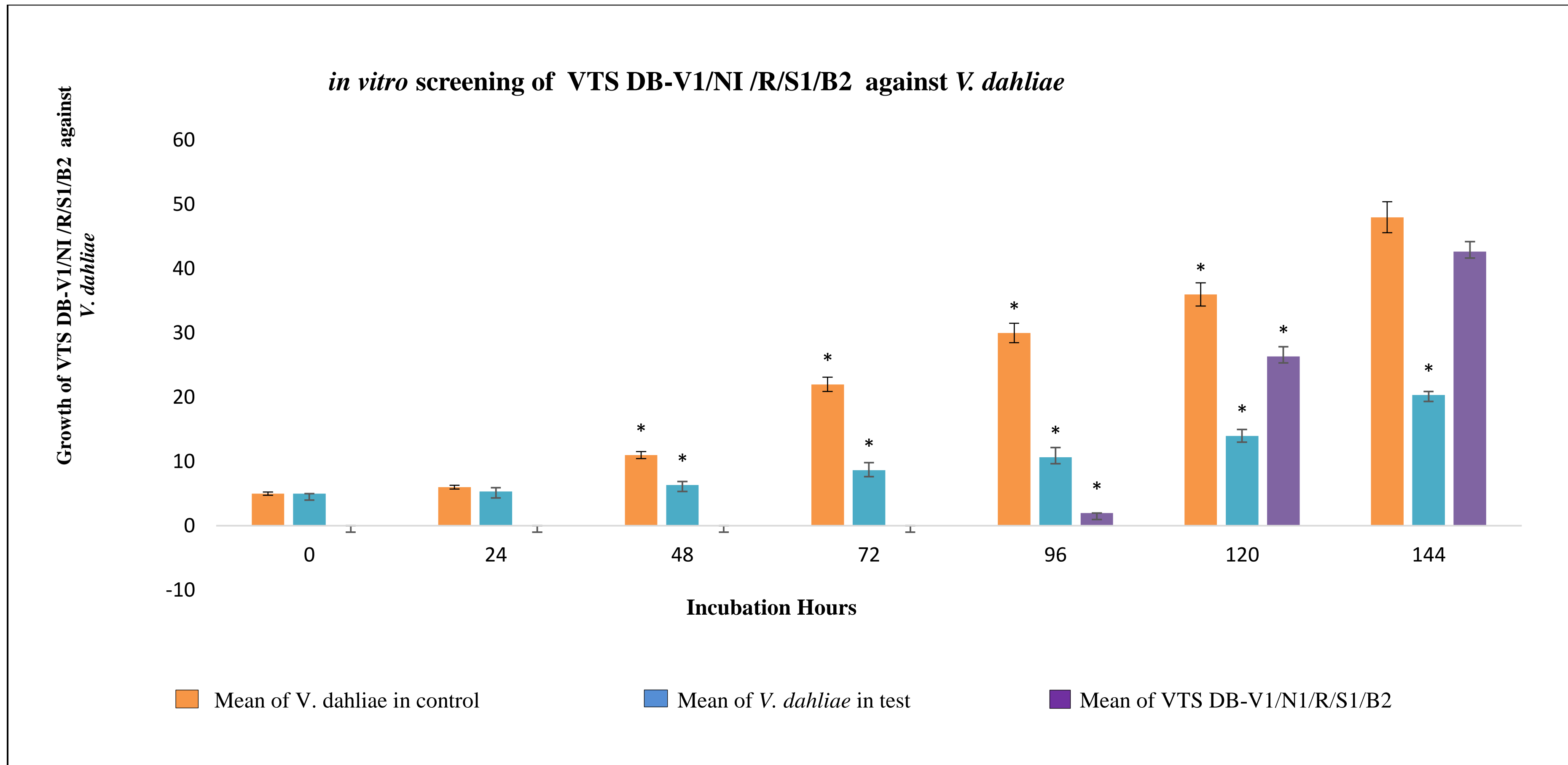


Figure 6.30: ***in vitro*** **screening of VTS DB-V1/NI /R/S1/B2 against** ***Vertcillium dahliae*** **after every 24 hours of incubation (Standard error bars are indicated. Mean with star denote a significant difference between compared producers; without star denote a lack of significant differences between compared means (ANOVA. p≥0.05))**

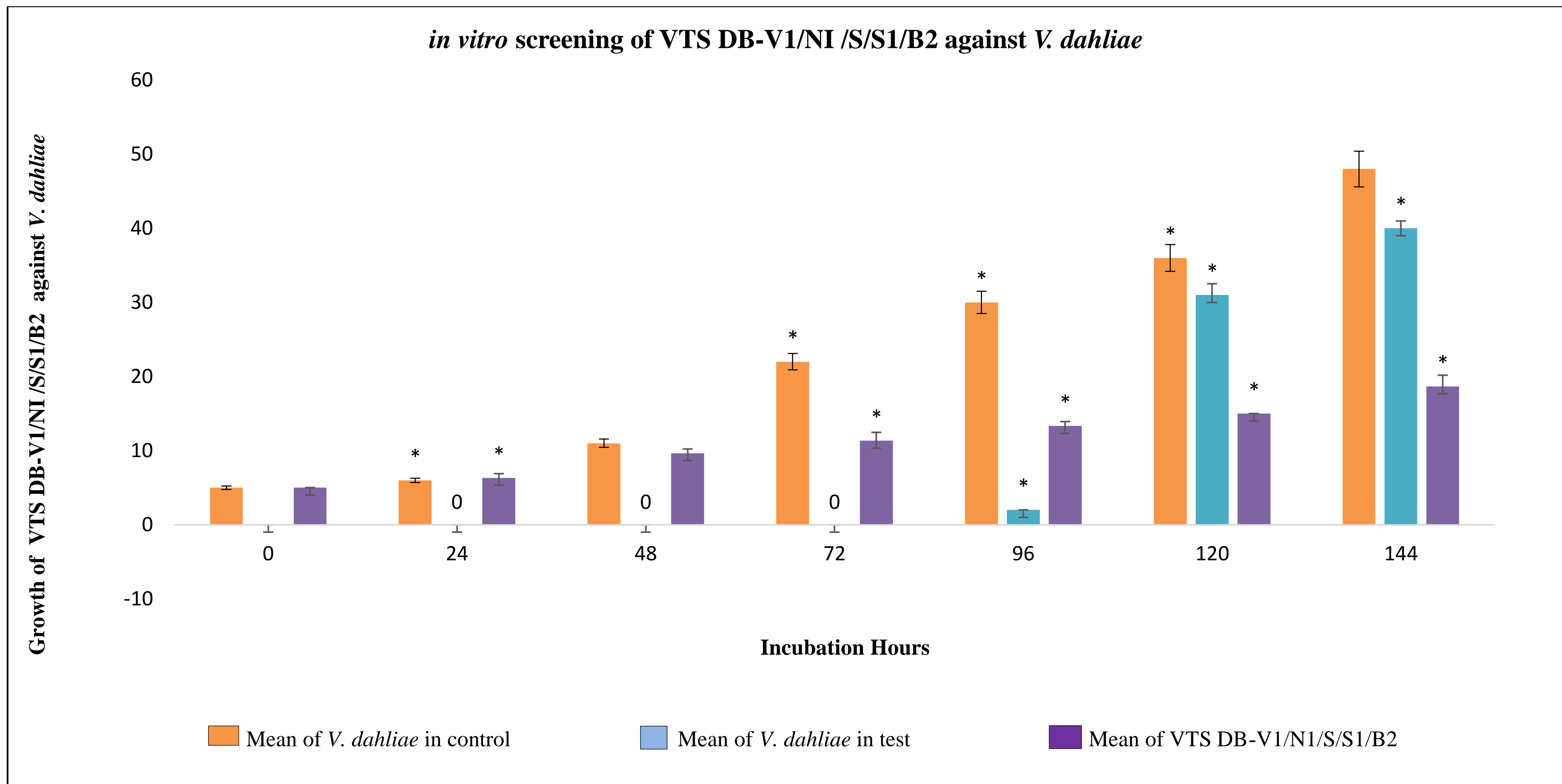


Figure 6.31: ***in vitro*** **screening of VTS DB-V1/NI /S/S1/B2 against *Vertcillium dahliae* after every 24 hours of incubation (Standard error bars are indicated. Mean with star denote a significant difference between compared producers; without star denote a lack of significant differences between compared means (ANOVA. p≥0.05))**

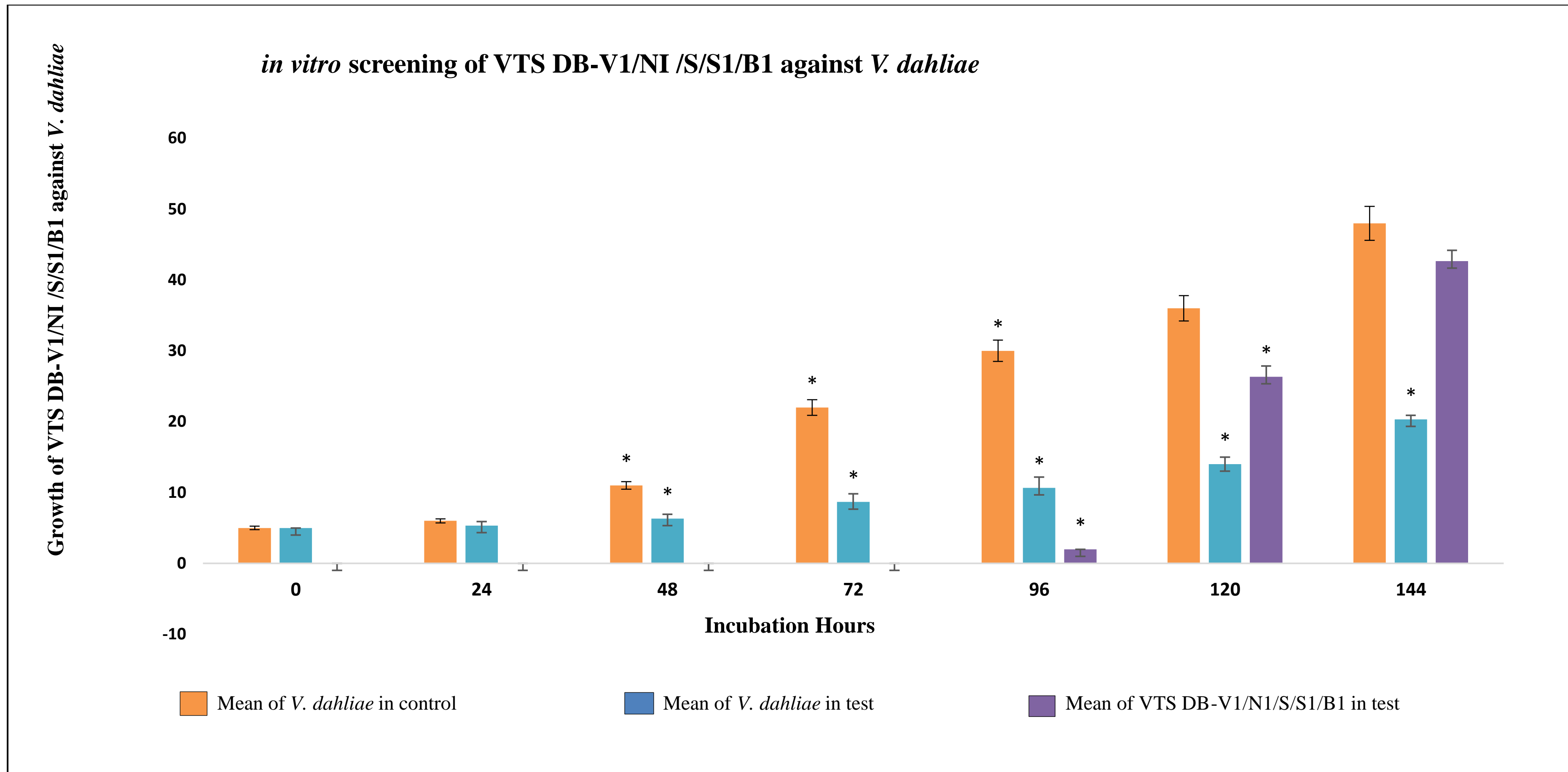


Figure 6.32- ***in vitro*** **screening of VTS DB-V1/NI /S/S1/B1 against** ***Vertcillium dahliae*** **in Dual Culture Assay after every 24 hours of incubation (Standard error bars are indicated. Mean with different letters denote a significant difference between compared producers; same letters in columns denote a lack of significant differences between compared means (ANOVA. p≥0.05))**

6.9.2 Determination of *in vitro* activities of cell wall degrading enzymes of endophytic bacterial strains

6.9.2.1 Semi Quantitative Assay of Cellulase Enzyme

Additional screening or second parameter of *in vitro* screening has advantage over the selection of biocontrol agents because it helps to degrade cell wall of pathogenic fungal strains during hydrolytic enzymatic activity. Additional screening of endophytic bacterial strains for evaluation of biocontrol agents have been done by checking it's *in vitro* antagonistic potential during hydrolytic enzymatic activity. Cellulase enzyme activity of antagonistic strains has performed by growing on liquid Czapek Mineral Salt Medium. Plates have been flooded with bromo cresol dye at 5th day of incubation. The result in **Figure 6.33** depicts the yellow opaque inhibition zones around the well during cellulase activity. The results in **Table 6.10** describes the inhibition zones produced by various endophytic bacterial strains at 5th day of incubation, in which *Pseudomonas aeruginosa* (VTS DB-V1/NI /S/S1/B2) has shown maximum growth of inhibition zone (8.56mm) followed by *Lysinibacillus macroides* (VTSDB-V1/N1/S/S1/B1) (7.3mm),VTSDBV1/N1/L/S1/B2(6.8mm), *Bacillus subtilis* (VTSDBV1/N1/R/S1/B2) (5.23mm), VG DB-V2/N1/L/S1/B2(5.2mm), VGDB-V2/N1/L/S1/B2(5.0mm) and VGDB-V1/N1/S/S1/B1(4.5mm) during cellulase activity. *Pseudomonas aeruginosa* (VTS DB-V1/NI /S/S1/B2) has shown most significant growth followed by *Lysinibacillus macroides.* Experiment has been performed in triplicates. **Figure 6.36** has shown the growth of all antagonistic bacterial strains as compared to control. Study of Kamala and Indira., 2011, describes that a clear inhibition zone around the well has been formed after adding Congo red solution., evidence of formation of cellulase enzymes from potential antagonistic strains. Statistical analysis of **Table 6.10** has shown no significant difference (LSD) in between compared means of VTS DB-V1/NI /L/S1/B2, VG DB-V1/NI /L/S1/B2, VG DB-V2/NI /L/S1/B1, VG DB-V1/NI /S/S1/B1 and VTS DB-V1/NI /R/S1/B2 hence denoted with same alphabet and different alphabets on compared means of VTS DB-V1/NI /S/S1/B2 and VTS DB-V1/NI /S/S1/B1 have shown least significant difference hence denoted with different alphabet (ANOVA. $p \geq 0.05$).

6.9.2.2 Semi Quantitative Assay of Protease Enzyme

in vitro screening of protease enzyme been performed on Casein Agar medium. During protease activity, endophytic bacterial strains shown transparent inhibition zone around the well as compared to control **(Figure 6.34)**. Experiment has been

performed in triplicates. The results in **Table 6.10** clearly describes that maximum inhibition zone has been recorded in *Pseudomonas aeruginosa* (VTS DB-V1/NI /S/S1/B2) (8.70mm) followed by *Lysinibacillus macroides* (VTS DB-V1/NI /S/S1/B1) (7.32mm), *Bacillus subtilis* (VTS DB-V1/NI /R/S1/B2) (6.66mm) , VG DB-V1/NI /L/S1/B2 (5.5mm), VG DB-V2/NI /L/S1/B1 (4.2mm), VG DB-V1/NI /S/S1/B1(3.9mm) and VTS DB-V1/NI /L/S1/B2 (3.2mm) during protease activity. **Figure 6.36** depicts the growth of all endophytic bacterial strains as compared to control. According to the study of Mata et al., 2001., lysis of cell wall has been occurred by catalysing the breakage of peptide bonds in proteins.Statistical analysis of **Table 6.10** has shown no significant difference (LSD) in between compared means of VTS DB-V1/NI /L/S1/B2, VG DB-V1/NI /L/S1/B2, VG DB-V2/NI /L/S1/B1, VG DB-V1/NI /S/S1/B1 and VTS DB-V1/NI /R/S1/B2 hence denoted with same alphabet and different alphabets on compared means of VTS DB-V1/NI /S/S1/B2 and VTS DB-V1/NI /S/S1/B1 have shown least significant difference hence denoted with different alphabet (ANOVA. $p \geq 0.05$).

6.9.2.3 Semi Quantitative Assay of Chitinase Enzyme

Chitinase enzyme activity of antagonistic strains been done on Chitinase Detection Medium comprising basal chitin medium. Experiment was carried out in triplicates and growth of all purple-coloured zones was measured. Endophytic bacterial strains have shown purple coloured inhibition zone around the well as significant inhibition activity **(Figure 6.35)**. The results of **Table 6.10** describes the maximum growth of purple coloured inhibition zone in *Pseudomonas aeruginosa* (VTS DB-V1/NI /S/S1/B2) (7.4mm) followed by *Lysinibacillus macroides* (VTS DB-V1/NI /S/S1/B1) (7.1mm), *Bacillus subtilis* (VTS DB-V1/NI /R/S1/B2) (6.3mm), VG DB-V1/NI /L/S1/B2 (6.1mm), VTS DB-V1/NI /L/S1/B2(5.7mm), VG DB-V2/NI /L/S1/B1(4.5mm) and VG DB-V1/NI /S/S1/B1(3.9mm) during chitinase activity. **Figure 6.36** shows the growth of all endophytic bacterial strains as compared to control. Statistical analysis of **Table 6.10** has shown no significant difference (LSD) in between compared means of VTS DB-V1/NI /L/S1/B2, VG DB-V1/NI /L/S1/B2, VG DB-V2/NI /L/S1/B1, VG DB-V1/NI /S/S1/B1, VTS DB-V1/NI /S/S1/B2 and VTS DB-V1/NI /R/S1/B2 hence denoted with same alphabet and different alphabets on compared means of and VTS DB-V1/NI /S/S1/B1 has shown significant difference hence denoted with different alphabet (ANOVA. $p \geq 0.05$).

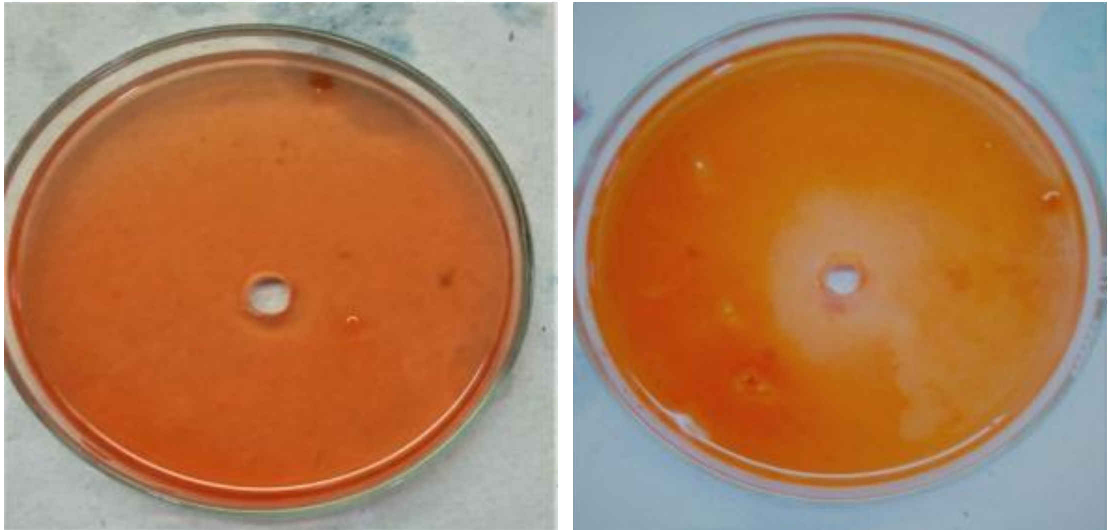


Figure 6.33: Cellulase Activity of Endophytic bacterial strain (a) Control (b) Test plate (yellow-opaque inhibition zone around well)

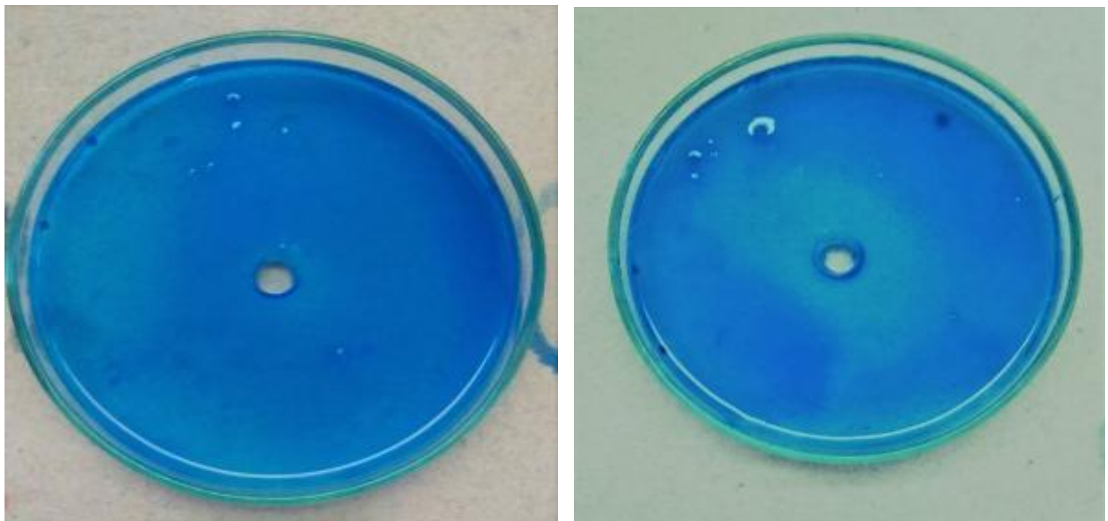


Figure 6.34: Protease Activity of Endophytic bacterial strain (a) Control (b) Test plate (clear inhibition zone around well)

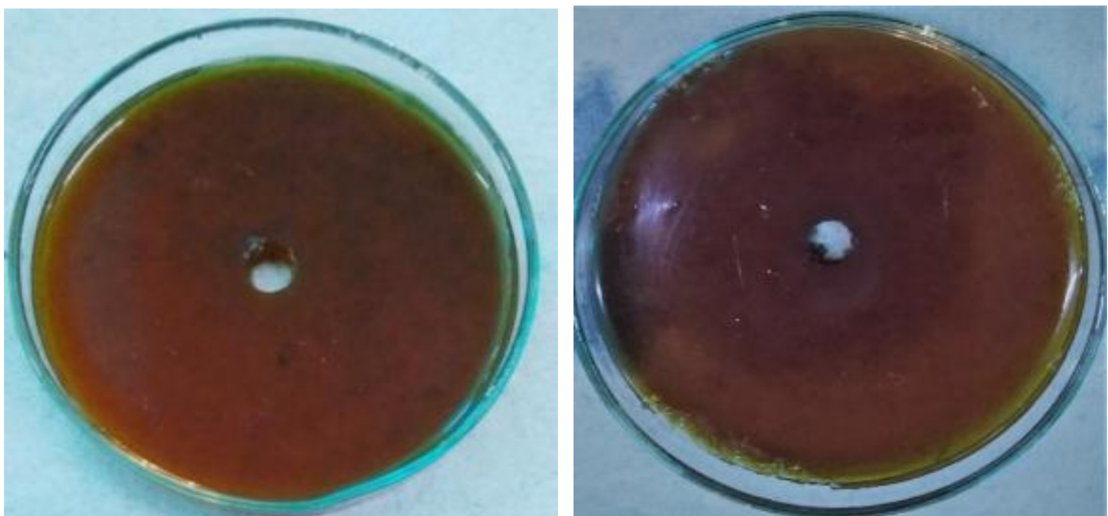


Figure 6.35: Chitinase Activity of Endophytic bacterial strain (a) Control (b) Test plate (clear inhibition zone around well)

Table 6.10: Zone formation during *in vitro* hydrolytic enzymatic activities of different endophytic bacterial strains

			Hydrolytic enzymatic activity of antagonistic strains(mm)		
S. No.	**Isolate**	**Origin**	**Cellulase activity**	**Protease activity**	**Chitinase activity**
1	**VTS DB-V1/NI /L/S1/B2**	Healthy stem of cotton plant	6.8±0.5de	3.2±1.5a	5.7±0.3c
2	**VG DB-V1/NI /L/S1/B2**	Healthy stem of cotton plant	5.2±0.2bc	5.5±0.5cd	6.1±0.5cd
3	**VG DB-V2/NI /L/S1/B1**	Healthy leaves of cotton plant	5.0±0.8ab	4.2±0.3bc	3.9±0.9a
4	**VG DB-V1/NI /S/S1/B1**	Healthy stem of cotton plant	4.5±0.5a	3.9±1.2ab	4.5±0.5ab
5	**VTS DB-V1/NI /S/S1/B2**	Healthy stem of cotton plant	8.56±0.9g	8.70±0.5g	7.4±0.7g
6	**VTS DB-V1/NI /R/S1/B2**	Healthy cotton root	5.23±0.3bcd	6.66±0.6de	6.3±0.4cde
7	**VTS DB-V1/NI /S/S1/B1**	Healthy stem of cotton plant	7.30±0.2f	7.32±0.8f	7.1±0.5ef

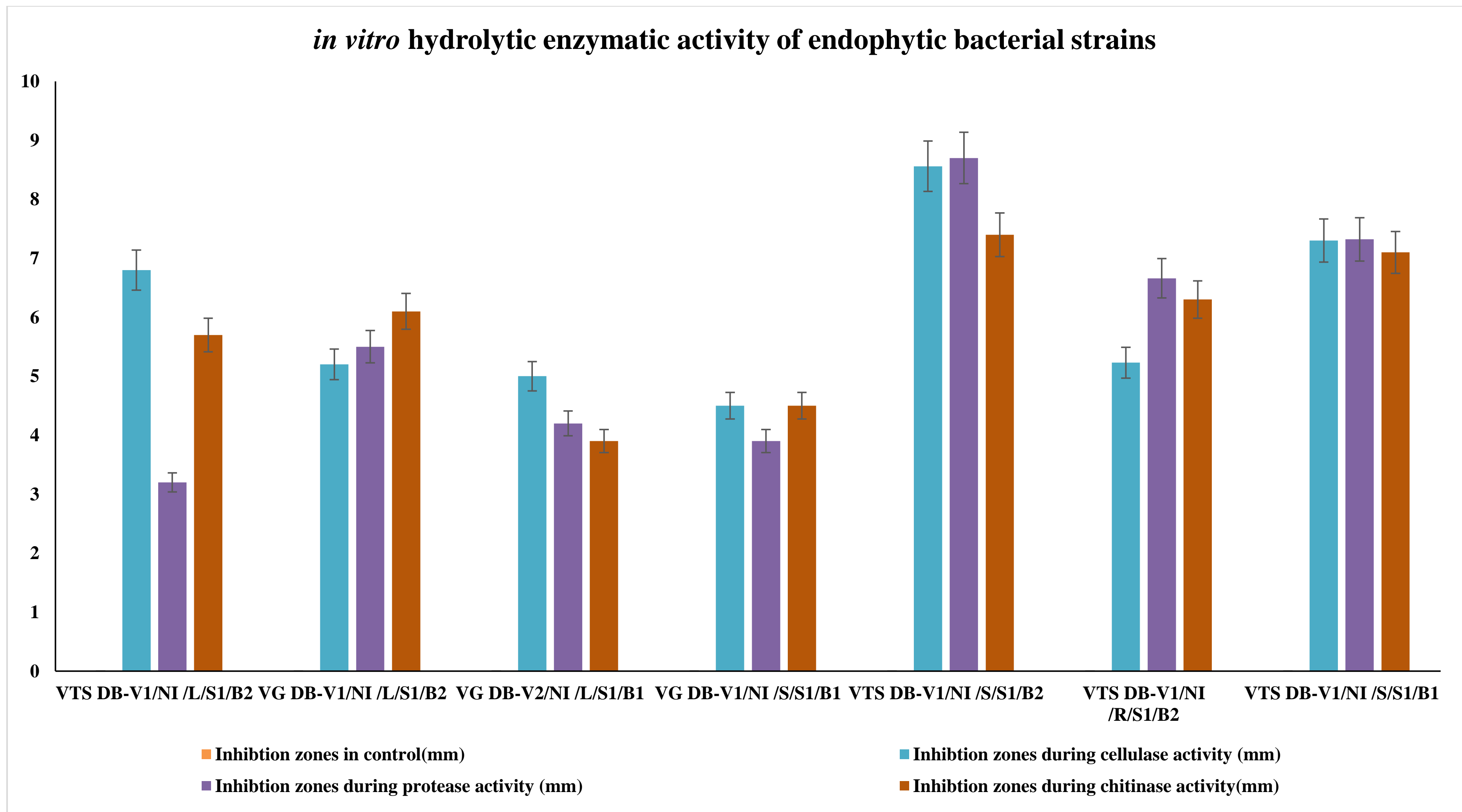
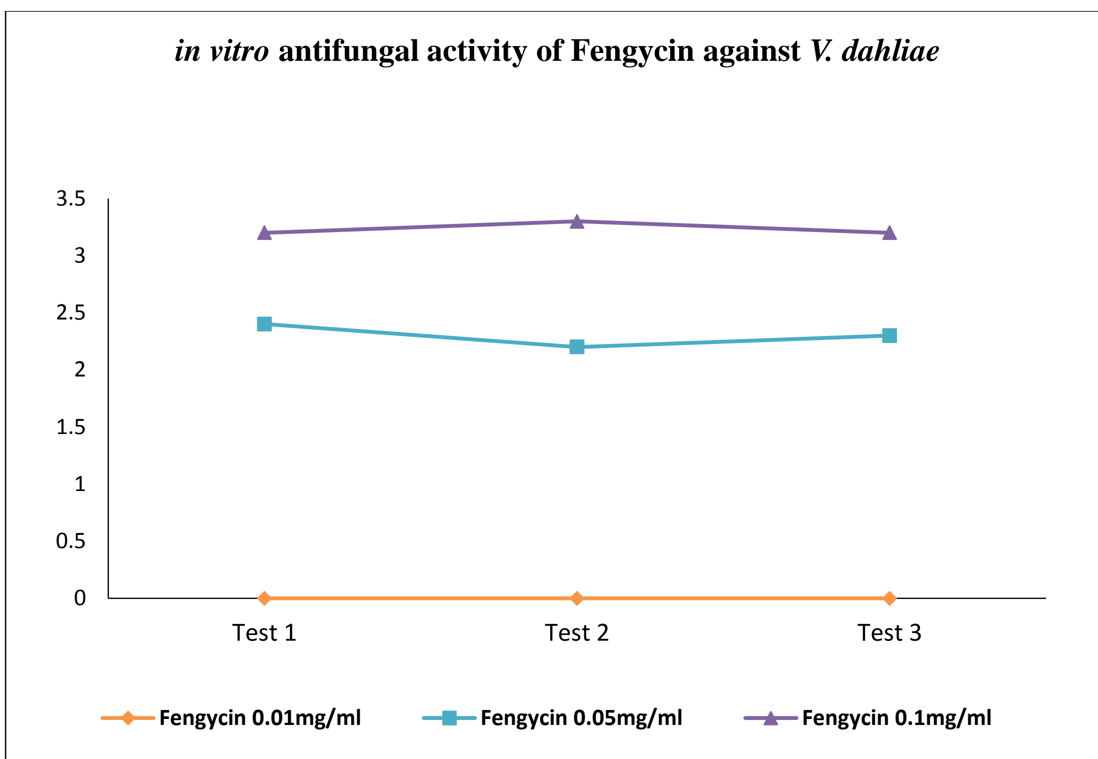


Figure 6.36: Zone formation during *in vitro* hydrolytic enzymatic activities (Standard error bars are indicated)

6.10 Antifungal activity of biosurfactants against *V. dahliae*

Surfactants are amphiphatic molecules, containing both hydrophobic and hydrophilic group. Surfactants can reduce the surface and interfacial tension in water and hydrocarbon system due to the presence of these two groups in the same molecule (Desai and Banat., 1997). Biosurfactants are defined primarily as amphipathic compounds produced on the surface of microbial cells or on biological surfaces on secreted extracellular hydrophobic and hydrophilic moieties and the ability to accumulate between liquid phases. Reduces surface or interfacial surface and interfacial tension (CD et al., 2004). Biosurfactants isolated from different microbial strains have shown positive biocontrol action against various pathogenic microorganism as effectively being used as biocontrol agent for management of various diseases and for achieving sustainable agriculture. Therefore, in the present investigation., Fengycin and Iturin have been evaluated as potential biocontrol agent against *V. dahliae* by checking it's *in vitro* antifungal activity by well diffusion assay.

