Kuwait J. Sci. **46 (4)** pp. 83-92, 2019

Response of *Rhizobium* to spent engine-oil contamination in the rhizosphere of legumes (*Arachis hypogaea* and *Phaseolus vulgaris*)

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Abstract

Indiscriminate releases of spent-engine oil into the environment mean danger to legume nodulation activities by *Rhizobium*. To ascertain this, responses of *Rhizobium* to spent engine-oil in the rhizosphere of *A. hypogaea* and *P. vulgaris* were studied by growing viable seeds in spent engine-oil contaminated soil at 0, 1.0 and 3.0 % w/w for 120 days. The influence of oil on total populations of heterotrophs and *Rhizobium* in rhizosphere and nodule were estimated by the soil dilution plate method on nutrient agar (NA) and yeast extract mannitol agar (YEMA), respectively. Plant height and root growth were assessed using a graduated-meter rule nodule and pod numbers by counting. Results showed that the population of *Rhizobium* in rhizosphere and nodule decreased with increased oil concentrations. Results were in contrast to observed increases in bacterial heterotrophs for tested legumes ($P < 0.05$). Plant height, root length, nodule and pod formation decreased with increased oil concentrations in soil ($P < 0.05$). Spent engine-oil impeded the *Rhizobium* response in the rhizosphere of legumes.

Keywords: Contamination; legume; *Rhizobium*; rhizosphere; nodule; spent engine-oil.

1. Introduction

The sporadic release of huge volumes of hydrocarbons in the form of petroleum into the environment often attracts global attention. However, small chronic releases of by-products of petroleum, such as spent engine-oil from automobile and mechanic workshops are frequently overlooked. Yet these events pose a similar threat to the environment and human health.

The impacts of petroleum hydrocarbon released into the environment are well documented. Contamination of arable lands through run-off arising from improper handling or indiscriminate disposal of spent engine-oil could portend serious danger to agriculture, especially legumes and their associated rhizosphere microorganisms. This includes *Rhizobium*. Legumes provide food for human and animals and serve as an alternative source of protein in the absence of animal protein (Redden *et al*., 2005 as cited by Grandawa, 2014). These plants constitute the third largest class of flowering plants (Madigan *et al*., 2012) and typically form a symbiotic relationship with *Rhizobium*. The results of this are the fixation of atmospheric nitrogen, enhancement of soil fertility, increasing organic matter, and the promotion of soil's overall structure (Christiansen & Graham, 2002).

Twenty-five percent of yearly nitrogen fixation on earth takes place in root nodules of legumes (Madigan *et al*., 2012). This process is of great importance to agriculture. This critically important activity of nodulation and fixation of nitrogen by *Rhizobium* could be hampered by the presence of spent engine-oil in soil. It is on this premise that the probable response of *Rhizobium* in the rhizosphere of *A. hypogaea* and *P. vulgaris* to spent engine-oil was investigated.

2. Materials and methods

2.1 Soil physicochemical analysis

The baseline physicochemical property of experimental soil was carried out for the textural component using the hydrometer method (Aliyu & Oyeyiola, 2011). pH, porosity, phosphorus, and total organic carbon were analyzed using the methods of Black (1965), Ezzati *et al*. (2012), Bray & Kurtz (1971), and Black (1965), respectively. Nitrogen in the soil was determined by micro-Kjeldahl procedure (Hesse, 1979).

2.2 Preparation of soil for planting

The soil used for plant propagation was collected from a depth of 0-15 cm in the top soil layer. Forty grams and 120 g of spent engine-oil were obtained from a mechanic's workshop and added to 4,000 g of soil. The two were thoroughly mixed to attain 1.0 and 3.0% w/w oil contamination, respectively. In addition, 4,000 g of uncontaminated soil (the control at 0 % w/w) were also prepared. Triplicates pots of the respective concentrations of 0.0, 1.0 and 3.0% w/w of oil in soil were prepared.

