

Research Article

Microbes in the *Datura* rootzone contribute to an antioxidant support system of flavonoids and other aromatic compounds

Savanah Senn ^{1,2*}, Kelly Pangell ¹ and Adrianna L. Bowerman ¹

¹ Agriculture Sciences Department, Los Angeles Pierce College, Woodland Hills, CA

² Environmental Sciences Graduate Program, Oregon State University, Corvallis, Oregon

* Correspondence: stclais@piercecollege.edu

Abstract: The purpose of this paper is to elucidate the roles that microbes may be playing in the rootzone of the medicinal plant *Datura inoxia*. We hypothesized that rhizospheric and endophytic microbes would be found that were capable of performing the same secondary metabolic functions of the plant rootzone they inhabited. We also hypothesized that the microbial functions would be cooperative with and supportive to plant secondary metabolite production, for example, by providing precursors to important plant bioactive molecules. The methods employed were microbial barcoding, tests of essential oils against antibiotic resistant bacteria and other soil bacterial isolates, 16S Next Generation Sequencing (NGS) metabarcoding, and Whole Genome Shotgun (WGS) taxonomic and functional. A few of the main bacterial genera of interest that were discovered in the *Datura* root microbiome were *Flavobacterium*, *Chitinophaga*, *Pseudomonas*, *Streptomyces*, *Rhizobium*, and *Bacillus*. In the context of known interactions, and current results, plants and microbes influence the flavonoid biosynthetic pathways of one other, in terms of the regulation of the phenylpropanoid pathway. This is important because these compounds are phyto-protective antioxidants and are precursors to many aromatic bioactive compounds that are relevant to human health. There was strong evidence to support the notion that synergistic production of plant derived secondary metabolites by microbes occurred, as well as the ability for the compounds to enter plant cells. There are possible biopharmaceutical and agricultural applications of the natural interplay that was discovered during this study of the *Datura inoxia* rhizosphere.

Keywords: Weed science, Plant-microbe interactions, Medicinal plants, Shotgun metagenomics, Soil metabarcoding

1. Introduction

The Significance of the Plant

The *Datura* plant genus produces several important cyclic compounds, most notably the tropane alkaloids. *Datura* produces alkaloids for anti-herbivory including scopolamine and atropine. Both of these alkaloids have medicinal uses, however at high doses they are toxic and can lead to hallucinations, or even death. Atropine is an antidote for nerve gas because it blocks acetylcholine activity. Atropine can also be used to treat asthma and low heartrate. Scopolamine is used for prevention of nausea and motion sickness.

Datura has analgesic properties [1]. Traditionally, the flowers have been worn around the ears to treat earache. Furthermore, the plant contains antioxidants such as flavonoids, and phenols. Quercetin is a flavonol with anticancer properties that has free radical scavenging activity. Other compounds may include scopoletin [2], which is a fluorescent coumarin that has antifungal properties against antibiotic resistant strains of *Candida* [3]. The plant does not produce nicotine like some other members of *Solanaceae*.

This plant is sometimes referred to as an entheogen due to its use by native people in the United States such as the Chumash. *Datura* has been used for rituals and ceremonies in

Hindu culture [4]. Since the flower is valued for its aesthetic properties, it is also grown as an ornamental plant and there are fertilizers that are developed specifically to promote flowering. It is also occasionally used as a hallucinogen in a non-spiritual setting.

Datura inoxia has grey-green, flat, simple foul-smelling leaves that typically lack toothed margins. The leaves resemble wild tobacco leaves and may contain ornithine and putrescine, which attracts predatory birds. The leaves are slightly downy. It is most readily identified by the white, sweet-smelling inflorescence, which blooms in the evening and attracts pollinators such as Sphinx moths. The blooms resemble *Brugmansia*. It is an outcrossing plant, like many of the *Solanaceae*. The flower exhibits heterostyly; the female part sticks out of the flower and may be receptive before the pollen is dehiscent, contributing to heterozygosity. The round seed pods are spiny and lignified and can be dehiscent. The seeds pods have a round fleshy placenta within, which is covered by numerous flat seeds, resembling a bell pepper on the inside. The seeds are highly heterozygous and spontaneous mutations are common in tissue culture [5].

A close relative of *Datura inoxia*, *D. wrightii* was recently recognized in rock art paintings by the native Chumash at Pinwheel Cave [6]. The nature of the altered state of consciousness theory was recently challenged by the discovery of rock art from Pinwheel Cave in Kern County, CA. Anthropologists had theorized that rock art paintings were created in an altered state of consciousness. Although anthropologists long believed that the rock art paintings depicted a vision or hallucination, the recent discoveries paint a different picture.

The cave art also depicts an anthropomorphic sphinx moth, which is known to pollinate the plant. Interestingly, sphinx moths have often been seen flying erratically after visiting *Datura* flowers (Ibid). Rather than depicting a hallucination, the rock art depicts the sacrament itself and reflects the natural cycles of flowering and pollination that are essential to this dynamic outcrossing plant. The cave was stuffed with at least 15 quids of *Datura* flowers that had been chewed by groups of up to 10 people. Groups also used the cave to prepare for hunting expeditions for several generations, and as a seasonal food preparation place. Toloache is a native drink preparation that is well-known as part of an adolescent initiation ceremony; these quids were chewed like tobacco and then stuffed into the ceiling of the cave. Beyond adolescence, *Datura* was used by native people to gain personal power, including for doctoring and hunting trips, and to treat ailments.

The Significance of the Plant Microbiome

Recently, a few studies have elucidated the interaction between plants and microbes during secondary metabolite production. It is known that in some species, such as *Crotalaria*, nodulation is required for alkaloid production [7]. Functional analysis of soil metagenomic data has expanded as a field, and is used as a way to consider how plants are influencing the microbiome [8]. The microbiome is equally important in understanding plant secondary metabolism.

Shotgun metagenomics approaches in applied soil science provide the advantage of amplifying sequences from throughout the genome, including coding regions, yielding a high sequencing depth but at a higher cost. 16S metabarcoding is used as a diagnostic tool in clinical sciences and plant ecology, and its forte is identification of bacteria and archaea. However, metagenomics and metabarcoding are culture independent, meaning that no viable accessions are maintained. For that reason, microbial barcoding of streak plates isolated from the soil is also a useful tool that provides material for later wet lab experiments involving plant inoculation or antimicrobial trials, and helps to validate NGS results with tangible materials.

