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EFFECT OF CRUDE OIL SPILLAGE ON THE FOLIAGE ANATOMICAL CHARACTERISTICS OF PIGEON PEA (*CAJANUS CAJAN* (L.)) MILLSP IMPLICATION FOR PHYTOREMEDIATION

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Abstract

The effect of crude oil pollution on the foliage anatomical characteristics of C. cajan Millsp was investigated. Seedlings were polluted with 20 ml, 40 ml, 150 ml and 750 ml (separate) of crude oil, 60 days after planting (DAP). Sections (μ) microns of the leaves of both polluted and unpolluted (control) plants, harvested at 63 and 106 (DAP), were examined (x 400 magnification) to determine the thickness of the leaf, thickness of the adaxial and abaxial epidermises, the number of stomata and the stomatal pore dimensions at the adaxial and abaxial leaf surfaces. The number of palisade and spongy cells per field view and the number of chloroplasts per palisade and spongy cells were counted, while the length and the diameter of xylem lumen were measured. The results showed that pollution with 20 ml to 150 ml of crude oil significantly ($p < 0.05$) increased the leaf thickness, number of spongy cells (40 and 150 ml), chloroplasts (40 – 750 ml), stomata (150 and 750 ml), stomatal pore width at abaxial leaf surface (150 and 750 ml), size of xylem lumen length and width (40 and 150 ml) for 106 DAP but not for 63 DAP. However, 750 ml of crude oil significantly ($p < 0.05$) reduced the thickness of the leaf, adaxial and abaxial epidermal thickness, number of spongy and palisade cells, chloroplast per spongy cell (106 DAP only), number of stomata at abaxial leaf surface (106 DAP only) than other treatments. C. cajan neither showed any serious toxicity symptoms nor was it poisoned or killed by crude oil pollution, instead, the growth in different cells was enhanced. The ability of C. cajan to withstand crude oil pollution is an indication of the possible use of the plant for phytoremediation of crude oil polluted soil.

Keywords: Crude oil, Cajanus, Phytoremediation, Leaf anatomy

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Introduction

The response of plants to oil pollution can manifest itself at various levels (physiological, biochemical and molecular) (Achuba and Ja-anni, 2018). The adverse effects of crude oil spillage on land and economic plants have been reported by Essien and John, 2010. Ramirez *et al*. (2017) reported that at high concentrations of oil in soil, most plant species suffer serious depression in growth. According to these workers, this problem has been attributed to poor soil conditions, dehydration and impaired nutrient uptake by the roots, created by the presence of crude oil. In Nigeria, a substantial quantity of crude oil is spilled annually, for example, Vidal (2010) reported that 1.89 million barrels of crude oil spilled into the Niger Delta between 1976 and 1996, out of a total of 2.4 million barrels that spilled in 4,835 incidents.

Bruederle and Hodler (2019) reported that 5400 spills occurred between 2000 and 2004, an average of 1080 spills per year in Niger Delta. Baird (2010) reported that between 9 million and 13 million barrels (1.5 million tons) of crude oil have been discharged in the Niger Delta since 1958 from over 7000 oil spill incidents, a yearly average of about 240,000 barrels.

Considering this large quantity of crude oil spillage into the environment, especially farm lands, and the fact that the inhabitants of these areas are subsistent farmers, there is an urgent need for the various agencies involved with oil production in Nigeria to pay urgent attention without delay to the problems of oil leakage in the future. It is known that root stress reduces leaf growth via stomata conductance (Yanisa *et al*, 2021). Furthermore, Oladayo *et al.* (2019) reported that mineral ions absorbed initially by the root are finally received by the mesophyll cells of the leaves.

According to Elena *et al*. (2020), growth of plants in oil polluted soil was generally retarded and chlorosis of leaves results, coupled with dehydration of the plants indicating water deficiency. Leaf anatomy is an important feature for internal water balance of plants.

Gilyazov *et al*. (2019), reported that the leaf anatomical characteristics of spring wheat were drastically reduced due to application of crude oil. The structure and ontogeny of stomata in different plant will vary with the application of abattoir waste water in crude oil (Achuba and Ja-anni, 2018).

