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EFFECT OF PERCHLORATE ON SEED GERMINATION AND ROOT ELONGATION OF PLANT SYSTEMS: A PHYTOTOXIC APPROACH

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ABSTRACT

Perchlorate (ClO_4^-) is a soluble anion, which is widely used in rocket fuel, demilitarization of weaponry and various explosive industries. Overuse of this anthropogenic environmental pollutant leads to bioaccumulation in plant systems and creates serious health implications in human by impairing the function of thyroid gland. Present work deals with study the phytotoxicity effect of ClO_4^- in various seeds germination and root elongation activity. *In vitro* studies were carried out against four plant systems (*Lycopersicum esculentum*, *Vigna mungo*, *Vigna radiate* and *Zea mays*) at ClO_4^- of varying initial concentrations ranges from 0 to 100 mg/L. Perchlorate of greater than 50 mg/L was observed to have inhibitory action on *Vigna radiate* and *Lycopersicum esculentum* compared to lower concentrations used for the study. Perchlorate of less than 25 mg/L was found to be non toxic against seed germination and plant growth. The phytotoxic effect was more in *Z. mays* compared to other three seeds analyzed for this study. The ClO_4^- compound was qualitatively identified from the spectra of the FTIR absorption in the characteristic band region 1090.55 ± 30 cm^{-1} and analyzed for all the plant systems. From the present study, it was confirmed that ClO_4^- use within the permissible limits, less than 25 mg/L would be safe to plant systems.

Key words: Perchlorate, Toxicity, Germination, Root elongation, FTIR.

INTRODUCTION

Perchlorate is widely used in rocket propellants and explosives where an energetic oxidant is required[1]. Due to its high solubility, little attenuation and stability in water, it persists in nature for long period and contaminate large amount of ground and surface water[2]. Perchlorate has been found in ground and surface water in 35 states in the US[3-5], China[6] and India[7]. In New York and Arizona established advisory levels for perchlorate are 6 and 14

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µg/L, respectively as a maximum contaminant level[8,9]. Due of its extensive usage, ClO_4^- salt in various forms leads to bioaccumulation in the environment and indirectly it reaches the human beings via accumulation in agricultural products[10-12]. The exposure to high concentrations of ClO_4^- cause adverse health effects to human body. The health concern associated with ClO_4^- is that it inhibits the iodine uptake by the thyroid gland and prevents the normal growth and metabolism resulting in clinical disorders such as goiter and developmental defects in fetuses[13-15] leading to hypothyroidism[16].

Various treatment technologies such as ion exchange, biodegradation, phytoremediation, membrane filtration, electrodialysis etc were used for removing ClO_4^- from drinking water and other contaminated sources[17]. Phytoremediation is an *in situ* mechanism and used as a promising technology for the cleanup of ClO_4^- contaminated soils, surface water and groundwater. It was found out that plant species are able to absorb perchlorate present in soil and irrigation water and it has been detected in food crops[11,18,19] like lettuce[20,21] and some beverages[22]. The phytoremediation mechanism may occur by phytodegradation or rhizotransformation (degradation in the root sphere primarily due to microbial activity) and phytoextraction (accumulation in the branches and leaves)[13,23]. Studies by van Aken and Schnoor showed that ClO_4^- has been shown to accumulate and slowly reduce inside poplar tree tissues[23].

The Fourier transform infrared (FTIR) spectroscopy allows the analysis of a relevant amount of compositional and structural information concerning environmental samples[24]. The analysis can be performed both on pure compounds and complex multicomponent mixtures, without separation into individual components. IR spectrometry is more sensitive and selective than colorimetric methods. Moreover, FTIR spectroscopy is an established time-saving method to characterize and analyze microorganisms and monitor biotechnological processes[25].

The overall objective of this study was to determine the phytotoxicity nature of ClO_4^- on various seeds germination activity of different plant systems. *In vitro* studies were carried out against *Lycopersicum esculentum, Vigna mungo, Vigna radiate* and *Zea mays* at varying initial ClO_4^- concentrations. Seed germination activity and root length was measured and qualitative confirmation of the presence of perchlorate was performed by FTIR spectroscopy. The FTIR spectroscopy can be used for determination of chlorate derivatives, using the vibration bands of perchlorate group[26]. The successful demonstration of the uptake of ClO_4^- by test systems could be used in further research on removal of perchlorate under field conditions.

