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Germination, physiological and biochemical responses of acacia seedlings (*Acacia raddiana* and *Acacia tortilis*) to petroleum contaminated soils☆

Thanh Hoai Tran [a, b, 1], Einav Mayzlish Gati [c], Amram Eshel [d], Gidon Winters [b, *]

[a] Manna Center Program for Food Safety & Security, School of Plant Sciences and Food Security, Tel-Aviv University, Tel-Aviv 69978, Israel
[b] The Dead Sea Arava Science Center, Tamar Regional Council, Neve Zohar 86910, Israel
[c] The Israel Gene Bank (IGB), Agricultural Research Organization (ARO), Volcani Center, P.O. Box 15159, Rishon LeZion, 7505101, Israel
[d] Department of Molecular Biology and Ecology of Plants, Tel-Aviv University, Tel-Aviv 69978, Israel

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ABSTRACT

Along the arid Arava, southern Israel, acacia trees (*Acacia raddiana* and *Acacia tortilis*) are considered keystone species. Yet they are threatened by the ongoing aquifer depletion for agriculture, the conversion of natural land to agricultural land, seed infestation by bruchid beetles, and the reduction in precipitation level in the region. In the acacia dominated Evrona reserve (southern Arava), adding to these threats are recurrent oil spills from an underground pipeline. We report here a study of the effects of contaminated soils, from a recent (December 2014) and a much older (1975) oil spills.

The effects of local petroleum oil-contaminated soils on germination and early growing stages of the two acacia species were studied by comparisons with uncontaminated (control) soils from the same sites. For both acacia species, germination was significantly reduced in the 2014 oil-contaminated soils, whereas delayed in the 1975 oil-contaminated soil. There was no significant effect of oil volatile compounds on seed germination. At 105 days post transplanting (DPT), height, leaf number, stem diameter, and root growth were significantly smaller in the oil-contaminated soils. While photosynthetic performance (quantum yield of photosystem II) did not differ considerably between treatments, reductions of chlorophylls content and protein content were found in seedlings growing in the contaminated soils. Significant increases in superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities were found in roots of seedlings growing in oil-contaminated soils. These results demonstrate that seed germination and seedling growth of both acacia species were strongly restricted by oil contamination in soils, from both recent (2014) and a 40-year old (1975) oil spills.

Such long-term effects of oil spills on local acacia seedlings could shift the structure of local acacia communities. These results should be taken into account by local authorities aiming to clean up and restore such polluted areas.

1. Introduction

Within the arid Negev and Arava deserts, *Acacia raddiana* and *Acacia tortilis* trees are considered keystone species (Munzbergova and Ward, 2002) that perform several important ecological services. These trees provide vital forage (leaves and pods) and water sources (leaf water content) throughout the year. Through their shade and shelter creating canopy, nitrogen-fixing qualities and their ability to act as hydraulic pumps that bring water closer to the surface, they improve soil conditions for other nearby plant species, increasing plant biodiversity under the tree canopies (Milton and Dean, 1995; Munzbergova and Ward, 2002; Abdallah et al., 2012) compared to the nearby surrounding areas.

The ongoing mortality of acacia trees in the region continues to be of major concern (Groner et al., 2015; Isaacson et al., 2017). Acacias are threatened by human activities such as road-building and ongoing aquifer depletion for agriculture (Ward and Rohner, 1997; Shrestha et al., 2003), the conversion of natural land to

☆ This paper has been recommended for acceptance by Dr. Hageman Kimberly Jill.
* Corresponding author.
E-mail address: wintersg@adssc.org (G. Winters).
[1] Present address: Nong Lam University, Ho Chi Minh City, 70000, Vietnam.

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agricultural land. Other threats to these trees include seed infestation by bruchid beetles (Rohner and Ward, 1999; Or and Ward, 2004), and the reduction in precipitation level in the region (Ginat et al., 2011). Loss of acacia trees in arid regions is associated with the loss of much of the biota and ecosystem services associated with these trees, as well as enhanced erosion of soils (Ward and Rohner, 1997; Shrestha et al., 2003).

The Evrona reserve is a hyper-arid saline and sandy nature reserve in southern Israel, that is dominated by *A. raddiana* and *A. tortilis*, with tree densities of up to 200 trees per km^2 (Golan et al., 2016). In Evrona, adding to the threats mentioned above are the unknown effects of oil-contaminated soils, the result of both relatively recent (December 2014; Groner et al., 2015) and much older (July 1975; Braster, 1975) crude oil spills. The recent spill (3rd December 2014) of 5000 m^3 crude oil in the Evrona reserve was well documented (Groner et al., 2015) (https://www.youtube.com/watch?v=q7QSkRVwXbA; Fig. 1). However the older oil spill that occurred in 1975 (Braster, 1975) was rediscover only while surveing that current spill by the Israeli Nature and Parks Authority (INPA) some 1 km south of the 2014 event (Nothers et al., 2017). This forty-years-old oil spill was twice the amount of oil the 2014 event (10,000 m^3) but, surprisingly, this event did not draw much attention and had never been treated and the oil effects have been mostly ignored (Golan et al., 2016; Nothers et al., 2017). Both oil spills were caused by damage to local underground oil pipes.

The potential long term effects of oil spills on their surroudning ecosystems are important for decision makers regarding the remedation efforts after such events (Nothers et al., 2017). Most of the post-spill studies have focused on marine environments (Martínez-Gómez et al., 2010) (Supplementary materials Fig. S1A), while not much is known about the effects of oil spills in terrestrial environments, and even less in plants growing in terrestrial arid ecosystems (Nothers et al., 2017; Supplementary materials Fig. S1B).

One tool that has been used in terrestrial pollution event studies has been eco-toxicity assessments (Hentati et al., 2013). Oil-contaminated soils were shown to reduce seed germination, plant growth and yields of various crop species at different levels of oil contaminations (Agbogidi and Eshegbeyi, 2006; Bona et al., 2011) (Supplementary Table S1). The negative effects of oil-contaminated soils on seed germination was also shown in mangrove species *Aegiceras corniculatum* and *Acanthus ilicifolius* grown in sandy and muddy sediments, contaminated by both fresh and used lubricating oil (Zhang et al., 2007; Ke et al., 2011). Studies have demonstrated the negative effects of oil contamination on above-ground biomass in grains of cereals, legumes and other crops (De Jong, 1980; Ekundayo et al., 2001; Merkl et al., 2004; Issoufi et al., 2006; Kisic et al., 2009). Negative effects of oil pollution were also shown for the plant's belowground compartment, caused by the changes in root structure, reduction in root length, density, surface area of the finest diameter root class and root biomass of many terrestrial species such as *Cyperus aggregatus* (Merkl et al., 2005a), ryegrass (*Lolium perenne*) (Kaimi et al., 2006; Kechavarzi et al., 2007), corn and wheat (Ekundayo et al., 2001; Tang et al., 2011), and mangroves (Cui et al., 2016). The application of petroleum oil emulsions either as insecticides on plants or through accidental oil spills, were shown to reduce photosynthesis and respiration rate in citrus species (Wedding et al., 1952; Rosso et al., 2005), and *Salicornia virginica* (Rosso et al., 2005). Biochemical responses such as decreases in protein content (Odjegba and Sadiq, 2002; He et al., 2005; Achuba, 2006; Fazeli et al., 2007), and increases in the activity of the antioxidant enzyme superoxide dismutase (SOD) (Zhang et al., 2007; Ke et al., 2011; Cui et al., 2016) were also reported (Supplementary Table S1).

Most of these studies were related to marine, tropical, or crop plants, with relatively little experimental work on the effects of oil pollution on desert plants (however, see Golan et al., 2016).

