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Rhizoremediation of residual sulfonylurea herbicides in agricultural soils using *Lens culinaris* and a commercial supplement

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ABSTRACT

Sulfonylureas (SU) are a popular herbicide used today for controlling weeds. While beneficial for this purpose they present a persistent problem in agricultural treated areas, with this treatment proving detrimental for successive crops. This study assessed the phytoremediative properties of lentils (*Lens culinaris*) grown in uncontaminated and chlorsulfuron-contaminated soil, with and without the addition of a growth supplement, PulseAider™. The results show that in the presence of lentils the degradation of chlorsulfuron is enhanced and this degradation rate is significantly increased when the PulseAider™ supplement was included during seed sowing. The supplement PulseAider™ also significantly increased shoot and root biomass, root branching, and nodule number under control conditions. While this was not so for plants grown in contaminated soils, the PulseAider™ supplement seemed to alter root branching and morphology. Most Probable Number (MPN) assays showed increased numbers of potential chlorsulfuron-degrading bacteria in soil treated with PulseAider™, although this was found to be significant only in the control soil. Sequencing of the 16S ribosomal gene showed the presence of *Pseudomonas fluorescens* bacterial species which is a known chlorsulfuron-degrading bacterium. This study is one of the first to address the remediation of residual SU herbicides and offers an economically feasible solution that may have an impact on global food security.

KEYWORDS

Chlorsulfuron; phytoremediation; lentil

General introduction

In order to support the world's expanding population, it is imperative that dryland agriculture is made more sustainable and efficient, ensuring that the farm sector can increase productivity. In Australia, the practice of using herbicides as a replacement to tillage in terms of weed control is increasing. A popular class of herbicides currently used is the sulfonylurea (SU) herbicides (Saeki *et al.* 2016).

Sulfonylurea herbicides (SU) were first used in 1982 and can control a wide variety of weeds with a significantly increased toxicity to invasive plants (100 times greater than previous herbicides). They can be also applied at very low rates (2–78 g ha^{-1}) and show greatly reduced toxicity to humans and other animals. By inhibiting the acetolactate synthase (ALS) enzyme, they are able to block the synthesis of three amino acids, leucine, isoleucine, and valine, thus interrupting plant growth and cell division (Brown 1990). SUs have also been believed to decrease cell cycle-specific RNA synthesis and block the progression of the cell cycle through the G1 and G2 transition points (Rost 1984). Through the use of SUs, long-term weed control has been established while also increasing crop yields, conserving moisture, minimizing soil erosion, and decreasing the use of fossil fuels in crop management (Ferris *et al.* 1995). While this practice creates long-term soil productivity, it also increases the use of persistent herbicides accumulated in the soil that can damage sensitive crops where there is a wider diversity of rotation in the next season after use of SU. While farmers are encouraged to use grain legumes for rotational crops, these legumes are quite sensitive to SUs that may be persistent in the soil (Haigh and Ferris 1991). In lentil and chickpea crops, root growth inhibition, yield reduction, and early death are some of the symptoms observed that are believed to be a result of SUs (Ferris *et al.* 1995).

The pH of soils can significantly affect the chemical hydrolysis of SUs, with rapid hydrolysis taking place in acidic soils as compared to alkaline soils (Wilhelm and Hollaway 1998). The hydrolysis half-life ($t_{1/2}$) of chlorsulfuron, for example, is remarkably different at pH 3 (1 day) as compared to pH 7.5 (>500 days) at 25°C. Similarly, the $t_{1/2}$ of bensulfuron-methyl at pH 5 is 7 days compared to a $t_{1/2}$ of 460 days at pH 8 (Sarmah and Sabadie 2002). Alkaline soils are representative of agricultural areas in South Eastern (SE) Australia, where there is extensive residual SU contamination, thus the persistence of SU is further exacerbated in these regions.

Fortunately, in parallel with the increasing herbicide contamination in the soil, there is also an increase in the development of procedures that use chemical and biological methods to remediate these contaminants. The strategy of using phytoremediation (especially rhizoremediation) is an increasingly inviting prospect for the improvement of these contaminated areas (Truu *et al.* 2015) . Rhizoremediation involves using

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plants and their associated microorganisms to remove, transform, immobilize, or degrade any toxic compounds present in soils or water. This process can be used to alleviate the effects of contaminants such as pesticides, herbicides, heavy metals, and explosives (Khan *et al.* 2004). When using this method, the main aim is to help the plant to increase both the microbial activity and biomass in order to encourage remediation (Reynolds *et al.* 1999).

Some studies have shown that the degradation of SUs occurs at a greater rate and more efficiently in non-sterile soil in comparison to sterile soil (Reynolds *et al.* 1999; Brown e al. 1997; Li *et al.* 1999; Miller *et al.* 1997), therefore, suggesting that the microbial population present in non-sterile soil may have the potential to degrade SU and reduce the herbicide contamination level. Other studies have demonstrated the ability of specific bacterial and fungal species to degrade chlorsulfuron under co-metabolic conditions *e.g.*, *P. fluorescens, Streptomyces griseolus*, and *Aspergillus niger* (Romesser and O'Keefe 1986; Zanardini *et al.* 2002a, b). Co-metabolism is the process by which a contaminant is fortuitously degraded by an enzyme or co-factor that is produced in response to the microbial metabolism of another compound of similar structure (Hazen 2010). Co-metabolism has been shown to greatly influence the degradation of SUs by microbes.

