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Tolerance and Sodium Ion Relations of *Paspalum conjugatum* Bergius (Sour Grass) to Water Soluble Fractions of Crude Oil

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Abstract: Plants not close to the sea could be exposed to water soluble fractions of crude oil by rainfall which could carry dissolved hydrocarbons to the groundwater by erosion and leaching, thereby making hydrocarbons available for plant uptake through roots. *Paspalum conjugatum* was exposed to different levels of Artificial Sea Water (ASW), Water Soluble Fraction (WSF) of oil and Sea Water Soluble Fraction (SWSF) of oil. Apart from decrease in tiller numbers, WSF had no significant (ANOVA, $p>0.05$) effect on growth parameters of *Pasplaum conjugatum*. However, 30% ASW and SWSF significantly ($p<0.05$) decreased, tiller numbers, height, shoot moisture content, shoot and root dry weight and mortality occurred at 50% ASW/SWSF. Results of sodium concentrations in ASW and SWSF showed that after 35 days there was no significant (ANOVA, $p = 0.07$) difference in Na^+ concentration between 10 to 50% ASW, while in SWSF, shoot Na^+ concentration increased with increasing salinity and at 50% SWSF, Na^+ concentrations exceeded that of 50% ASW by 23%. This suggests that PAHs present in WSF may modify the availability, absorption and/or passive uptake of Na^+ in *Paspalum conjugatum*.

Key words: Water soluble fraction, crude oil, tolerance, sodium, *Paspalum conjugatum*

INTRODUCTION

The adverse effect of oil on plants has been discussed by various researchers (Pezeshki and DeLaune, 1993; Lin and Mendelssohn, 1996, 2009; Pezeshki *et al*., 2000; Rosso *et al*., 2005). When spills of crude oil occur on bodies of water or land, even after the clean up, water-soluble components from the oil might remain and continue to exert their effects on the environment (Lee *et al*., 1974; Ziolli and Jardim, 2002). In small lakes, rivers and other inland ecosystems that receive frequent but relatively small spillages or continuous low level of oily discharges, the surrounding vegetation may be exposed to water soluble fractions (WSF, also referred to as SWSF if dissolved in seawater) of oil rather than the more spectacular oil slick. Sea Water Soluble Fraction (SWSF) of oil is an oil-seawater mixture containing a spectrum of dissolved, partially solvated or dispersed molecular and particulate aggregations of hydrocarbons, resulting from turbulence and wave action in oil polluted seawater (Amakiri, 1985). Water-soluble fractions is the organic enriched aqueous phase in contact with the oil spill and are phytotoxic (Ziolli and Jardim, 2002; Saco-Alvarez *et al*., 2008) however, information of toxicity effects of WSF to plants is limited. For example, Noyo *et al*. (2008) reported a decrease in the population of *Azolla africana* exposed to WSF, while Youssef (2002) observed irregular stomatal behavior and weak stomatal control during transpiration in leaves of *Avicennia marina* sprayed with WSF. Plants not close to the sea could also be exposed to WSFs of crude oil by oil activities carried out by man and rainfall which could carry dissolved hydrocarbons to the groundwater by erosion and leaching, thereby making hydrocarbons available for plant uptake through roots.

The importance of *P. conjugatum* has been discussed by Ibemesim (2010). In an earlier study, Ibemesim, (2010) showed that *Paspalum conjugatum* (sour grass) tolerated salinity levels up to 29% ASW (Na ~ 131 mM and K ~ 3.2 mM) and a combination of both salt and oil significantly decreased K^+ concentrations.

As *P. conjugatum* is also exposed to WSFs of crude oil, the present study hypothesizes that saline (SWSF) and non-saline water soluble fractions (WSF) of oil has an adverse effect on growth and affects uptake of Na^+ concentrations in *P. conjugatum*.

MATERIALS AND METHODS

Plant Stock Culture and Growth Conditions

Paspalum conjugatum seeds were obtained from the Royal Botanical Gardens, Kew, Wakehurst Place, Ardingly in 2004. Seeds were pre-germinated in trays filled with Sinclair Horticulture Limited (SHL) professional all purpose compost, obtained from William Sinclair Horticulture Limited. Seeds were watered with tap water for 2 weeks, after which germinated seedlings were transplanted into 180 mm (diameter)×200 mm (height) pots filled with John Innes No. 2 soil and silver sand mix (ratio 1:3). Pots were placed in ebb and flow tanks (a flood and drain system) measuring 1000 mm (length)×1000 mm (breadth)×200 mm (depth). Culture solution used was Hoagland solution and full strength contained (in mM) KNO_3, 1.2; $NH_4H_2PO_4$, 0.2; $MgSO_4$, 0.4 and $CaCl_2$, 1.0, together with micronutrients (in μM): H_3BO_3, 18.5; $MnCl_2$, 3.7; $ZnSO_4$, 0.3; $CuSO_4$, 0.13; $(NH_4)_6Mo_7O_{24}$, 0.14 and FeNaEDTA, 113.