The importance of acacia trees in the arid desert in general and in the Evrona nature reserve in particular, alongside a large number of studies demonstrating negative effects of oil-contaminated soils on a range of other plant species, were the main motivators to

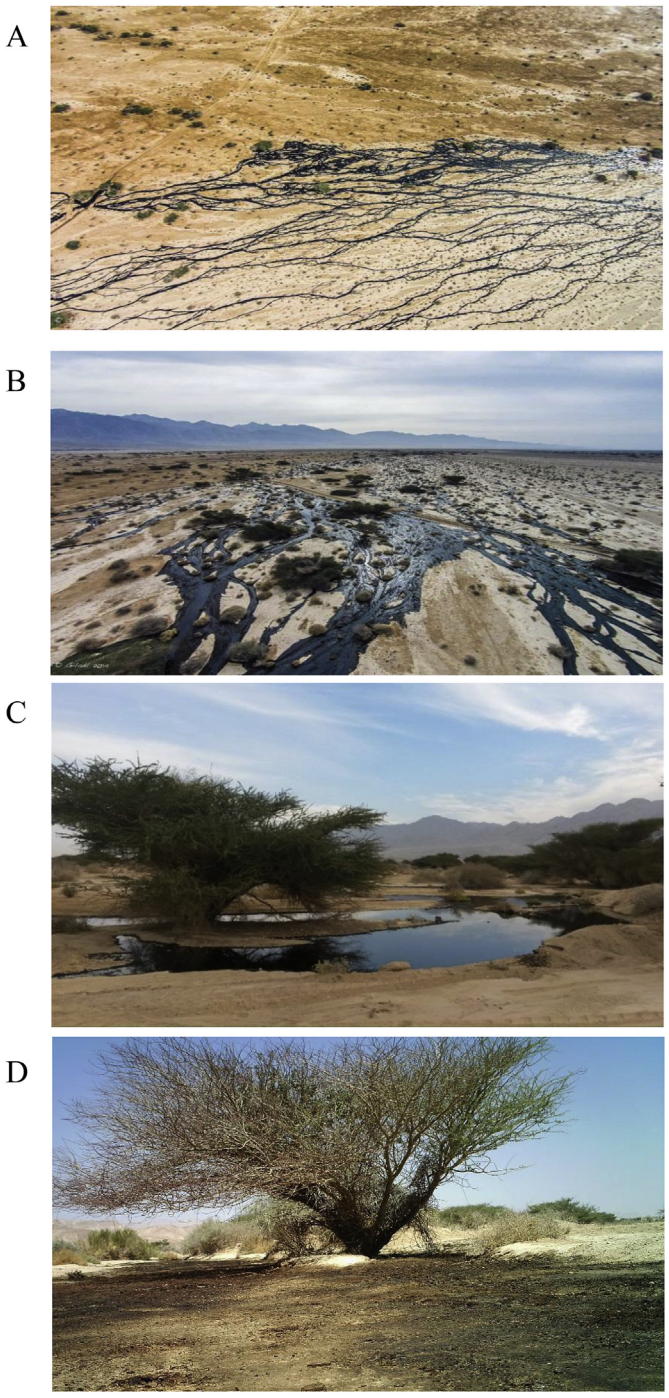

Fig. 1. Aerial (A, B) photographs of the December 2014 petroleum oil spill in the Evrona nature reserve, southern Israel. Photographed were taken on 4th Dec 2014. Shown also are ground photographs of *Acacia tortilis* sitting in a pool of oil (C). Most of this oil has been removed, but there is still a 30–50 cm layer of oil-contaminated top soil in the site (D). A similar oil-contaminated top soil exists in the 1975 polluted site (not shown). Photograph credits: A-B were taken Dr. Roi Talbi – The Israeli Nature and Parks Authority (INPA), C - taken by Nitzan Segev, D - by Gidon Winters.

commence this work. Our objectives were to evaluate responses of *A. raddiana* and *A. tortilis* seedlings from the Evrona reserve to the oil spills that occurred there in 1975 and 2014, and with remediation in mind, find a possible threshold under which the contaminated soils were diluted enough to prevent it from having negative effects on the seedlings.

2. Materials and methods

2.1. Plant material

Seeds from *A. raddiana* and *A. tortilis* trees (also known as *Vachellia tortilis* subsp. *raddiana* and *Vachellia tortilis* var. *tortilis*, respectively; Kyalangalilwa et al., 2013), were collected from the Evrona reserve, southern Arava, Israel (29°40′38.76″N, 35°0′55.12″E) during the 2011 and 2015 seed seasons by the Israel Plant Gene Bank (Volcani Center, ARO). The seeds were maintained according to gene-bank standards (dried to 15% humidity and kept in −20 °C). For breaking dormancy and synchronizing germination, the seed coat was mechanically scarified using a nail clipper. Seeds were used for both indoor and outdoor experiments (detailed below).

2.2. Soils collection and experimental setups

Non-contaminated (control) and contaminated soils were collected from both the 1975 and 2014 oil spill sites in the Evrona reserve (April 2016) and sieved (0.5 × 2 cm holes) to remove stones. Although the 1975 oil spill is over forty years old, no remediation has taken place in these contaminated soils (Nothers et al., 2017). Soil treatments included control (uncontaminated) soils from the two oil spill sites 1975 (1975–0.0) and 2014 (2014–0.0) and contaminated soils from the 1975 and 2014 oil spills considered as 100% contaminated soils (1975–1.0 and 2014–1.0; Fig. 2A). The 2014 oil-contaminated soil (2014–1.0 soil) was also mixed with the control soil (2014–0.0) to create soils that contained (by volume) 30% and 70% contaminated soils (2014–0.3 and 2014–0.7, respectively; Fig. 2A). With future remediation in mind, the aim of using diluted oil-contaminated soils (2014–0.3 and 2014–0.7), was to find a threshold under which the contaminated soils were diluted enough to prevent any negative effects on the seedlings.

Data regarding the soil composition and concentrations of the oil contaminations in Evrona soils from both the 1975 and 2014 oil spill sites is limited to one technical report (LDD, 2016; Supplementary material, Fig. S2) with very few replications, and one recent and more thorough study (Gordon et al., 2018). These resources shed light on some of the characteristics of the soils in both the 1975 and 2014 sites. We have summarized the main relevant findings of these resources in Supplementary material (Fig. S2).

2.2.1. Indoor germination experiments

Seeds were sown (each species separately) in 8 × 10 cm pots (three seeds per pot) filled with 500 cm^3 soil up to 1 cm of the pot's edges.

Experiments were laid out in a completely randomized design (CRD), that included two factors (six soil treatments by two acacia species) in six replications (n = 6 pots for each treatment and species). Pots were irrigated four times daily (1 min of water sprayed on the pots every 3 h). All pots were illuminated with cool daylight T5 fluorescent lamps (Osram Lumiux, 54 W/865) 14 h/day (300 μmol photons m^{-2} s^{-1} at the soil surface). Temperature was kept at 25 °C by setting up the germination experiment within a temperature controlled growth chamber.

Aiming to separate between the potential effects of the oil in the soil (direct contact) and the potentially damaging effect of the volatile chemicals that evaporate from the oil (indirect contact; Fingas and Brown, 2013), a second experiment was run in the same growth chambers. For this, soils (50 cm^3) from the different treatments (2014–0.0, 2014–1.0, 1975–0.0, 1975–1.0; Fig. 2A) were put in plastic containers that were placed at the bottom of 1L glass jars (Supplementary materials Fig. S3). Five acacia seeds of both species were put inside the top part of folded filter a paper that was inserted into the jars (n = 4 jars for each treatment and species). Water (120 ml) was added to each jar and the filter paper containing the seeds was positioned upright to allow water to reach the seeds through the filter paper. To prevent evaporation of the volatile chemicals jars were kept closed during 10 days of this experiment (Supplementary materials Fig. S3).

2.2.2. Seedlings growth and root development – outdoor experiments

Outdoor experiments were set up using two vertical systems: 1) polyvinyl chloride (PVC) pipes (7.1 cm diameter x 100 cm height, 60 pipes; Fig. 2B,D,F), and 2) rhizoboxes with transparent plastic fronts (30 cm length x 2.5 cm width x 100 cm height, 24 boxes; Fig. 2C,E,G). Both systems were used to measure aboveground and belowground seedling development; while the pipe systems (Fig. 2B,D,F) allowed for measuring the biomass and root development only at the end of the experiment (by harvesting the plants out of the pipes), the transparent side of the rhizoboxes (Fig. 2C,E,G) provided an opportunity to measure below ground development over time. In both systems, the bottom 70 cm were layered with control soils (2014–0.0 or 1975–0.0; Fig. 2B,C), on which we layered another 30 cm of either control (2014–0.0, 1975–0.0) or oil-contaminated (2014–1.0, 2014–0.3, 2014–0.7, or 1975–1.0) soils (Fig. 2A). Seeds were germinated indoors in germination trays filled with uncontaminated soil in a temperature-controlled growth chamber (25 °C). Seedlings with open cotyledons were transplanted into the pipes (one seedling per pipe, n = 5 pipes per each treatment and species) or rhizoboxes (four seedlings per box, two from each species, n = 4 boxes per each treatment and species).

Outdoor experiments were set up in CRD, with two factors (six soil treatments x two acacia species) under the natural irradiance and temperature conditions at the Yair R & D station at Hatzeva, 100 km north of the Evrona reserve. During the period of experiments (April-October 2016), air temperatures ranged between 14.7 and 45.2 °C (average air temperatures was 30.1 ± 0.13 °C), average relative humidity was 36.7 ± 0.09% and the accumulated precipitation during this period was 20.2 mm (Israel meteorological service, https://ims.data.gov.il; accessed 15th Dec 2016). In both systems, seedlings were irrigated 6 times a day (100 ml each time, 600 ml in total, between 06:00–18:00). Both the pipe and the rhizobox systems were totally covered by plastic sheets, black on the inside and white on the outside to provide total darkness, with only the upper tip of the pipes and boxes sticking out of the plastic and exposed to sunlight (Fig. 2D and E). This way the pipes and rhizoboxes were also protected from direct heating by the sun.