Mostafa *et al*. (2021), observed that leaf anatomical structures of *Azolla Pinnata* R.Br. were adversely affected by crude oil pollution. Crude oil pollution reduces the overall photosynthetic activity and chlorophyll contents in plants (Athar, *et al*, 2016; Hajihashemi *et al,* 2020). The most dangerous disorder that resulted from the effect of pollutants, including crude oil and petroleum hydrocarbons on plant is oxidative stress (Maslennikov *et al*, 2018;

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Zerid and Wani 2019). According to these authors, stress leads to the formation of many reactive oxygen species (ROSs) with high oxidizing capacity in cells (superoxide radical (O_2), H_2O_2, hydroxyl radical (OH), etc. Poljsak and Fink (2014) showed that, the ROSs destroy cell membrane complexes, disrupt transport processes and intracellular reactions, and thereby inhabit growth activity. Kreslavski *et al*. (2017) reported that when poly cyclic aromatic hydrocarbon enters through the stomata of leaves, it interferes with the metabolic processes in plants thereby reducing transpiration and photosynthetic rates.

Cajanus cajan is indigenous to India. It is a drought resistant, heat tolerant member of Fabaceae. Uses of *Cajanus* includes its use both as food and forage crop. It is also used in the prevention and treatment of many diseases. *Cajanus* can as well be used for cleaning contamination such as land fill leachate containing chromium and spent engine oil.

Phytoremediation is the use of certain plants to clean up soil, sediment and water contaminated with metals and / or organic contaminants such as crude oil, solvents, and poly aromatic hydrocarbons. It is an aesthetically pleasant mechanism that can reduce remedial costs, restores habitat and clean up contamination in place rather than entombing it in place or transporting the problem to another site (Marchiol *et al.*, 2007). Odiyi *et al*. (2020) emphasized that plants that have the ability to phytoremediate a polluted site should exhibit little or no toxicity symptoms under pollutions, whereas those that cannot be used for Phytoremediation are easily poisoned and killed by little pollution. The objective of this work was to investigate the effects of crude oil pollution on the foliage anatomical characteristics of *C. cajan*

MATERIALS AND METHODS

A total of 240 blacks, perforated polybags (34, 570 cm^3) were each filled with 16 kg of top soil, collected at a dept of 10 cm, from the Botanic Garden, University of Nigeria Nsukka. The top soil was mixed with poultry manure at a rate of 2:1. Two sets of 120 Polybags were prepared and labelled A and B respectively. Each bag of A had one seedling while B had no seedlings.

Sixty days after seed sowing, 24 polybags each (in experiment A) except the control were spilled with 20 ml (0.1 v/w), 40 ml (0.2 v/w), 150 ml (0.6 v/w) and 750 ml (3.0 v/w) of crude oil respectively. Also in experiment B, the first four batches of 24 polybags each were spilled with the same volumes of crude oil as in A, while the last batch of 24 poly bags served as the control. The crude oil used was abstained from shell Petroleum Development Company, Igurunta Port Harcourt, River State, Nigeria.

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The experiment was carried out in 2022 in a completely randomized design in 24 replicates. The leaves of polluted and unpolluted plants were collected randomly at 63 and 106 DAP, washed thoroughly with water and fixed in a formalin. The leaves were embedded vertically and transverse sections (10μ) were cut with the aid of a sledge microtone (Reichert Austria Nr 335, 674) and stained with safaranin for 10 minutes. The safaranin was drained off, washed 3 times with distilled water, then washed again with 70% alcohol, counter stained with fast green for 10 minutes, washed with absolute alcohol for 3 to 4 times and washed with pure xylene until they became clear.

The sections were observed under calibrated (using stage and ocular micrometer) photo microscope. The thickness of the leaf, thickness of the adaxial and abaxial epidermises of leaf, size of the xylem vessel lumen of leaf were measure in microne (N), while the number of palisade and sponge cells per field x400 magnification were counted. The epidermal peels were obtained by smearing both the adaxial and abaxial epidemics of leaves with neril vanisher and allowed to stay for 30 minutes. Thereafter, the surfaces of the leaves were marked with transparent cello tapes and gently pressed. The cello tapes were carefully removed with the epidermal layers and mounted on a clean microscopic slide and then viewed under microscope. The number of stomata in adaxial and abaxial leaf surfaces per unit area at 400 magnification was counted using the formula of Metcalfe and Chalk (1979). The length and width of the stomatal pores of adaxial and abaxial leaf epidermises of eight (8) randomly selected stands from each treatment was measured. The data obtained were subjected to one-way analysis of variance (ANOVA) using the IBM STATISTICS SPSS (Statistical Products Services Solution) software, version 20. The significantly different means were separated using the least significant difference (LSD) ($P < 0.05$). The results were presented in tables