Brimecombe *et al.* (2000) found that the presence of plant roots and their exudates in soil increases the activity of soil microbes. The concentration of microbes in the root area (the rhizosphere), the development of soil structure, and the production of root exudates, have all been hypothesized as being causes for this increased microbial activity. The root exudates (*i.e.*, amino acids, sugars, organic acids, and secondary metabolites such as flavonoids, alkaloids, and isoprenoids), can stimulate co-metabolism in microbes in the rhizosphere (Bais *et al.* 2006). Contaminants that are structurally analogous to plant exudates can be preferentially selected for degradation by the microbial population (Singer *et al.* 2003). The plant roots can also provide an ecological niche for the proliferation of bacteria and fungi supporting plant growth. Root exudates can promote bacterial growth in areas surrounding the root, such as zones of elongation and lateral root occurrence (Brimecombe *et al.* 2000).

In this study, we have used the principle of phytoremediation and co-metabolism to assess the impact of growing a leguminous crop such as, the lentil, *L. culinaris*, on SU levels in contaminated soils. Further, we have assessed the impact of a commercially available plant growth supplement, PulseAider^{TM}, a preparation made by Injekta Field Systems Inc (Australia) on lentil growth and chlorsulfuron degradation under glasshouse conditions. PulseAider^{TM} is a supplement that contains a cocktail of complex macro- and micronutrients, carbohydrates, secondary metabolites, and amino acids, designed to enhance seed germination and root growth in legume crops. While there is limited published documentation of the benefits of this supplement, a recent field trial assessing nodulation of broad beans in acid soils suggested that when applied with the seed, PulseAider^{TM} promoted better nodulation and soil health (Ryder and Dohle 2016). It was therefore proposed that the inclusion of this supplement may further enhance plant growth, microbial number, and potentially SU degradation in alkaline soils. This study found that in uncontaminated soil the inclusion of PulseAider^{TM} supplement enhanced plant root and shoot growth and nodulation. It was also found that the presence of lentils in chlorsulfuron-contaminated soil significantly increased the degradation of chlorsulfuron compared to unvegetated contaminated soil, which was enhanced with the inclusion of PulseAider^{TM} at seed sowing.

Materials and methods

Chemicals

Chlorsulfuron, triasulfuron, indole, and sodium salicylate were purchased from Sigma−Aldrich (Castle Hill, NSW). The chemicals and reagents used in this study were of analytical grade. Stock solutions of chlorsulfuron (10,000 μgL^{-1}) and triasulfuron (10,000 μgL^{-1}) were prepared in pH-adjusted MilliQ Water (pH 11.5). A growth supplement, PulseAider^{TM} (http://www.rowloader.com.au/products/), was prepared and donated by Injekta Field Systems Inc (South Australia, Australia).

Soil preparation

Soil was obtained from Debco Pty Ltd (Australia) (vege cust 650701 formulation) and pH determined to be 7.2. Soil moisture content was assessed by the oven-dried soil method (Rowell 1994). To simulate SE Australian agricultural soil, 50 L of the soil was further mixed with 50 L of sand in a 550-W 125L Ozito Cement Mixer (Ozito Pty Ltd). MilliQ water adjusted to pH 11.5 was added to alkalize the soil mix, achieving a water capacity of 70%.

Table 1 outlines the different soil treatments, if the soil was to be contaminated with chlorsulfuron, the herbicide was added to the pH-adjusted Milli Q water prior to mixing with the soil to a final chlorsulfuron concentration of $\sim 75\ \mu gL^{-1}$.

Soil was mixed regularly to ensure maximum absorbance of Milli Q water and uniform distribution of chlorsulfuron. Chlorsulfuron-contaminated soil was kept at 4°C until preparation of pots to minimize microbial activity and degradation of the herbicide.

Plant growth and effect of chlorsulfuron contamination

Lentil variety PBA Hurricane XT^{TM} was selected and obtained from Pulse Breeding Australia (PBA) which is a breeding program within Grains Research and Development Corporation,

Table 1. Summary of the soil treatments for analyses.

Soil treatment	Contamination	Plants	PulseAider^{TM}
C/P-/S-	x	x	x
C/P+/S+	x	✓	✓
C/P+/S-	x	✓	x
Co/P-/S-	✓	x	x
Co/P-/S+	✓	x	✓
Co/P+/S-	✓	✓	x
Co/P+/S+	✓	✓	✓

The treatments were denoted as follows: C, alkaline soil alone; Co, alkaline soil plus Chlorsulfuron; P-, No Plants; P+, With Plants; S-, No PulseAider^{TM}; S+, With PulseAider^{TM}.

Australia. This variety has been recommended to show improved tolerance to SU (http://www.pbseeds.com.au/seed_var_broch.html). For the assessment of growth and SU degradation, twelve pots (diameter = 14 cm, height = 20 cm) with five plants per pot were prepared per treatment. Six pots were used for plant sampling and the other six were used for soil sampling. Seeds were planted 2 cm below the soil line. In treatments involving the addition of PulseAider™, the PulseAider™ (200 μl) was added to the bottom of the planting hole, prior to addition of seed as per manufacturer's recommendations. Pots were kept in a 10 cm × 1 m × 2 m tray filled with water. Water levels in trays were maintained at >5 cm at all times during the experiment. The experiment was performed in a temperature-regulated greenhouse (average temp: 27.6°C) during the summer months. The seeds were not inoculated with *Rhizobium* before planting.

Plants were destructively sampled 7, 14, 21, 28, and 35 days after initial seed germination. Shoots were excised from the roots at the seed. Plant roots were carefully washed under running water to remove all adhering soil. Shoot and root material was dried for 48 hours at 80°C to constant weight in pre-weighed sample tubes. Shoot length was measured as the length of the central stem from base at soil surface to tip of the highest leaf. Root length was measured from the base at the soil surface to the tip of the longest root. Nodules associated with roots were counted individually.

For SU level determination and MPN analysis, soil was sampled at a depth of approximately one inch below the soil surface adjacent to the root systems, then immediately stored at 4°C for later analysis.