2.3 Testing propagated seeds for viability

The floatation method was used to test viability of the seeds of *P. vulgaris* and *A. hypogaea* before propagation. Seeds were soaked in lukewarm water for a period of 12 hours. Thereafter, the floating seeds (non-viable) were discarded and submerged seeds (viable) were preferentially selected for propagation. Five seeds were planted per pot.

After germination, a thinning process was carried out to permit two plant stands per pot (best growth). The plants were watered regularly throughout the study. Watering was done manually to avoid overwatering.

2.4 Isolation and enumeration of *Rhizobium* and heterotrophic bacteria in the rhizosphere

Rhizosphere soil for *Rhizobium* and heterotrophic bacterial analysis was obtained after 120 days of propagation using the method outlined by Abdel-Rahim *et al*., (1983) as modified by Ikediugwu & Ubogu (2012). Plants were uprooted from respective pots and gently shaken to free soil not firmly attached to the roots. Subsequent vigorous shaking was carried out in sterile polythene bags for the collection of about 10 g of soil from replicate pots. These were then pooled together.

One gram of rhizosphere soil was added to 9.0 ml of sterile physiological saline in test tubes for the preparation of a fivefold serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}). Next, 0.1 ml of the sample of the various dilutions was plated out in triplicate. The spread plate method on YEMA (to which Congo dye was incorporated) was used for *Rhizobium* and NA for the total heterotrophic bacterial population estimation in the rhizosphere. Plates were incubated at room temperature at 30° ± 2°C for 48-72 hours. Plates with 30 to 300 colonies were counted, and the bacterial populations were estimated. Elevated, convex, viscous colonies were counted. They had smooth, circular edges with a characteristic musky odor which absorbed the incorporated dye weakly as *Rhizobium* colonies.

2.5 Isolation and enumeration of *Rhizobium* in the root nodules

Rhizobium was isolated from the root nodules by adapting the method of Ben-Gweirif *et al.* (2005) and Deka &Azad (2006). Well-formed, healthy nodules (big and pinkish) were plucked from the roots. One gram of the nodules was obtained, washed in tap water, surface sterilized for 2 minutes with 70% ethanol, and then rinsed with distilled sterile water. Nodules were further surface sterilized with 3.5% w/v of sodium hypochlorite for 2 minutes and rinsed thrice with distilled sterile water before the nodules were crushed.

One gram of the surface sterilized nodules was crushed in a Mac Cartney bottle with a few drops of sterile water. Thereafter, sterile physiological saline was used to make up a volume of crushed nodules to 10.0 ml. Fivefold serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) was then carried out with subsequent plating out of 0.1 ml of dilutions. *Rhizobium* isolation and enumeration were performed as described above.

2.6 Characterization of isolated *Rhizobium*

The isolated *Rhizobium* from the rhizosphere and nodules of legumes was identified using cultural, morphological and biochemical characterization in accordance with Bergey's Manual of Determinative Bacteriology (John *et al*., 1994).

2.7 Evaluation of the nodule and pod formation

The effect of spent engine-oil on nodule and pod formation was determined after a 120-day period of propagation. Legumes were harvested and the roots were washed in slow-running tap water in order to remove all adhering soil. This also ensured no damage to the plant roots. Roots were air-dried for 30 minutes. Thereafter, the numbers of nodules and pods per plant were counted.

2.8 Evaluation of plant height and root growth

Plant height and root length growth under the influence of spent engine-oil contamination were determined at day 120 of the study period. Using a graduated meter rule, plant height was determined by taking measurement from the base of plant to the tallest leaf, while root length was taken from the base to the tip of the longest root.

2.9 Data analysis

Data were analyzed using measures of central tendency (mean) and dispersion (standard deviation), Student's *t*-test, and an analysis of variance ($P < 0.05$).

3. Results

3.1 Baseline physicochemical properties of experimental soil

The textural composition of the experimental soil from the baseline data indicates that the soil is loamy sand with a pH tending toward neutrality. The soil had moderate water holding capacity and a reasonable nitrogen content (Table 1).