Flavobacterium and Chitinophaga

According to Sang et al 2012, *Flavobacterium johnsoniae* produced 2,4-di-tert-butylphenol which had biocontrol activity against *Phytophthora* in *Capsicum* [9]. This references one of a few studies in *Solanaceae* where *Flavobacterium* has been shown to suppress disease. Another example is Carrion et al 2019, where *Rhizoctonia solani* was suppressed by the endophytic root microbiome [10]. Some of the most important natural products involved were phenazines, polyketides, siderophores, and chitinases. Aryl polyenes, which are structurally similar to carotenoids, were enriched by *Flavobacteriaceae*; terpenes and resorcinol were enriched by *Chitinophaga*. Similarly, in 2020 Lucke et al reported that as biocontrol agents, the secretion of secondary metabolites triggered the induction of systemic resistance in plants [11]. In particular, they pointed out the importance of the chitinase from *Flavobacterium* and the Polyketide Synthase (PKS) gene cluster from *Chitinophaga* that is essential for disease suppression.

Mycobacteriaceae

Del Barrio-Duque et al 2019 found that 17/19 *Mycobacteriaceae* strains tested had fungal growth-promoting properties on *Serendipita indica* in vitro and in tomato; they were plant growth-promoting and helped to alleviate symptoms of *Rhizoctonia solani* [12]. In this research, *Mycobacterium* was in a consortium with *Rhizobium* and *Paenibacillus*; in some instances, the isolations worked better than combinations of inoculants. Surprisingly, *Burkholderia* had a negative impact in vitro on *S. indica* fungal growth, although it has been shown to be plant growth promoting. An interesting review by Morris et al 2019 highlights the fact that benzylisoquinoline alkaloid methyltransferases are functionally similar to the cyclopropane methyltransferase from *Mycobacterium tuberculosis* [13]. *Mycobacterium* cyclopropanation could contribute to tropane alkaloid biosynthesis in *Datura*, or provide an intermediate toward the same.

Rhizobium and Streptomyces sp.

Banuelos-Vazquez reported that endophytes can receive nodulation genes, and in some cases, the ability to fix nitrogen, from *Rhizobium etli*. Plasmids are transferred to other endophytes in plant roots [14]. Another interesting example is from Kado and Kelly 2006, where they reported a successful protocol for transforming *Streptomyces* with *Agrobacterium* [46]. Lucke 2020 also noted the root nodulating capabilities and transfer of plasmids by *R. etli* and *A. tumefaciens*, and their ability to induce plant defenses [11].

Furthermore, the plant growth-promoting activities of endophytes may include production of plant growth regulators such as Indole Acetic Acid (IAA), and siderophore production for iron acquisition. *Streptomyces* spp. within *Bruguiera gymnorhiza* and *Boesenbergia rotunda* increased flavonoids and cyclopeptides with anti-HIV and anticancer activities [15]. Endophytic bacteria, as well as fungi, can produce paclitaxel [16]. Within *Taxus baccata*, *Streptomyces* produced 0.01-0.02 ng/mL, and *Bacillus subtilis* produced 1-25 ng/mL [16].

Indeed, as Wu et al pointed out, the relationship between medicinal plants and endophytes is a long-term, symbiotic relationship [15]. Endophytes can strongly regulate the synthesis of secondary metabolites in plants. For example, for abiotic stress tolerance, in *Pteris vittata*, *Agrobacterium* and *Bacillus* spp. reduced arsenate to arsenite.

Pseudomonas and Bacillus sp.

Volatile organic compounds can also help bacteria that are physically separated communicate with one another. Endophytic bacteria can protect plants by quenching quorum sensing molecules. For example, *Pseudomonas aeruginosa* could degrade 3-

hydroxy palmitic acid methyl ester, which is a quorum sensing molecule of *Rhizoctonia solanacearum*. The inoculation of eggplant with *P. aeruginosa* reduced bacterial wilt caused by *R. solanacearum* [15]. In tomato, *Bacillus aureus* and *Serratia nematodiphilia* were applied to seeds along with *Ralstonia syzigi* sub-infection. The treatment under inoculation had increased jasmonic acid concentration in leaves and roots until 12 days post-infection [17].

Antioxidant enzymes are activated by endophytes, such as phenylalanine ammonia lyase, tyrosine ammonia lyase, and polyphenol oxidase (PPO) [17]. Rhizobacterial root colonization significantly impacted phenolic compounds, terpenes, and essential oils in plants. *Pseudomonas putida* colonization root colonization altered benzoxaninone levels three days after inoculation. Colonization by *Rhizobium* changed the levels of phenolics, flavonoids, and anthocyanins, in blackberry, which was associated with delayed fungal postharvest growth [17].

In terms of the accumulation of plant secondary metabolites, production of plant sesquiterpenoids was enhanced in *Atractyloides macrocephala* by *Pseudomonas fluorescens*. Essential oil production was also increased [17]. According to Sang et al 2012, endophytes can increase resistance to insects, while the plants provide nutrition and protection [9]. In *Nethapodytes fortida* and *Apodytes dimidiata*, two grasses, *Fusarium solanii* fungi were found to produce campothecin. This quinoline compound was previously taken from the roots of *Nethapodytes* directly.

Purpose of the Study

The purpose of this paper is to elucidate the roles that microbes may play in the root-zone of the medicinal plant *Datura inoxia*. We hypothesize that we will find rhizospheric and endophytic microbes performing the same secondary metabolic functions of the plant rootzone they inhabit. We also hypothesize that the microbial functions will be cooperative with and supportive to plant secondary metabolite production, for example, by providing precursors to important plant bioactive molecules.

2. Materials and Methods

Soil samples were collected in triplicate from the field and arboretum at Los Angeles Pierce College. Replicated samples were taken from plant rootzones in the top 3 cm within a 1-foot radius of the plant subjects including *Alnus rhombifolia*, *Datura inoxia*, Ethiopian *Eragrostis tef* (Teff) grass, *Opuntia* cactus rootzone, and fallow conditions. They were stored at -20°C and DNA was extracted using the Qiagen Power Soil DNA kit and sent to Cold Spring Harbor Laboratory for 16S amplification, library preparation, and pooled 16S amplicon NGS on the Illumina MiSeq platform. Soil from the same sampling event was sent to Beijing Genomics Institute America, San Jose for logistics and processing, for the replicated *Datura*, *Alnus*, and fallow rhizospheric soil samples.

According to the USDA NRCS Web Soil Survey historic data, the fields related to the *Datura*, *Alnus*, and fallow samples are all classified identically as Cropley-Urban Land Complex 0-2% slopes, and the reported measurements for pH, texture, cation exchange capacity (CEC), and percent organic matter are identical, as shown in Table 1 [18].

Table 1. Soil Physical and Chemical Properties of the Fields sampled based on Historical USDA data.

Map Unit Name	pH	CEC	%Sand	%Silt	%Clay	%OM
Cropley-Urban land complex, 0 to 2%	7.9	37.5	22.1	27.9	50	1.5

Whole genome shotgun sequencing, DNA extraction, and library preparation was subsequently performed by Beijing Genomics Institute on the DNBseq platform. Preliminary trim and QC were carried out using SOAPnuke [19]. MG-RAST was used to generate functional and taxonomic profiles [20]. Taxonomic identification was performed using RefSeq and functional profiles were built from Subsystems identifiers. In order to determine which taxa were differentially abundant between categories, DESeq2 was used [21]. STAMP was used for functional analysis [22].

