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De novo transcriptome analysis of *Parthenium hysterophorus* L. and insights into its potential uses

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ABSTRACT

Parthenium hysterophorus L. is a notorious weed, which significantly reduce yield and quality of crops and causes several problems to human health. In, present study an attempt was made to understand the economic value, and survival nature of *Parthenium* through deep transcriptome analysis. Transcriptome analysis of leaf and root tissue of *P. hysterophorus* had resulted 7,832,143 reads in case of leaves, and 9,646,830 reads in case of roots sample with longest read length of 300 and 298 nucleotides, respectively. A total of 35,719 contigs were produced with an average length of 548bp after an assembly in all two samples. The Blastn of the above generated contigs with 61,901 sequences of *P. argentatum* resulted in the identification of 25,947 novel contigs specific to *P. hysterophorus*. The Kyoto Encyclopedia of Genes and Genomes pathway based analysis showed the expression of genes associated with pathways pertaining to biosynthesis of Glucosinolate, Amino acids, and Aminobenzoate degradation etc. The expression pattern of genes like Artemisinic aldehyde Delta (11(13)) reductase, Codeine O-demethylase, Taraxerol synthase, and Curculin-2 related to biosynthetic of therapeutic importance pathways was also evidenced. Further, the heavy metal accumulator property of *P. hysterophorus* was also studied. Expression analysis of heavy metal transporters such as ferrous ion transport protein B, and zinc transporter in roots was also validated with its heavy metal transport activity. This investigation provides new insights for functional studies of *P. hysterophorus* genes involved in biosynthesis of therapeutically important secondary metabolites, and other possible uses such as raw material for rubber industry.

Introduction

Parthenium hysterophorus is considered as one of the worst weed challenging sustainable yield of the crops and poses threat to human health as well as the environment. There are about 12 species of *Parthenium* viz., *P. alpinum* (Nutt.) Torr. and A. Gray, *P. argentatum* A.Gray, *P. cineraceum* Rollins, *P. confertum* A.Gray, *P. fruticosum* Less., *P. hysterophorus* L., *P. incanum* Kunth, *P. integrifolium* L., *P. ligulatum* (M.E. Jones) Barneby, *P. rollinsianum* Rzed., *P. schottii* Greenm. ex Millsp. & Chase, *P. tomentosum* DC grows around the world. Among the species, *P. argentatum* is only species having the economical value (Ponciano et al. 2012). *P. hysterophorus* L. is also a serious weed, growing across the agricultural lands and barren fields in Asia, Africa, Australia and other part of world (Gupta, 1990). The plant parts of *P. hysterophorus* contain growth inhibitors which are allergenic to animals and allelopathic to other plant species. This weed is considered to be a cause of several health issues like allergic respiratory problems, contact dermatitis, mutagenic to human and livestock (Patel, 2011).

Various approaches including cultural practices (Hand pulling, crop rotation, Mulching, etc), biological agents (via *Zygomorpha bicolorata*, *Puccinia abrupta* var), chemical treatment (herbicide like 2,4-D, Simazine, Atrazine, Alachlor and Butachlor) have been attempted to restrict the growth and multiplication of this weed, but the problem still remains as such. Further, *P. hysterophorus* is also an alternative host for the herbivorous insects like mealy-bug, aphids, etc. In spite of many drawbacks of this weed several reports have shown their uses in production of biogas and green manures, preparation of herbal medicines, insecticides, and recovery of phytochemicals for the industries (Bhojar and Gavkare, 1970; Javaid and Shah, 2008). Isolation of several useful secondary metabolites including Phenolic, Flavonoids, Pseudoguaianolides, Alkaloids, Histamine, Saponin, Glucosides, and Triterpene (sesquiterpene) have been also been reported from *P. hysterophorus* plant (Kushwaha and Maurya, 2012; Saini et al. 2014). The plant parts of *P. hysterophorus* also possessed with nematocidal, antifungal, antiamebic, antimalarial, trypanocidal, antibacterial and antiviral properties (Kushwaha and Maurya, 2012). *P. hysterophorus* has been used as a medicine for ulcerated sores, skin diseases, fever etc. in the Caribbean and Central America (Navie et al. 1996; Oudhia, 2001). Herbal boiled soup used to cure various skin disorders, and it has also been used as a remedy for a wide variety of ailments (Domínguez and Sierra, 1970; Morton, 1981). In Jamaica, its extract was used as flea repellent for dogs and other animals (Morton, 1981). It has also been reported the plant parts are source of anticancer, antioxidants and anti HIV compounds (Pandey et al. 2012; Kumar et al. 2013). Thus, this plant could be used as source of several medicinally important bioactive molecules by exploring the metabolic pathways present in *P. hysterophorus* as

previously reported (Kushwaha and Maurya, 2012). Among other approaches genome wide transcriptome analysis of *P. hysterophorus* is a novel approach to explore the metabolic pathways.

Heavy metal contamination has a large share in the environmental pollution, and several weed plants have great potential in heavy metal management and detoxification due their high growth and reproductive rate, efficient dispersal and rapid establishment in unutilized lands (Sharma and Pant, 2015). Some plants have hyperaccumulator property and they can accumulate 50-500 times greater than average plants (Lasat, 2000; Bhargava et al. 2012). Hyperaccumulator property of *Parthenium* spp. has also been reported and is particular *P. hysterophorus* hyperaccumulate Cu, Zn, and Cd from soil (Sharma and Pant, 2015; Essiett et al. 2011; Malik et al. 2010; Sanghamitra et al. 2012). Considering the manifold potential of *P. hysterophorus*, the present study accentuated upon *de novo* transcriptome analysis with an objective to explore its bioactive potential vis-a-vis metal hyperaccumulation activity.

Material and Methods

Selection of plant material, RNA isolation

P. hysterophorus plants (before flowering) were selected from the nursery of Central University of Rajasthan, India, and tagged. Total RNA was isolated from leaf and root tissues of three different plants by using Total RNA purification kit (Jena Biosciences, Germany). The RNA of three plants were pooled and treated as one sample for transcriptome analysis.

Library preparation, high throughput sequencing, de novo assembly and sequence annotation

The integrity and quality of the RNA was checked on agarose gel; quantified by Nanodrop-1000 and used for generation of RNA-Seq libraries. For construction of the RNA-Seq libraries, 1 µg of the total RNA was used from each sample. Total mRNA was isolated from total RNA sample using Dynabeads™ mRNA Purification Kit (Invitrogen) as described in the manufacturer's protocols. Total isolated mRNA was again quality checked on qubit 2.0 fluorometer. cDNA, library preparations and sequencing were carried out with manufacturer's protocols at Ion Torrent platform (Thermo Fisher Scientific).

The raw reads generated from sequencing of each samples were subjected to filtering and trimmed by CLC work bench (www.clcbio.com) for removal of low complexity sequences using the parameters- removal of reads with length of ≤100 bp, removal of low-quality Phred sequences of ≤20, removal of exact duplicate sequences and trimming of reads. The RNA-Seq raw reads from leaf and root libraries were used for quality-filtering algorithms and filtered for weak signals, low-

quality sequences, and the read ends were screened and trimmed for adaptor sequences to yield high quality (HQ) sequences (99.5% accuracy on single base reads). Because no reference genome exists for *P. hysterophorus*, reads were assembled *de novo*. The pre-processed high-quality reads were merged and utilized for *de novo* assembly using CLC Assembler 9.1 genomics workbench (www.clcbio.com) with default parameters for assembling into transcripts. These contigs were further aligned with BioEdit software (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) at default setting, 61, 901 sequences of *P. argentatum* L. (http://compgenomics.ucdavis.edu/data/cwassy_2012/guayule.unigenes) to get unique sequences of *P. hysterophorus* L. The contigs of *de novo* assembly of leaf and root were annotated using Blastx program of Blast2GO nonredundant protein (Nr) database.

Functional annotation, biological pathway assignment and KEGG pathway analysis of de novo assembly

To assign function to each unigene, gene ontology (GO) analysis was performed using GO annotation cellular component, molecular function and biological process. Each annotated sequence may have more than one GO term, either assigned in the different GO categories (biological process, molecular function and cellular component) or in the same category. GO enrichment tests were performed using GO enrichment tool of the Blast2GO. To obtain the functional classification of DE genes between root and leaf, Fisher's exact tests were performed for the determination of significant biological, molecular or cellular components in both the tissues, respectively ($q\text{value} < 0.05$). To gain an overview of gene metabolic pathway networks, the assignment of polypeptides encoded by unigenes from leaf and root transcriptome into metabolic pathways were mapped according to the Kyoto Encyclopedia of Genes and Genomes (KEGG). Enzyme commission (EC) numbers were assigned to unique sequences, based on the Blastx search of protein databases, GO annotation and mapping. Further, KEGG mapping was performed using KEGG plugin of Blast2GO. The output of KEGG analysis includes KEGG orthology (KO) assignments using KEGG automated annotation server (KAAS).

