



Investigation of molecular and elemental changes in rice grains infected by false smut disease using FTIR, LIBS and WDXRF spectroscopic techniques

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Received: 20 February 2020 / Accepted: 9 June 2020 / Published online: 22 June 2020
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Abstract

The primary aim of this work was to study the diseased and healthy rice plant samples at the elemental and molecular level to get the proper understanding of the causes and the chemical changes occurred in the healthy rice grains with the infestation due to false smut disease. We have used microscopic and isolation methods to detect and identify the presence of the species of pathogen involved in the diseased rice plant samples. Elemental analyses of rice grains were carried out using laser-induced breakdown spectroscopy (LIBS) and wavelength dispersive X-ray fluorescence (WDXRF) spectrometry. This revealed the mineral changes appeared in diseased rice plant samples due to the invasion of pathogens. The results from LIBS and WDXRF showed that the mineral elements such as calcium (Ca), magnesium (Mg), silicon (Si), copper (Cu) and iron (Fe) decreased in diseased rice grains than those of healthy rice grains whereas some elements showed their variable behavior in diseased rice grains than those of healthy grains. The results obtained by LIBS is in close agreement with the results obtained by WDXRF. We have employed Fourier-transform infrared (FTIR) spectroscopy to study the molecular changes appeared in the active bio compounds of the rice grains due to the infection caused by false smut. The present spectroscopic studies would provide the proper understanding of the rice false smut disease and also help in developing the proper treatment strategies to prevent the further yield loss caused by the rice false smut disease.

1 Introduction

Rice (*Oryza Sativa* L.) belongs to Gramineae (Poaceae) family and is the most important and widely cultivated human food crop worldwide. Being highly rich in carbohydrates, proteins and other minerals, this staple food crop is consumed by more than two-thirds of the population of India. Rice crop production also plays an important role in growth and development of the national economy. According to

the annual 2015–16 report by Department of Agriculture, United States [1], India is the second largest producer of rice and contributes about 22% of the whole world's rice production. In India, it is grown in almost every state with West Bengal (WB) leading in the way of production of about 14.71 million tons and Uttar Pradesh (UP) is the second largest rice producing state with the production of about 12.5 million tons under the cultivation area of about 5.86 million hectare land as per the agriculture statistics of 2014–2015 (A survey report, 2018). Despite being the second largest rice producing state, the productivity of the UP has been about 2082 kg/hectare which is much less than the national average (a survey report, 2018) [2]. The reason behind the low productivity is the considerable amount of rice crop which is damaged every year by the pathogens like fungus, bacteria and viruses. Among various fungal diseases of rice, such as rice blast, sheath blight, sheath rot, brown spot, and scald disease, rice false smut disease is the most prevalent and destructive due to the widespread use of chemical fertilizers which destroys tons of crops every year [3].

Rice false smut is one of the serious crop diseases which affect million tons of rice crops worldwide. Its infestation

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not only causes yield loss but also reduces the quality of grains due to the production of multiple toxins by the pathogens. The first description of false smut disease, *Ustilago-niidea virens*, on rice was given by Cooke in 1878 from Tirunelveli, Tamil Nadu in India. Firstly, it was considered as a minor disease which occurred sporadically in certain regions. But, epidemics of the disease was observed spreading more frequently in different parts of India and caused major yield loss every year [4, 5]. Atia [6] reported this as a crucial devastating disease which causes yield loss upto 10.91%. Ladhakshmi et al. [7] also reported the disease incidence of 10–20% in Punjab and 5–85% in Tamil Nadu on different rice cultivars. About 0.2–49% of the yield loss has been estimated due to false smut disease in different regions of India [8]. Ladhakshmi et al. [7] reported that a major destruction has been seen in the year 2009–2010 in the fields of Tamil Nadu due to the false smut disease. The authors also mentioned that the intensity of false smut disease in the fields of Tamil Nadu was so high that the air above the infested field appeared in the form of black and smoky layer when observed from some distance.

The symptoms produced by false smut (*U. virens*) become visible after flowering after the transformation of fungus into individual grains of the panicle into a yellowish smut ball. This yellowish smut ball then changes to yellowish orange, green, olive green and finally to greenish black (as can be seen from Fig. 1). *U. virens* produces both sexual (ascospores) and asexual (chlamydo-spores) stages in its life cycle [9]. Recently, *Villosiclava virens* has been proposed as the new name for the teleomorph of the false smut fungus [10]. Usually, the smut balls go unnoticed at its early stage and at the ultimate induced considerable losses of yield and quality in the rice crop production [11, 12].