2.3. Measurements

2.3.1. Germination

The germination of seeds in the various soil treatments was detected by cotyledon emergence above the soil surface and recorded after 6 days post sowing (DPS). Percent of germination at 6, 15, and 25 days post sowing (DPS) (n = 6, ±SE) was calculated as in Scott et al. (1984).

2.3.2. Growth analysis

In the pipes system (Fig. 2B,D,F), the seedling height was

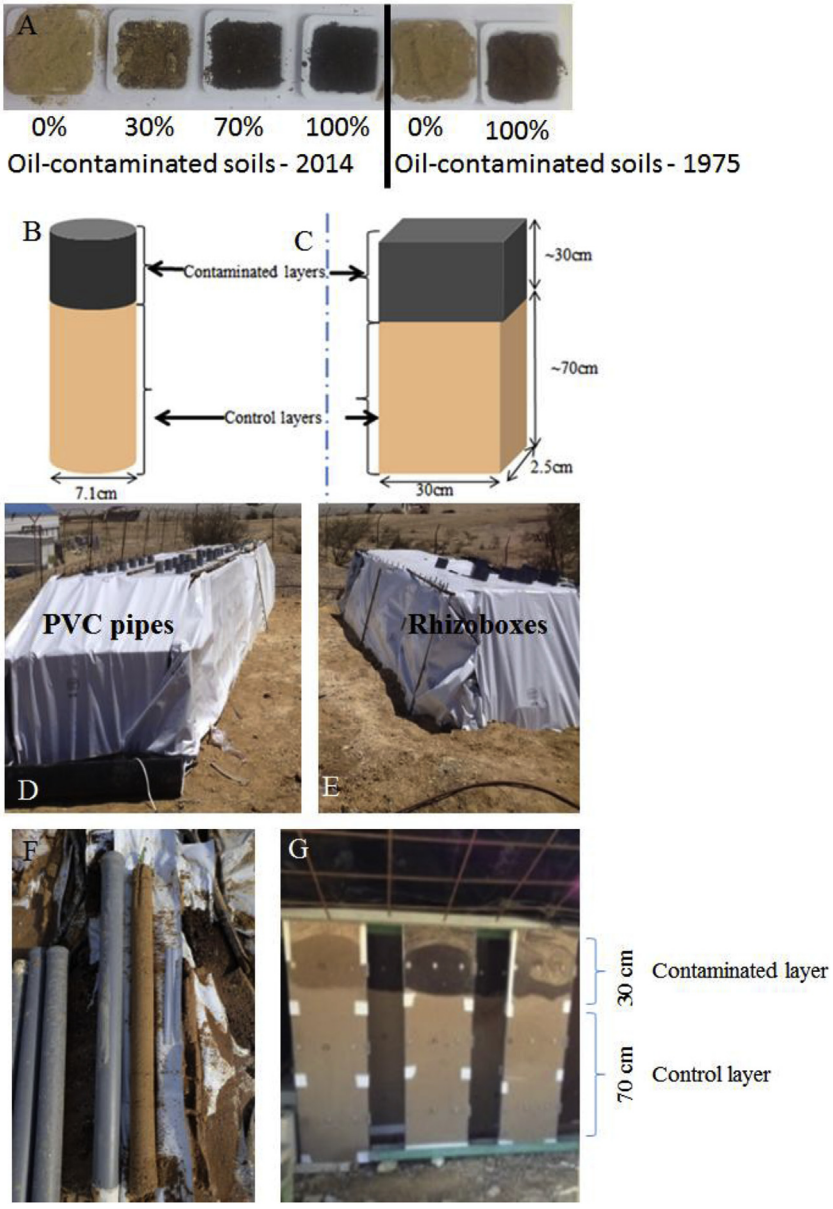

Fig. 2. Outdoor experimental setups used to test the effects of the oil-contaminations on above and below ground growth parameters. Shown are examples of the 6 soil treatments used that included soils from both the 2014 (control [0%], 30%, 70% and 100% contaminated soils) and the 1975 (control [0%] and 100% contaminated soils) spill events (A). Soils were layered on the top 30 cm of both setups (B,C), with the appropriate control (soil from either the 2014 or 1975 control) underneath (B,C). Shown are the two outdoor setups used in this study, these included the PVC pipe (B, D, F) and rhizoboxes (C, E, G) systems. Each of these outdoor setups was covered by white plastic sheets (black in the inside) to protect pipes/rhizoboxes from direct heating by the sun (D–E). Roots of seedlings in the pipe system were measured by harvesting at the end of the experiment (F), while they were measured twice a week in the rhizoboxes system (G).

measured from the ground to the apical bud after transplanting (beginning of the experiment), and at the end of the experiment, 105 days post transplanting (DPT). Similarly, leaves were counted at the beginning of the experiment (once the first leaves were completely open) and 50 DPT. Stem diameter was measured using a manual caliper at the end of the experiment (105 DPT).

In order to allow following the changes in root growth, roots were traced every four to five days by attaching, acetate transparent sheets to the transparent side of the rhizoboxes (Fig. 2C,E,G). Traced sheets were digitized (Canon LiDE 120 scanner with the resolution of 300 DPI) and analyzed using SmartRoot plugin in Image J (Schneider et al., 2012; Lobet et al., 2011). The experiment was terminated when the first root in any of the rhizoboxes reached the bottom. Upon ending the experiment, roots were washed from the soil, scanned and analyzed (as mentioned above).

The aboveground and belowground parts of seedlings of root growth experiment were separately dried at 80 °C for 72 h and their dry biomass was recorded.

2.3.3. Plant photosynthesis

Maximal photochemical quantum efficiency of photosystem II (Y) was measured 1 h after sunset according to Beer and Björk (2000), using a pulse amplitude modulated (PAM) chlorophyll fluorescence (Diving-PAM; Walz, Germany), equipped with a leaf distance clip.

2.3.4. Chlorophyll measurement

Chlorophyll *a* and *b* were extracted from the leaves by methanol

and measured according to Wellburn (1994) adapted to a 96 well microplate reader (Warren, 2008). For this, frozen (−80 °C) leaf samples were ground to fine powder in liquid nitrogen using pre-cooled mortar and pestle. Grinded leaf powder (10 mg) was transferred into 2 ml Eppendorf tube with 1 ml of methanol and 3 metal balls (3 mm), and homogenized further using a bullet blender (Next Advance, USA) homogenizer (2 min). Samples were then centrifuged (2 min, 13,500 RPM) and the supernatants were collected. Chlorophylls were re-extracted by adding another 1 ml of methanol to the pellet, shaking for another 2 min, centrifuging followed by the supernatant removal. The two supernatants were pooled in order to measure the chlorophylls content.

Samples or blanks of 200 μL of extract were transferred into 96-well plates and absorbance was read on a microplate reader (EPOCH2, Biotek, USA) at 652 and 665 nm. The absorbance was converted into a 1 cm path-length corrected absorbance using the following equations (Warren, 2008)

$$A_{652,\ 1cm} = (A_{652,\ microplate} - \text{blank})/\text{path-length} \quad (1)$$

$$A_{665,\ 1cm} = (A_{665,\ microplate} - \text{blank})/\text{path-length} \quad (2)$$

Where path-length is 0.51 for leaf extraction in methanol (Warren, 2008).

Chlorophyll (Chl) concentration was calculated using Wellburn (1994) equations:

$$\text{Chl a } (\mu g.\ ml^{-1}) = 16.72\ A_{665,\ 1cm} - 9.16\ A_{652,\ 1cm} \quad (3)$$

$$\text{Chl b } (\mu g.\ ml^{-1}) = -15.28\ A_{665,\ 1cm} + 34.09\ A_{652,\ 1cm} \quad (4)$$

$$\text{Total Chlorophyll } (\mu g.\ ml^{-1}) = 1.44\ A_{665,\ 1cm} + 24.93\ A_{652,\ 1cm} \quad (5)$$

Final results were converted to milligram chlorophyll per gram leaves fresh weight.

2.3.5. Total protein content and antioxidant enzyme activities

The extractions for protein content, superoxide dismutase (SOD) and ascorbate peroxidase (APX), were performed according to Elavarthi and Martin (2010). Fresh frozen tissues (leaf or root) were grinded to fine powder in liquid nitrogen using a precooled mortar and pestle. A sample (100 mg) of the powder was dispersed in 1.2 mL of 0.2 M potassium phosphate buffer (pH 7.8) containing 0.1 mM Ethylenediaminetetraacetic acid (EDTA) and 4% polyvinylpyrrolidone was added to neutralize phenol effects (Zhang et al., 2007). Samples with buffer were centrifuged (15,000 RPM, 20 min, 4 °C) and the supernatant was collected. Another 0.8 mL of the same buffer was added to the pellet and centrifuged again for another 15 min. The combined supernatants were stored at 4 °C until analysis of protein content, and superoxide dismutase (SOD), ascorbate peroxidase (APX) activity.