Table 1. Baseline physicochemical properties of experimental soil

Soil Properties	Values
Texture	
Sand (%)	74.0
Silt (%)	17.0
Clay (%)	9.0
pH	6.9
Porosity (%)	71.2
Nitrogen (%)	0.09
Phosphorus (mg/kg)	0.5
Total Organic Carbon (%)	1.2

The heterotrophic bacterial population in the rhizosphere of *A. hypogaea* ranged from 2.5 x 10^6 to 3.5 x 10^6 cfu/g of soil. On the other hand, the population of *P. vulgaris* ranged from 3.0 x 10^6 to 3.6 x 10^6 (white) and 3.4 x10^6 to 4.3 x10^6 cfu/g of soil (brown) from 0 to 3.0 % w/w concentrations, respectively. Populations of heterotrophic bacteria in the rhizosphere of the two varieties of *P. vulgaris* did not differ significantly in the presence of spent engine-oil ($P < 0.05$).

3.3 Effect of spent engine-oil on the total *Rhizobium* populations in the rhizosphere and nodules of legume

Rhizobium populations in both rhizosphere and the nodule of legumes decreased significantly with increased spent-engine oil concentration ($P < 0.05$) (Figure 2 and 3). In the

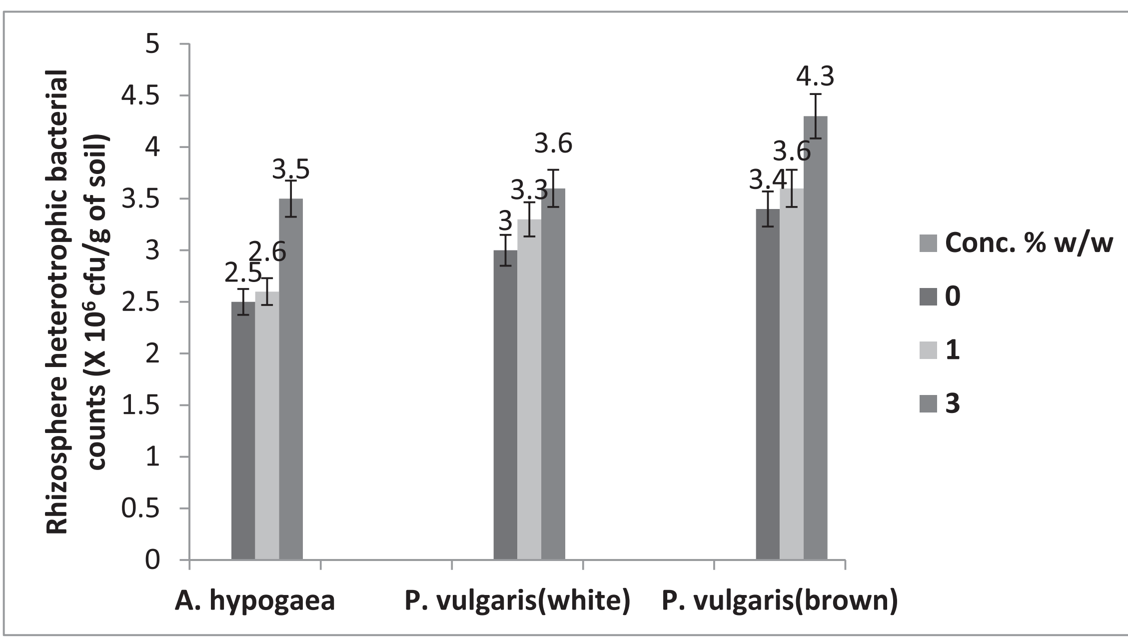

Fig. 1. Effect of spent engine-oil on the total heterotrophic bacterial populations in the rhizosphere of *A. hypogaea* and *P. vulgaris* (white and brown variety)

3.2 Effect of spent engine-oil on the total heterotrophic bacterial populations in the rhizosphere of legumes

The heterotrophic bacterial populations in the rhizosphere increased with increased spent-engine oil concentration for all the investigated legumes (Figure 1).