EXPERIMENTAL

Materials and methods

Sodium perchlorate monohydrate ($NaClO_4$, H_2O) and potassium bromide (KBr) for FTIR spectroscopy were procured from Himedia Pvt. Limited, Mumbai and stored at room temperature in lab condition. Deionized water (Milli-Q water, Millipore Corporation) was used for all experiments. Sodium perchlorate and Milli-Q water were used in the preparation of different perchlorate stock solutions (10, 25, 50, 75 and 100 mg/L) and all chemicals were of analytical grade.

Selection test plant seeds

Selections of plant test system were based on US Environment Protection Act protocol[27]. Seeds of *Lycopersicum esculentum, Vigna mungo*, *Vigna radiate* and *Zea mays* were selected because of its availability, low chromosome number, low germination time and high germination rate. The test species were selected because of their routine use in phytotoxicity tests and their importance as in food plants. It also acts as a best bio-indicator to check the environmental pollution and toxicant. All the seeds were obtained from a nursery (Vellore district, Tamilnadu, India) and were stored in the dark under room temperature until use.

Seedling exposure

The seeds were first checked for their viability by suspending them in deionized water and those seeds which settled to the bottom were selected for further study. The seeds were then soaked for 10 min. in a 10% sodium hypochlorite solution, which acts as a surface sterilizing agent[27]. Then the seeds were rinsed in deionized water thrice and stirred for 2 h in various initial perchlorate level (0, 10, 25, 50, 75 and 100 mg/L) using a magnetic stirrer. A control was kept for respective concentrations of perchlorate injection. Whatman No. 1 filter paper was then placed into each Petri dish (100 mm x 15 mm) and 5 mL of the respective perchlorate from the stock were added using a Pasteur pipette. The seeds were then transferred to the petri dish, with 10 seeds per dish and they were placed equidistant from one another. Subsequently the dishes were covered and sealed with sealing tape at a controlled temperature of 25 ± 1^oC and placed in dark condition. The end points of the experiment were when at least 80% of the control seeds had germinated, i.e., 80-85 hours for *L. esculentum*; 52 hours for *V. mungo, V. radiate* and 48-50 hours for *Zea mays*[28]. All the experiments were carried out in triplicates for further analysis.

Seed germination test

This test was conducted by following the standard method[27]. The test was performed

on the seeds of *L. esculentum, V. mungo, V. radiate* and *Z. mays*. The Relative Seed Germination rate (RSG), Relative Root Growth (RRG) and Germination Index (GI) were calculated using the following Eqs. (1), (2) and (3) respectively.

$$\text{Relative Seed Germination rate} = (Ss / Sc) \times 100 \quad \ldots(1)$$

$$\text{Relative Root Growth} = (Rs / Rc) \times 100 \quad \ldots(2)$$

$$\text{Germination Index} = (RSG \times RRG) / 100 \quad \ldots(3)$$

Where Ss is the number of seeds germinated in sample; Sc is the number of seeds germinated in control; Rs is the average root length in sample; Rc is the average root length in control.

FTIR characterization

Seed growth was observed after respective hours of incubation at room temperature and the percentage of seed germination and root length were measured. Then the air dried roots were separated from the seeds and dried in hot air oven (SEREVELL, India) at 60^{o}C for 24 h until constant weight. Subsequently they are ground to powder form for FTIR analysis. 300 mg sample was mixed with 900 mg of KBr and ground it in an agate mortar. The homogenous mixture was transferred into a diffuse reflectance cup (12 mm) without any pressure and levelled with a microscope glass slide. The FTIR spectra were measured on FT/IR-4100 type A (Sr. No: C191061016) spectrometer equipped by standard light source connected with a TGS detector. The FTIR absorption spectra were collected for each sample with data interval of 0.964233 cm^{-1} at a scanning speed of 2 mm/sec. For each spectrum, the average of 100 successive scans, over the range of 400-4000 cm^{-1} with a resolution of 4 cm^{-1}. The heights of the absorption bands in the FTIR spectra of all the test samples were compared with the standared control with the help of software ORIGIN (Version 8). The wave number (cm^{-1})/ %T ratio was calculated from the normalized spectra. The sodium perchlorate monohydrate salt mixed with the KBr pellets is used as a positive control for the experiment. The FTIR spectrogram for positive control compared with the experimental ones which contain KBr mixed with respective seeds in powder form. According to Mosier-Boss26 the range covered for perchlorate band peak at 1092 cm^{-1}. The experiments were carried out in triplicates and the mean value reported.