Determination of soil chlorsulfuron content

The soil samples were extracted twice with a 1:1 ratio of soil: solvent (40% (v/v) Methanol). The solvent was spiked with 50 μgL^{-1} of triasulfuron as an Internal Standard. Extraction samples were sonicated in an ultrasonic water bath for 10 minutes and centrifuged for 12 minutes at 4500 rpm. The extracts were combined and filtered with a 0.45-μm membrane. Portions of 2 mL of extract were transferred to glass vials for Liquid chromatography-mass spectrometry (LCMS) analysis. The LCMS analysis was performed on a Waters Quattro micro (Waters, Manchester, UK). Liquid chromatography (LC) separation was provided by a Waters Acquity UPLC (Waters, Milford, USA). A Phenomenex Kinetex-C18 column (50 mm length × 2.0 mm, id × 2.6 μm) was used as the analysis column. The column was maintained at a temperature 30°C. The chromatographic data were collected by the Masslynx Software (v4.1); this software was also used for quantification processing using the ratio of the chlorsulfuron to triasulfuron peak areas. An appropriate aliquot (10 μL) was injected. The mobile phase was composed of 0.1% formic acid in water (solvent A) and methanol (solvent B) with flow rate at 0.2 mL/minute. The following gradient profile was used: initial conditions: 95% A/5% B; then to: 5% A/95% B over 5 minutes, held for 1 minute, returned to 95% A/5% B over 0.5 minute and held at 95% A/5% B to equilibrate column. Under these conditions, the retention time for chlorsulfuron was 3.9 minutes and was 4.3 minutes for triasulfuron.

Determination of most probable number (MPN) in soil

To determine bacterial numbers in the soil sample an MPN assay based on a substrate of similar chemical nature to the SU contamination was used, following an adapted method from Wrenn and Venosa (1996) using a 96-well microtitre plate. Each well contained Bushnell-Haas broth, 1 mmol L^{-1} indole-3-acetic acid, 2 mmol L^{-1} sodium salicylate, and a sample of soil suspension (diluted 10^{-1} to 10^{-6}). Plates were incubated at 28°C for 3 weeks. Bacteria that express the dioxygenase enzyme cleave the aromatic ring of indole, which produces indigo. This is seen as an insoluble blue pigment in the wells of the plate. Salicylate is a degradation intermediate of aromatic compounds that can stimulate induction of dioxygenase enzymes. Wells with positive production of indigo were counted and used to calculate MPN indices obtained from 5-tube MPN tables for three successive 10-fold dilutions as described by Oblinger and Koburger (1975).

Identification of bacterial genera present in SU-contaminated soil

Soil solution was incubated in 1/100 dilution of Nutrient broth (1 g Bacto-tryptone, 0.5 g Yeast Extract, 1 g NaCl in 1 L) at 28°C under shaking conditions (Ohta and Hattori 1980). Bacterial cultures were pelleted and resuspended in Bushnell-Haas media (Zimbro 2003), spread onto Indole plates (1.5% Agar, 1 mM Indole, 2 mM sodium salicylate dissolved in Bushnell-Haas media) and incubated at 28°C (Liste and Prutz 2006). Blue colonies (indicating indigo production from indole) were selected and grown and plated under the previous conditions. Isolated blue colonies were then selected for PCR analysis. PCRs were carried out using primers designed previously, 16S ribosomal DNA was amplified (Iwamoto *et al.* 2000). Cycling conditions were: 1 cycle at 94°C for 4 minutes (initial denaturation) followed by 30 cycles for 1 minute at 94°C (denaturing), 1 minute at 54°C (annealing) and 90 seconds at 72°C (extension) followed by a final extension cycle at 72°C for 10 minutes. PCR products were column-purified (Wizard PCR Preps, Promega) and sequenced by the Australian Genome Research Facility (AGRF, Adelaide, Australia). Sequencing was performed using both forward and reverse primers (EUB f933 and EUB r1387). The consensus sequence was entered into the National Centre for Biotechnology Information (NCBI) nucleotide database (http://www.ncbi.nlm.nih.gov/) and the Mega BLAST (Basic Logical Alignment Search Tool) program (Zhang *et al.* 2000) was used to identify nearest matches.

Identification of nodule rhizobia

Soil solution was added to 1/100 dilution of nutrient broth (see above for composition of media) and incubated at 28°C under shaking conditions. Bacterial cultures were

pelleted and resuspended in diluted Nutrient media and spread onto Yeast extract media-Congo Red plates (Hahn 1966) and incubated at 28°C. White/pale pink colonies were selected and grown and plated under the previous conditions. Isolated white/pale pink colonies were then selected for PCR and sequence analyses. All analyses of the nodule rhizobia were performed as described for soil bacterial identification.

Statistical analyses

The significant differences in the chlorsulfuron degradation and all phenotypic measurements between different treatments over time were evaluated by three-way analysis of variance. All statistical analyses were performed using SPSS 23.0 for Windows (SPSS Inc.).

Results

The presence of PulseAider^{TM} enhances plant growth phenotypes in the absence of chlorsulfuron

Lentils were selected as candidate plants with potential for rhizoremediation for several reasons. The foremost reason is that the use of a marketable rotational crop species makes rhizoremediation of agricultural soils an attractive proposition economically (Black *et al.* 1999). Further, lentils are a major legume crop in SE Australia with the ability to fix nitrogen, which would improve soil properties and health. Lastly, as a legume, lentils are known to exude aromatic compounds, flavonoids that are analogous in structure to SU. The variety, PBA Hurricane XT^{TM} was selected as it has shown better tolerance to growth in SU-contaminated soils (Pulse Breeding Australia, 2013). The combination of nitrogen fixation, SU analogues, and a good potential to access inorganic phosphates provides a strong theoretical basis for the co-metabolism of SU. Co-metabolism was the goal of this investigation as SU concentrations in soils are probably insufficient for them to be an energetically viable substrate for direct metabolism.

