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Investigation of the manganese stress on wheat plant by attenuated total reflectance Fourier transform infrared spectroscopy

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Abstract

The present paper deals with the investigation of the changes in the chemical contents of the leaf of wheat seedling stressed by excess manganese using attenuated total reflectance Fourier transform infrared spectroscopy technique. The infrared spectra of the leaves of control and manganese treated wheat plants have been recorded in the spectral region 485 - 4000 cm⁻¹ at a resolution of 4 cm⁻¹. The recorded spectrum has been analyzed with the help of curve fitting method for the quantitative estimation of chemical contents and conformational changes. The study indicated changes arising in the polysaccharide, lignin, amino acid, secondary structure of protein, and lipid in the leaves of control and manganese treated wheat plants. Manganese treatment increased the amount of cellulose, lignin, and amide II till 200 µM concentration while a decrease was observed at 1000 µM concentration. Manganese induced conformational changes in the secondary structure of protein which was indicated by shifting of the bands to the higher wavenumber and change in the β sheet/α helix ratio. In addition, manganese stress decreased lipid content in the leaves of the wheat seedlings. The study demonstrates the potential of attenuated total reflectance Fourier transform infrared spectroscopy for the non-invasive and rapid monitoring of the plants stressed with heavy metals.
KEYWORDS: ATR-FTIR spectroscopy, biochemical analyses, curve fitting method, manganese toxicity

INTRODUCTION

Manganese is one of the most abundant metals in the earth crust and is a component of many minerals. It is present in soil due to weathering and atmospheric deposition arising from both natural and anthropogenic sources. Natural sources of manganese in soil include the parent mineral material from which the soil is derived. Anthropogenic sources of manganese are sewage sludge, municipal discharge, mineral ore processing and mining (especially nickel), and emissions from iron, steel, and ferroalloy industries [1]. Use of organomanganese compound methylcyclopentadienyl manganese tricarbonyl (MMT) as antiknocking agent in unleaded gasoline is another source of manganese in the environment. The addition of Manganese sulfate (MnSO₄) to the soil as fertilizer and as a livestock supplement and use of broad spectrum contact fungicides like Maneb (manganese ethylene-bis-dithiocarbamate) are a potential source of manganese in the soil [2].

Manganese is an essential micronutrient for the plant but when present in excess concentration to that of the threshold level, it is regarded as a toxic element. Manganese is involved in the hill reaction where water splitting mechanism of photosystem II (PSII) occurs in the chloro plast which produces necessary electrons and assimilates carbon dioxide during photosynthesis [3]. In addition, it plays an essential role in the activity of many enzymes that are involved in the oxidative reactions, carbohydrate metabolism,
phosphorylation, citric acid cycle, nitrogen assimilation and in the formation of photosynthetic proteins and enzymes\textsuperscript{[4]}. But excess manganese disturbs the morphological, physiological, biochemical functions in the plant and inhibits their overall growth and developmental process either directly or indirectly. The high concentration of manganese disturbs the absorption, translocation, and utilization of other essential elements like calcium, magnesium, iron, and phosphorus. When manganese is present beyond the threshold level, it shows an injurious effect on the photosynthetic apparatus present in the chloroplast, thus inhibiting the process of photosynthesis\textsuperscript{[5]}. One of the major consequences of heavy metal interaction with plant is the production of reactive oxygen species (ROS). Heavy metal generates ROS by mechanisms like stimulating the activity of NADPH oxidases, and by displacing essential cations from binding sites of enzymes\textsuperscript{[6]}. ROS species cause oxidative damage by reacting with the cellular component like lipid, protein and nucleic acid and causes lipid oxidation and membrane damage\textsuperscript{[7]}.

In order to detect the chemical changes arising in the plant due to the heavy metal, such techniques are required that can identify changes prior to the appearance of visual signs of toxicity in the plant so that the damage can be ameliorated and the growth of the plant can be restored. The current methods of plant diagnostics require extensive sample preparation which are time consuming and laborious. Also, multiple accessories are required for the simultaneous analysis of the chemical and elemental constituents of the plants. Since biological samples are complex biochemical matrixes which have spatial heterogeneous distribution, extraction procedures disturbs the entire cascade of
biochemical and makes them homogenous. Therefore in this scenario, such techniques are required which are fast, reliable, free from sample preparation and have simultaneous chemical detection capability. Analytical spectroscopy techniques can be successfully applied to study the physiological and metabolic status of plants under stress conditions. One of the versatile spectroscopic techniques available for tissues analysis in order to gain information about the structure of biological molecules is attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) technique. Simultaneous multi-component analysis of the biochemical profile of the plant tissue is the pivotal feature of ATR-FTIR. This technique can detect molecular vibrations arising due to inter and intra molecular forces. Through characteristic molecular vibrations one can explore information about the molecular conformation and intermolecular interaction of biological molecules under changing environment. By determining the position, intensity, width, and area of the infrared band, the configuration and the metabolic changes taking place within the plant tissue can be investigated \[8\]. Another advantage of using ATR-FTIR technique comprises minimal sample preparation and its ability to access the configuration of plant tissue components and their relative amount in situ as it does not lead to the disorganization of molecular conformation through solvent extraction \[9\].