Statistical analysis

Each concentration of the respective treatments was conducted in triplicate and results expressed as mean values ± standard error (SE). Experimental data of treatments

(perchlorate + seeds) were compared to their corresponding control (seeds + water) and statistically significant difference was reported.

RESULTS AND DISCUSSION

Phytotoxicity of perchlorate

Phytotoxicity of perchlorate was carried out according to USEPA protocol[27]. Seed germination and root elongation is a rapid and widely used acute phytotoxicity test. They have several advantages like sensitivity, simplicity, low cost and suitability for unstable chemicals or samples[29,30]. Toxicity studies of perchlorate were done on four seeds (*L. esculentum, V. mungo, V. radiate* and *Z. mays*) to find out the effects of perchlorate on seed germination and root elongation in different perchlorate level. The results of the formulated perchlorate doses (10, 25, 50, 75 and 100 mg/L) on treated seeds of *L. esculentum, V. mungo, V. radiate* and *Z. mays* were reported in Table 1.

Table 1: Root elongation and germination index test for L. esculentum, V. mungo, V. radiate and Z. mays after treatment with varying initial perchlorate concentrations (Control = 0, A = 10, B = 25, C = 50, D = 75 and E = 100 mg/L)

Test organism with varying perchlorate concentrations (mg/L)	Root Elongation (cm)	Germination Index	RSG*	RRG**	Germination (%)
L. esculentum					
Control (0 mg/L)	4.1 ± 0.21	100 ± 0.0	100	100	100
A (10 mg/L)	3.48 ± 0.62	80.22 ± 7.29	93.33	84.79	93.33
B (25 mg/L)	3.33 ± 0.52	76.7 ± 2.86	93.33	81.3	93.33
C (50 mg/L)	3.35 ± 0.46	76.09 ± 1.3	93.33	81.6	93.33
D (75 mg/L)	2.86 ± 0.45	62.77 ± 1.81	90	69.75	90
E (100 mg/L)	2.59 ± 0.28	53.19 ± 1.18	83.33	63.25	83.33
V. mungo					
Control (0 mg/L)	4.52 ± 0.19	100 ± 0.0	100	100	100
A (10 mg/L)	3.43 ± 0.33	73.07 ± 2.66	96.67	75.88	96.67
B (25 mg/L)	2.9 ± 0.19	64.15 ± 4.42	100	64.15	100

Cont…

Test organism with varying perchlorate concentrations (mg/L)	Root Elongation (cm)	Germination Index	RSG*	RRG**	Germination (%)
C (50 mg/L)	2.69 ± 0.81	59.58 ± 6.81	100	59.58	100
D (75 mg/L)	2.55 ± 0.18	56.41 ± 4.09	100	56.41	100
E (100 mg/L)	1.99 ± 0.18	39.95 ± 8.2	90	44.09	90
V. radiate					
Control (0 mg/L)	4.02 ± 0.42	100 ± 0.0	100	100	100
A (10 mg/L)	3.41 ± 0.69	82.70 ± 8.76	96.67	84.91	96.67
B (25 mg/L)	3.23 ± 0.56	78.29 ± 5.3	96.67	80.51	96.67
C (50 mg/L)	3.13 ± 0.43	74.95 ± 6.3	96.67	77.93	96.67
D (75 mg/L)	2.62 ± 0.17	65.08 ± 4.32	100	65.08	100
E (100 mg/L)	2.27 ± 0.68	54.52 ± 9.13	96.67	56.63	96.67
Z. mays					
Control (0 mg/L)	2.14 ± 0.03	100 ± 0.0	100	100	100
A (10 mg/L)	2.13 ± 0.14	89.55 ± 5.49	90	99.35	90
B (25 mg/L)	1.38 ± 0.13	53.49 ± 2.01	83.33	64.48	83.33
C (50 mg/L)	1.26 ± 0.2	50.9 ± 6.31	86.67	59.03	86.67
D (75 mg/L)	1.01 0.11	35.19 ± 3.18	73.33	47.34	73.33
E (100 mg/L)	0.72 ± 0.08	22.56 ± 4.25	66.67	33.64	66.67

