Microbial community dynamics and modulation of defence responses in black pepper by *Trichoderma harzianum*

Thesis Submitted

to the

Faculty of Science, University of Calicut

in Partial fulfillment for the award of

Doctor of Philosophy in Biotechnology

by

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ACKNOWLEDGEMENTS

With deep sense of gratitude and obligation from the core of my heart I express my sincere thanks to my supervisor Dr. M. Anandaraj, Former Director ICAR-IISR, Kozhikode, Kerala, for bringing me into the world of Phytophthora research. His innovative concepts "Trichorhizosphere, tripartite proteomics" and deep involvement in designing the study, made me to acquire new knowledge and skills. Any word of gratitude will be inadequate for his constant guidance and fatherly love during the course of the research.

I am highly grateful to my co guide Late Dr. Sailas Benjamin, Professor, Enzyme technology Lab, University of Calicut, Kerala for his concern and guidance. His critical comments and specific pinpointing of course correction especially on the manuscript preparations will be evergreen in my memories. I extend my cordial thanks to my present co-guide Dr. Santhosh J Eapen, Head, Crop Protection, ICAR-IISR, Kozhikode, Kerala for his encouragement during the later part of my thesis preparation.

I render my sincere thanks to Dr. K. Nirmal Babu, Director, ICAR-IISR, Kozhikode, Kerala for providing the favorable environment in completion of my thesis and Dr. B. Sasikumar, Head, Crop Improvement & Biotechnology, ICAR-IISR, for extending all help during the course of my study.

I feel highly elated to record my sincere thanks to Dr. R. Srinivasan (Principal Scientist –Soil Science) and Dr. Kandiannan (Principal Scientist –Agronomy) for helping me in green house studies. I place my heartfelt thanks to Mrs. Chandravally (Technical Assistant- Crop Protection) and Mrs. K. Sreeja (Research Scholar) for helping me during the course of culturing Phytophthora.

I am pleased to record my gratefulness to Dr. Hamza (Technical Assistant- Soil Science), Dr. R. Praveena (Scientist- Plant Pathology), Dr. Suseela Bhai (Principal Scientist

–Plant Pathology), Dr. D. Prasath (Principal Scientist –Horticulture) for helping me in carrying out the research.

My heartfelt thanks goes to Dr. K.S. Krishnamurthy (Principal Scientist –Plant Physiology), Dr. Jayashree (Principal Scientist - Agrl Engineering), Dr. Rema (Principal Scientist- Horticulture), Dr. Johnson K. George, Dr. R. Ramakrishnan Nair (Principal Scientists- Cytogenetics) and Dr. Rashid Parvez (Principal Scientist – Nematology) for their concern during the course of my research.

Words fail to express my deep love and gratitude from the bottom of my heart for my loving and caring husband Dr. S. Manivannan (Dean), Dept. of Horticulture, Central University, Sikkim, my mother Mrs. V. Kasthuri (Retd. Teacher), my mother in law Mrs. S. Samboornam and my son, Master. S. M. Puvan Adithya. It is their sacrifice, relentless encouragement and unmatched affection which made my work to reach its fruitful destination. My sincere thanks are due to my family members who were always with me to help and encourage throughout the course of the study.

Last but not the least, I thank my father Late Shri. A Palaniyandi , (Retd. Teacher), father in law, Late Shri. M. Subramanian (Chief Goods Superintendent, Southern Railways) and my forefathers for their blessings.

Financial assistance rendered through the Outreach Project on Phytophthora, Fusarium and Ralstonia Diseases of Horticultural and Filed Crops (PhytoFuRa) by Indian Council of Agricultural Research is deeply acknowledged.

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Abbreviation

%	-percentage
µl	-micro liter
1D gel	- One dimensional gel
B	-Boron
Ca	- Calcium
C	-Carbon
Cfu	-colony forming unit
CID	-Collision induced dissociation
cm	-centi meter
CO_2	-carbon di oxide
Cu	-Copper
ddH_20	- double distilled water
DNA	-Di nucleic acid
dpi	-days post infection
EST	-Expressed sequence tags
et al	-and others
Fe	-Ferrous
Fig	-Figure
G	-gram
GC	-Guanine Cytosine
H	-hour
HR	-Hyper sensitive reaction
KDa	-Kilo Dalton
Kg	- Kilogam
LC	- Liquid chromatography
LTQ	- Linear Trap Quadrupole
Mg	- Magnesium
Min	-Minutes
ml	-mille liter

mm - milli meter

Mn -Mangnese

MS - Mass spectrometry

N - Nitrogen

NCBI - National Center for Biotechnology information

Ng -nano gram

No -Number

^{o}C - Degree Celcious

P - Phosphorus

pH - Negative logarithm of hydrogen ions

ppm -parts per million

PR -Pathogenesis related

S - Sulphur

Sec - seconds

Sp. -Species

TMV -Tobacco mosaic virus

T-ISR - *Trichoderma* induced systemic resistance

Zn - Zinc

Table 10.b: Peptides with the sequence, score, ion and mass of T-ISR proteins from tripartite interaction

Protein name of peptide	Peptide sequence under quantitation	Peptide ion	Score	Mass
RPP13	AQELLMSLLK	1651	53.91	1144.6340
Germin like Protein	VTFLDDAQVK	3955	44.37	1134.5923
Subtilisin like protease	LADPFDYGGGLVNPNK	876	65.01	1675.8218
Carbonic anhydrase like protein	NPELYGELAK	611	39.02	1132.5760
Methionine synthase	YLFAGVVDGR	1525	52.51	1095.5709
NADP malic enzyme	SIQVIVVTDGER	1073	64.34	1314.7144
	VLIQFEDFANHNAFVLLAK	1514	34.30	2218.1445
Malate dehydrogenase	DDLFNINAGIVIC	2776	51.82	1317.6929
Protein transport inhibitor 1	KLEMLSIAFAGDGDLGLHHVISG CESLR	927	33.80	2983.4453
Aldolase type TIM	VTSVASFFVSR	2892	62.06	1198.6349
Nucleoside diphosphate kinase 2	GLVGEIISR	939	63.09	942.5501
	IIGATNPADSAPGTIR	807	58.25	1552.8217
Isocitrate dehydrogenase	DQYLNTEEFIDAVAEELK	3919	120.51	2126.0075
Succinate semialdehyde dehydrogenase (mitochondrial)	VETLLQDATSK	1512	58.64	1203.6349
NmrA like (-)ve transcriptional regulator family protein	FFPSEFGNDVDR	2059	51.01	1428.6317
Isoflavone reductase	YLPSEFGNDVDR	3843	30.90	1410.6416
WIN 2 wound induced protein	YGWTAFCGPVGPR	4112	55.70	1466.6764
Translationally controlled tumor like protein	VVDIVDVFR	3194	74.12	1060.5917
2-methylene furane 3 one reductase	VAAAALNPUDSK			
Porin	SLFTISGEVDTR	2783	61.75	1323.6673

Table 10.b: Peptides with the sequence, score, ion and mass of T-ISR proteins from tripartite interaction

Cinnamoyl Co-A	DVAEALILLYEK	4323	48.79	1375.7609
Leucine amino peptidase	EVFAASCVSGEK	871	43.56	1251.5983
	TIEVNNTDAEGR	3101	49.12	1317.6165
Dehydrogenase Family protein	TAEQTPLSALYAAK	2137	40.25	1462.7674
2-cys peroxiredoxin BAS1	APDFEAEAVFDQEFINVK	1561	46.20	2067.9801
	GLFIIDKEGVIQHSTINNLAIGR	2701	59.83	2507.3898
	LNTEVLGVSIDSVFSHLAWVQTDR	1865	35.37	2685.3773
Superoxide dismutase (Cu-Zn)	AFVVAELEDDLGKGGHELSLTTGNAGGR	1613	70.04	2878.4228
Superoxide dismutase (Fe-Mn)	LVVETTANQDPLVTK	2789	72.16	1626.8832
Catalase1	EGNWDLVGNNFPVFFIR	683	67.41	2022.9972
Catalase 2	EGNWDLVGNNFPVFFIR	809	66.86	2022.9985
Catalase 3	DLYSISAGNYPEWK	979	37.36	1756.7952
Peoxisomal (S)-2-hydroxy-acid oxidase GLO1 like	AIALTVDTPLLGR	519	79.73	1338.7872
	VPVFLDGGVR	1048	62.58	1057.5919
Peroxidase 12	IVS**C**ADITAIAAR	483	100.61	1359.7189
Peroxidase 12 like precursor	QGLFTSDQDLYTDC	3336	73.68	1657.7589
Peroxidase 5-like	GCDGSVLIDSTASVSEK	2764	41.33	1809.8604
Peroxidase 16	FSQTFVTAPATLR	2194	50.14	1437.7616
Peroxidase 60	GVVSCADIIAMAAC	163	75.66	1448.7446
Glutathione S tranferase F13 like	NPFGQIPVLDGDLTLFESR	977	55.60	2246.7268
	VLDVYEER	232	40.24	1021.5090
	VLDYYEGR	753	44.27	949.4865

Table 10.b: Peptides with the sequence, score, ion and mass of T-ISR proteins from tripartite interaction

Reactive intermediate Deaminase A chloroplastic	FVSDTIEEQTEQVLK	4049	48.41	1764.8408
Peroxiredoxin	LPFTLLSDEGNK	2578	70.05	1332.6933
	NGVVQLIYNNQFQPEK	3498	28.41	1889.9637
Ascorbate peroxidase 2 cytosolic	TGGPFGTIR	1213	43.09	904.4763
Ascorbate peroxidase 1 cytosolic	ALLSDPVFRPLVDK	2317	55.87	1568.8929
Ascorbate peroxidase 6 cytosolic	RDEDLLVLPTDAVLFEDPSFK	3648	51.95	2418.2370

Table 1: Growth parameters of black pepper *Tirchoderma harzianum* treated (T1) and Control (T2)

S. No	Parameter	T1 Mean	T2 Mean	Pr> (*t*)
1	Shoot weight (Fresh) (g)	7.7	3.0	<.0001
2	Root weight (Fresh) (g)	44.5	26.6	0.0050
3	Leaf area index (LAI)	802.5	430.4	0.0028
4	Stem Girth (cm)	0.1225	0.1400	0.3896
5	Height of the plant (cm)	78.5	44.4	0.0023
6	Root weight (Dry) (g)	1.7	0.7950	0.0018
7	Shoot weight (Dry) (g)	9.9	4.3	0.0003

Table 2: Enzymes with its EC number, pathways from metagenomes

Enzyme with EC Number (*Trichoderma harzianum* inoculated metagenome)	**Pathway involved**
cob(II)yrinic acid a,c-diamidereductase EC: (1.16.8.1):	Porphyrin and chlorophyll metabolism
nicotinamidase EC: (3.5.1.19)	Nicotinate and nicotinamide metabolism
dihydropteridinereductase [EC:1.5.1.34]	Folate biosynthesis
uracil reductase EC:3.5.4.261.1.1.193	Riboflavin metabolism
1.14.13.178 theophylline:oxygen oxidoreductase	Caffeine metabolism
3.5.4.17 adenosine-phosphate deaminase.	Purine metabolism
EC 2.7.1.53 L-xylulokinase	Ascorbate and aldarate metabolism
glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase [EC:2.4.1.122]	Mucin type O-Glycan biosynthesis
heparan-alpha-glucosaminide N-acetyltransferase [EC:2.3.1.78]	Heparan sulfate degradation
arylsulfatase B [EC:3.1.6.12]	Dermatan sulfate degradation
1.14.13.64	Benzoate degradation
protocatechuate,NADH:oxygenoxidoreductase, EC 1.17.99	Toluene degradation
PAH dioxygenase large subunit EC:1.13.11	Xenobiotics biodegradation and metabolism
EC 5.5.1.7 chloromuconatecycloisomerase	Fluorobenzoate degradation and Chlorocyclohexane and chlorobenzene degradation,
EC 1.1.1.288 xanthoxin dehydrogenase	Carotenoid biosynthesis,
EC 1.1.1.195 cinnamyl-alcohol dehydrogenase	Phenylpropanoid biosynthesis
Enzyme with EC Number (Un inoculated)	
beta-hydroxy-delta5-steroid dehydrogenase / steroid delta-isomerase [EC:1.1.1.1455.3.3.1]	Steroid hormone biosynthesis
chlorophyll synthase [EC:2.5.1.62]	Porphyrin and chlorophyll metabolism
chorismate--pyruvate lyase [EC:4.1.3.40),	Ubiquinone and other terpenoid-quinone biosynthesis
D-erythrose 4-phosphate dehydrogenase [EC:1.2.1.72]	Vit B6 metabolism
L-gulonate 3-dehydrogenase [EC:1.1.1.45]	uronate pathway
phosphatidylinositol 4-kinase type 2 [EC:2.7.1.67]	Inositol phosphate metabolism
peroxiredoxin 6, 1-Cys peroxiredoxin [EC:1.11.1.71.11.1.15 3.1.1]	Phenyl propanoid pathway
EC 2.7.7.14ethanolamine-phosphate cytidylyltransferase	Phosphonate and phosphinate metabolism

Table 3: Protein dynamics in Black pepper – *Trichoderma harzianum* interaction at 24 hai

Up regulated	Fold Change	Down regulated	Fold Change	Completely down regulated (Fold change : Infinity)	New proteins	Fold Change
Structural maintenance of chromosomal protein 2	1.33e+004	ATP synthase CF1 alpha subunit	64.26	PREDICTED: elongation factor Tu, chloroplastic-like	PREDICTED: peroxisomal (S)-2-hydroxy-acid oxidase GLO1-like	Infinity
Hypothetical protein SELMODRAFT	1.24	Ribulose- 1,5 bisphosphate carboxylase large chain	4043.63	PREDICTED: photosystem II CP43 reaction center protein-like	PREDICTED: probable LRR receptor-like serine/threonine-protein kinase RLK	Infinity
Rpp 4C4	123.55	ATP synthase beta chain	3.51e+005	PREDICTED: LOW QUALITY PROTEIN: ATP synthase gamma chain 1, chloroplastic	PREDICTED: protein transport protein SEC16B homolog isoform X1	Infinity
		Glyceroldehyde 3 PO4 dehydrogenase A subunit	49.16	PREDICTED: 17.8 kDa class I heat shock protein-like		
		Chloroplast sedoheptulose, 1, 7 bisphosphatase	39.36	2-cys peroxiredoxin BAS1		
				PREDICTED: peroxidase 12		

Table 3: Protein dynamics in Black pepper – *Trichoderma harzianum* interaction at 24 hai

Up regulated	Fold Change	Down regulated	Fold Change	Completely down regulated (Fold change : Infinity)	New proteins	Fold Change
		Rubisco activase	3.76	hypothetical protein ARALYDRAFT_909725		
		Peroxisomal glycolate oxidase	7.71	20 kDa chaperonin, chloroplastic-like		
		Catalase isozyme	412.94	harpin binding protein 1		
		Predicted protein	105.64	Transketolase		
		Superoxide dismutase	8.39	PREDICTED: triosephosphate isomerase, cytosolic		
		Peroxidase 2 like	1005.21	photosystem II protein V (chloroplast)		
		Aspartate amino transferase	2452.52	PREDICTED: cysteine synthase-like		
		Glutathione S- transferase F13	6.25	PREDICTED: V-type proton ATPase catalytic subunit A		
		Fructose-bisphosphate aldolase 1	35.39	hypothetical protein SORBIDRAFT_02g011260		

Table 3: Protein dynamics in Black pepper – *Trichoderma harzianum* interaction at 24 hai

Up regulated	Fold Change	Down regulated	Fold Change	Completely down regulated (Fold change : Infinity)	New proteins	Fold Change
		5- methyl tetra hydropteroyltriglutamate	5.79	PREDICTED: uncharacterized protein At5g02240-like		
		homocysteine methyl transferase 2	5.79	chlorophyll a-b binding protein 3, chloroplastic		
		Germin like prtoein	5287.68	heat shock protein 70B		
		Ascorbate peroxidase 1	63.40	PREDICTED: peroxidase 5		
				hypothetical protein SORBIDRAFT_02g011260		
				PREDICTED: uncharacterized protein At5g02240-like		
				chlorophyll a-b binding protein 3, chloroplastic		
				heat shock protein 70B		
				PREDICTED: peroxidase 5		
				hypothetical protein SELMODRAFT_270693		

Table 3: Protein dynamics in Black pepper – *Trichoderma harzianum* interaction at 24 hai

Up regulated	Fold Change	Down regulated	Fold Change	Completely down regulated (Fold change : Infinity)	New proteins	Fold Change
				60S ribosomal protein L12-like		
				PREDICTED: RUBISCo small chain, chloroplastic-like		
				O-acetylserine (thiol)lyase family protein		
				Aldolase-type TIM barrel family protein		
				predicted protein, partial		
				PREDICTED: ferredoxin--NADP reductase, leaf isozyme, chloroplastic-like		
				triosephosphate isomerase, cytosolic		
				PREDICTED: disease resistance protein RPP13-like		

Table 3: Protein dynamics in Black pepper – *Trichoderma harzianum* interaction at 24 hai

Up regulated	Fold Change	Down regulated	Fold Change	Completely down regulated (Fold change : Infinity)	New proteins	Fold Change
				PREDICTED: ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplastic		
				hypothetical protein CHLNCDRAFT_31033		
				UTP-glucose-1-phosphate uridylyltransferase		
				alanine:glyoxylate aminotransferase		
				glutamate synthase 2		
				carbonic anhydrase family protein		
				PREDICTED: bromodomain and WD repeat-containing protein 3-like isoform X1 [		

Table 3: Protein dynamics in Black pepper – *Trichoderma harzianum* interaction at 24 hai

Up regulated	Fold Change	Down regulated	Fold Change	Completely down regulated (Fold change : Infinity)	New proteins	Fold Change
				glyceraldehyde-3-phosphate dehydrogenase C2		
				hypothetical protein SELMODRAFT_408500		
				H(+)-transporting ATPase, subunit 1 (mitochondrion)		
				Peroxidase 16 precursor family protein		
				chaperonin 60A		
				ATPase, V1 complex, subunit B protein		

Table 4: Protein dynamics in black pepper – *Trichoderma harzianum* at 48 hai

Infinite in control and shut down in treatment
REDICTED: heat shock-related 70 kDa protein 2
PREDICTED: elongation factor 1-alpha 1
PREDICTED: ATP synthase subunit beta, mitochondrial
PREDICTED: glyceraldehyde-3-phosphate dehydrogenase
PREDICTED: rap guanine nucleotide exchange factor 2
PREDICTED: ATP synthase subunit alpha, mitochondrial
PREDICTED: collagen alpha-6(VI) chain-like
PREDICTED: heat shock 70 kDa protein 13
PREDICTED: nebulin-related-anchoring protein
PREDICTED: platelet-derived growth factor receptor beta-like
PREDICTED: histone H2A-beta, sperm

Table 5: Protein dynamics in black pepper – *Trichoderma harzianum* at 72 hai

Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Infinite in control , completely down regulated**
Splicing factor U2 large subunit B-like isoform X1	19.80	ATP synthase CF1 beta subunit	215.40	PREDICTED: ATP synthase subunit beta, mitochondrial-like
		E3- ubiquitin protein ligase HOS 1 like	272.09	ATP synthase CF1 alpha subunit (chloroplast)
				PREDICTED: ruBisCO large subunit-binding protein subunit alpha, chloroplastic-like
				catalase isozyme 2
				PREDICTED: photosystem II CP43 reaction center protein-like
				PREDICTED: ruBisCO large subunit-binding protein subunit alpha
				PREDICTED: ribulose bisphosphate carboxylase large chain
				uncharacterized protein LOC100281701
				heat shock protein 70B
				PREDICTED: peroxidase 12
				P-loop containing nucleoside triphosphate hydrolases superfamily protein
				ATPase, F1 complex, gamma subunit

Table 5: Protein dynamics in black pepper – *Trichoderma harzianum* at 72 hai

				protein
				glyceraldehyde-3-phosphate dehydrogenase C2
				photosystem II protein V (chloroplast)
				20 kDa chaperonin, chloroplastic-like
				predicted protein
				superoxide dismutase [Cu-Zn], chloroplastic
				uncharacterized protein LOC103831934
				ruBisCO large subunit-binding protein subunit beta
				PREDICTED: cysteine synthase-like
				Transketolase
				PREDICTED: ribulose bisphosphate carboxylase small chain, chloroplastic-like
				O-acetylserine (thiol)lyase family protein [Populus trichocarpa]
				predicted protein
				PREDICTED: photosystem I reaction center subunit IV B, chloroplastic-like
				fructose-bisphosphate aldolase 1
				chlorophyll a-b binding protein AB80,

Table 5: Protein dynamics in black pepper – *Trichoderma harzianum* at 72 hai

				chloroplastic-like
				glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic
				UDP-GLUCOSE PYROPHOSPHORYLASE 1
				heat shock protein 60
				PREDICTED: probable fructose-bisphosphate aldolase 3, chloroplastic
				peroxisomal glycolate oxidase
				catalase
				photosystem I P700 apoprotein A2 (chloroplast)
				methionine synthase
				alanine:glyoxylate aminotransferase
				PREDICTED: ferredoxin--NADP reductase, leaf isozyme, chloroplastic-like
				heat shock protein 82
				PREDICTED: ribulose bisphosphate carboxylase small chain 1, chloroplastic
				PREDICTED: cysteine synthase
				harpin binding protein 1
				PREDICTED: small heat shock protein,

Table 5: Protein dynamics in black pepper – *Trichoderma harzianum* at 72 hai

				chloroplastic
				carbonic anhydrase family protein
				ascorbate peroxidase 1, cytosolic
				PREDICTED: germin-like protein subfamily 1 member 17
				lactate/malate dehydrogenase family protein
				ATPase, V1 complex, subunit B protein
				PREDICTED: subtilisin-like protease SBT3.8

Table 6: Protein dynamics in black pepper – *Trichoderma harzianum* at 96 hai

Down regulated	Fold Change	Infinite in control and completely down regulated
Rubisco large subunit binding protein	2409.84	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast)
		PREDICTED: ATP synthase subunit beta, mitochondrial-like
		PREDICTED: ruBisCO large subunit-binding protein subunit alpha
		uncharacterized protein LOC100281701
		ATP synthase CF1 beta subunit (chloroplast)
		bisphosphate carboxylase large chain
		heat shock protein 70-3
		ATP synthase CF1 beta subunit (chloroplast)
		PREDICTED: photosystem II CP43 reaction center protein-like
		PREDICTED: serine hydroxymethyltransferase 1, mitochondrial
		carboxylase/oxygenase activase 2, chloroplastic-like isoform X1
		catalase isozyme 2
		PREDICTED: transketolase, chloroplastic
		aspartate aminotransferase
		PREDICTED: peroxidase 12
		aspartate aminotransferase
		PREDICTED: peroxidase 12
		PREDICTED: 17.8 kDa class I heat shock protein-like
		PREDICTED: cysteine synthase-
		RAB GTPase homolog E1B
		peroxisomal glycolate oxidase
		ATPase, F1 complex, gamma subunit protein
		harpin binding protein 1
		photosystem II protein V (chloroplast)
		chlorophyll a-b binding protein 3, chloroplastic
		glyceraldehyde-3-phosphate dehydrogenase C2
		20 kDa chaperonin, chloroplastic-like

Table 6: Protein dynamics in black pepper – *Trichoderma harzianum* at 96 hai

		photosystem II 47 kDa protein (chloroplast)
		ruBisCO large subunit-binding protein subunit beta
		PREDICTED: uncharacterized protein At5g02240-like
		hypothetical protein SELMODRAFT_411087
		predicted protein
		NADP malic enzyme 4
		methionine synthase
		PREDICTED: ribulose bisphosphate carboxylase small chain, chloroplastic-like
		Ribosomal protein L11 family protein
		PREDICTED: cysteine synthase
		O-acetylserine (thiol)lyase family protein
		predicted protein
		PREDICTED: photosystem I reaction center subunit IV B, chloroplastic-like
		heat shock protein 60
		chlorophyll a-b binding protein AB80, chloroplastic-like
		3-phosphate dehydrogenase A subunit 2
		PREDICTED: glutathione S-transferase F13
		UDP-GLUCOSE PYROPHOSPHORYLASE 1
		uncharacterized protein LOC100501719
		PREDICTED: probable fructose-bisphosphate aldolase 3, chloroplastic
		catalase
		photosystem I P700 apoprotein A2 (chloroplast)
		vacuolar ATP synthase subunit A
		PREDICTED: ferredoxin--NADP reductase, leaf isozyme, chloroplastic-like
		protein CP24 10A, chloroplastic
		PREDICTED: ribulose bisphosphate carboxylase small chain 1, chloroplastic
		hypothetical protein PRUPE_ppa010963mg
		PREDICTED: leucine aminopeptidase 1-like
		sedoheptulose bisphosphatase 1

Table 6: Protein dynamics in black pepper – *Trichoderma harzianum* at 96 hai

		PREDICTED: ferredoxin-dependent glutamate synthase, chloroplastic
		PREDICTED: UTP--glucose-1-phosphate uridylyltransferase
		uncharacterized protein LOC100193491
		H(+)-transporting ATPase, subunit 1 (mitochondrion)
		carbonic anhydrase family protein
		ascorbate peroxidase 1, cytosolic
		superoxide dismutase [Cu-Zn], chloroplastic
		PREDICTED: germin-like protein subfamily 1 member 17
		malate dehydrogenase, cytoplasmic
		dehydrogenase family protein
		ATPase, V1 complex, subunit B protein
		PREDICTED: subtilisin-like protease SBT3.8

Table 7: **Protein dynamics in Black pepper – *Phytopthora capsici* interaction at 12, 24 hai**

P12					P24				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
Histone H2A	241.00	Ribulose, 1,5, bis phosphate carboxylase/oxygenase large subunit	2.73	ATP synthase CF1 alpha subunit (chloroplast)	2-cys peroxiredoxin BAS 1	4.71	ATP synthase CF1 alpha subunit (Chloroplast)	13.71	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
Heat shock protein 70B	1.69	Rubisco large subunit bnding protein subunit beta	2.64	PREDICTED: ribulosebisphosphate carboxylase large chain	Photo system II CP43 reaction center protein kinase	1.37	ATP synthase subunit beta, Mitochondria - like	11.85	ruBisCO large subunit-binding protein subunit alpha
Histone H4	31.35	ATP synthase CF1 beta subunit	72.32	catalase isozyme 2	Low quality protein: ATP synthase gamma chain 1	2.51	Rubisco oxygenase large subunit	2.36	ruBisCO large subunit-binding protein subunit alpha
Chlorophyll a-b binding protein AB80	1.49	Uncharacterized protein LOC 100382265	3.38	ruBisCO large subunit-binding protein subunit alpha	Heat shock protein 70B	4.65	Uncharacterized protein LOC 100281701	3.57	serine hydroxymethyltransferase 1, mitochondrial
Photosystem II	1.22	Uncharacterized protein LOC	633.5	ruBisCO large subunit-binding	Cytosolic isocitrate	9.30	Heat shock	41.46	catalase isozyme 1

Table 7: **Protein dynamics in Black pepper – *Phytopthora capsici* interaction at 12, 24 hai**

P12					P24				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
47 KDa protein		100281701		protein subunit alpha, chloroplastic-like	dehydrogenase		protein 70		(New protein)
Ubiquitin Carboxyl – terminal hydrolase 5-like isoform X1	27.35	Glyceroldehyde -3- phosphate dehydrogenase C2	4.18	Transketolase	17.8 KDa class heat shock protein-like	1.04	Peroxisomal (s) 2 hydroxy acid oxidase	1.97	ribulosebisphosphate carboxylase large chain
		Peroxisomal glycolate oxidase	6.03	peroxidase 12	Malate dehydrogenase, glyoxysomal	4.06	Chlorophyll a –b binding protein of LHC II type 1	11.09	ATP synthase CF1 alpha subunit (chloroplast)
		Photosystem I protein V	2.45	17.8 kDa class I heat shock protein-like	Peroxisomal (s)-2-hydroxy-acid oxidase GLO1	184.37	Transketolase	145.41	20 kDachaperonin, chloroplastic-like
		Photosystem I protein D1	3.67	ATP synthase F1 subunit alpha	Mono dehydroascorbate reductase, seedling	2.57	Chaperonin CPN 60-2 mitochodrialp	48.15	superoxide dismutase [Cu-Zn], chloroplastic

Table 7: **Protein dynamics in Black pepper – *Phytopthora capsici* interaction at 12, 24 hai**

P12					P24				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
					isozyme		recursor		
		Fructose bisphosphatealdolase1	2.14	ATPase, F1 complex, gamma subunit protein	Predicted protein	2.96	Chlorophyll a-b binding protein AB80	1.09	ATP synthase CF1 epsilon subunit (plastid)
		Hypotheticalprotein POPTR-0008s08410g	2.14	uncharacterized protein At5g02240-like	NADP malic enzyme 4	2.91	Glyceraldehyde-3-Po4 dehydrogenase C2	2.51	cysteine synthase-like
		Chlorophyll a-b binding protein	5.21	ribulosebisphosphate carboxylase small chain, chloroplastic-like	Predicted-subtilisin –like protease	6.17	Aspartate amino transferase	3.16	Ribosomal protein L11 family protein
		Predicted protein	15.18	cysteine synthase	UDP –Glucose phosphorylase	1.02	Peroxidase 12	2.08	O-acetylserine (thiol)lyase family protein
		Heat shock protein	361.68	superoxide dismutase [Cu-Zn],	Enolase	1.70	Ferredoxin-NADP reductase	1.38	chlorophyll a-b binding protein of LHCII

Table 7: **Protein dynamics in Black pepper – *Phytopthora capsici* interaction at 12, 24 hai**

P12					P24				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
				chloroplastic					
		Glyceroldehyde -3- phosphate dehydrogenase A subunit 2	418.38	fructokinase 3	Trios phosphate isomerase	23.87	Chlorophyll a- b binding protein 151	1.07	probable fructose-bisphosphatealdolase 3, chloroplastic
		Germin like protein subfamily1 member 17	6.54	RAB GTPase homolog E1B	Glutathione S-transferase F13	1.13	Peroxidase precursor family protein	2.85	methionine synthase
				photosystem I reaction center subunit IV B, chloroplastic-like	Heat shock protein 82	6.90	Photo system II 47 KDa protein	1.51	UTP-glucose-1-phosphate uridylyltransferase
				UDP-GLUCOSE PYROPHOSPHORYLASE 1	Un characterized protein LOC 100501585	1.82	Ribulose bisphosphate carboxylase small chain	43.79	hypothetical protein CHLNCDRAFT_31033
				catalase	Hypothetical	1.02	Predicted	2.41	leucineaminopeptidase

Table 7: **Protein dynamics in Black pepper – *Phytopthora capsici* interaction at 12, 24 hai**

P12					P24				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
					protein CICLE-V 10013933		protein		1-like
				methionine synthase	probable L-type lectin-domain containing receptor kinase S.5 (New protein)	New Protein	Photo system reaction center Subunit IVB	3.71	
				alanine:glyoxylate aminotransferase	Photo system 1 subunit H2	8.17	Glyceraldehyde-3- Po4 dehydrogenase A	182.27	UTP--glucose-1-phosphate uridylyltransferase
				ferredoxin--NADP reductase, leaf isozyme, chloroplastic-like	Small heat shock protein chloroplastic	3.46	Catalase	3.37	uncharacterized WD repeat-containing protein C2A9.03-like isoform X1
				harpin binding protein 1			Predicted germin like protein subfamily 1	41.06	protein TRANSPORT INHIBITOR RESPONSE 1

Table 7: **Protein dynamics in Black pepper – *Phytopthora capsici* interaction at 12, 24 hai**

P12					P24				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
							member 17		
				small heat shock protein, chloroplastic			Vacuolar ATP synthase subunit A	48.39	lactate/malate dehydrogenase family protein
				carbonic anhydrase family protein			ATP ase, V1 complex, subunit B protein	2.59	peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase A-like isoform X1
				probable fructose-bisphosphatealdolase 3, chloroplastic			Ribulose bisphosphate carboxylase small chain 1	1.22	
				ascorbate peroxidase 1, cytosolic					
				uncharacterized WD repeat-			Cysteine	82.20	

Table 7: **Protein dynamics in Black pepper – *Phytopthora capsici* interaction at 12, 24 hai**

P12					P24				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
				containing protein C2A9.03-like isoform X1			synthase		
				protein TRANSPORT INHIBITOR RESPONSE 1			Catalase isozyme 3	3.88	
				ATPase, V1 complex, subunit B protein			Carbonic an hydrase family protein	1.49	
							Ascorbate peroxidase 1 cytosolic	35.26	
							Hypothetical protein POPTR-0008s08410g	8.16	

Table 7: **Protein dynamics in Black pepper – *Phytopthora capsici* interaction at 12, 24 hai**

P12					**P24**				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
							E3 ubiquitin –protein ligase HOS 1	2.34	
							Predicted protein	1.00	

Table 8: Protein dynamics in Black pepper – *T. harzianum*- *P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
ruBisCO large subunit-binding protein subunit alpha	1.74	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast)	1.21	cysteine synthase-like	ATP synthase CF1 beta subunit (chloroplast)	1.14	ATP synthase beta chain	1.07	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
ruBisCO large subunit-binding protein subunit alpha isoform X1	1.82	ATP synthase CF1 beta subunit (chloroplast)	1.06	photosystem II protein V (chloroplast)	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	New protein	ATP synthase CF1 beta subunit	9.85	malate dehydrogenase, glyoxysomal
probable mediator of RNA polymerase II transcription subunit 37c	3.7	SORBIDRAFT_01g000380 [Sorghum bicolor]	1.12	chlorophyll a-b binding protein 3, chloroplastic	probable mediator of RNA polymerase II transcription subunit 37c	1.96	ribulose bisphosphate carboxylase large chain	1051.25	chlorophyll a-b binding protein 3, chloroplastic
CARUB_v10028629mg	New protein	ATP synthase CF1 beta subunit (chloroplast)	5.11	photosystem II 47 kDa protein	O2 evolving complex 33kD family protein	9.07	ribulose bisphosphate carboxylase/oxygenase activase,	1.05	photosystem II protein V (chloroplast)

Table 8: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
							chloroplastic		
dehydrogenase family 2 member B4, mitochondrial isoform X1	6.5	ribulose bisphosphate carboxylase large chain	230.27	photosystem I reaction center subunit IV B, chloroplastic-like	oxygen-evolving enhancer protein 1, chloroplastic-like	5.41	catalase isozyme 2	2.72	chlorophyll a-b binding protein AB80, chloroplastic-like
dehydrogenase family 2 member B7, mitochondrial-like	2.43	ATP synthase CF1 beta chain	3.77	predicted protein	catalase isozyme 1	11.79	transketolase, chloroplastic	1.42	hypothetical protein CHLNCDRAFT_31033
2-cys peroxiredoxin BAS1	2.89	photosystem II CP43 reaction center protein-like	23.23	photosystem I P700 apoprotein A2	ruBisCO large subunit-binding protein subunit alpha	2.67	hypothetical protein POPTR_0008s08410g	1.59	PREDICTED: protein TRANSPORT INHIBITOR RESPONSE 1
superoxide dismutase [Cu-Zn], chloroplastic	3.96	Hydroxyl methyl transferase 1, mitochondrial	1.50	chlorophyll a-b binding protein CP24 10A, chloroplastic	superoxide dismutase [Cu-Zn], chloroplastic	16.80	uncharacterized protein LOC100384473	1.01	

Table 8: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
methionine synthase	4.24	carboxylase/oxygenase activase 2, chloroplastic-like isoform X1	6.93	UTP--glucose-1-phosphate uridylyltransferase	2-cys peroxiredoxin BAS1	4.91	ribulose bisphosphate carboxylase small chain 1, chloroplastic	1.49	
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	11.31	Transketolase	3.31		ruBisCO large subunit-binding protein subunit alpha	2.21	cysteine synthase	1.09	
catalase isozyme 2	37.11	catalase isozyme 1	1.14		uncharacterized protein LOC100281701	1.04	17.8 kDa class I heat shock protein-like	133.85	
glyceraldehyde 3-phosphate dehydrogenase A subunit 2	1.29	ATP synthase gamma chain 1, chloroplastic	1.34		glyceraldehyde-3-phosphate dehydrogenase, cytosolic	2.28	uncharacterized protein LOC100799358	2.62	
ferredoxin-dependent	2.00	ruBisCO large subunit-binding	1.78		ruBisCO large subunit-binding	2.21	RAB GTPase	2.70	

Table 8: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
glutamate synthase, chloroplastic		protein subunit beta			protein subunit beta, chloroplastic		homolog E1B		
peroxisomal (S)-2-hydroxy-acid oxidase GLO1-like	1.93	leucine amino peptidase 1-like	1.13		ruBisCO large subunit-binding protein subunit alpha, chloroplastic	1.04	photosystem II protein D1 (chloroplast)	125.90	
PRUPE_ppa010963mg	1.52	sedoheptulose-1,7-bisphosphatase, chloroplastic-like	1.06		glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic	34.64	predicted protein	1.28	
fructose-bisphosphate aldolase cytoplasmic isozyme	35.83	17.8 kDa class I heat shock protein-like	13.50		hypothetical protein POPTR_0014s13660g	20.84	Mono dehydroascorbate reductase, seedling isozyme	1.06	
glyceraldehyde-3-phosphate dehydrogenase	1.29	uncharacterized protein LOC100799358	1.24		CARUB_v10010551mg	312.42	catalase	1.01	