The results in **Figure 6.37** depicts the *in vitro* antifungal activity of both biosurfactant Fengycin and Iturin against *V. dahliae* in which Fengycin at concentration of 5ppm and 10 ppm have shown 2.3±0.1ab mm and 3.2± 0.05b mm inhibition zones respectively and Iturin at same concentrations have shown 2.9±0.15b mm, 3.8±0.2ab mm inhibition zones respectively as compared to control plate. Both Fengycin and Iturin have shown no inhibition zones against *V. dahliae* at 1ppm concentration. Mean with different letters denote a significant difference between compared means and same letters denote a lack of significant differences between compared means **(ANOVA. p≥0.05).** Similarly, Nihorimbere et al., (2011)., reported biosurfactants showing positive antagonism against pathogenic disease from rhizobacterial strains. Singh et al., 2007 has also reported the application of chemo surfactants and biosurfactants helps to facilitates plant growth and promotion, reduced competition, helps in parasitism and antibiosis activity.



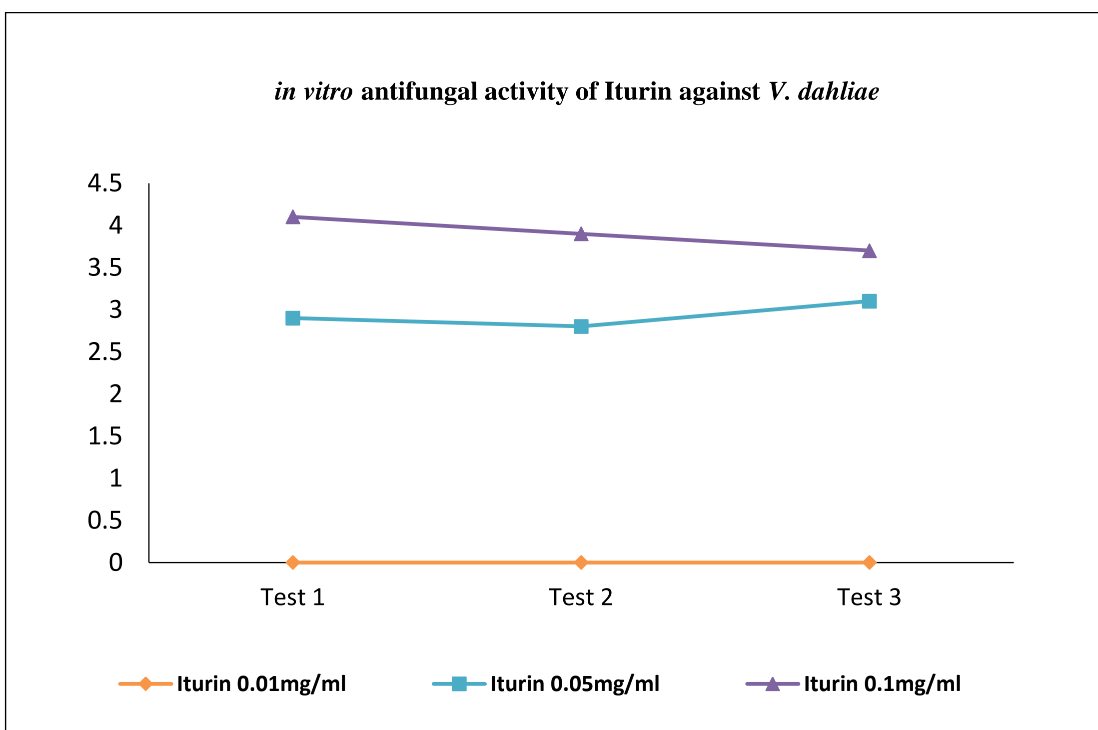


Figure 6.37: *in vitro* screening of antifungal activity of Fengycin and Iturin at 1ppm, 5ppm and 10 ppm concentrations against pathogenic fungus *V. dahliae* during "Well diffusion assay"

6.11 Preparation of Biochemical formulation and its antifungal activity against *V. dahliae*

Biochemical formulations are best defined as biologically active products containing one or more beneficial microbial strains in an easy to use and economical carrier

material with some added chemicals into it and plant growth promotion continues to inspire research and development in many fields. Long term use of harmful pesticides or fertilizers leads to toxic environment and reduce soil fertility, increase plant pathogens resistivity, and negative impacts on beneficial microorganism in soil and on humans (El-Tarabily et al., 2000; Adebanjo and Bankole., 2004). Therefore, production of bioformulations is need of an hour for increased application of microbial based formulations for sustainable agriculture and eliminates plant pathogens and helps to reduce the use of chemical pesticides. Hence, Biochemical formulation has been prepared by using different medicinal plant parts along with antagonistic fungus and endophytic bacterial strains. Plant parts of *Ricinus communis, Tinospora cardifolia, Withania somnifera, Datura stramonium, Clotropis procera, Azadiaracta indiaca, Citrullus colocynthis, Solanum xanthocarpum,* and Copper sulphate has been used for preparation of formulation. End product (crude plant extract) has been prepared after 30 days. *in vitro* antifungal activity of biochemical formulation has been done against *V. dahliae* by well diffusion method wherein biochemical formulation has shown positive *in vitro* antagonism against *V. dahliae* as compared to control. Inhibition zones have been recorded at 10, 50 and 100ppm. Inhibition zone of 7.6±0.8mm and 12±0.2mm has been recorded after 5 days of incubation at 27^0C around the well at 50ppm and 100ppm concentration respectively when compared to control. No inhibition zone has been formed at 10ppm concentration. Biochemical formulation been used in ratio of 1:9 (biochemical formulation: water) in the form of spray mixed with 1x108/ml of each antagonistic fungal strains and bacterized seed with biochemical formulation for *in vivo* evaluation against *V. dahliae*. According to the study of (Rouphael et al., 2015; Ruzzi and Aroca., 2015), microbial based bioformulations or bio stimulants like fungal based bioformulations with *Trichoderma sp*. and other endophytic fungal strains including mycorrhizal fungal strains and different species of PGPRs (*Azospirillum*, *Azotobacter* and *Rhizobium spp*.) have been used as positive biocontrol nutrients and also increased soil productivity under nutrient challenged conditions with nitrogen and phosphorus but also avoid abiotic and biotic stress. The use of powder bioformulations for the treatment of some plant diseases has already been demonstrated. For example, Amer and Utkhede., 2000 has developed several bioformulations and used them to successfully treat root rot.

6.11.1 LCMS of biochemical formulation:

LCMS has been greatly applied in the field of both proteomics and metabolomics research. This spectrometry is considered to highly advanced and analytical technology. LCMS is used for profiling of different compounds in samples with further structural elucidation. The different compounds in samples can be detected relatively or absolutely through analysing spectral features from LCMS data. The compounds identification in LCMS chromatograph is carried out based on mass-to-charge ratio and retention time. In our study, profiling of phytoconstituents in aqueous extract of different plants parts of *Ricinus communis, Tinospora cardifolia, Withania somnifera, Datura stramonium, Clotropis procera, Azadiaracta indiaca, Citrullus colocynthis, Solanum xanthocarpum* was carried out through positive and negative ionization MS-ESI mode of LCMS method. The LCMS chromatograms in positive and negative ionization mode are shown in **Figure 6.38**. In comparison with the previous literature review, the bioactive compounds were characterized by their mass spectrum, retention time, and fragmentation profiles. The list of identified phytocompounds with their molecular weight, retention time, monoisotopic mass and chemical formula has been shown in **Table 6.11** and **Table 6.12**.

In the present investigation, the positive ESI mode was found to be highly significant than negative ESI mode because more compounds have been detected from different plant extracts. It was observed that the different peaks were obtained at different retention times. In our results of the LC chromatogram, different compounds with their pharmacological were assigned on their peaks produced at different retention times (RT) as below **(Table 6.11-6.12).**

- ❖ The retention time (RT) 4.14 min of the peak was corresponding to 3-Glc-campesterol compound that is reported by Santos et al., 2018. It has antioxidant and antimicrobial activity (Santos et al., 2018).

- ❖ The retention time (RT) 6.65 min of the peak was corresponding to 2-O-β-D-glucopyranosyl-cucurbitacin E compound with antibacterial activity and is reported by Chawech et al., 2015.

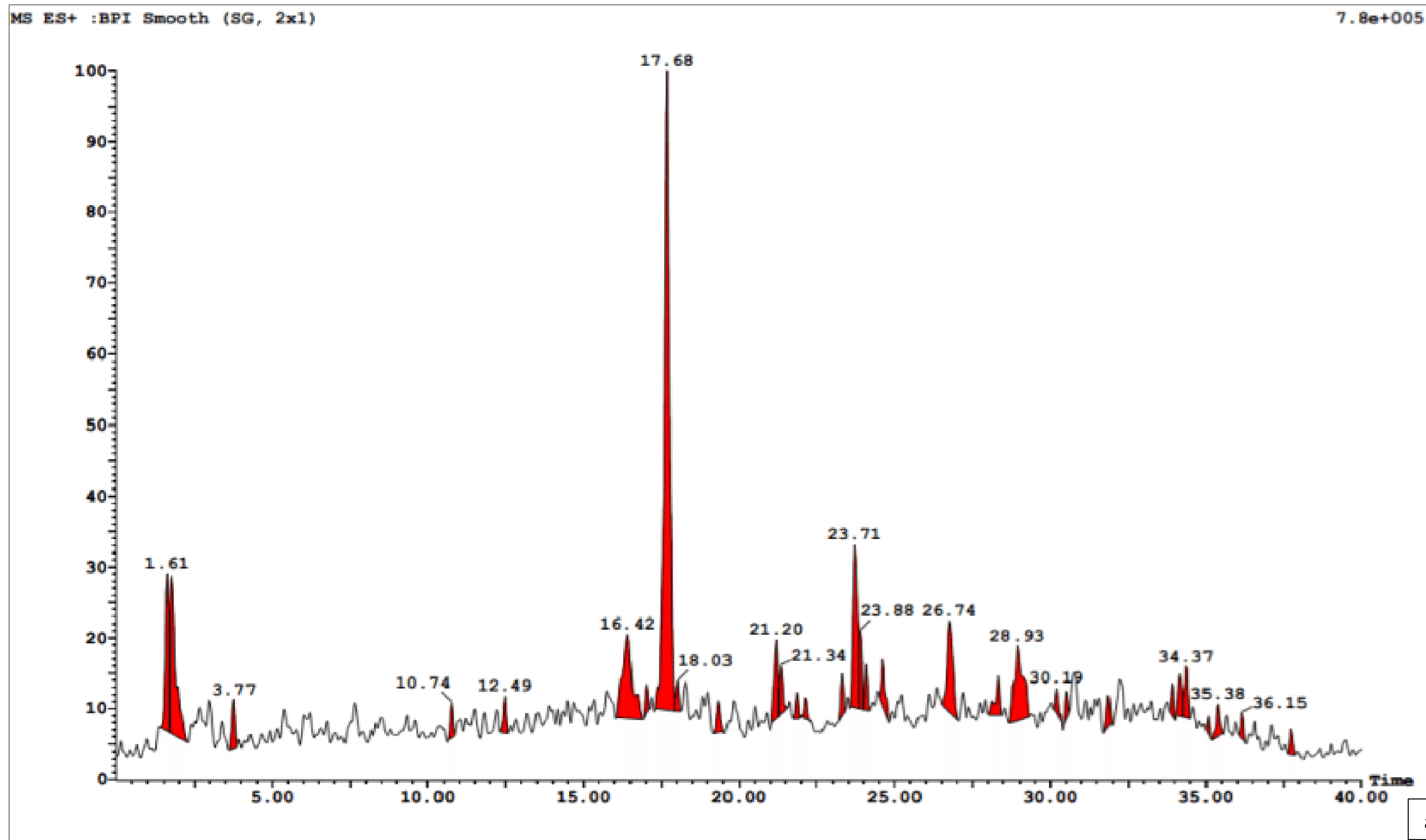
- ❖ The retention time (RT) 9.61 min of the peak was corresponding to Momordol compound that is reported by Santos et al., 2018. It is widely used in

antimicrobial activity, and it has great antioxidants present in it (Santos et al., 2018).

- ❖ The retention time (RT) 17.02 min of the peak was corresponding to Dihydroisocucurbitacin B-25-acetyl compound with antibacterial activity that is reported by Chawech et al., 2015.
- ❖ The retention time (RT) 15.99 min of the peak was corresponding to 2-O-β-D-glucopyranose- (22-27)- hexanocucurbitacin I compound that is reported by Chawech et al., 2015 and having antibacterial activity.
- ❖ The retention time (RT) 22.96 min of the peak was corresponding to 2-O-β-D-glucopyranosyl-cucurbitacin L compound with antibacterial activity and is reported by Chawech et al., 2015.
- ❖ The retention time (RT) 38.47 min of the peak was corresponding to Arvenin I compound that is reported by Chawech et al., 2015. It has antifungal activity against plant pathogens (Wang et al., 2013).
- ❖ The retention time (RT) 29.96 min of the peak was corresponding to 6'-acetyl-2-O-β-D- glucopyranosyl-cucurbitacin E compound showing antibacterial activity against according to the study of Chawech et al., 2015.
- ❖ The retention time (RT) 29.09 min of the peak was corresponding to 2,3-Dihydrowithaferin A3β-O-sulfate compound with different biological activities and is reported by Xue et al., 2009.
- ❖ The retention time (RT) 33.41 min of the peak was corresponding to 23, 24-dihydrowithagenin compound that is reported by Bolleddula et al., 2012. According to the study of Khan and Nasreen., 2010 it has antifungal activity against plant pathogens.
- ❖ The retention time (RT) 37.75 min of the peak was corresponding to Dihydrowithanolide compound that is reported by Jayaprakasam et al., 2004. According to the study of Khan and Nasreen., 2010 it has antifungal activity against plant pathogens.
- ❖ The retention time (RT) 14.77 min of the peak was corresponding to Dihydrowithferin A compound with antifungal activity according to the study of Trivedi et al., 2017.

- ❖ The retention time (RT) 17.68 min of the peak was corresponding to Petunidin 3-O-rhamnoside compound with great antioxidants and antibacterial potential according to Rothwell et al., 2015.
- ❖ The retention time (RT) 21.86 min of the peak was corresponding to Adenylosuccinate compound that is reported by Rothwell et al., 2015. Study of Silva et al., 2018., Adenylosuccinate has antibacterial activity.
- ❖ The retention time (RT) 18.67 min of the peak was corresponding to Lysoplasmenylcholine compound with antimicrobial activity., reported by Rothwell et al., 2015.
- ❖ The retention time (RT) 29.72 min of the peak was corresponding to GDP-L-galactose compound that is reported by Huang et al., 2013.
- ❖ The retention time (RT) 37.0 min of the peak was corresponding to Verbascoside compound that is reported by Boudjelal et al., 2012. According to the study of Oyourou et al., 2013, Verbascoside has shown antifungal activity.
- ❖ The retention time (RT) 19.58 min of the peak was corresponding to Deoxycholic acid 3- glucuronide compound and is used as antifungal compound reported by Satpute and Vnmare., 2018.
- ❖ The retention time (RT) 13.55 min of the peak was corresponding to 3 β - Hydroxydeoxo dihydrodeoxygedunin compound that is reported by Satpute and Vnmare., 2018.
- ❖ The retention time (RT) 10.74 min of the peak was corresponding to Hydrocortisone cypionate compound that is reported by Satpute and Vnmare., 2018.
- ❖ The retention time (RT) 22.23 min of the peak was corresponding to Dihydro deoxy streptomycin compound that is reported by Satpute and Vnmare., 2018.
- ❖ The retention time (RT) 28.34 min of the peak was corresponding to Withanolide S compound with great antifungal activity, reported by Trivedi et al., 2017.

- ❖ The retention time (RT) 37.14 min of the peak was corresponding to Solasonine compound that is reported by Paul et al., 2008. It helps to inhibit fungal growth according to the study of Fewell et al., 2014.
- ❖ The retention time (RT) 35.07 min of the peak was corresponding to β -2 solamargin compound that is reported by Paul et al., 2008.
- ❖ The retention time (RT) 22.14 min of the peak was corresponding to Germanicol cinnamate compound that is reported by Santos et al., 2018.
- ❖ The retention time (RT) 19.35 min of the peak was corresponding to Pubesenolide compound that is reported by Santos et al., 2018.
- ❖ The retention time (RT) 30.34 min of the peak was corresponding to Cucurbitacin E compound with antibacterial activity according to the study of Chawech et al., 2015.
- ❖ The retention time (RT) 31.87 min of the peak was corresponding to 2-O-β-D-glucopyranosyl-cucurbitacin I compound with antibacterial activity that is reported by Chawech et al., 2015
- ❖ The retention time (RT) 34.66 min of the peak was corresponding to 25-p-coumaroyl-3'-acetyl-2-O-β-D glucopyranosyl-cucurbitacin I compound with antibacterial activity that is reported by Chawech et al., 2015.
- ❖ The retention time (RT) 21.64 min of the peak was corresponding to Petunidin 3-O-rhamnoside compound that is reported by Baenas et al., 2014.
- ❖ The retention time (RT) 24.22 min of the peak was corresponding to Aswagandhanolide compound that is reported by Subbaraju et al., 2006.
- ❖ The retention time (RT) 24.64 min of the peak was corresponding to Sitoindoside X compound that is reported by Ghosal et al., 2006.
- ❖ The retention time (RT) 25.65 min of the peak was corresponding to 4-Deoxywithaperuvin compound that is reported by Abou-Douh et al., 2006.
- ❖ The retention time (RT) 33.7 min of the peak was corresponding to Solanesol compound that is reported by Takshak and Agrawal., 2015.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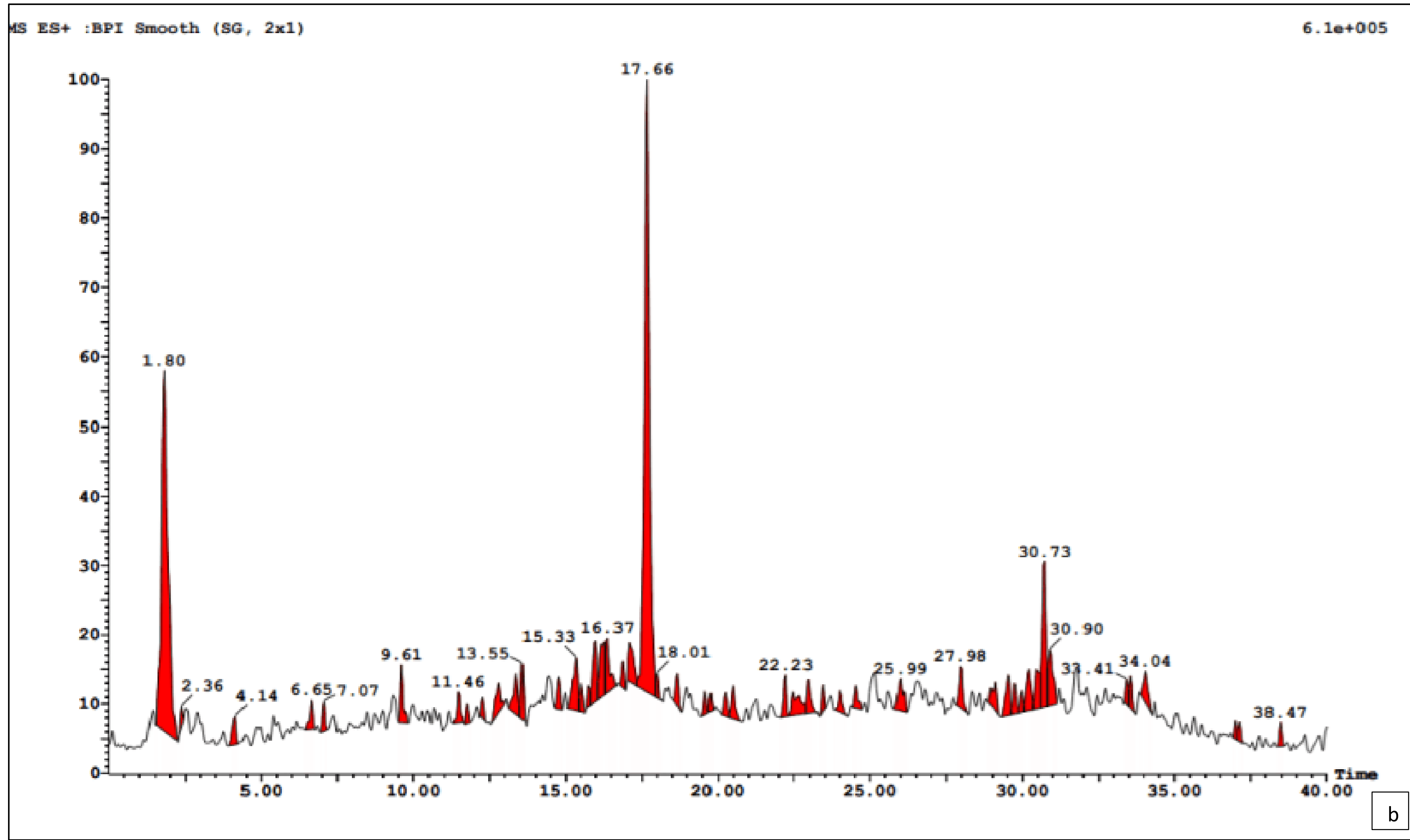
17.68
1.61
3.77
10.74
12.49
16.42
18.03
21.20
21.34
23.71
23.88
26.74
28.93
30.19
34.37
35.38
36.15
Time
a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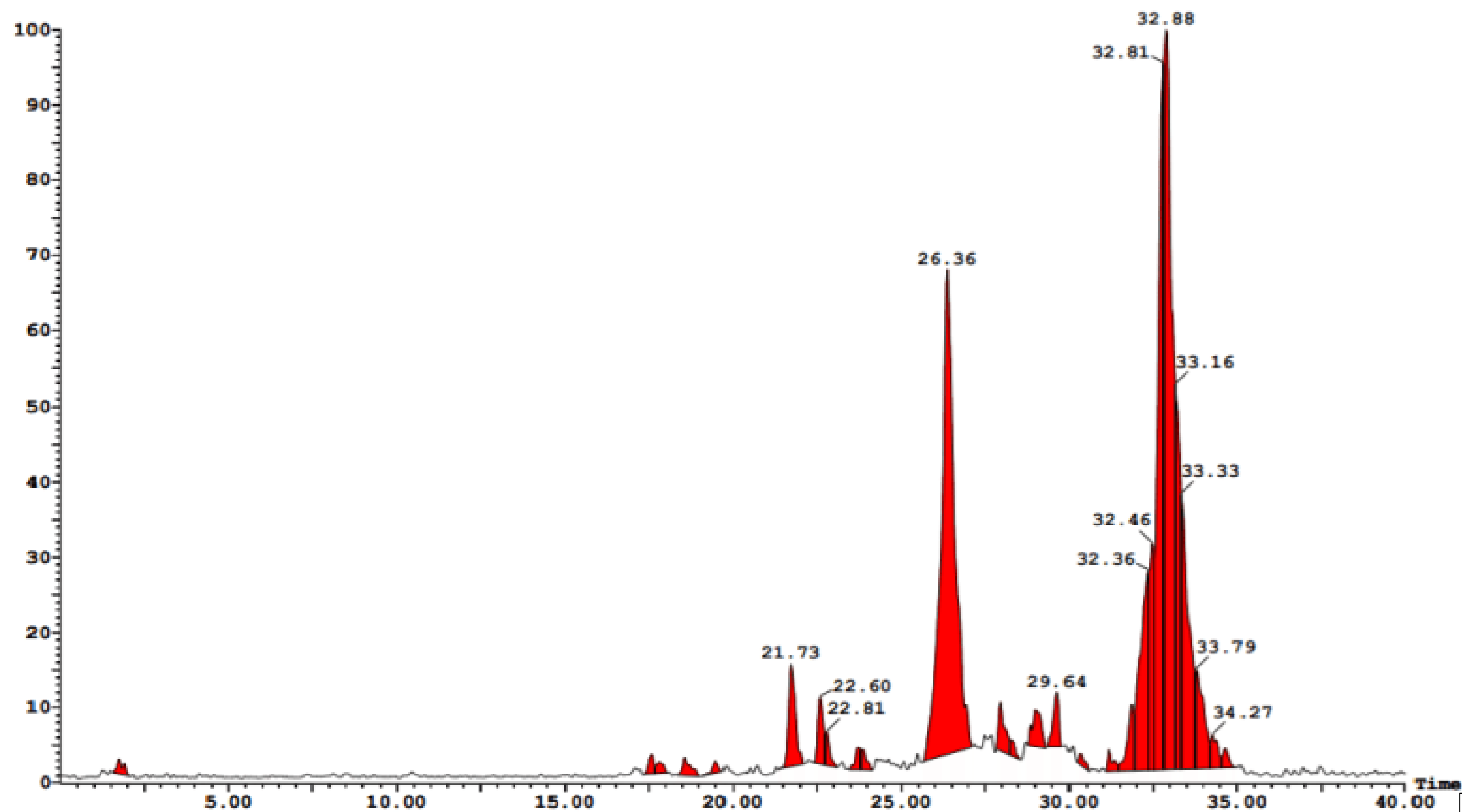
17.66
1.80
30.73
30.90
16.37
15.33
18.01
9.61
13.55
22.23
27.98
25.99
33.41
34.04
11.46
2.36
6.65
7.07
4.14
38.47
Time
b


MS ES- :BPI Smooth (SG, 2x1)
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100
90
80
70
60
50
40
30
20
10
0
32.88
32.81
26.36
33.16
33.33
32.46
32.36
21.73
33.79
22.60
29.64
22.81
34.27
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35.00
40.00
Time
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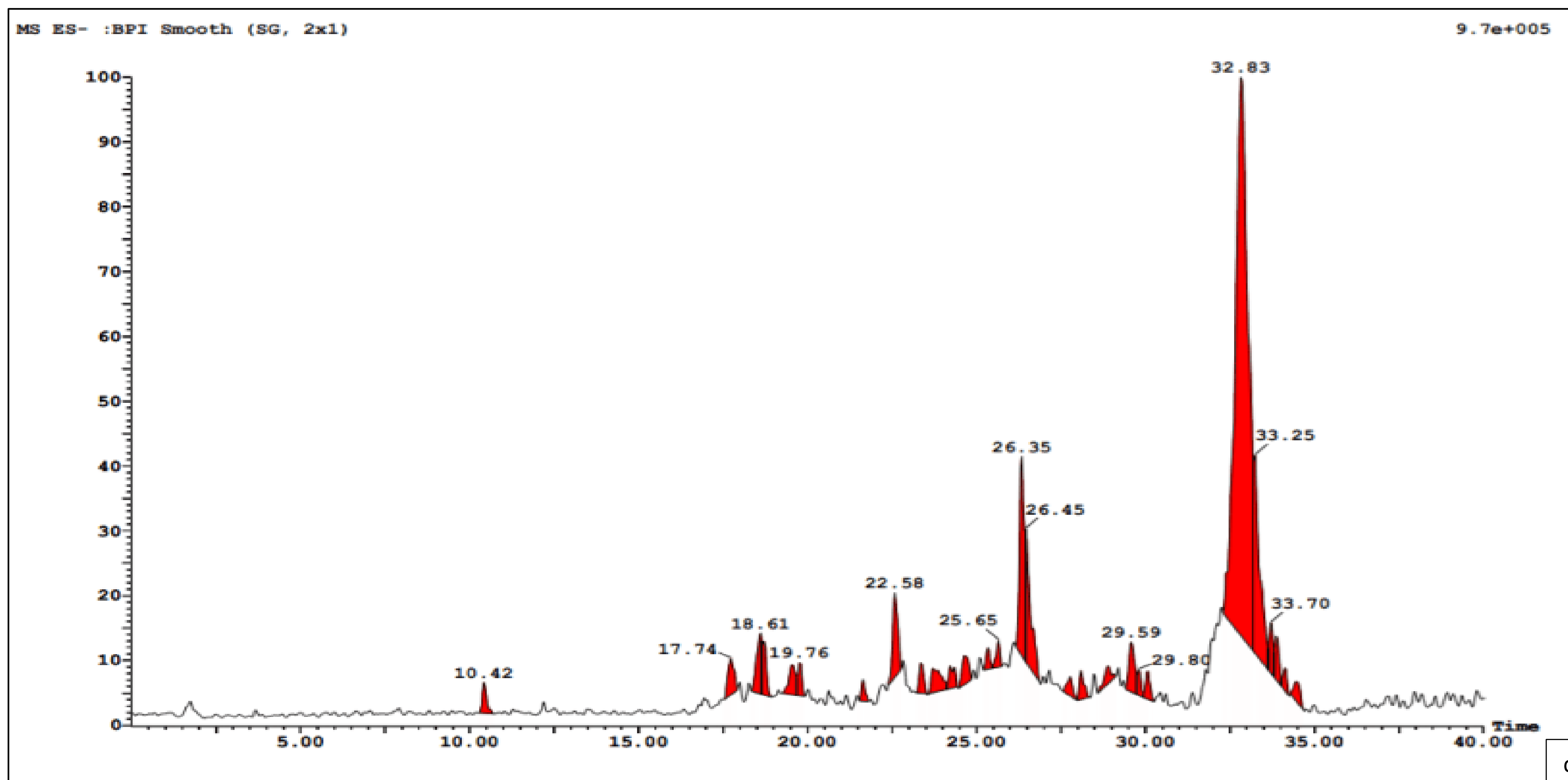


Figure 6.38: (a,b) LCMS chromatogram of biochemical formulation in positive mode showing peaks with identified compounds listed in Table 6.11 (c, d) LCMS chromatogram of biochemical formulation in negative mode showing peaks with identified compounds listed in Table 6.12

Table 6.11: Identification of active compounds in biochemical formulation by LCMS of in positive ionization mode

Sr No	Rt	Mol wt	Monoisotropic mass	Chemical formula	Compounds	References
1	4.14	878	882.73125546	$C_{56}H_{94}O_7$	3-Glc-campesterol	Santos et al., 2018
2	9.61	440	440.350174646.	$C_{26}H_{48}O_5$	Momordol	
3	17.02	560	146.05790880.	$C_{32}H_{48}O_8$	Dihydroisocucurbitacin B-25-acetyl	Chawech et al., 2015
4	15.99	562	29803-94-9.	$C_{30}H_{42}O_{12}$	2-O-β-D- glucopyranose- (22-27)-hexanocucurbitacin I	
5	22.96	678	676.34587709	$C_{36}H_{54}O_{12}$	2-O-β-D-glucopyranosyl-cucurbitacin L	
6	6.65	718	29803-94-9.	$C_{38}H_{54}O_{13}$	2-O-β-D- glucopyranosyl-cucurbitacin E	
7	38.47	720	146.05790880.	$C_{38}H_{56}O_{13}$	Arvenin I	
8	29.96	760	18444-66-1	$C_{40}H_{56}O_{14}$	6'-acetyl-2-O-β-D- glucopyranosyl-cucurbitacin E	
9	29.09	567	472.28249	$C_{28}H_{40}O_6$	2,3-Dihydrowithaferin A3β-O-sulfate	Xu et al., 2009
10	33.41	817	436.350174646	$C_{20}H_{27}O_9$	23, 24-dihydrowithagenin	Bolleddula et al., 2012
11	37.75	785	472.28248899	$C_{28}H_{40}O_7$	Dihydrowithanolide	Jayaprakasam et al., 2004
12	14.77	490	472.28248899	$C_{28}H_{40}O_6$	Dihydrowithferin A	Trivedi et al., 2017

