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Development of Nondestructive Measurement Technology for Detecting the Salinity Stress of Jatropha under Saline Irrigation Levels

Ahmed A. Afifi

Department of Soils and Water Use, National Research Centre, Dokki, Giza, Egypt

Abstract: Salinity problems are becoming more and more severe in management, which can cause physiological stress to plant and deteriorate the soil structure. Jatropha is a high tolerance to salinity. The aim of this study was to adopt the approach through the use of Fourier transform infrared (FT-IR) spectroscopy and chemometrics to detect Jatropha responses to salinity stress. Salinity treatment was applied by adding a diluted Mediterranean Sea water to attain salinity levels 2500 and 5000 ppm (two irrigation by saline water and the next was by tap water alternatively) and tap water was used with Jatropha. Amino acid proline at the rate of 100 and 200 ppm was sprayed. At least three indices estimated based on single leaf reflectance spectrum (400-4000 cm-1). The unsupervised clustering method, principal component analysis (PCA) showed no discrimination between the Jatropha and salt-treated plants. The supervised method, discriminate function analysis (DFA) was capable to classify Jatropha and salt-treated plants. Key regions were identified within the spectra corresponding to amino radicals. The application of IR enabled the identification of functional groups of potential importance in relation to the response of Jatropha to salinity.

Key words: Forier Infrared Spectrosopy · Jatropha · Salinity stress · Sea water

INTRODUCTION

High salinity causes polio-tropic effects on plant growth such as reduced cell expansion, decreased protein synthesis and accelerated cell death. Soil salinity is one of the major limitations of crop productivity worldwide. Salinity affects plants in different ways such as osmotic effects, specific-ion toxicity and/or nutritional disorders [1-4]. The extent by which one mechanism affects the plant over the others depends upon many factors including the species, genotype, plant age, ionic strength and composition of the salinizing solution and the organ in question.

Most of studies indicated that the effect of salinity on crops has been conducted in Jatropha led laboratory and greenhouse environments, allowing scientists to better understand detailed responses and determine possible mechanisms the plant uses to cope with this stress. However, such experimental conditions do not reflect the natural conditions the plant encounters in salt-affected areas. There are a number of additional abiotic and biotic stresses that plants may endure in the field such as extreme temperatures, water deficits, flooding, nutritional inadequacies, poor soil physical conditions, pathogens and pests [5]. Moreover, these stresses are not constant, but vary both spatially and temporally.

The vibration of chemical bond absorbs radiation in the IR region between 400 and 4000 cm^{-1}. Each fun using Fourier transform infrared (FT-IR) spectroscopy. This technique is highly suitable for the acquisition time of FT-IR spectrometry can be shorter than a second [6].

The examination of the initial response to stress because of the functional group in a molecule has characteristic absorption frequencies in the IR spectrum [6]. The sensitivity of IR spectroscopy has been successfully applied in vitro and in vivo detection of biological systems. During a chemical extraction of plant cell walls, components and possible cross links of each fraction were identified by FT-IR micro-spectroscopy [7]. The IR method provides a unique way to study the conformation of proteins [8]. The C-O, NH_2 and C-N bonding of the peptide linkage absorb radiation in the 1,800 to 1,200 cm^{-1} region. The absorption band of C-O

Corresponding Author: Ahmed A. Afifi, Soils and Water Use Dept. National Research Centre, Dokki, Giza, Egypt.
E-mail: a.afifinrc@gmail.com.

stretching vibrations of the amide group depends on the nature of hydrogen bonding between C-O and N-H moieties and is particularly useful for determining the secondary structure of a polypeptide chain [9]. In addition to the purified proteins, the secondary structures of proteins in complex biological samples have also been analyzed.

Mathematical approaches such as deconvolution and curve fitting are generally applied to extract information from the raw IR spectra to resolve the overlapping band components in the heterogeneous matrix. Deconvolution is a band-narrowing technique that can enhance small features buried in an overlapped band. Comments about this technique can be found in the review article by Surewicz and Mantsch [10]. The curve-fitting technique enables further quantitative analysis of individual bands buried in an overlapping band. A series of Gaussian or Lorenzian curve shapes are used to compose a synthesized spectrum. By subtracting the raw spectrum from the synthesized spectrum, the residual can be used to determine the fitness between synthesized and raw spectra. In a statistical manner, the number of synthesized spectra with low residual may be more than one. To reduce the possibility of fitting the curves in unwanted directions, the band positions obtained by deconvolution spectrum are typically used [10]. Decomposition of the amide I band by curve fitting into its constituents and the assignment of these components of a protein structure has been successfully applied in predicting the structure of membrane proteins [11].

