International Journal of Phytoremediation

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/bijp20

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To cite this article: Sana Dardouri, Asma Jedidi, Sabrine Mejri, Sabrine Hattab & Jalila Sghaier (2021) Morphological effect of dichloromethane on alfalfa (*Medicago sativa*) cultivated in soil amended with fertilizer manures, International Journal of Phytoremediation, 23:3, 263-271, DOI: 10.1080/15226514.2020.1810205

To link to this article: https://doi.org/10.1080/15226514.2020.1810205

Published online: 27 Aug 2020.

INTERNATIONAL JOURNAL OF PHYTOREMEDIATION
2021, VOL. 23, NO. 3, 263–271
https://doi.org/10.1080/15226514.2020.1810205

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Morphological effect of dichloromethane on alfalfa (*Medicago sativa*) cultivated in soil amended with fertilizer manures

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ABSTRACT

In this work, we investigated the morphological effect of dichloromethane (DCM) on alfalfa (*Medicago sativa*) plant. We studied *in vitro* the influence of its concentration on alfalfa germination. The plants were placed in pots for 15 weeks, and exposed to increasing concentrations of DCM (50 µg L^{-1} and 84 mg L^{-1}). In addition, we examined the effect of two manures (cow and sheep), which were applied to a contaminated soil, on alfalfa plant growth. The effect of the presence of dichloromethane is obvious even in plant–soil manure system. In fact, in the event of contamination, the soil–cow manure mixture represents the best setting medium for the Alfalfa plant compared to other environments, regardless of the contamination level. Indeed, the presence of two types of manure does not allow the suppression of the inhibitory effect of dichloromethane on the mass of the dry matter of the aerial part which is 18.38% for the cow manure-amended soil and 13.96% for the sheep manure-amended soil.

KEYWORDS
Adsorption; alfalfa; dichloromethane; manures; morphological effect

Introduction

Contamination by dense chlorinated solvents (DNAPL) is one of the most critical threats to the environment that humanity faces and is commonly related to the industrial activities. Among the most used solvents in the industry are trichlorethylene (TCE), tetrachlorethylene (PCE), methylene chloride or dichloromethane (DCM) and chloroform (TCM). It mainly uses these products in the pharmaceutical industry, adhesives, and aerosols and in paint stripping. These solvents are also used as chemical intermediates for the production of plant protection products and smoke bombs, as extraction liquids, as heat transfer fluids and as solvents for glue and ink production. Because of its characterization DCM has become a very useful solvent in many chemical processes, such as paint removal, metal cleaning, extraction of essential oils, pharmaceuticals, and flexible polyurethane foam.

Dichloromethane is one of the most hazardous pollutant threatening human health and ecosystems. It is a toxic compound potentially carcinogenic for humans (Shestakova and Sillanpää 2013). DCM penetrates the human body orally via water, food or by adsorption through the skin and can cause chemical burns, liver and kidney dysfunctions, pulmonary edema (Cayot *et al.* 2016). Besides neurotoxic effects and effects on the kidneys and lungs, exposure of laboratory animals to high concentrations of DCM causes toxicity in their central nervous systems, while exposure to low concentrations of DCM can damage the liver. In the case of animals, a single high dose of DCM, whether inhaled (up to 50,000 ppm) or ingested (up to 3825 mg/kg), was discovered to have adverse effects on the cardiovascular and nervous systems.

As a remedy for DNAPL pollution, many techniques were employed, including adsorption processes, air stripping and surfactant-enhanced dissolution (Khachikian and Harmon 2000; Huang *et al.* 2011). However, during the last decade, phytoremediation has been rapidly developing as a potential "green technology" for the cost-effective removal of DNAPL from soils and waters (Gerhardt *et al.* 2009; Cruz *et al.* 2014; Moccia *et al.* 2017). Several plants species have been tested for the DNAPL remediation, such as poplars (Gordon *et al.* 1998; Shang and Gordon 2002), Zea mays (Moccia *et al.* 2017), tobacco (Shang *et al.* 2001), fruit trees (Chard *et al.* 2006; Doucette *et al.* 2007), leguminous trees (Doty *et al.* 2007) and grass-like alfalfa (Zhang *et al.* 2013).

In fact, alfalfa (*Medicago sativa* L) was applied for soil phytoremediation of both inorganic (Bonfranceschi *et al.* 2009; Vamerali *et al.* 2011; Zaefarian *et al.* 2013, Marchand *et al.*, 2016) and organic pollutants (Wei and Pan 2010; Hechmi *et al.* 2014; Marchand *et al.* 2016), such as heavy metals (Zaefarian *et al.* 2013; Marchand *et al.* 2016), landfill leachate (Yang *et al.* 2017). This plant has a root framework appropriate for the petroleum hydrocarbons (PHC) rhizodegradation (Wang *et al.* 2012) and can add to trace elements (TE) phytostabilisation (Zribi *et al.* 2015). Alfalfa is widely developed as feedstock for its high shoot yield and life span (Campanelli *et al.* 2013).