Mineral nutrients are the basic need for the growth and development of the plants and are equally important for the disease control and management in the plant system [13]. It has been reported that there are two primary ways by which mineral nutrients affect the severity of the plant diseases. One of the ways is the formation of mechanical barriers to develop the thickness of the cell walls to restrict the entry of pathogens and the second way is the synthesis of various antioxidants like flavonoids, terpenoids, phenols, and jasmonic acids that provide a protection against the invasion of pathogens. Some nutrients like silicon (Si), calcium (Ca), potassium (K), magnesium (Mg), manganese (Mn), iron (Fe), phosphorous (P), and sulfur (S) have a greater impact on the plant diseases than other nutrients [14–16]. After infecting the host plants, pathogens immobilize the nutrients in the infected tissues like roots and stem, and interfere with their translocations and utilizations within the tissues which further cause nutrient deficiency and/or hyperaccumulation [17]. Thus, to know the whole mechanism of the plant diseases, it is very important to get the proper understanding of



Fig. 1 Photographs of rice plants and grains with healthy (X_1), low infected (X_2) and highly infected (X_3) by false smut disease of rice grains

how nutrients are relatable to the processes associated with the resistance and the tolerance of plants to diseases.

Incidences of the plant diseases pose a serious threat towards the sustainable agricultural production systems throughout the world. Thus, it is very important that more and more scientific studies should be geared up in the plant science to develop some robust diagnosing methods which can further help to maintain a sustainable production system worldwide. A little initiative towards the sustainable agriculture has been carried out in the present work employing advanced spectroscopic techniques, laser-induced breakdown spectroscopy (LIBS), wavelength dispersive x-ray fluorescence (WDXRF), and Fourier-transform infrared (FTIR) spectroscopy, to assess the proper understanding of the changes happened in the mineral nutrients and complex bio compounds of the diseased rice crop plant due to the infestation caused by false smut disease.

2 Material and methods

2.1 Collection and isolation of rice samples (healthy and infected)

Random survey was conducted at farmer's fields in districts Varansi (Paniyara, Babatpur, Jansa, Mohan Sarai, Singhai, Dhanapur) and Mirzapur (Adalpura, Kushha, Kachhawa and Adalhat) of state Uttar Pradesh (UP), India during Kharif

2018–2019. The healthy, low infected and highly infected parts of the rice grain studied are of the same variety. The healthy, low infected and highly infected parts of the rice grain parts were carefully removed from the rice crop plants (as can be seen from Fig. 1) and studied using WDXRF, LIBS and FTIR. The surface of smut balls was sterilized by dipping them in 1% sodium hypochlorite solution for 1 min and washed by 70% ethanol for 1 min. Finally, they were washed three times with sterilized distilled water. For comparison, conventional sterilizing methods were carried out with 0.1% mercuric chloride solution, 1% sodium hypochlorite treatment chloride solution, 1% sodium hypochlorite treatment for 1–2 min and 70% ethanol wash 1–2 min. The control samples were dipped in sterilized distilled water for 2 min only. Then, the smut balls were dried between two sterilized filter papers. The outer portion of dark powdery mass of spores was teased out into small pieces which were then inoculated into Petri dish and incubated at 27 °C. Further, Streptomycin @ 100 PPM was added to the medium at lukewarm stage before pouring into Petri plates in order to avoid the bacterial contamination. We have used hyphal tip method to get the pure culture of the fungus and for sub-culturing the fungus in media slants/Petri plates. The culture was periodically transferred to fresh media.

2.2 FTIR, LIBS and WDXRF spectroscopic techniques

FTIR spectra were recorded for the ground rice grains samples in the transmittance mode between 4000 and 400/cm with spectral resolution of 1/cm using Perkin-Elmer RXZ spectrophotometer. Only few milligrams of each rice sample were mixed with KBr to make a pellet being used in this FT-IR spectrophotometer in transmission mode.