Given the complex biological matrix, the changes of a specific functional group cannot be assigned to a particular molecule in the cells. However, the changes in chemical composition reflect the overall changes in the metabolic processes and can be detected more sense than the traditional methods.

The most sensitive mechanism to abiotic stress is photosynthesis and when plants are subjected to adverse environmental conditions such as drought, salinity, heat or cold, to name a few, carbon assimilation and the primary metabolism are largely affected. Among all primary metabolites: sugars, sugar alcohols and amino acids are the most important metabolites which concentration in plant tissues is affected by stress, usually as a downstream result of an impairment in the CO_2 assimilation process, but also as a result of a complex regulatory network [12and13]. Nevertheless, due to the great differences in concentration (usually several orders of magnitude) changes in secondary metabolite levels cannot be simply inferred from variations in their primary metabolite precursors and is usually a result of a complex regulatory process. For this reason, stress-associated changes in secondary metabolites will be considered and reviewed separately.

In this work, the early salt-induced changes in chemical constituent in Jatropha plants were detected by FT-IR spectrometry. The leaves were dried to remove the spectral interference caused by blanket absorption of water over most of the IR region. The changes in the amount of cell wall pectin and carbohydrate were calculated according to the band intensities of the original IR spectra. The IR signals were further processed by deconvolution and curve fitting to reveal the rapid changes in protein conformation. The differential responses to salt stress underline the different strategies to high salinity levels.

MATERIALS AND METHODS

Experiments were carried out on Jatropha seeds grown in pots 30 cm in diameter, that were filled with sandy soil under greenhouse conditions of National Research Centre, Dokki, Giza, Egypt. The soil was sandy in texture (Table 1) and the mechanical and chemical analysis of soil was carried out according to method described by [14]. Seeds of Jatropha were sown during the two summer seasons of 2010 and 2011. The seeds were irrigated regularly with tap water for three weeks until seedling emergence to avoid the effect of salinity on seedlings. All pots were supplied with the recommended dose of nitrogen (29 NP_2O_5), phosphorus (50) and potassium (62.5 K_2O) fertilizers. Irrigation with diluted Mediterranean Sea water was done after twenty one days from sowing to attain salinity levels 2500 and 5000 ppm (two irrigation by saline water and the next was by tap water alternatively) and tap water was used with Jatropha. Amino acid proline in 100 and 200 ppm was sprayed twice 156 days from sowing and the second two weeks later in addition control plant (sprayed with distilled water). The irrigation with either fresh and/or saline water reach the level of 70% of the total field capacity (F.C.) of the soil by weighing every pots and the needed amount was added.

The experiment included 9 treatments which were the combination of two levels of saline water (2500 and 5000 ppm) in addition to control plants (sprayed withtap water). As well as, all samples will be treated with two concentrations (100 and 200 ppm)and without proline. The experiments were arranged factorial experiment in complete randomized design (CRD) with three replicates with 3 replicate in each treatment.

Table 1: Physical and chemical analysis of the tested soil (average of the two seasons (2010 and 2011).

Soil analysis	Mean of the two seasons (2010 and 2011)	
Physical analysis:	Clay %	18.00
	Sand %	57.25
	Silt %	24.75
	Soil texture	Sandy
Chemical analysis	pH (1:2.J)	7.25
	E.C.(1:5.J)	1.1 dSm^{-1}
Available macro nutrient (ppm)	N	189.10
	P	3.14
	K	259.75
	Ca	65.15
	Mg	73.18
Available micro nutrient (ppm)	B	3.42
	Fe	15.14
	Mn	21.81
	Zn	1.18
	Cu	1.31
	Al	0.78

IR Spectroscopy: After 186 days from sowing representative samples from 3 replicates of treatments were used for infrared spectroscopy analysis. The leaves were taken from different plants and were pooled as one sample. Then the samples were immediately dried in an oven for 2 days at 60°C. Tablets for FT-IR spectroscopy were prepared in an agate mortar, by mixing leaves powder (2 mg) with KBr (1:100 p/p). The absorbance spectra were measured between 400 and 4000 cm^{-1}. At least three leaves were collected and a minimum of three spectra were obtained from each sample.