Table 8: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
C2									
uncharacterized protein LOC103974064	2.47	RAB GTPase homolog E1B	1.58		ATPase, V1 complex, subunit B protein	2.33	ribulose bisphosphate carboxylase small chain, chloroplastic-like	5.93	
NADP-dependent malic enzyme	2.13	2-methylene-furan-3-one reductase	1.83		peroxisomal (S)-2-hydroxy-acid oxidase GLO1-like	2.12	PREDICTED: photosystem I reaction center subunit IV B, chloroplastic-like	3.16	
V-type proton ATPase catalytic subunit A	2.10	20 kDa chaperonin, chloroplastic-like	2.99		glutathione S-transferase F13-like	2.14	heat shock protein 60	2.07	
phosphoglycerate kinase	2.65	uncharacterized protein At5g02240-like	17.19		ATP synthase CF1 epsilon subunit	3.55	ascorbate peroxidase 1, cytosolic	1.04	

Table 8: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
					(chloroplast)				
ARALYDRAFT_909725	1.56	Enolase	1.16		ferredoxin--NADP reductase, leaf isozyme, chloroplastic-like	1.81	germin-like protein subfamily 1 member 17	2.47	
aspartate aminotransferase	1.55	ferredoxin--NADP reductase, leaf isozyme, chloroplastic-like	1.23		ATP synthase gamma chain 1, chloroplastic	1.18	PREDICTED: disease resistance protein RPP13-like	32.48	
cytosolic isocitrate dehydrogenase	6.41	Ribosomal protein L11 family protein	1.04		ATP synthase F1 subunit alpha (mitochondrion)	2.66	probable fructose-bisphosphate aldolase 3, chloroplastic	1.37	
NmrA-like negative transcriptional regulator family protein	7.44	ribulose bisphosphate carboxylase small chain, chloroplastic-like	1.79		nucleoside diphosphate kinase 1	4.31	UTP-glucose-1-phosphate uridylyltransferase	46.73	

Table 8: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
peroxidase 12	1.37	UTP-glucose-1-phosphate uridylyltransferase	1.32		latex plastidic aldolase-like family protein	1.22	germin-like protein precursor	1.96	
malate dehydrogenase, glyoxysomal	4.41	O-acetylserine (thiol)lyase family protein	1.06		cytosolic isocitrate dehydrogenase	5.14	ferredoxin-dependent glutamate synthase, chloroplastic	8.22	
Malate dehydrogenase 1	1.28	phytochrome B-like, partial	6.01		Phospho glycerate kinase, cytosolic-like	1.73	uncharacterized mitochondrial protein AtMg00810-like	11.39	
peroxiredoxin Q, chloroplastic	4.35	heat shock protein 82	1.39		aspartate aminotransferase	1.12	retrotransposon protein	1.29	
Phosphoglycerate kinase family protein	9.61	ribulose bisphosphate carboxylase small chain 1,	1.70		catalase isozyme 3	New protein	hypothetical protein SORBIDRAFT_01g007230	2.51	

Table 8: Protein dynamics in Black pepper – *T. harzianum*- *P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
		chloroplastic							
glutathione S-transferase F13-like	1.84	catalase isozyme 3	1.17		peroxidase 12	2.06	hypothetical protein SELMODRAFT_408500	72.08	
Malate dehydrogenase family protein	12.28	predicted protein	5.90		enolase 2	2.47			
peroxidase 12	1.32	oxygen-evolving enhancer protein 2-1, chloroplastic-like	1.03		Malate dehydrogenase 1	1.97			
harpin binding protein 1	1.26	carbonic anhydrase family protein	1.18		NADP-dependent malic enzyme	2.83			
nucleoside diphosphate kinase 2	9.10	germin-like protein subfamily 1 member 17	1.12		cysteine synthase-like isoform X1	1.11			
Triose	3.15	lactate/malate	1.31		harpin binding	1.38			

Table 8: Protein dynamics in Black pepper – *T. harzianum*- *P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
phosphate isomerase, cytosolic		dehydrogenase family protein			protein 1				
Aldolase-type TIM barrel family protein	6.69				leucine aminopeptidase 1-like	2.48			
peroxisomal (S)-2-hydroxy-acid oxidase GLO1-like	5.58				triosephosphate isomerase, cytosolic	New protein			
peroxidase 12-like precursor	41.53				peroxidase 12	1.93			
malate dehydrogenase, cytoplasmic	1.66				predicted protein	1.71			
40S ribosomal protein S5	5.21				peroxidase 60	2.34			
hypothetical protein	4.08				20 kDa chaperonin,	1.40			

Table 8: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
POPTR_0016s00260g					chloroplastic-like				
hypothetical protein SELMODRAFT_411087	1.17				2-methylene-furan-3-one reductase	3.35			
uncharacterized protein LOC100305513	17.02				Iron/manganese superoxide dismutase family protein	5.93			
Phospho enol pyruvate carboxylase 1	New Protein				peroxisomal (S)-2-hydroxy-acid oxidase GLO1-like	New protein			
cysteine synthase	1.53				20S proteasome alpha subunit E1	8.44			
Triose phosphate isomerase,	6.91				ascorbate peroxidase 2	36.54			

Table 8: Protein dynamics in Black pepper – *T. harzianum*- *P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
cytosolic									
copper/zinc superoxide dismutase 1	15.76				photosystem I subunit VII chloroplast	New protein			
predicted protein	2.59				uncharacterized protein LOC103831934	11.78			
cysteine synthase	3.84				subtilisin-like protease SBT3.8	2.12			
succinate-semi aldehyde dehydrogenase , mitochondrial	3.37				triosephosphate isomerase, cytosolic	8.82			
Phosphoribulo kinase	12.42				mitochondrial outer membrane protein porin of 36 kDa	8.66			
hypothetical protein	1.44				DUF810-domain-	New Protein			

Table 8: Protein dynamics in Black pepper – *T. harzianum*- *P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
CHLNCDRAFT_31033					containing protein				
probable fructose-bisphosphate aldolase 3, chloroplastic	2.28				Nucleic acid-binding, OB-fold-like protein	7.18			
protein plastid transcriptionally active 16, chloroplastic	9.21				hypothetical protein PHAVU_007G074200g	New Protein			
wound-induced protein WIN2 precursor	7.10				predicted protein	2.99			
20S proteasome alpha subunit PAD1	New protein				uncharacterized protein LOC100501585	2.11			

Table 8: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
glucose-1-phosphate adenylyltransferase small subunit 2, chloroplastic-like	4.92				Ribosomal protein L11 family protein	3.14			
uncharacterized protein LOC100501719	1.58				Aldolase superfamily protein	New protein			
Catalase	1.10				hypothetical protein PRUPE_ppa011053mg	New protein			
Peroxidase 16 precursor family protein	8.59				hypothetical protein POPTR_0008s19410g	New Protein			
alanine:glyoxylate	2.74				macrophage migration	New			

Table 8: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
aminotransferase					inhibitory factor	Protein			
reactive Intermediate Deaminase A, chloroplastic	12.47				probable L-ascorbate peroxidase 6, chloroplastic	2.29			
Mono dehydroascorbate reductase	23.45				low-temperature-induced cysteine proteinase	2.39			
oxygen-evolving enhancer protein 1, chloroplastic-like	3.20				alanine:glyoxylate aminotransferase	3.61			
succinate-semialdehyde dehydrogenase, mitochondrial-	2.31				vacuolar ATP synthase subunit A	2.09			

Table 8: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
like									
peroxidase 5-like	2.06				cinnamoyl-CoA reductase 2-like isoform X1	2.42			
photosystem I subunit H2	1.24				ADP,ATP carrier protein 1, mitochondrial precursor	4.82			
subtilisin-like protease SBT3.8	2.18				hypothetical protein CICLE_v10022104mg	2.71			
eif4a-2	1.32				ATP synthase CF0 subunit I	4.00			
macrophage migration inhibitory factor	3.21				translationally-controlled tumor-like protein	New protein			
20 kDa	1.28				photosystem I	8.70			

Table 8: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
chaperonin, chloroplastic					subunit H2				
ascorbate peroxidase 1, cytosolic	1.10				uncharacterized protein At5g08430-like	24.61			
uncharacterized protein LOC104798653	New protein				succinate-semialdehyde dehydrogenase, mitochondrial	2.10			
hypothetical protein CICLE_v10022104mg	2.84				PREDICTED: uncharacterized protein At2g37660, chloroplastic-like	New Protein			
isoflavone reductase-like protein .	New protein				peroxidase 5-like	325.12			
ATPase, V1 complex, subunit B	3.60				carbonic anhydrase family	2.43			

Table 8: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
protein					protein				
ATP-dependent DNA helicase PIF1-like	3.08				Thio redoxin superfamily protein	New protein			
					uncharacterized protein At5g02240-like	80.93			
					photosystem II oxygen-evolving complex protein 2 precursor	11.30			
					plastocyanin A, chloroplastic	10.46			
					uncharacterized protein LOC100783304	15.57			
					malate dehydrogenase,	3.62			

Table 8: Protein dynamics in Black pepper – *T. harzianum*- *P. capsici* interaction at T72P12, T72P24 hai

T72P12 (T8)					T72P24 (T9)				
Up regulated	**Fold Change**	**Down Regulated**	**Fold Change**	**Completely down**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down**
					cytoplasmic				
					PREDICTED: uncharacterized protein At2g33490-like	New protein			
					alpha-mannosidase isoform X2	22.05			
					probable plastid-lipid-associated protein 3, chloroplastic	1.01			

Table 9: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T96P12, T96P24 hai

T96P12 (T10)					T96P24 (T11)				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
Ribulose 1,5 bisphosphate carboxylase/oxygenase large subunit	3.55	Ribulose bisphosphate carboxylase large chain	1.95	Ribulose bisphosphate carboxylase/oxygenase activase chloroplastic like	Super oxide dismutase (Cu-Zn)	42.02	ATP synthase subunit beta	2.59	ribulose bisphosphate carboxylase large chain
ATP synthase CF 1 beta subunit	2.63	Un characterized protein LOC 100281701	1.19	photosystem II protein V (chloroplast)	Copper/zinc super oxide dismutase	1302.44	ATP synthase CF1 alpha subunit	2.33	ruBisCO large subunit-binding protein subunit alpha, chloroplastic-like
Probable mediator of RNA polymerase II transcription	2.33			chlorophyll a-b binding protein 3, chloroplastic	Uncharacterized protein LOC 100191684	New Protein	ATP synthase CF1 beta subunit	2.10e+006	LOW QUALITY PROTEIN: ATP synthase gamma chain 1, chloroplastic

Table 9: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T96P12, T96P24 hai

T96P12 (T10)					T96P24 (T11)				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
Ribulose large subunit binding protein subunit alpha	1.27	Glyceroldehyde -3- PO4- dehydrogenase A subunit 2	1.15	photosystem II 47 kDa protein (chloroplast)	Fructokinase 3	31.38	Glyceraldehyde 3 PO4 dehydrogenase C2	1.05	ruBisCO large subunit-binding protein subunit alpha
ATP synthase CF1 beta subunit	1.37	Photosystem II CP43 reaction center protein - like	158.02	chlorophyll a-b binding protein AB80, chloroplastic-like	Glutathione-S-transferase F13	2.01	Catalase isozyme 2	95.53	heat shock protein 70B
Rubisco large subunit-binding protein beta	1.01	Aldehyde dehydrogenase family 2 member B7	1.51	photosystem I P700 apoprotein A2 (chloroplast)	Plastocyanin A	77.59	PhotosystemII CP43 reaction center protein like	9.47	peroxidase 12
Glyceraldehyde-3-Po4- dehydrogenase C2	2.38	Serine hydroxymethyl transferase 1	1.47	chlorophyll a-b binding protein CP24 10A,	Oxygen evolving enhancer	12.61	Uncharacterized protein LOC100281701	427.02	photosystem II protein V (chloroplast)

Table 9: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T96P12, T96P24 hai

T96P12 (T10)					T96P24 (T11)				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
				chloroplastic	protein 2-1				
2-cys peroxiredoxin BAS1	5.37	Transketolase	1.48	PREDICTED: 20 kDa chaperonin, chloroplastic	Chaperonin CPN 60-2 mitochondrial precursors	1.31	Catalase enzyme 1	28.19	peroxidase 60
Aldehyde dehydrogenase family 2 member B7	5.56	Ascorbate peroxidase 1 cytosolic	110.39	lactate/malate dehydrogenase family protein	Alpha mannoside isoform X2	New Protein	Peroxisomal glycolate oxidase	4.28	uncharacterized protein At5g02240-like
ATPase V1 complex subunit B protein	1.45	17.8 KDa class I heat shock protein-like	3.71				Chlorophyll a-b binding protein 3	7392.90	Transketolase
Peroxisomal oxidase GLO1 like(s)-2-	1.80	Cysteine synthase like	61.02				20 KDa chaperonin	79.53	NADP malic enzyme 4

Table 9: Protein dynamics in Black pepper – *T. harzianum*- *P. capsici* interaction at T96P12, T96P24 hai

T96P12 (T10)					T96P24 (T11)				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
hydroxy-acid									
Catalase 1	1.34	20 KDa chaperonin	1.63				Fructose-bisphosphate aldolase 1	3.18	ribulose bisphosphate carboxylase small chain, chloroplastic-like
Low quality protein ATP synthase gamma chain 1	2.20	Cysteine synthase	1.11				Rubisco large subunit binding protein	17.85	O-acetylserine (thiol)lyase family protein
ATP synthase f1 subunit alpha	3.06	Heat shock protein 60	1.26				Uncharacterised mitochondrial protein At mg00810mg Predicted protein	4.00	photosystem I reaction center subunit IV B, chloroplastic-like

Table 9: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T96P12, T96P24 hai

T96P12 (T10)					T96P24 (T11)				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
L-ascorbate peroxidase 2-cytosolic	110.39	Uncharacterized protein LOC 100799558	2.04				catalase	2.54	chlorophyll a-b binding protein AB80, chloroplastic-like
Superoxide dismutase (Cu- Zn)	2.33	Predicted un characterised protein At 5g02240-like	2.42				Photosystem II 47 kDa protein	9.75	glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic
Malate dehydrogenase, glyoxysomal	2.12	Peroxidase 60	1.34						UDP-GLUCOSE PYROPHOSPHORYLASE 1
Peroxidase 12	1.71	Heat shock protein 60	1.26				Catalase isozyme 2	12.01	probable fructose-bisphosphate aldolase 3,

Table 9: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T96P12, T96P24 hai

T96P12 (T10)					T96P24 (T11)				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
									chloroplastic
Nucleoside diphosphate kinase1	4.51	Nucleic acid binding OB-fold-like protein	1.31				Lactate/malate dehydrogenase family protein	12.13	aspartate aminotransferase
Ferredoxin-NADP reductase	1.22	Dehydro ascorbate reductase	New Protein				Carbonic anhydrase family protein	573.79	methionine synthase
Cytosolic isocitrate dehydrogenase	1.76	Heat shock protein 82	1.2				Ascorbate peroxidase	41.86	alanine:glyoxylate aminotransferase
Leucine aminopeptidase1 like	2.42	In 2-1 family protein	New Protein				Oxygen evolving enhancer protein 3	851.92	ferredoxin--NADP reductase, leaf isozyme,

Table 9: Protein dynamics in Black pepper – *T. harzianum*- *P. capsici* interaction at T96P12, T96P24 hai

T96P12 (T10)					T96P24 (T11)				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
									chloroplastic-like
Hairpin binding protein1	1.76	Ferredoxin – dependent glutamate synthase	1.06						sedoheptulose bisphosphatase 1
Ribosomal protein L11 family protein	1.70	UTP-glucose -1phosphate uridylyl transferase	5.82						PREDICTED: cysteine synthase
ATP synthase CF1 epsilon subunit	2.24	Small heat shock protein	181.29						harpin binding protein 1
Trios phosphate isomerase	2.75	Protein transport inhibitor response 1	2.07						PREDICTED: ribulose bisphosphate carboxylase small chain 1, chloroplastic

Table 9: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T96P12, T96P24 hai

T96P12 (T10)					T96P24 (T11)				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
Fe/Mn superoxide dismutase family protein	5.77								lactate/malate dehydrogenase family protein
ADP,ATP carrier protein	5.07								ATPase, V1 complex, subunit B protein
Uncharacterized protein LOC 103831939									
Predicted protein	124.18								
Photosystem I reaction center center subunit IV B, chloroplastic	1.35								
Catalase	1.26								
Macrophage	1.50								

Table 9: Protein dynamics in Black pepper – *T. harzianum*- *P. capsici* interaction at T96P12, T96P24 hai

T96P12 (T10)					T96P24 (T11)				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
migration inhibitory factor									
Ribulose bisphospahte carboxylase small chain	3.12								
UDP- glucose pyrophosphoryase	2.70								
NADP malic enzyme 4	2.73								
Peroxidase 12	1.92								
Predicted protein	1.77								
Aldolase- type TIM barrel family protein	82.70								
Predicted protein	2.84								
Glucose 1 Po4	1.65								

Table 9: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T96P12, T96P24 hai

T96P12 (T10)					T96P24 (T11)				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
adenyltransferase small subunit									
Subtilisin like protease SBT 3.8	2.20								
UTP-glucose-1 –4 uridyl transferase	7.59								
Peroxidase 16 precursor family protein	4.41								
Eif 4a -2	3.74								
Alanine: glyoxylate amino transferase	2.24								
Vacuolar ATP synthase subunitA	1.43								
Germin like protein subfamily 1 member	2.59								

Table 9: Protein dynamics in Black pepper – *T. harzianum- P. capsici* interaction at T96P12, T96P24 hai

T96P12 (T10)					T96P24 (T11)				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
17									
Phosphoglycerate kinase	1.33								
O2 evolving enhancing protein1 chloroplastic 1	9.83								
Photosystem I subunit H2	5.16								
Pyruvate kinase	3.15								
Cytochrome p 450 734 A6- like isoform	4.72								
Carbonic anhydrase family protein	2.40								
Enolase 2	1.59								
Nuclear pore complex protein	1.44								

Table 9: Protein dynamics in Black pepper – *T. harzianum*- *P. capsici* interaction at T96P12, T96P24 hai

T96P12 (T10)					T96P24 (T11)				
Up regulated	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**	**Up regulated**	**Fold Change**	**Down regulated**	**Fold Change**	**Completely down regulated**
NUP-1 like isoform X1									
O-acetyl serine	4.92								
Hypothetical/protein SORBIDRAFT-03g025780	9.48								
Malate dehydrogenase,cytoplasmic	2.81								
Catalase isozyme 3	New Protein								

Table 10.a . Dynamics of T-ISR proteins in tripartite interaction

S.No	Name of the Protein	T72	T96	P12	P24	T72P12 (T8)	T72P24 (T9)	T96P12 (T10)	T96P24 (T11)
	A. Defense Related Proteins								
1	RPP13						32.48		
2	Germin like Protein	∞	∞	6.54	41.06	1.12	2.47	2.59	
3	Subtilisin like protease	∞	∞		6.17	2.18	2.12	2.2	
4	Carbonic anhydrase like protein	∞	∞	∞	1.49	1.18	2.43	2.4	573.79
5	Methionine synthase	∞	∞	∞	∞	4.24			
6	NADP malic enzyme		∞		2.91	2.13	2.83	2.73	∞
7	Malate dehydrogenase		∞		4.06	4.41	3.62	2.81	∞
8	Protein transport inhibitor 1			∞	∞		∞	2.07	
9	WD repeat containing proteins			∞	∞				
10	Aldolase type TIM					6.69		82.7	
11	Nucleoside diphosphate kinase 2					9.1	4.31	4.51	
12	Isocitrate dehydrogenase				9.3	6.41	5.14	1.76	
13	Succinate semialdehyde dehydrogenase (mitochondrial)					3.37	2.1		
14	NmrA like (-)ve transcriptional regulator family protein					7.44		1.7	
15	Isoflavone reductase					∞			
16	WIN 2 wound induced protein					7.1			
17	Translationally controlled tumor like protein						∞		
18	2-methylene furane 3 one reductase					1.83	3.35	1.23	

Table 10.a . Dynamics of T-ISR proteins in tripartite interaction

19	Porin								8.66
20	Cinnamoyl Co-A						2.42		
21	Leucine amino peptidase		∞		∞	1.13	2.48	2.42	
22	Fructose bis phosphate aldolase (cytoplasmic)			2.14	∞	35.83	1.35		∞
	B. ROS Scavenging Proteins								
1	Dehydrogenase family protein					6.5		5.56	
2	2-cys peroxiredoxin BAS1	∞			4.71	2.39	4.91	5.73	
3	Superoxide dismutase (Cu-Zn)		∞	∞	∞	3.96	16.80	2.33	1320.44
4	Superoxide dismutase (Fe-Mn)			∞	∞		5.93		
5	Catalase isozyme 1,2,3		Cat2- ∞	Cat2- ∞	Cat1 - ∞ Cat 3- ∞	Cat 2 -37.71	Cat1- 11.79 Cat 2- 2.72 Cat 3- ∞		
6	Pexoxisomal(S)-2-hydroxy-acid oxidase GLO1-like				184.37	1.93	2.12		
7	Peroxidase 12	∞	∞		∞	1.50	2.06	1.71,1.92	
8	Peroxidse 12 like precursor				∞	41.53			
9	Peroxidase 5-like					2.06	325.12	-	
10	Peroxidase 16					8.59		4.41	
11	Peroxidase 60								1.34
12	Glutathione S-transferase F13 like				1.13	1.84	2.14	3.24	2.01
13	Reactive intermediate Deaminase A chloroplastic					12			
14	Monodehydro ascorbate reductase,					23.45			

Table 10.a . Dynamics of T-ISR proteins in tripartite interaction

15	Peroxiredoxin Q					4.35			
16	Ascorbate peroxidase 2 cytosolic			∞	∞	1.10	36.54	110.39	
17	Ascorbate peroxidase 1 cytosolic	∞	∞				1.04	1.04	
18	Ascorbate peroxidase 6 cytosolic						2.29		

(Green color – Up regulation; b) Brown color – Down regulation)

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BRAZILIAN JOURNAL OF MICROBIOLOGY xxx (2017) xxx–xxx

BRAZILIAN JOURNAL OF MICROBIOLOGY

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Environmental Microbiology

Trichoderma harzianum MTCC 5179 impacts the population and functional dynamics of microbial community in the rhizosphere of black pepper (*Piper nigrum* L.)

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ARTICLE INFO

Article history:
Received 27 September 2016
Accepted 16 May 2017
Available online xxx

Associate Editor: Jerri Zilli

Keywords:
Rhizosphere
Population abundance
Functional abundance

ABSTRACT

Employing Illumina Hiseq whole genome metagenome sequencing approach, we studied the impact of *Trichoderma harzianum* on altering the microbial community and its functional dynamics in the rhizhosphere soil of black pepper (*Piper nigrum* L.). The metagenomic datasets from the rhizosphere with (treatment) and without (control) *T. harzianum* inoculation were annotated using dual approach, *i.e.*, stand alone and MG-RAST. The probiotic application of *T. harzianum* in the rhizhosphere soil of black pepper impacted the population dynamics of rhizosphere bacteria, archae, eukaryote as reflected through the selective recruitment of bacteria [*Acidobacteriaceae bacterium* ($p = 1.24e{-}12$), *Candidatus koribacter versatilis* ($p = 2.66e{-}10$)] and fungi [(*Fusarium oxysporum* ($p = 0.013$), *Talaromyces stipitatus* ($p = 0.219$) and *Pestalotiopsis fici* ($p = 0.443$)] in terms of abundance in population and bacterial chemotaxis ($p = 0.012$), iron metabolism ($p = 2.97e{-}5$) with the reduction in abundance for pathogenicity islands ($p = 7.30e{-}3$), phages and prophages ($p = 7.30e{-}3$) with regard to functional abundance. Interestingly, it was found that the enriched functional metagenomic signatures on phytoremediation such as benzoate transport and degradation ($p = 2.34e{-}4$), and degradation of heterocyclic aromatic compounds ($p = 3.59e{-}13$) in the treatment influenced the rhizosphere micro ecosystem favoring growth and health of pepper plant. The population dynamics and functional richness of rhizosphere ecosystem in black pepper influenced by the treatment with *T. harzianum* provides the ecological importance of *T. harzianum* in the cultivation of black pepper.

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https://doi.org/10.1016/j.bjm.2017.05.011

Please cite this article in press as: Umadevi P, et al. *Trichoderma harzianum* MTCC 5179 impacts the population and functional dynamics of microbial community in the rhizosphere of black pepper (*Piper nigrum* L.). *Braz J Microbiol.* (2017), https://doi.org/10.1016/j.bjm.2017.05.011

Introduction

Plants contribute to the establishment of specific ecological niches of microbes in the rhizosphere by playing key role as ecosystem engineers.[1] The microbial community at the rhizosphere reflects its functional specificity at the level of plant–microbe interactions. It suggests that taxonomically-contrasted plant growth promoting strains may coexist in soil and colonize the same rhizosphere. The probiotic community enrichment by the plant is the major element in plant response to various biotic and abiotic stresses, coupled with the application of plant growth promoting microbes.[2] In the plant rhizosphere, the plant growth-promoting microbes play main roles such as modifying the root functioning, improving plant nutrition and its intake, and influencing the physiology of entire plant. Secondary metabolites secreted by the soil microbes has role in controlling biotic interactions.[3] The chemical ecology research field that focus on the understanding the specific interaction mediated by the producer organism with the target microbe and with the microbial community is of immense importance in rhizosphere microniche. The experimental approaches on the role of secondary metabolites suggests that they can act to slow down the germination of spores in order to bring less competitive environment for the growth, act as agents of symbiosis and competitive weapons against other competing organisms.[4] Hence, integrating functional and ecological knowledge on microbial populations in soil will be a prerequisite in developing novel management strategies for sustainable agriculture for which the population abundance of soil microbiome is an important component.

Trichoderma (telemorph Hypocrea) is an asexual fungal genus inhabiting the soil of all climatic zones; many of its species are used as effective biofertilizer and biocontrol agents for plants grown in greenhouse as well as fields.[5–7] The mechanism mediated by *Trichoderma* spp. includes the antibiotic activity,[8] mycoparasitism,[9] cell wall-lytic enzyme action,[10] competition for nutrients,[11] the induction of systemic resistance to pathogens in plants[5]; and nutrient supply through the degradation of biomass.[6,7]

Black pepper (*Piper nigrum* L.) – a native to India and popularly known as the king of spices – is an export oriented important spice crop grown in tropical countries. The foot rot disease caused by *Phytophthora capsici*, an oomycete pathogen contributes to the major crop loss as it infects the vine both in nursery and fields.[12] The elegant studies on *Trichoderma harzianum* (MTCC 5179) toward its growth promotion[13,14] and disease suppression[15–17] activities made this fungus an important component in the integrated disease management module of the cultivation strategy of black pepper in India. Thus, we hypothesized that the probiotic application of *Trichoderma* would alter the community composition or dynamics of other soil fungi and bacteria at the rhizosphere of black pepper; and that might contribute to the plant health in a better way than the rhizosphere community without *Trichoderma*. In the light of this hypothesis, this study is designed with three objectives: (a) to inoculate the rhizosphere of black pepper with *T. harzianum* (MTCC 5179) for assessing its impact on microbial community dynamics in the rhizosphere, (b) to subject the rhizhosphere soil to whole genome metagenomics analysis, and (c) to bring out the taxonomic and functional abundance for understanding the community dynamics.

Materials and methods

Raising of explant

Single node cuttings from *Sreekara* variety of black pepper were washed with Tween 20 for 15 min, followed by running tap water. The cuttings were subsequently surface sterilized with copper oxychloride (0.2%) for 15 min, and washed twice with sterile double distilled water (ddH_2O). The cuttings were again surface sterilized with mercuric chloride (0.1%) for 5 min, followed by wash with ddH_2O twice. The cut ends of the cuttings were quick dipped in indole-3-butyric acid (8000 ppm), and planted in protray on sterile perlite medium fortified with sterile Hoagland's solution.[18]

The protrays with the preparation as above were maintained in greenhouse with top portion sealed with aluminum foil. The cuttings were sprayed with Hoagland solution once in a day. After 2 months of growth (when plants attained 24–26 cm height with 4–5 leaves), the rhizosphere (perlite) samples from the plants were collected and analyzed for the presence or absence of *Trichoderma* spp. by plating (spread/pour plate method). Subsequently, saplings with no association of *Trichoderma* spp. were transferred to the pots filled with top soil (composition: 197 Ca; 173 K, 71 Mg; 18 S; 11.38 Fe; 5.56 Mn; 3.24 Zn; 1.64 P; 0.92 Cu; 0.16 B (all in ppm); and 1.6% organic carbon, pH: 4.35). Two sets of experiments [inoculated with *T. harzianum* (MTCC 5179), the treatment and without inoculation of *T. harzianum*, the control] with 4 replicates having 3 plants per replica were designed for the study. Talc formulation of *T. harzianum* (MTCC 5179) (3.5 g/3 kg soil) was used for inoculating the soil. Growth parameters *viz.*, height of the plant, stem girth (1 cm above from the soil region) and the leaf area index (LAI) were recorded. The LAI was calculated using the formula: length (cm) × width (cm) × 0.6. After 120 days, plants were uprooted, the rhizosphere soil (adhered to the roots of pepper plants) sample were collected from 3 biological replicates of both treatment and control, and stored at −80 °C. The weights of shoot and root (fresh and dry) were also recorded.

Extraction of rhizosphere soil DNA and sequencing

The rhizosphere soil DNA from the treated and control plants were extracted from 100 mg of soil using MoBio kit (MO BIO Laboratories, Inc. USA), according to the instruction of the manufacturer. DNA from three biological replicates was pooled for the downstream analysis. The integrity of the DNA was assessed by nanodrop spectrophotometer (2000/2000C, Thermo Scientific, USA), and 2 μL of each sample was subjected to electrophoresis on 1% agarose gel using 1× tris-borate-EDTA buffer. Gels were stained with ethidium bromide and viewed using Gel imaging System (Syngene Technologies Inc, USA). DNA library was prepared using NEB Next ultra DNA library prep kit for Illumina. Sequencing of the paired end library was done using Illumina Hiseq sequencing platform.

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Read quality assessment

The paired end reads generated were examined for read length, total number of reads, percentage of GC content and mean base quality distribution using FastQC tool kit. All reads were quality filtered with an average Phred quality of 20, and cutadapt (version 1.8.3) was used for adapter removal from the sequences.

De novo assembly and annotation

Assembly was performed with default k-mer length (31-size) using de-bruijn graph method. Inhouse PERL and Python code were used to parse the fastq files for the downstream analysis. The sequences were assembled with RayMeta[19] using a k-mer size of 31. The contigs with more than 150 bp were filtered and taken as pre-processed reads for downstream analysis. Glimmer-MG v 0.3.2[20] was used to predict the protein coding regions in the contigs. Each sample reads was completely assembled in about 5 days. This run time included *de novo* contig and scaffold assembly process.

Taxonomy/functional analysis

The taxonomy tree was generated based on neighbor-joining method using MEGAN software. The hierarchy of comparative taxonomic abundance in all the samples was based on the contig abundance with the number of reads assigned to the taxonomy. Functional annotation was performed using DIAMOND version 0.7.9[21] for predicted genes against the protein database using the BLAST version 2.2.29+[22] with an *e*-value of 1*e*−5. The functional analyses of all hits were analyzed using the KEGG and SEED options provided in the MEGAN software.[23]

Analysis by MG-RAST

The results from the standalone workflow were compared with MetaGenome Rapid Annotation using Subsystem Technology (MG-RAST).[24] Taxonomic classification was performed to view the taxonomic level in the samples against the M5NR public database using best fit classification with 1*e*−5 as maximum *e*-value cutoff, and 60% as minimum identity cutoff. Functional analysis for the distribution of functional categories using subsystems was carried out using the hierarchical classification with 1*e*−5 as maximum *e*-value cutoff, and 60% as minimum identity cutoff. Alpha diversity present in the treatment and control samples were estimated.

Statistics

For the growth parameters, the experimental design adopted was completely randomized design, and the data were analyzed by *t*-test. Analyses of differential/relative abundance features (of metagenome data) were done using STAMP software package.[25] The differential abundance between the samples was calculated using G-test (w/Yates') + Fisher's test for two sample analysis in STAMP tool.

Results

Growth parameters

The pH of *T. harzianum* treated soil was 5.2, after 120 days of inoculation; while that of control was 4.6. Growth parameters, *viz.*, the fresh root, fresh shoot, dry root, dry shoot, LAI (Leaf Area Index), height of the plant were significantly increased in the treatment (Table 1).

Metagenomics: sequencing and assembly

Paired End (251 bp × 2) sequencing yielded 2,121,934 and 2,123,836 reads for treatment and control samples, respectively. Majority of the sample reads had 40–70% GC content. The Phred score distribution (≥Q30) of the paired-end metagenome reads for treatment was 79.22%, while 80.82% was for the control. The assembly of reads formed 1,827,461 and 1,879,703 contigs and N50 of 210 and 212, respectively in treatment and control.

Analysis by MG-RAST

Out of 4,121,006 (97.1%) sequences that passed quality control, 93.5% sequences produced 3,389,349 predicted protein coding regions of the metagenome in the treatment. Of these, 33.7% sequences were assigned with annotation by M5NR database; 76.0% of annotated features from M5NR database were assigned with functional categories. From control sample, out of 4,162,647 sequences passed quality control (98%), 94.5% produced 3,558,779 predicted as protein coding region. Of these, 33.9% were assigned with annotation by M5NR database, and 74.7% of annotated features were assigned to functional categories. The mean sequence length, mean

Table 1 – Table showing the growth parameters of black pepper: with (treatment) and without (control) inoculation of *T. harzianum*. The growth parameters at 120 days are shown in the table (*n* = 12).

S. No	Parameters observed	T1 mean (with *Trichoderma*)	T2 mean (without *Trichoderma*)	Pr > (*t*)
1	Shoot weight (fresh)	7.7	3.0	<0.0001
2	Root weight (fresh)	44.5	26.6	0.0050
3	Leaf area index (LAI)	802.5	430.4	0.0028
4	Stem girth	0.1225	0.1400	0.3896
5	Height of the plant	78.5	44.4	0.0023
6	Root weight (dry)	1.7	0.7950	0.0018
7	Shoot weight (dry)	9.9	4.3	0.0003

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GC content for treated and control were 248 ± 13 bp, 63 ± 7% and 249 ± 12 bp, 62 ± 8%, respectively. The double approach we used (stand alone and MG-RAST) for the analysis of metagenome yielded coherent results in both taxonomy and functional categories. The comparative analysis on these metagenomes using MG-RAST is discussed further.

Population dynamics

The alpha diversity (Shanon diversity index) of the metagenome of both treatment and control samples were 489,569 and 455,862 species, respectively. From the analysis of relative abundance (percentage proportion) for top 10 bacterial species, *viz.*, *Acidobacteriaceae bacterium* KBS 96, *Candidatus koribacter versatilis*, *Ktedonobacter racemifer*, *Candidatus solibacter usitatus*, *Pedosphaera parvula*, *Sphingomonas* sp., URHD0057, *Gemmatimonadates bacterium*, *Pyrinomonas methylalipathogens*, *Chthonomonas calidirosea* and uncultured bacteria [of which *A. bacterium* ($p = 1.24e{-}12$) and *C. koribacter versatilis* ($p = 2.66e{-}10$) showed statistical significance] were found abundant in the treatment, while uncultured bacteria found were more in control sample ($p = 0.024$) (Fig. 1). The abundance of these bacteria suggests that probiotic application of *T. harzianum* in black pepper imparted the rhizosphere competence for the bacteria to colonize the roots as the presence of *A. bacterium* and *C. koribacter versatilis* has proven as the major rhizosphere competent bacteria involving unique metabolic pathway at the rhizosphere. Analysis of the relative abundance of top 10 fungi, *viz.*, *Rhizophagus irregularis*, *Fusarium oxysporum*, *Oidiodendran maius*, *Pseudogymnoasus pannorum*, *Talaromyces stipitatus*, *Pestalotiopsis fici*, *Mortierella verticillata* and *T. harzianum* showed that *F. oxysporum* ($p = 0.013$), *T. stipitatus* ($p = 0.219$) and *P. fici* ($p = 0.443$) were high in treatment, while the control showed higher abundance of *R. irregularis* ($p = 0.034$), *Pseudogymnoasus pannarum* (a human pathogenic fungus, $p = 0.488$) and *Oidiodendran* ($p = 0.484$). The *Trichoderma* reads were recorded only in treatment sample. The higher abundance of *F. oxysporum*, *T. stipitatus* and *P. fici* in treatment suggests that *T. harzianum* selectively enriches the biocontrol fungi in the rhizosphere. The reduction of pathogenic fungi, in turn, provides strong evidence that *T. harzianum* is able to reduce the human pathogenic effect of the amended soil, in comparison to the control.

Functional level dynamics

Functional abundance (Fig. 2) between the treatment and control samples using hierarchical classification with subsystem annotation sources showed that rhizosphere in the treatment was with abundant reads for virulence, disease and defense (54,857), motility and chemotaxis (11,992), and ion acquisition and metabolism (8151); while the control recorded 51,271 reads for virulence, disease and defense, 11,564 for motility and chemotaxis, and 7276 for ion acquisition and metabolism.

The relative abundance (percentage proportion) for the specific features (iron acquisition and bacterial chemotaxis) from stamp tool analysis is given in Fig. 3. The heme and hemin uptake and utilization systems in Gram negative bacteria ($p = 0.036$) and iron acquisition in red pigmented *Vibrio* ($p = 2.97e{-}5$) were abundant in treatment metagenome than in control. This indicates that the probiotic application of *T. harzianum* increased the microbial action for the metabolism and absorption of iron by the plant. The bacterial chemotaxis was higher in treated sample ($p = 0.012$), which shows the active/increased interaction of rhizosphere microbes on the black pepper roots by the application of *T. harzianum*. The treated sample recorded reduced abundance on pathogenicity islands, phages and prophages ($p = 7.30e{-}3$) (Fig. 3).