Protein content (C) was determined using the Bradford (1976) assay adapted for microplate reader using bovine serum albumin (BSA) as the protein standard. For this, a linear range of this protein standard was prepared from 0.05 to 0.5 mg/ml 10 μl of each standard or sample solution were transferred into separate microplate wells, followed by the addition of 200 μl of diluted (×4) Bio-Rad dye reagent. Samples were incubated at room temperature for 20 min and absorbance was read at 595 nm.

SOD activity was measured following Beyer and Fridovich (1987) method. The assay is based on the inhibition of the reduction of photochemical nitroblue tetrazolium (NBT) to formazan by SOD. The 250 μL (final volume) assay mixture consisted of 50 mM phosphate buffer (pH 7.8), 9.9 mM methionine, 57 μM NBT, 0.1 mM EDTA-Na_2, 0.025% Triton ×100, 0.02 mM riboflavin and 40 μl enzyme extract which was placed under fluorescent light (Osram Lumiux, 54 W/865) at light intensity 175 μmol m^{-2} s^{-1} for 20 min. Two other wells were prepared without adding the enzyme and treated as controls, one placed in the similar light condition as with samples while the other one placed in the darkness and treated as the blank. Absorbance was measured at 560 nm.

APX activity was determined using a modified method for microplate from Murshed et al. (2008). For this, 185 μl reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.0) and 0.25 mM ascorbate were dispensed into all microplate wells, and 10 μl of sample extract were added to the wells afterwards. The microplate was shaken for 5 s and absorbance (A_0) was measured at 290 nm for 3 min at 25 °C to determine nonspecific ascorbate degradation as the correction for the enzyme activity together with H_2O_2 blank (A_0'). In order to start APX reaction, 5 μl of 200 mM H_2O_2 was added to all wells. Samples were shaken again and the absorbance (A) measured for 5 min. Specific APX activity was calculated using the 2.8 mM cm^{-1} extinction coefficient (k) and normalized to mg. g^{-1} fresh weight (FW) of protein content (C) in tissues (Eq. (6)).

$$\text{APX activity } (\mu mol\ min^{-1}\ g^{-1}\ FW) = (A - A_0 - A_0')/(k \times C) \quad (6)$$

2.4. Statistical analysis

The $\arcsin\sqrt{x}$ transformation was applied to the germination percentage data in order to comply with the normality requirement of the statistical analysis.

All statistical analyses were performed using R software version 3.2.2 for Windows (R Core Team, 2015). Parametric two-way ANOVA was used to test the effects of oil-contaminated soils on the two acacia species, linear model optional for missing values was used due to the death of seedlings. Tukey's multiple comparisons were applied when $p < 0.05$ using Lsmeans (Lenth, 2016) and multcompView (Graves et al., 2015) packages. Graphs were created with the ggplot2 package (Wickham, 2016).

3. Results

3.1. Indoor germination experiments

At 6 DPS, both acacia species started germinating in control and 30% contamination soils (Fig. 3A). Percentage of germination 10 DPS in 2014–0.0 and 1975–0.0 soils, was significantly higher than in contaminated soils (two-way ANOVA, $p < 0.01$). Gradual germination was obtained in 1975–1.0 and 2014–0.3 while in the other treatments maximal germination was observed already at 6 DPS (Fig. 3A). A reduction in the germination was observed in oil-contaminated soils of 2014 proportional to oil contamination, whereas there was no significant difference in germination between control and contaminated soil of 1975 (1975–1.0; Fig. 3B). The highest percentage of germination was obtained in the 2014–0.0 (control) soil of *A. tortilis* (68.48 ± 9.5%) and slight reduction of seedling survival, presented by lower germination at 25 DPS compared to 6 and 15 DPS, was recorded in control and 2014–0.3 soils.

In the closed jar system, used to test the effects of volatiles on germination, seed germination percentage 10 DPS did not differ significantly between contaminated and control soils (two-way ANOVA, $p > 0.05$). Average germination rates ranged from 60.26 ± 3.16% to 88.71 ± 0.0%. Across treatments, there was a significant difference between the two species (two-way ANOVA, $p < 0.01$), with the higher average percentage of germination

recorded for *A. tortilis* (88.71 ± 0.0%), compared with *A. raddiana* (72.91 ± 9.48%) (data not shown).

3.2. Aboveground development

Changes in height, number of leaves and stem diameter of acacia seedlings grown in the pipe systems were measured. Seedlings grown in control soils were found to be significantly higher compared with the ones grown on contaminated soils in both species (two-way ANOVA, $p < 0.01$). Significant differences in the change of seedling height between the two acacia species were also observed ($p < 0.01$), with the highest value recorded in *A. raddiana* species at 6.65 ± 3.88 cm compared to 5.27 ± 4.13 cm of *A. tortilis* (Fig. 4A).

Changes in leaf number (Fig. 4B) were calculated by subtracting the values leaf number at 6 DPT from those counted at 50 DPT. The seedlings of both species grown in 1975–0.0 and 2014–0.0 control soils, had significantly larger changes in leaf growth than the changes that took place in seedlings growing in contaminated soils (two-way ANOVA, $p < 0.01$).

Results of stem diameter measurements taken 105 DPT (Fig. 4C) indicated a significant difference between stem diameter of seedlings grown in different soils (two-way ANOVA, $p < 0.01$). Stem diameter of both *A. raddiana* and *A. tortilis* seedlings grown in 2014–0.0 and 1975–0.0 soils was significantly larger than the seedlings grown in all others contaminated soils.

3.3. Belowground development

Compared with the pipe system (Fig. 2B,D,F), that allowed for measuring the biomass and root development only at the end of the experiment, the main advantage of the rhizoboxes (Fig. 2C,E,G) was that they allowed for continuous observation of the roots during the experiments (Fig. 2G; Supplementary video "Acacia root growth"). Experiments in the rhizoboxes system were terminated when the roots of the seedlings grown in the uncontaminated soil reached the bottom of the box. Root extension was measured by attaching transparent sheets to the root boxes, tracing roots 15, 19, 24, 28, 32 and 45 DPT and digitizing these images (Supplementary video "Acacia root growth"). The highest total root length was recorded in the seedlings grown in control treatments (1975–0.0, 2014–0.0), followed by the seedlings grown in 2014–0.3 soil

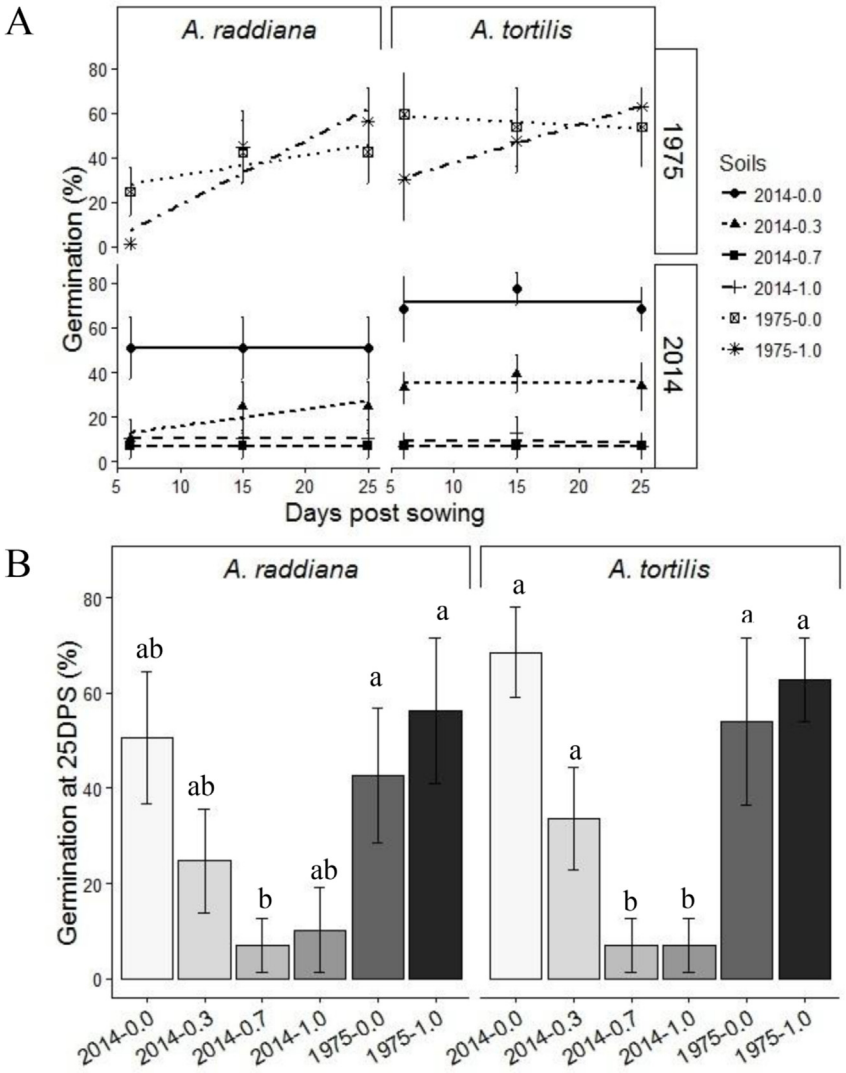

Fig. 3. Effect of petroleum contaminated soils on germination of *A. raddiana* and *A. tortilis* measured at 6, 15, and 25 days post sowing (DPS) (A), with detailed results shown for the end of the germination experiment (25 DPS, B) (n = 6, ±SE). Mean values with the same letters are not significantly different at $p < 0.05$ according to two-way ANOVA and Tukey's HSD tests.

