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THE EFFECT OF PETROLEUM HYDROCARBONS ON SEED GERMINATION, DEVELOPMENT AND SURVIVAL OF WILD AND CULTIVATED PLANTS IN EXTREME DESERT SOIL

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ABSTRACT

Long and short-term effects of soil contamination by of Petroleum hydrocarbons spills that occurred in 1975 and 2014 in a hyper-arid desert in the Arava Valley, Israel, were studied by assessing soil toxicity on seed germination, and seedling survival of wild local species (*Acacia raddiana* Savi, *Rumex cyprius* Murb., *Malva parviflora* L. and *Astragalus eremophilus* Boiss.) and. cultivated crops (Cucumber-*Cucumis sativus,* Tomato-*Solanum lycopersicum,* and Pepper-*Capsicum annuum*). Seed size and seed-coat structure strongly affected germination ability to soil contaminated with petroleum hydrocarbons in both the old (1975) and new (2014) spill. Species seeds with thick seed-coat and well developed macrosclereid cells were more tolerant to contamination than species with thin seed-coat and without macrosclereid cells. Furthermore, soils containing high were more toxic than soil containing low amounts of volatile compounds (old spill). Seedlings survival and morphology was strongly affected by the contamination in both old and new spill, and was leading to dwarf plants with few leaves and high mortality rate of the seedlings. We strongly recommend that actions to rehabilitate this hyper-arid ecosystem should take into consideration long term effects of oil spills that constrain the recovery of the native vegetation across time.

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INTRODUCTION

Due to the increasing frequency of petroleum hydrocarbons (oil) contamination in a wide range of terrestrial ecosystems, more attention is being paid to understand how organic pollutants affect the environment, mainly through their toxic effects on native vegetation, wildlife and human health (Rahbar et al. 2012). Petroleum hydrocarbons are recognized as being destructive and threatening to above- and below-ground active biota. Moreover, the presence of petroleum hydrocarbons in the soil affects seed germination, plant growth and development (Das and Mukherjee 2007; Bona et al. 2011; Asghar et al. 2013). Studies in different agro-ecosystems in Mexico and Brazil showed the effects of soil contamination by hydrocarbons on seed germinability of gramineous, herbaceous and leguminous species and found it depend on the time transcurred (Bossert and Bartha (1985), Trujillo and Gutirrez-Rojas (2000), Adam and Duncan (2002) and Bona et al. (2011).

Well-established plants respond differently to hydrocarbon toxicity (Crafts and Reiber 1948; Currier 1951; Baker 1970; Gauvrit and Cabanne 1993; and Chaineau et al. 1997). While members of the Umbelliferae family are notably tolerant to slight damage by oils, grasses are intolerant to oil contamination and, therefore, oils are sometimes used as post-emergence herbicides (Gauvrit and Cabanne 1993). Exposure of seeds to diesel fuel for more than one week significantly reduced their ability to germinate (Amakiri and Onofeghara 1984). However, seeds with a thick seed-coat reduce embryo exposure to contaminants and were viable after 32 weeks, even though rate of germination was lower by a factor of three..

Here we studied long- and short-term effects of soil contamination by oil spills on seed germination and seedling development. On December 3, 2014, ca. 5,000 $m^3$of crude oil leaked from the Eilat Ashkelon Pipeline Company's oil pipeline near Be'er Ora, a settlement in the southern Arava Valley in Israel. The oil flowed above ground for about six km throw existing stream-beds and sub-stream beds (Wadies), creating sub-channels of oil pollution and causing extensive ecological damage to about 144 km^2in the Evrona Nature Reserve. Forty years earlier, in 1975, a similar oil leak occurred somewhat further south than the 2014 spill. The earlier leak spilled ca 10,000 $m^3$of crude oil into the environment, nearly twice the amount of the 2014 spill. In 1975, the oil reached the southern part of the Evrona Reserve. The sub-channels in both spills ranged in width from 50 cm to five meters, with a depth of about 30 cm. The 1975 oil spill was never remedied (Ministry of Environmental Protection reports), therefore providing an unique time perspective to examine the carry-over effects of old compared to recent contamination events.

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The Evrona Nature Reserve at the south of the Arava Valley is a unique sanctuary that represents an extreme saline sandy desert ecosystem. The reserve, home to the last fully preserved salt marsh in the Arava Valley, features a density of up to 200 trees/km^2 of Acacia trees, together with other halophytic species and a grove of *Hyphaene thebaica* trees. In an arid environment, plants act as ecosystem engineers and play a significant role in the function of the ecosystem by serving as landscape modulators and shaping landscape patchiness. Changes in plant density due to lack of germination or restricted survival affect vegetation richness and composition (Boeken 2008). Therefore, following the severe ecological spill disturbance in 2014 in the Evrona Nature Reserve, it was important to assess short- and long-term eco-toxicity effects of soil contamination by petroleum hydrocarbons, by studying its effects on seed germination and seedling establishment in comparison to the oil spill in 1975.

The goal of the present study was to investigate how soil contaminated by petroleum hydrocarbons over different periods of time affects the germination and development of natural local species and cultivated crops. Seeds of plant species present in the Evrona Reserve were collected in missions previous to the 2014 spill, were preserved at the Israel Gene Bank and were used in the current study.

The study's results will contribute to the assessment regarding the urgency of treating the contamination in the reserve, as well as how the spill has affected the ability of the vegetation to regenerate and develop in contaminated areas.

MATERIAL AND METHODS

1. Soil sampling

Two sites affected by the oil spills and two control locations in the vicinity of the polluted sites were selected for soil sample collection. The first site accounts for the 1975 oil spill and the second for the 2014 spill. In each site soil was collected in four 25 m^2 plots, from five random points in each plot, and pooled together to create one large sample of about 25 kg per plot. The four different soil samples were labelled Ctrl 2014, Contamination 2014, Ctrl 1975, and contamination 1975.

2. Bioassay

Germination ability, seedling morphology and survival were examined in three cultivated crops (Cucumber-*Cucumis sativus,* Tomato-*Solanum lycopersicum,* and Pepper- *Capsicum annuum*) and four wild species typical to the Evrona Reserve – the tree *Acacia raddiana* Savi, and the annuals *Rumex cyprius* Murb., *Malva parviflora* L. and *Astragalus eremophilus* Boiss.. The species were chosen after a preliminary experiment for germination ability in the control soils.

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The crops and the wild species were sown separately in planting trays with 13x8 holes, 50ml volume. Four planting trays were used for the crop species and another four trays for the wild species, each tray containing one type of soil: Ctrl 2014, contamination 2014, Ctrl 1975 and Contamination 1975- , with a total of eight trays for the entire experiment. For each species in the planting tray, 4x6 holes were marked and two seeds were sown in each hole for a total of six replicates, where each replicate contained eight seeds with two seeds per hole (n=48). The trays were placed in a climate-controlled greenhouse for 2 month during July-August 2015 with natural light (average of 13.5 hours during these months) and two minutes of tap water spray irrigation four times a day.

Seed coats of the wild species were mechanically scarified to break dormancy and synchronize germination. Seeds of the cultivated crops, as well as *Rumex cyprius* were not scarified before sowing.

Germination ability (measured by emergence of the seeds) and seedling survival were measured during the experiment. Germination was calculated in each replicate as a percentage of the total seeds sown in that replicate. Seedling survival was calculated as a percentage of the maximum number of seedlings emerged in each replicate. Both germination and survival percentages were plotted against the log of days post sowing (DPS) and a linear regression analysis was perform using GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Statistical differences were calculated for Arcsine transform data.