13	17.68	447	479.11895116	$C_{22}H_{23}O_{10}$	Petunidin 3-O-rhamnoside	Rothwell et al 2015
14	21.86	462	463.07404340	$C_{14}H_{18}N_5O_{11}P$	Adenylosuccinate	
15	18.67	479	299.11338904	$C_{26}H_{54}NO_7P$	Lysoplasmenylcholine	
16	29.72	604	605.07715372	$C_{16}H_{25}N_5O_{16}P_2$	GDP-L-galactose	Huang et al., 2013
17	37.0	623	624.205444	$C_{29}H_{36}O_{15}$	Verbascoside	Boudjelal et al., 2012
18	19.58	568	568.32474772	$C_{30}H_{48}O_{10}$	Deoxycholic acid 3- glucuronide	Satpute and Vnmare., 2018
19	13.55	470	470.26683893	$C_{28}H_{38}O_6$	3 β -Hydroxydeoxo dihydrodeoxygedunin	
20	10.74	486	486.29813906	$C_{29}H_{42}O_6$	Hydrocortisone cypionate	
21	22.23	567	583.28131977	$C_{21}H_{41}N_7O_{11}$	Dihydro deoxy streptomycin	
22	28.34	505	504.27231823	$C_{28}H_{40}O_8$	Withanolide S	Trivedi et al 2017
23	37.14	884	883.49293524	$C_{45}H_{73}NO_{16}$	Solasonine	Paul et al., 2008
24	35.07	722	867.49802062	$C_{45}H_{73}NO_{15}$	β -2 solamargine	
25	22.14	556	556.428031036	$C_{39}H_{56}O_2$	Germanicol cinnamate	Santos et al., 2018
26	19.35	458	458.3032	$C_{28}H_{42}O_5$	Pubesenolide	

Table 6.12: Identification of active compounds in biochemical formulation by LCMS of in negative ionization mode

Sr. No.	Rt	Mol weight	Monoisotropic mass	Chemical formula	Compounds	References
1	30.34	556	556.303618384	$C_{32}H_{44}O_8$	Cucurbitacin E	Chawech et al., 2015
2	31.87	676	514.29305367	$C_{36}H_{52}O_{12}$	2-O-β-D- glucopyranosyl-cucurbitacin I	
3	34.66	864	514.29305367	$C_{47}H_{60}O_{15}$	25-p-coumaroyl-3'-acetyl-2-O-β-D glucopyranosyl-cucurbitacin I	
4	21.64	951	479.11895116,	$C_{22}H_{23}O_{10}$	Petunidin 3-O-rhamnoside	Baenas et al., 2014
5	24.22	975	974.521423	$C_{56}H_{78}O_{12}S$	Aswagandhanolide	Subbaraju et al., 2006
6	24.64	871	814.66865521	$C_{34}H_{48}O_{11}$	Sitoindoside X	Ghosal et al.,1988
7	25.65	504	290.11338906	$C_{22}H_{27}O_{11}$	4-Deoxywithaperuvin	Abou-Douh., 2002
8	33.7	631	630.57396698	$C_{45}H_{74}O$	Solanesol	Takshak and Agrawal., 2015

6.12 Soil solarization of natural field before sowing of seeds

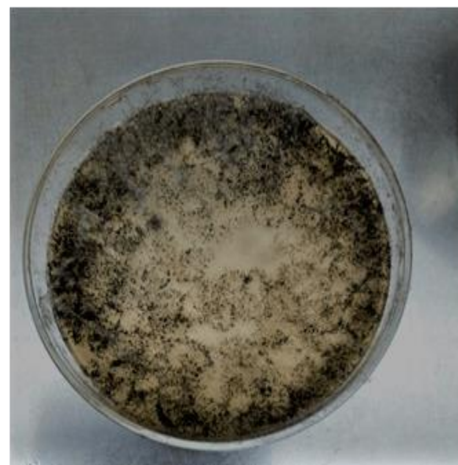
Soil solarization technique has been followed before sowing of seeds under natural field conditions**.** The field was prepared by ploughing before sowing of seeds. Dry weeds and stubble were removed by deep ploughing. Field has been prepared according to the experimental field layout as earlier mentioned. Four equal ridges have been prepared with wooden leveler and properly covered with polythene sheets for 30 days. After soil solarization treatment, sterility of soil has been evaluated for non-presence of pathogenic strains. **Figure 6.39** depicts that *Aspergillus niger* has been isolated from solarized soil but no growth of *V. dahliae* has been recorded after soil solarization. Hence, soil solarization plays an important role to control wilt disease, no growth of *Verticillium dahliae* has been recorded when soil sample has been tested by Serial dilution method.

(a)

(b)

(c)

(d)



(e)

Figure 6.39: (a, b) Preparation of field layout for soil solarization before sowing of seeds (c) Soil solarization with polythene sheets (d, e) Isolation of fungal strains from soil samples collected after soil solarization in experimental field layout

6.13 Soil sterilization and seed bacterization for Poly house experiments

Evaluation of potential antagonistic microbial strains against *Verticillium dahliae* has been performed in poly house study. Soil has been tested for the non-presence of *V. dahliae* periodically after soil sterilization before sowing of seeds. *Verticillium dahliae* has not been recorded in any PDA plate after soil sterilization. Bacterization of delinted seeds of *Verticillium dahliae* sensitive local variety Rasi 134 Bt has been done for both field and pot experiments. **Figure 6.40** depicts the growth of endophytic bacterial strains in nutrient broth medium after 24 hours at 37^0C and **Figure 6.41** depicts the seed bacterization process in laminar flow hood with 3 endophytic bacterial strains.

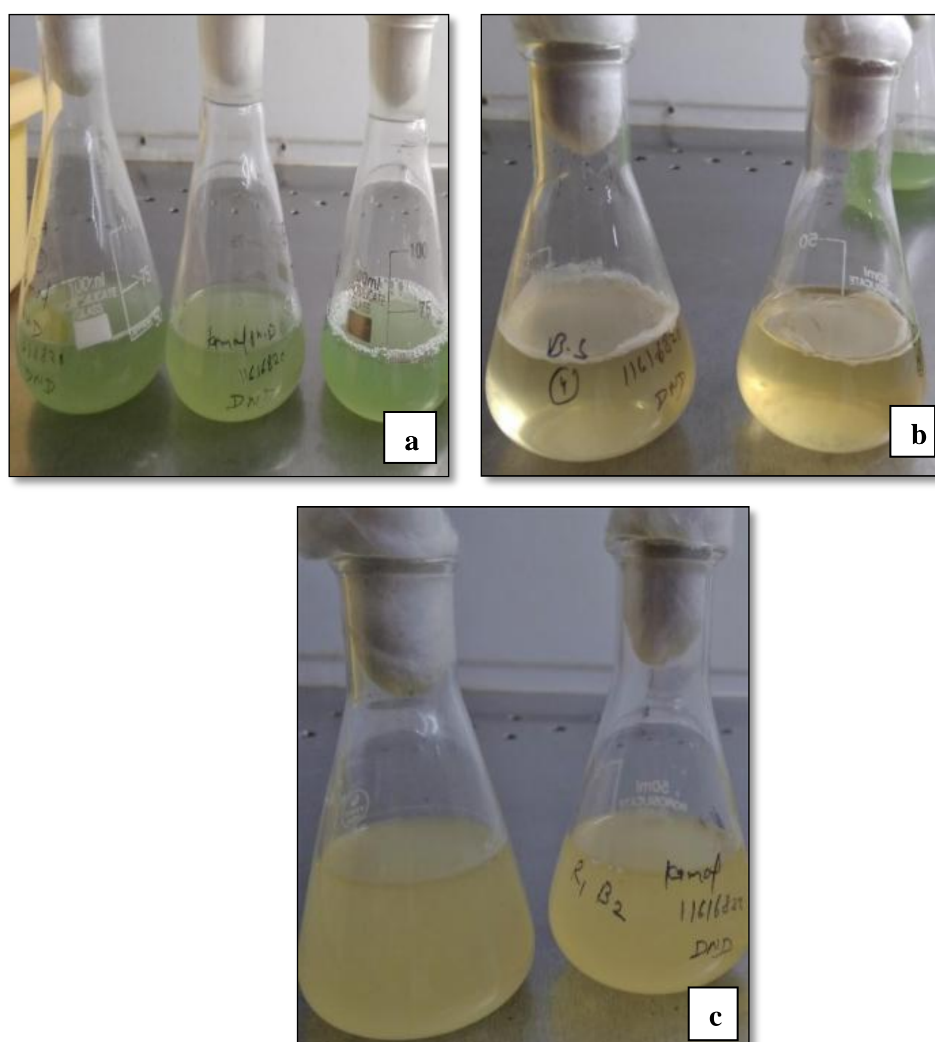


Figure 6.40: Growth of Endophytic bacterial strains on NA medium (a) (*Pseudomonas aeruginosa*) VTS DB-V1/NI /S/S1/B2 (b) (*Bacillus subtilis*) VTS DB-V1/NI /R/S1/B2 (c) (*Lysinibacillus macroides*) VTS DB-V1/NI /S/S1/B1

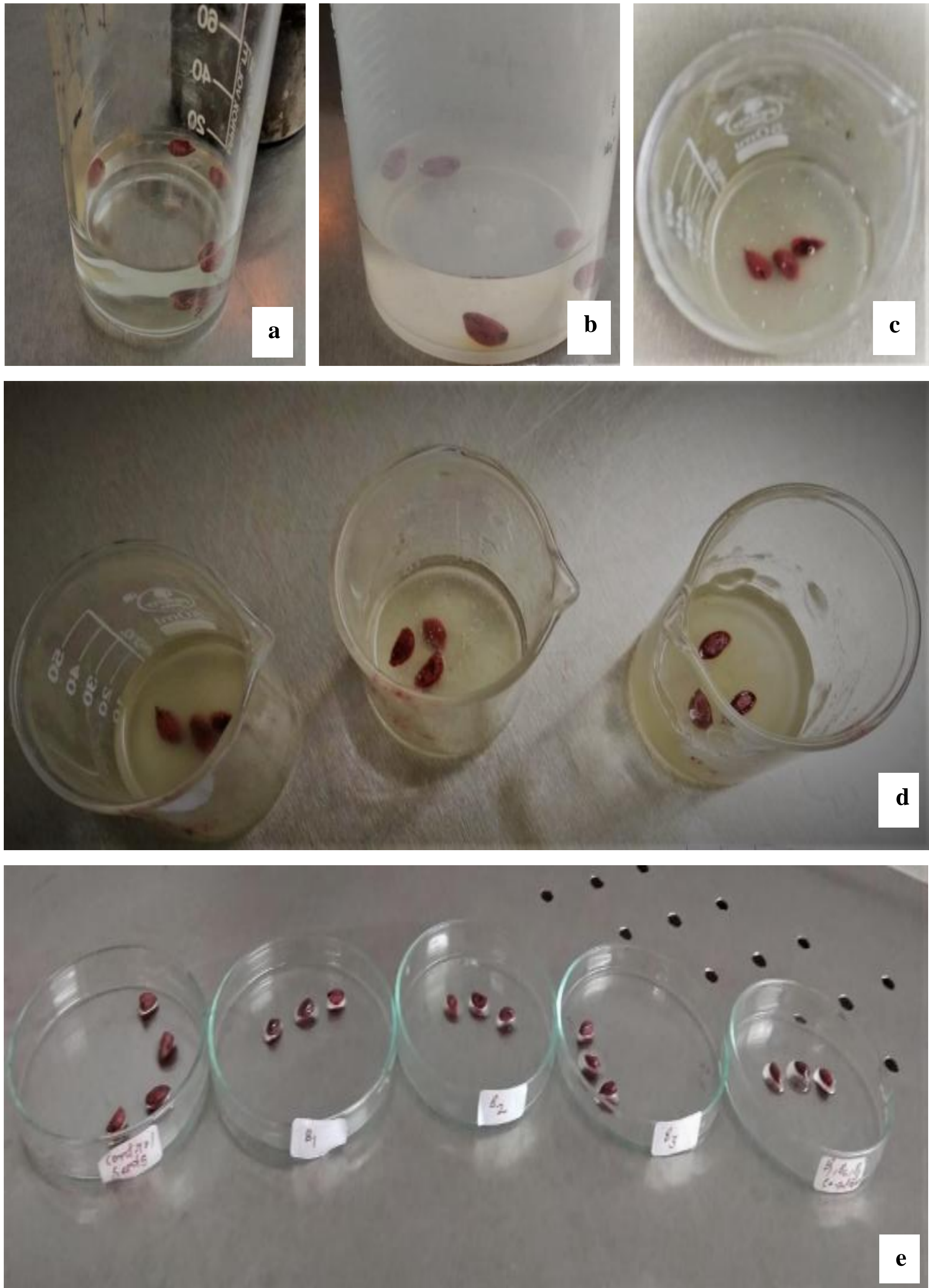


Figure 6.41: Process of Seed bacterization with endophytic bacterial strains (a) Surface sterilization of seeds (b) Washing with 0.5 M phophate buffer (c,d) Soaking of seeds in 1.5% CMC solution with endophytic bacterial strains (e) Bacterized seeds were kept separately in sterile condition in laminar flow for 12 hours

6.14: Biological control of *Verticillium* wilt of Cotton during field experiments

6.14.1 Biological control of *Verticillium* wilt of Cotton under field conditions with antagonistic fungal strains (monoculture experiment)

Antagonistic fungal strains have been used in the present investigation to control *Verticillium* wilt biologically under natural filed conditions. The experiment has been done under monoculture condition. **Figure 6.45** depicts the different growth stages of cotton plant during natural field conditions. Among 8 strains, 4 strains of *Aspergillus* viz. *A. niger*, *A. nidulans*, *A. fumigatus*, *A. terreus* and *Trichoderma harzianum* was selected for *in vivo* evaluation after checking their *in vitro* antagonistic potential. Disease symptoms have been appeared in control 2(inoculated with *V. dahliae*) after first blooming of cotton growth in August. **Figure 6.42** depicts first symptoms of wilt disease as yellow discolouration or chlorotic area between the veins of leaves in control plants when compared with test plants. Disease severity index has been recorded and calculated wherein control 2 plant (inoculated with *V. dahliae*) has shown wilting (87%) and control1 (inoculated with water only) has shown 46%, 32%, 28%, 19% and 32% disease severity during *A. nidulans*, *A. terreus*, *T. harzianum*, *A. fumigatus* and *A. niger* respectively. Cotton plants treated with *A. nidulans* have shown acropetal chlorosis (28%) and 0% disease severity (healthy) after 135 days of sowing has been recorded in test plants treated with *A. niger*, *A. fumigatus*, *A. terreus* and *Trichoderma harzianum*. **Table 6.15** describes the biocontrol protection of *Aspergillus terreus* (74.5%) followed by *Trichoderma harzianum* (72.3%), *Aspergillus fumigatus* (68.8%), *A. niger* (66.2%), *A. nidulans* (61.2%) as compared to control 1 (43.2), (41.3), (36.7), (41.3), (33.8) and control 2 (26.5), (24.2), (39.5), (22.6) and (39.5) respectively.

Figure 6.42: (a) Control plant of cotton inoculated with *V. dahliae* showing symptoms of wilt (b) Test plant treated with antagonistic fungal strain showing no disease symptoms

6.14.2 Biological control of *Verticillium* wilt of Cotton under natural field conditions with endophytic bacterial strains (Monoculture experiment)

In the present investigation*, Pseudomonas aeruginosa, Lysinibacillus macroides* and *Bacillus subtilis* were selected as biocontrol agent under natural field conditions after evaluating their significant *in vitro* effect against *Verticillium dahliae*. This experiment has been done under monoculture condition. Symptoms of wilt disease has been observed and recorded after every fortnight. Disease symptoms have been appeared after first blooming of cotton growth in August. Yellow discolouration or chlorosis between the veins of leaves were first symptoms of wilt disease in control (non-bacterized) plants when compared with test plants (bacterized). Seed treated with strain of *Pseudomonas aeruginosa* had significantly reduced the symptoms of wilt disease followed by *Lysinibacillus macroides* and *Bacillus subtilis* as compared to non-bacterized seeds. After first blooming stage of cotton plant, control plant has shown symptoms of wilting compared to test plants. Seed treated with *Pseudomonas aeruginosa* and *Lysinibacillus macroides* has shown 0% disease severity (healthy) and *B. subtilis* has shown acropetal chlorosis with 28% disease severity at 0-4 disease severity index rating scale. **Figure 6.43** depicts that control plants (non-bacterized) have shown wilting or death (88%) after 135 days of sowing. **Table 6.13** describes that most significant biocontrol efficacy has been recorded in *Pseudomonas aeruginosa* (73.6%) followed by *Lysinibacillus macroides* (68.5%) and *Bacillus subtilis* (62.5) as compared to control plant with (40.3), (36.7) and (39.2) biocontrol protection percent, respectively. Results indicates the reduction of wilt disease after direct inoculation of microsclerotia of *V. dahliae* into stem of bacterized cotton plants during *in vivo* studies. Microsclerotia of *V. dahliae* directly penetrates into roots and responsible for causing infection. Bacterial strains are expected to supress the action of *V. dahliae* through root penetration. Therefore., previous researchers conducted *in vivo* trials by artificial inoculation of microsclerotia of *V. dahliae* into soil. Endophytic bacteria such as *Pseudomonas aeruginosa* considered as most significant bacteria acts as a biocontrol agent and helps in growth of plant. Disease severity and symptoms ware significantly reduced after seed bacterization with *Pseudomonas aeruginosa* followed by *Lysinibacillus macroides* and *Bacillus subtilis* in poly house and field trials. Landa et al.,1997 have reported that biocontrol bacterial strains isolated from rhizosphere soil have shown approximately 32% inhibition against *F.*

oxysporum. Microsclerotia has been transferred directly through roots into the cotton plants during natural infection hence biocontrol bacterial strains would be directly penetrating through roots to reduce the effect of *V. dahliae* during evaluation of biocontrol bacterial strains. Different researchers had done *in vivo* trials with rhizobacteria by using artificially inoculated soil with microsclerotia or conidia (Berg et al., 2001; Mercado-Blanco et al., 2004; Tjamos et al., 2004; Uppal et al., 2008) on different crop plants against *V. dahliae*.

6.14.3 Biological control of *Verticillium* wilt of Cotton under natural field conditions with three endophytic bacterial strains and five antagonistic fungal strains (Co culture experiment)

Endophytic bacterial strains have been used in a combinatorial form under natural field conditions to suppress the effect of soil borne pathogen *V. dahliae*. *Pseudomonas aeruginosa, Lysinibacillus macroides* and *Bacillus subtilis* were used as seed bacterization along with five antagonistic fungal strains and evaluated as biocontrol agent under natural field conditions. Symptoms of wilt disease has been observed and recorded after every fortnight. Disease symptoms have been appeared after first blooming of cotton growth in August. Yellow discolouration or chlorotic between the veins of leaves were first symptoms of wilt disease in control (non-bacterized) plants compared with test plants (bacterized). After first blooming stage of cotton plant, control plant has shown symptoms of wilting compared to test plants. Control plants (non-bacterized) have shown wilting or death (81%) after 135 days of sowing and treated plants have shown no disease symptoms (0% healthy) at 0-4 rating scale and 71.3% biocontrol protection percent **(Table 6.14)**.

Figure 6.43: (a) Control plant (Non bacterized) of cotton inoculated with *V. dahliae* showing symptoms of wilt (b) Healthy cotton plant showing no wilt symptoms treated with *P aeruginosa*, *B subtilis* and *L. macroide*

6.14.4 Biological control of *Verticillium* wilt of Cotton under natural field conditions with endophytic bacterial strains and biochemical formulation (Monoculture experiment)

Endophytic bacterial strains with biochemical formulation have been evaluated in a combinatorial form under natural field conditions to suppress the effect of soil borne pathogen *V. dahliae*. *Pseudomonas aeruginosa, Lysinibacillus macroides* and *Bacillus subtilis* were selected for used as biocontrol agent under natural field conditions along with biochemical formulation after evaluating their significant *in vitro* effect against *Verticillium dahliae*. Biochemical formulation has been used in a spray form after every fortnight and symptoms of wilt disease has been observed and recorded carefully. Disease symptoms have been appeared after first blooming of cotton growth in August. Yellow discolouration or chlorotic between the veins of leaves were first symptoms of wilt disease in control (non-bacterized) plants when compared with test plants (bacterized). After first blooming stage of cotton plant, control plant has shown symptoms of wilting than test plants. Seed treated in combination with *Pseudomonas aeruginosa* and *Lysinibacillus macroides* have shown no symptoms of wilt 0% disease severity (healthy) at 0-4 disease severity index rating scale and *B. subtilis* has shown necrosis with 43% disease severity. Control plants (non-bacterized) have shown wilting or death (82%) after 135 days of sowing. **Table 6.13** shows the percentage of biocontrol protection for *Pseudomonas aeruginosa* (76.6%) followed by *Lysinibacillus macroides* (69.5%) and *Bacillus subtilis* (58.3%) as compared to control plants (35.5%), (39.7%) and (36.6%) respectively.

6.14.5 Efficacy of antagonistic fungal strains along with biochemical formulation under natural field experiments (monoculture experiment)

Five antagonistic fungal strains were evaluated against *V. dahliae* with biochemical formulation. Biochemical formulation has been used along with antagonistic fungal strains in spray form. After inoculation of *V. dahliae* symptoms were developed on *Verticillium*-inoculated plants (control). **Figure 6.44** depicts the acropetal chlorosis in cotton plants treated with biochemical formulation along with *A. nidulans* with 26% disease severity. 0% disease severity (healthy) after 135 days of sowing has been recorded in test plants treated with *A. niger*, *A. fumigatus*, *A. terreus* and *Trichoderma*

harzianum under natural field conditions. **Table 6.15** shows the most significant biocontrol efficacy in *Trichoderma harzianum* (79.5%) followed by *Aspergillus terreus* (78.3%), *A. fumigatus* (72.8%), *A. niger (69.2%)* and *A. nidulans* (68.2%) as compared to control 1 (35.6%), (33.6%), (39.7%), (31.5%) and (32.8%) and control 2 plants (24.2%), (26.5%), (39.5%), (22.6%) and (38.5%) respectively. Statistical analysis of biocontrol protection percent during field trials, mean values are followed by the same letter are not significantly different and different letter denotes significant difference of means according to Tukey test at least significant difference (P = 0.05 level). Some beneficial fungi have also been reported to correlate with plant roots in different ways., from mutually beneficial relationships to parasitism (Behie and Bidochka., 2014). The main fungal bio stimulants are mainly composed of the genus *Trichoderma*. Mycorrhizal fungi are known to improve nutrient absorption and plant growth in sustainable and ecological ways, improving crop productivity (Johnson and Gilbert, 2015). The genus *Trichoderma* has become very important as a microbial biostimulant for plants in agriculture due to its multifunctional role in controlling the adverse effects of biological and abiotic stress and their response in vegetable crops (Simard et al., 2012).

Figure 6.44: (a) Control plant of cotton inoculated with *V. dahliae* showing symptoms of wilt without biochemical formulation (b) Test plant treated with antagonistic fungal strain along with biochemical formulation showing no disease symptom

6.14.6 Efficacy of antagonistic strains and endophytic bacterial strains along with biochemical formulation under natural field conditions experiments (Coculture experiment)

Five antagonistic fungal strains were evaluated against *V. dahliae* along with biochemical formulation, used in spray form. After inoculation of *V. dahliae* symptoms were developed on *Verticillium*-inoculated plants (control 2) but were not observed on any of the Control 1. Control plants have shown wilting with 82% after 135 days of sowing than test plants (inoculated with both *V. dahliae* and antagonistic fungal strains, biochemical formulation, and bacterized seeds) after first blooming stage. During natural field condition trials, 78.2 percent significant biocontrol efficacy has been recorded in test plants as compared to control plants **(Table 6.14)**.

a

b

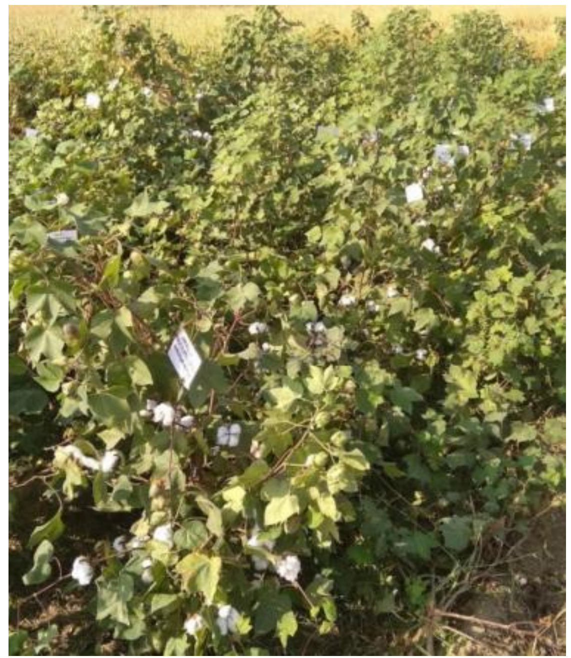
c

d

e

f

g



h **i**

j

Figure 6.45: Stages of growth of cotton plant during natural field conditions in experimental filed layout (a) Preparation of field layout (b) Seed establishment (c) First true leaf stage (d) Growth of cotton plant after 30 days of sowing (e) Growth of cotton plant after 60 days of sowing or canopy development stage (f) Growth of cotton plant after 90 days of sowing (g) First flowering stage of cotton plant (h, i) Development of cotton plants (j) Maturation stage

6.15: Biological control of *Verticillium* wilt of Cotton during polyhouse experiments

6.15.1: Biological control of *Verticillium* wilt of Cotton during polyhouse experiments with endophytic bacterial strains (Monoculture experiment)

Sowing of bacterized seeds with non-bacterized (control) also has been done and symptoms of wilt disease have been observed in non- bacterized plants as compared to bacterized plants. First symptoms of disease have been observed as light-yellow discolouration between veins of leaves after inoculation of *Verticillium dahliae* in control (non-bacterized) plants as compared to bacterized plants. **Figure 6.46** depicts the significant suppression of wilt disease recorded in treatment of seeds with *Pseudomonas aeruginosa* as compared to control plants. Disease severity index has been recorded and calculated wherein control plant (inoculated with *V. dahliae*) has shown wilting (89%) and *Pseudomonas aeruginosa* with 0% (healthy) disease severity, *Lysinibacillus macroides* and *Bacillus subtilis* have shown 19% and 32% acropetal chlorosis, respectively at 0-4 rating scale. **Table 6.13** describes the biocontrol protection percent in *Pseudomonas aeruginosa* (69.6%) followed by *Lysinibacillus macroides* (65.2%) *Bacillus subtilis* (62.5%) as compared to control with (36.4%), (29.7%) and (32.2%) respectively. In similar study of Edwards et al., 1994, the most effective biocontrol bacterial strains have been used from genera belongs to *Bacillus* including *Streptomyces* and *Pseudomonas.* Study of Landa et al., 1997 have shown that biocontrol bacterial strains isolated from rhizosphere soil have shown approximately 32% inhibition against *F. oxysporum*. Similar study has been done by AR et al., 2011, in which the genus *Bacillus* have shown 80.0% of inhibition activity and acts as an antagonist. Result of El-zik, 1985., have described that seed bacterization with four strains of *Pseudomonas* and *S. plymuthica* have Microsclerotia has been transferred directly through roots into the cotton plants during natural

infection hence biocontrol bacterial strains would be directly penetrating through roots to reduce the effect of *V. dahliae* during evaluation of biocontrol bacterial strains. Reduction of wilt disease was 39.2% to 50.9% and 22.1% to 36.8% in trials of 2005 and 2006 respectively when seed was bacterized with *Pseudomonas sp*. isolated from *X. strumarium* (FP22), *Convolvulus arvensis* (FP35*), Portulaca sp.* (FP23), *G. hirsutum* (FP30) (Erdogan and Benlioglu., 2009). According to the study of Leben et al., 1987; Berg et al., 2001; Mercado-Blanco et al., 2004; Tjamos et al., 2004; Malandraki et al., 2008; Uppal et al., 2008) in *vivo* trials have been done with rhizobacterial strains by using soil inoculated with conidia or microsclerotia against *V. dahliae* on different crop plants.

6.15.2 Biocontrol efficacy of potential antagonistic strains under poly house (Monoculture experiment)

During polyhouse trials, antagonistic fungal strains were evaluated against *V. dahliae*. Symptoms of wilt has been developed in *Verticillium*-inoculated plants (control 2) but not developed on any Control 1 plants (water-inoculated plants). Light yellow areas between the veins of leaves were the first disease symptoms appeared in control 2 plants and also in second mature leaves of control 2 plants. After first blooming stage of cotton plant, control 1 has shown acropetal chlorosis and control 2 plants shown symptoms of wilting than test plants (inoculated with both *V. dahliae)*. Plants treated with *Trichoderma harzianum, Aspergillus terreus* and *A. niger* has shown 0% disease severity (healthy) and test plants treated with *A. fumigatus* and *A. nidulans* have shown acropetal chlorosis with 22 and 28% respectively at 0-4 disease severity index rating scale. Control 2 plants have shown wilting or death (81%) after 135 days of sowing. **Table 6.15** describes that during pot trials, most significant biocontrol efficacy has been recorded in *Trichoderma harzianum* (70.3%) followed by *Aspergillus terreus* (66.5%), *A. niger* (64.2%), *A. fumigatus* (61.5%) and *A. nidulans* (59.2%) as compared to control 1 plants with (38.1), (36.2), (38.4), (42.7) and (35.8) and control 2 plants (22.1), (28.8), (22.6), (39.5) and (38.5) respectively. Results of Harman et al., 2004; González-Cárdenas et al., 2005; Benítez et al., 2006 have described that some of the *Trichoderma sp*. have shown antagonistic activity and hyperparasitic ability and act as biocontrol agent. Statistical analysis of biocontrol protection percent during pot trials, mean values are followed by the same letter are

not significantly different and different letter denotes significant difference of means according to Tukey test at least significant difference (P = 0.05 level).

6.15.3 Efficacy of antagonistic fungal strains and bacterial strains under poly house experiments (Co culture)

During polyhouse trials, five antagonistic fungal strains and three endophytic bacterial strains were evaluated against *V. dahliae*. Symptoms of *Verticillium* wilt has been recorded in *Verticillium*-inoculated plants (control 2) as compared to Control 1. The first disease symptoms appeared in control 2 plants as light-yellow areas between veins of leaves and on cotton bolls, stem. After first blooming stage of cotton plant, control 1 has shown acropetal chlorosis and control 2 plants shown symptoms of wilting than test plants. Test plants have shown 0% disease severity (healthy) at 0-4 disease severity index rating scale. Control 2 plants have shown wilting or death (83%) after 135 days of sowing during pot trials and 69.5% biocontrol protection percent **(Table 6.14)**.