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In conjunction with the previously mentioned solutions, the addition of farmyard manures, such as cow and sheep manures, as well as compost, can reduce the mobility and uptake of heavy metals in soils and crops (Pichtel and Bradway 2008). Cow manure is rich in organic materials and contains essential nutrients for crop production. Its use as a source of fertilizer is a very common practice. Therefore, it has been widely used as a natural amendment to enhance soil fertility (Kapkiyai *et al.* 1999) and increase crop yield The use of farm manure, cow and sheep manure and compost reduces mobility and absorption of pollutants in soils and crops (Pichtel and Bradway 2008; Kiran *et al.* 2017). Cow manure is rich in organic matter and contains nutrients essential for crop production. The application of manure as a source of fertilizer is a very common practice. As a result, it has been extensively used as a natural amendment to improve soil fertility and increase crop yields (Jokela 1992; Kapkiyai *et al.* 1999). Manure amendment can improve also, the biodegradation of natural mixes and decreases the versatility of certain metals (Ruttens *et al.* 2006).

No question organic matter (OM) is good for alfalfa, in absence of other fertilizer. But Zhang *et al.* (2001) grew good yields of alfalfa with only water and essential nutrients added in small amount on a silty sand with 0.5% OM. Comparing DCM and MTBE, DCM is 60 times more volatile than MTBE at 25 °C. Zhang *et al.* (2001) quote a half-length of MTBE in the range of 20 cm from soil surface, so DCM would be gone likely below ground. At most, it might affect the root zone.

In this work, we evaluated the performances of alfalfa plant and its capability to resist and remove DCM from a polluted soil. Studies of germination, root and shoot measurements and biomass were carried out regarding DCM, to give a better understanding on growth and morphological effect processes in alfalfa plant in the presence of organic fertilizers (cow and sheep manure), and for two different concentrations of DCM which are 50 μgL^{-1} and 84 mg L^{-1}.

Materials and methods

Soils, manures and solutions

The sample of soil used in the experiments, as well as the manures, were collected from an agricultural area near the industrial zone in the region of Sousse in Tunisia. The soil sample was taken from the upper 10 cm thickness layer under the first soil layer. Immediately after collection, it was transported to the laboratory in dry self-sealing and clean bags, where it was firstly sieved to remove large particle and get a homogeneous texture. Afterwards, the particle size distribution of the sample was measured by laser diffraction particle size analyzer (Mitrotrac S3500). And the textural analysis was determined using the Robinson pipette method and the hydraulic parameters of were obtained using cell compression and mini disk infiltrometer (Decagon Devices). As for The seeds, they were obtained from the Tunisian Seed Control Agency

The dichloromethane (DCM) used as a chemical pollutant was purchased from Sigma-Aldrich. It is a highly volatile and colorless liquid with an ethereal odor that can be detected at 200–300 ppm. It is also slightly soluble in water (13–20 g L^{-1} at 20 °C) but miscible with several organic solvents. It dissolves in many products like oils and resins.

Seed germination

The seeds germination capacity of alfalfa plants is the first physiological process affected by the presence of pollutants in soils (Mihoub *et al.* 2005). Therefore, we conducted a seed germination test to study the effect of dichloromethane concentrations on plant seeds. The test was established using uniform-sized, disinfected and no *n*-contaminated seeds (Control), and DCM contaminated seeds with concentrations of 84 mg L^{-1} (C_1), 10 mg L^{-1} (C_2) and 50µg L^{-1}(C_3). The contaminated seeds were placed in flasks filled with DCM solutions at different concentrations for 24 h. The vials were tightly closed to prevent volatilization of DCM. Three replicates had been created for each experiment. We deposited 63 seeds in each Petri dish which were kept under continuous light conditions in grow room for a six days. Germination length in each Petri dish was counted and the corresponding phenotypes were photographed (Figure 1).

Considered germination parameters are calculated as follows:

- Germination percentage (GP) is an estimation of germinated seeds versus total seeds.

$$GP = \left(\text{germinated seeds/total seeds}\right) \times 100$$

- Germination index (GI) is an index of seed germination speed and was calculated according to the following equation:

$$GI = \sum D_i N_i / S$$

where D_i is the number of days after sowing; N_i is the number of seeds germinated in day i; S is the total number of seeds planted.

- Seedling mortality (SM) is calculated using the following formula:

SM = (number of non-germinated seeds/numbers of days)*100

2.3. Pot experiment

For these experiments, we placed plastic pots filled with 2 kg dry soil and soil-manure mixture in the greenhouse for duration of 15 weeks. Next, the dichloromethane was diluted in ethanol and poured into the pots. Planting of the alfalfa seeds, was carried out 4 days after soil contamination.