Functional annotation Clusters of Orthologous genes

For functional annotation of 35719 contigs with Clusters of Orthologous the EggNOG platform was used as explained by Huerta-Cepas et al. (2015).

cDNA preparation and qRT-PCR analysis

Total RNA was isolated from leaves, roots and Phytoplasma infected plants by using Total RNA purification kit (Jena Biosciences, Germany), and the cDNA was synthesized using Verso cDNA

synthesis kit following manufacturer's instructions (Thermo Scientific, USA). qRT-PCR of selected contigs were performed by using diluted cDNA products (five- fold with deionized water) as a template, and the DyNamo Color Flash SYBR Green qPCR kit (Thermo Fisher Scientific). Each reaction mixture (10 μ L) was prepared by adding 5 μ l of 2X SYBR Green PCR Master mix, 1 μ l (10 pmol) of each forward and reverse primer (Table 1), and 1 μ L of diluted cDNA. The amplification condition was as follows: initial denaturation at 95°C for 7 min, followed by 45 cycles of 95°C for 10s, 55°C for 10s and 72°C for 10s. The expressions of the genes were normalized with *P. argentatum* Actin (>Contig12870) as internal reference gene. The ' $2^{-\Delta\Delta Ct}$ ' method was applied to analyze the qRT-PCR results (Livak and Schmittgen, 2001).

Extraction of heavy metals from root and shoot of P. hysterophorus and their analysis

From different city of India viz,-Ajmer, Allahabad, Bhopal, Bilaspur, Delhi, Kanpur, Nagpur and Patna, *P. hysterophorus* plants (ecotypes) were collected and dried at room temperature. Dried tissues were chopped into small pieces, and 250 mg of those chopped tissues were transferred to 50 ml beaker followed by incubation with 3 ml of HNO₃ for overnight. Subsequently, the samples were stirred continuously by placing in hot plate at 90°C by adding one ml of HNO₃, till the sample become whitish. The wet sample were added with 3 ml of H₂O, and filtered by Whatman filter paper 41, and this process was repeated thrice. About 9 ml of filtrate were stored at 4°C, and were sent to Central Instrumental Facility, National Botanical Research Institute, Lucknow, India for heavy metal analysis. The heavy metal content in these samples was estimated using ICP-MS (7500cx, Agilent, Japan).

Results and Discussion

Transcriptome sequencing of P. hysterophorus L. and de novo assembly

Transcriptome sequences were generated from the cDNA libraries constructed using leaf and root samples of *P. hysterophorus*. The paired-end sequencing-by-synthesis on Ion Torrent platform (Thermo Scientific) generated a raw data of 500.8 Mb and 500.9Mb from leaf and root sample libraries, respectively. Maximum read length was 298 and 300bp for root and leaf samples, respectively. After quality checking and processing of the raw reads data, 7,832,143 and 9,646,830 reads of leaf and root samples were retained for further assembly (Table 2). These high-quality reads were assembled and a total of 35,719 contigs with average length of 548 bp (Range 250-6132) were generated (Table 2). Blastn of these contigs with 61,901 sequences of *P. argentatum* resulted in the identification of 25,947 novel contigs unique to *P. hysterophorus* L. These

transcriptome derived data was deposited at BioSample database with the BioSample accessions SAMN09374370 and SAMN09374371.

Table 1. Primer used in qRT-PCR (5'-3').

Primer sequences			
Selected Condition	Contig Id	Forward (5'-3')	Reverse (5'-3')
Medicinally important genes	Contig_10251	AGTCGATGAAAGGGCACATCTT	AATAAGTGTCCGTAGCCCGAG
	Contig_10682	TCGGTTATGAGAAAGCCACC	TTGGCTCCGATGACACGATG
	Contig_10995	AGGCGCAAGGACTGTTATG	ACCGCGGTTTCTTGCTGAT
	Contig_11288	CAGAGTGAGACTCGATGCGTT	CTCAGAAAGCATGGCAAGG
	Contig_11932	ATGGCGTTTGGATCCCTGTG	GTCGCCCTATGCTCAATGCT
	Contig_14549	TCATGGCCTCTACCACCATTTC	CCCAAATGTAAGCATAGCCGT
	Contig_16230	CGCCGATCAGTTACATTGCC	ACCGGGAGATTAAACCCGTC
	Contig_17636	AACCCATTTGCATACATGCCT	GTTTCGTGCACGTAATTCGGAG
	Contig_18305	TGCTTTGTGACGCATTGTGG	CCCGTTCCCATAACGTCTTC
Pathogenesis related gene	Contig_16342	GTCAGGCATCTACCATCGAG	CATACACGGGGACATGTCTGG
	Contig_17254	TCCCTTCACGGGTGTTTCTG	ACCACCCACGATTATTGGCG
	Contig_1727	TCGAACACGCTTCGAACAAC	AGGAAGTCGTCCGGATCCAT
	Contig_17868	CAAGGGACCACCGGACAATC	TTGGGGGTGTGAGTGCATTA
	Contig_18095	GTTCAAGTTAGCCAAGGTGG	TTCCCATCCCCTTTTAGCTC
	Contig_1955	GCTATCGACTTCACTTCTGGC	TGGATAGCACTTGAGACGGG
	Contig_21931	TCGCGGTTTCAACGGGAA	AATCGATAACCACGGGCCAA
	Contig_29314	TGCCTTTCTAACCAGCTC	GTCGAAGCCAGCTCATGTTG
Genes involved in Rubber biosynthesis	Contig_4082	GGAGCTGATGCACGTTATGG	TGAGTCGACGTGGGTGAGAT
	Contig_41	CAGCGCAGAACATTGAGAGC	CATTGGATCCCGCCGATTCT
	Contig_3114	TGCTCCGTGACACTTGTGAG	AGAGATGTTAGCAACCGGGG
	Contig_5630	GGCCGAGAAACAGATCCCTG	GTCAAGAGCTCGAAGGACCA
	Contig_5711	CTTCCGAACCAACATGCCAC	GTGTCCGCCAGTGAGTTCAG
	Contig_34679	TCCAATCCCACAACACATTGC	CTAGGAATCCGGCTTTGTGGC
Gene involved in Heavy metal transportation	Contig_23348	AGAGGCATTCGTCAGCGTAG	TACGACTACAAGCCAAGCCG
	Contig_9290	TTGGGAGCTGGTTTGCTAGG	GGATGGTTAGGATTGCGGCT
	Contig_364	AAGTGGCATAACCCATGTTG	GTGCGCCTAACAATGGTCTG
	Contig_19948	TGGCTCTAGTTGACCTGCTG	AAAGGACATACACCCAGCCC
	Contig_2731	TCTACTCGGCCAGTTTGCTC	TTCGGAGTCTGGTTCGATGC
	Contig_20841	TCCAGACGGTGGCTGTTTAG	AGCAGTAACCCTCTCCGTTG
	Contig_23236	TTCAGTGATCCCTCCCGAGC	CACTCAACCCACCAACTCCA
	Contig_14801	TGGGTTTCGAGCCTAGAGAG	TCCACATTGTCTGGTTGTGGT
Actin(<i>Parthenium argentatum</i>)	Contig12870	GAAGAGAACCTCAGGGCAAC	CGAGCAAGAGCTTGAGACTG

Table 2. The summary of sequencing and assembly data output.

Events	Leaf	Root	Assembly result
Data output (Mb)	500.8	500.9	-
Number of reads/contigs	7,832,143	9,646,830	35,719
Maximum read length	300	298	548 (Averages length)

Functional annotation of the *de novo* assembly

The *de novo* assembly of *P. hysterophorus* was annotated with Blastx, 33,765 contigs were successfully mapped by Blast2x annotations and used to classify the functions of the contigs of *P. hysterophorus*. 22,069 contigs were mapped to GO terms by Blast2GO mapping and were grouped to three main categories (biological processes, molecular functions, and cellular components) of the GO classification. In case of the contigs grouped under cellular component, high proportion of transcripts belonged to cell organelle, membrane extracellular region and macromolecular complex (Figure 1). Transcripts, categorized under molecular functions, have possessed with attributes of domain binding, catalytic activity, transporter activity, signal transduction, molecular regulation, and transcription factors etc. (Figure 1). While some processes like antioxidant activity, nutrient reservoir activity, metallochaperone activity and chemorepellant activity were also reported. In the group of biological processes, transcripts with putative role in cellular, metabolic, biological regulation, developmental, and cellular component biogenesis were abundant (Figure 1).

