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Oil Pollution Affects the Central Metabolism of Keystone *Vachellia* (*Acacia*) Trees

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Abstract: *Vachellia* (formerly *Acacia*) trees are native to arid environments in Africa and the Arabian Peninsula, where they often support the local animal and plant communities acting as keystone species. The aim of this study was to examine whether oil pollution affected the central metabolism of the native keystone trees *Vachellia tortilis* (Forssk.) and *V. raddiana* (Savi), as either adults or seedlings. The study was conducted in the Evrona Nature Reserve, a desert ecosystem in southern Israel where two major oil spills occurred in 1975 and in 2014. Leaf samples were collected to analyze the central metabolite profiles from oil-polluted and unpolluted adult trees and from *Vachellia* seedlings growing in oil-polluted and unpolluted soils in an outdoor setup. We found that oil pollution had a stronger effect on one-year-old seedlings than on adult trees, reducing the levels of amino acids, sugars, and organic acids. While adult trees are mildly affected by oil pollution, the effects on young seedlings can cause a long-term reduction in the population of these keystone desert trees, ultimately threatening this entire ecosystem.

Keywords: amino acid; desert; environmental impact; Evrona Nature Reserve; oil spill; petroleum; recovery

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1. Introduction

Although renewable energy sources are growing in popularity, human societies still heavily rely on fossil fuels [1]. Unfortunately, fossil fuels are associated with several environmental threats, including climate change and pollution [2]. Accidental oil spills have dispersed millions of tons of oil into the environment, jeopardizing ecosystems around the world. For instance, from 1958–2016, ca. 5.5 million tons of oil polluted mangrove ecosystems alone [3]. While examples of oil spills in marine and coastal environments are numerous [4,5], oil spills also occur in terrestrial ecosystems, often seriously damaging them. Oil can persist in terrestrial environments for decades [6], with dramatic effects on the vegetation [7,8]. Plants can suffer both lethal and sublethal effects, which may already be evident during the contamination event or shortly thereafter. Additionally, oil pollution can reduce plant recruitment, affecting the community in the long term, and if its negative effects are not mitigated, this can ultimately cause habitat loss, dramatically altering the landscape [3].

Most terrestrial oil spills occur in deserts, where wells are often located [9]. Desert ecosystems are characterized by extreme abiotic conditions, such as high temperatures and low precipitation levels [10], which limit the presence of species and curtail most biological processes [11]. For this reason, desert ecosystems are particularly sensitive to anthropogenic disturbances and often take a long time to recover [12]. While there is ample evidence for the negative effects of oil pollution in marine, coastal, and tropical ecosystems [7,8,13,14], the consequences of oil pollution for desert communities, including plant species, are less understood.

By changing the soil's physical and chemical characteristics, oil pollution can induce abiotic stress in plants [15]. Oil pollution was shown to reduce transpiration and photosynthetic rates and change plant metabolism [16]. It can also cause oxidative stress and alter the concentration of phenols and flavonoid compounds [17,18], compromise the synthesis of sugars [19], cause the accumulation of starch in the leaves [20], and increase the levels of free amino acids and total protein [21]. It can also reduce seeds' germination success and hinder their root development [22]. The impact of oil pollution on plants may depend on the oil type, characteristics of the polluted soil, the environmental conditions in which the pollution took place, and the plant species affected [16]. The response of plants to oil pollution can be family specific; for instance, Arellano et al. [14] showed that Melastomataceae, Fabaceae, Rubiaceae, and Euphorbiaceae were more susceptible to oil than other common plant families in the Amazon region. Evaluating the effects of oil pollution on four desert Asteraceae, Malallah et al. [18] showed that even within the same family, the responses to oil pollution were not always consistent. Finally, the effect of oil may depend on the plant developmental stage, as young plants are usually more susceptible to stress factors than mature trees [23].

When protecting natural areas, conservationists often need to identify which species should be prioritized in terms of conservation and management efforts [24]. Some species are regarded as worthy of protection due to their population decline, while others, even though not endangered, are considered important for conservation because they contribute to protecting the community and the entire ecosystem. Species with a disproportionally beneficial effect on the community are termed keystone species [25]. High mortality of keystone species often damages ecosystems, such that they are no longer recognizable [26,27]. Therefore, keystone species are particularly important for maintaining functional ecosystems and are usually given high priority in conservation programs [28].

In this study, the effect of terrestrial oil spills on two native keystone species in a desert ecosystem was investigated. In 1975 and 2014, two large oil spills affected the Evrona Nature Reserve, a protected desert ecosystem located in southern Israel [29]. *Vachellia* (formerly *Acacia*) *tortilis* (Forssk.) and *V. raddiana* (Savi) are the only tree species growing in this area, where they act as keystone species [30,31]. *Vachellia tortilis* is distributed across tropical and subtropical regions in Africa and in the Arabian Peninsula. *Vachellia raddiana* is frequently considered to be a subspecies of *V. tortilis*, except in Israel where the two species are morphologically and genetically distinguishable, in addition to having different local distributions [32–34]. The populations of both *V. tortilis* and *V. raddiana* in southern Israel are declining, mostly due to anthropogenic factors [35–37].

