

Comparative Meta-Transcriptome Analysis of *Arabidopsis thaliana* Under Organic Stress and Its Validation

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Abstract. Globalization and industrialization had led to the production of large quantities of waste with complex contents; these are prone to cause serious impact on sustainable development. Plant-microbe integrated system was found to be effective in treating specific waste streams in constructed wetlands or in rhizo-remediation. The molecular basis for this effective treatment vis-à-vis the interaction between plant and microbe in any such bio-augmented phytoremediation system is not well understood. Towards this end, a meta-analysis of transcriptomes of the model plant *Arabidopsis thaliana* (Col-0) was performed by analysing 36 microarray samples belonging to seven microarray studies under various organic stresses. To understand the metabolic impact of the stress over the plant and their influence on root exudation, the genes involved in metabolic pathways of the root exudates were analysed. Totally 1313 genes involved in 26 metabolic pathways that in-turn corresponds to 55 root exudates were identified. Comparison of the DEG list of all stress conditions and the genes involved in root exudates, resulted in the short listing of four candidate genes that were highly influential both, in terms of expression pattern, leading to microbial enrichment and statistical significance. Further, to validate this in-silico result, a real time experimental set-up of *Arabidopsis thaliana* (Col-0) under simulated dye and microbial stress was designed. After treatment, Real Time Quantitative PCR was done for the four candidate genes and the expression pattern of those genes in real time conditions was obtained to corroborate their in-silico result.

Keywords: Plant-microbe interaction; organic stress; root transcriptome; root exudates; *Arabidopsis thaliana*; rhizo-remediation; meta-analysis of root transcriptome..

1 Introduction

Anthropogenic activities as part of economic development have led to the release of huge amount of waste containing hazardous components into the environment and have deleterious impact (Gudelj et al., 2011). Various remediation strategies are adopted to overcome the effects caused by these hazardous wastes. Bio-remediation especially, phytoremediation is one among them which has gained its global importance because of its cost-effectiveness and eco-friendly approach (Glick, 2010). Plants release root exudates into their rhizosphere which varies depending upon various biotic and abiotic factors (Cheng and Gershenson, 2007). These root exudates contribute to selective enrichment of microbial community (Yang and Crowley, 2000). The microbial concentration in the rhizosphere may reach up to 10^{10} - 10^{12} cells per gram, as compared to often $<10^8$ cells per gram in bulk soil (Cheng and Gershenson, 2007;

Lynch and Whipps, 1990). Microorganisms in the rhizosphere contribute to different functions including organic and inorganic pollutant degradation (Burken and Schnoor, 1996). Bio-augmentation is another strategy in which the remediation can be tailored to a specific and dominant pollutant in the surrounding environment (Nzila et al., 2016). Studies have shown that plants along with microorganisms (bio-augmented-phytoremediation) can improve the efficacy of pollutant remediation (Agarwal et al., 2020). Still the molecular mechanism in the bio-augmented phytoremediation system is not well understood. This work aims to bridge the gap between the effectiveness of the augmented phytoremediation system and its molecular schema. The correlation between different organic stresses and the pathways that are modified, especially the biosynthesis of various root exudates was analysed. Based on the most significantly modulated genes in this pathway four candidate genes were selected and were analysed for their expression levels under a simulated plant-microbe treatment system for azo dyes. This study was selected as such a plant-microbe integrated system was found to be more effective in treating azo dyes (Jayapal et al., 2018).

2 Materials and Methods

2.1 Chemicals, microbes, plants and reagents

All chemicals used in this study were analytical grade from either HIMEDIA or SIGMA. *Arabidopsis thaliana* (Col-0) seeds were obtained from Department of Plant Biotechnology, Madurai Kamaraj University, Madurai, India. Real time Quantitative PCR reagents were procured from Thermo Scientific and Takara Biosciences. Cow dung slurry was suspended in sterile water and filtered with sterile muslin cloth to remove the suspended particles. The cow dung slurry filtrate was used as source of microbes. All the experiments and assays were done in triplicates.

