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SCREENING ALFALFA "*MEDICAGO SATIVA* L." GENOTYPES FOR PETROLEUM CONTAMINATION TOLERANCE; AN APPROACH TO DEVELOP A STRESS TOLERANT GENOTYPE

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ABSTRACT: This recent study aimed to evaluate the impact of petroleum-contaminated soil on the growth and productivity of seven alfalfa (Medicago sativa L.) genotypes. Three levels of petroleum contamination were used (zero, 40, and 60ml.kg^{-1}). A range of morphological and physiological traits were assessed, including germination percentage, tolerance index, chlorophyll content, plant height, dry weight, and survival rate. The obtained results indicated that petroleum stress significantly reduced most measured traits, with the highest impact observed at the 60 ml kg^{-1} level. Tolerance index and chlorophyll content were reliable indicators of genotype resilience. A strong genotype × environment interaction was observed due to a significant interaction between genotype and the level of contamination. However, genotypes such as "Rammah" and "Australian" presented a notable resilience to petroleum contamination, since they maintained a relatively higher performance under stress. Therefore, it was assumed that these two genotypes might be utilized in selective breeding for phytoremediation and soil restoration programs.

Keywords: Alfalfa (*Medicago sativa* L.) genotypes, Petroleum contamination, Tolerance index, chlorophyll content, genetic variability.

INTRODUCTION

Many crop species are less resilient to petroleum contamination. That is expressed by poor germination, reduced shoot, and root growth, and suppressed biomass (Adam and Duncan, 2002, and Ali *et al.*, 2013).

Contamination with petroleum hydrocarbons is a serious problem worldwide, especially in regions with petroleum production and/or refining (EPA, 2020). That type of pollution is harming agricultural productivity and environmental sustainability in North Africa. The accumulation of petroleum residues matches with its persistent nature that results in lowering soil-water-holding capacity, raising soil salinity, limiting nutrients availability, leading to disturbed crop-plants growth (Adam & Duncan, 2002; Song *et al*., 2004; Afzal *et al*, 2011; Ali *et al*., 2013; and Ahmed *et al*., 2025).

Members of the legume family have proven to be promising species with good resilience to environmental stresses, including soil and water salinity, drought, and heavy metal contamination (Ahmed *et al*., 2025). Alfalfa, "Medicago Sativa, L.," is the most common forage legume in temperate and tropical regions of the world. It was proposed as an efficient Phytoremediator because of its perennial habit, deep-rooting system, and good ability to fix nitrogen (Zhou & Song, 2004; Radovic *et al*., 2009; Malik *et al*., 2019; Simeonova & Georgieva, 2002; Jiang *et al*., 2021).

Genotypes of alfalfa respond differently to petroleum contamination effects depending on genetic, consequently physiological differences (Zhou and Song, 2004, and Simeonova and Georgieva, 2020). Although the potential of alfalfa to resist petroleum pollution is well

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recognized, variations among available genotypes remain poorly understood or underutilized in breeding programs. The number of studies aimed to enhance petroleum contamination tolerance in forage legumes generally or in alfalfa specifically is still very limited. So, breeding alfalfa for petroleum contamination resilience might empower its use for phytoremediation and sustainable cultivation in petroleum-polluted soil.

Many approaches have been used to improve alfalfa sustainability under stress. Screening under greenhouse or controlled field conditions represented a direct approach under the target environment (Malik *et al*., 2019). Recurrent selection within tolerant genotypes, due to alfalfa's nature as a heterozygous and outcrossing crop, is successful in breeding drought- and salinity-tolerant cultivars (Radovic *et al*., 2009). The modern method of selection, depending on maker-assistance, has not been used for improving petroleum contamination tolerance (Kumar & Singh, 2022).

Breeding alfalfa for petroleum tolerance is of valuable importance in regions with recent or potential petroleum contamination hazards. The first step in such a program should focus on screening germplasm phenotypically for resistance to petroleum contamination. The second step might be selecting individuals within elite genotypes to generate a cross-pollinated population among tolerant individuals. This approach can develop a petroleum-contamination-tolerant genotype suitable for such polluted soil or similar stresses.

The aims of this recent study were to screen the available alfalfa germplasm as a prime step toward developing a petroleum contamination-tolerant genotype.

MATERIALS AND METHODS

A greenhouse experiment was conducted to screen alfalfa “Medicago sativa L.” genotypes under petroleum-contamination conditions. Seed of alfalfa genotypes was obtained from the forage research section, Field Crops Research Institute, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt.