Fifty percent Hoagland solution was pumped into these tanks every 30 min, which were allowed to remain flooded for 15 min before draining. The flood and drain action allowed for continuous aeration of the solution, which was changed every 2 weeks. The plants were illuminated with a minimum of 300 μmol/m/sec PAR, provided by supplemental lighting from 400-W HPI/T lamps and measured with a Licor-Quantum sensor (Li-185B) 30 cm above the pots, for up to 16 h light. Temperature ranged from 24-32°C during the day and 15-18°C at night. These conditions were maintained throughout all the experiments.

Source of Crude Oil

Wytch farm crude oil was provided by Oil Spill Response Limited (OSRL) Southampton. It had a specific gravity of 0.83 and was classified as light crude oil.

Preparation of SWSF and WSF

In all cases, SWSF and WSF were prepared from Wytch farm crude oil. In literature there is no uniformity in the experimental steps used by different researchers to obtain WSF from a crude oil sample and experiments are conducted in different ways. For the purpose of this study, a routine method was developed from a combination of methods used by Amakiri (1985), Ali *et al.* (1995) and Saeed and Al-Mutairi (2000). Full strength artificial seawater (ASW-salinity 35‰, chlorinity 19%, pH 6.5) was prepared, using Harvey (1966) recipe, in clean flat-bottomed 2 L Pyrex flasks. The 35‰ ASW contained 36 g L^{-1} of total dissolved salts and approximately 20 g L^{-1} of Chloride (Table 1 in Harvey, 1966). Only glass, Teflon coated or stainless steel equipment was used in sample preparation as these were easier to

Table 1: Concentration of Na and K in ASW/SWSF treatments for *Paspalum conjugatum* and concentrations (μg L^{-1}) of BTEX and PAHs in SWSF and WSF of fresh Wytch farm crude oil used in polluting seedlings of *Paspalum conjugatum*

	Salt concentration (mM L^{-1}) ASW/SWSF (%)		SWSF (μg L^{-1})		WSF (μg L^{-1})	
Treatments	Na	K	BTEX	PAH	BTEX	PAH
Control	0.3	3.00	0.0	0.0	0.0	0.0
10	45.8	1.12	1.4	0.5	1.4	0.2
30	137.0	3.36	4.3	1.5	4.1	0.4
50	229.0	5.60	7.1	2.5	6.8	0.7

Crude oil was extracted in 100% ASW for SWSF and distilled water for WSF. Control is fifty percent Hoagland solution. ASW = Artificial seawater, WSF = Water soluble fraction of oil and SWSF = Seawater soluble fraction of oil

clean and did not absorb oil. Nine hundred milliliters of full strength (100%) ASW was poured in 1 L brown glass reagent flasks (190 mm in height and 95 mm in bottom diameter) and then 100 mL of fresh Wytch farm crude oil was carefully poured with the aid of a glass beaker to form a thin layer (2 mm thick) on the surface of the seawater. A Teflon coated magnetic stirrer bar was added and flask was immediately sealed with a suba seal (Aldrich Chemical Co. Ltd., Dorset, UK) to minimize loss by evaporation of the more volatile components. A 20 mL syringe needle (50 mm long-gauge stainless steel) was inserted into the Suba seal close to the surface of the seawater to allow the addition of nitrogen. Another U shaped stainless steel tube (1000 mm long×1 mm outer diameter, from Advent Research Materials Limited, Eynsham, Oxford, OX29 4JA, UK) for the collection of SWSF, was inserted through the Suba Seal deep into the ASW until it was close to the bottom of the flask. The collection flask was placed on a support next to the fume cupboard. The flask containing the oil/seawater was placed on a magnetic stirrer inside a fume cupboard and stirred at an ambient temperature of 23± 2°C for 24 h. Stirring speed was adjusted so that the vortex did not extend more than 50% of the distance to the bottom of the flask (this was to avoid spillage). After stirring, oil and water phases were allowed to separate for 24 h. The SWSF was collected by applying a slight nitrogen pressure to the top of the oil/water surface through the needle, this caused the SWSF to rise through the stainless steel tube into the collection flask. The emulsion phase was not collected. When each collection was complete the flow of nitrogen was stopped and the stainless steel tube was raised to be above the oil/seawater surface. The WSF extracts were obtained by following the same procedure using distilled water as the extractant. Sample extracts from both WSF and SWSF were further analyzed by Gas chromatographic-Mass spectrometry (GC-MS) for BTEX concentration (Ibemesim, 2008).