We examined whether oil pollution affected the central metabolite profiles of adult *Vachellia* trees and seedlings. Our hypothesis was that in both cases, these profiles would be adversely modified as the result of oil spill contaminations. In accordance with previous observations, we predicted that plant metabolism would be affected by oil pollution [22] and that the seedlings would be more susceptible to the effects of the oil-polluted soil than the adult trees.

2. Materials and Methods

2.1. Evrona Nature Reserve

The Evrona Nature Reserve (29°40′38.76′′ N, 35°0′55.12′′ E) is a protected natural area located in the Arava Rift Valley, in southern Israel. The area is a hyperarid desert that experiences 25–50 mm annual precipitation and average summer air temperatures near 31 °C [38]. The soils here are saline and vary in their texture and composition (for details, see [29,39]). In July 1975, a leak in the Eilat Ashkelon pipeline caused a spill of 8000–10,000 m^3 of crude oil into the reserve. No remediation was attempted, except for pumping some of the oil out of the area. At the time of the spill, the composition of the oil was unknown; however, a detailed description of the residual fuel was given by Nothers et al. [40]. In December 2014, about 5000 m^3 of crude oil leaked from the same pipeline, and in 2019, the soil was tilled 10 cm deep to break the impermeable crust created by the oil and improve the soil's hydrological properties. However, this measure was not effective [39].

2.2. Leaf Collection, Adult Trees

Two adjacent study sites affected by the 2014 oil spill were selected: the first (Evrona North) where both *V. raddiana* and *V. tortilis* occur, and the second (Evrona South) with *V. tortilis* only. See Möller et al. [41] for additional details on the study sites. From 24 to 27 September 2017, 10 *V. raddiana* trees that were not in the main route of the oil spill (i.e., unpolluted soil) and eight trees that were directly exposed to the oil (i.e., growing in oil-polluted soil) were selected. Similarly, 10 unpolluted and 10 oil-polluted *V. tortilis* trees in the Evrona North site and 10 unpolluted and 9 oil-polluted *V. tortilis* trees in the South site were selected. Leaves were collected randomly from each tree, up to 2 m above ground during the morning–midday hours (9:00–13:00) to fill a 50-mL falcon tube. The samples were immediately frozen in liquid nitrogen, and later, back in the laboratory, they were stored at -80 °C.

2.3. One-Year-Old Seedlings

In April 2016, both oil-polluted and unpolluted soils were collected from the sites affected by the 1975 and 2014 oil spills. These soils were used to produce four treatments: unpolluted soil (0% oil) and 30%, 70%, and 100% oil-polluted soils. The 30% and 70% treatments were prepared by mixing unpolluted and oil-polluted soils in the desired proportions [22]. *Vachellia* seeds were sown inside PVC pipes (7.1 cm diameter × 100 cm height, ~4 L in volume) [22] and filled with one of the four soil treatments (Figure S1). Each *Vachellia* species, site, and soil treatment combination was replicated five times for a total of 80 seedlings (two plant species × two research sites [1975 and 2014] × four soil treatments x five biological replicates). However, some seeds failed to germinate, particularly *V. tortilis* seedlings growing in soil polluted during the 2014 oil spill, and thus they were excluded from the analysis, thereby reducing the sample size (see Table S1). This is consistent with the findings of Tran et al. [22], who found reduced germination in soil polluted by the 2014 oil spill and a germination delay in soil polluted by the 1975 oil spill, although such effects were detected in both tree species. In May, two to three leaves per seedling were collected, placed in vials, and stored in liquid nitrogen. The experiment was conducted during December 2019 at the Yair Research and Development Station in Hatzeva (Southern Israel).

2.4. Targeted Metabolic Analysis Using Gas Chromatography-Mass Spectrometry (GC-MS)

For extraction of central (i.e., primary) metabolites, the collected frozen leaf tissue was completely ground into a fine powder with liquid nitrogen using a mortar and pestle. From each sample, 100 mg of frozen ground plant tissue was mixed with solvents containing a ratio of methanol/water/chloroform of 55:23:22 v/v, following previously described protocols [42]. After phase separation, 100 µL of the top hydrophilic layer was collected and dried in a speed vac. Then, the dried samples were derivatized by adding 40 µL of 20 mg/mL metoxyamine-hydrochloride (Sigma-Aldrich, IL, USA), dissolved in pyridine, following incubation for 2 h in an orbital shaker at 1000 rpm at 37 °C. Next, we added to each sample N-methyl-N-(trimethylsilyl) tri-fluoroacetamide, including standard mix (alkanes) in a volume of 77 µL, followed by 30 min incubations in an orbital shaker at 37 °C. Lastly, 1 µL of each sample was injected into the GC-MS single quote instrument (Agilent 5977B). For data acquisition, the Mass Hunter software was used, as well as the NIST mass spectral library normalized to the internal standard, 13-sorbitol (Sigma-Aldrich, IL, USA). In addition, retention index (RI) libraries (provided by the Max-Planck Institute for Plant

Physiology in Golm, Germany; http://gmd.mpimp-golm.mpg.de/ (accessed on 11 June 2021)) were used for validation as previously described [43,44]. For the full list of central metabolites and their classifications, see Tables S2 and S3.

