Characterization of jute endophytes with inhibiting activity against *Macrophomina phaseolina*

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By

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CERTIFICATE

This is to certify that the research work embodied in this thesis entitled **"Characterization of jute endophytes with inhibiting activity against *Macrophomina phaseolina*"** has been carried out both in the Molecular Biology Lab, Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh and in the Regional Centre for Biotechnology, Faridabad, Haryana (NCR Delhi), India.

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Declaration

I hereby declare that the research work embodied in this thesis entitled **"Characterization of jute endophytes with inhibiting activity against *Macrophomina phaseolina*"** has been carried out by me under the supervision of Dr. Haseena Khan, Molecular Biology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh and co-supervision of Dr. Mohammad Riazul Islam, Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh and Dr. Tushar K Maiti, Regional Centre for Biotechnology, NCR Biotech Science Cluster, Faridabad, Haryana (NCR Delhi), India.

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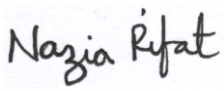

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Dedicated to

My Family

For their unconditional love and enormous support

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Abstract

An endophytic bacterium *Burkholderia contaminans* NZ; isolated from jute, an important fibre producing plant, shows significant growth promotion activity in *in vivo* pot experiments. Ability to fix nitrogen, production of indole acetic acid (IAA) and siderophore together with ACC (1-aminocyclopropane-1-carboxylate) deaminase activity make this endophyte a potent plant growth-promoting agent. Moreover, it proved to be an efficient bio control agent active against devastating plant pathogenic fungi *Macrophomina phaseolina* and several other fungi. Gas chromatography-mass spectroscopy (GC-MS) allowed the identification of some volatile biocontrol metabolites such as 2-bromo-2-cyano-N, N-dimethylacetamide, S-[4-cyanophenyl]-N, N-dimethylthiocarbamate and isoproturon that are known for their antimicrobial activities. The bacterium is also catalase, cellulase, lipase and protease positive which too could be responsible for the antifungal activity. The complete NZ genome analyses revealed the safe to use nature of this bacterium. It has been found to harbor multiple antimicrobial and plant growth promotion related genes but it lacks major virulence-related gene loci. For the better understanding of the mechanism of bacterium-fungi antagonism which should assist in the successful application of bacteria as biological control agents against fungal pathogens of plants, a combination of approaches including total fungal protein identification and comparative proteomics of bacteria-induced differential protein expression in *M. phaseolina* has been outlined in this study. Using the iTRAQ LC-MS/MS method for quantitative proteomics study, a comparative analysis of the whole proteome of *M. phaseolina* under both *B. contaminans* NZ challenged and unchallenged conditions were made and 2204 different proteins were identified of which 141 were considered to have significant deviation in expression. KEGG pathway analysis identified most of the upregulated proteins to be functionally related to energy production (26.11%), as well as defense and stress response (23.45%) while there was a significant down regulation in oxidative stress protection pathways (42.61%), growth and cell wall integrity (30.95%) and virulence (23.81%). A tough battle appears to ensue between the fungus and the bacterium, where *B. contaminans* NZ manages to arrest the growth of the fungus and decrease its pathogenicity but, the latter apparently survives under ‘hibernating’ conditions by up-regulating its energy metabolism. The findings of the study

indicate that *B. contaminans* NZ can be considered as a completely safe, sustainable eco-friendly bioinoculant for crop improvement.

Key words: endophyte, *Burkholderia contaminans* NZ, *Macrophomina phaseolina*, bio-control, growth promotion, genome analyses, iTRAQ.

Characterization of jute endophytes with inhibiting activity against *Macrophomina phaseolina*

Content of the thesis

Chapter 2: Characterization of jute endophyte *Burkholderia contaminans* NZ and evaluating its ability for plant growth promotion.

2.1 Overview

2.2 Materials and Methods

2.2.1 Isolation of endophytic bacteria and collection of plant materials

2.2.2 Molecular characterization with 16S rRNA

2.2.3 General characterization

2.2.3.1 Growth Curve

2.2.3.2 Influence of pH and temperature on antifungal activity

2.2.3.3 Catalase test

2.2.4 Characterization of exo-enzymes produced by endophytic bacteria

2.2.4.1 Screening of cellulolytic enzyme-production

2.2.4.2 Xylanase assay

2.2.4.3 Lipase assay

2.2.4.4 Chitinase assay

2.2.4.5 Qualitative and quantitative protease assay

2.2.5 Plant growth promotion

2.2.5.1 Siderophore production assays

2.2.5.2 ACC deaminase activity test

2.2.5.3 *In vitro* screening for indole-3-acetic acid production

2.2.5.4 Biological nitrogen fixation assay

2.2.5.5 Biocontrol against phyto pathogenic fungus

2.2.6 *In vivo* growth promotion study

2.2.6.1 Jute seed inoculation with bacteria

2.2.6.2 In vivo pot experiment in hydroponic culture system

2.2.6.3 Statistical analysis

2.3. Result

2.4 Discussion

2.5 References

Chapter 3: Identification of antimicrobial compounds of *B. contaminans* NZ for antagonism of *Macrophomina phaseolina*

Chapter 4: Characterization of *B. contaminans* NZ as non-pathogenic safe bioinoculant through whole genome analysis

Chapter 5: Quantitative proteomic study for the identification of total proteins and the comparative analysis of the change at the proteomic level in *M. phaseolina* when challenged by *B. contaminans* NZ

5.1 Overview

5.2 Materials and Methods

5.2.1 In-vitro dual culture assays

5.2.2 Change in pathogenicity of *B. contaminans* challenged *M. phaseolina*

5.2.3 Cell culture and preparation of protein extracts

5.2.3.1 Trypsin digestion and iTRAQ labeling for LC-MS/MS

5.2.3.2 Peptide fractionation by high pH RP HPLC and LC-MS/MS

5.2.6 Peptide purification and determination of specific activity

5.2.3.3 Protein identification and data analysis

5.2.3.4 Statistical and bioinformatics analysis

5.3 Results

5.3.1 Effects of B. contaminans on growth and morphology of *M. phaseolina*

5.3.2 Reduction of pathogenicity in *M. phaseolina*

5.3.3 Identification of *M. phaseolina* proteome

5.3.4 Quantitative proteomics of *M. phaseolina* under bacterial stress

5.3.5 Profile of differentially expressed proteins

5.3.6 Functional classification of differentially expressed proteins

5.3.6.1 Up regulated differentially expressed proteins (DEPs)

5.3.7 Protein–protein interaction

5.4 Discussion

5.5 References

Abbreviations

%	Percentage
°C	Degree Celcius
µg	Microgram
µl	Microliter
µM	Micromolar
β-ME	Beta-mercaptoethanol
ACC 1-	Aminocyclopropane-1-carboxylate
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
DEPs	Differentially expressed proteins
dH_2O	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
FAO	Food and agriculture organization
g	Gram
GC-MS	Gas Chromatography Mass Spectrometry
h	Hour
IAA	Indole-3-acetic acid
iTRAQ	Isobaric tags for relative and absolute quantitation
Kb	Kilo base pair
kDa	Kilo dalton
L	Liter

LB	Luria Bertani medium
LC-MS	Liquid chromatography mass spectrometry
M	Molar
mg	Milligram
min	Minutes
ml	Milliliter
mM	Mili molar
ng	Nano gram
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting Rhizobacteria
PPM	Parts per million
ROS	Reactive oxygen species
RNA	Ribonucleic acid
rpm	Rotation Per Minute
sec	Seconds
TE	TRIS-EDTA
Tm	Melting Temperature
TSA	Tryptone soy agar
TSB	Tryptone soy broth
U	Unit
VOC	Volatile organic compound

CHAPTER 1 GENERAL INTRODUCTION

Pages 1 - 9

Preface

With an increase in the world population in an alarming rate, global food insecurity is one of the major problems faced by humanity. According to a report of Food and Agriculture Organization (FAO, 2012), agricultural production has to be increased by 60% over the next 40 years to fulfill the increasing food consumption. Improvement of agricultural productivity is the major approach to address this problem over the coming decades.

To increase agricultural productivity, plant yield and growth are needed to be stimulated and the crops should be protected from phyto-pathogens. Due to the increasing cost of chemical fertilizers and their negative environmental impacts, safe use of microorganisms that improve soil fertility and enhance plant growth is getting immense attention (Adesemoye, Torbert et al. 2009). Use of fungicides to limit phyto-pathogenic fungus is also facing increasing restriction due to their harmful effect on human health and environment (Qi, Aiuchi et al.). Chemical control measures damage the balance of the environmental microbial community and may also lead to the evolution of resistant pathogen strains (Qi, Aiuchi et al.).

Therefore, as low cost, specific, efficient and eco-friendly option major efforts are being taken to exploit beneficial microorganisms that enhance plant growth and protect plants from phyto-pathogens.

At present, the majority of the registered bacterial products are based on species of *Bacillus* and *Pseudomonas* (EU Pesticides Database, 2012). These species are widely known for their versatile metabolic activity and diverse beneficial effects on plant vigor and health. Numerous examples highlight the use of bio-control agents in combating fungal phytopathogens, among them the control of *M. incognita* by *B. velezensis* in cotton (Xiang, Lawrence et al. 2017), *Fusarium* by *Bacillus* in cumin (Gajera, Savaliya et al. 2016) and by *Psuedomonus putida* in tomato (Pastor, Masciarelli et al. 2016) are three from a list of many latest progresses.

However, despite these positive characteristics, bacterial products can show some inconsistency between trials (Montesinos, 2003). This is assumed to be due to the short persistence of bacterial cells in the rhizosphere/soil environment and their susceptibility to unfavorable environmental conditions. One possible way to overcome these drawbacks is to develop biopreparations based on beneficial endophytic bacteria. The term endophyte was first used by De Bary to represent the endosymbiont micro-organisms (bacteria and fungi) that live inside the living plant tissues for at least a part of their lives without causing any apparent harm to the host (Christina, Christapher et al. 2013). A healthy plant host and these resident dynamic communities of microbiome are so functionally and anatomically intertwined with each other that they can build a dense network of multitrophic interactions (Braga, Dourado et al. 2016).

Since bacterial endophytes colonize the plant interior, which is a stable and protected environment, their interaction with a plant can grow into a longer relationship. In addition to housing endophytic bacteria, plants provide them with nutrients. Although it may seem plants are too generous in harboring the microorganisms but in reality, the endophytes are more like paying guests. Some endophytes repay their hosts by promoting plant growth, vigor and suppressing phytopathogens (Hardoim, Van Overbeek et al. 2015). For a long time healthy plants were thought to be free of bacteria but over the past few decades our understanding of the role of bacteria in the plant has radically advanced (Mitter, Petric et al. 2013). Endophytic microorganisms have been the focus of intense study for years in terms of their diversity, metagenomics, multipartite interaction, combinatorial biosynthesis, plant growth promotion and bioremediation etc. (Kaul, Sharma et al. 2016).

Plant growth promotion and biocontrol activity are promising aspects of endophytes. Endophytes confer plant growth promotion either directly by producing or making available growth factors and nutrients or indirectly by suppressing biotic diseases and predators as well

as by providing tolerance against unfavorable abiotic conditions (Mitter, Petric et al. 2013). Plant growth promoting rhizobacteria (PGPR) use different mechanisms of action to promote plant growth. These mechanisms have been grouped into three clusters according to the PGPR effects on plant physiology. These groups are: (i) biofertilization including biological fixation of atmospheric nitrogen, phosphate solubilization, siderophore and exo-polysaccharide production; (ii) phyto-stimulation including production of indole acetic acid, gibberellin, cytokinin and ethylene; and (iii) biocontrol activity including induction of systemic resistance, competition for iron, nutrient and space, production of antibiotics, lytic enzymes, hydrogen cyanide and volatile compounds (Mitter, Petric et al. 2013, Tsegay, Gizaw et al. 2016).

Many studies conducted on endophytes, emphasize the ability of these microorganisms to promote plant growth and their additive/synergistic effects on plant growth and protection (Adesemoye, Torbert et al. 2009). Among them *Streptomyces* spp enhances the growth of a number of crops (Vurukonda, Giovanardi et al. 2018), *B. subtilis* (Rajamanickam, Karthikeyan et al. 2018) and *Pseudomonas parafulva, Pantoea agglomerans* (Verma, Kingsley et al. 2018) are few of many recent developments.

Jute is an important fiber producing crop of South-east Asia (Babu, Saxena et al. 2007). Initially, it was used only as a packaging material however with time a number of other uses of jute have reinforced the importance of this crop (Haidar, Ferdous et al. 2018) such as a source of paper pulp, textile raw material and biofuels (Kumar, Barrett et al. 2009, Alfermann 2010).

Corchorus olitorius and *Corchorus capsularis* are two popular jute species cultivated mostly for their fiber quality. The main challenges for jute production are the availability of soil nutrients and adverse conditions arising from different biotic and abiotic stresses. Jute is attacked by various phytopathogen throughout its life cycle. Among them the most

devastating necrotrophic fungal pathogen is *Macrophomina phaseolina* (Tassi) Goid. causing charcoal rot disease and can infect more than 500 plant species of about 100 families including major food crops (maize, sorghum), pulse crops (common bean, green gram), fiber crops (jute, cotton), and oil crops (soybean, sunflower, sesame) (Islam, Haque et al. 2012).

M. phaseolina hyphae initially invades the cortical tissue of jute plants, followed by sclerotia formation. A large amount of sclerotia produced by the pathogen blocks plant vessels leading to stem rot disease and consequently wilting of the plants (Biswas, Dey et al. 2013). It can attack any part of the host at any stage of growth and spreads rapidly within the infected plants resulting in a high economic burden to growers (Biswas, Dey et al. 2013). It is difficult to control *M. phaseolina* due to its persistence as sclerotia in the soil and plant debris which is resistant to extreme environments, enabling the pathogen to survive for months or years in the soil (Willetts 1971). In Bangladesh, annually the fiber yield of jute is reduced by 30% due to this pathogen (Islam, Haque et al. 2012).

M. phaseolina can be effectively controlled by fumigating soil with methyl bromide but as a sustainable, safe and eco-friendly option, biocontrol can be an efficient alternative to protect plants from phytopathogens. Endophytes have such a promising aspect as biocontrol agents that a huge number of researches are being directed to them.

Burkholderia, *Rahnella*, *Pseudomonas*, and *Curtobacterium* are major endophyte genera that have shown bio-control activities in the *in-vitro* assays. The bio-control activities of *Burkholderia* strains were stronger across all tested plant pathogens as compared to other strains (Kandel, Firrincieli et al. 2017).

The molecular and physiological characteristics of both culturable and non-culturable endophytic bacteria and fungi present in different parts of a jute (*Corchorus olitorius*) plant have already been identified (Najnin, Shafrin et al. 2015).

In the present study, while screening jute endophytes with potential inhibiting action against *M. phaseolina,* a jute seed endophytic bacterium showed significant inhibitory effect which was later identified by 16S rRNA sequencing as *Burkholderia contaminans* NZ*;* a gram-negative, obligatory aerobic, rod-shaped beta-proteobacteria. The bacterium was found to have promising influence on plant growth promotion and possessed all the characteristics of a plant growth promoting bacteria (PGPB) and was proven to be an efficient bio control agent against several plant pathogenic fungi.

Nature of the bio-control metabolite(s) characterized by a number of tests and gas chromatography-mass spectroscopy (GC-MS) showed the fungal growth inhibition to be intimately associated with the volatile compounds produced by *B. contaminans* NZ. When GC-MS using sterile activated charcoal was carried out, 25 volatile compounds, including known antimicrobial compounds were identified.

However, this β -proteobacterial genus *Burkholderia* comprises many phyto-pathogenic species and some members also pose a major hazard to animal and human health. *Burkholderia cepacia* complex (Bcc) includes several closely related *Burkholderia* species and these opportunistic pathogens frequently colonize lungs of cystic fibrosis and immune compromised patients (Ghequire and De Mot 2015). There are also a limited number of reports implicating certain isolates as plant pathogens (Li, Roberts et al. 2002). The ecological and genomic versatility of different *Burkholderia* species is likely due to their unusually large genomes (Lessie, Hendrickson et al. 1996). Therefore, the use of *Burkholderia* species as a bio-control agent in agriculture is disputed due to the difficulties in distinguishing between plant growth-promoting and the pathogenic bacteria (Deng, Wang et al. 2016).

Therefore, before considering the application of *B. contaminans* NZ in agriculture as a promising plant growth promotion and biocontrol agent, its pathogenicity and virulence properties were needed to be studied thoroughly.

Antagonistic fungal–bacterial interactions are mostly determined by the competitive survival for the limited nutrient and resources in the environment. This competition for existence has been a long-term focus for the better understanding of bionetwork and also for the development of improved biological control agents against fungal diseases in plants. An enhanced knowledge of the response these fungal pathogens exhibit in the presence of such bio control agents will possibly shed light on how a stable and long-lasting antagonism can be established. Studies on *in vitro* antagonism of bacterial strains against fungal pathogens of plants have revealed important information on the different compounds and genes involved (Ballhausen and de Boer 2016). However, holistic investigations of such antagonisms are very limited.

Since a proteome provides comprehensive information about a given cellular environment, a detailed study of proteomics is expected to contribute greatly to our understanding when focused on an organism's response to stimuli.

The genome of *M. phaseolina* has been sequenced in 2012 (Islam, Haque et al. 2012), but till date there is no proteomic study data on this pathogenic fungus. However, an identification of the total protein of an organism is needed to link an annotated genome sequence to gene expression data at the proteomic level.

The endophytic bacteria *B. contaminans* NZ has shown strong antagonistic activity against the plant pathogenic fungus *M. phaseolina*. But how does it impede *M. phaseolina* from infecting plants, or what is the molecular basis of the fungal response to inhibition? In order to understand these, it is necessary to assess the changes at the protein level in *M. phaseolina* after bacterial inhibition.

There are many studies on plant growth promoting and biocontrol agents but most lack a holistic approach which comprises of studies to understand how a plant growth promoting microorganism improves plant growth, how it acts as a biocontrol agent, how safe it is for real world applications and how actually it combats the pathogens. The present study has addressed the utility of a jute endophyte *B. contaminans* NZ as a PGPR organism in all possible aspects through the following aims and objectives and found it to be a completely safe and efficient.

Aims of the thesis

This Ph.D. thesis focused on the isolation and characterization of a novel beneficial endophytic bacteria with plant growth promotion and biocontrol abilities. The aims of the thesis were as follows:

1. To test jute endophyte *Burkholderia contaminans* NZ for its ability to promote plant growth by identifying potential plant-beneficial traits
2. To characterize the role of *B. contaminans* NZ in controlling phyto-pathogenic fungi and to identify the antimicrobial compounds secreted by the bacteria
3. To evaluate *B. contaminans* NZ as a non-pathogenic safe bioinoculant through whole genome analysis
4. A holistic study of antagonism by *B. contaminans* NZ to suppress *M. phaseolina* by quantitative proteomic study for the identification of total proteins and the proteomic changes in *M. phaseolina* after bacterial inhibition by iTRAQ method.

The thesis comprises of two parts: (i) analyzing the antifungal and growth promoting activities of *B. contaminans* NZ and how safe it is to use this bacterium in the fields, (ii) understanding how *B. contaminans* NZ antagonizes *M. phaseolina* through a proteome-based approach.

Part one is sub divided into three individual chapters each with its overview, materials and methods, results and discussion.

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CHAPTER 2

Characterization of Jute Endophyte *Burkholderia contaminans* NZ And Evaluating Its Ability for Plant Growth Promotion.

Pages 10 - 40

Characterization of jute endophyte *Burkholderia contaminans* NZ and evaluating its ability for plant growth promotion

2.1 Overview

Plant growth promotion activity of endophytes is a very promising prospect. The main reason for the attention on endophytes is the realization that, if these bacteria can be reintroduced in the endophytic stage, a more stable and constant relationship can be established between plant-beneficial endophytic bacteria and plants than for rhizospheric or epiphytic bacteria and plants (Malfanova 2013). Therefore, endophytes with plant-beneficial traits are potentially excellent plant growth promoters and/or biological control agents for sustainable crop production (Di Fiore and Del Gallo 1995).

Endophytes confer plant growth promotion by directly producing or making available growth factors and nutrients or indirectly by suppressing biotic diseases and predators as well as providing tolerance against unfavorable abiotic conditions (Bruto, Prigent-Combaret et al. 2014). Using these mechanisms, some endophytic bacteria can significantly contribute to the growth of plants on soils with low-fertility (Sevilla, Burris et al. 2001).

Burkholderia is a versatile, widely distributed bacterial genus with complex taxonomy whose members occupy diverse ecological niches including soil, water, plants and animals (Paungfoo-Lonhienne, Lonhienne et al. 2016)

Bacterial species in the plant-beneficial-environmental clade of *Burkholderia* represent a substantial component of endophytic microbes in many plant species (Paungfoo-Lonhienne, Lonhienne et al. 2016). It has been reported that members of the genus *Burkholderia* are

highly versatile bacteria that can stimulate growth of inoculated plants and induce physiological changes enhancing their adaptation to environmental stresses (Barka, Nowak et al. 2006, Gasser, Cardinale et al. 2011)

Plant growth-promoting bacteria (PGPB) are microorganisms which can influence the health, growth and stress response of their host plants. PGPB can greatly assist sustainable agriculture by improving plant health and biomass while reducing fertilizer usage up to 50% (da Costa, Granada et al. 2014). PGPB affect plant growth by directly acquiring nutrients (phosphate, nitrogen, iron) or modulating plant hormone levels (auxins, ethylene), and also by indirectly inhibiting pathogenic bacteria, fungi or insects (Glick 2012) and lytic enzyme production (Velivelli, Sessitsch et al. 2014).

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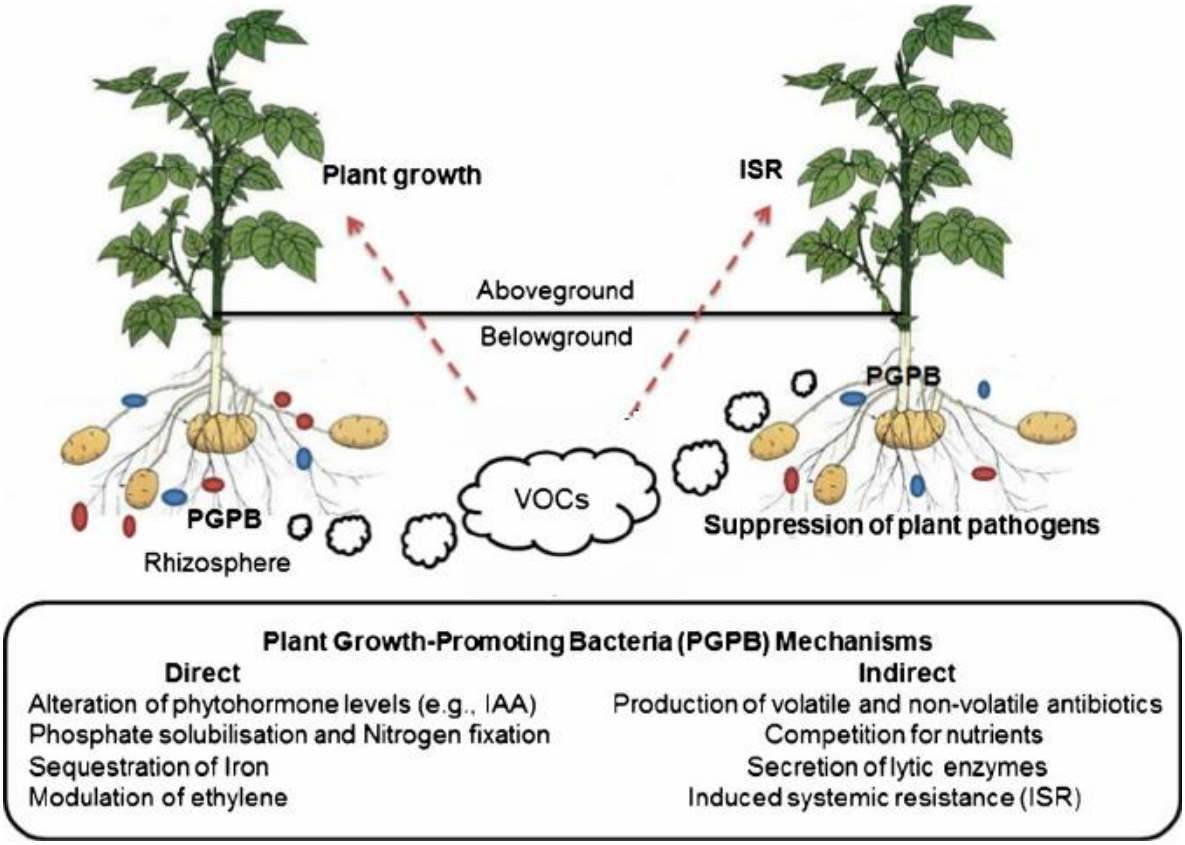


Figure: Mechanisms of plant-growth-promoting bacteria (PGPB) (Velivelli, Sessitsch et al. 2014)

The objective of this chapter was to explore the capabilities of our isolated jute endophyte *B. contaminans* NZ as a plant growth promoting bacteria and it was found to have promising role in plant growth promotion as it possesses all the characteristics of a PGPB.

2.2 Materials and Methods

2.2.1 Isolation of endophytic bacteria and collection of plant materials

Fresh seeds of jute variety (*Corchorus olitorius* var. O-9897 were collected from the Bangladesh Jute Research Institute (BJRI).

Endophytic bacteria were isolated from the seed of jute plant. For isolation of bacteria, surface sterilization techniques were used as described by Coombs and Franco (2003) with some modifications.

Samples were washed under running tap water, soaked in ethanol for 1 min and then treated with 3% sodium hypochlorite for 3 min, 30s wash in 70% ethanol. Finally, samples were rinsed with autoclaved dH_2O twice and dried on sterile tissue paper.

After surface disinfection, seeds, leaves, stems, roots and seedlings were ground with a sterile mortar pestle and dissolved in sterile phosphate buffer saline (PBS, 100 ml containing Na_2HPO_4 0.144 g; KH_2PO_4 0.24 g; KCl 0.02 g; NaCl 0.8 g; pH 7.4). Serial dilutions were prepared from the ground plant parts, and 100 µl aliquots from each dilution of 1×10^{-2}, 1×10^{-3}, and 1×10−6, 1×10−9 were spread on solid growth media (TSB, LB and MRS agar) and incubated at 37 °C for 24‑72 h. Control plates were carefully prepared by spreading water (the same that was used in the final step of surface sterilization) and PBS buffer only.

Distinct morphological appearances such as color, shape and growth pattern of cultured colonies were carefully examined. Distinctly different bacterial species were thus identified and sub-cultured carefully in fresh petri dishes containing the respective media in which the original colony had appeared. Bacterial isolates were preserved temporarily in 20% glycerol solution at −80°C. The isolated bacteria were screened for their potential biocontrol activity against the model phyto-pathogenic fungi *Macrophomina phaseolina.* And a jute seed endophytic bacterium showing a potential inhibiting effect was selected for this study.

Media preparation

The isolated bacterial strain was grown in, and maintained on TSA (HiMedia, India) media in petri plates. 55.1g media were dissolved in 850 ml of distilled water. Final pH: 7.3 ± 0.2 at 25°C and volume 1000 ml.

2.2.2 Molecular characterization with 16S rRNA

For the isolation of genomic DNA from the isolate, bacterium was grown overnight in 5 mL of tryptic soy broth in a rotatory shaker at 37°C. Cells were harvested by centrifugation for 10 min and 6000 rpm/min (5000x g) at 4°C. Bacterial genomic DNA was isolated using a DNA isolation kit, ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research).

PCR was performed for the 16S rRNA region with forward primer 27F, 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 1492R, 5′-GGTTACCTTGTTACGACTT-3′ (Ludwig 2008).

For nucleotide sequence determination, PCR products were separated on a 1% agarose gel, recovered and purified from agarose using a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). Sequencing was performed by 1st base (Malaysia). Similarity

searches in GenBank were performed using BLAST (http://www.ncbi.nlm.nih.gov/blast/; (Altschul, Gish et al. 1990).

2.2.3 General characterization

2.2.3.1 Growth curve

The growth curve of the bacterial culture was constructed by incubating the bacterium in TSB media at 37^0C for 72 hours in an orbital shaking incubator with shaking at 150 rpm.

2.2.3.2 Influence of pH and temperature on antifungal activity

To evaluate the influence of pH on bacterium activity, the pH of TSA media solutions was adjusted to 3, 5, 7, and 9 with 5N NaOH or 5N HCl aseptically. All pH values were measured with a pH meter (Orion 410A+; Thermo Electron Corp., New York, USA). Each medium with different pH values was inoculated with 1% volume of the bacterial suspensions. The cultivation was carried out on a test tube shaker at 37^0C for 24 h. then tested against the indicator fungal strain in PDA plate by well diffusion method. 20 µL of the overnight bacterial liquid culture of different pH was introduced into the 6 mm diameter well punched aseptically with a micropipette tip in the opposite side of a PDA agar plate where a 5-mm plug taken from the plate of an actively growing fungal colony was inoculated in the centre of that petri dish.

Thermostability of the metabolite(s) was carried out using aliquots of 25 ml of bacterial liquid culture in TSB media. Each was treated for 10 min to 5 different temperature conditions: 37, 60, 80,100 and 121^0C (1 atm) and tested against the fungus in the same way as described above.

2.2.3.3 Catalase test

For routine check of aerobic bacteria, catalase test was done by transferring a small amount of bacterial colony to the surface of a clean, dry glass slide using a sterile loop. A drop of 3% H_2O_2 was added on to the slide and mixed. The result was positive when a rapid evolution of oxygen (within 5-10 sec.) as evidenced by bubbling was observed.

2.2.4 Characterization of exo-enzymes produced by endophytic bacteria

2.2.4.1 Screening of cellulolytic enzyme-production

Single colonies of the bacterium from fresh TSA agar plates were inoculated on yeast extract peptone agar media supplemented with 1.0% carboxy methylcellulose (CMC) (Sigma-Aldrich, USA) as the substrate. The media was also provided with essential salts such as $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 1.0 g, $CaCl_2$ 1.0 g, $MgSO_4$ 0.5 g per litre and 1.5% agar at pH 5.5. After inoculation the plates were incubated at 37^0 C for 24 h and were then stained with 0.1% Congo red for 30 min flooded with 1N NaCl for color development and 2N HCl was added to visualize the clear zone. Cellulase production was indicated by the appearance of a halo around the hydrolysed area (Florencio, Couri et al. 2012).

2.2.4.2 Xylanase assay

Xylanase activity was assayed by growing the bacterium onto minimal agar plates (0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.1% KCl, 0.05% yeast extract and 1.5% agar supplemented with 1.5% xylan (SIGMA, USA) at pH 5.5.

After 24h of incubation the plates were stained with 0.1% Congo red for 30 min flooded with 1N NaCl for color development and 2N HCl was added to visualize the clear zone (Meddeb-Mouelhi, Moisan et al. 2014).

2.2.4.3 Lipase assay

For screening of lipase activity, the bacterium was grown on media supplemented with 1% tween 20 (SIGMA, USA) made in tris buffered saline. The growth media also included yeast extract 1g/L, peptone 0.5 g/L, NaCl 1.0 g/L, KH_2PO_4 1.0 g/L , $CaCl_2$ 1.0 g/L and agar 1.5% (Salihu, Alam et al. 2011).

After incubation for 48h, appearance of a zone of intensification due to the formation of insoluble calcium salts by the released free fatty acids due to the action of the lipase enzyme was considered positive.

2.2.4.4 Chitinase assay

Chitinase enzyme assay was performed by growing the bacterium on colloidal chitin agar media. Chitin from shrimp cells (SIGMA, USA) was used to prepare colloidal chitin by the modified method of Hsu and Lockwood (Hsu and Lockwood 1975).

Chitinase detection medium was directly supplemented with colloidal chitin (4.5g/l) and bromocresol purple (0.15g/l). The medium also contained KH_2PO_4 2.0 g/L, $CaCl_2$ 1.0 g/ L, $MgSO_4$ g/L, NaCl 1g/L, peptone 0.5 g/L, yeast extract 0.1g/L and 1.5% agar. The pH of the media was adjusted to 4.7 and sterilized at 121°C for 15 min.

Inoculation of colloidal chitin media containing bromocresol purple (pH 4.7) with chitinolytic bacterium results in the breakdown of chitin into N-acetylglucosamine causing a corresponding shift in pH towards alkalinity and a change in the color of the pH indicator dye to purple from orange around the inoculation site.

2.2.4.5 Qualitative and quantitative protease assay

Qualitative protease assay on agar plate

For protease assay of the bacterium, 1.5% gelatin (Merck, Germany) was used (Vermelho, Meirelles et al. 1996). The other components of the media included KH_2PO_4 2.0 g/L,$CaCl_2$ 1.0 g/L ,$MgSO_4$ g/L, NaCl 1g/L, peptone 0.5 g/L, yeast extract 0.1g/L and 1.5% agar. The pH was set at 6.5.

After inoculation of bacterium, the plates were incubated at 37°C for 2 days. Then the plates were flooded with 100% saturated ammonium sulphate to precipitate unhydrolyzed gelatin, making the clear zones easier to see.

Quantitative protease activity assay

For measuring protease activity, we followed the procedure described by Carrie Cupp-Enyard (Cupp-Enyard 2008) in triplicates.

To the reaction mixture (5ml) containing 0.65% weight/volume casein solution, prepared by mixing 6.5 mg/ml of 50 mM potassium phosphate buffer (pH 10.5), 0.5ml of enzyme solution was added and incubated at 37°C for exactly 10 min. The reaction was stopped by the addition of 5 ml TCA reagent. Then the enzyme solution was added to each tube, even in the blank, so that the final volume of the enzyme solution in each tube was 1 ml. The solutions were incubated at 37°C for 30 min and each of the test and blank solutions were filtered using a 0.45 um polyethersulfone syringe filter. Tyrosine standard in different dilutions were used to calculate the protease activity. To all of the vials containing the standard, test and blank, 5ml of sodium carbonate was added, and for best results, 1 ml of Folin's reagent was added immediately afterwards and incubated at 37ºC for 30 min. 2ml of these solutions were filtered using a 0.45 um polyethersulfone syringe filter into suitable cuvettes. The absorbance of the blank and test samples were measured by a spectrophotometer at a wavelength of 660nm.

One unit of alkaline protease activity (1 APU) is defined as the amount of enzyme liberating 1 μg of tyrosine per min according to the following equation:

$$\text{Units/ml enzyme} = \frac{(\text{umole tyrosine equivalents released}) \times (11)}{(1) \times (10) \times (2)}$$

11= Total volume (in milliliters) of assay

10= Time of assay (in min) as per the unit definition

1= Volume of enzyme (in milliliters) of enzyme used

2= Volume (in milliliters) used in colorimetric determination

2.2.5 Plant growth promotion

2.2.5.1 Siderophore production assays

Siderophore production was determined by the universal chemical assay method of Schwyn and Neilands on chrome azurol S (CAS) agar plates (Schwyn and Neilands 1987).