Wheat is one of the most important cereals consumed worldwide. Due to increased level of manganese in the agricultural soil, the study of the effect of manganese on wheat plant is essential. Manganese is expected to interfere with the metabolic and physiological processes of the wheat plant. Investigation of the biochemical changes in the control (plants without metal toxicity) and manganese treated plants has been performed using
ATR-FTIR spectroscopy technique combined with curve fitting. The ATR-FTIR technique has been applied for the non-invasive and non-destructive determination of changes in the composition of cell wall components (cellulose, pectin, hemicelluloses, and lignin), amino acids, proteins, and lipid due to the pretreatment of manganese with the wheat plant. Curve fitting method has been applied for the quantitative estimation of the relative proportion of the biochemical constituents and to demonstrate the changes arising in the secondary structure of protein as a result of manganese pretreatment. The absorbance ratios are used to determine the extent of change in the secondary structure of protein and to assess the level of lipid saturation and lipid peroxidation. The aim of this study is to demonstrate the potential of ATR-FTIR combined with curve fitting method for the non-destructive, non-invasive, rapid and sensitive monitoring of the plants stressed with heavy metals.

MATERIAL AND METHODS
All the chemicals used in this experiment were purchased from Merck, India (Merck Specialties Private Limited, Mumbai, India) of analytical grade and were used without further purification. The seeds of *Triticum aestivum* L. (wheat) were procured from certified supplier of local seed market of Allahabad, India. Uniform size seeds were selected and washed with distilled water. Seeds were surface sterilized by soaking them in 4% sodium hypochlorite solution for 20 minutes and washed again with distilled water. The sterilized seeds were transferred on wet filter paper placed in petridis and kept in dark for 3 days. Uniformly germinated seeds were transferred in plastic pots containing acid washed sand and were grown on 1/4 Hoagland nutrient solution containing: KNO₃
(0.6 mM), Ca(NO₃)₂ (0.4 mM), NH₄H₂PO₄ (0.2 mM), MgSO₄ (0.1 mM), KC1 (50 μM), H₃BO₃ (25 μM), FeNa EDTA (20 μM), MnSO₄ (2 μM), ZnSO₄ (2 μM), CuSO₄ (0.5 μM) and (NH₄)₆Mo₇O₂₄ (0.5 μM). Seedlings were grown under a photosynthetic photon flux density (PPFD) of 350 μmol photon m⁻² s⁻¹ and relative humidity of 50–60% with a light/dark cycle of 16/8 h. After three days of growth, manganese stress was introduced in the plants at different concentration (50 μM, 100 μM, 200 μM, 500 μM, and 1000 μM) for 4 days, each on alternate days. MnCl₂·4H₂O containing Mn (II) was used to prepare metal solutions of different concentration in ¼ strength Hoagland solution. After 4 days of treatment, shoots and roots were harvested for determining growth and biomass parameters. Untreated wheat plants were regarded as control.

The ATR-FTIR experiments were conducted using ABB Bomem FTIR MB 3000 spectrometer (ABB Analytical measurements, Canada) equipped with ZnSe beam splitter (non-hygroscopic), deuterated triglycine surface (DTGS) detector, Horizon MB™ FTIR processing software and PIKE-Miracle ATR unit attached with a zinc selenide internal reflection element as a sample holder. The ceramic globar with electric stabilization was used as a source of infrared radiation. This experimental setup can produce spectrum in the spectral range 485 - 10500 cm⁻¹ with lowest resolution 1 cm⁻¹. Prior to performing ATR-FTIR experiment, second set of the leaf of wheat plants were collected from the control and treated plants. The leaves were washed with double distilled water to clean visual impurities then dried at room temperature. Before running each sample, the ATR crystal was cleaned thoroughly with acetone. The adaxial surface of the leaf of control and treated plant was placed on sample platform of the ATR crystal.
and spectrum was recorded in the spectral region 485 - 4000 cm$^{-1}$ at a resolution of 4 cm$^{-1}$. Each leaf was scanned at four adjacent places to acquire the infrared spectrum. To improve signal to noise ratio, twenty five scans were collected and average intensity was analyzed. Since base line slope was not linear across the entire wavenumber range, base line correction was performed for each acquired spectrum using Horizon MB$^{\text{TM}}$FTIR software. The wavenumber and position of the spectral peak were identified with the help of secondary derivative which was generated using Horizon MB$^{\text{TM}}$FTIR software. Spectral peak fitting or curve fitting process was performed using origin 8.0 package for quantitative comparison. The area of Gaussian fitted peaks was used to quantify the chemicals present in the specimen. The experimental data were subjected to analysis of variance (ANOVA) using origin 8.0 package. Mean values were compared by the Tukey’s multiple range test at the 5% significance level. The results presented are the means ± standard error of three independent experiments with three replicates in each experiment to check the reproducibility of the results.