Seedling height was measured in the cultivated crops at 7 days post-germination (*Cucumis sativus*, *Capsicum annuum*) and 9 days post-germination (*Solanum lycopersicum*) for each of the different treatment plots, since germination time was affected by the contamination. Seedling hypocotyl length was measured from the soil surface to the cotyledon.

The number of leaves on the wild plants was counted and calculated as number of leaves per plant. The height of *Acacia raddiana* seedlings was measured in 2-month after sowing , from soil level to the apical bud.

A one-way ANOVA followed by Tukey's multiple comparisons test (for the comparison of all four soil treatment groups, i.e. 1975 control and contamination, and 2014 control and contamination) or t-test (for pair comparison) was performed using GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) and JMP statistical package.

3. Seed size and seed-coat thickness

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Seed size and seed-coat structure were measured in the studied wild species. Seed size (length and width) was measured with a stereomicroscope (Leica MZFLIII, Leica Microsystems GmbH) equipped with a Nikon DS-Fi1 camera. The measurements were performed using NIS elements BR 3.1 software.

For seed coat measurements, seeds were cut by hand with a razor blade in transverse orientation and attached to a metal stub by double-sided carbon tape, and coated with gold palladium (Quorum SC7620 mini sputter coater). Images were taken with a JEOL JCM-6000 bench top SEM. Analysis was performed using the SEM software.

Means of replicates were subjected to statistical analysis by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test ($P < 0.01$) using the JMP statistical package.

4. Soil water-holding capacity

Water holding capacity (WHC) was calculated for the soil collected (sub-samples) based on the weight of the water held in the sample vs. the dry weight of the sample. The data were subjected to statistical analysis of variance using the SAS model (ANOVA). Duncan's multiple range test and Pearson correlation coefficients were used to evaluate differences between means.

RESULTS

Seed germination of cultivated crops

Cucumis sativus seeds germinated in both the 1975 and 2014 control soils at 2 DPS, with a higher germination percentage in the 2014 control soil compared to the 1975 control soils (80% and 50% respectively). At 4 DPS, germination in both control soil plots reached a maximum of 100%. Soil contaminated in the 1975 oil spill inhibited the germination of *Cucumis sativus* seeds by 5 days, and significantly reduced ($p<0.001$) both germination rate and ability to a final 80% maximum at 14 DPS. Almost no germination was observed in the 2014 oil spill soils, with a maximum of 5% at 11 DPS. (Online Resource1A)

Solanum lycopersicum seeds germinated at similar rates in both control plots starting at 4 DPS with 15-20% and increased to a maximum germination rate of 80% and 90% at 9 DPS in the 1975 and 2014 control plots respectively. A germination inhibition of 9 days was observed in the 1975 oil spill plots compared to the control plots, with a significant reduction in both germination rate ($p<0.001$) and ability, resulting in a maximum of only 60% at 42 DPS. The *Solanum lycopersicum* seeds did not germinate at all in the 2014 oil spill plots. (Online Resource1B)

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Different patterns were observed in the germination percentages and rates of *Capsicum annuum* seeds. Initially, germination was observed only in the 1975 control plots, starting with 15% at 10 DPS and reaching maximal levels of 50% at 18 DPS. In the 1975 oil spill plots, however, germination was delayed until 7 DPS but the rate of germination was significantly higher than in the control plot ($p<0.001$) and reached higher maximal germination ability of 75% at 25 DPS. The 2014 control soils significantly inhibited the *Capsicum annuum* seeds' germination compared to the 1975 plots, with 30% maximal germination at 25 DPS. In the 2014 oil spill plots, like the *Solanum lycopersicum* seeds, the *Capsicum annuum* did not germinate throughout the experiment. (Online Resource1C)

Survival of cultivated crop seedlings

Except for the *Cucumis sativus* seedlings, which survived at a level of nearly 100% in all treatments (data not shown), the survival rate of *Solanum lycopersicum* and *Capsicum annuum*was strongly affected by the treatment ($p<0.0001$ and 0.025 respectively; Online Resource2). Because no germination was observed in either of these two crops in the 2014 oil spill plots, seedling survival was calculated and presented only for the 1975 plots. Of the *Solanum lycopersicum* seedlings that successfully germinated in the 1975 oil spill plots, 75% survived up to 95 DPS whereas almost 100% survived in the control plot. The mortality levels of *Capsicum annuum* seedlings were higher, with about 50% survival at 45 DPS compared to greater than 90% in the control plot.

Height of cultivated crop seedlings

The heights of 7-day old *Cucumis sativus* seedlings (Online Resource3) were significantly affected ($p<0.0001$) by the plot in which they were planted. The seedlings' heights in the control plots were similar, with an average value of 3.2 cm. However, the seedlings in both contaminated plots were significantly ($p<0.0001$) shorter, with an average height of 1.35 and 1.175 cm in the 1975 and 2014 spill oil plots respectively.

The heights of *Solanum lycopersicum* seedlings were measured at 9 days post-germination and those of the *Capsicum annuum* seedlings were measured at 7 days post-germination in the 1975 plots only, since no germination was detected in the 2014 oil spill plot. The heights of the seedlings of both crops were significantly lower ($p<0.0001$) in the oil spill plot, with an average value for *Solanum lycopersicum* of 1.6 cm compared to 3.5 cm in the control plot. The average height of the *Capsicum annuum* seedlings was 1.2 cm in the spill plot compared to 2.1 cm in the control plot (Fig 3 B;C).

Wild plants seed germination

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Acacia raddiana, a keystone species in the Arava valley, was the only species in this study to germinate in both the 1975 and 2014 oil spill soils within the same timeframe as in the control soils (Fig 1A). In all soil plots, the initial germination was observed at 3 DPS, but significant differences were found in the germination percentages between the plots: 50-60% in the control plots and 12-20% in the oil spill plots. The germination rate was significantly lower in the oil spill plots compared to the controls ($p<0.002$ for the 1975 spill and $p<0.0001$ for the 2014 spill). The maximal germination percentages in the 1975 spill soils were reached at 21 DPS, with levels similar to those of the control plots. In the 2014 spill soils, however, maximal germination reached only 35%.

Astragalus eremophilus Boiss. is a rare annual plant, limited to arid environments, and is listed as a near-threatened species on the "Red List" of Israel flora (Shmida et al., 2007; 2011). In this experiment, the maximal germination levels of the plant were reached within few days. In both control plots, 60-70% germination was found at 8 DPS, but in the 1975 contamination plot the germination reached a lower maximal value of 30% at 5 DPS and the calculated germination rate was significantly lower than in the control ($p<0.0001$). In the 2014 contamination plot, the few seedlings that germinated by 8 DPS represented 10% of all seedlings in the plot (Fig 1B).

Malva parviflora L. is a common annual grass with edible leaves that is used by the local population. In the 1975 control plot, the germination rate of this species was the highest, starting at 4 DPS with 20% germination and reaching a maximal value of 50% at 19 DPS. A different pattern was found in the 1975 contamination plot, with initial germination of 4% at 7 DPS and a maximal germination of 20% at 22 DPS. The germination rate was significantly ($p<0.00001$) lower in the contaminated soil compared to the control. *Malva parviflora L.* seeds germinated differently in the 2014 control plot than in the 1975 control plot. The germination rate in the 2014 control soils was significantly lower ($p<0.0001$) with maximal levels of 25% at 22 DPS. In the 2014 contamination soil, only one seed germinated during the entire experiment, at 7 DPS (Fig 1C).