For soil metabarcoding and 16S barcoding analyses, DNA Subway was used [23]. For soil metabarcoding, DNA extraction was performed with the Qiagen PowerSoil kit. Library preparation followed the Earth Microbiome Project 16S Illumina Amplicon protocol [24, 25]. Sequencing was performed at Cold Spring Harbor Laboratories. The Greengenes identifier (utilizing 515F/806R primers) was used.

Bacterial isolations were also generated for the *Alnus*, *Datura*, and fallow rhizosphere samples in order to provide material for future in vivo plant experiments according to the Soil Science protocols published by St. Clair et al [26]. Isolates from the soil solutions at 10^{-3} , 10^{-4} , and 10^{-5} dilutions were plated and grown for three days on various media, summarized in Table 2. Selected isolates were streaked on plates of the same media and colony PCR was performed after three days of isolated growth. For bacterial isolations, DNA was extracted by boiling colonies with chelex beads for 10 minutes, followed by 16S rRNA amplification. Amplicon sizes were > 500 bp. Sanger sequencing for bacterial isolates was performed by Genewiz. QC was performed and consensus sequences were generated in the Cyverse DNA Subway Blue Line. The Phylophil Maximum Likelihood tree was generated in Cyverse. Bacterial putative identifications for the isolates were generated using the EZBioCloud 16S-based ID application [27].

Lemon balm (*Melissa officinalis*) and Tea tree (*Maleleuca alternifolia*) essential oils are well-characterized. Vegetative plant material was harvested from and air dried at room temperature for one week. The samples were handled separately. Soxhlet extraction was carried out for three cycles using reagent ethanol as the solvent. The extracts were subsequently dewaxed by running them through a Buchner funnel and filter paper.

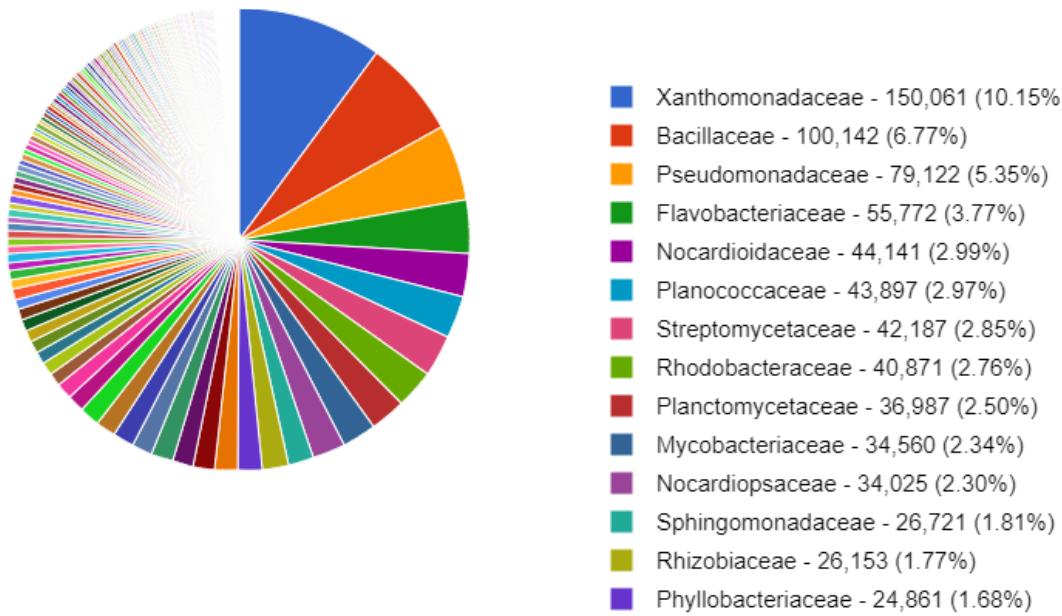
Bacteria were cultured in 1.5 mL tubes of nutrient broth for 48 hours at room temperature. Separate sterile glass tubes filled with 2mL of nutrient broth were inoculated with 100 μ L of each bacterial broth culture. The glass tubes were inoculated with 20 μ L of either lemon balm oil or tea tree oil from the soxhlet extractions. The clarity of the solution, which was a proxy for bacterial colonization levels, was measured as % transmission at 600 nm. Transmittance at 600nm was measured using a spectrophotometer after culturing at room temperature overnight. The data was analyzed using R.

3. Results

Soil samples WGS and MG-RAST taxonomic and functional assignments

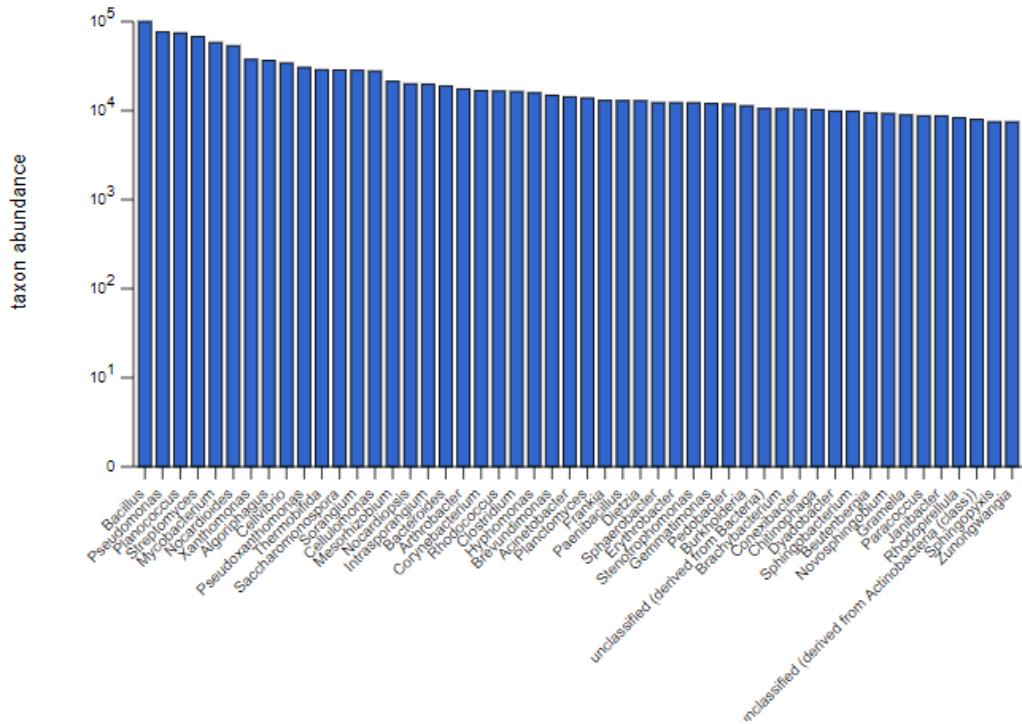
The sequence count ranged from 4,608,913 to 34,551,930 sequences. Interestingly, and in contrast to the DNA Subway Purple line results, each category between the *Datura* rootzone, Fallow field near the compost pile, and the Alder rootzone soil had a sample in the top 3 best performers for read counts, but the Fallow field tended to have lower read counts overall, which is more of what one would expect considering no plants were growing there.