RESULTS

The results of the means thickness of the leaf of *C. Cajan* polluted with different volumes of crude oil stored that 40 – 750 ml, reduced significantly ($P < 0.05$) the mean thickness of the leaf at 63 DAP) while 40 ad 150ml significantly ($P < 0.05$) increased it at 106 DAP (Table 1.) Plants of the control and 20ml had leaf thickness that were the same at 63 DAP. At 106 DAP, the plants of the control and 20ml had leaf thickness that did not differ from each other. Table 1 showed that the mean thickness of the adaxial epidermal leaf of plants treated with 40ml to 750 ml of crude oil at 106 DAP were higher than the mean thickness of the leaves in 63 DAP for the same treatment. Control was significantly ($P < 0.05$) different from the plants treated with crude oil at 63 DAP, while at 106 DAP, control and 20ml produced adaxial epidermal thickness that did not differ from each other but significantly different from those treated with 40 – 750 ml of crude oil. At 63 DAP (Table 1), the mean thickness of the abaxial epidermis of the control and plants treated with 20ml were significantly ($P < 0.05$) more than that of the

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other polluted plants, so, crude oil pollution significantly reduced the thickness of the abaxial epidermis 3 days after pollution, especially with heavier pollution (150ml and 750ml). However, at 106 DAP, the heavier pollution (150ml) had mean abaxial leaf thickness that was the same with the control. Also at 106 DAP, both the abaxial epidermis of the control and other treated plants were significantly thicker than those of 63 DAP. Plant polluted with 750ml had abaxial epidermis lower than the control and the other polluted plants at 106 DAP. At 63 DAP, crude oil had no effect on both the xylem lumen length and width, but at 106 DAP, increasing the volume of crude oil from 40 – 150ml significantly increased both the length and width of xylem lumen. Also at 106 DAP, the control and the plants treated with 750ml had xylem lumen length and width that were the same.

Table I: Thickness of leaf, epidermis and number of spongy and palisade cells of *C. cajan* polluted with different volumes of crude oil at 63 and 106 DAP.

Thickness of leaf (N)

Growth Stage DAP	O ml	20 ml	40 ml	150 ml	750 ml
63	0.043 ± 0.01^{a}	0.43 ± 0.00^{a}	0.41 ± 0.00^{b}	0.40 ± 0.00^{b}	0.38 ± 0.00^{c}
106	0.44 ± 0.01^{cd}	$0.47 + 0.001^{c}$	0.57 ± 0.01^{b}	0.60 ± 0.01^{a}	0.42 ± 0.01^{d}

Thickness of Adaxial epidermis (ju)

63	$0.05\pm 0.0o1^{a}$	0.04 ± 0.00^{b}	0.04 ± 0.00^{b}	0.40 ± 0.00^{b}	0.3 ± 0.00^{c}
106	0.52 ± 0.01^{c}	0.48 ± 0.00^{c}	0.62 ± 0.00^{b}	0.68 ± 0.00^{a}	0.46 ± 0.0^{d}

Thickness of abaxial epidermis (N)

63	0.04 ± 0.001^{a}	0.04 ± 0.00^{a}	0.03 ± 0.00^{b}	0.02 ± 0.00^{c}	0.02 ± 0.00^{c}
106	0.66 ± 0.00^{a}	0.05 ± 0.00^{b}	0.6 ± 0.00^{a}	0.06 ± 0.00^{a}	0.04 ± 0.00^{c}

Length of xylem lumen (N)

63	0.24± 0.01	0.21± 0.02	0.21±0.01	0.23±0.01	0.24± 0.05
106	0.25 ± 0.00^{b}	0.24 ± 0.00^{c}	0.27 ± 0.00^{a}	027 ± 0.00^{a}	0.25 ± 0.00^{b}

Width of xylem lumen (N)

63	0.15± 0.01	0.13± 0.00	0.12±0.00	0.13±0.01	0.15± 0.25
106	0.158 ± 0.02^{ab}	0.150 ± 0.00^{ab}	0.170 ± 0.00^{ab}	0.171 ± 0.00^{a}	0.147 ± 0.00^{b}

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Values represent means and standard error. Significant means were separated using LSD at PL 0.05. While means with the same letter (s) in the same column are not significantly different.