Treatment

Treatments consisted of ASW, SWSF and WSF applied at the following concentrations: 0, 10, 30 and 50% for *P. conjugatum*. Each treatment had four replicates and a total of 48 potted plants were used. A maximum of half strength (50%) WSF and salt solution was used as previous results (Ibemesim, 2010) indicated that *P. conjugatum* could not tolerate saline levels above this concentration. The treatment concentrations were arrived at by serially diluting stock solutions of 100% ASW, SWSF and WSF with Reverse Osmosis (RO) water. The treatments were designed to reflect natural dilutions of oil-contaminated seawater with freshwater, where the concentration of both the compounds extracted from the oils and the salts change in concert-rather than changing salt concentrations in a constant concentration of oil extract or vice versa. Diluted neutral stock solutions were then amended by adding appropriate salts for half strength Hoagland or Yoshida solution. The change in concentrations of salts in ASW/SWSF and WSFs (BTEX and PAH) of crude oil in SWSF is shown in Table 1. The WSFs were applied directly to the soil surface and allowed to drain into the trays holding the pots. Plants were irrigated every 2 days with the treatment solutions during the experimental period (35 days). As the effect of salinity on growth parameters had already been determined in Ibemesim (2010) the following parameters were measured only in WSF treatments to determine if the water soluble extract of oil (without salt) had any effect on plant height, number of tillers, fresh weight (used in the calculation of moisture content) and dry weight. The Na^+ concentration was determined in shoots. In WSF treatments neither Na^+ nor K^+ concentration was determined as plants were not exposed to salt and it was assumed that WSF did not contain appreciable amount of salt. Plants were 56 days old at time of harvest.

Plant Height

Height of *P. conjugatum* was measured with a measuring tape, from soil to the tip of the longest leaf and expressed in centimeters (cm).

Number of Tillers

Number of tillers produced in each pot were counted and recorded.

Fresh Weight Determination

P. conjugatum plants were harvested and separated into shoots and roots with the aid of a scissors. Plant parts were rinsed with Reverse Osmosis (RO) water (to remove dirt and any adherence of WSF components on plant surface), blotted dry with paper towels (to remove excess water) and weighed on a Mettler 2000 weighing machine. Results were used in the calculation of moisture content (Ibemesim, 2010).

Dry Weight Determination

After fresh weight determination, plants parts were put in brown paper bags and dried in an oven at 80°C for 48 h. Dried plant material were weighed on a Mettler 2000 weighing balance and expressed in grams (g).

Statistical Analysis

In all experiments, results were analyzed statistically by using SPSS version 14 computer program, using one way Analysis of Variance (ANOVA), after conducting Levene's test of homogeneity of variance. Significant differences between means (at the 95% level) were then determined using the Tukey HSD test (Field, 2000).

RESULTS

Growth Response of *Paspalum conjugatum* to ASW, SWSF and WSF

At 30- 50% WSF, height of *P. conjugatum* was significantly ($p<0.05$) reduced by 14-20% compared to control (Fig. 1) however, WSF had no significant ($p>0.05$) effect on tiller production (Fig. 2). There was a 12 to 15% significant ($p<0.05$) decrease in shoot moisture content at 30 and 50% ASW and 50% SWSF relative to control (Fig. 3a). On the other hand, shoot moisture content remained constant across increasing concentrations of WSF and was significantly ($p<0.05$) higher than ASW and SWSF at 30-50% pollution level. Root moisture content increased significantly ($p<0.05$) with increase in WSF concentrations, but in ASW and SWSF there was no difference in root moisture content except at 50% SWSF (Fig. 3b).