2.2 Data retrieval, DEG prediction and sorting

Microarray data sets of *Arabidopsis thaliana* roots under various abiotic stresses were retrieved from Gene Expression Omnibus (GEO) platform of NCBI. The microarray platform selected was GPL198. Datasets consisted probes that represents approximately 25,000 genes. Studies under these platforms were queried using “*Arabidopsis thaliana*” AND “Root” as keywords. The datasets were normalized using “limma” package of GEO2R tool (Lu et al., 2021). Multiple-testing corrections using Benjamini & Hochberg (FDR) method was done to obtain adjusted P-values for the datasets. Differential Expressed Gene was sorted out with a cut-off of logarithmic Fold Change (log FC) more than +2 and P-value less than 0.05 using R-programming.

2.3 Exudate gene retrieval and candidate gene selection

Root exudates are important for microbial enrichment in the rhizosphere. *Arabidopsis thaliana* root exudates are comprised of about 55 different bio-molecules, including different classes of carbohydrates, amino acids and phenolics (Chaparro et al., 2013). The pathways which are involved in these exudate productions were annotated from KEGG Pathway database. The genes involved in the pathways were retrieved using KEGGREST (R- package).

The seven datasets used in this study were microarray datasets pertaining to roots of *Arabidopsis thaliana* under organic stress. The abiotic stresses were, 4-chloro- 6 methyl- 2- phenyl pyrimidine (CMP), 2- (4- carboxyphenyl) tetra methyl imidazoline-1 oxyl- 3- oxide (cPTIO), Fenclorim, Norflurazone, Phenanthrene, Dicamba (3, 6 dichloro 2 methoxy benzoic acid) and Probenazole. The genes involved in the exudation pattern were sorted from the set of

up-regulated gene for all seven datasets along with their Fold change value using spreadsheets. Based on the Standard deviation of the expression fold and Count of Occurrence of up-regulation for each gene in each condition, all those genes were sorted and a heat map was generated using “ggplot” module. Top four genes were selected as candidate genes based on their statistical significance among various organic stress conditions and were up-regulated in four out of seven organic stress conditions

2.4 *Arabidopsis thaliana* (Col-0) plant cultivation

The *Arabidopsis thaliana* seeds were surface sterilized using 1% Bleach solution for 5 minutes and 75% ethanol for 3 minutes and then rinsed with sterile water. Sterilized seeds were sown on plant tissue culture (PTC) bottles containing 0.5X MS (Murashige and Skoog, 1962) supplemented with 2% (w/v) of sucrose and 1% (w/v) of solidifying agent (Kazachkova et al., 2013). The seeds were incubated two days at 4 °C for stratification. After stratification, the bottles were incubated in the growth room under controlled conditions (16-h light (120 mmol m⁻² s⁻¹) / 8-h dark) for 22 days. The 22 days old plantlets were used for further studies.

2.5 Transfer of plants to hydroponics

Twenty-two days old plants were removed from MS medium and were then rinsed with distilled water and sterile distilled water to remove the remains of MS medium on the root surface. The plants were transferred to 0.1X Hoagland's nutrient solution. 1.5ml Eppendorf tube lids (holed at centre) were used as floaters to hold the plant in the surface of the liquid medium. The tissue culture bottles containing the plants were maintained in tissue culture growth chamber under conditions of 16/8 hrs light/dark cycles, temperature at 25 ± 2°C and humidity 60% till the plants get adapted. The plant was maintained in hydroponics for four days, to stabilize its growth. The Hoagland's nutrient solution was replaced periodically (Sasikaladevi, 2017).