Soil preparation

Sandy soil was sieved by a 20-mesh sieve to exclude particles other than sand. The sieved sand was washed three times with distilled water before being mixed with crude petroleum. Three rates of contamination represented by control (no petroleum was added), medium (40 $mL.kg^{-1}$), and high (60 $mL.kg^{-1}$) were added to two-kilogram pots filled with prepared sand. Petroleum was completely mixed with sand before seeding the proposed alfalfa genotypes.

Screened alfalfa genotypes

The screened alfalfa genotypes included: Nubaria 1, Rammah, Wahat, Ismaelia 1, Farafra, Hasawii, and Australian. Sowing started on September 15th, 2023, with ten seeds per pot. Plants were allowed to grow through vegetative and flowering stages. The same treatments were repeated on September 10^{th}, 2024. The average of measured characters across the two experiments was used for statistical analysis.

Statistical design

A factorial combination of three levels of contamination and seven genotypes of alfalfa was tested in a randomized complete block design with eight replicates. Each character was scored from four replicates to ensure enough plants for each measurement.

Measured characters

Data were recorded for germination percentage after 14 days from sowing for all replicates, and then the most uniform four replicates were used for statistical analysis. Tolerance index (T.I.) was estimated for each genotype as the difference in germination percentage between values obtained under any of the studied pollution levels and the value obtained under no pollution (control). After 30 days from sowing, three seedlings were uprooted from four replications, to determine shoot and root length as well as fresh weights. Samples were dried at 70 °C until weight constancy, to estimate the dry weight of the shoot and root. Total produced biomass was estimated as the sum of the dry weight of the shoot and root for each plant. The average of the three plants was used for statistical analysis. Seeding vigor index (SVI) was estimated by multiplying shoot dry weight × shoot length divided by hundred.

Biomass reduction was estimated by subtracting the total biomass value obtained in each replicate under any of the pollution levels from the respective value obtained under the zero-pollution level (control), expressed as a percentage of the control value. Chlorophyll content was measured by the SPAAD-502 apparatus 60 days after sowing. After 90 days, the number of surviving plants was used to estimate the plant survival rate (PSR) based on the number of plants exposed to contamination.

Data were analyzed using Mstat-c v. 20 software (Mstat-c, Miteligen, USA). Numerical data were transformed before analysis. Differences among means were compared using the least significant difference (LSD) test at the appropriate level of significance.

RESULT AND DISCUSSION

Germination percentage and Tolerance index

Table 1 shows the mean squares of germination percentage and tolerance index as affected by petroleum contamination level, alfalfa genotype, and their interaction. Data reveal significant (P≥ 0.01) effects of petroleum contamination level, alfalfa genotypes, and their interaction on both characters.

Table 1: Mean squares of germination percentage and tolerance index as affected by petroleum contamination level, alfalfa genotypes, and their interaction.

Source of variations	d.f.	Mean squares	
		Germination percentage %	Tolerance index
Reps	3	0.080 ^{NS}	1.476 ^{NS}
Contamination Level (A)	2	10.74**	774.5 **
Genotypes (B)	6	1.669**	0.014 **
A×B	12	0.554**	0.005 **
Error	60	0.105	2.068

* and **; indicates significance at 0.05 and 0.01 levels, respectively.

NS; not significantly different.

Means of germination percentage as affected by petroleum contamination levels, alfalfa genotypes, and their interaction are presented in Table 2. Overall, germination percentage had declined with each increase in contamination level. The highest germination percentage was recorded under uncontaminated control treatment (85.14%), while the high level of contamination (60 $mL.kg^{-1}$) gave the least value of germination percentage (64.14%). The “Rammah” genotype exhibited the highest germination percentage (85.25%) across all examined levels of petroleum contamination. This value closely resembled that of the “Australian” genotype (82.58%). In the meantime, the “Hasawii” genotype showed the least significant germination percentage (63.17%). The interaction between genotypes and contamination level proved that both “Rammah” and “Australian” genotypes consistently outperformed the other studied alfalfa genotypes under each of the studied contamination levels.

Table 2: Means of germination percentage as affected by petroleum contamination levels, alfalfa genotypes, and their interaction.