The bacterium was checked on CAS blue agar medium which contained 100 ml of MM9 salt solution (KH_2PO4 15 g, NaCl 25 g, NH_4Cl 50 g), 32.24 g piperazine- N, N'- bis 2- ethane sulfonic acid (PIPES) [pH adjusted to 6.0], 1.5% agar, 30 ml 10% casamino acid solution, 10 ml of 20% glucose solution and 100 ml of the dye solution (50 ml of 0.06 g chrome azurol S with 9 ml of 0.0027 g of $FeCl_3 \cdot 6$ H2O dissolved in 10 ml of 10mM HCl), 0.073 g of HDTMA. The bacterial strain was inoculated as a point on CAS agar plate and incubated at 28 °C for 72 h. Siderophore sequestration of iron on CAS agar changes the color of the medium from

blue to orange around the bacterial colonies and appearance of a halo zone indicates siderophore activity.

2.2.5.2 ACC deaminase activity test:

Both molecular and biochemical approaches were used for detection of ACC deaminase production by the endophytic bacterium as described by Penrose et al (Penrose, Moffatt et al. 2001).

Molecular detection of ACC deaminase gene (acdS)

Degenerate primers acdS F (5′- GCC TTY GGC GGC AAC AAG AC−3′) and acdS R (5′- CGA GCASACSACGATGTAGTCGA −3′) for acdS were designed from available sequences for acdS genes of other fungi. PCR amplification of the acdS gene was carried out for the strains. The sequence of the 470 bp amplicons so obtained was checked against GenBank database by using NCBI BLAST tool.

Screening for ACC deaminase activity

Modified DF minimal salt medium (Dworkin and Foster 1958) was used to screen *B. contaminans* NZ for its ACC deaminase synthesizing activity on solid plates (Ali, Sandhya et al. 2014). The bacterium was streaked on DF salt solid media (DF salts per liter: 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 2.0 g glucose, 2.0 g gluconic acid and 2.0 g citric acid with trace elements:100 mg $FeSO_4 \cdot 7H_2O$, 10 mg H_3BO_3, 11.2 mg $MnSO_4 \cdot H_2O$, 124.6 mg$ZnSO_4 \cdot 7H_2O$, 78.22 mg $CuSO_4 \cdot 5H_2O$, 10 mg MoO_3, pH 7.2, 15 g agar) supplemented with filter sterilized 3 mM ACC, and incubated at 30°C. for 72 h (Haidar, Ferdous et al. 2018). DF salt media without ACC was used as a negative control. Growth of the bacterium in DF media when compared to negative controls indicated bacterial ACC deaminase activity.

Colorimetric assay for quantitative screening of ACC deaminase

ACC deaminase assay was done as described by Haidar et al (Haidar, Ferdous et al. 2018). For colorimetric quantification, toluene treated cells were prepared for the bacterium according to the process of Penrose and Glick (Penrose, Moffatt et al. 2001). ACC deaminase activity was determined by measuring the production of α-ketobutyrate and the OD was measured at 595 nm. The amount of protein for toluene treated cells was determined by the Bradford method using BSA as a standard (Bradford 1976).

2.2.5.3 *In vitro* screening for indole-3-acetic acid production

In vitro production of indole-3-acetic acid (IAA) was determined by the colorimetric method described by Gordon and Weber (Gordon and Weber 1951). We followed the detailed method described by Haidar et al (Haidar, Ferdous et al. 2018). 30 ml sterile Luria- Bertani (LB) media was inoculated with an overnight bacterial culture (~3×108 CFU/ml) in the absence of L-tryptophan and incubated at 30 ± 1 °C with continuous shaking at 180 rpm for 96 h. After incubation, the liquid culture was centrifuged at 12,000g at 4° C and 3 ml of the supernatant was mixed with 2 ml of Salkowski reagent (2.0 ml of 0.5M $FeCl_3$ in 98.0 ml of 35% $HClO_4$) and incubated for 30 min in the dark for color development at room temperature. Intensity of the color was measured at 535 nm by using a spectrophotometer (Shimadzu uv1800). Standard curve ranging from 5 µg/ml to 100 µg/ml was used for comparison to calculate IAA production. The procedure was done in triplicates.

Standard Curve: Standards were made in LB medium at 0, 5, 10, 20, 50, and 100 µg/ml (ppm). IAA is soluble in ethanol or acetone.

To a glass beaker of 10 ml acetone in the fume hood, 10 mg IAA was added and stirred with metal spatula until completely dissolved. This is the 1000 µg/ml stock

1 ml of the 1000 µg/ml stock was added to 9 ml medium (LB) and mixed well by inversion. This is the 100 µg/ml standard. 5 ml of the 100 µg/ml standard was transferred to 5 ml of LB media. This is the 50 µg/ml standard. 1 ml of the 100 µg/ml standard was added to another vial with 9 ml LB. This is the 10 µg/ml standard. 2 ml of the 100 µg/ml standard was transferred to a vial of 8 ml LB. This is the 20 µg/ml standard. 1 ml of the 50 µg/ml standard was added to 9 ml LB. This is the 5 µg/ml standard. Now 2 ml of the Salkowski Reagent was added into 6 test tubes labeled with each standard.

1 ml of each standard, including a no-IAA control of pure LB were transferred into the test tubes and incubated at room temperature for 25 min and then the OD was taken at 530 nm.

2.2.5.4 Biological nitrogen fixation assay

Nitrogen utilizing bacteria were identified by their growth on nitrogen free media (Kuklinsky-Sobral, Araújo et al. 2004). Growth in nitrogen free media indicated nitrogen fixing ability of the bacterium.

2.2.5.5 Biocontrol against phyto pathogenic fungus

In order to evaluate the bacterium as a bio-control agent, which is an important trait of plant growth promoting bacteria, antagonism assays were performed under sterile conditions with jute seeds (*Corchorus olitorius* var O4) using 1.0% sodium hypochlorite. The seeds were allowed to germinate on a 110 mm Whatman filter paper (moistened with sterile water) in petri plates under four different conditions: (1) in the presence of the *B. contaminans* NZ strain, (2) in the presence of *M. phaseolina* (3) in the presence of the bacteria and fungus co-inoculums and (4) in the absence of any inoculum (used as a negative control).

2.2.6 *In vivo* growth promotion study

2.2.6.1 Jute seed inoculation with bacteria

At first the bacterium was cultured on TSA plates and then inoculated in TSB media and incubated at 37° C at 180 rpm for 24–48 hours. At the same time scarification was done for jute seeds (*Corchorus olitorius* var O-4) with sandpaper and seeds were surface sterilized by treating with 5% NaOCl for 1 min and then treated with autoclaved water three times for 2 min. Sterilized seeds were dipped into the bacterial suspension. From McFarland standard (used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range to standardize microbial testing.), bacterial cells of 10^8 CFU/ml were determined for each seed by a serial dilution method (Islam, Akanda et al. 2016).

2.2.6.2 *In vivo* pot experiment in hydroponic culture system

Jute seeds inoculated with bacterial suspension were grown under controlled environmental conditions in a 'Fitotron' plant growth chamber (Weiss Technik India Private Limited) at 28^0C, 70% relative humidity and 16-h light/8-h dark cycle. The seeds were allowed to germinate for three days and later grown in a hydroponic culture system. The seedlings were supplied with a modified Yoshida medium (NH_4NO_3 91.4g, K_2SO_4 71.4g, $NaH_2PO_4.2H_2O$ 40.3g, $CaCl_2.2H_2O$ 88.6g, $MgSO_4.7H2O$ 324.0g per litre and micronutrients ($MnCl_2.4H_2O$ 1.5g, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.074g, H_3BO_3 0.934g, $ZnSO_4.7H_2O$ 0.035 g, $CuSO_4$.$5H_2O$ 0.31g, $FeCl_3$.$6H_2O$ 7.70 g, citric acid 11.9 g and $H_2SO_4$50.0g per liter) (Cock, Yoshida et al. 1976). 1.25 ml was added per liter of the medium. The pH of the solution was adjusted to 5.5 with NaOH. 30 seeds were sown 1 cm deep in the cork sheet in separate pots. Jute plants were grown for 10 days in a hydroponic culture system and 5 plants were collected for the

measurement of fresh weight, dry weight, shoot length and root length on days 4, 7 and 10 after transfer of the seedlings into the hydroponic solution.

2.2.6.3 Statistical analysis

Data obtained from *in vivo* pot experiment tests were subjected to t-test against the control. The results presented as average means, standard deviation (SD) and standard error (SE) were determined by following the standard procedures.

2.3 Results

Identification of endophytic bacteria

BLAST searches in the GenBank database using 16S rDNA sequences revealed that the bacterium is *Burkholderia contaminans* and we named it NZ strain.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments | Download | GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Burkholderia contaminans DNA, scaffold: scaffold02, strain: CH-1	60212	6.555e+06	82%	0.0	99%	AP018358.1
Burkholderia contaminans strain FL-1-2-30-S1-D0 chromosome 1, complete sequence	47709	4.874e+06	65%	0.0	98%	CP013390.1
Burkholderia contaminans strain MS14 chromosome 1, complete sequence	47626	3.002e+06	41%	0.0	98%	CP009743.1
Burkholderia lata strain FL-7-5-30-S1-D0 chromosome 1, complete sequence	45273	4.548e+06	63%	0.0	97%	CP013404.1
Burkholderia lata strain 383 chromosome 1, complete sequence	44540	5.198e+06	74%	0.0	96%	CP000151.1
Burkholderia cepacia strain INT3-BP177 chromosome 1, complete sequence	42669	3.941e+06	58%	0.0	95%	CP013375.1
Burkholderia contaminans DNA, scaffold: scaffold01, strain: CH-1	42618	8.108e+05	10%	0.0	99%	AP018357.1

Figure 1: BLAST search result for the endophytic bacterial 16s rDNA sequence

General characterization

Temperature sensitivity test showed that the bacterium was active up to 80^{0}C and it also showed antifungal activity in a broad spectrum of pH ranging from pH 3 to 9. Growth curve showed stationary phase of *B. contaminans* NZ after 37 hour.

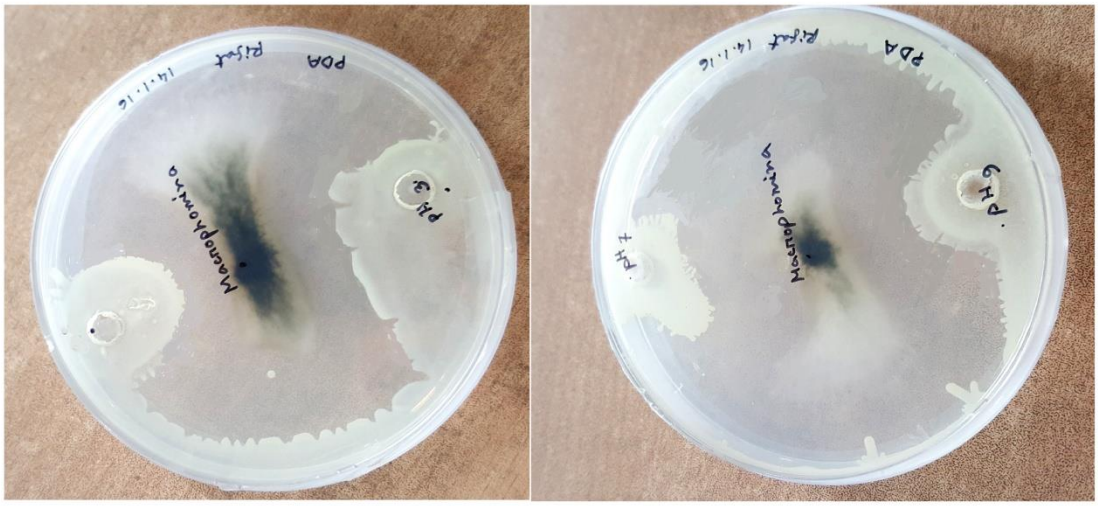


Figure 2: Activity of *B. contaminans* NZ in different pHs ranging from 3, 5, 7 and 9.

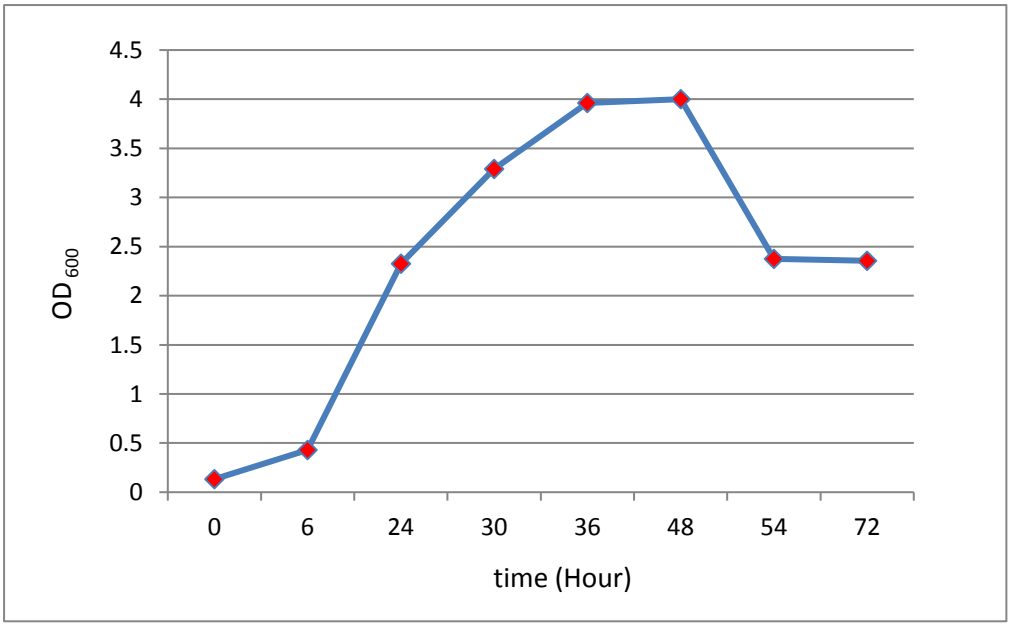


Figure 3: Growth Curve of *B. contaminans* NZ

Exo –enzyme production by *B. contaminans* NZ

B. contaminans NZ has catalase activity. This was ascertained by the formation of bubbles when 3% H_2O_2 was used indicating the bacterium to be an obligate aerobe.

The endophyte was also found to be cellulase, lipase and protease positive (Fig.4). However, the bacterium did not produce lytic enzymes like chitinase and xylanase under our culture conditions.

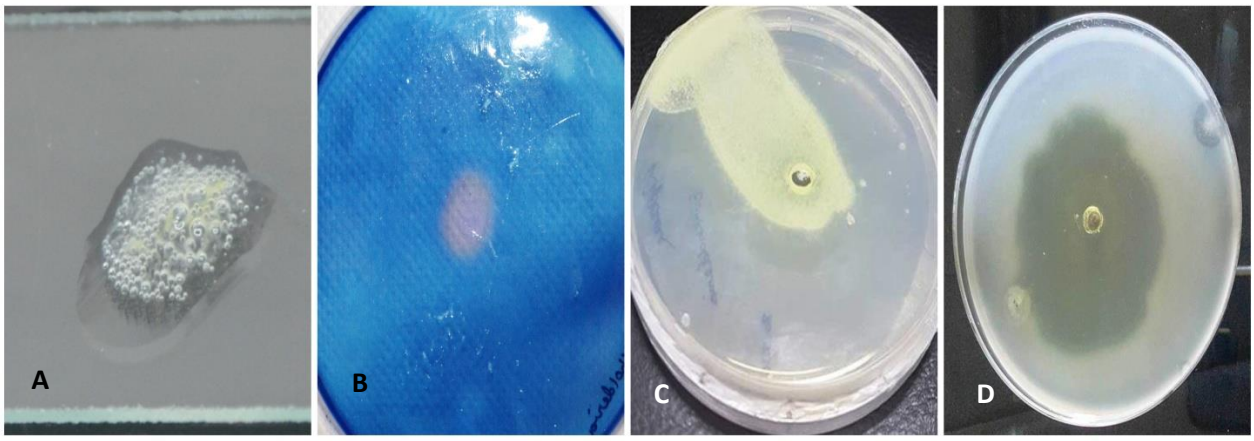


Figure 4: Positive enzyme production of (A) catalase (B) cellulose (C) lipase and (D) protease

Protease the most important hydrolytic enzyme was selected for quantitative estimation. Culture supernatant exhibited strong protease activity potentially capable of degrading fungal cell walls. Bacterial protease activity was found to be 89.9 µg/mL/min calculated from the standard curve of tyrosine in quantitative protease assay.

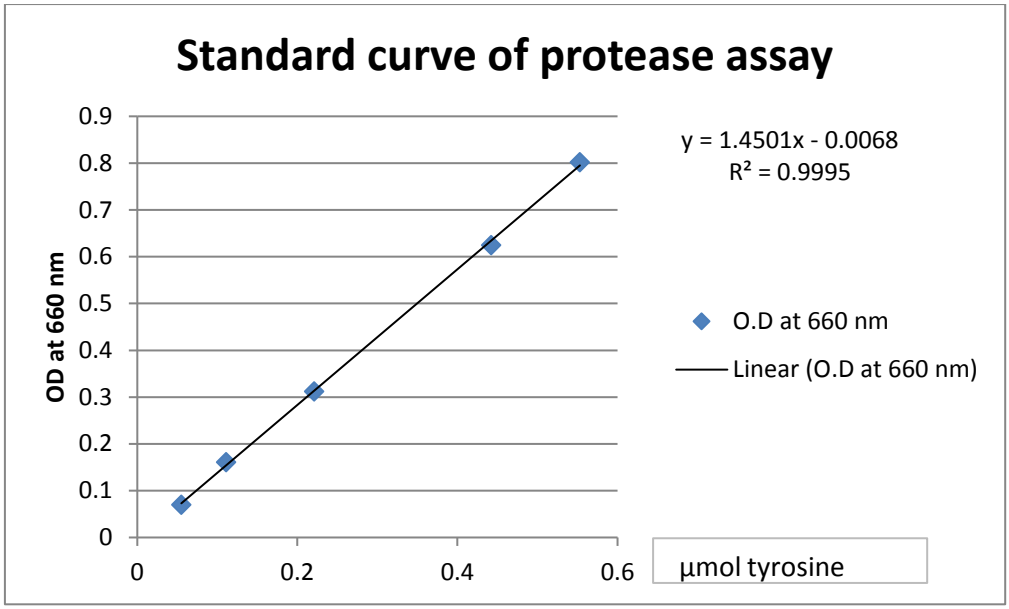


Figure 5: standard curve for quantitative protease assay

Plant growth promotion

Siderophore production

A yellow halo of 6 mm formed around the bacterial colony on a CAS plate, suggested siderophore production to be a common characteristic of *B. contaminans* (Figure 6).

Nitrogen fixation

Bacterial growth on nitrogen free media (NFb solid media) indicates the nitrogen fixing ability of *B. contaminans* NZ (Figure 6) and a cross check on semisolid media confirmed the same.

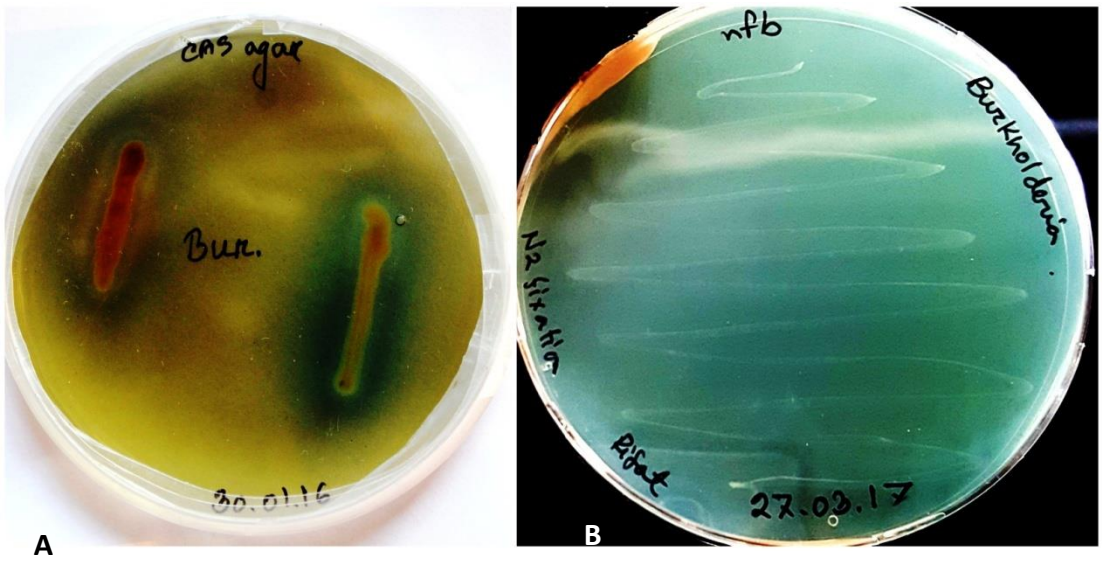
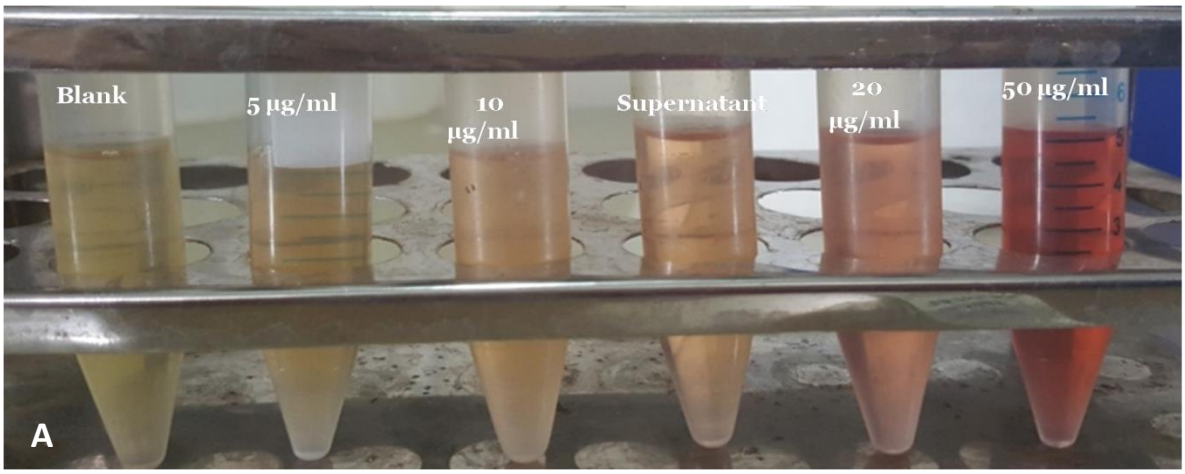


Figure 6: Plant growth promotion features of *B. contaminans* NZ (A) positive siderophore activity (B) Bacterial growth on nitrogen free media confirms the nitrogen fixing ability

Indole acetic acid (IAA) production

Bacterial culture supernatant produced red color after the addition of Salkowski's reagent indicating the ability of the bacterium for IAA production. By quantitative assay, from standard curve, 12.22 µg/ml IAA was found to be produced by *B. contaminans* NZ.



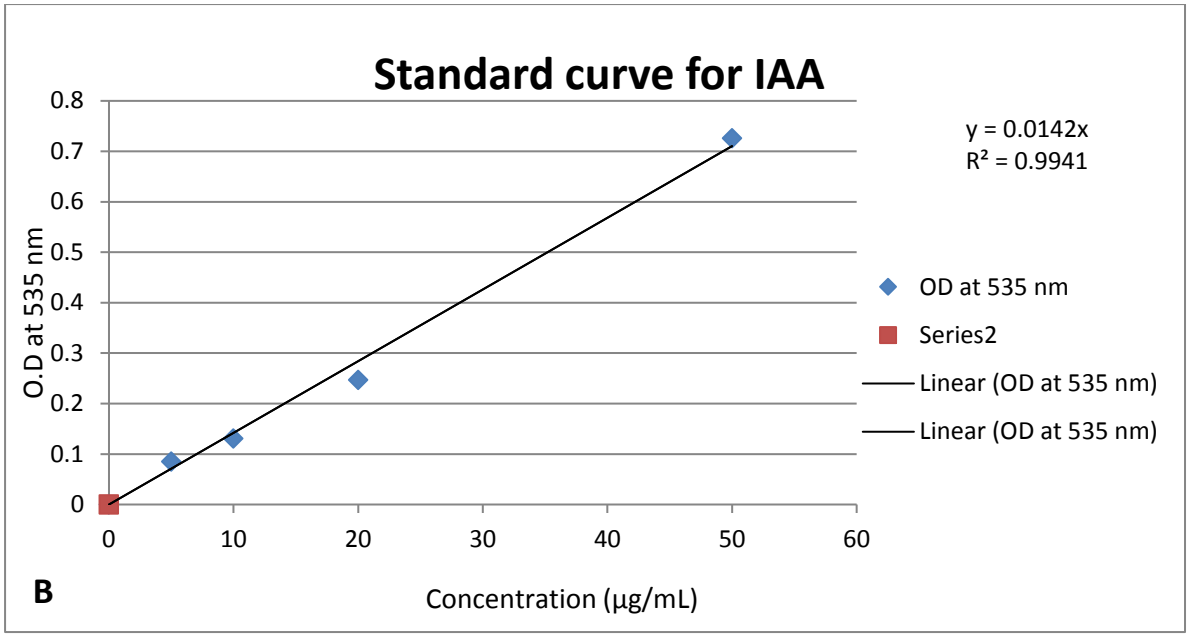


Figure 7: Indole acetic acid assay A) colorometric assay of IAA B) Standard curve for IAA production for determination of IAA concentration in the isolated endophytic sample.

ACC deaminase activity

B. contaminans NZ was found positive in genetic screening for acdS (ACC deaminase synthase) gene, showing sequence similarity to acdS gene from other *Burkholderia* species. In a plate assay, growth of the bacterium in DF media indicated positive ACC deaminase activity.

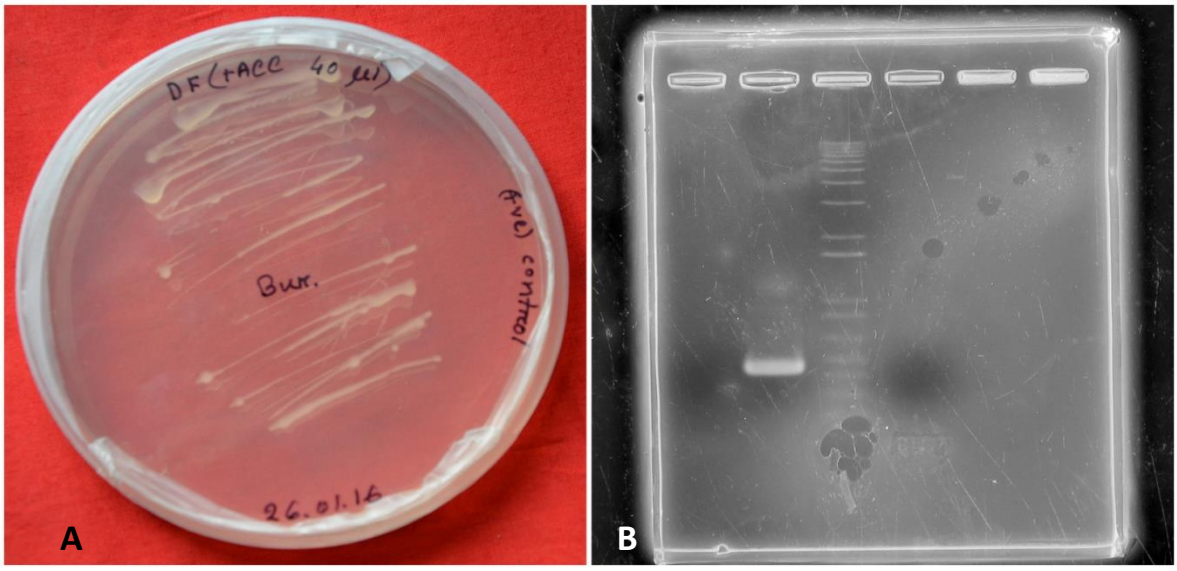


Figure 8: ACC deaminase assay (A) positive ACC deaminase activity in qualitative plate assay (B) presence of 470 bp acdS gene in *B. contaminans* NZ

In a quantitative colorimetric assay, it was found to be efficient in hydrolyzing ACC, producing 54.2 µM mg–1 h−1 of α-ketobutyrate.

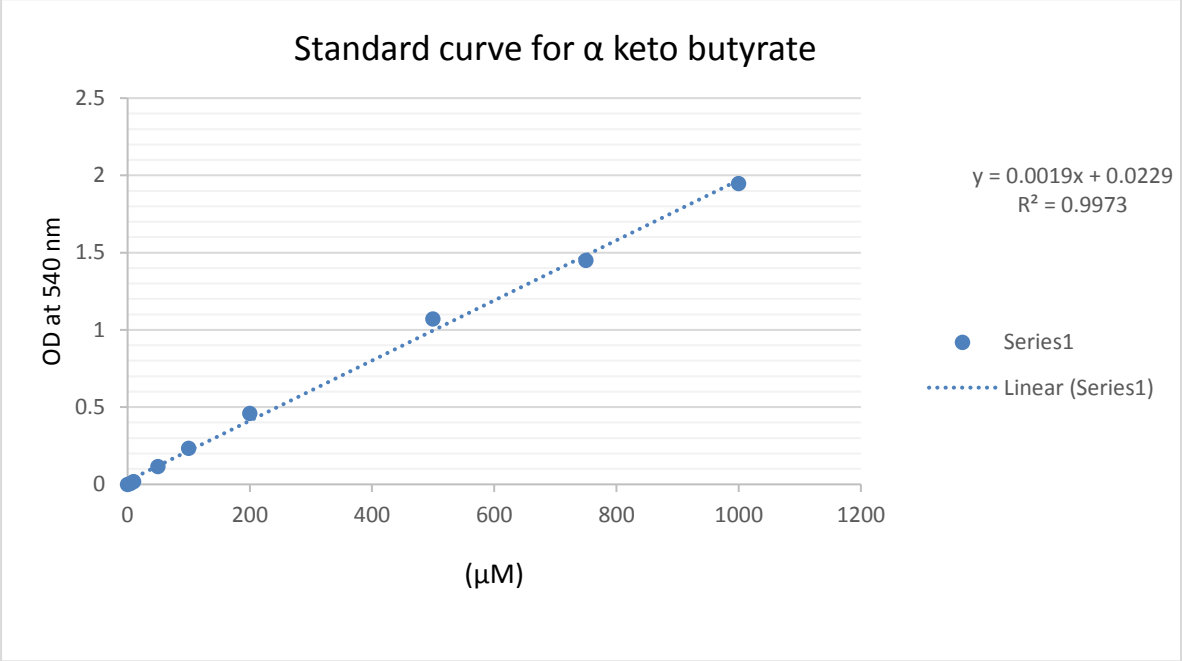


Figure 9: Standard curve for ACC deaminase activity using α-ketobutyrate

Biocontrol against phyto pathogenic fungus

Jute seedlings inoculated with the phyto pathogenic fungus *M. phaseolina* showed intense seedling death. In contrast, when plants were inoculated with a mixture of the fungus and bacterium *B. contaminans* NZ, the infectious effect associated with the fungus was almost absent and growth of the jute seedling was as good as the control healthy seedlings (Figure 10). This result suggests that *B. contaminans* NZ has the ability to antagonize the growth of phyto pathogenic fungi indicating its biocontrol feature which is important to be considered as a plant growth promoting bacteria.

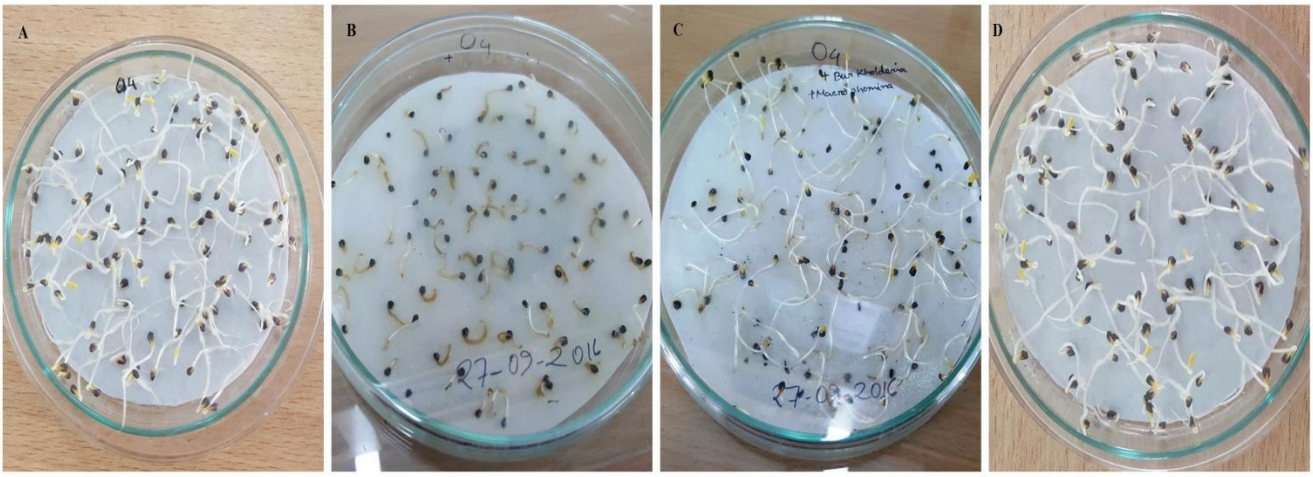


Figure 10: Antagonistic effect of endophytic bacteria *B. contaminans* NZ against a phyto pathogenic fungus on plant: jute seedlings (A) in the presence of the *B. contaminans* NZ strain, (B) in the presence of *M. phaseolina* (C) in the presence of the bacteria and fungus co-inoculums and (D) in the absence of any inoculum (used as a negative control).

Pot tests for *in vivo* assay of plant growth promotion

B. contaminans NZ has significant effect in increasing the root and shoot length and number of jute seedlings when compared with the control jute plants. In hydroponic solution, using 10^8 bacterial cell per microliter for 4, 7 and 10 days, the bacterium was found to promote growth of jute plants effectively and showed significant effect on total biomass, leaf area, plant height and root shoot length exhibiting increment in fresh and dry weights when compared to the control.