Plant growth was assessed by differences in shoot and root length, dry weight (DW), and nodule number between days 7 and 35 after germination (Figure 1). In uncontaminated soil, the addition of PulseAider^{TM} produced a significant increase in all the phenotypic characteristics of the C/S+ plants after 35 days of growth, ($p > 0.05$) (shoot DW, $p = 0.015$, root DW, $p = 0.011$, shoot length, $P = 0.015$, nodules, $p = 0.003$, root length, $p = 0.739$), with the exception of root length (Figure 1). A stimulating effect on root length of the PulseAider^{TM} was evident early in the growth phase of the plants at day 7 and continued through the first 28 days of growth. However, by day 35, plants in the absence of PulseAider^{TM} (C/S-) had root lengths similar to the C/S+ plants (Figure 1B and D). At day 35, though, the C/S+ plants had higher root DW even though the roots were of similar length, suggesting that the C/S+ roots had more biomass, which is suggestive of improved branching. Increased branching and the presence of more fine lateral roots emerging from the tap root were evident from visual observations (Figure 2A and B).

Unlike the root effect of the PulseAider^{TM}, the CS+ plants had a significantly higher shoot length and shoot DW

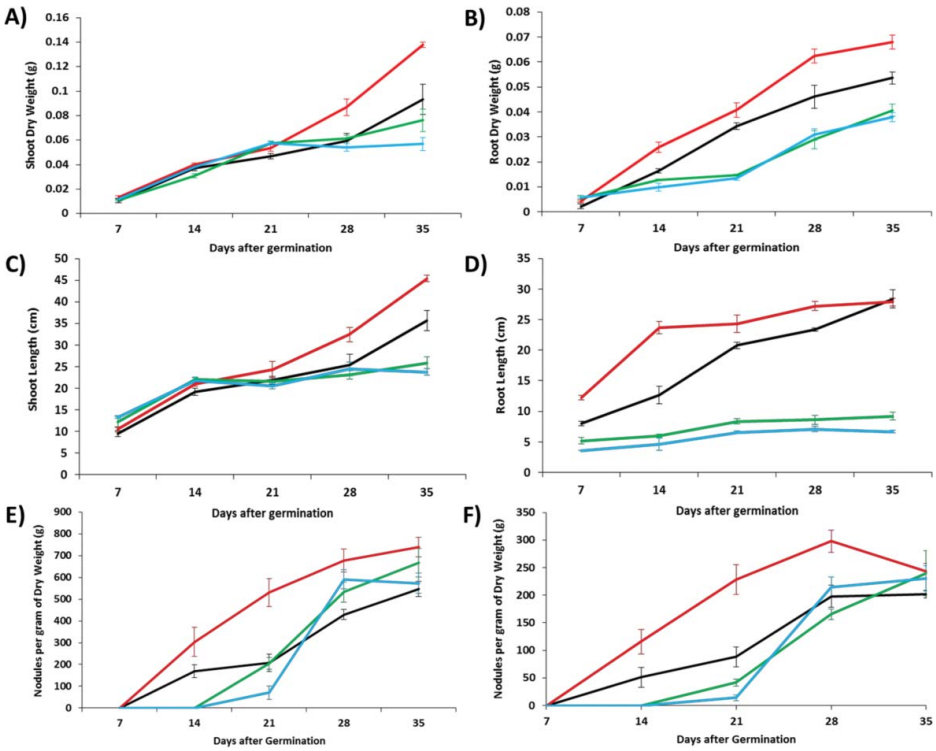

Figure 1. Effect of supplement and/or chlorsulfuron contamination on plant growth. Plants were grown in the treatments as outlined in Table 1 over a 35-day period. (A) Shoot dry weight, (B) root dry weight, (C) shoot length, (D) root length, (E) nodules per gram of plant dry weight, and (F) nodules per gram of root dry weight. Error bar represents SD for n = 5. Asterisks indicate the significance of differences in the growth phenotype between soil treatments on the level of $p < 0.05$ (– C/S-, – C/S+, – Co/S-, – Co/S+).

Figure 2. Lentil root morphology in uncontaminated and contaminated soil. Plants were harvested 35 days after germination. Contaminated soil contained 75 ppb Chlorsulfuron. (A) Lentil roots grown in Uncontaminated soil with no PulseAider^{TM} treatment, (B) lentil roots grown in Uncontaminated soil with PulseAider^{TM} treatment, (C) lentil roots grown in Chlorsulfuron-contaminated soil with no PulseAider^{TM} treatment, and (D) lentil roots grown in Chlorsulfuron-contaminated soil with PulseAider^{TM} treatment.

throughout the growth period, although the DW only became significantly higher toward the end of the growth phase. Interestingly, in the CS+ plants, the shoot DW became significantly greater than the CS- shoot DW at approximately the same time as the root length values ceased to become significantly different (Figure 1A and D). This suggests that the increased root mass early in growth, allowed for a greater root surface area to be exposed to the soil, potentially increasing the nutrient uptake and in turn, creating more biomass in the shoots.

The presence of PulseAider^{TM} did not improve plant biomass but did improve root morphology in chlorsulfuron-contaminated soils

Lentil plants were germinated and grown in uncontaminated soil (C) and chlorsulfuron-contaminated soil (Co) with either no PulseAider^{TM} (S-) or PulseAider^{TM} added (S+). The germination rate of PBA Hurricane XT seeds was unaffected by chlorsulfuron-contaminated soil as compared to uncontaminated soil (data not shown). At the concentration of chlorsulfuron used in this study (75 μgL^{-1}), plants did not flower for the duration of the experiment, however lentils did produce flowers within 6–8 weeks at lower contamination levels which are more similar to those found in contaminated soils in the field (up to 10 μgL^{-1}). Also, the presence of PulseAider^{TM} did not affect the number of flowers or the flowering times of plants grown in uncontaminated soil (data not shown).