Functional characterization using KEGG

Functional classification and pathway assignment was performed by the Kyoto Encyclopedia of Genes and Genomes (KEGG). To identify the biological pathways that are functional in the leaf and root tissues of *P. hysterophorus*, and 13,301 contigs were mapped to enzymes and their enzyme code distribution is represented in Supplementary file 1. Hydrolases and transferases were the most abundant enzymes represented by 4687 and 4428 contigs, respectively. In total, all contigs of *P. hysterophorus* were assigned to 156 KEGG pathways (Supplementary file 1) involved in biosynthesis of various metabolites. The pathways responsible for the basic metabolism processes, such as arginine and proline metabolism (PATH 00330), cysteine and methionine metabolism (PATH 00270), alanine, aspartate and glutamate metabolism (PATH 00250), glycolysis (PATH00010), citrate cycle (PATH 00020), pentose and glucuronate interconversion (PATH 00040), purine metabolism (PATH 00230), valine, leucine and isoleucine degradation (PATH 00280) were most abundant (Supplementary file 1). Even many pathways related to secondary metabolism including isoflavonoid (PATH 00943) and isoquinoline alkaloid biosynthesis (PATH

00950), aminobenzoate degradation (PATH 00627), biosynthesis of antibiotics (PATH 01130), monoterpene biosynthesis (PATH 00902), phenylpropanoid biosynthesis (PATH 00940), terpenoid backbone biosynthesis (PATH 00900), and glucosinolate biosynthesis pathway (PATH 00966) were present (Supplementary file 1). Interestingly antibiotic biosynthesis pathway (PATH 01130) was also more abundant than other pathways (Supplementary file 1).

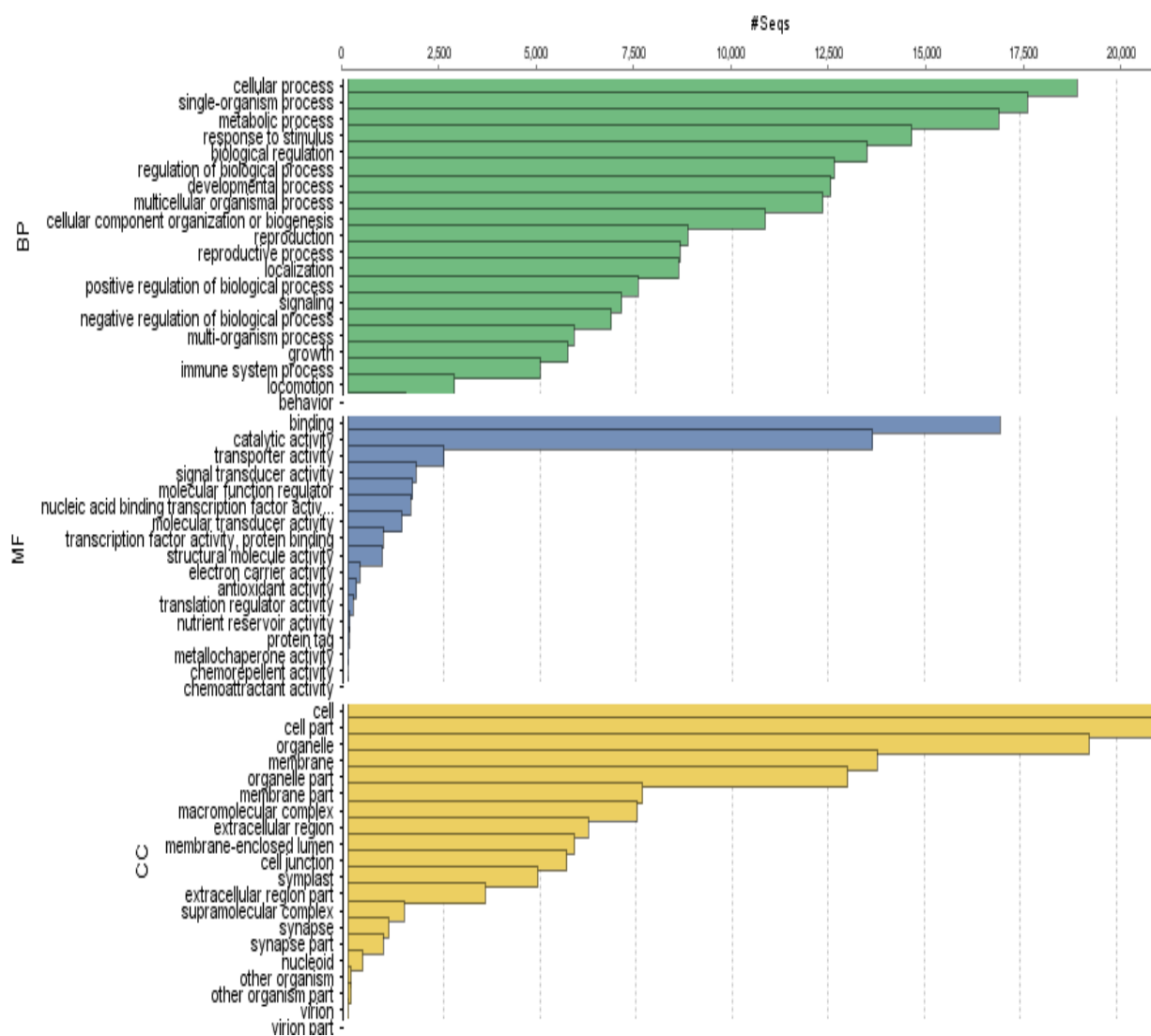


Figure 1. Classification of the contigs in three main categories: cellular component (CC), molecular function (MF) and biological process (BP).

Functional annotation of Orthologous genes Clusters

Out of 35719 *de novo* assembled contigs, 11572 showed significant homology and were successfully analyzed by EggNOG. 10818 contigs were successfully annotated and separated into 24

categories abbreviated as A to Z. (Table 3 and Figure 2). Because some of these unigenes were annotated with multiple COG functions, so total of 12,401 functional annotations were produced (Table 3). These COG annotations were grouped into 24 functional categories (Figure 2) and among them five categories include “Unknown function” (22.69%); “Signal Transduction” (10.38%), “Posttranslational modification, protein turnover and chaperones” (8.7%), “Transcription” (6.79%); and “Carbohydrate metabolism and transport” (6.39%). 754 contigs which were not assigned to any of the category and were kept separate as not categorized. The major category of the genes with unknown function can be studied to know further about this weed’s behavior and other important pathways.