The experiments consist of 3 treatments with 6 replicates for each treatment and the soils used are as follows: (i) soil (*S*), (ii) soil mixed with cow manure (1.3%w/w) (SCM), (iii)

S	S+CM	S+SM	C_1-S	C_1-S+ CM	C_1-S+ SM	C_2-S	C_2-S+ CM	C_2-S+ SM
S	S+CM	S+SM	C_1-S	C_1-S+ CM	C_1-S+ SM	C_2-S	C_2-S+ CM	C_2-S+ SM
S	S+CM	S+SM	C_1-S	C_1-S+ CM	C_1-S+ SM	C_2-S	C_2-S+ CM	C_2-S+ SM
S	S+CM	S+ SM	C_1-S	C_1-S+ CM	C_1-S+ SM	C_2-S	C_2-S+ CM	C_2-S+ SM
S	S+CM	S+ SM	C_1-S	C_1-S+ CM	C_1-S+ SM	C_2-S	C_2-S+ CM	C_2-S+ SM
S	S+CM	S+ SM	C_1-S	C_1-S+ CM	C_1-S+ SM	C_2-S	C_2-S+ CM	C_2-S+ SM
Without DCM treatment			C_1=84 mg l^{-1}			C_2=50 µg l^{-1}		

Figure 1. Effects of different concentrations of DCM pretreatments for 12 h on the inhibition of alfalfa seed germination: (A) without treatment, (B) C = 10 mg/l, (C) C = 50 µg/l and (D) C = 84 mg/l.

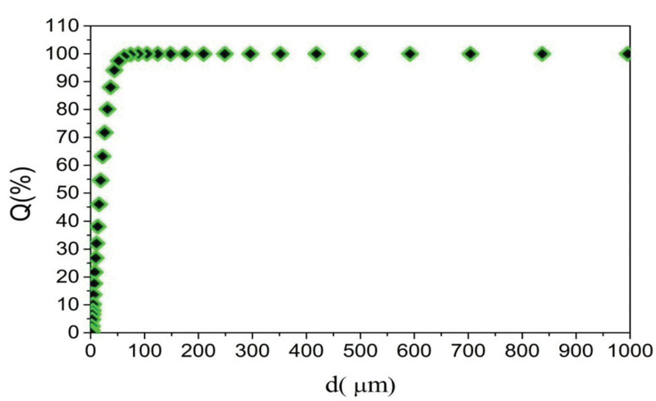

Figure 2. Pot experiment.

soil mixed with sheep manure (1.3%w/w) (SSM). Eighteen plants were maintained under optimum growing conditions without being contaminated with DCM. A second set of plants was implanted in a contaminated soil with a concentration of 80 mg L^{-1} (C_1), where we conducted treatments SCM-C_1 and SSM-C_1. And the third was treated with 50 µg L^{-1}(C_2), which constituted treatments SCM-C_2 and SSM-C_2 (Figure 2).

After filling the pots with the different types of soil, the dichloromethane were diluted in ethanol and added to the pots. Seedling of alfalfa seeds were carried out 4 days after pots contamination. The experiment was performed under normal conditions with day temperature ranging from 19–22 °C and light exposure for a period of 12 h. The shoots lengths of alfalfa plants for all treatments were measured every week. After growing for 105 days, the roots and shoots of control and DCM exposed were collected and the primary roots lengths as well as shoots length were determined.

2.4. Measurement of chlorophyll content

By means of spectrophotometer analyses (ZUZI spectrophotometer model 4201/50) we measured the chlorophyll and carotenes contents in leaves. The samples were homogenized with acetone (0.05/2 w/v), filtrated in a dark environment, and then measured at the wavelengths of 646.8 nm ($A_{646.8}$), 663.2 nm ($A_{663.2}$) and 470 nm (A_{470}). In order to estimate the chlorophyll a (Chl^{-a}), b (Chl^{-b}) and carotenes contents in the leaves, we used the following equations and the results are expressed as µg g^{-1} of fresh weight.

$$C_{Chl-a} = 12.25A_{663.2} - 2.79A_{646.8}$$

$$C_{Chl-b} = 21.5A_{646.8} - 5.10A_{663.2}$$

$$C_{(x+c)} = (1000A_{470} - 1.82C_{Chl-a} - 85.02C_{Chl-b})/198$$

Statistical analyses

The data illustrated in the figures below, represents the average of three replicates of different treatments (average ± SD). All investigated parameters were expressed as averaged values ± standard deviations: statistical significance was accepted at $p < 0.05$.

Sorption experiment and analyses data

To evaluate the adsorption capacity of dichloromethane in three adsorbents (soil, soil–cow manure mixture and soil–-sheep manure mixture) we designed an experiment to get adsorption isotherms. The isotherms were achieved by using a set of glass flasks (60 ml) containing 20 ml of dichloromethane solutions. 1 g of adsorbents added to the DCM solutions with different concentrations (10, 50, 80, 100, 150, 200 and 250 mg/L) was kept under stirring speed of 450 rpm for 2 hours. Then the supernatants were filtered, centrifuged and analyzed using gas chromatography (Agilent technologies 7890A). All analyses were carried out in triplicate. The amount of DCM adsorbed per unit weight of adsorbent at equilibrium, q_e(mg g^{-1}) was calculated as follows:

$$q_e = \frac{(C_0 - C_e)V}{m} \quad (1)$$

where C_0 and C_e are the initial concentration and liquid-phase concentrations of DCM solution at equilibrium (mg L^{-1}) respectively, V is the DCM volume (L) and m is the mass of the adsorbent (g). Adsorption isotherm models provide important information about physicochemical process, sorption capacity and the solution molecules distribution between the liquid and solid phases. The isotherm models represent a relation between the amount of a solute adsorbed at a constant temperature and its concentration in the equilibrium solution. To examine the performance of models to predict the adsorption data, several isotherm models were employed, such as: Langmuir (Langmuir 1918), Freundlich (Freundlich 1947), Brouers–Sotolongo (Brouers *et al.* 2005) and Hill–Sips (Sips 1948). The non-linear forms of the Freundlich (Equation (2)), Langmuir (Equation (3)), Brouers–Sotolongo (Equation (4)) and Hill–Sips (Equation (5)) models are given as follow:

$$q_e = k_f(C_e)^{1/n} \quad (2)$$

$$q_e = \frac{q_m b c_e}{1 + b c_e} \quad (3)$$

$$q_e = q_m(1 - \exp(-k_{BS} C_e^{n_{BS}})) \quad (4)$$

$$q_e = q_m(1 - \left(1 + (\frac{C_e}{b_{hs}})^{n_{hs}}\right)^{-1}) \quad (5)$$

k_F: Freundlich isotherm constant [(mg g^{-1})(L mg^{-1})((1 − n)/n)]; n: Freundlich exponent; q_m: Langmuir adsorption capacity (mg g^{-1}); b: Langmuir isotherm constant (L mg^{-1}); k_{BS}, n_{BS}: Brouers–Sotolongo constants; k_{hs}, n_{hs}: Hill–Sips constants.

The Freundlich isotherms model predicts multilayer adsorption and describes equilibrium on heterogeneous surfaces (Freundlich 1947; Gimbert *et al.* 2008). This Langmuir model assumes that the forces of interaction between the adsorbed molecules are negligible (Gimbert *et al.* 2008), there is a single layer of adsorbate on the outer surface of the adsorbent (Langmuir 1918) and no further adsorption will take place if the molecule occupies the adsorption site.

The BS model suggests the existence of a heterogeneous adsorption energy landscape (Ncibi *et al.* 2008). Hill–Sips (HS) isotherm model (Equation (5)) is a combined form of Langmuir and Freundlich expressions deduced for predicting the heterogeneous adsorption systems and circumvent the limitation of the rising adsorbate concentration associated with Freundlich isotherm model (Ahmad *et al.* 2015). At high-adsorbate concentration, it predicts monolayer adsorption characteristics of Langmuir, while in low adsorbate concentration, it reduces to Freundlich isotherm (Sips 1948).

Results and discussion

Soil characterization

The grain size distribution curve of the sample tested is shown in Figure 3. The particle size analysis of soil sample shows that the fifty percent passing particle size (d50) was calculated as19μm.This shows that is a silty soil. This result is confirmed by the Robinson pipette method analysis which proves that the soil sample contains 6% of sand, 19% of clay and 72% of silt. The hydrodynamic parameters of three soils are recapitulated in Table 1.

Effect of DCM concentration on germination efficiency and growth

Figure 1 shows the germination test results. Indeed, the numbers of germinated seeds in relation to the total numbers of seeds are almost equal for the three concentrations and close to those found for the control seeds. The result shows the low susceptibility of germination capacity of the alfalfa seeds to the DCM contamination and that it is the concentration of contaminant. So, both concentrations (the strongest and the lowest) were chosen for the rest of the work in order to test the effect of dichloromethane on the growth of alfalfa plants.

Data of germination percentage (GP), seed germination index (SGI) and seeds mortality (SM) of Alfalfa plant are illustrated in (Figure 4). GP of seeds was affected upon applying different concentrations of DCM (El-Darier and Zein El-Dien 2011). However, the increase in concentration does not have a significant effect on the germination percentage but, they affect significantly the germinal length of alfalfa plant (Figure 5).