The reduction of pathogenicity island and phages in treatment, when compared to control, provides strong evidence for the selective community recruited by the *T. harzianum* toward the beneficial use in the cultivation system of black pepper. Though metagenome of control sample showed higher abundance (reads) globally for other functional category (Fig. 2), specific features were observed at the highest functional distribution classification in treatment, which includes metabolism of aromatic compounds, *viz.*, benzoate transport and degradation ($p = 2.34e{-}4$) and degradation of heterocyclic aromatic compounds ($p = 3.59e{-}13$). The increased abundance for these metabolism of aromatic compounds brings out that the probiotic application of *T. harzianum* in black pepper is capable of creating the unique community for the phytoremediation.

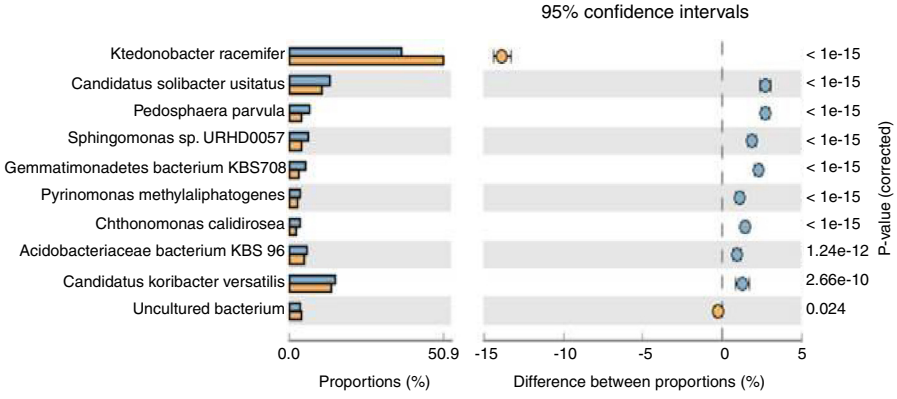

Fig. 1 – Species level extended error bar chart profile for top 10 bacteria from STAMP tool. *T. harzianum* treatment is denoted by blue bar and control by orange bar. The differential abundance between the samples were calculated with G-test (w/Yates') + Fisher's test for two sample analysis in STAMP tool.

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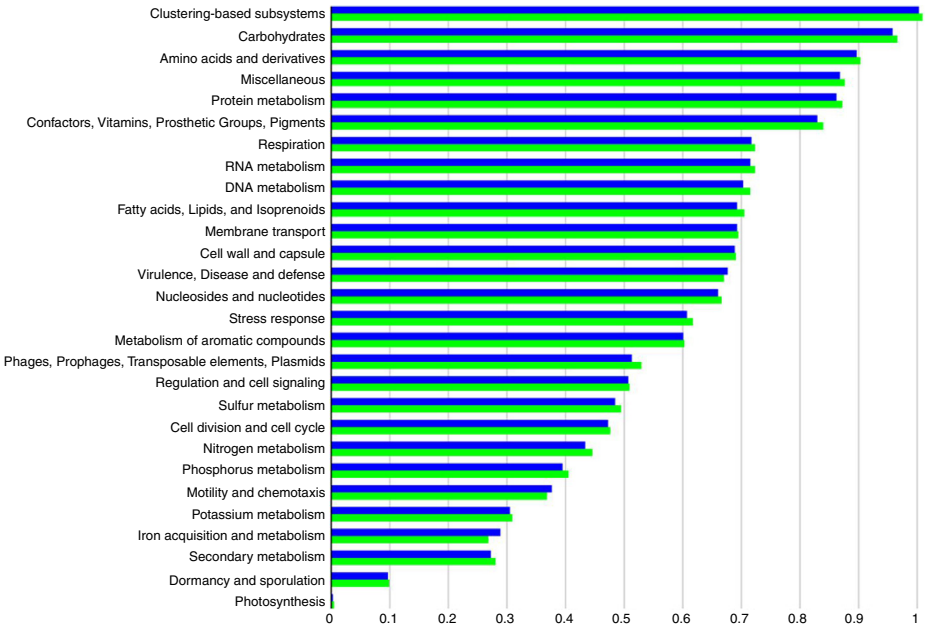

Fig. 2 – Classification based on functional abundance by MG-RAST. Blue line: *T. harzianum* treatment is denoted by blue bar and control by green bar. Motility and chemotaxis, iron acquisition, and virulence and disease functions are with high abundance in treatment.

Discussion

The prime objectives of this study was to assess the community changes at the rhizosphere of black pepper pursuant to the inoculation of *T. harzianum*, and also to unveil the significant effects of *T. harzianum* on the selective recruitment of specific microbes, and their functional assignments in rhizosphere of black pepper. The results clearly showed that *T. harzianum* significantly influenced in the selective abundance of beneficial bacteria and fungi, and subsequent growth promotion in black pepper; and the impact at functional level was identified as increased bacterial chemotaxis, virulence, disease and defense, ion metabolism. From the results, increase in the growth parameters, *viz.*, fresh root, fresh shoot, dry root, dry shoot, leaf area index, height of the plant reveals the growth promotion activity of *T. harzianum* in black pepper, as indicated by other authors too. Anandaraj and Sarma[14] reported that the application of *T. harzianum* (MTCC 5179) resulted in enhanced growth in black pepper with increased number of nodes, and consequently the number of cuttings. Sibi[13] also showed the positive influence of *T. harzianum* (MTCC 5179) on the improvements in the formation of fresh root and shoot, followed by increase in the dry weight of root and shoot in black pepper. Treatment with *T. harzianum* (MTCC 5179) individually imparted better growth promotion and disease suppression than that of a consortia of plant growth-promoting rhizobacteria alone or in combination with *T. harzianum* (MTCC 5179).[13] These studies indicated growth promotion and the organism was recommended as a component of integrated disease management and without a clear understanding of other mechanisms. The present study unravels the underlying microbial dynamics and major functional processes.

Though the population abundance of bacteria, archea and eukaryote were a little less in treatment than in control, it showed selective abundance (more percentage proportion) of bacteria, *viz.*, *A. bacterium* and *C. koribacter versatili* – out of top 10 bacterial species; these bacteria belong to the phylum Acidobacteriaceae, the avid colonizer of the rhizosphere with potent rhizosphere competence.[26] *A. bacterium* is capable of growing on diverse collection of complex organic compounds including xylan, cellulose, methyl cellulose, syringate, pectin and ferulate.[27] *Candidatus* sp. contains abundance of carboxylase active enzymes (CAZyme) family and are involved in the breakdown, utilization and biosynthesis of diverse structural and storage polysaccharides and resistance to fluctuating temperature and nutrient deficient conditions.[28] This selective abundant recruitment of these beneficial bacteria in the treatment might be the major impact for the growth promotion activity by the active breakdown of complex organic compounds by these organisms, thereby creating microclimates for the colonization of microbes in the roots and subsequent interaction with the communities at the rhizosphere. Further, the analysis of black pepper root exudates and action of these bacteria on the root metabolites would give the specific role of these bacteria at the rhizosphere of black pepper.

Unlike in control, the metagenome of treatment showed abundant reads of the beneficial fungi, *viz. F. oxysporum*, *Talaromyces* sp. *Pestalotiopsis* sp. and *T. harzianum*; a positive correlation with expected beneficial activities as pointed out by different authors: Eparvier and Alabouvette[29] showed that increased population of *F. oxysporum* was better for the biocontrol and disease suppression activity in Flax; many isolates of *Talaromyces* spp. were shown to promote plant growth.[30] Elegant studies have demonstrated that *T. flavus* antagonizes plant pathogenic fungi.[31,32] In present study, higher

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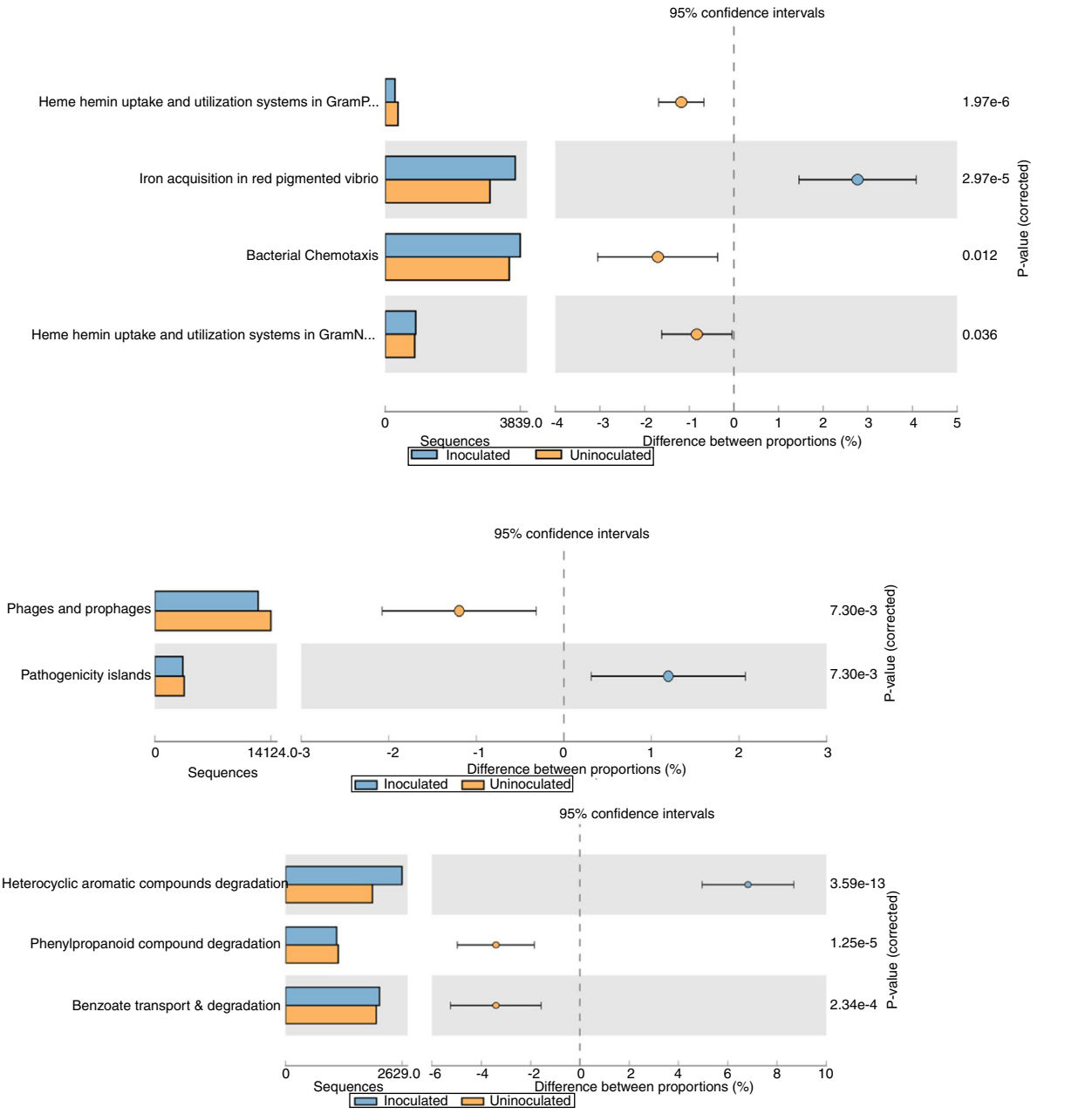

Fig. 3 – Functional level extended error bar chart profile for iron acquisition and chemotaxis, phages and prophages, pathogenecity islands and heterocyclic aromatic compounds degradation from STAMP tool. *T. harzianum* treatment is denoted by blue bar and control by orange bar. The differential abundance between the samples were calculated with G-test (w/Yate's) + Fisher's test for two sample analysis in STAMP tool.

abundance of the species of *Fusarium* and *Talaromyces* in treatment indicates the ecological significance on their population abundance driven by the addition of *T. harzianum* toward the fitness of black pepper growth and subsequent yield.

Rajan et al.[15] showed the biocontrol and disease suppression activities of *T. harzianum* (MTTC 5179) in black pepper against foot rot disease at field conditions; which was found to be efficiently proliferating in the soil and remained in the soil for long time, apart from imparting protection to the root system against *P. capsici*. In the present study, the metagenome analysis was performed after four months of treatment, and proved that *T. harzianum* (MTCC 5179) was able to remain in soil for a long time. Interestingly, the proportion of *R. irregularis* was higher in the control than in treatment, which indicates the interaction of *Trichoderma* with the native Vesicular Arbuscular Mycorrhiza (VAM) and modulation of its population. The spore germination and hyphal growth of *G. mosseae* was stimulated by *T. harzianum* with the production of volatile compounds.[33] In present study, the less abundance of Arbuscular mycorrhizal fungi (AMF) in treated soil might be due to the stimulated growth of AMF by the community recruited by *T. harzianum* thereby increased colonization inside

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the plant[34] rather than their physical presence in the rhizosphere and *vice versa* in control. Application of *T. harzianum* improved better growth of black pepper, which was at par with *T. harzianum* in combination with AMF. The treatments with AMF alone and in combination with *Pseudomonas* sp. failed to enhance the growth.[13] *P. fici*, an endophyte of tea produces bioactive metabolites and natural products,[35] and the analyses of its genome and transcriptome showed that it harbors efficient genes responsible for the synthesis of various secondary metabolites.[36] Further functional analysis of the reads on *P. fici*, from the present metagenome data would give significant insight into its role on black pepper through interaction at rhizosphere.

The metagenome of the treatment in the present study showed higher abundance for iron acquisition and metabolism in red pigmented *Vibrio*, coupled with heme and hemin uptake and utilization systems in Gram negative bacteria than control; which evidences the influence of *T. harzianum* in rhizosphere–microbe interaction. Rhizosphere microbiome facilitates the uptake of specific trace elements such as iron. Iron in soil, exists primarily in the insoluble ferric oxide form, which is not available for microbial growth. Based on the scarcity of available irons as well as the toxicity of free iron at elevated concentrations in the environment, bacteria employ a variety of mechanisms to regulate the intracellular iron concentrations.[37] On the other hand, plants also play crucial role in increasing the solubility of inorganic iron in the rhizosphere, which may be due to the interaction with microbiome.[38] Rhizobacteria are generally motile, and the motility is either random or chemotactic for interacting with the plants.[39] In fact, the bacterial chemotaxis was found as abundant in treatment than in control, suggesting that the probiotic application of *T. harzianum* in black pepper would enable active interaction of the recruited bacterial community in the root system. Anatomical data from the treatment and control also provide ample evidences for the aforesaid inference.[34] The abundance of reads on pathogenicity islands, functionality of phages and prophages were found to be less in treatment than in control. The less abundance of human pathogenic fungi as evidenced from the analysis of taxonomy abundance is highly related to the results of functional analysis, which suggests the beneficial effect of probiotic application of *T. harzianum*, especially in the context of human health.

Rhizoremediation is a specific form of phytoremediation involving plants and their associated rhizospheric microorganisms (bacteria and fungi). Rhizoremediation can either occur naturally or could be facilitated by inoculating soil with microorganisms capable of degrading environmental contaminants. The plant associated non-pathogenic endophytic and the rhizospheric bacteria are the major players in the degradation of toxic metabolites present in soil.[40] Heterocyclic aromatic compounds and benzoates are toxic compounds persist for a long time in soil, that leads to ill effects in animals and humans. In the present study, metagenome of treatment recorded higher abundance of reads for the degradation of heterocyclic aromatic compounds, benzoate transport and its degradation. This information would give the positive impact of *T. harzianum* in the cropping system of black pepper. Further, the functional metagenomics would give more information on bacteria involved in the rhizo remediation through the rhizoecosystem in black pepper.

In conclusion, the population dynamics and functional richness of rhizosphere ecosystem in black pepper influenced by the treatment with *T. harzianum* provides the ecological importance of *T. harzianum* in the cultivation of black pepper. On the basis of the present report and previous studies on effect of *T. harzianum* in the fitness of black pepper; it can be suggested that as mycorhizosphere, another microecological niche, *viz.*, 'trichorhizosphere' is also coexists in altering the community dynamics of bacteria and soil fungi; and thus, the rhizosphere microecosystem developed by *T. harzianum* might contribute a pivotal role in imparting plant health, which is unlike the lone effect of *T. harzianum*. The methods employed in this study show a significant step toward possible implementation of metagenomics for the functional elucidation of *T. harzianum* – the valuable biocontrol, growth promoting fungus in the production system of black pepper. The rhizosphere and the trichorhizosphere metagenomes of black pepper elucidated in this study would become important factors in developing any IDM modules in the root ecosystem of black pepper. Further, targeted studies based on the present metagenomic read on each organism and at each component would give enormous information on this microclimate.

Conflicts of interest

The authors declare that there exists no conflict of interest.

Acknowledgements

This study was funded by Indian Council of Agricultural Research, India, through outreach project "PhytoFuRa (*Phytophthora*, *Fusarium* and *Ralstonia* diseases of Horticultural and Field Crops". www.phytofura.net.in). The sequencing service was hired from Scigenome, Kochi, Kerala. The authors acknowledge Drs. Devasahayam and Sasikumar, ICAR – Indian Institute of Spices Research, Kozhikodeode, Kerala for their support in carrying out the experiments. PU is grateful to Drs. V. Srinivasan, Hamza, SJ Eapen, K. Kandiannan and D. Prasath, ICAR – Indian Institute of Spices Research, Kozhikode, Kerala for their valuable suggestion in implementing this work.

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Endophytic interactions of *Trichoderma harzianum* in a tropical perennial rhizo-ecosystem

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Abstract
This study demonstrates the endophytic interaction of the well-known growth promoting and biocontrol agent in black pepper. Trichoderma harzianum, coupled with its rhizosphere fungal flora was evidenced from metagenomics. We employed short-term and long-term strategies to study the interactions of T. harzianum in black pepper rhizosphere. In short-term strategy, T. harzianum was co-cultivated with axenic plantlets while pot culturing of plants in soil mixed with T. harzianum was performed in the long-term strategy. The colonization was investigated by light microscopy and scanning electron microscopy (SEM).

The co-cultivation of T. harzianum with black pepper showed the intercellular colonization at 24 h and formation of intracellular hyphae with vesicles at 48 h of interaction. The long term strategy inferred that T. harzianum was able to colonize the black pepper roots along with the AMF inter- and intra-cellularly. The whole genome metagenomic sequencing brings out the population abundance of the entire rhizosphere fungal flora.

Keywords: Fungal endophyte, Arbuscular micorhizal fungi, *Trichoderma harzianum* and *Piper nigrum*.

Introduction

Black pepper (*Piper nigrum* L.), popularly known as king of spices or black gold, is a highly valued perennial spice crop grown in tropical world. It is propagated vegetative means through stem cuttings. This export oriented spice climber succumbs to several diseases caused by fungi, bacteria and viruses.[29] Among these, foot-rot caused by the soil-borne oomycetous fungal pathogen, *Phytophtora capsici* is a major constraint for the healthy maintenance of this plant.[5] Crop loss due to this disease alone in Kerala, India (major centre of black pepper production) was estimated to range from 3.4 to 9.4%[3].

Infected plant debris in the soil and dried vines in the gardens are the primary source of inoculum of the pathogen.[2] Some of the black pepper associated bacteria such as *Psuedomonas aeruginosa*, *P. putida* and *Bacillus megaterium* were identified as effective antagonistic endophytes against the foot-rot disease[8]. But the mycelial fungus, *Trichoderma harzianum* is being widely used both in the nursery and field as successful integrated disease management component in India.[7,28,35]

Growth promotion[6,28,32,35] and disease suppression[26,28] activities of *Trichoderma* spp. on black pepper are manifold both in the nursery and field conditions. Despite beneficial claims, studies on the interactions of *T. harzianum* with the rhizosphere of black pepper are less attempted. Therefore, such interactions must be brought to the limelight so as to understand the nature of interaction of *Trichoderma* with the black pepper roots and its impact on other fungal population at the rhizosphere towards the expected beneficial effects.

Thus, the specific objectives of the present study were set as: (a) Examination of colonization behavior and the nature of interaction of *T. harzianum* at the rhizosphere of black pepper using the techniques of microscopy and (b) Correlation studies of rhizosphere soil metagenomics on fungal population pursuant to the inoculation of *T. harzianum*.

Material and Methods

Fungal inoculum: Talc formulation of *T. harzianum* MTCC 5179 obtained from the biocontrol laboratory, ICAR- Indian Institute of Spices Research, Kozhikode, Kerala was used for the pot culture study by mixing 3 g of talc with 3.5 kg of top soil. For co-cultivation study (liquid culture of *Trichoderma*), 72 h old culture on potato-dextrose-agar (PDA) plates was cut into 5 mm^2 discs and one such disc was inoculated in conical flasks containing 50 ml PD medium. After 10 days, 100 ml sterile double distilled water (ddH_2O) was added to the flasks and spore mass was scraped out to be used as inoculum for co-cultivation studies.

Co-cultivation (Short-term colonization)

Plant material: Single node cuttings from black pepper variety namely '*Sreekara*' were washed with tween-20 for 15 min and washed under running tap water. The cuttings were immersed in 0.2% copper oxychloride for 15 min followed by wash in sterile ddH_2O twice. The cuttings were surface sterilized with 0.1% mercuric chloride for 5 min on clean bench and then washed twice with sterile ddH_2O.

The cut ends were quick dipped in 8000 ppm IBA (indole butyric acid) and planted in plantons (7.5 × 7.5 × 10 cm, Himedia) filled with pre-sterilized perlite medium and fortified with sterile Hoagland's solution. The plantons were maintained in tissue culture room at 22 +/- 25°C and 3000 lux for the production of saplings.

Co-cultivation and Microscopy: Under aseptic conditions, the saplings were transferred to sterile petri-dish and roots were cleaned with sterile ddH_2O so as to remove the adhered perlite. Liquid culture of *T. harzianum* (10^9cfu/ml) was added to the *in vitro* saplings (only water added to control). The plantlets were maintained in the incubator shaker (Remi CIS 24 Plus, India) at 25ºC under constant shaking (115 rpm). Root samples (from replicas) were collected after 12, 24 or 48 h incubation and then rinsed in sterile water. They were fixed in 25% ethanol and stored at 4ºC. Toluidine blue and cotton blue staining techniques were performed to observe the extra- and intra-cellular colonization. These samples were subjected to SEM analysis for observing the interactions of *T.harzianum* with black pepper roots during exorhizal colonization. Root clearings were used to verify the endophytic colonization.[27]

Pot culture study (Long-term colonization)

Plant material: Cuttings were prepared as described for the short-term study. The cut ends of the cuttings were quick dipped in 8000 ppm IBA and planted on pre-sterilized perlite medium in protray fortified with sterile Hoagland's solution.[21] The protrays with the preparation as above were maintained in greenhouse with the top portion of protray sealed with aluminum foil. The cuttings were sprayed (foliar) daily with Hoagland's solution thrice. After 2 months of growth, when plants were with 4-5 leaves and 24 - 26 cm height; the perlite adhered to the rhizosphere was collected by gentle tapping and analyzed for the presence or absence of *Trichoderma* by plating (spread/pour plate method).

Subsequently, saplings free of *Trichoderma* were transferred to the pots filled with field collected top soil. The nutrient content of the soil was analyzed[34] as (minerals in ppm): 1.6% organic carbon; 1.64 P, 173 K, 197 Ca, 71 Mg, 11.38 Fe, 18 S, 5.56 Mn, 3.24 Zn, 0.92 Cu, 0.16 B and pH, 4.35. Two treatments (with and without *T. harzianum*) with 4 replicates having 3 plants per replicate were designed for the study. Growth parameters *viz.* height of the plant, stem girth (1 cm above from the soil region), leaf area index (LAI) and number of leaves were recorded.

The LAI was calculated using the formula: length (cm) × width (cm) × 0.6. After 120 days, plants were uprooted, the rhizosphere soil (adhered to the roots of pepper plants) samples were collected from 3 biological replicates of both treatment and control for metagenomics using Illumina hiseq. The weights of shoot and root (fresh and dry) were also recorded.

Root clearing: After 120 days of growth in the pots, the plants were uprooted; root samples were collected by cutting the roots at the collar region of the stem, washed in sterile ddH_2O, fully dried in hot air oven (at 60ºC for 16h) and stored in paper bags at 25ºC. For the analysis of colonization frequency, 25 root bits (~1cm in length) were taken randomly from *T. harzianum* treated and non-treated samples. Dried roots were rehydrated with sterile water for 1 h and then 10% (w/v) of KOH was added to roots and boiled in microwave oven for 10 min followed by rinsing with sterile ddH_2O.

Post clearing was performed with alkaline hydrogen peroxide [0.5% NH_4OH and 0.5% H_2O_2 (v/v) in ddH_2O] by boiling the roots in microwave oven for 5 min followed by rinsing with sterile ddH_2O and acidification using 1% HCl.[27] The roots were stained with 0.05% tryphan blue in lactophenol stain for 15 min, followed by destaining (lactic acid: glycerol: water in the ratio; 40:40:20) for 30 min and examined under microscope (Leica DM 5000 B, USA).

Sample preparation for SEM: Dried root bits were rehydrated with sterile ddH_2O for 1 h and two methods were adopted subsequently:

Method 1: The root bits were fixed with 2.5% glutaraldehyde for 2 h followed by wash with sterile ddH_2O twice for 30 min. Secondary fixation was done in 2 % paraformaldehyde in 1.0 M KH_2PO_4 and Na_2HPO_4 buffer (pH 7.2) and washed with the same buffer twice. Samples were dehydrated using a series of ethanol in ascending concentrations (25, 50, 75 and absolute alcohol for 30 min each).

Method 2*:* Root bits were fixed in 100% methanol for 1 h followed by dehydration using a series of ethanol in ascending concentrations (25, 50, 75 and absolute alcohol for 30 min each).

Processed root samples by either method were cut into thin sections (1 - 2 mm) using a fine scalpel and mounted onto the aluminum specimen stubs using double-adhesive coated carbon tabs and gold sputtering was performed using ion gold sputtering unit (20 sec). The samples were then viewed and the images were micro graphed using Hitachi SU6600 field emission scanning electron microscope (Hitachi, Japan).

Metagenome sequencing, assembly and annotation: The rhizosphere soil DNA was extracted from 100 mg of rhizosphere soil using MoBio kit (MO BIO Laboratories, Inc USA) according to the manufacturer's instruction. DNA was isolated from three biological replicas (from control and *T. harzianum* inoculated plants) pooled for analyses**.** Two µg of DNA from each sample was used for the library preparation using NEB Next ultra DNA library prep kit for Illumina. Sequencing of the paired end library was done in illumina Hiseq platform. The sequences were assembled with RayMeta[10] using a k-mer size of 31 using de-bruijn graph method. Filtered contigs with more than 150 bp length were used with Glimmer-MG v 0.3.2[23] to predict the protein coding regions in the contigs. Functional annotation was done using Diamond v 0.7.9[11] for predicted genes against the protein database using the BLAST version 2.2.29+, with an e value of 1e-5. [4]

Statistical Analysis: For the growth parameters, the experimental design adopted was CRD and the data were analyzed by *t*-test. The differential abundance of fungi (metagenome) between the samples was calculated using G-test (w/Yates') + Fisher's test for two sample analysis in STAMP tool.

Results

Growth Promotion: Growth parameters *viz*. the fresh root, fresh shoot, dry root, dry shoot, LAI and height of the plant were significantly increased upon probiotic application of *Trichoderma harzianum* MTCC 5179 when compared to control (Table 1).

Co-cultivation (Short-term colonization study)

Bright field Microscopy and SEM: The external colonization of *T. harzianum* occurred on the roots of the *in vitro* derived pepper plants as surface adherence at 12 h of co-cultivation. The mycelial spread was increased with increasing time (at 24 h and at 48 h) with profusely growing mycelia bearing chlamydospores as evidenced from toludine blue staining (Figure 1 a, b andc). Upon cotton blue staining, intercellular colonization was found at 24 h incubation (Figure 1 d). *T. harzianum* established endophytic colonization inside the cell at 48 h with luxurious intracellular mycelia (Figure 1 e). No fungal growth was observed (external or internal) on the root tissues of control plant.

Root clearings of plants inoculated with *T. harzianum* showed fungal mycelium in the intercellular spaces at 24 h and hyphal tips as dark blue granules inside the cell (Figure 2 a) and intracellular chlamydospores were observed at 48 h (Figure 2 b). Analysis by SEM showed intact cell structures in control roots (Figure 3a) but *T. harzianum* inoculated samples taken at 24 h and 48 h showed an indication of the interaction with the root cells (Figure 3 b and c) and hyphal growth on the surface at 24 h (Figure 3d). At 12 h, the enlargement of hyphal tip as papillae showed its interaction for intracellular colonization (Figure 3e). Massive colonization was observed on root surface at 48h (Figure 3 f). No fungal growth was observed on the surface of the root tissues of control plant (data not shown).

Pot culture study (Long-term colonization)

Bright field microscopy: Roots of control plants showed no fungal colonization externally but *T. harzianum* treated plants showed invasion of mycelia. Root sections of treated plants stained with cotton blue showed AMF vesicles and arbuscules while the control samples showed a few arbuscules with no vesicles (Figure 4 a and b). The arbuscules in control were localized to the zone of elongation of the root. In general, presence of mycelia was found increasing with maturation of the root tissue.

The root tip meristem showed no colonization; however, the elongation zone showed intercellular colonization with more number of vesicles and the maturation zone was densely colonized by inter- and intra-cellular hyphae with comparatively less number of vesicles and large number of arbuscules.

The AMF mycelia were intercellular i.e. along the tangential plane of the cortex in the elongation zone. The mycelia in this region were larger in size and rarely septate were only of AMF, no *Trichoderma* mycelia were seen in this region but it was observed only in the maturation zone of the root as septate mycelium along with the AMF (Figure 4 b and c). *Trichoderma* in this region was found with conidia (Figure 4 d and e). This zone of maturation of root had structurally differentiated AMF mycelia with prominent septation along with monilioids hyphae (Figure 5c).

Microsclerotia were also observed inside the root cells (Figure 5b). The colonization of AMF was found to be that of Arum type (Figure 5a). This portion of roots had vesicles with round, oval, ellipsoidal and irregular shapes (Figure 5d). Some vesicles were originated from monioloid hyphae. The size of the AMF vesicles was ranged from 40 - 147µm.

Compared to control, the treated plants showed higher mycorhizal frequency (100%) after four months of pot culture. The average number of vesicles was ~ 40 per 1 cm root tissue. Interestingly, the root hairs showed no internal mycelium in both the samples though AMF mycelia were present on the external surfaces (data not shown).

Analyses using SEM: Comparing two sample preparation methods employed for the SEM, the methanol fixation was found better in terms of good cell structure which aided visual observation of bacteria and fungus whereas glutaraldehyde fixation distorted the surface structures (Figure not shown). Hence, methanol fixation was taken for further analysis of black pepper root samples by SEM. The root sample from the control showed weak adherence of organisms on the surface (Figure 6a) while the treated roots showed abundant adherence of organisms on the surface upon imaging with SEM (Fig. 6b).

Population abundance evidenced from metagenomics: From the entire profile of fungal flora of metagenome, only 10 most abundant species were taken for the analysis in this study (Fig. 7). Among them, four were endophytes *viz*. *Pestalotiopsis fici, Oidiodendranmaius, Rhizophagus* sp. and *T. harzianum* in which the *Trichoderma* reads were present only in *Trichoderma* inoculated soil and not in the control.

The population abundance of the fungal species with biocontrol potential showed that *Fusarium oxysporum* ($p = 0.013$), *Pestalotiopsis fici* ($p = 0.443$) and *Talaromyces stipitatus* ($p = 0.219$) were high in *T. harzianum* treated soil metagenome. The metagenome of the control sample was high in *Rhizophagus irregularis* ($p=0.034$), *Pseudogymnoasus pannarum* (human pathogenic fungus) ($p=0.488$) and *Oidiodendran* sp. ($p=0.484$).

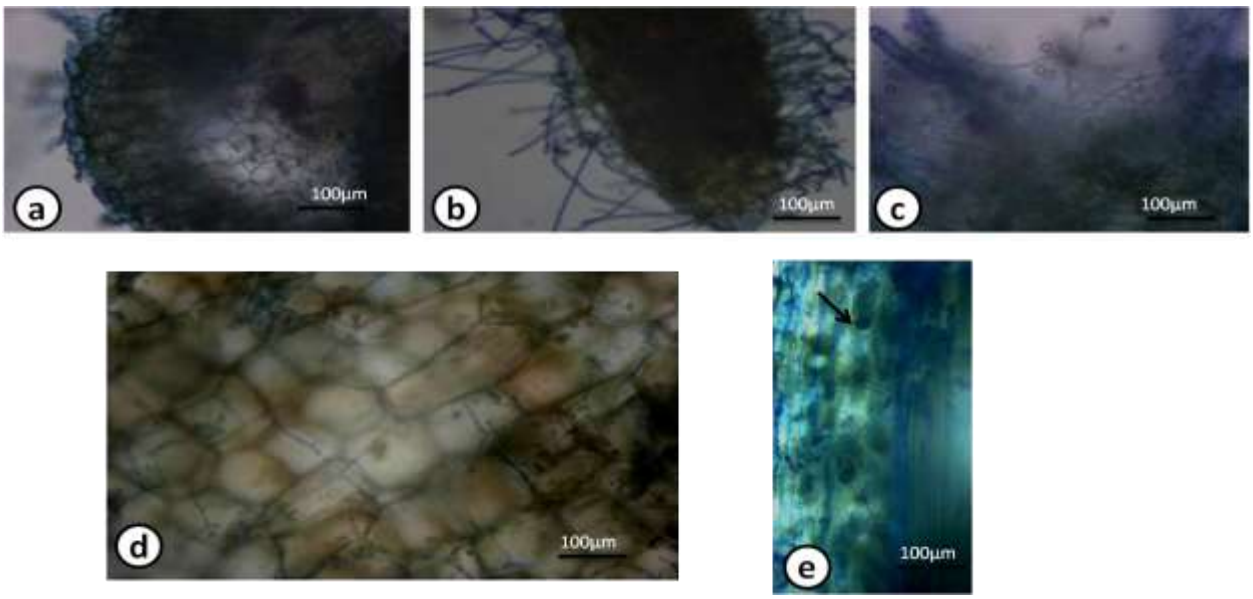

Fig. 1: Extracellular attachment and intracellular interaction of *T. harzianum* during co-cultivation (short-term colonization) with *in vitro* derived black pepper plants. Fig. a to c: toludine blue staining of root tissue - (a) control, (b) after 24 h incoculation and (c) after 48 h inoculation showing hyphae and chlamydospores on the surface of the root section. Fig. d and e: cotton blue staining of root sections - (d) intercellular growth at 24 h and (e) intracellular colonization (arrows) at 48 h.

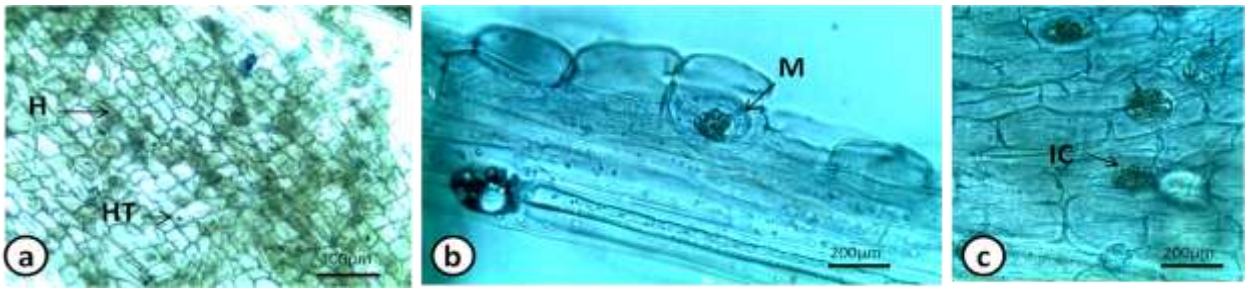

Fig. 2: Bright field micrographs on root clearing of black pepper after co-cultivation with *T.harzianum* showing endophytic interaction. Samples were taken at 12, 24 and 48 h on inoculation, and stained with cotton blue. (a) Inter- and intra-cellular colonization at 24h; (b) and (c) Intracellular colonization at 48h. Hyphae (H), hyphal tips (HT), mycelium (M) and intracellular structure (IC) are marked.

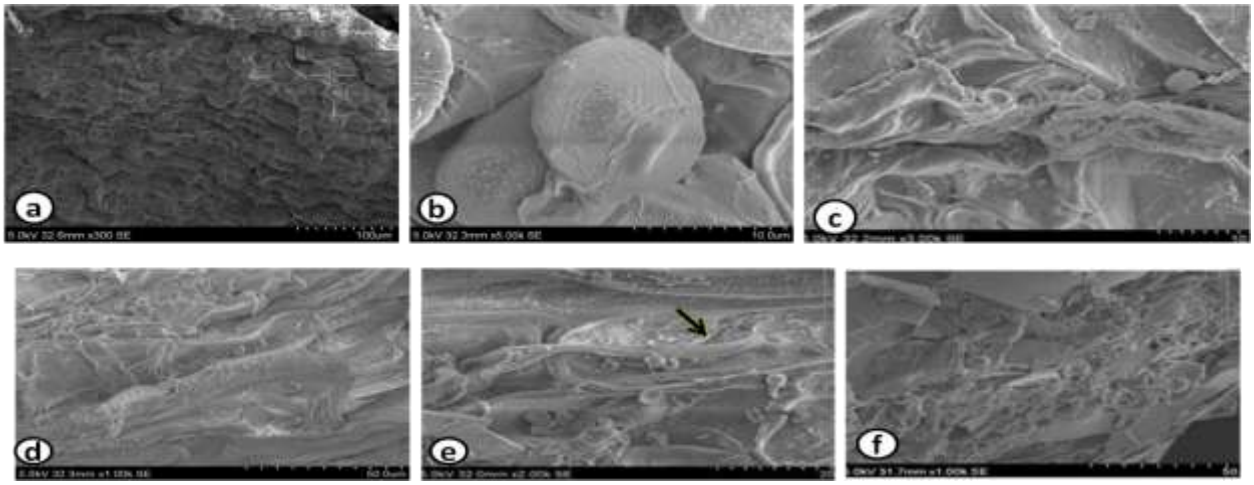

Fig. 3: SEM micrographs taken after co-cultivation of *T. harzianum* 5179 with *in vitro* derived black pepper plant roots; samples taken at 12, 24 and 48 h of inoculation fixed with methanol. (a) control (without *T. harzianum*); (b) spore on cell suface; c) surface attachment at 12 h; (d) hyphal growth at 24 h; (e) enlargement of hyphal tip (arrow-papilla) and (f) dense colonization at 48h.