RESULTS AND DATA ANALYSES

Growth and Biomass Pattern

Table 1 and figure 1(a) and (b) illustrates the effect of different concentrations of manganese on the growth parameters (shoot length and root length) of the wheat seedlings. The observed results indicated sensitivity of wheat seedlings towards different concentration of manganese. The shoot height of the plant decreased followed by all the manganese treatments. The retardation in the height of the plants with respect to the control was 8.42%, 17.91%, 21.45%, 24.24, and 31.07% (p<0.05) for the 50 μM, 100
µM, 200 µM, 500 µM, and 1000 µM manganese treatments respectively. Similarly, the root height was also affected in response to manganese treatment. The root height declined significantly after manganese treatment. The decrease was 5.24%, 10.63%, 18.23%, 26.08%, and 30.73% (p<0.05) respectively. The reduction in growth of the plant was found to be severe for 1000 µM manganese concentration. The fresh mass of shoot was decreased by 1.95%, 4.64%, 9.77%, 11.45%, and 30.04%, (p<0.05) and that of the root decreased by 7.66%, 17.44%, 23.43%, 33.69%, and 34.47% (p<0.05) after 50 µM, 100 µM, 200 µM, 500 µM, and 1000 µM treatment of manganese respectively.

**ATR-FTIR Analyses**

The ATR-FTIR spectra were acquired to investigate alterations and conformational transformations of the functional groups contributed from different biochemicals such as carbohydrates, proteins, lipids, and lignin of wheat leaves treated with different concentrations of manganese. Figure 2 shows the recorded infrared spectra of the control and manganese treated leaves of wheat plants in the spectral range of 485 - 4000 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\). The recorded spectrum shows a number of absorption bands due to molecular vibrations of different functional groups of varying intensity representing different biochemical present in the leaf of the wheat plant. The enlarged fingerprint region of polysaccharides, lignin, protein, and lipid are shown in Figures 3 and 5.

**Analyses of the Infrared Region 1000-1250 Cm\(^{-1}\)**

The recorded infrared spectral region 1000 – 1250 cm\(^{-1}\) belongs to fingerprint region of polysaccharides. The presence of xyloglucan was detected at wavenumber 1041 cm\(^{-1}\)\(^{[10]}\).
For the different treatments of manganese, peak area of the band at 1041 cm\(^{-1}\) representing xyloglucan showed a considerable decrease. The assignment of the different bands and their peak area obtained from curve fitting analysis are incorporated in Table 2. For 50 µM treatment of manganese, there was a slight increase \((p<0.05)\) of 1.08% in xyloglucan content of the leaf of wheat plant. While for treatment with 100 µM, 200 µM, 500 µM, and 1000 µM manganese, spectral peak area showed a continuous decrease \((p<0.05)\) of 17.62%, 20.56%, 24.11%, and 31.31% respectively. The peak located around 1099 cm\(^{-1}\) represents the asymmetric vibration of C-O-C band pertaining to cellulose \[^{[11]}\].

The analysis of band at 1099 cm\(^{-1}\) in control and manganese treated plants revealed that after an initial rise \((p<0.05)\) of 3.12%, 12.92%, and 7.69% in the peak area for 50 µM, 100 µM, and 200 µM manganese treatment; there was a continuous decrease for further treatments of manganese with respect to control. The peak area decreased \((p<0.05)\) by 4.62% and 16.92% for the 500 µM and 1000 µM manganese treatment respectively. The infrared band at about 1130 - 1160 cm\(^{-1}\) was dominated by the glycosidic linkage ν(C-O-C). The band at 1155 cm\(^{-1}\) represents cellulosic component of the leaf of wheat plant \[^{[11]}\].

Peak area analysis of this band for the treatment with 50 µM, 100 µM, and 200 µM manganese revealed that there was an increase \((p<0.05)\) in the peak area of 1.01%, 4.73%, and 1.69% respectively with respect to control plant. For the further increase in the concentration of manganese to 500 µM and 1000 µM, a decrease in the peak area was observed to 10.14% and 20.94% respectively. A deformation of C-O-H and asymmetric stretch C-O-C of esters was observed at 1249 cm\(^{-1}\). It could be assigned to hemicelluloses present in the leaf of the wheat plant \[^{[11]}\]. The peak area of this band also declined as the concentration of manganese was increased. It decreased \((p<0.05)\) to about 12.77%,
17.93%, 15.76%, 33.97%, and 44.56% with respect to control for the 50 μM, 100 μM, 200 μM, 500 μM, and 1000 μM manganese respectively.