The mean number of spongy cells per field decreased 3days after pollution (63 DAP) in plants treated with 150ml and 750 ml, while control, 20 ml and 40ml had mean number of spongy cells per field that were the same. At 106 DAP, 46 days after pollution, control, 20ml and 40ml had mean spongy cells that were the same while 750ml decreased the mean spongy cells. As for the palisade cells per field at 63 DAP, 40ml and 150ml significantly ($P < 0.05$) increased the mean number while control and plants treated with 20ml and 750ml decreased it (Table 2). Crude oil did not have any effect on the control, 20ml and 40ml at 106 DAP but decreased significantly ($P < 0.05$) the mean palisade cells of plants treated with 750ml. However, judging from the values obtained at both 63 and 106 DAP respectively, plants of the 106 DAP, performed better in terms of palisade number.

Crude oil pollution with 150ml significantly ($P < 0.05$) decreased the mean number of chloroplasts per palisade cell when compared with the control. All other treatments did not change the number of chloroplasts per palisade cell at 63 DAP (Table 2). However, at 106 DAP, crude oil treatment with 150ml significantly ($P < 0.05$) increased the number of chloroplasts per palisade cell while all other treatments including the control did not change the mean number of chloroplasts per palisade cell. Crude oil treatment did not have any effect on the number of chloroplasts per sponge cell at 63 DAP but crude oil treatment with 40ml to 750ml significantly ($P < 0.05$) increased the number of chloroplasts per sponge cell at 106 DAP when compared with the control and plants treated with 20ml of crude oil.

Table 2: Number of spongy, palisade cells and chloroplasts in *C. cajan* polluted with different volume of crude oil at 63 and 106 DAP.

Number of spongy cells per field

Growth stage DAP	Oml	20ml	40ml	150ml	750ml
63	69.60 ± 0.00^{bc}	69.60 ± 0.25^{a}	69.60 ± 0.25^{a}	67.02 ± 0.15^{b}	60.38 ± 0.01^{c}
106	69.32 ± 0.01^{cd}	68.94 ± 0.01^{c}	71.34 ± 1.73^{ab}	71.78 ± 0.00^{a}	63.50 ± 0.00^{d}

Number of Palisade cells per field

63	15.36 ± 0.05^{c}	14.79 ± 0.08^{d}	17.58 ± 0.07^{b}	18.58 ± 0.01^{a}	13.94 ± 0.01^{d}
106	29.85 ± 0.10^{a}	29.74 ± 0.01^{a}	$26.94+1.01^{a}$	28.92 ± 0.26^{b}	27.76 ± 0.11^{c}

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Number of chloroplasts per palisade cell

63	13.13± 0.01[a]	11.60±0.02[ab]	12.13± 0.00[ab]	10.75± 0.03[b]	11.50± 0.01[ab]
106	16.13± 0.00[ab]	15.50±0.01[b]	15.88± 0.02[b]	17.88± 0.00[a]	15.00± 0.03[b]

Number of chloroplasts per spongy cell

63	4.50± 0.00[a]	4.25± 0.00[a]	4.38± 0.00[a]	4.60± 0.00[a]	4.13± 0.00[a]
106	4.00± 0.00[a]	4.00± 0.00[a]	5.01± 0.00[b]	5.06± 0.00[b]	5.07± 0.01[b]

Values represent means and standard error. Significant means were separated using LSD at PL 0.05. While means with the same letter (s) in the same column are not significantly different.