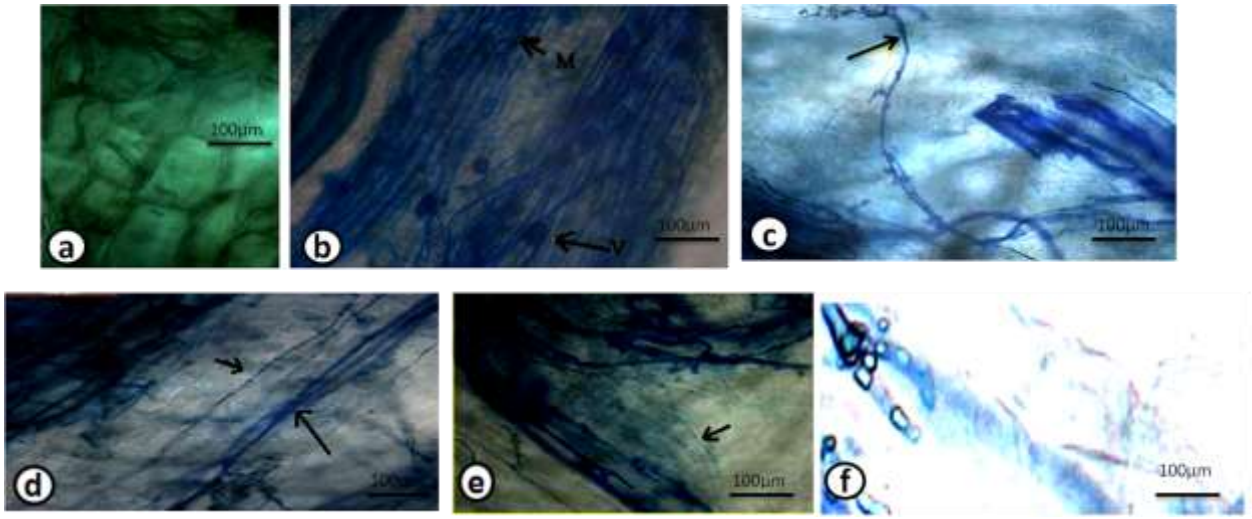

Fig. 4: Images of *T. harzianum* inoculated plant roots with bright field microscopy after 4 months of growth (long-term colonization) in pot culture. The dried roots were cleared and stained with cotton blue. (a) control; (b) *T. harzianum* inoculated; (c) arrow showing extra radicular AMF hyphae; (d) *T. harzianum* (short arrow), AMF (long arrow) hyphae; (e) arrow showing *T. harzianum* mycelium and (f) conidiophore with conidia. Vesicles (V) and mycelium (M) are marked.

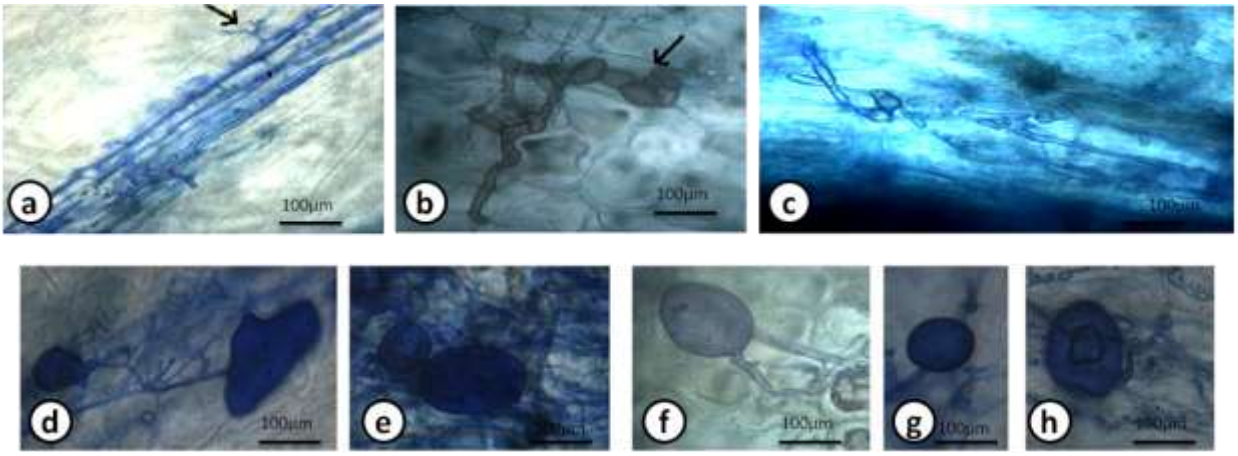

Fig. 5: Maturation zone of black pepper roots showing different AMF structures during long-term colonization (4 months) of *T. harzianum* (a) Tree-like intracellular arbuscules (arrow showing arum type growth of AMF mycelium, (b) intracellular microsclerotia; c) moniliod hyphae of AMF and (d) different shapes of AMF vesicles at the maturation zone of roots.

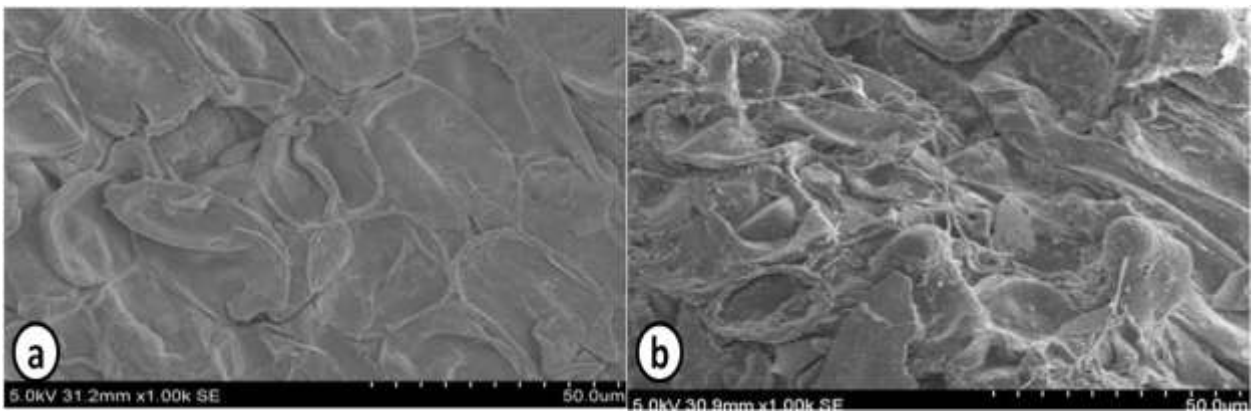

Fig. 6: Scanning electron micrograps of black pepper roots fixed with methanol. (a) Root sample from pots without *T. harzianum* inoculation showing few microbes on the surface and (b) with *T. harzianum* inoculation showing abundant microorganisms on the surface (samples taken after 120 days with or without inoculation of *T. harzianum*).

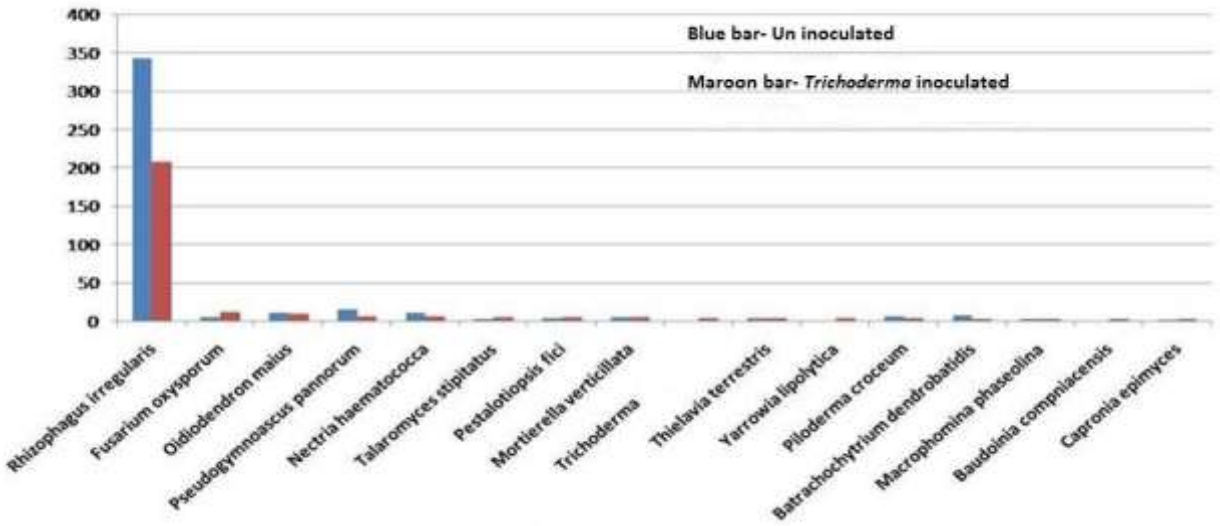

Fig. 7: Abundance of fungal species as observed from the metagenome of black pepper rhizosphere soil using illumina hiseq whole genome metagenomics sequencing.

Table 1
Growth parameters of black pepper with and without *Trichoderma* inoculation

S.N.	Parameters observed	T1 Mean (with *Trichoderma)*	T2 Mean (without *Trichoderma*)	Pr> (*t*)
1	Shoot weight (Fresh)	7.7	3.0	<.0001
2	Root weight (Fresh)	44.5	26.6	0.0050
3	Leaf area index (LAI)	802.5	430.4	0.0028
4	Stem Girth	0.1225	0.1400	0.3896
5	Height of the plant	78.5	44.4	0.0023
6	Root weight (Dry)	1.7	0.7950	0.0018
7	Shoot weight (Dry)	9.9	4.3	0.0003

Discussion

The prime objective of this study was to elucidate the nature of colonization of *T. harzianum* in black pepper both at the anatomical level using microscopic techniques and at molecular level employing metagenomics. When we attempted the study, to our surprise, its endophytic colonization accompanied by AMF was luxuriant upon application of *Trichoderma*.

Co-cultivation study showed that *T. harzianum* is efficiently colonizing the plant roots endophytically. Chacon et al[13] demonstrated intercellular ramification of *T. harzianum* hyphae in the root cells of tomato. They found that some cells were colonized intracellularly only after 48h and occurrence of yeast like structures after 72h treatment. Many *Trichoderma* spp. infecting cocoa plants *viz.T. ovalisporum*[22], *T. paucisporum*[31], *T. evansii*[30] and *T. martiale*[19] were identified as endophytes. TEM[39] showed direct root penetration of *Trichoderma* spp. in cocoa plant. Papillae - the swollen hyphal tips - were also reported in the interactions of *T. harzianum* with tomato roots during adherence[13]. We showed the appearance of papillae on root surfaces at 24 h of inoculation suggesting its quick interaction with the black pepper root system.

The phenomenon of biocontrol and growth promotion does not occur in all *Trichoderma*-host interactions; for instance[16] endophytic colonization of cocoa by *T. stromaticum* was unable to induce plant growth and was resistant to *Magnoporthe perniciosa*. However, some species of *Trichoderma viz. viride, harzianum* and *pseudokoningii* were found promoting growth in cucumber, corn, petunia and pea.[20,37,38] Apart from growth promotion, the endophytic colonization of *T. hamatum* in cocoa was found inducing drought tolerance in the plant[9]. In the present study, the growth promotion was also found to be enhanced by the co-colonization as evidenced from increase in growth parameters (fresh root, fresh shoot, dry root, dry shoot, LAI and height of the plant) in *Trichoderma* treated plants when compared to control which was not showing any co-colonization.

The maturation zone of the black pepper roots showed moniliod hyphae along with structurally differentiated AMF mycelium with prominent septation. Moniliod hyphae of dark-septate fungus in the aquatic angiosperm, *Eorhiza arnoldii* could produce diverse moniliod assemblages[24]. Microsclerotia were also observed inside the cells of pepper and the colonization of AMF was found as Arum type [1]. The region of maturation showed vesicles with different shapes.

We were able to show the *Trichoderma* mycelium and conidia along with the AMF mycelium and vesicles - suggesting co-colonisation inside the roots of black pepper.

The interaction between AMF and *Trichoderma* has been elucidated in many studies: Filion et al[18] reported that *Glomus intraradices* stimulated the conidial germination of *T. harzianum* and Datnoff et al[14] observed a synergistic interaction between them in tomato. Co-inoculation of *T. harzianum* and *T. aureoviride* decreased the time to vegetative sporulation in axenic cultures of these mycorhizal species. Synergistic effect between *G.intraradices* and *T. aureoviridae* in enhancing the growth in citrus in organic substrate has been reported which was higher than the individual effect of *G. intraradices.*[12]

Since the experiment was set up in field soil, the observation is that the roots inoculated with *Trichoderma* had abundant VAM colonization as indicated by the presence of both vesicles and arbuscules. It indicates the native mycorhiza colonizing pepper roots without any hindrance by inoculated *Trichoderma harzianum* MTCC 1579 suggesting the inoculated *Trichoderma* has facilitated mycorhizal colonization where as in the control soil though there was AMF colonization, it was sparse. The native beneficial microbes like mycorhiza had equal opportunity of colonizing black pepper roots the fact that in *Trichoderma harzianum* inoculated roots, the presence of more AMF suggests its active role in helping mycorhizal colonization.

As no choice experiment with insect pests, DeJaeger et al[15] indicated mycoparasitism in one to one interaction. *Trichoderma harzianum* being a saprophyte and opportunistic antagonist [20] in the absence of other nutrient source for its survival perhaps would have colonized AMF. In an experiment by Sibi[32] where selected compatible (*in vitro*) consortia of PGPR (*Pseudomonas mendocina, Bacillus pumilus, Serratia marcescens* and *Rhizbium* sp) inoculated on black pepper rhizosphere, the population of *S. marcescens* was declined to zero when compared to the population in *in vitro* experiments in which the PGPR consortia was compatible suggesting the role of rhizosphere in selecting and maintaining the organisms.

Methanol fixation was found to be good for sample processing for SEM. The attachment of bacterial/fungal population on the surface of *Trichoderma* treated roots showed the rhizosphere competence of soil organisms with *Trichoderma*. Many bacterial cells were found adhering to the surface which was absent in the control roots. With *Arabiodpsis* and barley, Talbot and White[33] also found that methanol-based method was superior to other fixation methods of samples for analysis by SEM.

Reports on growth promotion effect of VAM on black pepper showed that *G. fasciculatum* incorporation as infective propagules (cultured on Rhodes grass) increased the rooting of black pepper at nursery condition.[6] The authors showed more than 80% colonization of *G. fasciculatum* in black pepper roots than control. Detailed study on the effect of biocontrol agent on hardening of tissue cultured black pepper with VAM demonstrated that the treatments wherein *G. fasciculatum* and *T. harzianum* were inoculated showed higher root and shoot mass compared to control.[32]

The growth promotion effect on plantlets inoculated with species of *Pseudomonas, Rhizobium* and *Trichoderma* was checked in the presence or absence of VAM and it was found that the overall growth with VAM was higher than that without VAM.[32] Compared to the effect of *Trichoderma* in black pepper, the treatments inoculated with *Pseudomonas* or *Rhizobium* alone with or without VAM recorded low profile on growth promotion which further indicated the synergistic effect of this fungus.

The comparison of *Trichoderma* treatments with or without VAM recorded higher growth promotion in *Trichoderma* (alone) without VAM suggests the principal action of *T. harzianum* in helping the native VAM fungi present in the soil to colonize the black pepper plants. This was evident from our results with microscopy wherein we showed the endophytic colonization (100% colonization frequency) of AMF along with *Trichoderma* mycelium in *Trichoderma* inoculated soil compared to control.

Metagenomics on fungal population showed population abundance of beneficial fungi *viz*. *Fusarium oxysporum*¸ *Pestalotiosis fici* and *Talaromyces stipitatus* which may impart biocontrol property in *T. harzianum* inoculated plant rhizosphere than in control. Increased population of *Fusarium* spp. showed biocontrol and disease suppression in the rhizosphere of flax.[17] *P. fici*, an endophyte could produce bioactive metabolites and natural products in tea.[36] *Talaromyces* spp. are reported as biocontrol agents against species of *Verticillium and Rhizoctonia* in tomato and potato.[25] Metagenome analysis showed that the AMF *(Rhizophagus irregularis)* was higher in control but less in *Trichoderma* inoculated soil. Microscopic observation on the internal colonization of AMF between these treatments shows increased endophytic colonization of AMF upon *Trichoderma* inoculation.

Although the *Rhizophagus irrregularis* was abundant in rhizosphere soil of control, it had not colonized the tissue to get the benefit of symbiosis from the plant and also the high abundance of this AMF species (*Rhizophagus irregularis)* was ineffective in increasing plant growth in control. The reason for non-colonization in control is not clear where as when *Trichoderma* inoculated root AMF colonization was also facilitated as *Trichoderma harzianum* was known as helper organism for VAM with increase in plant growth. Further targeted studies are needed to understand the time bound interaction of AMF in rhizosphere upon inoculation of *Trichoderma harzianum* MTTC 5179 towards the AMF species abundance.

Conclusion

This study demonstrated the localization and endophytic colonization of *T. harzianum* MTCC 5179 in black pepper. Enhanced AMF root colonization by the *Trichoderma* inoculation in black pepper indicates that *T. harzianum*acts as helper organism in the root ecosystem of black pepper for colonizing AMF on the plant. Moreover, the native microbes that are selectively recruited by black pepper under the *Trichoderma* influenced rhizosphere would have helped to mobilize nutrients and enhanced, the growth. Further, detailed studies on *Trichoderma*, AMF and native microflora with the host in a multipartite interaction would help in developing targeted biocontrol strategy to overcome soil borne pathogens.

Acknowledgement

This study was funded by Indian Council of Agricultural Research, India, through outreach project "PhytoFuRa (*Phytophthora*, *Fusarium* and *Ralstonia* diseases of Horticultural and Field Crops". www.phytofura.net.in). The sequencing service was hired from Scigenome, Kochi, Kerala. The authors acknowledge Drs. Devasahayam and Sasikumar, ICAR - Indian Institute of Spices Research, Kozhikodeode, Kerala for their support in carrying out the experiments. PU is grateful to Drs. V. Srinivasan, Hamza, SJ Eapen, K. Kandiannan and D. Prasath, ICAR- Indian Institute of Spices Research, Kozhikode, Kerala for their valuable suggestion in implementing this work.

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(Received 29th August 2016, accepted 17th October 2016)

1. Introduction

The term “rhizosphere” was coined by Lorenz Hiltner in the year 1904 and the idea on rhizosphere by Hiltner was centered that plant nutrition is considerably influenced by the microbial composition of the soil adhering to the roots. His concept of rhizosphere envisioned that not only beneficial microbes are attracted by the root exudates but also the pathogens which are able to adjust to the specific root exudates. He hypothesized that the resistance of plants towards pathogenesis is dependent on the composition of the rhizosphere microflora. He even envisioned that the quality of plant products may be dependent on the composition of the root microflora (Hartmann *et al.,* 2008).

Though the importance of the rhizosphere microbiome for plant fitness is greatly recognized, for majority of rhizosphere microorganisms the recorded information is meager. To enhance the plant fitness to various biotic/abiotic stresses, it is essential to know the nature of microorganism present in the rhizosphere microbiome and the role they are playing towards the plant fitness. A number of studies have demonstrated that many plant-associated microorganisms can have profound effects on seed germination, seedling vigor, plant growth and development, nutrition, diseases, and productivity (Gevers *et al.,* 2012), the collective communities of plant-associated microorganisms are referred to as the plant microbiome or as the plants other genome. Wagg *et al.,* (2011) suggested that rhizosphere microbial diversity might act as bio indicators for maintaining plant productivity under different environmental conditions.

Rhizosphere engineering is nothing but the reshaping of the rhizosphere in the sense that modulating the soil rhizosphere microbial community. This could be done in two ways one towards breeding of crops for better beneficial microbe association at rhizosphere and the other

is towards probiotic application of beneficial microbes in soil. Smith *et al.,* 1999 from the initial studies using several inbred lines of tomato, identified three quantitative trait loci (QTL) associated with disease suppression by a strain of *B. cereus*. They detected significant phenotypic variation among recombinant inbred lines of tomato and made an attempt to identify loci associated with resistance to *Pythium torulosum*, disease suppression by *B. cereus*, and with growth of *B. cereu*s on the seed. These results indicated that genetic variation in host plant species can be exploited to enhance the beneficial associations between plants and rhizosphere soil microorganisms. Studies with transgenic plants that were manipulated to secrete specific signal molecules had shown that plants communicate with microorganisms in the rhizosphere (Oger *et al.,* 1997; 2004). Reshaping the rhizosphere microbiome by introducing beneficial microorganisms that protect the host plant against pathogen infections is in many ways comparable with the use of probiotics in humans. Zengler and Palsson (2012) indicated that top-down approaches such as metagenomics and bottom-up approaches that targets individual species or strains need to be integrated and combined with modeling approaches towards understanding the disease suppressiveness. To reduce the impact of plant diseases, Mendes *et al.,* (2013 proposed 'core microbiome' that is effective against soil borne pathogens in different agro-ecosystems which is very much similar to the concept of the core microbiome in human microbiology (Huse *et al.,* 2012). This approach of going 'back to the roots' would lead to the identification of new rhizosphere microorganisms, genes, and traits which may be exploited for other applications.

Plants contribute to the establishment of specific ecological niches of microbes in the rhizosphere by playing key role as ecosystem engineers (Hartmann *et al.,* 2009). The microbial community at the rhizosphere reflects its functional specificity on plant-microbe interactions. It

suggests that taxonomically-contrasted plant growth promoting strains may coexist in soil and colonize the same rhizosphere. The probiotic community enrichment by the plant is the major element in plant response to various biotic and abiotic stresses, coupled with the application of plant growth promoting microbes (Vacheron *et al.,* 2013). In the plant rhizosphere, the plant growth-promoting microbes play major roles such as modifying the root functioning, improving plant nutrition and its intake, and influencing the physiology of entire plant. Secondary metabolites secreted by the soil microbes has role in controlling biotic interactions (Karlovsky, 2008). The chemical ecology research field that focus on the understanding the specific interaction mediated by the producer organism with the target microbe and with the microbial community is of immense importance in rhizosphere microniche. Hence, integrating functional and ecological knowledge on microbial populations in soil will be a prerequisite in developing novel management strategies for sustainable agriculture for which the population abundance of soil microbiome is an important component.

Black pepper (*Piper nigrum* L.), popularly known as king of spices or black gold, is a highly valued perennial, export oriented spice crop grown in the tropical world. It is propagated vegetatively through stem cuttings. This spice climber succumbs to several diseases caused by fungi, bacteria and viruses (Ravindran 2000). Among these, foot-rot caused by the soil-borne oomycetous fungal pathogen, *Phytophtora capsici*, is a major constraint for this crop (Anandaraj *et al.,* 1996). Crop loss due to this disease alone in Kerala, India (major center of black pepper production) was estimated to range from 3.4 to 9.4% (Anandaraj *et al.,* 1989). Infected plant materials in the soil and dried vines in the gardens are the primary source of inoculum of the pathogen (Anandaraj 1997). There are no varieties of black pepper at present, which is completely resistant against this disease and the progress in understanding the molecular

components of this pathosystem is hampered due to lack of protein or transcriptome databases (Gordo *et al.,* 2012). Though some of the black pepper associated bacteria, such as *Pseudomonas aeruginosa*, *P. putida* and *Bacillus megaterium* were identified as effective antagonistic endophytes against the foot-rot disease (Arvind *et al.,* 2009; Anith *et al.,* 2003), the mycelial fungus, *Trichoderma harzianum* is being widely used both in the nursery and field as successful integrated disease management component in India (Rajan *et al.,* 2002; Thankamani *et al.,* 2005). Though growth promotion (Rajan *et al.,* 2002; Anandaraj and Sarma 2003; Thankamani *et al.,* 2005; Sibi 2013) and disease suppression (Rajan *et al.,* 2002; Paul *et al.,* 2005) activities of *Trichoderma* spp. on black pepper are manifold, both in the nursery and field conditions. Information on the interactions of *T. harzianum* with the rhizosphere microbes of black pepper, nature of colonization in black pepper roots and on the molecular mechanism of plant defense responses induced by this fungus in black pepper is not yet attempted.

During Plant- *Trichoderma* interaction the elicitors released by the fungus induces the different type of signals by Salicylic acid, Jasmonic acid & Reactive oxygen species (SA, JA& ROS) thereby triggering expression of defense response. Though the processes on this interaction at the biochemical and molecular level are well studied, the nature of resistance of this fungus on each crop would not be the same. The type of resistance by the fungi on each crop species is one of the main parameter to determine the kind of resistance. Though the *Trichoderma* mediated defense is said to be induced systemic resistance (ISR), in many cases the Plant –*Trichoderma* interaction had resulted in systemic acquired resistance (SAR) with major expression of pathogenesis related proteins (PR 1, 2 and 5) (Mathys *et al.,* 2012, Hermosa *et al.,* 2012). Contreras-Cornejo *et al*., (2011) and Salas-Marina *et al*., (2011) demonstrated ISR/SAR in their study using *Arabidopsis*. Being the only bio control agent in black pepper cultivation

system in India it is of paramount important to elucidate the pattern of resistance mechanism mediated by this fungus. Transcriptomics have been successfully used in studying various Plant-*Phytophthora* interactions. This not only provides understanding the molecular mechanism as well as to identify candidates for resistance against pathogen. Till now studies on the two way interaction (Plant-*Phytophthora*) were restricted only to either on roots or in planta inoculated/detached leaf only (Ali *et al.,* 2014; Zuluaga *et al.,* 2015; Jupe *et al.,* 2013). The label free proteomics was used to study the apoplastic secretome of potato during compatible and incompatible interaction (Ali *et al.,* 2014) and total leaf protein profiling of black pepper (var Panniyur-1) during *Phytophthora capsici* interaction (Mahadevan *et al.,* 2016). The detached leaf was used to inoculate the *Phytophthora* and samples were analyzed only at 24 hours after inoculation in case of black pepper by Mahadevan *et al.,* (2016). Most *Phytophthora* species are soil borne pathogens, and hence, the most direct way infecting host plants is via the fine feeder root system. The difference in leaf protein expression of black pepper+ pathogen from protein expression of Black pepper + *Trichoderma* + Pathogen would become the major component in deciding the beneficial action of *T. harzianum* in induced systemic resistance (ISR).

The three way interaction (Plant-*Trichoderma*-Pathogen) is less attempted (Marra *et al.,* 2006; Keswani *et al.,* 2016) in comparison to two way interactions (Plant+*Trichoderma*) (Harman *et al.,* 2004). Understanding the tripartite interaction at molecular level is needed in order to enhance the applicability in agro ecosystem and to unveil the cross talks involved in the beneficial association (Keswani *et al.,* 2016). When compared to gene expression and transcriptomics approach, the application of proteomics in this area of research is very minimal. The nature and composition of ISR strongly depend on the tripartite combination plant-BCA-pathogen (Duijff *et al.,* 1998; Tjamos *et al.,* 2005). Velazquez-Robledo *et al.,* (2011), Sagara *et*

al., (2007) and Karolev *et al.,* (2008) reported the induction of ISR-like resistance by *T. asperellum* or *T. harzianum* T39 against *Botrytis cinerea* in tripartite interaction. Mathys *et al.,* (2012) also found that the induction of ISR happened only after pathogen infection in the Arabidopsis- *Trichoderma- B. cinera*. The ISR in plants is regulated by JA and or ET synthesis unlike in SAR which is regulated by SA. (Vallad and Goodman 2004).Gel based proteomics was attempted by Marra *et al.,* (2006) on *Trichoderma*-Bean-*Botrytis* and *Trichoderma* –Bean-*Rhizoctonia* interaction. This was the only study on tripartite interaction on *Trichoderma* using proteomics. There is no study using proteomics even in *Arabidopsis* –*Trichoderma* –pathogen interaction.

Thus, in this study it was hypothesized that the probiotic application of *Trichoderma* sp. would alter the community composition or dynamics of other soil fungi and bacteria at the rhizosphere of black pepper; and that might contribute to the plant health in a better way than the rhizosphere community without *Trichoderma*. That is attempted in the present thesis as part1 using whole genome soil metagenomics. The two way (Plant- *Trichoderma*) has been attempted only in certain monocots, solanaceae and in model crop *Arabidopsis*. No proteomics based study in woody perennial crop- *Trichoderma* interaction is reported till now. Label free proteomics is attempted in this thesis to elucidate the black pepper –*T. harzianum* interaction to bring out the ISR reaction in plant leaves which are inoculated with *T. harzianum.* The ISR in case of Black pepper - *Phytophthora capsici* is also attempted using proteomics. These two way interactions (Black pepper- *T. harzianum* and Black pepper – *Phytophthora capsici)* form the part 2 in the present thesis. In order to fingerprint the *T. harzianum* induced systemic resistance related proteins (T-ISR) in leaf we had attempted the Tripartite interaction (Back pepper – *T. harzianum* – *P. capsici*) using proteomics which is discussed in Part 3 of the thesis.

Hence the objectives are

1. To analyze the microbial community in black pepper rhizosphere and to record the community dynamics with inoculation of *Trichoderma harzianum* MTCC 5179

2. I a. To elucidate Black pepper- *T harzianum* interaction

   I b. Endophytic growth analysis using microscopy

   II. To elucidate Black pepper –*Phytophthora capsici* interaction

3. To elucidate Black pepper-*T. harzianum-Phytophthora capsici* interaction.

2. Review of Literature

2.1. *Trichoderma*

Trichoderma spp. is free-living, spore producing ascomycetes fungi (Harman *et al.*, 2004). They are proved to be opportunistic, avirulent plant symbionts. (Mukherjee *et al.,* 2012). They live in soil and show close association with the rhizosphere. The colonization of *Trichoderma* in the rhizosphere leads to significant effect in plant metabolism and hormones, soluble sugars, phenolic compounds and amino acids, photosynthetic rate and transpiration (Yedidia *et al.*, 2003; Brotman *et al.,* 2012). The secondary metabolites secreted in the rhizosphere by *Trichoderma* mediate plant growth and nutrition, induction of systemic resistance (ISR) and the biocontrol activity against pathogens. The biocontrol activity includes competition for space and nutrients and production of antibiotics and hydrolytic enzymes (Harman *et al.*, 2004). This fungus triggers systemic resistance and enhances root growth and development in plants (Contreras-Cornejo *et al.*, 2016). The ability to produce antibiotics, to induce systemic resistance in plants, and to parasitize plant pathogens makes *Trichoderma* spp. useful as biopesticide and biofertilizer (Solanki *et al.*, 2011).The ISR is mediated by JA and ET signal transduction due to the effect of beneficial microbes like *Trichoderma* spp. (Pieterse *et al*., 2014). They control wide range of plant pathogens through elicitation of ISR or localized resistance (Harman *et al*., 2004). Root colonization with *Trichoderma* primes leaf tissues activates JA-regulated defense responses leading to higher resistance to pathogens (Martínez-Medina *et al*., 2013) , the enzymes and metabolites produced by this fungus are able to modify ethylene levels in the plant (Viterbo *et al*., 2010).

Omics studies have greatly contributed to the development of translational research in *Trichoderma*. The first member of the genus whole genome sequenced was *T. reesei*/*H. jecorina* (Martinez *et al.,* 2008) which is industrially important producer of cellulases but not a biocontrol agent. In the recent past year, the genomes of biocontrol species viz., *T. harzianum* and *T. asperellum*, have been sequenced (Kubicek *et al.,* 2011). The comparative genomics of these two species explained the presence of genes in these mycoparasites to attack other fungi and interact with plants (Kubicek *et al.,* 2011). Till date seven *Trichoderma* spp. were whole genome sequenced (Baroncelli *et al.,* 2016; Srivastava *et al.,* 2014). Genomics on *Trichoderma* spp. are regarded as a successful case of translational research, where data are quickly applied to develop new active principles for commercial products, new types of formulations and development of safer application protocols (Lorito *et al.*, 2010).

2.2. Rhizosphere and its engineering

The rhizosphere is the most complex habitat consisting an integrated network of plant roots, soil and diverse microbial groups of bacteria, archaea, viruses, and microeukaryotes. Rhizosphere engineering permits improvement of plant and soil health. Microbial engineering and plant engineering are the strategies to engineer the rhizosphere. The microbial engineering strategy involves population engineering rather than single strain engineering. Soil amendment remains the most important technique, even though recent progress in microbial and plant engineering proves to be viable strategies for rhizosphere engineering (Dessaux *et al.,* 2016). The most direct way to alter the microbiome is through inoculation (probiotic application of PGPR) which comes under microbial engineering strategy. Bioengineering of synthetic microbial communities for plant/crop growth promotion, disease resistance, and stress tolerance/regulation also an attractive opportunity. By utilizing the knowledge obtained from naturally occurring

microbial communities containing PGPRs it is possible to create synthetic microbial community. Many beneficial microbes which colonize the rhizosphere, have publically available genome sequences which can be used for genetic engineering efforts (Ahkami *et al.*, 2017). Another strategy to engineer the rhizosphere is through breeding for plant traits. Specific root exudates, root architecture, or other plant traits that support beneficial microbiomes could be used to engineer those traits into crops through CRISPR and other gene editing tools (Nogales *et al.*, 2015).

2.3. Whole genome - soil metagenomics -as a component of rhizozphere studies

Recent high-throughput sequencing methods viz., Roche 454, Illumiona hiseq, SOLid, Ion Torrent and PacBio RSII enable identification, relative quantification and functional dynamics of microbial community in the soil sample which thereby provides information on community ecology. Attempt on soil rhizosphere whole genome metagenomics is very limited. Novel integrated bioinformatics platforms (MEGAN, MGRAST) offers ways to find the community level taxomic affiliation, functional enrichment and the interaction network. MG-RAST (Meyer *et al.*, 2008) is one of the prominent platforms which supports deposition and analysis of metagenomic datasets.

Xu *et al.*, (2014) used 33 metagenome datasets derived from different soil sites viz., forest, desert, grass land, Arctic and mangrove sediment through whole genome metagenome shotgun sequencing using Roche 454 and Illumina platforms. With the use of integrated bioinformatics tools the phylogeny and functional characteristic of the microbial population were analyzed. Along with the profiling of microbial community from each soil type an array of metagenomic biomarkers with 46 taxa and 33 metabolic modules were derived as indicators for differentiating the soil communities.

The comparative metagenomics approach was employed to compare the Loktak the largest freshwater lakes of India represents an exclusive rhizospheric microbial community for biogeochemical cycling of nutrients. The soil metagenomic data with available metagenomes from four other aquatic habitats, varying from pristine to highly polluted eutrophic habitats. It was found that the *Candidatus Solibacter, Bradyrhizobium, Candidatus Koribacter, Pedosphaera, Methylobacterium, Anaeromyxobacter, Sorangium, Opitutus* and *Acidobacterium* genera are selectively dominant in fresh water habitat. This selective microbial enrichment has been attributed to the phenomenon of bioremediation at Loktak Lake (Puranik *et al.,* 2016). Bhattacharyya *et al.,* (2016) studied the bacterial diversity and population dynamics under ambient CO_2 (a-CO_2) and elevated CO_2 + temperature (e-CO_2T) in low land rice rhizosphere using whole genome metagenomic approach. The dominant bacterial communities were found to be *Proteobacteria, Firmicutes, Acidobacteria, Actinobacteria* and *Planctomycetes*. Genera related to methane production viz., *Methanobacterium, Methanosphaera, Methanothermus* and *Methanothermococcus* were absent in a-CO_2. The enzymes involved in acetoclastic methanogenesis pathway and serine pathways of methanotrophy were with abundant reads in e-CO_2T compared to CO_2. High bacterial diversity and abundances of C and N decomposing bacteria in the rhizosphere were found under e-CO_2T, which suggested the possible exploration microbes for their specific role in nutrient cycling, sustainable agriculture and environment management.

The rhizosphere microbiome metagenomics of gray mangroves in the red sea using 454 GS FLX Titanium technology showed predominance by Proteobacteria, Bacteroidetes and Firmicutes, with high abundance of sulfate reducers and methanogens, although specific groups were selectively enriched in the rhizosphere compared to the bulk soil. MG-RAST functional

analysis observed the enrichment in metabolism of aromatic compounds and potassium metabolism along with the enrichment of pathway that utilize osmolytes (Alzubaidy *et al.*, 2016). Simoes *et al.*, (2015) showed Ascomycota was the dominant phylum (76%–85%) in fungi in the rhizosphere metagenome of gray mangroves of the Red sea. They also detected several commercially-used fungi viz., producers of secreted cellulases and anaerobic producers of cellulosomes in the datasets using MG-RAST platform.

Castaneda and Barbosa (2016) characterized the taxonomic and functional diversity of bacterial and fungal communities present in soil using metagenomic approach from vineyards in Central Chile and the native forest soil prior to the establishment of the vineyard as comparative datasets. Analysis showed that bacteria were more abundant than fungi in both types of habitats. Functional diversity was observed on genes for metabolism of amino acids, fatty acids, nucleotides and secondary metabolism as enriched in forest soils, while genes for metabolism of potassium as enriched vineyard soils.