Figure 1. MG-RAST classification of the NCBI RefSeq identification of the soil organisms at the family level, for a representative sample of *Datura* rootzone soil.



In the *Datura* rootzone, some of the most abundant genera were *Pedobacter* and *Flavobacterium*. *Flavobacterium johnsoniae* is well-known for its tyrosine ammonia lyase gene, which has been cloned and used in genetic transformations to optimize production of aromatic compounds of pharmaceutical value in microbial expression systems. Some potential applications are production of tropane alkaloids and p-coumaric acid [28, 29].

Figure 2. Relative abundance of the top genera in a representative sample of *Datura* rootzone soil from the Pierce Farm in Los Angeles, California.



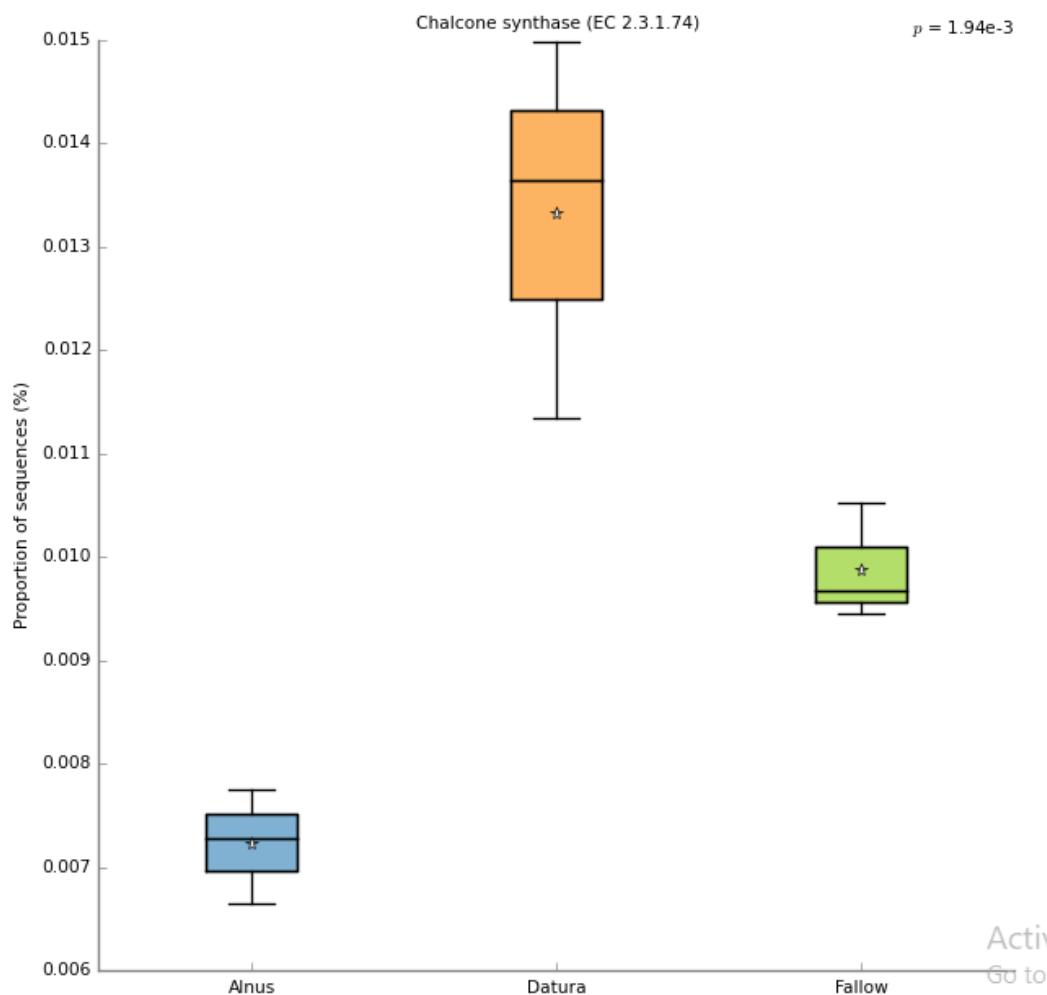
Functional Analysis in STAMP

Using a cutoff of alpha=0.05, the following were significantly higher in the *Datura*-associated soil samples: Alpha-xylosidase ($p<0.017$), chalcone synthase ($p<0.002$) (Figure 3), putrescine utilization ($p=0.05$), Aromatic amino acid transport protein AroP ($p<0.003$), polyols ABC transporter permease component ($p<0.003$), Quinate permease ($p=0.002$), ubiquinone biosynthesis enzyme COQ 7 ($p<7*10^{-3}$), Heavy metal sensor histidine kinase ($p<6.4*10^{-3}$), flavin reductase family ($p=0.022$), para-hydroxybenzoate- polyprenyl transferase ($p<0.004$), Siderophore achromobactin ABC transporter ($p<0.0021$), NADPH quinone oxidoreductase 2 ($p<0.0021$), vanillate O-demethylase oxygenase subunit ($p<0.0014$),

Phenylacetic acid degradation protein PaaN ($p<0.0013$), clavulanic acid biosynthesis ($p=0.027$), cobalamin synthesis ($p<0.005$), and MAP kinase pathways ($p<0.0045$).

Figure 3. Differential abundance of chalcone synthase genes was detected in the *Datura* rootzone based on STAMP analysis of the MG-RAST generated Subsystems metagenomic functional profile.

Box plot



On the other hand, the Na^+ translocating NADH-quinone reductase subunit was differentially abundant in Marquis C ($p<7*10^{-4}$), the fallow field near the compost pile. Gram positive cell wall components were differentially abundant in Marquis C ($p<0.008$), although gram negative cell wall components were equally likely to be present in all samples ($p=0.244$). The CRISPR associated protein TM1812 was enriched in the fallow field near the compost pile ($p<0.0014$). Phenylpropanoid compound degradation functional genes were most abundant in the *Alnus* samples ($p<6.4*10^{-5}$). Tocopherol biosynthesis genes were also differentially abundant in the *Alnus* rhizosphere-associated soil ($p<0.004$).