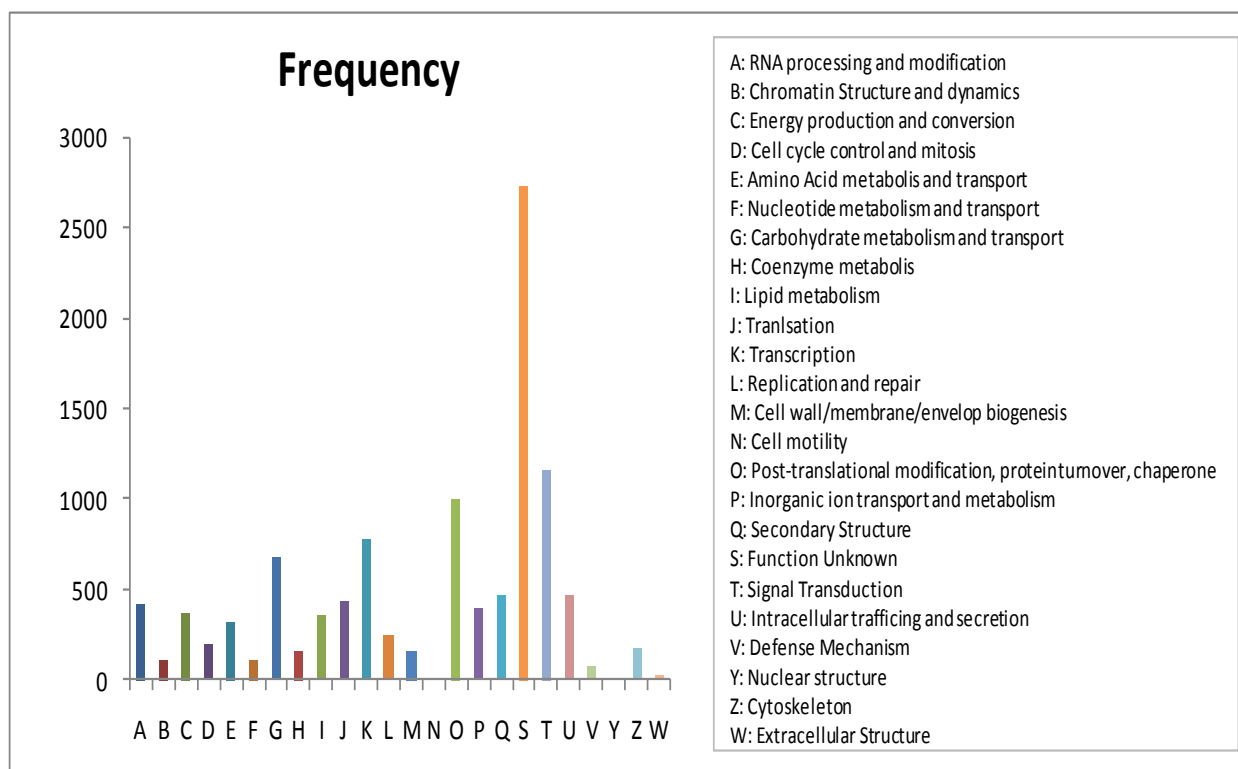


Figure 2. Function Classification of the *Parthenium hysterophorus* transcriptome by Cluster of Orthologous Genes. A total of 11,572 contigs showing significant homology to the COGs database at NCBI (E-value $\leq 1.0e-5$) have a COG classification among the 24 categories.

Universal expression pattern of nutrient exporter and defense-related transcripts

P. hysterophorus plant is supposed to be one of the best survivors among other known weeds across tropical and subtropical countries. The expression pattern of gene related to nutrient transport, heat and water stress management in transcriptome of *P. hysterophorus*, were analyzed and found that heat stress transcription factor, peptide transporter PTR1, nitrate transporter, urease accessory protein UreH, urea-proton symporter, nitrate reductase, desiccation-related

protein PCC13-62, senescence/dehydration-associated protein and early-responsive genes to dehydration stress were showed higher expression in the root tissue (Supplementary file 2). The results showed that expression of these genes might help in avoiding the abiotic stresses by regulating metabolic activity as well as nutrient uptake to the plants (Supplementary file 2). Further, the expression patterns of genes related to management of biotic stress were also assessed and the genes conferring resistance and tolerance to biotic stress, and hypersensitive response were universally expressed in leaf and root tissues. Constituent expression of pEARL1-like lipid transfer protein 3, pathogenesis-related genes transcriptional activator PTI5, pathogenesis-related protein PRB1-3, probable disease resistance proteins, leaf rust 10 disease-resistance locus receptor, defensin-like protein 1 and A3, putative disease resistance family protein like RPP4, RPP8, RPP13, MLO-like protein 11, protein overexpression of cationic peroxidase 3, disease resistance protein (RGA2), disease resistance protein (ADR1), putative disease resistance protein (At4g19050 and At4g27220), BTB/POZ domain-containing protein (At5g66560), pathogenesis-related protein 1 and nematode resistance protein-like HSPRO2 were also observed (Supplementary file 2).

Table 3. Cluster of Ortholog Genes (COG) analysis by EggNOG.

Category Description	Abbreviation	Annotations	Percentage %
RNA processing and modification	A	413	3.43
Chromatin Structure and dynamics	B	106	0.90
Energy production and conversion	C	371	3.08
Cell cycle control and mitosis	D	198	1.76
Amino Acid metabolis and transport	E	320	2.66
Nucleotide metabolism and transport	F	112	0.93
Carbohydrate metabolism and transport	G	683	6.39
Coenzyme metabolis	H	164	1.40
Lipid metabolism	I	360	3.03
Tranlsation	J	427	3.58
Transcription	K	777	6.79
Replication and repair	L	246	2.19
Cell wall/membrane/envelop biogenesis	M	154	1.60
Cell motility	N	3	0.02
Post-translational modification, protein turnover, chaperone functions	O	1005	8.70
Inorganic ion transport and metabolism	P	390	3.26
Secondary Structure	Q	471	4.04
Function Unknown	S	2732	22.69
Signal Transduction	T	1163	10.38
Intracellular trafficking and secretion	U	468	4.18
Defense Mechanism	V	74	0.61
Nuclear structure	Y	2	0.34
Cytoskeleton	Z	174	1.76
Extracellular Structure	W	17	0.14
Not categorized	-	754	6.26
Total		12041	

Similarly, fungal resistance genes, such as chitin elicitor receptor kinase 1, enhanced disease resistance 2, chitinase1, chitinase 2, endochitinase 4 were showed predominant expression (Supplementary file 2). Majority of these genes associated with either host plant resistance mechanisms against various pathogens and pests or the biotic stresses. Thus, the *P. hysterothorus* plants might have showed greater endurance to biotic and abiotic stresses, and showed its best survival nature baring a few such as witches broom disease (Li et al. 2011; Keshwal, 1982; Yadav et al. 2015; Fauzi, 2009). Further, the expression pattern of some of the pathogenesis related genes viz; pathogenesis-related protein 1 (PR-1), nematode resistance protein-(HSPRO2), rust resistance kinase Lr10, chitinase1, leaf rust 10 disease-resistance locus receptor-like protein kinase (Like 1.3), and defensin-A3 were evaluated among different tissue of healthy plant as well as phytoplasma infected plants (Table 4; Figure 3) showing symptoms of witches broom disease. On comparison between leaf and root tissue of healthy plants, expression of TMV Disease resistance protein RPP4, Pathogenesis-related protein PR-1, Rust resistance kinase Lr10, Leaf Rust 10 Disease-Resistance Locus Receptor-Like Protein Kinase-Like 1.3 were significantly higher in leaves as compared to roots (Table 4). Contrasting to this, PR-1, Chitinase 1, Nematode resistance protein-like HSPRO2 and Defensin-A3 showed higher expression in roots (Table 4).

Table 4: Expression pattern of transcript related to Pathogenesis related gene

Gene name	Contigs ID	Leaf (RPKM)	Root (RPKM)	Fold change expression in leaves (compare to root) at transcriptome	Fold change expression in leaves (compare to root) at qRT-PCR (\pm showed STDEV)
Pathogenesis-related protein 1	Contig_17254	32.48	6.83	-7.9	1.64 \pm 0.16
Nematode resistance protein-like HSPRO2	Contig_1727	25.67	134.86	9.05	1.03 \pm 0.07
Pathogenesis-related protein PR-1	Contig_17868	6.54	30.58	2.76	6.36 \pm 0.79
Rust resistance kinase Lr10	Contig_18095	6.61	2.60	-4.22	2.63 \pm 0.08
Chitinase 1	Contig_1955	5.58	2.42	2.4	1.83 \pm 0.01
Leaf Rust 10 Disease-Resistance Locus Receptor-Like Protein Kinase-Like 1.3	Contig_21931	9.4	18.16	1.14	2.21 \pm 0.23
Defensin-A3	Contig_29314	14.58	3.25	-7.4	1.74 \pm 0.28

We have also compared the expression pattern of above pathogenesis related genes between control and phytoplasma infected *P. hysterothorus* plants' leaf and observed that the expression of

TMV resistance protein RPP4, PR-1, and Like 1.3 were significantly suppressed in the leaf tissue of infected plants (Figure 3).

