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Original Research

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Assessment of Combination of Biocontrol Strains on the Fusaric Acid and other Toxins Secreted from *Fusarium oxysporum* by HPLC-MS/MS Method and Differential Expression Profiling in *Arachis hypogaea* L

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Abstract

The ability of *Fusarium oxysporum* (Schlecht Emend. Snyder and Hansen) in *Arachis hypogaea* L to produce mycotoxins i.e. Fusaric Acid (FA), Deoxynivalenol (DON), Nivalenol (NIV), Zearalenone, (ZEN), Aflatoxin B1, B2, G1 and G2 in *Arachis hypogaea* L. leaves in vivo was evaluated in relation to combination of three biocontrol agents, *Trichoderma viride* + *Pseudomonas fluorescens, Trichoderma harzianum* + *Pseudomonas fluorescens, Trichoderma viride* + *Trichoderma harzianum*. Among the toxins tested, only FA was identified in plants infected with *Fusarium oxysporum* by LC-MS/MS and quantified using HPLC (4 µg/Kg) Fusaric acid, Deoxynivalenol, Nivalenol, Zearalenone, Aflatoxin B_1, B_2, G_1 and G_2 toxins were not detected in plants treated with the combinations of biocontrol agents. The results demonstrate that this procedure is suitable for simultaneous determination of mycotoxins in *Fusarium oxysporum* of groundnut and the toxin (FA) identified which contributes to the pathogenicity of the fungus during infection. Further differential expression of genes of three leaf samples of control, infected with *Fusarium oxysporum* and treated leaf sample using combinations of biocontrol agents (*Trichoderma viride* + *Pseudomonas fluorescens*) depicted 5559 genes in control specific, 4316 genes as infected specific and 4264 genes are treated specific. In treated samples 1265 up and 850 down regulation genes were depicted where as in infected sample 605 up and 509 down regulatory genes were depicted. Gene oncology and pathways were found from Uniprot data base. These findings provide new insights into the genetic and biochemical processes required for FA production of *Fusarium oxysporum* infecting *Arachis hypogaea* L.

Keywords: *Arachis hypogaea* L, *Fusarium oxysporum*, HPLC-MS/MS Method, RNA Transcriptome Sequencing

1. Introduction

Arachis hypogaea L. (Groundnut) is an important food legume and edible oil seed crop in the world[1]. *Fusarium* wilt of groundnut caused by *Fusarium oxysporum* (Schlecht. Emend. Snyder and Hansen) is a disease of having considerable economic loss in the production of groundnut. *F. oxysporum*, a soil-borne saprophyte with specificity to more than 150 different plant hosts[2]. The pathogen *F. oxysporum* infect its host plant through the roots, invade the xylem vessels and cause a lethal wilting of the infected plant. [3]Found that infected plants produce phytotoxins and they penetrate into host tissues under auspicious environmental conditions causing wilt disease or death.

[4]Documented that 50% of Groundnut diseases are due to *Fusarium* species. Most of the fungi produces secondary metabolites which includes toxins, pigments, plant growth regulators and antibiotics[5]. Mycotoxins often present harmful effects and are toxic to humans,

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plants and animals[6]. [7]Found that the fungal toxin Fusaric acid (5-butyl picolinic acid) is one of the most important mycotoxin that is produced by many *Fusarium* spp. particularly pathogenic strains of *F. oxysporum*, a cause of wilt diseases in a great variety of plants. It was first discovered in laboratory from *Fusarium heterosporum* Nees culture. This fungal metabolite compound was responsible for pathogenesis of tomato wilt symptoms caused by *F. osxysporum* f. sp. *Lycopersici* Schlecht. emend. Snyd and Hans[8]. FA is a natural mycotoxin produced by numerous *Fusarium* species under in vitro and in vivo conditions[9–11]. [12,13]Who found that fusaric acid (5-*n*-butylpicolinic acid; FA)—which is produced by *Fusarium oxysporum* is associated with wilt in banana, cotton, pea, tomato and other plants, affects membrane permeability (increasing the leakage of potassium and other electrolytes) and inhibits respiration.