The pellets of rice samples (X_1 , X_2 and X_3) were formed by pressing the mixtures under 8 tons for 5 min. LIBS spectra of rice samples (in pressed pellet form) were recorded using a commercial LIBS instrument (RT100-EC, Applied Spectra, Inc.). A Q-switched Nd:YAG laser beam was focused on the surface of the sample pellets by an objective lens (5× magnifications, 35 mm working distance). The wavelength, pulse duration, pulse energy, and repetition rate were 1064 nm, 10 ns, 17 mJ/pulse, and 10 Hz, respectively. The detector used was the multichannel charge-coupled device (CCD) spectrometer. The laser spot size on the sample surface was 100 μm in diameter. For the LIBS measurements, the optical emission from the laser-induced plasma was collected by two lenses through the top quartz window and sent to the CCD spectrometer with ~ 0.1 nm spectral resolution and wavelength coverage between 190 and 1040 nm. The CCD detection gate with 1.05 ms width was delayed from the laser pulse by 0.5 μs . The number of laser shots accumulated for a single LIBS spectrum was 50 and for one sample 20 line scans have been conducted.

The concentrations of elements present in different kinds of rice grains were measured using WDXRF spectrometer (Model: S8 TIGER, Make: Bruker, Germany). In this WDXRF spectrometer, an end-window X-ray tube with ^{45}Rh anode is adjusted to a voltage of 60 kV and a current of 170 mA, correlating to a maximum power supply of 4 kW to generate the primary beam. For the more accurate and reproducible results, the rice samples was analyzed three times. Lighter and heavy elements were detected and quantified using flow proportional counter and scintillation counter detector respectively. The acquisition time taken to capture a single spectrum was 20–25 min. Using the analyzer crystal LiF220 in combination with the 0.23° collimator, the best separation was achieved for adjacent elements. The mask size was 23 mm and the spectrum was recorded in vacuum. In the present work, while analyzing data, the maximum standard deviation (SD) obtained was obtained 5%. The concentration of trace and heavy elements were measured using standard-less semi-quantitative Spectra^{plus} software (Quant Express) package already installed in the spectrometer. The advantage of this software is that it does not need any standard reference materials to check its reliability and reproducibility with respect to the given samples.

3 Results

3.1 FTIR spectroscopy of rice plant

The FTIR spectra of different plant extracts give the structural information of various biomolecules present in the plant extracts and are mainly used to identify the functional groups of organic active compounds in the plant samples. The FTIR analysis of healthy (X_1), low infected (X_2), and highly infected (X_3) rice grains were recorded between 450 and 4000/cm to observe the compositional differences that may take place in the internal structure of the rice grains due to the action of the fungal attack. FTIR spectra show absorption bands at different wavelengths reflecting the functional groups associated with them. This will further help us distinguish both the healthy and infected rice plant spikelet's on the basis of their spectral differences. The absorption band appeared in the range of 3600–3200/cm is assigned to the –OH stretching and may be taken as an indication of either organic contents or moisture present in the rice plant samples. This moisture-affected region was not used to observe the spectral changes regarding fungal attack since this may lead to some confusion in the interpretation of the results [18]. From the FTIR spectra shown in Fig. 2, it has been cleared that not only the fingerprint region (1500–500/cm), but also the other regions like fatty acids, amides, and polysaccharides band regions reveals the maximum information related to spectral changes related to fungal attack.

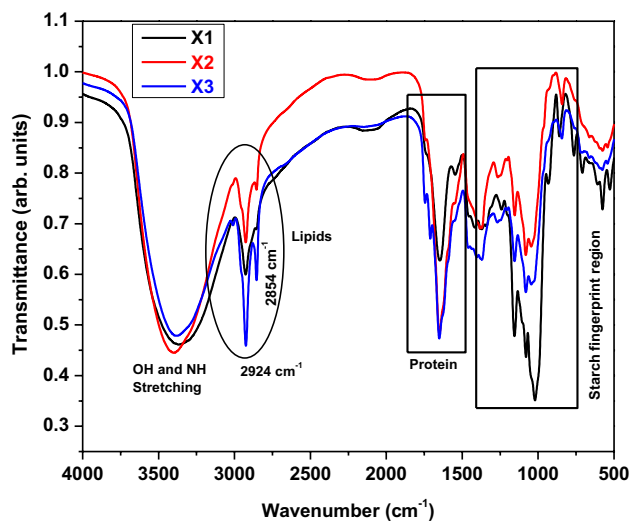


Fig. 2 FTIR spectra of healthy rice grains (X_1), low infected rice grains (X_2) and highly infected rice grains (X_3)