6.15.4 Efficacy of antagonistic fungal strains along with biochemical formulation under poly house experiments (Monoculture experiment)

Five antagonistic fungal strains were evaluated against *V. dahliae* with biochemical formulation, used in spray form. After inoculation of *V. dahliae* symptoms were developed on *Verticillium*-inoculated plants (control 2) but were not observed on any of the Control 1. Control (inoculated with *V. dahliae*) plants have shown symptoms of wilting (89%) after 135 days of sowing than test plants (inoculated with both *V. dahliae* and antagonistic fungal strains and biochemical formulation) after first blooming stage. No disease symptoms have been recorded in cotton plants treated with *T. harzianum* + BCF, *A. terreus*+ BCF and *A. fumigatus*+ BCF (0%= healthy plant). Test plants treated with *A. niger* and *A. nidulans* have shown acropetal chlorosis with 19 and 21% severity during polyhouse experiments. Control1 has shown 35%, 39%, 26%, 21% and 33% disease severity during *A. nidulans*, *A. terreus*, *T. harzianum*, *A. fumigatus* and *A. niger* respectively. **Table 6.15** describes., most significant biocontrol efficacy has been recorded in *Trichoderma harzianum* (73.2%) followed by *Aspergillus terreus* (68.5%), *A. fumigatus* (66.2%), *A. niger* (61.3%) and *A. nidulans* (57.2%) as compared to control 1 (36.5), (33.2), (37.5), (39.5) and (34.6) and control 2 plants (25.1), (26.6), (33.5), (20.4) and (36.5) respectively. Different

researchers have used bioformulations in powdered form or liquid for control of plant diseases. *Verticillium* wilt diseases have been effectively controlled by bioformulations (Naraghi et al. 2012). Amer and Utkhede., 2000 have successfully control root rot disease by using liquid bioformulations. So, microbial based bioformulations have shown positive biocontrol effect against various pathogenic diseases.

6.15.5: Biological control of *Verticillium* wilt of Cotton during polyhouse experiments with endophytic bacterial strains and biochemical formulation (Monoculture experiment)

Symptoms of wilt disease have been observed in non- bacterized plants without biochemical formulation when compared to bacterized plants. Light-yellow discolouration between veins of leaves was first disease symptoms recorded after inoculation of *Verticillium dahliae* in control (non-bacterized) plants without biochemical formulation. Most positive significant suppression of wilt disease was recorded in treatment of seeds with *Pseudomonas aeruginosa*. Disease severity index has been recorded and calculated wherein control plant (inoculated with *V. dahliae*) has shown wilting (86%) and *Pseudomonas aeruginosa* with BCF and *Lysinibacillus macroides* with BCF has shown no disease symptoms 0% (healthy) and *Bacillus subtilis* have shown 32% acropetal chlorosis, at 0-4 rating scale. **Table 6.13** describes the biocontrol protection in *Pseudomonas aeruginosa* (70.6%) followed by *Lysinibacillus macroides* (68.6%) *Bacillus subtilis* (64.5%) as compared to control plant with (37.5%), (36.5%) and (39.2%) respectively. Bioformulations are best defined as biologically active products containing one or more beneficial microbial strains in an easy to use and economical carrier materials. *Verticillium* wilt diseases have been effectively controlled by bioformulations (Naraghi et al. 2012).

6.15.6 Efficacy of antagonistic strains and endophytic bacterial strains along with biochemical formulation under poly house experiments (Coculture experiment)

Five antagonistic fungal strains were evaluated against *V. dahliae* with biochemical formulation, used in spray form. After inoculation of *V. dahliae* symptoms were developed on *Verticillium*-inoculated plants (control) plants but were not observed on test plants. Control (inoculated with *V. dahliae*) plants have shown symptoms of

wilting (85%) after 135 days of sowing than test plants (inoculated with both *V. dahliae* and antagonistic fungal strains, biochemical formulation and bacterized seeds) after first blooming stage. During pot trials, 72.5% significant biocontrol efficacy has been recorded in test plants as compared to control plants **(Table 6.14)**.

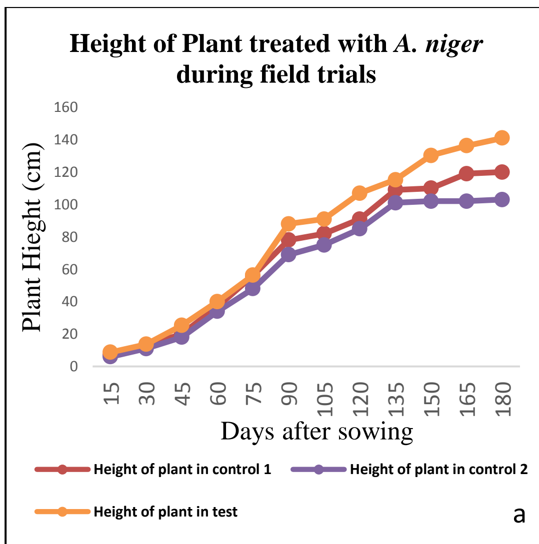
Figure 6.46: **(a,b)** Control plant of cotton inoculated with *V. dahliae* showing symptoms of wilt during pot trials along with test plants showing no symptoms of wilt **(c)** Control (Non bacterized) plant of cotton inoculated with *V. dahliae* showing symptoms of wilt during pot trials along with test plants (bacterized) showing no symptoms of wilt

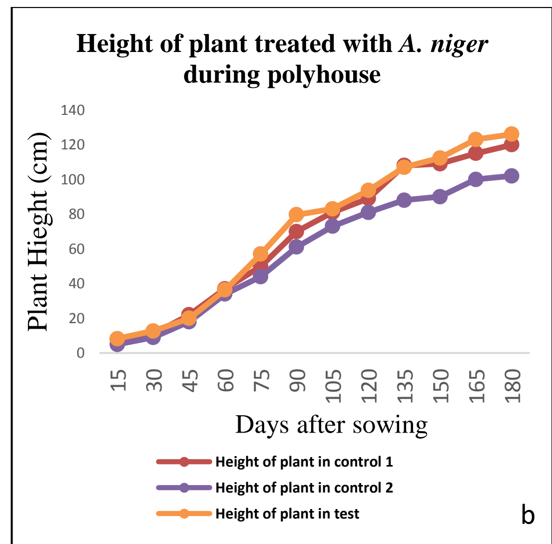
6.16 Assessment of plant growth promoting potential of potential biocontrol strains

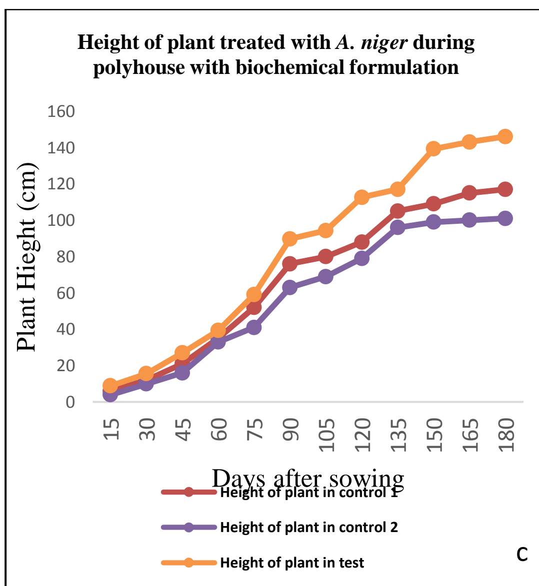
Antagonistic fungal strains that showed positive biocontrol efficacy in the greenhouse and field experiments were analyzed for their potential effects on growth on cotton plant. However, their biocontrol efficacy of potential antagonistic strains had relationship with their *in vitro* antagonistic activity against *V. dahliae* or their activities of cell wall degrading enzymes. On the other hand, antagonistic strains help to increase biomass or cotton yield. Different growth factors such as height of plant, number of nodes and root length has been studied after every fortnight during both poly house and field experiments. **Figure 6.47** describes height of plant treated with antagonistic fungal strains as compared to control during both poly house and natural field conditions along with biochemical formulation. **Figure 6.48** depicts the height of seed bacterized cotton plants during both poly house and natural field conditions have also been recorded after every fortnight from day of sowing to till harvesting along with biochemical formulation as compared to non-bacterized plants. **Figure 6.49-6.50** depicts the height of cotton plants with 5 antagonistic fungal strains, 3 bacterial strains along with biochemical formulation during both polyhouse and natural field conditions. **Table 6.16** describes the number of nodes per plant when treated with antagonistic fungal strains as compared to control during natural field conditions along with biochemical formulation. Maximum number of nodes have been recorded in *A. terreus* (38± 0.5) followed by *T. harzianum* (35± 0.7), A. niger (31± 0.2), and *A. nidulans* (29± 0.5). **Table 6.17** describes the number of nodes per plant in seed bacterized cotton plants during under natural field conditions after every fortnight from day of sowing to till harvesting along with biochemical formulation as compared to non-bacterized plants. Maximum number of nodes have been recorded in *P. aeruginosa* with 33±1.5 followed by *Lysinibacillus macroides* (31± 1.2) and *Bacillus subtilis* (29±0.3) along with biochemical formulation. **Table 6.18** describes the number of nodes per plant with 5 antagonistic fungal strains, 3 bacterial strains

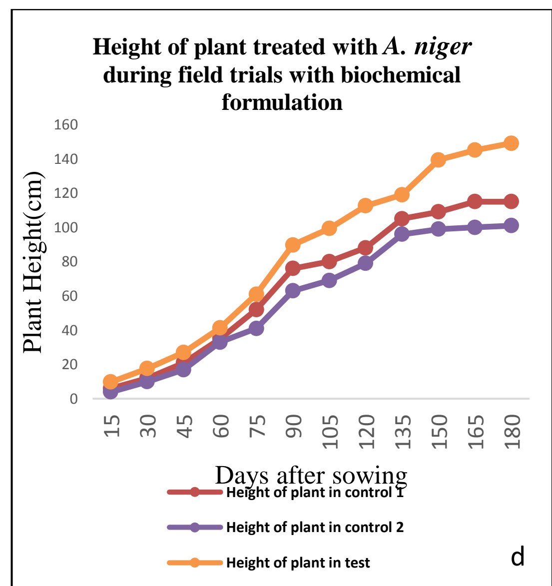
along with biochemical formulation during natural field conditions. Maximum number of nodes has been recorded in fungal strains along with biochemical formulation (36± 0.5) followed by antagonistic fungal strains without biochemical formulation (32± 0.5), endophytic bacterial strains with biochemical formulation (29± 0.2) and endophytic bacterial strains without formulation (25± 0.7). **Table 6.19** describes the number of nodes per plant with 5 antagonistic fungal strains, 3 bacterial strains along with biochemical formulation during pot experiments in polyhouse. Maximum number of nodes have been recorded in antagonistic fungal strains along with biochemical formulation., number of nodes recorded in *A. terreus* (29± 1.2) were followed by *A. fumigatus* (26±1.2), *T. harzianum* (24±0.5), *A. niger* (22±0.5) and *A. nidulans* (21±0.8). **Table 6.20** describes the number of nodes per plant during seed bacterized cotton plants during pot trials have also been recorded after every fortnight along with biochemical formulation as compared to non-bacterized plants. Maximum number of nodes have been recorded in *P. aeruginosa* along with biochemical formulation (26±1.2) followed by *L. macroides* (23±0.2) and *B. subtilis* (22±1.5). **Table 6.21-6.22, 6.23-6.24** describes the yield per plant during poly house and natural field conditions along with biochemical formulation in poly house and natural filed conditions. Highest cotton yield has been recorded in plants treated with *A. terreus* and also with biochemical formulation during both field experiment and poly house followed by *T, harzianum*. **Figure 6.51** depicts the maximum growth and length of root after harvesting in treated plants as compared to control plants. However, the antagonistic strain of *Aspergillus terreus* has shown most positive antagonistic activity during *in vitro* screening as well as bio control efficacy and helps in growth promotion of cotton plants. Endophytic bacterial strains that showed positive biocontrol efficacy in the greenhouse and field experiments were analysed for their potential effects on growth on cotton plants. However, their biocontrol efficacy of endophytic bacterial strains had relationship with their *in vitro* antagonistic activity against *V. dahliae* or their activities of cell wall degrading enzymes. Yield and growth of seed bacterized plants with *Pseudomonas aeruginosa* under natural field conditions was more than *Lysinibacillus macroides* followed by *Bacillus subtilis* when compared with control (non-bacterized). Results of growth parameters discussed above clearly indicate the significant increase in cotton yield when compared to control plants. Suppression of wilt symptoms was also recorded during pot and field trials in bacterized cotton plants. Major effect of *V. dahliae* was

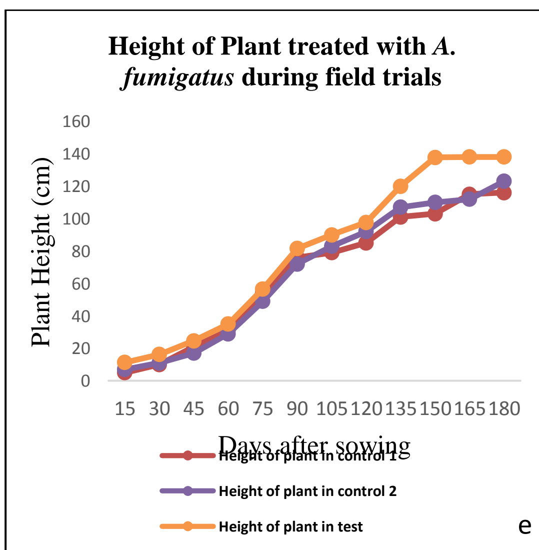
defoliation and poor growth and development of cotton plant was also significantly suppressed by *Pseudomonas aeruginosa, Lysinibacillus macroides* and *Bacillus subtilis*. Wilt symptoms was recorded after three months of non-bacterized seeds sowing under controlled and natural field conditions as compared to bacterized seeds in soil solarized fields. The combinatorial study of soil solarization and potential biocontrol bacterial strains provides a promising alternative method for control of *Verticillium* wilt of cotton disease from previously cotton growing fields and helps to increase cotton yield along with growth and development of cotton plants. Maximum root length was recorded in treated plant than control plants which shows the symptoms of wilting after first blooming stage. Study of various researchers have described that growth of cotton plant and development was reduced because of *Verticillium* wilt. Similar work of Pulman and DeVay 1982 was reported that reduction in cotton lint has been observed if foliar symptoms have been recorded after mid-august. Hence, cotton yield has been correlated with first foliar symptoms. Similar study of Bejerano-Alcazar et al. (1997) has also indicated that reduction in cotton yield has been recorded in plants, showing symptoms of disease before first flowering stage and effect of wilt disease was less in those plants where disease symptoms were recorded after boll opening stage. Pulman and DeVay (1982) was also found that different growth parameters such as height of plant and number of nodes were significantly highest in seed bacterized plants as compared to control plants. Results of Duan et al., 2010 have also indicated that phosphate-solubilizing bacterial stains significantly increased plant height, dry mass of treated plants as compared to control plants. Therefore, in this study 5 antagonistic fungal strains and 3 endophytic bacterial strains along with biochemical formulation helps to increase cotton plant height, dry weight, canopy and root length in treated plants as compared to control plants. Hence, cotton growth and yield were also significantly increased in treated plants when compared with control plants.

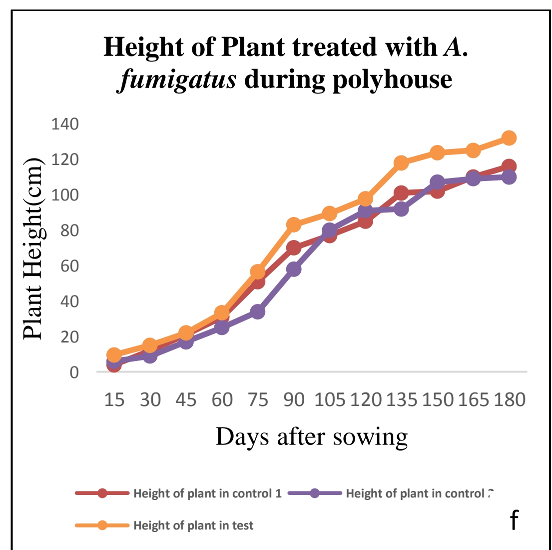

Height of Plant treated with A. niger during field trials
Plant Hieght (cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
a

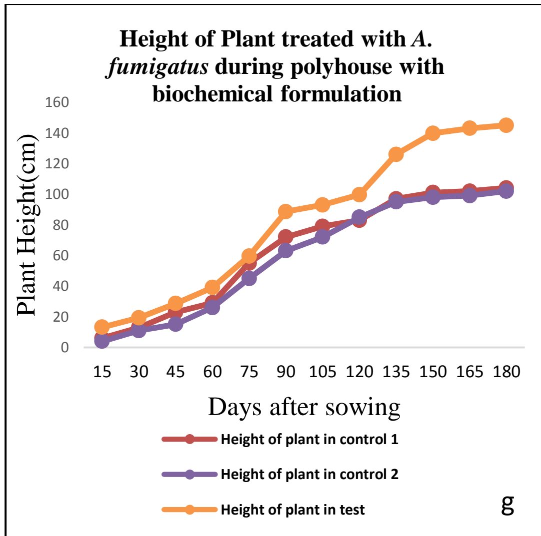

Height of plant treated with A. niger during polyhouse
Plant Hieght (cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
b

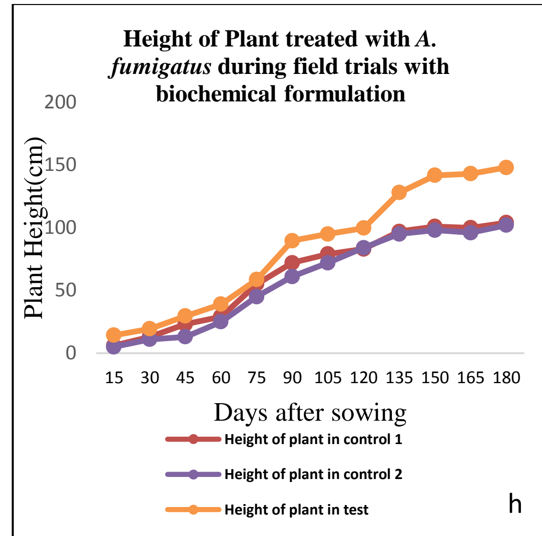

Height of plant treated with A. niger during polyhouse with biochemical formulation
Plant Hieght (cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
c


Height of plant treated with A. niger during field trials with biochemical formulation
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
d


Height of Plant treated with A. fumigatus during field trials
Plant Height (cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
e


Height of Plant treated with A. fumigatus during polyhouse
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in test
f


Height of Plant treated with *A. fumigatus* during polyhouse with biochemical formulation
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
g


Height of Plant treated with *A. fumigatus* during field trials with biochemical formulation
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
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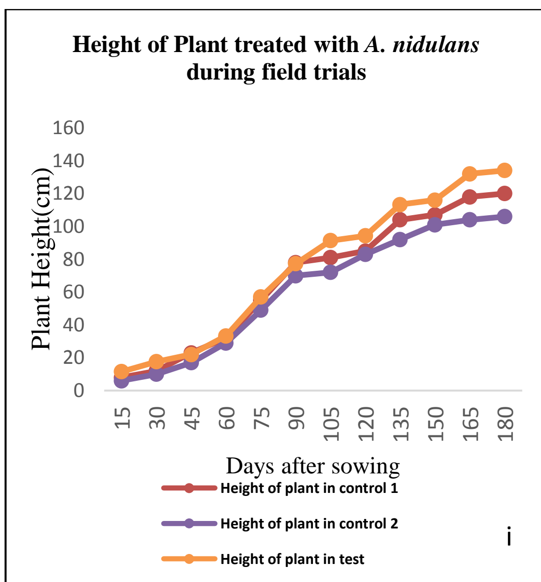
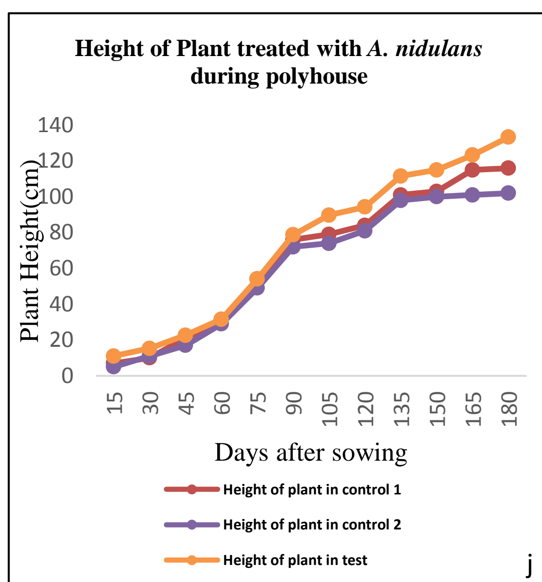
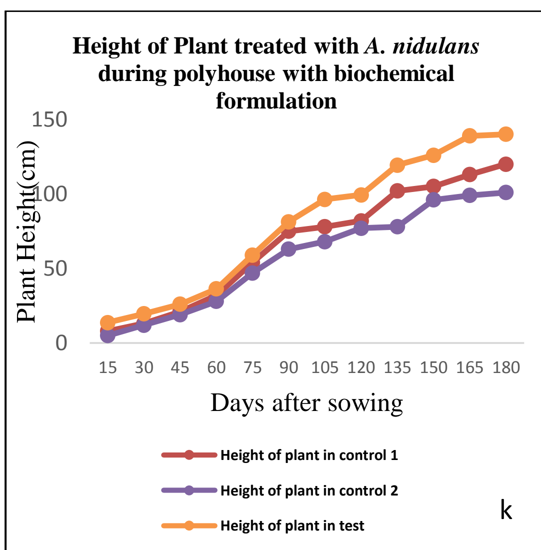
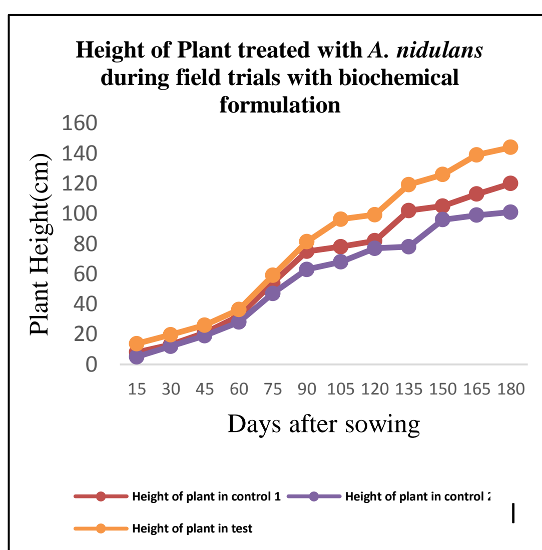

Height of Plant treated with *A. nidulans* during field trials
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
i


Height of Plant treated with *A. nidulans* during polyhouse
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
j


Height of Plant treated with *A. nidulans* during polyhouse with biochemical formulation
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
k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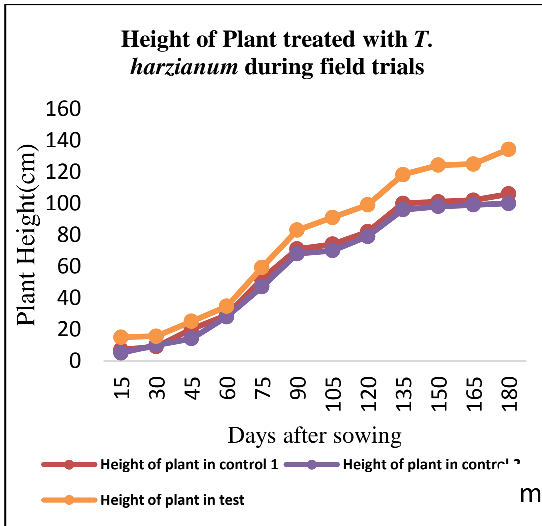
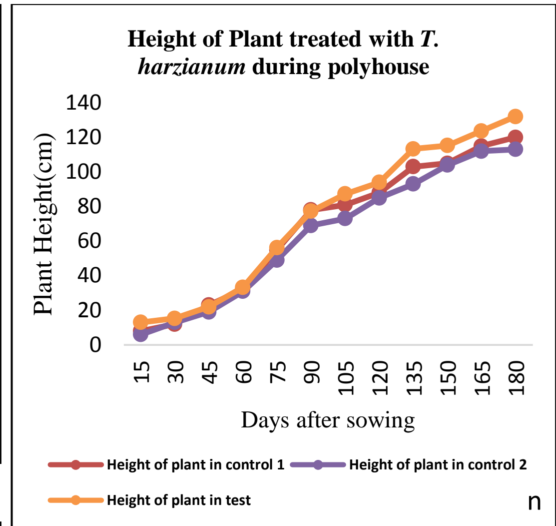
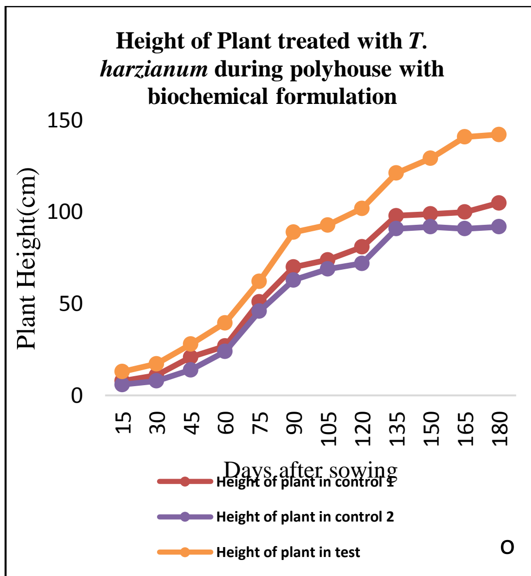
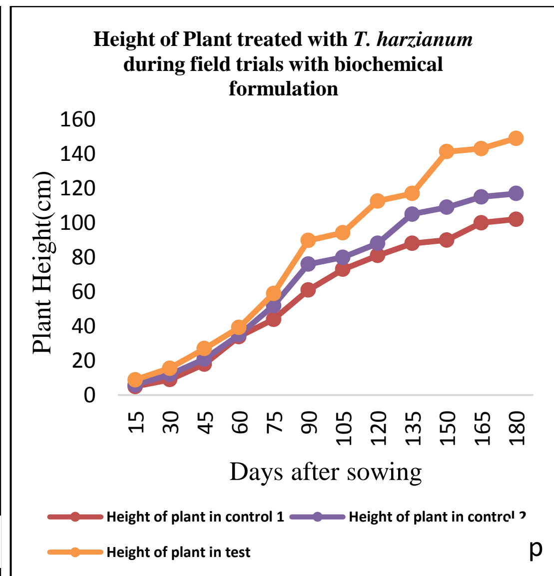
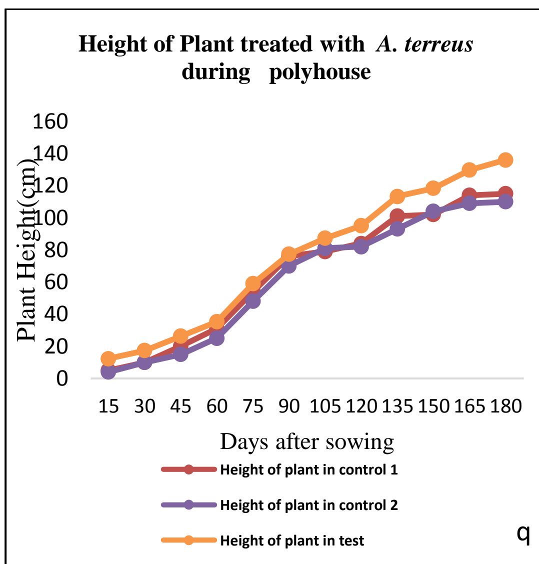
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in test
l

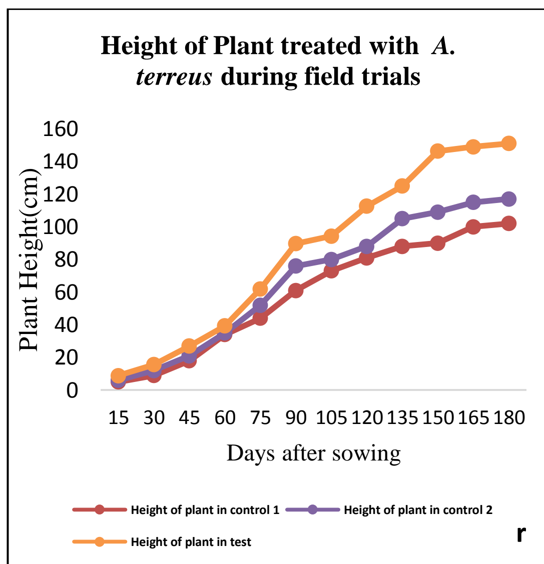

Height of Plant treated with T. harzianum during field trials
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
m


Height of Plant treated with T. harzianum during polyhouse
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
n


Height of Plant treated with T. harzianum during polyhouse with biochemical formulation
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
o


Height of Plant treated with T. harzianum during field trials with biochemical formulation
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
p


Height of Plant treated with A. terreus during polyhouse
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
q


Height of Plant treated with A. terreus during field trials
Plant Height(cm)
Days after sowing
Height of plant in control 1
Height of plant in control 2
Height of plant in test
r

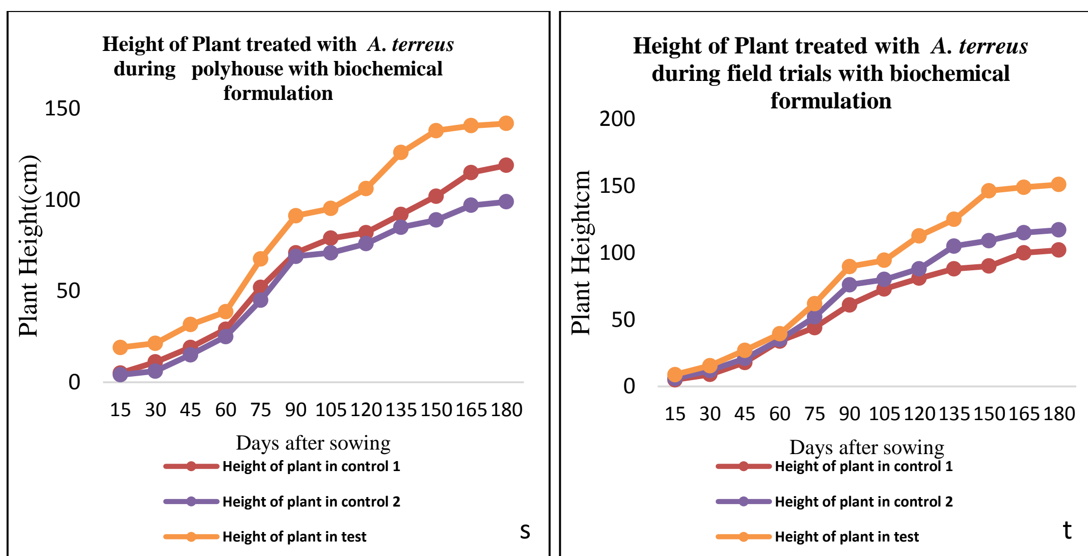


Figure 6.47: Effect of antagonistic strains on plant height (cm)- (a) Effect of *A.* niger during field trials **(b)** Effect of *A.* niger during poly house **(c)** Effect of *A. niger* during polyhouse along with biochemical formulation **(d)** Effect of *A. niger* during field trials along with biochemical formulation **(e)** Effect of *A. fumigatus* during field trials **(f)** Effect of *A. fumigatus* during poly house **(g)** Effect of *A. fumigatus* during polyhouse along with biochemical formulation **(h)** Effect of *A. fumigatus* during field trials along with biochemical formulation **(i)** Effect of *A. nidulans* during field trials **(j)** Effect of *A. nidulans* during poly house experiment **(k)** Effect of *A. nidulans* during poly house with biochemical formulation **(l)** Effect of *A. nidulans* during field trials along with biochemical formulation **(m)** Effect of *T. harzianum* on plant height during field trials **(n)** Effect of *T. harzianum* on plant height during poly house **(o)** Height of plant treated with *T. harzianum* during poly house with biochemical formulation **(p)** Height of Plant treated with *T. harzianum* during field trials with biochemical formulation **(q)** Height of Plant treated with *A. terreus* during polyhouse **(r)** Height of Plant treated with *A. terreus* during field trials **(s)** Height of Plant treated with *A. terreus* during polyhouse with biochemical formulation **(t)** Height of Plant treated with *A. terreus* during field trials with biochemical formulation