2.6 Design of Experiment (DOE)

Each trial was carried out in triplicates, with each tissue culture bottle containing three plantlets. For the experimental simulations, methyl red of 40 mg/L in 0.1X Hoagland's nutrient solution and 1% (v/v) of cow dung slurry filtrate in the Hoagland solution as a source of microbes were used. The treatment set up was maintained in tissue culture chamber overnight under conditions of 16/8 hrs light/dark cycles, temperature at 25 ± 2°C and humidity 60%. (Table 1).

Table 1. Design of the study (P: Plant alone system; PM: Plant with microbe system; PP: Plant with pollutant system; PPM: hybrid system containing plant, pollutant and microbe)

DOE	P	PM	PP	PPM
Plant	+	+	+	+
Microbes	-	+	-	+
Methyl red	-	-	+	+

2.7 Total RNA Extraction and total RNA quantification

RNA was isolated from the roots of the treated plants as per the standard protocol (Ahn, 2009). Briefly, the root tissue of each experimental study was ground separately in mortar and pestle using Liquid Nitrogen and total RNA isolated using Trizol reagent. Isolated RNA was washed with 75% ethanol and air dried. RNA was re-suspended in TE buffer and stored at -80 °C till further use. RNA was quantified using The SpectraMax® QuickDrop™ Micro-Volume Spectrophotometer.

2.8 Reverse Transcription

Revert Aid First Strand cDNA synthesis kit was used for Reverse transcription as per the manufacturer's instruction. All reactions were initiated with 100ng of total RNA.

2.9 Real Time Quantitative PCR and Data analysis

All experiments were performed in Bio-Rad CFX96 system using TB® Green premix Ex TaqII. Each 10 µL of reaction mixture contained, cDNA corresponding to 100ng of total RNA. Tubulin beta chain was used as internal control gene (Hong et al., 2010). Primers for all four candidate genes and internal control genes were designed using Primer Pick of NCBI. The primers were designed targeting the reference sequence of individual genes. To eliminate non-specific amplification, the primers were designed over exon-exon junction, with an amplicon size cut-off of 150bps. Also primers were obtained from previous works (Gunning et al., 2014). PCR thermo cycles program was programmed as 95.0 °C: 30secs for Pre-denaturation, 40 cycles of Denaturation at 95.0 °C for 5 seconds followed by amplification at 60.0 °C for 30 seconds.

The stability of reference gene expression under different conditions was evaluated by comparing C_q values between treatment and control studies performed with an identical amount of purified cDNA. The expression fold change was determined by $\Delta\Delta C_q$ method (Livak and Schmittgen, 2001).

3 Results and Discussion

Plant – microbe interaction is one of the inevitable mechanisms that take place in a phytoremediation process. Complete understanding of the molecular background and biochemical signalling between plant and microbe in remediating the given pollutant stress is important to annotate the complete picture of a plant-microbe integrated phytoremediation system. Results of the meta-analysis done on *Arabidopsis thaliana* root transcriptome under various stress and real time validation of the candidate gene expression are discussed below.

3.1 Data collection and curation

Open access datasets of microarray experiments pertaining to roots of *Arabidopsis thaliana* subjected to various abiotic stresses, performed in ATH1-121501 Affymetrix *Arabidopsis thaliana* Genome Array were retrieved (Table 2).

Table.2. Summary of Datasets retrieved from GEO database

S. No	Type of stress	No. of datasets
1	Organics – Aromatic ring / amine	7
2	Organics - others	11

The collected datasets were screened on studies that involved organic stress (especially compounds consisting aromatic rings and amine groups), as the aim is understand the molecular background of plant-microbe integrated remediation, especially under azo dye stress. The short-listed studies included those with 4-chloro- 6 methyl- 2- phenyl pyrimidine (CMP), 2-(4- carboxyphenyl) tetra methyl imidazoline-1 oxyl- 3- oxide (cPTIO), Fenclorim, Norflurazone, Phenanthrene, Dicamba (3, 6 dichloro 2 methoxy benzoic acid) and Probenazole as abiotic stresses and data relevant to roots of *Arabidopsis thaliana* were taken for further analysis.