In the present study, under contaminated soil conditions, plant growth was significantly inhibited when compared to plants grown in uncontaminated soil over the 35-day period analyzed (Figures 1 and 2). The presence of chlorsulfuron in the soil caused reduced root DW and root length and branching (Figure 2C). The presence of PulseAider^{TM} did not increase any of the quantitative growth parameters measured for plants grown in the contaminated soil (Figure 1). In the Co/S+ plants, there was no significant difference in root DW; however, the presence of the supplement resulted in a small but significant decrease in root length ($p = 0.006$) (Figure 1D). However, as with the plants in the uncontaminated soil, the presence of PulseAider^{TM} appeared to increase root branching and alter the root morphology, giving the branching roots the appearance of being more abundant and finer as compared to the roots in the absence of supplement (Figure 2C and D). Although this change in morphology did not result in a change in biomass (Figure 1B), it has resulted in the development of more of the smaller finer roots.

The presence of PulseAider^{TM} stimulates increased nodule formation in uncontaminated soil compared to chlorsulfuron-contaminated soil

As well as stimulating plant growth in uncontaminated soil, PulseAider^{TM} caused a dramatic increase in nodule formation in both uncontaminated and contaminated soil despite a reduced root length phenotype observed with the contaminated soil (Figure 2). The presence of more nodules may be a contributing factor in the better growth observed with PulseAider^{TM} in uncontaminated soils. These nodules were pinkish in color and to assess if these were nitrogen-fixing nodules, the microbes extracted from nodules were cultured and identified using sequencing of the 16S ribosomal gene (Lindemann and Ham 1979; Tas *et al.* 1996). Due to bacterial contamination of the C/S- sample, the data for this treatment are not shown.

Sequence analysis determined that two different varieties of rhizobia were present, *Rhizobium sp* and *Bradyrhizobium* sp. Sequence analysis of the 16S RNA PCR products revealed a product showing highest homology to *Rhizobium leguminosarum* which was not unexpected for lentils (Table 2). Other PCR products detected were from *Bradyrhizobium* species. The *Bradyrhizobium* species are most commonly found in the nodules of tropical legumes such as soybean (Guimarães *et al.* 2015), and so their presence here was surprising. The *R. leguminosarum* species is the most common *Rhizobia* spp. associated with lentils (Rashid *et al.* 2014). The presence of a *Bradyrhizobium* sp may suggest that this species may have been already present in the soil and available for nodule formation. To determine the exact species, a deeper level of identification, Multilocus Sequence Typing would have to be performed, amplifying a number of specific housekeeping genes for further sequence analysis.

Soil from PulseAider^{TM}-treated plants grown in contaminated soil show increased indole-degrading bacterial numbers

Soil from the seven different treatments was assessed for the presence of indole-degrading bacteria by extraction of the microbes from the soil sample, followed by culture in the presence of indole (MPN assay). The Bushnell-Haas medium is composed of ingredients that can support the growth of microorganisms but is missing a carbon source (Zimbro 2003). The carbon source in this experiment was indole, and also included was sodium salicylate, which is an inducer of dioxygenase

Table 2. 16S ribosomal gene sequence analysis of *Rhizobium* species isolated from root nodules.

NCBI database accession number	Aligned sequence description	% Similarity
Control-With PulseAider™		
NR_135877.1	*Bradyrhizobium erythrophlei*	99
NR_117513.1	*Bradyrhizobium lablabi* strain CCBAU 23086	99
NR_112927.1	*Bradyrhizobium elkanii* strain NBRC 14791	99
NR_133707.1	*Bradyrhizobium icense* strain LMTR 13	99
NR_043037.1	*Bradyrhizobium pachyrhizi* strain PAC48	99
Contaminated-No PulseAider™		
NR_135877.1	*Bradyrhizobium erythrophlei*	99
NR_117513.1	*Bradyrhizobium lablabi* strain CCBAU 23086	99
NR_112927.1	*Bradyrhizobium elkanii* strain NBRC 14791	99
NR_133707.1	*Bradyrhizobium icense* strain LMTR 13	99
NR_043037.1	*Bradyrhizobium pachyrhizi* strain PAC48	99
Contaminated-With PulseAider™		
NR_137257.1	*Rhizobium ecuadorense* strain CNPSo 671	99
NR_137229.1	*Rhizobium anhuiense* strain CCBAU 23252	99
NR_103919.1	*Rhizobium leguminosarum bv. viciae* 3841 strain 3841	99
NR_113671.1	*Rhizobium phaseoli* strain NBRC 14785	99
NR_113895.1	*Rhizobium indigoferae* strain NBRC 100398	99

Nodules were isolated from plant roots grown under four individual soil treatments; Uncontaminated soil with PulseAider™ treatment, Chlorsulfuron-contaminated soil with no PulseAider™ treatment, and Chlorsulfuron-contaminated soil with PulseAider™ treatment.

transcription. With this experimental design, microbes that can utilize indole as a carbon source should survive and grow whereas microbes that cannot metabolize indole will be nutritionally starved. As indole has a similar chemical structure to chlorsulfuron, it is hypothesized that bacterial enzymes capable of degrading indole will also be capable of degrading chlorsulfuron. These soil samples were those collected from the growth study (Figure 1), but included an additional treatment of soil without plants, contaminant, and PulseAider.