Of all examined wild plants, the seeds of *Rumex cyprius* were the most sensitive to the contamination. The seeds germinated at very low percentages and only in the 1975 contamination plot. The germination occurred initially at 45 DPS and reached maximal levels of only 7% by 86 DPS. In the control plots, however, the germination started at 6 DPS (2014 control plot) and 7 DPS (1975 control plot) at levels of 8-10%, reaching maximal levels of 40% by 27 DPS in the 2014 control plot and 33% by16 DPS in the 1975 control plot (Fig 1D).

Survival of wild plant seedlings

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Although the germination rates of *Acacia raddiana* seeds reached similar levels in the control and contaminated plots, the survival rate of the seedlings differed and a high mortality level was recorded under the contamination conditions. While seedlings that germinated in the 1975 control plot survived throughout the experiment, at 24 DPS the seedlings growing in the 1975 contaminated plot began to die; of the 100% seedlings recorded at 21 DPS, the survival percentage for *Acacia raddiana* fell to 55% by 58DPS, the end of the experiment for *Acacia raddiana*. The mortality rate was significantly higher ($p<0.0001$) under the 2014 contamination conditions, with an average survival of 10% by 41 DPS. A slow mortality was observed in the 2014 control plot, with a mean survival of 80% at 73 DPS (Fig 2A).

Astragalus eremophilus Boiss. seedlings survived at a level of 90% in both control plots, with no significant differences observed between the plots. However, the seedlings' survival rates were significantly ($p<0.0001$) lower for both contamination plots, with only 30% survival at the end of the experiment under the 1975 contamination conditions. In the 2014 contamination plot, the few seedlings that were germinating on the eighth day of the experiment collapsed the following day and 0% survival was recorded (Fig 2B).

A similar pattern was observed in the survival of *Malva parviflora L*. seedlings. In both control plots, the average survival level was 95-100%, but only 60% of the seedlings under the 1975 contamination conditions survived. The few seedlings that germinated under the 2014 contamination conditions collapsed the day after germination with a 0% survival (Fig 2C).

The few seedlings of *Rumex cyprius* that germinated in the 1975 contamination plot collapsed the day after germination, compared with 90-100% survival in both control plots (data not shown).

Leaves of wild plants

Because *Astragalus eremophilus Boiss.* and *Malva parviflora L*. seeds hardly germinated in the 2014 contaminated plot and the seedlings did not survive to grow leaves, only the results from the 1975 control and contaminated plots for these species are shown. Furthermore, since the seedlings of *Rumex cyprius* in both contaminated plots did not survive to grow leaves, this plant is not presented in this section.

The number of leaves in each *Acacia raddiana* seedling was counted at 36 DPS (Fig 3A). The average number of leaves per plant in the 1975 control plot was significantly ($p<0.0001$) higher than the number of leaves in the 1975 contaminated plot, with 6.6 leaves compared to 0.86 leaves. In the 2014 control plot, the average number of leaves was not significantly different from in the 1975 control plot, reaching a value of 5.19. The number of leaves in the 2014 contamination plot, however, was not significantly different than the number of leaves in the

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1975 contamination plot, with a mean value of 2 leaves per plant but significantly lower than the 2014 control plot plants ($p<0.05$).

The same pattern of results was found for both *Astragalus eremophilus Boiss.* and *Malva parviflora L.* with significantly more leaves per plant in the 1975 control plot than in the contaminated plot ($p<0.0001$). The average number of leaves per plant in the *Malva parviflora L.* seedlings was 4.075, whereas in the contaminated plot there were only 1.44 leaves per plant. In the 1975 control plot, an average of 4.06 leaves grew on the *Astragalus eremophilus Boiss.* seedlings, compared to 0.66 found on the plants growing in the 1975 contaminated plot (Fig 3B;C).

Height of Acacia raddiana seedlings

The heights of the *Acacia raddiana* seedlings (Fig 4) were measured two months after initial germination. The heights of the seedlings in the 2014 control plot were the highest, reaching an average of 13.5 cm compared to the significantly lower height of 5 cm in the 2014 contaminated plot ($p<0.0001$). In the 1975 control plot, the average height of the seedlings was 10 cm, significantly higher than the average height of 5.7 cm ($p<0.001$) in the 1975 contaminated plot.

Wild plants'seed size and seed coat thickness

The seed coat of the *Acacia raddiana* were the thickest observed in the study, with a mean value of 340µm and was composed from at least three layers of cuticle, Macrosclereid cells and mesophyll layer (Fig 5A,B; table 1). The seed coats of both *Malva parviflora L.* and *Astragalus eremophilus Boiss.* were significantly thinner; both had similar values, 35.6 and 34.6 respectively, with a clear layer of Macrosclereid cells (Fig 5C,D,E,F; table 1). The seed coat of *Rumex cyprius Murb.* was the thinnest and significantly different than the other wild plants' seed coats, with a mean value of 16.3 (table 1). The Macrosclereid cells were absent from the *Rumex cyprius Murb.*seed coat (Fig 5G,H).

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Table 1: seed coat thickness measured in µm using JEOL JCM-6000 benchtop SEM. Analysis was performed using the SEM software. Seed length and width measured in mm using stereomicroscope (Leica MZFLIII, Leica Microsystems GmbH) equipped with a Nikon DS-Fi1 camera. The measurements were performed using NIS elements BR 3.1 software

	Seed coat thickness (µm)	Seed length (mm)	Seed width (mm)
Acacia raddiana	340.9±1.8 (a)	6.2±0.3 (a)	4.4±0.09 (a)
Malva parviflora L.	35.6±1.6 (b)	1.2±0.03 (c)	1.25±0.03 (b)
Astragaluseremophilus Boiss.	34.6±0.8 (b)	1.17±0.14 (c)	1.26±0.06 (bc)
Rumex cyprius Murb.	16.3±0.6 (c)	2.65±0.08 (b)	1.08±0.03 (c)

The width and length of *Acacia raddiana* were two to four times significantly bigger than the other seeds examined in this study. *Malva parviflora L.* and *Astragalus eremophilus Boiss.* were similar in size, while *Rumex cyprius Murb.* was significantly longer than *Malva parviflora L.* and *Astragalus eremophilus Boiss.* but not as wide (Fig 5I; table 1).

Soil WHC

In the present study, the WHC in the control soils was found to range between 18.1% and 19.7% for 1975 and 2014 years sampling sites. These values were found to be eight and three time significantly higher in comparison to the 1975 contaminated sites (mean 2.83%; $p<0.001$) and the 2014 contaminated sites (6.2% $p<0.0001$), respectively.

DISCUSSION

Oil that enters the environment poses risks to the ecosystems with which it comes into contact. The components of oil are toxic and some are even known mutagens and carcinogens (Banks and Schultz 2005). Chemical analysis is conventionally used to assess the level of contamination, and the concentration of components in the soil is tested, although this test does not provide a complete picture of the potential impact of the pollution. Biological tests (bioassay) to determine the eco-toxicity of the ground contamination have been widely used in recent years, particularly when the source of pollution, like oil, is a complex mixture of several components.

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When the contamination is found in the soil itself, plants have been used for biological assessment and different tests have been proposed, such as short-term germination tests and the longer-term plant development tests that require several weeks to complete. (Wang 1991a, b; Wang et al. 2001).