2.5. Statistical Analysis

All the analyses were carried out in R version 4.0.3 [45] through RStudio [46]. To visualize how oil pollution, study site, and tree species affected selected organic compounds, we performed a principal component analysis (PCA) using the package *ggbiplot* [47]. For this analysis, data were standardized by dividing the log-transformed values by the median value for each organic compound. Since many *V. tortilis* seedlings did not survive (see Table S1), the data were insufficient to visualize the PCA for this species. In two cases, the level of the compound 4-hydroxyphenyl-beta-glucopyranoside in *Vachellia* seedlings was zero, and we substituted, for this value, half of the lowest value recorded. To test for significant differences in the concentrations of specific metabolites, we log-transformed the data and performed a one-way analysis of variance (ANOVA) and post hoc tests, with user-defined contrast, using the package *lsmeans* [48].

3. Results

3.1. Metabolic Profile of the Adult Trees

The PCA was used to learn about differences in metabolite profiles in relation to tree species (*V. raddiana* vs. *V. tortilis*), study sites (North vs. South sites), and pollution effects (oil-polluted vs. unpolluted soils). As shown in Figure 1, the first two principal components explained 38.7% of the variance combined. Sample sites and species were separated according to PC2 (16.2%), while no separation was seen for oil pollution. The relative low values of the two principal components variance potentially indicates many other unknown factors that could contribute to the variance between factors. This suggests that both these factors (sample sites and species) had a small effect on the plant primary metabolic profile (Table S4).

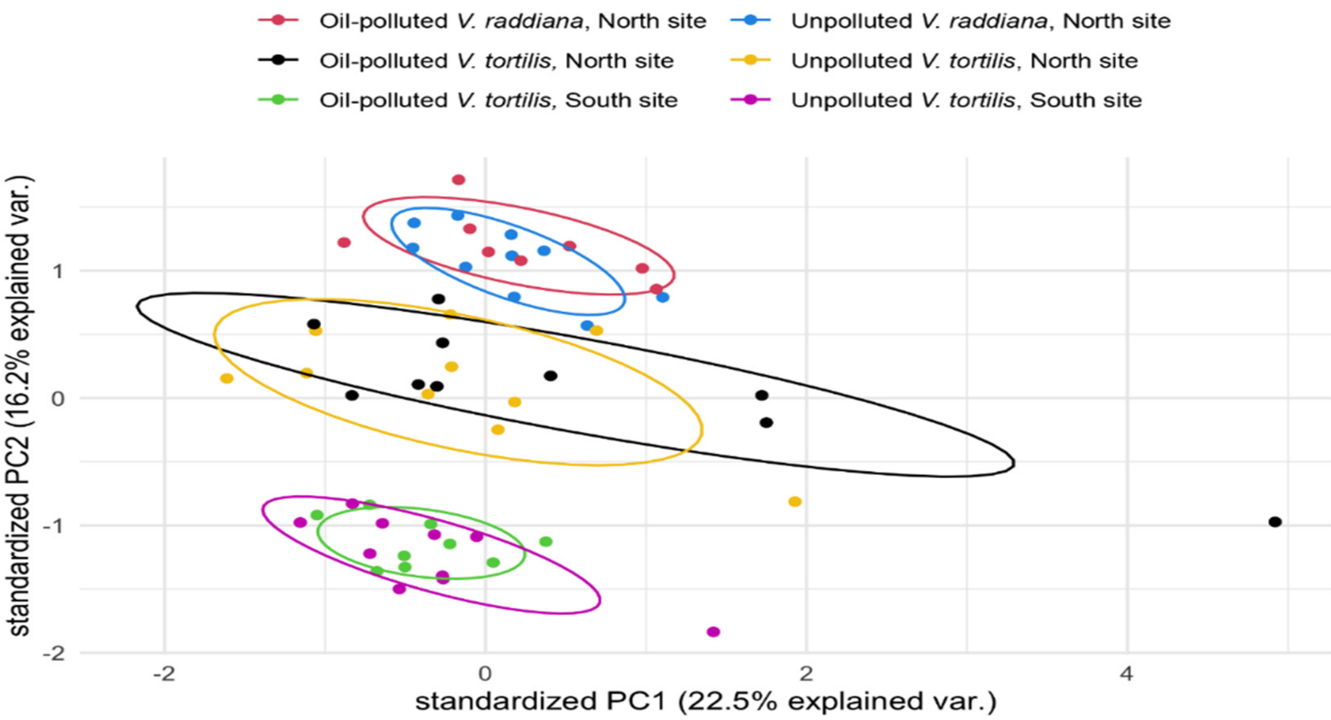

Figure 1. Principal component analysis (PCA) plot based on 49 metabolites detected in *Vachellia* adult trees growing in oil-polluted and unpolluted soils in two sites located in the Evrona Nature Reserve, southern Israel.