rhizosphere, the *Rhizobium* populations in *A. hypogaea* ranged from 6.0 x 10^3 to 3.4 x 10^3 cfu/g of soil. In contrast, that of *P. vulgaris* ranged from 2.8 x 10^3 to 1.6 x 10^3 (white) and 3.2 x10^3 to 1.4 x10^3 cfu/g of soil (brown) from 0 to 3.0 % w/w concentrations, respectively. Similarly, in nodule, the *Rhizobium* populations in *A. hypogaea* ranged from 9.0 x 10^4 to 5.0 x 10^4 cfu/g of nodule, while that of *P. vulgaris* ranged from 3.5 x 10^4 to 1.6 x 10^4 (white) and 1.6 x10^4 to 1.2 x10^4 cfu/g of nodule (brown) from 0 to 3.0% w/w concentrations, respectively.

For each of the legumes investigated, the population of *Rhizobium* was consistently higher in the nodule than the rhizosphere in the respective concentrations of oil tested ($P < 0.05$). While the populations of *Rhizobium* in rhizosphere of the two varieties of *P. vulgaris* did not differ significantly, nodule populations in the white variety were higher than that of brown variety in the respective oil concentrations tested, including the control (0 % w/w) ($P < 0.05$).

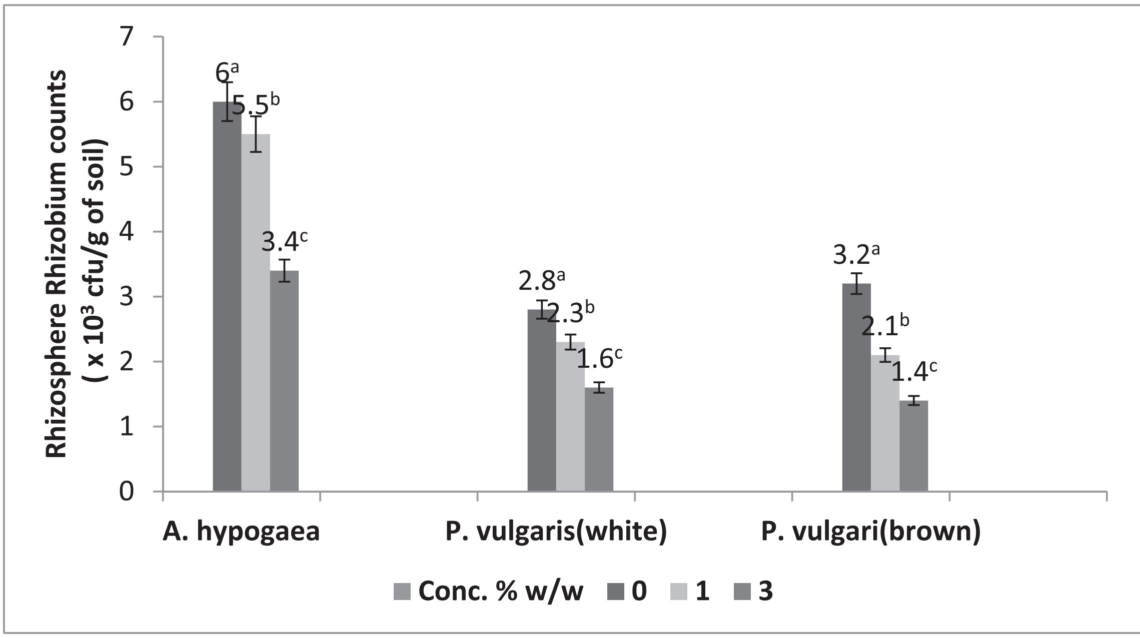

Fig. 2. Effect of spent engine-oil on the total *Rhizobium* populations in the rhizosphere of *A. hypogaea* and *P. vulgaris* (white and brown variety)

*Values having same superscript alphabet for the same plant did not differ significantly ($P < 0.05$).