(Fig. 5). The seedlings of both species grown in 1975–0.0 soil showed significantly higher values in all measured points compared to the 1975–1.0 soil (two-way ANOVA, $p < 0.01$). For seedlings grown in 2014 soils, the root extension in the 2014–1.0 soil did not significantly differ from those grown in 2014–0.0 during the first 24 days of measurements. After 28 DPT, the root extension in 2014 soils started to deviate between treatments with the significantly higher values observed in 2014–0.0 soil compared with contaminated soils, especially at the final point of measurement (45 DPT).

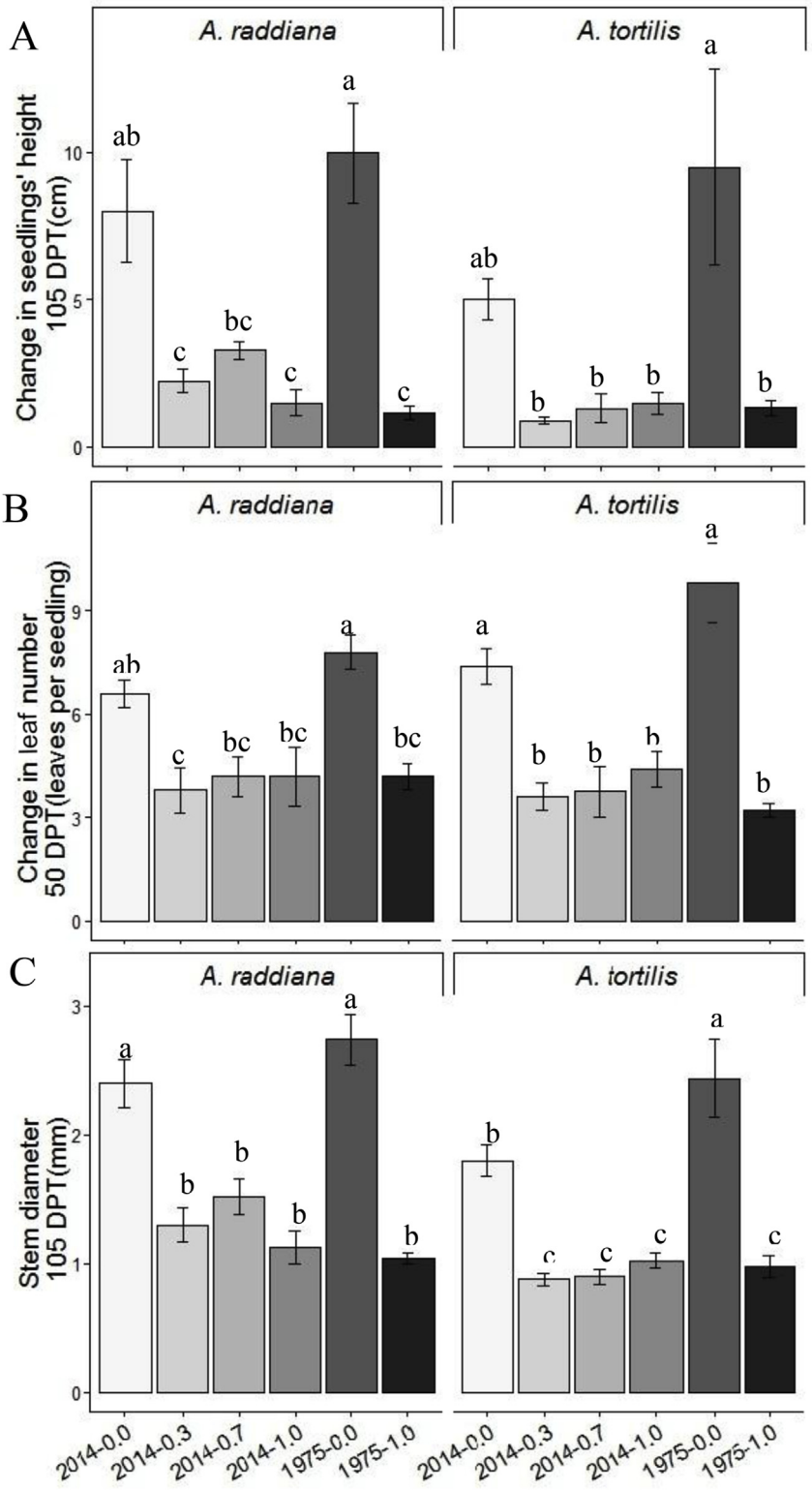

Fig. 4. Change in height (A, 105 DPS), leaf number (B, 50 DPS) and stem size (C, 105 DPS) of *A. raddiana* and *A. tortilis* seedlings (n = 5, ±SE) grown outdoors in the pipe system in different soil conditions. Mean values with the same letter are not significantly different at $p < 0.05$ according to two-way ANOVA and Tukey's HSD tests.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.envpol.2017.11.067.

3.4. Photosynthetic performance and chlorophylls content

PAM fluorometry indicated a non-significant difference between treatments in the maximum photochemical quantum efficiency (F_v/F_m) of photosystem II (PSII) at 40 DPT (two ways ANOVA, $p > 0.05$). However, average F_v/F_m in *A. tortilis* growing in control soils (0.79 ± 0.01) was slightly higher compared with their respective oil-contaminated treatments values at 0.71 ± 0.05 to 0.77 ± 0.01 (data not shown).

Significant differences in chlorophyll content (Fig. 6) were found for *A. raddiana* (two-way ANOVA, $p < 0.05$). *A. raddiana* seedlings grown on contaminated soils (2014–0.3, 2014–0.7, and 1975–1.0 soils) experienced a strong reduction in chlorophyll contents (Fig. 6), and this reduction was the strongest in seedlings grown on the 1975–1.0 soil (a reduction of up to 70.79% of the control) (Table 1). In *A. tortilis*, while their was a slight reduction in chlorophylls of seedlings grown in 1975–1.0 soil compared to the controls, we did not find significant differences between treatments in this species (Fig. 6).

3.5. Protein content

Overall, there was a reduction in root protein content proportional to oil–contaminated soils (Fig. 7A). The protein amount in roots of seedlings grown in 1975–0.0 soil was significantly higher than the protein content of roots grown in 1975–1.0 soil (two-way ANOVA, $P < 0.1$). We found higher protein concentration in roots from *A. raddiana* compared with roots of *A. tortilis* ($p < 0.05$). For the protein content in leaves (Fig. 7B), a significant reduction was found only in *A. raddiana* leaves of seedlings grown in the different soil treatments ($p < 0.05$). While a similar trend was found in *A. tortilis* grown in 2014–0.3 and 2014–0.7 soils, these were not significantly different.

3.6. SOD and APX activity

There was a significantly different response in terms of SOD activity in the roots of seedlings grown on the different soils (two-way ANOVA, $p < 0.05$, Fig. 8A). *A. raddiana* seedlings showed a strong activity of SOD in roots exposed to 2014–1.0 and 1975–1.0 soils. SOD activity slightly increased with the increasing of 2014 contaminated soils concentration. While there was no dose-dependent response in *A. tortilis*, also in this species, the SOD values were found to be higher in roots of seedlings grown on contaminated soils, especially in 2014–1.0 and 1975–1.0 soils.

The response of SOD activity in leaves (Fig. 8B) was opposite to that of the roots, with the highest SOD activity found in both the control soils (2014–0.0 and 1975–0.0) ($p < 0.01$), and the lowest SOD activity found in contaminated soils, especially at the higher concentrations of the 1975 event (1975–1.0 soil; Fig. 8B). There was a significant difference between the two species in the terms of SOD activity in leaves (two-way ANOVA, $p < 0.05$; Fig. 8B) with the higher SOD concentration achieved in leaves of *A. raddiana* (Fig. 8B).