Out of 49 metabolites detected in this analysis (Table S3), only five had levels that were significantly different between trees growing in oil-polluted and unpolluted soils. The heatmap presented in Figure 2 shows the fold change values of these five metabolites. Both oil-polluted *V. tortilis* and *V. raddiana* trees showed an increase in the amino acid alanine in both sites. The increase in β-alanine was only significant in *V. raddiana* trees in the North site. When comparing the metabolic changes between oil-polluted and unpolluted trees, we found that oil-polluted *V. tortilis* trees in the South site had higher levels of phosphoric acid and gallic acid. Oil-polluted *V. tortilis* trees in the North site had lower maltose levels. Overall, these results revealed a mild effect of the oil spill on the metabolic profile of mature trees, where alanine was increased in all three comparisons, while the other four metabolites were significantly different, either by site or by species.

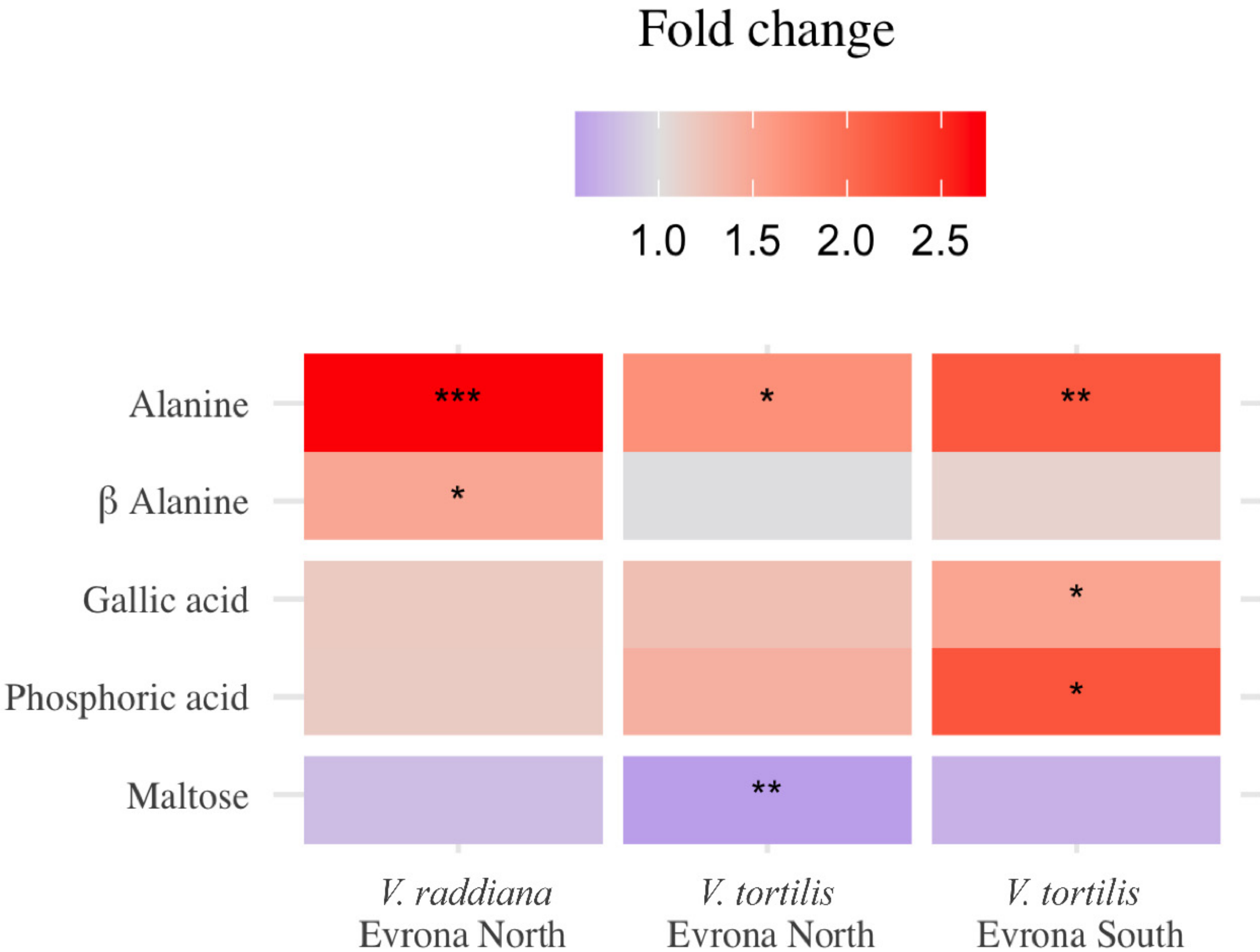

Figure 2. Heatmap of central metabolites detected in adult *Vachellia* trees in the Evrona Nature Reserve, southern Israel, in the Evrona North and Evrona South sites. The metabolites were selected using a one-way ANOVA and lsmeans post hoc test and presented in fold change comparing oil-polluted and unpolluted soil from the same site. Color coding indicates fold change of induction (red) or repression (blue) of the metabolite level. Asterisks indicate significant differences between oil-polluted and unpolluted adult trees (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.2. *Metabolic Profile of Vachellia Raddiana Seedlings*

A second PCA was used to examine differences in the metabolite profiles of seedlings in relation to the study site (1975 and 2014 oil pollution events) and pollution effect (0%, 30%, 70%, and 100% oil-polluted soils). For seedlings grown in soil polluted during the 1975 oil spill, the first two principal components explained 47.5% of the variance. The cluster of the *V. raddiana* seedlings grown in unpolluted soil partially overlapped with the cluster of seedlings grown in 30% oil-polluted soil, while the clusters for 70% and 100% oil-polluted soils were separated from the control (Figure 3a). For seedlings grown in soil polluted during the 2014 oil spill, the first two principal components explained 59.4% of the variance. The clusters of seedlings grown in unpolluted soil and 30% oil-polluted soil mostly overlapped, while the cluster of seedlings grown in 70% oil-polluted soil was further away from the control (Figure 3b). Seedlings grown in 100% 2014 oil-polluted soil did not survive (see Table S1) and, as mentioned, were not included in this analysis.