FA produced by *Fusarium oxysporum* which mainly disturbs the metabolism of growing plants[14]. [15]Reported that Fusaric acid is capable of conjugating with Cu, Co, Fe and Zn, forming chelates which make these minerals unavailable to plants. It further inhibits the activities of plant defensive enzymes and leads to a reduction in plant cell viability[16]. These processes quickly provoke other physiological reactions such as the production of reactive oxygen species[17]. Some studies have been reported on the toxic effect of FA on some endogenous enzymes of plants, alteration of cell growth, mitochondrial activity and membrane permeability[18,19], inhibition of respiratory activity[20,21] and decreasing ATP levels[22,23] and in several plant species. Toxins such as Deoxynivalenol, Nivalenol have been found in grains. Zearalenone or F-2 toxin [6-(10-hydroxy-6-oxo-trans-1-undecenyl) βresorcyclic acid lactone] is a non-steroidal, oesterogenic toxin produced by various Fusarium spp. including *F. graminearum, F. oxysporum*, *F. roseum*, *F. semitectum* and *F. tricinctum* in various feed stuffs and in mainly cereals. The presence of zearale none in groundnuts has been reported by earlier workers[24].

Aflatoxins, are another group of potential mycotoxins that infects the groundnut. Groundnuts are susceptible to infection both by *Aspergillus flavus* and *Aspergillus parasiticus*[25]. Aflatoxins produced by *Aspergillus flavus* Link ex Fries, are the major mycotoxins that contaminate groundnuts and affect their quality. Aflatoxins as potent carcinogenic metabolites are the most toxic among mycotoxins contaminating food and feed commodities[26]. The primary source of these fungi is the soil. The groundnuts that are not harvested remains in the ground and acts as a main source[27]. Aflatoxins are also hepatotoxic and teratogenic. *A. flavus* invades groundnut seeds both at pre-harvest growth stages and at post-harvest drying curing and storage, producing aflatoxins B1, B2, G1 and G2.

Transcriptomic information is used in a wide range of biological studies and provides fundamental insight into biological processes and applications, such as the levels of gene expression[28], the gene expression profiles during development[29]. RNA-seq technology has been successfully applied quite ubiquitously to species such as humans, yeast, mice, grape, Arabidopsis, rice, soybeans, sesame and sweetpotato[30]. However, transcriptomic information is lacking for the peanut plant because this information is difficult to obtain and to date, there has been little interest in such data regarding *Fusarium oxysporum* infecting *Arachis hypogaea* L. The present study was taken to determine the suitable production of toxic metabolites of *Fusarium oxysporum* which cause *Fusariumwilt* in *Arachis hypogaea* L and to identify transcripts related to secondary metabolism and their detection of pathogenicity and virulence factors in *Arachis hypogaea* L.

2. Materials and Methods

2.1 Fungal and Bacterial Cultures

Trichoderma viride, *Trichoderma harzianum*, *Pseudomonas fluorescens* and *Fusarium oxysporum* were obtained from Institute of Microbial Technology (IMTECH, Chandigarh, MTCC Nos. 2047, 3112, 664.2087, respectively). *Fusarium oxysporum* was grown on Potato Sucrose Agar for 30 days and further grown in Czapek's medium for 7 days and filtrate was taken. *Trichoderma viride* and *Trichoderma harzianum* were grown on Malt Extract agar and *Pseudomonas fluorescens* on Antartic Biotic Medium and grown on Czapek-Dox medium in conical flask. It was further centrifuged and culture filtrate was taken.

2.2 Plant Material

Four plants of *Arachis hypogaea* raised from the seeds (JLR–variety) were grown in each of twenty earthen pots (25 cm diameter) upto 75 DAS and grouped into three sets. Control Plants - First set of two pots was sprayed with distilled water (sample 1). Infected Plants - Second set of four pots was sprayed with culture of pathogen, *Fusarium oxysporum* on 30 DAS and left without any

treatment (sample 2). Infected-treated plants - Third set of twelve pots was sprayed with pathogen on 30 DAS. These infected plants were sprayed with OIC of culture filtrates of combinations of biocontrol agents, *Trichoderma* spp. and *Pseudomonas fluorescens*:*Trichoderma viride* + *Pseudomonas fluorescens* (*Tv* + *Pf)* as sample 3, *Trichoderma harzianum* + *Pseudomonas fluorescens* (*Th* + *Pf)* as sample 4 and *Trichoderma viride* + *Trichoderma harzianum (Tv* + *Th*) *as* sample 5 on 40 DAS. On 50 DAS, the leaves of control, infected and infected treated plants were collected for this method.