The results are reported as mean ± S.D; *RSG-relative seed germination rate,
**RRG- relative root growth

Kordan have reported that germination is normally known as a physiological process which begins with water inhibition by seeds and culmination in the emergence of the rootlet[31]. The germination (%) for the sample treated with 100 mg/L of perchlorate in case of *L. esculentum* and *Z. mays* were 83.33% and 66.67%, respectively where as control seeds showed 100% germination on both the cases. The summary of effect of perchlorate on seed germination of *L. esculentum* (A), *V. mungo* (B), *V. radiate* (C) and *Z. mays* (D) are showed in Fig. 1. All the control treated seeds showed 100% germination in all the test systems. Results reveal that *Z. mays* showed the lowest germination % compared to other test plant seeds.

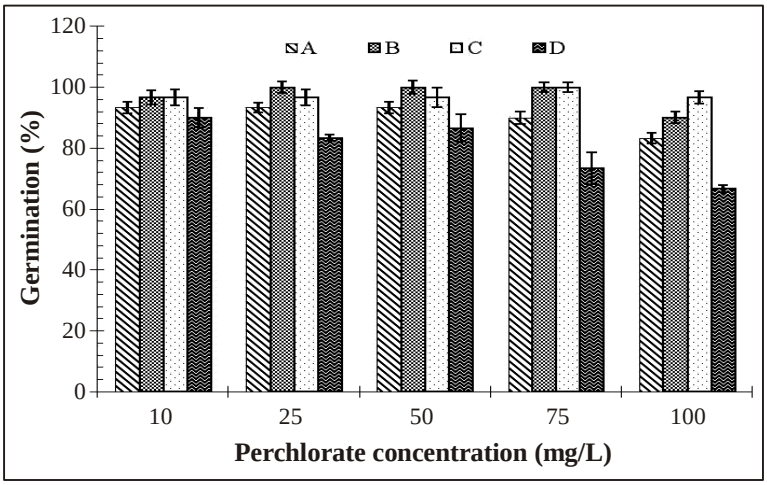

Fig. 1: Effect of perchlorate on germination (%) of *L. esculentum* (A), *V. mungo* (B), *V. radiate* (C) and *Z. mays* (D)

The results of the root elongation for the sample treated with 100 mg/L of perchlorate in case of *V. mungo* and *Z. mays* were 1.99 cm and 0.72 cm respectively, where as control seeds showed a visible growth difference (4.1 cm and 2.14 cm) in both the test systems. The summary of effect of perchlorate on root length of *L. esculentum* (A), *V. mungo* (B), *V. radiate* (C) and *Z. mays* (D) are showed in Fig. 2. The measurement of root elongation study also reveals that the toxicity effect of perchlorate was predominant in *Z. mays* compared to other test seeds.

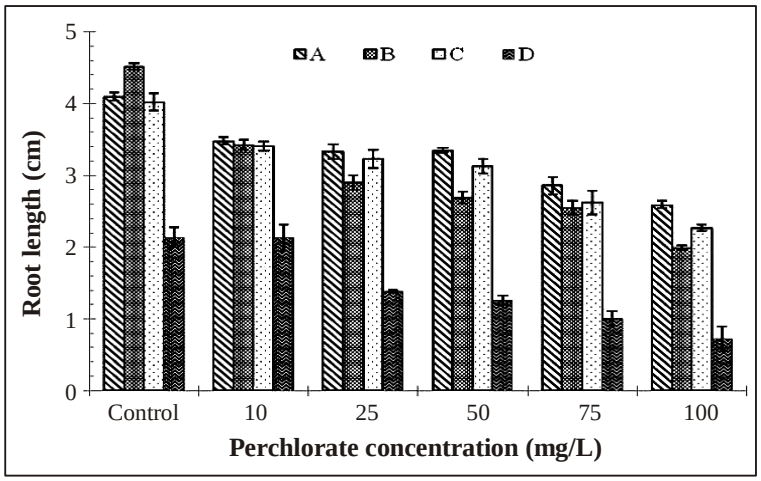

Fig. 2: Effect of perchlorate on root length of *L. esculentum* (A), *V. mungo* (B), *V. radiate* (C) and *Z. mays* (D)