Genotypes	Contamination levels			Mean
	Control (zero)	**Moderate (40 mL.kg^{-1})**	**High (60 mL.kg^{-1})**	
Nubaria 1	84.50	75.75	55.25	71.83
Rammah	91.75	84.25	79.75	85.25
Wahat	80.50	71.00	61.50	71.00
Ismaelia 1	82.00	73.50	66.00	73.83
Farafra	86.75	62.75	57.00	68.83
Hasawii	81.25	56.25	52.00	63.17
Australian	89.25	81.00	77.50	82.58
Mean	85.14	72.07	64.14	

L.S.D$_{0.01}$ (Genotypes) = 0.35
L.S.D$_{0.01}$ (Contamination levels) = 0.23
L.S.D$_{0.01}$ (Gen ×cont. interaction) = 0.61

The tolerance index that relates the germination percentage of each studied alfalfa genotype under each contamination level to the respective value under uncontaminated conditions was summarized in Table 3. Generally, overall, the tolerance index values of the studied alfalfa genotypes were significantly reduced with each increase in petroleum contamination level (100, 84.60, and 72.50% for uncontaminated, 40 mL.kg^{-1}, and 60 mL.kg^{-1}, respectively). Highly adapted genotypes of alfalfa exhibited the highest significant values of tolerance index compared to contamination levels. Those were represented by "Rammah" and "Australian" genotypes (92.93 and 92.77, respectively). The aforementioned genotypes expressed the highest significant tolerance indices under each studied contamination level, significantly surpassing the other studied genotypes. The most responsive genotype to contamination was the Hasawi genotype with the least tolerance index values under each studied contamination level, and overall, the studied contamination levels (69.00, 44.00, and 71.00% under 40 mL.kg^{-1}, 60 mL.kg^{-1}, and overall contamination levels, respectively).

Table 3: Means of tolerance index as affected by petroleum contamination levels, alfalfa genotypes, and their interaction.

Genotypes	Contamination levels			Mean
	Light (zero)	**Moderate (40 mL.kg^{-1})**	**High (60 mL.kg^{-1})**	
Nubaria 1	100	89.80	66.30	85.37
Rammah	100	91.80	87.00	92.93
Wahat	100	88.30	76.50	88.27
Ismaelia 1	100	89.80	80.50	90.10
Farafra	100	72.80	66.00	79.60
Hasawii	100	69.00	44.00	71.00
Australian	100	91.00	87.33	92.77
Mean	100	84.60	72.50	

L.S.D$_{0.01}$ (Genotypes) = 1.56
L.S.D$_{0.01}$ (Contamination levels) = 1.02
L.S.D$_{0.01}$ (Gen ×cont. interaction) = 2.70

The revised literature indicated a strong adverse effect of petroleum contamination on germination percentage as well as tolerance index (Adam & Duncan, 2002). Such a reference explained the decrease in germination percentage of alfalfa due to petroleum contamination because of the physical coating of seeds that reduced oxygen availability, besides the toxic effect of hydrocarbons on embryonic tissue. Genetic diversity among alfalfa genotypes might be responsible for variation in the stress response to the petroleum contamination mechanism. Cheema *et al.* (2009) and Chaimeau *et al.* (1997) explained the superiority of alfalfa genotypes under stress (Likely petroleum contamination) to enhanced physiological tolerance, seed coat permeability, and root emergence vigor. Rezek *et al* (2008) supposed that significant cultivar × contamination level interaction indicates that genotypic response to petroleum pollution is not uniform, so that there is a need for genotype-specific identification to be used for phytoremediation in petroleum contaminated environments (Oliveira *et al*, 2009).

Depending on the recent result, “Rammah” and “Australian” alfalfa genotypes might be proposed for cultivation in petroleum-contaminated soils, depending on their high germination percentage and tolerance index values. Such genotypes represent a good potential for use in phytoremediation programs.

Growth parameters

After 30 days from sowing, shoot length, and dry weight of shoot and root were measured and/or estimated. Table 4 showed the mean squares of shoot length, shoot dry weight, and root dry weight as affected by petroleum contamination level, alfalfa genotype, and their interaction. All three measured characters were significantly (P≥ 0.01) affected by contamination level and alfalfa genotype. Also, the interaction between contamination level and alfalfa genotype was significant for all three traits.

Table 4: Mean squares of shoot length, shoot dry weight, and root dry weight as affected by petroleum contamination level, alfalfa genotype, and their interaction.

Source of variations	d.f.	Mean squares		
		Shoot length	Shoot dry weight	Root dry weight
Reps	3	37.36**	7.775**	1.547**
Contamination Level (A)	2	624.7**	13.57**	8.157**
Genotypes (B)	6	484.7**	5.677**	6.485**
A×B	12	5.735**	0.162*	0.087**
Error	60	0.460	0.071	0.007

* and **; indicates significance at 0.05 and 0.01 levels, respectively.
NS; not significantly different.