The absorption band in the region of 2800–3000/cm can be assigned to the lipids and fatty acids present in the rice plant samples. The band at 1739/cm generally shows the C=O stretching vibration of ester group linking in lipids and fatty acids. In our results the absorption bands appeared at 2928, 2855 and 1731/cm all are reflecting the presence of lipids and fatty acids in the healthy and infected rice plant samples. The content of lipids and fatty acids changes as clearly seen from these three absorption bands and is getting more and more with the infection in the rice samples. The absorption bands at 1451/cm can be assigned to the methyl and methylene groups of lipids and protein presents in the rice samples.

The presence of protein content in the rice crop plant samples has been confirmed by the appearance of two absorption bands of the amide group. The absorption band of amide-I group appeared at 1650/cm and amide-II group at 1545/cm, thereby indicate the presence of protein content in all the three rice plant samples. The absorption band of amide I group at 1650/cm can be attributed to the C=O stretching of the peptide bonds of protein molecules in the three rice plant samples whereas the peak at 1545/cm for the amide II group arises due to the coupling of two N–H bonding and CN stretching vibrations. It is clearly seen that the area under the absorption bands corresponding to protein content is more for the infected plant samples and is less for the healthy rice plant samples. This increasing pattern of the protein content in the infected plant samples can be considered as one of the consequences of the fungal attack on the rice crop plants.

Starch is the major component of the rice crop plant, so its presence should be highlighted in the FTIR spectrum. It is mainly composed of two polymeric components, amylose in soluble form and amylopectin in insoluble form, both these

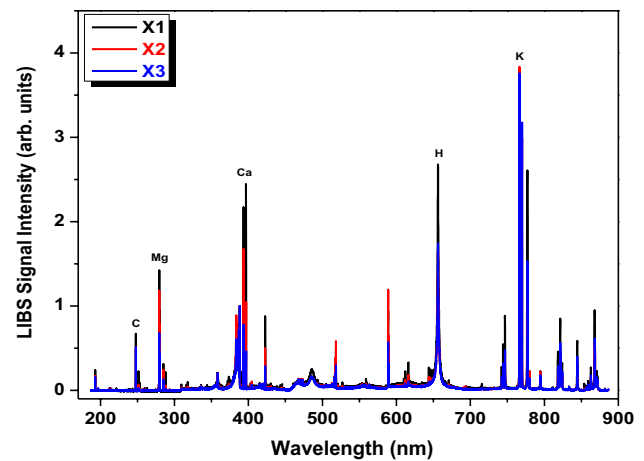


Fig. 3 Typical LIBS spectra of rice grains (healthy X_1 , low infected X_2 , and highly infected X_3) in the spectral region 200–900 nm

bands show their presence through the absorption bands in the FTIR spectrum. The absorption bands observed at 1023, 1052, 1081, 1104 and 1154/cm are all arising due to the C–O stretching vibrations of glycogen and carbohydrates present in the rice crop plant samples [19]. It is clearly seen from the relative changes in the intensities of those bands falls under the fingerprint region 1200–800/cm of the FTIR spectrum of rice samples that a decreasing pattern is seen in the peaks with high infection than the healthy ones. This decreasing pattern is more in initial stage of infection i.e. with the initiation of the fungal infection the content of starch degrades fast and it increases at a slow rate after the infection spreads out in the whole rice crop plant.

The increasing pattern of the protein and lipid contents and the decrease in the carbohydrates are the major consequences of the fungal attack reflected from the three respective spectra of the rice crop plants when compared with one another. The spectral changes that appeared in the FTIR spectra of three rice crop plant samples implies the major changes in the important nutrients like protein, fatty acids, lipids and carbohydrates of the plants body as serious consequence of the fungus attack that will later degrade the whole quality of the concerning crop plant.