2.4. Defense mechanism in plants

Plants have innate immunity which is recognized and activated by the invading organism. Recognition of non-self-molecules is the first step towards an active immune response and is mediated by pattern-recognition receptors (PRRs) in the host cells. These PRRs are able to recognize microbe-associated molecular patterns (MAMPs), which are also often termed as pathogen associated molecular patterns (PAMPs) (Boller and Felix 2009).The recognition of MAMPs/PAMPs by plant PRRs leads to so called PAMP-triggered immunity/pattern triggered immunity (PTI) / non-host resistance which provides a first line of defence against most of the non-adapted pathogens (Jones and Dang 2006). When PTI is suppressed by pathogen effectors delivered into the host cell, plants can overcome pathogen suppression of PTI and bring back the

pathogen resistance through effector-triggered immunity (ETI). This branch of innate immune signaling is activated when individual pathogen effectors are recognized by disease resistance (R) proteins (Nomura *et al.,* 2011). In addition to PTI and ETI the plant immune responses are regulated by several plant hormones, including salicylic acid (SA), jasmonic acid (JA), and ethylene. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are the two forms of induced resistance. In both SAR and ISR plants are preconditioned by prior infection or treatment that results in resistance (or tolerance) against subsequent challenge by a pathogen. SAR can be triggered by exposing plant to virulent, avirulent, and nonpathogenic microbes (Sticher *et al.,* 1997). SAR is established by the co-ordinated expression of pathogenesis related proteins and salicylic acid in plants (Cameron *et al.,* 1994). Where in ISR is initiated by plant growth promoting bacteria (PGPR) in plant system (van Loon *et al.,* 1998). It does not involve accumulation of salicylic acid and PR protein unlike SAR, but relies on synthesis of jasmonate and ethylene (Pieterse *et al.,* 1996).

2.5. Molecular mechanism of Plant- *Trichoderma* Interaction

Induced systemic responses (ISR) are not only initiated by pathogens, but also from interactions with avirulent microbes. The colonization of the plant rhizosphere by certain strains of plant growth-promoting rhizobacteria (PGPR) results in enhanced state of resistance to subsequent pathogen attack which is generally known as systemic ISR. PGPR-mediated ISR can occur in many plant species and demonstrated to be effective against wide range of pathogens. (van Loon *et al.,* 1998). In contrast to SA-dependent SAR the ISR depends primarily on jasmonic acid (JA) and ethylene which was evidenced from the extensive work with Arabidopsis (Ton *et al.,* 2002). Findings also indicate that there is a cross talk and certain degree of overlap between SA and JA (Dong 2001; Glazebrook *et al.,* 2003). In addition to PGPR, certain group of

root-colonizing beneficial fungi also found to induce/enhance plant resistance to pathogens. They belong to the anamorphic stages of fungi, including *Trichoderma* spp., *Fusarium* spp., binucleate *Rhizoctonia*, and *Pythium oligandrum* that are commonly found in most soils throughout the world (Harman *et al.*, 2004; Le Floch *et al.*, 2005). Among these fungi, it is now widely accepted/demonstrated that the biocontrol potential of *Trichoderma* spp. resides on their ability to induce both local and systemic resistance responses.

Howell *et al.*, (2000) and Yedidia *et al.*, (2003) have reported that the root colonization by *Trichoderma* spp. resulted in the accumulation of antimicrobial compounds both locally in the roots and systemically in the leaves respectively. The JA/ET signaling from plant and a mitogen-activated protein kinase signaling pathway from the fungus were identified to be the major players for the *Trichoderma* mediated ISR in *Cucumis sativus* (Shoresh *et al.*, 2006; Viterbo *et al.*, 2005). Cellulase and xylanase have been described as proteinaceous elicitors in *Trichoderma* spp. (Bailey *et al.*, 1992; Calderon *et al.*, 1993). Many reports indicate that the metabolites capable of elicitation of plant defense are produced by *Trichoderma* (Hanson & Howell 2004; Harman *et al.*, 2004). Djonovic *et al.*, (2006) characterized Sm1, a novel proteinaceous nonenzymatic elicitor from the rhizosphere-competent fungi *Trichoderma virens*. The purified Sm1 effectively induced the plant defense responses and systemic resistance against a foliar pathogen of *Gossypium hirsutum* and the Sm1 response was associated with the accumulation of reactive oxygen species, phenolic compounds, up regulation of genes from SA and JA/ET pathway and genes involved in the biosynthesis of sesquiterpenoid phytoalexins . The induction of Ca2+-mediated signal perception as an early step during the interaction of soybean cells with *Trichoderma* metabolites was demonstrated by Navazio *et al.*, (2007). Quantitative increase in total phenol, total protein, major three fatty acids and the induction of jasmonic acid (JA) was

found after treatment of mustard with powder form of *Trichoderma* (Dave *et al.,* 2013). In general, the molecular mechanisms on *Trichoderma* - mediated induced resistance have been studied in dicot plants to certain extent (Shoresh *et al.,* 2005; Viterbo *et al.,* 2005; Djonovic *et al.,* 2006) when compared monocots where it is very limited. But many lines of similarity in various aspects of induced resistance occur between monocots and dicots had been observed (Dong 2004; Chem *et al.,* 2005).

Root colonization (Shoresh & Harman 2008) of *Trichoderma harzianum* Rifai strain 22 (T22) induces changes in the proteome of shoots of 7 day old *Zea mays* seedlings, though T22 is infected only on roots. Two-dimensional gel electrophoresis followed by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry and nano spray ion-trap tandem mass spectrometry was used to identify proteins that are differentially expressed in response to colonization of maize plants with T22 identified that 91 spots out of 114 were up-regulated and 30 out of 50 down-regulated proteins . Grouping of these proteins showed that a large portion of the up-regulated proteins are involved in carbohydrate metabolism, photosynthesis and up and down regulated proteins from stress and defense responses. The stress response proteins glutathione S-transferase (up-regulated spot), glutathione-dependent formaldehyde dehydrogenase (up-regulated spot), peroxidase (up-regulated spot), and heat shock proteins (two up- and three down-regulated spots). The nucleotide-binding site (NBS)/Leu-rich repeat (LRR) resistance protein-like proteins, Phenyl ammonium lyase (PAL) protein, were found to be up regulated.

Shoresh *et al.,* (2006), demonstrated in cucumber, that a MAPK is activated by inoculation of the roots with *T. asperellum*. The activation of this gene was found to be necessary for the plant's *Trichoderma* conferred defense against bacterial pathogen and silencing of this

MAPK completely eliminated this protection (TIPK). Sequence analysis showed that the TIPK is homologous to MPK3a, WIPK, and MPK3 which are the wound induced genes. Systemic expression of the gene in leaves post *Trichoderma* inoculation was also demonstrated in their study. The level of expression of TIPK during post *Trichoderma* inoculation was much higher and more prolonged when plants were inoculated with *Trichoderma* prior to pathogen challenge.

Though there are remarkable works on monocot and dicots the research attempt and the knowledge on perennial crop-*Trichoderma* is in naïve state. Palmieri *et al.*, (2012) analyzed proteomic changes in grapevine leaves in response to T39 treatment and *P. viticola* inoculation using the high-throughput eight-plex iTRAQ platform. Proteins involved in stress response showed increased abundance in T39-treated plants .Increase in abundance of two receptors (a leucine-rich repeat receptor-like protein kinase and a receptor like protein kinase precursor), a guanine nucleotide-binding protein (GTPase-activating protein), resistance RPP protein and two TMV resistance proteins N, proteins involved in abscisic acid and auxin signaling and metabolism, and thioredoxin and a ferredoxin-thioredoxin reductase along with proteins involved in signal transduction (a *Pseudomonas syringae* resistant protein, a Rabgap/TBC domain-containing protein, and two disease resistance proteins) and redox balance (aglutaredoxin, a copper/zinc superoxide dismutase, and a glutathione reductase) were induced in T39-treated plants after *P. viticola* inoculation.

2.6. Colonization of plant roots by *Trichoderma*

Though the members of the genus *Trichoderma* are saprophytes, the root colonization by certain strains is common. Some rhizosphere competent strains of *Trichoderma* also colonize the intercellular region in roots (Yedidia *et al.,* 1999). Colonization studies using light microscopy and SEM (De Souza *et al.,* 2008) showed that *T. stromaticum isolates* used in cacao plantations

as a biocontrol agent of witches' broom diseases were able to make endophytic colonization. The endophytic colonization was found better in seedlings under sterile condition than under non sterile condition. The conidia of *T harzianum* CECT 2413 on tomato roots showed profuse adhesion of hyphae and colonization of the epidermis as well as the cortex. The confocal microscopy of *Trichoderma* transformant with GFP tag showed hyphal growth in intercellular region and the plant induced papilla –like hyphal tip formation (Chacon *et al.,* 2007). Grandular trichomes were demonstrated to be the entry point for four *Trichoderma* species (*T. ovalisporum, T. hamatum, T. koningiopsis and T. harzianum*) into cacao stem that leads to systemic colonization of the tissue (Bailey *et al.,* 2009). SEM analysis revealed swellings resembling appresoria on the grandular trichome tips in cacao.

2.7. *Trichoderma*- Arbuscular mycorrhizal fungi Interaction

The information on interactions between the roots associated beneficial microbes is very important. Studies have demonstrated that certain *Trichoderma* strains might influence AMF activity. The presence of *T. harzianum* significantly increased the root colonization of AMF species *G. intraradices, G. constrictum* and *G. claroideum* compared to the colonization of the most efficient *G. mosseae* inoculated alone (Martinez *et al.,* 2004) in melon plants. Camprubi *et al.,* (1995) observed nil effect by *Trichoderma* on AMF in Citrus. Antagonistic effect was observed by Martinez *et al.,* (2004). Green *et al.,* (1999) observed that the growth and P uptake by external mycelium of AMF were not inhibited by *Trichoderma* rather the adverse effect of AMF on growth of *Trichoderma* was recorded. Mycoparasitic action of *Trichoderma* on AMF mycelium has been demonstrated (De Jaeger *et al.,* 2010). The presence of *Trichoderma* was found to affect the P transloation into host plant by the *Glomus* sp (De Jaeger *et al.,* 2011). The

spore germination and hyphal growth of *G. mosseae* was stimulated by *T. harzianum* with the production of volatile compounds (Calvet *et al.,* 1992).

2.8. Plant –*Phytophthora* Interaction (Two way Interaction)

Zhang *et al.,* (2013) identified CaRGA 2 gene from *Capsicum annum* and showed its involvement in defense response against *P. capsici*. Quantitative RT-PCR showed rapid induction of this gene during pathogen invasion. Based on the VIGS technique it was demonstrated that this CaRGA2 has functional role in HR to *P. capsici* infection.

1220 deferentially expressed genes were explored using RNA-seq (Wang *et al.,* 2015) and found many of these genes were involved in defense responses. Gene encoding lignin-forming anionic Peroxidase showed higher fold-increase at 24 hpi in PI 2012334 which is resistant cultivar of *Capsicum annum* than in Qiemen the susceptible cultivar. The level of expression did not peak in susceptible until 5 dpi. The phytoalexin terpene synthase was highly expressed in resistant line at 24 hpi. The phytohormone gene viz., JAZ-like gene was significantly up-regulated at 24 hpi in both lines with rapid up regulation at 5 dpi in susceptible showed the JAZ-like gene as major player in altering JA signaling and disease symptom development. The study also showed that six differentially expressed genes were on chromosome 5. These six genes were up-regulated post *P. capsici* infection in resistant line while susceptible showed very lower level of expression throughout.

Purified 15 KDa proteinaceous elicitor from *P. colocasiae* when infiltrated into the taro leaves followed by pathogen inoculation induced SAR. The tolerant Muktakeshi cultivar did not take up infection up to 2 weeks, while in susceptible *Telia* cultivar the infection started within a week (Mishra *et al.,* 2009).

Zuluaga *et al.,* (2015) analyzed the expression of three *P. infestans* genes as markers for biotrophy (*IpiO* and *SNE1*) and necrotrophy (*PiNPP1.1*) and defined the time frame of thetomato–*P. infestans* interaction. The study showed that 48 h after inoculation (hai), the interaction was biotrophic, at 144 hai, the interaction was necrotrophic and 96 hai was identified as the time point representing the transition. RNA seq analysis using 454 sequencing found both PTI and ETI at 48 hai, indicating the responses during biotrophy. The transcript abundance of putative receptors increased after *P. infestans* infection compared to mock inoculated samples. Transcripts with differential abundance included 108 putative genes encoding resistance-like proteins with Toll-interleukin-like receptor/coiled coil (TIR/CC)-NBS-LRR domains. SA and JA levels also increased after inoculation, suggesting the phytohormones mediated defense upon *Phytophthora* infection.

Ali *et al.,* (2014) employed 1D-gel separation followed by MS/ MS analysis on a LC-coupled Orbitrap mass spectrometer to determine quantitative changes in apoplastic protein levels in resistant variety Sarpo Mira and SW93-1015 the susceptible genotype to *P. infestans*. In incompatible interactions (Sarpo Mira) found to have the high expression of candidates for hypersensitive response initiation which includes Kunitz-like protease inhibitor, transcription factors and an RCR3-like protein along with MYB transcription factors, glutaredoxins, RING zinc finger proteins and U-box proteins, that were associated with resistance. The subtilisin-like protease, lipid transfer proteins, defensins and strictosidine synthase were also expressed. These genes and proteins were referred as candidate involved in initiation of HR and resistance against *P. infestans.*

Induced expression of glucanase by *P. capsici* in black pepper was evidenced (Nazeem *et al.,* 2008) by western blot analysis using resistant (Kalluvally) and the susceptible (Panniyur-1)

varieties. In both the varieties the expression of glucanase was observed only after infection by *P. capsici*. The glucanase expression was absent in the healthy or uninfected plants.

Jebakumar *et al.,* (2001) reported the presence of glucanase at 48 hpi during infection by *P. capsici* in resistant variety IISR Shakthi but the susceptible varieties (Subhakara & Panniyur 1) failed to get the reaction.

Mahadevan *et al.,* (2016) reported the label-free proteome profile of black pepper (*P.nigrum* L. variety –Panniyur 1) the susceptible variety to *Phytophthora* during 24 hpi. The comparison with the mock inoculated control resulted in 151,189 peptides of which 5870 were unique peptides which belonged to 532 novel protein hits. The identified proteins belonged to 49 bio chemical pathways.

2.9. Plant- *Trichoderma* -Pathogen Interaction (Tripartite Interaction)

Studies on the three-way interaction with Plant-*Trichoderma* - pathogen are aimed at unraveling the mechanisms involved in partner recognition and the molecular cross-talk to maintain the beneficial association between the fungal antagonist and the plant. Using proteomics (Marra *et al.,* 2006) and gene reporter systems (Lu *et al.,* 2004) the molecular cross-talk during three-way interactions have been attempted. Tripartite interaction studies require experiments that investigate the changes in gene expression dynamics in each partner involved, singly and also in all possible combinations. Proteomic approach (Marra *et al.,* 2006) indicated that in the plant specific PR proteins and other disease related factors (i.e. potential resistance genes) regulates the three-way interaction. The presence of *Trichoderma* modified quantitatively and qualitatively the plant response to a pathogen attack.

Gfp-tagged mutants of T. *atroviride* were used to study the in situ *Trichoderma* – plant – pathogen interaction by using different promoters of bio control-related genes to drive the

expression of the living producer (Lu *et al.*, 2004). In particular, induction of *Trichoderma* genes in the presence of the soil-borne pathogens *R. solani* and *P. ultimum* was monitored by confocal and fluorescence microscopy which allowed for the first time a direct visualization of the mycoparasitic gene expression cascade in vivo. It was found that specific compounds released by the host cell walls were involved in mycoparasitism induction in addition to the involvement of *T. atroviride* endo- and exochitinases (nag1 and chit42). Analysis of ISR through transcriptome study, more specifically of the responses induced before (ISR-prime) and after addition (ISR-boost) (Primed plant inoculated with pathogen) of *B. cinerea*, was done by Mathys *et al.,* (2012). The primed defense response of *T. hamatum* T382-treated plants upon pathogen inoculation is demonstrated by the faster induction of defense processes, JA-synthesis and the production of secondary metabolites viz., anthocyanins, flavonoids and galactolipids. Defense– related ROS-production was down regulated while the phenyl propanoid pathway was induced during ISR-boost.

Brotman *et al*., (2012) found that T203 primed *Arabidopsis* plants challenged with the bacterial pathogen showed quantitative differences in gene expression showing priming activity of the beneficial fungus. Lipid transfer protein (LTP) 4, a member of PR-14 pathogenesis-related family was up regulated, while the WRKY40 transcription factor, the susceptibility factor to bacterial infection with reduced expression. Transcription factors with significant expression variation in T203 treated plants were HIG1/MYB51, a regulator of indolic glucosinolate biosynthesis plant secondary metabolites and MYB77 which modulates plant responses to auxin, for controlling lateral root growth and development under changing environmental conditions. T203 root pretreatment showed increase in expression of PR1, PR2 and Et/JA responsive genes (eir1, eto3, lox2). Et-responsive transcription factor ERF13 was strongly induced in primed

plant inoculated with pathogen *Pseudomonas syringae*, showed the Et signaling cascades activation by T203.

Contreras-Cornejo *et al.,* (2011) reported that colonization of *Arabidopsis* roots by *T. virens* or *T. atroviride* reduced disease symptoms and plant death caused by *Botrytis cinerae* in leaves. The defense gene expression, induction of H_2O_2, accumulation of SA and JA, camalexin production when correlated to reduced disease symptoms in *Arabidopsis* colonized by *Trichoderma*, suggests that the combined activation of these defense pathways in conferring the plant immunity against a fungal necrotizing pathogen.

Yedadia *et al.,* (2003) observed Pal1 activation both locally and systematically after application of *Trichoderma* to the root system. Thus, *Pal1* activation peaked at 48 h post elicitation with *T. asperellum* in the leaves. A similar time course based *Pal1* expression was observed (Martinez *et al.,* 2001) using active cellulase from *T. longibrachiatum* as an inducer. HPL and lox1 the defense enzymes belonging to a distinct pathogen-induced metabolic pathway were found induced by *T. asperellum* treatment. The expression of HPL in the *Trichoderma*-pre elicited plants was further increase by challenge inoculation with *P. syringae* pv. *lachrymans*.

Reduction of symptom due to inhibition of disease related processes and up regulation of defense mechanism after *P. viticola* in T39 –primed grape vine has been demonstrated (Perazzolli *et al.,* 2012). Induced resistance by *Trichoderma* in susceptible variety was found to be partially mimicking the resistant variety of grape vine. The symptom reduction in susceptible genotype was achieved by specific up regulation of 59 receptor kinases, 10 protein kinases, two bHLHs genes, one MYB gene and also the NPR1.1 gene. The Avr/cf9, Hin1, Hsr203i (HR related genes were induced and the HR necrosis was not present in T 39 primed plants upon pathogen infection.

The defensive reaction of the pepper to *P. capsicum* primed with *T. harzianum* was found to be systemic on the leaves with the involvement of PR proteins exhibiting peroxidase activity (Ezziyyani *et al.,* 2007). Khan *et al.,* (2004) conducted the split root experiments which evidenced the induction of the systemic response in cucumber by *Trichoderma harzianum* 382 against *P. capsici*. *Trichoderma harzianum* 382 consistently reduced the severity of root rot in plants harvested from the infested heated peat mix.

Transient expression of Ca LTP-N, an inducible protein derived from *Trichoderma* in *N. benthamiana* leaves reduced the disease development by *P. nicotianae* infection which proved this LTP gene function in *Trichoderma* induced resistance against *Phytophthora* spp (Bae *et al.,* 2011).

3. Materials and Methods

3.1 Whole genome soil rhizosphere metagenomics

3.1.1 Raising of black pepper cuttings

The plants of black pepper variety Sreekara were collected from ICAR-IISR nursery, Kozhikode, Kerala. Single node cuttings from the plants were washed with Tween 20 for 15 min, followed by washing under running tap water. The cuttings were surface sterilized with copper oxychloride (0.2%) for 15 min, rinsed twice with sterile double distilled water. The cuttings were treated with mercuric chloride (0.1%) for 5 min, followed by wash with sterile ddH_2O twice. The cut ends of the cuttings were quick dipped in indole-3-butyric acid (8000 ppm), and planted in protray (cavity thickness 0.8mm, L x W x D = 550x300x5.2 cm) having sterile perlite medium fortified with sterile Hoagland's solution (Hoagland & Arnon 1938).

The protrays were maintained in greenhouse with top portion sealed with aluminum foil. The cuttings were sprayed with Hoagland solution once in a day. After 2 months of growth when plants attained 24 - 26 cm height with 4-5 leaves, the rhizosphere perlite samples were collected and analyzed for the presence or absence of *Trichoderma* spp. by spread/pour plate method.

3.1.2. Planting in pots

The plantlets with no association of *Trichoderma* spp. were transferred to the pots filled with top soil (composition: 197 Ca; 173 K, 71 Mg; 18 S; 11.38 Fe; 5.56 Mn; 3.24 Zn; 1.64 P; 0.92 Cu; 0.16 B (all in ppm); and 1.6% organic carbon, pH: 4.35. The pots were divided into two sets, inoculated with *T.harzianum* (MTCC 5179), the treated and without inoculation of *T. harzianum*, the control. In each set 4 replicates having 3 plants per replica were kept for the

study. Talc formulation of *T. harzianum* (MTCC 5179) (3.5g/ 3 kg soil) was mixed in the soil to serve as the treated set. Growth parameters *viz.*, height of the plant, stem girth (1 cm above from the soil region) and the leaf area index (LAI) were observed at 30, 60, 90 and 120 days. The LAI was calculated using the formula: length (cm) × width (cm) × 0.6. After 120 days, plants were uprooted, the soil adhered to the roots of pepper plants were collected from 3 biological replicates from both treatment and control, and stored at -80^{o}C. The shoot and root (fresh and dry) weights were also recorded.

3.1.3 Workflow of Metagenomics

The shotgun metagenomics sequencing approach provides community-level information in complex environments. Illumina hiseq is a high throughput sequencing platform used for whole genome metagenomics. Illumina sequencing technology offers short reads, 2x250 or 2x300 bp but generates high sequencing depth. Longer reads are preferred as they overcome short contigs and other difficulties during assembly. Illumina reads are recommended in metagenomics studies where the difference between rare and abundant cells is significant. Assembly involves the merging of reads from the same genome into a single contiguous sequence (contig). Many available tools were built upon a traditional de Brujin graph approach to genome assembly.

Once assembled, genes can be predicted and functionally annotated. Functional annotation is performed by classifying predicted metagenomics proteins into protein families using sequence or hidden Markov models (HMM) databases. Frequently used sequence databases for functional annotation include subsystem approach to genome annotation (SEED) & Kyoto encylopedia of genes and genomes (KEGG).

The paired–end read refers to the reading of both the forward and reverse template strands of the same receptor sequence during sequencing. The overall read length of the sequence can be increased by using the sequence read from both strands. The overlap between both reads increases confidence in the paired-read.

In a next generation sequencing (NGS), the data sequence artifacts, including read errors (base calling errors and small insertions/deletions), poor quality reads and primer/adaptor contamination are common. These errors impose significant impact on the downstream sequence analysis. The quality of data is very important for various downstream analyses, such as sequence assembly. FASTQC tool kits are widely used for this purpose. This toolkit is available as either standalone or open source.

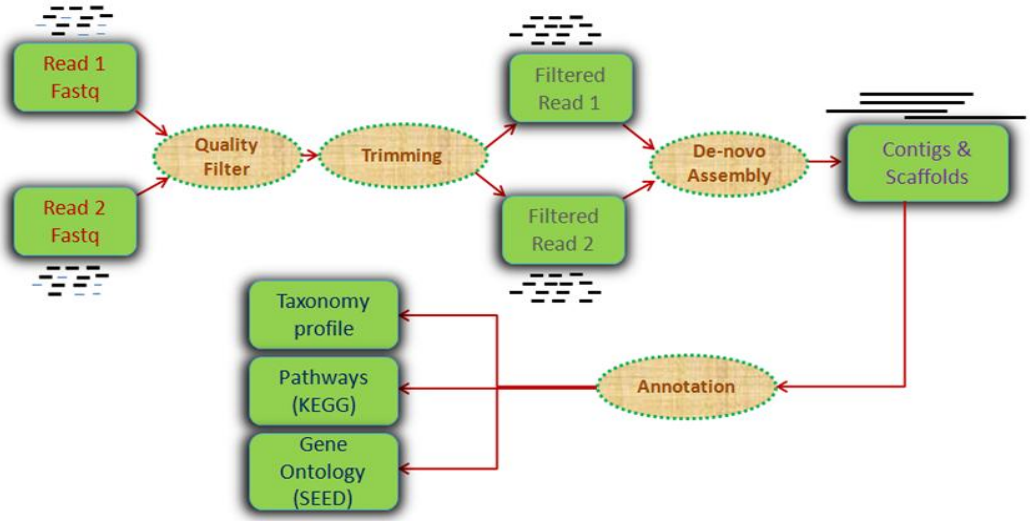

Fig 1: Workflow of Illumina Hiseq whole genome metagenomics analysis

3.1.4. Extraction of rhizosphere soil DNA and sequencing

The rhizosphere soil 100 mg was used to extract DNA from the treated and control plants using MoBio kit (MO BIO Laboratories, Inc. USA). The quality and integrity of the DNA was analyzed by nanodrop spectrophotometer (2000/2000C, Thermo Scientific, USA), and 2 µl of DNA from each sample was subjected to electrophoresis on 1% agarose gel using 1X tris-borate-EDTA buffer. Ethidium bromide stained gels was imaged using Gel imaging System (Syngene Technologies Inc, USA). Paired end DNA library was made using NEB Next ultra DNA library prep kit for Illumina. Sequencing was done using Illumina Hiseq sequencing platform.

3.1.5. Read quality assessment

The paired end reads generated were analyzed for read length, total number of reads, percentage of GC content and mean base quality distribution using FastQC tool kit. All reads were quality filtered with an average Phred quality of 20. The cutadapt (version 1.8.3) was used for adapter removal from the sequences.

3.1.6 De novo assembly and annotation

Denovo assembly was performed with default k-mer length (31-size) using de-bruijn graph method. In house PERL and Python code were used to parse the fastq files for the downstream analysis. The sequences were assembled with RayMeta (Boisvert *et al.,* 2012) with the k-mer size of 31.The contigs with more than 150 bp were filtered and grouped as pre-processed reads for downstream analysis. The protein coding regions in the contigs was predicted by Glimmer-MG v 0.3.2 (Kelley *et al.,* 2012). Each sample reads was completely assembled in 5 days. This run time includes *de novo* contig and scaffold assembly process.

Shotgun metagenomic reads are often aligned to a database of known genes. This homology-based annotation critically relies on the mapping of short reads to orthologous genes of similar function. Functional annotation for the samples from this study was done using DIAMOND version v 0.7.9 for predicted genes against the protein database using the BLAST version 2.2.29+ with an E-value of 1e-5.

3.1.7. Taxonomy/ functional analysis

The taxonomy tree was generated based on neighbor-joining method using MEGAN (Huson *et al.,* 2007). The hierarchy of comparative taxonomic abundance in all the samples was based on the contig abundance with the number of reads assigned to the taxonomy. Functional annotation was performed using DIAMOND version 0.7.9 (Buckfink *et al.,* 2015) for predicted genes against the protein database using the BLAST version 2.2.29+ (Altschul *et al.,* 1990) with an E-value of 1e-5. The functional analyses of all hits were analyzed using the KEGG and SEED options provided in the MEGAN software.

3.1.8. Analysis by MG-RAST

The results from the standalone workflow were compared with MetaGenome Rapid Annotation using Subsystem Technology (MG-RAST) (Meyer *et al.,* 2008). Taxonomic classification was done to bring the taxonomic level in the samples against the M5NR public database using best fit classification with 1e-5 as maximum e-value cutoff, and 60% as minimum identity cutoff. Functional analysis for the distribution of functional categories was done using subsystems under hierarchical classification with 1e-5 as maximum e-value cutoff, and 60% as minimum identity cutoff. Alpha diversity present in the treatment and control samples were also estimated.

3.1.9. Statistics

Completely randomized design was adopted for the analysis of growth parameters, and the data were analyzed by *t*-test. Analyses of differential/relative abundance features of metagenome data were done using STAMP software package (Park & Beiko 2010). The differential abundance between the samples was calculated using G-test (w/Yates') + Fisher's test for two sample analysis in STAMP tool.

3.2. Colonization of plant roots by *T. harzianum*

3.2.1. Fungal inoculums preparation

T. harzianum MTCC 5179 in talc formulation, obtained from the biocontrol laboratory, ICAR- Indian Institute of Spices Research, Kozhikode, Kerala was used for the pot culture study by mixing 3 g of talc with 3.5 kg of top soil. For co-cultivation study the liquid culture of *T. harzianum* was used. The 72 h old culture on potato-dextrose-agar (PDA) plates was cut into 5 mm^2 discs, and one disc was inoculated in conical flasks containing 50 ml potato- dextrose (PD) medium. After 10 days, 100 ml sterile double distilled water (ddH_2O) was added to the flasks, and spore mass was scraped out to be used as inoculum for co-cultivation studies.

3.2.2. Co-cultivation (Short-term colonization)

3.2.2.1. Plant material

Single node cuttings from black pepper variety 'Sreekara' were washed with tween-20 for 15 min, and washed in running tap water for 30 min. The cuttings were treated in 0.2% copper oxychloride for 15 min, followed rinsing in sterile ddH_2O twice. The cuttings were sterilized using 0.1% mercuric chloride for 5 min on clean bench, and then washed twice with sterile ddH_2O. The cut ends were quick dipped in 8000 ppm IBA (indole butyric acid) and planted in plantons (7.5 × 7.5 × 10 cm, Himedia) having pre-sterilized perlite medium, fortified

with sterile Hoagland's solution. The plantons were maintained in tissue culture room at 22 +/- 25^{o}C, and 3000 lux for the production of saplings.

3.2.2.2 Co-cultivation and Microscopy

The cuttings were transferred under aseptic conditions, to sterile petri-dish, and roots were washed gently with sterile ddH_2O so as to remove the perlite adhering to the roots. *T. harzianum* (10^9cfu/ml) spores were added to the *in vitro* grown plants. The control was with water. The plantlets were kept in the incubator shaker (Remi CIS 24 Plus, India) at 25 ^{o}C with constant shaking at 115 rpm. Root samples (from replicas) collected after 12, 24 or 48 h incubation. The root samples were rinsed in sterile water, fixed in 25% ethanol and stored at 4^{o}C. Toludine blue and cotton blue staining techniques were used to observe the extra- and intra-cellular colonization. The samples were also subjected to Scanning Electron Microscopy (SEM) (Hitachi SU 600, Japan) analysis for observing the interactions of *T.harzianum* with black pepper roots during colonization. Root clearing followed by staining was done to check the endophytic colonization.

3.2.3. Pot culture study (Long-term colonization)

3.2.3.1 Plant material

Cuttings were prepared as described for the short-term study. The cut ends of the cuttings were quick dipped in 8000 ppm IBA, and planted on pre-sterilized perlite medium in protray fortified with sterile Hoagland's solution as described under 2.2.1. The protrays were maintained in greenhouse with top portion sealed with aluminum foil. The cuttings were sprayed with Hoagland solution once in a day. After 2 months of growth when plants attained 24 - 26 cm height with 4-5 leaves, the rhizosphere perlite samples were collected and analyzed for the presence or absence of *Trichoderma* spp. by spread/pour plate method. The plantlets with no

association of *Trichoderma* spp. were transferred to the pots filled with top soil as described under 1.1.2. The pots were divided into two sets, one inoculated with *T. harzianum* (MTCC 5179) and another without inoculation of *T. harzianum,* the control.

3.2.4. Root clearing

Root samples were collected after120 days by cutting the roots at the collar region of the stem, washed in sterile ddH_2O, fully dried in hot air oven (at 60 ^oC for 16h). Colonization frequency was analyzed using 25 root bits (~1cm in length) taken randomly from *T. harzianum* treated and control samples. Dried roots were rehydrated with sterile water for 1 h; then 10% (w/v) of KOH was added to roots and boiled in microwave oven for 10 min, followed by rinsing with sterile ddH_2O. Post clearing was done using alkaline hydrogen peroxide [0.5% NH_4OH and 0.5% H_2O_2 (v/v) in ddH_2O] by boiling the roots in microwave oven for 5 min. The root bits were rinsed with sterile ddH_2O and acidification was done using 1% HCl. The cleared roots were stained with 0.05% tryphan blue in lactophenol stain for 15 min, de stained using lactic acid: glycerol: water in the ratio; 40:40:20 for 30 min following the procedure described by Philips & Hayman (1970) and examined under microscope (Leica DM 5000 B, USA).

3.2.5. Sample preparation for SEM

Dried root bits were immersed in sterile ddH_2O for 1 h to rehydrate. The following two methods were adopted.

3.2.5.1. *Method 1*:

The root bits were fixed with 2.5% glutaraldehyde for 2 h, followed by wash with sterile ddH_2O twice for 30 min. Secondary fixation was done in 2 % paraformaldehyde in 1.0 M KH_2PO_4 and N_2HPO_4 buffer (pH 7.2), and washed with the same buffer twice. Samples were dehydrated using 25%, 50%, and 75% and absolute alcohol for 30 min each (Hess 1966).

3.2.5.2. Method 2:

Root bits were fixed using 100% methanol for 1 h and dehydrated using 25%, 50%, and 75% and absolute alcohol for 30 min each (Neinhuis & Edelmann 1996). Processed root samples from both the methods were cut into thin sections (1 - 2 mm) using a fine scalpel and mounted on the aluminum specimen stubs using double-adhesive coated carbon tabs. The gold sputtering was done using ion gold sputtering unit (20 sec). The processed samples were micrographed using Hitachi SU6600 field emission scanning electron microscope (Hitachi, Japan).

3.3. Black pepper- *T. harzianum* Interaction

3. 3.1. T. harzianum liquid culture Preparation

T. harzianum MTCC 5179 in talc formulation, obtained from the biocontrol laboratory, ICAR- Indian Institute of Spices Research, Kozhikode, Kerala was used for the pot culture study by mixing 3 g of talc with 3.5 kg of top soil. For co-cultivation study the liquid culture of *T. harzianum* was used. The 72 h old culture on potato-dextrose-agar (PDA) plates was cut into 5 mm^2 discs, and one disc was inoculated in conical flasks containing 50 ml potato- dextrose (PD) medium. After 10 days, 100 ml sterile double distilled water (ddH_2O) was added to the flasks, and spore mass was scraped out to be used as inoculum for bipartite interaction studies.

3.3.2 T. harzianum inoculation

Single node cuttings from black pepper variety 'Sreekara' were washed with tween-20 for 15 min, and washed in running tap water for 30 min. The cuttings were treated in 0.2% copper oxychloride for 15 min, followed rinsing in sterile ddH_2O twice. The cuttings were sterilized using 0.1% mercuric chloride for 5 min on clean bench, and then washed twice with sterile ddH_2O. The cut ends were quick dipped in 8000 ppm IBA (indole butyric acid) and

planted in plantons (7.5 × 7.5 × 10 cm, Himedia) having pre-sterilized perlite medium, fortified with sterile Hoagland's solution. The plantons were maintained in tissue culture room at 22 +/- 25^{o}C, and 3000 lux for the production of saplings. The plantlets with 4-5 leaves were used for the interaction study. The plantlets were inoculated with the *T. harzianum* spore culture in sterile plantons and were maintained at 24±1 ºC with intermittent shaking manually.

3.3.3 Defining the Time frame

The roots were examined by light microscopy to ascertain the colonization of *T. harzianum* to define the time frame for sampling. Root sections stained with cotton blue at 72 and 96 hpi showed the yeast like structure inside the cells showing the good growth of *T. harzianum* and hence the leaf samples were taken from the plants at 72 (T72) and 96 (T96) hpi for proteomics. Control plants were kept with sterile water.

3. 4. Black pepper- *P. Capsici* Interaction

3. 4.1 Zoospore inoculums Preparation

The virulent *Phytophthora capsici* isolate 05-06 maintained in National repository of Phytopthora , ICAR-IISR, Kozhikode was sub-cultured in carrot agar medium (200g Carrot; 16 g Agar; 1 L Water) and incubated at 24±1 ºC for 72 h. After 72 h mycelial discs (5mm) were cut using cork borer, placed gently to float in Petri plates containing sterile distilled water (avoiding immersion of disc into the water) and incubated in laminar flow chamber at 24±1 ºC for 72 h. After the incubation period the plates were taken and kept at 4^{0}C for seconds in order to liberate zoospores. The zoospores 10^{6}cfu/ml was used to infect the axenic plants. The zoospore suspension was added to the plants in a planton and kept at 24±1 ºC for infection of

Phytophthora to the black pepper roots. The leaf samples from biological replicates were taken at 12 (P12) and 24 (P24) hpi along with the control sample for the proteomics.

3.5. Black pepper- *T. harzianum- P.capsici* Inoculation

The *T. harzianum* primed plants from 72 and 96 hpi were used for tripartite interaction experiment. The primed plants were kept in sterile planton and *P. capcisi* zoospores (10^6cfu/ml) was added. The setup was kept at 24±1 ºC in sterile environment. The unprimed plants as defined in 3.3 were used as control. The leaf sampling was done from biological replicates at control, 12 (T72P12 & T96P12) and 24 (T72P24 & T96P24) hpi for proteomics.

3.6. Proteomics

3.6.1. Protein extraction

The leaf samples T72, T 96, P12, P24 and T72P12, T72P24, T96P12, T96P24 were used to extract the total leaf protein by following Umadevi & Anandaraj (2015) .

3.6.2. Protein fractionation and Quantification

The whole protein extract was fractionated using 50KDa Amicon filters. 500 ul of sample was loaded in the filters, centrifuged at 1000g for 2 min. The concentrate was removed separately and Lowry method (Lowry *et al.*, 1951) of protein quantification was done.