A FT-IR spectrometer (FTIR-NEXUS 670, Thermo Nicolet Corporation, America) was used to collect spectra. Spectra were obtained in 32 scans co-added, 4000 resolution and 2.0 gains. The parameters for the Fourier self-deconvolution were a smoothing factor of 15.0 and a width factor of 30.0 cm^{-1}. De-convolved and second-derivative spectra were calculated for Fourier self-deconvolution and the bands were selected and normalized to unity with Omnic 7 software. Curve-fitting of the original spectra was performed with Origin 7 software. The band position of functional groups was monitored with Knowitall 7.8 software (http://www.knowitall.com). The spectral region between 3000 and 2800 cm^{-1} was selected to analyze lipids. The spectral region between 1800 and 1500 cm^{-1} was selected to analyze proteins. The spectral region between 1200 and 1000 cm^{-1} was selected to analyze carbohydrates.

RESULTS AND DISCUSSION

IR Monitors the Changes in the Leaves under Salt Stress: In this study, FT-IR spectroscopy was used for analysis of changes in carbohydrate, protein and cell wall. The IR absorption spectrum between 4000 and 400 cm^{-1} was revealed on the leaves IR spectroscopy (Fig. 1). In mid-IR region (4000 and 400 cm^{-1}) appeared large numbers of sharp peaks, indicating that the leaves have a rich chemical composition, such as carbohydrates, proteins and lipids. However, this region yielded broad and overlapped bands.

The bands around 3370 cm^{-1} represent O-H and N-H stretching vibrations that are mainly generated by proteins and carbohydrates [15]. The bands between 3000 and 2800 cm^{-1} represent C-H stretching vibrations that are mainly generated by lipids [16]. The protein absorption bands mainly located between 1800 and 1500 cm^{-1} contained amide-I and amide-II bands [9 and17], but overlapped with other absorption bands within this region. Amide III, the functional group of nucleic acid and carbohydrates contributed to these absorption bands in the leaves. Absorption spectrums were further processed with Fourier self-deconvolution. After this process, the weak feature bands buried in some overlapped band can be enhanced and band positions corresponding to protein, lipid, carbohydrate and cell wall pectin were clearly distinguished.

The changes in protein under salt stress: Amide-I and amide-II bands are particularly useful for determining the protein IR absorption changes. Amide-I region (1700-1600 cm^{-1}) mainly represent C=O stretching vibrations of polypeptide, which can detect changes of the overall protein conformation and content [9]. After de-convolution and curve fitting process, three bands composed in amide-I region between 1700 and 1600 cm^{-1} were distinguished and these bands can give additional information about the protein structure, the band around 1685 cm^{-1} assigned to the turn structure, the band around 1656 cm^{-1} assigned to the á-helix structure and the band around 1621 cm^{-1} assigned to the β-sheet structure. The relative band area of turn structure was decreasing and the absorption strength was lower; but the corresponding value of β-sheet structure was increased and the absorption strength was enhanced. In addition, the curve-fitting results showed the amide I total band area decreased 23% which meaning the total protein contents declining remarkable, indicated that protein

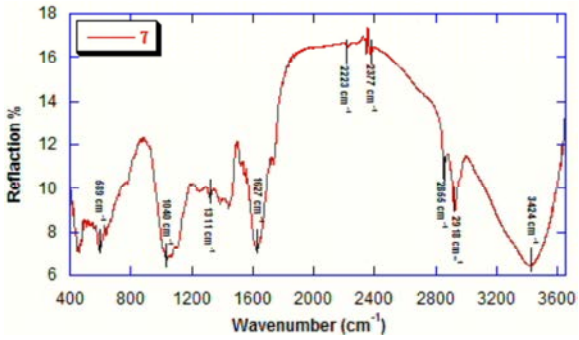

Fig. 1: Mid-infrared spectra for Jatropha irrigated with tap water.