Petroleum contamination significantly reduced shoot length in all of the alfalfa genotypes studied (Table 5). The average of the studied alfalfa genotypes showed a 48.07% reduction in shoot length at high levels of petroleum contamination. Across all the studied levels of contamination, “Rammah” (23.63 cm) and “Australian” (23.31cm) genotypes had enjoyed the tallest shoots. Additionally, the “Hasawii” genotype exhibited the shortest shoot length (9.185 cm). That value was not significantly different from values presented by the “Farafra” genotype (9.674 cm). Significantly moderate values of shoot length were presented by “Nubaria” (13.15 cm) and “Wahat” (11.73 cm) genotypes. As for the interaction between genotype and contamination level, two genotypes (“Rammah” and “Australian”) significantly maintained the tallest shoot under a high level of contamination (21.25 and 19.55 cm for the former and the latter, respectively).

Table 5: Means of shoot length (cm) as affected by petroleum contamination levels, alfalfa genotypes, and their interaction.

Genotypes	Contamination levels			Mean
	Control (zero)	Moderate (40 mL.kg^{-1})	High (60 mL.kg^{-1})	
Nubaria 1	17.63	13.44	8.385	13.15
Rammah	26.75	22.88	21.25	23.63
Wahat	16.04	12.04	7.005	11.73
Ismaelia 1	16.09	8.883	5.013	9.995
Farafra	16.03	8.343	4.648	9.674
Hasawii	15.03	7.673	4.853	9.185
Australian	28.56	21.83	19.55	23.31
Mean	19.45	13.58	10.10	

L.S.D$_{0.01}$ (Genotypes) = 0.74
L.S.D$_{0.01}$ (Contamination levels) = 0.48
L.S.D$_{0.01}$ (Gen ×cont. interaction) = 1.28

Shoot dry weight (g) was significantly lower across the studied alfalfa genotypes, with higher levels of petroleum contamination (Table 6). A significant reduction of 21.18% was recorded under a moderate level of contamination (from 3.234 to 2.549 g); meanwhile, the significant reduction in shoot dry weight reached 27.78% under the high level of contamination (from 2.549 to 1.841 g). Again, both of “Rammah” and “Australian” genotypes significantly presented the heaviest dry shoots (3.538 and 3.492 g for the former and the latter, respectively). Meanwhile, “Hasawii” had presented the least significant dry shoot (1.798 g). Also, significantly, the heaviest dry shoot was presented by any of “Rammah” or “Australian” alfalfa genotypes, under any of the studied contamination levels. The previous results highlighted genotypic variability among the tested alfalfa genotypes, as well as expressed the impact of petroleum contamination on plant growth.

Table 6: Means of shoot dry weight (g) as affected by petroleum contamination levels, alfalfa genotypes, and their interaction.

Genotypes	Contamination levels			Mean
	Control (zero)	Moderate (40mL.kg^{-1})	High (60 mL.kg^{-1})	
Nubaria 1	3.073	2.265	1.728	2.355
Rammah	3.928	3.755	2.930	3.538
Wahat	2.900	1.993	1.483	2.125
Ismaelia 1	3.000	2.198	1.575	2.258
Farafra	3.253	2.120	1.301	2.225
Hasawii	2.548	1.805	1.040	1.798
Australian	3.935	3.708	2.833	3.492
Mean	3.234	2.549	1.841	

L.S.D$_{0.01}$ (Genotypes) = 0.29
L.S.D$_{0.01}$ (Contamination levels) = 0.19
L.S.D$_{0.05}$ (Gen ×cont. interaction) = 0.38

The root dry weight (g) of all alfalfa genotypes in the studies exposed to moderate levels of petroleum contamination (40 mL.kg^{-1}) decreased by 20.10%, from 2.617 to 2.091 g. (Table 7). At the same time, the rate of substantial reduction in root dry weight increased to 26.45% when alfalfa genotypes were exposed to a high level of petroleum contamination (60 mL.kg^{-1}) (from 2.091 to 1.539 g). The "Rammah" genotype also exhibited the heaviest root (3.197 g) over contamination levels among the genotypes that were studied. The second significant rank of contamination tolerance was occupied by the "Australian" genotype (2.971 g). The third rank was that of "Nubaria" genotype. Meanwhile, other studied genotypes had significantly limited dry root. It was worth noting that the "Rammah" genotype had the heaviest dry root under any of the studied petroleum contamination levels. Additionally, the "Hasawii" genotype produced the least root dry weight under all studied contamination levels.

Table 7: Means of root dry weight (g) as affected by petroleum contamination levels, alfalfa genotypes, and their interaction.