3.2 LIBS analysis of healthy, low infected, and highly infected rice samples

LIBS spectra of healthy (X_1), low infected (X_2), and highly infected (X_3) rice samples were recorded in the spectral range of 200–900 nm in order to get the information regarding the changes that appeared in the elemental composition of the rice plant due to the infestation by false smut disease. Figure 3 shows the typical LIBS spectra (average of 20 laser scans) of rice grains (X_1 , X_2 , and X_3) and thereby reveals the relative presence of major

and trace minerals present in rice samples. In order to check the variation in the LIBS signal intensities of different element, we have analyzed the variations in the LIBS signal intensities for different line scans for most of the elements. In Fig. 4a and b, we have plotted for Ca II line (393.366 nm) and Mg II line (279.553 nm) intensities. This clearly reveals that the LIBS signal intensities are quite consistent and constant for every line scans. Thus, the LIBS data is significant and reliable that can be used to predict the relative concentrations of the mineral elements present in the samples. The major and trace mineral elements detected in rice samples were magnesium (Mg), silicon (Si), aluminum (Al), calcium (Ca), sodium (Na), potassium (K), zinc (Zn), iron (Fe), titanium (Ti), chromium (Cr), manganese (Mn), copper (Cu), and chlorine (Cl). In addition to this, lighter element such as carbon (C), hydrogen (H), nitrogen (N), and oxygen (O) were also detected in rice samples which indicates the presence of organic constituents of rice samples and that is a great advantage over other techniques like atomic absorption spectroscopy (AAS), inductively coupled plasma optical emission spectroscopy (ICP-OES), and WDXRF. The identification of the elements is totally based on the presence of the strong and persistent lines of the elements appeared in the LIBS spectra of targeted rice plant samples and these persistent lines were assigned by using the NIST Atomic Spectra Database (https://physics.nist.gov/PhysRefData/ASD/lines_form.html) and the result reported by Sansonettia and Martin [20]. From Fig. 4, it is confirmed that the LIBS signal intensities of these elements appeared in the LIBS spectra is significantly different in different rice samples (X_1 , X_2 , and X_3) with the appearance of

smutty balls due to the fungal infection on the rice spikelet (as can be seen in Fig. 1).

Figure 5a shows the LIBS spectra of rice samples (X_1 , X_2 , and X_3) in the wavelength region between 276 and 291 nm which indicate the presence of Mg (279.55, 280.27 and 285.21 nm) and Si (288.15 nm). The spectra shown in Fig. 5a and b are the averages of 20 laser scans. The resonance lines of Ca and Al can be easily identified by the emission peaks appeared at 393.38, 396.84, 394.40, and 396.15 nm (Fig. 5b) in the wavelength region of 390–406 nm. Since the characteristic line intensities provide a profile of relative concentrations of the elements, so we can relate the emission line intensities of the elements to their concentrations in the rice samples [21]. The emission line intensities of Mg, Si, Ca and Al follow the same trend in the rice samples as $X_1 > X_2 > X_3$ i.e. the healthy part of the rice plant spikelet accumulates more content of Mg, Si, Ca and Al than those in the diseased part of the rice plant spikelet and can be easily seen in Fig. 5a and b. Figure 5b also shows the presence of Mn and K in all the rice samples with the corresponding emission peaks at 403.3 and 404.41 nm. The intensities of both the elements shows increasing trend with the association of false smutty disease in the rice samples i.e. the contents of K and Mn increase with the fungal infection. The presence of Na and N emission lines were easily identified from the next parts of the LIBS spectra which cover the wavelength ranges of 554–640 nm and 725–766 nm and are shown in Fig. 5c and d thereby indicate the presence of Na and N in all the rice plant samples. From the LIBS spectra shown in Fig. 5c and d, it is clearly shown that the intensity of the Na is found to be higher in the diseased rice plant sample than the healthy whereas the content of N is found to be higher in healthy part of the rice plant samples. LIBS spectra in