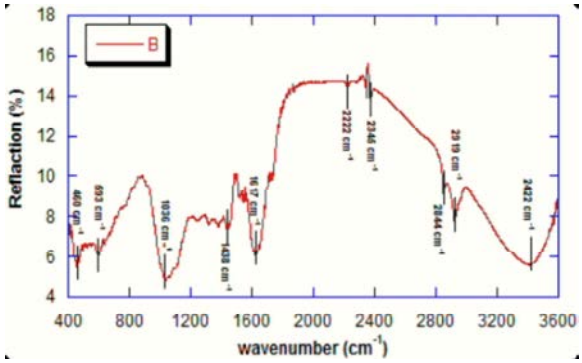

Fig. 2: Mid-infrared spectra for Jatropha under salt stress.

synthesis is sensitive to salt stress. Similar salt-stressed changes were also observed in the amide II band located at around 1544cm^{-1} (Fig. 2).

The results showed that the protein synthesis is sensitive to salt stress in Jatropha leaves at the earliest stage and the stability of protein secondary structures were capable to change after salt treatment. But it was not enough to breach protein synthesis pathway and the injury was not irreversible with salt stress and this restores phenomena may be largely due to physiological adjust. FT-IR spectroscopy studies of protein structure are increasingly widespread used. The IR spectra demonstrated that salt stress can inflect the protein structure and protein became less ordered, but following prolonged stress, this response did not persist and protein refolded slowly [18].

The Changes in Lipids under Salt Stress: The IR spectrum between 3000 and 2800 cm^{-1} mainly occur from lipids. De-convolution was used to enhance the resolution of IR spectra and then showed five bands uncovered, which located at approximately 2960, 2921, 2900, 2873 and 2850 cm^{-1}, respectively. The bands around 2850 and 2921 cm^{-1} represents C-H asym- or sym-stretching vibration, which belongs to the -CH_2 group of lipids and the bands around 2873 and 2960 cm^{-1} also represent C-H asym- or sym- stretching vibration, but it belongs to the -CH_3 group of lipid. The IR spectra were curving fitted. After of salt stress, compared with the Jatropha, the absorption strength around 2850 cm^{-1} was enhanced and the bandwidth was decreased, whereas the absorption strength around 2921 cm^{-1} was lower and the bandwidth was increased; around 2873 and 2960 cm^{-1}, the bands whose absorption strength were lower and bandwidth were increased. On the other hand, Fig. 2, at 2935 cm^{-1}, de-convolution enhances the resolution of small band buried in the original spectra and it belongs to -OCH_2-group stretching vibration (http://www.chem.unipotsdam.de), this group represents peroxides and hydro-peroxides and could be considered as biomarker for indicating the lipid. The emerging of this peak and addition of a variety of other absorption peak located in this region reflected the relative declining of -CH_2, indicated that the oxidative stress of lipid was severed after salt exposure. After stress all bands returned to the Jatropha level and the small band at 2935 cm^{-1} disappeared. Through curve fitting analysis, the total band area between 2980 and 2845 cm^{-1} returned to the Jatropha level and the band at 2935 cm^{-1} also disappeared. In stress, the fitting results show the total burned areas (3000-2800 cm^{-1}) were similar to Jatropha. This implies that lipid have not accumulated in the Jatropha leaf tissue. About the FT-IR spectroscopy studies of plant, there was few reports on -OCH_2 group stretching vibration. This provides more direct evidence for lipid peroxidation damage of salt stress on plants.

IR Monitor the Changes in Carbohydrate under Salt Stress: The IR spectra between 1200 and 1000 cm^{-1} mainly occur from carbohydrates. De-convolution showed four bands represented C-O-C stretching vibration (Fig. 1), located at approximately 1199, 1155, 1106 and 1037 cm^{-1}, respectively. After salt stress, the total band areas between 1200 and 1000 cm^{-1} only was increased by 0.29% compared with the Jatropha by curve fitting analysis (Fig. 2), implying the carbohydrate did not accumulate obviously. But all bands strength was enhanced, indicating the carbohydrate structure has changed. After of stress, the total band areas was decreased by 19% and the bands at 1199 and 1060 cm^{-1} lowered gradually, indicated the carbohydrate synthesis decreased and the

structure kept changing, showing salt stress changed the structure of carbohydrate in the plant leaf. Taken together, the above results means carbohydrate synthesis is sensitive to salt stress in the Jatropha leaves.

Literature data showed salt stress can stimulate the metabolism of carbohydrate in the apricot leaves [19], this indicated the carbohydrate synthesis pathway or some carbohydrate may play a critical role in the anti-oxidative response to salt stress. Compared with these results, a significant difference was exiting on carbohydrate changing profile, inflecting the dissimilar salt resistance ability which arising from different capacity of metabolism adjustment to salt stress.