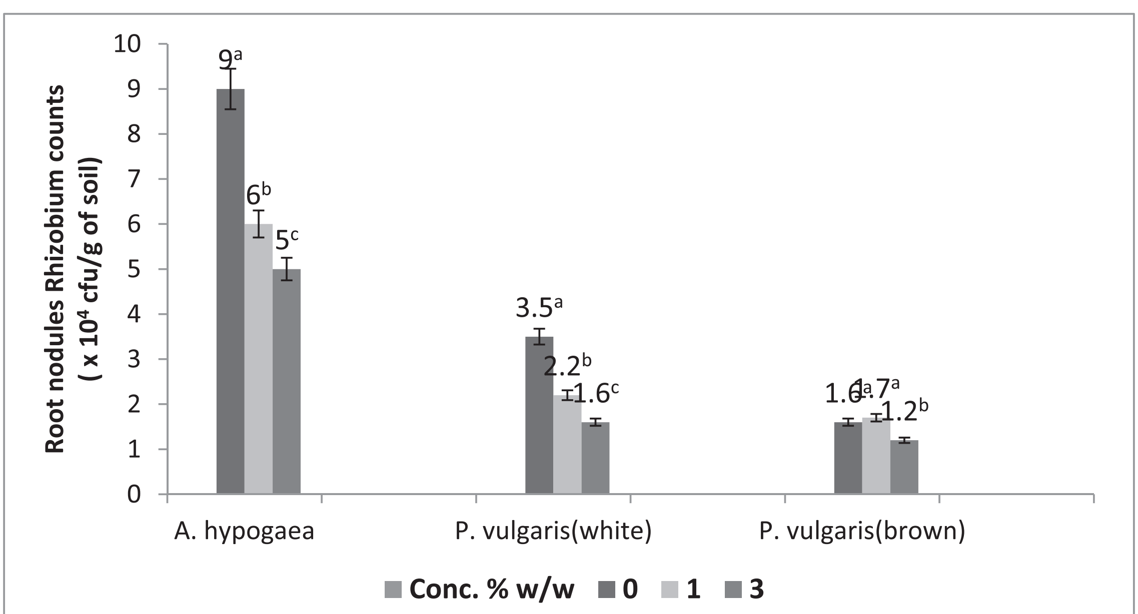

Fig. 3. Effect of spent engine-oil on the total *Rhizobium* populations in the root nodules of *A. hypogaea* and *P. vulgaris* (white and brown variety)

*Values having same superscript alphabet for the same plant did not differ significantly ($P < 0.05$).

3.4 Effect of spent engine-oil on nodulation and pod formation in legumes

Nodulation in *A. hypogaea* and the two varieties of *P. vulgaris* were affected by the presence of spent engine-oil. The numbers of nodules formed decreased with increased oil concentrations for all the legumes in this study ($P < 0.05$) (Table 2). The number of nodules for *A. hypogaea* ranged from 90.0 to 21.0 per plant. For of *P. vulgaris,* it ranged from 75.0 to 10.7 (white) and 30.0 to 15.4 (brown) per plant from 0 to 3.0 % w/w oil concentrations, respectively. Similarly, the number of pods formed also decreased with increased oil concentrations in *A. hypogaea* ($P < 0.05$) (Table 3). Pod number ranged from 12.3 to 3.3 per plant from 0 to 3 % w/w oil concentrations, respectively. However, the effect of oil on pod formation in *P. vulgaris* was not determined.

3.5 Effect of spent engine-oil on root length growth in legumes

Save for the brown variety of *P. vulgaris*, root length growth of legumes was simulated by 1% w/w oil concentration. Nonetheless, these increases over the control were not statistically significant (Table 4). Furthermore, with the exception of the white variety of the *P. vulgaris*, root length of legumes decreased with increased oil concentration ($P < 0.05$). Root length of *A. hypogaea* ranged from 21.7 to 10.3 cm, while that of *P. vulgaris* ranged from 23.2 to 23.6 (white) and 24.3 to 19.0 cm (brown) from 0 to 3 % w/w oil concentrations, respectively.