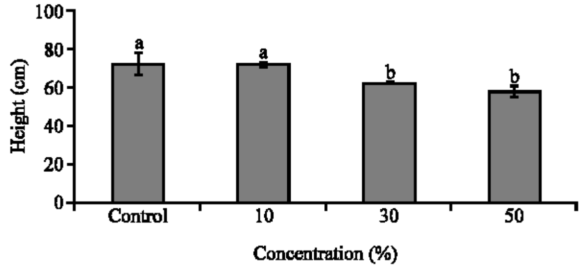

Fig. 1: Shoot height (cm) of *Paspalum conjugatum* treated with 0 to 50% WSF for 35 days. Values are Mean±SE n = 4. For each pollution level, means followed by different letters are significantly different at the 0.05 level (Tukey HSD). No plant death was recorded. WSF = Water soluble fraction of oil

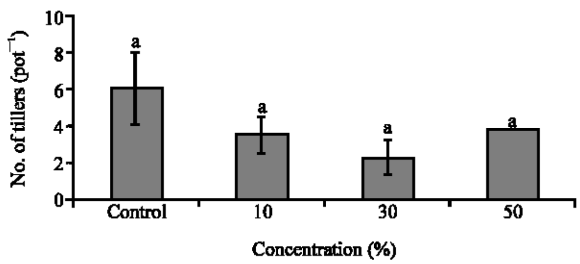

Fig. 2: Number of tillers (pot^{-1}) of *Paspalum conjugatum* treated with 0 to 50% WSF for 35 days. Values are Mean±SE n = 4. For each pollution level, means followed by different letters are significantly different at the 0.05 level (Tukey HSD). No plant death was recorded. WSF = Water soluble fraction of oil

After 35 days of growth in a range of salinities, there was no significant ($p>0.05$) reduction in shoot dry weight at 10% ASW and SWSF compared to control. But at higher concentrations of ASW and SWSF, shoot dry weight decreased significantly ($p<0.001$) with increase in salinity, being reduced by approximately 58% in 30% ASW and SWSF and by 78 to 79% in 50% ASW and SWSF, respectively (Fig. 4a). The pattern of response for root dry weight (Fig. 4b) was practically identical to that of shoot dry weight. On the other hand, there was no significant ($p>0.05$) reduction in shoot dry weight in comparison to control in WSF treatments compared to control. Shoot dry weights were significantly higher at 30 and 50% WSF pollution levels compared to ASW and SWSF (Fig. 4a). This meant that *P. conjugatum* was able to tolerate up to 50% WSF pollution, without any record of mortality. Root dry weight in WSF followed the same trend as shoot dry weight except at 10% WSF where root dry weight was significantly ($p>0.05$) increased (Fig. 4b). Increased root dry weight in 10% WSF conversely decreased its shoot:root in comparison to 10% ASW and SWSF (Fig. 5).

Ion Balance of *Paspalum conjugatum* Exposed to ASW and SWSF

Due to WSF samples not containing salt, the results were not presented as Na^+ concentration were similar to those of control. Meanwhile Na^+ concentrations in shoots of *P. conjugatum* were different between ASW and SWSF (Fig. 6). After 35 days there was no significant difference ($p = 0.07$) in Na^+ concentration between 10 to 50% ASW, but the increase was significant relative to control, while in SWSF the increase in shoot Na^+ was gradual and at 50% SWSF (2.73±0.39 μmol mg^{-1} dw) concentrations exceeded that of 50% ASW (2.10±0.09 μmol mg^{-1} dw) by 23%.

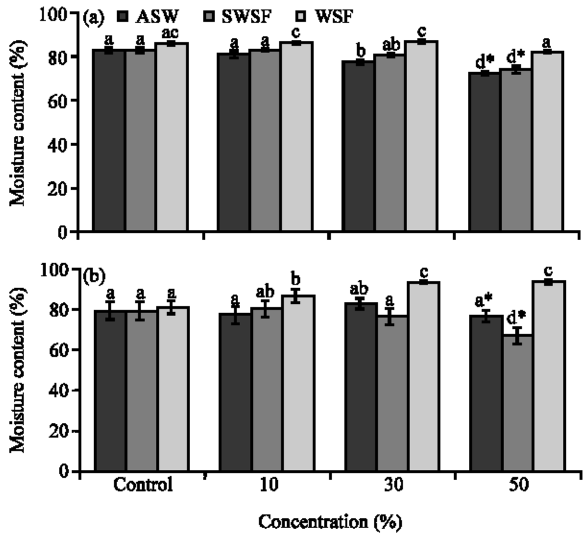

Fig. 3: Moisture content (%) for (a) shoots and (b) roots of *Paspalum conjugatum* treated with 0 to 50% ASW, SWSF and WSF for 35 days. Values are Mean±SE n = 4. For each pollution level, means followed by different letters are significantly different at the 0.05 level (Tukey HSD). Letters marked by an *specify all plants died at indicated treatment. ASW = Artificial seawater, SWSF = Seawater soluble fraction of oil and WSF = Water soluble fraction of oil