Genotypes	Contamination levels			Means
	Control (zero)	Moderate (40 mL.kg^{-1})	High (60 mL.kg^{-1})	
Nubaria 1	2.528	2.2	1.575	2.101
Rammah	3.555	3.198	2.838	3.197
Wahat	2.353	1.643	0.964	1.653
Ismaelia 1	2.473	1.815	1.160	1.816
Farafra	2.278	1.598	0.929	1.602
Hasawii	1.705	1.31	0.681	1.232
Australian	3.428	2.87	2.616	2.971
Means	2.617	2.091	1.538	

L.S.D$_{0.01}$ (Genotypes) = 0.1
L.S.D$_{0.01}$ (Contamination levels) = 0.1
L.S.D$_{0.01}$ (Gen ×cont. interaction) = 0.16

Reduction in shoot length and dry weight of root proved the significant suppression of petroleum contamination on the growth of alfalfa seedlings. The recent findings are in accordance with the results reported by Adam and Duncan (2002) and Cheema *et al.* (2009), who published that hydrocarbons interfere with root development, cell division, and nutrient absorption.

"Rammah" and "Australian" genotypes demonstrated remarkable tolerance to petroleum contamination, which may be attributed to their robust antioxidant responses and efficient allocation of energy to roots and shoots under stress conditions. These results are supported by the findings of Rezek *et al.* (2008) and Chaineau *et al.* (1997), who emphasized the importance of cultivar selection in phytoremediation programs.

Significant interaction effects (Genotype × petroleum contamination level) across all growth parameters confirm that the genotypic response to petroleum contamination is complex and treatment-specific, emphasizing the importance of screening genotypes under petroleum contamination conditions.

Total biomass

Mean squares of total biomass and biomass reduction as affected by petroleum contamination level, alfalfa cultivar, and their interaction are presented in Table 8. Total alfalfa plant biomass differed significantly (P≥ 0.01) with different levels of petroleum contamination.

Additionally, genotypes had significantly different biomass responses to contamination levels (P≥ 0.01). However, there was no significant interaction between genotype and contamination level.

Table 8: Mean squares of total biomass and biomass reduction as affected by petroleum contamination level, alfalfa genotype, and their interaction.

Source of variations	d.f.	Mean squares	
		Total biomass	Biomass reduction
Reps	3	17.08 **	4.387 NS
Contamination Level (A)	2	48.42 **	83.61**
Genotypes (B)	6	20.22**	13.59**
A×B	12	0.390 NS	14.94**
Error	60	1.191	2.350

* And **; indicates significance at 0.05 and 0.01 levels, respectively.
NS; not significantly different

The total biomass (g) (Table 9) of the studied genotypes was reduced by 10.85% (from 5.205 to 4.640 g) when the contamination level increased to 40 $mL.kg^{-1}$. While the magnitude of reduction reached 11.85% (from 4.640 to 2.698 g) when the contamination level reached 60 $mL.kg^{-1}$. Over contamination levels, "Rammah" and "Australian" genotypes significantly produced the highest plant biomass (6.142 and 5.848 g, respectively). A significantly moderate plant biomass was presented by the "Nubaria" genotype (4.034 g). While the other studied alfalfa genotypes significantly produced lower biomass, with the lowest figure presented by the "Hasawii" genotype (2.764 g).

Table 9: Means of total biomass (g) as affected by petroleum contamination levels, alfalfa genotypes, and their interaction.

Genotypes	Contamination levels			Means
	Control (zero)	Moderate (40mL.kg^{-1})	High (60 mL.kg^{-1})	
Nubaria 1	5.010	4.465	2.628	4.034
Rammah	6.635	6.953	4.838	6.142
Wahat	4.690	3.635	1.862	3.396
Ismaelia 1	4.850	4.013	2.064	3.642
Farafra	4.935	3.718	1.665	3.439
Hasawii	3.768	3.115	1.410	2.764
Australian	6.548	6.578	4.419	5.848
Means	5.205	4.640	2.698	

$L.S.D_{0.01}$ (Genotypes) = 1.19
$L.S.D_{0.01}$ (Contamination levels) =0.78

The reduction in alfalfa plant biomass due to exposure to petroleum contamination, and the genotypic differences among genotypes, match the results reported by Adam and Duncan (2002) and Merkl *et al*. (2005), which showed that petroleum hydrocarbons decrease soil microbial activity that is related to nutrient availability to plants and reduce plant growth.