3.6.3. Mass Spectrometry

For LC-LTQ Orbitrap MS analysis samples were re-solubilized in 2% [v/v] acetonitrile,0.1%[v/v] formic acid in water and injected onto an Agilent1200 (Agilent, Santa Clara,CA,USA) nano-flow LC system that was in-line coupled to the nano-electrospray source of a LTQ-Orbitrap discovery hybrid mass spectrometer (Thermo Scientific, SanJose,

CA,USA).Peptides were separated on Zorbax 300SB-C18 (Agilent, Santa Clara, CA,USA) by a gradient developed from 2% [v/v] acetonitrile, 0.1% [v/v] formic acid to 80% [v/v] acetonitrile, 0.1% [v/v] formic acid in water over 70min at a flow rate of 300nl/min. Full MS in a mass range between m/z 300and m/z 2000 was performed in an Orbi trap mass analyser with a resolution of 30,000at m/z 400 and an AGC target of 2 $X10^5$. The strongest five signals were selected for CID–MS/MS in the LTQ ion trap at normalized collision energy of 35% using an AGC target of 1 x 10^5 and two microscans. Dynamic exclusion was enabled with one repeat counts during 45s and an exclusion period of 120s. All the 6 samples were included in the analysis where control samples were chosen as reference and all other ion intensity maps from other samples were automatically aligned to the reference. The peptide ion detection method was high resolution. Considering the good initial alignment quality, the data set was not subjected to any further manual correction such as vector editing. Relative quantification using Hi-3 was selected for automatic processing of the software. After successful alignment, no further filtering was applied to subsequent quantification steps in the software. Parameter settings such as no protein grouping and quantitation from non-conflicting features were used for protein building. Peptide identification was performed by CID-based MS/MS of the selected precursors.

3. 6.4. Peptide Identification

Homology-driven proteomics is the viable option to characterize proteomes of organisms with un sequenced genomes. The peptides are generated from a precursor protein through cleavage by the enzymes (eg. Trypsin).The tandem mass spectrometry (MS/MS) is commonly used for peptide identification. An unknown peptide undergoes fragmentation, and its fragment masses are registered as peptide fragmentation spectrum (also called peptide mass spectrum or MS/MS spectrum). The computational methods (database searches) further infer the peptide

sequence from its spectrum. Database searches are done for the unknown protein using the uninterrupted peak lists deduced from MS/MS spectra whose sequences are accurately represented in protein, nucleotide or EST databases. The sequence tag search against phylogenetically related species identifies peptides that share a stretch of identical sequence of few amino acid residues which is complemented by the masses of corresponding fragment ions. De novo interpretation of peptide tandem mass spectra relies on dedicated sequence-similarity searching engines.

For protein/peptide identification, MS/MS data were searched against the Viridiplantae database (as the black pepper genome is not sequenced and the availability of EST and protein sequence information is very meager) using an in-house Mascot server (version2.4) through the ProteomeDiscoverer1.4 software. The search was set up for full tryptic peptides with a maximum of three missed cleavage sites. Carbamidomethyl on cysteine and oxidized methionine were included as variable modifications. The precursor mass tolerance threshold was 110 ppm, and the maximum fragment mass error was 0.8Da.The significance threshold of the ion score was calculated based on a false discovery rate of < 1%, estimated by the peptide valid at or node of the Proteome Discoverer software.

3.6.5. Functional annotation using Blast2Go

The most important aspects in mining omics data is to associate individual sequences and expression information with biological function. Functional annotation allows categorization of genes into functional classes which is useful to understand the physiological meaning of genes and to know the functional differences among the subgroups of sequences. Blast2GO (Conesa & Gotz 2008) is a bioinformatics tool that enables Gene Ontology (GO) based data mining on

sequence data for which no GO annotation is yet available. This tool is a suitable platform for functional genomics research in non-model species. Its species-independent character and different data input options bring it a valuable mining resource for all organism.

The accessions from the peptide data of the present study were used to query the NCBI protein BLAST to retrieve the protein sequences. These sequences were used in Blast2Go (for functional annotation of the proteins with the e value set as e-10. The functional annotations for all proteins were done for different categories viz., biological process (BP), cellular component (CC) and molecular functions (MF).

3.6.6. STRING analysis

To bring out the full information of a protein's function, knowledge about its specific interaction partners is important. STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis tool integrates protein-protein interactions, including direct (physical) as well as indirect (functional) associations. STRING covers more than 2000 organisms, which has necessitated scalable algorithms for transferring interaction information between organisms (Szklarczyk *et al.*, 2014).

In the present study, the protein – protein interaction networks were deduced using STRING v10 for the up regulated proteins (with the confidence level 0.40) of tripartite interaction was done to bring the KEGG path way enrichment in *T. harzianum* induced systemic resistance.

4. Results

4.1. Whole genome rhizosphere soil metagenomics

The black pepper plants (*Trichoderma harzianum* treated and control) were maintained in the green house and the growth parameters were recorded at 30, 45, 60, 90 and 120 days. After this the plants were up rooted; fresh and dry weight of shoot, root and height were recorded. All the growth parameters were significantly increased in the treatment (Table 1). The pH of *T. harzianum* treated soil was increased as 5.2, after 120 days of inoculation; while that of control was 4.6.

4.1.1. Extraction of Soil DNA

The DNA was extracted from the rhizosphere soil adhering to the roots. The DNA quality was checked in agarose gel (Fig 2).

4.1.2. Metagenomics: sequencing

The rhizosphere soil DNA (*T. harzianum* treated and Control) were sequenced using illumine hiseq sequencing platform. The paired end reads generated were examined for read length, total number of reads, percentage GC and mean base quality distribution using Fast QC tool kit. Paired End (251bp × 2) sequencing yielded 2,121,934 and 2,123,836 reads for treatment and control samples, respectively. Majority of the sample reads had 40-70% GC content. All reads were quality filtered with an average Phred quality (Phred quality scores is the automatic determination of accurate, quality-based consensus sequences) of 20. The cutadapt, version 1.8.3 was used for adapter removal for the sequences. The Phred score distribution (>=Q30) of the paired-end metagenome reads for treatment was 79.22%, while 80.82% was for control.

4.1.3. Assembly

The assembly of reads formed 1,827,461 and 1,879,703 contigs and N50 of 210 and 212, respectively in treatment and control. The assembled reads were analyzed by stand alone approach and MG-RAST. The double approach we used (stand alone and MG-RAST) for the analysis of metagenome yielded coherent results in both taxonomy and functional categories. Statistical analysis was done for the population and functional enrichment of metagenomics reads form the MG-RAST based analysis results.

Denovo assembly was performed with default k-mer length (31-size) using de-bruijn graph method. In-house PERL and Python code were used to parse the fastq files for the downstream analysis.A brief explanation would be appropriate. The sequences were assembled with RayMeta using a k-mer size of 31.Filtered all contigs with less than 150 bp length. The protein coding regions in the contigs were predicted using Glimmer- MG v0.3.2.

4.1.4. Annotation

The functional analyses of all hits were analyzed using the KEGG and SEED options provided in the MEta Genome ANalyzer (MEGAN) software. Rarefaction analysis (Rarefaction is the calculation of species richness for an individual samples which is based on the construction ofrarefaction curves) was performed for all the samples at the most resolved taxonomic level of the NCBI taxonomy in MEGAN. The curves were created for all taxa include bacteria, archaea, eukaryote, viruses, unclassified and other sequences (Fig 3). The term denoted as leaves in the graph corresponds to the samples under analysis viz., inoculated and uninoculated.

4.1.5. Taxonomic changes at rhizosphere

Taxonomic profiling was performed using NCBI taxonomy data sets using MEGAN. The taxonomy tree was generated based on neighbor-joining method. The diversity at phylum level and species level (Fig 4, 5) show the hierarchy of comparative taxonomic abundance in all the samples based on contig abundance. The fungal population both in order and species are shown in Fig 6 & 7. The alpha diversity (Shanon diversity index) in inoculated and uninoculated was 489,569 and 455,862 species respectively indicating the high species diversity in *T. harzianum* treated inoculated rhizosphere soil.

4.1.6. Analysis by MG-RAST

The MG-RAST server is a SEED -based environment that allows the researcher to upload metagenomes for automated analyses. The server provides the annotation of sequence fragments, their phylogenetic classification, functional classification of samples, and comparison between multiple metagenomes. In the present study**,** out of 4,121,006 (97.1%) sequences that passed quality control, 93.5% sequences produced 3,389,349 predicted protein coding regions of the metagenome in the *Trichoderma harzianum* treated. Of these, 33.7% sequences were assigned with annotation by M5NR database; 76.0% of annotated features from M5NR database were assigned with functional categories. From control sample, out of 4,162,647 sequences passed quality control (98%), 94.5% produced 3,558,779 predicted as protein coding region. Of these, 33.9% were assigned with annotation by M5NR database, and 74.7% of annotated features were assigned to functional categories. The mean sequence length, mean GC content for treated and control were 248 ± 13bp, 63 ±7% and 249 ± 12bp, 62±8%, respectively.

4.1.7. Population dynamics

The alpha diversity (Shanon diversity index) of the metagenome of both *T. harzianum* treated and control samples were 489.5 and 455.8 species, respectively (Fig 8). STAMP is a graphical software package that provides statistical tests and exploratory plots for analysing taxonomic and functional profiles. It supports tests for comparing pairs of samples or samples organized into two or more treatment groups. From the analysis of relative abundance (percentage proportion) using STAMP tool for top 10 bacterial species, *viz*., *Acidobacteriaceae bacterium* KBS 96, *Candidatus koribacter versatilis, Ktedonobacter racemifer, Candidatus solibacter usitatus, Pedosphaera parvula, Sphingomonas* sp., URHD0057, *Gemmatimonadates bacterium, Pyrinomonas* methylali phatogenes, *Chthonomonas calidirosea* and uncultured bacteria [of which *Acidobacteriaceae bacterium* (*p* =1.24e-12) and *Candidatus koribacter versatilis*(*p*=2.66e-10)showed statistical significance] were found abundant in the treatment, while unculturable bacteria found were more in control sample (*p*=0.024) (Fig 9). Analysis of the relative abundance of top 10 fungi, *viz., Rhizophagus irregularis, Fusarium oxysporum, Oidiodendran maius, Pseudogymnoasus pannorum, Talaromyces stipitatus, Pestalotiopsis fici, Mortierella verticillata* and *Trichoderma harzianum* showed that *Fusarium oxysporum* (*p* =0.013), *Talaromyces stipitatus* (*p* = 0.219) and *Pestalotiopsis fici* (*p* = 0.443) were high in treatment, while the control showed higher abundance of *Rhizophagus irregularis* (*p* = 0.034), *Pseudogymnoasus pannarum* (a human pathogenic fungus, *p* = 0.488) and *Oidiodendran* (*p* = 0.484). The *T. harzianum* reads were recorded only on treatment sample.

4.1.8. Functional level dynamics

Functional abundance (Fig 10) between *T. harzianum* treated and control samples using hierarchical classification with subsystem annotation sources showed that rhizosphere in the treatment was with abundant reads for virulence, disease and defense (54857), motility and chemotaxis (11992), ion acquisition and metabolism (8151); while the control recorded 51271 reads for virulence, disease and defense, 11564 for motility and chemotaxis, and 7276 for ion acquisition and metabolism. The relative abundance for the specific features (iron acquisition and bacterial chemotaxis) from stamp tool analysis is given in Fig 11. The heme and hemin uptake and utilization systems in Gram negative bacteria (p=0.036) and iron acquisition in red pigmented *Vibrio* (p=2.97 e-5) were abundant in treatment metagenome than in control.The treated sample recorded reduced abundance on pathogenicity islands, phages and prophages (p=7.30 e-3) **(**Fig 11**).**

4.1.9. Impact of T harzianum on metabolic pathway

Metabolism of aromatic compounds were rich (p-value 0.242) in *T. harzianum* inoculated rhizosphere metagenome. It had many metabolic pathways (Table 3) enriched to it viz., triacylglycerol metabolism, poly cyclic aromatic hydro carbon degradation pathway, toludene degradation, biphenyl degradation, benzoate degradation, dioxin degradation, sphingolipid metabolism, styrene degradation, glycan biosynthesis, purine metabolism, histidine biosynthesis and riboflavin metabolism. The uninoculated rhizosphere metagenome had bile acid biosynthesis, steroid hormone biosynthesis, plastid biosynthesis, starch, sucrose metabolism, inositol metabolism, ubiquinone biosynthesis and catechol degradation pathway genes.

4.2 Colonization of *T. harzianum* on black pepper roots

4.2.1. Co-cultivation

Short time strategy was applied to study the colonization of *T harzianum* in black pepper roots. *Trichoderma harzianum* was co-cultivated with axenic plantlets under aseptic condition and the colonization pattern was observed as follows.

4.2.1.1 Bright field Microscopy

The external colonization of *T. harzianum* has occurred on the inoculated roots of the *in vitro* derived pepper plants as surface adherence at 12 h of co-cultivation. The mycelial spread has increased with increasing time (at 24 h and at 48 h) with profusely growing mycelia bearing chlamydospores, as evidenced from toludine blue staining (Figure 12 a, b,c). Upon cotton blue staining, intercellular colonization was found at 24 h incubation (Figure 13 d). *T. harzianum* established endophytic colonization inside the cell at 48 h with luxurious intracellular mycelia (Figure 12 e). No fungal growth was observed (external or internal) on the root tissues of control plant. Root clearings of plants inoculated with *T. harzianum* showed fungal mycelium in the intercellular spaces at 24 h and hyphal tips as dark blue granules inside the cell (Figure 13 a); and intracellular chlamydospores were observed at 48 h (Figure 13 b, c).

4.2.1.2 Scanning Electron Microscopy

Comparing two sample preparation methods employed for the SEM, the methanol fixation was found better in terms of good cell structure, which aided visual observation of bacteria and fungus; whereas glutaraldehyde fixation distorted the surface structures (Fig 14). Hence, methanol fixation was taken for further analysis of black pepper root samples by SEM.

Analysis by SEM showed intact cell structures in control roots (Figure 15 a); but *T. harzianum* inoculated samples taken at 24 h and 48 h showed an indication of the interaction

with root cells (Figure 15 b & c), and hyphal growth on the surface at 24 h (Figure 15 d). At 12 h, the enlargement of hyphal tip as papillae showed its interaction for intracellular colonization (Figure 15 e). Massive colonization was observed on root surface at 48h (Figure 15 f).

4.2.2 Pot culture Study

This long term strategy was used to study the colonization of *T. harzianum* at balck pepper roots after a period of 4 months from the initial pot cultivation of axenic plantlets in soil mixed with *Trichoderma harzianum.*

4.2.2.1 Bright filed microscopy

Roots of control plants showed no fungal colonization externally, but *T. harzianum* treated plants showed invasion of mycelia. Root sections of treated plants stained with cotton blue showed AMF vesicles and arbuscules; while the control samples showed a few arbuscules with no vesicles (Figure 16 a &b). The arbuscules in control were localized to the zone of elongation of the root. In general, presence of mycelia was found increasing with maturation of the root tissue. The root tip meristem showed no colonization; however, the elongation zone showed intercellular colonization with more number of vesicles, and the maturation zone was densely colonized by inter- and intra-cellular hyphae with comparatively less number of vesicles and large number of arbuscules. The AMF mycelia were intercellular, i.e., along the tangential plane of the cortex in the elongation zone. The mycelia in this region were larger in size and rarely septate were only of AMF, no *T. harzianum* mycelia were seen in this region; but it was observed only in the maturation zone of the root as septate mycelium along with the AMF (Figure 16 b & c).*T. harzianum* in this region was found with conidia (Figure 16 d & e). This zone of maturation of root had structurally differentiated AMF mycelia with prominent septation. along with monilioids hyphae (Figure 17 c). Microsclerotia were also observed inside the root

cells (Figure 17 b). The colonization of AMF was found to be that of Arum type (Figure 17 a). This portion of roots had vesicles with round, oval, ellipsoidal and irregular shapes (Figure 17 d). Some vesicles were originated from monioloid hyphae. The size of the AMF vesicles was ranged from 40 - 147µm. Compared to control, the treated plants showed higher mycorhizal frequency (100%), after four months of pot culture. The average number of vesicles was ~ 40 per 1cm root tissue. Interestingly, the root hairs showed no internal mycelium in both the samples, though AMF mycelia were present on the external surfaces.

4.3. Black pepper -*T. harzianum* Interaction (Bipartite)

This two way or bipartite interaction was done with black pepper – *T. harzianum harzianum. T. harzianum* inoculated plants at different time points (24, 48, 72 and 96 hai denoted in this study as T24, T48, T72 and T96) showed differential protein profiles. It was observed that most of the proteins which were of constitutive in control (uninoculated) were found to get completely down regulated in inoculated plants in general. Apart from this both up regulated and down regulated proteins were also recorded.

4.3.1. Black pepper - T. harzianum interaction at 24 hai (T24)

At 24 hai 43 proteins were completely down regulated, which were in infinite quantity in control based on Annova at P>_ 0.05. Six proteins were up regulated and 19 proteins were down regulated. Blast2Go analysis for all proteins with e value e-10 (Fig 18 a, b, c) was done. Functional annotations are given for different categories viz. biological process (BP), cellular component (CC) and molecular functions (MF). The up regulated, down regulated, the proteins that were Infinite in control and completely down regulated in treatment(here after we refer them as completely regulated) and newly produced proteins that were having the peptides under regulation is given in the Table.3.

The black pepper- *T. harzianum* interaction at root showed the complete down regulation of some primary metabolism in the defense responding leaves. The redox regulation against ROS during the *T. harzianum* colonization was also recorded. At 24hai two upregulated proteins viz., structural maintenance of chromosomal protein2 with the role in chromosomal dynamics and RPP4c4 the candidate gene for Rpp-4 mediated resistance were observed. The new proteins emerged were of Peroxisomal (S)-2- hydroxy-acid oxidase GLO1-like, probable LRR receptor-like serine-threonine protein kinase RLK and protein transport protein SC16B homolog isoform X1 with the role in endoplasmic reticulum localization.The down regulated proteins were falling into different metablic activities viz., photosynthesis related (ATP synthase F1 alpha subuit, beta chain, rubisco activase), calvin cycle (chloroplast seoheptulase 1,7 bisphosphatase, fructose-bisphosphate aldolase 1), amino acid metabolism (aspartate amino transferase, 5-methyltetra hydropteroyltriglutamate homocysteine methyl transferase-2), photorespiration (Peroxisomal glycolate oxidase) and the defense proteins against ROS (catalase, super oxide dismutase, peroxidase 2-like, glutathione - s - tranferase, ascorbate peroxidase 1, glyceraldehyde 3 PO4 dehydrogenase).

The expression of proteins which are under complete down regulation at 24hai were again falling into different metabolic activities viz., Photosynthesis (photosystem CP43 reaction center protein like, ATP synthase gamma chain1 chloroplastic, photosystem II protein V (chloroplast), chlorophyll a-b binding protein 3,chloroplastic, rubisco small chain, ferredoxin-NADP reductase leaf isozyme, rubisco activase 1 chloroplastic), defense proteins against ROS (2-cys peroxiredoxin BAS1, peroxidase 12, peroxidase 5, peroxidase 16 precursor family protein, carbonic anhydrase, glyceraldehyde 3- phosphate dehydrogenase) , heat shock proteins (chaperonin 60A, heat shock protein 70 B, 20 KDa chaperonin chloroplastic like,17.8 KDa class

I heat shock protein like), calvin cycle (Transketolase), Cystein pool (O-acetylserine (thiol) lyase family protin, cysteine synthase-like), carbohydrate & amino acid metabolism (UTP glucose-1-phosphate uridylyltransferase, alanine:glyoxylate aminotransferase, glutamate synthase 2,triosphosphate isomerase), ATPase (ATPase V1 complex, H(+) transporting ATPase, subunit 1 mitochondrial,V-type proton ATPase catalytic subunit A), R gene (Rpp13 like), others (elongation factor Tu, 60 ribosomal protein L12 –like, aldolase type TIM barrel family protein, bromodomain and WD repeat containing protein 3-like isoform X1).

4.3.2. Black pepper - T. harzianum interaction at 48 hai (T48)

At T 48 hai 11 proteins which were of constitutive in control were found completely down regulated. The Blast2Go analysis on functional ontology is showed in Fig 19 a, b & c. The peptides corresponding to the proteins with various biological processes were found to be not under quantifiable expression.

The proteins with cellular activity (elongation factor 1 alpha, rap guanine nucleotide exchange factor 2, collagen alpha-6 (VI) chain like, nebulin- related-anchoring protein, platelet-derived growth factor receptor beta- like, Histone H2 A- beta) are the major group showing shut down in the expression at 48hai of black pepper- *T. harzianum* interaction.

4.3.3. Black pepper - T. harzianum interaction at 72 hai (T72)

At 72 hai 49 proteins which were constitutive in control were found to be completely down regulated. One up regulated and 2 down regulated proteins were observed. The Blast2Go analysis on functional ontology is showed (Fig 20 a,b,c).

The up regulated protein was splicing factor U2 large subunit b-like isoform X1 and the down regulated proteins were ATP synthase CF1 beta subunit and E3 ubiquitin ligase HOS1 like (mediator of cell death and defense). Major proportion of proteins with shut down in expression at 72 hai belonged to photosynthesis (ATP synthase subunit beta mitochondrial- like, ATP synthase CF1 alpha subunit -chloroplast, rubisco large subunit- binding protein subunit alpha, photosystem II CP43 reaction center protein like, ATP ase F1 complex gamma subunit protein, photosystem II protein V, rubisco small chain chloroplastic like, photo system I reaction center subunit IV B chloroplastic – like, photosystem I P700apoprotein A2), Calvin cycle (Transketolase, fructose- bisphosphate aldolase 1, fructose bisphosphate aldolase 3 chloroplastic, Ferredoxin-NADP reductase leaf isozyme chloroplastic like), protein against ROS (catalase isozyme 2, peroxidase 12, glyceroldehyde-3-phosphate dehydrogenase C2, superoxide dismutase (Cu-Zn) chloroplastic, carbonic anhydrase family protein), photorespiration (Peroxisomal glucolate oxidase, alanine:glyoxylate aminotransferase), cysteine pool (O-acetylserine (thiol) lyase family protein, cysteine synthase), cell death (UDP- glucose phosphorylase), defense response (P-loop containing nucleoside triphosphate hydrolases super family protein, germin like protein subfamily 1 member 17,lactate/malate dehydrogenase family protein, subtiltisin-like protease SBT 3.8), secretary systems (ATPse V1 complex subunit B protein), heat shock proteins (heat shock protein 70B, 20 KDa chaperonin chloroplastic like, heat shock protein 60,heat shock protein 82).

4. 3.4. Black pepper - T. harzianum interaction at 96 hai **(T96)**

At 96 hai 65 proteins which were constitutive in control were found to be shut down in treatment. One down regulated protein was observed, but no up regulated protein was observed. Blast2Go analysis on functional classification is given in Fig 21 (a, b, c) & Table 6.

The proteins with complete down regulation in expression at 96 hai were falling into photosynthesis (rubisco large subunit, ATPsynthase CF1 beta subunit mitochondrial like,photosystem II CP43 reaction center protein -like, rubiscoactivase 2, ATPase F1 gamma subunit protein, photosystemII protein V, chlorophyll a-b binding protein 3 chloroplastic, photosystem II 47KDa protein chloroplast, rubisco small chain, photosystem I reaction center subunit IV B chloroplastic-like, chlorophyll a-b binding protein AB 80 chloroplastic –like, photosystem I P700 apoprotein A2 chloroplast,protein CP24 10A chloroplastic, ascorbate peroxidase), proteins against ROS (catalase isozyme 2, peroxidase 12, glyceraldehyde 3 phosphate dehydrogenase, ascorbate peroxidase 1 cytosolic,super oxide dismutase (Cu-Zn) chloroplastic), calvin cycle (sedoheptulose bisphosphatase 1, UTP-glucose-1-phosphate uridylyltransferase, ferreoxin-NADP reductase leaf isozyme chloroplastic, fructose-bisphosphate aldolase 3 chloroplastic, fructose bisphosphate aldolase, sedoheptulose bisphosphatase 1), defense (aspartate amino transferase, RAB GTPase homolog E 18,NADP malic enzyme 4,O-acetylserine (thiol) lyase family protein, germin –like protein sub family 1 member 17,subtilisin-like protease SBT 3.8), photorespiration (transketolase, peroxisomal glycolate oxidase, serine hydroxyl methyl trnasferase1 mitochondrial, ferredoxin dependent glutamate synthase chloroplastic, malate dehydrogenase cytoplasmic), cell death (UDP-glucose pyrophosphrylase), heat shock proteins (heat shock protein 70-3, heat shock protein 60)

4.4. Black pepper- *P. capsici* (Bipartite) Interaction

4.4.1. Black pepper – Phytophthora capsici (P12)

The proteome pattern of black pepper plants inoculated with *Phytophthora* at 12 hai showed 52 differential proteins with 6 up regulated, 16 down regulated and 30 proteins which

were completely down regulated. The functional annotation for biological function, molecular function and cellular component of all the peptides usingBlast2Go is shown in Fig 22 a, b & c.

The up regulated proteins at 12 hai were of histones (Histone H2A, H4), heat shock protein 70B, Photosynthesis related (Chlorophyll a-b binding protein AB80, Photo system II 47KDa protein) and ubiquitin carboxyl terminal hydrolase 5-like isoform X1.

Down regulated protein groups were belonging to photosynthesis related (Rubisco large subunit, ATP synthase beta subunit- binding protein subunit beta, ATP synthase CF1 beta subunit, Photosystem I protein V, Photosystem I protein D1, Chlorophyll a-b binding protein), Photo respiration (Peroxisomal glycolate oxidase, Fructose bisphosphate aldolase 1), defense related (Glyceraldehyde -3- phosphate dehydrogenase A subunit 2, Germin like protein subfamily member 17) and others (uncharacterized protein LOC 100281701, 100382265). Completely down regulated protein groups were photosynthesis related (ATP synthase CF1 alpha subunit (chloroplast), Rubisco large chain, ATP ase F1 complex gamma subunit protein, Rubisco small chain, chloroplastic- like, photosystem 1 reaction center subunit IV B, chloroplastic- like, Calvin cycle (Transketolase, fructo kinase 3, ferredoxin NADP reductase, leaf isozyme, chloroplastic-like, probable fructose –bisphosphate aldolase 3, chloroplastic), amino acid metabolism (methionine synthase, alanine: glyoxylate amino transferase) , Cystein pool (Cysteine synthase), defense proteins against ROS (peroxides 12, Superoxide dismutase (Cu-Zn) chloroplastic, catalase, carbonic anhydrase family protein, ascorbate peroxidase 1 cytosolic), heat shock proteins (Small heat shock protein chloroplastic, 17.8 K Da class 1 heat shock protein- like), cell death regulator (UDP-Glucose pyrophosphorylase 1), others (Uncharacterized WD repeat containing protein C2A9.03 –like isoform X1, Protein Transport Inhibitor response 1, hairpin binding protein, RAB GTPase homolog E1B).

4.4.2. Black pepper – Phytophthora capsici (P24)

The proteome pattern of black pepper plants inoculated with *Phytophthora* at 24 hai showed 81 differential proteins with 22 up regulated, 34 down regulated and 24 proteins which were completely down regulated. The functional annotation for biological function, molecular function and cellular component of all the peptides using Blast2Go is shown in Fig 23 a, b & c.

The protein expression pattern at 24 hai showed many proteins with up, down and fully down regulation compared to 12 hai. The up regulated proteins belonged to the groups viz., Photosynthesis (Photo system II CP43 reaction center protein kinase, ATP synthase gamma chain 1, Photosystem 1 subunit H2), Heat shock proteins (Heat shock protein 70B, 17.8 k Da class heat shock protein – like, Heat shock protein 82, small heat shock protein chloroplastic), cell death regulator (UDP-Glucose pyrophosphorylase 1), defense response (2cys peroxiredoxin BAS1, Malate dehydrogenase glyoxysomal, peroxisomal (s)-2-hydroxy-acid oxidase GLO1, mono dehydro ascorbate reductase, NADP malic enzymes 4, Subtilisin –like protease, Glutathione S- transferase F 13), Probable type lectin domain containing receptor kinase S.5 (new protein),others(Cytosolic iso citrate dehydrogenase, Enolase).

The down regulated protein groups were of photosynthesis (ATP synthase CF1 alpha subunit Chloroplast, ATP synthase subunit binding protein mitochondria-like, Rubisco oxygenase large subunit, chlorophyll a-b binding protein of LHC II type 1, chlorophyll a-b binding protein AB80, chlorophyll a-b binding protein 151, photosystem II 47 K Da protein, Photosystem reaction center subunit IVB), Heat shock proteins (heat shock protein 70,Chaperonin CPN 60-2 mitochondrial precursor), defense related proteins against ROS (Glyceraldehde -3-PO4 dehydrogenase C2, aspartate amino transferase, Peroxidase 12,

Peroxidase precursor family protein, catalase, catalase isozyme 3, carbonic anhydrase fmily protein, ascorbate peroxidase 1 cytosolic, defense related protein (ferredoxin –NADP reductase, germin like protein sub family 1 member 17), , others (ATPase V1 complex subunit B protein, Vacuolar ATP synthase subunit A , cysteine synthase, E3 ubiquitin – protein ligase HOS 1).

The completely down regulated protein groups were of Photosynthesis (Rubisco large subunit, rubisco large subunit binding protein subunit alpha, ATP synthase CF1 alpha subunit chloroplast), Carbohydrate metabolism & Calvin cycle (UTP-glucose-1- phosphate uridylyltransferase, fructose- bisphospahte aldolase 3 chloroplastic), cysteine pool (O-acetylserine (thiol) lyase family protein, cysteine synthase like), defense response(lactate/malate dehydrogenase family protein), amino acid metabolism (methionine synthase), others (leucine amino peptidase 1 like, ribosomal protein L11 family protein, WD repeat containing protein C2A9.03-lie isoform X1, protein transport inhibitor response 1, peptide-N4-(N-acetyl-beta-glucosminyl)asparagine amidase A-like isoform X1.

4.5. Black pepper – *T. harzianum* – *P. capsici* Interaction

The tripartite interaction was facilitated at 72T12P (72 h *T. harzianum* primed and 12h of *Phytophthora*) (T8) showed 78 up regulated proteins, 36 down regulated proteins and 9 fully down regulated proteins, 72T24P (T9) showed 86 up regulated proteins, 31 down regulated and 7 fully down regulated proteins (Table 8). T96P12 (T10) showed 60 up regulated, 27 down regulated, 9 fully down regulated proteins; T96P24 (T11) recorded 8 up regulated, 19 down regulated, 19 fully down regulated proteins (Table 9). Functional annotation for all proteins is given as different categories viz., biological process (BP), cellular component (CC) and molecular functions (MF) below (Fig. 24 a - l).

4. 5.1. Black pepper –T. harzianum (72 hai) – P. capsici (12 hai) (T8)

The proteins with up regulation were of photosynthesis related (Rubisco large subunit binding protein alpha, oxygen evolving enhancer protein 1 cytoplasmic like, photosystem I subunit H2, ATP synthase subunit beta), Calvin cycle (glyceroldehyde 3 Phosphate dehydrogenase A subunit, NADP dependent malic enzyme, phospho glycerate kinase family protein, triose phosphate isomerase, PEP caboxylase), Photo respiration (alanine: glyoxylate amino transferase), Amino acid synthesis (Methionine synthase, Cysteine synthase, 5-methyltetrahydropteroyl tri glutamate homocysteine methyltransferase, ferredoxin dependent glutamate synthase, aspartate aminotransferase, isocitrate dehydrogenase cytosolic), Defense response against ROS (Dehydrogenase family 2 protein, 2-cys peroxiredoxin BAS1, superoxide dismutase (Cu-Zn), catalase isozyme 2, pexoxisomal (S)-2-hydroxy-acid oxidase GLO1-like, peroxidase 12, peroxiredoxin Q, glutathione S-trnasferase F13 like, peroxidase 16, reactive intermediate Deaminase A chloroplastic, monodehydro ascorbate reductase, peroxidase 5-like, ascorbate peroxidase 1 cytosolic), Defense related (fructose bishosphate aldolase, cytosolic isocitrate dehydrogenase, malate dehydrogenase, Nmr-like negative transcriptional regulator family protein, succinate semi aldehyde dehydrogenase mitochondrial like, wound –induced protein WIN2 precursor, nucleoside diphosphate kinase 2, subtilisin-like protease SBT3.8, isoflavone reductase like protein) and others (probable mediator of RNA polymerase II transcription subunit 37c,V type proto ATPase catalytic subunit A, hairpin binding protein1,40Sribosomal protein S5, protein plastid transcriptionally active 16, 20S proteasome alpha subunit PAD1, ATPase V1 complex subunit B protein, ATP dependent DNA helicase

PIF1-like, macrophage migration factor,20 kDa chaperonin, eif4a-2,hypothetical protein and uncharacterized protein).

Down regulated proteins were of photosynthesis (rubisco subunit, ATP synthase CF1 beta subunit, photosystem II CP43 reaction center protein-like, Rubisco activase isoform X1 like, ATP synthase gamma chain 1, phytochrome B-like partial, oxygen evolving enhancer protein 2-1 chloroplastic like), Calvin cycle (sedoheptulose-1,7-bisphosphatase chloroplastic like, Enolase, ferredoxin-NADP reductase leaf isozyme chloroplastic like, UTP-glucose-1-phosphate uridylyltransferase), photorespiration (Tansketolase), heat shock proteins (20kDa chaperonin chloroplastic like, heat shock protein 82, 17.8 kDa class I heat shock protein-like), defense related (Leucine amino peptidase 1-like, RAB GTPase homolog E1B, 2-methylene-furan-3-one-reductase, O-acetylserine (thiol) lyase family protein, catalase isozyme 1,3, carbonic anhydrase family protein, germin like protein sub family 1 member 17, lactate/malate dehydrogenase family protein), others (ribosomal protein L11, uncharacterized protein).

Fully down regulated proteins were majorly of photosynthesis related (photosystem II protein V, chlorophyll a-b binding protein 3 chloroplastic, photosystem II 47 kDa protein, photosystem I reaction center subunit IV B chloroplastic like, photosystem I P700 apoprotein A2, chlorophyll –b binding protein CP24 10A), the cysteine synthase and UTP-glucose-1-phosphate uridylyltransferase.

4. 5.2. Black pepper –T. harzianum (72 hai) – P. capsici (24 hai) (T9)

The up regulated proteins were of Photosynthesis related (ATP synthase CF1 beta subunit (chloroplast), O2 evolving complex 33kD family protein, oxygen-evolving enhancer protein 1, chloroplastic-like, rubisCO large subunit-binding protein subunit alpha, rubisCO large subunit-

binding protein subunit alpha, rubisCO large subunit-binding protein subunit beta, chloroplastic ruBisCO large subunit-binding protein subunit alpha, chloroplastic ATP synthase CF1 epsilon subunit (chloroplast), ATP synthase gamma chain 1, chloroplastic, ATP synthase F1 subunit alpha (mitochondrion), photosystem I subunit VII (chloroplast), ATP synthase CF0 subunit I, photosystem I subunit H2, photosystem II oxygen-evolving complex protein 2 precursor, plastocyanin A, chloroplastic, plastid-lipid-associated protein 3, chloroplastic) calvin cycle (ferredoxin--NADP reductase, leaf isozyme, chloroplastic-like, phosphoglycerate kinase,, cytosolic-like, NADP-dependent malic enzyme, triosephosphate isomerase, cytosolic (New protein),) photorespiration(alanine:glyoxylate aminotransferase, Thioredoxin superfamily protein,), defense againstROS(peroxisomal (S)-2-hydroxy-acid oxidase GLO1-like, glutathione S-transferase F13-like, catalase isozyme 3(New protein), peroxidase 12, peroxidase 60, Iron/manganese superoxide dismutase family protein, ascorbate peroxidase 2, probable L-ascorbate peroxidase 6, chloroplastic, peroxidase 5-like,) defense related (nucleoside diphosphate kinase 1, cytosolic isocitrate dehydrogenase, Malate dehydrogenase 1, leucine aminopeptidase 1-like, 2-methylene-furan-3-one reductase, subtilisin-like protease SBT3.8, Aldolase superfamily protein (New protein), low-temperature-induced cysteine proteinase, cinnamoyl-CoA reductase 2-like isoform X1, translationally-controlled tumor-like protein (New protein), succinate-semialdehyde dehydrogenase, mitochondrial, carbonic anhydrase family protein, malate dehydrogenase, cytoplasmic) amino acid synthesis (aspartate aminotransferase, cysteine synthase-like isoform X1,) and others (harpin binding protein 1, 20 kDa chaperonin, chloroplastic-like, 20S proteasome alpha subunit E1, mitochondrial outer membrane protein porin of 36 kDa, DUF810-domain-containing protein, Nucleic acid-binding, OB-fold-like protein, Ribosomal protein L11 family protein, macrophage migration inhibitory factor, vacuolar

ATP synthase subunit A, ADP,ATP carrier protein 1, mitochondrial precursor, alpha-mannosidase isoform X2,

Down regulated proteins were of photosynthesis related (ATP synthase beta chain, ATP synthase CF1 beta subunit, ribulose bisphosphate carboxylase large chain, ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic, ribulose bisphosphate carboxylase small chain 1, chloroplastic, photosystem II protein D1 (chloroplast), ribulose bisphosphate carboxylase small chain, chloroplastic-like, PREDICTED: photosystem I reaction center subunit IV B, chloroplastic-like), calvin cycle (UTP-glucose-1-phosphate uridylyltransferase), photorespiration (transketolase chloroplastic), defense against ROS (catalase isozyme 2, ascorbate peroxidase 1), amino acid synthesis (cysteine synthase, ferredoxin-dependent glutamate synthase) heat shock proteins (heat shock protein 60, 17.8 kDa class I heat shock protein-like), defense related (germin-like protein subfamily 1 member 17, PREDICTED: disease resistance protein RPP13-like, probable fructose-bisphosphate aldolase 3, chloroplastic, , germin-like protein precursor chloroplastic, uncharacterized mitochondrial protein AtMg00810-like, retrotransposon protein, mono dehydro ascorbate reductase seedling isozyme, RAB GTPase homolog E1B).