Table 2. Effect of spent engine-oil on nodulation in *A. hypogaea* and *P. vulgaris* (white and brown variety)

Nodule Numbers per Plant			
Spent engine-oil conc. (% w/w)	**Legume**		
	A. hypogaea	*P. vulgaris* (white)	*P. vulgaris* (brown)
0	90.0 ± 2.0^{a}	75.0 ± 2.0^{a}	30.3 ± 1.5^{a}
1	34.3 ± 1.5^{b}	30.0 ± 3.5^{b}	16.0 ± 1.0^{b}
3	21.0 ± 2.0^{c}	10.7 ± 1.2^{c}	15.4 ± 1.7^{b}

*Key: Values having same superscript alphabet in the same column did not differ significantly ($P < 0.05$).

Table 3. Effect of spent engine-oil on pod formation in *A. hypogaea* and *P. vulgaris* (white and brown variety)

Numbers of Pods per Plant			
Spent engine-oil conc. (% w/w)	**Legume**		
	A. hypogaea	*P. vulgaris* (white)	*P. vulgaris* (brown)
0	12.3 ± 2.5^{a}	ND	ND
1	5.0 ± 1.0^{b}	ND	ND
3	3.3 ± 0.6^{c}	ND	ND

*Key: Values having same superscript alphabet in the same column did not differ significantly ($P < 0.05$).
ND: Not determined

Table 4. Effect of spent engine-oil on root length growth in *A. hypogaea* and *P. vulgaris* (white and brown variety)

Root Length Growth (cm)			
Spent engine-oil conc. (% w/w)	**Legume**		
	A. hypogaea	*P. vulgaris* (white)	*P. vulgaris* (brown)
0	21.7 ± 2.5^{a}	23.2 ± 3.0^{a}	24.3 ± 1.5^{a}
1	23.1 ± 2.0^{a}	25.0 ± 1.7^{a}	20.0 ± 2.3^{ab}
3	10.3 ± 1.5^{b}	23.6 ± 2.0^{a}	19.0 ± 1.0^{b}

*Key: Values having same superscript alphabet in the same column did not differ significantly (P < 0.05).

3.6 Effect of spent engine-oil on the height growth of legumes

With the exclusion of the brown variety of *P. vulgaris* were 1% w/w oil concentration produced a higher height growth over control and 3 % w/w concentration, height growth in legumes decreased with increased oil concentrations (P < 0.05) (Table 5). The height growth of *A. hypogaea* ranged from 33.0 to 24.0 cm, while that of *P. vulgaris* ranged from 140.0 to 66.2 (white) and 142.3 to 36.0 cm (brown) from 0 to 3 % w/w oil concentrations, respectively.

Table 5. Effect of spent engine-oil on height growth in *A. hypogaea* and *P. vulgaris* (white and brown variety)

Height Growth (cm)			
Spent engine-oil conc. (% w/w)	**Legume**		
	A. hypogaea	*P. vulgaris* (white)	*P. vulgaris* (brown)
0	33.0 ± 1.0^{a}	140.0 ± 5.0^{a}	142.3 ± 1.5^{a}
1	31.0 ± 2.0^{ab}	96.2 ± 3.0^{b}	161.0 ± 3.5^{b}
3	24.0 ± 1.7^{b}	66.2 ± 2.0^{c}	36.0 ± 2.0^{c}

*Key: Values having same superscript alphabet in the same column did not differ significantly (P < 0.05).

4. Discussion

The soil used in the propagation of legumes in this study is adequately suitable on the basis of its physicochemical analysis. Soil baseline pH in this study is 6.9, which falls within the ideal pH range (5.3-7.3) for *Rhizobium* and legume growth (Nyambok *et al*., 2011; Ajeigbe *et al*., 2014; Okello *et al*., 2014). *Rhizobium* growth response in soil is contingent on soil pH (Vincent, 1965). In spite of the fact that *Rhizobium* and legume can tolerate low pH, it varies with species, pH values of lower than 5.0 are usually inhibitory to legume nodulation and symbiotic N_2 fixation (Lowendorf *et al*., 1981; Munns *et al*., 1981; Grahamp, *et. al*., 1994; Mohammadi *et al*., 2012).