Seyfferth and Parker[32] showed that, the uptake rate of perchlorate in different genotypes of lettuce exhibited a linear relationship of perchlorate accumulation in tissues with increasing perchlorate concentration in culture medium. They also predicted that the perchlorate accumulation had a larger influence on geological location rather than the genotype. Perchlorate of greater than 50 mg/L showed considerable level of phytotoxicity action against all the four tested seeds and the effect was more in *Z. mays* compared to other test seeds analyzed for this study. Perchlorate of less than 25 mg/L, showed negligible inhibitory effect on seed germination and root elongation in all the tested plant seeds. Control treated seeds showed 100% root germination in all cases and this suggests that as perchlorate concentration increases, the toxicity effect towards test plant systems also increases. It is evident from Fig. 3 that, exposure to perchlorate, at 100 mg/L, did affect seed germination and there was visible or textural difference in the 100 mg/L plant seeds and the control plant seed samples of *Z. mays*. Germination of *Z. may*s was shown, since it was more sensitive to perchlorate.

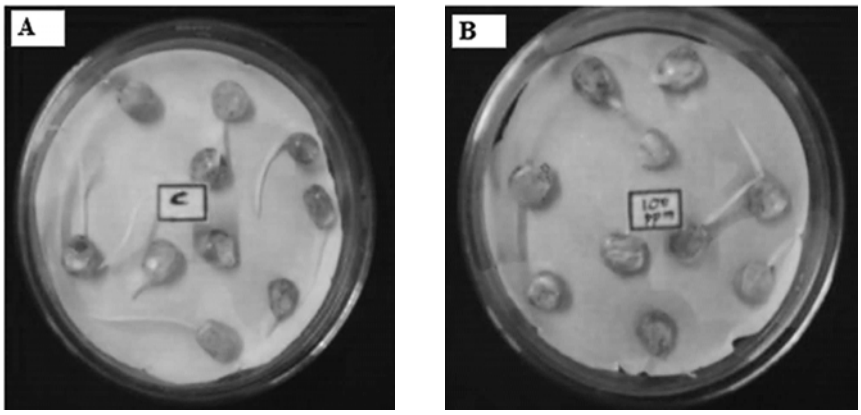

Fig. 3: The visible growth difference observed with perchlorate exposure in *Z. mays* (A) control seeds (0 mg/L perchlorate) (B) Seeds treated with 100 mg/L perchlorate

Perchlorate was reported to be phytotoxic in nature and bioaccumulated in several plant systems like spinach (99.4-175 µg/Kg), Romaine lettuce (13.9-46.7 µg/Kg), *L. esculentum* etc and found in many fruits like orange from US (ND-2.89 µg/Kg), green grape from Chile (26.6-62.1 µg/Kg), empire apple from Canada (1.17-1.60 µg/Kg) etc.[33] Study conducted by Ha et al.[34] reported that the ratio of perchlorate accumulation was more in spinach leaves compared to that in lettuce leaves. From the obtained results, 50 mg/L, 75 mg/L and 100 mg/L of perchlorate were found to be toxic against plant systems. Our results suggests that a linear relationship between ClO_4^- concentration and bioaccumulation rate which causes the inhibitory effect on seed germination and root elongation processes. In general, ClO_4^- of higher concentration has found to have growth inhibitory effect towards plant community in the environment.