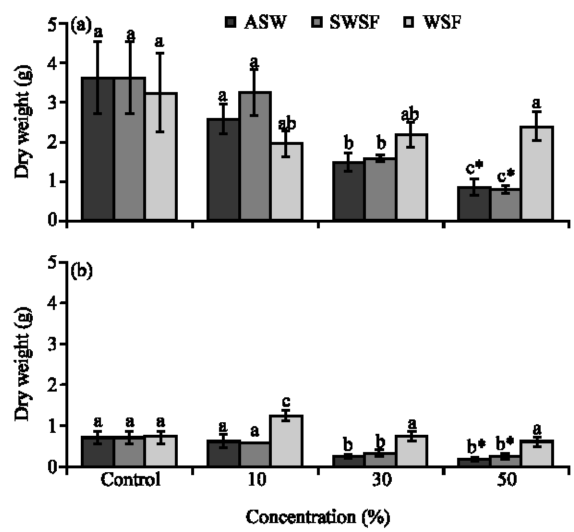

Fig. 4: Mean dry weight (g) of (a) shoots and (b) roots for *Paspalum conjugatum* treated with 0 to 50% ASW, SWSF and WSF for 35 days. Values are the Means±SE n = 4. For each pollution level, means followed by different letters are significantly different at the 0.05 level (Tukey HSD). Letters marked by an *specify all plants died at indicated treatment. ASW = Artificial seawater, SWSF = Seawater soluble fraction of oil and WSF = Water soluble fraction of oil

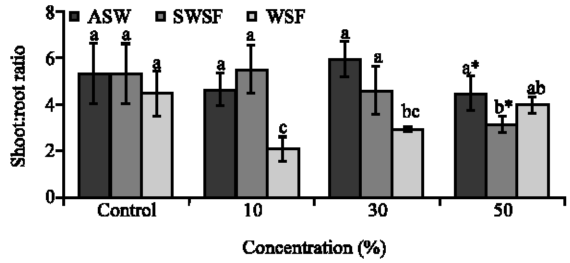

Fig. 5: Shoot: Root ratio for *Paspalum conjugatum* treated with 0 to 50% ASW, SWSF and WSF for 35 days. Values are the Mean±SE n = 4. For each pollution level, means followed by different letters are significantly different at the 0.05 level (Tukey HSD). Letters marked by an *specify all plants died at indicated treatment. ASW = Artificial seawater, SWSF = Seawater soluble fraction of oil and WSF = Water soluble fraction of oil

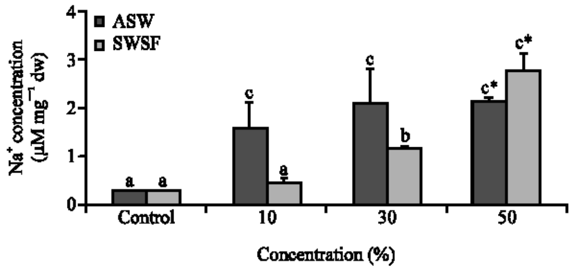

Fig. 6: Shoot Na^+ concentration (μM mg^{-1} dw) for *Paspalum conjugatum* treated with 0 to 50% ASW and SWSF for 35 days. Values are the Mean±SE n = 4. For each pollution level, means followed by different letters are significantly different at the 0.05 level (Tukey HSD). Letters marked by an *specify all plants died at indicated treatment. ASW = Artificial seawater, SWSF = Seawater soluble fraction of oil

DISCUSSION

The effects of SWSF and WSF of Wytch farm crude oil on *P. conjugatum* was studied and results were compared with saline (ASW) and non-saline (50% Hoagland solution) culture solutions. Yellowing of leaves was noticed in visual observations of both plants grown in SWSF or WSF. Similarly, Agboghidi and Bamidele (2009) reported yellowing of leaves in water lettuce (*Pistia stratiotes*) exposed to 50 and 100% WSF of crude oil. Yellowing of leaves, may be due to the fact that oil hydrocarbons gained entry to the plant through the root system where they were translocated to the shoot by the transpiration stream (Getter *et al*., 1985). Youssef (2002) demonstrated that although the uptake of water soluble hydrocarbons was possible through the foliage, perhaps through stomatal openings or dissolution in the cuticle, there was no difference in Net Assimilation Rate (NAR) between control and WSF treated *Avicennia marina* plants treated for 6 weeks.