While most soil conditions tested showed an increase in microbial growth rate, both soil samples in the absence of plants showed either a decrease in growth or a non-significant increase in bacterial number (C/P-/S- and Co/P-/S- soil, respectively) (Figure 3; Table 3). In the C/P+/S+ soil, there was a significant increase in the growth rate of microbes as compared to the C/P+/S- soil ($p = 0.019$), suggesting that the supplement PulseAider with the plants enhances microbial population. A similar increase in the rate of growth of microbes was also seen in the Co/P+/S+ soil when compared to the Co/P+/S- soil, however under these conditions this increase was not significant ($p = 0.61$). Interestingly, a slight increase in microbial growth was observed in the Co/P-/S+ soil as compared to the Co/P-/S- soil, although this increase was also not significant ($p = 0.57$), this may reflect an increased nutrient supply in the presence of PulseAider in the soil which has been observed to enhance microbial growth in other studies (Knelman *et al.* 2014).

Previously, it has been observed that microbial numbers increased in soil with plants, when a contaminant was present (Khan *et al.* 2013; Miya and Firestone 2000). The MPN data seem to support this, although only the Co/P+/S+ soil showed a significant difference compared to the C/P+/S+ soil. This effect was further enhanced by the addition of plants as a contributing factor.

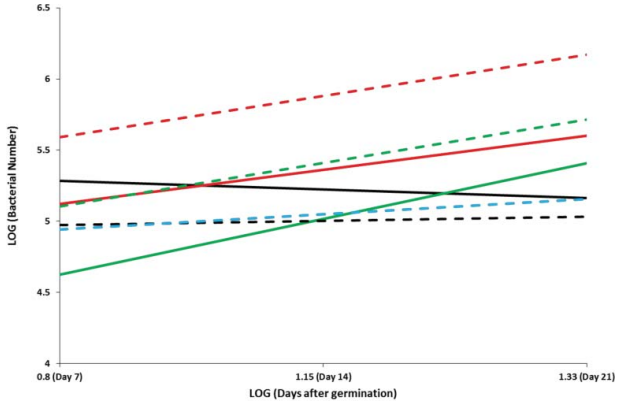

Figure 3. Effect of plants, Chlorsulfuron, and PulseAider™ on microbial population. Microbial population effects were assessed using MPNs determined in soil samples from the following treatments after 7, 14, and 21 days of plant growth: Uncontaminated soil containing plants/no PulseAider™ (—C/P+/S-), Uncontaminated soil containing plants/with PulseAider™ (— C/P+/S+), uncontaminated soil containing no plants/no PulseAider™ (— C/P-/S-), Contaminated soil containing plants/no PulseAider™ (- - - Co/P+/S-), contaminated soil containing plants/with PulseAider™ (- - -Co/P+/S+), contaminated soil containing no plants/no PulseAider™ (- - - Co/P-/S-) and contaminated soil containing no plants/with PulseAider™ (- - - Co/P-/S+).

Identification of Pseudomonas bacterial species in contaminated soil samples

By applying the developed indole-based MPN method, bacterial strains potentially capable of chlorsulfuron degradation have been isolated from the soil and identified by bacterio-diagnostic tests (Iwamoto *et al.* 2000). While four soil samples were tested, only one bacterial strain was identified from each sample in this study. A *Pseudomonas* strain was isolated from each sample and although the exact strain was not determined, the top five strains were all part of the *P. fluorescens* family (Table 4).

PulseAider™ and the presence of lentils increase chlorsulfuron degradation

The concentration of chlorsulfuron under different soil treatment conditions (Co/P-/S-, Co/P-/S+, Co/P+/S-, and Co/P+/S+) was monitored over 35 days, the duration of the growth study and it was found that the majority of degradation occurred over the first 21 days, thus only these data were analyzed (Figure 4).

While there was chlorsulfuron-degradation in soil alone, the presence of plants significantly increased this rate ($p = 0.046$). Also, in the presence of plants, the addition of PulseAider™

Table 3. Effect of interaction of soil, plants, and chlorsulfuron on microbial growth rates.

Conditions	Growth rate
C/P-/S-	−0.0597
C/P+/S-	0.2408
C/P+/S+	0.391
Co/P-/S-	0.0298
Co/P+/S-	0.2903
Co/P+/S+	0.3063
Co/P-/S+	0.108

Growth rates were calculated from the data in Figure 3.

Table 4. 16S ribosomal gene sequence analysis of bacterial species isolated from treated soil.

NCBI database accession number	Aligned sequence description	% Similarity
NR_114481.1	Pseudomonas tolaasii strain ATCC 33618	100
NR_114227.1	Pseudomonas tolaasii strain NBRC 103163	100
NR_117826.1	Pseudomonas corrugata	100
NR_117823.1	Pseudomonas tolaasii	100
NR_042541.1	Pseudomonas reinekei strain MT1	100

Bacteria were isolated from four individual soil treatments; Uncontaminated soil with PulseAider™ treatment, Chlorsulfuron-contaminated soil with no PulseAider™ treatment, and Chlorsulfuron-contaminated soil with PulseAider™ treatment.

during seed sowing significantly ($p < 0.005$) increased the degradation rate until the level of chlorsulfuron reached a threshold concentration where it may no longer be a viable carbon source for the microbes (Figure 4A and B).

The degradation of chlorsulfuron in soil with plants, in our analysis was significantly greater than their degradation in the absence of plants (vegetated soil without PulseAider™, $p = 0.046$: vegetated soil with PulseAider™, $p < 0.0005$). This supports the previous findings that vegetated soil enhances the degradation of soil contaminants and highlights the importance of rhizosphere interactions in contaminant degradation.