Crude oil pollution affected the production of stomata in the adaxial leaf surfaces at 63 and 106 DAP respectively. Only plants of the control and the plants treated with 20ml produced stomata while plants treated with 40ml, 150ml and 750ml had no stomata (Table 3). At 63 DAP, pollution with 20ml of crude oil significantly. ($P < 0.05$) increased the number of stomata while treatment with 40ml, 150ml and 750ml, significantly ($P < 0.05$) decreased the stomata number in the abaxial leaf surface. Pollution with 40ml and 150ml significantly ($P < 0.05$) increased the number of stomata in the abaxial leaf source while 20ml and 750ml decreased stomata number significantly ($P < 0.05$) when compared with the control at 106 DAP. At 63 and 106 DAP, as there were no stomata produced in the adaxial leaf surface, with the treatment of 40ml to 750ml, no stomata pore length and width were equally observed.

However, the control and plants polluted with 20ml of crude oil had stomata pore length and width that were wider at 106 DAP for length and 63 DAP for width. There were no significant changes in the stomatal pore length of the abaxial leaf surface at 106 DAP. Pollution with 750ml significantly ($P < 0.05$) decreased the stomatal pore length at 63 DAP when compared with the control and plant polluted with 40ml of crude oil. Crude oil pollution decreased the width of the stomatal pore significantly especially when the volume of the crude oil increased at 63 DAP. At 106 DAP, increase in the volume of crude oil from 150ml to 750ml also widened the stomatal pore width more than the control and plant polluted with 20ml and 40ml of crude oil.

Table 3: Number of stomata and stomatal pore length and width of *Cajanus cajan* polluted with difference volume of crude oil at 63 and 106 DAP

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Number of Stomata in the adaxial leaf surface

Growth stage DAP	Central	20ml	40ml	150ml	750ml
63	2.00±0.00 [a]	1.00+0.00[b]	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]
106	2.00±0.00 [a]	1.00+0.00[b]	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]

Number of Stomata in the abaxial leaf surface

63	7.00±0.33[b]	9.00±0.43[a]	1.00±0.33[a]	3.00±0.36[c]	2.00±0.39[d]
106	9.83±0.94[b]	1.92±0.26[d]	13.00±0.35[a]	12.00±0.46[a]	5.00±0.71[c]

Stomatal pore length of adaxial leaf surface (µ)

63	0.01±0.00[a]	0.01±0.00[a]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]
106	0.013±0.00[b]	0.014±0.00[a]	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]

Stomatal pore width of adaxial leaf surface (µ)

63	0.004±0.00[a]	0.003±0.00[b]	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]
106	0.01±0.00[a]	0.01±0.00[a]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]

Stomatal pore length of abaxial leaf surface (µ)

63	0.012±0.00[a]	0.011±0.00[a,b]	0.012±0.00[a]	0.011+0.00[a,b]	0.010±0.00[b]
106	0.01±0.00[a]	0.01±0.00[a]	0.01±0.00[a]	0.01±0.00[a]	0.01±0.00[a]

Stomatal pore width of abaxial leaf surface (µ)

63	0.004±0.00[a]	0.003±0.00[b]	0.002±0.00[c]	0.002±0.00[c]	0.002±0.00[c]
106	0.005±0.00[b]	0.005±0.00[b]	0.005±0.00[b]	0.006±0.00[a]	0.006±0.00[a]

Values represent means and standard error significant means were separated using LSD at PL 0.05, which means with the same letter (s) in the same column are not significantly different.

DISCUSSION

The results showed that the *C. cajan* was able to overcame the shock it might have experienced at 3 days of exposure to crude oil and increased in parameters such as thickness of leaf, thickness of adaxial leaf epidermis, length and width of xylem Lumen vessel, number of sponge cells, chloroplasts in both spongy ad palisade cells, number of stomata at abaxial leaf surface

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at, 106 DAP stomata pore length at abbatial leaf surface (63 DAP) and stomata pore width at 106 DAP. The ability of the plant to withstand high levels of crude oil (up to 750ml) spilled on it is an indication that it tolerated those levels of pollution. Since the plant did not die, it showed that it might have degraded the crude oil into non-toxic molecules which it utilized for growth. Therefore, crude oil might have demonstrated growth regulating properties of the plant. This is in agreement with work of Punwong *et al*. (2017) who showed that crude oil increased the cuticle thickness epidermal cell diameter at both adaxial and abaxial epidermis and palisade thickness of Terminalis catapa L. Daniel *et al*. (2019) reported that the number of sponge and palisade cells, size of stomata pore length and width and the stem thickness of Polypogonaustralis exposed to crude oil pollution increased within few days of exposure. Achuba and Ja-anni (2018), observed that the structure and ontogeny of stomata in different plants vary with the application of abattoir waste water in crude oil.