No mortality of *P. conjugatum* was recorded at the highest level (50%) of WSF used in this study. The low toxicity of WSF maybe as a result of the low aqueous solubility of oil which decreased from approximately 30 mg L^{-1} for un-weathered crude oil to a negligible value after evaporation of the more soluble low-molecular-weight aromatics (e.g., benzene, toluene and the xylene). Ralph and Burchett (1998) reported that *Halophila ovalis* (seagrass) exposed to crude oil-sea water mixture (1% w/v) resulted in only minor reductions in chlorophyll fluorescence response even at the 100% treatment.

The experiments with non saline WSF provided some information on the toxicity of the water extracts of Wytch farm crude oil. Though there was a significant decrease in shoot height at 30-50%, however, other parameters measured were not significantly affected. The apparent lack of significant effect on the growth parameters measured in *P. conjugatum* showed that *P. conjugatum* was tolerant to 50% WSF of Wytch farm crude oil. Literature on growth patterns of plants exposed to WSF is limited but there is an abundance of literature on effects of WSF on marine fauna and micro-organisms. Siron *et al.* (1991) demonstrated that the diatom *Phaeodactylum tricornutum* and the chlorophyte *Dunaliella tertiolecta* tolerated exposure to WSF (Arabian light crude oil) up to 1.6 and 3.6% WSF, respectively. Also, Ohwada *et al.* (2003) did not detect acute toxicity in phytoplankton exposed to low concentrations of WSF, but it should be noted the medium was enriched with fertilizer to promote photosynthesis in the phytoplankton. Higher concentrations of WSF were not used in *P. conjugatum* plants as one of the aims of this study was to determine what caused the death of *P. conjugatum* plants at 50% SWSF (i.e., is it salt, WSF or both).

The effects of SWSF on *P. conjugatum* were similar to those of ASW as shoot and root dry weight decreased significantly from 30% ASW/SWSF treatment. The SWSF significantly decreased root dry weight with increase in salinity. Plant mortality occurred at 50% ASW/SWSF. The SWSF significantly decreased Na^+ concentrations in 10-30% SWSF treatments in comparison to 10-30% ASW this confirmed previous results obtained by Ibemesim (2010) that oil pollution interfered with Na^+ uptake in *P. conjugatum*. However, at 50% SWSF shoots had higher Na^+ concentrations than in 50% ASW which may have resulted in plants dying a few days (3 d) earlier than their 50% ASW counterparts. Studies on SWSF affecting nutrient uptake in plants are scarce. Almeida *et al.* (2008) reported an increase in the amounts of metal accumulated in both roots and stems of salt marsh plants when the 10^4 μg L^{-1} of Cu enriched elutriate was amended with PAHs. Similar results were obtained by Noyo *et al.* (2008). This suggests that PAHs present in WSF may modify the availability, absorption and/or passive uptake of Na^+ in plants.

The major cause of plant mortality in *P. conjugatum* at 50% SWSF appears to be mainly due to saline stress rather than the toxic effect of water soluble fractions of oil. However, any reduction in shoot and root dry weight caused by WSF would have reduced the ability of *P. conjugatum* to accommodate Na^+ (and Cl^-) transported from roots to shoots. According to Ralph and Burchett (1998), the primary phytotoxic effect of petrochemical exposure is the absorption of WSF of crude oil, followed by the incorporation of sub-lethal quantities of hydrocarbons, which reduces the tolerance of plants to other stress factors. It is also expected that some fractions of petroleum can dissolve biological membranes and as a consequence, disrupt the plant root architecture (Chaineau *et al.*, 1997; Adam and Duncan, 2002). Finally, the energetic costs that may be employed to degrade those petroleum fractions reaching the plant cell could also represent a stress source (Harvey *et al.*, 2002).

CONCLUSION

In summary, the negative effect observed in *P. conjugatum* plants grown in 50% SWSF was mainly due to the presence of salt (ASW) rather than the toxic effects of WSF of crude oil. However, an interactive effect cannot be ruled out as WSF caused a reduction in plant height and shoot: root ratio. Decrease in tiller number (Fig. 2), howbeit not significant, was also evident. Furthermore, SWSF decreased the concentration of Na^+ in *P. conjugatum* between 10-30% pollution level, meaning that WSF of oil exerted an influence on ion uptake. In future, higher concentrations of WSF need to be used to determine at what concentration it becomes detrimental to the growth of *P. conjugatum* and the mechanism WSF restricts ion uptake in plants needs to be investigated.

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