Table 6.13: Biocontrol of *Verticillium da hliae* from endophytic bacterial strains during pot trials, field trails and biochemical formulation + antagonistic strains (Pot and field trails)

Name of Endophytic bacterial strains	Biological control under pot trials without biochemical formulation		Biological control under field trials without biochemical formulation		Biological control under pot trials with biochemical formulation		Biological control under field trials with biochemical formulation	
	B.C in control (Pot Trials)	B.C in TEST (Pot trials)	B.C in control (Field Trials)	B.C in TEST (Field trials)	B.C in control (Pot Trials + Biochemical formulation)	B.C in Test (Pot Trials + BCF)	B.C in control (Field Trials + BCF)	B.C in Test (Field Trials + BCF)
Pseudomonas aeruginosa	36.4ab	69.6bc	40.3bc	76.3.2c	37.5ab	70.6bc	35.5a	76.6c
Bacillus subtilis	32.2b	62.5ab	39.2bc	62.5a	39.2c	64.5a	36.6ab	58.3a
Lysinibacillus macroides	29.7ac	65.2b	36.7ab	68.5ab	36.5a	68.6ab	39.7bc	69.5.8ab

Table 6.13 indicates: **Control 1 (inoculated with water only) in both poly house and field trials, and trials with biochemical formulation + antagonistic strains under polyhouse and field trials, Control 2 (inoculated with *V. dahliae*) in both poly house and field trials, and trials with biochemical formulation + antagonistic strains under polyhouse and field trials, Test plants (inoculated with both *V. dahliae* and antagonistic strain during each experiment) in both poly house and field trials, and trials with biochemical formulation + antagonistic strains under polyhouse and field trial**

Table 6.14: Biocontrol of *Verticillium dahliae* from endophytic bacterial strains during pot trials, field trails and biochemical formulation + antagonistic strains along with co- culture (Pot and field trails)

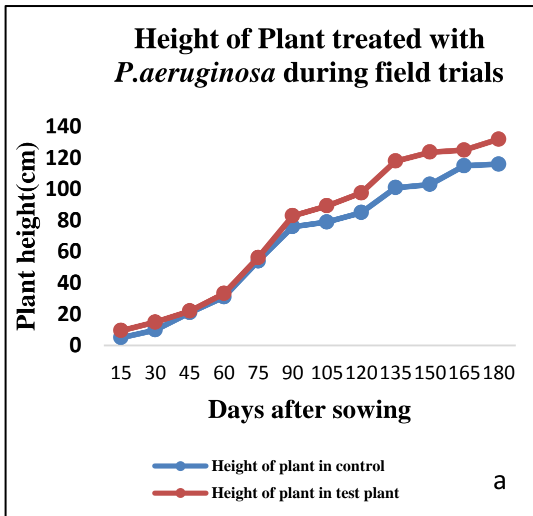
Name of Endophytic bacterial strains	Biological control under pot trials without biochemical formulation (monoculture experiment)		Biological control under pot trials without biochemical formulation (co-culture experiment)	Biological control under field trials without biochemical formulation (monoculture experiment)		Biological control under field trials without biochemical formulation (co-culture experiment)	Biological control under pot trials with biochemical formulation (monoculture experiment)		Biological control under pot trials with biochemical formulation (co-culture experiment)	Biological control under field trials with biochemical formulation (monoculture experiment)		Biological control under field trials with biochemical formulation (co-culture experiment)
	B.C in control (Pot Trials)	B.C in TEST (Pot trials)	B.C in TEST (Pot trials)	B.C in control (Field Trials)	B.C in TEST (Field trials)	B.C in TEST (Field trials)	B.C in control (Pot Trials + Biochemical formulation)	B.C in Test (Pot Trials + BCF)	B.C in Test (Pot Trials + BCF)	B.C in control (Field Trials + BCF)	B.C in Test (Field Trials + BCF)	B.C in Test (Field Trials + BCF)
Pseudomonas aeruginosa	36.4ab	69.6bc	69.5	40.3bc	76.3.2c	71.3	37.5ab	70.6bc	72.5	35.5a	76.6c	78.2
Bacillus subtilis	32.2b	62.5ab		39.2bc	62.5a		39.2c	64.5a		36.6ab	58.3a	
Lysinibacillus macroides	29.7ac	65.2b		36.7ab	68.5ab		36.5a	68.6ab		39.7bc	69.5.8ab	

Table 6.14 indicates: **Control 1 (inoculated with water only) in both poly house and field trials, and trials with biochemical formulation + antagonistic strains under polyhouse and field trials, Control 2 (inoculated with *V. dahliae*) in both poly house and field trials, and trials with biochemical formulation + antagonistic strains under polyhouse and field trials, Test plants (inoculated with both *V. dahliae* and antagonistic strain during each experiment) in both poly house and field trials, and trials with biochemical formulation + antagonistic strains under polyhouse and field trial**

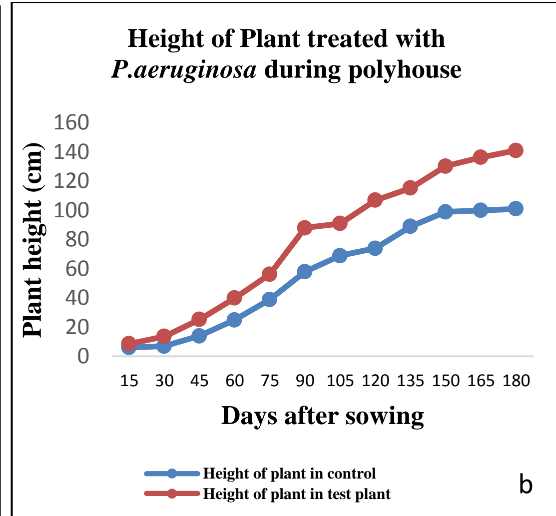
Table 6.15: Biocontrol of *Verticillium dahliae* from antagonistic fungal strains during pot trials, field trials and biochemical formulation + antagonistic strains (Pot and field trials)

Name of Antagonistic fungal strains	Biological control under pot trials without biochemical formulation			Biological control under field trials without biochemical formulation			Biological control under pot trials with biochemical formulation			Biological control under field trials with biochemical formulation		
	B.C in control 1 (Pot Trials)	B.C in control 2 (Pot Trials)	B.C in TEST (Pot trials)	B.C in control 1 (Field Trials)	B.C in control 2 (Field Trials)	B.C in TEST (Field trials)	B.C in control 1(Pot Trials + Biochemical formulation)	B.C in control 2(Pot Trials + BCF)	B.C in Test (Pot Trials + BCF)	B.C in control 1(F.T + BCF)	B.C in control 2(F.T + BCF)	B.C in Test (F.T + BCF)
Aspergillus niger	38.4a	22.6e	64.2cd	41.3b	22.6a	66.2d	39.5a	20.4a	65.2cd	31.5e	22.6e	69.2e
A. terreus	36.2b	28.8a	66.5be	43.2a	26.5d	74.5a	33.2b	26.6ae	69.5de	33.6b	26.5d	78.3bc
A. fumigatus	42.7ac	39.5ab	61.5cd	36.7bc	39.5ab	68.8cd	37.5bc	33.5ab	68.5cd	39.7ad	39.5ab	72.8cd
A. nidulans	35.8d	38.5bc	59.2bcd	33.8d	38.5bc	61.2de	34.6d	36.5bc	31.2bd	32.8cd	38.5bc	68.2d
Trichoderma harzianum	38.15ad	22.1d	70.3ad	41.3b	24.2d	72.3b	36.5e	25.1d	74.3ae	35.6be	24.2d	79.5ab

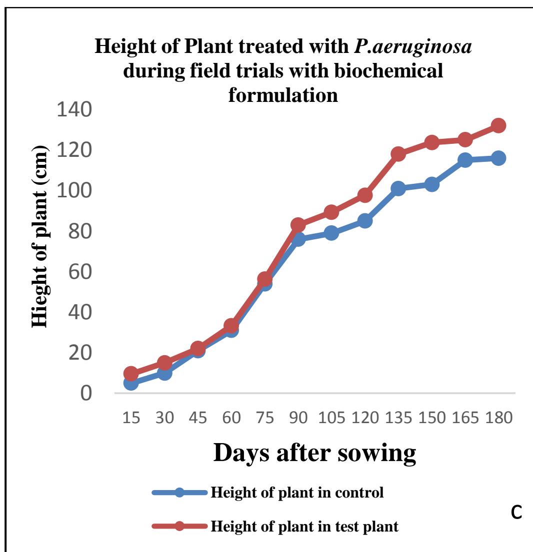
Table 6.15: Biocontrol of *Verticillium dahliae* from antagonistic fungal strains during pot trials, field trails and biochemical formulation + antagonistic strains (Pot and field trails). Mean values are followed by the same letter are not significantly different and different letter denotes significant difference of means according to Tukey test at least significant difference (P = 0.05 level)



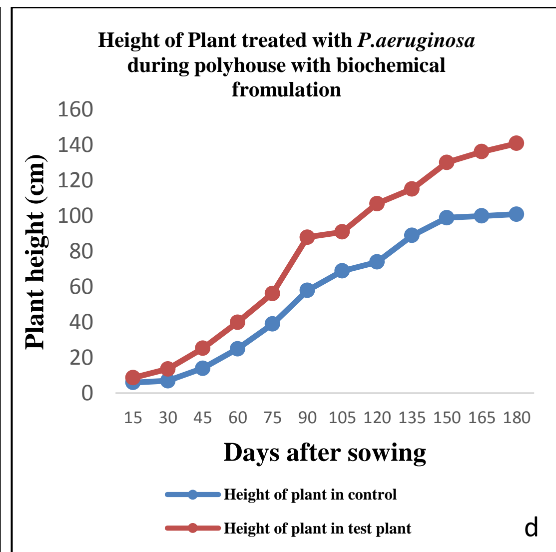
Height of Plant treated with
P.aeruginosa during field trials
Plant height(cm)
140
120
100
80
60
40
20
0
15 30 45 60 75 90 105 120 135 150 165 180
Days after sowing
Height of plant in control
Height of plant in test plant
a



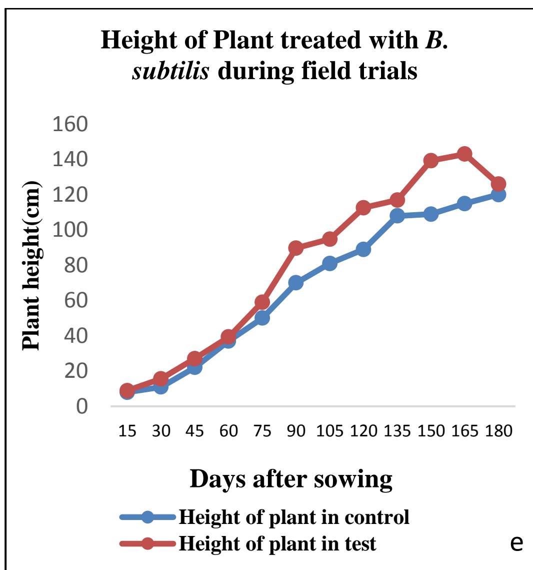
Height of Plant treated with
P.aeruginosa during polyhouse
Plant height (cm)
160
140
120
100
80
60
40
20
0
15 30 45 60 75 90 105 120 135 150 165 180
Days after sowing
Height of plant in control
Height of plant in test plant
b



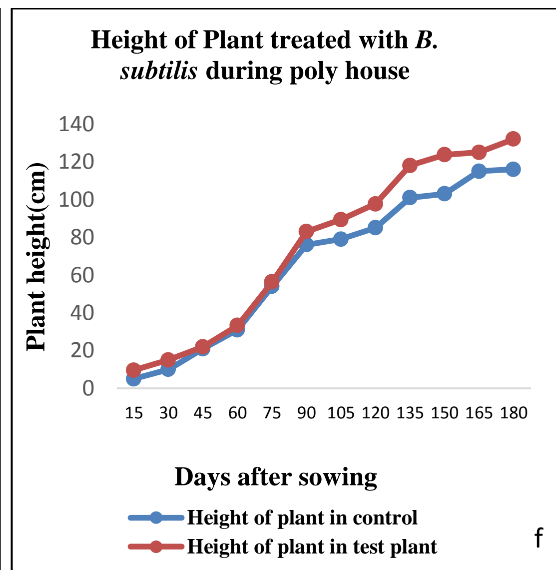
Height of Plant treated with *P.aeruginosa*
during field trials with biochemical
formulation
Hieght of plant (cm)
140
120
100
80
60
40
20
0
15 30 45 60 75 90 105 120 135 150 165 180
Days after sowing
Height of plant in control
Height of plant in test plant
c



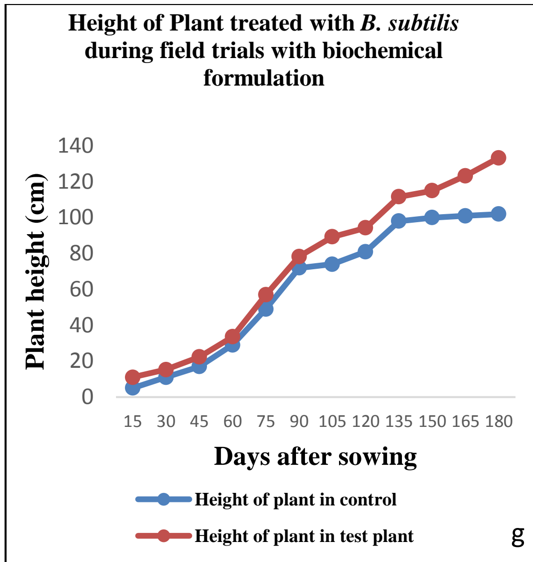
Height of Plant treated with *P.aeruginosa*
during polyhouse with biochemical
fromulation
Plant height (cm)
160
140
120
100
80
60
40
20
0
15 30 45 60 75 90 105 120 135 150 165 180
Days after sowing
Height of plant in control
Height of plant in test plant
d

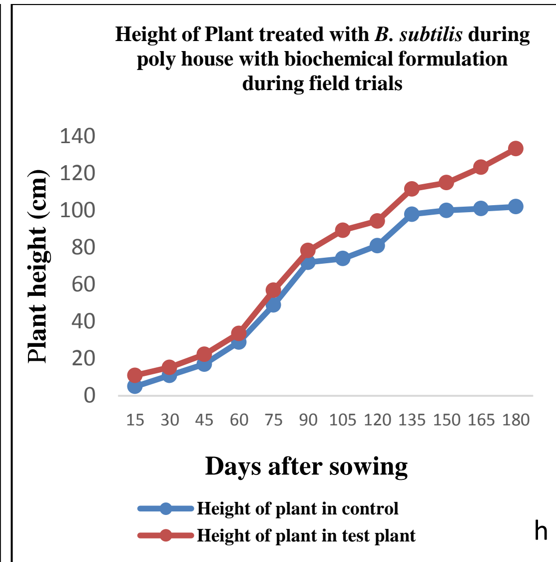


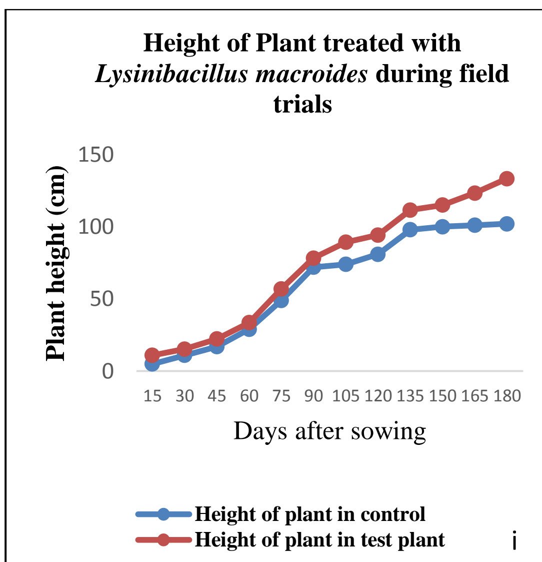
Height of Plant treated with *B.
subtilis* during field trials
Plant height(cm)
160
140
120
100
80
60
40
20
0
15 30 45 60 75 90 105 120 135 150 165 180
Days after sowing
Height of plant in control
Height of plant in test
e

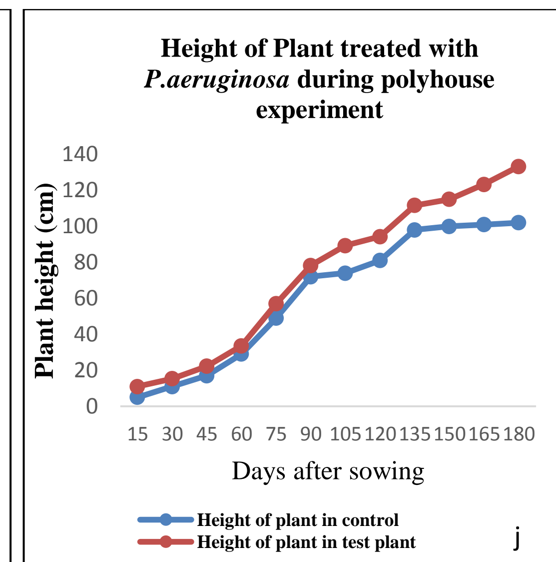


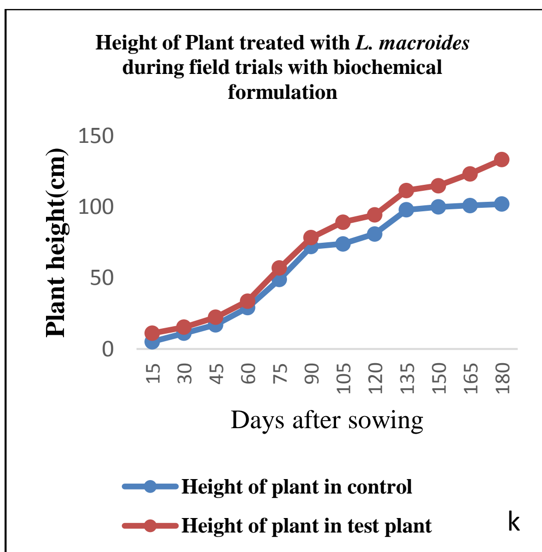
Height of Plant treated with *B.
subtilis* during poly house
Plant height(cm)
140
120
100
80
60
40
20
0
15 30 45 60 75 90 105 120 135 150 165 180
Days after sowing
Height of plant in control
Height of plant in test plant
f


Height of Plant treated with B. subtilis during field trials with biochemical formulation
Plant height (cm)
140
120
100
80
60
40
20
0
15 30 45 60 75 90 105 120 135 150 165 180
Days after sowing
Height of plant in control
Height of plant in test plant
g


Height of Plant treated with B. subtilis during poly house with biochemical formulation during field trials
Plant height (cm)
140
120
100
80
60
40
20
0
15 30 45 60 75 90 105 120 135 150 165 180
Days after sowing
Height of plant in control
Height of plant in test plant
h


Height of Plant treated with Lysinibacillus macroides during field trials
Plant height (cm)
150
100
50
0
15 30 45 60 75 90 105 120 135 150 165 180
Days after sowing
Height of plant in control
Height of plant in test plant
i


Height of Plant treated with P.aeruginosa during polyhouse experiment
Plant height (cm)
140
120
100
80
60
40
20
0
15 30 45 60 75 90 105 120 135 150 165 180
Days after sowing
Height of plant in control
Height of plant in test plant
j


Height of Plant treated with L. macroides during field trials with biochemical formulation
Plant height(cm)
150
100
50
0
15 30 45 60 75 90 105 120 135 150 165 180
Days after sowing
Height of plant in control
Height of plant in test plant
k

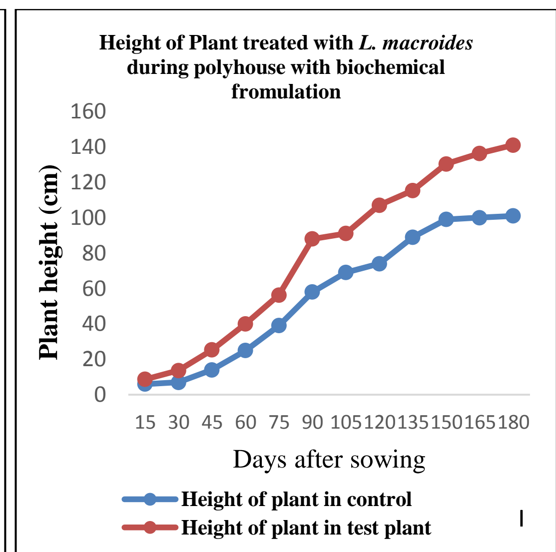

Height of Plant treated with L. macroides during polyhouse with biochemical fromulation
Plant height (cm)
160
140
120
100
80
60
40
20
0
15 30 45 60 75 90 105 120 135 150 165 180
Days after sowing
Height of plant in control
Height of plant in test plant
l

Figure 6.48: Effect of endophytic bacterial strains on plant height (cm) (a) Effect of *Pseudomonas aeruginosa* during field trials **(b)** Effect of *P. aeruginosa* during poly house **(c)** Effect of *P. aeruginosa* during field trials along with biochemical formulation **(d)** Effect of *P. aeruginosa* during polyhouse experiment along with biochemical formulation **(e)** Effect of *B. subtilis* during field trials **(f)** Effect of *B. subtilis* during poly house **(g)** Effect of *B. subtilis* during field trials along with biochemical formulation **(h)** Effect of *B. subtilis* during poly house experiment along with biochemical formulation **(i)** Effect of *Lysinibacillus macroides* during field trials **(b)** Effect of *L. macroides* during poly house **(c)** Effect of *L. macroides* during field trials along with biochemical formulation **(d)** Effect of *L. macroides* during polyhouse experiment along with biochemical formulation

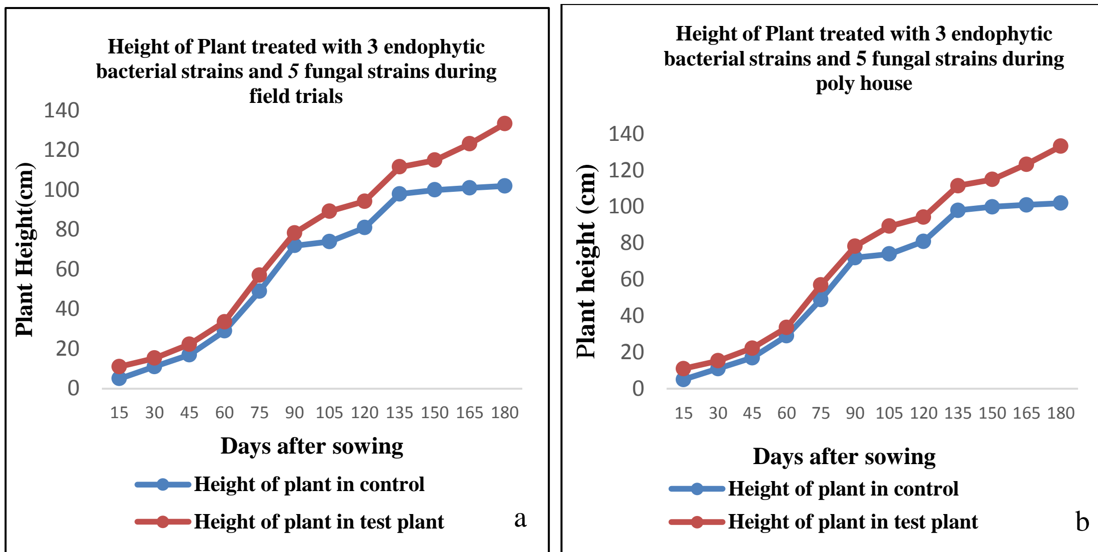


Figure 6.49: Height of plant treated with 3 endophytic bacterial strains and 5 antagonistic fungal strains during polyhouse experiments (a) Effect of 3 endophytic bacterial strains and 5 antagonistic fungal strains on plant height during field trials **(b)** Effect of 3 endophytic bacterial strains and 5 antagonistic fungal strains on plant height during poly house experiment

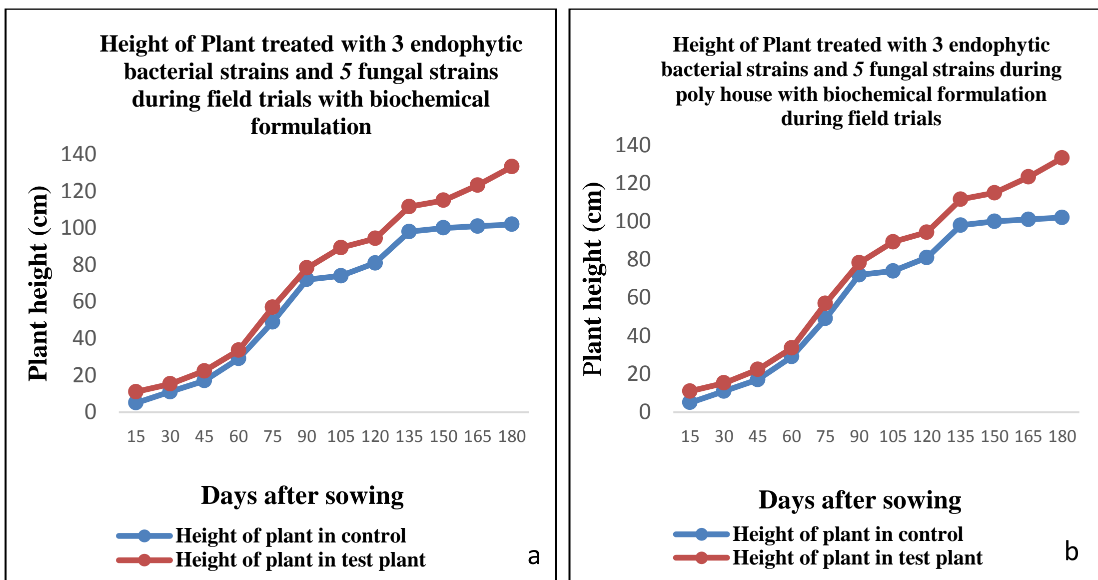


Figure 6.50: Height of plant treated with 3 endophytic bacterial strains and 5 antagonistic fungal strains with biochemical formulation under natural field conditions (a) Effect of 3 endophytic bacterial strains and 5 antagonistic fungal strains on plant height during field trials with biochemical formulation **(b)** Effect of 3 endophytic bacterial strains and 5 antagonistic fungal strains with biochemical formulation on plant height during poly house experiment

Table 6.16: Effect of antagonistic fungal strains on number of nodes per plant under natural field conditions as compared to control (Monoculture experiment) (BCF indicates – Biochemical formulation) Mean values are followed by the same letter are not significantly different and different letter denotes significant difference of means according to Tukey test at least significant difference (P = 0.05 level)

Name of Antagonistic fungal strains	**Number of nodes per plant in Control 1(uninoculated with *V. dahliae*)**	**Number of nodes per plant in Control 2 (inoculated with *V. dahliae*)**	**Number of nodes per plant test (inoculated with both *V. dahliae* and antagonistic strain)**	**Number of nodes per plant test (inoculated with *V. dahliae* and BCF with antagonistic strain)**
Aspergillus niger	18±0.5de	11±0.7ab	31±0.2ab	32±0.5ab
A. terreus	15±0.3ab	14±1.5cd	38±0.5d	41±0.1de
A. fumigatus	17±0.8bc	11±0.5ab	35±1.2c	36±0.4bcd
A. nidulans	17±1.2d	10±0.3a	29±0.5a	29±0.5a
Trichoderma harzianum	13±0.5a	13±0.6c	35±0.7c	37±0.7cd

Table 6.17: Effect of endophytic bacterial strains (Seed bacterization) on number of nodes per plant under natural field conditions as compared to control (Monoculture experiment) (BCF indicates – Biochemical formulation) Mean values are followed by the same letter are not significantly different and different letter denotes significant difference of means according to Tukey test at least significant difference (P = 0.05 level)

Name of Endophytic bacterial strains	**Number of nodes per plant in Control (uninoculated with *V. dahliae*)**	**Number of nodes per plant test (inoculated with both *V. dahliae* and endophytic bacterial strain)**	**Number of nodes per plant in test (inoculated with *V. dahliae* and BCF with endophytic bacterial strain)**
Pseudomonas aeruginosa	13±0.3bc	31±1.2c	33±1.5abc
Bacillus subtilis	11±0.5b	26±0.3a	29±0.3a
Lysinibacillus macroides	10±0.7ab	29±0.5ab	31±1.2ab

Table 6.18: Effect of microbial strains along with biochemical formulation on number of nodes per plant under natural field conditions as compared to control (Co-culture experiment) (BCF indicates – Biochemical formulation) Mean values are followed by the same letter are not significantly different and different letter denotes significant difference of means according to Tukey test at least significant difference (P = 0.05 level)

Sr No.	**Number of nodes per plant in Control (uninoculated with *V. dahliae*)**	**Number of nodes per plant test (inoculated with both *V. dahliae* and 3 endophytic bacterial strain)**	**Number of nodes per plant test (inoculated with *V. dahliae* and BCF with 3 endophytic bacterial strain)**	**Number of nodes per plant test (inoculated with *V. dahliae* and 5 antagonistic fungal strains)**	**Number of nodes per plant test (inoculated with *V. dahliae* and BCF with 5 antagonistic fungal strains)**
1	11±0.5a	25±0.7bc	29±0.2cd	32±0.5de	36±0.5def

Table 6.19: Yield per plant during field trials (in gm) (Monoculture experiment) (BCF indicates – Biochemical formulation)

Mean values are followed by the same letter are not significantly different and different letter denotes significant difference of means according to Tukey test at least significant difference (P = 0.05 level)

Name of strains	**Yield per plant in Control (uninoculated with *V. dahliae*)**	**Yield per plant test (inoculated with both *V. dahliae* and antagonistic strains)**	**Yield per plant in test (inoculated with *V. dahliae* and BCF with antagonistic strain)**
Aspergillus niger	1590±0.3fg	2230±0.5b	2340±0.5bc
A. terreus	1490±0.5cd	2630±0.4cde	2740±1.5de
A. fumigatus	1580±1.5ef	2890±0.5ef	3060±1.2ef
A. nidulans	1470±0.5b	2040±1.2ab	2290±0.7bc
Trichoderma harzianum	1490±0.7bcd	2590±0.5cd	2670±0.5cd
Pseudomonas aeruginosa	1380±0.5a	2290±1.5bc	**2240**±0.2b
Bacillus subtilis	1560±0.3de	2030±0.5a	**2080**±0.5a
Lysinibacillus macroides	1480±1.2bc	2190±0.7bc	**2270**±0.8bc

Table 6.20: Yield per plant during field trials (in gm) (Coculture experiment) (BCF indicates – Biochemical formulation) Mean values are followed by the same letter are not significantly different and different letter denotes significant difference of means according to Tukey test at least significant difference (P = 0.05 level)

Sr. No.	**Yield per plant in Control (uninoculated with *V. dahliae*)**	**Yield per plant test (inoculated with both *V. dahliae* and 3 endophytic bacterial strain)**	**Yield per plant test (inoculated with *V. dahliae* and BCF with 3 endophytic bacterial strain)**	**Yield per plant test (inoculated with *V. dahliae* and 5 antagonistic fungal strains)**	**Yield per plant test (inoculated with *V. dahliae* and BCF with 5 antagonistic fungal strains)**
1	1590±0.3a	2230±0.5b	2390±0.5bc	2630±0.5cd	2980±0.5e