Mean magnitude of reduction in alfalfa biomass was significantly (P≥ 0.01) affected by petroleum contamination level, genotype, and their interaction. Under a moderate level of contamination, the plant biomass of the "Rammah" and "Australian" genotypes was improved by 4.79% and 0.45%, respectively (Table 10). While an overall genotype biomass reduction reached 10.83%, with a range starting with 55.21% biomass reduction for the "Hasawii" genotype to 10.08% reduction for the "Nubaria" genotype. Exposure to a high level of contamination resulted in a reduction of over 41.85% in the biomass of 41.85%. The least significant reduction in biomass under the high level of petroleum contamination was presented by any of "Rammah" or "Australian" genotypes (30.41 and 32.62%, respectively). While significantly maximum biomass reductions were recorded by any of "Farafra" or "Hasawii" genotypes (55.22 and 54.73%, respectively). It was worth noting that both "Rammah" and "Australian" genotypes expressed the least significant biomass reduction overall the studied petroleum contamination levels (8.540 and 10.72, respectively). Meanwhile, the most significantly sensitive alfalfa genotype was "Hasawii" with an overall biomass reduction of 36.64%.

Table 10: Mean of biomass reduction (%) as affected by petroleum contamination levels, alfalfa genotypes, and their interaction.

Genotypes	Contamination levels			Means
	Control (zero)	Moderate (40 mL.kg^{-1})	High (60 mL.kg^{-1})	
Nubaria 1	0.00	10.08	41.14	17.07
Rammah	0.00	+04.79	30.41	8.54
Wahat	0.00	22.50	48.77	23.75
Ismaelia 1	0.00	17.26	48.51	21.92
Farafra	0.00	24.66	55.22	26.62
Hasawii	0.00	55.21	54.73	36.64
Australian	0.00	+0.450	32.62	10.72
Means	0.00	10.85	41.85	

L.S.D$_{0.01}$ (Genotypes) = 1.7
L.S.D$_{0.01}$ (Contamination levels) = 1.1
L.S.D$_{0.01}$ (Gen ×cont. interaction) = 2.9

The recent results match those published by Coulon *et al.* (2010), which showed different sensitivities of genotypes to petroleum hydrocarbons. So, selecting genotypes with lower biomass reduction under contamination, such as "Rammah" and "Australian", might promote phytoremediation or cultivation in contaminated soil.

Chlorophyll content

The analysis of variance for chlorophyll content (Table 11) revealed a significant ($P\geq 0.01$) difference among contamination levels, alfalfa genotypes, and their interaction. This might indicate that chlorophyll content is a good indicator of contamination effects and genetic differences among affected genotypes.

Table 11: Mean squares of chlorophyll content as affected by petroleum contamination level, alfalfa genotype, and their interaction.

Source of variations	d.f.	Mean squares
Reps	3	57.10**
Contamination Level (A)	2	1284**
Genotypes (B)	6	295.6**
A×B	12	11.15**
Error	60	0.929

* And **; indicates significance at 0.05 and 0.01 levels, respectively.
NS; not significantly different.

Chlorophyll content (Table 12) had significantly decreased with higher levels of contamination overall than the tested alfalfa genotypes (38.08, 32.39, and 25.14 for no contamination, moderate, and high levels of contamination, respectively). Meanwhile, “Rammah” genotype significantly presented the highest chlorophyll content overall the studied contamination levels, followed by “Australian” genotype (39.58 and 38.42 for the former and the latter, respectively). Also, “Hasawii” genotype significantly expressed the least chlorophyll content overall contamination level (27.00). Under uncontaminated conditions, “Rammah” and “Australian” genotypes scored over 45 chlorophyll content, indicating strong growth and high content of chlorophyll, whereas other genotypes scored values over 30, indicating good growth, but require additional fertilizers. Under high levels of contamination (60 $mL.kg^{-1}$), all tested genotypes expressed low values of chlorophyll content (less than 30), except for “Rammah” and “Australian”, which were more tolerant to contamination (values of chlorophyll content over 30).

Table 12: Means of chlorophyll content as affected by petroleum contamination level, alfalfa genotypes, and their interaction.

Genotypes	Contamination levels			Mean
	Control (zero)	Moderate (40 $mL.kg^{-1}$)	High (60 $mL.kg^{-1}$)	
Nubaria 1	34.00	30.25	22.00	28.75
Rammah	45.75	38.50	34.50	39.58
Wahat	39.75	31.50	23.00	31.42
Ismaelia 1	39.25	31.25	21.75	30.75
Farafra	35.50	30.00	20.25	28.58
Hasawii	32.00	27.50	21.50	27.00
Australian	44.50	37.75	33.00	38.42
Mean	38.68	32.39	25.14	

$L.S.D_{0.01}$ (Genotypes) = 1.05
$L.S.D_{0.01}$ (Contamination levels) = 0.69
$L.S.D_{0.01}$ (Gen ×cont. interaction) = 1.81