CMP is an herbicide safener used to increase tolerance to the chloroacetanilide group of herbicides (Steel et al., 2011). CMP probably induces Glutathione S transferase genes in both root and suspension culture of *Arabidopsis thaliana* (Edwards et al., 2005). cPTIO belongs to nitronyl nitroxides (NNO) compounds. These NNO compounds being stable organic radicals, react with nitric oxides (D'Alessandro et al., 2013). Hence, these cPTIOs are also termed as nitric oxide scavenger. Norflurazone is a fluorinated pyridazinone herbicide and is a Delta – 6 desaturase inhibitor (Sopeña et al., 2011). Dicamba is a commonly used house-hold herbicide (Behrens et al., 2007). It is chlorinated derivative of o-anisic acid. Probenazole is widely used anti-fungal agent in rice cultivation. It protects the rice plant against *Magnaporthe grisea*, a rice blast fungus (Yoshioka et al., 2001). Phenanthrene is a poly aromatic hydrocarbon (PAH) (Weisman et al., 2010), and are known to induce oxidative stress and leaf deformation (Alkio et al., 2005; Liu et al., 2009). All the above-described compounds have aromatic rings or amine groups, which is also similar while considering the stereo-chemical structure of azo dyes. Thus, these microarray studies were analysed for differential gene expression using GEO2R. The up regulated genes from each study were sorted out with a cut-off on log₂FC greater than two and P- value lower than 0.05, using R programming. Among the 27,416 annotated protein coding genes of *Arabidopsis thaliana* (TAIR10, Gene annotation data), 2298 were up regulated cumulatively in the above stated stress conditions.

3.2 Exudate gene retrieval and candidate gene selection

Root exudates of *Arabidopsis thaliana* comprises of various organic compounds like carbohydrates, proteins, amino acids, phenolics, organic acids etc. Previous study on *Arabidopsis thaliana* (Col- 0) wild type grown in Murashige and Skoog medium (MS) supplemented with 3% sucrose and 0.9% Bactoagar at 25 °C with a photo period of 16hr light/ 8 hrs dark had shown to release around fifty five compounds in its root exudates, including various classes of sugars, amino acids, phenolics and organic acids (Chaparro et al., 2013). Each of the compound reported were taken for pathway analysis in KEGG pathway database. Totally 26 pathways were involved in the listed root exudate biosynthesis. Major pathways were amino acid and nucleotide synthesis pathway, fatty acid biosynthesis pathway, fructose and mannose biosynthesis pathway, Pentose phosphate pathway, pentose and glucuronate inter-conversion pathway. The relevant pathways annotated with the rate limiting enzymes were retrieved from KEGG database (Supplementary material).

Of the 26 annotated exudation pathways, five pathways noted in Supplementary material are involved in the exudation of predominant exudates. The genes involved in the pathways were retrieved using KEGGREST module of R (Tenenbaum, 2016). After compiling all the genes for individual pathways around 1312 protein coding genes were identified to be involved in the regulatory and anabolic pathway of the reported 55 root exudate compounds.

The selection of candidate gene was performed using Microsoft spreadsheets. The list of up-regulated gene sets for individual study were compared against the list of 1312 genes involved in exudation pattern and a heat map was generated using “ggplot” module of R. The heat map was generated based on the Standard deviation of the expression fold and Count of Occurrence of up-regulation for each gene in the various organic stress conditions (Fig. 1).