The heterotrophic bacterial population responses in the rhizosphere of *A. hypogaea* and *P. vulgaris* to spent engine-oil were similar. Heterotrophic bacterial populations expanded with increased oil input. This finding is in concordance with previous reports that the population of heterotrophic bacteria in soil is amplified with increased oil concentrations (Atlas, 1978; Sparrow *et al*., 1978; Pinholt *et al*., 1979) up to levels as high as 39.2% (Odu, 1972). This population increase is attributed to the selective enrichment and proliferation of that section of soil microorganisms capable of utilizing the contaminating oil (Atlas 1984; Coulon *et al*., 2006; Hamamura, 2006).

Spent engine-oil decimated *Rhizobium* populations in the rhizosphere and nodules of both legumes with more impact at higher concentrations. Symbiotic N_2 fixing bacteria connected with legumes have been reported to be highly sensitive to crude oil contamination (John *et al*., 2011). Similarly, Ekpo and Nkanang (2010), reported reduced population of nitrifying bacteria in the rhizosphere of *A. hypogaea* and *Vigna unguiculata* at concentrations as low as 1% v/w of diesel oil in soil. The researchers attributed this to the bacteria's inability to effectively compete in the contaminated soil with other microorganisms, which swiftly increased and caused the depletion of available nutrients. In the same way, the reduced population of *Rhizobium* in the rhizosphere and nodules of *A. hypogaea* and *P. vulgaris* in this study may be the result of the direct toxic effect of the oil together with their inability to effectively compete with other rhizosphere microorganisms with a higher capacity to tolerate and utilize the contaminated oil. The struggle for existence among microbial soil components is intensified in the rhizosphere (Parkinson & Waid, 1960) with microbial degradation of organic compounds being compelled by both energy requirement and the need to decrease toxicity (Chaudhry *et al*., 2005).

The reduced numbers of nodules in *A. hypogaea* and the two varieties of *P. vulgaris* with increased concentrations of spent engine-oil in comparison with uncontaminated soil, indicates that oil contamination impedes nodulation activities of *Rhizobium* in legumes. This assertion verified research from previous studies. Reduced nodulation have been reported in *Vigna unguiculata*, *Sphenostylis stenocarpa* (Ihimikaiya & Tanee, 2014); *Glycine max* (Onuh *et al*., 2010); *Vicia sativa* (Adams & Duncan (2010) and *Calopogonium mucoides*, *Centrosena pubescens* (John *et al*., 2011) in soil contaminated with spent engine-oil, spent motor-oil, diesel oil and crude oil, respectively. The reduced nodulation activities in legumes observed in this study is the resultant effect of a lowered population of *Rhizobium* witnessed in the rhizosphere in the presence of spent engine oil. Reduced nodulation in legume in sympathy to *Rhizobium* population declined in the rhizosphere have been reported (Ubogu *et al*., 2017). Nodulation and fixation of nitrogen is impaired by diminished *Rhizobium* population in soil (Chauhan *et al*., 1988).

Pod formation in *A. hypogaea* was significantly affected by the presence of spent engine-oil. Although the effect of oil on pod formation in the two varieties of *P. vulgaris* was not determined, Nwoko *et al*. (2007) showed that formation of pods in *P. vulgaris* diminished with increased concentrations of spent engine-oil in soil. Similarly, Okon and Mbong (2013) reported complete failure in pod (fruit) formation in *Abelmoscus esculentus* in the presence of spent engine-oil at concentrations as low as 2% in soil. Pod formation impairment observed in this study is due to oil-impeded nodulation. There is a correlation between the level of nodulation and pod formation in legumes. Higher levels of nodulation in legumes have been reported to be associated with increased pod numbers (Ubogu *et al*., 2017).