2.3 LC-MS/MS Analysis

Secondary metabolites in the extracts were analysed using a Liquid Chromatography-mass Spectrometry (LC-MS/MS) system equipped with HPLC analytical column (SRI/INST(FF)/LCMS/Col-18) maintained at 40°C with an electrospray source in positive electrospray ionization mode. The mobile phase eluted at 0.3 ml/min consisted of (A) - Acetonitrile: water: formic acid (5:95:0.2) (B) - Acetonirile: water: formic acid (95: 5: 0.2). The linear gradient elution programme was applied as follows 0 min 98% A, 2% B; 6 min 60% A, 40% B; 10 min 60% A, 40% B; 12 min 0% A, 100% B; 13 min 60% A, 40% B; 15 min 98% A, 2% B; 18 min 98% A, 2% B. The injection volume was 20 µl. The ionization source conditions were set as follows Capillary voltage of 3.5 KV, Cone voltage of 22 V, Source temp 120°C, Resolvation temp 350°C. The cone and desolvation gas flow were 50 and 900 L/h. Mycotoxins used as standards included Fusaric acid (FA), Deoxynivalenol, Nivalenol, Zearalenone, Aflatoxin B_1, B_2, G_1 and G_2, all were purchases from Sigma Aldrich (St. Louis, MO USA) chemical company. These toxins were selected because of their occurrence in F. *oxysporum* and other phytopathogenic *Fusarium* species.

2.4 RNA Isolation

To investigate the comprehensive gene expression profiles of *Arachis hypogaea* L. and its pathogen *F. oxysporum* simultaneously at the early stages of infection, we analysed transcriptomes obtained from sequencing RNA from three samples, which included three biological replicates for each condition (control/infected and treated). RNA was isolated from three leaf samples and to 100 ul of RNA solution, add 10 ul of 3 M sodium Acetate (NaAcetate, pH 7 to 8) and 220 ul of 100% ethanol to RNA solution and mix well gently.

2.5 RNA-Seq Analysis of Host Transcriptome

The three leaf samples of Control, Infected and Treated were aligned to *Arachis hypogaea* L genome from National Centre for Biotechnology Information (NCBI). Read count for aligned reads were fetched using tool feature count. Unaligned reads of Infected and Treated samples were aligned to *Fusarium oxysporum* genome from NCBI using Bowtie2. Read count for aligned reads that aligned to fusarium were fetched soft ware using feature count. Top Hat is a fast splice junction mapper for RNA-Seq reads. It aligns RNA-Seq reads to genomes using the ultra-high-throughput short read aligner Bowtie2. High quality reads (fastq format) were aligned to *Arachis hypogaea* L genome (from NCBI) using Tophat [2] splice aware aligner. Alignment percentage of control, infected and treated samples to *Arachis hypogaea* L. were depicted.

2.6 Differential Expression Analysis w.r.t Control (Host Specific Expression)

Differential expression analysis was performed using DESeq R package. The package DESeq provides methods to test for differential expression by use of the negative binomial distribution.

Differential expression analysis was done as "Treated vs. Control" and "Infected vs, Control". The transcripts with log2foldChange >=2 and p-value<=0.05 were considered as significantly Up regulated and the transcripts with log2foldChange <= -2 were and p-value<=0.05 considered as significantly Down regulated. Sample specific transcripts were identified as well. HeatMaps were created using Cluster 3.0 and Java TreeView (v 1.1.6) for Top 30 significantly up regulated and Top 30 significantly down regulated genes. Following are the Heat Maps for Treated vs. Control and Infected vs. Control were depicted.

2.7 Gene Ontology and Pathways

Gene ontology and Pathways were found from Uniprot database (Infected_vs_Control.xlsx and Treated_vs_Control.xlsx sheet). Pie-donut representation for Top 5 GO and Pathways w.r.t Host for significantly differential genes are depicted

3. Statistical Analysis

Data has been analysed according to standard analyses of variance by a one -way ANOVA with statgraphics Tukey's test was used to compare differences between different treatments.