APX activity in the roots was higher in 2014–1.0 and 1975–1.0 soils and reduced in control soils (2014–0.0 and 1975–0.0), but this difference was not significant (two-way ANOVA, $p > 0.05$, data not shown). In contrast, the response of APX in the leaves of *A. raddiana* was significantly higher in control soils (two-way ANOVA, $p < 0.05$) at least in *A. raddiana* seedlings. The APX enzyme activity in *A. tortilis* (Table 1) was also slightly lower at 13.03% and 10.66% in 2014–0.3 and 2014–0.7 soils, respectively compared to that of seedlings grown on the 2014–0.0 control soils. The reduction of this enzyme activity reached 46.16% in 1975–1.0 soil. However, all these reductions in APX activity in leaf tissues were not statistically significant ($p > 0.05$).

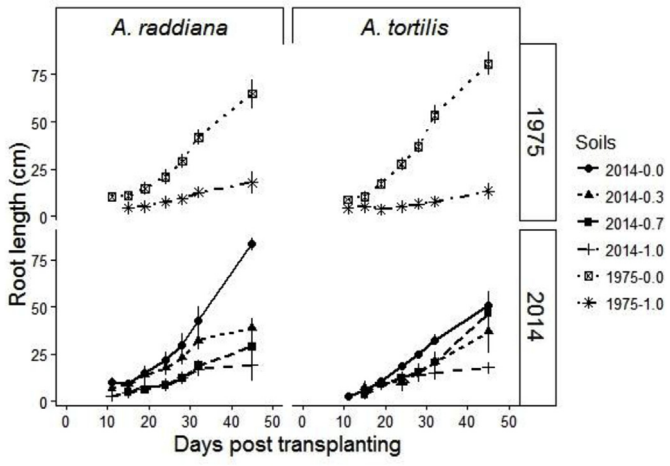

Fig. 5. Root extension of *A. raddiana* and *A. tortilis* seedlings (n = 4, ±SE) grown outdoors in the rhizoboxes system in different soil conditions and measured over time (0–50 DPS). Mean values with the same letter are not significantly different at $p < 0.05$ according to two-way ANOVA and Tukey's HSD tests.

4. Discussions

In Southern Israel, *Acacia raddiana* and *Acacia tortilis* are keystone species, and they both dominate the Evrona reserve. Studying the short and long terms effects of petroleum oil contamination from the recent (2014) and 40 years old (1975) oil spills were the main objectives of this study. In particularly, we were interested in studying the effects of oil on germination and seedling development both above and belowground.

The reduction in the ability of acacia seeds to germinate in oil-contaminated soils, is similar to the findings of studies on several other plant species such as corn (Udo and Fayemi, 1975; Amakiri and Onofeghara, 1983; Ogboghodo et al., 2004; Issoufi et al., 2006; Tang et al., 2011), wheat (Salanitro et al., 1997; Banks and Schultz, 2005), grasses and legumes (Merkl et al., 2004, 2005b; Supplementary Table S1). However, the work presented here showed a slight increase of seed germination (16–32% of control) in both *A. raddiana* and *A. tortilis* grown in soils contaminated 40 years ago. These results are consistent with the study of Adam and Duncan (2002) conducted on twenty five different plant species and the recent study of Golan et al. (2016) on *A. raddiana*. The difference between seed germination rates in the 1975–1.0 soil and those measured in soils contaminated by the much more recent oil spill event (2014) might be explained by the evaporation of volatile hydrocarbons over the long period that separates these two oil spills. Kroening et al. (2001) and Sharonova and Breus (2012) indicated that these fractions of carbon components in oil could act as a physical barrier for water and oxygen penetrating the seed. This could be one of the possible explanations for the low germination rates found in the soils contaminated by the 2014 oil spill event. Further soil chemical and physical properties analysis is necessary to confirm these assumptions.

The fact that in the closed jar experiment (where there was no direct contact between the seeds and oil-contaminated soils) there was no difference in germination rates compared with control soils, indicates that it is not the petroleum carbohydrate volatiles that were preventing germination in our experiments. These closed jar results concur with those of Kroening et al. (2001) that also found

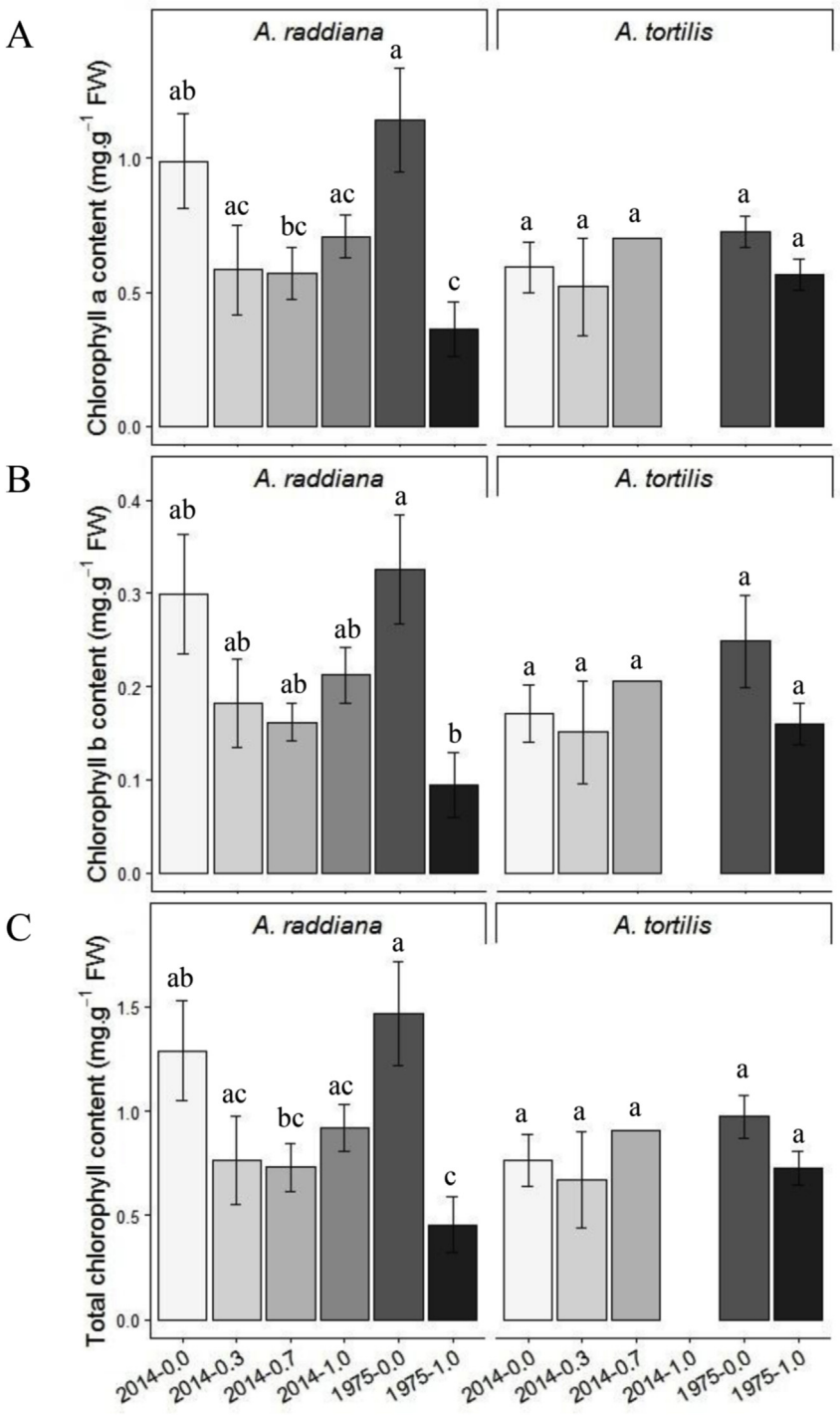

Fig. 6. Chlorophyll content (mg.g^{-1} FW) in leaves of seedlings grown outdoors in the pipes system at 105 days post transplanting (DPT; n = 5, ±SE). Chlorophyll a (A), chlorophyll b (B) and total chlorophyll (C). Mean values with the same letter are not significantly different at $p < 0.05$ according to two-way ANOVA and Tukey's HSD tests. There is no data for *A. tortilis* seedlings growing in the 2014–1.0 treatment, since there was not enough leaf tissue at this time point (105 DPT) to do the analysis.

no significant differences of germination of white clover and ryegrass exposed to oil volatiles.