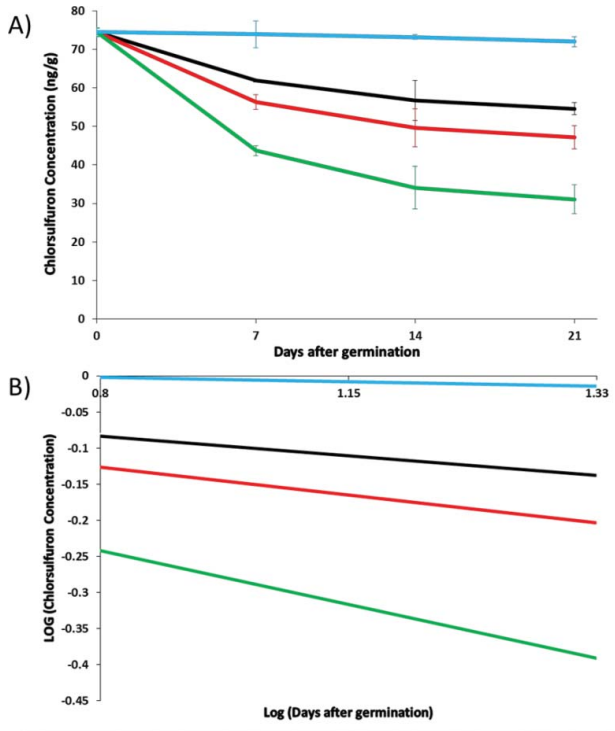

Figure 4. Effect of plants and PulseAider™ on Chlorsulfuron degradation over 21 days. (A) Degradation curves of Chlorsulfuron under no plants/no PulseAider™ (– P-/S-), no plants/with PulseAider™ (– P-/S+), with plants/no PulseAider™ (– P+/S-) and with plants/with PulseAider™ (– P+/S+) soil treatments over 21 days. Error bar represents one standard deviation of triplicate assays. Asterisks indicate the significance of differences in the Chlorsulfuron degradation between soil treatments on the level of $p < 0.05$. (B) LOG degradation curves under no plants/no PulseAider™ (– P-/S-), no plants/with PulseAider™ (– P-/S+), with plants/no PulseAider™ (– P+/S-) and with plants/with PulseAider™ (– P+/S+) soil treatments over 21 days. (P- = no plants, P+ = with plants, S- = no PulseAider™, S+ = with PulseAider™).

Discussion

In this study, the use of lentils and a commercially available growth supplement which has been used to promote lentil growth were explored for their use in reducing the levels of SU-contaminated soil. The principle behind this strategy relies on the phenomenon of co-metabolism to promote SU-degrading bacteria in the soil. Legumes were chosen for this study as these plants exude compounds with similar structure to that of SU to attract nitrogen-fixing bacteria and this was hypothesized to provide a strong theoretical basis for the co-metabolism of SU. The choice of legumes would also enhance soil quality for future agricultural use. Co-metabolism was the goal of this investigation as SU concentrations in soils are probably insufficient for them to be an energetically viable substrate for direct metabolism. From the data presented it is clear that the presence of lentils in the contaminated soil enhanced the degradation of SU and altered the microbial population.

Further, the use of a commercially available supplement during the seed sowing of legumes further enhanced this degradation and significantly altered the soil microbial population. From the growth studies it was evident that PulseAider™ has a positive effect on both root and shoot growth in uncontaminated soil and dramatically improved nodulation. This is the first documented evidence for the positive effect of PulseAider™, which appeared to alter the lentil root morphology. This is consistent with the design of this product to improve root cell division as claimed. Further, root morphology in plants grown in SU-contaminated soil in the presence of PulseAider™ was altered; however, plant growth was not significantly better than it was in its absence. However, in the presence of PulseAider™ there was an impact on the soil microbial population and did result in enhanced SU degradation. From this study it is not possible to distinguish whether the effect of altered root morphology in the presence of PulseAider™ and SU is a result of a direct effect on the plant, or a consequence of lower SU levels in the soil. Further work on changes within the plant in the presence of PulseAider™ is needed to resolve this.

Studies on various crops such as lentil, wheat, and chickpea have shown a variety of metabolic, proteomic, and transcriptomic changes in the plant when chlorsulfuron has been applied (Al-Quraan *et al.* 2015; Anderson *et al.* 2004; Zulet *et al.* 2015). The target for SU herbicides is the enzyme ALS which is involved in the synthesis of branched-chain amino acids. The SU herbicides are well documented to result in the cessation of root cell division and growth (Brown 1990; Rost 1984; Zabalza *et al.* 2004). The meristematic tissue is particularly sensitive and in wheat and barley it has been noted that these herbicides inhibit the growth of the smaller fine roots, which play a major role in nutrient absorption (Rengel and Wheal 1997). ALS can catalyze either the formation of acetolactate from two pyruvate molecules, or the formation of 2-aceto-2-hydroxybutyrate from pyruvate and 2-ketobutyrate (Singh 1999). When ALS is inhibited, this then allows for an increase in the available pyruvate levels. These elevated levels of pyruvate can lead to the induction of the fermentation and alternative respiratory pathways (Vanlerberghe *et al.* 1995; Zabalza

et al. 2005). These pathways are low-ATP-producing pathways and so this may be a factor in the reduction of growth of both roots and shoots (Zulet *et al.* 2015). Other metabolic consequences have been observed, such as carbohydrate accumulation in roots and leaves due to inhibited phloem transport, increased free amino acid content causing imbalances in carbon/nitrogen metabolism, and increased respiration causing acute hypoxia (Zabalza *et al.* 2004; Orcaray *et al.* 2012; Zabalza *et al.* 2009, 2011). At present, the role that these pathways may play in the phytotoxicity of chlorsulfuron has not been fully established.

These biochemical changes in the lentil plant caused by chlorsulfuron application consequently create phenotypic changes in the plant. The observed changes in root morphology are consistent with that reported in other studies, where it has been demonstrated that chlorsulfuron causes a severe decrease in root growth, density, and a thickening of roots becomes evident (Zabalza *et al.* 2011).

As it has been documented that SU herbicides inhibit the development of the branching roots (Rengel and Wheal 1997), the increased presence of these roots, may be indicative of lower levels of the herbicide contamination in the presence of PulseAider^{TM}. Further, the presence of these finer roots may enhance changes in the rhizosphere, which would influence and promote changes in the soil microbiome.