4. Results and Discussion

In the present study, among the leaf samples tested FA was detected in the leaves infected with *Fusarium oxysporum* (4 µg/Kg) (Table 1). Toxins produced by plant pathogens are known to be involved in plant disease development[31]. It has been reported that production of FA may vary greatly among different isolates of the *F. oxysporum* species complex Fusaric acid has a broad spectrum and is considered to be directly related to the severity of damping off, vascular wilt and root rot diseases of numerous vegetable crops[32,33]. The toxicological interest in species of *Fusarium* arises from their production of a range of chemically different toxic compounds, such as fusaric acid, fumonisins and also with other toxins (Table 2).

In order to increase the understanding of transcriptomic responses produced by infection with one of the most important pathogens, *F. oxysporum*, the present research work was concentrated on RNA-Seq analysis to compare the global gene expression patterns in *Arachis hypogaea* L. leaves inoculated with pathogen. Our results revealed that the Alignment percentage of control, infected and treated samples to *Arachis hypogaea* L. (93.35, 93.20, 82.10%) were recorded respectively (Table 3).

The RNA-Seq method is particularly robust for gene expression studies and allows the identification of plant-targeted control strategies, as well as the detection of pathogenicity and virulence factors. Our results revealed that 1265 genes were upregulated in treated sample when compared with infected leaf sample (605). (Table 4 and Figures 2, 3, 4, 5). Our results substantiate earlier reports that in management of plant diseases the use of biocontrol agents in combinations were more effective than individual agents. [34,35]Biodegradation of FA by different micro-organisms has been reported in the literature[36]. Work on peanuts has also shown that

Table 1. Effect of culture filtrates of *T. viride+P. fluorescens*, *T. harzianum* + *P. fluorescens* and *T. viride* + *T. harzianum* on Fusaric acid, Deoxynivalenol, Zearalenone of *Arachis hypogaea* leaves infected with *F. oxysporum*

S. No	Sample weight (g)	Dilution (ml)	Concentration (µg/l)	Fusaric acid (µg /Kg)	Deoxy nivalenol (µg /Kg)	Nivalenol (µg /Kg)	Zeara lenone (µg /Kg)
2957A 2957B (Control)	3.6706 5.2103	50 50	ND ND	ND	ND	ND	ND
2958 A 2958 B (Infected)	3.7454 5.3368	50 50	BDL-0.4ppb BDL-o.4ppb	BDL- 4 BDL- 4	ND	ND	ND
2959A 2959B Infected Plants treated with *Tv+Pf*	2.8547 5.0147	50 50	ND ND	ND	ND	ND	ND
2960A 2960B Infected Plants treated with *Th+Pf*	3.8583 5.3318	50 50	ND ND	ND	ND	ND	ND
2961A 2961B Infected plants treated with *Tv+Th*	50 50	ND ND	ND	ND		ND	ND

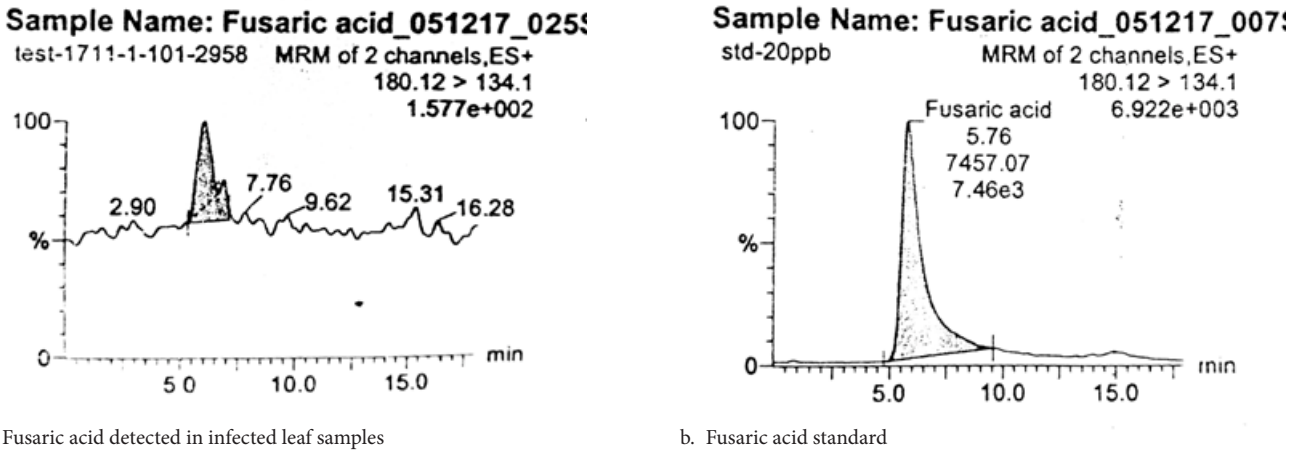
ND - Not Detected.
BDL- Below Detection limit- 4 µg /Kg.
LOD - 10 µg/Kg.