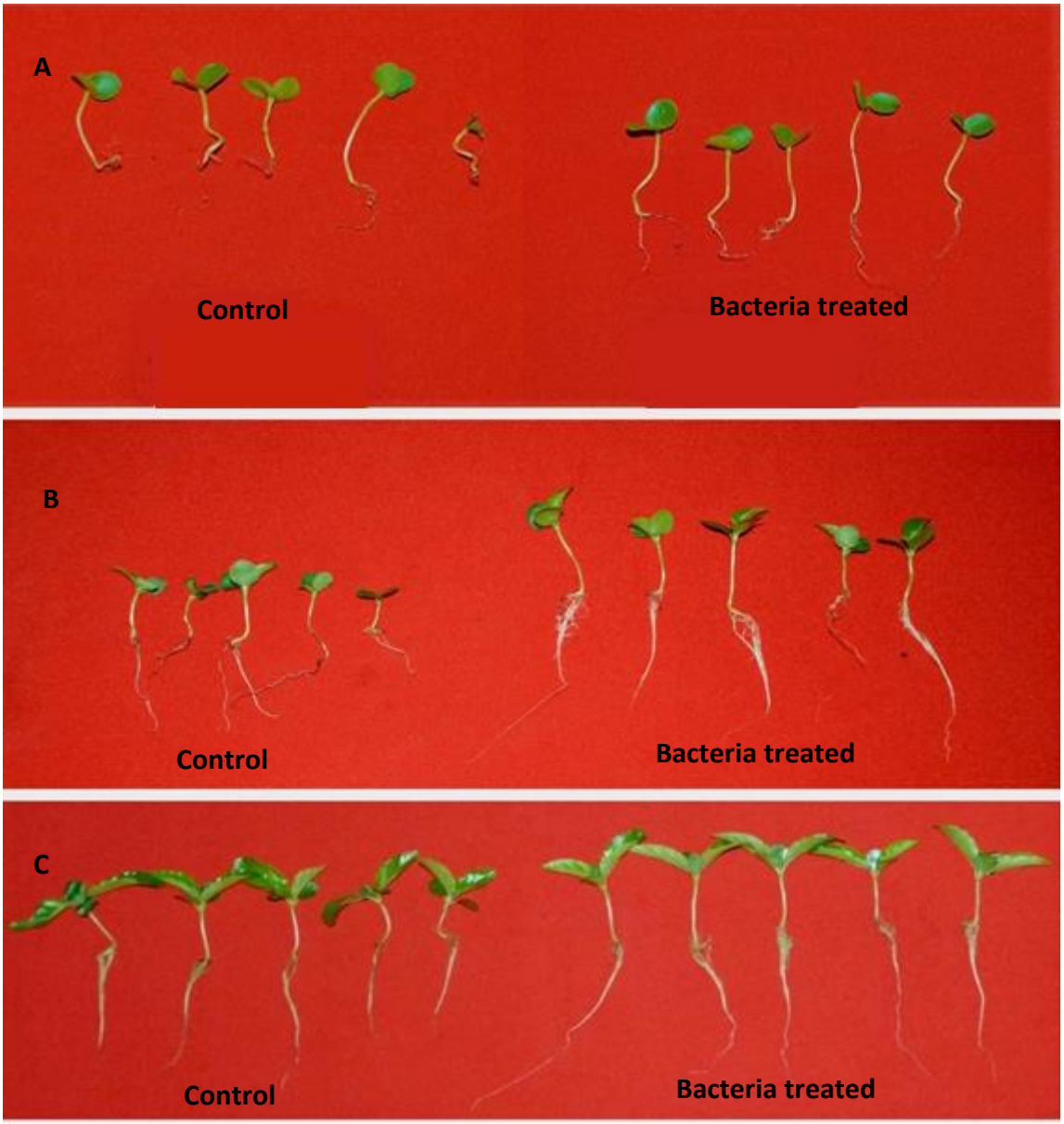


Figure 11: *In vivo* effect of plant growth promoting endophytic bacteria *B. contaminans* NZ. Significant effect of the bacterium in increasing the root and shoot length of jute seedlings when compared with the control jute plants in (A) 4 days (B) 7 days and (C) 10 days.

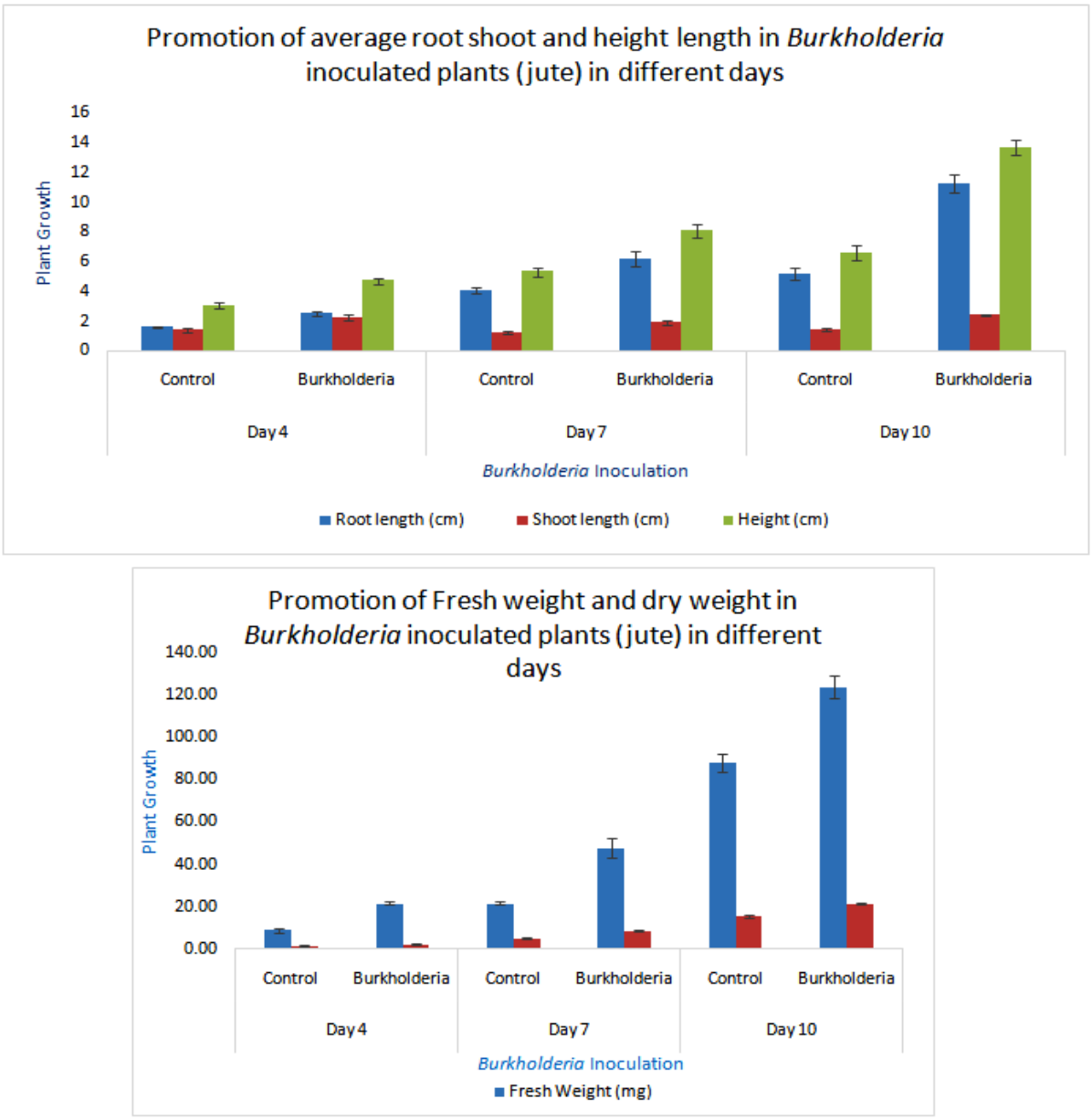


Figure 12: Comparison of (A) shoot and root length, plant height (B) fresh and dry weights of *B. contaminans* NZ treated jute seeds and non-treated control seeds. Error bar represents the standard error of means from replicates. Data represents shoot and root length in cm and fresh and dry weights in mg. The experiment was repeated three times.

Statistical analysis of the *in vivo* plant growth promotion activity

B. contaminans NZ showed significant effect (P value <0.005) in increasing the root length, shoot length and average height of jute seedlings (Table 1 and 2).

Table 1: Average root length, shoot length and plant height of bacteria treated jute seedlings Vs untreated control in pot experiments in 4, 7 and 10 days. Mean and standard deviation of three replicates per experiment are presented.

		Average Root Length (cm)	SD	SE	Average Shoot Length (cm)	SD	SE	Average Height (cm)	SD	SE
Day 4	Control	1.64	0.11	0.05	1.40	0.32	0.14	3.04	0.42	0.19
	Burkholderia	2.5	0.34	0.15	2.22	0.46	0.21	4.72	0.44	0.20
Day 7	Control	4.06	0.52	0.23	1.26	0.22	0.10	5.32	0.68	0.31
	Burkholderia	6.18	1.11	0.49	1.92	0.36	0.16	8.10	1.01	0.45
Day 10	Control	5.2	0.95	0.42	1.42	0.18	0.08	6.62	1.04	0.46
	Burkholderia	11.28	1.35	0.60	2.44	0.15	0.07	13.72	1.09	0.48

Table 2: Fresh weight and dry weight of the bacteria treated jute seedlings vs untreated control in pot experiments in 4, 7 and 10 days with mean and standard deviation of three replicates.

		Average Fresh wt (mg)	SD	SE	Average Dry wt (mg)	SD	SE
Day 4	Control	8.98	2.44	1.09	1.66	0.43	0.19
	Burkholderia	21.64	1.60	0.69	2.40	0.50	0.23
Day 7	Control	21.64	1.69	0.75	5.20	0.75	0.33
	Burkholderia	47.96	10.08	4.51	8.80	0.52	0.23
Day 10	Control	87.98	8.82	3.94	15.68	1.32	0.59
	Burkholderia	123.86	11.27	5.04	21.26	0.69	0.31

Highest root length was found to be 11.28 cm, which was 116.9% longer than the control.

The effect on shoot length was also significant. On an average it was found to be 50.7% longer than the control jute plants. Fresh weight increased significantly measuring 141%, 122% and 41% than the control in 4, 7 and 10 days respectively.

Dry weight of jute plants in the presence of *B. contaminans* NZ increased by 49.8% on an average for all three replicates of each 4, 7 and 10 day measurements.

2.4 Discussion

Application of plant growth promoting bacteria could be an efficient strategy to improve plant productivity, particularly in soils with low fertility and reduce the use of chemical fertilization with the consequential reduction of the environmental pollution problems associated with commercial nitrogen fertilization (Parra-Cota, Peña-Cabriales et al. 2014). Moreover, chemical control and most cultural practices do not represent effective tools to

limit the growth of phyto-pathogen and its spread (Torres, Brandan et al. 2016). Therefore, the use of biocontrol organisms with plant growth promoting features can be the most potentially attractive disease management approach as it is able to promote plant growth, enhance tolerance to abiotic stress (Berendsen, Pieterse et al. 2012), suppress pathogens locally and induce systemic resistance (ISR) against a broad range of diseases in crops (Shanmugam, Kanoujia et al. 2011, Pineda, Dicke et al. 2013).

While studying a microbiome of jute, *Burkholderia contaminans* NZ, isolated as a jute endophyte was shown to be a potent biocontrol agent effective against a number of pathogenic fungi and a resourceful plant growth promoting agent.

The bacterium also possesses an array of exo-enzymes namely cellulase, lipase and protease. They may contribute to growth inhibition of the pathogenic fungi through degradation of their cell walls. Strong protease activity found for *B. contaminans* NZ may play a major role in inhibiting pathogens. Bacterial proteases are also known to play a significant role in mycoparasitism (Rabha, Sharma et al. 2016).

This *B. contaminans* NZ strain has been screened and characterized, *in vitro*, for its potential plant growth-promoting (PGP) traits.

Direct plant growth promotion includes:

(1) the ability to produce a vital enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase which reduces the indigenous ethylene level in the root of developing plants thereby increasing the root length and growth as elevated levels of ethylene caused by some stresses are known to inhibit root elongation and lateral root emergence (Negi, Ivanchenko et al. 2008).

(2) The ability to produce phyto-hormones like auxin, i.e. indole acetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA) and cytokinins. Phytohormones, in particular indole acetic acid (IAA) is one of the most physiologically active auxins, known to control organogenesis, cellular responses such as cell expansion, division, and thus increase the root length, which gives larger root surface allowing plants to get more access to nutrients from the soils (Boiero, Perrig et al. 2007) . IAA production and its possible involvement in plant growth promotion has been well reported for endophytic *Burkholderia* (Govindarajan, Balandreau et al. 2008, Tallapragada, Dikshit et al. 2015).

(3) A symbiotic nitrogen fixating ability; after water, nitrogen is the major limiting compound for crop production. Many plants can obtain nitrogen through a process known as BNF (biological nitrogen fixation). PGPB retain more soil organic N, and other nutrients in the plant–soil system, thus reducing the need for fertilizers.

(4) Improved defense against pathogens by producing siderophores, ß-1, 3-glucanase, chitinases, antibiotic, fluorescent pigment and cyanide (Hayat, Ali et al. 2010). Usually, these low molecular weight siderophores bind Fe3+, transport it back to the microbial cell, and make the same available for growth. Plants can utilize microbial siderophores for iron acquisition (Sessitsch, Reiter et al. 2004). Several bacterial endophytes have been reported for growth promoting potentiality through siderophore production in sugarcane (Saccharum sp.), rye grass (Lolium perenne) Thai jasmine rice plant and jute (Haidar, Ferdous et al. 2018). Iron deficient tomato plants supplemented with microbial siderophores have also been shown to produce high crop yields, and had increased chlorophyll and iron content in the leaves (Radzki, Mañero et al. 2013).

In accordance with these findings, *B. contaminans* NZ was evaluated for IAA and siderophore production, ACC deaminase activity, nitrogen fixation and antifungal activities. It was found to possess all these characteristic PGPB features.

The growth performance was measured for the endophytic bacterium on the basis of their shoot and root length, dry and fresh weight, leaf and root number in comparison to the non-inoculated controls. An *in vivo* assay demonstrated that both yield and biomass were significantly increased like, plant height, root/shoot length, dry weight, fresh weight and number of leaves and leaf area when jute seedlings were inoculated with the endophytic PGPB *B. contaminans* NZ. This could be the combined effect of ACC deaminase production (18.1 μMmg−1 h−1), nitrogen fixation and siderophore production.

It is likely that the application of endophytes could be an advantage since they are present in a much more protected environment than rhizospheric bacteria and therefore less susceptible to the changing ecological environment (Haidar, Ferdous et al. 2018). The bacterial endophyte *B. contaminans* NZ identified in this study can therefore be a strong alternative of synthetic fertilizers and chemical biocontrol agents.

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CHAPTER 3

Identification of antimicrobial compounds of *B. contaminans* NZ for antagonism of *Macrophomina phaseolina*

Pages 41 - 57

Identification of antimicrobial compounds of *B. contaminans* NZ for antagonism of *Macrophomina phaseolina*

3.1 Overview

Fungicidal chemicals can be an effective means of controlling phyto pathogenic fungi like *Macrophomina phaseolina*. But chemical control measures are hazardous and can lead to the evolution of resistant strains of the pathogen and may destroy balances in the microbial community, which may harm beneficial organisms (Gill and Garg 2014). For these reasons, biological control strategies are emerging as promising alternatives.

However, *in vivo* bio-control agent selection is not a simple task due to the diversity of agents and interactions with the host plant, and therefore, efficient search methods are required. Thus, it is necessary to develop efficient selection strategies to reduce costs and increase the possibility of selecting organisms that can be produced in a large scale at low cost and that maintain their viability and efficiency for long periods. In 1997, Schisler and Slininger divided the selection process into three categories: (i) choosing the appropriate pathosystem, (ii) choosing the adequate method, and (iii) characterizing the isolates and evaluating efficiency (Mota, Gomes et al. 2017).

As mentioned earlier a search carried out among endophytic bacteria isolated from various part of jute plant (*Corchorus olitorius*) for a suitable biocontrol agent effective against the phyto-pathogen *M. phaseolina* led to the identification of *Burkholderia contaminans* from

jute seed. It was found to substantially inhibit the growth of *M. phaseolina* in *in vitro* culture conditions hinting at an effective alternative to hazardous bio-insecticides. The bacterium showed strong antagonistic properties against several other plant fungal pathogens as well, namely *Nigrospora sphaerica, Xylaria sp. Aspergillums fumigates, Aspergillus niger, Penicillium oxalicum, Rhizoctonia solani.*

In order to investigate the nature of the antifungal compound(s) produced by *B. contaminans* NZ*;* supernatant of the bacterial liquid culture, intra and extracellular extracts and precipitated secreted proteins of bacteria were screened without success. However, an assay of the volatile organic compounds (VOCs) produced by *B. contaminans* NZ in the presence of *M. phaseolina* was found to be the probable antifungal source.

Microorganisms produce a wide range of infochemicals, frequently secondary metabolites, both in soluble and volatile forms. Positive, negative or neutral interactions can occur between a very wide range of soil bacteria and fungi. These effects include both stimulation and inhibition of growth (Wheatley 2002).

Gas chromatography coupled to mass spectrometry is a versatile tool to separate, quantify and identify unknown (volatile) organic compounds and permanent gases. By combining sensitivity and a high resolving power, complex mixtures can be analyzed. In order to investigate the antifungal compounds produced by *B. contaminans*, Gas Chromatography-Mass Spectrometry (GC-MS) was used to determine the bioactive compounds and we found three potent antimicrobial compounds in the volatile compound profile. Details of the present study establish *B. contaminans* NZ as an efficient antagonist of plant pathogenic fungus for which VOCs may play an important role.

3.2 Methods and materials

3.2.1 Bacterial and fungal strains used in this study and collection of plant materials

The phyto-pathogenic fungi *Macrophomina phaseolina* was obtained from the Bangladesh Jute Research Institute (BJRI), Dhaka. *Nigrospora sphaerica, Xylaria sp. Aspergillums fumigatus, Aspergillus niger* and *Penicillium oxalicum* were isolated as jute endophytic fungi in the Molecular Biology Lab, department of Biochemistry and Molecular Biology, University of Dhaka. *Rhizoctonia solani* was obtained from the Bangladesh Agricultural University. All the fungi were grown and maintained on potato dextrose agar (PDA) (HiMedia, India) at 28°C.

3.2.2 Antifungal dual culture assay

In-vitro bacterial-fungal dual-culture assays were established in both agar well diffusion and cross streak method in 9 cm diameter petri dish systems containing PDA medium (Balouiri, Sadiki et al. 2016). A 5-mm plug taken from the plate of an actively growing fungal colony was inoculated in one side of the petri dish. Fresh cells of *B. contaminans* NZ were either streaked in 3-cm length parallel lines on the other side of the fungal plug or 20µL of the overnight bacterial liquid culture was introduced into the 6 mm diameter well punched aseptically with a micropipette tip in agar plates. Control treatments with fungus only were also set up. The plates were incubated at 28° C for 4 to 5 days. After incubation, the diameter of the fungal colonies were scored and measured in mm.

The antifungal activity of *B. contaminans* NZ was determined against all studied phyto-pathogenic fungi and the toxicity was expressed as percentage of growth inhibition (PGI) and calculated according to Zygadlo et al. (Elshafie, Camele et al. 2012) formula,

$$PGI\ (\%) = 100\ (GC - GT)/GC$$

where GC represents the average diameter of fungi grown in PDA (control); GT represents the average diameter of fungi co-cultivated on the PDA dish with the antagonistic bacteria.

3.2.3 Microscopic analysis

In order to evaluate the morphological changes associated with volatile compounds, a dual culture assay was performed as previously described, and fungal samples of *M. phaseolina* was taken from the borders of inhibition zone showing high levels of inhibition. Photographs of fungal hyphae from control and *B. contaminans* NZ challenged were taken using a fluorescent microscope (EVOS FL, ThermoSci, USA) version 3.6.0 at (bright field,60x)

3.2.4 VOC test

3.2.4.1 Antagonistic assay of bacterial VOCs

Volatile compounds were detected by the method reported by Yuan et al (Yuan, Raza et al. 2012) with some media modifications. Two compartments of the three-part plates containing TSA media were inoculated with *B. contaminans* NZ, except for the control plates. Another compartment containing PDA medium was used for *M. phaseolina* to test growth inhibition. The plates were wrapped with parafilm and incubated at 28°C for 4 days. Production of volatile compounds was then determined based on inhibition of the radial growth of the fungi and the diameters of the fungal colony in control and treated plates.

3.2.4.2 Extraction of volatile compounds

Volatile compounds were extracted by the method reported by Jayaswal et al (Jayaswal, Fernandez et al. 1993). In order to collect the volatile compounds produced by *B. contaminans* NZ, it was inoculated on PDA plates with the fungus and the lids were replaced with a bottom plate that contained 3 g of sterile activated charcoal (Sigma). These two plates

were sealed with adhesive transparent tape and incubated at 28°C for 5 days. The experiments were performed in triplicates.

After the incubation, the activated charcoal was collected and washed with 5 ml of ethyl acetate to extract all trapped volatile compounds, which were then analyzed by gas chromatography-mass spectrometry (GC-MS).

3.2.4.3 GC -MS analyses

Ethyl acetate extract of extracellular components was subjected to GC-MS analysis using Perkin Elmer Clarus@6890 gas chromatograph with a Perkin Elmer Clarus@SQ8C mass detector connected with a capillary column Perkin Elmer, Elite-5 MS (60 m ×0.25 mm, film thickness 0.25 µm). 1 μL of the sample with a split ratio of 20:1 was injected. Helium was used as the carrier gas and the flow rate was set at 1 mL/min. The oven temperature set at 80°C (held for 5 min), was raised by 5°C per min to 272°C (held for 10 min). The analysis was carried out in the EI (electron impact) mode with 70e V of ionization energy. The analysis was performed in full scan mode, ranging from *m*/*z* 2 to 600. The compounds were detected after analyzing the mass spectrum of each component using the NIST98 library.

3.3 Results

3.3.1 Antagonistic activity of *B. contaminans* NZ in dual culture assay

When *M. phaseolina* was co cultured with *B. contaminans* NZ, clear inhibition of fungal mycelial growth was evident after the fourth day of incubation compared to the control treatments without bacteria. This inhibition persisted for more than sixteen weeks.

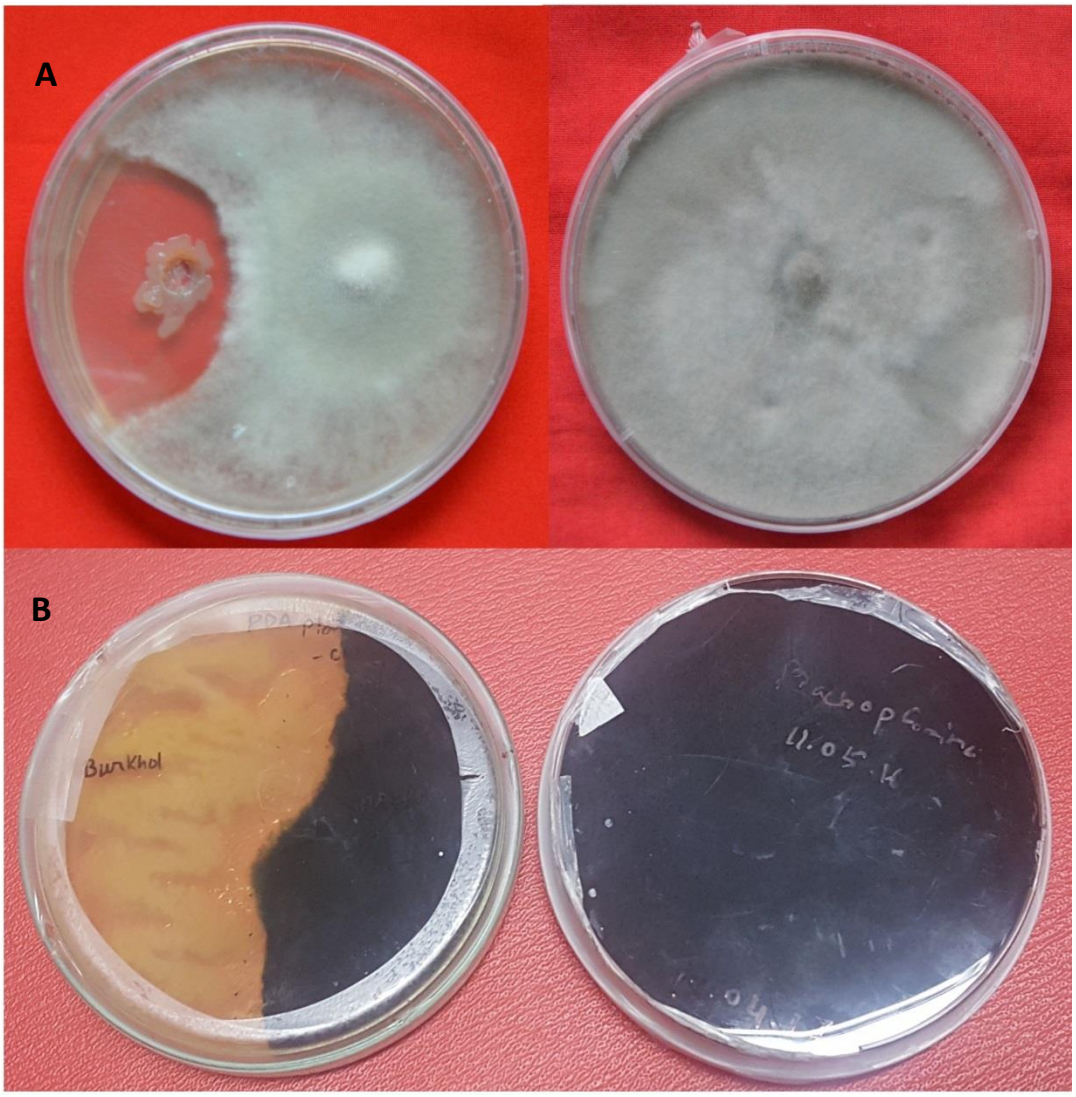

Figure 1: *In vitro* dual culture bacterial-fungal assay. (A) Agar well diffusion method: *M. phaseolina* challenged with *B. contaminans* NZ Vs control *M. phaseolina* monoculture (B) Inhibition of *B. contaminans* NZ against *M. phaseolina* by cross streak method.

B. contaminans substantially inhibited the growth of six other plant fungal pathogens in dual culture. These fungi are *Nigrospora sphaerica, Xylaria sp. Aspergillums fumigatus, Aspergillus niger, Penicillium oxalicum, Rhizoctonia solani*.

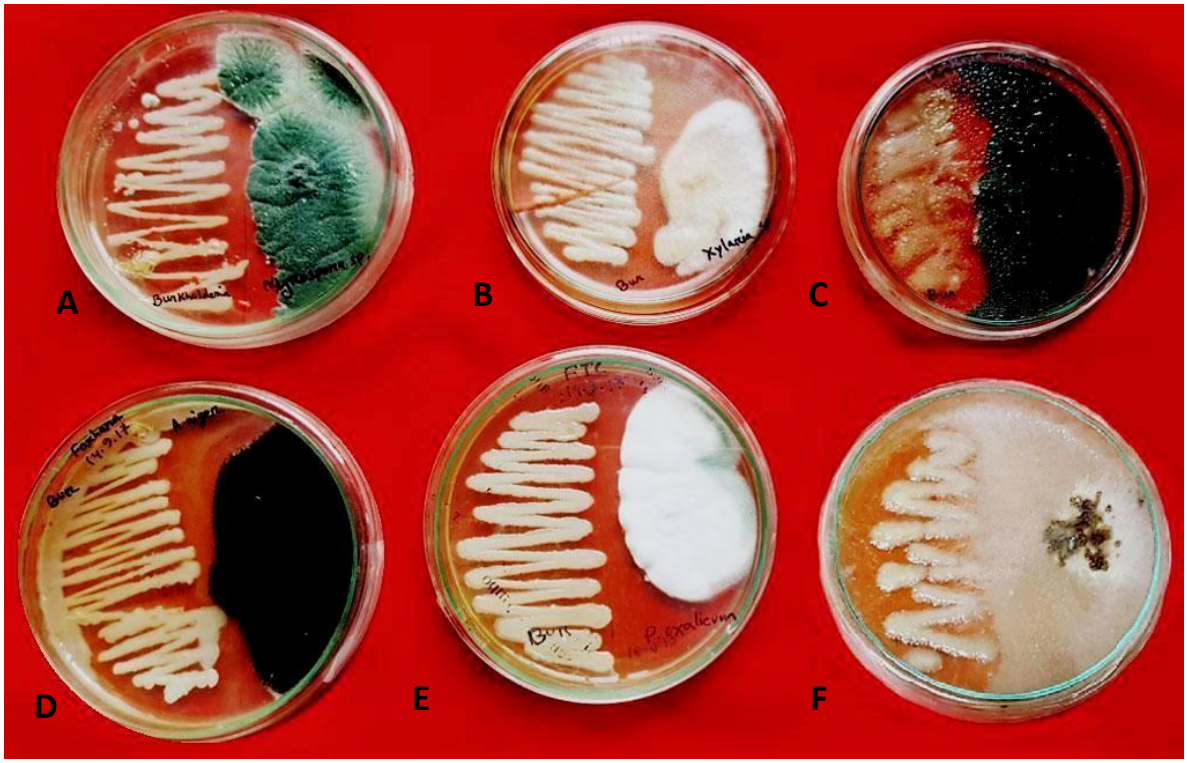

Figure 2: Antagonistic properties of *B. contaminans* NZ against other phyto-pathogenic fungi (A) *Nigrospora sphaerica* (B) *Xylaria sp.* (C) *Aspergillums fumigatus* (D) *Aspergillus niger* (E) *Penicillium oxalicum* (F) *Rhizoctonia solani*

B. contaminans NZ showed significant growth inhibition in front of the bacterial streak ranging from 44 to 58% in comparison to the control, when it was co-cultured with all the tested fungi in dual-culture assays. Growth inhibition rates observed for each fungal species are shown in Table 1.

Table 1: Growth inhibition rate

Fungus name	Growth inhibition (%) (Mean ± SD)
Xylaria.sp	57.71 ± 1.88
Aspergillus fumigatus	45.94 ± 1.45
Aspergillus niger	54.38 ± 4.10
Penicillium oxalicum	55.64 ± 2.96
Macrophomina phaseolina	49.04 ± 3.41
Nigrospora sphaerica	52.99± 3.67
Rhizoctonia solani	44.31 ± 4.36

3.3.2 Microscopic analysis

Microscopic analysis of *M. phaseolina* co-cultivated with *B. contaminans* was found to exhibit morphological changes as a consequence of the volatile organic compounds (VOCs) produced by *B. contaminans* NZ.

Structural changes in the mycelium of *M. phaseolina* were visible on the intersection zone between the inhibition areas generated by the diffusion of metabolites compared to the fungus control. *M. phaseolina* in presence of *B. contaminans* NZ had greater density of sclerotia characterized by a repeated branching pattern, hyphal swelling and hyphae intertwinement, which could be due to the compaction and collapse of the outer cells of the peripheral hyphae.

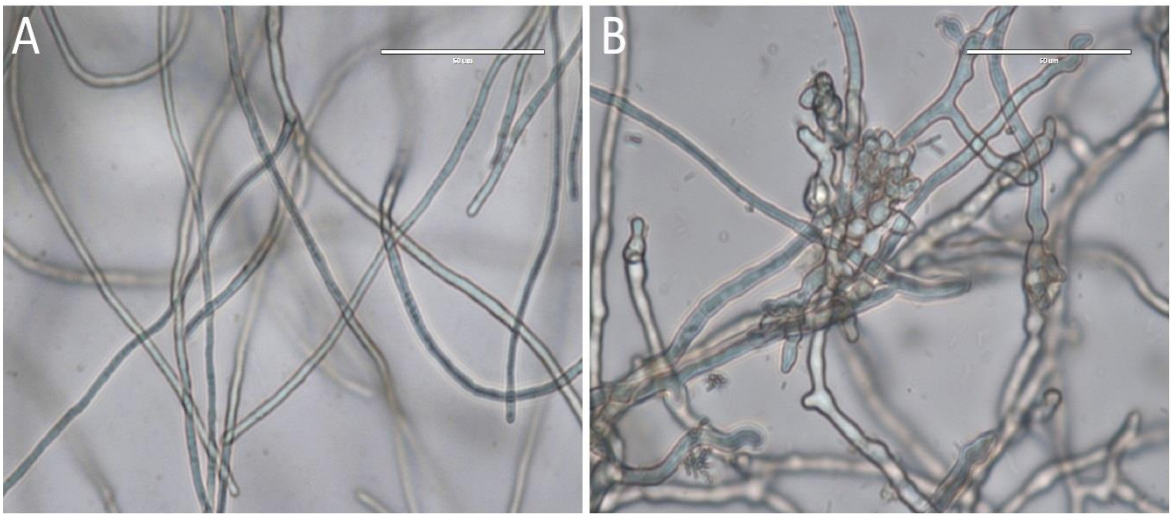

Figure 3: Microscopic analysis. (A) Control *M. phaseolina* having extended straight mycelia with normal branching and septation. (B) *M. phaseolina* challenged with *B. contaminans* NZ showing increased frequency of septa, branching and swollen mycelia.

3.3.3 Antagonistic assay of bacterial VOCs

VOC activities were checked in three compartment plates (Fig 4). Leading edges of *M. phaseolina* colonies failed to reach the petri plate perimeters in the presence of *B. contaminans* NZ in the different compartments of the petri plates relative to the no-bacteria control fungal plate. In this volatile inhibitor activity test, significant decrease in the radii of the pathogen colonies were observed (Fig 4) which clearly indicated that in contrast to the control, fungal growth was inhibited in the presence of bacteria through VOCs emitted by *B. contaminans* NZ.

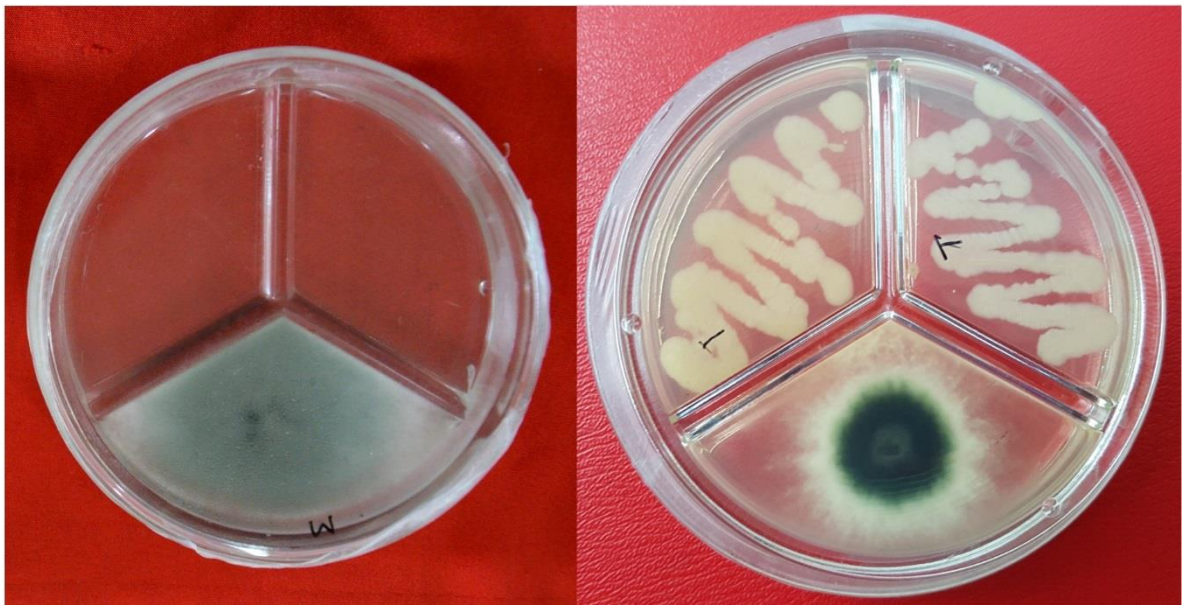

Figure 4: Inhibition of *M. phaseolina* by volatile compounds: volatile compound assay in three partition petri plates (A) Full growth of control *M. phaseolina* (B) Shorter leading edge of the fungal hyphae than that of control plates in presence of *B. contaminans* NZ, separated by partition.