**Analyses of the Infrared Region 1300-1500 Cm⁻¹**

Lignin content was represented by the absorption at a wavenumber 1321 cm⁻¹ in the infrared spectrum of leaves of wheat plant. It developed due to vibrations of C-C and N-H [12]. The amount of lignin was estimated by the peak area of its characteristic bands. The peak area comparison of 1321 cm⁻¹ band revealed that after treatment with 50 μM manganese, peak area increased (p<0.05) by 18.36%. An increase in the concentration of manganese till 200 μM did not change the peak area and the increase was maintained. But treatment beyond 200 μM drastically decreased the peak area of lignin band. For the 500 μM and 1000 μM manganese treatment, retardation (p<0.05) in the peak area was up to 67% and 75.51% respectively. Another band corresponding to lignin was observed at 1515 cm⁻¹ due to the vibration of C=C and C=O aromatic system (semicircle ring stretch) [12]. The area under this curve also increased upon the treatment with manganese. Increment in the peak area was 15% (p<0.05) for the 50 μM and 100 μM manganese treatments. More intensive treatments impaired the spectra in this region and distorted spectral features indicating the lignin damage in the leaves of wheat plant. The increase in the peak area of lignin band indicated that degree of lignin cross-linking in these plants was increased.

**Analyses of The Infrared Region 1500-1700 Cm⁻¹**
The infrared signal at 1550 cm\(^{-1}\) was assigned to N-H bending and C-N stretching vibrations of the amide II band\(^{[13]}\). Manganese treatment did not induce any structural change in the N-H band of the amide II. The peak area comparison revealed that manganese treatment drastically increased the area of the C-N stretching vibration under amide II band. The peak area altered (p<0.05) from 0.06 (control) to 0.47 followed by treatment with 50 \(\mu\)M and 100 \(\mu\)M manganese treatment respectively. Further treatment with 200 \(\mu\)M decreased (p<0.05) peak area to 0.11. More intensive treatments of manganese impaired the spectral features of this band indicating the drastic decrease in the amide II content of the leaves of wheat plants. The peak area obtained from curve fitting analysis and assignment of the bands are listed in Table 3.