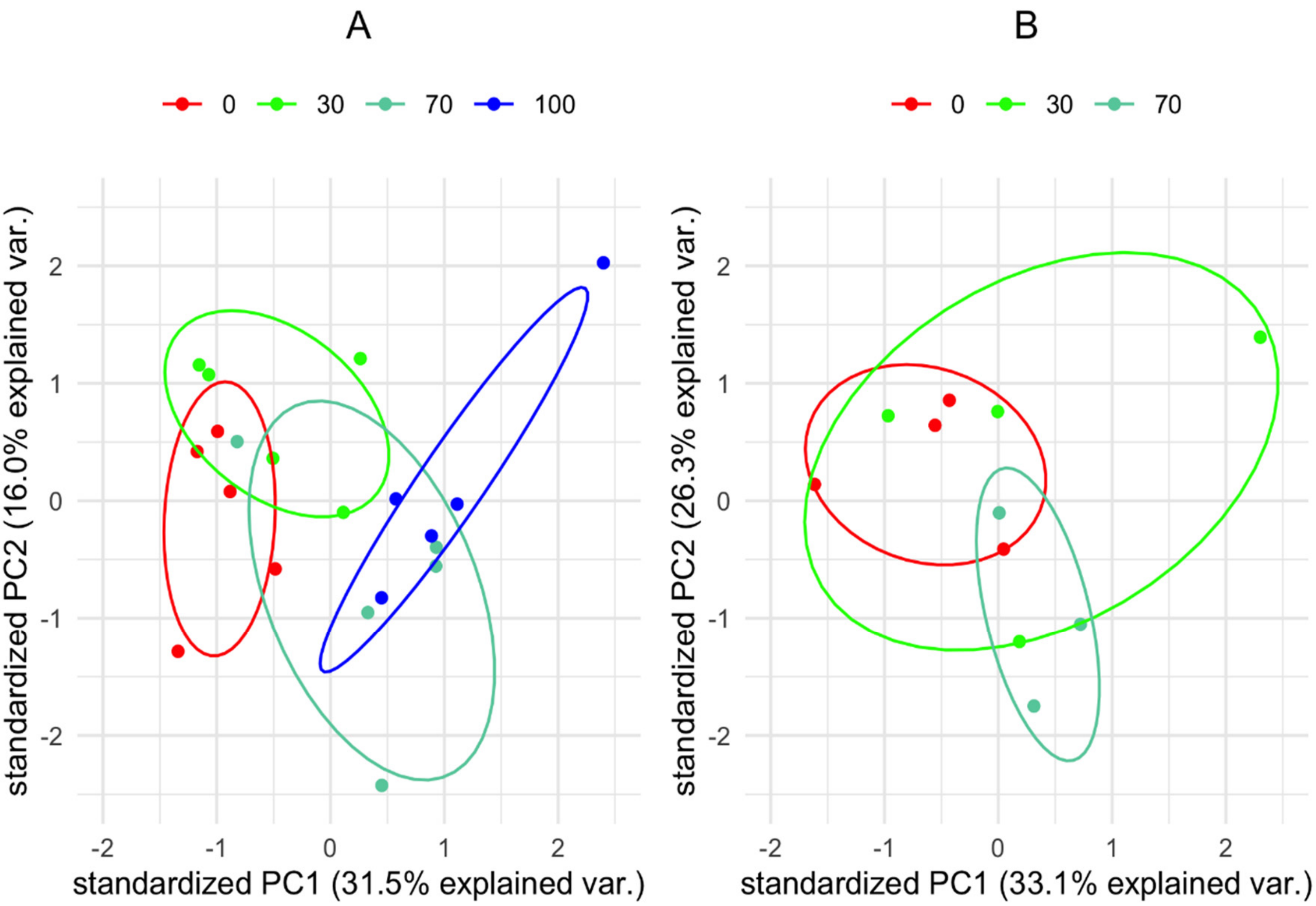

Figure 3. Principal component analysis plots of 58 metabolites detected in Vachellia raddiana seedlings according to the oil concentration (0%, 30%, 70%, and 100%) in 1975 (**A**) and 2014 (**B**) oil-polluted soils.

Changes in the levels of specific metabolites were most evident for *V. raddiana* seedlings grown in soil collected from the site affected by the 1975 oil spill. The levels of several amino acids, amino acid derivatives, sugars, and organic acids were significantly reduced in seedlings grown in oil-polluted soil, while amino alcohols were increased. Moreover, the levels of other classes of compounds were altered by the oil-polluted soils (Figure 4, Tables S5 and S6).