IR Monitors the Changes in Cell Wall Pectin under Salt Stress: The band around 1743 cm^{-1} represents -COOR stretching vibration (Fig. 2), which belongs to characteristic group of cell wall pectin. After salt stress, the total band areas at 1743 cm^{-1} decreased by 12% compared with the Jatropha irrigated with tap water by curve fitting analysis and the band intensity was lowered, indicating the pectin synthesis decreased. After stress, this band just had a little change compared with the Jatropha (conotrol), indicating the pectin synthesis returned to the Jatropha level. The absorption bands of phosphodiester group were buried with other absorption bands, so, to argue whether there are produces banding with salt, clear spectra of phosphodiester group is necessary.

In this study, the significant recovery phenomena was observed in 24h treated sample, we speculated that this due to plant native physiological adjustment mechanism. It is well known that plants can alleviate the stress of salinity by accumulating salt, in other words, salts are present as no activity or non-toxic form in plants, i.e. combined with cell wall, ion active transport into the vacuole, banding organic acids or protein [20]. Because of salt banding with certain plants products, the amount of salt retained in active site can be small, then salt toxicity is alleviated [21]. In this research, only the macroscopical and simultaneous data on biological macromolecular was obtained and the in-depth analysis of salt-responding second compositions need be conducted with mathematical model construction based native FT-IR data. It is very likely that salt tolerance mechanisms may differ depending on the plant species [22] and this difference would be demonstrated by FT-IR technique to further understand the responding and adjustment mechanisms to salt accumulation and sensitive plant species, ultimately accelerating plant breeding process.

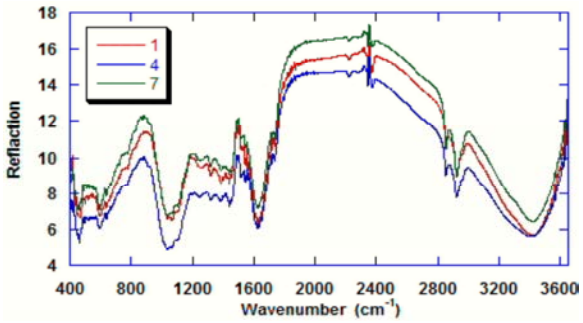

Fig. 3: Mid-infrared spectra for Jatropha leaves irrigated with fresh water.

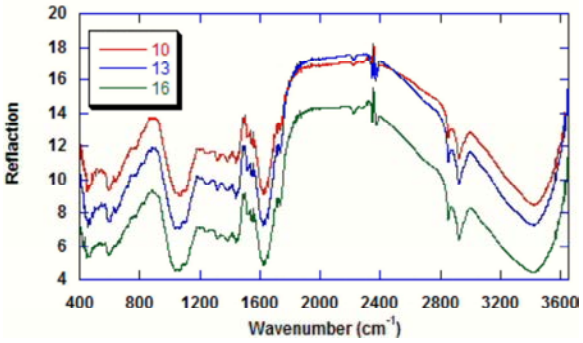

Fig. 4: Mid-infrared spectra for Jatropha irrigated with saline water (5000 ppm) with and without praline

The Role of Proline: During abiotic stress conditions, plants induce the synthesis of osmolytes such as soluble sugars and amino acids which contribute to turgor maintenance by osmotic adjustment [23 and 24]. Among amino acids, Proline is the main effector in this response (in addition to hexoses), contributing to around 50% of the osmotic adjustment in maize root tips [25]. Indeed, increases in Pro content have been reported in response to different abiotic stress conditions like salt stress [26 and 24], soil flooding [27], drought [28] or extreme temperatures [29]. However, whether Pro can counteract and protect against abiotic stress or not is still a question of debate. As illustrated in Fig. 3 the infrared spectra for the use of normal water for irrigation in the case with and without proline, that the spectra is almost the same except for the range from 400 to 1600 cm^{-1}. While, for the use of saline water with different concentrations, it is noticed that the use of 100 proline is similar to without proline. However the use of 200 proline is not comparable with the others as seen in Fig. 4. Regarding the application of 100 ppm proline, under different irrigation treatment, some shift for the location of the functional groups (Fig.5) were observed.