Table 2. Effect of culture filtrates of *T. viride* + *P. fluorescens*, *T. harzianum* + *P. fluorescens* and *T. viride* + *T. harzianum* on Aflatoxin B_1, B_2, G_1, G_2 toxins of *Arachis hypogaea* leaves infected with *F. oxysporum*

S. No	Sample weight (g)	Dilution (ml)	Concentraton (µg/l)	Aflatoxin (µg/Kg) B_1, B_2, G_1, G_2
2957A 2957B (Control)	2.5349 2.0182	12.5 12.5	ND ND	ND
2958 A 2958 B (Infected)	2.1819 5.1813	12.5 12.5	ND ND	ND
2959A 2959B Infected Plants treated with *Tv+Pf*	2.2692 2.8854	12.5 12.5	ND ND	ND
2960A 2960B Infected Plants treated with *Th+Pf*	2.5381 2.0485	12.5 12.5	ND ND	ND
2961A 2961B Infected plants treated with *Tv+Th*	2.5985 2.3141	12.5 12.5	ND ND	ND

$$\text{LOD} = \frac{1(\mu/l) \times 12.5\ (mL)}{2.5g} = 5\ \mu g\ /Kg$$

ND - Not Detected.



a. Fusaric acid detected in infected leaf samples

b. Fusaric acid standard

Figure 1. Chromatogram of fusaric acid detected by HPLC and by fragmentation upon LC- MS/MS analysis **(a)** *Arachis hypogaea* L leaves, infected with *Fusarium oxysporum* **(b)** standard Fusaric acid.

Trichoderma spp. may inhibit FA production in roots and leaves of peanut or maize infected with *Fusarium* spp. [37,38]Besides, the efficacy of 6PAP in suppression of synthesis and degrading of FA has been described for the first time in *Pseudomonas fluorescens*[39].

5. Conclusion

Results from the present study illustrates that only *Fusarium oxysporum* infected leaves are able to produce mycotoxin Fusaric acid. The present study concluded that suitable mycotoxin production from *Fusarium*

Table 3. Alignment to *Arachis hypogaea* L. Genome

Sample	HQ Reads	Aligned Reads	Unaligned Reads	Alignment%
Control	55,760,238	**52,052,676**	**3,707,562**	**93.35**
Infected	52,549,146	48,978,076	**3,571,070**	**93.20**
Treated	**52,948,332**	**43,473,648**	**9,474,674**	**82.10**