Fletcher *et al. (*2000), further emphasized that plant growth regulating properties of triazole are mediated by their interference with isoprenoid pathway and shift in the balance of plant hormones. The results therefore, showed that *C. cajan* may be efficient in phytoremediation, since most of these anatomical characteristics were enhanced especially when higher volumes of crude oil are used (at 106 DAP only) rather than being adversely affected by crude oil pollution. Plants that have the ability phytoremediate a polluted site should exhibit little or no toxicity symptoms under pollution, whereas those that cannot be used for phytoremediation are easily poisoned and killed by little pollution (Odiyietal, 2020). In the present study, *C. cajan* neither showed any toxicity symptoms nor was it poisoned or killed by crude oil pollution. *C. cajan* may be used for phytoremediation of crude oil polluted site. In phytoremediation or phyto-transformation, organic contaminants are absorbed inside the plant and metabolized to non-toxic molecules by natural chemical processes within the plant. These non-toxic molecules may be utilized by plant for growth (Nwadinigwe and Olawole, 2020). As the age of the plant advanced, the thickness of the leaves treated with 150ml and 40ml of crude oil increased more than that of the control in the present study. This is in agreement with the reported that crude oil pollution increased the thickness of Sorghum vulgare. The number of spongy cells in the leaves polluted with crude oil was higher than that of the control (40ml and 150ml at 106 DAP). This is in conformity with the work Daniel *et al*. (2019), who reported that the number of spongy and palisade cells of Polypogonautralis exposed to crude oil were increased. Crude oil also increased the thickness of cell wall of the parenchyma tissues in Mucuna (Omosun *et al*, 2009). This increase in the thickness of the cell wall of Mucuna caused the parenchyma cells to have thicker cell walls as a drought avoidance mechanism to reduce water loss (Omosun *et al*, 2009). The concentration of crude oil of 40ml and 150ml at 106 DAP increased the stomatal number in the abaxial leaf surface. This showed that the plant might have degraded the crude oil into non-toxic compounds and utilized them for increase in the number of stomata at that

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high concentration of crude oil. This may prove as well that *C. cajan* may be used for remediating crude oil polluted sites. This is in agreement with that of Verma and Chandra (2015) who showed that auto-pollution increased the number and size of epidermal cell and stomata of Sida cordifolia. However, the result is inconsistent with the report of Olaranont *et al*. (2018) who reported that adaxial stomatal densities of common beach grass were reduced on exposure of crude oil pollution compared to the control. At the abaxial leaf surface, the stomatal pore length was not affected by crude oil pollution. This is in agreement with the work of Dipu and Salom (2014), who reported that the stomatal pore length, cortex area, pith and fibre length of Abutilon indicum exposed to air pollution were the same as the control. However, the results showed that at the abaxial leaf surface, crude oil pollution of 150ml and 750ml increased the stomatal pore width more than the control. This may be as a result of the growth of the stomata or wider opening of the stomatal pore due to crude oil pollution. A wider stomatal pore allows more water and gases to pass through than a narrower pore. This is in agreement with the report of Daniel *et al* (2019), who reported that the stomatal pore width of Poly pogon australis increased on exposure to crude oil pollution. The result is in contrast with the report of Yanisa *et al.* (2021), who showed that a common coastal plant (Ipomoea Pre-Caprae) subjected to crude oil pollution was indifferent to the pollutant. The use of *C. cajan* remediating soil polluted with crude oil may be recommended, since crude oil pollution did not have adverse effect on the foliage anatomical characteristics. Rather the plant might have degraded and utilized the crude oil for growth in terms of leaf thickness, epidermis, length and width of xylem lumen, number of spongy cells, number of chloroplasts per spongy cell, number of stomata at abaxial leaf surface and stomatal pore width at abaxial leaf surface. Crude oil also may be useful as a growth regulator for *C. cajan*

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