Overall, the severity of spent engine-oil's impact on the two legumes' root length growth was low at the tested concentrations. However, root growth measurement indicated that *P. vulgaris* tolerated spent engine-oil better than *A. hypogaea*, and the white variety was more tolerant than the brown. While 3% w/w of oil inhibited root length growth, 1% w/w produced marginal root increases in *A.* hypogaea and in the white variety of *P. vulgaris.*It is important to note that these findings concur with those of Okon and Mbong (2013),Ihimikaiye and Tanee (2014),and Onuh *et al*. (2010), all of whom reported a negative impact of spent engine-oil oil/motor-oil on the root length growth of *A. esculentus*; *V. unguiculata*, *S. stenocarpa*; and *Glycine max,* respectively. The findings also lend credence to Tereke *et al*. (2015) who reported that 5% crude oil stimulated root length growth of *Carex hirta* and *Faba bona* by 17 and 64% respectively but reduced that of *Medicago lupulin* and *Trifolium pretense*. These divergent reports invariably suggest that root growth response to oil contamination is a function of concentration and species.

Disregarding the result that 1% w/w of the spent oil stimulated height growth of the brown variety of *P. vulgaris* over the control and 3 % w/w, generally legume heights decreased with increased concentrations of oil. These outcomes agree with previous research. Onuhet *et al*. (2010), Nwoko *et al*. (2007), Terek *et al*. (2015) reported a diminishing height growth in legumes with the presence and with increasing concentrations of oil in the soil. Reduction in height growth with increasing oil concentration may be ascribed to any elevated stress imposed on the plant with an accompanying higher quantity of oil in the soil (Odokuma & Ubogu, 2014). Microorganisms capable of breaking down oil compete for mineral nutrients and O_2 utilization with the plant. Oxygen depletion eventually leads to anaerobic conditions which then result in a microbial production of phytotoxins, such as hydrogen sulfide. The soil's physical structure is also impacted by the oil, diminishing its ability to retain air and moisture (Dejong, 1980).

5. Conclusion

The results of this study showed that there is an impeded response of *Rhizobium* in the rhizosphere of *A. hypogaea* and *P. vulgaris* in the presence of spent engine-oil. This had a negative impact on the overall growth and yield of the legumes.

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Submitted : 03/11/2017
**Revised : **17/04/2018
Accepted : 18/04/2018

استجابة ريزوبيوم للتلوث الناتج عن احتراق زيوت المحركات في جذور البقوليات (الفول السوداني والفاصوليا)

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الملخص

إن الانبعاثات العشوائية من زيوت المحركات المحترقة في البيئة تُنذر بالخطر على أنشطة الريزوبيوم في جذور البقوليات. وللتأكد من ذلك، تم دراسة استجابة ريزوبيوم لزيت المحرك المحترق في جذور نباتات الفول السوداني (A. hypogaea) والفاصوليا (P. vulgaris) عن طريق زراعة بذور قابلة للنمو في التربة الملوثة بزيوت المحركات المحترقة عند 0 و 1.0 و 3.0% بالوزن الرطب لمدة 120 يوماً. تم تقدير تأثير الزيوت على إجمالي الكائنات غير ذاتية التغذية والرايزوبيوم في الجذور والعقد من خلال العزل بطريقة الصحاف لتمييع التربة على أجار المغذيات (NA) وأجارمانيتول خلاصة الخميرة (YEMA) على التوالي. تم قياس ارتفاع النبات وطول الجذور باستخدام مسطرة مترية مُدَرَجة؛ كما تم عد العقيدات والقرنة. أظهرت النتائج أن عدد ريزوبيوم في الجذور والعقيدات قد انخفض مع زيادة تركيز الزيت على عكس الزيادة الملحوظة في الكائنات البكتيرية غير ذاتية التغذية في البقوليات موضوع الدراسة (P < 0.05). وانخفض ارتفاع النبات، وطول الجذور، وتكوين العقيدات والقرنة بزيادة تركيزات الزيت في التربة (P < 0.05). أعاق زيت المحرك المحترق استجابة ريزوبيوم في جذور البقوليات.