3.3.4 Chemical analysis of volatile compounds

In order to identify the chemical nature of the volatile compounds, we used GC coupled to MS for *B. contaminans*. The volatile profile of this strain was compared against non-inoculated medium control profiles. Using sterile activated charcoal, we identified 25 volatile compounds, including 2-bromo-2-cyano-N, N-dimethylacetamide,S-[4-cyanophenyl]-N,N-dimethylthiocarbamate and isoproturon which are known for their antimicrobial activities suggesting that they have important roles in the antifungal property associated with this bacterium (Table 2, Fig 5).

Table 2: list of 25 compounds identified in GC-MS

Name	RT	MW	Formula
Benzyl alcohol alpha 1 dimethyl amino ethyl/ Methylephedrin	14.83	179	C11H17NO
Carbamic acid	17.26	261	C10H9O3NCl2
Ethyl 4 dimethyl thiocarbamoyloxy 3 methoxy 5 nitro cinnamate	17.65	354	C15H18O6N2S
DIMETHYLAMINO]PROPYL]AMINO-4-[TRICHLOROMETHYL	19.56	402	C17H21ON4Cl3
LEVACETYLMETHADOL	21.4	353	C23H31O2N
2-[[2-[DIMETHYLAMINO]PROPYL]AMINO-4-[TRICHLOROMETHYL]-6-[.ALPHA.,.AL	22.49	489	C16H17N5Cl6
1,4-BENZENEDITHIOL, 2,5-BIS(ETHOXYCARBONYL)-, BIS(DIMETHYLTHIOCARBAMIDE	23.9	428	C18H24O6N2S2
NORVALINE, ETHYL ESTER/ L-VALINE, ETHYL ESTER	24.31	145	C7H15O2N
L-VALINYL-S-BENZYL-L-CYSTEINE, BENZYL(ESTER	26.33	400	C22H28O3N2S
2,4-DIAMINO-5,6,7,8-TETRAHYDRO-5-PHENYL[1]BENZOTHIENO[2,3-D]PYRIMIDINE	27.61	296	C16H16N4S
N N-dimethyl-3 4-methylenedioxyamphetamine	28.48	207	C12H17NO2
5H-FURO[2,3-C]PYRAN-3-CARBONITRILE, 2-AMINO-5,5-DIMETHYL	31.2	192	C10H12O2N2
BAMBUTEROL, N-TRIFLUOROACETYL-O-TRIMETHYLSILYL DERIV	32.31	535	C23H36F3N3O6Si
3,4-METHYLENEDIOXY-N-ETHYLAMPHETAMINE	36.03	207	C12H17NO2
ISOPROTURON	38.72	206	C12H18N2O
Amphibine I	41.53	406	C21H34N4O4
Formic acid, 1-dimethylamino, [2-methoxy-4-(3-oxobutyl)]phenyl ester	43.36	265	C14H19NO4
ATENOLOL, TBDMS DERIVATIVE	44.11	380	C20H36O3N2Si
2-BROMO-2-CYANO-N,N-DIMETHYLACETAMIDE	45.31	190	C5H7ON2Br
S-(4-Cyanophenyl) dimethylthiocarbamate	45.57	206	C10H10N2OS
5H-DIBENZ(B,F)AZEPINE, 10,11-DIHYDRO-5-(2'-(N,N-DIMETHYLAMINO)-2'-MET	46.39	280	C19H24N2
S-[4-CYANOPHENYL]-N,N-DIMETHYLTHIOCARBAMATE	47.13	206	C10H10N2OS
Formic acid, 1-dimethylamino, [2-methoxy-4-(3-oxobutyl)]phenyl ester	48.43	265	C14H19NO4
ACEBUTOLOL	49.79	336	C18H28O4N2
2-ACETYLAMINO-3-(3,4,5-TRIMETHOXYPHENYL)-N,N-DIMETHYLPROPENAMIDE	51.662	322	C16H22O5N2

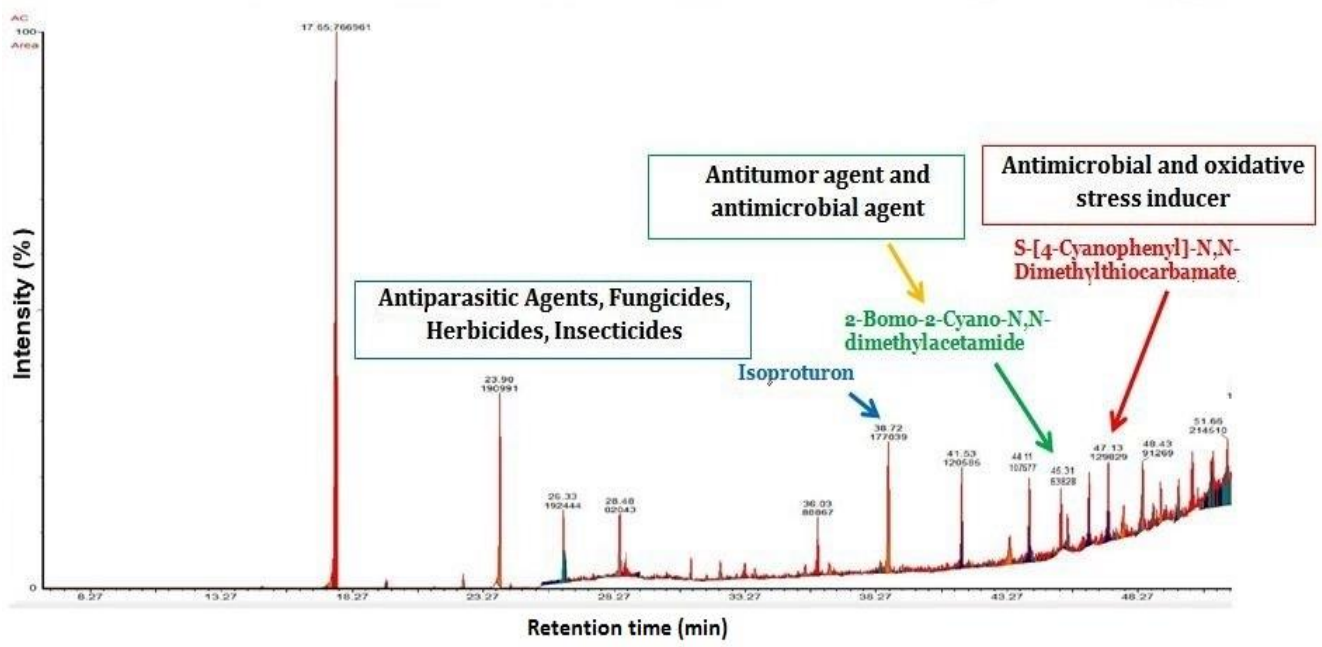

Figure 5: Important peaks identified in *B. contaminans* NZ volatome by GC-MS

3.4 Discussion

Concerns regarding human health and the environment have led to reduced use of agrochemicals and the development of sustainable agriculture. Thus we see an increased number of biological control studies as the focus of investigations which reflects the desire to develop sustainable methods for plant disease control (Mota, Gomes et al. 2017) . However, efficient antagonists must be obtained for biological control to become a reality. Different *Burkholderia* species have been reported to protect crops against phyto-pathogens through the production of a number of different antifungal substances (Li, Quan et al. 2007).

B. phenazinium, *B. megapolitana*, and *B. bryophila*, are all reported to possess antifungal activities and *B. phenazinium* has been found to produce phenazineiodinin, which has both anti-microbial and cytotoxic activities (Eberl and Vandamme 2016). *B. cepacia* complex has been reported to produce pyrrolnitrin which is an inhibitor of the electron transport system

(Schmidt, Blom et al. 2009). Another *Burkholderia* species *B. gladioli* strain NGJ1 has recently been reported to demonstrate antifungal activity against *R. solani* and various other fungi through mycophagy.

In this chapter, we report that *B. contaminans* NZ is an extremely versatile biocontrol agent capable of suppressing the growth of a number of plant-pathogenic fungi (Fig 1, 2). We also found that *B. contaminans* NZ can antagonize these fungi under a broad range of temperature and pH.

B. contaminans NZ causes marked morphological changes of *M. phaseolina*. Excessive hyphal branching with swollen and balloon shaped cells close to the bacterial colonies was noticeable under a microscope (Fig 3). Such morphological changes in the cell membrane have also been reported for *F. solani* and *C. dematium* following inhibition by *B. cepacia* (Narayanasamy 2013). *Burkholderia* CF66I has been found to reduce hyphal extension rates and induce profound changes in cell morphology resulting in swelling and formation of very short hyphae with multiple branches in *R. solani* (Quan, Zheng et al. 2006).

Various reports have demonstrated that volatile organic compounds (VOCs) produced by some bacteria can influence the growth of fungi (Wheatley 2002, Schalchli, Hormazabal et al. 2011). *Burkholderia gladioli* pv. agaricicola strain is known for producing volatile organic compounds (VOCs), which inhibit the growth of *F. oxysporum* and *R. solani*.

This study identified twenty-five volatile compounds emitted by *B. contaminans* NZ. Among them ethyl 4 dimethyl thiocarbamoyloxy 3 methoxy 5 nitro cinnamate is a derivative of cinnamic acid which possesses antibacterial, antifungal and parasite combating abilities (Ferenc, Sadowski et al. 2013), 1,4-benzenedithiol, 2,5-bis(ethoxycarbonyl)-, bis(dimethylthiocarbamide ($C_{18}H_{24}O_6N_2S_2$), a derivative of bis (ethoxycarbonyl) methylthio, is reported for its antimicrobial and antioxidant activities (Padmavathi, Reddy et al. 2011) and 5h-furo[2,3-c]pyran-3-carbonitrile, 2-amino-5,5-dimethyl, a pyran derivative used for

drug synthesis is known for its antimicrobial, mutagenic, anti-proliferative, and antitumor activities (Sandaroos, Nazif et al. 2015).

B. contaminans NZ also emits ethyl ester norvaline which is a component of novel heterocyclic compounds with moderate activity against bacteria and fungi (Sankar, Kumar et al. 2007). Another volatile compound found in GC-MS analysis is 2,4-diamino-5,6,7,8-tetrahydro-5-phenyl[1]benzothieno[2,3-d]pyrimidine, a thienopyrimidine derivative having anticancer, antiviral, antitumor, anti-inflammatory and antimicrobial activities (El-Gazzar, Hussein et al. 2007).

B. contaminans NZ emits another significant volatile compound, S-[4-cyanophenyl]-N,N-dimethylthiocarbamate which is a derivative of N,N dimethyldithiocarbamate, a component of an useful insecticide cyanofenphos and other antimicrobial compositions. It is also a potent oxidative stress inducer that can lead to oxidative stress and fungal tissue injury by inhibiting superoxide dismutase (SOD) (Zhu, Wang et al. 2013). Multiple studies have shown that inhibition of Cu, Zn SOD by N, N -diethyldithiocarbamate leads to oxidative stress and tissue injury. It should be noted that N, N - diethyldithiocarbamate may also affect other enzymes and cause oxidative stress through different mechanisms. These chemical properties and biological activities are utilized in pesticidal, industrial, and therapeutic applications (Zhu, Wang et al. 2013). This finding is specifically important because when the total proteome of *M. phaseolina* under *B. contaminans* NZ stress was analyzed (Chapter 5), an array of proteins involved in oxidative stress response in *M. phaseolina* was found to be down regulated. Finding a oxidative stress inducer as a volatile compound suggests that s-[4-cyanophenyl]-n,n-dimethylthiocarbamate extruded by *B. contaminans* NZ may inhibit the enzymes responsible for combating oxidative stress leading to tissue injury and growth inhibition of *M. phaseolina.*

From the volatile compound profile of *B. contaminans* NZ we found two other important compounds that are known for their antimicrobial activities. 3-(4-isopropylphenyl)-1,1-dimethylurea industrially known as isoproturon is a pesticide and more precisely a herbicide. It is known for its anthelmintics, antiparasitic, fungicidal activity too [ref: http://www.phenomenex.com/Compound?id=3-%284-Isopropylphenyl%29-1%2C1-Dimethylurea].

Lastly the compound 2-bromo-2-cyano-n,n-dimethylacetamide has been reported as a antitumor and a antimicrobial agent (Steiner and Himwich 1964).

[ref: https://patents.google.com/patent/US20020147235].

It can therefore be reasonably argued that many of the compounds found in the volatome of *B. contaminans* NZ could be responsible for its antifungal activity, in agreement with the work carried out by other researchers.

In addition, we do not exclude the possibility that other molecules beyond volatile compounds could also be involved in causing morphological changes and growth arrest of the phytopathogen. In this regard, sequence analysis of *B. contaminans* genome (discussed in chapter 4) led to the identification of gene clusters responsible for the biosynthesis of antifungal compounds.

Furthermore, cellulase, lipase and protease enzymes may also contribute to growth inhibition of the pathogenic fungi through degradation of their cell walls. Strong protease activity found for *B. contaminans* NZ (chapter 2) along with the secondary metabolites may play a major role in inhibiting pathogens. Bacterial proteases are also known to play a significant role in mycoparasitism (Rabha, Sharma et al. 2016).

Production of siderophores is an additional mode of action for fungal growth inhibition by endophytic bacteria (Vurukonda, Giovanardi et al. 2018). *B. contaminans* NZ was evaluated

for siderophore production and found positive for such ability. Thirty siderophore related genes were also found to be present in the *B. contaminans* NZ genome.

Overall, our findings indicate that *B. contaminans* NZ a proficient antagonist of phyto-pathogenic *M. phaseolina* and other fungi, exerts its antifungal activity through the volatile compounds it produces.

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CHAPTER 4

Characterization of *B. contaminans* NZ as non-pathogenic safe bioinoculant through whole genome analysis

Pages 58 - 80

Characterization of *B. contaminans* NZ as non-pathogenic safe bioinoculant through whole genome analysis

4.1 Overview

This study has identified *Burkholderia contaminans* NZ to have significant growth promotion activity and strong antagonistic activity against several plant pathogenic fungi, making it an eligible bio-control and bio-fertilizer candidate in agriculture. Even with tremendous biotechnological potential of the *Burkholderia* genus, their agricultural and industrial use is severely limited due to the potential threat that some strains pose to human health. A group of 20 closely related bacterial species in particular, referred to as the *Burkholderia cepacia* complex (Bcc), which have emerged as opportunistic pathogens that can cause severe infections in cystic fibrosis (CF) and immunocompromised patients (De Smet, Mayo et al. 2015). It can cause a severe decline in lung function possibly by causing a life-threatening systemic infection (Scoffone, Chiarelli et al. 2017).

However, the number of novel *Burkholderia* species that exhibit plant-beneficial properties and isolated from the natural environment, often from soil samples or from the rhizosphere of various plants has increased vastly over the past few years (Eberl and Vandamme 2016). The use of *Burkholderia* in agricultural applications is therefore considered a double-edged sword, and a lot of effort has been invested into discriminating between the beneficial environmental and the clinical *Burkholderia* strains (Mahenthiralingam, Baldwin et al. 2008). Recently some

species are believed to be safe for applications, as there are very rarely clinical reports of posing a risk to human health.

Therefore, before the use of *B. contaminans* NZ in agriculture as a plant growth promotion and biocontrol agent, it is necessary to establish its safety.

Analysis of the complete genome sequence and a study of the phylogenetic relatedness of *B. contaminans* NZ to 12 other *Burkholderia* species including plant growth-promoting, cystic fibrosis opportunistic and plant pathogenic strains provide important information regarding the safety of *Burkholderia contaminans* NZ as a PGPR and bio-control agent. This is based on their capacity to produce siderophore and secondary metabolites and the absence of virulence genes. Moreover, *B. contaminans* NZ is not a member of genomovar III of the *B. cepacia* complex which contains the highly epidemic strains isolated from patients with cystic fibrosis (Mahenthiralingam, Vandamme et al. 2001)

Details of the present study establish *B. contaminans* NZ as a harmless/safe alternative for hazardous chemical fertilizer and fungicide.

4.2 Methods and materials

4.2.1 Genome sequencing of *B. contaminans* NZ

B. contaminans NZ was cultured overnight in TSB medium (Himedia, India) in a shaker at 37°C. The genomic DNA was extracted using the GenElute™ Bacterial Genomic DNA Kit (Sigma, Germany). DNA quality and quantity were assessed by using Nanodrop 2000.

(Thermo Fisher Scientific, Wilmington, DE, United States). The purified DNA was delivered to the sequencing facilities by using the DNAstable R Plus (Biomatrica, San Diego,CA, United States)

A genomic library was constructed and employed for 300-bp paired-end whole-genome sequencing using an Illumina MiSeq platform (Illumina, San Diego, CA, USA) according to the manufacturer's instructions at the Genome Research Institute of North South University, Dhaka, Bangladesh. The total generated raw reads (10x coverage) were assembled using SPAdes version 3.11 (Bankevich, Nurk et al. 2012). The scaffold generated was mapped and ordered using ABACAS (Assefa, Keane et al. 2009) and included the reference genome of *B. contaminans* CH1 (GenBank accession no AP018357 to AP018360 (four entries)). Structural gene prediction and functional annotation were performed using the Rapid Annotations using Subsystems Technology (RAST) server (Aziz, Bartels et al. 2008).

4.2.2 Data analysis and Bioinformatics tool

Gene clusters for the biosynthesis of secondary metabolites were identified by using antiSMASH v.4.0.1 (Weber, Blin et al. 2015). Assembly and annotation CGview tools were used to generate the map of circular genomes (Grant and Stothard 2008).

Gene function prediction was performed by the SEED database [23] followed by the annotation of the complete genome of *B. contaminans* strain NZ using the (RAST) server (http://rast.nmpdr.org) (Aziz, Bartels et al. 2008). SEED also provides subsystems (collections of functionally related protein families) and their derived FIGfams (protein families), which represent the core of the RAST annotation engine (Overbeek, Olson et al. 2013).

Function and pathway analysis were performed using KEGG (Kyoto encyclopedia of genes and genomes; http://www.genome.jp/kegg/) database (Kanehisa and Goto 2000). Genome annotations through PIFAR annotation tool was performed for the identification of plant-associated bacteria and genetic factors involved in bacterial interactions with plant-hosts (Martínez-García, López-Solanilla et al. 2016). The average nucleotide identity (ANI) (Richter and Rosselló-Móra 2009) was calculated by a script developed by Kostas's lab

(http://enve-omics.gatech.edu/), considering a minimum length of 700 bp and a minimum identity of 70%. The fragment options were set to 1000 bp for window size and 200 bp for step size.

4.2.3 Comparison with genomes of other *Burkholderia* strains

Twelve previously sequenced *Burkholderia* strains were selected for comparative genome analysis based upon their characteristics and distinctive biological properties (Deng, Wang et al. 2016). The selected strains, their characteristics and accession numbers are given in table 1.

Table 1. List of strains used in comparative analysis.

***Burkholderia* strain**	**Features**	**Accession no**
B. contaminans CH1	Plant growth promoting (Choi, Sugiura et al. 2017)	AP018357, AP018358, AP018359, AP018360
B. contaminans MS14	Plant growth promoting (Deng, Wang et al. 2016)	CP009743, CP009744, CP009745
B. ambifaria AMMD	Plant growth promoting (Coenye, Mahenthiralingam et al. 2001)	NC_008390.1, NC_008391.1, NC_008392.1
B. lata 383	Plant growth promoting (Vanlaere, Baldwin et al. 2009)	NC_007510.1, NC_007511.1, NC_007509.1
B. oklahomensis EO147	Opportunistic pathogens (Glass, Steigerwalt et al. 2006)	CP008726.1, CP008727.1
B. pseudomallei K96243	Opportunistic mammalian pathogens(Glass, Steigerwalt et al. 2006)	NC_006350.1, NC_006351.1
B. vietnameinsis LMG 10929	Opportunistic mammalian pathogens (LiPuma, Dulaney et al. 1999)	CP009631.1, CP009630.1, CP009632.1
B. multivorans ATCC 17616	Opportunistic mammalian pathogens(Biddick, Spilker et al. 2003)	NC_010804.1, NC_010805.1, NC_010801.1
*B. cenocepacia*J2315	Opportunistic mammalian pathogens (Holden, Seth-Smith et al. 2009)	NC_011000.1, NC_011001.1, NC_011002.1

B. mallei ATCC 23344	Opportunistic mammalian pathogens (Ulrich and DeShazer 2004)	NC_006348.1, NC_006349.2
B. gladioli BSR3	Plant-pathogen (Seo, Lim et al. 2011)	NC_015381.1, NC_015376.1
*B. glumae*BGR1	Plant-pathogen (Lim, Lee et al. 2009)	NC_012724.2, NC_012721.2

4.2.4 Phylogenetic Analysis

Phylogenetic analyses based on 16s rRNA was used to evaluate the taxonomic affiliation of *B. contaminans* NZ. To construct the phylogenetic tree, 16s rRNA sequences of NZ, were searched through nucleotide blast and the top hits of the reference strains were downloaded as a aligned FASTA format and phylogenetic and molecular evolutionary analyses were conducted by using MUSCLE (Multiple Sequence Comparison by Log- Expectation) tool (Edgar 2004) by neighbor-joining method.

4.2.5 Data Deposition

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession QRBC00000000. The version described in this paper is version QRBC01000000.

4.3 Results

4.3.1 Whole genome data analysis

4.3.1.1 Overview of *B. contaminans* NZ genome

Whole genome of *B. contaminans* NZ was sequenced using Illumina MiSeq technology and a final assembly of 2099 scaffolds resulting in 87,42,352 bases was obtained. The genome was found to consist of three circular chromosomes (3740964 bp, 3513214 bp, 1488174 bp respectively) with an average of 66.6% GC, 8075 protein coding DNA sequences (CDS) and 76 tRNAs and no plasmid.

Organism Overview for Burkholderia sp (292.128)

Genome	Burkholderia sp (Taxonomy ID: 292)
Domain	Bacteria
Taxonomy	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia; Burkholderia cepacia complex; Burkholderia sp
Neighbors	View closest neighbors
Size	8,742,352
GC Content	66.6
N50	6566
L50	403
Number of Contigs (with PEGs)	2099
Number of Subsystems	534
Number of Coding Sequences	8075
Number of RNAs	76

Figure 1: General overview of the whole genome data of *B. contaminans* NZ

Circular chromosome and GC skew were computed using CGViewer Server.

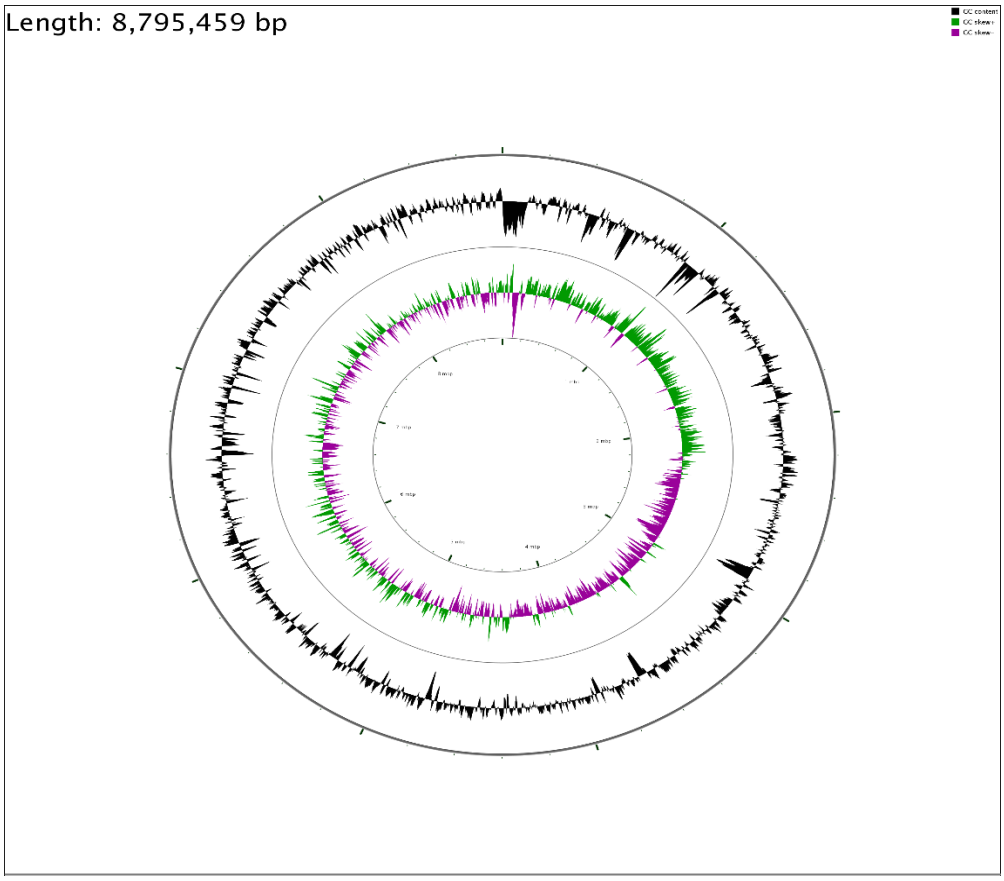
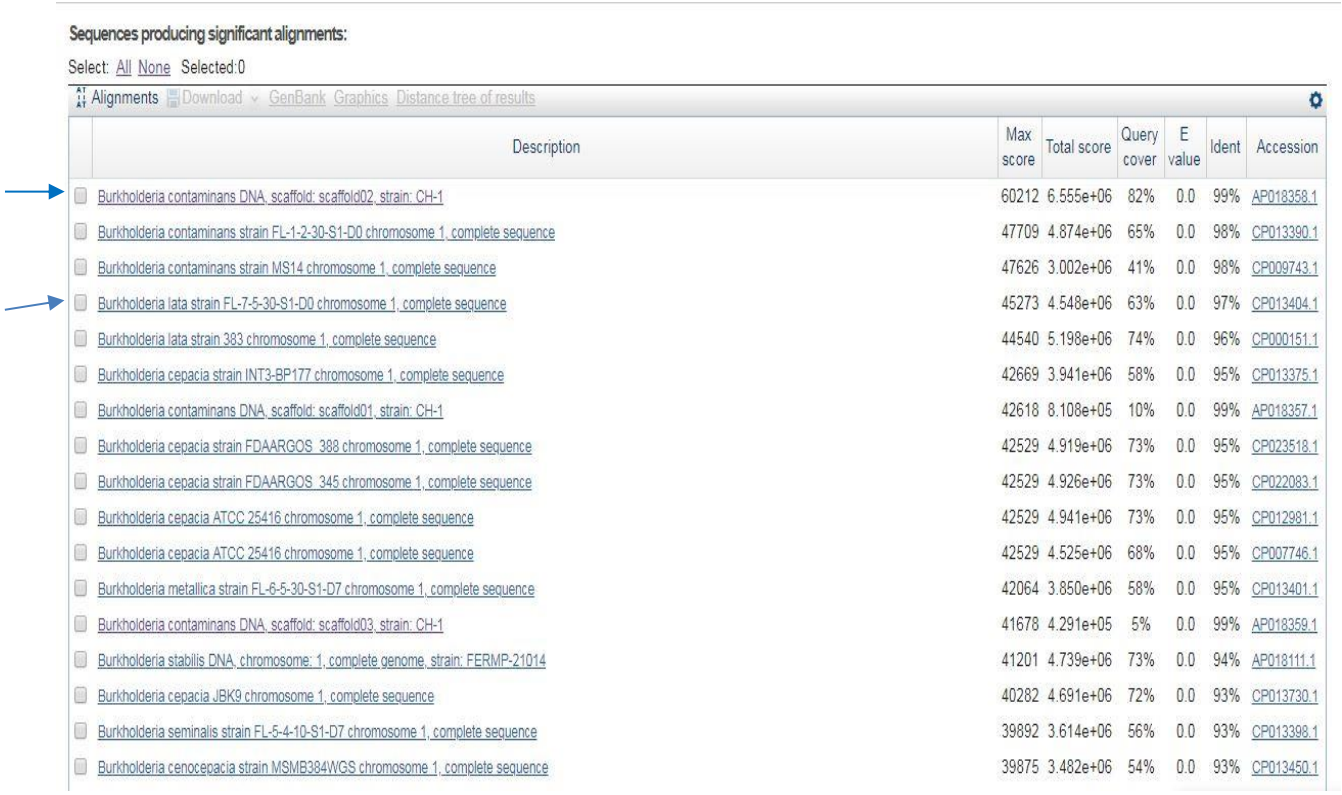


Figure 2: Circular representation of the *B. contaminans* NZ genome.

Rings from outside to inside: (1) GC content (black), (2) GC skew (purple and green)

4.3.1.2 Blast search and AntiSMASH analysis

According to the nucleotide BLAST (BLASTn), *B. contaminans* NZ chromosome has a maximum of 99% similarity with *B. contaminans* strain CH-1.



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Burkholderia contaminans DNA, scaffold: scaffold02, strain: CH-1	60212	6.555e+06	82%	0.0	99%	AP018358.1
Burkholderia contaminans strain FL-1-2-30-S1-D0 chromosome 1, complete sequence	47709	4.874e+06	65%	0.0	98%	CP013390.1
Burkholderia contaminans strain MS14 chromosome 1, complete sequence	47626	3.002e+06	41%	0.0	98%	CP009743.1
Burkholderia lata strain FL-7-5-30-S1-D0 chromosome 1, complete sequence	45273	4.548e+06	63%	0.0	97%	CP013404.1
Burkholderia lata strain 383 chromosome 1, complete sequence	44540	5.198e+06	74%	0.0	96%	CP000151.1
Burkholderia cepacia strain INT3-BP177 chromosome 1, complete sequence	42669	3.941e+06	58%	0.0	95%	CP013375.1
Burkholderia contaminans DNA, scaffold: scaffold01, strain: CH-1	42618	8.108e+05	10%	0.0	99%	AP018357.1
Burkholderia cepacia strain FDAARGOS_388 chromosome 1, complete sequence	42529	4.919e+06	73%	0.0	95%	CP023518.1
Burkholderia cepacia strain FDAARGOS_345 chromosome 1, complete sequence	42529	4.926e+06	73%	0.0	95%	CP022083.1
Burkholderia cepacia ATCC 25416 chromosome 1, complete sequence	42529	4.941e+06	73%	0.0	95%	CP012981.1
Burkholderia cepacia ATCC 25416 chromosome 1, complete sequence	42529	4.525e+06	68%	0.0	95%	CP007746.1
Burkholderia metallica strain FL-6-5-30-S1-D7 chromosome 1, complete sequence	42064	3.850e+06	58%	0.0	95%	CP013401.1
Burkholderia contaminans DNA, scaffold: scaffold03, strain: CH-1	41678	4.291e+05	5%	0.0	99%	AP018359.1
Burkholderia stabilis DNA, chromosome: 1, complete genome, strain: FERMP-21014	41201	4.739e+06	73%	0.0	94%	AP018111.1
Burkholderia cepacia JBK9 chromosome 1, complete sequence	40282	4.691e+06	72%	0.0	93%	CP013730.1
Burkholderia seminalis strain FL-5-4-10-S1-D7 chromosome 1, complete sequence	39892	3.614e+06	56%	0.0	93%	CP013398.1
Burkholderia cenocepacia strain MSMB384WGS chromosome 1, complete sequence	39875	3.482e+06	54%	0.0	93%	CP013450.1

Figure 3: Blast analysis result identified the bacterium as *B. contaminans* and with second closest similarity to *B. lata*

At least 85% of the annotated CH-1genes are also present in our assembled contigs. Nucleotide BLAST has been performed between the CH-1genes against the assembled contigs and 6380 genes were identified in the contigs with an e value of at least 1e-10.

B. contaminans NZ also has significant similarity with the *B. lata* strain FL-7-5-30-S1-D0. This is not surprising since both belonged to the taxon K of the Bcc prior to their relocation as two different species.

AntiSMASH analysis revealed 19 clusters for the biosynthesis of secondary metabolites (Table 2). Among them, biosynthetic clusters were identified for D-alanine-poly (phosphoribitol) ligase, terpene, type I polyketide synthase, beta-ketoacyl synthase and non-ribosomal peptide synthetase.

Table 2. Secondary metabolite biosynthesis gene cluster of NZ

Antismash result
Gene Cluster 1: (NRPS): D-alanine--poly(phosphoribitol) ligase subunit DltA [*Burkholderia contaminans*]
Gene Cluster 2: (NRPS): D-alanine--poly(phosphoribitol) ligase subunit DltA [*Burkholderia contaminans*]
Gene Cluster 3: (0ther) MULTISPECIES: amino acid adenylation protein [*Burkholderia*]
Gene Cluster 4: (Type 1 PKS) type I polyketide synthase, partial [*Burkholderia contaminans*]
Gene Cluster 5: (t1pks-nrps) KR domain-containing protein [*Burkholderia contaminans*]/ type I polyketide synthase, partial
Gene Cluster 6: (Type = terpene)
Gene Cluster 7: (Type = terpene)
Gene Cluster 8: (Type = other)
Gene Cluster 9: (Type = terpene)
Gene Cluster 10: (Type = t1pks) MULTISPECIES: type I polyketide synthase [*Burkholderia*]
Gene Cluster 11: Type = cf_fatty_acid.
Gene Cluster 12: Type = terpene
Gene Cluster 13: Type = terpene
Gene Cluster 14: (Type = other) D-alanine--poly(phosphoribitol) ligase subunit DltA [*Burkholderia contaminans*]
Gene Cluster 15: (Type = nrps) MULTISPECIES: non-ribosomal peptide synthetase [*Burkholderia*]
Gene Cluster 16: Type = other. MULTISPECIES: KR domain-containing protein
Gene Cluster 17: Type = other beta-ketoacyl synthase [*Burkholderia contaminans]*
Gene Cluster 18: Type = other non-ribosomal peptide synthetase [*Burkholderia* sp]
Gene Cluster 19: Type = nrps non-ribosomal peptide synthetase [*Burkholderia*]

4.3.2 Functional analysis of the genome sequence

The complete genome of *B. contaminans* NZ was annotated using the SEED database and gene functions were predicted by the Rapid Annotations using Subsystems Technology (RAST) server (http://rast.nmpdr.org) providing the mapping of genes to subsystems.