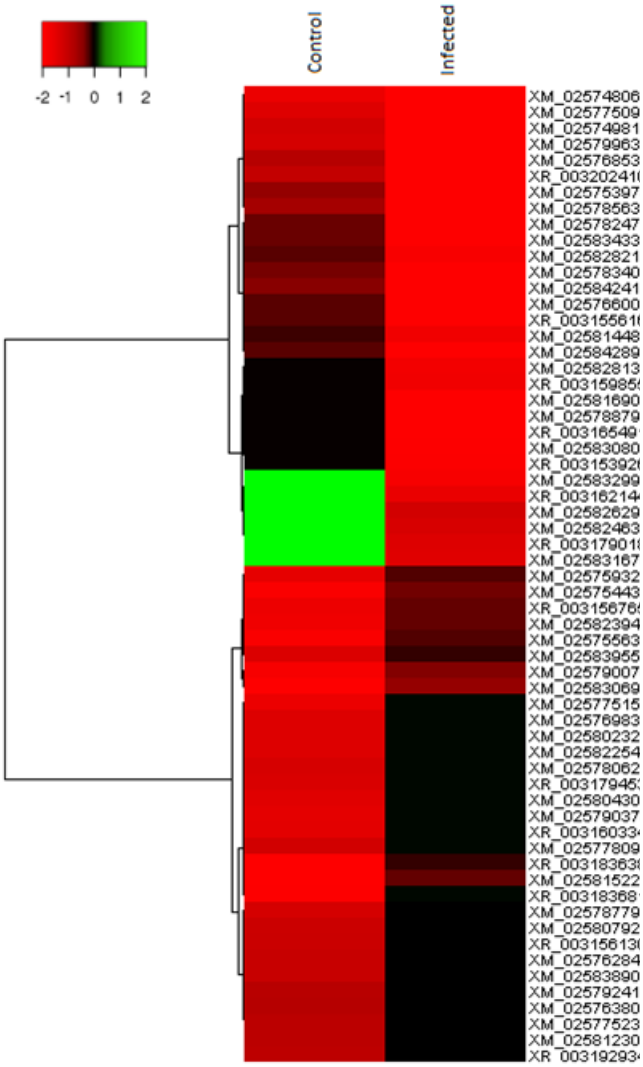


Figure 2. HeatMaps were created using Cluster 3.0 and Java TreeView (v 1.1.6) for Top 30 significantly up regulated and Top 30 significantly down regulated genes for Control vs. Infected.

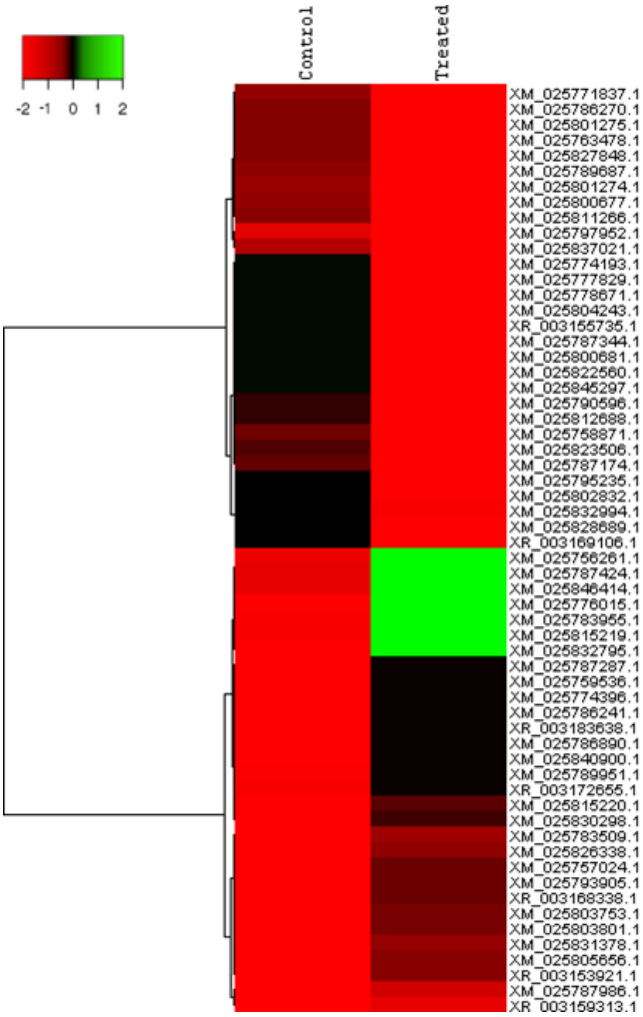


Figure 3. HeatMaps were created using Cluster 3.0 and Java TreeView (v 1.1.6) for Top 30 significantly up regulated and Top 30 significantly down regulated genes for Treated vs. Control.