The recorded spectral region 1600 - 1700 cm\(^{-1}\) was specific to amide I band which arose due to C=O stretching vibration of the amide group coupled with in plane N-H bending \(^{[14]}\). The exact frequency of vibration of this band depends on the nature of hydrogen bonding involving the C=O and N-H moieties. The spectrum of water is also reported in this region with the peak at 1644 cm\(^{-1}\) due to bending frequency of O-H. This O-H band has impact on amide I profile and induce changes in relative area of the amide I. The altered area of the amide I band can be correlated to the presence of hydrogen bonding induced by the interaction with water. Due to the lack of sufficient data; we are unable to account it in the present analyses. Due to the low resolution of the spectrometer only main peak of amide I appear, but the amide I bands are a convoluted conglomeration of \(\alpha\)-helix, \(\beta\)-sheet (parallel and anti parallel pleated sheets), turns and random coil structures \(^{[14]}\). High sensitivity of amide I bands to small variations in terms of
geometrical configuration of molecules and hydrogen bonding pattern makes it useful for investigating the compositional and conformational changes in the secondary structure of proteins \[^{15}\]. The recorded spectra in the region of 1600 - 1700 cm\(^{-1}\) yielded relatively feature less bands due to the extensive overlap of the component bands buried beneath the broad band. These component bands lie in close proximity to one another and are beyond the resolving power of the instrument. To enhance the resolution of the infrared spectra and to precisely determine the individual band component corresponding to specific secondary structure for further qualitative analysis, curve-fitting analysis was used. Curve fitting narrows down the band width of individual component and separates them beyond the instrumental resolution. Figure 4 shows the curve fitted spectra of amide I band of the leaf of wheat plant without and with treatment of various concentrations of manganese. Four bands were found between the spectral region 1600 - 1700 cm\(^{-1}\) in the spectra of control and manganese treated plants \[^{16}\]. The bands developing in the region 1620 - 1630 cm\(^{-1}\) were assigned as \(\beta\)-sheet structure, while the bands arising in the region 1645 - 1660 cm\(^{-1}\) were assigned as \(\beta\)-sheet structure of protein. The bands centered in the region of 1670 - 1700 cm\(^{-1}\) were assigned as turn structure \[^{16}\]. The band components changed their maxima and positions followed by manganese treatment. The conformation of \(\beta\)-sheet was seen at 1618 cm\(^{-1}\) in control as well as in manganese treatments of 50 \(\mu\)M and 100 \(\mu\)M. Further treatment with 200 \(\mu\)M manganese shifted the \(\beta\)-sheet structure to higher wavenumber (1627 cm\(^{-1}\)). Peak area analysis of the \(\beta\)-sheet revealed a continuous decrease in the area. Its reduction (p<0.05) was 25.41\%, 35.36\%, and 43.09\% with respect to control for 50 \(\mu\)M, 100 \(\mu\)M, and 200 \(\mu\)M manganese treatment respectively. Treatments of 500 \(\mu\)M and 1000 \(\mu\)M caused a further shift in the position of \(\beta\)-sheets to
1631 cm\(^{-1}\). The area under these curve increased in comparison to the treatments with 50-200 µM manganese. The structure of amide I band at 1646 cm\(^{-1}\) was assigned as \(\alpha\)-helix. The peak position remained unchanged till the treatment with 200 µM while the peak area increased (p<0.05) slightly to 2.31% and 15.02% for 50 µM and 100 µM manganese treatments. For the 200 µM treatment with manganese, the peak area decreased (p<0.05) to 21.96% with respect to control. More extensive treatments of manganese (500 µM and 1000 µM) caused a shift of this band to 1660 cm\(^{-1}\) which was assigned to \(\alpha\)-helix conformation of protein. The absorption signal developed at 1677 cm\(^{-1}\) represented by \(\beta\)-turn of the amide band. The area beneath the band 1677 cm\(^{-1}\) showed a continuous decrease following the treatment with 50 µM, 100 µM, and 200 µM manganese. In addition to these, an inter \(\beta\)-sheet configuration of amide I band was seen at 1699 cm\(^{-1}\). The area under this band also retarded for the treatments with 50 µM and 100 µM manganese and it altogether disappeared when the plants were treated beyond 100 µM. In addition signature of side-chain absorption was seen to be developing at 1602 cm\(^{-1}\) when the plants were treated with 200 µM, 500 µM, and 1000 µM manganese. The band at 1602 cm\(^{-1}\) was assigned to ring vibration (phenyl) of tyrosine. The peak area analysis of this band which started developing for 200 µM manganese treatments showed a continuous increase for further treatments of 500 µM and 1000 µM manganese. The effect of heavy metal stress on the protein molecular structure demonstrated the decrease in the ratio of area of \(\beta\)-sheet to \(\alpha\)-helix. In the untreated plant leaf, the ratio of the area between \(\beta\)-sheet and \(\alpha\)-helix (\(A_{1618}/A_{1648}\)) was 1.91 but the treatment with 50 µM and 100 µM manganese increased the ratio to 2.62 and 3.40, further treatment with 200 µM decreased the \(\beta\)-sheet and \(\alpha\)-helix to 2.62. As there was shifting in the \(\beta\)-sheet and \(\alpha\)-
helix for the 500 µM and 1000 µM manganese treatments, therefore β-sheet and α-helix was now calculated for (A_{1630}/A_{1660}). The ratio decreased to 2.10 and 1.63 for the 500 µM and 1000 µM manganese treatment respectively. The shifting was a result of hydrogen bond weakening between the peptide bonds.