The results detailed in this study provide a broad view of the germination capacity and survivability of various seeds in response to the 2014 and 1975 oil spills. The fact that two spill events occurred within 40 years of each other in the same area, and the first event was never treated, gives us the unique opportunity to study the short and long-term effects of oil contamination. Furthermore, the ecosystem in this study is a hyper-arid environment, with uniquely slow decomposition and turnover processes, which gives us an undisturbed view of the possibilities for ecosystem rehabilitation following the aforementioned ecological disasters.

We chose short-term tests to assess seeds' germination ability, together with long-term tests to evaluate the acute chronic effects of pollution on plant development. Germination tests reported in the literature (Perez et al. 1986; Fletcher et al. 1985; Banks and Schultz 2005; Adam and Duncan 2002; Sharonova and Breus 2012)used the most sensitive possible seeds to examine the nature of the damage following a ground contamination event. Therefore, part of the experiment was conducted on cultivated seeds known to be susceptible to hydrocarbon contamination (Sharonova and Breus 2012). The selected seeds can also germinate in saline desert soil, which is prevalent in the area of the pollution (Pen-Mouratov et al. 2010). In this study, cultivated crop seeds were severely affected by the recent spill, with almost no germination recorded. The first stage of petroleum hydrocarbons pollution was found to be toxic to the sensitive cultivated seeds, and the results from the 1975 spill plot demonstrated that the effects continue even 40 years after a pollution event. To determine the effect of pollution on the local flora found in the Evrona Reserve and the surrounding area, however, it was important to examine germination capacity together with plant development of annual and perennial plants native to the region.

Adam and Duncan (2002) found that as the concentration of volatile substances falls, the percentage of seeds that germinate increases. Their findings are consistent with those presented in this report, which show that the rate of germination is higher in the areas contaminated by the 1975 oil spill than in the area contaminated in 2014 where volatile substances are still numerous and significant. Volatile hydrocarbons (petroleum, benzene, gasoline), particularly the smallest and lightest of these, can easily pass through the plant's cell membrane and cause toxicity (van Overbeek and Nlondeau 1954). As well, these components react chemically with components in the cell and interfere with vital processes. This is apparently the reason for the low rates of germination observed in soils contaminated in 2014 and the low survival rate in plants that

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managed to germinate. It appears that the rate of seed germination increases as the polluting components in the soil evaporate.

Both the results of this report and the work of Adam and Duncan seem to indicate that even the few volatile substances in the soil contaminated in 1975 do not permit the seeds to germinate at rates and levels similar to those observed in the control plots. According to Sharonova and Breus(2012)this observation can be explained by the medium-sized components of oil (e.g., diesel fuel, naphtha, kerosene, gas-oil), which act as a physical barrier and prevent water and oxygen from penetrating the seed, as well as the intake of nutrients through the developing roots. Water-holding capacity (WHC), the amount of water that soils can hold, is known to be of great importance. An increase in the amount of water supported will allow more plant growth and are less susceptible to leaching losses of nutrients, pesticides or pollution. For both polluted sites, the WHC was lower than normal (determined by the control),while the oily sludge on the surface prevented the water from penetrating the soil layers and the seeds from germinating and developing. Mishraet al. (2001) showed that as bioremediation and oil degradation increase, WHC was increased, leading to grass germination in their study site. As the oil was never removed from the 1975 spill area and, therefore, the WHC remained low, water could not penetrate the soil, probably causing the low level of seed germination and seedling survival observed in the 1975 contaminated sample, together with the toxic effects of crude oil components. In addition, the intervention of the microbial component in the ground that supports seedling development is also influential in the ability of the seedling to grow into a mature plant (Anoliefo and Vwioko1995; Kummerova et al. 2008).

The results demonstrate that different species respond differently to oil pollution. Cultivated plants did not germinate at all (pepper and tomato) or at a rate of only a few percent (cucumber) in soils contaminated in 2014, but germinated with a delay of several days in soils polluted 40 years ago. Wild flora that were examined showed a different pattern, where *Rumex roseus L*. did not germinate at all in soils contaminated in 2014 and 1975, while *Malva parviflora L*. and *Astragalus remophilus Boiss.* sprouted only in soils that suffered from the 1975 spill (apart from one Astragalus seedling that did germinate in the area polluted in 2014 but did not survive longer than one day). In contrast, *Acacia raddiana* germinated in areas polluted in 2014 and 1975.

Many studies show that species plays a role in the sensitivity of a plant to hydrocarbon pollution, where different species of the same taxonomical family will, for the most part, respond in the same way, and that the weight and size of the seed, its morphology, and its anatomic structure are influential (Sharonova and Breus 2012). In this study, the different patterns of sensitivity in the germination ability of the wild plants reflect the findings of seed coat anatomy as well as seed size. Seeds with macrosclereids cells in the seed coat layers (*Acacia raddiana*, *Malva*

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parviflora L. and *Astragalus remophilus Boiss*) were more tolerant to the 1975 soil pollution, whereas the absence of this layer, as was found in the seeds of *Rumex roseus L*., made the seeds more sensitive to the oil.

Macrosclereid cells form the outer seed coat layer of physiologically dormant seeds and prevent water intake. These cells are not alive and are characterized by an extensive secondary wall formation that make the seed impervious to water (Rolston 1978). The physiologically dormant seeds are not sealed only against water. In this case, the mechanism also effectively prevented oil penetration through the outer layer of the seed.

Acacia raddiana, *Malva parviflora L*. and *Astragalus remophilus Boiss* were treated pre-sowing by mechanical scarification of the seed coat by chipping. We assumed that the wound in the seed coat was specific to a small point and most of the seed coat was still sealed, thus most probably allowing only slight oil penetration. However, no pre-sowing treatment was needed for *Rumex roseus L*. seeds because water can penetrate its seed coat; oil, too probably, penetrates its seed coat, severely injuring the seeds' ability to germinate. The seed coat anatomy of the physiologically dormant seeds is thicker in the *Acacia raddiana* seeds and somewhat similar for *Malva parviflora L*. and *Astragalus remophilus Boiss*. These findings are consistent with the ability of *Acacia raddiana* to germinate in the 2014 polluted soil, probably also due to its larger dimensions as well as thicker seed coat. As was demonstrated by Robson et al. (2004), large seed mass is correlated with tolerance to hydrocarbon-contamination in soil. In the current study, the low surface-area-to-volume ratio of *Acacia raddiana* seeds compared to the other tested seeds, decreased its exposure to the oil in the soil and thus increased its ability to germinate with lower injury to the seeds than what was observed in other species.

Furthermore, pollution does not only affect seed germination. As our results suggest, and as shown by Achuba(2006), oil pollution also affects other processes in plant growth and development, such as cell division and enzyme activity. The significantly short length of the hypocotyl in plants that grew in contaminated soils, which generates dwarfed and underdeveloped plants, is most probably the result of the damage to cell division, a cessation of mitotic activity and a reduction in cell size, as reported by Achuba. A decrease in the number of leaves that the plant develops following exposure to oil is caused by native cell division and damage to the normal enzymatic activity necessary for the proper development of the growing plant. In addition, the toxicity of the oil is linked with a decrease in the amounts of phosphorus and nitrogen available to plants (Benka-Coker and Ekundayo 1995) and interferes with the chemotaxis in the ground (Rosenberg et al. 1993).