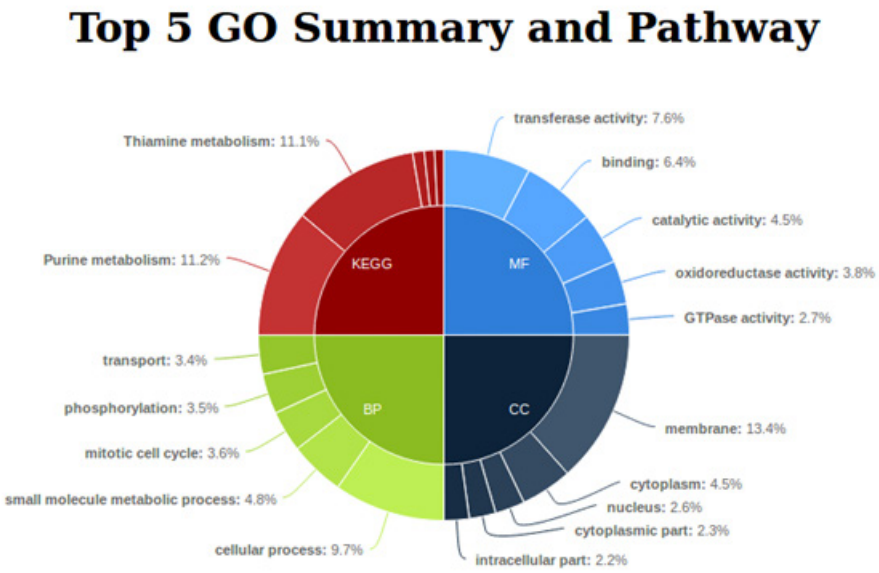


Figure 4. Pie-donut representation for Top 5 GO and Pathways w.r.t Host for significantly differential genes are depicted for Infected_vs._Control.

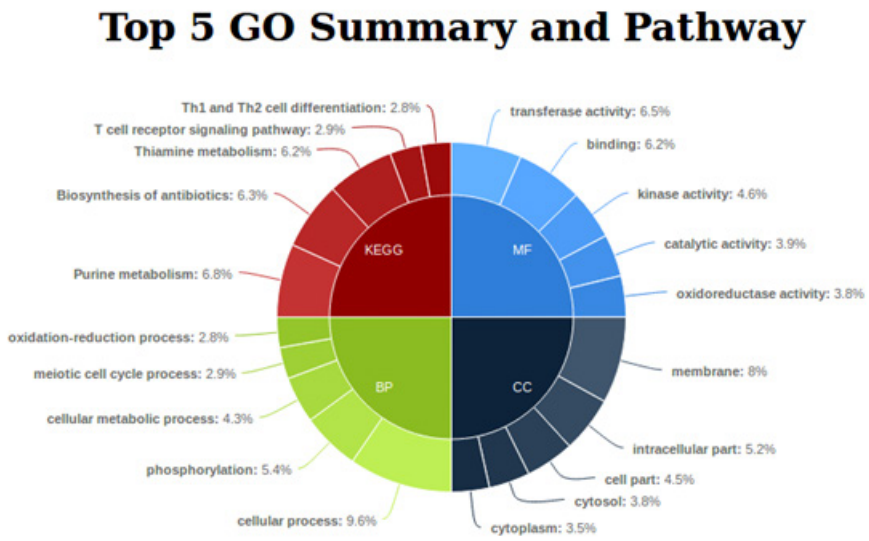


Figure 5. Pie-donut representation for Top 5 GO and Pathways w.r.t Host for significantly differential genes are depicted for Treated_vs. Control.

Table 4. Read count of the reads aligned to peanut were used for differential analysis

Expression Status (Treated vs Control)	Treated vs Control	Infected vs Control
Up regulated (FC >=2 , p <=0.05)	1,265	605
Down regulated (FC <= -2, p <=0.05)	850	509
Control Specific	5,559	4,242
Treated Specific	4,264	**4316**

oxysporum infecting groundnut. Our results highlight the contribution and limitations of an integrated approach in *Arachis hypogaea* L of both functional (pathogenicity and plant toxicity) and genomic (transcriptomic) analyses to understanding in the differential expression of genes to Fusaric acid in fungi and these results may be useful in the developing new methods of broadening resistance of groundnut to *F. oxysporum*, including the over-expression of *Arachis hypogaea* L key genes. Further our study aimed on the identification of the *FUB* cluster and the and the biochemical pathway required for FA

formation as well as the impact of FA on plant-parasite interactions and plant-associated microbial communities

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