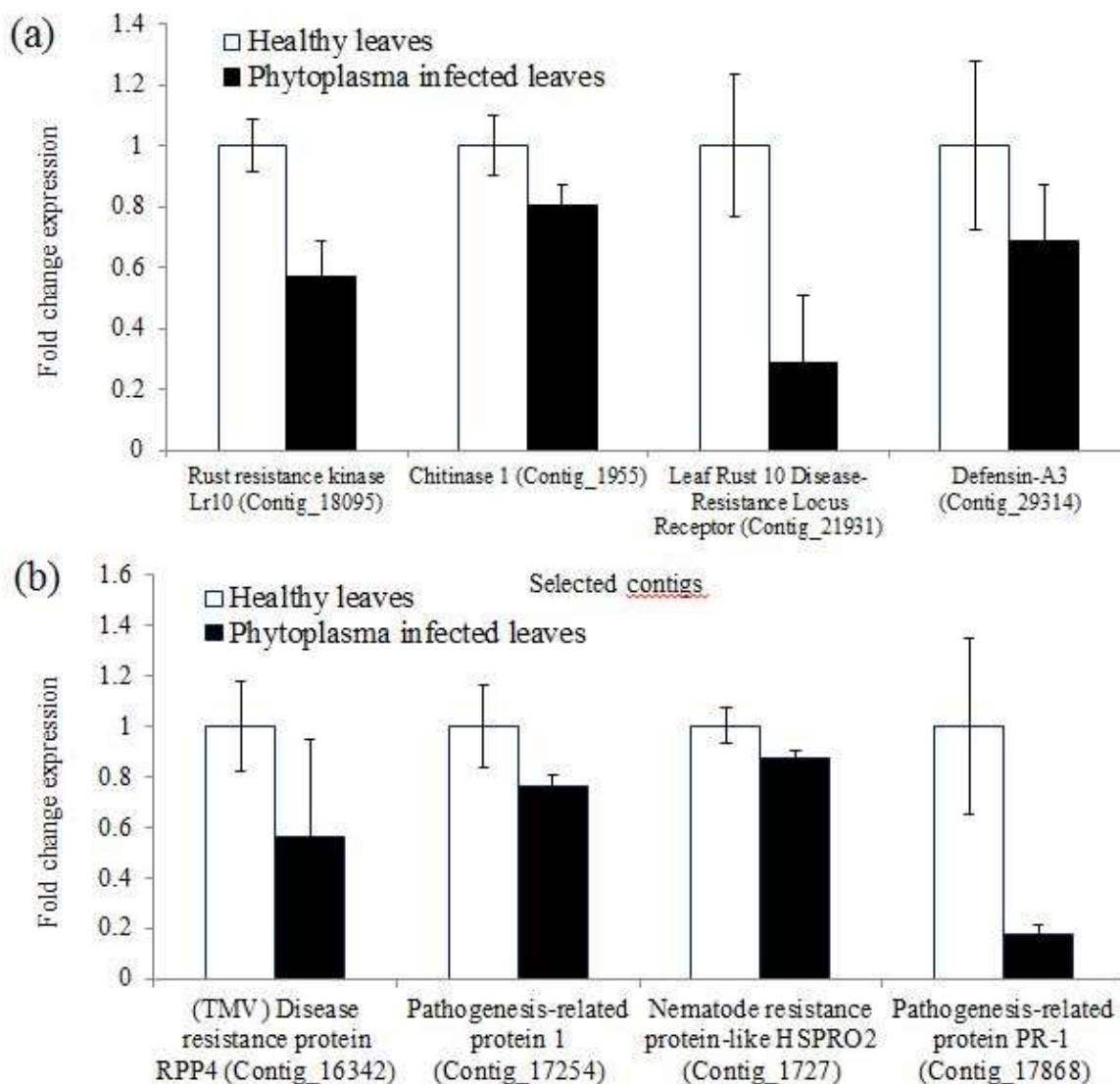


Figure 3. Expression pattern of pathogenesis related genes in Phytoplasma infected and healthy plants.

Expression pattern of medicinal and nutritional value related transcripts

The plant parts of *P. hysterophorus* were used in various traditional systems of medicines including skin diseases, fever and anemia etc. (Kushwaha and Maurya, 2012). Thus, the expressions patterns of various genes contributing biosynthesis of metabolites were also evaluated. The expression of genes including Hyoscyamine 6-dioxygenase, Neomenthol dehydrogenase, GDSL esterase/lipase LIP-4, Artemisinic aldehyde Delta (11(13)) reductase, Codeine O-demethylase,

Taraxerol synthase, Betaine aldehyde dehydrogenase 2, Curculin-2 were quite evident from their corresponding transcript abundance which evidenced towards their biosynthesis in *P. hysterophorus* (Table 5).

Table 5. Expression pattern of transcript related to Medicinally important genes.

Gene name	Contigs ID	Leaf (RPKM)	Root (RPKM)	Fold change expression in leaves (compare to root)
Hyoscyamine 6-dioxygenase	Contig_3338	464.06	29.79	91.70
(+)-Neomenthol dehydrogenase	Contig_10251	361.7	40.12	-15.17
GDSL esterase/lipase LIP-4	Contig_10267	2.8	6.4	1.3
Artemisinic aldehyde Delta(11(13)) reductase	Contig_10682	84.5	21.1	-6.73
Betaine aldehyde dehydrogenase 2	Contig_5906	27.26	61.33	1.33
Secologanin synthase	Contig_17636	2.5	11.3	2.5
Crocetin glucosyltransferase	Contig_18305	203.2	1.01	-320.26
Codeine O-demethylase	Contig_11932	5.1	39.6	4.58
Taraxerol synthase	Contig_14549	16.7	6.1	-4.5
Curculin-2	Contig_18744	2.6	9.7	2.13
Folylpolyglutamate synthase	Contig_16262	13.2	13.5	-1.6
Tocopherol cyclase	Contig_31026	13.05	2.03	-10.6
Hyaluronidase-3	Contig_10758	44.96	36.79	-2.05
Hydroxymethylglutaryl-CoA synthase	Contig_150	40.59	36.72	4.34
3-hydroxy-3-methylglutaryl-coenzyme A reductase	Contig_41	129.33	2850.17	13.07
Hyaluronoglucosaminidase-3	Contig_10758	44.96	36.79	-2.05
Nicotinamidase 1	Contig_10995	132.4	39.6	-5.6
Nicotianamine aminotransferase B	Contig_4322	34.44	36.03	-1.6
Biotin synthase	Contig_11288	6.4	15.05	1.3
Thiamine thiazole synthase	Contig_69	1396.79	4659.93	1.9
Farnesyl pyrophosphate synthase	Contig_3114	5.21	17.48	1.98
Farnesyl diphosphate synthase	Contig_267	102.67	120.74	-1.43
Biotin carboxyl carrier protein of acetyl-CoA carboxylase	Contig_645	32.06	41.73	-1.29
Anthranilate synthase	Contig_1344	10.20	14.07	-1.22
Nicotinate-nucleotide adenyltransferase	Contig_8792	21.69	15.28	-2.39
Homolog of Tropinone reductase	Contig_12789	13.41	5.70	-3.94
Retinol dehydrogenase 13	Contig_13097	0	11.25	179.91
Patatin-like protein 6	Contig_776	11.21	152.19	8.08
Thaumatococin-like protein	Contig_16230	0	6.05	84.4

In addition, expression analysis of the genes involved in vitamin biosynthesis viz., Folylpolylglutamate synthase, Tocopherol cyclase, Hyaluronidase-3, Hydroxymethylglutaryl-CoA synthase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, Hyaluronoglucosaminidase-3, Nicotinamidase 1, Nicotianamine aminotransferase B, Biotin synthase, Thiamine thiazole synthase, Farnesyl pyrophosphate synthase, Farnesyl diphosphate synthase, Biotin carboxyl carrier protein of acetyl-CoA carboxylase, Anthranilate synthase, Nicotinate-nucleotide adenyltransferase, homolog of Tropinone reductase, and Retinol dehydrogenase 13 have also affirmed in leaf and root tissues of *P. hysterophorus* (Table 5). Expression of Patatin-like protein 6 and Thaumatin-like protein were also found in transcriptome of *P. hysterophorus*.

To validate their existence in *P. hysterophorus*, expression of some selected gene viz., (+)-neomenthol dehydrogenase, Artemisinic aldehyde Delta(11(13)) reductase, Nicotinamidase 1, Biotin synthase, Codeine O-demethylase, Taraxerol synthase, Thaumatin-like protein, Secologanin synthase, and Crocetin glucosyltransferase were assessed by using qRT-PCR analysis (Figure 4). The results showed that (+)-neomenthol dehydrogenase was highly expressed in leaves as compared to roots (Figure 4 a) while the expression of Artemisinic aldehyde Delta (11(13)) reductase, Biotin synthase, Taraxerol synthase, Thaumatin-like protein and Crocetin glucosyltransferase was expressed in both root and leave tissues without much variation (Figure 4 a-b). The expression of AaDR and subsequent phytochemical analysis substantiated towards presence of a compound very similar to artemisinin in *P. hysterophorous* suggest its potential use as a source for development of antimalarial compound (Supplementary file 3). The expression of Nicotinamidase 1, Codeine O-demethylase, and Secologanin synthase was comparatively more in roots than leaves (Figure 4 a-b). Thus, these findings on expression of many genes related to biosynthetic pathways of several medicinally and nutritionally important bioactive molecules in *P. hysterophorus*, substantiated the scope for its use as a source of these metabolites to enrich the herbal industry.