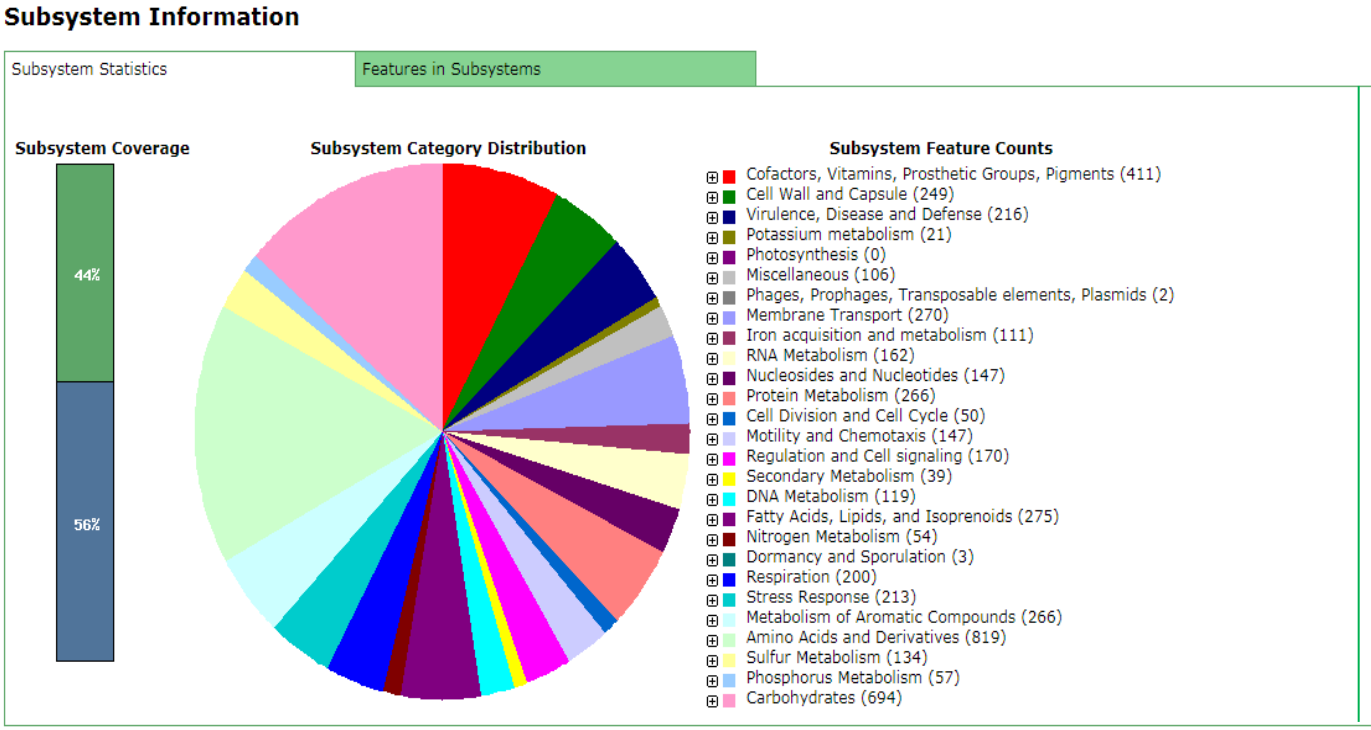


Figure 3: Mapping of *B. contaminans* NZ genes to subsystems

Functional analysis was also performed using KEGG which identified 2059 genes involved in one of the many metabolic pathways (Figure 4). Among the genes classified under the KEGG pathway categories, the largest number was found to be involved in the metabolism of carbohydrate (18%) and amino acids (18%), biosynthesis of antibiotic and secondary metabolites (11%), transport and cellular community (10%) [ABC transporters 6.31%, phosphotransferase systems (PTS) 0.243%, secretion systems 2.28%] and cofactors and vitamins (9%). Most of the remaining genes are involved in processes related to translation

and transcription (8%), lipid metabolism (6%), and signal transduction (5%) [two component systems 4.37%].

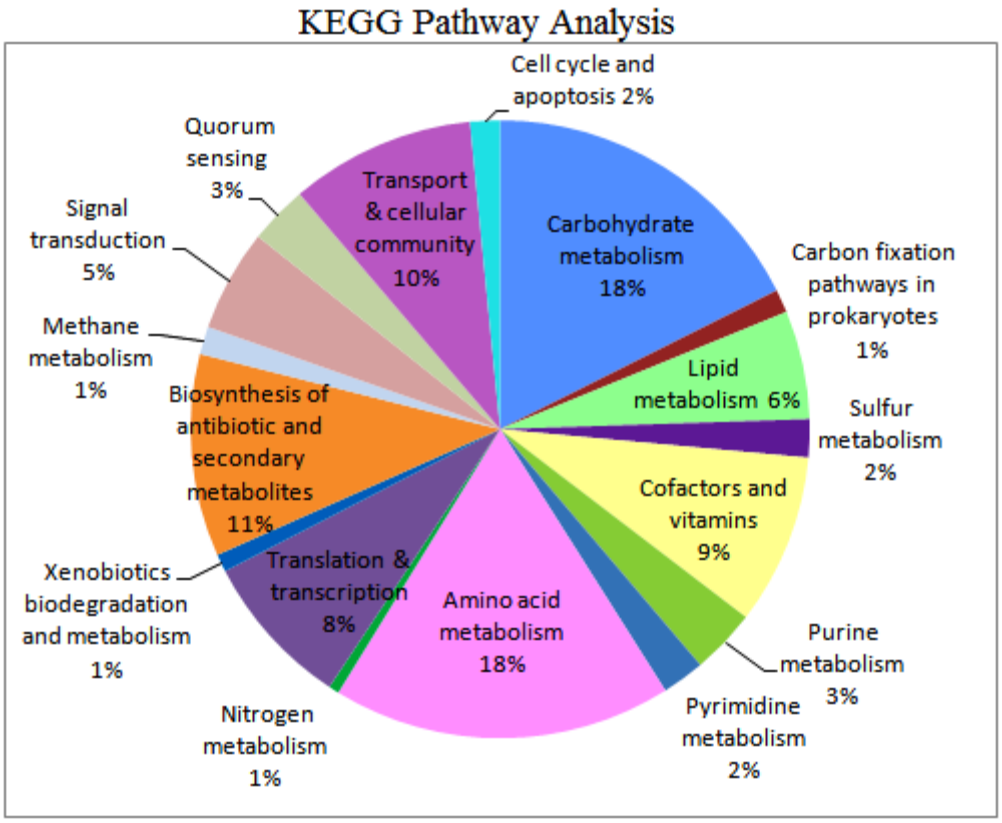


Figure 4: KEGG pathway analysis of *B. contaminans* NZ genes

B. contaminans NZ carry putative genes responsible for characteristics related to plant growth promotion. As observed for other PGPR *Burkholderia* strains (Parra-Cota, Peña-Cabriales et al. 2014), the nifHDK operon required for nitrogen fixation was detected in *B. contaminans* NZ along with 1-aminocyclopropane-1-carboxylate (ACC) deaminase coding sequence, gene coding for iron(III) ABC transporter substrate-binding protein, phytoene synthase gene, coding sequence for phosphotransferase system (PTS) and major facilitator superfamily (MFS) transporter gene. *In silico* analysis using RAST also revealed the presence of siderophore biosynthesis gene cluster and genes like indole acetamide hydrolase involved in the production of indole acetic acid (IAA), a plant hormone associated with plant growth

(Esmaeel, Sanchez et al. 2018). In addition, the genome sequence indicates the presence of pyrroloquinolinequinone synthase and glucose dehydrogenase implicated in the production of gluconic acid and 2-ketogluconic acid, required for mineral phosphate solubilization. The annotated genome also has genes related to 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which potentially plays a role in promoting plant growth.

4.3.3 Distinguishing between plant growth-promoting and pathogenic bacteria

Pathogenic *Burkholderia* species including CF opportunistic isolates have more virulent genes like biosynthesis genes for cable pili, toxoflavin, O-antigen (O-Ag) biosynthetic cluster and the type VI secretion system VgrG-5 protein, necessary for initiating and causing an opportunistic infection. But all these virulence related genes are absent in NZ.

Table 3: List of antibiotic biosynthesis and virulent genes and their presence or absence in *B. contaminans* NZ genome

Properties	Name	Biosynthesis Gene	Presence
Antibiotic and siderophore	Occidiofungin	OcfD-ocfJ	--
	Pyrrolnitrin	prnA-prnD	++
	Pyochelin	pchR	++
	Ferric siderophore transport	pchD-pchA	++
	ABC-type siderophore export system	FeoB	++
	Siderophore Pyoverdine	Pvd	++
Virulence metabolics	Cepacian	bceA-bceK, bceN-bceT	--
	Toxoflavin	toxR, toxA-toxE	--
	Hydrogen Cyanide	hcnA-hcnC	--
	2-heptyl-3-hydroxy-4(1H)-quinolone	pqsA-pqsE	--
	Type VI secretion system	VgrG	--
	Cable pili gene	cblA	--
	Zinc metaloprotease	Zmp	--
	Cu2+ and Zn2+ containing periplasmic SOD	apaH-reG	++
	O-antigen (OAg) biosynthetic cluster	OAg	--

By screening the bacterial sequences through PIFAR (Plant–bacteria interaction factors resource) annotation tool, no phytotoxin and biofilm biosynthesis genes or fungal plant cell wall-degrading enzymes were found suggesting harmless bacterial interactions with the plant-hosts.

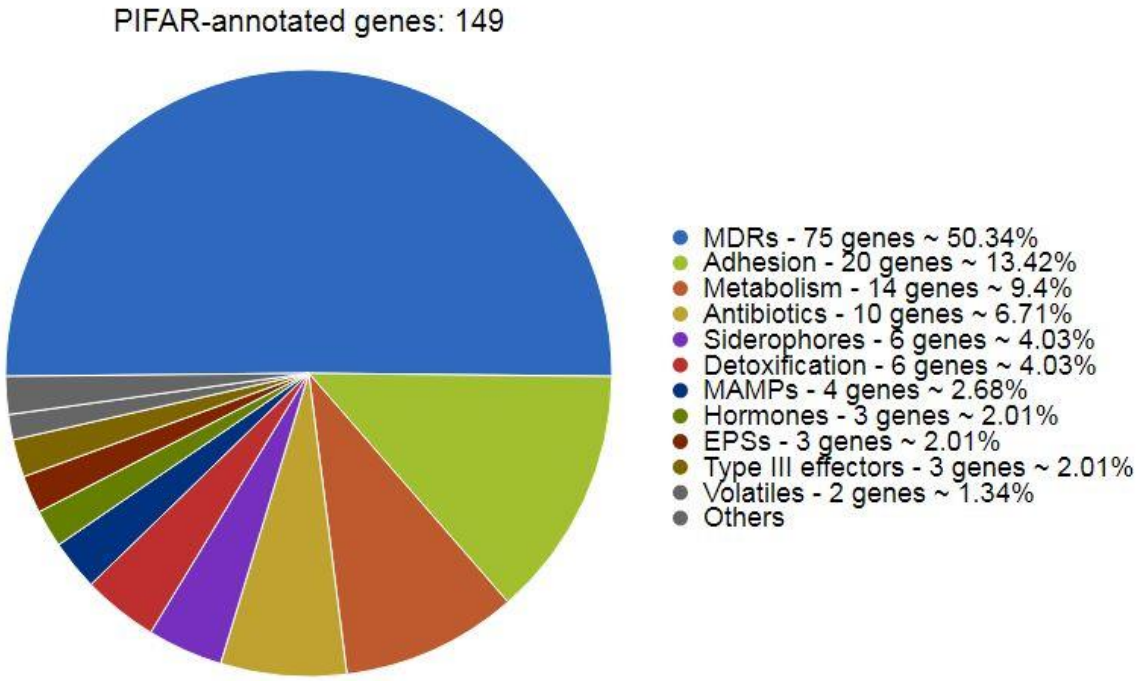


Figure 5: Genes involved in bacterial interactions with plant-hosts.

From this annotation data it was also found that *B. contaminans* NZ majorly possesses multiple drug resistance (MDRs) gene families containing 75 genes that is ~ 50.34% of all the plant–bacteria interaction factors available till date. This suggests that this elaborate MDR mechanism found in *B. contaminans* NZ helps it to adapt and survive in different environments (Deng, Wang et al. 2016).

While searching for the core biosynthesis genes, siderophore and antibiotic production-related gene loci were also found in *B. contaminans* NZ as indicated in table 3.

Pyrrolnitrin, which is produced by some Bcc and several other gram-negative bacteria, inhibits growth of a wide range of fungi and some Gram-positive bacteria. Complete pyrrolnitrin biosynthesis gene family has also been found in NZ. Several *Burkholderia* species have been reported to produce occidiofungin. However, the occidiofungin biosynthesis locus was not found in *B. contaminans NZ.*

Thirty siderophore related genes are present in the genome of *B. contaminans* NZ. Among them ferric siderophore transport, ABC-type siderophore export system, arthrobactin, siderophore pyoverdine and intact siderophore pyochelin biosynthesis pch gene cluster are the prominent ones (Table 4). From PIFAR data, it is apparent that, biosynthesis of pyochelin in *B. contaminans* NZ is associated with 5 genes (PMID: 22261733) all of which (100%) were identified and found to spread over 5 regions of the bacterial genome.

Table 4: pyochelin biosynthetic gene distribution in *B. contaminans* NZ genome

# Gene	Uniprot	Hit gene	E-value	Location
1	Q4KAY2	or_2382	1e-139	Region 1 (2247159..2249288)
2	Q4KAZ2	or_5154	3e-110	Region 2 (4743658..4745097)
3	Q4KAY8	or_6343	2e-174	Region 3 (5818323..5821178)
4	Q4KAY7	or_7353	0	Region 4 (6748678..6751764)
5	Q4KAZ0	or_8746	1e-43	Region 5 (7971262..7971804)

4.3.4 Phylogenetic relationship to other *Burkholderia* species

Strains with ANI values greater than 96%, which equate to a DNA–DNA hybridization value of 70%, are considered to be the same species. Strains with ANI value greater than 90% are considered to have high genome relatedness. Strains with ANI value lower than 90% are considered to have divergent genomes (Deng, Wang et al. 2016). As shown in Table 5, strain NZ has the greatest nucleotide identity to other plant growth promoting *Burkholderia* and it has less than 90% similarity with genomes from CF opportunistic and mammalian pathogens and plant-pathogenic strains.

Table 5: Average nucleotide identity (ANI) values for different *Burkholderia* strains

Category	*Burkholderia* strain	ANI number (%)
Plant growth promoting bacteria	***B. contaminans CH1 strain***	**99.85**
	B. contaminans MS14	97.32
	B. ambifaria AMMD	91.31
	B. lata	94.37
CF opportunistic pathogens and mammalian pathogens	*B. oklahomensis EO147*	84.26
	B. pseudomallei K96243	84.16
	B. vietnameinsis LMG 10929	88.51
	B. multivorans ATCC 17616	89.0
	B. cenocepacia J2315	89.82
	B. mallei ATCC 23344	84.21
Plant-pathogenic strains	*B. gladioli BSR3*	82.90
	B. glumae BGR1	83.21

To obtain a more accurate classification within the genus *Burkholderia*, a phylogenetic tree was constructed based on 16s rRNA using the neighbour-joining method (Figure 6).

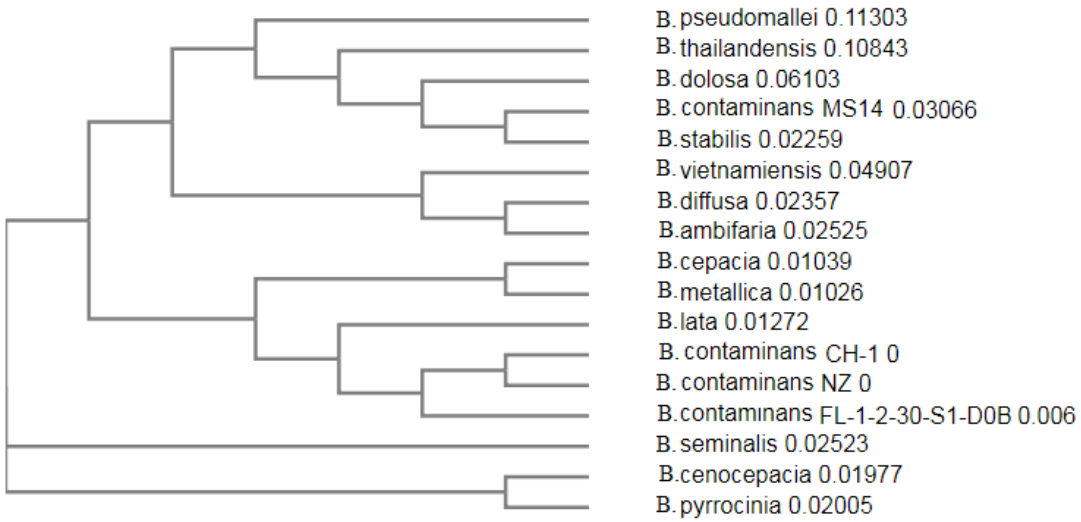


Figure 6: Phylogenetic tree based on 16S rRNA sequences of different Burkholderia species.

4.4 Discussion:

In the previous chapters (chapters 2 and 3), we have established the jute endophytic bacterium *B. contaminans* NZ as a versatile biocontrol agent capable of suppressing the growth of a number of plant-pathogenic fungi and an efficient plant growth promoting agent.

However, as the genus *Burkholderia* contains a large number of diverse species which include many clinically significant organisms, plant-pathogens, as well as environmental species (Sawana, Adeolu et al. 2014), in the present study, we aim at discerning pathogenic strains from our isolated beneficial *Burkholderia* strain.

The decisions on the industrial, agricultural or biotechnological use of a *Burkholderia* strain can be made only on a case-by-case basis after careful molecular and phenotypic characterization of the strains (Eberl and Vandamme 2016).

Genomic similarity between different *Burkholderia* species and phenotypic dissimilarity within the strains of a single species make it a complicated task to differentiate among *Burkholderia* species (Peddayelachagiri, Paul et al. 2016). Conventional classification of *Burkholderia* species is not able to distinguish between pathogenic and nonpathogenic species and a whole genome comparison as was conducted with *B. contaminans* NZ is required to make this distinction (Deng, Wang et al. 2016).

B. contaminans NZ has three chromosomes with a genome size of 8.7 Mb. By investigating NZ's genome, we found loci contributing to biosynthesis of several plant growth promotion related agents, which may partially explain its plant growth-promoting activity. In addition, biochemical characterizations (chapter 2) attest that *B. contaminans* NZ is a plant growth promoting rhizobacteria (PGPR).

On KEGG pathway analysis it was found that *B. contaminans* NZ possesses the zeatin biosynthesis gene. Zeatin is a plant hormone derived from the purine adenine. It is a member of the plant growth hormone family known as cytokinins that promotes cell division in plant roots and shoots (Lee, Yang et al. 2012). The bacterium also contains ansamycin biosynthesis genes. Ansamycin is a family of bacterial secondary metabolites that have antifungal and antibacterial properties (SCHIEWE and Zeeck 1999). The annotated genome also has genes related to 1-aminocyclopropane-1-carboxylate (ACC) deaminase, siderophore biosynthesis gene cluster, and genes involved in the production of indole acetic acid (IAA).

Burkholderia is reported as one of the most significant phosphate solubilizing bacteria that solubilizes inorganic P sources and enhances growth and yield of crop plants (Rodríguez and

Fraga 1999). Genome sequence also indicates the presence of pyrroloquinoline quinone synthase and glucose dehydrogenase genes. These genes are associated with the biosynthesis of gluconic acid and 2-ketogluconic acid, metabolites involved in mineral phosphate solubilization (Chhabra, Brazil et al. 2013). Expression analysis of these key genes, hints at their possible role in plant growth promotion.

There are multiple gene regions related to antimicrobial production or antagonistic activity explaining its antifungal activity too. The wide spectrum of multi-drug resistance and antibiotic biosynthesis genes possessed by *B. contaminans* NZ may contribute to its survivability as an endophyte. In this regard, sequence analysis of *B. contaminans* genome led to the identification of gene clusters responsible for the biosynthesis of antifungal compounds pyrrolnitrin which inhibits growth of a wide range of fungi and of some Gram-positive bacteria (Nunvar, Kalferstova et al. 2016). Interestingly biosynthetic genes for another antifungal compound, occidiofungin, first described in *B. contaminans* MS14 (Gu, Smith et al. 2009) were not found to be present in *B. contaminans* NZ.

Production of siderophores is an additional mode of action for fungal growth inhibition by endophytic bacteria (Vurukonda, Giovanardi et al. 2018). *In silico* analysis using RAST also revealed the presence of thirty siderophore related genes in the *B. contaminans* NZ genome including ferric siderophore transport, ABC-type siderophore export system, arthrobactin, siderophore pyoverdine and intact siderophore pyochelin biosynthesis pch gene cluster. These results show that the PGPB strains have multiple mechanisms for inhibiting competing microorganism that increase their survivability.

A whole-genome comparative approach has identified jute endophytic *B. contaminans* NZ to be less virulent by comparing the antibiotic and siderophore biosynthesis genes and virulence loci among twelve other *Burkholderia* species.

It has been reported that *B. cenocepacia* strains expressing both cable (Cbl) pili and the 22-kDa adhesin bind strongly to cytokeratin 13 (CK13) and invade squamous epithelium efficiently to infect a host (Urban, Goldberg et al. 2005). *B. contaminans* NZ lacks the cable pili biosynthesis gene cluster indicating their inability to attach to the host cell to initiate infection.VgrG-5, a *Burkholderia* type VI secretion system 5-associated protein, required for full mammalian virulence (Schwarz, Singh et al. 2014) is also absent in the genome of *B. contaminans* NZ. Cystic fibrosis (CF)-related O-antigen of lipopolysaccharides associated with transmissible infections in CF patients (Deng, Wang et al. 2016) as well as zinc metallo proteases that may be involved in overall virulence of several Bcc strains (Corbett, Burtnick et al. 2003) are also missing in *B. contaminans* NZ.

Among virulence associated reported proteins of *Burkholderia*, only the *SodC* gene encoding for a Cu^{2+} and Zn^{2+} containing periplasmic superoxide dismutase (SOD) that contributes to intracellular survival indicating self-protectionability in CF patients, is present in the genome.

In various reports plant pathogenic *Burkholderia* species have been shown to commonly produce plant-toxic secondary metabolites, polysaccharides and other toxins, such as rice grain rot and wilt causal agent toxoflavin, exo-polysaccharide toxin cepacian, hydrogen cyanide (HCN) and 2-heptyl-3-hydroxy-4(1H)-quinolone (Deng, Wang et al. 2016). Fortuitously these pathogenic genes are not present in the jute endophytic bacterium. Based on functional gene analysis, this *Burkholderia* species is unlikely to be pathogenic to either plants or mammals.

Furthermore, data for average nucleotide identity (ANI) and phylogenetic analysis suggest its close relation to other plant growth-promoting *Burkholderia* strains.

The average nucleotide identity is one of the most robust measurements of genomic relatedness between strains (Kim et al. 2014). The ANI data show that the nonpathogenic and pathogenic isolates are clustered separately.

Comparing the ANI values of different *Burkholderia* species confirm that distinct lineages exist among *Burkholderia* species (Angus, Agapakis et al. 2014). The ANI values between *B. contaminans* and *B. lata* were shown to be above 90%. Therefore, these isolates can be considered in the same non pathohgenic group according to Konstantinidis et al (Konstantinidis, Ramette et al. 2006).

Overall, from the whole genome analysis it is apparent that *B. contaminans* NZ lacks major virulence-related genes like the biosynthesis genes for cable pili, adhesin, and the VgrG-5 protein and contains genes which contribute to the biosynthesis of several antimicrobial and growth promotion agents making it a versatile and safe to use bio-inoculant.

4.5 References

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CHAPTER 5

Quantitative proteomic study for the identification of total proteins and the comparative analysis of the change at the proteomic level in *M. phaseolina* when challenged by *B. contaminans* NZ

Pages 81 - 127

Quantitative proteomic study for the identification of total proteins and the comparative analysis of the change at the proteomic level in *M. phaseolina* when challenged by *B. contaminans* NZ

5.1 Overview

The earlier findings of the present study established *Burkholderia contaminans* NZ as a potent biocontrol and plant growth promoting agent that is safe to use and ecofriendly in nature. In this present chapter, the prime objective was to identify through a proteomic analysis of *Macrophomina phaseolina* under *B. contaminans* NZ the mode of action and response of the fungus in the presence of the antifungal action of *B. contaminans* NZ.

Antagonistic fungal–bacterial interactions lie at the very heart of competitive survival for the limited resources in the bio-ecosystem. This paradigm for existence has been a long-term focus of researchers desperate for an enhanced understanding of bionetwork functions so as to develop potent biological control agents against fungal pathogens, providing alternatives to chemicals for practical agronomic purposes. Numerous examples highlight the use of bio-control agents in combating fungal phytopathogens (Gajera, Savaliya et al. 2016, Pastor, Masciarelli et al. 2016). However, the resistance mechanisms of pathogens are so diverse that an effective biological arsenal is still at large. Many pathogens are capable of exploiting a wide array of mechanisms in order to counteract and compete against antagonism from both microbial antagonists and other pathogens (Duffy, Schouten et al. 2003).They appear to be

affected at some point by the bio-control agents but soon functionalizes its genomic supply to overpower the latter (Gerbore, Benhamou et al. 2014). Therefore, fungal-bacterial interactions are very complex and a more comprehensive mechanistic understanding of the molecular communication between pathogens and biological control agents is needed to elucidate these intricated relations. This knowledge can be used in the application of microorganisms for the biological control of plant diseases, complementing or replacing traditional chemical treatments (Gkarmiri, Finlay et al. 2015).

Studies on *in vitro* antagonism of bacterial strains against fungal pathogens of plants have revealed important information on the different compounds and genes involved (Ballhausen and de Boer 2016). Studies on the transcriptomic responses to stressors based on systems biology tools, such as RNA-seq or microarray, have yielded important insights into the stress adaptation of any organism (Zhang, Sun et al. 2014).

Transcriptomic analysis of the phytopathogen *Rhizoctonia solani* AG-3 in response to the antagonistic bacteria *Serratia proteamaculans* and *Serratia plymuthica* [5] and a dual transcriptional profiling of *Collimonas fungivorans* versus *Aspergillus niger* [6] were made some years ago.

However, transcriptional profiling may only partially contribute to the understanding of stress adaptation because not all transcripts can be translated and mRNA abundances may not correspond to protein expression level due to pre-, co- and post-translational modification, and proteins, not mRNA, are the effectors of biological functions (Feder and Walser 2005). In addition, critical regulatory signaling events downstream of transcription will not be detected by transcript analysis (Tomanek 2011).

Proteins are the final products of gene expression and the main executive molecules in catalytic and metabolic activities in all organisms. Therefore, a detailed study of proteins is expected to contribute greatly to our understanding of gene function in the post-genomic era.

However, proteomic and bioinformatics studies particularly for phyto-pathogenic fungi are still in their formative stages (Uranga, Ghassemian et al. 2017). Over the last two decades, an emergence of vast genomic databases have completely revolutionized the way in which mass spectrometry is used to analyze proteins (Florencio, Cunha et al. 2016, Zhang and Elias 2017). Recent advances in proteomics and bioinformatics have allowed profiling of widespread protein expression and further relative quantification (Florencio, Cunha et al. 2016). Since a proteome provides comprehensive information about a given cellular environment, analyses of proteomics is more pertinent when focused on an organism's response to stimuli. However, holistic investigations of such response or antagonisms are scant. High throughput isobaric tag for relative and absolute quantification (iTRAQ)-based LC-MS/MS proteomics is popular for quantitative proteome study. This technique has a high degree of sensitivity, and the amine specific isobaric reagents permit identification and quantitation of up to 8 different samples simultaneously (Becker and Bern 2011).

The only species under *Macrophomina* genus, *M. phaseolina* is a phyto-pathogenic fungus that affects a diverse range of host plants in comparatively low moisture conditions. This poses a major worldwide threat to crop yield (Kumari, Shekhawat et al. 2015). A number of reports have been made on the bio-control of *M. phaseolina* some of which are control by *Bacillus* and *Trichoderma* in strawberry (Pastrana, Basallote-Ureba et al. 2016), by *Trichoderma* in sunflower (Reetha, Pavani et al. 2014), mung bean (Hussain, Ghaffar et al. 1990), and chickpea, by *Trichoderma* and *Pseudomonas* (Pandey, Gohel et al. 2017) in gerbera etc. However, not much is known as to how they impede *M. phaseolina* from infecting plants, or what the molecular basis of the fungal response to inhibition is.

Post genomic studies namely transcriptomic and proteomic analyses are increasingly being used to gain insights of cellular processes and functions of an organism (Graham, Graham et al. 2007). The genome of *M. phaseolina* sequenced in 2012 (Islam, Haque et al. 2012),

revealed the fungus's armor of a large array of hydrolytic enzymes which possibly allows it to infect a diverse host type. However, an identification of the total protein of an organism is needed to link an annotated genome sequence to gene expression data at the proteomic level. One of the objectives of this study was to obtain an extensive coverage of the *M. phaseolina* proteome. This led to the identification of up to 82.4 % of total identified proteins with LC-MS/MS.

The key focus of this chapter was to elucidate the molecular mechanisms by which the fungus *M. phaseolina* respond to the challenge posed by the plant-endophytic bacterium *B. contaminans* NZ, an effective fungal antagonist.

Another *Burkholderia* species *B. gladioli* strain NGJ1 has recently been reported to demonstrate antifungal activity on *Rhizoctonia solani* and various other fungi. The bacteria exhibited mycophagy, growing and multiplying at the cost of fungal biomass (Swain, Yadav et al. 2017). Contrary to this report on *B. gladioli, B. contaminans* NZ while exhibiting fungal inhibition was not found to be mycophagic even for *Rhizoctonia solani.*

For *M. phaseolina* it has been shown that the antifungal activity of *B. contaminans* NZ does not have the killing prowess as exhibited by *B. gladioli*. *B. contaminans* NZ is unable to kill the fungus as a steady inhibition zone was found to be maintained around the fungal mycelia and the bacterium was unable to grow over the *M. phaseolina* biomass. Even the fungal mycelia were able to germinate when transferred from the bacteria challenged plate onto fresh medium albeit with a loss of pathogenicity. This bacterium - fungal interaction demonstrates that, *M. phaseolina* is capable of withstanding bacterial stress and develops strategies to remain static in the face of adversity, avoiding being killed by the bacteria.

Proteome analysis of *M. phaseolina* identified 2204 proteins of which 141 were found to be differentially regulated upon *B. contaminans* NZ challenged condition. Interestingly, most of these proteins with altered expression are related to defense, virulence, cell proliferation

and cell wall composition together with the proteins of redox and metabolic pathways. Overall, the proteomic data of *M. phaseolina* has provided important information as to how the fungus responds to the bio-control environment.

5.2 Materials and methods

Unless mentioned otherwise, all the chemicals were obtained from Sigma-Aldrich, (St. Luis, MO, USA). Culture media Potato Dextrose Agar (PDA) and Tryptic Soya Broth (TSB) were obtained from HiMedia (HiMedia, India). Trypsin (Mass Spectrometry Grade), RIPA Lysis and Extraction Buffer and BCA Protein Assay Kit were purchased from Thermo Scientific (Thermo Scientific Pierce, Rockford, IL). iTRAQ 4-plex multiplex kit were purchased from AB Sciex (Framingham, MA). Protease inhibitor cocktail was purchased from Roche Diagnostics (Indianapolis, IN).

5.2.1 *In-vitro* dual culture assays

In-vitro bacterial-fungal dual-culture assays were established in 9 cm diameter petri dish systems containing PDA medium. A 5-mm plug taken from the plate of an actively growing colony of *M. phaseolina* was inoculated in one side of the petri dish. Fresh cells of *B. contaminans* NZ were streaked in 3-cm length parallel lines on the other side of the fungal plug. Plates with only fungus were also set up as controls. The plates were incubated at 28 ± 2° C for 4 to 5 days.

5.2.2 Change in pathogenicity of *B. contaminans* challenged *M. phaseolina*

To assess any changes in fungal pathogenicity, an assay was performed with surface sterilized jute seeds (*Corchorus olitorius* var O4) using 1.0% sodium hypchlorite. The seeds were allowed to germinate on a 110 mm Whatman filter paper (moistened with sterile water) in petri plates under two different conditions. In one plate a control 1% fungal mycelia solution

was inoculated and in the other same amount of *B. contaminans* NZ challenged *M. phaseolina* was used. A seedling plate without any inoculum was used as the control.

5.2.3 Cell culture and preparation of protein extracts

M. phaseolina cells were incubated in 50 mL Potato Dextrose Broth (PDB) for 5 days in an incubator shaker at 28°C at 180 rpm. 20 mL of overnight grown culture of *B. contaminans* NZ in TSB was added to 50 mL of 3 day old *M. phaseolina* culture in PDB. The fungal mycelia were collected after 2 days of bacterial contact.

Pure and co-cultured fungal mycelia were filtered and washed three times with ice cold 10mM phosphate buffer to remove bacterial contamination and were ground in liquid nitrogen in a pre-cooled mortar. Crushed fungal cell samples were homogenized in RIPA buffer containing 1 x concentration of protease inhibitor cocktail. Supernatant was collected by centrifugation and subjected to three pulses of sonication on ice. Total soluble protein was recovered by centrifugation at 14,000 x g for 30 min at 4^{o}C, precipitated with 6 volumes of (vol/vol) ice-cold acetone overnight at 4°C, and centrifuged at 10,000 x *g* for 10 min at 4°C. The resulting protein precipitate was washed twice with cold acetone, air-dried, and stored at –80^{o}C until use. Semi dried pellet was dissolved using 8 M urea (Sigma-Aldrich). Total protein concentration was determined by the BCA Protein Assay kit (Thermo Scientific Pierce, Rockford, IL) as per manufacturer's instructions. The protein concentration was calculated using bovine serum albumin (BSA) as standard. Three such experiments were carried out; a total of three biological and three technical replicates were analyzed for differential proteomic elucidation.

5.2.3.1 Trypsin digestion and iTRAQ labeling for LC-MS/MS

A total of 100 µg of proteins from each sample were reduced using 10 mM DTT at 60°C for 60 min. and alkylated using a 20 mM IAA at room temperature for 30 min to block the cysteine. The proteins were then digested overnight by sequencing grade trypsin (1:20 w/w in 50 mMTEAB) at 37°C according to the manufacturer's instructions (ABsciex Inc. USA). Then the peptides from each sample were first re-suspended in100 mM TEAB and iTRAQ reagents were dissolved in 70 µL of ethanol by vortexing for 1 min. Peptides from control and stressed samples were labelled with iTRAQ (AB Sciex, Framingham, MA, USA) mass tag 114, and 116 respectively at room temperature for 2 h. The reaction was stopped by adding 120 µL H_2O, followed by centrifugation at 13,800 × g for 1 min. The samples were then pooled together into one fresh tube as illustrated in Figure 4 and dried in a speedvac. Except for the iTRAQ labeling, similar digestion protocol was followed to prepare fungal protein samples for total protein identification.

5.2.3.2 Peptide fractionation by high pH RP HPLC and LC-MS/MS

Prior to mass spectrometric analysis, the pooled peptides were re-suspended in 80 µL of buffer A (98 % H_20, 2% ACN, pH 10.0) and were fractionated using an Agilent 1200 HPLC system on high-pH reverse-phase Zorbax 300 Extend-C18 column (2.1 × 100 mm, 3µm, 150 Å, C18, Agilent Technology, Santa Clara, CA, USA). The 60 min liner gradient was composed of 96% buffer A for 1 min; 4–19% buffer B (98% ACN, 2% H_20, pH 10.0) for 30 min; then 19–95% buffer B for 23 min; followed by 95% buffer B for 5 min. The eluted fractions were collected in every 1 min interval into 48 fractions, and then pooled to give a final total of 12 fractions. The collected fractions were then lyophilized and stored at -20°C until MS analysis.