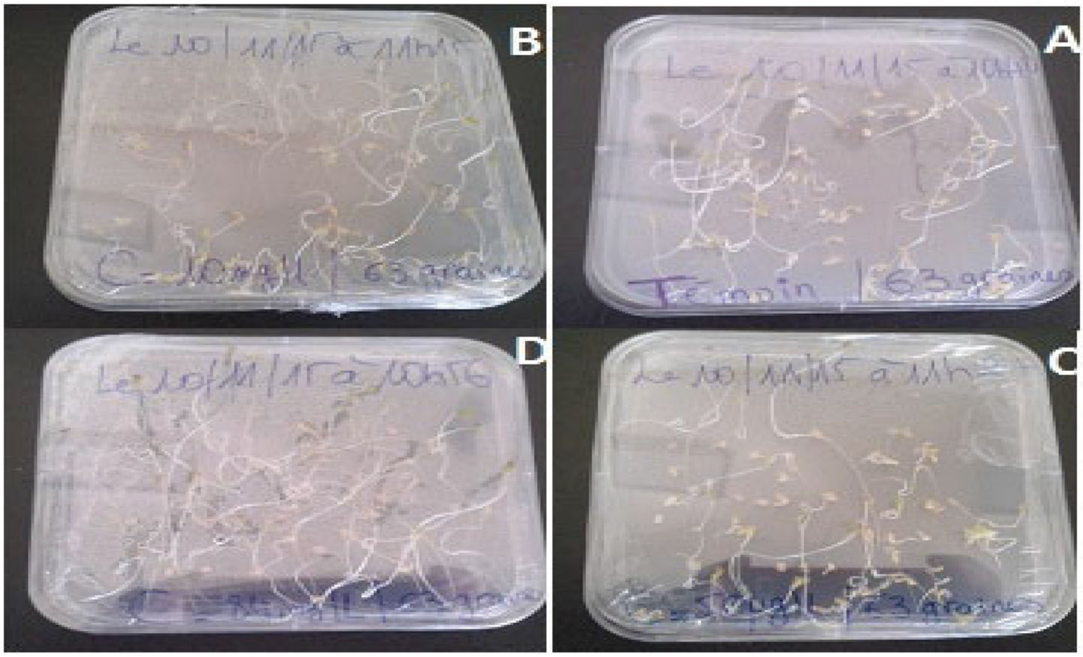

Figure 3. Particle size analysis of soil.

Table 1. Hydrodynamic parameters of soil.

Sand (%)	Clay (%)	Silt (%)	θ_r	θ_s	K_s (m/s)	α (cm^{-1})	n
6	19	72	0.0847	0.483	$5.6.10^{-7}$	0.01466	2.351

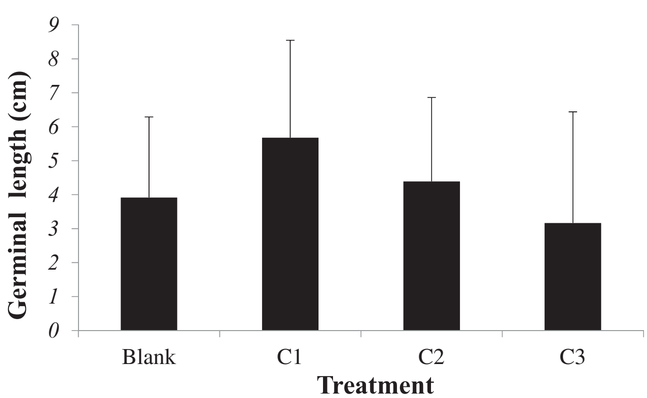

Figure 4. Germination parameters for all treatment.

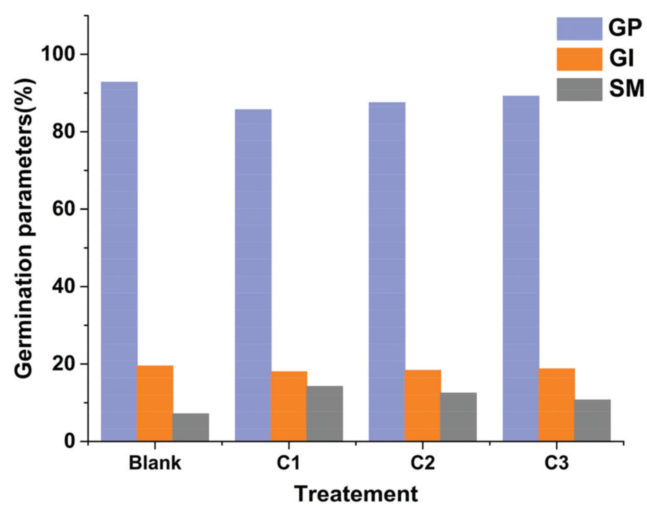

Figure 5. Germination length of alfalfa for all treatment.

Regarding GI, the value decreased distinctly as DCM concentration increased. Initially, GI began with value of about 56 at 84 mg L^{-1} DCM concentration. Continuously, higher GI values (57.16 and 58.33) were obtained at 10 mg/l and 50 µg L^{-1}DCM concentrations respectively. SM is proportional with increasing DCM concentration (Figure 4) as Rekik *et al.* (2017) found in the case of irrigation of alfalfa with wastewater.

Morphological effects of DCM on alfalfa seedlings development

Alfalfa seedlings growth, root length ($p < 0.05$) and shoot length ($p < 0.05$) were significantly decreased when 80 mg L^{-1} DCM concentration were applied. Root and shoot length, fresh and dry weight of roots and shoots were examined in the plants after 15 weeks of culturing. The size and mass of alfalfa plants was affected by the exposure to DCM. The inhibitory effects were more pronounced with the highest dose of DCM. Sharifi *et al.* (2016) have shown that the toluene concentration of 450 mg L^{-1} has caused significant detrimental effects on the root growth of alfalfa plants.

The shoot length was decreased in alfalfa plant exposed to DCM when compared to unspiked control soil (Figure 6a). The plant grown in DCM spiked soil exhibited a more significant reduction in shoot length than in manures spiked soil. Also, the root length was significantly reduced in comparison with the solvent-free control treatment (Sharifi *et al.* 2016).