FTIR analysis

Perchlorate is a polyatomic anion with tetrahedral symmetry which exibit four vibrational modes at the followng frequencies: 935 (Cl-O), 460 (O-Cl-O), 1050-1170 (Cl-O) and 630 (O-Cl-O) cm^{-1}. Previous reports show that Raman spetroscopy was used to detect perchlorate in lettuce extracts[35] and the band intensity at 1092 and 1331 cm^{-1} are assigned to be for perchlorate and nitrate, respectively[26]. Present study FTIR spectra (sodium perchlorate + KBr pellets) showed the band width maximum at 1090.55 cm^{-1} for perchlorate (control) and experimental ones (seed powdered sample) obtained the peaks with in 1090.55 ± 31 cm^{-1}. In all the test samples, we have analyzed the FTIR spectra for the higest (100 mg/L) and lowest (10 mg/L) concentration of perchlorate treated in plant seeds.

The band width observed for L. esculentum was 1059.69 cm^{-1} and 1060.66 cm^{-1} at 10 and 100 mg/L of perchlorate concetration, respectively are showed in Fig. 4. The band width observed for *V. mungo* was 1062.59 cm^{-1} and 1076.08 cm^{-1} at 10 and 100 mg/L of perchlorate concetration respectively (Fig. 5). The band width observed for *V. radiate* was 1077.05 cm^{-1} and 1077.05 cm^{-1} at 10 and 100 mg/L of perchlorate concetration, respectively (Fig. 6). The band width observed for Zea mays was 1078.98 cm^{-1} and 1075.12 cm^{-1} at 10 and 100 mg/L of perchlorate concetration respectively (Fig. 7). The small changes in peak position may be due to the effect of various environmental conditions like weathering, mechanical treatment such as grinding, pressure and temperature changes[36].

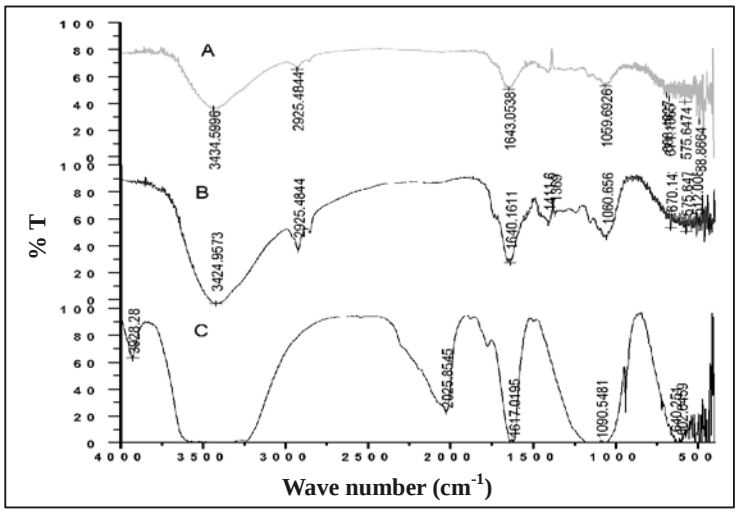

Fig. 4: FTIR spectra of *Lycopersicum esculentum* at 10 and 100 mg/L of perchlorate concentration: A – 10 mg/L spectra, B – 100 mg/L spectra, C – control spectra (perchlorate + KBr)

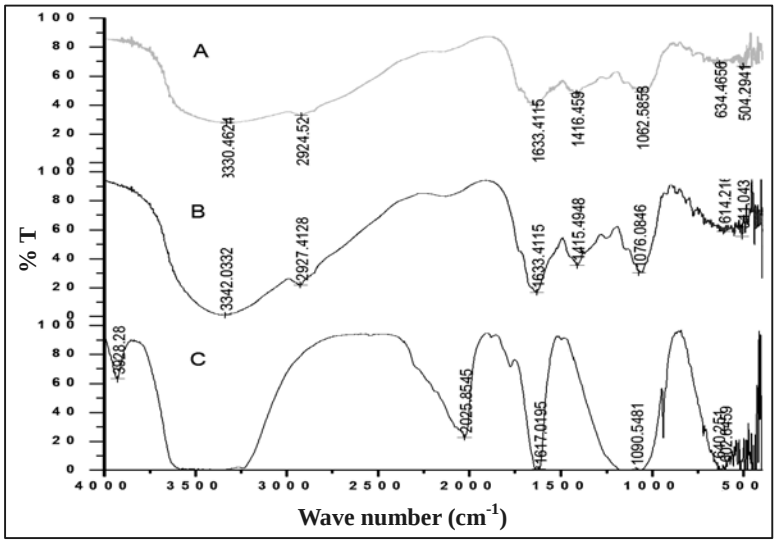

Fig. 5: FTIR spectra of *Vigna mungo* at 10 and 100 mg/L of perchlorate concentration: A – 10 mg/L spectra, B – 100 mg/L spectra, C – control spectra (perchlorate + KBr)