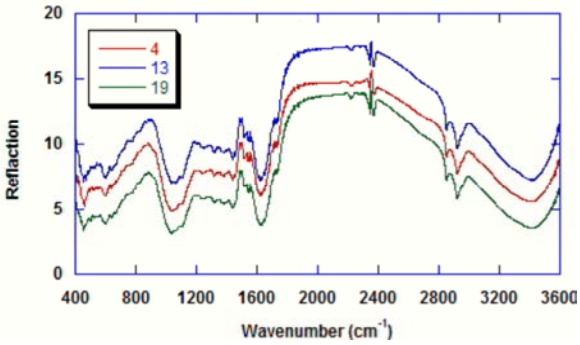

Fig. 5: Mid-infrared spectra for Jatropha irrigated withdifferent water treatment with 100 ppm praline.

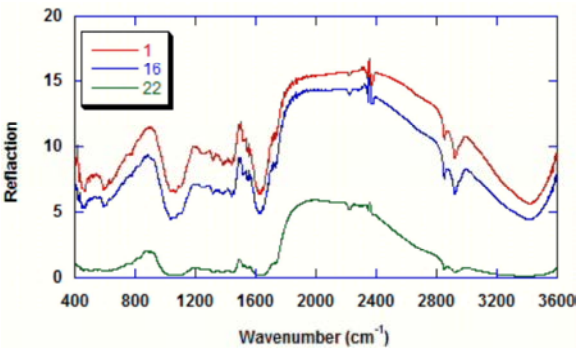

Fig. 6: Mid-infrared spectra for Jatropha irrigated withdifferent water treatment with 200 proline.

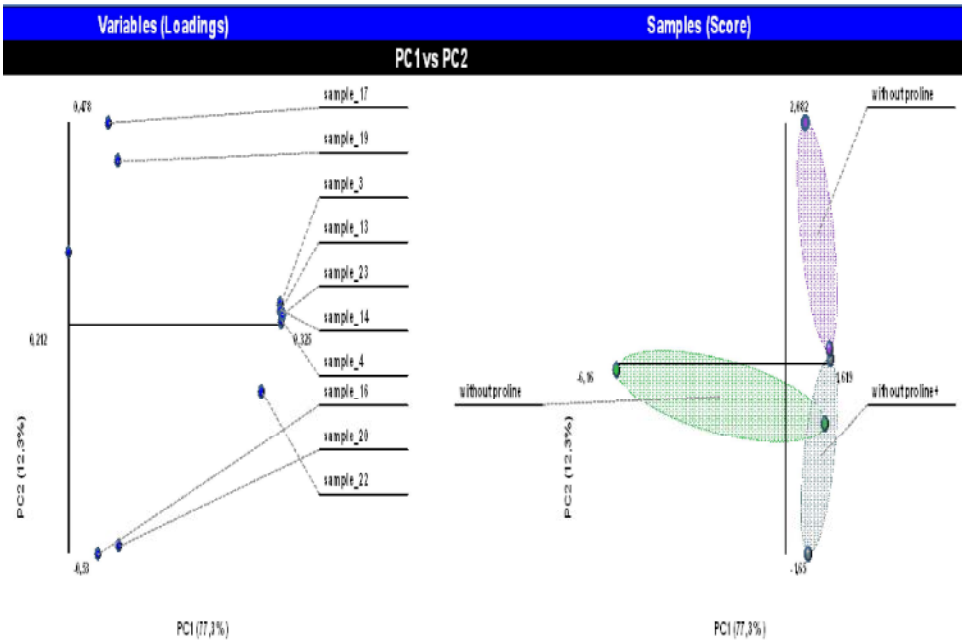

Fig. 7: PCA for the different treated plants.

For the use of proline with 200 with the different cases of irrigation water, it shows that the sample 1 (fresh water treated) and sample 16 (saline water 5000) are similar. While, sample 22 (saline water 2500 ppm) is different. Which illustrates the role of proline should be under some condition (Fig. 6).

Cluster Analyses of FT-IR Spectra: The tissue extracts of Jatropha leaf samples were analyzed using FT-IR. It should be noted that the two varieties Jatropha irrigated with (tap water and salt-treated) were analyzed individually as separate experiments. clustering is the task of grouping a set of objects in such a way that objects in the same group (called a cluster) are more similar (in some senseor another) to each other than to those in other groups (clusters). These FT-IR spectra and all the others collected look very similar, they all show broad and complex contours and it is difficult to identify key features by eye. Such spectra readily illustrate the need to employ chemometric techniques for their analysis.