A significant difference could be noted between the growth rates of non-DCM-contaminated plants and DCM-treated plants. In the absence of dichloromethane in the

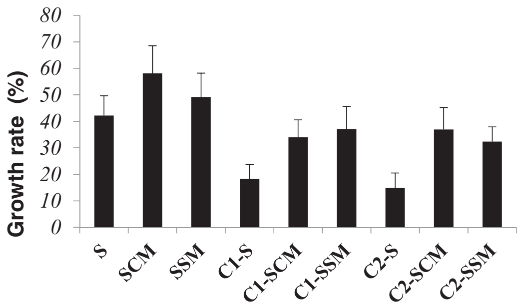

Figure 6. Growth rate of alfalfa.

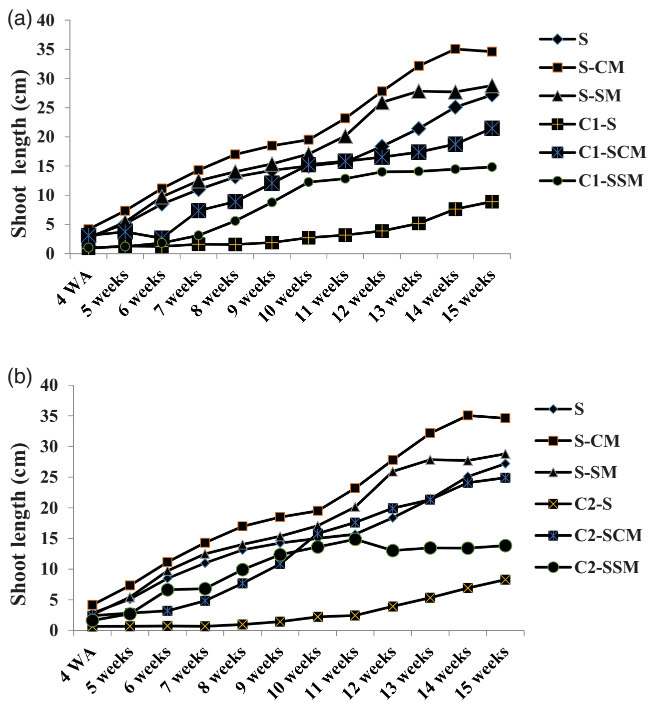

Figure 7. Shoot length of alfalfa plant contaminated with (a) C_1-DCM and (b) C_2-DCM.

pots containing the soil-manure mixture, the growth of the plants is better compared to that of existing plants in pots containing manure-free soils. Similarly, in the case of contamination with dichloromethane, there is a significant difference between the growth of plants planted in the soil-manure and those implanted in the soil without manure. In fact, in the event of contamination, the soil-cow manure mixture represents the best setting medium for the Alfalfa plant compared to other environments, whatever the level of contamination. Thus, the presence of manure in the control and contaminated soils promotes plant growth (Figure 7). Kiran *et al.* (2017) have shown that the application of cow manure have a positive effects on plant growth and biomass accumulation. Also, Elouear *et al.* (2016) have proved that the application of sheep manure could increase nutrients for plant growth.

Even for contaminated soil and concentration, soil rich in organic fertilizer is more resistant to the pollutant in the soil and adsorbs less polluting particles. Therefore, minimizes the amount of pollutant absorbed by plants on the one hand and enriches the plants to be more resistant to contamination on the other hand (Zhongqi *et al.* 2016). Indeed, the

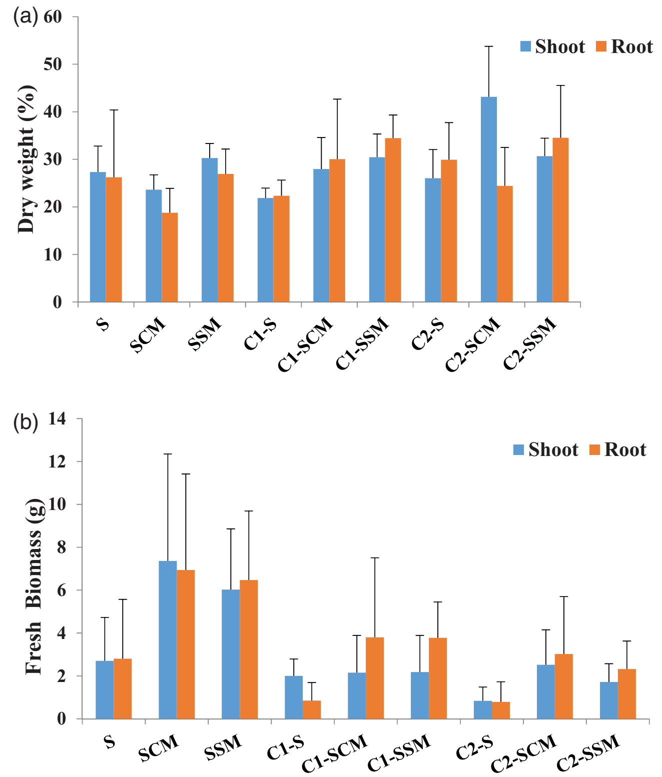

Figure 8. (a) Dry weight of shoot and root part of alfalfa, (b) Fresh biomass of shoot and root part of alfalfa.