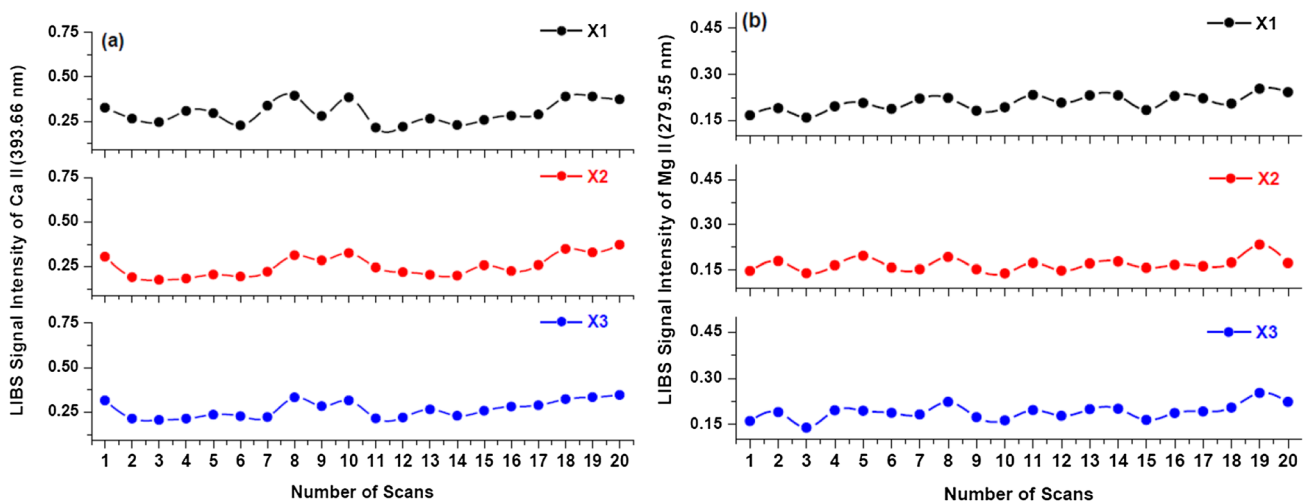


Fig. 4 Variations in the LIBS signal intensities for different line scans for **a** Ca lines observed at 393.66 nm; and **b** Mg lines observed at 279.55 nm in rice samples (X_1 , X_2 and X_3)