Completely down regulated proteins were mainly of photosynthesis related (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, , glyoxysomal, chlorophyll a-b binding protein 3, chloroplastic, photosystem II protein V (chloroplast), chlorophyll a-b binding protein AB80, chloroplastic-like) and defense related (malate dehydrogenase, PREDICTED: protein TRANSPORT INHIBITOR RESPONSE 1)

4. 5.3. Black pepper –T. harzianum (96 hai) – P. capsici (12 hai) (T10)

The up regulated proteins in this particular hai were of photosynthesis related (Rubisco large subunit, ATP synthase CF1 beta subunit, epsilon subunit, Ribulose large subunit binding protein subunit alpha, beta, ATP synthase F1 subunit alpha, photosystem I reaction center subunit IV B chloroplastic, Rubisco small chain, photosystem I subunit H2, cytochrome P450 734 A-6like isoform, photosystem I reaction center subunit IV B chloroplastic, Rubisco small chain, O2 evolving enhancing protein 1, photosystem 1 subunit H2, cytochrome P450 734 A6 like isoform,), Calvin cycle (Glyceroldehyde 3 Po4 dehydrogenase, Ferredoxin NADP reductase, Triosphospahte isomerase, UDP glucose pyrophosphorylase, UTP-glucose 1-4-uridyl transferase, phosphor glycerate kinase, phosphoglycerate kinase, enolase 2,) Defense against ROS (2-cys peroxiredoxin BAS1, peroxisomal oxidaseGLO1 like(s)-2-hydroxy-acid, catalase1,ascorbate peroxidase 2, Cu-Zn/Fe-Mn SOD,peroxidase12,aldolase type TIM barrel family protein, subtilisin like protease, peroxidase 16 precursor family protein), Defnese related (Aldehyde dehydrogenase, malate dehydrogenase (glyoxylate/cytoplasmic),nucleoside diphospahte kinase 1,leucine amino peptidase 1 like,NADP-malic enzyme, Germin like protein, carbonic anhydrase,) and others (probable mediator of RNA Polymerase II transcription, ATPase V1 complex subunit B protein, hairpin binding protein 1, Ribosmal protein L11 family protein, ADP/ATP carrier protein, macrophagemigration inhibitory factor, Eif 4a-1, alanine:glyoxylate amino transferase, vaculor ATP synthase subunit A, nuclear pore complex protein NUP-1 like isoformX1)

The down regulate proteins were of Photosynthesis (Ribulose bisphosphate carboxylase large chain, Photosystem II CP43 reaction center protein – like), HSPs (17.8 KDa class I heat shock protein-like, 20 KDa chaperonin Heat shock protein 60, Small heat shock protein), calvin cycle/photorespiration(Glyceroldehyde-3- PO4-dehydrogenase A subunit 2, Transketolase ,UTP-

glucose -1phosphate uridylyl transferase), amino acid synthesis (Serine hydroxymethyl transferase 1 Cysteine synthase like, Cysteine synthase, Ferredoxin –dependent glutamate synthase) Defense against ROS (Peroxidase 60, Ascorbate peroxidase 1 cytosolic, Aldehyde dehydrogenase family 2 member B7), others (Nucleic acid binding OB-fold-like protein, Dehydro ascorbate reductase, In 2-1 family protein, Protein transport inhibitor response 1, RAB GTPase homolog E1B)

Completely down regulated proteins include Photosynthesis (Ribulose bisphosphate carboxylase/oxygenase activase chloroplastic like, photosystem II protein V (chloroplast), chlorophyll a-b binding protein 3, chloroplastic, photosystem II 47 kDa protein (chloroplast), chlorophyll a-b binding protein AB80, chloroplastic-like, photosystem I P700 apoprotein A2 (chloroplast), chlorophyll a-b binding protein CP24 10A, chloroplastic) and others (PREDICTED: 20 kDa chaperonin, chloroplastic, lactate/malate dehydrogenase family protein)

4.5.4. Black pepper –T. harzianum (96 hai) – P. capsici (24 hai) (T11)

The up regulated proteins were of photosynthesis (Plastocyanin A, Oxygen evolving enhancer protein 2-1) Defense against ROS (Super oxide dismutase (Cu-Zn), Copper/zinc super oxide dismutase), Others (Fructokinase 3, Glutathione-S-transferase F13, Chaperonin CPN 60-2 mitochondrial precursors).

The down regulated proteins were of photosynthesis (ATP synthase subunit beta, ATP synthase CF1 alpha subunit , ATP synthase CF1 beta subunit, PhotosystemII CP43 reaction center protein like, Chlorophyll a-b binding protein 3, Rubisco large subunit binding protein, Photosystem II 47 kDa protein, Oxygen evolving enhancer protein 3) defense against ROS (Catalase isozyme 2, Catalase enzyme 1, Ascorbate peroxidase) defnse related (Fructose-

bisphosphate aldolase 1, Probable fructose- bisphosphate aldolase, Carbonic anhydrase family protein), others(Glyceraldehyde 3 PO4 dehydrogenase C2 , Peroxisomal glycolate oxidase, 20 KDa chaperonin, Lactate/malate dehydrogenase family protein, Alpha mannoside isoform X2)

Fully down regulated proteins were of Photosynthesis (ribulose bisphosphate carboxylase large chain, rubisCO large subunit-binding protein subunit alpha, chloroplastic-like, LOW QUALITY PROTEIN: ATP synthase gamma chain 1, chloroplastic, ruBisCO large subunit-binding protein subunit alpha, photosystem II protein V (chloroplast), ribulose bisphosphate carboxylase small chain, chloroplastic-like, photosystem I reaction center subunit IV B, chloroplastic-like, chlorophyll a-b binding protein AB80, chloroplastic-like, PREDICTED: ribulose bisphosphate carboxylase small chain 1, chloroplastic)

Photorespiration (Transketolase, alanine:glyoxylate aminotransferase), amino acid synthesis(aspartate aminotransferase, methionine synthase, PREDICTED: cysteine synthase) defense against ROS (peroxidase 12, peroxidase 60), defense related (O-acetylserine (thiol)lyase family protein, probable fructose-bisphosphate aldolase 3, chloroplastic , NADP malic enzyme 4), calvin cycle (glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic, UDP-GLUCOSE PYROPHOSPHORYLASE 1, ferredoxin--NADP reductase, leaf isozyme, chloroplastic-like, sedoheptulose bisphosphatase 1), others (harpin binding protein 1, lactate/malate dehydrogenase family protein ATPase, V1 complex, subunit B protein, heat shock protein 70B.

4. 5.5. Protein Interaction network

STRING analysis for the tripartite interaction was done to understand the functional enrichment and protein – protein interaction network. Medium confidence level 0.400 was kept for the analysis. The analysis revealed that KEGG pathway enrichment for T8 were of Cysteine,

methionine metabolism, Carbon fixation in photosynthetic organisms & Biosynthesis of secondary metabolism whereas the T9 showed Glyoxylate & dicaboxylate metabolism, Carbon fixation, Metabolic pathways, Carbon metabolism & Peroxisome. In T10 condition the KEGG pathways for carbon fixation in photosynthetic organisms, metabolic pathways, Carbon metabolism, Peroxisome & Biosynthesis of secondary metabolites whereas T11 showed only the enrichment of Peroxisome (Fig 25 a - d).

5. Discussion

5.1. Metagenomics

The first objective of this thesis was to assess the microbial community changes at the rhizosphere of black pepper up on inoculation of *T. harzianum,* and also to unravel the significant effects of the introduced *T. harzianum* on the selective recruitment of specific microbes, and their functional assignments in black pepper rhizosphere. The results showed that *T. harzianum* significantly influenced rhizosphere microbial community and there was selective abundance of beneficial bacteria and fungi which resulted in growth promotion in black pepper. The impact at functional level was identified as increased bacterial chemotaxis, virulence, disease and defense and ion metabolism. Increase in the growth parameters, such as fresh and dry weight of shoot, root, leaf area index and height reveals the growth promotion activity of *T. harzianum* in black pepper. This was reported earlier by Anandaraj and Sarma (2003). They demonstrated that the application of *T. harzianum* (MTCC 5179) resulted in enhanced growth in black pepper with increased number of nodes, and thereby the number of cuttings. Sibi, 2013 reported the positive influence of *T. harzianum (*MTCC 5179) on the fresh root and shoot, thereby on the dry weight of root and shoot in black pepper. Treatment with *T. harzianum* (MTCC 5179) alone imparted better growth promotion and disease suppression compared to that of a consortia of *plant* growth-promoting rhizobacteria alone or in combination with *T. harzianum* (MTCC 5179). But the exact change in microflora was not investigated so far. The present study on whole genome metagenomics of rhizosphere soil form control and treament revealed that the population abundance of bacteria, archea and eukaryote were less in treatment than in control in general, it showed selective abundance (more percentage proportion) of

bacteria, *viz*., *Acidobacteriaceae bacterium* and *Candidatus koribacter versatili* - out of top 10 bacterial species*viz*., *Acidobacteriaceae bacterium* KBS 96, *Candidatus koribacter versatilis, Ktedonobacter racemifer, Candidatus solibacter usitatus, Pedosphaera parvula, Sphingomonas* sp., URHD0057, *Gemmatimonadates bacterium, Pyrinomonas methylal pathogens, Chthonomonas calidirosea* and uncultured bacteria from the STAMP analysis on population abundance. The microbial community is reported to be different in bulk and rhizosphere soil (Lundberg *et al.,* 2012; Peiffer *et al.,* 2013). Every plant recruits its own community for its benefit. Though the diversity is less, the abundance of specific organims in the *T. harzianum* treated rhizosphere suggests that selection of microorganisms. These bacteria belong to the phylum Acidobacteriaceae, are the strong colonizer of the rhizosphere having rhizosphere competence (Nunes da Rocha *et al.,* 2013). *Acidobacteriaceae bacterium* is capable of growing on diverse collection of complex organic compounds including xylan, cellulose, methyl cellulose, syringate, pectin and ferulate (Eichorst *et al.,* 2011). *Candidatus* sp. contains abundance of carboxylase active enzymes (CAZyme) family and are involved in the breakdown, utilization and biosynthesis of diverse structural and storage polysaccharides and resistance to fluctuating temperature and nutrient deficient conditions (Rawat *et al.,* 2012).This selective abundance of these beneficial bacteria in the *T. harzianum* treated metagenome might be the major impact for the growth promotion activity by the active breakdown of complex organic compounds by these organisms, which created microclimate for the colonization of microbes in the roots and subsequent interaction with the communities at the rhizosphere. Further, the analysis of black pepper root exudates and action of these bacteria on the root metabolites would give the specific role of these bacteria at the black pepper rhizosphere.

Unlike in control, the metagenome of treatment showed abundant reads of the beneficial fungi, *viz*. *Fusarium oxysporum, Talaromyces*sp. *Pestalotiopsis* sp. and *T. harzianum*; with expected beneficial activities as reported by different authors: Eparvier and Alabouvette (1994) showed that increased population of *F. oxysporum* was better towards the biocontrol and disease suppression activity in Flax; many isolates of *Talaromyces* spp. have been demonstrated to promote plant growth (Naraghi *et al* 2010). Elegant studies also demonstrated that *T. flavus* antagonises plant pathogenic fungi (Naraghi *et al.,* 2013; 2012). In our present study, the higher abundance of *Fusarium* and *Talaromyces* species in the treatment indicates the ecological significance on their population abundance driven by the addition of *T. harzianum* towards the fitness of black pepper.

Rajan *et al.,* (2002) reported the biocontrol and disease suppression activities of *T. harzianum* (MTTC 5179) in black pepper against foot rot disease at field conditions. This isolate was found efficiently proliferating in the soil and remained in the soil for long time, also imparted protection to the root system against *P.capsici*. In the present study, the metagenome analysis was performed after four months of treatment which again proved that *T. harzianum* (MTCC 5179) was able to remain in soil during the experimental time with the abundance of *T. harzianum* specific metagenome reads. Noticing, the proportion the *Rhizophagus irregularis* was higher in the control than in treatment, which indicated the interaction of *T. harzianum* with the native VAM and regulation of its population. The spore germination and hyphal growth of *G. mosseae* was stimulated by *T. harzianum* with the production of volatile compounds (Calvet *et al.,* 1992). In present study, the less abundance of AMF in treated soil might be due to the stimulated growth of AMF by the community helped by *T. harzianum* thereby increased colonization inside the plant rather than their physical presence in the rhizosphere and *vice versa*

in control. Application of *T. harzianum* imparted better growth of black pepper, which was at par with *T. harzianum* in combination with AMF. The treatments with AMF alone and in combination with *Pseudomonas* sp. failed to enhance the growth (Sibi 2013). *Pestalotiopsis fici*, an endophyte of tea produces bioactive metabolites and natural products (Liu 2011), and the analyses of its genome and transcriptome showed that it harbors efficient genes responsible for the synthesis of various secondary metabolites (Wang 2015). Further functional analysis of the reads on *Pestalotiopsis fici*, from the present metagenome data would give significant insight into its role on black pepper through interaction at rhizosphere.

Statistical analysis on functional abundance showed higher abundance for iron acquisition and metabolism in red pigmented *Vibrio*, coupled with heme and hemin uptake and utilization systems in Gram negative bacteria in treatment than control; which evidences the influence of *T. harzianum* in rhizosphere - microbe interaction. Rhizosphere microbiome facilitates the uptake of specific trace elements such as iron. Iron in soil, exists primarily in the insoluble ferric oxide form, which is not available for microbial growth. Based on the scarcity of available irons as well as the toxicity of free iron at elevated concentrations in the environment, bacteria employ a variety of mechanisms to regulate the intracellular iron concentrations (Hider and Kong 2010). On the other hand, plants also play crucial role in increasing the solubility of inorganic iron in the rhizosphere, which may be due to the interaction with microbiome (Walker and Connolly 2008). Rhizobacteria are generally motile, and the motility is either random or chemotactic for interacting with the plants (Broek and Vanderleyden 19995). The bacterial chemotaxis was found as abundant in treatment than in control, suggesting that the probiotic application of *T. harzianum* in black pepper would enable active interaction of the recruited bacterial community in the root system. Anatomical data by scanning electron microscopy from

the treatment and control also provide ample evidences for the aforesaid inference. The root sample from the control showed weak adherence of bacteria on the surface; while the treated roots showed abundant adherence on the surface upon imaging with SEM (Umadevi *et al.*, 2017a).

The abundance of reads on pathogenicity islands, phages and prophages were found to be less in treatment than in control. The less abundance of these functional groups as evidenced from the analysis of taxonomy abundance is highly related to the results of functional analysis (Umadevi *et al.,* 2017 b), suggests the beneficial effect of probiotic application of *T. harzianum*, especially in the context of antibiotic resistance gene pool. In terms of microbial ecology, soil microorganisms interact with and eventually resist diverse chemical arsenal produced either from plant roots or from pathogenic organisms to bring unlikely organism as a community to improve plant fitness. This emphasizes the ecological significance of resistome. The resistome denotes the totality of antibiotic resistance genes of all pathogenic and non-pathogenic microorganisms in the given soil. Functional annotation using Blast2Go yielded contigs those codes for antibiotic resistance genes with its enzyme codes. The *T. harzianum* inoculated soil rhizosphere was enriched with 127 sequence contigs with 86 enzymes codes from the non-pathogenic organisms with the role in antibiotic metabolic pathway and many novel contigs while the soil rhizosphere metagenome without *T. harzianum* recorded 92 sequence contigs with 69 enzymes codes.

The plant associated non-pathogenic endophytic and the rhizospheric bacteria are the major players in the degradation of toxic metabolites present in soil (McGuinness 2009). Heterocyclic aromatic compounds and benzoates (synthetic compounds) are toxic compounds persist for a long time in soil, that leads to ill effects in animals and humans. In the present study, metagenome of treatment recorded higher abundance of reads for the degradation of heterocyclic

aromatic compounds, benzoate transport and its degradation. This information would give the positive impact of *T. harzianum* in the cropping system of black pepper. Further, the functional metagenomics would give more information on bacteria involved in the rhizoremediation through the rhizoecosystem in black pepper.

5.2. Colonization of *T. harzianum* on black pepper roots

In the treatment involving *T. harzianum,* the endophytic colonization of *T. harzianum* accompanied by AMF was luxuriant. Co-cultivation study showed that *T. harzianum* efficiently colonizing the plant roots endophytically. Chacon *et al.,* (2007) demonstrated intercellular ramification of *T. harzianum* hyphae in the root cells of tomato; they found that some cells were colonized intracellularly only after 48 h, and occurrence of yeast like structures after 72 h treatment. Many *T. harzianum* spp. infecting cocoa plants, *viz*., *T. ovalisporum* (Holmes *et al.*, 2004), *T. paucisporum* (Samuels *et al.*, 2006), *T. evansii* (Samuel and Ismaiel 2009) and *T. martial* (Hanada *et al.*, 2008) were identified as endophytes. Using Transmission Electron Microscopy (TEM) (Yedidia *et al.,* 2000), showed direct root penetration of *Trichoderma* spp. in cocoa plant. Papillae - the swollen hyphal tips - were also reported in the interactions of *T. harzianum* with tomato roots during adherence (Chacon *et al.,* 2007). We observed the appearance of papillae on root surfaces at 24 h of inoculation, suggesting its quick interaction with the black pepper root system. The phenomenon of biocontrol and growth promotion does not occur in all *Trichoderma*-host interactions; for instance (De souza *et al.,* 2008), found that endophytic colonization of cocoa by *T. stromaticum* was unable to induce plant growth and resistance to *Magnoporthe perniciosa* . However, some species of *Trichoderma*, *viz*., *viride, harzianum* and *pseudokoningii* were found promoting growth in cucumber, corn, petunia and pea (Windham *et al.*, 1986; Harman 2004; Yedidia *et al.*, 1999). In the present study, the growth

promotion was also found to be enhanced by the co-colonization as evidenced from increase in growth parameters (fresh root, fresh shoot, dry root, dry shoot, Leaf Area Index (LAI) and height of the plant) in *T. harzianum* treated plants when compared to control which was not showing any co-colonization.

The maturation zone of the black pepper roots showed moniliod hyphae along with structurally differentiated AMF mycelium with prominent septation (Refer to Fig 16 b, c). Moniliod hyphae of dark-septate fungus in the aquatic angiosperm, *Eorhiza arnol dii* could produce diverse moniliod assemblages (Klymiuk *et al.,* 2013). Microsclerotia were also observed inside the cells and AMF colonization was found as Arum type (Alexopolous *et al.,* 2004). The region of maturation showed vesicles with different shapes. *T. harzianum* mycelium and conidia along with the AMF mycelium and vesicles were noticed- suggesting co-colonization inside the roots of black pepper. The interaction between AMF and *Trichoderma* has been elucidated in many studies: Filion *et al.,* (1999) reported that *Glomus intraradices* stimulated the conidial germination of *T. harzianum*; and Datnoff *et al.,* (1995) observed a synergistic interaction between them in tomato. Co-inoculation of *T. harzianum* and *T. aureoviride* decreased the time to vegetative sporulation in axenic cultures of these mycorhizal species (Calvet *et al.,* 1992). Synergistic effect between *G. intraradices* and *T. aureoviridae* in enhancing the growth in citrus in organic substrate has been reported, which was higher than the individual effect of *G. intraradices* (Camprubi *et al.,* 1995). Since the experiment was set up in field soil, the observation that the roots inoculated with *Trichoderma* had abundant VAM colonization as indicated by the presence of both vesicles and arbuscules indicates the native mycorhiza colonizing pepper roots without any hindrance by inoculated *Trichoderma harzianum* MTCC 1579 suggesting the inoculated *Trichoderma* has facilitated mycorhizal colonization, where as in

the control soil though there was AMF colonization it was sparse. The native beneficial microbes like mycorhiza had equal opportunity of colonizing black pepper roots; the fact that in *Trichoderma harzianum* inoculated roots the presence of more AMF suggests its active role in helping mycorhizal colonization.

As no choice experiment with insect pests, the report by De jaeger *et al.,* (2010) indicated mycoparasitism in one to one interaction. *Trichoderma harzianum* being a saprophyte and opportunistic antagonist (Harman *et al.,* 2004) in the absence of other nutrient source for its survival perhaps would have colonized AMF. In an experiment Sibi, (2013) where selected compatible (*invitro*) consortia of PGPR (*Pseudomonas mendocina, Bacillus pumilus, Serratia macescens* and *Rhizbium sp*) inoculated on black pepper rhizosphere, the population of *S. marcescens* was declined to zero when compared to the population in *invitro* experiments in which the PGPR consortia was compatible suggesting the role of rhizosphere in selecting and maintaining the organisms.

Comparing two sample preparation methods employed for the SEM, the methanol fixation was found better in terms of good cell structure, which aided visual observation of bacteria and fungus; whereas glutaraldehyde fixation distorted the surface structures. The attachment of bacterial/fungal population on the surface of *T. harzianum* treated roots showed the rhizosphere competence of soil organisms with *T. harzianum*. Many bacterial cells were found adhering to the surface, which was absent in the control roots. With *Arabiodpsis* and barley, Talbot and White (2013) also found that methanol-based method was superior to other fixation methods of samples for analysis by SEM.

Reports on growth promotion effect of VAM on black pepper shows that *G. fasciculatum* incorporation as infective propagules (cultured on Rhodes grass) increased the rooting of black

pepper at nursery condition (Anandaraj and Sarma 1994).The authors showed more than 80% colonization of *G.fasciculatum* in black pepper roots than control. Detailed study on the effect of biocontrol agent on hardening of tissue cultured black pepper with VAM demonstrated that the treatments; wherein *G. fasciculatum* and *T. harzianum* were inoculated showed higher root and shoot mass compared to control (Sibi 2013). The growth promotion effect on plantlets inoculated with species of *Pseudomonas, Rhizobium* and *Trichoderma* was checked in the presence or absence of VAM, and found that the overall growth with VAM was higher than that without VAM (Sibi 2013). Compared to the effect of *Trichoderma* in black pepper, the treatments inoculated with *Pseudomonas* or *Rhizobium* alone with or without VAM recorded low profile on growth promotion, which further indicates the synergistic effect of this fungus. The comparison of *T. harzianum* treatments with or without VAM recorded higher growth promotion in *T. harzianum* (alone) without VAM suggests the principal action of *T.harzianum* in helping the native VAM fungi present in the soil to colonize the black pepper plants. This was evident from our results with microscopy; wherein, we showed the endophytic colonization (100% colonization frequency) of AMF along with *T. harzianum* mycelium in *T. harzianum* inoculated soil, compared to control (Refer to Figure 17).

Metagenome analysis showed that the AMF *(Rhizophagus irregularis)* was higher in control, but less in *T. harzianum* inoculated soil, microscopic observation on the internal colonization of AMF between these treatments shows increased endophytic colonization of AMF upon *T. harzianum* inoculation. Although the *Rhizophagus irrregularis* was abundant in rhizosphere soil of control it had not colonized the tissue to get the benefit of symbiosis from the plant and also the high abundance of this AMF species (*Rhizophagus irregularis)* was ineffective in increasing plant growth in control. The reason for non-colonization in control is not clear

where as when *T. harzianum* inoculated roots AMF colonization was also facilitated as *Trichoderma harzianum* was known as helper organism for VAM with increase in plant growth (Calvet *et al.,* 1992).This study demonstrated the localization and endophytic colonization of *T. harzianum* MTCC 5179 in black pepper. Enhanced AMF root colonization by the *Trichoderma* inoculation in black pepper indicates that *T. harzianum* acts as helper organism in the root ecosystem of black pepper for colonizing AMF on the plant. Moreover, the native microbes that are selectively recruited by black pepper under the *T. harzianum* influenced rhizosphere would have helped to mobilize nutrients and enhanced the growth. Further, detailed studies on *T. harzianum,* AMF and native microflora with the host in a multipartite interaction would help in developing targeted biocontrol strategy to overcome soil borne pathogens.

5.3. Black pepper-*T. harzianum* Interaction

Colonization of plant roots by the beneficial microbes brings unique sensitized state to the plant. This state can be induced by broad range of pathogen infections. The colonization initiates mobilization of defense which is often associated with enhanced immunity and stress tolerance. Earlier studies have shown that though the *T. harzianum* interaction is confined to roots, the leaves also showed resistance to plant pathogens (Yedidia *et al.,* 1999; 2000). Studies till date focused mainly on metabolic changes occurred on roots (Chacon *et al.,* 2007) and at the late stage (after 7 days) (Shoresh & Harman 2008) of *Trichoderma* colonization in tomato and maize plants respectively. Information on molecular changes on leaf at early stages of interaction of *Trichoderma* colonization at root is meager. One particular study (Seggera *et al.,* 2007) demonstrates the metabolic changes in leaf during the *Trichodema* interaction at roots of cucumber, but only during 24 hai. In the present bipartite study (Black pepper- *Trichoderma harzianum*) we have profiled the proteome under expression during during 24, 48, 72 & 96 hai

for the first time. We found that there was a well-orchestrated maintenance of many metabolic pathways and defense proteins during the infection at various hours after inoculation (hai).

5.3.1. T24 Black pepper- T. harzianum (24hai)

The shotgun proteomics of leaves at 24 hai of *T. harzianum* inoculation in roots of black pepper showed 43 proteins with complete down regulation, six proteins with up regulation and 19 with down regulation. Structural maintenance of chromosomal protein 2 was up-regulated. This protein has the role in chromosomal dynamics. The Rpp 4 C4, the candidate R gene was also up regulated. This gene was proved to be involved in Rpp-4 mediated resistance (Meyer *et al.,* 2009). There were 3 Induced proteins identified. LRR-receptor–like serine/threonine-protein kinase RLK. This group of serine/threonine receptor kinase was proven to be important in symbiosis (Krusell *et al.,* 2002) Transport protein SEC 16B homolg isoform X1 was also induced. SEC 16 is the component of COPII which is required for transport of membrane proteins from ER to Golgi such as ST-GTP, AtERD2-GFP, secretory and vacuole-targeted forms of GFP (Andreeva *et al.,* 2000).

GLO 1 interacts with TGA transcription factors. GLO proteins are also proved to be involved in non-host defense response in *Arabidopsis* to *P. syringae* and for Pto/AvrPto mediated defense response (Chern *et al.,* 2013).

The proteins for chromosomal dynamics, RLK for symbiosis and SEC 16 for signal transduction suggests that these proteins has the major role for favoring the colonization of *T. harzianum* in black pepper and also the systemic response in leaf.

The metabolic shift in source-sink is the major plant response. We found shut down of many primary metabolism related proteins in the defense responding leaves (Refer to Table 3).

The photosynthesis related proteins were strongly reduced. Many proteins falling into these functional groups were down regulated at 24 hai. Irrespective of the mechanism, the down regulation of photosynthesis is towards reducing the energy expenditure by the plant during the colonization. We can expect that the photosynthesis related proteins to be down-regulated in a state in which the plant is switching to non-photosynthetic-metabolism. Proteomic studies on cucumber – *T. asperellum* (Segarra *et al.*, 2007) showed the down regulation of rubisco activase at 24 hai. The down regulation of peroxisomal glycolate oxidase, the enzyme for photorespiration also confirms the reduction in photosynthesis in present study. An inhibition of photosynthesis was found in rice with low GOX activities (Xu *et al.*, 2009). This result also establishes that the photo respiration also gets modulated by *T. harzianum* interaction with plants. Calvin cycle related proteins were also down during the interaction.

Amino acid metabolism was down regulated; the proteins were of ethylene biosynthetic pathway proteins. Ethylene is stress induced hormone and also has role in senescence. The down regulation of the proteins in the present study strongly suggest that the ethylene synthesis was inhibited in black pepper –*T. harzianum* interaction and thereby the senescence of plant during colonization. It could be the otherway also by reducing the precursor ethylene root elongation would have occured resulting in increased biomass. In contrast to this in Cucumber –*T asperellum* (Segarra *et al.*, 2007) interaction ethylene synthesis was noticed with the up regulation of ACC oxidase.

The ROS can be stated as threat to cells or as a secondary messenger involved in the stress-response signal transduction pathway (Dat *et al.*, 2000). If complete reduction does not occur, the result may be the oxidative stress leading to the oxidation of lipids, proteins, and DNA thereby the cell damage. The damaging level of ROS is reduced by enzymatic and non-

enzymatic components which are defense proteins against ROS. Superoxide dismutases (SOD, ascorbate peroxidases, and catalases (CAT) are the enzymatic scavengers of superoxide (O2–) and hydrogen peroxide (H2O2). Glutathione (GSH) and ascorbic acid (ASA) are the non-enzymatic antioxidants which maintain cellular redox homeostasis (Noctor and Foyer 1998). We found these proteins (SOD, CAT) including glyceraldehyde 3-PO4 dehydrogenase and 2-cys peroxiredoxin BAS 1 are also down regulated during 24 hai. This suggests that the *T. harzianum* is maintaining the redox state in such a way that during its interaction/colonization the ROS is not produced to the damaging level in the plant. Segarra *et al.,* (2007) also found that the peroxiredoxin, 2-cys peroxiredoxin, Glyceraldehyde-3-phosphate dehydrogenase, and cytosolic ascorbate peroxidase were down-regulated at 24hai.

Heat shock proteins were down regulated in leaf at 24hai of *Trichoderma* with black pepper roots. Some family of HSPs was found to be expressed only when there are environmental stresses. In plants role of HSP redox in defense is little known only. The good maintenance of oxidative and temperature state by the *Trichoderma* during its interaction might be the reason for keeping the HSP shut down in our study also. On contrary to this Segarra *et al.,* (2007), noticed up regulation of HSP70 in Cucumber-*T. harzianum* system at 24hai.

The Cys pool is important in maintenance of the cellular redox state. Proteome analysis of Arabidopsis leaf peroxisomes revealed that the presence of the cytosolic OAS-A1 isoform within this compartment was involved primarily in oxidative metabolic reactions (Lopez Martin *et al.,* 2008). Cys pool may serve as an independent node for redox signaling and control in plants. Each compartmental Cys pool and their biosynthesis should be crucial under transient stress situation in the plant as a consequence of environmental changes. In the present study we found down regulation of proteins involved in cysteine pool. This suggests that the *T. harzianum*

colonization did not make the plant to produce any stress unlike the pathogenic organism interaction in plant. Hence the redox state is maintained to avoid the stress related response.

The R gene RPP13 – like protein was the first *Arabidopsis* R gene to act via a novel signaling pathway independent of salicylic acid-mediated response (Bittener-Eddy *et al.,* 2001) and the major R gene against fungus. This disease resistant protein was found to be down regulated during interaction of *T. harzianum* in black pepper. This suggests that the *T. harzianum* colonization suppressed the plant immunity and hence the plants did not show any defense resistance unlike in pathogen infection.

5.3.2. T48 Black pepper -T. harzianum (48hai)

Information on metabolic changes on leaf at 48hai of *T. harzianum* colonization at root is nil. Our study provides insight into the proteome change in leaves up on root colonization of *T. harzianum*. Our anatomical studies showed that at 48 hai the *T. harzianum* establishes the intracellular interaction in black pepper roots. At this particular time point, the proteomic analysis was done in leaves. The dynamics of protein expression is discussed below. Of the proteins that were differentially expressed at 48hai 11 were down regulated. Apart from heat shock protein, glyceraldehyde 3 phosphate and elongation factor 1 alpha some new cellular proteins were also found in this 48hai. Collagen alpha-6 (VI) chain like protein, the cell binding protein was down regulated. Collagen VI has been reported to connective tissue innate immunity (Abdillahi 2016). Rap guanine nucleotide exchange factor 2 was down regulated. This protein is involved in Rap & Ras family of GTPase. It serves as a link between cell surface receptors and GTPase in intracellular signaling cascades (Ohtsuka *et al.,* 1999). Platelet derived growth factor receptor beta –like protein which is involved in cell proliferation & migration was also down regulated. Histone H2 A-beta was down regulated at 48 hai. Priming treatment makes covalent

modifications of histones which reduces the ionic interaction with DNA and thus provide open chromatin for the binding of other effector proteins which leads to activation of particular gene (Conrath 2011). *T. harzianum* interaction did not alter proteins of any cellular activity (cell proliferation & migration, histones) that would result in defense response rather it maintained the normal condition by down regulating these important proteins at this hour of interaction in order to make successful colonization inside the plant roots.

5.3.3. T72 Black pepper - T. harzianum (72hai)

This time point of interaction did not have much up regulated proteins; but the splicing factor U2 large subunit up regulation was observed. Alternative splicing is to reshape the transcriptome towards biotic and abiotic stresses. This pre-mRNA splicing leads to impaired plant defense and altered developmental progammes (Staiger *et al.,* 2013)

E3 Ubiquitin ligase HOS1 like which is the mediator of cell death and defense (Duplan and Rivas, 2014) was down regulated suggesting the avoidance of cell death and defense in the plant leaf by the *T. harzianum* during the interaction. Apart from complete down regulation of proteins falling into different functional groups viz., photosynthesis, photorespiration, Calvin cycle, cysteine pool, ethylene synthesis, proteins against ROS and heat shock proteins as observed in 24 hai, some defense related proteins like Peroxidase, Catalase, Super oxide dismutase were also identified at 72hai

UDP-glucose pyro phosphorylase1 protein, the cell death regulator (Chivasa *et al*., 2013) was also found to be down regulated at 72 hai. Germinlike protein subfamily 1 member 17 was down regulated. The germin like protein gene family are involved in plant defense (Manosalva *et al.,* 2009). Subtilisin –like protease SBT 3.8 the protein for the pathogen recognition and immune

priming against pathogen (Figueirdo *et al.,* 2014) was down regulated, since *T. harzianum* is not a pathogen this protein was not activated perhaps.

5.3.4. T 96 Black pepper - T. harzianum (96 hai)

Of the expressed proteins at 96 hai the proteins belongs to defense RAB GTPase homolog E 18 and the ROS related protein ascorbate peroxidase1 cytosolic are the proteins that are new down regulated proteins compared to other early (hai) of interaction. Ascorbate peroxidase is the key antioxidant during SAR following pathogen infection in plants (Gara *et al.,* 1996). RAB GTPase functions in hypersensitive cell death during immunity – associated programmed cell death (Kwon *et al.,* 2009)

Taken together, the expression pattern of proteins at all time of sampling shows that there is no play of SA as this phyto hormone is needed for the synthesis of PR proteins. In all hai the PR proteins are down regulated to greater degree so SA involvement can be ruled out. The other phyto hormone ethylene also not having the role as the important proteins (methionine synthase, alanine: glyoxylate aminotransferase) needed for ethylene biosynthesis is down regulated. The up regulation and new protein induced group had PTI related receptors and R genes which sends the clue that the pattern recognition step itself the *Tirchoderma* might be shifting the plant activities favorable towards its own establishment by suppressing the plant immunity. Some important down regulated proteins were found to be the marker proteins for the suppression of immunity in black pepper by *T. harzianum*. At 24 hai the SOD, CAT, Glyceroldehde -3 Po4; at 48 hai Rap guanine nucleotide exchange factor 2, Histone 2A; at 72 hai, the germin like protein and subtilisin marker proteins for plant defense; at 96 hai, the SAR marker ascorbate peroxidase and RAB GTPase which is immunity associated programmed cell

death. Thus, *T. harzianum* inoculation on the roots alters the host physiology so much at the farther end of leaves.

Many findings from transcriptomics and metabolomicsapproach (Moran *et al.,* 2012; Alfano *et al.,* 2007; Brotman *et al* ., 2013, Segarra *et al.,* 2007) suggest that *T. harzianum,* like the plant beneficial fungus *Piriformospora indica,* has to cope with plant defense responses during the initial stages of the interaction by broad-spectrum suppression of innate immunity, to allow colonization of *Arabidopsis* roots (Jacobs *et al.,* 2011). The Cytochrome P450 monooxygenases (*CYP712A2*, *CYP712A1*, *CYP93D1* and *CYP76G1*), the genes mediate synthesis and metabolism of many physiologically important primary and secondary compounds that are related to plant defense against a range of pathogenic microbes and insects were found to be down regulated in *Trichoderma asperelloides* T203 –*Arabidopsis* interaction at root). The WRKY group III transcription factors (WRKY41, WRKY53, and WRKY55) are up-regulated at first 24 hours of *Trichoderma* root colonization and went down along with the expression of other defense related transcripts. These results also suggested that *Trichoderma* can temporarily repress local defense plant immune response. The induced expressions of the transcription factor ANAC081, which has been shown to be a repressor of the expression of genes that encode pathogenesis-related proteins also showed the suppression of plant immunity during *Trichoderma* colonization (Brotman *et al.,* 2013). The microarray analysis by Moran-Diez *et al.,* (2012) showed global gene expression changes in the leaf of *Arabidopsis* 24 hours after roots inoculation with *Trichoderma harzianum* T34. The JA and SA mediated defense –related genes were found to be down regulated T34. Alfano *et al.,* (2007) found that except PR-5 other marker genes of SAR pathway were not upregulated in root in T. hamatum382 –Tomato interaction

which was similar to the report by Segarra *et al., (*2007) in which the concentration of SA and JA in cucumber roots and cotyledons were not altered significantly by *T. asperellum* T-34.

Our work presents the leaf proteomics on 48, 72 & 96 hai as first time in Plant - *T. harzianum* interaction. The results presented in this thesis add information to how the fungi could maintain the plant metabolism and suppress the plant immunity for its own establishment.