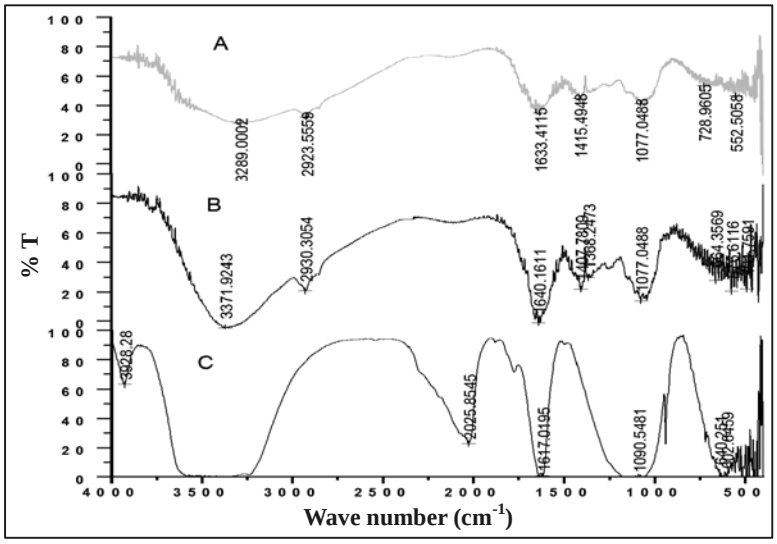

Fig. 6: FTIR spectra of *Vigna radiate* at 10 and 100 mg/L of perchlorate concentration: A – 10 mg/L spectra, B – 100 mg/L spectra, C – control spectra (perchlorate + KBr).

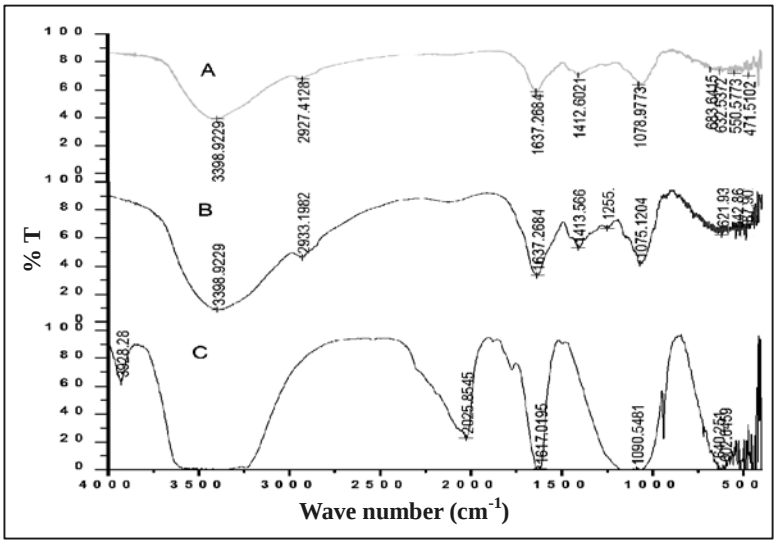

Fig. 7: FTIR spectra of *Zea mays* at 10 and 100 mg/L of perchlorate concentration: A – 10 mg/L spectra, B – 100 mg/L spectra, C – control spectra (perchlorate + KBr)