Table 6.21: Effect of antagonistic fungal strains on number of nodes per plant during pot trials as compared to control (BCF indicates – Biochemical formulation) Mean values are followed by the same letter are not significantly different and different letter denotes significant difference of means according to Tukey test at least significant difference (P = 0.05 level)

Name of Antagonistic fungal strains	**Number of nodes per plant in Control 1(uninoculated with *V. dahliae*)**	**Number of nodes per plant in Control 2 (inoculated with *V. dahliae*)**	**Number of nodes per plant test (inoculated with both *V. dahliae* and antagonistic strain)**	**Number of nodes per plant test (inoculated with *V. dahliae* and BCF with antagonistic strain)**
Aspergillus niger	11±0.7a	8±1.2a	17±0.5bc	22±0.5a
A. terreus	14±1.5bcd	10±1.0bc	23±0.7d	29±1.2c
A. fumigatus	12±0.8ab	11±0.5d	20±1.0bc	26±0.5bc
A. nidulans	15±0.25de	10±0.75bc	16±1.2a	21±0.8c
Trichoderma harzianum	13±0.5bc	9±1.5ab	19±0.5bc	24±0.5b

Table 6.22: Effect of endophytic bacterial strains (Seed bacterization) on number of nodes per plant during pot trials as compared to control (BCF indicates – Biochemical formulation) Mean values are followed by the same letter are not significantly different and different letter denotes significant difference of means according to Tukey test at least significant difference (P = 0.05 level)

Name of Endophytic bacterial strains	**Number of nodes per plant in Control (uninoculated with *V. dahliae*)**	**Number of nodes per plant test (inoculated with both *V. dahliae* and endophytic bacterial strain)**	**Number of nodes per plant test (inoculated with both *V. dahliae* and 3 endophytic bacterial strain)**	**Number of nodes per plant test (inoculated with *V. dahliae* and BCF with endophytic bacterial strain)**	**Number of nodes per plant test (inoculated with *V. dahliae* and BCF with 3 endophytic bacterial strain)**
Pseudomonas aeruginosa	13±1.5c	19±0.7c	24±0.2bc	28±0.7c	26±1.2c
Bacillus subtilis	11±0.2ab	16±0.3a	21±0.7a	25±0.5a	22±1.5a
Lysinibacillus macroides	10±1.2a	18±0.2ab	24±0.5b	26±0.3ab	23±0.2ab

Table 6.23: Effect of microbial strains along with biochemical formulation on number of nodes per plant during pot trials as compared to control (Monoculture experiment) (BCF indicates – Biochemical formulation) Mean values are followed by the same letter are not significantly different and different letter denotes significant difference of means according to Tukey test at least significant difference (P = 0.05 level)

Sr No.	Number of nodes per plant in Control (uninoculated with *V. dahliae*)	Number of nodes per plant test (inoculated with both *V. dahliae* and 3 endophytic bacterial strain)	Number of nodes per plant test (inoculated with *V. dahliae* and BCF with 3 endophytic bacterial strain)	Number of nodes per plant test (inoculated with *V. dahliae* and 5 antagonistic fungal strains)	Number of nodes per plant test (inoculated with *V. dahliae* and BCF with 5 antagonistic fungal strains)
1	10±0.5a	18±0.7bc	22±0.2cd	25±0.5de	27±0.5ef

Table 6.24: Yield per plant during pot trials (in gm) (Monoculture experiment) (BCF indicates – Biochemical formulation) Mean values are followed by the same letter are not significantly different and different letter denotes significant difference of means according to Tukey test at least significant difference (P = 0.05 level)

Name of strains	Yield per plant in Control (uninoculated with *V. dahliae*)	Yield per plant test (inoculated with both *V. dahliae* and antagonistic microbial strains)	Yield per plant in test (inoculated with *V. dahliae* and BCF with antagonistic microbial strain)
Aspergillus niger	1170±0.3fg	1910±0.5b	2260±0.5bc
A. terreus	1230±0.5cd	1930±0.4cde	2395±1.5de
A. fumigatus	1210±1.5ef	2090±0.5ef	2280±1.2ef
A. nidulans	1190±0.5b	1980±1.2ab	2075±0.7bc
Trichoderma harzianum	1495±0.7bcd	2065±0.5cd	2360±0.5cd
Pseudomonas aeruginosa	1090±0.5a	1760±1.5bc	2185±0.2b
Bacillus subtilis	1050±0.3de	1390±0.5a	1480±0.5a
Lysinibacillus macroides	1080±1.2bc	1695±0.7bc	2270±0.8bc

Table 6.25: Yield per plant during pot trials (in gm) (Coculture experiment) (BCF indicates – Biochemical formulation) Mean values are followed by the same letter are not significantly different and different letter denotes significant difference of means according to Tukey test at least significant difference (P = 0.05 level)

Sr. No.	Yield per plant in Control (uninoculated with *V. dahliae*)	Yield per plant test (inoculated with both *V. dahliae* and 3 endophytic bacterial strain)	Yield per plant test (inoculated with *V. dahliae* and BCF with 3 endophytic bacterial strain)	Yield per plant test (inoculated with *V. dahliae* and 5 antagonistic fungal strains)	Yield per plant test (inoculated with *V. dahliae* and BCF with 5 antagonistic fungal strains)
1	**1280**±0.3a	**1530**±0.5b	**1790**±0.5bc	**2230**±0.5cd	**2386**±0.5e

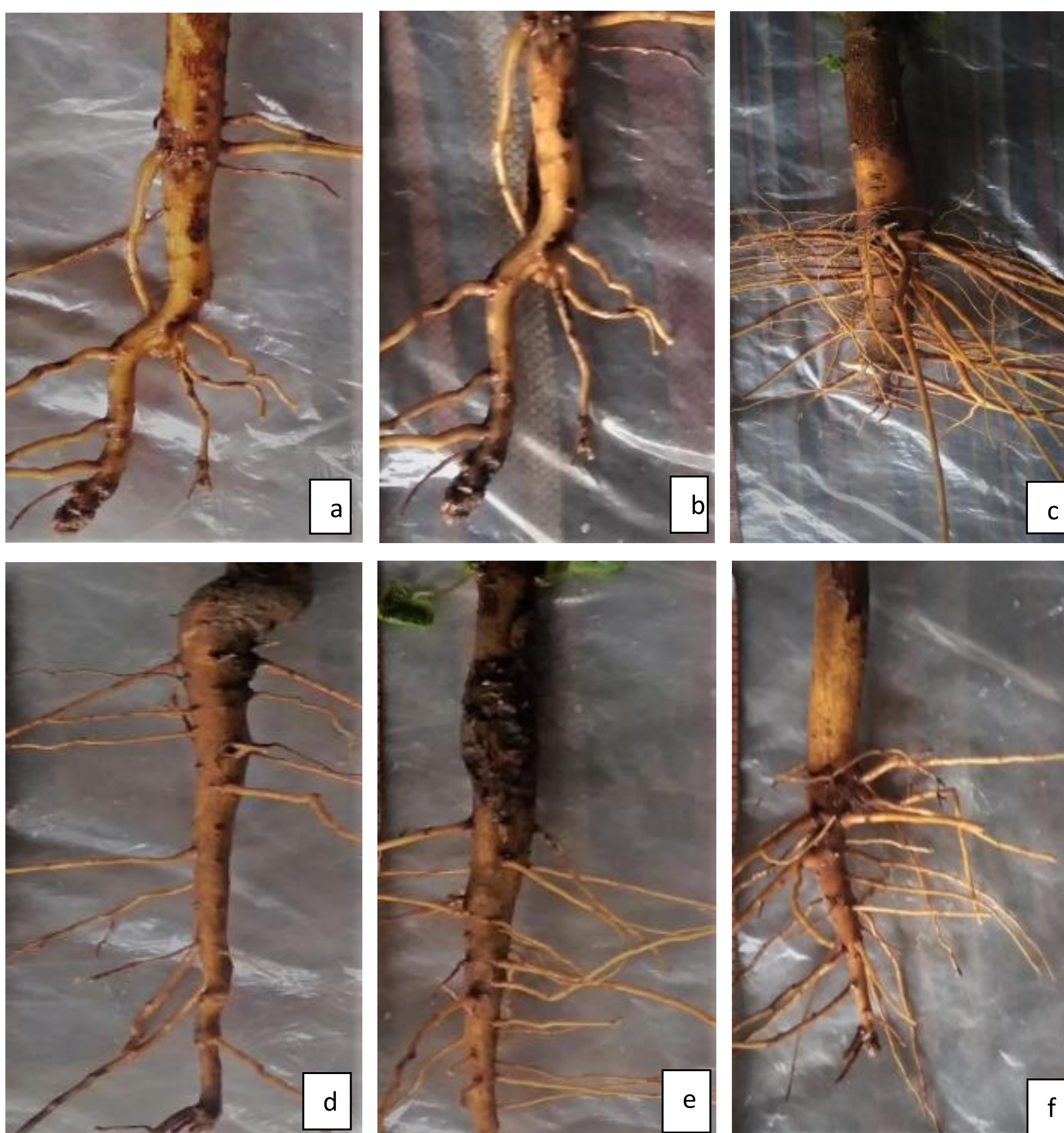


Figure 6.51: Roots of cotton plant after harvesting (with and without treatment) (a) Root of control 1 (un inoculated with *V. dahliae*) cotton plant (b) Root of Control 2 (inoculated with *V. dahliae*) (c) Roots of Test cotton plant tretaed with anatgonistic fungal strain (d) Root treated with endophytic bacterial strain (Seed bacterization) (e) Root of Control plant (inoculated with *V. dahliae*) without biochemical fromulation (f) Roots of Test cotton plant tretaed with anatgonistic fungal strain along with biochemical formulation

CHAPTER VII

SUMMARY AND CONCLUSION

7.1 Summary

Cotton is an economically important crop grown throughout the world. According to the Ministry of Agriculture and Farmers Welfare cotton 118.7 million bales, has been cultivated during 2019-2020. Cotton plants are being affected by the most devastating soil borne pathogen *Verticillium dahliae*. Symptoms of *Verticillium* wilt generally appears at the first flowering stage. The strategy to develop wilt free cotton plants viz. usage of fungicides, chemical fumigation exists but more intensified, powerful, and effective strategy to control the disease is the need of an hour. Biological control is the most effective and sustainable method to reduce the effect of *Verticillium dahliae* on cotton and safe, environment friendly and convenient approach to be used. Studies conducted earlier have reported the use of microbial strains for control the *Verticillium* wilt of cotton. Bioformulation developed from different medicinal plants along with antagonistic strains and chemicals has been used against *V. dahliae* is also effective approach to control wilt disease. The primary aim of this research is to develop a strategy to control *Verticillium* wilt of cotton by evaluating the antagonistic fungal strains as potential biocontrol agents along in combination with biochemical formulation. Bathinda is major cotton producing district of Punjab, hence major focus of this research is to explore antagonistic fungi from different organic manures and endophytic bacteria from healthy cotton plant parts; followed evaluating *in vitro* antagonistic activity and biosurfactant based studies with enzymatic screening for evaluation of potential biocontrol agents to suppress *Verticillium* wilt of cotton under both poly house and natural field conditions in Bathinda, Punjab, India.

7.1.1 Isolation of microbial strains from collected samples

Verticillium dahliae, most devastating pathogenic fungus of cotton has been isolated from infected cotton plant parts and rhizospheric and bulk soil. Prevalence of *Verticillium dahliae* is 73%. Isolation of antagonistic fungal strains was carried out by using PDA plates from different types of organic manures with Serial dilution method. Nine antagonistic fungal strains have been isolated from different organic manures using PDA medium supplemented with streptomycin as antibiotic. Three antagonistic fungal strains have been isolated and purified from rhizospheric soil of healthy plant parts. Endophytic bacterial strains have been isolated from healthy

cotton plant parts viz. roots, stems and leaves. Seven endophytic bacterial strains have been isolated on NA medium through streaking method. Different bacterial strains were purified by streaking using NA plates.

7.1.2 Identification of microbial strains

Verticillium dahliae has been preliminary identified by their morphological colony characters, hyphal structure and microsclerotia and 18S rRNA gene identification by PCR was conducted by outsourcing through Science Farmers, Bangalore India by DNA extraction method. Two strains of *Aspergillus* viz. *A. niger, A. nidulans*, were identified by making LCB mounts under light microscope. *Cladosporium sp., Trichoderma harzianum,* and *Gliocladium sp*. were identified by studying their resting spore size, structure, hyphae and identified under light microscope. *Fusarium solani, Aspergillus fumigatus* and *Aspergillus terreus* were identified by Indian Type Culture Collection (ITCC), Division of Plant Pathology, Indian Agriculture Research Institute, New Delhi. Molecular techniques based identification through 16S rRNA gene identification by PCR was conducted by outsourcing through Barcode Bioscience Bangalore India by DNA extraction method. Integrated Transcribed Spacer (ITS1 5´-GTAGTCATATGCTTGTCTC-3´) and (ITS4 5´-CTTCCGTCAATTCCTTTAAG-3´) primers were amplified by 18S rRNA gene region, sequenced and analyzed for similarity among species in NCBI data base. Three endophytic bacterial strains have been identified through 16s rRNA and named as VTS DB-V1/NI /S/S1/B2 (*Pseudomonas aeruginosa)*, VTS DB-V1/NI /R/S1/B2 (*Bacillus subtilis*)**,** VTS DB-V1/NI /S/S1/B1(*Lysinibacillus macroides*).

7.1.3 Preliminary screening of antagonistic fungal strains through Dual Culture Assay

Preliminary screening of antagonistic fungal strains was performed against *V. dahliae* by Dual culture assay in which all strains have shown different antagonism towards *Verticillium dahliae*. Percentage inhibition of radial growth (PIRG) of *V. dahliae* towards *Aspergillus niger, Aspergillus nidulans, Gliocladium species, Aspergillus terreus, Trichoderma harzianum* and *Cladosporium sp., Aspergillus fumigatus and Fusarium solani* was 63.20%, 61.12%, 61.81%, 59.90%, 72.91% and 6.95%,73.46% and 47.22% respectively.

7.1.4 Cellulase, Protease, Chitinase activity in Antagonistic fungi

7.1.4.1 Semi Quantitative Assay of Cellulase Enzyme

Cellulase enzyme activity of antagonistic strains has performed by growing on liquid Czapek Mineral Salt Medium and strains shown positive cellulase activity produced yellow opaque inhibition zone around the well in comparison to control. *Aspergillus niger, Aspergillus nidulans, Gliocladium sp., Aspergillus fumigatus, Trichoderma harzianum, Aspergillus terreus* and *Fusarium solani* have produced 10.4, 7.5, 10.83, 9.83, 8.5,11.5 and 5.0 mm yellow-opaque zones, respectively.

7.1.4.2 Semi Quantitative Assay of Protease Enzyme

Screening of protease enzyme been done on Casein Agar medium. Antagonistic fungal strains have shown clear inhibition zones around the well as compared to control plates. Screening of protease enzyme separated from *Aspergillus niger, Aspergillus nidulans, Gliocladium sp., Aspergillus fumigatus*, *Trichoderma harzianum*, *Cladosporium sp., Aspergillus terreus* and *Fusarium solani* produced 9.5, 8.1, 9.06, 9.33, 6.46, 3.56, 10.7 and 6.7 mm clear zones respectively

7.1.4.3 Semi Quantitative Assay of Chitinase Enzyme

Chitinase enzyme activity of antagonistic strains has been performed on Chitinase Detection Medium comprising basal chitin medium. Chitinase activity was shown by *Aspergillus niger, Aspergillus nidulans, Gliocladium sp., Aspergillus fumigatus*, *Trichoderma harzianum, Cladosporium sp., Aspergillus terreus* and *Fusarium solani* have produced 9.66, 8.5, 8.43, 8.5, 8.1, 3.2, 9.5 and 6.6-mm zones respectively around the well.

7.1.5 Preliminary screening of endophytic bacterial strains by Dual Culture Assay

Endophytic bacterial strains have been isolated form healthy cotton plant parts growing under natural field conditions and evaluated as biocontrol agent against *V. dahliae* by through Dual culture assay. A total seven bacterial strains been isolated, purified and preserved for further *in vitro* screening. VTS DB-V1/NI /L/S1/B2, VG DB-V1/NI /L/S1/B2, VG DB-V2/NI /L/S1/B1, VG DB-V1/NI /S/S1/B1, VTS DB-V1/NI /S/S1/B2 (*Pseudomonas aeruginosa*), VTS DB-V1/NI /R/S1/B2 (*Bacillus subtilis*), VTS DB-V1/NI /S/S1/B1(*Lysinibacillus macroides*) have shown 48.62%, 56.25%, 45.2%, 40.29%, 61.12%, 57.64% and 59.2% respectively PIRG.

7.1.6 Determination of *in vitro* activities of cell wall degrading enzymes of Antagonists

7.1.6.1 Quantitative Assay of Cellulase Enzyme

Cellulase enzyme activity of antagonistic strains has performed by growing on liquid Czapek Mineral Salt Medium. Control plates have shown 0 mm growth and test plates (endophytic bacterial strains) have shown different yellow opaque inhibition zones around the well (Figure 6.30). After 5 days of incubation period, VTS DB-V1/NI /L/S1/B2, VG DB-V1/NI /L/S1/B2, VG DB-V2/NI /L/S1/B1, VG DB-V1/NI /S/S1/B1, VTS DB-V1/NI /S/S1/B2, VTS DB-V1/NI /R/S1/B2, VTS DB-V1/NI/S/S1/B1 have produced 6.8,5.2, 5.0,4.5 8.56, 5.23 and 7.3 mm yellow- opaque inhibition zones respectively.

7.1.6.2 Quantitative Assay of Protease Enzyme

Screening of protease enzyme been done on Casein Agar medium. VTS DB-V1/NI /L/S1/B2, VG DB-V1/NI /L/S1/B2, VG DB-V2/NI /L/S1/B1, VG DB-V1/NI /S/S1/B1, VTS DB-V1/NI /S/S1/B2, VTS DB-V1/NI /R/S1/B2, VTS DB-V1/NI /S/S1/B1 have shown 3.2, 5.5, 4.2, 3.9, 8.70, 6.66 and 7.32 mm inhibition zones respectively.

7.1.6.3 Quantitative Assay of Chitinase Enzyme

Chitinase enzyme activity of antagonistic strains has been performed on Chitinase Detection Medium comprising basal chitin medium. Endophytic bacterial strains, VTS DB-V1/NI /L/S1/B2VG DB-V1/NI /L/S1/B2, VG DB-V2/NI /L/S1/B1, VG DB-V1/NI /S/S1/B1, VTS DB-V1/NI /S/S1/B2, VTS DB-V1/NI /R/S1/B2 and VTS DB-V1/NI /S/S1/B1 have produced 5.7, 6.1, 3.9, 4.5, 7.4, 6.3 and 7.1mm purple coloured inhibition zones, respectively.

7.1.7 Antifungal activity of biosurfactants against *V. dahliae*

Antifungal activity of biosurfactant viz. Fengycin and Iturin have been evaluated against *V. dahliae* in which Fengycin at concentration of 5ppm and 10 ppm have shown 0mm, 2.3±0.1mm and 3.2± 0.05 mm inhibition zones respectively. Iturin at same concentrations have shown 2.9±0.15, 3.8±0.2 mm inhibition zones respectively as compared to control plates.

7.1.8 Preparation of Biochemical formulation and its antifungal activity against *V. dahliae*

Biochemical formulation has been prepared by using combinatorially from different medicinal plant parts along with antagonistic fungus and endophytic bacterial strains. Plant parts of *Ricinus communis, Tinospora cardifolia, Withania somnifera, Datura stramonium, Clotropis procera, Azadiaracta indiaca, Citrullus colocynthis, Solanum xanthocarpum.* Copper sulphate has also been used for preparation of formulation. Product (crude plant extract) has been obtained after 30 days and further used for field trial. *in vitro* antifungal activity of biochemical formulation has been evaluated against *V. dahliae* by well diffusion method wherein biochemical formulation has shown positive *in vitro* antagonism against *V. dahliae* as compared to control. Inhibition zone of 12 mm has been recorded after 5 days of incubation at 28^0C around the well at 100ppm concentration compared to control. Biochemical formulation has been used in ratio of 1:9 (biochemical formulation: water) in the form of spray mixed with $3.4x10^5$ conidia/ml of antagonistic fungal strains for *in vivo* evaluation against *V. dahliae.*

7.1.8.1 LCMS of biochemical formulation

Biochemical formulation has been characterized from CDRI, Lucknow for LCMS analysis**.** Total twenty six active compounds viz. 3-Glc-campesterol, Momordol , Dihydroisocucurbitacin B-25-acetyl, 2-O-β-D- glucopyranose- (22-27)-hexanocucurbitacin I, 2-O-β-D-glucopyranosyl-cucurbitacin L, 2-O-β-D-glucopyranosyl-cucurbitacin E, Arvenin I, 6’-acetyl-2-O-β-D- glucopyranosyl-cucurbitacin E, 2,3-Dihydrowithaferin A3β-O-sulfate, 23, 24-dihydrowithagenin, Dihydrowithanolide, Dihydrowithferin A, Petunidin 3-O-rhamnoside, Adenylosuccinate, Lysoplasmenylcholine, GDP-L-galactose, Verbascoside, Deoxycholic acid 3- glucuronide, 3 β -Hydroxydeoxo dihydrodeoxygedunin, Hydrocortisone cypionate, Dihydro deoxy streptomycin, Withanolide S, Solasonine, β -2 solamargine, Germanicol cinnamate, Pubesenolide have been identified in positive ionization mode. Total eight active compounds viz. Cucurbitacin E, 2-O-β-D-glucopyranosyl-cucurbitacin I, 25-p-coumaroyl-3’-acetyl-2-O-β-D glucopyranosyl-cucurbitacin I, Petunidin 3-O-rhamnoside, Aswagandhanolide, Sitoindoside X, 4-Deoxywithaperuvin and Solanesol have been identified by LCMS of biochemical formulation in negative ionization mode based on literature survey.

7.1.9 Soil solarization of natural field

Soil solarization technique has been performed before sowing of seeds under natural field conditions**.** The field was prepared by ploughing once before sowing of seeds and dry weeds and stubble were removed by deep ploughing. Soil solarization technique has been used before sowing of seeds in already prepared experimental field. Four equal ridges have been prepared with wooden leveller and properly covered with polythene sheets. After 30 days, serial dilution technique has been used for isolation of pathogenic strains but no growth of *V. dahliae* has been recorded after soil solarization.

7.1.10 Soil sterilization and seed bacterization in Poly house experiments

Evaluation of potential antagonistic microbial strains against *Verticillium dahliae* has been performed in poly house experiments. Soil, sand, peat and vermiculite have been used in ratio of 1:1:1:1 respectively for each pot. Soil was collected from previously cotton growing fields in polythene bags. Soil was air dried in hot air oven and sieved to remove the debris. Soil and sand were sterilized separately in autoclave for at least 30 minutes by using saturated steam. Bacterization of delinted seeds of *Verticillium dahliae* sensitive local variety Rasi 134 Bt has been done for both field and pot experiments. Cotton seeds are first treated with 1% NaOCl for 1 min for surface sterilization followed by three times washing in sterile phosphate buffer having concentration 0.05 M (pH 7.4) and endophytic bacterial strains. Nutrient Broth medium has used for antagonistic bacterial growth for 24 hours at 37^0C. Pellets of bacterial strains after centrifugation have been collected and suspended in sterile phosphate buffer with 1.5% CMC solution.

7.1.11 Biological control of *Verticillium* wilts of Cotton in polyhouse experiments

7.1.11.1 Biological control of *Verticillium* wilt of Cotton in polyhouse experiments with endophytic bacterial strains (Monoculture experiment)

Bacterized seeds and non-bacterized (control) were sown for recording the symptoms of wilt disease. Disease severity index has been recorded in comparison with control plant. Control plant (inoculated with *V. dahliae*) has shown wilting (89%) and *Pseudomonas aeruginosa* with 0% (healthy) disease severity, *Lysinibacillus macroides* and *Bacillus subtilis* have shown 19% and 32% acropetal chlorosis, respectively at 0-4 rating scale. Biocontrol protection was recorded 69.6% in

Pseudomonas aeruginosa followed by *Lysinibacillus macroides* (65.2%) *Bacillus subtilis* (62.5%) as compared to control.

7.1.11.2 Biocontrol efficacy of potential antagonistic strains under poly house (Monoculture experiment)

During polyhouse trials, antagonistic fungal strains were evaluated against *V. dahliae*. Wilt symptoms were developed on *Verticillium*-inoculated plants (control 2) but were not observed on any of the water-inoculated plants (Control 1). The first disease symptoms appeared in control 2 plants as light-yellow areas between veins of leaves and on the first and second mature leaves of the control plants of Rasi 134 Bt. During pot trials, most significant biocontrol efficacy has been recorded in *Trichoderma harzianum* followed by *Aspergillus terreus*, *A. niger*, *A. fumigatus* and *A. nidulans* with 70.3, 66.5, 64.2, 61.5 and 59.2 % biocontrol protection percent respectively as compared to control plants.

7.1.11.3 Efficacy of fungal and bacterial antagonistic strains under poly house experiments (Co culture)

During polyhouse trials, five antagonistic fungal strains and three endophytic bacterial strains were evaluated against *V. dahliae*. Symptoms of *Verticillium* wilt has been recorded in *Verticillium*-inoculated plants (control 2) as compared to Control 1. Test plants have shown 0% disease severity (healthy) at 0-4 disease severity index rating scale. Control 2 plants have shown wilting or death (83%) after 135 days of sowing during pot trials and 69.5% biocontrol protection percent.

7.1.11.4 Efficacy of antagonistic strains along with biochemical formulation under poly house experiments (Monoculture)

Five antagonistic fungal strains were evaluated against *V. dahliae* with biochemical formulation as spray form. Control (inoculated with *V. dahliae*) plants have shown symptoms of wilting (89%) after 135 days of sowing than test plants (inoculated with both *V. dahliae* and antagonistic fungal strains and biochemical formulation) after first blooming stage. Test plants treated with *A. niger* and *A. nidulans* have shown acropetal chlorosis with 19 and 21% severity during polyhouse experiments. Control 1 has shown 35%, 39%, 26%, 21% and 33% disease severity during *A. nidulans*, *A. terreus*, *T. harzianum*, *A. fumigatus* and *A. niger* respectively. During pot trials, most significant biocontrol efficacy has been recorded in *Trichoderma harzianum* followed

by *Aspergillus terreus*, *A. fumigatus*, *A. niger* and *A. nidulans* with 73.2, 68.5, 66.2, 61.3 and 57.2 biocontrol protection percent respectively as compared to control plants.

7.1.11.5 Biological control of *Verticillium* wilt of Cotton in polyhouse experiments with endophytic bacterial strains and biochemical formulation (Monoculture experiment)

Bacterized seeds with non-bacterized (control) were performed after sowing. Symptoms of wilt disease have been observed in non- bacterized plants without biochemical formulation compared to bacterized plants. Most positive significant suppression of wilt disease was recorded in treatment of seeds with *Pseudomonas aeruginosa*. Disease severity index has been recorded and calculated in comparison to control plant (inoculated with *V. dahliae*) has shown wilting (86%) and *Pseudomonas aeruginosa* with BCF and *Lysinibacillus macroides* with BCF has shown no disease symptoms 0% (healthy) and *Bacillus subtilis* have shown 32% acropetal chlorosis, at 0-4 rating scale. Biocontrol protection was recorded 70.6% in *Pseudomonas aeruginosa* followed by *Lysinibacillus macroides* (68.6%) *Bacillus subtilis* (64.5%) as compared to control

7.1.11.6 Efficacy of antagonistic strains and endophytic bacterial strains along with biochemical formulation under poly house experiments (Coculture experiment)

Five antagonistic fungal strains were evaluated against *V. dahliae* with biochemical formulation, used in spray form. After inoculation of *V. dahliae* symptoms were developed on *Verticillium*-inoculated plants (control) plants but were not observed on test plants. Control (inoculated with *V. dahliae*) plants have shown symptoms of wilting (85%) after 135 days of sowing than test plants (inoculated with both *V. dahliae* and antagonistic fungal strains, biochemical formulation and bacterized seeds) after first blooming stage. During pot trials, 75.2 percent significant biocontrol efficacy has been recorded in test plants as compared to control plants.

7.1.12 Biological control of *Verticillium* wilt of Cotton in field experiments

7.1.12.1 Biological control of *Verticillium* wilt of Cotton under field conditions with antagonistic fungal strains (monoculture experiment)

Among eight strains, four strains of *Aspergillus* viz. *A. niger*, *A. nidulans*, *A. fumigatus* and *A. terreus* and *Trichoderma harzianum* also selected for *in vivo* evaluation after checking their *in vitro* antagonistic potential. Disease severity index has been recorded and calculated in comparison to control 2 plant (inoculated with *V. dahliae*) has shown wilting (87%) and control 1 (inoculated with water) has shown disease severity in *A. nidulans* (46%), *A. terreus*(32%), *T. harzianum*(28%), *A. fumigatus(*19%) and *A. niger* (32%) respectively. *Aspergillus terreus* was significantly reduced the symptoms of wilt disease with biocontrol protection of 74.5% followed by *Trichoderma harzianum* (72.3%), *Aspergillus fumigatus* (68.8%), *A. niger* (66.2%), *A. nidulans* (61.2%) as compared to control 1 and control 2.

7.1.12.2 Biological control of *Verticillium* wilt of Cotton under natural field conditions with endophytic bacterial strains (Monoculture experiment)

Pseudomonas aeruginosa, Lysinibacillus macroides and *Bacillus subtilis* were selected for used as biocontrol agent under natural field conditions after evaluating their significant *in vitro* effect against *Verticillium dahliae*. Most significant biocontrol efficacy has been recorded in *Pseudomonas aeruginosa* followed by *Lysinibacillus macroides* and *Bacillus subtilis* with 73.6%, 68.5% and 62.5% biocontrol protection percent, respectively**.**

7.1.12.3 Biological control of *Verticillium* wilt of Cotton under natural field conditions with endophytic bacterial and fungal strains (Co culture)

Pseudomonas aeruginosa, Lysinibacillus macroides and *Bacillus subtilis* were used as seed bacterization along with five antagonistic fungal strains and evaluated as biocontrol agent under natural field conditions. Control plants (non-bacterized) have shown wilting or death (81%) after 135 days of sowing and treated plants have shown no disease symptoms (0% healthy) at 0-4 rating scale and 71.3% biocontrol protection percent.

7.1.12.4 Biological control of *Verticillium* wilt of Cotton under natural field conditions with endophytic bacterial strains and biochemical formulation (Monoculture)

Endophytic bacterial strains with biochemical formulation have been evaluated used in a combinatorial form under natural field conditions to suppress the effect of soil borne pathogen *V. dahliae*. *Pseudomonas aeruginosa, Lysinibacillus macroides* and *Bacillus subtilis* were selected for used as biocontrol agent under natural field conditions along with biochemical formulation after evaluating their significant *in vitro* effect against *Verticillium dahliae*. Control plants (non-bacterized) have shown wilting or death (82%) after 135 days of sowing. Most significant biocontrol efficacy has been recorded in *Pseudomonas aeruginosa* (76.6%) followed by *Lysinibacillus macrolides* (69.5%) and *Bacillus subtilis* (58.3%) with 76.6%, 69.5% and 58.3% biocontrol protection percent, respectively.