5.4. Black pepper – *P. capsici* Interaction

The host proteins involved in susceptible or resistant genotype of black pepper – *Phytophthora* interaction is rarely attempted. Though recent studies on this interaction using transcriptomics and proteomics (Johnson *et al.,* 2012; Mahadevan *et al.,* 2016; Anandaraj and Umadevi 2016) is attempted, the pattern of interaction studied were only on the leaf inoculation (detached/ In planta) of *Phytophthora*. There are no reports till now on proteins/genes expressed in leaves upon root inoculation of *Phytophthora*. We attempted this study to bring out the proteins involved in systemic response in black pepper leaves at 12 and 24 hai.

Earlier report on *Phytophthora* –woody plant interaction with the aim to understand the status of physiological parameters on leaves upon *Phytophthora* infection in roots is included in this discussion below to show that this pathogen is able to induce systemic response in above ground portion of the plants.

Covalent modifications of histone which reduces the ionic interaction with DNA and thus provide open chromatin for the binding of other effector proteins which leads to activation of particular gene (Conrath 2011). The up regulation of histones suggests there would be transient expression of many genes which are of systemic in nature at leaf during root infection of the pathogen. HSP 70 B was found to get up regulated in *Phytophthora* stress in black pepper. The

up regulation of certain photosynthesis related proteins were of only minimum fold change (1-2 fold only). The infection of *P. capsici* at root of black pepper plants showed the down regulation of various metabolic pathways proteins. Less *et al.,* 2011 demonstrated the association of primary metabolism and defense in *Arabidopsis* stressed with virulent pathogen of *P. infestans*. The expression analysis showed that the up regulated transcripts were involved in energy production (glycolysis, TCA, ATP biosynthesis, biosynthesis of amino acids and amino acids associated with photorespiration). Down regulated transcripts were associated with assimilatory processes (photo synthesis, starch, lipid metabolism, C1 metabolism etc).

The suppression of genes involved in photosynthesis, carbon fixation, and secondary metabolites (involved in the biosynthesis of phytohormones) during a compatible interaction have earlier been reported in *P. infestans*– potato pathosystem (Restrepo *et al.,* 2005). The Co_2 assimilation rate and stomatal conductance of *P. citricola*- root infected beech seedlings were significantly reduced in leaf compared with the healthy control seedlings only after 2 days of infection. Both parameters further decreased to almost zero at 7dpi. Between 3 and 4dpi, the decrease in photosynthetic quantum yield of photosystem II was rather observed as weak and was less than 15% compared to the control, but by 6 and 7dpi it strongly decreased to about 50% (Fleischman 2005). The concentration of chlorophyll was found to get reduced in leaves of plants infected by *P. citricola* and *P. cambivora*, indicating that photosynthesis is getting affected in leaves during infection at roots of beech plants (Fleischman 2004). The protein profile of black pepper- *P. capsici* showed much photosynthetic related protein down regulated suggesting that the photosynthesis is altered upon infection. Anandaraj (2000) suggested that there are no visible aerial symptoms till a substantial feeder root system is damaged. The altered

physiology certainly adds to the debilitating nature of the disease. The down regulation of photosynthetic activity is perhaps the reason for reduced vigor of infected plants.

One of the earliest plant responses to a pathogen is the induction of oxidative stress and ROS, that have been associated with HR induction (Richberg *et al.,* 1998) and SAR (Bolwell and Daudi, 2009). After pathogen infection, transcript abundance of genes associated with the production of ROS was decreased in highly compatible *P. infestans*–tomato (susceptible) interaction (Cai *et al.,* 2013) while it showed contrasting results in *P. infestans* (US17)– tomato interaction involving the partially resistant IL6-2, where ROS were highly expressed as early as 36 hai. This is in corroboration with our results. In the present study we used the susceptible genotype for *Phytophthora* - the variety Sreekara, hence the ROS scavenging proteins were down regulated. Our proteomics study on resistant genotype IISR Shakthi showed up regulation of ROS related proteins (Anandaraj and Umadevi 2016). The concentration of a-tocopherol and xanthophyll cycle pigments were increased in plants infected by *P. citricola* and *P. cambivora*, indicated that several reactive oxygen species might have been formed in leaves during infection at roots of beech plants (Fleischman 2004).

Germin like protein subfamily 1 member 17 was down regulated at 12 hai. The down regulation of germin-like proteins which gives broad-spectrum disease resistance (Manosalva *et al.,* 2009) clearly suggests that the pathogen infection at root did not impart resistance at leaves.

Apart from major metabolic process (Photosynthesis, Calvin cycle, ROS) related proteins UDP-glucose pyro phosphorylase1 protein, the cell death regulator (Chivasa *et al.,* 2013) was also found to get down regulated at 12 hai. The methionine synthase protein involved in ethylene biosynthesis was also down regulated. This suggests that the senescence related processes was

not activated in leaf at 12hai. ACC-oxidase 1 (ACO1) was transiently expressed in leaves of *F. sylvatica* seedlings infected with *P. citricola* in the roots. The relative expression pattern showed the highest level of expression 2 days after infection (Portz 2010).

The transport inhibitor protein 1 (TIR1) gene encodes an F-box protein containing 16 degenerate leucine-rich repeats (LRRs) (Ruegger *et al.,* 1998). Gray, 1999 also showed that over expression of TIR1 in transgenic plants results in enhanced auxin response including an increase in auxin dependent gene expression. In our study we found the down regulation of this protein in both 12 and 24 hai. This results shows that there is no auxin dependent defense regulation in black pepper system.

When comparing the protein profiles of 12hai, at 24 hai the SAR seems to get activated in leaves to certain extent. The number of heat shock proteins expression was increased. The cell death regulator, certain ROS related defense proteins were found to have up regulations which were of under down regulation at 12hai.

A new protein Probable type lectin domain containing receptor kinase S.5 was found expressed suggesting that this protein might be involved in signaling for SAR. In *Arabidopsis*, many L-type lectin receptor kinases (LecRKs) have been identified as putative immune receptors. Compared with control plants suppression of CaLecRK-S.5 expression significantly enhanced the susceptibility to *Phytophthora capsici* (Woo *et al.,* 2016). Systemic acquired resistance was also abolished in CaLecRK-S.5-silenced plants indicated that CaLecRK-S.5 positively regulates plant immunity at the transcriptional level. In the present study, the peptides corresponding to this protein are expressed at 24 hai at leaf indicating its involvement in SAR at this particular hour. But the effect on SAR either positive or negative is needed to be investigated

further. Since the expression is induced in leaf at this hour after root infection of pathogen place this protein as important from this study.

The down regulated proteins in 24 hai also included the peroxidase, catalase proteins which are important PR proteins during pathogen infection. These proteins were found to be at fully down regulated stage at 12hai. The expression of these proteins showed improved expression from full shut down to comparable down regulation at 24 hai suggesting the reaction of plant against the pathogen at 24 hai. But in general the down regulation of these proteins suggests that there is not much resistance developed in leaf during root infection of *Phytophthora* since the plant under study was the susceptible type. Apart from proteins involved in assimilatory processes, proteins associated with leucine were found to be down regulated at this hai. Less *et al.*, 2011 also demonstrated the down regulation of transcripts on leucine synthesis during Arabidopsis –*Phytophthora* interaction. The innate defense is also compromised when *P. capsici* is actively infecting the roots in the aerial part and makes the whole plant susceptible. When the weather is favorable, the total plantation is wiped out in few days.

Taken together the SAR reaction had initiated at 24 hai than 12 hai. The earliest reaction in plant against pathogen viz., activation of ROS scavengers and the strong indicators of SAR, the PR proteins were not present in both the hour to a greater extent could be attributed to the susceptible genotype (Sreekara) of the present study. Our data on protein dynamics in the resistant genotype IISR Shakthi recorded up regulation of ROS scavengers and PR proteins at 12 and 24hai (Anandaraj and Umadevi 2016).

5.5. Black pepper – *T. harzianum* –*P. capsici* Interaction

The systemically altered "defense readiness" was demonstrated by Waller *et al.*, (2005) in barley plants primed with *Piriformospora indica*. They found the reduction in powdery mildew infection in leaf in *P. indica*- infested plants. The systemic elevated antioxidants in leaves from *P. indica*-infested plants. The glutathione pool (GSH and oxidized glutathione) was found to be enhanced in leaf. Glutathione reductase activity was also enhanced in leaves during the first 3 weeks of *P. indica* infestation, corroborated the systemic induction of antioxidant capacity by *P. indica*.

Systemic induced resistance proteins whose expression dynamics would serve as a valuable marker in analyzing the induction of resistance. The activation of defense proteins is induced only upon infection of the pathogen. Our study is an attempt to profile the systemic defense proteins induced in leaf of *T. harzianum* treated plants. The plants were treated with of *T. harzianum* at root for 72 hai and 96 hai and the proteins of systemic response related were profiled in leaf after inoculation of the plant at root with *Phytophthora capsici* at 12 hai and 24 hai.

In general in the tripartite interaction (Black pepper- *T. harzianum* – *P. capsici*) the proteins that are up regulated is very high in number at the time of examination (T72P12, T72P24 and T96P12) when compared to two way interaction (Black pepper- *T. harzianum*; Black pepper- *P. capsici*) indicating the systemic response mediated by the *T. harzianum* in upper plant part in the black pepper.

We observed a complex response from proteins of photosynthesis related pathway with the distribution of the proteins in up, down and fully down regulated groups. The T72P24 and

T96P12 recorded more number of up regulated proteins than T7212 and T96P24. Less *et al.*, 2011 reported using transcriptomics the down regulation of photosynthesis related genes in many biotic stresses in *Arabidopsis* including their infection by virulent pathogen *P. infestans*. We also observed that more number of down regulated proteins in two way interaction (plant-pathogen; black pepper - *Phytophthora*). But in contrary to that in tripartite interaction the increase in number of up regulated proteins suggests that the protein of photosynthesis which is of assimilatory process is also getting induced. These proteins might be the *T. harzianum* induced systemic response proteins against *Phytophthora capsici.* That means the *T. harzianum* inoculated plants reverses the activities of *P.capsici* and reponses normal of physiology. That could be the reason that T. harzianum inoculated plants are healthy despite pathogen inoculation (Sibi 2013).

The photo respiration and carbohydrate metabolism, amino acid synthesis related genes were also found to show the dynamics in regulation. The plant defense is the complex process which requires abundant supply of energy from primary metabolic processes (Bolton 2009). The defense signaling mediated by carbohydrate, amino acid metabolism and photorespiration used to get negatively regulated when they are no longer needed.

The proteomics of tripartite interaction study revealed many T-ISR proteins from ROS scavenging proteins, defense related protein groups, HSHs and other important protein groups as marker proteins of tripartite interaction. The details are elaborated below.

5.5.1. ROS scavenging proteins as T-ISR in Black pepper

The ROS scavenger proteins as the markers for T-ISR in black pepper were identified with their differential expression (Refer Table 10). Mono dehydro ascorbate reductase is one of

the key anti- oxidant enzyme responsible for scavenging ROS. The activity has been shown to be up regulated under several stresses. This protein showed 23.45 fold expression in T72P12 suggesting the early involvement of ISR in black pepper. The expression of this protein was absent in all other treatments of tripartite interaction and also in both two way interaction (Black pepper-*T. harzianum* and Black pepper –*P. capsici*).

Superoxide dismutase (MnSOD, Cu/ZnSOD and FeSOD) belongs to the group of metallo enzymes and functions as an important enzyme in the first line of antioxidant defense. (Tuna *et al.,* 2008). Increased SOD activity often appears to enhance plant tolerance to oxidative stress. We observed the up regulation of SOD as ISR protein in all tripartite interaction which was found to be down regulated in black pepper infected only with pathogen suggesting that SOD is playing important role in defense which is being identified as T-ISR protein.

Tobacco class I and II catalases are differentially expressed during elicitor-induced hypersensitive cell death and localized acquired resistance (Dorey *et al.,* 1998). In Arabidopsis cat2, a knockout mutant for the major leaf catalase has elevated H2O2 (Han *et al.,* 2013). In maize 3 biochemically distinct catalase enzymes 1, 2 and 3 with temporal and spatial specificity in expression had been demonstrated (Scandalios *et al.,* 1984) and they exhibited variably in its expression to different environmental stresses. In the present study also the differential expression of catalase isozymes was recorded. This important antioxidant protein was down regulated in B.P + *Phytophthora* (Two way) and absent in B.P + *T. harzianum* (two way) interaction while in tripartite it showed up regulation suggesting this as T-ISR protein in black pepper defense system. The temporal expression also was observed as T72P12 with up regulation of Catalase 2 isozyme, T72P24 with down regulation of catalse2, up regulation of catalase 1 and 3 isozymes, T96P12 with only up regulation of catalase 1 and 3.

In pathogen-infected plants, stress-inducible GSTs play key roles in the suppression of necrosis caused by pathogen attack by detoxifying organic hydro peroxides of fatty acids produced from peroxidation of membranes (Gullner & Komives 2001; Dixon *et al.*, 2002). This is in accordance with the results obtained from our study. The up regulation of GST was noticed only in BP +*Phytophthora* and the tripartite interaction which has pathogen infection component. But this enzyme expression was absent in B.P + *T. harzianum*. This proves that GST are induced by the pathogen infection not by the beneficial organism *T. harzianum*. The fold change expression was higher in tripartite than in B.P+ *Phytophthora* interaction (Two way).

Organic peroxides occur in response to pathogen attack (Mauch and Dudler 1993) and detoxification of microbial toxins during hypersensitive response (HR). If not reduced, peroxides will be converted to cytotoxic aldehyde derivatives, which can result in cell death (Dean *et al.*, 2005). Dehydrogenase family proteins are stress related proteins, important for detoxifying the aldehydes. The present study recorded the expression of dehydrogenase family protein as present and up regulated only in tripartite interaction suggesting this as T-ISR protein. The expression of this protein seems to be early hours of pathogen infection (12hai) as it showed its up regulation only in T72P12 and T92P12 while it was found to be absent in late hai (24hai) viz.,T72P24 and T96P24.

Plant cells express many peroxidase isozymes with different organs/ tissues and different subsets of isozymes are translated for different environmental stresses. We observed peroxidase 5, 12, 16, 60 and peroxides like precursor protein as up regulated only in tripartite interaction which makes this enzyme group to fall into T-ISP proteins in black pepper. The expression dynamics is different for each isozyme. The T72P12 had up regulation of all isoforms while

T72P24 was with isoform 5 & 12, T96p12 was with 12, 16 and 60 and T96P24 was absent in having peroxidase expression.

Reactive intermediate Deaminase A chloroplastic (RidA) is responsible for the clearance of reactive species (Lambrecht *et al.*, 2013). In 2014, RidA proteins were identified in *Arabidopsis thaliana* and maize. *Arabidopsis thaliana* RidA (AtRidA) is targeted to chloroplasts. By converting the reactive enamine/imines to harmless 2-oxoacids, RidA preempts damage to branched chain amino transferase and makes the isoleucine biosynthesis to proceed (Niehaus *et al.*, 2014). The present study observed RidA as T-ISR protein in black pepper against *Phytophthora* as it showed very early expression (12 fold) in T72P12.

2-Cys peroxiredoxin BAS1 has demonstrated functions in antioxidative defense system of the chloroplast in plants (Baier & Dietz 1997). This protein was found to get upregulation only on 24 hai during BP - *Phytophthora* (Two way interaction), but due to the effect of *T. harzianum* this protein was found to be expressed at 12 hai itself (T72P12).

Ascorbate peroxidase exists as isozymes with important role in metabolism of H2O2 in plants. This enzyme activity generally increases along with other antioxidant enzymes viz., catalase and SOD in response to various environmental stresses (Shigeoka *et al.*, 2002). In the present study, we observed 3 isoforms of ascorbate peroxidase 1, 2 and 6. The expression of this protein was down regulated in the two way interactions while it was up regulated in tripartite interaction suggests that this protein is also an important T-ISR protein in black pepper.

5.5.2. Defense related proteins as T-ISR in black pepper

We also grouped the other important group of protein in this interaction study. The defense related proteins of T-ISR are discussed here (Refer Table 10). The cytosolic isocitrate

dehydrogenase (cICDH) is the most abundant isoform in leaves, responsible for up to 90% of the NADP+-dependent activity in leaf extracts (Kruse *et al.,* 1998; Hodges 2002). Mhamdi *et al.,* 2010 demonstrated that this protein also plays a role in redox signaling linked to pathogen responses in Arabidopsis. In our study the up regulation of this protein was observed in two way (B.P + *Phytophthora)* and also in tripartite interaction. In two way interaction this protein got expressed only at 24 hai while the tripartite recorded this protein in up regulation at 12 hai itself. The fold change expression was decreasing with increase in the *Trichodema* priming and also the time of pathogen infection. This suggests that this protein is one of the early inducible proteins in black pepper upon *Phytophthora* infection.

Malic enzyme is a multifaceted protein. It is demonstrated in defense-related deposition of lignin by providing NADPH for the two NADPH-dependent reductive steps in monolignol biosynthesis. On the other hand, it can supply NADPH for flavonoid biosynthesis as many steps in the flavonoid biosynthesis pathway require reductive power. Pyruvate, another product of NADP-ME reaction, can be used for obtaining ATP through respiration in the mitochondria; and may serve as a precursor for synthesis of phosphoenolpyruvate (PEP). PEP is utilized in the shikimate pathway, leading to the synthesis of aromatic amino acids including phenylalanine, the common substrate for lignin and flavonoid synthesis. Moreover, NADP-ME can be involved in mechanisms producing NADPH for synthesis of activated oxygen species that are produced in order to kill or damage pathogens (Casati *et al.,* 1999). In the present study the up regulation was noticed only in two way interaction (B.P +*Phytophthora*) at late hai viz., 24 hai whereas in tripartite it was induced in 12 hai itself suggesting this as a inducible protein with early expression by the action of T-ISR.

Nucleoside diphosphate protein kinase (NDPK) gene TAB2 of tomato was earlier considered as a non-regulatory housekeeping enzyme. But protein pull-down assays showed that TAB2 interacts with LeMPK3, but not with other members of tomato MAPKs. Overexpression of the wild type TAB2 also enhanced resistance to virulent *Pseudomonas syringae* pv. tomato. The phosphoproteomics approach on the tMEK2 defense pathway study resulted in the identification of TAB2 as a downstream protein of LeMPK3 and as an effective pathway component in tMEK2-mediated disease resistance (Xing *et al.,* 2008). The present study identified this protein as T-ISR protein with the enhanced expression in tripartite. This was absent in both two way interaction (B.P + *Phytophthora* and BP + *T. harzianum*) indicating this as important early responsive protein in induced systemic resistance as the T96P24 lacks its expression.

The transport inhibitor protein 1 (TIR1) gene encodes an F-box protein containing 16 degenerate leucine-rich repeats (LRRs) (Ruegger *et al.,* 1998). Gray, 1999 also showed that overexpression of TIR1 in transgenic plants results in enhanced auxin response including an increase in auxin dependent gene expression. In our study we found this protein to be absent in two way interaction (B.P + *T. harzianum)* the B.P +*Phytophthora* two way and tripartite interactions showed the down regulation of this protein. This results shows that there is no auxin dependent defense regulation in black pepper system.

Win proteins (win 1, 2) has been demonstrated as defense related proteins and ethylene plays role in regulating the transcription of these genes in a systemic response (Stanford *et al* 1989). WIN2 precursors were highly expressed in response to fungal (*Aspergillus flavus*) inoculation (Dhakal *et al.,* 2017). Harris *et al.,* (1997), showed rapid expression of win peptide in tomato leaf abscission tissue may contribute to the protection of the exposed tissue surface

from bacterial and fungal attack. In the present study the WIN2 precursor showed 7.10 fold up regulation in the treatment T72P12 suggesting this as an important signature as T-ISR and also for the participation of ethylene pathway of defense in black pepper against *Phytophthora*.

Iso flavone reductase is an important enzyme in synthesis of isoflavanoid phytoalexin (glyceollins) in plants. Cheng *et al.,* 2015 demonstrated that the over expression of GmIFR transgenic soybean enhanced the resistance to *Phytophthora sojae*. It also increased the transcriptional level of genes involved viz., PAL, 4CL, CHS in phenylpropanal pathway. The relative expression levels of ROS of transgenic plants were significantly lower than those of non-transgenic plants after incubation with *P. sojae* showed that GmIFR might function as an antioxidant to reduce ROS in soybean. Our study showed the up regulation (Infinite) of this protein at T72P12 condition suggesting the role of isofalvanoid pathway in defense against *Phytophthora*. The enzyme expression was found only in tripartite (T72P12) shows it as *T. harzianum* induced systemic response proteins.

Nmr like negative transcriptional regulator family protein was found to get up regulation in tripartite condition T72P12. NmrA is a repressor of genes involved in nitrogen metabolism (Stammers *et al.,* 2001).

Leucine amino peptidase was shown as an inducible component in the defense response. Lap A RNAs, proteins, and activities increase in response to oomycete pathogens *Phytophthora parasitica* (Pautot *et al.,* 1993). This particular protein was expressed and up regulated only in tripartite interaction in our study while it was absent in two way interactions (B.P + *T. harzianum* and B.P+ *Phytophthora*) hence it might be the ISR protein expressed by the action of *T. harzianum* against *P. capsici.*

Germin like proteins had been shown to exert broad-spectrum disease resistance (Manosalva *et al.,* 2009) in plants. This protein was found to be down regulated to the major fold in two way interactions. P12 and P24 recorded 6.54 and 41.06 fold respectively. But it was reduced to certain extent in tripartite suggesting that this broad spectrum disease resistance protein expression was improved by the action of *T. harzianum*. The down regulation fold change in T72P12, T72P24 and T96P12 were of 1.12, 2.47 and 2.59 respectively.

Subtilisin like protease is demonstrated for its role in pathogen recognition and immune priming against pathogen in plants (Figueirdo *et al.,* 2014). Our present study found that only in two way interaction of B.P +*T. harzianum* this protein was down regulated. But in other two way interaction with pathogen and in tripartite this protein was up regulated. This clearly indicates that this is the specific protein associated with pathogen recognition.

Lu *et al.,* (2012) Identified 8 family genes of fructose 1.6 bisphosphate aldolase with the localization in cytoplasm and plastids. These proteins have highly conserved TIM barrel domain and C- terminal domain with variable N terminal domain. The expression pattern of each protein showed difference in response to various abiotic stresses. In the present study its up regulation only in tripartite interaction suggests that it might be involved in pathogen resistance and it is an T-ISR protein. The up regulation of fructose bisophophate aldolase protein was recorded only in tripartite condition T72P12 (35.83 fold) and the aldolase TIM barrel family protein was recorded at T72P12 (6.69 fold) and T96P12 (82.70 fold).

The R protein RPP 13 has been demonstrated to act via a novel signaling pathway independent of salicylic acid- mediated pathway in *Arabidopsis* (Bittner- Eddy *et al.,* 2000). In our present study we observed the down regulation of this protein only in two way interaction

with *T. harzianum* (T24) with full down regulation and in one tripartite condition T72P24 with 32.48 fold. This protein was found to be absent in rest of the conditions. The *T. harzianum* action shows the positive effect on this protein expression as the down regulation is improved from fully down to the 32.48 fold.

Lignin and lignin-related compounds are induced by infection with pathogens. Cinnamoyl CoA reductase (CCR) catalyzes the first step of the monolignol-specific branch from the phenylpropanoid pathway and is considered as a potential control point regulating the overall carbon flux toward lignin production (Chabannes *et al.,* 2001; Lacombe *et al.,* 1997). H2O2 induced as one of the defense responses may stimulate polymerization of monolignols in the infected regions. Kawasaki, (2006) found that OsRac1 (small GTPase) controls lignin synthesis through regulation of both NADPH oxidase and OsCCR1 activities during defense responses in rice. The up regulation of CCR was noticed only in tripartite condition T72P24 shows this as an important T-ISR protein and suggests that the *T. harzianum* mediates the lignin production in leaf as an ISR activity.

The GABA shut is the metabolic pathway that bypasses two steps of TCA cycle. This pathway is composed of succinate semi aldehyde dehydrogenase. Boucher *et al.,* (2003), showed the role of this pathway in prevention of accumulation of reactive oxygen and also cell death in *Arabidopsis.* Our study found that this protein is induced as T-ISR protein in black pepper. Only the T72P12 and T72P24 condition showed the up regulation suggesting this protein involvement in defense.

The proteins involved in ethylene biosynthesis are found to get up regulated in tripartite interaction which gives strong indication on the involvement of ethylene pathway in induced

systemic resistance in our study. The methionine synthase (4.24 fold in T72P12) and 5 - methyltetrahydropteroyltriglutamate - homocysteine methyl transferase (11.31 fold in T72P12 and Infinite in T72P24) were recorded. Hence the *T. harzianum* might induce ethylene mediated defense pathway in black pepper against *Phytophthora*.

2-methylene furan -3-one –reductase was identified as T-ISR protein which showed its up regulation only in tripartite at T72P12 and T72P24 while it was absent in both two way interaction. Translationally Controlled Tumor Protein has the negative role in HR induction. The NbTCTP in HR induction was investigated by studying the effect of NbTCTP silencing on HR induction by *Agrobacterium*-mediated transient expression of HR-inducible elicitors, including INF1 from *Phytophthora infestans* (Gupta *et al.,* 2013). Induction of cell death by INF1 expression accelerated significantly in NbTCTP -silenced plants in comparison with control plants 24–48 HAI. Therefore, NbTCTP might have a role in cell death regulation during HR to fine-tune programmed cell death-associated plant defense responses. The present study found the up regulation of this protein in only tripartite interaction as induced protein at T72P24 condition. The expression of this protein suggests that it might have a role in reducing the HR reaction (reduced necrosis) in black pepper and this resistance is mediated by *T. harzianum*. Although the hypersensitive cell death is efficient defense against pathogens, the disease resistance can be achieved by a number of mechanisms, or that in some cases, a subset of defense mechanisms may be sufficient to stop pathogen growth (Kushallappa *et al.,* 2016). Recently an *Arabidopsis* mutant (*dnd1)* was isolated that exhibits resistance to virulent pathogens in the absence of HR (Yu *et al.,* 1998).

Martinez-Medina *et al.,* (2013) studied the *Trichoderma* induced resistance in tomato plants. The fourth and fifth leaves of *Trichoderma* treated plants after 5 weeks were used to

inoculate with the pathogen *Botrytis cinerea* to analyze the induced gene expression. They found the enhanced expression of known marker genes viz.,, proteinase inhibitor II, multicystatin and pro systemin involved in JA pathway. In contrast there was no significant difference in the genes responsible for either SA or ET. *Trichoderma* inoculated plants did not show any induction of the genes in the absence of pathogen, suggest that the JA dependent defense underlies the systemic induction by *Trichoderma* in tomato *against B. cinerea.*

Marra *et al.,* (2006) demonstrated the dynamics of proteins in *Trichoderma*-Bean-*Botrytis* and *Trichoderma* –Bean- *Rhizoctonia* interaction using proteomics. The study provided the protein profile in *Trichoderma*-bean-*Botrytis* interaction with 57 spots as new, 93 spots absent, 25 up regulated and 62 down regulated when compared to two way plant-*Trichoderma* interactions. This suggested that the differential proteins are the systemic resistance proteins induced against the pathogen uponits infection. The same way tripartite interaction with *Trichoderma* – Bean -*Rhizoctonia solani,* 63 spots emerged as new, 116 as absent, 27 up regulated and 29 down regulated when compared with *Trichoderma* – Bean interaction, suggesting the systemic response by the *Trichoderma* in the plants. The *Trichoderma* inoculated in roots elicited the systemic defense response in leaf of pepper plants (Ahmed *et al.,* 2000). The percentage of *P. capsici* isolated from leaf at 9 days after inoculation was higher in control than in *Trichoderma* treated plants. This suggested that the inoculation of *Trichoderma* at root could induce the systemic response in the upper part of the plants. Velazquez- Robeldo *et al.,* (2011); Segarra *et al.,* (2010) and Karolev *et al.,* (2008) showed the induction of ISR-like resistance in *Arabidopsis* by *T. asperellum* or *T. harzianum* T39 against *B. cinerea*.

5.5.3. Other proteins as T-ISR

The proteins which are important in T-ISR but not coming under the ROS and defense related protein category are explained below. These proteins are also the prominent marker for the T-ISR in black pepper. Alpha mannosidase are involved in turnover of plant complex type N-glycans. This protein was found to be up regulated at T72P24 (22.05) and T96P24 (Infinite). Hairpin binding protein was up regulated only in tripartite condition (T72P12, T72P24) while in other two way interaction it was found to get down regulated. Among the heat shock proteins, the 20Kda chaperonin showed 1.4 fold up regulation in T72P24 and CPN-60-2 mitochondrial protein showed 1.13 fold up regulation in T96P24. The tripartite condition T72P24 recorded the up regulation of mitochondrial outer membrane protein porin (8.66 fold), DUF 810 domain containing protein (infinite) and nucleic acid binding OB fold like protein (7.18 fold). The enhanced expression of above proteins in only in the tripartite interaction suggests that these proteins also might play a role in induced systemic resistance mediated by *T. harzianum* in black pepper.

5.5.4. Functional enrichment of proteins involved in tripartite interaction

STRING functional enrichment showed the T72P12 and T96P12 with KEGG pathway enrichment for secondary metabolites suggest that the production of defense related metabolites might be at the early hours of infection of *Phytophthora* which is due to the action of *T. harzianum.* The enrichment note on peroxisome from all condition of tripartite suggest that the leaf peroxisome is acting as a major pathogen defense mechanism in black pepper (susceptible genotype) which is induced by *T. harzianum.*

Taken together our data on expression dynamics of proteins in tripartite interaction while comparing the (two) two way interactions clearly shows that the *T. harzianum* had manifested induced systemic resistance in black pepper against *Phytophthora*. Many recognized *Trichoderma* strains are able to elicit JA and ET synthesis involved in development of ISR (Salas-Marina *et al.*, 2011; Contreras-Cornejo *et al.*, 2011, Hermosa *et al.*, 2012). From our study we could identify the enhanced ET synthesis in the ISR development in black pepper against *Phytophthora.* The enrichment of strong ROS related activity suggested that the ROS mediated signaling as major component in T-ISR in our study. It is also noted that the auxin mediated defense signaling is not present in T-ISR in black pepper. The isoflavanoid pathway and lignin synthesis got enhanced in T-ISR. The ISR of *Trichoderma* had been shown to be genotype-dependent. The *B. cinerea* infection of plants pre-treated with *Trichoderma* leading to enhanced activation of JA-responsive genes boosting systemic resistance was found to be genotype-dependent with certain tomato lines showing absence of ISR by *Trichoderma* (Tucci *et al*., 2011). From our study it is proved that *T. harzianum* induced systemic resistance is the major player to mediate defense in Sreekara, the susceptible variety of black pepper for *Phytophthora capsici.*

6. Summary and Conclusions

6.1. Rhizosphere and "Trichorhizosphere" of black pepper

It is well known that the plant rhizosphere along with the associated microbial communities plays a vital role in the health of the plant and protection from soilborne pathogens. Linderman (1998) coined the term Mycorhizosphere as the roots colonized by arbuscular mycorhiza exhibits a different community structure when compared to non-mycorrhizal roots. *Trichoderma* being an opportunistic endophyte (Harman *et al.,* 2004) improves growth of the inoculated plants besides suppressing the diseases caused by soil borne pathogens especially *Phytophthora*. In black pepper this was elucidated by using metagenomic tools and the results showed the presence of differential microbial communities and thus justifying the use of the term "trichorhizosphere" (Umadevi *et al.*, 2017). The illumina hiseq sequenced soil metagenome assembled reads, when analyzed with double approach viz., stand alone and MG-RAST yielded coherent results in both taxonomy and functional categories. STAMP tool analysis of relative abundance on top ten bacteria and fungi showed statistically higher proportion of *Acidobacteriaceae bacterium, Candidatus koribacter versatilis* in *Trichoderma* inoculated sample, uncultured bacteria in control and *Fusarium oxysporum*, *Talaromyces stipitatus, Pestalotiopsis fici* in *Trichoderma* inoculated sample, *Rhizophagus irregularis*, *Pseudogymnoasus pannarum* (Human pathogenic fungi), *Oidiodendran* in control sample respectively. The relative abundance for the specific functional features showed the high abundance of heme and hemin uptake, iron acquisition, metabolism of aromatic compounds in *Trichoderma* treated soil metagenome and with the reduced abundance on pathogenicity islands, phages and prophages than untreated soil (control).

The population dynamics and functional richness of rhizosphere ecosystem in black pepper influenced by the treatment with *T. harzianum* provides evidence for the ecological importance of *T. harzianum* in the cultivation of black pepper. On the basis of the present report and previous studies on effect of *T. harzianum* in the fitness of black pepper; it can be suggested that as mycorhizosphere, another micro ecological niche, viz., 'trichorhizosphere' is also exists in altering the community dynamics of bacteria and soil fungi; and thus, the rhizosphere micro ecosystem developed by *T. harzianum* might contribute a pivotal role in imparting plant health, which is unlike the individual effect of *T. harzianum*. The methods employed in this study show a significant step toward possible application of metagenomics for the functional elucidation of *T. harzianum* - the valuable biocontrol, growth promoting fungus in the production system of black pepper.

The rhizosphere and the trichorhizosphere metagenomes of black pepper elucidated in this study would become important factors in developing any IDM modules in the root ecosystem of black pepper.

6.2. Root colonization of *T. harzianum*

The co colonisation and microscopy especially electon microscopic study demonstrated the localization, endophytic colonization and helper activity of *T. harzianum* MTCC 5179 in black pepper. This endophytic interaction of the fungus underwent several morphological changes during interaction with the root system of the host. Scanning electron microscopy showed the enlargement of hyphal tip as papillae at 12 hai on the black pepper roots. The root clearing and bright filed microcopy showed fungal mycelium in the intercellular spaces at 24 hai with hyphal tips as dark blue granules inside the cells. The intracellular chlamydospores were

observed at 48 hai. Enhanced AMF root colonization by the *T. harzianum* inoculation in black pepper form pot culture study indicates that *T. harzianum* acts as helper organism in the root ecosystem of black pepper for colonization by native AMF present in the soil on the plant.

6.3. Black pepper -*T. harzianum* interaction

The immune suppression by *T. harzianum* in black pepper during its colonization has been demonstrated by the label free proteomics. The expression pattern of proteins during *T. harzianum* interaction showed the down regulation of PR proteins to the greater degree indicating the absence of SA involvement at all the time intervals studied. The other phyto hormone ethylene biosynthesis is also down regulated. The up regulation and new protein induced group with PTI related receptors and R genes shows that the pattern recognition step itself the *Trichoderma* might be altering the plant activities favorable towards its own establishment. Some important down regulated proteins were found to be the marker proteins for the suppression of immunity in black pepper by *T. harzianum.* At 24 hai the SOD, CAT, Glyceroldehde -3 Po4; at 48 hai Rap guanine nucleotide exchange factor 2, Histone 2A; at 72 hai, the germin like protein and subtilisin marker proteins for plant defense; at 96hai, the SAR marker ascorbate peroxidase and RAB GTPase which is immunity associated programmed cell death.

6.4. Black pepper - *Phytophthora* interaction

The protein profile of black pepper- *P. capsici* showed much photosynthetic related protein down regulated suggesting that the photosynthesis is altered upon infection. The SAR reaction was initiated at 24 hai than 12 hai. Most of the proteins that were fully down regulated were with slightly increased expression in 24 hai when compared to 12 hai. Activation of ROS

scavengers and the strong indicators of SAR, the PR proteins were not present in both the hour to a greater extent. This could be attributed to the susceptible genotype (Sreekara) of the present study. Our unpublished data on protein dynamics in the resistant genotype IISR Shakthi recorded up regulation of ROS scavengers and PR proteins at 12 and 24hai.This shows the genotype specific resistance in black pepper for *Phytophthora*.

6.5. Black pepper - *T. harzianum* - *Phytophthora*

Expression dynamics of proteins in tripartite interaction clearly showed the *T. harzianum* induced systemic resistance (T-ISR) the systemically modulated “defense readiness” in black pepper against *Phytophthora.* Twenty three defense related proteins and eighteen ROS scavenging proteins were identified as T-ISR proteins. The enrichment of strong ROS related activity suggests that the ROS mediated signaling as major component in T-ISR in black pepper and also the involvement of ET synthesis in the ISR development in black pepper against *Phytophthora.* The auxin mediated defense signaling component was absent in T-ISR in black pepper. The isoflavanoid pathway and lignin synthesis are also found to be important component of T-ISR in black pepper.

The functional enrichment of protein showed the involvement of peroxisome from all condition of tripartite which suggest that the leaf peroxisome is acting as a major pathogen defense mechanism in black pepper (susceptible genotype) during early hours of defense which is induced by *T. harzianum*.

The hypersensitive response or cell death is the qualitative resistance, while the the reduced susceptibility is considered as quantitative resistance. The disease resistance is due to additive effects of several resistance metabolites and proteins. (Kushalappa *et al.*, 2016). The

proteins identified in this study are considered as quantitative resistance candidates mediated by *T. harzianum* in black pepper.

Our study is the first of its kind report on tripartite interaction using label free proteomics. We have elucidated the T-ISR in leaf of root primed black pepper plants upon *Phytophthora* infection.

This study developed the entire peptide signatures from the proteins involved in Black pepper + *T. harzianum*; Black pepper + *Phytophthora* and Black pepper + *T. harzianum* + *Phytophthora* interactions. The peptides from Black pepper + *T. harzianum*, can be used to understand the mode of suppression of immunity in this crop by this beneficial fungus using functional genomics studies.

The peptide signatures of these important host defense proteins from Black pepper + *Phytophthora* interaction could be the possible candidates which can be used to develop the protein based QTL in screening and developing resistant varieties against *Phytophthora* in black pepper using genome editing technology in future.

The T-ISR proteins from the tripartite interaction are the possible candidates for studying the defense signaling mechanism, designing the new molecules as inducers of defense and using it in field condition.