There was enrichment of cell wall degradation and depletion of gram-positive bacteria, stress response, siderophores, catabolism of tyrosine, enrichment for aromatic compounds and permeases, production and reduction of flavonoids, antibiotics and cobalamin, and degradation of oxidized products in the *Datura* rootzone. The presence of differentially abundant permease components for essential oils and quinates suggests that there is a secretion pathway that would allow these compounds to enter plant roots and contribute to flavonoid, alkaloid and terpenoid biosynthesis in plant hosts.

Purple Line analysis and comparison

Demultiplexed sequence counts for the DNA Subway Purple Line paired end data set ranged from 5,472 to 3.63 million. The lowest number of reads were for one of the

Datura rootzone soil samples and a tilled fallow sample nearby in Marquis C. The highest number of reads were from Field 17, a fallow field tilled with cow patties and Marquis C near the compost pile. Indeed, in Field 17 also had a trend toward the highest alpha diversity, followed by Marquis C. There appeared to be more within group variation reflected by the 16S workflow compared to WGS.

Enrichment of *Pedobacter* matched up with MG-RAST results, and with *Mycobacterium* which may be related to alkaloid biosynthesis. *Pedobacter* represented >32% of the reads for one of the *Datura* rootzone samples. *Blastococcus* sequences were abundant in all three *Datura* rootzone soil samples. *Streptomyces*, *Rubrobacter* and *Mycobacterium* were abundant in one of the *Datura* samples, but were not detected in the other two. There appeared to be a similar drought tolerant entourage of bacteria associated with *Datura*, Teffgrass, and the cactus-associated soil samples, based on the Principal Coordinates Analysis (DNA Subway Purple Line Public project 7102).

Some unexpected results were detected by the isolation of bacterial colonies and analysis on the Blue Line. Four *Chryseobacterium* accessions were isolated from the soil that did not show up on 16S amplicon sequencing for the same soil samples (Figure 4). However, the *Chryseobacterium* genus was detected with DNBseq WGS (see Table 4).

Table 2 Bacterial Strains Isolated and Culture Conditions

Sample No.	Field	Colony Color	Morphology	Antibiotic Used	Media	Absorbed CR
1	Marquis C	Transl yellow	Unkown	NA	Nutrient agar	NA
2	Marquis D	White/ tan	Unkown	Penicillin	Nutrient agar	NA
3	Field 28	White/ tan	Unkown	Penicillin	Nutrient agar	NA
4	Marquis A	Yellow	Unkown	Streptomycin	Nutrient agar	NA
5	Marquis A	Yellow	Raised	NA	Nutrient agar	NA
6	Arboretum	White	Spreading	NA	TYES- CR	absorbed cr
7	Marquis A	Transl yellow	Raised	NA	Nutrient agar	NA
8	Marquis A	White	Gliding	NA	Nutrient agar	NA
9	Marquis A	Transl yellow	Gliding	NA	Nutrient agar	NA
10	Marquis A	Yellow	Flat	NA	Nutrient agar	NA
11	Marquis A	Transl Yellow	Highly motile at 4°C	NA	Nutrient agar	NA
12	Arboretum	Clear/ tan	Mucoid	NA	TYES	Intense pink
13	Arboretum	White	Flat	NA	TYES	Dark red

					Nutrient agar	
14	Marquis A	Yellow	Raised	NA	NA	
15	Arboretum	Clear/tan	Small mucoid	NA	TYES	Light pink
16	Marquis A	Clear/tan	Mucoid	NA	TYES	NA
17	Marquis A	Transl yellow	Mucoid	NA	TYES	NA
18	Arboretum	Clear/tan	Gliding	NA	TYES	Abs cr
19	Arboretum	White	Spreading	NA	TYES	Abs cr ring
20	Marquis A	Transl yellow	Raised, wrinkled	NA	1/2 NA+AC	NA
21	Marquis A	Transl yellow	Small colony	NA	1/2 NA +AC	NA
22	Marquis A	Transl yellow	Small colony	Griseofulvin	ISP-6	NA
23	Marquis A	Yellow/orange	raised	Griseofulvin	ISP-6	NA
24	Marquis A	White/grey	Large colony	Griseofulvin	ISP-6	NA
25	Marquis A	Transl yellow	Wrinkled	Griseofulvin	ISP-6	NA

Figure 4. Phylogenetic Maximum Likelihood Tree.