It is clear, therefore, that the most recent oil spill prevents the vast majority of seeds from germinating and those that do manage to germinate in the oil-polluted channels do not survive

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for long. The ground still contains remnants of the 1975 spill and considerably reduces both the rate of germination and the capacity for survival and development of seedlings growing in the polluted channels. An ecosystem with vegetation that cannot regenerate eventually will be severely disrupted, since the entire composition and diversity of the community depend on the germination ability of seeds and survival of seedlings. Seedlings, especially in arid environments, play a significant role in the formation and maintenance of spatial heterogeneity and survive better in resource-rich patches than in open spaces (Boeken2008). Most vegetation in hyper-arid zones, like the Arava valley, is directly linked to rainfall and water redistribution through active stream channels (Jacoby et al. 2006; Shmida 1985). In both the 1975 and 2014 spills, the oil flowed into the reserve via above ground water sources, which are the resource-rich area of the reserve. An observation and monitoring study, conducted by Nothers et al. (in preparation-personal communication), in the same area clearly shows that only large acacia trees are found in the area of the 1975 oil spill. Their results clearly demonstrate that the existing mature acacia trees were probably not affected by the oil, mainly due to their deep root systems that did not come in direct contact with the contaminated surface. In contrast, new seedlings that were germinating in the oil did not develop into mature trees and, for this reason, trees smaller than 40 cm are not found in this area. The effect of oil on the germinating seedlings, as shown in the current study, persists even 40 years after an oil spill. In our study, both the 1975 and 2014 contaminated soils resulted in short plants with fewer leaves and reflect observations made in the field.

Vegetation native to the Arava and Evrona Nature Reserve has found it difficult to adapt to pollution. The *Acacia raddiana*, among the most important plants in this area, cannot germinate and develop properly in the contaminated soil, while plants grow significantly fewer leaves, are dwarfed, and do not survive for long. A plant with few leaves will be unable to perform long-term, substantial and quality photosynthesis and, therefore, probably will not survive. Other plant species that were investigated in this study failed to develop and survive. We must continue to examine the ability of plants to survive in these areas over the longer-term to learn how long before the effects of oil contamination dissipate and whether in soil without volatile fractions, as was the case in the 1975 oil spill, the abnormal seedlings (dwarfed with few leaves) will survive over time. In addition, the channels through which the oil flowed should be examined to determine plant dynamics, while changes in the nature of plant populations in contaminated and uncontaminated areas should be observed and compared. Any actions by the authorities to rehabilitate the reserve should take into consideration the results of this study, which clearly indicatethat the pollution in this arid environment inhibits the recovery of the native flora over time. Furthermore, prior to any rehabilitation actions, the bioassay conducted in this study should be considered to understand how to best help directly affected organisms.

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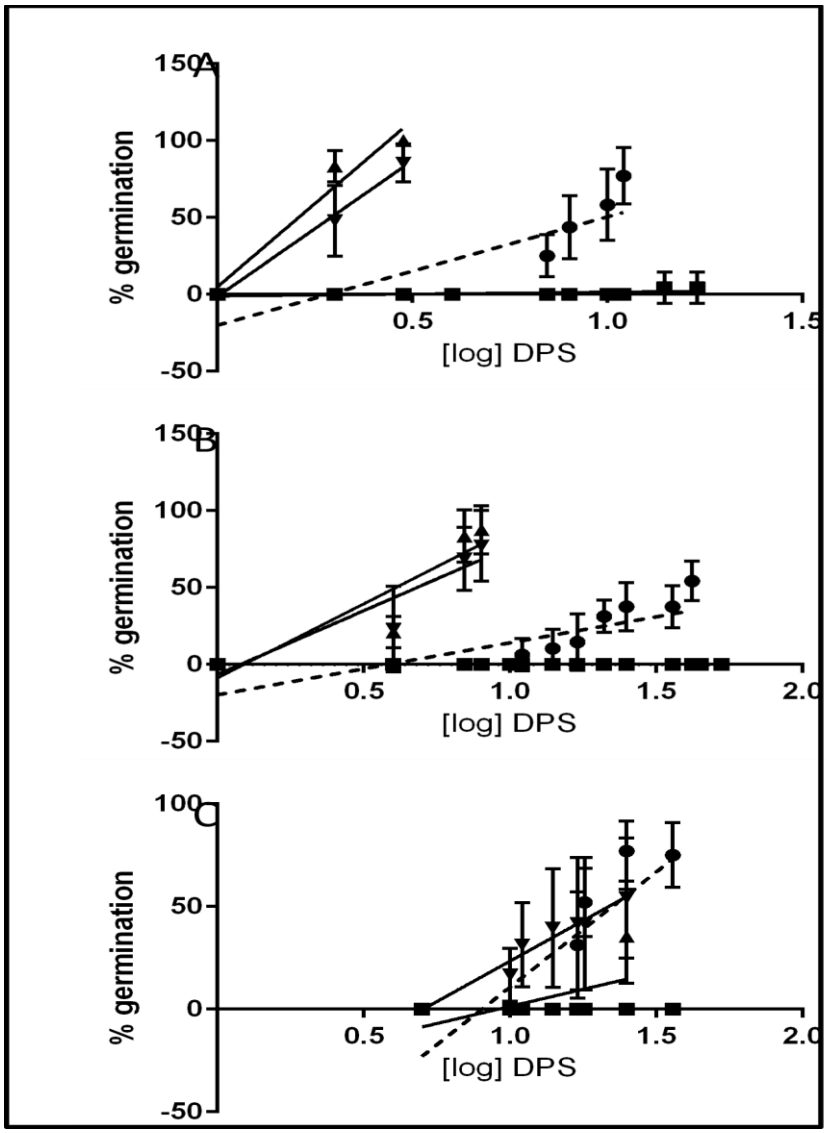
LEGENDS:

Online Resource1: Germination ability percentages for (A) Cucumber-*(Cucumis sativus*), (B) Tomato-*(Solanum lycopersicum*) and (C) Pepper- (*Capsicum annuum*) in Ctrl 2014(solid line with upright triangles), contamination 2014 (dotted line with squares), Ctrl 1975 (solid line with downward-facing triangles), and contamination 1975 (dotted line with circles) plots. Mean and SE of percentages were plotted against the log of DPS and a linear regression analysis was performed.