7.1.12.5 Efficacy of antagonistic strains along with biochemical formulation under natural field experiments (monoculture experiment)

Five antagonistic fungal strains were evaluated against *V. dahliae* with biochemical formulation. Biochemical formulation has been used along with antagonistic fungal strains in spray form. After inoculation of *V. dahliae,* symptoms were developed on *Verticillium*-inoculated plants (control). Most significant biocontrol efficacy has been recorded in *Trichoderma harzianum* with 79.5% percent followed by *Aspergillus terreus* (78.3%), *A. fumigatus* (72.8%), *A. niger (69.2%)* and *A. nidulans* (68.2%) as compared to control plants.

7.1.12.6 Efficacy of antagonistic strains and endophytic bacterial strains along with biochemical formulation under poly house experiments (Coculture)

Five antagonistic fungal strains were evaluated against *V. dahliae* with biochemical formulation, used in spray form. Control plants have shown wilting with 82% after 135 days of sowing than test plants (inoculated with both *V. dahliae* and antagonistic fungal strains, biochemical formulation, and bacterized seeds) after first blooming stage. During natural field condition trials, 78.2 percent significant biocontrol efficacy has been recorded in test plants as compared to control plants.

7.1.13 Assessment of plant growth promoting potential of endophytic bacterial strains and antagonistic fungal strains

Antagonistic fungal strains and endophytic bacterial strains that showed positive biocontrol efficacy in the greenhouse and field experiments were analyzed for their potential effects on growth on cotton plant. Antagonistic fungal strains and endophytic bacterial strains help to increase biomass or cotton yield. Different growth factors such as height of plant, number of nodes and root length has been studied after every fortnight during both poly house and field experiments.

7.2 Conclusion

Cotton is a crop of fibre, oil and protein and an economically important crop grown throughout the world. Cotton is the major vital kharif crop of Punjab, India but cotton yield starts reducing annually due to attack of soil borne pathogenic fungus *Verticillium dahliae* causing *Verticillium* wilt disease in cotton. Major focus of this research is to isolate antagonistic fungi from different organic manures and endophytic bacteria from healthy cotton plant parts; followed by *in vitro* antagonistic activity and biosurfactant based studies with enzymatic screening for evaluation of potential biocontrol agents to suppress *Verticillium* wilt of cotton under both poly house and natural field conditions in Bathinda, Punjab, India. Bathinda, major cotton producing district of Punjab is selected for this study. Pathogenic fungus has been isolated from collected samples growing under natural field conditions of Bathinda, Punjab, India. Eight antagonistic fungal strains such as *A. niger, A. nidulans*, *Cladosporium sp., Trichoderma harzianum,* and *Gliocladium sp., Fusarium solani, Aspergillus fumigatus* and *Aspergillus terreus* and seven endophytic bacterial strains have been isolated out of which, three endophytic bacterial strains have been identified through 16s rRNA and named as VTS DB-V1/NI /S/S1/B2 (*Pseudomonas aeruginosa)*, VTS DB-V1/NI /R/S1/B2 (*Bacillus subtilis*), VTS DB-V1/NI /S/S1/B1(*Lysinibacillus macroides*). Biocontrol potential of antagonistic isolates was assessed on following criteria: (1) *in vitro* inhibition of *Verticillium dahliae* by Dual culture assay, (2) activities of cell wall degrading enzymes including protease, cellulase, and chitinase. (3) *in vivo* field trials under poly house and natural field conditions. Most positive *in vitro* antagonism has been observed in *A. niger*, *A. nidulans*, *A. fumigatus* and *A. terreus* and *Trichoderma harzianum* and VTS DB-V1/NI /S/S1/B2 (*Pseudomonas aeruginosa)*, VTS DB-V1/NI /R/S1/B2 (*Bacillus*

subtilis), VTS DB-V1/NI /S/S1/B1(*Lysinibacillus macroides*). Based on their *in vitro* antagonism against *V. dahliae*, these strains have been evaluated during *in vivo* experiments.

Seed bacterization with *Pseudomonas aeruginosa* helps to reduced wilt symptoms in natural fields and pot trials more than *Lysinibacillus macroides* followed by *Bacillus subtilis* in comparison to control plants (non-bacterized). In both pot experiments and field trials, growth parameters and yield of cotton were significantly higher in seed bacterized plants (*Pseudomonas aeruginosa.)* than non-bacterized seeds (control). Most significant biocontrol efficacy has been recorded in *Trichoderma harzianum* followed by *A. terreus* and *A. niger* during pot trials. Most effective biocontrol efficacy has been observed in combinatorial study between biochemical formulation and *T. harzianum* under both poly house and natural field conditions. Effect of antagonistic strains helps to increases height and yield of cotton as compared to control plants. This investigation indicates that *T. harzianum* in combination with biochemical formulation have the potential to control *Verticillium* wilt significantly. Growth factors have also been studied after every fortnight under both poly house and natural field conditions. Highest cotton yield has been recorded in plants treated with *A. terreus* and also with biochemical formulation during both field experiment and poly house followed by *T, harzianum.* Antagonistic fungal strains, endophytic bacterial strains under poly house and natural conditions field trials of Bathinda, Punjab in combination with soil solarization and biochemical formulation have shown positive biocontrol efficacy in integrated management of wilt disease. This approach also effects on growth of plants and increases yield of cotton in Bathinda region.

REFERENCES

1. Abadias, M., Teixidó, N., Usall, J., Solsona, C., & Viñas, I. (2005). Survival of the postharvest biocontrol yeast Candida sake CPA-1 after dehydration by spray-drying. *Biocontrol Science and Technology*, *15*(8), 835-846.

2. Abou-Douh, A. M. (2002). New withanolides and other constituents from the fruit of Withania somnifera. *Archiv der Pharmazie: An International Journal Pharmaceutical and Medicinal Chemistry*, *335*(6), 267-276.

3. Adebanjo, A., & Bankole, S. A. (2004). Evaluation of some fungi and bacteria for biocontrol of anthracnose disease of cowpea. *Journal of Basic Microbiology: An International Journal on Biochemistry, Physiology, Genetics, Morphology, and Ecology of Microorganisms, 44*(1), 3-9.

4. Aghighi, S., Shahidi Bonjar, G. H., Rawashdeh, R., Batayneh, S., & Saadoun, I. (2004). First report of antifungal spectra of activity of Iranian actinomycetes strains against *Alternaria solani, Alternaria alternate, Fusarium solani, Phytophthora megasperma, Verticillium dahliae* and *Saccharomyces cerevisiae*. *Asian Journal of Plant Sciences, 3*(4), 463-471.

5. Agrawal, T., & Kotasthane, A. S. (2012). Chitinolytic assay of indigenous Trichoderma isolates collected from different geographical locations of Chhattisgarh in Central India. *Springer Plus*, *1*(1), 1-10.

6. Alamri, S., Hashem, M., & Mostafa, Y. S. (2012). *In vitro* and *in vivo* biocontrol of soil-borne phytopathogenic fungi by certain bioagents and their possible mode of action. *Biocontrol Science, 17(4)*, 155-167.

7. Amer, G. A., & Utkhede, R. S. (2000). Development of formulations of biological agents for management of root rot of lettuce and cucumber. *Canadian journal of microbiology, 46(9)*, 809-816.

8. Aneja, K. R. (2007). *Experiments in microbiology, plant pathology and biotechnology*. New Age International.

9. Ayele, A. G., Wheeler, T. A., & Dever, J. K. (2020). Impacts of Verticillium Wilt on Photosynthesis Rate, Lint Production, and Fiber Quality of Greenhouse-Grown Cotton (Gossypium hirsutum). *Plants*, *9*(7), 857.

10. Bailey, K. L., & Lazarovits, G. (2003). Suppressing soil-borne diseases with residue management and organic amendments. *Soil and tillage research, 72(2)*, 169-180.

11. Barakate, M., Ouhdouch, Y., Oufdou, K. H., & Beaulieu, C. (2002). Characterization of rhizospheric soil streptomycetes from Moroccan habitats and their antimicrobial activities. *World Journal of Microbiology and Biotechnology, 18(1)*, 49-54.

12. Behie, S. W., & Bidochka, M. J. (2014). Ubiquity of insect-derived nitrogen transfer to plants by endophytic insect-pathogenic fungi: an additional branch of the soil nitrogen cycle. *Applied and environmental microbiology, 80*(5), 1553-1560.

13. Bejarano-Alcázar, J., Melero-Vara, J. M., Blanco-López, M. A., & Jimenez-Diaz, R. M. (1995). Influence of inoculum density of defoliating and nondefoliating pathotypes of *Verticillium dahliae* on epidemics of Verticillium wilt of cotton in southern Spain. *Phytopathology*, *85*(12), 1474-1481.

14. Benítez, T., Rincón, A. M., Limón, M. C., & Codon, A. C. (2004). Biocontrol mechanisms of Trichoderma strains. *International microbiology*, *7*(4), 249-260.

15. Berg, G., Fritze, A., Roskot, N., & Smalla, K. (2001). Evaluation of potential biocontrol rhizobacteria from different host plants of *Verticillium dahliae Kleb. Journal of applied microbiology*, *91*(6), 963-971.

16. Biering-Sùrensen, F., Sùrensen, J. L., Barros, T. E. P., Monteiro, A. A., Nuseibeh, I., Shenaq, S. M., & Shibasaki, K. (1997). Pressure ulcer treatment. *Spinal cord*, *35*, 641-646.

17. Bolek, Y., Bell, A. A., El-Zik, K. M., Thaxton, P. M., & Magill, C. W. (2005). Reaction of cotton cultivars and an F2 population to stem inoculation with isolates *Verticillium dahliae*. *Journal of Phytopathology*, *153*(5), 269-273.

18. Bolleddula, J., Fitch, W., Vareed, S. K., & Nair, M. G. (2012). Identification of metabolites in *Withania sominfera* fruits by liquid chromatography and

high-resolution mass spectrometry. *Rapid communications in mass spectrometry*, *26*(11), 1277-1290.

19. Boudjelal, A., Henchiri, C., Siracusa, L., Sari, M., & Ruberto, G. (2012). Compositional analysis and in vivo anti-diabetic activity of wild Algerian Marrubium vulgare L. infusion. *Fitoterapia*, *83*(2), 286-292.

20. Brar, S. K., Verma, M., Tyagi, R. D., & Valéro, J. R. (2006). Recent advances in downstream processing and formulations of Bacillus thuringiensis based biopesticides. *Process Biochemistry*, *41*(2), 323-342.

21. Burges, H. D. (2012). *Formulation of microbial biopesticides: beneficial microorganisms, nematodes and seed treatments*. Springer Science & Business Media.

22. Cavaglieri, L. R., Passone, A., & Etcheverry, M. G. (2004). Correlation between screening procedures to select root endophytes for biological control of Fusarium verticillioides in Zea mays L. *Biological Control*, *31*(3), 259-267.

23. Cárdenas, J. C. G., García, J. M. M., & Acosta, A. G. (2005). Evaluación de diferentes concentraciones de *Trichoderma spp.* contra *Fusarium oxysporum* agente causal de la pudrición de plántulas en papaya (Carica papaya L.) en Tuxpan, Veracruz, México. *Revista Científica UDO Agrícola*, *5*(1), 45-47.

24. Çelik, S., Bardak, A., Erdoğan, O., Parlak, D., Uçar, R., Tekerek, H., ... & Hayat, K. H. (2017). Determination of the response of some cotton varieties to cotton wilt disease caused by *Verticillium dahliae Kleb*.

25. Cotxarrera, L., Trillas-Gay, M. I., Steinberg, C., & Alabouvette, C. (2002). Use of sewage sludge compost and *Trichoderma asperellum* isolates to suppress Fusarium wilt of tomato. *Soil Biology and biochemistry, 34*(4), 467-476.

26. Chawech, R., Mhalla, D., Trigui, M., Mihoubi, M., Fabre, N., & Jarraya, R. (2015). Chemical composition and antibacterial activity of extracts and compounds isolated from *Citrullus colocynthis* (L.) Schrad. *Journal of Pharmacognosy and Phytochemistry*, *4*(4), 197.

27. Cherkupally, R., Amballa, H., & Bhoomi, NR. (2017) In vitro screening for enzymatic activity of *Trichoderma* species for biocontrol potential. *A Plant Science, 6*,1784.

28. Cubukcu, N., & Benlioglu, K. (2007). Biological control of Verticillium wilt of cotton by endophytic bacteria. *IOBC WPRS BULLETIN*, *30*(6/2), 371.

29. Cun, M., Guiliang, J., & Chuanlin, Z. (2002). The advances in cotton breeding resistance to Fusarium and Verticillium wilts in China during past fifty years. *Zhongguo Nongye Kexue (China)*.

30. Compant, S., Duffy, B., Nowak, J., Clément, C., & Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and environmental microbiology*, *71*(9), 4951-4959.

31. Debode, J., Maeyer, K. D., Perneel, M., Pannecoucque, J., Backer, G. D., & Höfte, M. (2007). Biosurfactants are involved in the biological control of Verticillium microsclerotia by *Pseudomonas spp*. *Journal of applied microbiology, 103*(4), 1184-1196.

32. Daayf, F. (2015). Verticillium wilts in crop plants: Pathogen invasion and host defence responses. *Canadian Journal of Plant Pathology*, *37*(1), 8-20.

33. De Brito, A. M., Gagne, S., & Antoun, H. (1995). Effect of compost on rhizosphere microflora of the tomato and on the incidence of plant growth-promoting rhizobacteria. *Applied and Environmental Microbiology*, *61*(1), 194-199.

34. de Faria, M. R., & Wraight, S. P. (2007). Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biological control*, *43*(3), 237-256.

35. Deketelaere, S., Tyvaert, L., França, S. C., & Höfte, M. (2017). Desirable traits of a good biocontrol agent against Verticillium wilt. *Frontiers in microbiology, 8,* 1186.

36. Dervis, S., & Bicici, M. (2005). Vegetative compatibility groups in *Verticillium dahliae* isolates from cotton in Turkey. *Phytoparasitica, 33*(2), 157-168.

37. Desai, J. D., & Banat, I. M. (1997). Microbial production of surfactants and their commercial potential. *Microbiology and Molecular biology reviews*, *61*(1), 47-64.

38. Duan, Y. X., Chen, T., Chen, X., & Zhao, X. M. (2010). Overexpression of glucose-6-phosphate dehydrogenase enhances riboflavin production in *Bacillus subtilis*. *Applied microbiology and biotechnology*, *85*(6), 1907-1914.

39. Dung, J. K., Hamm, P. B., Eggers, J. E., & Johnson, D. A. (2013). Incidence and impact of *Verticillium dahliae* in soil associated with certified potato seed lots. *Phytopathology*, *103*(1), 55-63.

40. Droby, S., Cohen, L., Daus, A., Weiss, B., Horev, B., Chalutz, E., ... & Shachnai, A. (1998). Commercial testing of Aspire: a yeast preparation for the biological control of postharvest decay of citrus. *Biological control*, *12*(2), 97-101.

41. Eddouaouda, K., Mnif, S., Badis, A., Younes, S. B., Cherif, S., Ferhat, S., & Sayadi, S. (2012). Characterization of a novel biosurfactant produced by Staphylococcus sp. strain 1E with potential application on hydrocarbon bioremediation. *Journal of Basic Microbiology*, *52*(4), 408-418.

42. Eljounaidi, K., Lee, S. K., & Bae, H. (2016). Bacterial endophytes as potential biocontrol agents of vascular wilt diseases–review and future prospects. *Biological Control*, *103*, 62-68.

43. El-Mehalawy, A. A., Hassanein, N. M., Khater, H. M., El-Din, E. K., & Youssef, Y. A. (2004). Influence of maize root colonization by the rhizosphere actinomycetes and yeast fungi on plant growth and on the biological control of late wilt disease. *International Journal of Agriculture Biology*, *6*(4), 599-605.

44. El-Tarabily, K. A., Soliman, M. H., Nassar, A. H., Al-Hassani, H. A., Sivasithamparam, K., McKenna, F., & Hardy, G. S. J. (2000). Biological

control of *Sclerotinia* minor using a chitinolytic bacterium and actinomycetes. *Plant pathology, 49*(5), 573-583.

45. El-Tarabily, K. A., Nassar, A. H., Hardy, G. S. J., & Sivasithamparam, K. (2009). Plant growth promotion and biological control of *Pythium aphanidermatum*, a pathogen of cucumber, by endophytic actinomycetes. *Journal of Applied Microbiology, 106*(1), 13-26.

46. El-Zik, K. M., (1985). Integrated control of Verticillium wilt of cotton. *Plant Disease, 69*(12), 1025-1032.

47. Erdogan, O., Benlioglu, K., (2009). Biological control of Verticillium wilt on cotton by the use of fluorescent *Pseudomonas spp.* under field conditions. *Journal of Biological Control 53,* 39–45.

48. Fang, H., Zhou, H., Sanogo, S., Lipka, A. E., Fang, D. D., Percy, R. G., ... & Zhang, J. (2014). Quantitative trait locus analysis of Verticillium wilt resistance in an introgressed recombinant inbred population of Upland cotton. *Molecular breeding*, *33*(3), 709-720.

49. Fewell, A. M., Roddick, J. G., & Weissenberg, M. (1994). Interactions between the glycoalkaloids solasonine and solamargine in relation to inhibition of fungal growth. *Phytochemistry*, *37*(4), 1007-1011.

50. Fravel, D. R. (2005). Commercialization and implementation of biocontrol. *Annu. Rev. Phytopathol.*, *43*, 337-359.

51. Gao, F. K., Dai, C. C., & Liu, X. Z. (2010). Mechanisms of fungal endophytes in plant protection against pathogens. *African Journal of Microbiology Research, 4*(13), 1346-1351.

52. Gaur, V. K., Sharma, P., Sirohi, R., Varjani, S., Taherzadeh, M. J., Chang, J. S., & Kim, S. H. (2021). Production of biosurfactants from agro-industrial waste and waste cooking oil in a circular bioeconomy: *An Overview. Bioresource Technology,* 126059.

53. Ghosal, S., Kaur, R., & Bhattacharya, S. K. (1988). Chemistry and bioactivity of sitoindosides IX and X. *Planta medica*, *54*(06), 561-561.

54. Gill, H. K., McSorley, R., & Treadwell, D. D. (2009). Comparative performance of different plastic films for soil solarization and weed suppression. *HortTechnology*, *19*(4), 769-774.

55. Gilman, J.C., (1995) A manual of soil fungi. Rupa Offset Printers. Jaipur 363.

56. Göre, M. E., Caner, Ö. K., Altın, N., Aydın, M. H., Erdoğan, O., Filizer, F., & Büyükdöğerlioğlu, A. (2009). Evaluation of cotton cultivars for resistance to pathotypes of *Verticillium dahliae*. *Crop protection*, *28*(3), 215-219.

57. Guijarro, B., Melgarejo, P., & De Cal, A. (2007). Effect of stabilizers on the shelf-life of Penicillium frequentans conidia and their efficacy as a biological agent against peach brown rot. *International journal of food microbiology*, *113*(2), 117-124.

58. Guo, B., Dai, J. R., Ng, S., Huang, Y., Leong, C., Ong, W., & Carté, B. K. (2000). Cytonic acids A and B: novel tridepside inhibitors of hCMV protease from the endophytic fungus *Cytonaema species*. *Journal of Natural Products*, *63*(5), 602-604.

59. García-Mata, C., & Lamattina, L. (2001). Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. *Plant Physiology*, *126*(3), 1196-1204.

60. Haas, D., & Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature reviews microbiology*, *3*(4), 307-319.

61. Hagan, A. K., Bowen, K. L., Miller, B., Scott, S., & Nichols, R. L. (2017). Fungicide selection and nozzle arrangement impact target spot control and yield of cotton. *Plant Health Progress*, *18*(4), 211-218.

62. Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., & Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Canadian journal of microbiology*, *43*(10), 895-914.

63. Hampton, R. E., Wullschleger, S. D., & Oosterhuis, D. M. (1990). Impact of Verticillium wilt on net photosynthesis, respiration and photorespiration in field-grown cotton (Gossypium hirsutum L.). *Physiological and Molecular Plant Pathology*, *37*(4), 271-280.

64. Hanson, L. E. (2000). Reduction of Verticillium wilt symptoms in cotton following seed treatment with Trichoderma virens.

65. Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., & Lorito, M. (2004). *Trichoderma species*—opportunistic, avirulent plant symbionts. *Nature reviews microbiology*, *2*(1), 43-56.

66. Howell, C. R., (2003). Mechanisms employed by Trichoderma species in the biological control of plant diseases: the history and evolution of current concepts. *Plant disease, 87*(1), 4-10.

67. Huang, C. F., Ma, L., Sun, L. J., Ali, M., Arfan, M., Liu, J. W., & Hu, L. H. (2009). Immunosuppressive withanolides from *Withania coagulans*. *Chemistry & biodiversity*, *6*(9), 1415-1426.

68. Huang, J., Mi, J., Lin, H., Wang, Z., Chen, R., Hu, R., ... & Pray, C. (2010). A decade of Bt cotton in Chinese fields: Assessing the direct effects and indirect externalities of Bt cotton adoption in China. *Science China Life Sciences*, *53*(8), 981-991.

69. Huang, Y., Li, L., Smith, K. P., & Muehlbauer, G. J., (2016). Differential transcriptomic responses to *Fusarium graminearum* infection in two barley quantitative trait loci associated with Fusarium head blight resistance. *BMC genomics, 17*(1), 1-16.

70. Ibarra-Medina, J., & Pinkerton, A. J. (2010). A CFD model of the laser, coaxial powder stream and substrate interaction in laser cladding. *Physics Procedia*, *5*, 337-346.

71. Inderbitzin, P., Bostock, R. M., Davis, R. M., Usami, T., Platt, H. W., & Subbarao, K. V. (2011). Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. *PloS one*, *6*(12), e28341.

72. Iyriboz, N., (1941). Cotton Diseases, Ministry of Agriculture of Turkey, Publication No. 237. Marifet Press, Izmir, Turkey.

73. Jain, S., Tiwary, A. K., Sapra, B., & Jain, N. K. (2007). Formulation and evaluation of ethosomes for transdermal delivery of lamivudine. *Aaps Pharmscitech*, *8*(4), 249-257.

74. Jayaprakasam, B., Strasburg, G. A., & Nair, M. G. (2004). Potent lipid peroxidation inhibitors from *Withania somnifera* fruits. *Tetrahedron*, *60*(13), 3109-3121.

75. Johnson, D., & Gilbert, L. (2015). Interplant signalling through hyphal networks. *New Phytologist*, *205*(4), 1448-1453.

76. Kamala, T., & Indira, S. (2011). Evaluation of indigenous *Trichoderma* isolates from Manipur as biocontrol agent against *Pythium aphanidermatum* on common beans. *3 Biotech*, *1*(4), 217-225.

77. Kamala, T., & Indira, S., (2014). Molecular characterization of *Trichoderma harzianum* strains from Manipur and their biocontrol potential against *Pythium ultimum*. *International Journal of Current Microbiology and Applied Sciences, 3*(7), 258-270.

78. Kapat, A., Zimand, G., & Elad, Y., (1998). Effect of two isolates of *Trichoderma harzianum* on the activity of hydrolytic enzymes produced byBotrytis cinerea. *Physiological and Molecular Plant Pathology, 52*(2), 127-137.

79. Kavroulakis, N., Ehaliotis, C., Ntougias, S., Zervakis, G. I., & Papadopoulou, K. K. (2005). Local and systemic resistance against fungal pathogens of tomato plants elicited by a compost derived from agricultural residues. *Physiological and Molecular Plant Pathology, 66*(5), 163-174.

80. Karena, R. G., Sapovadiya, M. H., & Mehta, D. R. (2021). Characterization of American cotton (*Gossypium hirsutum L.*) genotypes based on morphological characters. *Journal of Pharmacognosy and Phytochemistry, 10*(4), 135-145.

81. Khadi, B. M., Santhy, V., & Yadav, M. S. (2010). Cotton: an introduction. In Cotton (pp. 1-14). Springer, Berlin, Heidelberg.

82. Khan, M. R., Li, L., Pérez-Sánchez, C., Saraf, A., Florens, L., Slaughter, B. D., ... & Si, K. (2015). Amyloidogenic oligomerization transforms Drosophila Orb2 from a translation repressor to an activator. *Cell*, *163*(6), 1468-1483.

83. Khan, M. R., Li, L., Pérez-Sánchez, C., Saraf, A., Florens, L., Slaughter, B. D., ... & Si, K. (2013). Amyloidogenic oligomerization transforms Drosophila Orb2 from a translation repressor to an activator. *Cell*, *163*(6), 1468-1483.

84. Khan, Z. S., & Nasreen, S. (2010). Phytochemical analysis, antifungal activity and mode of action of methanol extracts from plants against pathogens. *Journal of Agricultural Technology, 6*(4), 793-805.

85. Kim, P. I., Ryu, J. W., Kim, Y. H., & Chi, Y. T. (2010). Production of biosurfactant lipopeptides iturin A, fengycin, and surfactin A from *Bacillus subtilis* CMB32 for control of Colletotrichum gloeosporioides. *Journal of microbiology and biotechnology, 20*(1), 138-145.

86. Klimes, A., Dobinson, K. F., Thomma, B. P., & Klosterman, S. J. (2015). Genomics spurs rapid advances in our understanding of the biology of vascular wilt pathogens in the genus *Verticillium*. *Annual Review of Phytopathology, 53*, 181-198.

87. Klosterman, S. J., Atallah, Z. K., Vallad, G. E., & Subbarao, K. V. (2009). Diversity, pathogenicity, and management of *Verticillium species*. *Annual review of phytopathology, 47*, 39-62.

88. Knowles, A., (2008). Recent developments of safer formulations of agrochemicals. Environmentalist, 28,35–44.

89. Kobra, N., Jalil, K., & Youbert, G. (2009). Effects of three Glomus species as biocontrol agents against Verticillium-induced wilt in cotton. *Journal of Plant Protection Research, 49*(2), 185-189.

90. Kumar, A., & Yadav, D. S. (2001). Long-term effects of fertilizers on the soil fertility and productivity of a rice–wheat system. *Journal of Agronomy and Crop science*, *186*(1), 47-54.

91. Kuter, G. A., Nelson, E. B., Hoitink, H. A. J., & Madden, L. V. (1983). Fungal populations in container media amended with composted hardwood bark

suppressive and conducive to Rhizoctonia damping-off. *Phytopathology, 73*(10), 1450-1456.

92. Landa, B. B., Hervás, A., Bettiol, W., & Jiménez-Díaz, R. M. (1997). Antagonistic activity of Bacteria from the chickpea rhizosphere against *Fusarium Oxysporum* f. sp. Ciceris. *Phytoparasitica, 25*(4), 305-318.

93. Lang, C., & Polle, A. (2011). Ectomycorrhizal fungal diversity, tree diversity and root nutrient relations in a mixed Central European Forest. *Tree physiology, 31*(5), 531-538.

94. Larena, I., Melgarejo, P., & De Cal, A. (2003). Drying of conidia of *Penicillium oxalicum*, a biological control agent against Fusarium wilt of tomato. *Journal of Phytopathology*, *151*(11-12), 600-606.

95. Lawrence, K., Hagan, A., Norton, R., Hu, J., Faske, T., Hutmacher, R., ... & Mehl, H. L. (2015, January). Cotton disease loss estimate committee report, 2014. In *Proceedings of the 2015 Beltwide Cotton Conferences, San Antonio, TX. Cordova: National Cotton Council* (pp. 188-190).

96. Li, C. H., Shi, L., Han, Q., Hu, H. L., Zhao, M. W., Tang, C. M., & Li, S. P. (2012). Biocontrol of Verticillium wilt and colonization of cotton plants by an endophytic bacterial isolate. *Journal of applied microbiology*, *113*(3), 641-651.

97. Liu, H., Zeng, Q., Yalimaimaiti, N., Wang, W., Zhang, R., & Yao, J. (2021). Comprehensive genomic analysis of *Bacillus velezensis* AL7 reveals its biocontrol potential against Verticillium wilt of cotton. *Molecular Genetics and Genomics*, *296*(6), 1287-1298.

98. MacKerron, D. K. L., Young, M. W., & Davies, H. V. (1995). A critical assessment of the value of petiole sap analysis in optimizing the nitrogen nutrition of the potato crop. *Plant and soil, 172*(2), 247-260.

99. Makkar, R. S., & Rockne, K. J. (2003). Comparison of synthetic surfactants and biosurfactants in enhancing biodegradation of polycyclic aromatic hydrocarbons. *Environmental Toxicology and Chemistry: An International Journal, 22*(10), 2280-2292.

100. Mansoori, M., Heydari, A., Hassanzadeh, N., Rezaee, S., & Naraghi, L. (2013). Evaluation of *Pseudomonas* and *Bacillus* bacterial antagonists for biological control of cotton Verticillium wilt disease. *Journal of Plant Protection Research, 53*(2), 154-157.

101. McInroy J.A & Kloepper J.W. (1995). Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant Soil,173*,337–342.

102. Mansoori, M., Heydari, A., Hassanzadeh, N., Rezaee, S., & Naraghi, L. (2013). Evaluation of *Pseudomonas* and *Bacillus* bacterial antagonists for biological control of cotton Verticillium wilt disease. *Journal of Plant Protection Research*.

103. Mehnaz, S. (2016). An overview of globally available bioformulations. *Bioformulations: For Sustainable Agriculture*, 267-281.

100. Mendes, R., Kruijt, M., De Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H., ... & Raaijmakers, J. M. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science*, *332*(6033), 1097-1100.

101. Mercado-Blanco, J., Rodrıguez-Jurado, D., Hervás, A., & Jiménez-Dıaz, R. M. (2004). Suppression of Verticillium wilt in olive planting stocks by root-associated fluorescent Pseudomonas spp. *Biological Control, 30*(2), 474-486.

102. Mohan, P. K., Nakhla, G., & Yanful, E. K. (2006). Biokinetics of biodegradation of surfactants under aerobic, anoxic and anaerobic conditions. *Water Research*, *40*(3), 533-540.

103. Mohan, K. S., Kalia, V., & Gujar, G. T. (2021). Insect resistance management for Bt cotton in India. *Genetically Modified Crops in Asia Pacific, 31*.

104. Monte, E. (2001). Understanding *Trichoderma:* between biotechnology and microbial ecology. *International Microbiology*, *4*(1), 1-4.

105. Nagrale, D. T., Gawande, S. P., Gokte-Narkhedkar, N., & Waghmare, V. N. (2020). Association of phytopathogenic *Pantoea dispersa* inner boll rot of cotton (*Gossypium hirsutum L.*) in Maharashtra state, India. *European Journal of Plant Pathology, 158*(1), 251-260.

106. Naraghi, L., Heydari, A., Rezaee, S., Razavi, M., Jahanifar, H., & Khaledi, E. (2010). Biological control of tomato Verticillium wilt disease by *Talaromyces flavus*. *Journal of Plant Protection Research*.

107. Naraghi, L., Heydari, A., Rezaee, S., & Razavi, M. (2012). Biocontrol agent *Talaromyces flavus* stimulates the growth of cotton and potato. *Journal of plant growth regulation, 31*(4), 471-477.

108. Newman, M. A. (2002). New in-furrow fungicides for seedling disease control in cotton. In *The BCPC Conference: Pests and diseases, Volumes 1 and 2. Proceedings of an international conference held at the Brighton Hilton Metropole Hotel, Brighton, UK, 18-21 November 2002* (pp. 371-376). British Crop Protection Council.

109. Nielsen, T. H., & Sørensen, J. (2003). Production of cyclic lipopeptides by *Pseudomonas fluorescens* strains in bulk soil and in the sugar beet rhizosphere. *Applied and environmental microbiology, 69(2*), 861-868.

110. Nihorimbere, V., Ongena, M., Smargiassi, M., & Thonart, P. (2011). Beneficial effect of the rhizosphere microbial community for plant growth and health. *Biotechnologie, Agronomie, Société et Environnement*, *15*(2), 327-337.

111. Oka, Y., & Yermiyahu, U. (2002). Suppressive effects of composts against the root-knot nematode Meloidogyne javanica on tomato. *Nematology*, *4*(8), 891-898.