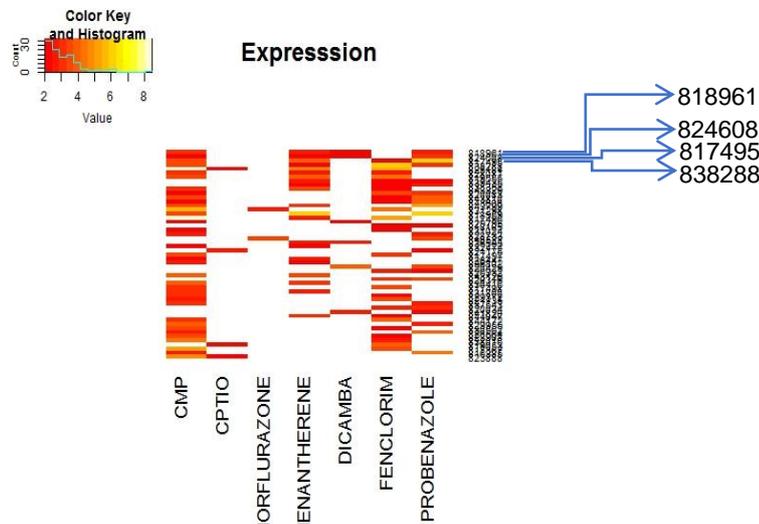


Fig.1. Heat map showing the top four candidate genes, based on Standard deviation of the expression fold and Count of Occurrence of up-regulation for each gene in the various organic stress conditions.

The top four candidate genes that have both higher expression and statistical significance were: 1. Glutathione S-transferase (GST) tau 4 (817495), 2. Glutathione S-transferase (GST) tau 24 (838288), 3. Chitinase family protein (818961), 3. Homolog of carrot EP3-3 chitinase (824608). The anabolic impact of those candidate genes in exudate metabolites are given in the Table 3

Table. 3. Significance of the candidate genes in exudate metabolism

S. No	Gene ID	Gene name	Pathways involved
1	817495	Glutathione S-transferase (GST)tau 4	Oxoproline
2	838288	Glutathione S-transferase (GST) tau 24	Oxoproline
3	818961	Chitinase family protein	Fucose, Rhamnose, Glu-1-Phosphate, Glucosamine, Mannosamine
4	824608	Homolog of carrot EP3-3 chitinase	Fucose, Rhamnose, Glu-1-Phosphate, Glucosamine

Glutathione S transferases (GSTs) are soluble proteins found in plants and animals. The GSTs have a molecular mass around 50kDA. These GSTs catalyses the conversion of the tripeptide glutathione to a polar S – glutathionylated product(Steel et al., 2011). They have a predominant role in metabolism as well as detoxification of drugs in animals (Wilce and Parker, 1994). Glutathione S transferases in plants were first identified on 1970's, in which it had a role in protecting maize crop from herbicide stress (Cobb and Kirkwood, 2000). Many GSTs have been identified and characterized both in plants and humans (Steel et al., 2011).

In this work, two classes of GSTs showed their metabolic significance in biosynthesis of Oxoproline. Oxoproline is a precursor of Glutamic acid. Oxoproline is a well-studied intermediate in eukaryotic gamma- glutamyl cycle. These oxoproline is metabolized to glutamate by an ATP- dependent oxoprolinase(Griffith et al., 1978) in eukaryotes. But most of the prokaryotes lack this oxoprolinase(Niehaus et al., 2017).. Studies have enumerated the importance of putative 5- oxoprolinase genes in bio-remediating microbes for tolerating the lower water potential stress (Coronado et al., 2014). Thus it can be hypothesized that this leads to an enrichment of oxoprolinase positive microbes in the rhizosphere of *Arabidopsis thaliana* under these type of organic stress like herbicides, poly aromatic hydrocarbons

Chitinase group of enzymes belong to glycosyl hydrolysis family that degrade chitin to 1-4 linked N- acetyl D- glucosamine (Passarinho and de Vries, 2002). These are also termed as pathogenesis-related proteins(PRPs); they are induced by various fungal, bacterial and viral infections (Graham and Sticklen, 1993). Plants do not contain chitin, but still, most of the agricultural pests contain chitin in their cellular component. Plant secretes these chitinase to degrade fungal cell wall which acts as a defence mechanism (Passarinho and de Vries, 2002).