For LC-MS/MS data was acquired using 5600 TripleTOF$^+$ (ABSciex, Concord, Canada). The instrument was coupled with an Eksigent NanoLC-2DPlus system (Eksigent, Dublin, CA,

USA), and the samples were loaded at a flow rate of 2 µl/ min for 10 min and eluted from the analytical column at a flow rate of 300 nl/min in a linear gradient of 5% solvent B to 35% solvent B in 60 min. Solvent A being composed of 0.1% (v/v) formic acid in water and solvent B comprising 95% (v/v) acetonitrile with 0.1% (v/v) formic acid. The TripleTOF 5600 system was run on an information dependent acquisition (IDA) mode with a TOF/MS survey scan (350–1250 m/z) where the accumulation time was 250 ms. For fragmentation, a maximum of 10 precursor ions per cycle were selected, with a total cycle time of roughly 2.3 seconds and each MS/MS spectrum was collected for 100 ms (100–1500 m/z). The parent ions with a charge state from +2 to +5 were included for the MS/MS fragmentation. The threshold precursor ion intensity was set at more than 120 cps and was not present on the dynamic exclusion list. After fragmentation of an ion by MS/MS, its mass and isotopes were excluded for 10 seconds. The MS/MS spectra were operated in high sensitivity mode with adjust collision energy when using iTRAQ reagent settings.

5.2.3.3 Protein identification and data analysis

All the wiff. files containing MS and MS/MS spectra generated from Triple TOF 5600 were submitted for database searching and quantitative analysis using the ProteinPilot™ V 4.5 software (ABSciex, Concord, Canada). ProteinPilot search engine was used for iTRAQ based quantitation in a data dependent mode. This search engine uses a sequence tag method plus protein database searching. Each MS/MS spectrum was searched against *Macrophomina* species from Uniprot/Swissport database (November 28, 2012; 14056 entries). The searching parameters were set as iTRAQ peptide label, cysteine alkylation with methyl methanethiosulfonate, trypsin digestion and identification focus for biological modifications. The resulting data set was auto bias-corrected to normalize any variations arising from unequal mixing of the differently labeled samples. False discovery rate (FDR) was estimated

using a target-decoy based strategy. The proteins and peptides were filtered with 1% global protein level FDR. For quantitation the ratio threshold was set to >1.3 (equivalent or more than 95% confidence) and p-value <0.05 to ensure that quantitation was based on at least two unique peptides. Proteins were considered only if they were significant in all independent biological and technical triplicate experiments. The average values of replicates were used to indicate the final protein abundance at a given time point. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno, Csordas et al. 2015) partner repository with the dataset identifier PXD009121

5.2.3.4 Statistical and bioinformatics analysis

A statistical analysis to compare the groups was performed using *t* test (Sigma Stat, Jandel Scientific, USA). For functional annotation and cellular location, the protein lists were analyzed according to the Blast2GO tool (https://www.blast2go.com/), Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg) (Kanehisa, Goto et al. 2011). Predicted interacting partners were analyzed using STRING v10 database (Szklarczyk, Franceschini et al. 2015).

5.3 Results

5.3.1 Effects of *B. contaminans* on growth and morphology of *M. phaseolina*

When *M. phaseolina* was co cultured with *B. contaminans* NZ, clear inhibition of fungal mycelial growth was evident at 4 days (Fig. 1B) compared to the control treatment without bacteria (Fig. 1A). This inhibition persisted for more than sixteen weeks (Fig 1C). The sclerotia collected from confrontation plates retains viability as the control sclerotia can

germinate when transferred onto a fresh PDA plate, even when it was obtained from a 3 month old fungus-bacteria co-culture plate.

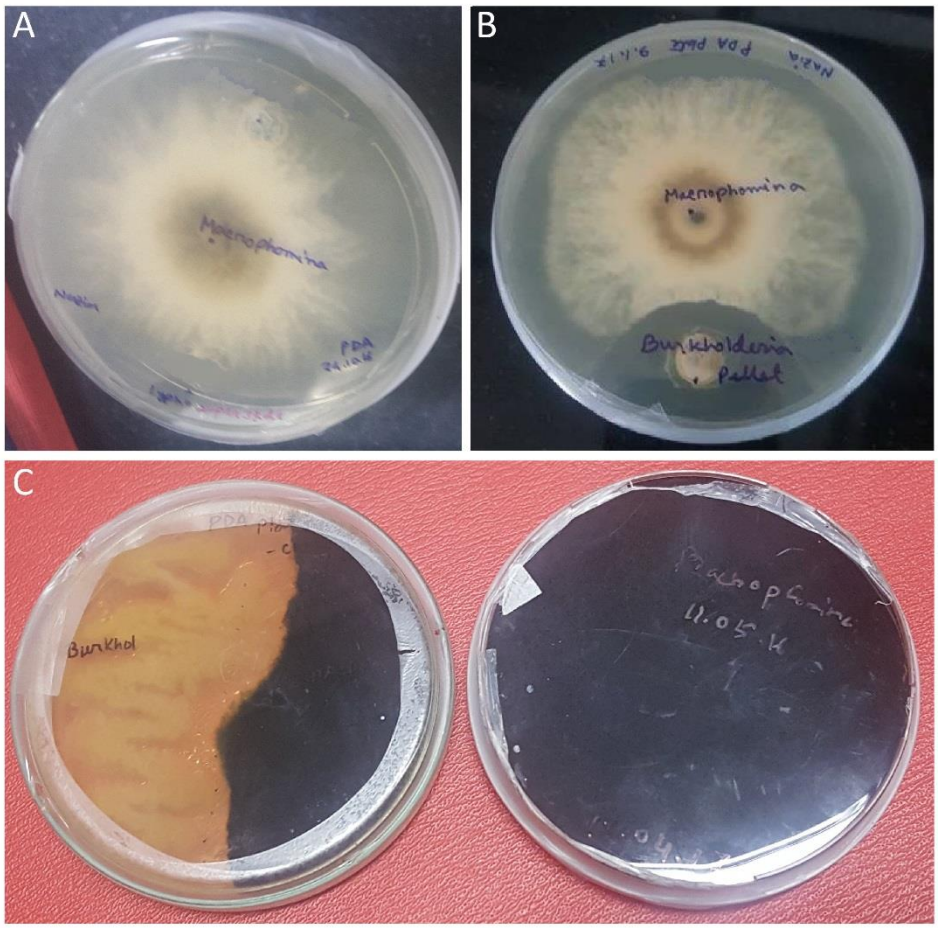


Figure 1: *In vitro* dual culture bacterial-fungal assay. (A) Control *M. phaseolina* monoculture, (B) *M. phaseolina* challenged with *B. contaminans* NZ (5 days) and (C) persistent inhibition of *B. contaminans* NZ against *M. phaseolina* (16 weeks).

5.3.2 Reduction of pathogenicity in *M. phaseolina*

When *B. contaminans* NZ challenged *M. phaseolina* was used to inoculate the jute seeds, the germinating seedlings looked healthy and were similar to the ones in which no *M. phaseolina* was inoculated. This indicated significant loss of virulence in the *Burkholderia* challenged

M. phaseolina. However, *M. phaseolina* without the stress appeared as virulent as expected with seedlings dying from heavy infection.

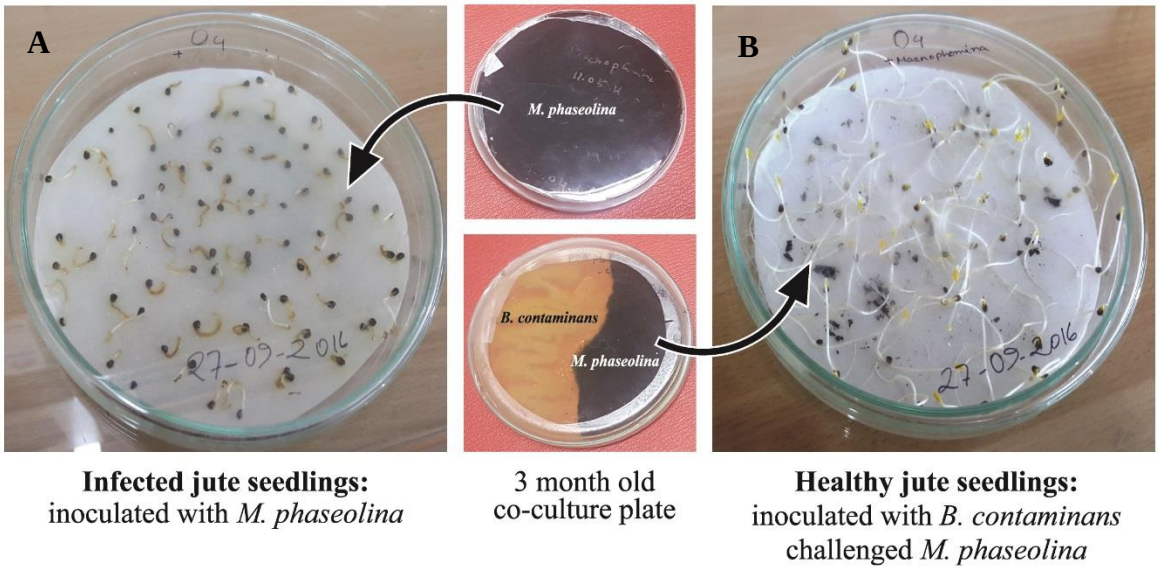


Figure 2: Pathogenicity reduction test. Jute (*Corchorus olitorius* var. O4) seedlings inoculated with (A) *M. phaseolina* and (C) *B. contaminans* NZ challenged *M. phaseolina*.

5.3.3 Identification of *M. phaseolina* proteome

A total of 2204 proteins common in both the biological replicates were identified.

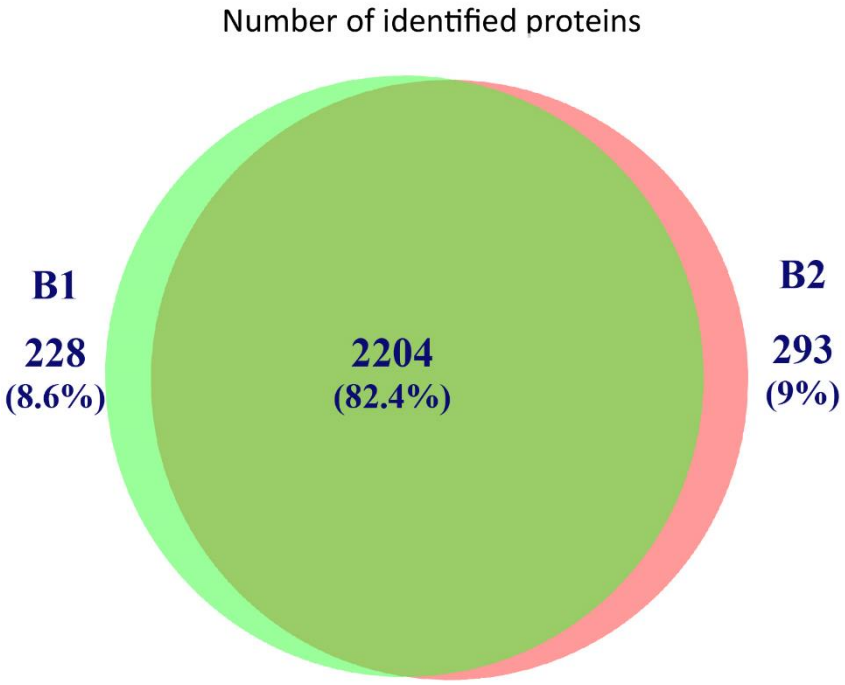


Figure 3: Venn diagram showing total proteins identified in *M. phaseolina* with two biological replicates.

The average length of the total identified proteins was 487 amino acids (Fig.4A). Enzyme distribution: *M. phaseolina* has a relatively high level of enzymes belonging to hydrolases, oxidoreductase and transferase family (Fig.4B).

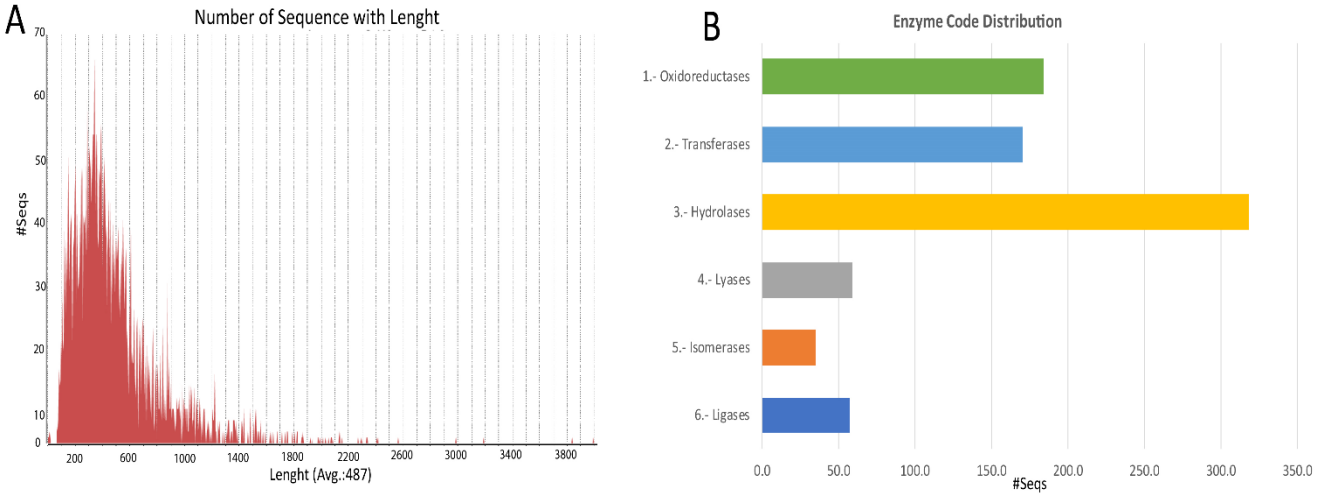
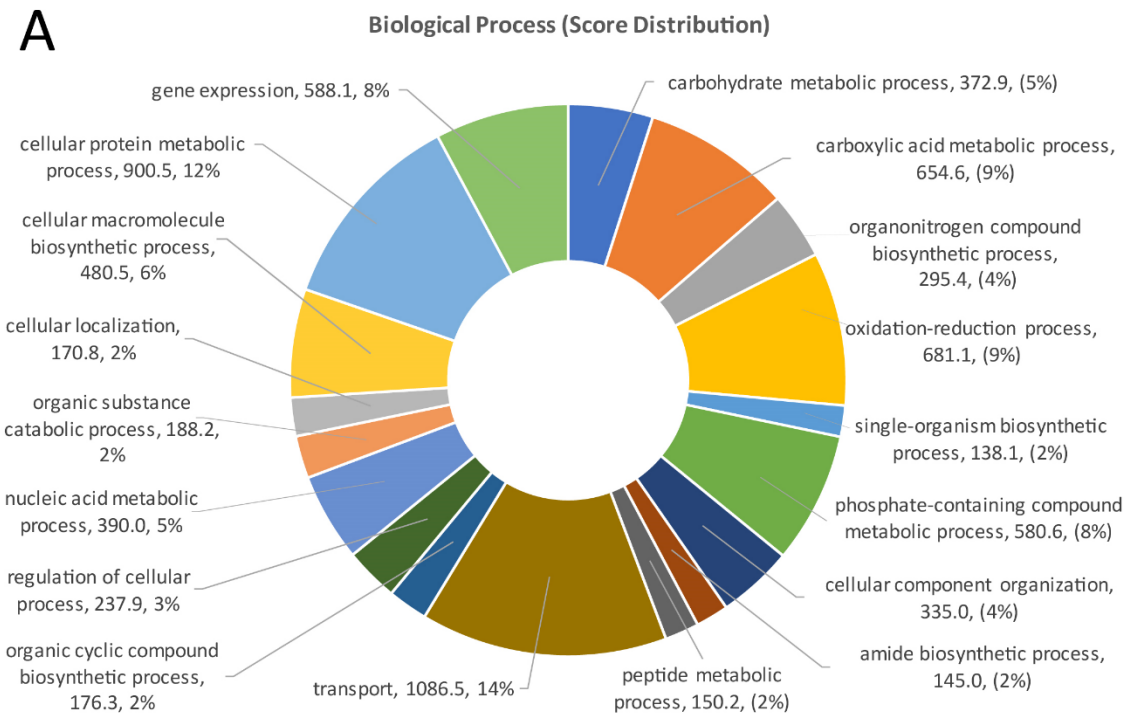
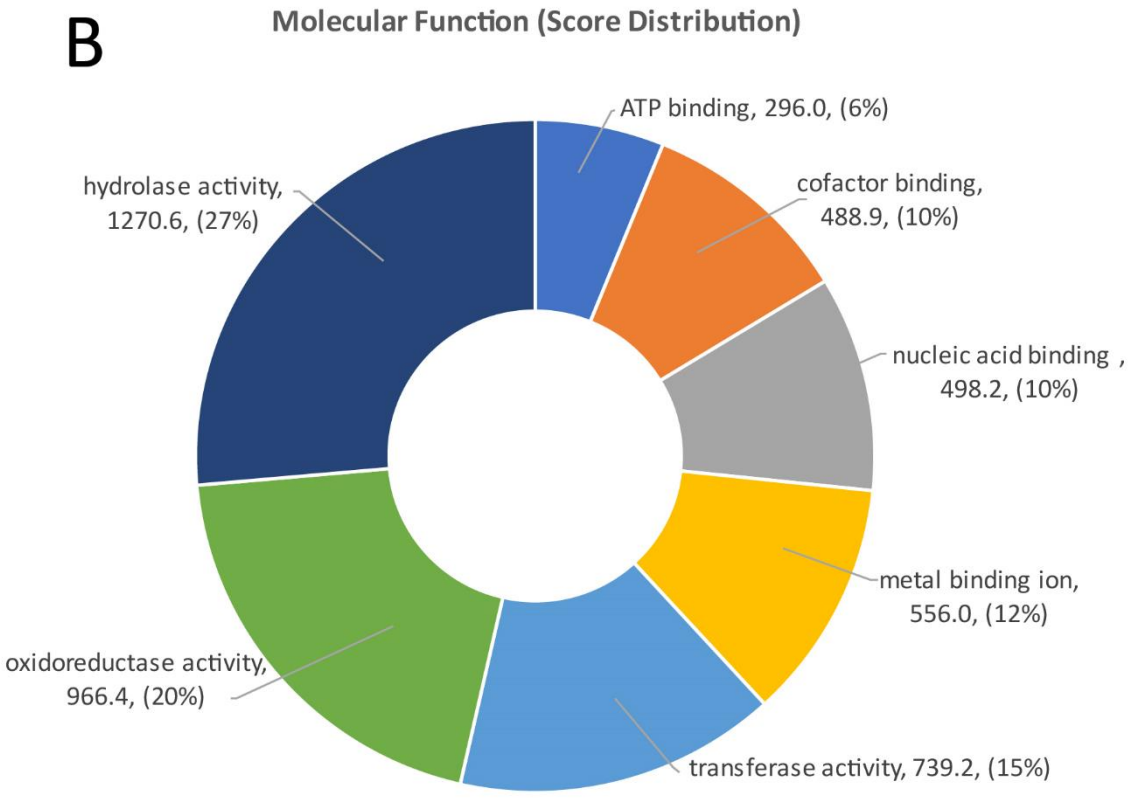


Figure 4: Analysis of total identified proteins in *M. phaseolina*. Graph showing (A) the score distribution with different lengths of identified proteins, and (B) enzyme distribution.

Homology-based function prediction was carried out for *M. phaseolina* proteome using Blast2GO. The major part of the protein belongs to transport (15%) followed by cellular protein metabolic process (12%), carboxylic metabolic process (9%) and oxidation-reduction process (9%) in the category of biological process *(*Fig.5A). Similarly, hydrolase (27%) and oxidoreductase activities (20%) were predicted in molecular function category (Fig.5B), and a major part of the proteins were found to be localized in the membranous part (Fig.5C).



B



C

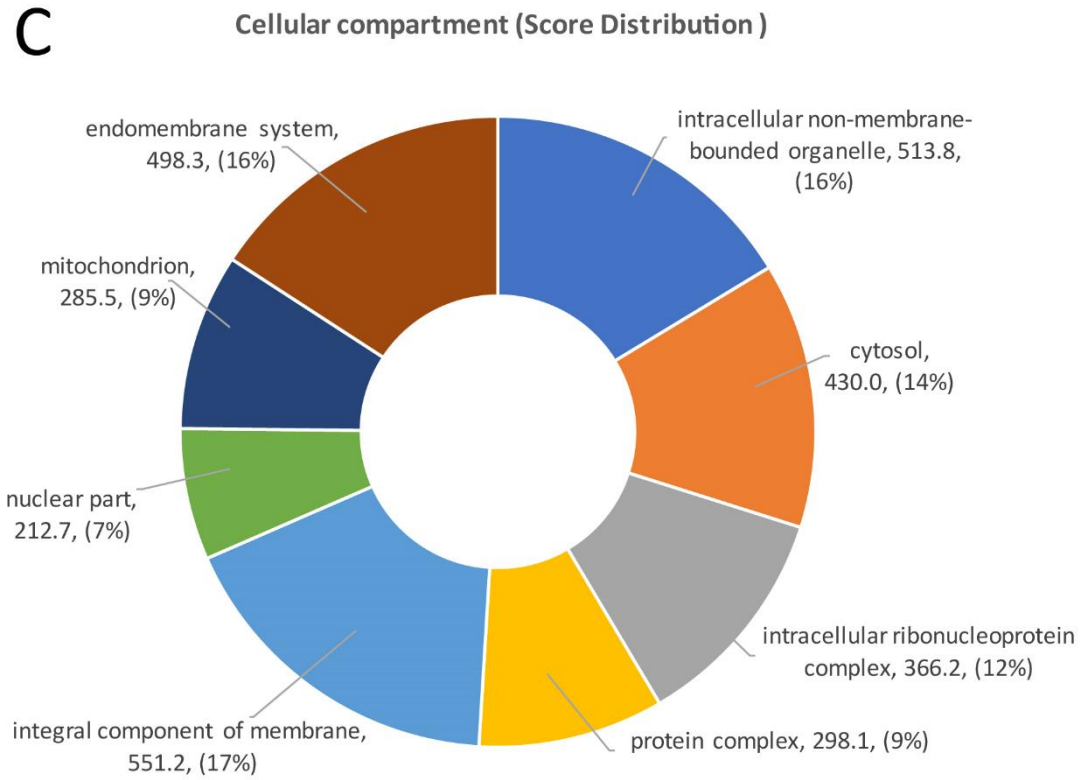


Figure 5: Classification of total identified proteins using Blast2GO based on (A) biological process, (B) molecular function, (C) cellular compartment.

Pathway annotation of total identified proteins was done using the KEGG database. Table 1 shows the no of protein involved in metabolic pathways in *M. phaseolina*.

Table 1: KEGG pathway annotation of total identified proteins

Pathway name	No of protein involved
Glycolysis / Gluconeogenesis	27
Citrate cycle (TCA cycle)	20
Pentose phosphate pathway	16
Pentose and glucuronate interconversions	11
Fructose and mannose metabolism	14
Galactose metabolism	13
Ascorbate and aldarate metabolism	3
Starch and sucrose metabolism	21
Amino sugar and nucleotide sugar metabolism	23
Pyruvate metabolism	24
Glyoxylate and dicarboxylate metabolism	19
Propanoate metabolism	15
Butanoate metabolism	11
C5-Branched dibasic acid metabolism	3
Inositol phosphate metabolism	9
Oxidative phosphorylation	51
Carbon fixation	16
Methane metabolism	18
Nitrogen metabolism	9
Sulfur metabolism	10
Fatty acid biosynthesis	5
Fatty acid elongation	4
Fatty acid degradation	13
Synthesis & degradation of ketone bodies	3
Steroid biosynthesis	4
Primary bile acid biosynthesis	2
Steroid hormone biosynthesis	1
Glycerolipid metabolism	13
Glycerophospholipid metabolism	13

Ether lipid metabolism	4
Sphingolipid metabolism	5
Arachidonic acid metabolism	2
Linoleic acid metabolism	2
alpha-Linolenic acid metabolism	2
Biosynthesis of unsaturated fatty acids	5
Purine metabolism (35)	35
Pyrimidine metabolism	23
Alanine, aspartate and glutamate metabolism	19
Glycine, serine and threonine metabolism	30
Cysteine and methionine metabolism	29
Val, leu and isoleu degradation	17
Val, leu and isoleu biosynthesis	9
Lysine biosynthesis	9
Lysine degradation	11
Arginine biosynthesis	13
Arginine and proline metabolism	18
Histidine metabolism	10
Tyrosine metabolism	14
Phenylalanine metabolism	11
Tryptophan metabolism	18
Phenylalanine, tyrosine and tryptophan biosynthesis	10
beta-Alanine metabolism	10
Taurine and hypotaurine metabolism	5
Phosphonate and phosphinate metabolism	1
Selenocompound metabolism	6
Cyanoamino acid metabolism	7
D-Arginine and D-ornithine metabolism	1
Glutathione metabolism	13
N-Glycan biosynthesis	10
Various types of N-glycan biosynthesis	8
Mannose type O-glycan biosyntheis	1
Other types of O-glycan biosynthesis	2
Glycosaminoglycan degradation	1
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	3
Glycosphingolipid biosynthesis - globo and isoglobo series	2
Glycosphingolipid biosynthesis - ganglio series	1
Other glycan degradation	4
Thiamine metabolism	3
Riboflavin metabolism	3
Vitamin B6 metabolism	5
Nicotinate and nicotinamide metabolism	7

Pantothenate and CoA biosynthesis	8
Biotin metabolism	3
Folate biosynthesis	3
One carbon pool by folate	7
Retinol metabolism	2
Porphyrin and chlorophyll metabolism	7
Ubiquinone and other terpenoid-quinone biosynthesis	2
Terpenoid backbone biosynthesis	10
Sesquiterpenoid and triterpenoid biosynthesis	1
Carotenoid biosynthesis	1
Insect hormone biosynthesis	1
Limonene and pinene degradation	1
Geraniol degradation	1
Biosynthesis of ansamycins	1
Phenylpropanoid biosynthesis	3
Indole alkaloid biosynthesis	1
Indole diterpene alkaloid biosynthesis (1)	1
Isoquinoline alkaloid biosynthesis	6
Tropane, piperidine and pyridine alkaloid biosynthesis	4
Caffeine metabolism	2
Betalain biosynthesis	1
Penicillin and cephalosporin biosynthesis	2
Carbapenem biosynthesis	1
Monobactam biosynthesis	2
Streptomycin biosynthesis	4
Neomycin, kanamycin and gentamicin biosynthesis (1)	1
Novobiocin biosynthesis	1
Staurosporine biosynthesis	1
Phenazine biosynthesis	1
Prodigiosin biosyntheses	1
Aflatoxin biosynthesis	2
Benzoate degradation	5
Aminobenzoate degradation	8
Fluorobenzoate degradation	2
Chloroalkane and chloroalkene degradation	4
Chlorocyclohexane and chlorobenzene degradation	3
Toluene degradation	3
Styrene degradation	6
Atrazine degradation	1
Caprolactam degradation	3
Dioxin degradation	1
Naphthalene degradation	3

Polycyclic aromatic hydrocarbon degradation	1
Metabolism of xenobiotics by cytochrome P450	6
Drug metabolism - cytochrome P450	5
Drug metabolism - other enzymes	6
RNA polymerase	6
Basal transcription factors	1
Spliceosome	28
Ribosome	68
Aminoacyl-tRNA biosynthesis	21
RNA transport	47
mRNA surveillance pathway	20
Ribosome biogenesis in eukaryotes	11
Protein export	11
Protein processing in endoplasmic reticulum	44
SNARE interactions in vesicular transport	7
Ubiquitin mediated proteolysis	17
Sulfur relay system	3
Proteasome	32
RNA degradation	21
DNA replication	7
Base excision repair	1
Nucleotide excision repair	7
Mismatch repair	4
Homologous recombination	3
Fanconi anemia pathway	2
ABC transporters	1
Bacterial secretion system	1
Two-component system	10
Ras signaling pathway	11
Rap1 signaling pathway	8
MAPK signaling pathway	11
MAPK signaling pathway - yeast	23
ErbB signaling pathway	6
Wnt signaling pathway	11
Hedgehog signaling pathway	4
TGF-beta signaling pathway	6
Hippo signaling pathway	7
VEGF signaling pathway	6
Apelin signaling pathway	10
Jak-STAT signaling pathway	3
NF-kappa B signaling pathway	2
TNF signaling pathway	2

HIF-1 signaling pathway	10
FoxO signaling pathway	10
Calcium signaling pathway	7
Phosphatidylinositol signaling system	8
Phospholipase D signaling pathway	10
Sphingolipid signaling pathway	14
cAMP signaling pathway	9
cGMP - PKG signaling pathway	9
PI3K-Akt signaling pathway	20
AMPK signaling pathway	14
mTOR signaling pathway	20
Endocytosis	35
Phagosome	21
Lysosome	17
Peroxisome	26
Autophagy - yeast	32
Autophagy - other eukaryotes	7
Mitophagy - yeast	11
Cell cycle	11
Meiosis - yeast	13
Apoptosis	9
Ferroptosis	6
Necroptosis	10
p53 signaling pathway	2
Cellular senescence	12
Focal adhesion	8
Adherens junction	5
Tight junction	15
Gap junction	9
Signaling pathways regulating pluripotency of stem cells	3
Quorum sensing	5
Regulation of actin cytoskeleton	15
Platinum drug resistance	5
Antifolate resistance	3
Endocrine resistance	5

5.3.4 Quantitative proteomics of *M. phaseolina* under bacterial stress

On quantitative analysis of *M. phaseolina* in normal and under *B. contaminans* NZ stress (Fig.6A), a total of 47282 spectra were obtained from the iTRAQ LC-MS/MS experiment.

After data filtering to eliminate low-scoring spectra, a total of 25429 unique spectra with 1% FDR (false discovery rate) that met the strict confidence criteria for identification were matched to 2204 unique proteins (Fig 6B). Raw data shows the normal distribution of relative abundance which reflects the majority of proteins to be unchanged (Fig.6C). Further data was normalized using bias correction feature in ProteinPilot v 4.5 software (Fig.6 D).

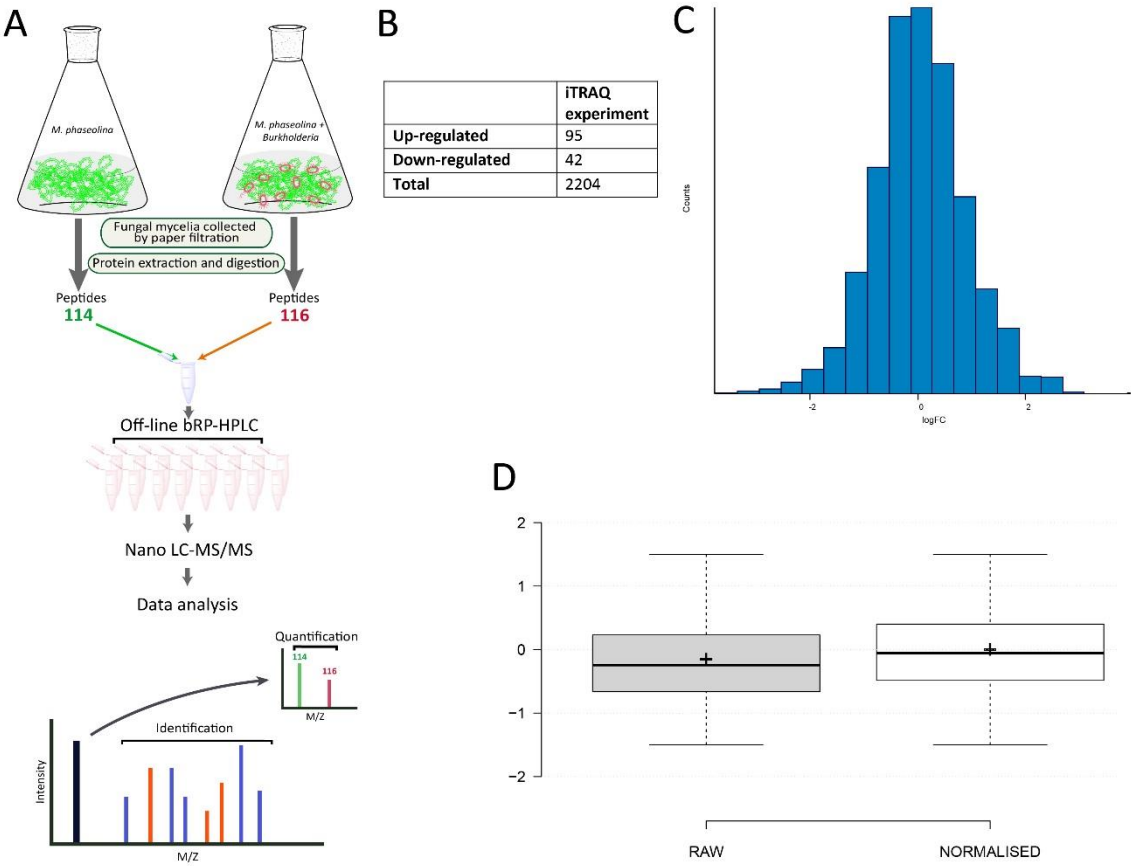


Figure 6: Overview of experimental design (A) Workflow of quantitative proteomic analysis, (B) table showing the number of modulated proteins and total identified proteins, (C) histogram displaying log2 ratios of all proteins, (D) box plot showing the effect of bias correction (normalization) based on total reporter ion intensity.

Spectral data were searched using Uniprot decoy database and proteins with at least two validated peptides were considered for quantitation. Results showed that the iTRAQ data in

all replicates were almost undisputed, suggesting that protein abundance in *M. phaseolina* changed with *B. contaminans* NZ stress.

5.3.5 Profile of differentially expressed proteins

Among the proteins which showed a significant change (P< 0.05) in abundance, 137 differentially expressed proteins (DEPs) were chosen by a ratio >1.40 or <0.8 under *B. contaminans* NZ stress. Among the total DEPs in all the replicates 95 were upregulated (Fig.7A) and 42 downregulated (Fig.7B). The list of up and down regulated proteins are given in table 2 and 3.