SPAD meter provides a non-destructive, real-time estimate of chlorophyll content, which is strongly associated with photosynthetic capacity, nitrogen status, and plant vigor (Peng *et al.*, 1995; Lunagaria & Patel, 2015; Santarosa *et al.*, 2016; Vishwakarma *et al.*, 2023). The significant reduction in chlorophyll level due to increasing petroleum contamination aligns with the results reported by El-Nahhal *et al.* (2013), who found that hydrocarbon contamination enhanced chlorophyll synthesis but disrupted thylakoid membrane stability. The high sensitivity of “Farafra” and “Hasawii” genotypes under high level of contamination suggested a lower adaptive capacity to petroleum toxicity, possible due to impaired uptake of essential nutrients or oxidative damage on the other side, the relatively stable chlorophyll levels in “Rammah” and “Australian” genotypes across petroleum contamination, suggest enhanced physiological tolerance, possibly linked to efficient antioxidant system or better hydrocarbon exclusion mechanism as proposed by Garcia- Sanchez *et al.* (2014). These genotypes may maintain cellular integrity and chloroplast function, allowing sustained pigment production even under moderate and high levels of petroleum contamination stress. The genetic variability detected among the tested genotypes highlights the potential for selecting alfalfa lines with higher resilience to petroleum pollution (Ashraf, 2010; Kang *et al*, 2011; Liu *et al*, 2011).

Seedling vigor

Seedling vigor index, which combines dry shoot weight and shoot length, and finally the number of surviving plants after 90 days of exposure to petroleum contamination, showed a significant (P≥ 0.01) response to contamination level, genotypes, and their interaction (Table 13).

Table 13: Mean squares of seedling vigor index and number of survival plants as affected by petroleum contamination level, alfalfa genotype, and their interaction.

Source of variations	d.f.	Mean squares	
		Seedling vigor index	Number of survival plants
Blocks	3	0.207 **	0.378 **
Contamination Level (A)	2	0.946 **	8.241**
Genotype (B)	6	0.564**	9.432**
A×B	12	0.008**	0.208**
Error	60	0.002	0.014

* and **; indicates significance at 0.05 and 0.01 levels, respectively.
NS; not significantly different

Seedling vigor index scored a significant reduction of about 40% with each rise in contamination level (from 0.660 to 0.393 to 0.236, for the three studied contamination levels, respectively) as reported in Table 14. Also, the “Rammah” genotype significantly expressed the highest value of seedling vigor index (0.852), followed by the Australian genotype (0.839). Meanwhile, the other studied alfalfa genotype scored an index value less than 0.5, which reflected sensitivity to petroleum contamination. That was true under the two tested contamination levels (40 and 60 $mL.kg^{-1}$), where any of “Rammah” or “Australian” genotypes significantly showed the highest seedling vigor index under both contamination levels. The stable performance of “Rammah” and “Australian” seedling index might indicate their efficient energy reserves or enhanced root and shoot allocation under petroleum contamination stress. Meanwhile, the obtained decrease in seedling vigor might reflect impaired seed metabolism and energy mobilization, as discussed by Achuba (2006), Coulon *et al.* (2010), and Bojimova and Velkova (2011).

Table 14: Means of seedling vigor index as affected by petroleum contamination levels, alfalfa genotypes, and their interaction.

Genotypes	Contamination levels			Means
	Control (zero)	Moderate (40 $mL.kg^{-1}$)	High (60 $mL.kg^{-1}$)	
Nubaria 1	0.55	0.307	0.148	0.335
Rammah	1.059	0.866	0.631	0.852
Wahat	0.47	0.244	0.108	0.274
Ismaelia 1	0.485	0.199	0.084	0.256
Farafra	0.531	0.178	0.059	0.256
Hasawii	0.387	0.142	0.052	0.194
Australian	1.136	0.813	0.567	0.839
Means	0.660	0.393	0.236	

$L.S.D_{0.01}$ (Genotypes) = 0.05
$L.S.D_{0.01}$ (Contamination levels) = 0.03
$L.S.D_{0.01}$ (Gen ×cont. interaction) = 0.1

Means of the number of survival plants as affected by petroleum contamination levels, alfalfa genotypes, and their interaction are presented in Table 15. The number of surviving plants after 90 days of exposure to petroleum contamination over all the tested genotypes reached 11.45% (from 77.61 to 68.75 plants) under a moderate level of contamination (40 $mL.kg^{-1}$), whereas, reached 20.24% (from 77.61 to 59.96 plants) under the high level of contamination (60 $mL.kg^{-1}$). Meanwhile, “Rammah” and “Australian” genotypes significantly maintained the least magnitude of reduction in the number of surviving plants over all tested contamination levels (3.238 and 3.667% reduction for the former and the latter under moderate contamination level, as well as 10.43 and 10.56% under high contamination level). In the meantime, the magnitude of reduction in the number of surviving plants for the most sensitive genotype “Hawaii” reached 19.84% under a moderate contamination level and 35.30% under a high level of contamination.