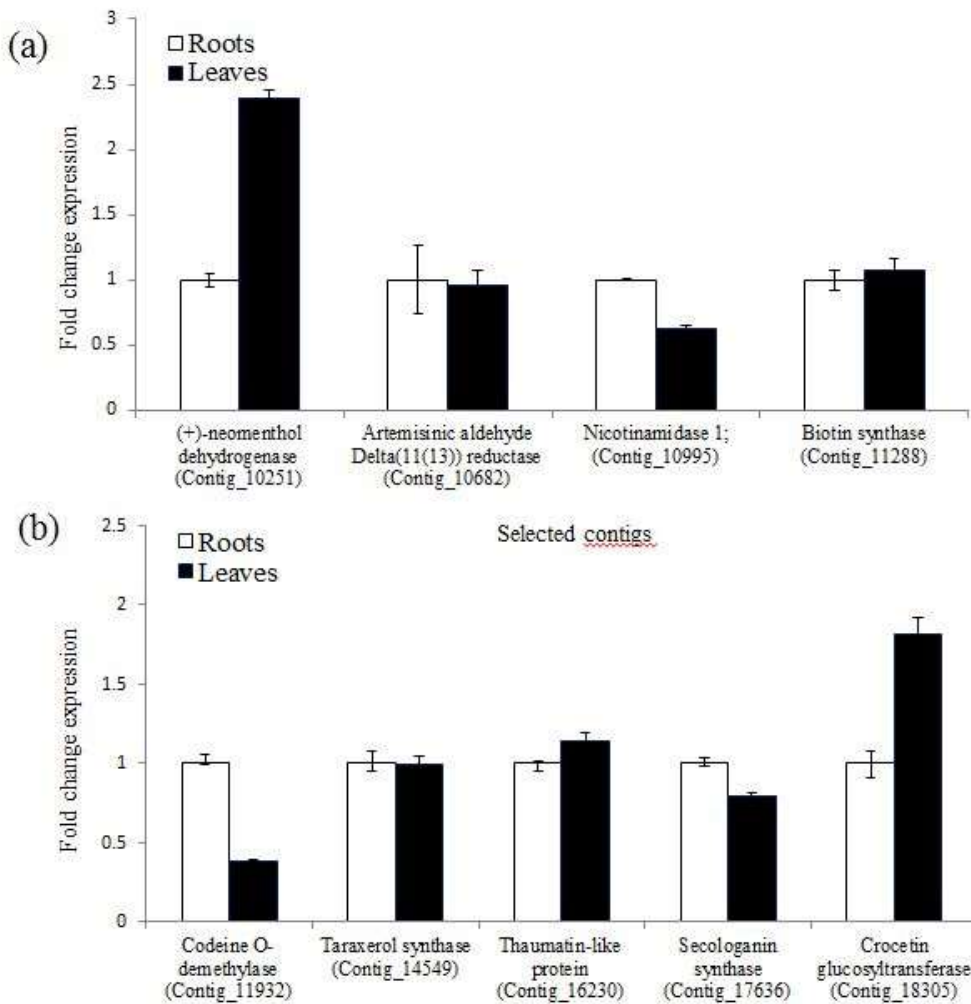


Figure 4. Expression pattern of medicinal and nutritional value related genes.

Expression pattern of rubber biosynthesis related genes

Expression analysis of major genes involved in rubber biosynthesis showed their expression in transcriptome of leaf and root of *P. hysterophorus*, which also suggests it's possible use of this plant as raw material for rubber biosynthesis. The genes responsible for the biosynthesis of proteins like Hydroxymethylglutaryl-CoA synthase (HMGS), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), Farnesyl pyrophosphate synthase (FPPS), Allene oxide synthase (AOS/RPP), and Rubber cis-polyprenyltransferase HRT2 (CPT) found their expression in transcriptome profile of *P. hysterophorus* (Table 6). Except Rubber cis-polyprenyltransferase HRT2 (CPT), Farnesyl pyrophosphate synthase (FPPS) and Allene oxide synthase (AOS/RPP) the other genes showed higher level of expression in leaves in comparison to roots (Table 6). Further, to confirm their expression with qRT-PCR, different primers were designed (Table 1) and assessed their expression

in different tissues. The qRT-PCR results also validated the in silico expression data except cis-polyprenyltransferase HRT2 (CPT) expression (Figure 5 a-b). The maximum expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) was found in roots and is followed by Hydroxymethylglutaryl-CoA synthase (HMGS; Figure 5 a-b), while other selected genes were more or less equivalently expressed in both root and leaves (Figure 5 a-b).

Table 6: Expression pattern of transcript related to rubber biosynthesis

Gene name	Contigs ID	Leaf (RPKM)	Root (RPKM)	Fold change expression in leaves (compare to root)
Hydroxymethylglutaryl-CoA synthase	Contig_4082	26.27	224.49	5.06
3-hydroxy-3-methylglutaryl-coenzyme A reductase	Contig_41	129.33	2850.17	13.07
Farnesyl pyrophosphate synthase	Contig_3114	5.2	17.48	1.98
Squalene synthase	Contig_5630	69.3	35.23	-3.2
Allene oxide synthase	Contig_5711	318.8	186.8	-2.8
Rubber cis-polyprenyltransferase HRT2	Contig_34679	45.71	0	-960.6

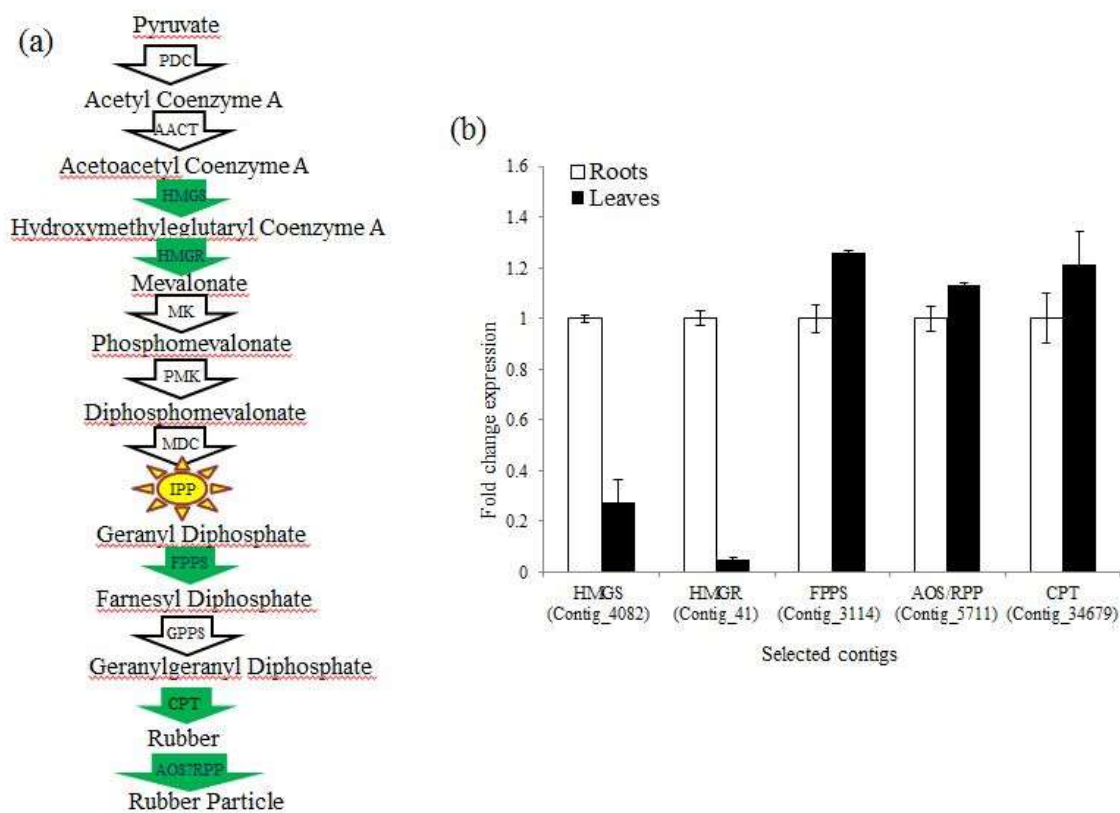


Figure 5. Generalized pathway and expression pattern of rubber biosynthesis related genes.

General cytosolic mevalonate (MVA) pathway of rubber biosynthesis (modified from Chow et al. 2011; Li et al. 2016) (a).qRT-PCR result of some selected genes (Green box of Figure a) viz., in roots and leaves tissue (b).PDC, pyruvate dehydrogenase complex; AACT, acetyl coenzyme A acetyltransferase; HMGS, hydroxymethylglutaryl coenzyme A synthase; HMGR, hydroxymethylglutaryl coenzyme A reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; MDC, diphosphomevalonate decarboxylase; IPP isopentenyl diphosphate; FPPS, farnesyl pyrophosphate synthase; GPPS, geranylgeranyl pyrophosphate synthase; CPT, cisprenyltransferase; AOS/RPP, Cytochrome P450 74A2 (allene oxide synthase/rubber particle protein).