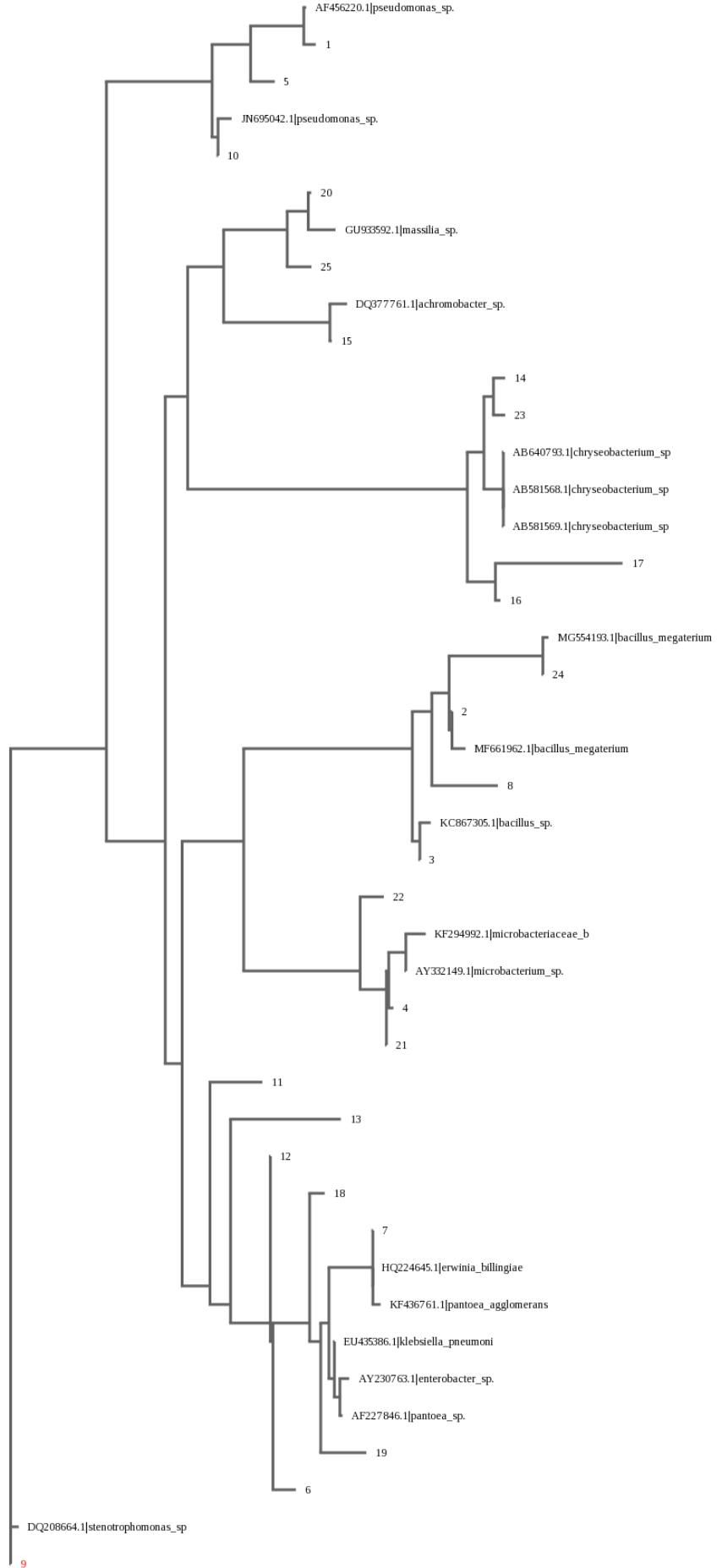


Table 3 Prokaryote Identification using EZBioCloud 16S-Based ID

Name	Top-hit taxon	Top-hit strain	Similarity (%)	Completeness (%)
Sample 1	<i>Pseudomonas mucoides</i>	P154a	84.46	52.2
Sample 2	<i>Priestia qingshengii</i>	G19	71.85	66.6
Sample 3	<i>NUQE_s</i>	AFS041167	83.45	52.8
Sample 4	<i>Luethyella okanaganae</i>	LBG B4405	82.3	51.7
	<i>Pseudomonas</i>			
Sample 5	<i>neuropathica</i>	P155	83.05	52.2
Sample 6	<i>Enterobacter huaxiensis</i>	90008	80.75	53.3
Sample 7	<i>Pantoea deleyi</i>	LMG 24200	86.08	52.6
	Marseille-			
Sample 8	<i>Bacillus mediterraneensis</i>	P2366	82.08	55.2
Sample 9	<i>Xanthomonas maliensis</i>	M97	81.03	53.5
Sample 10	<i>Pseudomonas bijieensis</i>	L22-9	70.53	70.4
Sample 11	<i>Erwinia billingiae</i>	CIP 106121	85.53	27.8
Sample 12	<i>CP009454_s</i>	ND04	72.2	70.3
Sample 13	<i>Klebsiella huaxiensis</i>	WCHKI090001	90.4	52.4
Sample 14	<i>Chryseobacterium tructae</i>	1084-08	81.03	55.4
Sample 15	<i>Achromobacter deleyi</i>	LMG 3458	82.94	52.5
	<i>Chryseobacterium</i>			
Sample 16	<i>vietnamense</i>	GIMN1.005	78.87	53.7
	<i>Chryseobacterium</i>			
Sample 17	<i>aurantiacum</i>	F30	59.61	78.2
Sample 18	<i>Pantoea dispersa</i>	LMG 2603	83.51	53.6
Sample 19	<i>Pantoea eucrina</i>	LMG 2781	85.74	52.4
Sample 20	<i>GU563773_s</i>	Bmc86	72.27	65.2
Sample 21	<i>Luethyella okanaganae</i>	LBG B4405	62.85	92.3
Sample 22	<i>Microbacterium binotii</i>	CIP 101303	63.59	92.5
	<i>Chryseobacterium</i>			
Sample 23	<i>phosphatilyticum</i>	ISE14	80.7	55.9
Sample 24	<i>Priestia megaterium</i>	NBRC 15308	82.65	53.7
Sample 25	<i>Massilia flava</i>	Y9	80.44	52.4

Differential Abundance Analysis in DESeq2

There were 223 virus, archaea, or bacterial genera that were differentially abundant between the *Datura* rootzone samples and the Fallow samples from Marquis Field near the compost pile. There were 140 species that were associated with the *Datura* rootzone and 83 species that were associated with the fallow samples, based on adjusted p-value<0.001. Of those, 124 genera were also significant based on the log fold change cut-off of 1.33, 34 were associated with the *Datura* rootzone, and the remaining genera were associated with the fallow samples. The results are summarized in Table 4 and visualized in Figure 5.

Table 4. The 34 genera with both high negative log fold change values, and significant adjusted p-values (p<0.001) representing those associated with the *Datura* rootzone are shown.

OTUID	Genus	log2FoldChange	p-adj.
OTU149	Cavemovirus	-8.74069944	1.46E-11
OTU494	Pantoea	-3.382739635	5.9E-18
OTU168	Chryseobacterium	-2.894493584	0.000172
OTU332	Janthinobacterium	-2.396801275	5.53E-06
OTU307	Herbaspirillum	-2.385130454	2.86E-08
OTU505	Pedobacter	-2.384332893	6E-05
OTU247	Erwinia	-2.324319999	3.84E-35
OTU738	unclassified (derived from Flavobacteriaceae)	-2.299754251	0.00078
OTU273	Gemmata	-2.299386567	3.76E-19
OTU308	Herminiiimonas	-2.1461305	2.3E-05
OTU693	Variovorax	-2.033564378	1.25E-07
OTU759	unclassified (derived from Verrucomicrobia subdivision 3)	-1.966909323	5.46E-18
OTU169	Chthoniobacter	-1.924054848	5.19E-11
OTU440	Muciluginibacter	-1.920113231	6.47E-05
OTU263	Fluoribacter	-1.819462381	0.006919
OTU569	Riemerella	-1.783874932	0.008796
OTU488	Oxalobacter	-1.775720869	1.2E-06
OTU339	Klebsiella	-1.680008901	0.00538
OTU277	Geodermatophilus	-1.67277933	0.000157
OTU463	Nitrospira	-1.638446217	0.000467
OTU262	Flavobacterium	-1.633855026	0.007125
OTU156	Chitinophaga	-1.588995265	0.000676
OTU696	Verrucomicrobium	-1.523905486	3.61E-05
OTU234	Dyadobacter	-1.505907596	0.005228
OTU480	Opitutus	-1.500266429	5.62E-05
OTU598	Serratia	-1.489698167	2.49E-10
OTU421	Methylobacterium	-1.448043272	2.59E-12
OTU136	Candidatus Solibacter	-1.428079685	1.1E-15
OTU624	Stigmatella	-1.392596868	6.52E-07
OTU751	unclassified (derived from Proteobacteria)	-1.373840383	0.003172
OTU180	Conexibacter	-1.373047754	0.000188
OTU651	Terriglobus	-1.362530369	6.27E-16
OTU532	Polaromonas	-1.340071098	9.35E-06
OTU727	unclassified (derived from Candidatus Poribacteria)	-1.330571533	0.00172