3.3 Validation of expression pattern using RT- qPCR

The candidate genes were analyzed for expression variation under four different conditions, Plant alone (P), Plant with microbial consortium (PM), Plant with pollutant (PP) and Plant with pollutant and microbial consortium (PPM) using RT-qPCR.

Equal amount of RNA sample from each test condition was taken for Reverse transcription and the synthesized cDNA was amplified in Real Time PCR. The Relative Fluorescence Unit (RFU) for four candidate genes under four different conditions with two primer sets each along with the two internal control genes were recorded and amplification graph was generated by the system. Non-template control (NTC) for all primer sets were included in the analysis. The amplification curve for each candidate gene in all four experimental conditions and the amplification curve for internal control gene (TUB2) are shown in Fig. 2. (a-e).

The amplification curves for all the four candidate genes clearly show the variability prevailing in different stress conditions. The amplification curves for internal control gene shows no variation among different stress condition, which indicates that the selection of internal control gene was satisfactory to perform relative quantification of expression variation in all candidate genes. The Cq value for each sample was obtained and further expression data analysis were performed using Delta-Delta Cq method and the results are tabulated in Table. 4.

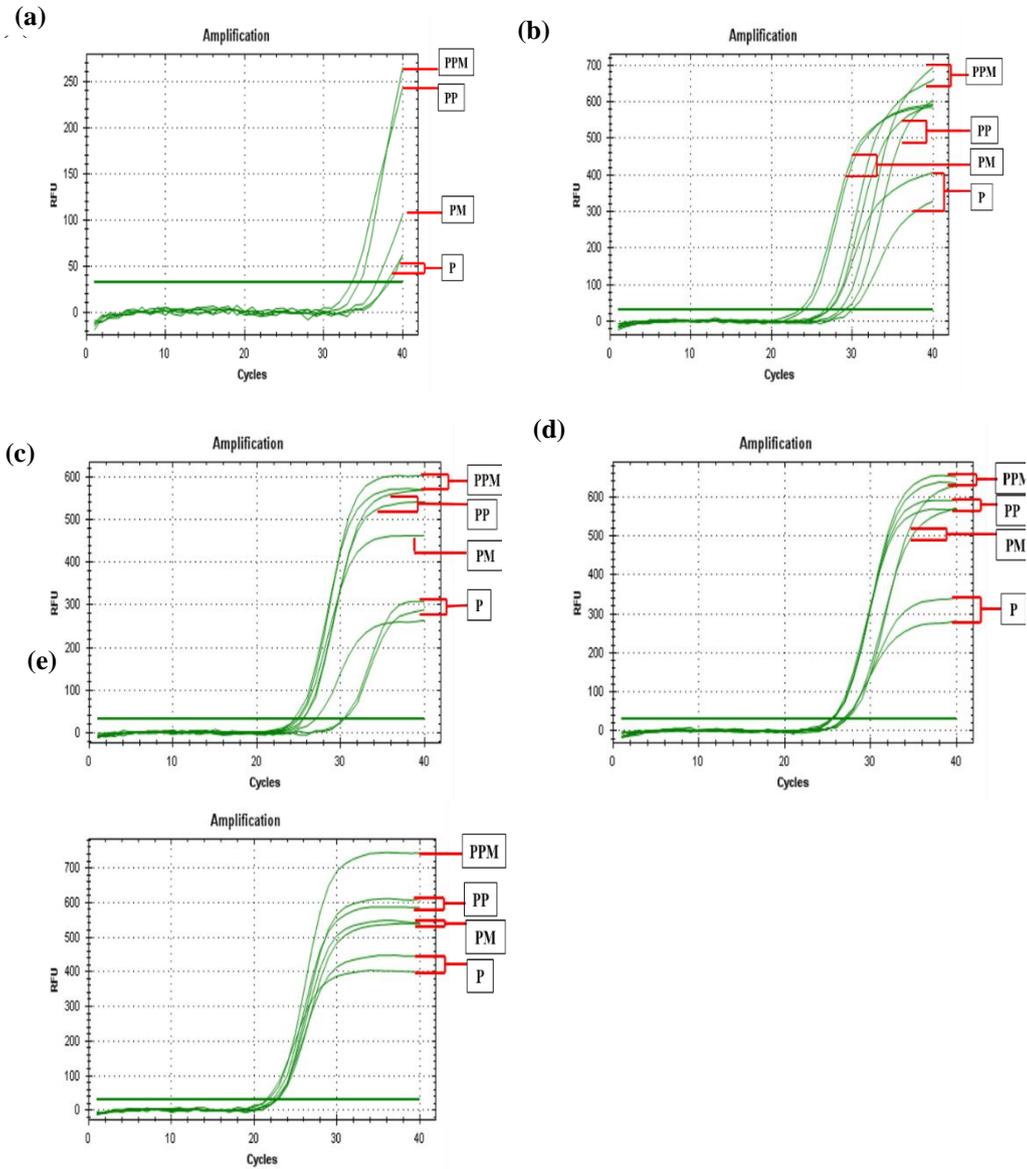


Fig.2. Amplification curve obtained by RT – q PCR under four different experimental conditions

(a) Homolog of carrot EP3-3 chitinase gene, (b) Chitinase family protein gene, (c) Glutathione S-transferase (GST) tau 24 gene, (d) Glutathione S-transferase (GST) tau 4 gene, (e) internal control (TUB2) gene

Table. 4. Expression fold change of candidate genes in plant-microbe, plant-pollutant and plant with pollutant and microbe conditions

Condition	Homolog of carrot EP3-3 chitinase	Chitinase family protein	GST Tau 24	GST Tau 4
PM	1.97	12.7	3.86	1.64
PP	5.43	7.26	2.206	0.942
PPM	0.243	0.42	1.75	1.01