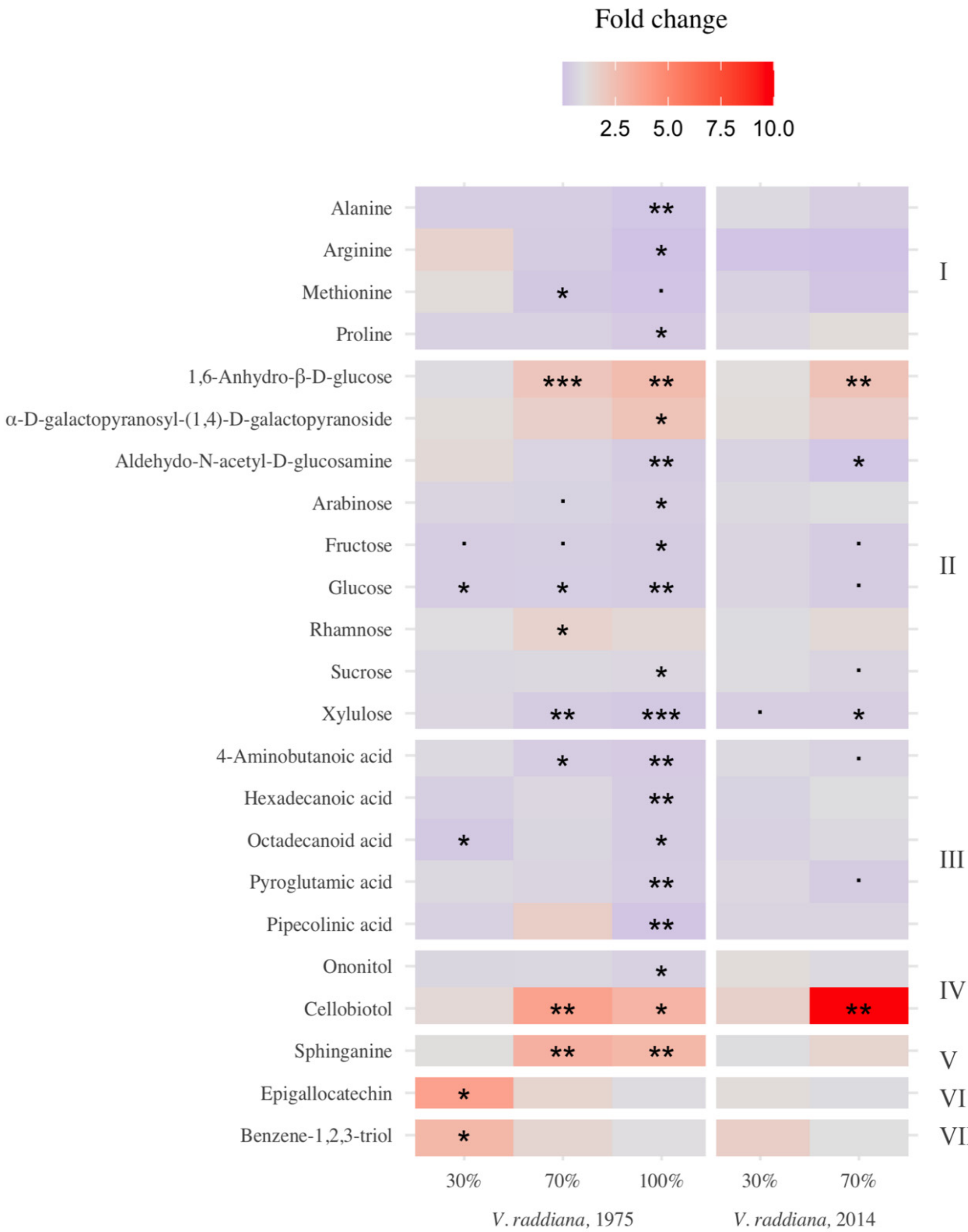

Figure 4. Heatmap of central metabolites detected in Vachellia seedlings grown in soil from the site polluted in 1975 and 2014. The metabolites were compared using a one-way ANOVA and lsmeans post hoc test and presented in fold change comparing oil-polluted and unpolluted soil. Only compounds with at least one significant comparison were included (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Dots indicate marginal significances, $p < 0.1$). The metabolites were divided according to their biochemical characterization as follows: (I) amino acids, (II) sugars, (III) organic acids, (IV) sugar alcohols, (V) amino alcohols, (VI) others, and (VII) pyragallols. Color coding indicates fold change of induction (red) or repression (blue) of the metabolite level.

4. Discussion

Environmental metabolic profiling is an increasingly important tool used to describe the chemical phenotypes of different organisms in relation to the abiotic environment. Recent applications of this tool include monitoring the health of bees [49], humans [50], and plants [51]. Additionally, the impact of several pollutants (the focus of our study), such air pollutants [52], metal pollutants [53], and solar filters [54], have also been investigated using metabolite profiling. Our study p resents a novel approach to environmental assessments and a stepping stone in the application of environmental metabolic profiling for monitoring the ecological status of plants affected by oil contamination. We found that oil pollution can alter the central metabolite profiles of two keystone tree species in a hyperarid desert ecosystem, particularly in young seedlings and, to a lesser degree, in adult trees.

The metabolic profiles of adult *V. raddiana* and *V. tortilis* trees responded mildly to oil pollution, and no major effect on any specific metabolic pathway was detected. However, in *V. tortilis* trees, we observed inductions in the amino acid alanine and two organic acids (gallic acid and phosphoric acid), parallel to a reduction in the sugar maltose. Increased amino acid levels may represent either an active response by the plant to pollution stress, as amino acids are used in the detoxification process [21], or enhanced proteolysis, leading to leaf senescence [55,56]. Similar to the results shown here, previous studies found that plants respond