**Analyses of the Infrared Region 2800-2900 Cm⁻¹**

The recorded infrared bands in the spectral region 2800 - 3000 cm⁻¹ developed primarily due to symmetric and asymmetric stretching vibrations of the methyl and methylene groups and these vibrations were caused mainly by lipid. The assignment of the different bands and their peak area obtained from curve fitting analysis are tabulated in Table 4. The absorption band observed at 2850 cm⁻¹ represented by the symmetric vibrations of methylene (CH₂) group while the strong band at wavenumber 2918 cm⁻¹ indicated the presence of asymmetric vibration of methylene (CH₂). The relatively weak absorption maxima of asymmetric stretching vibration of methyl (CH₃) developed at 2958 cm⁻¹[17]. In addition to these, band at 1734 cm⁻¹ was attributed to stretching vibration of the carbonyl group (C=O). It represents ester bonds within the lipid molecule[18]. Manganese treatment in the wheat plant did not cause any conformational change in the vibrations of CH₂ and CH₃ structure even at higher concentrations. But the band at wavenumber 1734 cm⁻¹ showed a shifting towards lower wavenumber. The treatments with the concentration of 500 µM and 1000 µM induced a shift to 1724 cm⁻¹ in the C=O bond from 1734 cm⁻¹ in control. Manganese interaction with wheat plant resulted in the reduction of the peak areas of the lipid bands. In order to get the information about peak area beneath the lipid band, curve-fitting method was used. With the increasing
concentration of manganese, peak area of the absorption signal at 2850 cm$^{-1}$ showed a declining trend. When the plant was treated with 50 µM manganese, there was a slight increase in the peak area of 4.11% with respect to control. But the treatment with 100 µM manganese decreased the peak area to 4.49%. On more intensive treatments with manganese, the peak area continuously declined (p<0.05) by 1.12%, 1.87% and 12.73% for 200 µM, 500 µM, and 1000 µM manganese respectively. The peak area comparison was same for the symmetric vibrations of methylene also. After an initial rise of 2.57% for the 50 µM manganese treated plants, the peak area continuously retarded for further manganese treatment. The peak area decreased (p<0.05) to 2.25%, 2.89%, 4.01%, and 14.14% for the 100 µM, 200 µM, 500 µM, and 1000 µM manganese treatments respectively. The decrease in the peak area of the lipid bands indicated that amount of lipid was affected by the manganese treatment. As the concentration of manganese increased within the plant, the amount of lipid reduced and the reduction was more severe for 1000 µM manganese. The curve fitting results indicated that peak area ratio of the CH$_2$ and CH$_3$ ($A_{CH2}/A_{CH3}$) ratios continuously increased following the treatment with varying concentration of manganese. For instance, the untreated plant areal ratio was 20.73. The 50 µM, 100 µM, 200 µM, and 500 µM manganese treatments increased the areal ratio to 26.58, 28.14, 30.20, and 33.16 respectively. The more rigorous treatment with manganese at 1000 µM drastically increased the ratio to 66.75. An idea about the extent of lipid oxidation can be obtained by the ratio of the intensities between the bands corresponding to the stretching modes of CH$_3$ group (2954 cm$^{-1}$) and the carbonyl band (1734 cm$^{-1}$). This method has been used to investigate oxidatively modified
triacylglycerols \(^{[19]}\). The intensity ratio of the bands at 2954 cm\(^{-1}\) and 1734 cm\(^{-1}\) (\(A_{2954}/A_{1734}\)) continuously increased from 0.58 to 16.83.

**DISCUSSION**

Results of the present investigation indicate that seedling growth in terms of growth and biomass parameters, protein, and lipid content of wheat seedlings are adversely affected when exposed to different concentrations of manganese. It has been reported that plant’s water status is indicative of fresh mass of the plant; therefore reduction in the fresh mass of the wheat seedlings is due to loss of water which results in poor growth of the wheat seedling \(^{[20]}\). Increase in the cell wall components like cellulose, xyl glucan and lignin content are observed after manganese treatment. Plant cells are covered by structural proteins and polysaccharides which are impregnated by the aromatic polymer lignin. These components are interwoven to form an intricate mesh and are called cell wall. The cell wall is the first structure to come in contact with the environment \(^{[21]}\). The presence of carboxyl, hydroxyl, and aromatic functional groups in the polysaccharides of the cell wall provides ion exchange sites for counter ions. This chemical characteristic makes cell wall a very good biosorbent of heavy metals. The cell wall polysaccharides are affected by the heavy metal ions, as alteration in synthesis and composition in cell wall is observed \(^{[22]}\). An increase in the cellulose and lignin component in the leaves of wheat plant up to 200 \(\mu\)M concentration is observed. This increase is due to the modifications in the physico-chemical properties of the cell wall which increase the binding capacity and at the same time lower the entry of heavy metals in the protoplast. This indicates that under mineral stress the composition of cell wall is modified. The
increase in polysaccharide and lignin content up to 200 µM indicates the stress tolerance mechanism of cell wall by chelating metal ions onto the surface of these molecules by electrostatic force attraction of the negatively charged functional groups. These modifications make plant cell wall less permeable to the heavy metals like manganese and inhibit their entry to the protoplasm [23]. But after 200 µM manganese treatment, the tolerance and resistance mechanism of plant is completely exhausted as indicated by the drastic reduction in the area of the band corresponding to lignin content and impairment of spectral signatures belonging to lignin. The decrease observed in the cellulose component of the leaves of wheat seedlings after 500 and 1000 µM manganese pretreatment indicates that these concentrations are highly toxic to the plants. This decrease would be due to the inhibition of conversion of sucrose into cellulosic components. The cell wall is affected by heavy metals, since its biosynthesis and composition can be altered [24]. The results indicate that manganese is not efficiently neutralized and becomes a toxic factor by affecting the composition of cell wall. The interaction of manganese with the polysaccharide component can interface with the enzymatic activities present in the cell walls by inhibiting the enzymatic reactions that give strength to the cell wall structure thereby negatively affecting their resistance mechanism and lead to the growth retardation in the plant [25].