Heavy metal accumulator property and expression pattern of related transporters

Heavy metal accumulation activity of *P. hysterophorus* ecotypes at rosette stage, were collected from different cities of India viz,-Ajmer, Allahabad, Bhopal, Bilaspur, Delhi, Kanpur, Nagpur and Patna were assessed by ICP-MS analysis of heavy metals in both leaf and root tissues. The heavy metals, such as, cadmium (Cd), chromium (Cr), copper (Cu), ferrous (Fe), manganese (Mn), nickel (Ni), and zinc (Zn) were detected in both leaves and roots of *P. hysterophorus* plants from different cities (Table 7). The leaves and roots of all ecotypes showed accumulation of Ni and Cr in minimal concentration (Table 7). Maximum concentration of Mn 142.2 ug/g was found in root tissue of the ecotype collected from Bhopal ecotype whereas minimal conc of Mn was noticed in Kanpur (Table 7). Further, Fe content was maximum in root tissues of the Allahabad ecotype (400.357 ug/g), and was followed by Bilaspur (134.3 ug/g) (Table 7). Most of the genotypes showed hyperaccumulation in root tissue as compared to leaves. The accumulation of Zn and Fe in leaves and roots was also quite evident, thus *P. hysterophorous* could be used as micronutrient enriched fertilizer with biocompost (Table 7) to nurture the upcoming crops in the respective agricultural fields. Further the heavy metal accumulation property of this species hypothesize it's use as a bioremediation agent for elimination of heavy metals from waste and left land of used mines.

To validate the heavy metal hyperaccumulation activity, expression analysis of transporters involved in heavy metal accumulation was carried out in root and leaf tissue of the ecotype collected from Central University of Rajasthan premises only (Supplementary file 4). The expression of transcripts corresponding to the transporter genes associated with Ferrous iron transport protein, Zinc transporter 4, Zinc transporter 8, and Manganese-transporting ATPase PDR2 were evaluated through qRT-PCR analysis in both root and leaf tissue (Figure 6). Except Zinc transporter 4, the expression patterns of other transcripts were more in roots in comparison to leaf tissues (Figure 6). Maximum expression of Manganese-transporting ATPase PDR2 of eightfold as compare to leaves was found in the ecotype tested here (Figure 6).

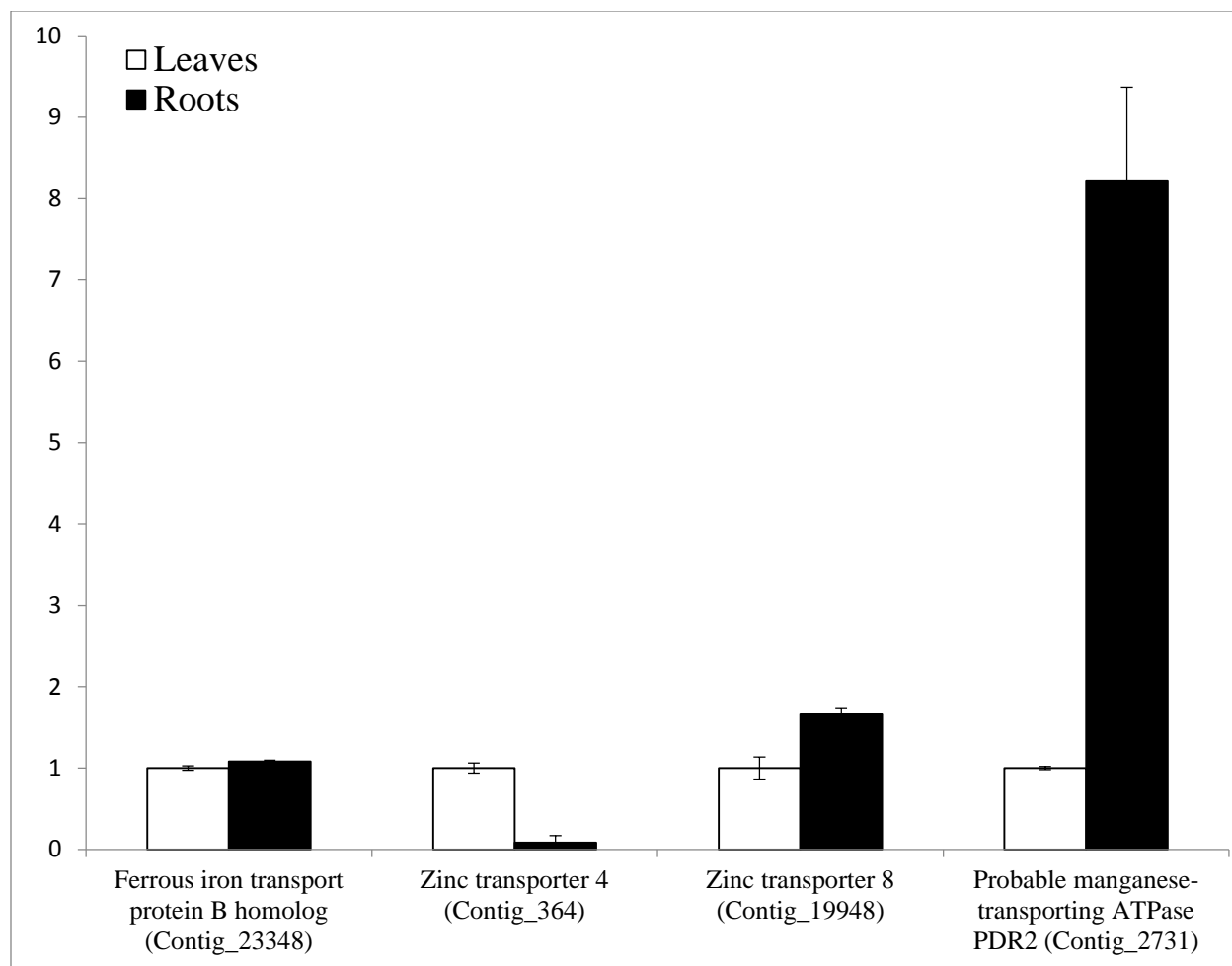


Figure 6. Expression pattern of transporters involved in heavy metal accumulation.

P. hysterophorus devastating weed, it seriously affect the productivity of many crops. Due to their vigorous growth, high multiplication with efficient seed dispersal mechanisms, and rapid establishment, they grow easily. Although *P. hysterophorus* is considered as notorious weed, its value in production of green manure, biogas, herbal medicines, insecticides, production of secondary metabolites have been reported (Bhoyar and Gavkare, 1970; Javaid and Shah, 2008; Kushwaha and Maurya, 2012; Saini et al. 2014). Considering the importance of this weed, in the present study the transcriptome of *P. hysterophorus* was assembled *de novo* to elucidate and validate its possible uses in future. Transcriptome sequencing revealed output was about 500.8 Mb and 500.9 Mb in leaf (L) and Root (R) tissues respectively and their assembly yielded about 35,719 contigs. Differential expression analysis using DEseq revealed many processes that are tissue-specific, in particular between roots and leaves. In general, there were 7355 transcripts that were found to be differentially expressed between leaf and root with the FDR $q < 0.05$ value. A total

of 3071 and 4283 transcripts were found to be differentially expressed in leaves and roots, respectively.

Table 7. Metals Concentration ($\mu\text{g/g}$) in leaves and roots of *Parthenium hysterophorus* L.