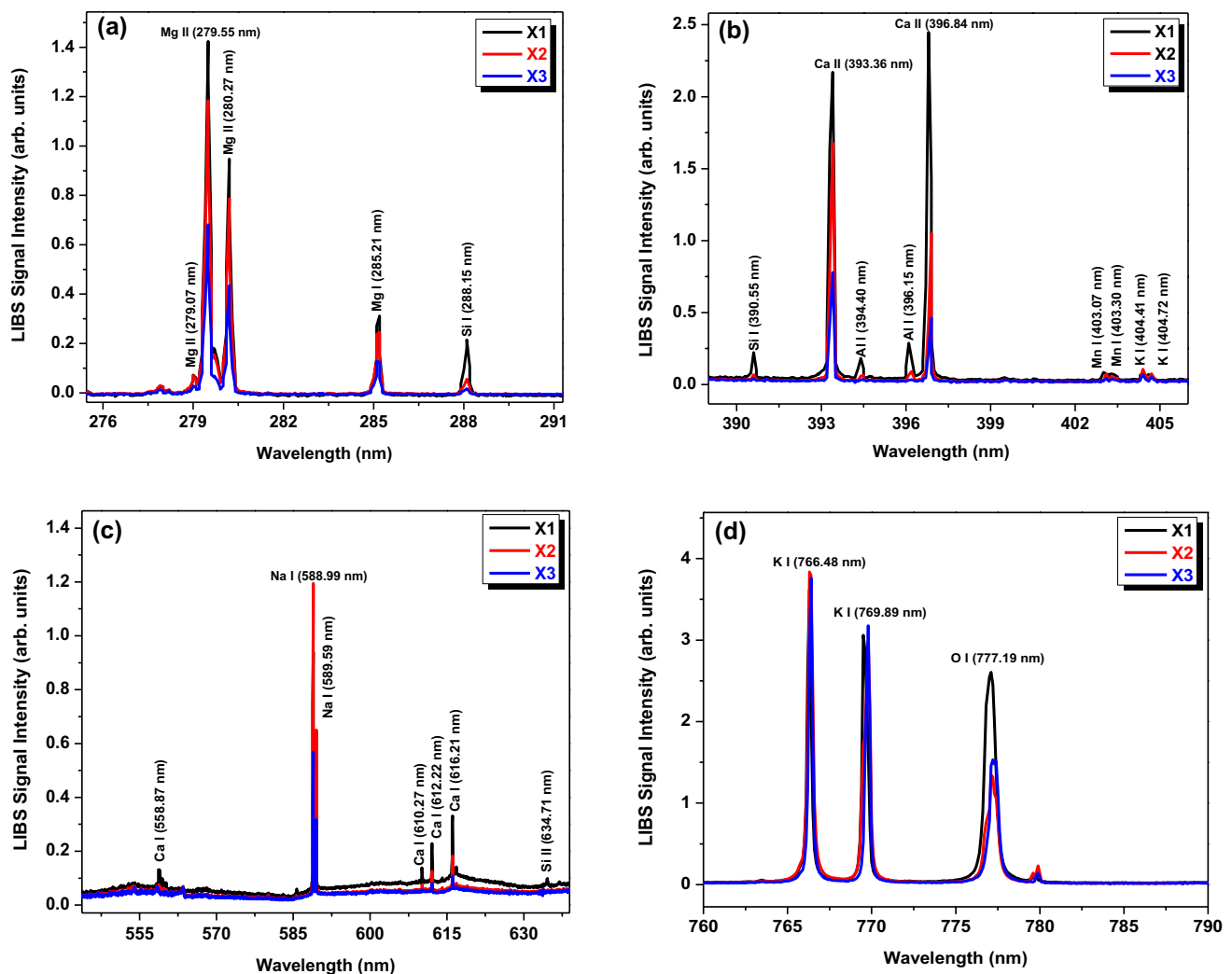


Fig. 5 LIBS spectra of rice grains (healthy X_1 , low infected X_2 , and highly infected X_3) in different spectra region **a** 276–291 nm, **b** 390–405 nm, **c** 550–635 nm, and **d** 760–790 nm indicating the relative

presence of the mineral elements like Mg, Si, Ca, Al, Mn, Na, and K and their pattern due to the infection caused by false smut

Fig. 5d shows the presence of K lines in all the rice samples and the content of K is highly present in the diseased rice plant samples than the healthy rice plant sample. In addition to all these elements, we have also detected some trace amount elements, Fe, Ti, Zn, S, Cl, Cr, and Cu in all the three rice samples with their different concentrations. The decreasing pattern of elements like Ca, Mg, Si, Al, Fe, Zn and Cu and increasing pattern of elements like K, P, S, Mn, Cl and Cr can be considered as the major changes in the elemental composition which appeared due to the infestation of false smut disease on rice plant sample.

We have also observed that the concentration of Cr, a highly toxic and non-essential element of plants, was higher in diseased part than that from healthy parts. There is a chance that it might be taken up by the fruits or flowers of the rice plant from the soil where it was grown as it is a

serious pollutant of the industrial wastages. The presence of Cr in rice plants were also reported in rice plants using LIBS [22] and ICP-MS [23]. To validate our LIBS results and also to quantify these elements, we have employed WDXRF to quantify major and trace elements in healthy and diseased rice samples.

3.3 WDXRF study of rice grain samples (healthy and infected)

The WDXRF spectra of healthy (X_1) and diseased rice samples (low infected X_2 and highly infected X_3) caused by false smut have been recorded in different energy ranges and analyzed carefully to monitor the changes in the elemental composition of the rice grains with the formation of yellow and black smutty balls on the rice spikelets due to false