presence of two types of manure does not allow the suppression of the inhibitory effect of dichloromethane on the mass of the dry matter of the aerial part which is 18.38% for the soil containing the cow manure and 13.96% for the soil containing sheep manure. In the absence of manure, the rate of reduction of the dry matter of the aerial part is 20.02% for the dichloromethane concentration treatment of 84 mg L^{-1} and 4.75% for the treatment of dichloromethane concentration of 50 μg L^{-1} (Figure 8). The low percentage of the dry matter (not more than 55%) can be explained by the dryness and the bad state of the plants. In fact, the presence of manure significantly increases the biomass of the aerial part and the root part of Alfalfa plants grown in soils contaminated with dichloromethane (1.18 and 1-fold higher for the dichloromethane concentration of 84 mg L^{-1}, 1.8 and 1.01 times higher for the dichloromethane concentration of 50 μg L^{-1}.

Physiological effects of DCM on alfalfa seedlings (chlorophyll content)

DCM effects on alfalfa chlorophyll pigment and carotenoid contents were estimated (Figure 9).The exposure of alfalfa seedlings to DCM resulted in a reduction of chlorophyll content in leaves. The deleterious effect of DCM became more pronounced with increasing concentrations .In plants exposed to 84 mg L^{-1} DCM concentration, Chl-aChl-band decreased by 31.34% and 54.38%, respectively compared to control plants cultivated in soil-cow manure mixture. The similar trend were displayed for the same DCM concentration, Chl-aChl-band decreased by 66.44% and 33.67%,

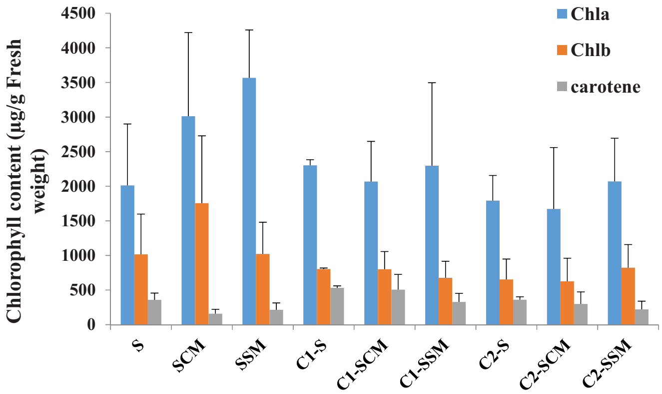

Figure 9. Effect of DCM treatment on Chl-a and Chl-b and carotene content of Alfalfa leaves after 15 weeks exposure to both concentrations of DCM.

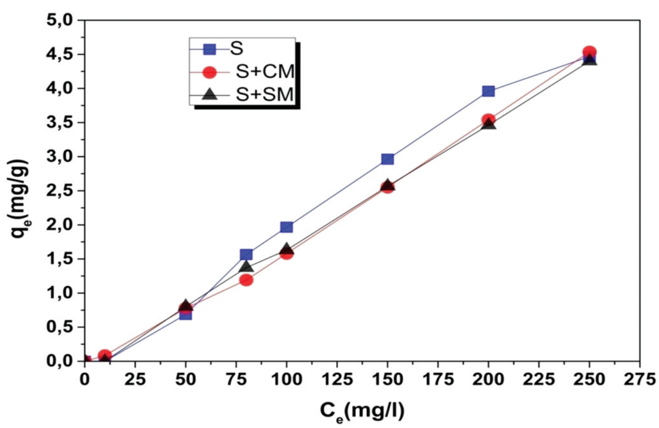

Figure 10. Adsorption isotherm of dichloromethane in three adsorbents.

respectively compared to control plants cultivated in soil-sheep manure mixture. However, the DCM damage effect appears significantly in alfalfa plants cultivated in soil sheep manure than cultivated in soil cow manure mixture.

Rekik *et al.* (2017) have shown that chlorophyll a (chl a) and chlorophyll b (chl b) levels in alfalfa ($p < 0.01$) were significantly different when irrigated with treated wastewater and untreated wastewater. Different behaviors of carotenoid contents have been noticed according to the medium used (Figure 9).

Adsorption experiments and data analyses

Figure 10 shows the adsorption isotherm of DCM in three adsorbents. The DCM adsorption in silty soil–cow manure mixture is the best compared to the others adsorbents.