The results indicate shifting in the positions of band components of amide I after the manganese treatment. The observed shift in peak at 1618 cm\(^{-1}\) to 1630 cm\(^{-1}\) and the peak at 1646 cm\(^{-1}\) to 1660 cm\(^{-1}\), indicates the weakening of hydrogen bonds. In proteins, hydrogen bonds are seen between peptide bond. The absorption position of amide I band
indicates the degree of hydrogen bonding, greater the wavenumber shift, weaker the hydrogen bonding. As the hydrogen bond weakens, the protein structure becomes less ordered [26]. Also the observed increase in the β-sheet/α-helix ratio is a possible indicator of increased disordered proportion of protein (weaker hydrogen bonding) in the plant leaf [27]. Thus shifting of position of bands to higher wavenumber and increase in the β-sheet/α-helix ratio are a possible signal of the overall protein disordering due to the treatment with manganese. While the decrease in the peak area of the bands of amide I and amide II with the increase in concentration of manganese indicate the protein damage caused by manganese within the plant. The decrease in protein content is due to the increasing activity of proteases. Proteins are hydrolyzed by proteases to release amino acid residues [28]. At higher concentration of manganese (200 µM-1000 µM) an amino acid residue develops at 1602 cm⁻¹ and its peak area gradually increases. This increase in the amino acid side chain which is possibly assigned to tyrosine is an indicative of the increase in the activity of proteases. These side chain amino acids are important in chelating the heavy metals and provide a resistance mechanism to the plants [29].

The observed decrease in the area of bands for lipid indicates that amount of lipid decreases after the manganese treatment while the observed increase in ratio of CH₂/CH₃ indicates the increased level of saturation of lipid molecule. The increase in the level of saturation in a fatty acid indicates disordered status of lipid within the plant and this makes the lipid molecule more susceptible to the oxidative damage [26]. An idea about the extent of lipid oxidation obtained from the ratio of the stretching modes of CH₃ groups and the carbonyl bands indicate an increasing trend. The absorbance ratio of the bands at 2954 cm⁻¹ and 1734 cm⁻¹ (A₂954/A₁734) continuously increases from 0.58 to 16.83. This
increase can be directly linked to the increased peroxidation of lipid within the leaf after the manganese treatment. It has been reported that heavy metals are often associated with the formation of ROS within the plant material. ROS is known to cause oxidative stress within the lipid and protein of the plant species. The ROS trigger the oxidation lipid by increasing activity of lipid peroxidases \[^{30}\].

**CONCLUSIONS**

The present investigation describes the potential of ATR-FTIR spectroscopy technique for the non-destructive and rapid interrogation of stress arising due to implanted heavy metals in plant seedlings. ATR-FTIR spectroscopy is a suitable technique for the detection of the conformational transformations and chemical changes in plants under various stress conditions. The obtained results indicate that plant growth was adversely affected when exposed to manganese at different concentrations beyond optimum level. Growth and biomass parameters in terms of shoot length, root length, and fresh weight significantly reduced with increasing concentration of manganese. The chemical constituents (cellulose, lignin, protein, and lipid) also responded negatively to the higher concentration of manganese.

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REFERENCES


Table 1: Effect of manganese on the growth (cm ± standard error) and biomass (g ± standard error) of wheat seedlings

<table>
<thead>
<tr>
<th>Growth and biomass parameters</th>
<th>Concentration of manganese</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Shoot length (cm)</td>
<td>23.3±0.56a</td>
</tr>
<tr>
<td>Root length (cm)</td>
<td>11.52±0.25a</td>
</tr>
<tr>
<td>Shoot weight (g)</td>
<td>0.122±0.005a</td>
</tr>
<tr>
<td>Root weight (g)</td>
<td>0.041±0.004a</td>
</tr>
</tbody>
</table>

*Data are the means ± standard error of three independent experiments. Values with different superscripts within the same row show significant differences at P<0.05 level between treatments according to the Tukey’s multiple range test.
**Table 2:** Peak area analyses of the spectral region 1000 - 1500 cm\(^{-1}\) of the recorded infrared spectrum of the leaves of control and manganese treated wheat plants

<table>
<thead>
<tr>
<th>Peak position (cm(^{-1}))</th>
<th>Conformations</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1041</td>
<td>xyloglucans</td>
<td>6.47±0.07a</td>
</tr>
<tr>
<td>1099</td>
<td>asymmetric vibration of C-O-C band of cellulose</td>
<td>0.33±0.01a</td>
</tr>
<tr>
<td>1155</td>
<td>asymmetric stretch of C–O–C group of cellulose</td>
<td>0.30±0.04a</td>
</tr>
<tr>
<td>1249</td>
<td>deformation of C-O-H and asymmetric stretch C-O-C of esters</td>
<td>0.37±0.05a</td>
</tr>
<tr>
<td>1321</td>
<td>Vibrations of C-C and N-H of lignin</td>
<td>0.49±0.03a</td>
</tr>
<tr>
<td>1373</td>
<td>(\delta(CH_3)) and (\delta(CH_2)) of proteins, lipids and lignin</td>
<td>0.76±0.03a</td>
</tr>
<tr>
<td>1462</td>
<td>asymmetric CH(_3) bending mode of end ethyl group of proteins/lignin</td>
<td>0.59±0.02a</td>
</tr>
<tr>
<td>1515</td>
<td>vibration of C=C and C=O aromatic system</td>
<td>0.01±0.00a</td>
</tr>
<tr>
<td>(semicircle ring stretch) of lignin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------------------</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Data are the means ± standard error of three independent experiments. Values with different superscripts within same row show significant differences at P<0.05 level between treatments according to the Tukey’s multiple range test.*
Table 3: Peak area analyses of the spectral region 1600 - 1700 cm\(^{-1}\) of the recorded infrared spectrum of the leaves of control and manganese treated wheat plants.