Among the more notable results were differentially abundant bacteria in the *Datura* rootzone including *Flavobacterium*, *Chryseobacterium*, and unclassified members of the *Flavobacteriaceae*. *Chitinophaga*, *Muciluginibacter*, and *Pedobacter* of the closely related *Sphingobacteriaceae* were also differentially associated with the *Datura* rootzone. These genera are associated with degradation of polysaccharides or chitin. There was also evidence that *Verrucomicrobium* that are typically associated with *Flavobacterium* as well as unclassified *Verrucomicrobium* were significantly associated with the *Datura* rootzone. These genera are known for hydrolysis of xylans. *Dyadobacter* of the *Cytophagaceae*, which has polysaccharide and amino acid degrading functions, was present in differentially large quantities;

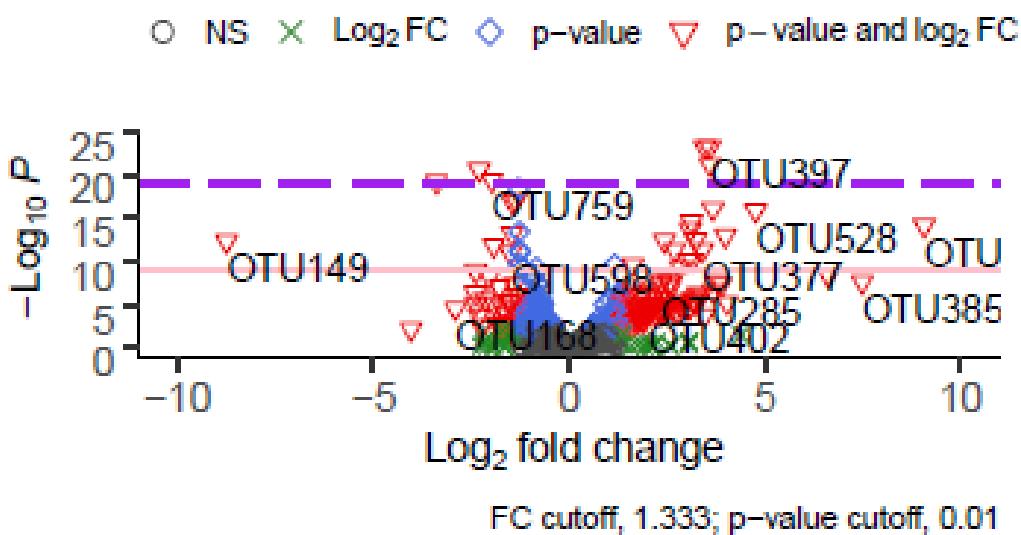
this species lives in glaciers and was formerly classified as a member *Flavobacteriales*. *Stigmatella*, which is responsible for breaking down insoluble debris, was also significantly associated with the *Datura* rootzone.

Herbasperillum and *Oxalobacter* from the *Oxalobacteriaceae* were differentially abundant, along with *Janthinobacterium*, which produces violacein, an antifungal, antiprotoist, antibacterial, and anti-tumor compound [30]. Interestingly, sequences from the plant pathogen *Erwinia* were also present in significantly high numbers, as well as the poultry disease *Riemerella*, and the infectious *Serratia* which is consistent with the animal bedding that is spread from the compost pile to all of the adjacent fields. *Variovorax* is involved in disrupting quorum sensing and has swarming motility, and was differentially abundant in the *Datura* rootzone. *Bradyrhizobium* and *Azorhizobium* abundance in the *Datura* rootzone was significant based on adjusted p-value ($p < 0.01$) but not based on log fold change values.

Figure 5. Differential Abundance Analysis in R shows that there were a large number of taxa that were differentially expressed in the fallow (positive log fold change) versus *Datura* (negative log fold change) samples.

DESeq2 results for Virus, Bacteria, & Archaea

Differential abundance: Fallow vs. *Datura inoxia*



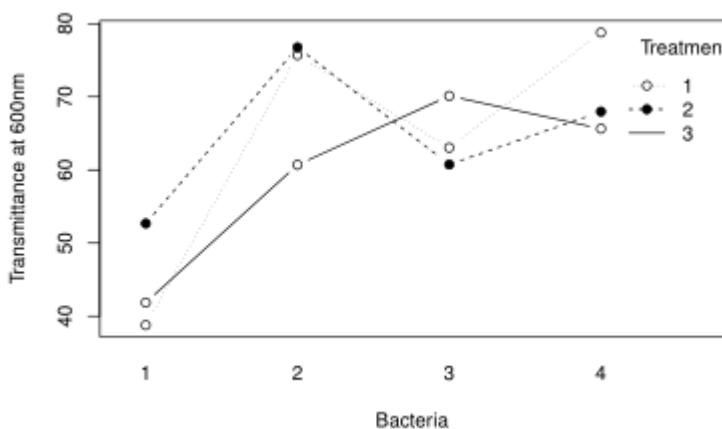
Microbial barcoding and essential oil trial

The PhyliP Maximum Likelihood phylogenetic tree is shown Figure 4. The results indicated that there are four main clusters of bacteria that were isolated. The first cluster includes isolates related to *Pseudomonas* sp. The second cluster consists of two groupings; one group is related to *Massilia* sp. and *Achromobacter* sp., while the second group is related to *Chryseobacterium* sp. The third cluster consisted of *Bacillus* sp. and *Microbacteriaceae*. Interestingly, *Priestia megaterium* is used as an herbicide in organic production [44]. The fourth cluster is made up of mostly pathogenic species such as *Erwinia*, *Pantoea*, and *Klebsiella*. Most of the pathogenic strains were associated with the Arboretum samples. *Stenotrophomonas* was used as the outgroup. As shown in Figure 4 and Table 3, there were four *Chryseobacterium* isolates that were detected with barcoding, which were not detected with 16S NGS metabarcoding. The ascertainment of the *Achromobacter* isolate agrees with NGS results. The *Xanthomonas* isolation agrees with the WGS results, since *Xanthomonadaceae* was the top family detected in the WGS samples for the *Datura inoxia* rootzone.

The main findings of the essential oil trials indicate that *Pseudomonas mucoides* was not susceptible to penicillin, and not susceptible to lemon balm essential oils. In

retrospect, since *P. mucoides* is a gram-negative bacterium it would not be expected to be susceptible to penicillin. *Bacillus pseudomycoides* was resistant to penicillin but grew even more with lemon balm and tea tree essential oils. It is interesting because *B. pseudomycoides* was tolerant of high alcohol content, possibly since it is a lactic acid fermenter. *Priestia qingshengii* was not susceptible to penicillin and grew less when lemon balm or tea tree essential oils were added. *Leuthyella okanaganae* was resistant to streptomycin; however, growth was controlled significantly when lemon balm or tea tree essential oils were added and the least growth occurred when the tea tree treatment was used.