All the 10 mg/L perchlorate treated seeds (10 mg/L perchlorate + seed + KBr) FTIR spectra showed lesser peak height than the respective seeds treated with perchlorate of higher concentration (100 mg/L perchlorate + seed + KBr). The spectral analysis of samples are made on the basis of the magnitude and relative intensities of the recorded spectra and in the analogy with the assignments made by other researchers on the similar type of perchlorate compounds. The accuracy of chlorate evaluation increase using the control derivative spectra. Our initial observation related to FTIR spectra revealed that all the solid seed samples are dominated by the –OH stretch which exhibit absorption bands at 3250-3350 cm^{-1}, 2940-2920 cm^{-1} dominated by methylene group ($–CH_2–$), 1660-1620 cm^{-1} dominated by nitrates group (–ONO) and 1420-1400 cm^{-1} dominated by primary amides group ($–CONH_2$), thus indicating the similarity of the chemical composition and qualitative identity. The C–H bands associated with methyl and methylene groups that usually occur at 2920 cm^{-1} (CH asymmetric stretch) and at 2860 cm^{-1} (CH symmetric stretch) are superimposed as a shoulder of the broad O–H band. Other bands characteristic to humic substances appear at 1633.41, 1640.16, 1643.05, 1630 and 1411.64 cm^{-1}. Also at 1630-1640 cm^{-1} appear the vibration band for absorbed water, C=C, C=O from amide and benzophenones from humic substances. Each seed sample spectrum appears to have distinctive spectra in the 400-4000 cm^{-1} region with maximum intensive peak at 3289-3434.59 cm^{-1}, most probably due to the presence of various organic constituents present in test seed[37].

The summary of common observed absorption frequencies (cm^{-1}) and its tentative assignment of bond vibrations for *L. esculentum*, *V. mungo*, *V. radiate* and *Z. mays* after treatment with 10 and 100 mg/L of perchlorate are showed in Table 2. In the control sample (sodium perchlorate + KBr) spectra, water has strong IR absorbance at three prominent bands around 3349.75 (O-H stretching), 2025.85 (water association), and 1617.02 cm^{-1} (H-O-H bending). Its broadness is generally attributed to hydrogen bonding. If the sample particle size is minimum, then the sample (seed in powder form) in most disordered state and it produces better FTIR spectra. The absence of some peaks from sample to sample, suggesting that perchlorate interacted with the seeds and created a change in its composition or due to the differnece in particle size which is used for FTIR characterization.

Table 2: Summary of the common observed absorption frequencies (cm^{-1}) in FTIR spectra and its tentative assignment of bond vibrations for Control, *L. esculentum*, *V. mungo*, *V. radiate* and *Z. mays* after treatment with 10 mg/L and 100 mg/L of perchlorate

Sample	Common observed frequency (cm^{-1})	Mode of assignment
Control (Sodium perchlorate + KBr)	640.25, 1617.02, 1090.55, 2025.85	O-Cl-O vibration, C-C stretch, Cl-O vibration, H-O-H bending, water association
L. esculentum	1059, 1640, 2925.48	Cl-O vibration, -ONO- stretch, $-CH_2-$ vibration
V. mungo	1415, 1633.41	C-S stretch, Ring Vibration (boat)
V. radiate	1077.05	Cl-O vibration mode
Z. mays	1412, 1637.27, 3398.92	C-N-C bend, -ONO- stretch, NH_2 stretch

CONCLUSION

The fate of pollutants uptake and bioaccumulation mechanism are not yet well understood which effects the seed germination and root elongation processes. This is because of the synergistic or antagonistic effects of complex physico-chemical or biochemical processes, such as adsorption, binding to components present in seeds; reaction with other compounds, particle size distribution, biodegradation etc. which can modify specific pollutants properties. Present study showed the toxicity effect of perchlorate in seed germination and root elongation in a concentration-dependent manner. From the obtained

results, greater than 50 mg/L of perchlorate was found to be toxic against plant systems. Among the four test systems (*L. esculentum, V. mungo, V. radiate* and *Z. mays*) selected for the studies, seeds of *Z. mays* were more sensitive to perchlorate than other test plant systems. The perchlorate compound can be qualitatively identified in the FTIR absorption spectra by the characteristic band 1090.55 ± 30 cm^{-1}. The limitation of study is that the detection of trace amount of perchlorate present in aqueous matrices is not possible due to the intense bands which may occur due to OH streching and bending modes of water that dominate large regions of the infrared spectrum[26]. The results also provide evidence of a linear relationship between perchlorate concentration and bioaccumulation rate, which leads to a growth inhibitory effect in plant systems. Nevertheless, it act as an effective remediae for removing perhlorate from the contaminated sources such as soil and water present in the environment.

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