The MPN method is commonly used for enumerating microorganisms in environmental samples (Escalante-Espinosa *et al.* 2005; Mikkonen *et al.* 2011; Toyama *et al.* 2011). Using a liquid media, microbial growth can be detected by creating a selective environment that allows only specific microbes to grow. In this study, an MPN method was developed allowing for the indirect estimation of populations of bacteria potentially capable of chlorsulfuron degradation. This was based on the hypothesis that the flavonoids produced by the lentil roots will allow for the co-metabolism of chlorsulfuron by the soil microorganisms. A chemical with a similar chemical structure to both chlorsulfuron and the flavonoids was needed for this method to be able to enumerate microbial numbers. Unfortunately, the breakdown products of chlorsulfuron are not detectable by a color change of the solution, which is essential in an MPN assay to identify positive and negative samples. To this end, indole was selected as all three chemicals share an aromatic ring that is believed to be the target of many flavonoid-degrading enzymes *e.g.*, dioxygenases. Indole is also degraded to form indigo, an insoluble purple product, which can be either visually observed or detected spectrophotometrically. A positive sample means that it contains microbes capable of degrading indole, suggesting that they might also be capable of degrading chlorsulfuron. Though this is not a conclusive test, combined with the other analyses it provides supporting evidence of the co-metabolic breakdown of chlorsulfuron.

The MPN results are consistent with previous findings where microbial populations increased in vegetated soils compared to non-vegetated soils (Grayston *et al.* 1998). The small increase in microbial growth rate in the absence of plants when chlorsulfuron was present in the soil indicates that the chlorsulfuron-degrading microbes are using chlorsulfuron as a carbon source whereas no such carbon source exists in the uncontaminated soil.

Previously, it has been observed that microbial numbers increased in soil with plants when a contaminant was present (Khan *et al.* 2013; Miya and Firestone 2000). The MPN data seem to support this, although only the contaminated soil with plants and PulseAider^{TM} present showed a significant difference compared to the control soil with plants and PulseAider^{TM} present. This effect was further enhanced by the addition of plants as a contributing factor. Siciliano *et al* (2001) found that plants can selectively increase the root-associated endophytic population expressing contaminant-degrading enzymes *e.g.*, dioxygenases and monooxygenases (Siciliano *et al.* 2001). This previous study showed that an increase in numbers of bacteria capable of contaminant degradation is dependent on both the presence of the contaminant and the plant itself. So while the data presented are informative about the relative numbers of bacteria contained in the soil from the various treatments, it does not give an indication of the composition of the microbial population in each soil type. An analysis of the soil microbiome using a metagenomics approach would need to be performed in order to determine the number of specific genera present and shed light on the actual effect of contamination, PulseAider^{TM} and plant presence on the microbial composition (Lakshmanan *et al.* 2014).

Several *Pseudomonas* strains, including *P.fluorescens* have been repeatedly shown to degrade SUs (Zanardini *et al.* 2002; Arabet *et al.* 2014; Valle *et al.* 2006). While it would have been interesting to isolate other bacterial species, the fact that a *Pseudomonas* strain was identified shows that our method of isolating potential chlorsulfuron-degrading microbes was possibly successful. A large-scale screening of the soil samples may lead to the isolation of further degradative microbial species.

The majority of recent studies have demonstrated that co-metabolic degradation of SUs by bacteria can occur, but this has been demonstrated in media under laboratory conditions (Zanardini *et al.* 2002; Luo *et al.* 2008; Wang *et al.* 2016; Zhang *et al.* 2013; Zhao *et al.* 2015). The Zanardini *et al* (2002a) study showed that the biodegradation of chlorsulfuron by *P. fluorescens* B2 strain, occurred preferentially under co-metabolic conditions in nutrient-rich medium (32% degradation of chlorsulfuron) as compared to a medium where chlorsulfuron was the sole carbon source (15% degradation of chlorsulfuron), suggesting that the degradation was increased in the presence of co-substrates.

Our results suggest these observations and interactions can be extended to soil and that the presence of plants in the soil can greatly enhance the degradation of chlorsulfuron and potentially other SUs, compared to soil without plants. The ability of vegetation in soils to increase contaminant degradation has been shown with polyaromatic hydrocarbons and the herbicide, atrazine, using fescue grass (Banks *et al.* 1999; Belden and Coats 2004; Liu *et al.* 2014) but the effect on SUs has not been documented.

The observed increase in chlorsulfuron-degradation in vegetated soil treated with PulseAider^{TM} is characteristic of biostimulation, in which the addition of nutrients can increase microbial populations, more specifically, the microbial populations needed to degrade contaminants (Dehghani *et al.* 2013). The increased chlorsulfuron degradation by the contaminated soil with plants and PulseAider^{TM} present was most likely due

to the interaction between plants, the growth supplement, and bacteria in a co-metabolic environment.

Results from this study suggest that the addition of a growth supplement (PulseAider™) induced significant increases in many growth parameters and root nodulation in lentils. In addition to this, there was also a significant increase in the degradation of chlorsulfuron in soil when plants where grown with PulseAider™. Bacterial numbers in soil treated with PulseAider™ also appeared to increase in uncontaminated soils, although this may be due to selection processes favoring the growth of chlorsulfuron-degrading bacteria. Bacterial species from the *P. fluorescens* family, which are commonly associated with SUs, were isolated, providing support for this hypothesis.

Using these findings, we have identified the positive effects of the PulseAider™ growth supplement for lentil crops and these effects may expand to other legume crops. Further, its effect on SU-contaminated soils in this study warrants further investigation of its use under field conditions in agricultural areas suffering from accumulated SU contamination.

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