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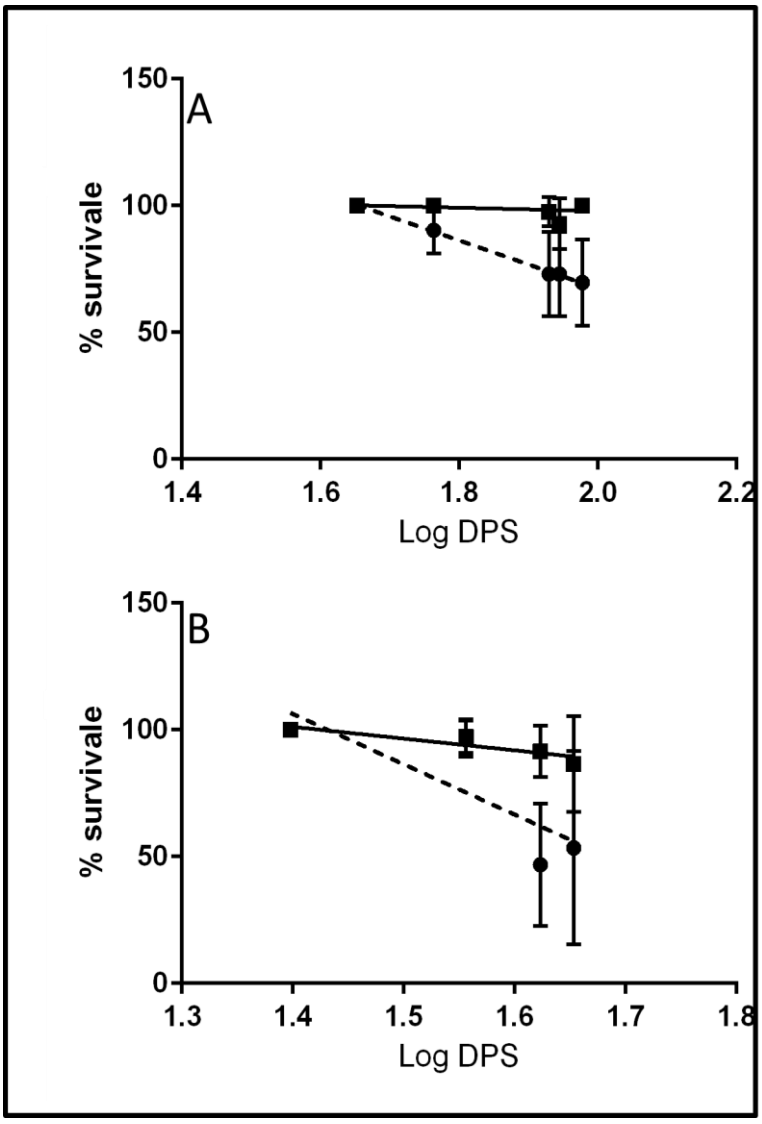
Online Resource2: Survival rate percentages for (A) Tomato-*(Solanum lycopersicum*) and (B) Pepper-
(*Capsicum annuum*) in Ctrl 1975 (solid line with squares), and contamination 1975 (dotted line with circles) plots. Mean and SE of percentages were plotted against the log of DPS and a linear regression analysis was performed.



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Online Resource3: Seedling height mean and SEfor (A) Cucumber-*(Cucumis sativus*), (B) Tomato-*(Solanum lycopersicum*) and (C) Pepper-(*Capsicum annuum*). Different letters indicate statistically significant differences between the means of the different experimental plots by one-way ANOVA with Tukey-Kramer multiple comparison test (A) or t-test (B, C).

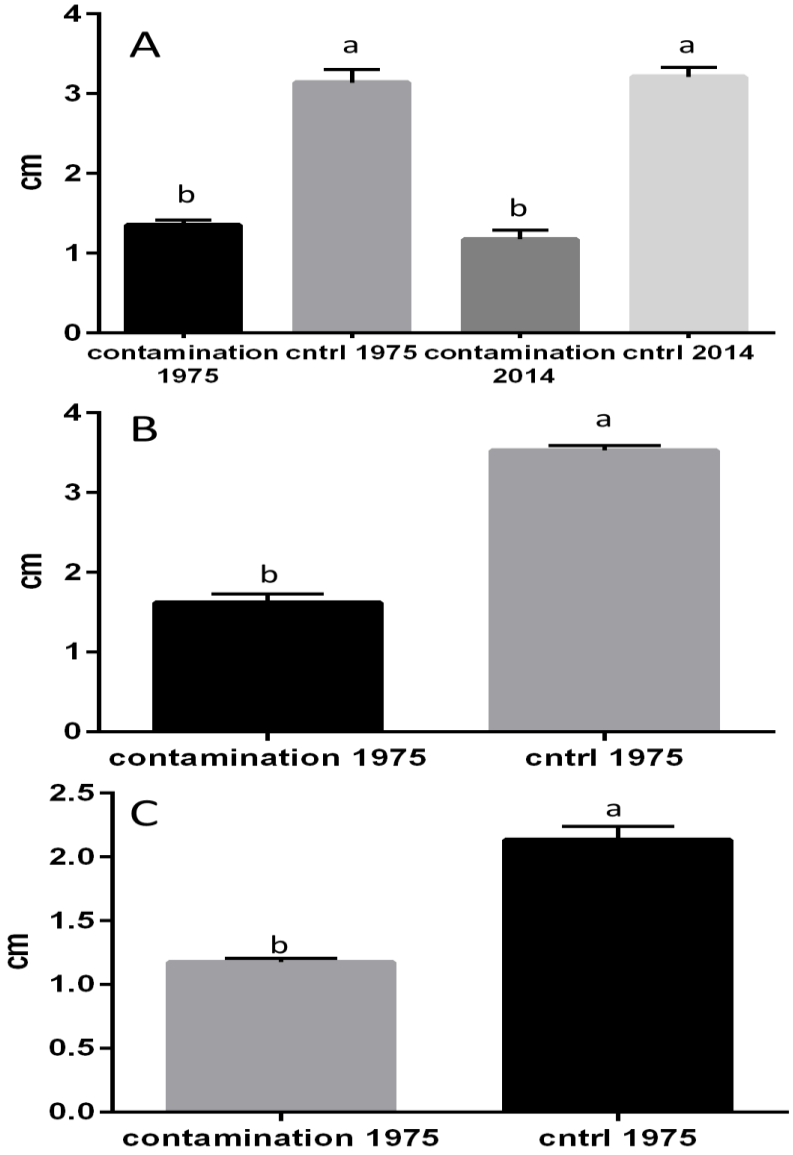

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Fig 1: Germination ability percentages for (A) *Acacia raddiana*, (B) *Astragalus eremophilus Boiss.*,(C) *Malva parviflora L,* and (D) *Rumex cyprius Murb.* in Ctrl 2014 (solid line with upright triangles), contamination 2014 (dotted line with squares), Ctrl 1975 (solid line with downward-facing triangles),and contamination 1975 (dotted line with circles) plots. Mean and SE of percentages were plotted against the log of DPS and a linear regression analysis was performed.