to oil contamination via physiological, biochemical, and molecular adjustments. For example, cowpea (*Vigna unguiculata* L.) seedlings exposed to crude oil showed induction in free sugar, total protein, and amino acid levels and a reduction in chlorophyll contents in the leaves [21]. Biomass and chlorophyll content in the leaves of two rye (*Secale cereale* L.) variants exposed to crude oil-polluted soil decreased, while the levels of the amino acid proline and oxidative stress parameters, such as enzyme activity and antioxidant content, increased in a variant-dependent manner [57]. Similarly, three-month-old jojoba (*Simmodsia chinensis* Link) plants, growing in oil-polluted soil, had reduced chlorophyll contents in their leaves and increased total carbohydrates, total proteins, amino acids, and proline in their plant shoots [58]. Organic acids are important in several metabolic pathways, particularly under water stress and soil nutrient deficiencies [59–61]. The fact that we only observed an increase in organic acid content in the South site, where soils are more saline, may indicate that the trees growing in these poorer soils may be even more susceptible to oil pollution than their North site counterparts. Considering the involvement of maltose in the protection of photosynthetic electrons and starch metabolism [62], an increase or decrease in maltose content can be associated with other forms of environmental stress, such as temperature shocks, and may have negative effects on plants [63]. The level of beta-alanine was only increased in the North site *V. raddiana*. β-alanine is a nonproteinogenic acid that is involved in multiple stress responses in plants, including temperature extremes, hypoxia, drought, heavy metal shock (such as cadmium ion exposure), and several biotic stresses [64]. Our results suggest the oil contamination has species- and site-specific effects on β-alanine abundance. Because, in our study area, *V. raddiana* and *V. tortilis* have different phenological traits, it was difficult to pinpoint whether the stronger effects on *V. tortilis* were the result of species-specific responses to the oil-polluted soils or some other factor. Moreover, the observed difference between trees in the different study sites may be explained by differential soil permeability to the oil due to contrasting soil textures and compositions [65].