Table 1 summaries the results of all measured effects of oil-contaminated soils on acacia seedlings. These results indicate the strong decrease in seedling height, leaf number and stem diameter. These results concur with many studies previously done on a wide range of species that have shown the effect of oil contamination on seedlings height, leaf number and area, length and number of roots (Klokk, 1984; Ekundayo et al., 2001; Merkl et al., 2005a; Supplementary Table S1). Moreover, the results presented here for seedling height and leaf number, are in agreement with the significantly negative effects recently found for *A. raddiana* seedlings grown in petroleum contaminated soils (Golan et al., 2016). Also, the inhibition of the development of seedling above and belowground parts of the soil contaminated some 40 years ago was not less, and sometimes even greater, than by the newly

Table 1
Percentage of decrease in measured parameters of acacia seedlings at the end of experiments [(% decrease = Control − Contaminated) x 100/(Control)].

Parameter	*Acacia raddiana*				*Acacia tortilis*				*F*-value of two-way ANOVA	
	2014–0.3	2014–0.7	2014–1.0	1975–1.0	2014–0.3	2014–0.7	2014–1.0	1975–1.0	Soil effect	Species effect
Germination and pipe systems										
germination (25 DPS)	51.12	86.29	79.89	−32.28	50.83	89.86	89.86	−16.53	9.21***	1.12
height change (105 DPT)	72.00	59.00	81.25	88.58	82.47	74.10	70.52	86.11	15.75***	2.24
leaf number (50DPT)	42.42	36.36	36.36	46.15	51.35	49.32	40.54	67.35	24.01***	0.43
stem diameter (105 DPT)	45.83	36.67	53.13	62.04	51.11	50.00	43.06	60.04	37.52***	15.24***
Transplanted seedlings in rhizoboxes (45 DPT)										
total root length	7.31	54.08	69.45	76.33	46.73	37.27	73.83	80.49	15.28***	1.39
root depth	48.77	61.87	80.24	72.64	32.87	14.99	67.36	83.62	19.57***	0.11
belowground dry biomass	27.82	58.53	66.99	53.74	19.86	9.62	55.56	69.40	13.52***	18.35***
aboveground dry biomass	29.61	54.59	49.63	14.04	36.05	8.56	43.87	57.71	10.73***	13.98***
Directly sowing seedlings in rhizoboxes (30 DPS)										
primary root length	47.22	49.10		64.14	42.47	70.88		78.76	14.92***	0.03
number of lateral roots	65.76	61.54		74.78	64.68	81.04		84.33	11.85***	0.49
total lateral root length	49.98	50.42		87.50	44.59	69.24		61.44	8.70***	6.43*
belowground dry biomass	28.22	66.98		59.79	55.89	73.56		62.30	56.08***	44.59***
aboveground dry biomass	12.09	69.82		34.20	60.55	68.89		56.80	26.68***	25.65***
Rhizobium measurements (105 DPT)										
number of nodules	42.62	45.90	48.77	100.00	−11.11	65.28	37.50	78.85	3.87***	6.62*
dry weight of nodules	62.72	59.62	59.62	100.00	80.85	81.28	63.40	80.08	1.62	0.14
Physiological and biochemical measurements										
seedlings photosynthesis (40 DPT)	4.68	1.08	0.45	0.45	3.17	10.28	4.34	5.55	1.75	3.36
chlorophyll a	40.93	42.37	28.43	68.38	12.41	−17.78		21.85	2.96*	4.71*
chlorophyll b	39.32	46.02	29.23	70.79	11.78	−20.09		35.57	2.69*	2.43
total chlorophyll a+b	40.56	43.22	28.62	68.92	12.27	−18.30		25.36	2.99*	4.19*
protein content in roots	31.81	23.14	61.60	71.15	42.78	60.66	50.58	68.05	12.75***	5.03*
protein content in leaves	14.98	36.22	51.79	21.55	22.28	35.73		−1.10	3.29*	3.57
SOD activity in roots	−91.90	−40.18	−332.34	−268.30	−11.91	−29.60	−75.14	−84.25	4.66***	1.35
SOD activity in leaves	67.32	57.92	62.57	85.65	61.74	69.48		83.45	7.68***	6.74*.
APX activity in roots	11.00	−1.02	−131.11	−135.90	−83.17	−120.89	−245.86	−227.12	3.85**	1.73
APX activity in leaves	56.07	50.44	52.55	63.89	13.03	10.66		46.16	3.86*	3.54

*, ** and *** indicate the *F*-values are significant at $p < 0.05$, 0.01 and 0.001 levels, respectively.

contaminated soil of 2014 (Table 1). This implies that the non-volatile components of the oil are responsible for these effects. According to Baker (1970), low-boiling compounds, unsaturated compounds, aromatics, and acids in petroleum oil may penetrate into the plant tissue and travel in the intercellular spaces, and possibly also in the vascular system. These hydrocarbon molecules may cause the damages to cell membranes leading to leakage of cell contents, or reducing transpiration rate by blocking intercellular spaces.

For both polluted sites, Golan et al. (2016) showed that the oily cover on the surface of these soils prevents water from penetrating the soil layers due to the hydrophobic properties of petroleum. This water deficiency affects leaf growth, the height of developing seedlings and root elongation (Merkl et al., 2005a). An additional physical factor that may influence the seedling growth both above and below ground is the high temperature associated with darker color of oil-contaminated soils. Bowen (1991) and Kaspar and Bland (1992) found that temperature had a considerable effect on the growth of plants and the development of root system including root elongation and the establishment of new lateral roots. The darker color of soils contaminated by oil together with the extremely hot air temperature (up to 46 °C) during the study period, enhanced high solar radiation absorption and brought about the higher temperature in the oil-contaminated soils (results not shown). Considering temperature effects on other species under oil-contaminated condition (Merkl et al., 2005b), it could be assumed that these conditions had a strong effect on the root growth of acacia seedlings even though they are adapted to arid conditions (Munzbergova and Ward, 2002).

In addition, the negative effect of oil contamination on the nutrient acquisition and the activity of microorganism associated with the roots of acacias (Fall et al., 2008) could contribute to the poor growth of acacia seedlings in contaminated soils. De Jong (1980), Ogboghodo et al. (2004), and Rosso et al. (2005) indicated that petroleum contamination could reduce the availability of nitrogen and phosphate in the soil, affecting both nutrient and water uptake ability of plants. Zahran (1999, 2001) described the crucial roles of rhizobia activity in acacia species growing in arid conditions and showed their importance as a source of nitrogen. Merkl et al. (2004), working on grasses and legume species showed the considerable inhibition of root rhizobia nodulation in oil-contaminated soils. Similarly, in the present study (Table 1) the limitation of nodulation was found in contaminated soils (results not shown). The lack of rhizobia nodules could reduce nutrient availability which would entail the lower seedling growth and development.

Several studies have pointed out to the mechanisms involved in the oil's effect on root growth. Achuba (2006) found that various concentrations of petroleum carbohydrates reduced the activities of amylase and starch phosphorylase within seedling's cotyledons, and the meristem mitotic activity. This might also explain the reduced plant growth and root development shown in the present study. Furthermore, as the crude oil concentration of soil increased, a decrease in the stomatal index of leaves was found together with the smaller size of the roots (Omosun et al., 2008). These may have also contributed to the poor seedling growth in oil-contaminated soil shown here.

The results of the present study showed a significant reduction in chlorophylls content in *A. raddiana*, as was found by Al-Hawas et al. (2012). However, it was found that petroleum contamination did not have any significant effect on the photosynthetic performance of existing photosynthetic units as measured by PAM