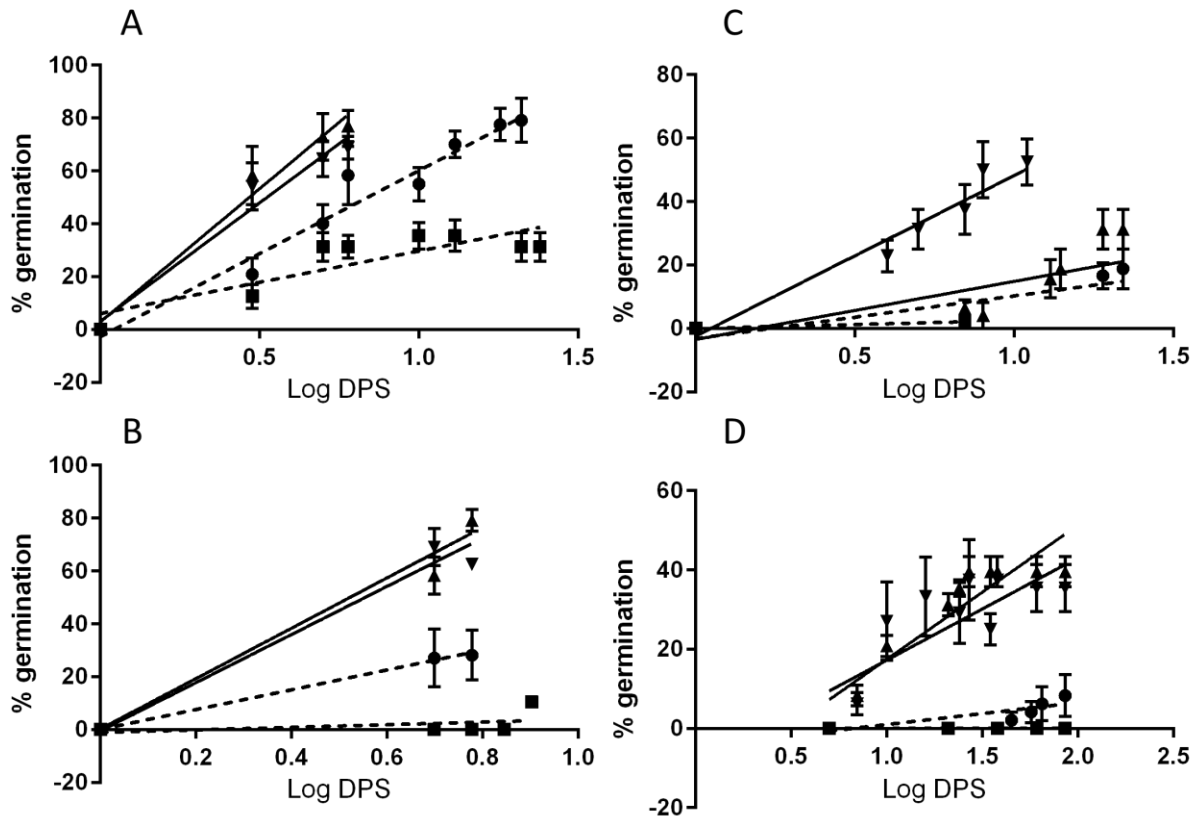

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Fig 2: Survival rate percentages for (A) *Acacia raddiana*, (B) *Astragalus eremophilus Boiss* and (C) *Malva parviflora L* in Ctrl 1975 (solid line with squares), and contamination 1975 (dotted line with circles) plots. Mean and SE of percentages were plotted against the log of DPS and a linear regression analysis was performed.

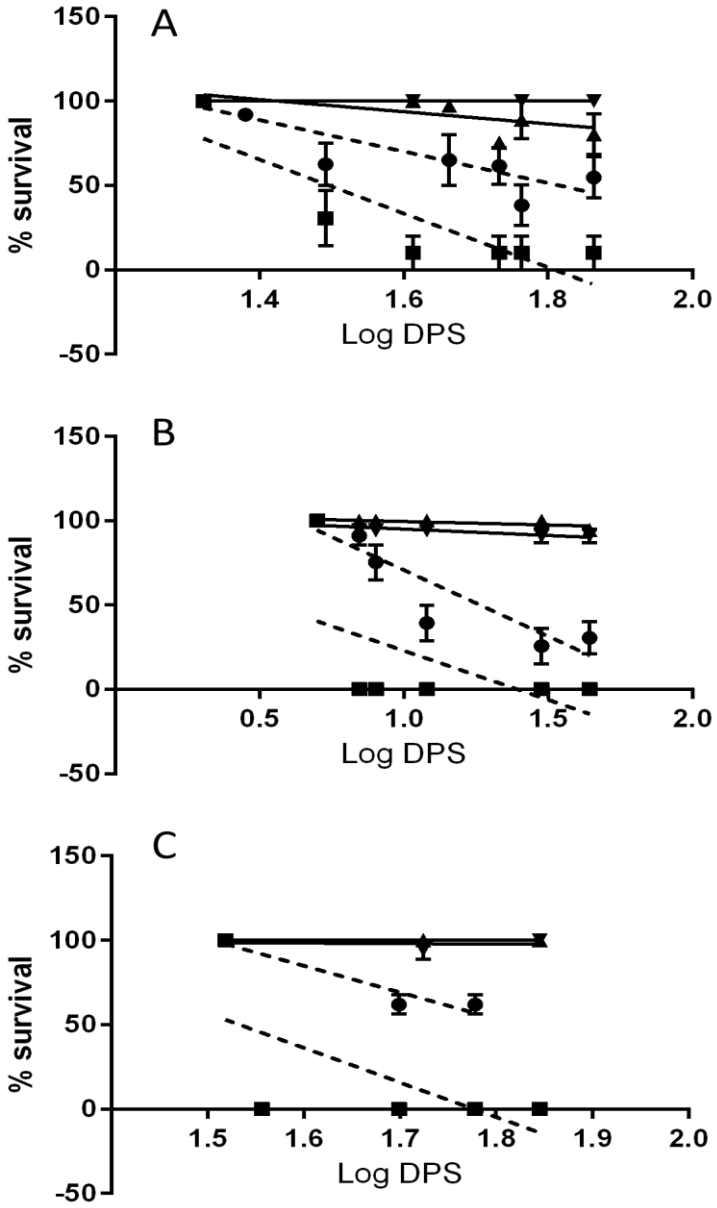

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Fig 3: Mean number and SE of leaves per plant for(A) *Acacia raddiana*, (B) *Astragalus eremophilus Boiss.* and (C) *Malva parviflora*. Different letters indicate statistically significant differences between the means of the different experimental plots by one-way ANOVA with Tukey-Kramer multiple comparison test (A) or t-test (B, C).

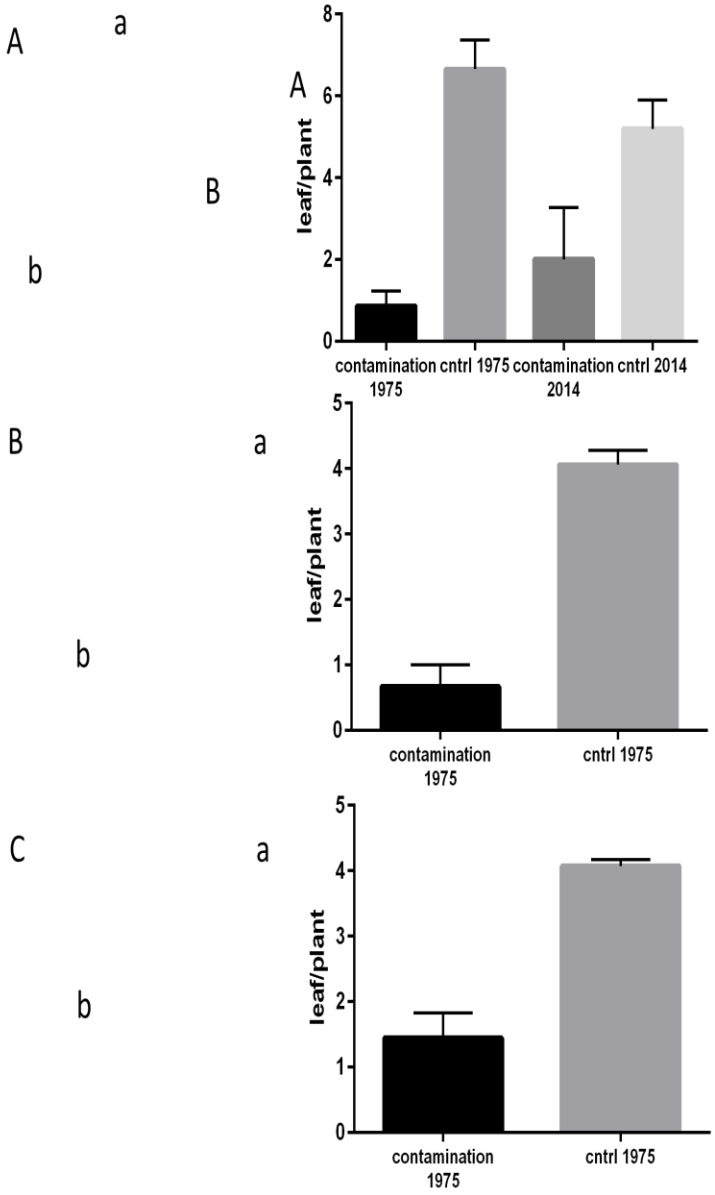

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Fig 4: Mean height and SE of two-month old *Acacia raddiana* seedling. Different letters indicate statistically significant differences between means for the different experimental plots (i.e. 2014 spill or 1975 spill) by one-way ANOVA using t-test pair comparison.