Table 15: Means of the number of survival plants as affected by petroleum contamination levels, alfalfa genotypes, and their interaction.

Genotype	Contamination levels			Means
	Control (zero)	Moderate (40 $mL.kg^{-1}$)	High (60 $mL.kg^{-1}$)	
Nubaria 1	83.00	77.00	63.00	74.33
Rammah	92.75	89.75	83.50	88.67
Wahat	73.25	60.75	54.00	62.67
Ismaelia 1	68.00	56.25	44.50	56.25
Farafra	75.25	62.00	53.75	63.67
Hasawii	60.50	48.50	40.00	49.67
Australian	90.50	87.00	81.00	86.17
Means	77.61	68.75	59.96	

$L.S.D_{0.01}$ (Genotypes) = 0.13
$L.S.D_{0.01}$ (Contamination levels) = 0.1
$L.S.D_{0.01}$ (Gen ×cont. interaction) = 0.22

These recent results support the use of survival rate after a period of exposure to contamination as a quick screening measure for petroleum contamination tolerance (Banks *et al*, 2003), since survival reflects contaminative responses to stress, including germination, root health, and shoot growth (Merkl *et al*., 2005, and Meng *et al*, 2011).

General conclusions

Petroleum contamination significantly reduced all growth traits, including shoot and root biomass and chlorophyll content. Alfalfa genotypes significantly differed in their response to contamination, with “Rammah” and “Australian” genotypes performing best under petroleum contamination stress. Tolerance index and chlorophyll content proved to be a reliable indicator of genotype resilience. The significant interaction effects suggested that a strong genotype × environment relationship was established due to a significant genotype × contamination level interaction, as well as a genotype × environment relationship under petroleum contamination stress. The recent findings support the use of specific alfalfa genotypes for cultivation or phytoremediation in soils contaminated with petroleum. However, genotypes such as “Rammah” and “Australian” presented a notable resilience to petroleum contamination, since they maintained a relatively higher performance under stress. So, it was supposed that these two genotypes might be used for selective breeding for phytoremediation and soil restoration programs.

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تقييم تراكيب وراثية من البرسيم الحجازي من حيث تحمل التلوث بالبترول: اسلوب لتطوير تراكيب وراثية مقاومة للإجهاد

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الملخص العربي

هدفت الدراسة إلى تقييم تأثير التربة الملوثة بالبترول على نمو وتطور سبعة تراكيب وراثية من البرسيم الحجازي وذلك تحت ثلاث مستويات من التلوث بالبترول (صفر، ٤٠ ، ٦٠ مل/كجم من التربة). شملت الدراسة قياس العديد من الصفات المورفولوجية والفسيولوجية وشملت: نسبة الانبات، دليل التحمل، طول النبات والوزن الجاف والمحتوي من الكلوروفيل ودليل البقاء في التربة الملوثة.

واشارت النتائج المتحصل عليها إلي أن إجهاد التلوث بالبترول قد أدي إلي انخفاض ملحوظ في معظم الصفات المقاسة، مع تسجيل أعلي تأثير سلبي عند مستوي التلوث المرتفع (٦٠ مل/كجم تربة). وقد وجد أن كلا من دليل التحمل ومحتوي الكلوروفيل تعتبر مؤشرات جيدة على قدرة التراكيب الوراثية على التكيف. كما اظهرت النتائج وجود علاقة قوية بين سلوك التركيب الوراثي وطبيعة البيئة التي ينمو فيها وذلك بدليل التفاعل المعنوي بين التراكيب الوراثية ومستوي التلوث بالبترول، وتلك النتائج تدعم امكانية استخدام بعض التراكيب الوراثية من البرسيم الحجازي في المعالجة النباتية للتربة الملوثة بالبترول. وبصفة عامة فان بعض التراكيب الوراثية مثل " رماح " و " الاسترالي " قد أظهرت تحمل واضح للتلوث البترولي حيث حافظت على مستويات جيدة من النمو تحت ظروف الاجهاد البترولي. ولذلك فقد يوصى بأن هاذان التركيبان الوراثيان من الممكن استخدامها في برامج الانتخاب لإنتاج تراكيب تستخدم في المعالجة النباتية وعلاج تدهور التربة.