The increase in expression pattern of the chitinase group of genes may be a defense mechanism adapted by the plant to tolerate the pathogenic stress which may include fungal species (Punja and Zhang, 1993). Also, up regulation was observed in GST family proteins. GSTs protect plant cells through detoxification, amelioration of oxidative stress and hormonal signaling in response to various biotic and abiotic stresses (Gullner et al., 2018; Mauch and Dudler, 1993). In plant with pollutant condition, the up-regulation of both chitinase and GST family proteins may be to enhance the level of oxoproline, Fucose, Rhamnose, Glu-1-Phosphate, Glucosamine, Mannosamine in the root exudates. These exudate compounds are amino acid precursors or derivatives of carbohydrates, which are well studied organic compounds in enhancing microbial enrichment (Chaparro et al., 2013). The expression of the candidate genes in plant with pollutant and microbial consortia condition was down regulated compared to the control (plant alone system). The chitinase group of proteins are a defensive response of the plant (Graham and Sticklen, 1993; Passarinho and de Vries, 2002; Punja and Zhang, 1993). In this scenario due to additional pollutant stress, the plant needs the associative function of microbes that has efficient remediation capabilities and therefore, a down regulation in chitinase family genes was observed. In addition, there was no significant, differential expression of GSTs in this treatment. Since microbe-associated remediation of the pollutant was in progress, the requirement for ROS scavenging GSTs family proteins might be reduced and hence no differential expression was observed in this system.

4 Conclusion

Bioremediation is a suitable technique to restore contaminated sites. Rhizoremediation with bio-augmentation could alleviate the limitations of phytoremediation. Understanding of the molecular mechanism behind the plant-microbe integrated pollutant remediation systems is still unclear. This study, on understanding the molecular mystery of plant-microbe integrated remediation system has revealed interesting insights on suppression of defence mechanism of plant to enhance microbial enrichment thereby tolerating the pollutant stress. Further detailed studies on the complete microbiome and transcriptome of both plant and microbes may help to the better understanding of this bio-augmented remediation strategy and thereby we can engineer this remediation system for enhanced efficiency, eco-friendliness and economic advantages.

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