112. Oluwaseun, A. C., Kola, O. J., Mishra, P., Singh, J. R., Singh, A. K., Cameotra, S. S., & Micheal, B. O. (2017). Characterization and optimization of a rhamnolipid from *Pseudomonas aeruginosa* C1501 with novel biosurfactant activities. *Sustainable Chemistry and Pharmacy, 6*, 26-36.

113. Oros-Sichler, M., Gomes, N. C., Neuber, G., & Smalla, K. (2006). A new semi-nested PCR protocol to amplify large 18S rRNA gene fragments for PCR-DGGE analysis of soil fungal communities. *Journal of Microbiological Methods*, *65*(1), 63-75.

114. Oyourou, J. N., Combrinck, S., Regnier, T., & Marston, A. (2013). Purification, stability and antifungal activity of verbascoside from *Lippia javanica* and *Lantana camara* leaf extracts. *Industrial Crops and Products*, *43*, 820-826.

115. Patki, S. S., & Sable, G. S. (2016). Cotton leaf disease detection & classification using multi SVM. *International Journal of Advanced Research in Computer and Communication Engineering, 5*(10), 165-168.

116. Paul, A. T., Vir, S., & Bhutani, K. K. (2008). Liquid chromatography–mass spectrometry-based quantification of steroidal glycoalkaloids from *Solanum xanthocarpum* and effect of different extraction methods on their content. *Journal of Chromatography A*, *1208*(1-2), 141-146.

117. Podgwaite, J. D., Reardon, R. C., Walton, G. S., Venables, L., & Kolodny-Hirsch, D. M. (1992). Effects of aerially applied Gypchek on gypsy moth *(Lepidoptera: Lymantriidae*) populations in Maryland woodlots. *Journal of economic entomology, 85*(4), 1136-1139.

118. Pratap Singh, D., & Prabha, R. (2017). Bioconversion of agricultural wastes into high value biocompost: a route to livelihood generation for farmers. *Adv. Recycl. Waste Manag*, *137*.

119. Presley, J. T. (1953). Verticillium wilt of cotton.

120. PReM, M., Swarnkar, R., Kantilal, V. D. K., Jeetsinh, P. S. K., & Chitharbhai, K. B. (2016). Combined tillage tools-a review. *Current Agriculture Research Journal, 4*(2), 179.

121. Pullman, G. S., DeVay, J. E., & Garber, R. H. (1981). Soil solarization and thermal death: a logarithmic relationship between time and temperature for four soilborne plant pathogens. *Phytopathology, 71*(9), 959-964.

122. Punja, Z. K., & Utkhede, R. S. (2003). Using fungi and yeasts to manage vegetable crop diseases. *TRENDS in Biotechnology*, *21*(9), 400-407.

123. Rekanovic, E., Milijasevic, S., Todorovic, B., & Potocnik, I. (2007). Possibilities of biological and chemical control of Verticillium wilt in pepper. *Phytoparasitica, 35*(5), 436.

124. Rodríguez, A., Gea, T., Sánchez, A., & Font, X. (2021). Agro-wastes and Inert Materials as Supports for the Production of Biosurfactants by Solid-state Fermentation. *Waste and Biomass Valorization, 12*(4), 1963-1976.

125. Rothwell, J. A., Perez-Jimenez, J., Neveu, V., Medina-Remon, A., M'hiri, N., García-Lobato, P., ... & Scalbert, A. (2013). Phenol-Explorer 3.0: a major update of the Phenol-Explorer database to incorporate data on the effects of food processing on polyphenol content.

126. Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., ... & Colla, G. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Scientia Horticulturae, 196*, 91-108.

127. Ruzzi, M., & Aroca, R. (2015). Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Scientia Horticulturae, 196*, 124-134.

128. Saikia, R. R., Deka, S., Deka, M., & Banat, I. M. (2012). Isolation of biosurfactant-producing *Pseudomonas aeruginosa* RS29 from oil-contaminated soil and evaluation of different nitrogen sources in biosurfactant production. *Annals of microbiology, 62(2),* 753-763.

129. Santos, P. M., Batista, D. L., Ribeiro, L. A., Boffo, E. F., de Cerqueira, M. D., Martins, D., ... & Ribeiro, P. R. (2018). Identification of antioxidant and antimicrobial compounds from the oilseed crop *Ricinus communis* using a multiplatform metabolite profiling approach. *Industrial Crops and Products*, *124*, 834-844.

130. Sardi, P., Saracchi, M., Quaroni, S., Petrolini, B., Borgonovi, G. E., & Merli, S. (1992). Isolation of endophytic *Streptomyces* strains from surface-sterilized roots. *Applied and Environmental Microbiology, 58*(8), 2691-2693.

131. Satpute, S.B., & Vanmare, D.J. (2018). Chemical profile of *Datura stramonium* L. by using HRLC-MS spectra. *Int. Res. Journal of Science & Engineering, 6* (6), 231-235.

132. Shamsi, S., & Naher, N. (2014). Boll rot of cotton (*Gossypium hirsutum L.*) caused by Rhizopus 0ryzae went & prins. Geerl. -A new record in Bangladesh. *Bangladesh Journal of Agricultural Research, 39(3)*, 547-551.

133. Shim, J., Mangat, P. K., & Angeles-Shim, R. B. (2018). Natural variation in wild Gossypium species as a tool to broaden the genetic base of cultivated cotton. *J. Plant Sci. Curr. Res*, *2*(005).

134. Silva, V., Igrejas, G., Falco, V., Santos, T. P., Torres, C., Oliveira, A. M., ... & Poeta, P. (2018). Chemical composition, antioxidant and antimicrobial activity of phenolic compounds extracted from wine industry by-products. *Food Control*, *92*, 516-522.

135. Simard, S. W., Beiler, K. J., Bingham, M. A., Deslippe, J. R., Philip, L. J., & Teste, F. P. (2012). Mycorrhizal networks: mechanisms, ecology and modelling. *Fungal Biology Reviews*, *26*(1), 39-60.

136. Singh, A., Van Hamme, J. D., & Ward, O. P. (2007). Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotechnology advances, 25*(1), 99-121.

137. Singh, S. K., Singh, M. K., Verma, H., Singh, P. P., Singh, A. V., Rashmi, K., & Kumar, A. (2021). Biosurfactant producing microbes for clean-up of soil contaminants. *In Microbe Mediated Remediation of Environmental Contaminants (pp.* 89-93). Woodhead Publishing.

138. Skidmore, A. M., & Dickinson, C. H. (1976). Colony interactions and hyphal interference between Septoria nodorum and phylloplane fungi. *Transactions of the British Mycological Society*, *66*(1), 57-64.

139. Sonkamble, V. V., & Kamble, L. H. (2015). Antidiabetic potential and identification of phytochemicals from *Tinospora cordifolia*. *American Journal of Phytomedicine and Clinical Therapeutics*, *3*(1), 097-110.

140. Stenersen, J. (2004). *Chemical pesticides mode of action and toxicology*. CRC press.

141. Stockwell, V. O., & Stack, J. P. (2007). Using *Pseudomonas spp*. for integrated biological control. *Phytopathology*, *97*(2), 244-249.

142. St John, L., Tilley, D. J., Ogle, D., Jacobs, J., Holzworth, L., & Wiesner, L. (2011). Principles of seedbed preparation for conservation seedings.

143. Subbaraju, G. V., Vanisree, M., Rao, C. V., Sivaramakrishna, C., Sridhar, P., Jayaprakasam, B., & Nair, M. G. (2006). Ashwagandhanolide, a bioactive dimeric thiowithanolide isolated from the roots of *Withania somnifera*. *Journal of natural products*, *69*(12), 1790-1792.

144. Swadling, I. R., & Jeffries, P. (1996). Isolation of microbial antagonists for biocontrol of grey mould disease of strawberries. *Biocontrol Science and Technology, 6(1)*, 125-136.

145. Tadros, F. (2005). Applied surfactants, principles and applications. Wiley, Weinheim, 187–256.

146. Takshak, S., & Agrawal, S. B. (2015). Alterations in metabolite profile and free radical scavenging activities of *Withania somnifera* leaf and root extracts under supplemental ultraviolet-B radiation. *Acta physiologiae plantarum*, *37*(12), 1-12.

147. Termorshuizen, A. J., Evan, R., Jvan der Gaag. D., Alabouvette, C., Chen, Y., Lagerlöf, J., Malandrakis, A.A., Paplomatas, E.J., Rämert, B., Ryckeboer, J., Steinberg, C., & Zmora-Nahum, S. (2006). Suppressiveness of 18 composts against 7 pathosystems: variability in pathogen response. *Soil Biology Biochemistry, 38*,2461–2477.

148. Thavasi, R., Jayalakshmi, S., Balasubramanian, T., Banat IM (2008) Production and characterization of a glycolipid biosurfactant from *Bacillus megaterium* using economically cheaper sources. *World Journal of Microbiology and Biotechnology, 24,* 917–925.

149. Tjamos, E. C., Tsitsigiannis, D. I., Tjamos, S. E., Antoniou, P. P., & Katinakis, P. (2004). Selection and screening of endorhizosphere bacteria from solarized soils as biocontrol agents against *Verticillium dahliae* of solanaceous hosts. *European Journal of Plant Pathology, 110(1)*, 35-44.

150. Trivedi, M. K., Panda, P., Sethi, K. K., & Jana, S. (2017). Metabolite profiling in *Withania somnifera* roots hydroalcoholic extract using LC/MS, GC/MS and NMR spectroscopy. *Chemistry & biodiversity*, *14*(3), e1600280.

151. Trivedi, M. K., Branton, A., Trivedi, D., Nayak, G., Nykvist, C. D., Lavelle, C., ... & Jana, S. (2017). Liquid chromatography-mass spectrometry (LC-MS) analysis of *Withania somnifera* (Ashwagandha) root extract treated with the energy of consciousness. *Am. J. Quantum Chem. Mol. Spectrosc*, *1*(1), 21-30.

152. Uppal, A. K., El Hadrami, A., Adam, L. R., Tenuta, M., & Daayf, F. (2008). Biological control of potato Verticillium wilt under controlled and field conditions using selected bacterial antagonists and plant extracts. *Biological Control*, *44*(1), 90-100.

153. Usami, T., Momma, N., Kikuchi, S., Watanabe, H., Hayashi, A., Mizukawa, M., ... & Ohmori, Y. (2017). Race 2 of *Verticillium dahliae* infecting tomato in Japan can be split into two races with differential pathogenicity on resistant rootstocks. *Plant Pathology, 66(2)*, 230-238.

154. Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., & Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytologist*, *206*(4), 1196-1206.

155. Vijayaraghavan, P., Flanetraj, S.R., & Vincent, S.G.P. (2011). Purification and Characterization of a Cysteine Proteinase for the Mid-gut Gland of Indian Lobster, Panulirus homarus. *World Journal of Chemistry, 6(1)*, 44-48.

156. Vijayaraghavan, P., & Vincent, S. G. P. (2013). A simple method for the detection of protease activity on agar plates using bromocresolgreen dye. *Journal of Biochemical Technology*, *4*(3), 628-630.

157. Wang, S., Wang, Z., Li, H., Zhang, C., & Zhao, B. (2009). Effect of soil on colonization and growth promotion-function of *Streptomyces sp*. S506. *Zhongguo Shengtai Nongye Xuebao/Chinese Journal of Eco-Agriculture*, *17*(2), 335-338.

158. Wang, X., Radwan, M. M., Taráwneh, A. H., Gao, J., Wedge, D. E., Rosa, L. H., ... & Cutler, S. J. (2013). Antifungal activity against plant pathogens of metabolites from the endophytic fungus *Cladosporium cladosporioides*. *Journal of agricultural and food chemistry*, *61*(19), 4551-4555.

159. Warrior, P., Rehberger, L. A., Beach, M., Grau, P. A., Kirfman, G. W., & Conley, J. M. (1999). Commercial development and introduction of DiTeraTM, a new nematicide. *Pesticide Science*, *55*(3), 376-379.

160. Wei, F., Feng, H., Zhang, D., Feng, Z., Zhao, L., Zhang, Y., ... & Xu, X. (2021). Composition of rhizosphere microbial communities associated with healthy and Verticillium wilt diseased cotton plants. *Frontiers in Microbiology, 12*.

161. Weller, D. M. (1988). Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annual review of phytopathology, 26(1),* 379-407.

162. Wolf, D. C., & Skipper, H. D. (1994). Soil sterilization. Methods of Soil Analysis: Part 2 Microbiological and Biochemical Properties, 5, 41-51.

163. Whitbread, A., Blair, G., Konboon, Y., Lefroy, R., & Naklang, K. (2003). Managing crop residues, fertilizers and leaf litters to improve soil C, nutrient balances, and the grain yield of rice and wheat cropping systems in Thailand and Australia. *Agriculture, Ecosystems & Environment*, *100*(2-3), 251-263.

164. Xu, Y. M., Marron, M. T., Seddon, E., McLaughlin, S. P., Ray, D. T., Whitesell, L., & Gunatilaka, A. L. (2009). 2, 3-Dihydrowithaferin A-3β-O-sulfate, a new potential prodrug of withaferin A from aeroponically grown Withania somnifera. *Bioorganic & medicinal chemistry*, *17*(6), 2210-2214.

165. Xue, L., Xue, Q., Chen, Q., Lin, C., Shen, G., & Zhao, J. (2013). Isolation and evaluation of rhizosphere actinomycetes with potential application for biocontrol of Verticillium wilt of cotton. *Crop Protection*, *43*, 231-240.

166. Yang, P., Sun, Z. X., Liu, S. Y., Lu, H. X., Zhou, Y., & Sun, M. (2013). Combining antagonistic endophytic bacteria in different growth stages of cotton for control of Verticillium wilt. *Crop protection*, *47*, 17-23.

167. Yescas-Romo, K. F., Molina-Freaner, F. E., Ramírez-Godina, F., Reyes-Valdés, M. H., Martínez, O., & Hayano-Kanashiro, C. (2021). Seed morphology and cytogenetic characterization of an endangered wild cotton, Gossypium turneri Fryxell, from Mexico. *Genetic Resources and Crop Evolution, 68(5),* 2119-2129.

168. Yoshida, S., Ohba, A., Liang, Y. M., Koitabashi, M., & Tsushima, S. (2012). Specificity of *Pseudomonas* isolates on healthy and Fusarium head blight-infected spikelets of wheat heads. *Microbial ecology, 64(1),* 214-225.

169. Yuan, Y., Feng, H., Wang, L., Li, Z., Shi, Y., Zhao, L., ... & Zhu, H. (2017). Potential of endophytic fungi isolated from cotton roots for biological control against verticillium wilt disease. *PLoS One, 12(1)*, e0170557.

170. Yin, H., Qiang, J., Jia, Y., Ye, J., Peng, H., Qin, H., ... & He, B. (2009). Characteristics of biosurfactant produced by *Pseudomonas aeruginosa* S6 isolated from oil-containing wastewater. *Process Biochemistry, 44(3),* 302-308.

171. Zheng, Y., Xue, Q. Y., Xu, L. L., Xu, Q., Lu, S., Gu, C., & Guo, & J. H. (2011). A screening strategy of fungal biocontrol agents towards Verticillium wilt of cotton. *Biological Control, 56(3),* 209-216.

172. Zhu, Q. H., Fan, L., Liu, Y., Xu, H., Llewellyn, D., & Wilson, I. (2013). miR482 regulation of NBS-LRR defense genes during fungal pathogen infection in cotton. *PLoS One, 8(12),* e84390.

173. Dr. N.B. Singh, 2009, Revolution in Indian Cotton, 14, Directorate of Cotton Development Ministry of Agriculture, Department of Agriculture & Cooperation Government of India, & National Center of Integrated Pest Management ICAR, Pusa Campus, New Delhi.

APPENDIX-I

ABBREVIATIONS

Sr. No.	Abbreviation	Full Meaning
1	%	Percentage
2	≥	Greater or equal
3	>	Greater than
4	<	Less than
5	-	Minus
6	+	Plus
7	()	Open and close bracket
8	β	Beta
9	µg	Microgram
10	±	Plus or Minus
11	^{O}C	Degree centigrade
12	CMC	Carboxymethyl Cellulose
13	cm	Centimeter
14	ANOVA	Analysis Of Variance
15	DNA	Deoxyribonucleic Acid
16	rRNA	Ribosomal Ribonucleic Acid
17	PCR	Polymerase Chain Reaction
18	mm	Millimetre
19	rpm	Revolution Per Minute
20	Min	Minute
21	nm	Nanometer
22	mg	Milligram
23	kg	Kilogram

24	CLCuD	Cotton leaf curl virus disease
25	PGR	Plant growth regulators
26	lb	Pound or Pound mass
27	OSI	Organic solvent infusion
28	m^2	Square meter
29	m	Meter
30	PIRG	Percentage inhibition of radial growth
31	rpm	Revolutions per minute
32	M	Molar
33	ITCC	Indian type culture collection
34	ml	Milliliter
35	LCMS	Liquid chromatography Mass Spectrometry
36	psi	Pound per square inch
37	cfu	colony forming unit
38	Lat	Latitude
39	Long	Longitude
40	I	Infected
41	N.I	Non Infected
42	RS	Rhizospheric soil
43	BS	Bulk soil
44	Vill	Village
45	PDA	Potato Dextrose agar
46	Na	Nutrient Agar
47	LSD	Least significance difference
48	VTS DB-V1/NI /L/S1/B2	Village Talwandi sabo District Bathinda- Village 1/Non Infected/Leaf/Sample 1/Bacteria 2
49	VG DB-V1/NI /L/S1/B2	Village Goniana District Bathinda- Village 1/Non Infected/Leaf/Sample 1/Bacteria 2

50	VG DB-V2/NI /L/S1/B1	Village Goniana District Bathinda- Village 2/Non Infected/Leaf/Sample 1/Bacteria 1
51	VG DB-V1/NI /S/S1/B1	Village Goniana District Bathinda- Village 1/Non Infected/Stem/Sample 1/Bacteria 1
52	VTS DB-V1/NI /S/S1/B2	Village Talwandi sabo District Bathinda- Village 1/Non Infected/Stem/Sample 1/Bacteria 2
53	VTS DB-V1/NI /R/S1/B2	Village Talwandi sabo District Bathinda- Village 1/Non Infected/Root/Sample 1/Bacteria 2
54	VTS DB-V1/NI /S/S1/B1	Village Talwandi sabo District Bathinda- Village 1/Non Infected/Stem/Sample 1/Bacteria 1
55	OSM/Y/F1	Oil seed manure/yellow/Fungus 1
56	BM/W/F1	Bio manure/white/Fungus 1
57	BM/DG/ F2	Bio manure/ Dull Green/F2
58	AM/BG/F1	Animal manure/ Black green/ Fungus 1
59	AM/B/F2	Animal manure/ Black green/ Fungus2
60	AM/GG/F3	Animal Manure/Greyish Green/Fungus 3
61	CM/DG/F1	Compost Manure/ Dark green/Fungus1
62	CM/B/F2	Compost Manure/ Brown/Fungus 2
63	PM/G/F1	Poultry Manure/ Green/Fungus1
64	VC/LB/F1	Vermicompost manure/light Brown/Fungus 1
65	ppm	Parts per million
66	PGPRs	plant growth-promoting rhizobacteria
67	MS-ESI	Mass spectrometry electron spray ionization
68	ESI	Electron Spray ionization
69	RT	Retention time

APPENDIX II

LIST OF CHEMICALS USED IN THE RESEARCH

Sr. No.	Chemicals
1	Congo Red
2	Deionized water
3	Peptone
	Carboxy Methyl Cellulose
	Potassium dihydrogen phosphate
	Magnesium sulphate
	Sodium nitrate
	Potassium chloride
	Peptone
	Potassium dihydrogen phosphate
	Beef extract
	Yeast extract
	Sodium chloride
	Casein
	Colloidal chitin
	Diazanium sulfate
	Citric acid monohydrate
	Bromo cresol purple
	Tween- 80
	Bromo Cresol Green Dye
	Congo red solution
	Copper sulphate
	Sodium hypochlorite

Sr. No.	Instruments
1	BOD Incubator MAC
2	Cooling Centrifuge
3	Compound Light Microscope
4	High precision water bath with digital temperature controller
5	Oven
6	Laminar Air flow
7	Remi Auto Mix blender
8	Microwave 30SC3
9	Microprocessor Digital pH meter
10	Refrigerator
11	Weighing balance LCD series
12	Autoclave

NAMES OF MICROORGANISMS AND THEIR ABBREVIATIONS

Sr. No	Abbreviation	Full Meaning
1	*G. arboreum,*	*Gossypium arboreum*
2	*G. herbaceum*	*Gossypium herbaceum*
3	*G. hirsutum*	*Gossypium hirsutum*
5	*T. harzianum*	*Trichoderma harzianum*
6	*P. fluorescence*	*Pseudomonas fluorescence*
7	*T. flavus*	*Trichoderma flavus*
8	*G. versiforme*	*Glomus versiforme*
9	*G. etunicatum*	*Glomus etunicatum*
10	*G. intraradices*	*Glomus intraradices*
11	*T. virens*	*Trichoderma virens*
12	*A. niger*	*Aspergillus niger*
13	*A. terreus*	*Aspergillus terreus*
14	*A. fumigatus*	*Aspergillus fumigatus*
15	*A nidulans*	*Aspergillus nidulans*

APPENDIX III

SEQUENCE OF MICROORGANISMS IDENTIFIED IN THIS RESEARCH USING 16S rRNA and 18SrRNA SEQUENCING

Forward sequence of *P. aeruginosa*

CCCAAAAGTCGGAACTGGGCCGTGTGGGGGATCCTTGAGATCTAGTGGCG

CAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAA

AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGGTT

AATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACT

TTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATG

GCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGC

GCAACCCTTGTCCTTATTTACCAGCACCTCGGGTGGGCACTCTAAGGAGA

CTGCCGCTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATG

GCCCTTACGCGCACGGCTACACACGTGCTACGATGGTCGGTACAAAGGGT

TGCCGA

Forward sequence of *B. subtilis*

CCCGAAGAGGATGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAG

CTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACT

CAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT

CGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCT

AGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTG

TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA

CCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCG

GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTA

TGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAA

ACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAG

TCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGC

ATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACC

ACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAG

CCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGG

TAACCGAA

Forward sequence of *L.macroides*

CCCGAAGAGGATGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAG

CTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACT

CAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT

CGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGTTGACCACTG

TAGAGATATAGTTTCCCCTTCGGGGGCAACGGTGACAGGTGGTGCATGGT

TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC

AACCCTTGATCTTAGTTGCCATCATTTAGTTGGGCACTCTAAGGTGACTGC

CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCT

TATGACCTGGGCTACACACGTGCTACAATGGACGATACAAACGGTTGCCA

ACTCGCGAGAGGGAGCTAATCCGATAAAGTCGTTCTCAGTTCGGATTGTA

GGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAG

CATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACAC

CACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAG

CCGCCGAAGGTGGGATAGATGATTGGGGTGAAGTCGTAACAAAG

GTAACCGAAGAGTTTGATCCTGGCTCAG

Forward Sequence of *Verticillium dahliae*

CGGCCGGCTCTAGTATAAGCAATTACAAAGCGAAACTGCGAATGGCTCAT

TATATAAGTTATCGTTTATTTGATAGTACCTTACTACATGGATAACCGTGG

TAATTCTAGAGCTAATACATGCTGAAAATCCCGACTTCGGAAGGGATGTA

TTTATTAGATACAAAACCAATGCCCTTCGGGGCTCTCTTGGTGATTCATGA

TAACTTCTCGAATCGCACGGCCCCGCGCCGGCGATGGTTCATTCAAATTTC

TTCCCTATCAACTTTCGATGCTACGGTATTGTCCTAGCATGGTTGCAACGG

GTGACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAGCCTGAGAAACGGC

TACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACTC

GGGGAGGTAGTGACGATAAATACCGATACAGGGCTCTTTTGGGTCCTGTA

ATTGGAATGAGTACAATTCAAATCCCTTAACGAGGAACAATTGGAGGGCA

AGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAA

AGTTGTTGTGGTTAAAAAGCTCGTAGTAGAAGCTCGGCCTCCGGTCGGCC

GGTCCGCCTCACCGCGTGCACTGGTTCGGCCGGGGCTTCACCTTCTGCGGA

ACCGCATCTCCTTCACTGGGGGTGTCGGGGAAACAGGACGTTTACTTTGA

AAAAATTAGAGTGCTCCAGGCAGGCCTATGCTCGAATACATTAGCATGGA

ATAATGGAATAGGACGTGTGGTCTTATTTCGTTGGTTTCTAGGACCGCCGT

AATGATTAATAGGGACAGTCGGGGGCATCAGTATTCAATCGTCAGAGGTG

AAATTCTTGGATCGATTGAAGACTAACTACTGCGAAAGCATTTGCCAAGG

ATGTTTTCATTTATCAGGAACGAAAGTTAGGGGATCGAAGACGATCAGAT

ACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGACGGTGT

TATTTCTTGACCCGTTCGGCACCTTACAAGAAATCAAAGGGCTTGGGCTCC

AGGGGGAATATGGTCGCAGGCTGAAATTAA

APPENDIX IV

Media preparation Procedure

1) Preparation of Potato dextrose agar

Ingredients	Gm/litre
Potato infusion	200 gm
Dextrose	20 gm
Agar	15 gm
Distilled water	1 litre

Preparation of medium

1. The medium was prepared by weighing 39 gm of PDA powder to 1 Litre of Distilled water.
2. The medium was boiled while mixing to dissolve.
3. The solution was sterilized by autoclaving at 15 lbs pressure for 15 minutes at 121°C.
4. Finally, medium was poured into petri plates.

2) Preparation of Nutrient Agar

Ingredients	Gm/litre
Peptic digest of animal tissue	5 gm
Sodium chloride	5 gm
Beef extract	1.5 gm
Yeast extract	1.5 gm
Agar	15 gm
Distilled water	1 litre

Preparation of medium

1. The medium was prepared by weighing 28 gm of PDA powder to 1 Litre of Distilled water.
2. The medium was boiled while mixing to dissolve.
3. The solution was sterilized by autoclaving at 15 lbs pressure for 15 minutes at 121°C.
4. Finally, medium was poured into petri plates.

3) Preparation of Nutrient Broth Medium

Ingredients	Gm/litre
Peptic digest of animal tissue	5 gm
Sodium chloride	5 gm
Beef extract	1.5 gm
Yeast extract	1.5 gm
Distilled water	1 litre

Preparation of medium

1. The medium was prepared by weighing 13 gm of NA powder to 1 Litre of Distilled water.
2. The medium was boiled while mixing to dissolve.
3. The solution was sterilized by autoclaving at 15 lbs pressure for 15 minutes at 121°C.
4. Finally, medium was poured into petri plates.

4) Preparation of Potato dextrose Broth Medium

Ingredients	Gm/litre
Potato infusion	200 gm
Dextrose	20 gm
Distilled water	1 litre

Preparation of medium

1. The medium was prepared by weighing 24 gm of PDA powder to 1 Litre of Distilled water.
2. The medium was boiled while mixing to dissolve.
3. The solution was sterilized by autoclaving at 15 lbs pressure for 15 minutes at 121°C.
4. Finally, medium was poured into petri plates.

5) Czapek Mineral Salt Agar Medium

Ingredients	Gm/litre
KH_2PO_4	1.00 gm
$MgSO_4.7H_2O$	0.50 gm
$NaNO_3$	2.00 gm
KCl	0.50 gm
Peptone	2.00 gm
Agar	15.0 gm
Distilled water	1 litre

Preparation of medium

1. The medium was prepared by weighing all the ingredients to 1 Litre of distilled water.
2. The medium was boiled while mixing to dissolve.
3. The solution was sterilized by autoclaving at 15 lbs pressure for 15 minutes at 121°C.
4. Finally, medium was poured into petri plates.

6) Casein Agar medium

Ingredients	Gm/litre
Peptic digest of animal tissue	5.00gm
Beef extract	1.50gm
Yeast extract	1.50gm
Sodium chloride	5.00gm
Casein	10.00gm
Agar	15.0 gm
Distilled water	1 litre

Preparation of medium

1. The medium was prepared by weighing all the ingredients and dissolved in 1 Litre of distilled water.
2. The medium was boiled while mixing to dissolve.

3. The solution was sterilized by autoclaving at 15 lbs pressure for 15 minutes at 121°C.
4. Finally, medium was poured into petri plates.

7) Basal chitin agar medium

Ingredients	Gm/litre
Colloidal chitin	4.5gm
$MgSO_4.7H_2O$	0.30gm
$NH4SO_4$	3.00gm
KH_2PO_4	2.00gm
Citric acid monohydrate	1.00 gm
Bromo cresol purple	0.15gm
Tween-80	0.15gm
Agar	15.0 gm
Distilled water	1 litre

Preparation of medium

1. The medium was prepared by weighing all the ingredients and dissolved in 1 Litre of distilled water.
2. The medium was boiled while mixing to dissolve.
3. The solution was sterilized by autoclaving at 15 lbs pressure for 15 minutes at 121°C.
4. Finally, medium was poured into petri plates.

APPENDIX V

LIST OF PUBLICATION AND PRESENTATIONS

Publication

1. Review article published entitled **“Microbial biosurfactants and its industrial applications”** in Everyman’s Science Journal, Oct-Nov. (2018), Vol No. 4**.**

2. Manuscript entitled “**Suppression of *Verticillium* wilt of cotton through liquid material and antagonistic fungal strains under natural field conditions**” has been published in “**Materials today: Proceedings”,** (2021).

   DOI https://doi.org/10.1016/j.matpr.2021.08.036

   Link
   https://www.sciencedirect.com/science/article/pii/S2214785321054638

   Patent

   Provisional patent number of Biochemical formulation has been received.

Presentations

1. Oral presentation entitled **“Biological control of *Verticillium* wilt of cotton by antagonistic fungal strains and biochemical formulation under poly house and natural field conditions”** in virtual International conference on Environmental, Agriculture, Animal and Human Health held from 5th- 6th June 2021.

2. Poster Paper presentation at 60th Annual Conference of Association of Microbiologist of India (AMI2019) and International Symposium on Microbial Technologies in Sustainable Development of Energy, Environment, Agriculture and Health titled **“Evaluation of potential biocontrol agents towards Verticillium wilt of cotton”** held between 15th to 18th November organized by Central University Haryana, Mahendergarh, India.

3. Oral presentation entitled **“Evaluation of potential biocontrol bacterial strains against *Verticillium* wilt of cotton”** in International conference on Environment and life science held on 23rd October, 2021.

4. Poster presentation in International Conference/Symposium on **“Innovative Strategies for sustainable Water Management”** held from 17 Nov.2018 to 18 Nov. 2018 at LPU, Phagwara in collaboration with McGill University Canada.

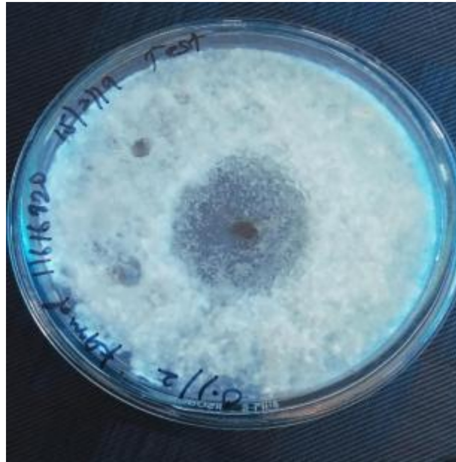
5. Poster Presentation in **DBT Sponsored National Conference** on “Recent Trends in Plant Sciences” held from 19th April to 20th April 2018 at Sanmati Government College of Science Education and Research, Jagraon, Ludhiana.

APPENDIX VI

PICTURES OF SOME WORK DONE DURING THE RESEARCH

Growth of antagonistic fungal strains in liquid medium

Crude enzyme extract



Biosurfactant Activity

Soil collection for poly house trials

Experimental field