Figure 6. The interaction plot for the Bacteria and Treatment variables of the essential oil trial are shown. Interestingly, there was an interaction between the essential oil used and the bacterial accession it was tested against.



It is interesting because Luna et al 2007 found that several *Bacillus pseudomycoides* strains were susceptible to beta-lactams, however the strain ascertained in this study was resistant to penicillin [31]. *Bacillus pseudomycoides* is related to *Bacillus anthracis* and *Bacillus cereus*; these species are notorious for being antibiotic resistant but *B. pseudomycoides* and *B. cereus* are particularly resistant to clindamycin [31].

Pseudomonas is also notoriously resistant to antibiotics, although its inclusion here was somewhat serendipitous since it was not selected with an antibiotic that would be typically used to combat gram negative bacteria. It was also found that tea tree and lemon balm oils were ineffective against the *P. mycoides* isolate.

4. Discussion

Cytophagaceae are known to degrade polysaccharides [32]. *Pedobacter* inoculation has been shown to significantly increase the antioxidant content of strawberries [33]. Closely related species are the *Flavobacterium*, which are famous for producing flavonoids including quinones and they play a role in disease suppression in soils [34]. Flavonoid and alkaloid production gene ontologies are closely related [35, 36]. *Rhizoctonia solani* is suppressed by *Flavobacterium* [37]. This is believed to be related to its chitinase activity which is also of interest for energy production from biomass [38].

As Fadiji 2020 pointed out, plant protective endophytes stimulate plant secondary metabolite production while inducing plant resistance to pathogens [39]. In addition, endophytic fungi produce strong antioxidants. In *Taxus cuspidata* paclitaxel production was elevated, and in *Euphorbia pekinensis* there was elevated terpene production in response to the *Fusarium* E5 elicitor; these are two examples from the article. Some examples of protection provided by the endophytes include antibiotics, competition for resources with pathogens, and lytic enzymes such as chitinases that break down fungi and plant cell walls.

The chitinase activity provides a possible answer to how endophytes and their products are transported into the plant. Furthermore, xylanase and other cell wall degrading

enzymes such as chitinases and cellobio-hydrolase were detected in the metagenome of several endophytes [17]. These lytic enzymes have the dual function of giving microbes access to plant cells, ducts, and intercellular spaces for colonization and transport, and the enzymes can also break down fungi cell walls to assist the plant host while fighting off infections.

Our preliminary evidence shows that the functional hits for Chalcone synthase in the microbes of the *Datura* rootzone are differentially abundant in contrast to the fallow sample. Chalcone synthase is involved in antioxidant production in bacteria and plants, and is homologous with polyketide synthase, which is part of the alkaloid production pathway in plants [40, 41]. There appears to be synergy between the plant and microbe functions in terms of secondary metabolite production in the rootzone.

Genetic Engineering and the Flavonoid Biosynthesis Pathway

Another way to look at this is that microbes may be engineered to produce the plant medicinal compounds directly [42]. Chemical synthesis of aromatic compounds uses benzene, toluene, and xylene as starting materials; these materials are derived from petroleum. Microorganisms are a renewable source of plant-derived secondary compounds. These compounds include phenolic acids, flavonoids, stilbenoids, coumarin, and conjugates of the same. A pathway of interest for the engineering of microbes for the production of plant metabolites, which occurs in nature, is the pathway by which tyrosine is converted to p-coumaric acid by tyrosine ammonia lyase and later converted to quercetin.

Yonekura et al 2019 shed light on the flavonoid biosynthesis pathway [35]. In the plant kingdom, flavonoids are widely distributed in all subclasses, except for hornworts. This suggests that chalcone synthase, the first enzyme in the pathway, was evolved many times in parallel. If the enzyme was universal, then it must have been lost by many species along the way, since not all species within the subclasses have it [35]. The first two enzymes on the biosynthetic pathway are chalcone synthase and chalcone isomerase, which are believed to also have evolved first from a common ancestor involved in lipid metabolism.

There are 9,000 flavonoid compounds defined strictly as:

1. Structural derivatives of phenyl-substituted propylbenzenes with a C15 backbone,
2. Phenyl-substituted propylbenzene derivatives with a C16 skeleton, or
3. Phenyl-substituted propylbenzenes condensed with C6-C3 lignan precursors to form flavonolignans.

More broadly, we can also include chalcones and dihydrochalcones, anthocyanins, and aurones [35]. It is believed that defense against UV radiation, and plant hormone regulation were the first flavonoid functions. Flavonoid accumulation could help with harsh field conditions in *Datura*. They are structurally related to carotenoids, which are part of the accessory light harvesting complex in plants. Interestingly, UV-B radiation of mosses increased flavonoid content, which supports this notion [35].

Chalcone synthase (CHS) is derived from beta-ketoacyl ACP synthase. It is in the Type III polyketide synthase (PKS) gene family. CHS may have evolved before chalcone isomerase (CHI), since CHS catalyzes the first step and the step catalyzed by CHI may happen spontaneously [35]. P-coumaroyl CoA is transformed to naringenin chalcone by CHS, then to naringenin by CHI. *Arabidopsis* has four Type III PKS genes and one functional CHS.

CHS is promiscuous in the sense that it can use many substrates, although it cannot function on bulky substrates [35]. *Flavobacterium johnsoniae* has a chalcone synthase gene according to the Uniprot database [43], which may be working synergistically with *Datura* to increase flavonoid content in the plant, supply acyl compounds to the plant for downstream production for plant secondary metabolites, or have a modified function that is specific to its plant host. It is worth mentioning that *Datura*, *Streptomyces*, and *Agrobacterium* also have this gene. In vitro, CHS from *Huperzia serrata* can produce other polyketides besides flavonoids, like aromatic tricyclic pyridoisoindoles [35].

Evolutionarily, CHS on the flavonoid biosynthetic pathway may have paved the way for biosynthesis of medicinal secondary metabolites by land plants. It expanded the chemical variety of the metabolites plants produced over time [35]. Type III PKS can also synthesize oxidosqualane and terpenes in other specialized metabolisms, for example. It is not known why plants and their endophytes have evolved this important set of enzymes, but it warrants further investigation.

There was strong evidence to support the hypothesis that synergistic production of plant derived secondary metabolites by microbes occurred in the *Datura* rootzone, as well as the ability for the compounds to enter plant cells. Many medicinal plants produce economically important compounds, and synthetic production is not possible because the pathways are poorly understood. Further elucidating the interaction between microbes and wild plants could also shed light on resilience mechanisms, which could be used for crop improvement. For example, it has been shown that beneficial microbes prime plant defenses, giving a competitive advantage to organic farming [45]. Furthermore, understanding how these plants adapt to their environment will empower weed control efforts, including biocontrol.

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