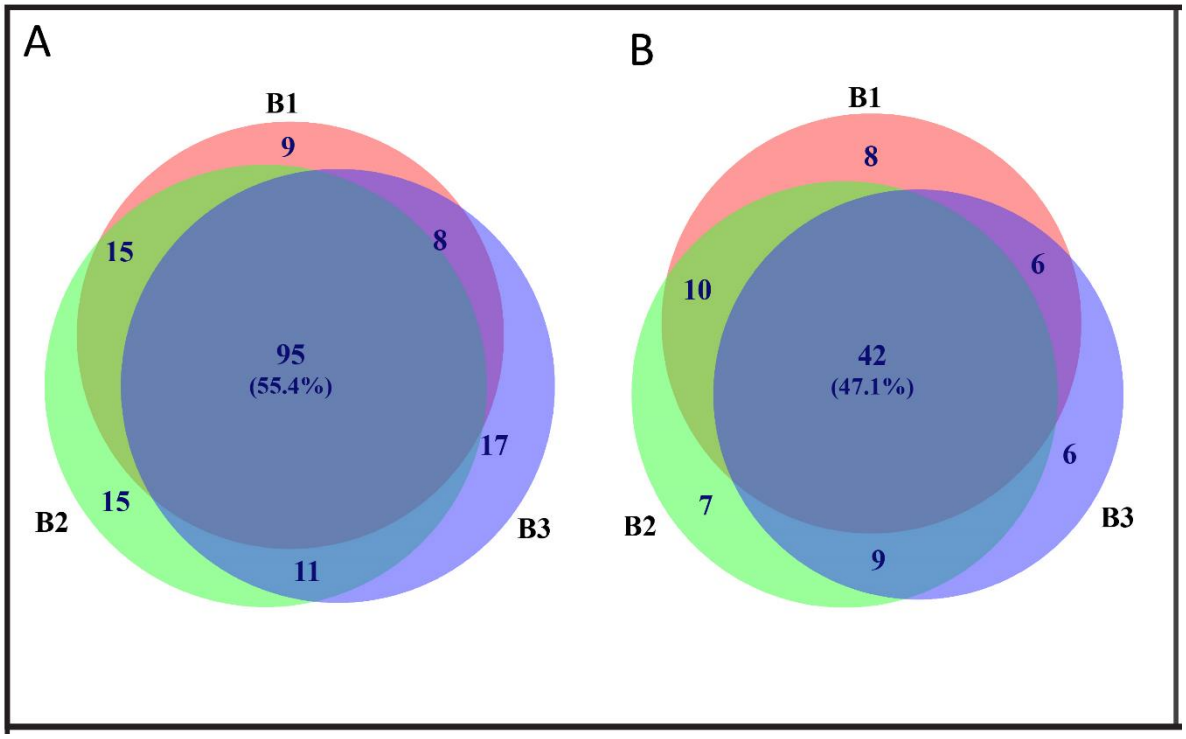


Figure 7: Differentially expressed proteins detected by quantitative iTRAQ mass spectrometry in *M. phaseolina* after *B. contaminans* NZ stress. Venn diagram showing (A) up-regulated and (B) down-regulated proteins obtained from three biological replicates.

Table 2: List of upregulated proteins

Accession	Name	Peptides	116:114	P Value
K2REE7	4-hydroxyphenylpyruvate dioxygenase	8	1.769	0.033
K2SRZ2	ABC transporter-like protein	63	1.441	0.039
K2SFB1	Actin-like protein	37	2.486	0.035
K2R862	Acyl-CoA dehydrogenase conserved site	5	2.893	0.049
K2R5D9	Adenosyl homocysteinase	42	2.783	0.000
K2RVH4	Alcohol dehydrogenase superfamily zinc-containing	83	3.472	0.017
K2R6A6	Aldo/keto reductase	13	2.612	0.018
K2SXE7	Alkyl hydroperoxide reductase subunit C	5	2.739	0.007
K2S027	Alpha-D-phosphohexomutase superfamily	47	2.029	0.028
K2S5D4	Alternative oxidase	9	2.143	0.046
K2RVG6	Amine oxidase	26	1.896	0.006
K2RWV0	Aminotransferase class-3	21	4.343	0.001
K2SRC1	Antibiotic biosynthesis monooxygenase	19	1.527	0.021
K2SF00	Arginase	5	2.368	0.025
K2SZB1	Aspartate aminotransferase	48	2.476	0.045
K2R9P7	ATP synthase subunit beta	117	3.105	0.001
K2T007	ATPase F0 complex subunit B mitochondrial	19	2.461	0.004
K2S0I9	ATPase F0 complex subunit G mitochondrial	5	1.921	0.029
K2S5T0	ATP-dependent (S)-NAD(P)H-hydrate dehydratase	6	2.269	0.039
K2SU19	ATP-dependent 6-phosphofructokinase	25	2.261	0.001
K2R2Y5	Calcium-binding EF-hand	7	2.027	0.004
K2S399	Carbohydrate-binding WSC	6	3.799	0.047
K2RH65	Chaperonin ClpA/B	30	2.107	0.016

K2SGX9	Chaperonin Cpn60	51	3.101	0.000
K2REF5	Citrate synthase	60	1.977	0.022
K2R4G6	Clustered mitochondria protein homolog	17	1.707	0.002
K2QMM6	Coproporphyrinogen III oxidase aerobic	19	2.431	0.001
K2S0M1	Cyanate hydratase	9	3.221	0.024
K2SQD9	Cytochrome c oxidase subunit Va	13	2.676	0.031
K2RA83	Delta-1-pyrroline-5-carboxylate dehydrogenase 1	30	2.873	0.000
K2RSR2	Dihydrolipoyl dehydrogenase	28	2.086	0.022
K2R7P4	Electron transfer flavoprotein beta-subunit conserved site	8	1.921	0.017
K2RX29	Elongation factor 1-alpha	89	2.375	0.008
K2SCR2	Enolase	178	6.203	0.000
K2SX20	Formate dehydrogenase	74	2.766	0.001
K2RQ38	Formate-tetrahydrofolate ligase FTHFS	15	2.386	0.002
K2S676	Fumarate lyase	12	1.852	0.013
K2RLJ8	Fumarate reductase/succinate dehydrogenase flavoprotein	20	2.258	0.048
K2S5T7	Fumarylacetoacetase	6	2.733	0.028
K2RRJ6	Glucose-methanol-choline oxidoreductase	19	2.546	0.005
K2RY42	Glutamate/phenylalanine/leucine/valine dehydrogenase	27	2.054	0.000
K2RNS8	Glutathione-dependent formaldehyde-activating family GFA	7	2.700	0.033
K2SSH4	Glyceraldehyde-3-phosphate dehydrogenase	160	6.362	0.001
K2STU8	Glycine cleavage system P protein homodimeric	9	1.843	0.012
K2REN2	Glycoside hydrolase family 15	7	1.836	0.022
K2RZJ7	GrpE protein homolog	8	1.616	0.048

K2RT72	Heat shock protein 9/12	6	2.632	0.038
K2RQC7	Heat shock protein Hsp20	7	2.393	0.041
K2QU21	Heat shock protein Hsp70	105	1.901	0.018
K2SAA2	Helicase	11	2.156	0.002
K2S774	Histidine biosynthesis trifunctional protein	23	1.865	0.006
K2QLH4	Histone H4	8	1.564	0.041
K2S675	Isocitrate lyase	21	1.734	0.026
K2QIW5	Isocitrate/isopropylmalate dehydrogenase	13	1.985	0.010
K2RZ68	Kelch-type beta propeller	9	2.447	0.032
K2RZT2	Ketose-bisphosphate aldolase class-2	50	3.136	0.010
K2R3V9	Mitochondrial carrier protein	58	1.930	0.012
K2RCX6	Mitochondrial glycoprotein	11	1.788	0.028
K2S5K3	Mitochondrial substrate/solute carrier	10	1.808	0.032
K2RPJ6	Monooxygenase FAD-binding protein	34	2.197	0.011
K2REA2	Mov34/MPN/PAD-1	6	2.128	0.048
K2R4Y3	Multicopper oxidase type 1	6	1.838	0.043
K2RSB8	Myo-inositol-1-phosphate synthase	12	2.320	0.001
K2RJY6	NLI interacting factor	5	2.170	0.012
K2RS06	O-methyltransferase family 3	4	2.235	0.038
K2RW54	Peptidase M18	16	2.329	0.013
K2R4N5	Peptidase M41	9	1.708	0.009
K2RXQ4	Phosphoglycerate kinase	70	4.501	0.000
K2RPR2	Phosphoglycerate mutase 23-bisphosphoglycerate-independent	79	2.869	0.000
K2RB27	Proteasome subunit alpha type	16	2.615	0.010

K2RXE1	Proteinase inhibitor I13 potato inhibitor I	16	3.592	0.005
K2QIT7	Pyruvate kinase	61	2.486	0.000
K2RPK6	Reverse transcriptase	10	2.526	0.029
K2RYI0	Ribosomal protein L10e	14	2.122	0.000
K2REH6	Ribosomal protein L14b/L23e	11	1.498	0.040
K2RZG1	Ribosomal protein L24e	10	2.055	0.007
K2QXJ1	Ribosomal protein L38e	7	2.633	0.043
K2RCA4	Ribosomal protein S11	19	3.049	0.008
K2T087	RNA helicase ATP-dependent DEAD-box conserved site	20	2.218	0.000
K2RHJ5	Six-bladed beta-propeller TolB-like protein	13	3.942	0.008
K2RZI3	Succinate-CoA ligase subunit beta	27	2.057	0.025
K2RB38	Sulfate adenylyltransferase	26	1.832	0.003
K2RKD6	Superoxide dismutase	14	2.420	0.044
K2REC7	Tetratricopeptide-like helical protein	26	1.989	0.000
K2QQI7	Thiamine pyrophosphate enzyme TPP-binding protein	112	5.293	0.000
K2RSS9	Thiolase	23	3.976	0.004
K2SJ45	Triosephosphate isomerase	87	1.619	0.001
K2S4V7	Uncharacterized protein	23	3.245	0.001
K2R790	Uncharacterized protein	15	1.785	0.001
K2SJF8	Uncharacterized protein	12	3.434	0.003
K2SLU4	Uncharacterized protein	13	1.551	0.003
K2SCF2	Uncharacterized protein	13	3.415	0.003
K2SGW0	Uncharacterized protein	7	1.556	0.004
K2SGC6	Uncharacterized protein	17	1.355	0.005

Table 3: list of down-regulated proteins

Accession	Name	Peptides	116:114	pValue
K2SZA6	Aldehyde oxidase/xanthine dehydrogenase a/b hammerhead	16	0.640	0.002
K2RHH3	Alpha-1,4 glucan phosphorylase	73	0.694	0.022
K2RVI7	Aminotransferase class V/Cysteine desulfurase	5	0.528	0.001
K2S0W9	Beta-ketoacyl synthase	36	0.633	0.003
K2SYZ6	Branched-chain-amino-acid aminotransferase	6	0.419	0.040
K2SEA6	Carboxyl transferase	41	0.539	0.000
K2R915	Cell division protein GTP binding protein	13	0.467	0.030
K2R5U7	Coatomer subunit alpha	11	0.465	0.019
K2RHV6	Cytochrome b5	29	0.532	0.001
K2SMM1	Cytochrome P450	70	0.610	0.001
K2RER8	EPS15-like protein	14	0.600	0.001
K2R2Y2	Eukaryotic translation initiation factor 3 subunit A	19	0.522	0.003
K2RP09	FAD-binding 8	11	0.200	0.013
K2SX89	Flavin-containing monooxygenase-like protein	7	0.292	0.011
K2QUS6	Flavodoxin	14	0.677	0.001
K2RMC4	Glucose-6-phosphate 1-dehydrogenase	41	0.534	0.000
K2RI17	Glutamate decarboxylase	30	0.419	0.026
K2SZ80	Glutamate dehydrogenase	20	0.311	0.001
K2RX14	Glutamine amidotransferase class-2	54	0.548	0.010
K2RKT7	Glutaredoxin	16	0.689	0.001
K2SDY4	Glutathione S-transferase	15	0.518	0.036
K2S3H7	Glycosyl transferase family 39	5	0.466	0.027

K2S0K4	Heterokaryon incompatibility Het-C	39	0.584	0.010
K2RM16	Kinesin-like protein	3	0.683	0.042
K2RT07	NADH:ubiquinone oxidoreductase 51kDa subunit conserved site	16	0.659	0.000
K2T0E2	NADH-quinone oxidoreductase subunit D	19	0.644	0.007
K2RP44	Negative transcriptional regulator	9	0.473	0.020
K2RXU4	NmrA-like protein	17	0.372	0.012
K2RWL0	NMT1/THI5-like protein	3	0.469	0.023
K2SCI0	Peroxidase	22	0.130	0.000
K2RWV5	Phosphomannomutase	6	0.491	0.016
K2QMU3	Ras GTPase	16	0.679	0.044
K2RH07	Recoverin	15	0.507	0.037
K2SAQ8	Ribonuclease II/R	23	0.465	0.007
K2QYF8	RNA-processing protein HAT helix	6	0.536	0.042
K2R7Y4	Scytalone dehydratase	11	0.133	0.051
K2S4A5	Tubulin alpha chain	27	0.385	0.000
K2S2J9	Tubulin binding cofactor A	5	0.532	0.034
K2RYE5	UDP-glucose:Glycoprotein Glucosyltransferase	6	0.330	0.016
K2SMR9	Uncharacterized protein	14	0.600	0.024
K2RJ97	Uncharacterized protein	18	0.591	0.035
K2SSQ4	Uncharacterized protein	28	0.370	0.002

Volcano plot showing the $\log_2$ ratio of gene expression levels between control and stressed condition; the colored dots in green and red represent the differentially expressed proteins (p value <0.01 represented by black horizontal line) and 1.4-fold expression difference

(represented by two red vertical lines) (Fig. 8 A). Cluster analysis based on biological process revealed majority of proteins belonging to the metabolic processes (Fig. 8B).

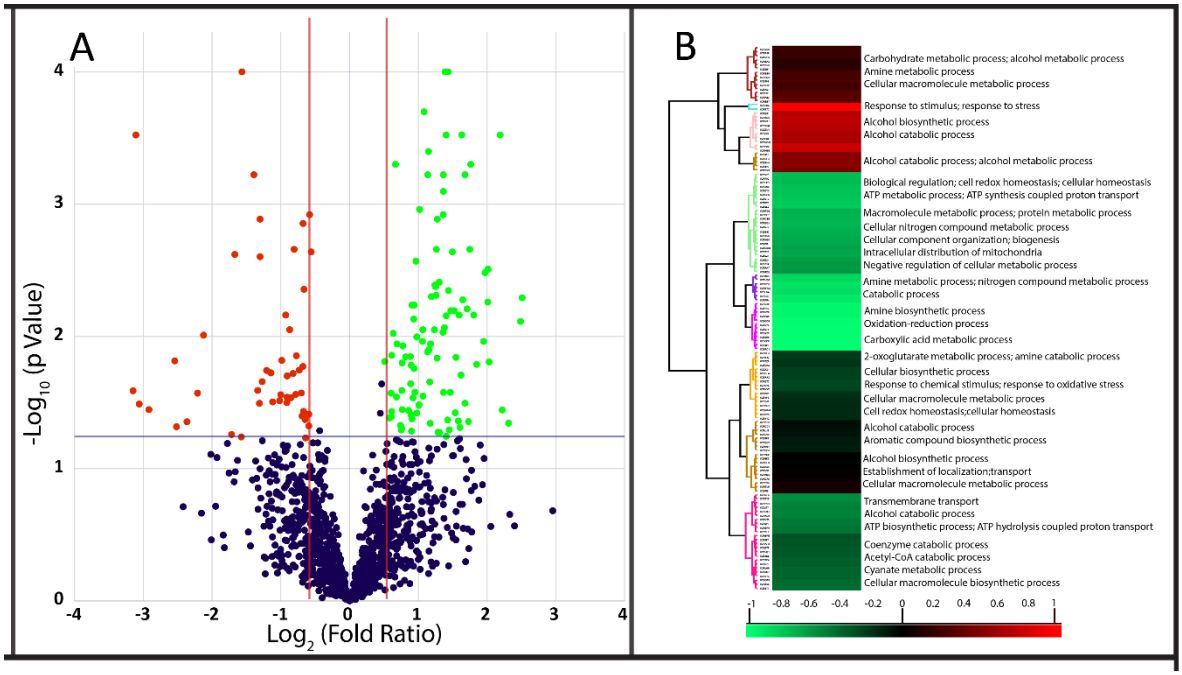
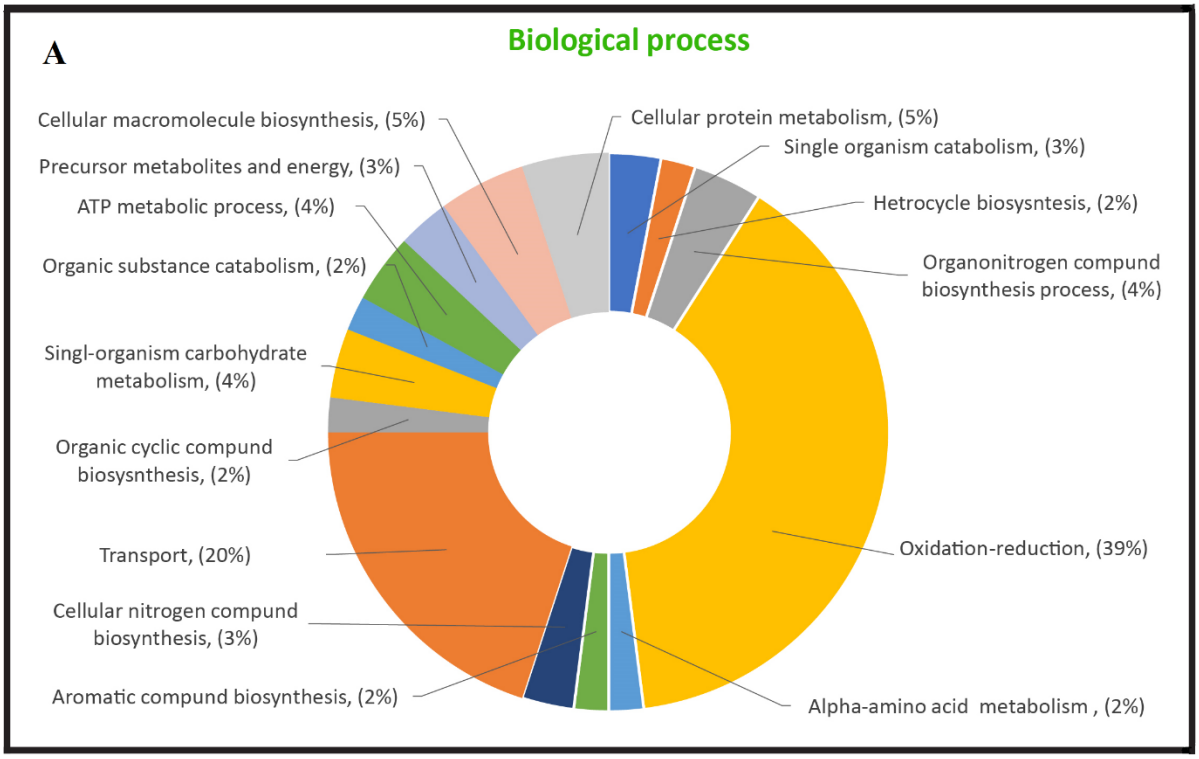
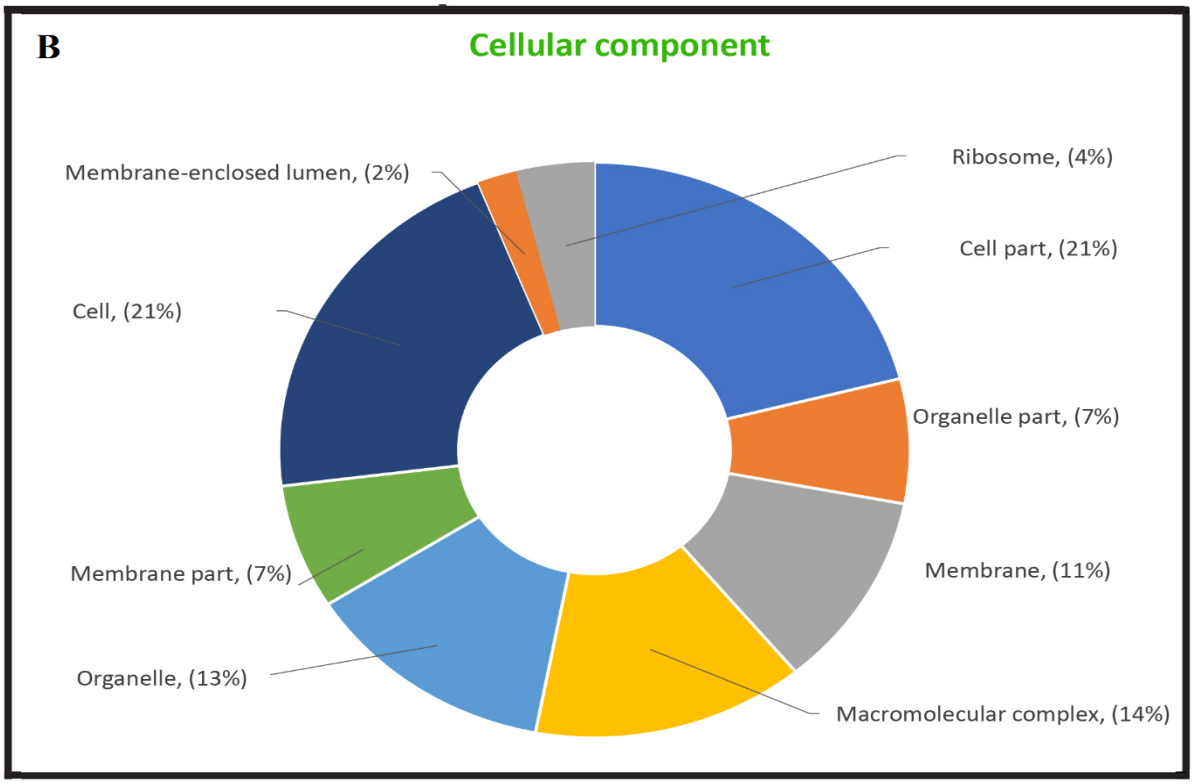


Figure 8: (A)Volcano plot showing the log2 ratio of gene expression levels between control and stressed *M. phaseolina*; the colored dots in green and red represent the differentially expressed proteins (p value <0.01 represented by black horizontal line) and 1.4-fold expression difference (represented by two red vertical lines). (B) Hierarchical clustering of differentially expressed proteins in *M. phaseolina* after *B. contaminans* NZ stress.

Differentially expressed proteins of *M. phaseolina* were further annotated using Blast2GO. Up regulated (Fig.9: A, B, C) and down regulated (Fig.10: A, B, C) proteins were analyzed separately into three categories belonging to 'biological process', 'cellular component' and molecular function'.


A
Biological process
Cellular macromolecule biosynthesis, (5%)
Precursor metabolites and energy, (3%)
ATP metabolic process, (4%)
Organic substance catabolism, (2%)
Singl-organism carbohydrate metabolism, (4%)
Organic cyclic compund biosysnthesis, (2%)
Transport, (20%)
Cellular nitrogen compund biosynthesis, (3%)
Aromatic compund biosynthesis, (2%)
Cellular protein metabolism, (5%)
Single organism catabolism, (3%)
Hetrocycle biosysntesis, (2%)
Organonitrogen compund biosynthesis process, (4%)
Oxidation-reduction, (39%)
Alpha-amino acid metabolism , (2%)


B
Cellular component
Membrane-enclosed lumen, (2%)
Cell, (21%)
Membrane part, (7%)
Organelle, (13%)
Ribosome, (4%)
Cell part, (21%)
Organelle part, (7%)
Membrane, (11%)
Macromolecular complex, (14%)

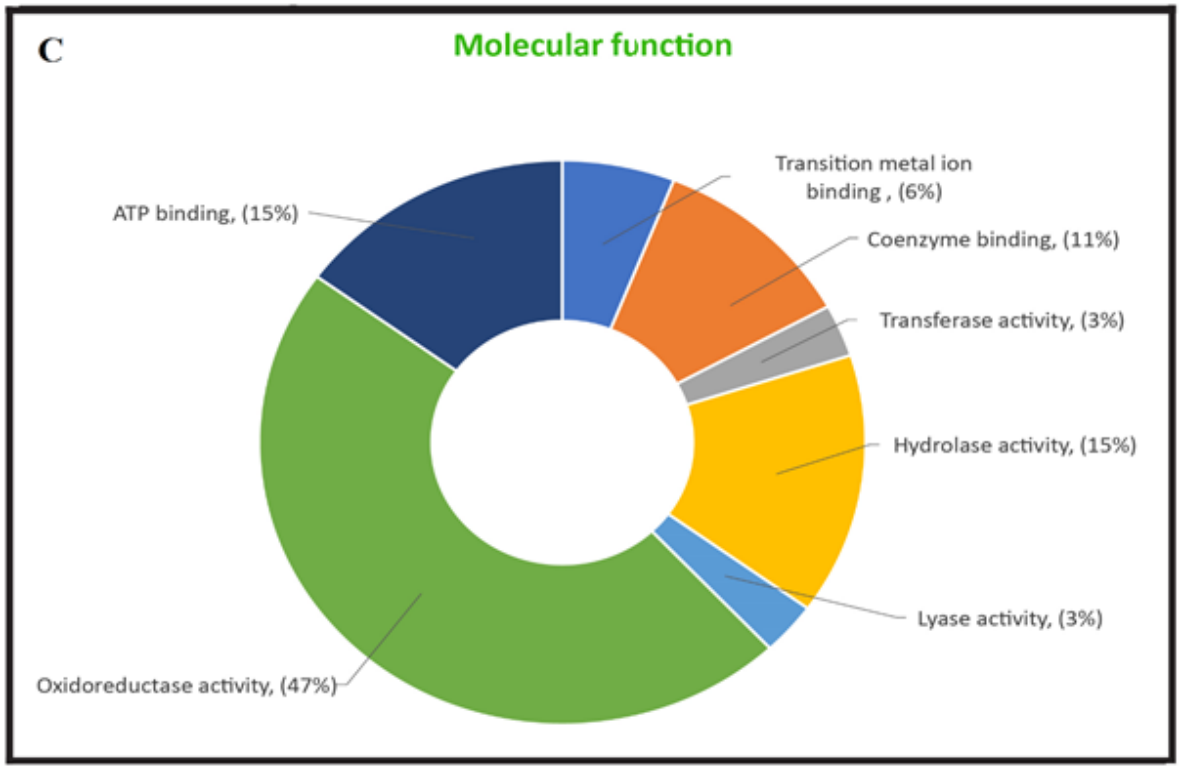
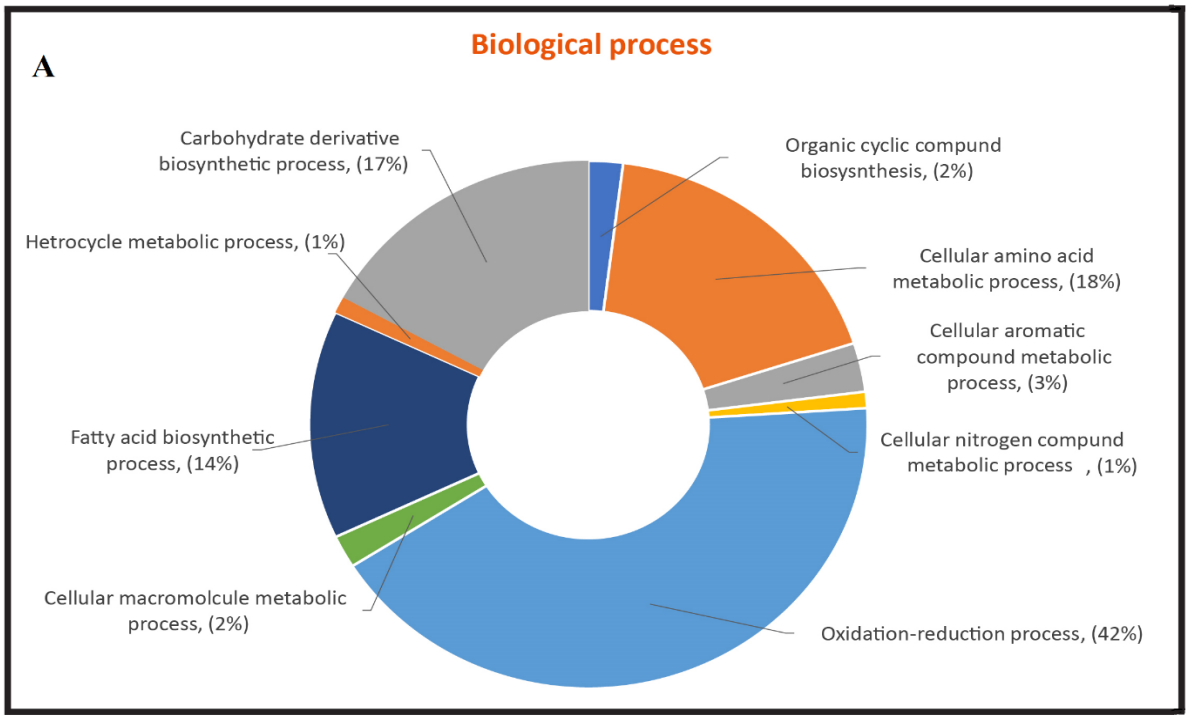


Figure 9: GO based functional annotation of up regulated proteins based on (A) biological process (B) cellular component (C) molecular function



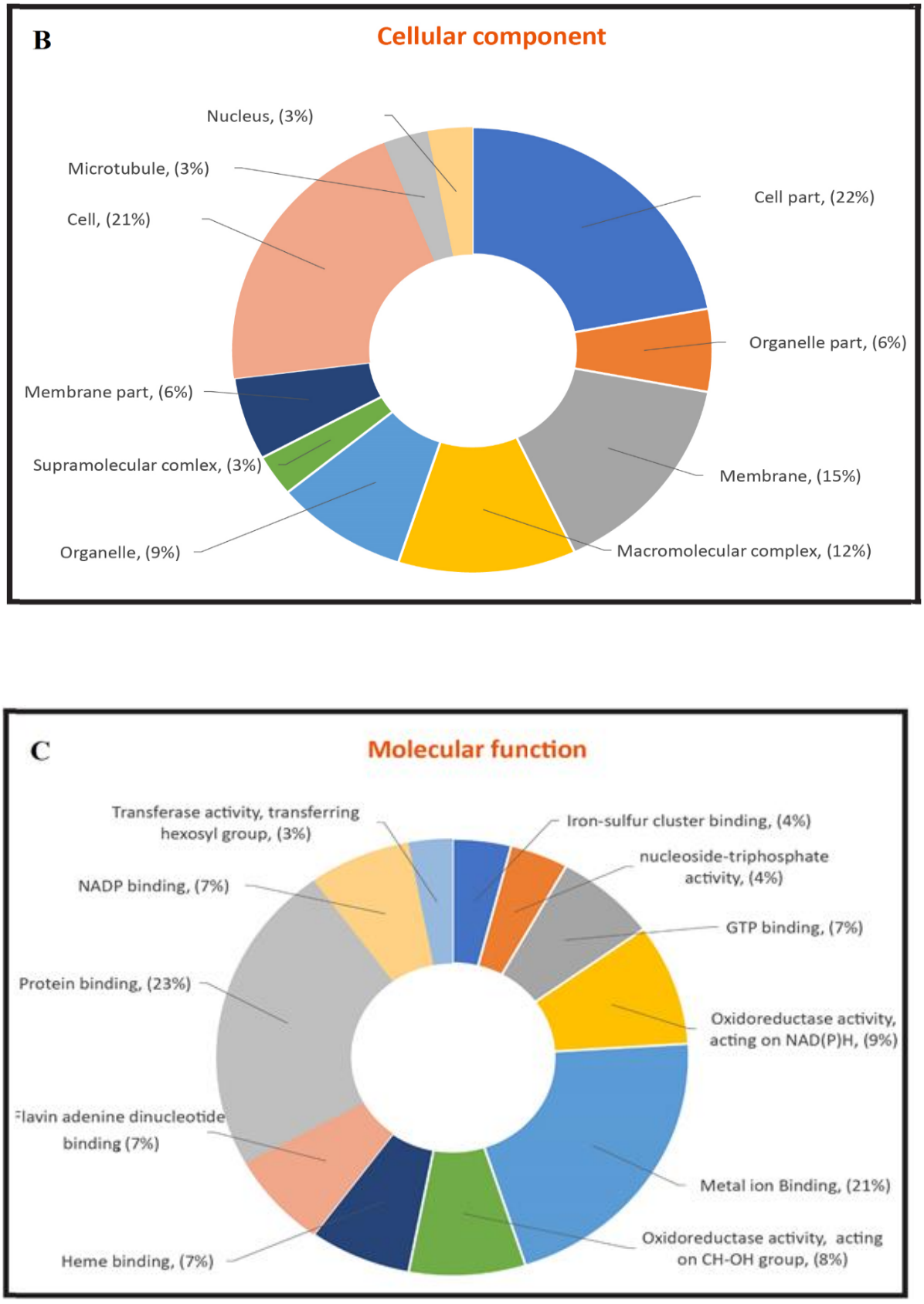


Figure 10: Annotated down regulated proteins into three categories using Blast2GO (A) biological process (B) cellular component and (C) molecular function.

5.3.6 Functional classification of differentially expressed proteins

Metabolic pathway enrichment analysis of responsive proteins was further conducted according to information from the KEGG Pathway Database. The DEPs are majorly classified into 12 categories according to their putative biological functions.

5.3.6.1 Up-regulated differentially expressed proteins (DEPs)

The majority of up-regulated DEPs were classified into 4 categories: energy and carbohydrate metabolism (26.11%), defense and stress response (23.45%) amino acid metabolism (19.91%) and genetic information processing/transcription, translation (6.64%) (Fig: 11).

The other categories are as follows: cell growth and death/ endocytosis/ apoptosis/ senescence (5.31%), signaling pathway (4.87%), folding, sorting and degradation (3.09 %), biosynthesis of other secondary metabolites (3.09 %), lipid metabolism (2.66%), metabolism of cofactors and vitamins (2.21%), nucleotide metabolism (1.32%), cellular community /cell motility (0.88%), and metabolism of xenobiotics (0.44%) (Fig 11).

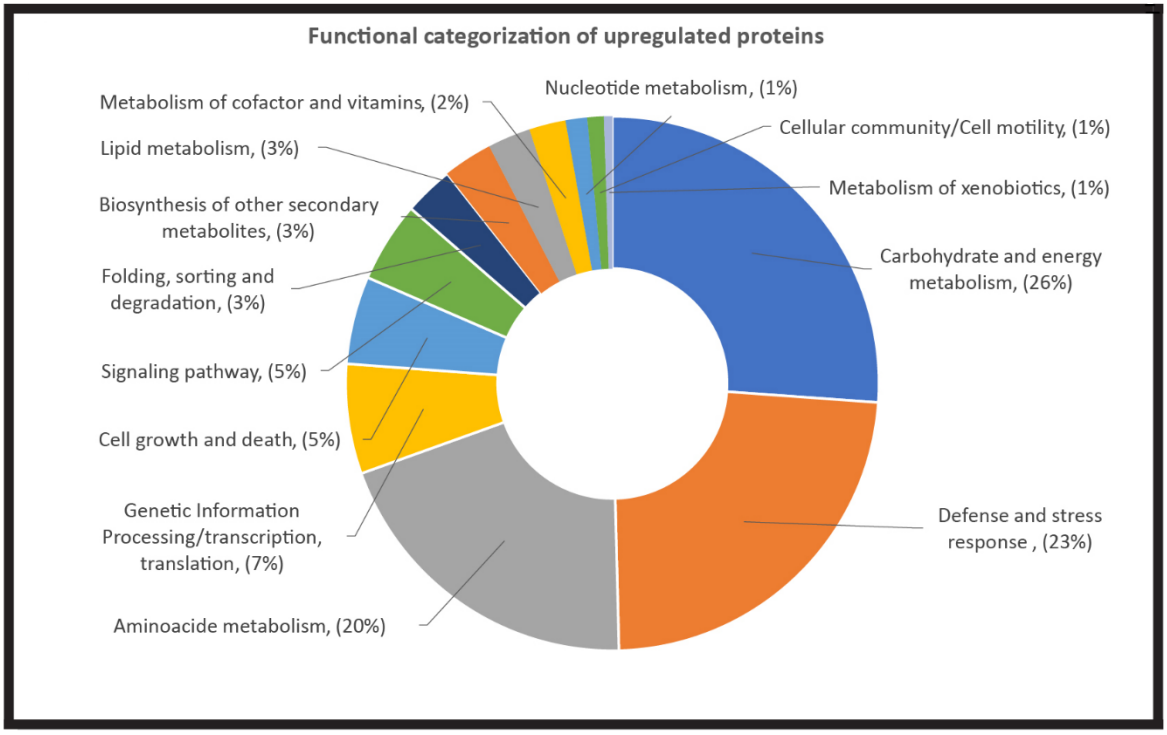


Figure 11: Functional categorization based on KEGG database of up-regulated proteins

The effect of *B. contaminans* resulted in the up-regulation of several proteins involved in energy and carbohydrate metabolism, glycolysis and citric acid cycle. Many proteins involved in defense response were up-regulated, such as the molecular chaperones, antioxidant enzymes and heat shock proteins indicating their crucial protective roles against biotic stress.