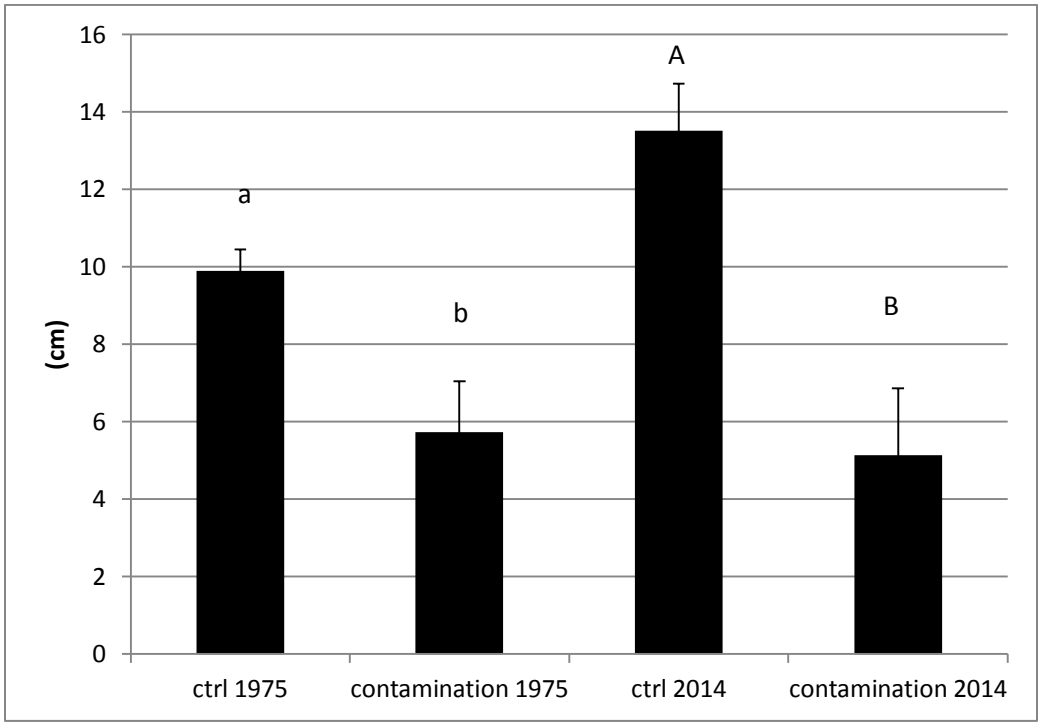

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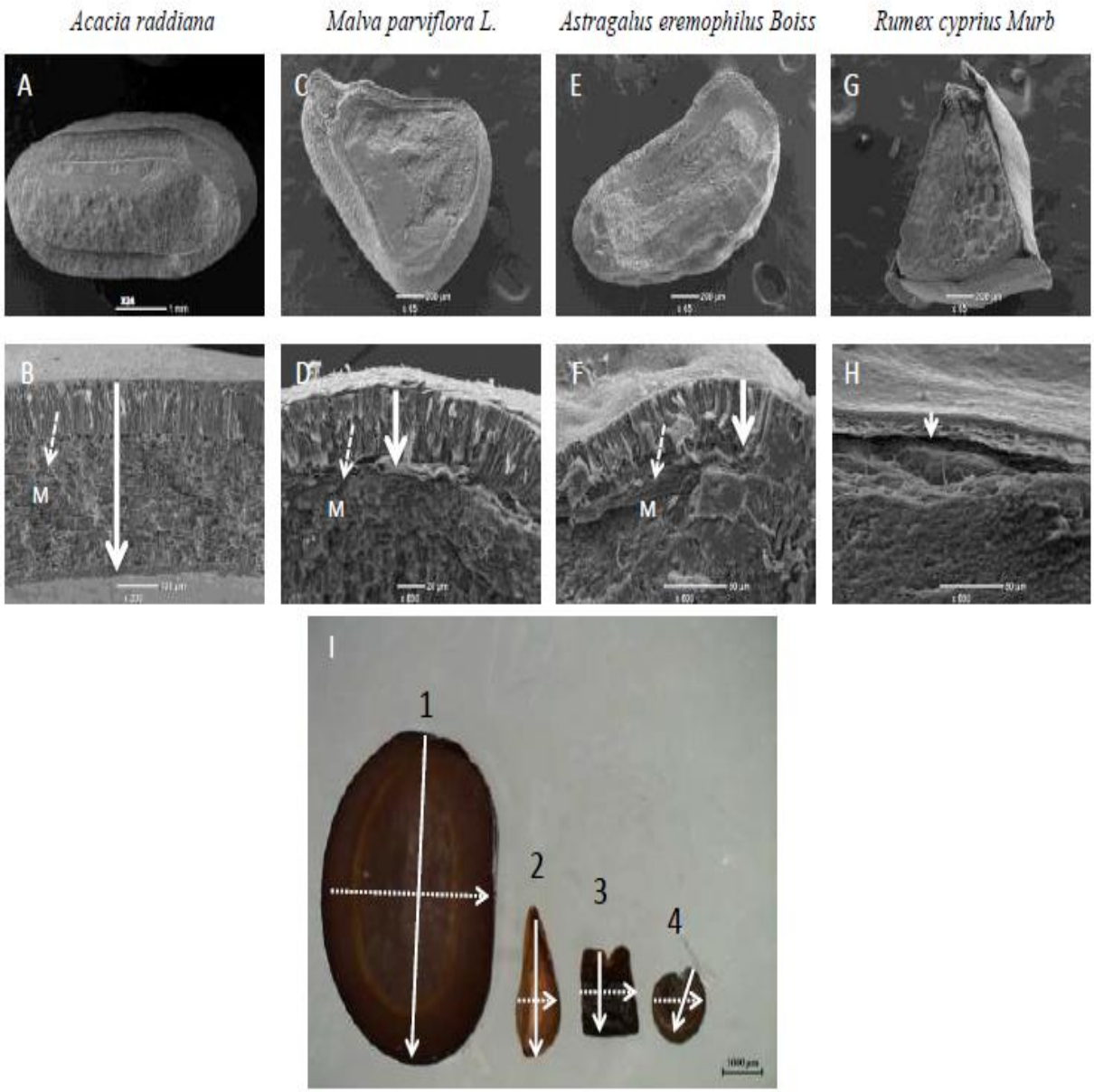
Fig 5: Seed coat thickness of (A,B) *Acacia raddiana*; (C,D) *Malva parviflora*; (E,F) *Astragalus eremophilus Boiss*.; and (G,H) *Rumex cyprius Murb*. using SEM microscopy. Overall view of transverse sections show the seed coat and inner seed parts (A,C,E,G) detailed view shows seed coat thickness; the green arrow indicates the measured area (B,D,F,H). Stereomicroscope image (I) of Acacia raddiana (1) Rumex cyprius Murb.(2) Astragalus eremophilus Boiss. (3) and Malva parviflora (4), where the solid line shows seed length and the dotted line shows seed width.



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