The good fitting of the model with the experimental data is represented by a high value of correlation coefficient. For soil adsorbent, the Hill-Sips model provides a better fit in the DCM adsorption isotherm. This model is a combined form of Langmuir and Freundlich equations (Foo and Hameed 2010). The adsorption removal of dichloromethane onto activated carbon composite were well described by Freundlich adsorption isotherm (Alhooshani 2019). Figure 11 demonstrates the fit of the adsorption data of DCM on silty soil, sheep and cow manures by the models

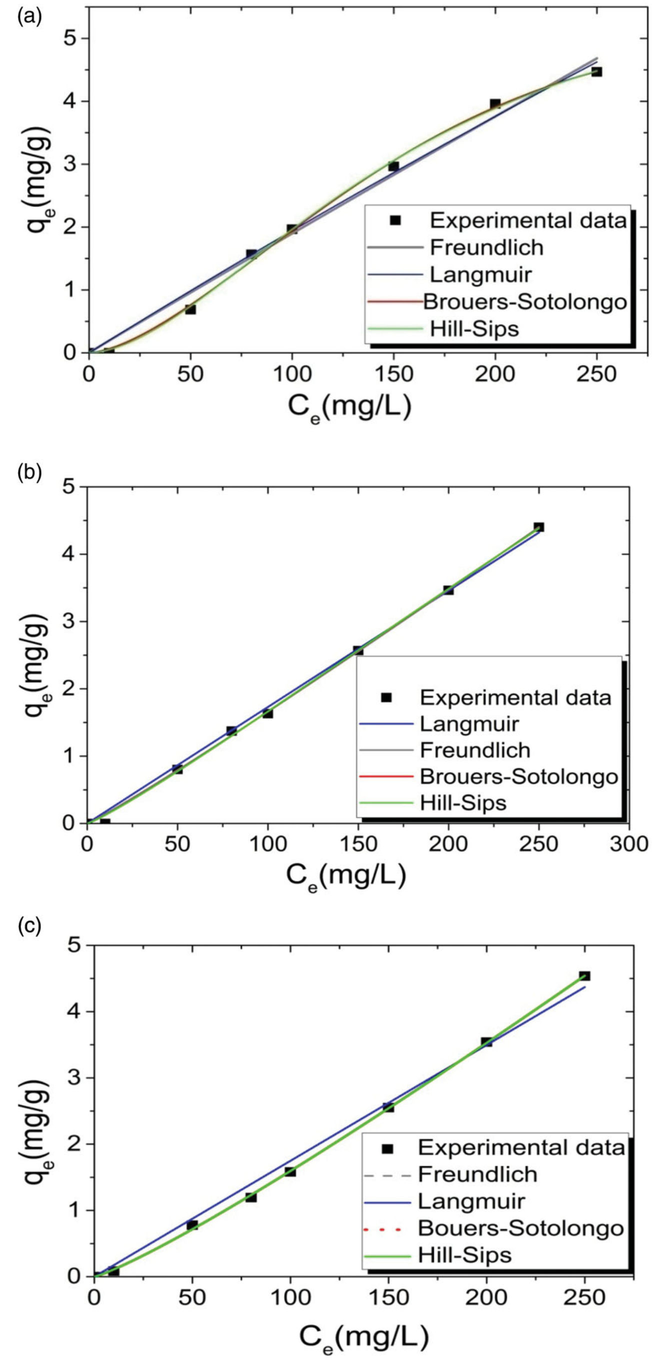

Figure 11. Non-linear fits of isotherm by several isotherm models for the adsorption of DCM in (a) silty soil, (b) soil–sheep manure mixture, (c) soil–cow manure mixture.

listed in the experimental section (i.e., Freundlich, Langmuir, BS and HS). The corresponding models parameters and the R^2 values are listed in Table 2. Based on the correlation coefficient all models were suitable for fitting MB adsorption isotherms. Freundlich and HS models seemed to be more adequate.

Conclusion

Data obtained during this 15 week experimental trial proved that the addition of manure in soil-plant system showed positive effects on alfalfa growth, survival rate and shoot

Table 2. Isotherm parameters of Freundlich, Langmuir, Brouers–Sotolongo and Hill–Sips models fitted to the adsorption data of dichloromethane in three adsorbents.

	Soil	Soil-SM	Soil-CM
Freundlich			
k_F	0.0203	0.0123	0.00825
n	1.014	0.939	0.874
R^2	0.9874	<u>0.9983</u>	<u>0.9995</u>
Langmuir			
q_m	5.838	4.591	6.745
b	3.442×10^{-4}	3.77×10^{-4}	2.593×10^{-4}
R^2	0.9884	0.996	0.9923
Brouers–Sotolongo			
q_m	5.198	21.507	7.891
k_{bs}	3.435×10^{-4}	4.324×10^{-4}	9.657×10^{-5}
n_{bs}	1.567	1.135	1.163
R^2	0.9978	0.9981	<u>0.9995</u>
Hill-Sips			
q_m	6.652	3.777	15.704
b_{hs}	0.006	6.752×10^{-4}	1.947×10^{-4}
n_{hs}	1.742	1.141	1.162
R^2	<u>0.998</u>	0.9981	<u>0.9995</u>

The underlined values represent the best correlation coefficient values.

DCM concentrations. In fact, in the event of contamination, the soil–cow manure mixture represents the best setting medium for the Alfalfa plant compared to soil and soil–sheep manure mixture. The information obtained at this laboratory scale provides important data for future studies on the phytoremediation of DNAPL contamination using the alfalfa plant.

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