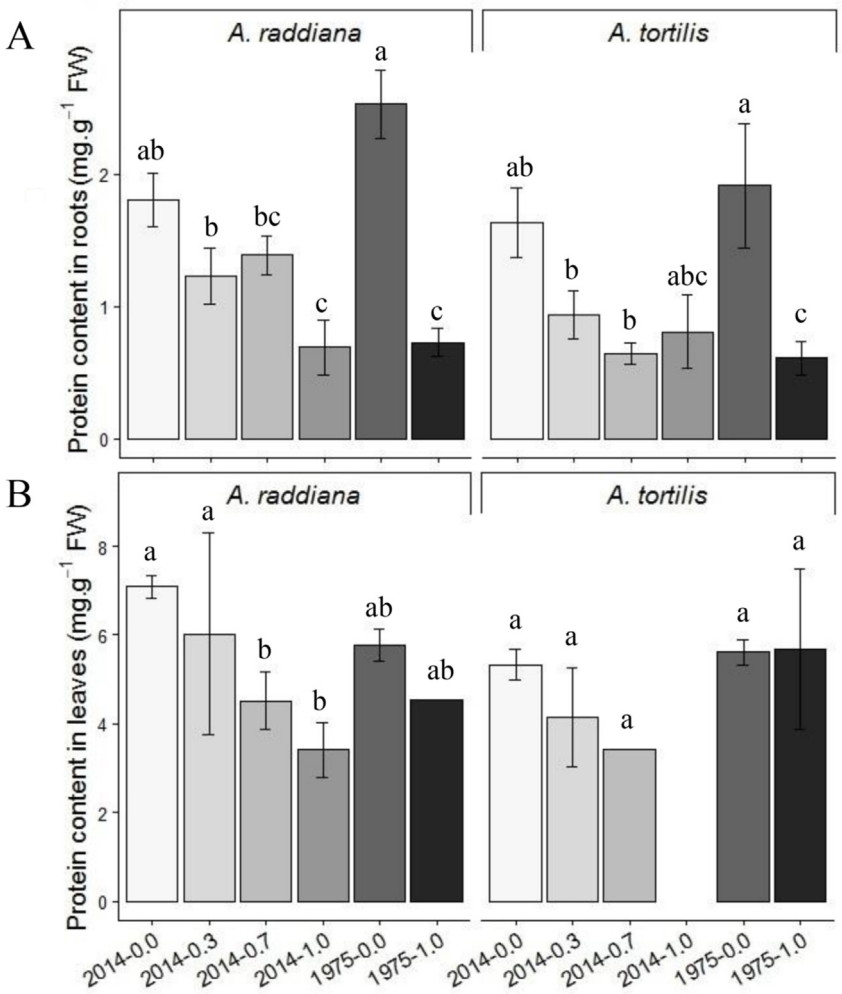

Fig. 7. Protein content (mg. g^{-1} FW) in roots (A) and leaves (B) of seedlings grown outdoors in the pipes system at 105 DPT (n = 1–5, ±SE). Mean values with the same letter are not significantly different at $p < 0.05$ according to two-way ANOVA and Tukey's HSD tests. There is no data for *A. tortilis* seedlings growing in the 2014–1.0 treatment, since there was not enough leaf tissue at this time point (105 DPT) to do the analysis.

fluorometry, which is in agreement with studies of Odjegba and Sadiq (2002) and Achuba (2006).

As far as we know, the present study is the first study to examine the effects of petroleum contamination on biochemical responses of acacia seedlings. The significant decrease of total protein content in roots and leaves of acacia seedlings grown in oil-contaminated soils concur with the work of Odjegba and Sadiq (2002) that studied the effect of petroleum oil on *Amaranthus hybridus*, but differ from Achuba (2006), results of which showed that total protein contents in leaf tissues of cowpea (*Vigna unguiculata*) seedlings actually increased under oil contamination conditions. The decreased protein contents shown in the present study could have also been at least partially, caused by differences in soil temperatures which were higher in the 2014–0.7 and 2014–1.0 soils compared with other soil treatments (data not shown, discussed above). This was similar to the effect of heat stress on the protein content of turf grasses (He et al., 2005) and sesame (Fazeli et al., 2007).

In addition to the decline in protein content in leaves and roots, the results presented here also demonstrated increased activity of SOD and APX in root tissues of acacia seedlings exposed to oil-contaminated soils. These results are similar to other studies on mangroves where SOD and APX contents increased in root tissues exposed to oil contamination (Zhang et al., 2007; Ke et al., 2011; Al-Hawas et al., 2012; Cui et al., 2016) (Supplementary Table S1). Interestingly in the study presented here, the activity of both SOD and APX enzymes was significantly reduced in the leaf tissue of seedlings grown in contaminated soil in comparison with those grown in control soils.

From these results, we can predict that in the Evrona reserve, both oil pollution events dramatically reduce the ability of new seedlings to be recruited into the local acacia population.

A surprising result of this study was the long-term effects (more than forty years) of the 1975 oil spill on local acacia seedlings growth, particularly in *A. tortilis*, the dominant acacia species in Evrona reserve. Indeed, in a recent survey of acacia tree size distribution in the 1975 oil spill and 1975 control areas, it was found that small/young trees (canopy size of less than 2 m, and between 4 and 5 m) were totally absent in the 1975 oil spill site (Nothers et al., 2017). Lack of younger trees in this site indicates the long-term effects of untreated oil spills.

While the current average total petroleum hydrocarbons (TPH) content of the 1975 site is only 25% of the TPH levels at the 2014 site (LDD, 2016; Supplementary materials Fig. S2A), Gordon et al. (2018) recently showed that the soil of the 1975 site was at least as hydrophobic as the soil of the site of the recent 2014 oil spill

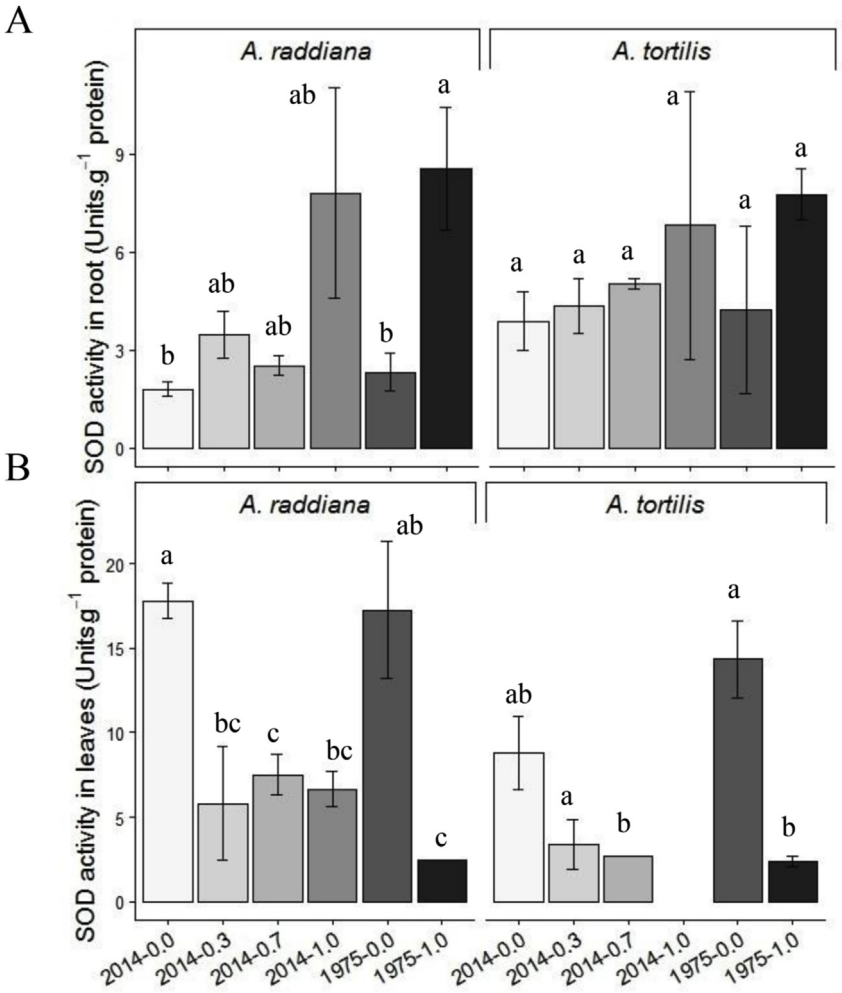

Fig. 8. SOD activity (units g^{-1} protein) in seedlings' roots (A) and leaves (B) grown outdoors in the pipe system at 105 DPT (n = 1–5, ±SE). Mean values with the same letter are not significantly different at $p < 0.05$ according to two-way ANOVA and Tukey's HSD tests.

(Supplementary materials S2), even though more than forty years have passed since the 1975 oil spill.

The possible mechanisms behind the effects of petroleum contaminated soils on plants such as the changes soil properties, toxicity from chemical compounds in the actual oil, or the reduction in the activity of microorganism in the soil, all still need to be clarified further.

The dilution of the oil-contaminated 2014 soil, slightly reduced the its negative effects on the seedlings, particularly at 30% soil mixture. Thus, decreasing oil concentration by mixing the local soil could be a possible way to reclaim the habitat. The results shown here must be taken into consideration of management and restoration efforts in the area.

5. Conclusions

Germination ability in both *Acacia raddiana* and *Acacia tortilis* were significantly lower in all oil-contaminated soil treatments. Seedlings growth parameters, above and below ground dry biomass were remarkable decreased as the function of the increased percent of the oil-contaminated soils. PAM fluorometry indicated that *A. tortilis* might be more sensitive to these oil contaminates than *A. raddiana*. From these results, we can predict that in the Evrona reserve, both oil pollution events will dramatically reduce the ability of new seedlings to be recruited into the local acacia population. The shift in the age structure of the local acacia communities caused by petroleum pollution could, if not mediated, lead to changes in the entire ecosystem in the research area. This prediction should guide management and restoration efforts in the area.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.11.067.

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