Oil pollution had a stronger effect on seedlings than on adult trees, and the levels of amino acids, most sugars, and organic acids were consistently reduced in plants growing in oil-polluted soils. While determining the exact mechanism through which oil pollution affects the seedlings was not the aim of our study, it is plausible that these patterns reflected an osmotic adjustment [15]. In plants, osmotic stress is the result of a water shortage, which in our case, may have been caused by the hydrophobic properties of the oil-polluted soils [15]. These results indicate that oil pollution can interfere with the seedling's central metabolism and impede growth. Negative effects of oil-contaminated soil on *Vachellia* seedlings were also detected by Tran et al. [22], who found reduced aboveground and belowground growth. Similarly, de Jong found that oil pollution can affect aboveground growth in cereals [66]. Moreover, reduced amino acid levels could have additional negative effects on the plant, as amino acids are major precursors of specialized (i.e., secondary) metabolites [67], which have many essential functions, including deterring herbivores [68] and seed predators [69].

The metabolic profiles of seedlings grown in soils polluted during the 1975 and 2014 oil spill events were similar (Figure 4). This suggests that oil pollution may have long-lasting (46 years so far) effects if no remediation measures are undertaken. Although a comparison between the 1975 and 2014 oil spills is limited by the lack of data from *V. raddiana* seedlings grown in 100% oil-polluted soil, the fact that no seedling germinated in recently oil-polluted soil (see also [22]) is another potential piece of evidence for the negative effects of oil contamination on young trees. Finally, we found that the concentration of the amino acid alanine in oil-polluted *Vachellia* trees changed (increased in adult trees and decreased in seedlings) compared with unpolluted trees, suggesting that alanine could be used as a biomarker for oil contamination in these (but probably also other) tree species.

The greater sensitivity of young seedlings to oil compounds may be due to the fact that seedlings have short roots, which are more likely to be in contact with the oil-polluted soil that is usually distributed within the upper 30–45 cm; however, the roots of mature and older *Vachellia* trees may extend deep into the soil (<10 m), reducing their direct contact with the actual oil compounds in the topsoil. Following the December 2014 oil pollution event, the Israel Nature and Park Authority initiated a massive five-year monitoring effort in the Evrona Nature Reserve. These efforts also included close monitoring of adult *Vachellia* trees growing in oil-polluted and unpolluted soils in the same sites studied here [70]. Changes in NDVI, greenness index, and tree circumference and height [71], alongside phenology, were monitored every three months for four years. Surprisingly, but in line with our results, no differences between trees growing in oil-polluted vs. unpolluted soils were observed for any of the monitored parameters, thus supporting the findings indicated here that neither oil spill markedly affected the adult trees. In contrast, our metabolite profiling of young seedlings showed distinct effects of the oil-polluted soils on young *Vachellia* trees. These results are consistent with previous work performed in the area affected by the 1975 oil spill by Nothers et al. (2017), which showed that while mature *V. raddiana* trees were present and equally distributed in different size classes (height class of 1–7 m), young trees (<1 m tall) were absent in the oil-polluted soils, even 42 years after the oil spill occurred. Those findings have important implications for restoration and management efforts in oil-polluted sites in general, since at least some of these efforts need to focus on the yet "unborn" individuals. This is particularly important for long-lived *Vachellia* trees that can survive for 600 years; without young seedlings, these mature "healthy" populations have no long-term future.

Recent efforts have begun in the Evrona Nature Reserve to eliminate the remaining oil in the polluted soils (using oil-eating bacteria, for example). A possible question in these efforts is just how much oil must be removed to "clean up" this fragile habitat. Our results point to a potential threshold, as there were no major differences between the metabolic profiles of seedlings exposed to 30% oil-polluted soil and unpolluted soil. These results have important implications for the efforts needed to return to a "clean" ecosystem in the Evrona Nature Reserve.

5. Conclusions

Oil contamination can negatively affect the central metabolism of *Vachellia* trees, although with stronger effects on young seedlings than on adult trees. Mechanical and chemical remediation strategies should be developed, as seedlings are essential to guarantee the continuity of the *Vachellia* tree population in this ecosystem. Our study is the first to use environmental metabolic profiling to assess the impacts of oil contamination on a tree species. Further research is needed to understand how changes in the metabolic profile translate to plant physiology and survival, and how this could affect the associated desert animal and plant communities. As desert ecosystems are particularly vulnerable to anthropogenic disturbance, we stress the importance of prevention measures to avoid oil spills from happening in the first place.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/su13126660/s1, Figure S1: One-year-old Vachellia raddiana seedlings growing in PVC pipes, Table S1: Number of biological replicates used for the metabolic analysis of one-year-old Vachellia seedlings; Table S2: Full list of central metabolites detected in adult trees using GC-MS; Table S3: Full list of central metabolites detected in young seedlings using GC-MS; Table S4: PCA contribution for each metabolite detected in adult Vachellia trees; Table S5: PCA contribution for each metabolite detected in Vachellia raddiana seedlings affected by the 1975 oil spill; Table S6: PCA contribution for each metabolite detected in Vachellia raddiana seedlings affected by the 2014 oil spill.

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Data Availability Statement: Data is contained within the article or supplementary material.

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