5.3.6.2 Down regulated differentially expressed proteins (DEPs)

The four major categories of down regulated DEPs are: defense and stress related (22.5%), energy and carbohydrate metabolism (16.9%), cell growth and death/apoptosis/senescence (15.5%) and amino acid metabolism (9.9%) (Fig: 12).

The other categories are as follows: xenobiotic biodegradation and metabolism (8.4%), translation and transcription (5.6%), lipid metabolism (4.2%), biosynthesis of other secondary metabolites (4.2%), transport and cellular motility (4.2%), signal transduction (2.8

%), metabolism of cofactors and vitamins (2.8 %) and folding, sorting and degradation (2.8%) (Fig: 12).

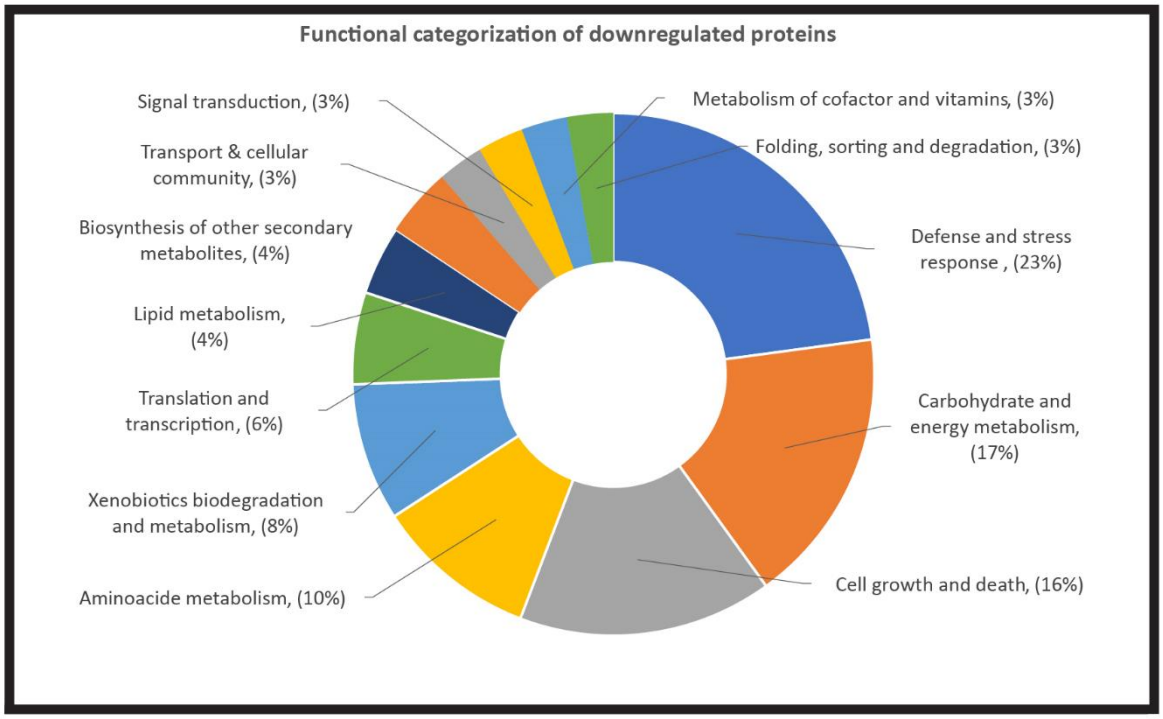


Figure 12: Functional categorization based on KEGG database of down-regulated proteins

The enzymes essential for cell wall rigidity and cell integrity were down regulated significantly in *M. phaseolina* after bacterial inhibition. It is clear that *B. contaminans* manifests profound stress on the fungal cell wall structure and organization.

Down-regulation was observed for major regulators of growth, cell division, motility and development; like RasGTPase, microtubules protein, tubulin alpha chain, kinesin-like proteins etc. These proteins can also be responsible for the reduction in growth and the marked morphological changes observed in *M. phaseolina* hyphae with swelling and subcellular abnormalities after *B. contaminans* stress.

Oxidative damage protecting antioxidant systems enzymes like phosphomannomutase, flavodoxin, glutaredoxin etc. were also down regulated suggesting *Burkholderia's* inhibitory effects on fungal growth.

Glucose-6-phosphate 1-dehydrogenase was down regulated in *M. phaseolina* after the bacterial stress. The enzyme catalyzes the rate-limiting step of the oxidative pentose-phosphate in which NADPH is generated. NADPH participates in the process of oxidation-reduction involved in protecting cells against the toxicity of reactive oxygen species (ROS), allowing the regeneration of the important antioxidant, glutathione (GSH). Glutathione S-transferase and glutaredoxin were also down-regulated. A steady supply of glutathione was apparently absent due to a down-regulation of glucose-6-phosphate 1-dehydrogenase. Reduced expression of proteins involved in the fungal oxidative stress management system appears to be a target for *B. contaminans* inhibition and as a result, an array of proteins involved in oxidative stress response in *M. phaseolina* was found to be down-regulated.

Strikingly, pathogenicity regulatory genes and metabolic and signaling responsive proteins, including those involved in ion transportation, and biosynthesis, were down-regulated, indicating that *M. phaseolina* resorts to energy conservation as an effective strategy for countering the stress imposed by *B. contaminans* NZ.

5.3.7 Protein–protein interaction

Protein–protein interaction networks are important for systems-level understanding of cellular processes. In order to assess such interactive network of up and down regulated fungal proteins, the most up-regulated protein, enolase was searched as the query protein in the STRING database. The first 3 predicted functional partners were found to be pyruvate kinase, phosphoglycerate kinase and triose phosphate isomerase. In *M. phaseolina* all these proteins were found to be up-regulated with enolase (Fig: 13A).

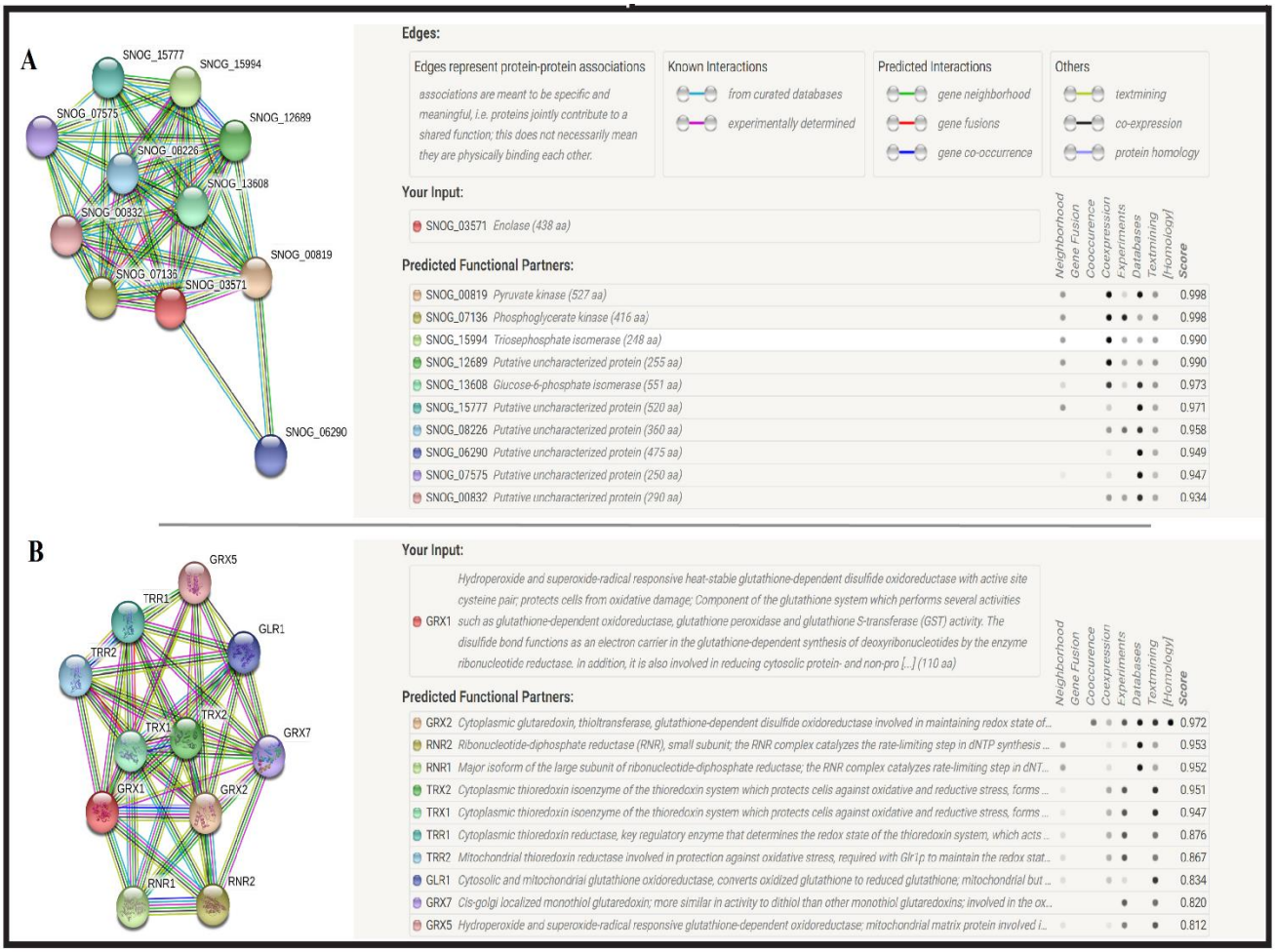


Figure 13: Interaction analysis of (A) Most upregulated protein enolase and (D) down regulated protein GRX1 (Glutathione S-transferase) using STRING database.

When the down-regulated protein glutathione S-transferase which has an important role in oxidative stress management was the input then it's predicted functional partners were found to be glutaredoxin and glutathione oxidoreductase, both of which were down-regulated along with the input protein (Fig: 13 B).

5.4 Discussion

Proteomics is now widely employed to recognize factors responsive to environmental or biotic stresses. Thus a comparison of both *B. contaminans* NZ challenged and unchallenged proteome of *M. phaseolina* was expected to provide an understanding of the physiological response to this biotic stress condition and highlight the variations in the protein profile following the biotic stress.

Besides the genome sequence of *M. phaseolina* that depicts its large arsenal of hydrolyzing enzymes (Islam, Haque et al. 2012), nothing much is known about the proteins it expresses. The abundance of such enzymes was also validated by our proteome data (Fig 4B). Upon analyses of the differentially regulated proteins, a pattern became apparent which allowed an understanding of how this killer pathogen while losing its killing prowess avoids being killed.

Up-regulation of energy production pathway

Most of the *M. phaseolina* proteins with higher expression in *B. contaminans* NZ induced stress have been found to be involved in carbohydrate and energy metabolism. The main pathways that were shown to be affected are the classical glycolytic pathway, TCA cycle, oxidative phosphorylation, pentose phosphate pathway and gluconeogenesis. Other carbohydrate metabolic processes that were also affected include fructose, sucrose, starch and some other monosaccharide metabolic pathways.

In a number of organisms, enolase, an enzyme involved in glycolysis is known to respond to stress by increasing its expression (Ji, Wang et al. 2016). Thus, a 6.2-fold up-regulation of enolase imply that *B. contaminans* causes substantial stress on *M. phaseolina*. This enzyme has been reported to be a multifunctional protein upregulated in heat shock (Li, Tian et al. 2017) and in response to hypoxic stress and glucose deprivation (Ji, Wang et al. 2016). It is possible that among the adverse conditions that *B. contaminans* NZ imposes on *M. phaseolina,* competition for glucose may account for one of them.

Studies by De Backer et al. (2001) and Lo et al. (2005) also reveal that knockout of enolase (ENO1) gene causes a decrease in the growth rate, drug sensitivity and mycelium development of *C. albicans* (Ji, Wang et al. 2016*)*. In response to *B. contaminans* NZ infection, *M. phaseolina* upregulates the formation of enolase. This asserts the fungal attempt to stay alive and corroborates why viable fungus is obtained even from a three month old bacteria-fungus co-culture plate.

Another upregulated enzyme, triosephosphate isomerase, crucial for glycolysis, has been reported to be regulated in response to various abiotic stresses and the expression is specially increased under oxidative stress (de Arruda Grossklaus, Bailão et al. 2013). Additionally, elevated expression of the glycolytic enzyme, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase observed for *M. phaseolina* was similar to that found for *P. umbellatus* where both the enzyme and the glycolytic pathway were found to be induced under oxidative stress condition during the initial sclerotia formation (Li, Tian et al. 2017).

Phosphoglycerate kinase, a housekeeping enzyme of the glycolytic pathway reported to protect cells against oxidants (Minic 2015), was found to be upregulated by 4.5 fold in *M. phaseolina*. Through a proteome analysis of *A. fumigatus*, where 117 proteins were identified with an altered abundance in response to hypoxia, the glycolytic pathway enzyme, phosphoglycerate kinase was shown to have increased activity (Minic 2015).

Most key enzymes of glycolysis have therefore been found to be elevated in *M. phaseolina* after *B. contaminans* challenge, namely ATP-dependent 6-phosphofructokinase which is a highly regulated enzyme of glycolysis along with other glycolytic enzymes ketose-bisphosphate aldolase class-2, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase which catalyzes the final step of glycolysis.

Up-regulation of the citric acid and oxidative phosphorylation pathways indicate a possible mechanism by which *M. phaseolina* fights against the impairments imparted by *B. contaminans*. One of the most significantly up-regulated proteins, citrate synthase, a key enzyme of the TCA cycle determines fungal ability to survive by reducing its competition for nutrient resources (Alekseev, Dubina et al. 2016). Formate dehydrogenase, also upregulated, has an important part in the respiration and oxidative phosphorylation and is known to provide alternative metabolic pathways for fungal survival under unfavorable conditions (Ponpinit and Pinchai).

For *P. lutzii* it has been reported that an antifungal compound, argentilactone down-regulates the entire primary carbohydrate and energy metabolism of the fungus (Prado, Bailão et al. 2015). In contrast, it can be deduced that the mechanism by which *B. contaminans* NZ imparts stress on *M. phaseolina* growth does not involve down-regulation or cessation of the glycolytic or energy cycle pathway but creates an environment deprived of energy sources (such as glucose etc.) which causes a rigorous up-regulation of the aforementioned pathways. Additionally, acyl-CoA dehydrogenase which transfers electrons to FAD to form the $FADH_2$ used in ATP production was found to be upregulated. This enzyme is also known to have a role in signaling by ROS (Poirier, Antonenkov et al. 2006).

Apparently for similar reasons other monosaccharide metabolism pathways (fructose, galactose, mannose) as well as the glyoxalate pathway enzymes were found to be up-regulated. The same was true for the genes of gluconeogenesis pathway. Moreover, fatty acid degradation pathway was also up-regulated. These findings are suggestive of an all-out *M. phaseolina* aggression, which tries to overcome the oxidative stress imposed by *B. contaminans* NZ. The fungus appears to achieve this through a heavy reliance on its energy

resources which ultimately leads to the observed 'hibernation' during the stress and springing to growth once the challenge is removed.

Down-regulation of oxidative stress response pathway

In fungal intracellular signaling, ROS is used to decide between growth and proliferation on one hand, and growth arrest and cell differentiation on the other (Breitenbach, Weber et al. 2015). Interestingly, most of the rate limiting enzymes related to oxidative stress response pathway were shown to be downregulated in *M. phaseolina* under *B. contaminans* NZ stress. The rate limiting enzyme of the pentose phosphate pathway (glucose-6-phosphate 1-dehydrogenase) responsible for NADPH production was down-regulated as well. NADPH, involved in the major oxidation-reduction pathway, acts as a reducing agent for maintaining cell integrity against the reactive oxygen species by regenerating glutathione, a major antioxidant (Berg, Tymoczko et al. 2002). NADH dehydrogenase was also found to be down regulated. So were many oxidative-damage protecting molecules like peroxidase, flavodoxin, glutaredoxin etc. (Zurbriggen, Tognetti et al. 2007, Iraqui, Kienda et al. 2009), suggesting the occurrence of a major imbalance in the cellular oxidation-reduction system in *M. phaseolina*. It matches with the result of the bacterial volatile compound assay (chapter 3). We identified an potent oxidative stress inducer s-[4-cyanophenyl]-n,n-dimethylthiocarbamate as volatile compounds emitted from *B. contaminans* NZ, which may inhibit the enzymes responsible for maintaining the oxidative stress response in *M. phaseolina* leading to tissue injury and growth inhibition (Zhu, Wang et al. 2013)

Down-regulation of cell growth and development:

Proteins essential for cell wall rigidity and cell integrity namely tubulin were greatly down-regulated, indicating a definite stress on fungal cell wall structure or organization. Glycosyl

transferase family 39, essential for cell wall rigidity, cell integrity and budding, growth and adaptation to environmental stress was also down-regulated (Klutts, Yoneda et al. 2006). Glutamine amidotransferase class-2, a downregulated enzyme is reported to be induced by cell wall stressors (Lee, Damsz et al. 2010). It catalyzes the first and rate-limiting step in the biosynthetic pathway for the synthesis of cell wall protein, chitin. Differential expression of cell-wall modification genes is expected when fungi are exposed to stress-related conditions (Gkarmiri, Finlay et al. 2015). Another down-regulated protein heterokaryon incompatibility Het-C reported for regulation of cell wall assembly is essential in fungal growth and development (Saupe, Kuldau et al. 1996).

Down-regulation of the main regulators of growth, cell division, motility and development; like RasGTPase, microtubule proteins, tubulin alpha chain, kinesin-like protein etc. can also be responsible for the observed reduction in colony growth, hyphal movement with swollen and balloon shaped cells and sub-cellular abnormalities (Rischitor, Konzack et al. 2004, Zhao, Liu et al. 2014, Fortwendel 2015). The growth of *M. phaseolina* was entirely inhibited by *B. contaminans* with marked morphological changes. Excessive hyphal branching close to the bacterial colony was also discernible (Chapter 4). Such morphological changes in the cell membrane have also been reported *for F. solani* and *C. dematium* following inhibition by *Burkholderia cepacia* (Narayanasamy 2013).

Up-regulation of defence related proteins

M. phaseolina does up-regulate some of its defense related proteins including chaperonins and heat shock proteins, which usually function as signals for biotic stress. Among them, Hsp 12 is solely responsible for stress tolerance; cell morphology and adhesion, Hsp 20 and 70 are involved in maintaining membrane and cellular proteins (Tiwari, Thakur et al. 2015). Up-regulation of such proteins indicates that *M. phaseolina* is frazzled by the presence of *B.*

contaminans NZ (as evident from the microscopic study of fungal filaments, fig 3, chapter 3).

Down-regulation of virulence proteins

In this study, downregulated proteins assert to a significant loss in the determinants of virulence. Fungi, especially filamentous fungi, produce a vast array of secondary metabolites through the involvement of various cytochrome P450s (CYPs) which are associated with pathogenicity (Chen, Lee et al. 2014). In *F. asiaticum* it has been reported that tubulin binding cofactor A (TBCA) plays crucial roles in the vegetative growth, conidiation, temperature sensitivity and virulence (Zhang, Chen et al. 2015). Down-regulation of both cytochrome P450s and TBCA explains the reason behind the observed loss of pathogenicity in case of challenged *M. phaseolina.*

The secondary metabolite melanin is important for fungal survival and essential for host infection. As melanin synthesis is essential for fungal pathogenicity, enzymes involved in melanin biosynthesis like scytalone dehydratase has been considered a good target for developing control agents against fungal diseases (Yamada, Motoyama et al. 2004). After bacterial interaction, scytalone dehydratase of *M. phaseolina* was found to be downregulated by 7.69 fold indicating severe impairment of melanin biosynthesis leading to decreased stress tolerance and virulence (Tseng, Chung et al. 2011).

Moreover, in fungal pathogens, GTP-binding proteins like septins (downregulated in *M. phaseolina* after the biotic stress) are generally necessary for virulence and are directly associated with host tissue adhesion and entry (Bridges and Gladfelter 2015). In *C. albicans,* a family of four flavodoxin-like proteins (FLPs) act as NAD(P)H quinone oxidoreductases, conferring important antioxidant effects. FLPs reduce ubiquinone (coenzyme Q), enabling it to serve as an antioxidant in the membrane and are critical for fungal virulence (Li, Naseem

et al. 2015). With the down-regulation of flavodoxin in *M. phaseolina*, *B. contaminans* appears to exude both oxidative stress and reduced fungal pathogenicity.

In a virulence study of *A. fumigatus*, ribosomal biogenesis proteins, RNA-processing protein HAT helix and signaling molecules (including G-protein, Ras protein RasGTPase and recoverin) have been reported to increase virulence through an alteration of metabolic response under stressed conditions (Ghazaei 2017). These same proteins found to be downregulated in *M. phaseolina* emphasize their effect on fungal virulence. The downregulated NmrA like protein is known to serve as a regulator of the sugar-sensor mechanism integrating carbon and nitrogen metabolism in order to control plant infection in the rice blast fungus *Magnaporthe oryzae* (Garciandia and Suarez 2013).

Based on the data collected through this experiment, *M. phaseolina* appears to be significantly affected by the presence of *B. contaminans* NZ. Its proteome changes considerably with elevated expression of defense responsive genes and it seems to apply a major emphasis on energy production through the recruitment of diverse pathways, and by decreasing the anabolic system (lipid synthesis etc.). *M. phaseolina* appears to engage its total efforts to survive even by giving up the production of its secondary metabolites like melanin, a pathogenicity curbing compound. The relative capacities of *M. phaseolina* to cause damage to its host therefore appear to be greatly compromised.

B. contaminans NZ also poses an oxidative stressed condition on the fungus. *M. phaseolina* fails to challenge this enforcement because of down-regulation of its major oxidative stress busting systems and the machinery that helps to maintain integrity and rigidity of its cell wall. This is evident from the observed arrest of mycelial spread.

M. phaseolina, a versatile organism in terms of sustainability employs its whole battery of combating mechanisms resulting in a tug of war that leads to an apparent inhibition of *M. phaseolina* growth. However, the fungus under siege manages to stay put in its own territory

and reverts from its arrested growth to an active life with reduced virulence once the challenge is removed. That this notorious fungus can be inhibited using an effective biocontrol agent like *Burkholderia* is not new, but as the first report of *M. phaseolina* proteome the findings from this work is important to understand the fungal defense arsenal which allows it to remain static in the presence of antifungal agents. The available genome data and now this proteomic analysis will together contribute to the development of a robust control system against this fungal strain.

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CHAPTER 6

General Discussion and Concluding Remarks

Pages 128 - 136

General Discussion

Every year, plant pathogenic fungi cause damage to crops, worth millions of dollars, all over the world despite the extensive use of chemical pesticides (Li, Quan et al. 2007).

Jute is a long soft and shiny fiber producing crop better known as golden fiber. It is one of the most affordable, 100% bio-degradable, recyclable and thus environment friendly natural fiber and is considered second only to cotton in the amount produced and the diverse use of vegetable fibers (Khan and Khan 2015). Food and Agriculture Organization of the United Nations (FAO) mentions it as the fiber of the future (ref: http://www.fao.org/economic/futurefibres/fibres/jute/en/). In Bangladesh, annually the fiber yield of jute is reduced by 30% due to the necrotrophic fungal pathogen *Macrophomina phaseolina*. The fungus can infect the root and lower stem of over 500 plant species and has a wide geographic distribution (Kunwar, Singh et al. 1986). *M. phaseolina* hyphae initially invades the cortical tissue of jute plants, followed by sclerotia formation, causing stem rot disease (Islam, Haque et al. 2012). Synthetic chemical fungicides do not provide adequate control of the pathogen, besides being toxic to soil microflora, and hazardous to human and animal health (Gupta, Dubey et al. 2002).

Screening of jute endophytic bacteria that can have a potential inhibiting effect against *M. phaseolina* led us to identify a jute seed endophytic bacterium *Burkholderia contaminans* NZ showing strong antifungal activity against the fungal pathogen.

Due to shared environmental niche and nutrient constraints, inter-species competition occurs between co-habiting bacteria and fungi (Frey-Klett, Burlinson et al. 2011). Several bacteria show antifungal properties by producing antifungal metabolites, chitinolytic enzymes, siderophores, toxins, etc. (Swain, Yadav et al. 2017).

The genus *Burkholderia* contains a large number of diverse species which include many clinically important organisms, phytopathogens, as well as environmental species (Sawana, Adeolu et al. 2014) with an effective association with plants. Different *Burkholderia* species have been reported to protect crops against phytopathogens through the production of a number of different antifungal substances (Li, Quan et al. 2007).

In the present manuscript, we report *B. contaminans* NZ as an extremely versatile biocontrol agent capable of suppressing the growth of a number of plant-pathogenic fungi namely *M. phaseolina, Nigrospora sphaerica, Xylaria sp. Aspergillums fumigatus, Aspergillus niger, Penicillium oxalicum, Rhizoctonia solani*. We also found *B. contaminans* NZ can antagonize these fungi under a broad range of temperature and pH.

B. contaminans NZ inhibits the growth of *M. phaseolina* and causes marked morphological changes with excessive hyphal branching with swollen and balloon shaped cells close to the bacterial colonies. Such morphological changes in the cell membrane have also been reported *for F. solani* and *C. dematium* following inhibition by *Burkholderia cepacia* (Quan, Zheng et al. 2006, Narayanasamy 2013).

In recent years, many reports have been published concerning to the antifungal compounds produced by *Burkholderia* for instance, pyrrolnitrin is effective against fungi, yeasts and gram-positive bacteria produced by *Burkholderia cepacia* complex (Schmidt, Blom et al. 2009). *B. cepacia* also produces cepaciamide an anti-fungal lipopeptide (Lee, Kim et al. 1994, Jiao, Yoshihara et al. 1996).

Burkholderia phenazinium has been found to produce phenazineiodinin, which has both anti-microbial and cytotoxic activities (Eberl and Vandamme 2016). Another *Burkholderia* species *B. gladioli* strain NGJ1 has recently been reported to demonstrate antifungal activity

against *Rhizoctonia solani* and various other fungi exhibiting mycophagy, growing and multiplying at the cost of fungal biomass (Swain, Yadav et al. 2017).

Various reports have demonstrated that volatile organic compounds (VOCs), including hydrocarbons, alcohols, ketones, aldehydes, ethers, esters, terpenes and several heteroaromatic compounds produced by some bacteria can influence the growth of fungi (Wheatley 2002, Schalchli, Hormazabal et al. 2011). *Burkholderia gladioli* pv. agaricicola strain is known for producing volatile organic compounds (VOCs), which inhibit fungal growth and reduce the growth rate of *Fusarium oxysporum* and *Rhizoctonia solani.*

Gas chromatography-mass spectroscopy using sterile activated charcoal, has identified 25 volatile compounds (VOC) produced by *B. contaminans* NZ, including 2-bromo-2-cyano-N, N-dimethyl acetamide, isoproturon and S-[4-cyanophenyl]-N, N-dimethyl thiocarbamate which are known for their antimicrobial activities. The compound 2-bromo-2-cyano-n,n-dimethylacetamide has been reported as a antitumor and a antimicrobial agent (Steiner and Himwich 1964), (ref: https://patents.google.com/patent/US20020147235) and isoproturon is a known pesticide reported for its anthelmintics, antiparasitic, fungicidal activity too [ref: http://www.phenomenex.com/Compound?id=3-%284-Isopropylphenyl%29-1%2C1-Dimethylurea]. *B. contaminans* NZ emits another important volatile compound, [4-cyanophenyl]-n,n-dimethylthiocarbamate, a potent oxidative stress inducer that can lead to oxidative stress and fungal tissue injury by inhibiting superoxide dismutase (SOD) (Zhu, Wang et al. 2013). From the analysis of the proteomics data of *M. phaseolina* under *B. contaminans* NZ stress, an array of proteins involved in oxidative stress response in *M. phaseolina* were found to be surprisingly down regulated. Finding s-[4-cyanophenyl]-n,n-dimethylthiocarbamate, an oxidative stress inducer as a volatile compound suggests that it

could be responsible for the down regulation of enzymes involved in combatting oxidative stress, leading to tissue injury and growth inhibition of *M. phaseolina.*

Bacterial species belonging to the genera *Burkholderia* have also been studied extensively from a biocontrol perspective and they were found to use a wide array of mechanisms to influence plant growth and health (Gkarmiri, Finlay et al. 2015).

Many studies conducted on endophytes, emphasize the ability of these microorganisms to promote plant growth and their additive/synergistic effects on plant growth and protection. Numerous examples highlight endophytic bacteria viz. *Streptomyces* spp (Vurukonda, Giovanardi et al. 2018), *B. subtilis* (Rajamanickam, Karthikeyan et al. 2018) and *Pseudomonas parafulva, Pantoea agglomerans* (Verma, Kingsley et al. 2018).

Since plant growth promoting bacteria (PGPB) are well known for their disease reduction and growth promotion abilities. Biocontrol by use of PGPB can be a potentially efficient approach for sustainable agriculture (Van, 2007). This study provides evidence that *B. contaminans* NZ owns all the qualities of PGPB e.g. IAA and siderophore production, ACC deaminase activity, nitrogen fixation and promoting plant seed germination, root and shoot elongation along with bioactivity against a range of plant pathogenic fungi.

Clarification of the taxonomy of the member of *Burkholderia* genus and the pathogenic potential of environmental isolates, such as *B. contaminans* NZ, is critical prior to intensive studies directed at commercial development as a bioinoculant.

In this study, the pathogenic potential of NZ has been determined through whole genome analysis which has identified the isolate jute endophytic *Burkholderia* to be less virulent by comparing antibiotic and siderophore biosynthesis genes and virulence loci among twelve other *Burkholderia* species. Bio-synthetic genes required for virulence associated proteins like cable (Cbl) pili, adhesin, VgrG-5, a *Burkholderia* type VI secretion system 5-associated

protein, cystic fibrosis (CF)-related O-antigen and zinc metalloprotease are absent in the genome of *B. contaminans* NZ. Moreover, *B. contaminans* NZ is not a member of genomovar III of the *B. cepacia* complex which contains the highly epidemic strains isolated from patients with cystic fibrosis (Mahenthiralingam, Coenye et al. 2000). In addition, data for average nucleotide identity and phylogenetic analysis suggest its close relation to other plant growth-promoting *Burkholderia* strains.

From the whole genome analysis, it is apparent that *B. contaminans* NZ contains genes which contribute to the biosynthesis of several antimicrobial and growth promotion agents making it a versatile and safe to use bio inoculant.

Greater insight into the mechanisms underlying natural antagonistic interactions between bacteria and fungi has the potential to yield significant knowledge that can be used in the application of microorganisms for the biological control of plant diseases, complementing or replacing traditional chemical treatments (Gkarmiri, Finlay et al. 2015). However, responses of fungal pathogens to antagonistic bacteria have not been studied extensively till date.

More recently, studies on the transcriptomic responses to stress based on systems biology tools, such as RNA-seq or microarray, have yielded important insights into the stress adaptation of many organisms (Zhang, Sun et al. 2014). The transcriptional profiling of the mycophagous bacterium *Collimonas fungivorans* and the fungus *Aspergillus niger* during interaction in *in-vitro* dual-culture assays has been reported with altered expression of genes related to lipid and cell wall degradation, cell defence and nitrogen deficiency (Mela, Fritsche et al. 2011). However, transcriptional profiling may only partially contribute to the understanding of stress adaptation because not all transcripts can be translated and mRNA abundances may not correspond to protein expression levels due to pre-, co- and post-translational modification, and proteins, not mRNA, are the effectors of biological functions

(Feder and Walser 2005). In addition, critical regulatory signaling events downstream of transcription will not be detected by transcript analysis (Tomanek 2011).

Proteomics, the large-scale study of protein structures and functions, has the potential to fill this gap and enhance our understanding of the molecular mechanisms of stress responses in *M. phaseolina* after bacterial stress.

We adopted the isobaric tags for relative and absolute quantitation (iTRAQ) coupled with two-dimensional LC-MS/MS approach, which is known to be a benchmarking technique in protein expression analysis with high throughput and high reproducibility (Gan, Chong et al. 2007), to identify the proteins and determine the change in fungal proteome profile after bacterial inhibition. A total of 2204 different proteins were identified in *M. phaseolina* of which 141 were considered to have significant deviation after *B. contaminans* NZ inhibition. Most of the upregulated proteins have been found to be involved in carbohydrate and energy metabolism and down regulated proteins are mostly involved in oxidative stress response, cell growth and virulence.

B. contaminans NZ poses an oxidative stressed condition on the fungus and *M. phaseolina* fails to overcome this challenge due to the down-regulation of its major oxidative stress busting systems and the machinery that helps to maintain integrity and rigidity of its cell wall. This is evident from the observed arrest of mycelial spread. However, *M. phaseolina* appears to engage its total efforts to survive even by giving up the production of its virulence associated compound. The relative pathogenic capacities of *M. phaseolina* to infect host therefore appear to be compromised heavily.

The notorious plant pathogenic fungus leaves no stone unturned to combat the bacterial stress and appears to achieve this through a heavy reliance on its energy production which

ultimately leads to the observed 'hibernation' during the stress and reverts to active growth once the bacterial challenge is removed.

The proteomics data has not only enhanced our knowledge on the overall protein distribution in *M. phaseolina* but also improved our understanding of molecular mechanisms underlying bacteria fungal antagonism. A foundation has now been laid for further studies on characterizing proteins associated with tolerance to biotic and abiotic stressors.

In summary, the findings of the present study indicate that, jute endophytic bacterium *B. contaminans* NZ is an effective plant growth promoting bacteria (PGPB) and a proficient antagonist of *Macrophomina phaseolina* and other plant pathogenic fungi, for which the activities of the volatile compounds seem to play an important role in fungal growth inhibition. Safe to use nature of this bacterium with the ability to improve soil fertility, enhance plant growth and limit phyto-pathogenic fungi can be applied in fields as sustainable eco-friendly bioinoculant for crop improvement. The first ever proteomic study of *M. phaseolina* provides an understanding of its physiological response and variations in the fungal protein profile following *B. contaminans* NZ inhibition.

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