<table>
<thead>
<tr>
<th>Peak position (cm(^{-1}))</th>
<th>Conformation</th>
<th>Peak area</th>
<th>Control</th>
<th>50 µM</th>
<th>100 µM</th>
<th>200 µM</th>
<th>500 µM</th>
<th>1000 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1550</td>
<td>The amide II (N-H deformation and C-N stretch of proteins)</td>
<td>0.06±0.00a</td>
<td>0.47±0.00b</td>
<td>0.47±0.29b</td>
<td>0.11±0.001c</td>
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<td>-</td>
<td></td>
</tr>
<tr>
<td>1600-1605</td>
<td>Side chain</td>
<td></td>
<td></td>
<td>1.13±47.29a</td>
<td>2.60±1.67b</td>
<td>3.57±1.67c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1618-1622</td>
<td>β-sheet</td>
<td>1.81±0.75a</td>
<td>1.35±0.32b</td>
<td>1.17±0.22c</td>
<td>-</td>
<td>-</td>
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<td></td>
</tr>
<tr>
<td>1627-1630</td>
<td>β-sheet</td>
<td>-</td>
<td>-</td>
<td>1.03±18.93a</td>
<td>1.63±.27b</td>
<td>2.10±.26c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1645-1650</td>
<td>α-helix</td>
<td>3.46±1.02a</td>
<td>3.54±0.59a</td>
<td>3.98±0.40b</td>
<td>2.70±53.27c</td>
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<td>-</td>
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</tr>
<tr>
<td>1655-1660</td>
<td>α-helix</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.41±.21a</td>
<td>3.74±.19d</td>
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</tr>
<tr>
<td>1677-1680</td>
<td>β-turn</td>
<td>1.14±0.43a</td>
<td>0.17±0.28a</td>
<td>0.11±0.10b</td>
<td>1.90±21.32c</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Turn</td>
<td>0.14±0.11a</td>
<td>2.24±0.68b</td>
<td>2.17±0.36c</td>
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</tbody>
</table>

*Data are the means ± standard error of three independent experiments. Values with different superscripts within same row show significant differences at P<0.05 level between treatments according to the Tukey’s multiple range test.
**Table 4:** Peak area analyses of the spectral region 2800 - 3000 cm\(^{-1}\) of the recorded infrared spectrum of the leaves of control and manganese treated wheat plants

<table>
<thead>
<tr>
<th>Peak position (cm(^{-1}))</th>
<th>conformations</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>2850</td>
<td>symmetric CH(_2) stretch</td>
<td>2.67±0.02a</td>
</tr>
<tr>
<td>2917</td>
<td>asymmetric CH(_2) stretch</td>
<td>6.22±0.02a</td>
</tr>
<tr>
<td>2958</td>
<td>asymmetric CH(_3) stretch</td>
<td>0.30±0.01a</td>
</tr>
</tbody>
</table>

*Data are the means ± standard error of three independent experiments. Values with different superscripts within same row show significant differences at P<0.05 level between treatments according to the Tukey’s multiple range test.*
**Figure 1(a):** Impact of different manganese treatments on root and shoot length (cm plant$^{-1}$) of the wheat plants. Data are mean ± standard error of three independent experiments.
Figure 1(b): Impact of different manganese treatments on the fresh weight of root and shoot (gm plant$^{-1}$) of the wheat plants. Data are mean ± standard error of three independent experiments.
**Figure 2:** Recorded infrared spectra of leaves of wheat seedlings pretreated with manganese at different concentrations.
**Figure 3:** Recorded infrared spectra of fingerprint region of carbohydrate and lignin present in the leaves of wheat seedlings pretreated with manganese at different concentrations.
Figure 4: Curve fitting of amide I band of leaves of wheat seedlings pretreated with manganese at different concentrations (A) control (B) 50 µM (C) 100 µM (D) 200 µM (E) 500 µM and (F) 1000 µM
**Figure 5:** Recorded infrared spectra of fingerprint region of lipid present in the leaves of wheat seedlings pretreated with manganese at different concentrations