The PCA plots show for each treatment (Fig. 7), based on the FT-IR spectra fingerprints of each. It can be seen in these Figures that there is a high discrimination between the Jatropha treated with tap water and salt-treated plants. Also, it can be seen that there is a large intrinsic variation within the data sets, 77% of which is accounted for by the first principal component (PC) (Fig. 8 and Table 2).

Table 2: Principle component analysis.

Component 1	Component 2	Component 3
77%	12%	8%

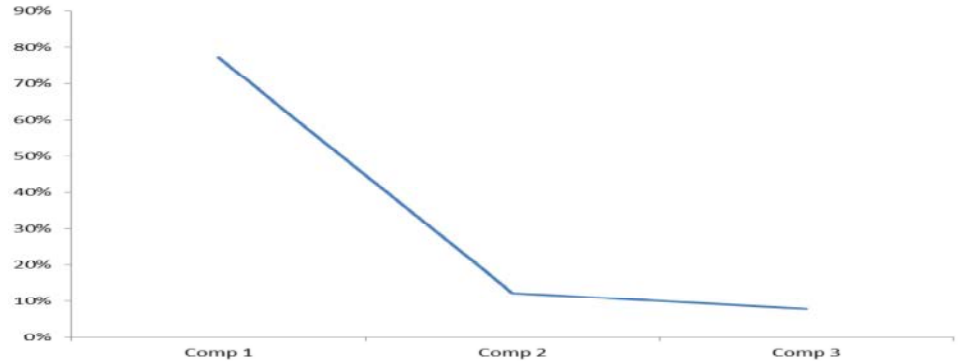

Fig. 8: Principle component analysis.

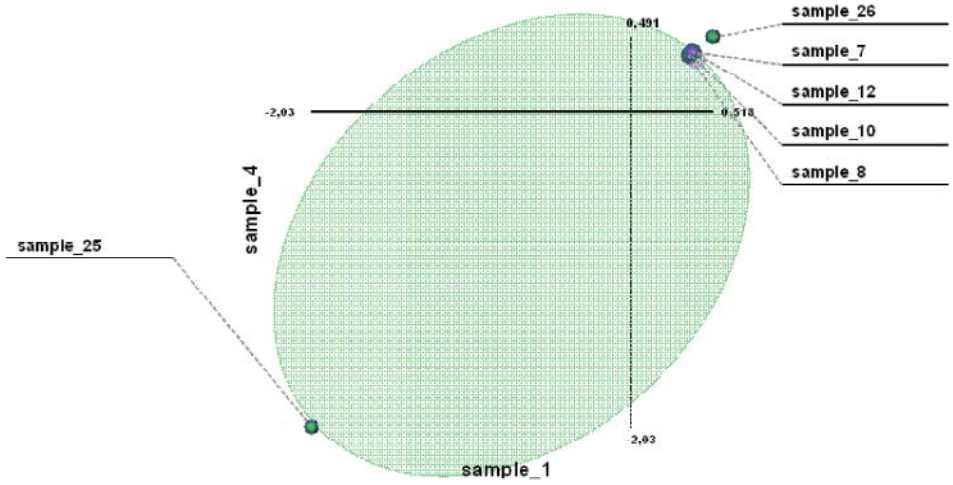

Fig. 9: PCA for the comparison between treated and un-treated samples with praline.

So, we try to see the effect of adding proline, as illustrated in (Fig. 9) we use PCA to differentiate between the use of proline and without. The Figure shows that all the samples without adding proline is almost the same except the case of using saline water at 2500ppm it was far from the others like the case in sample 25 and 26. However, the sample 26 was outlier from the sample 25, while sample 25 was close to the samples treated with fresh water with adding praline.

DFA models for all treated samples were then produced using knowledge of the treatment group structure of each data set, hence termed a supervised technique. The number of PCs used in each DFA model was optimized by cross-validation, using a training and test data set.

The Discriminant Factor Analysis (DFA) model was able to discriminate between the Jatropha and salt treated plant. However, the separation was more distinct between the Jatropha and salt-treated plants (Fig. 10 and 11). This trend may be due to a greater biochemical difference between the Jatropha and salt-treated plant as the DFA model was able to discriminate between the Jatropha and salt treated plants in each treatment. However, the separation was more distinct between the Jatropha and salt-treated plants, with only one un-Jatropha sample misclassified in the test set (sample 26). This trend may be due to a greater biochemical difference between the Jatropha and salt-treated plants.