Cities	Cd		Cr		Cu		Fe
	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves
Ajmer	4.341±0.25	2.147±0.2	2.956±0.05	2.216±0.04	9.796±0.14	11.788±0.2	67.547±0.73
Allahabad	5.797±0.3	6.237±0.03	0.404±0.04	0.202±0.001	1.408±0.1	2.039±0.69	12.905±1.08
Bhopal	1.012±1.01	9.436±0.1	0.632±0.05	0.856±0.04	2.58±0.45	3.42±0.1	30.265±1.24
Bilaspur	2.109±0.02	2.171±0.04	1.38±0.03	2.94±0.05	8.6030.08	8.132±0.17	47.307±0.67
Delhi	7.956±0.93	6.565±0.21	1.559±0.04	0.503±0.01	10.117±0.29	7.212±0.11	43.533±2.04
Kanpur	3.987±0.17	7.981±0.09	0.768±0.01	0.735±0.02	6.056±0.09	5.051±0.04	25.679±0.55
Nagpur	3.715±0.09	6.007±0.18	1.732±0.01	1.315±0.05	12.035±0.15	5.14±0.05	81.8±1.42
Patna	9.605±0.16	2.601±0.28	1.875±0.05	2.277±0.02	13.133±0.21	6.06±0.05	88.96±0.52
Cities	Fe		Mn		Ni		Zn
	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
Ajmer	89.707±.84	42.387±0.71	40.933±0.56	1.632±0.19	2.161±0.03	46.707 ±0.079	32.111±0.37
Allahabad	400.357±5.36	5.581±0.5	12.402±0.08	5.829±0.21	1.3360.43	10.315±0.47	18.668±2.41
Bhopal	27.729±0.81	18.391±3.39	142.2±3.01	1.011±0.11	1.596±0.05	11.576±1.44	39.351±0.57
Bilaspur	134.307±2.16	33.957±0.33	87.16±1.65	1.211±0.03	2.904±0.03	21.201±0.26	29.872±0.62
Delhi	15.367±0.18	17.953±0.4	7.219±0.12	1.467±0.03	1.952±0.05	29.744±0.52	14.352±0.23
Kanpur	26.631±0.22	17.353±0.26	8.524±0.13	1.595±0.03	1.303±0.03	24.465±0.39	16.784±0.18
Nagpur	30.303±0.34	47.067±0.55	14.811±0.14	6.355±0.09	3.237±0.01	29.528±0.39	21.844±0.07
Patna	94.707±1.03	38.015±0.37	50.227±0.42	3.039±0.11	3.967±0.04	31.613±0.29	34.003±0.31

The transcripts were well distributed into different functional groups: molecular function (MF), biological process (BP) and cellular component (CC), were associated with binding activity, membranes, catalytic, transporter, antioxidant, nutrient reservoir, metallochaperone, chemorepellant activity and cellular developmental processes in both leave and root tissues. The distribution pattern of the unigenes for all the two libraries under different GO terms exhibited similarity. The KEGG based classification suggested about 156 KEGG pathways mainly related to Hydrolase and Transferase, secondary metabolite production and amino acid metabolism. High expression of amino acid metabolism genes, Biotin synthase, Thiamine thiazole synthase, Biotin carboxyl carrier protein of acetyl-CoA carboxylase, Nicotinate-nucleotide adenyltransferase, and

Retinol dehydrogenase 13 and presence of 48-54% protein in plants (Khan et al. 2011) with easy growing nature in wide range of environment suggest it possible utilization in fodder industry after elimination of parthenin (Kushwaha and Maurya, 2012; Khan et al. 2011; Narasimhan et al. 1993) or by using parthenin mutants of *P. hysterophorus* L.

Expression of genes like Hyoscyamine 6-dioxygenase, Artemisinic aldehyde Delta (11(13)) reductase, Codeine O-demethylase, Taraxerol synthase, Betaine aldehyde dehydrogenase 2, Curculin-2 were analyzed in *P. hysterophorus*, and the findings substantiated their involvement in synthesis of important chemicals like Hyoscyamine, Artemisinin, Codeine, Taraxerol, and Betaine in *P. hysterophorus* (Zhang et al. 2008). Further, optimization of extraction of these bioactive compounds from *P. hysterophorus* and its subsequent scaling up could use this weed as an alternative source for production of these metabolites. Among 12 species of *Parthenium*, only *P. argentatum* is reported to have economic use for production of guayule rubber (Ponciano et al. 2012). Expression profile of genes pertaining to rubber biosynthesis in *P. hysterophorus* such as Hydroxymethylglutaryl-CoA synthase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, Farnesyl pyrophosphate synthase, Allene oxide synthase, Rubber cis-polyprenyl transferase HRT2 were also affirmed in transcriptome assembly as reported in *P. argentatum* (Ponciano et al. 2012). The qRT-PCR assay also confirmed the expression of rubber biosynthesis related genes and possible utilization of *P. hysterophorus* as source of raw material for the rubber industry.

P. hysterophorus, ecotypes collected from different cities of India showed accumulation of metal ions. However the ecotypes obtained from sites with putative heavy metal contamination showed more accumulation of corresponding metal ions in its roots and leaves. The chelation and accumulation of these metal ions depends upon the activity of transporters which import the metal ions into the cell and further sequester them to vacuoles. *In silico* expression analysis of these transporters related to heavy metal accumulation in the transcriptome of *P. hysterophorus* revealed its phytoaccumulator activity as expected. Similar kind of phytoremediation ability was also reported in *Louisiana iris*, *Sinapis alba*, *Salix matsudana*, and *Sedum alfredii* (Tian et al. 2015; Zhang et al. 2016; Yang et al. 2015; Gao et al. 2013). The metal accumulation activity of *P. hysterophorus* in case of Cu (59.3 g/kg) (Pandey et al. 2012), Zn (36.1- 414 µg/g in shoots and 17-417.2 µg/g in roots) (Kumar et al. 2013), and Cd (Sanghamitra et al. 2011) have been already reported. On the basis of earlier reports as well as the finding from this study substantiated the use of this plant to accumulate higher amount of heavy metal (Cr, Fe, Mn, Ni, and Zn). Expression of iron-storage protein ferritin, which is localized in the plastids of plants, is also involved in *P. hysterophorus* which might have played a significant role during development of the plant and under stress

environment, with wider adaptation (Briat, 1996; Batish et al. 2002; Kaur et al. 2014). The expression of genes/transporter related to nutrient transport, heat tolerance, drought tolerance, strigolactone biosynthesis for mycorrhizal associations specially CCDs (Carotenoid Cleavage Dioxygenases) (Supplementary file 5) (Aly et al. 2014), and defense systems etc. in constitutive manner could also be attributed towards its survival in diverse soil types as well as in diverse agroclimatic conditions (Aly et al. 2014). Expression of a Peptide transporter PTR1, Nitrate transporter, Urease accessory protein UreH, Urea-proton symporter, and Nitrate reductase responsible for nutrient transport were also observed. Among abiotic stress related genes the expression of heat stress transcription factor, desiccation-related protein PCC13-62, senescence/dehydration-associated protein and early-responsive to dehydration stress were also observed. Further to manage the biotic stresses, many pathogenesis related genes are constitutive expressed. Nematode resistance protein-like HSPRO2 gene was more expressed in roots in comparison to leaves and this might provide nematode resistance to these plants across the soil diversity.

In nature, it's very hard to detect any organism which are parasitizing on *P. hysterophorus*. Very rarely some fungus like rust causing and witches broom disease caused by phytoplasma are reported (Li et al. 2011; Keshwal, 1982; Yadav et al. 2015). Further, expression of some pathogenesis related genes like (TMV) disease resistance protein RPP4, pathogenesis-related protein PR-1, and leaf rust 10 disease-resistance locus receptor in phytoplasma infected plants were found to be suppressed. It has also been observed that the phytoplasma infestation causes suppression of genes related to floral development (*APETALA-1 (PhAP1)*) and seed setting (*RING-type E3 ubiquitin ligase (PhATL80)*) in *P. hysterophorus* (Dubey et al. 2019). On the basis of above results, it could be hypothesized that the phytoplasma infection not only suppresses the expression of genes related to plant immunity, but also altered the expression of many developmentally regulated genes. The KEGG pathway exploration also revealed the existence Glucosinolate biosynthesis pathway, which might be involved in the insect repellent properties of *P. hysterophorus* against insects and other pests (Radojčić Redovniković et al. 2008), and augment its growth in the tropical and subtropical regimes. Expression pattern of genes related to secondary metabolite and rubbers biosynthesis suggests its potential uses in pharmaceutical as well as rubber industry. Further, several useful property like nematocidal, antifungal, antiamoebic, antimalarial, trypanocidal, antibacterial and antiviral activity of *P. hysterophorus*, suggest its manifold use for the welfare of mankind (Kushwaha and Maurya, 2012).

Conclusion

The present study reported the transcriptome assembly of *P. hysterophorus*, and also identified expression of many genes which prevailed this weed an advantage in its growth and multiplication, and its possible use in agro industry. Several important genes regulating the heat stress, nutrient transporters, drought stress and defense were constitutively expressed that could explain the best survival nature of this weed. Expression pattern of candidate genes like Hyoscyamine 6-dioxygenase, Neomenthol dehydrogenase, GDSL esterase/lipase LIP-4, Artemisinic aldehyde Delta (11(13)) reductase, Codeine O-demethylase, Taraxerol synthase, Betaine aldehyde dehydrogenase 2, and Curculin-2 may suggest the presence of related biomolecules in the *P. hysterophorus* plants.

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Conflicts of Interest

Authors confirm that there is no conflict of interest to disclose.

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