However, when we compare the use of proline with two different samples of using fresh water like sample 7 and with saline water like sample 25 (both two samples

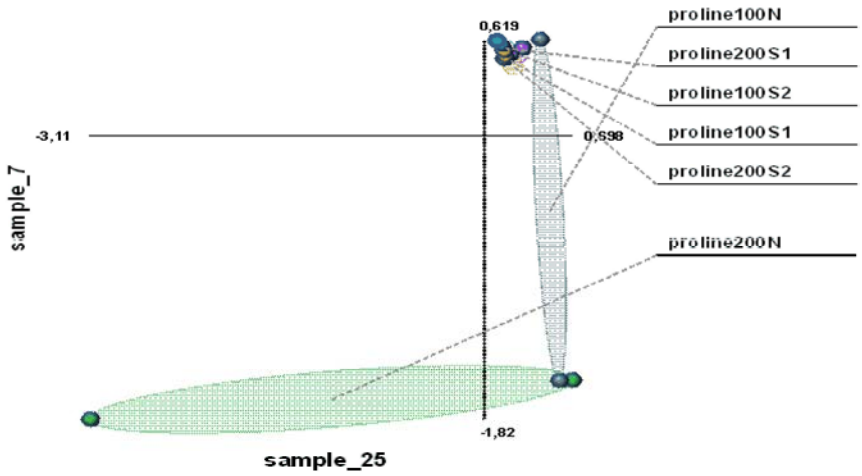

Fig. 10: Partial least square regression for the all treated samples.

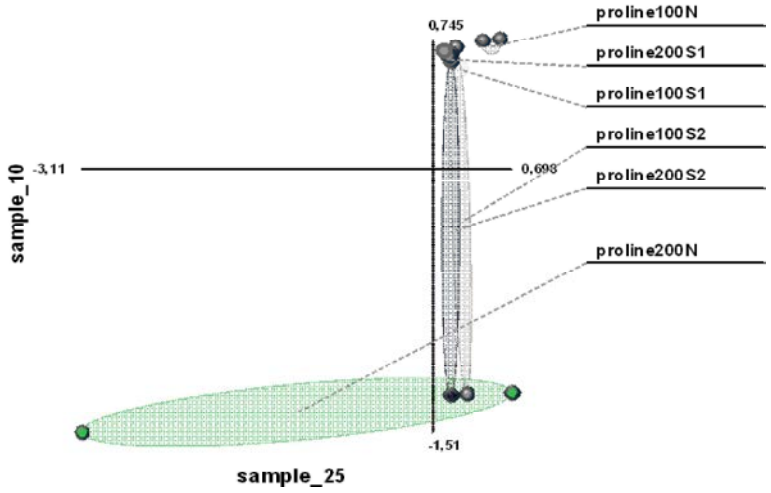

Fig. 11: Partial least square regression for the all treated samples.

used without proline) (Fig. 10), we realized that the use of proline 200 ppm with fresh water is close to the untreated sample 25 under irrigation with saline water (2500 ppm). While, in the case of comparison with proline and without for the case of saline water (samples 10 and 25) it is very easy to discriminate between the samples (Fig. 11).

CONCLUSIONS

The results indicated that salt stress influenced carbohydrate, protein, lipid and cell wall pectin synthesis pathway in Jatropha leaf, however, only carbohydrate kept changing and the metabolic balance of protein, lipid and cell wall pectin just changed. This result illuminates that salt stress can affect secondary metabolism and some compound structure have been changed.

Results obtained in this experiment suggested that FT-IR was capable to detect the chemical changes in salt-stressed plants at early stages and various spectral data can be used to analyze changes of various compounds in the plants. FT-IR is more quick and convenient than other techniques for detecting physiological indicators. Due to a few amount sample needed, the entire process of plants growth can be determined with this method. The FT-IR will be used extensively in the research on plant physiology due to its virtues of simple and efficient on manipulation. However, it is regrettable that we did not detect clear characteristic spectra of nucleic acid and the influence of salt on nucleic acid will be analyzed in the subsequent experiment by combinational using infrared microscope and FT-IR device. The absorption bands of phosphodiester group

were buried with other absorption bands, so, to argue whether there are produces banding with salt, clear spectra of phosphodiester group are necessary.

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