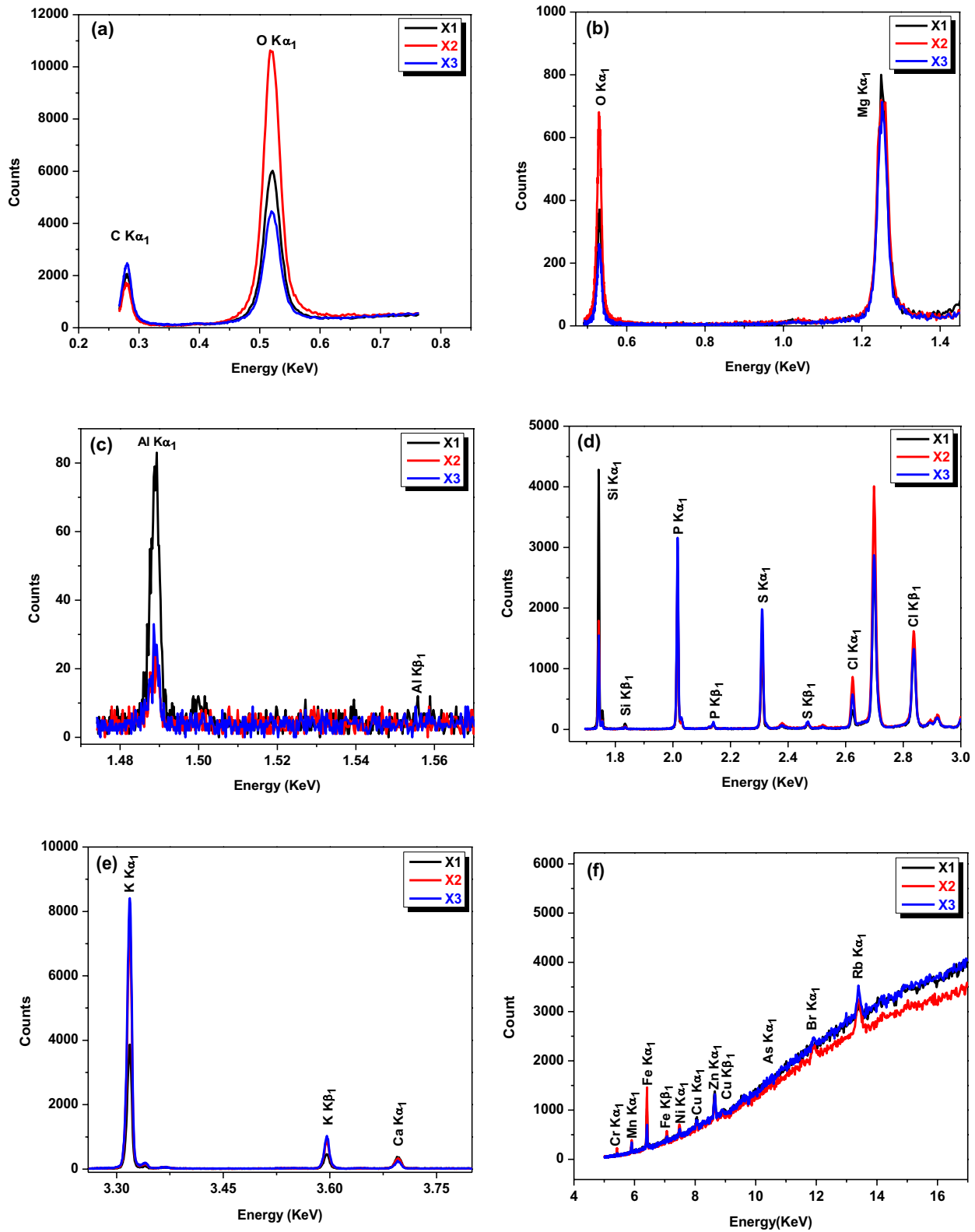


Fig. 6 Typical WDXRF spectra of healthy, low infected and highly infected rice grains

smut disease. Figure 6a–f show overlapped WDXRF spectra of the rice samples in different energy ranges and thereby indicate the presence of minerals elements like Al, Ca, Cl, Cu, Mg, Fe, Mn, Ni, K, Si, S, Zn, P, Rb and Cr. The XRF energies of the elements detected in the WDXRF spectra of rice grain samples are listed in Table 1. The concentrations

of elements detected in rice samples (X_1 , X_2 and X_3) can be found in Table 2.

We have compared the LIBS results with those obtained from WDXRF. The intensities of characteristic lines of Ca (393.36 nm), Mg (279.55 nm), K (766.48 nm), Si (288.15 nm), Cu (327.35 nm), Fe (238.40 nm), Cr (425.43 nm) and Mn (403.07 nm) were calculated from LIBS data, which indicate their relative presence in rice samples. The data present (Fig. 7) is the average value of 20 spectra. We have plotted the intensity values with respect to the concentrations of the elements present in rice samples. Bar graphs as shown in Fig. 7 show the comparison of intensity variation of mineral elements from the LIBS spectra and the concentration variation of minerals elements by WDXRF of rice grains (X_1 , X_2 and X_3). We have observed the same patterns of relative presence of mineral elements (as indicated from Fig. 7) using LIBS and WDXRF techniques.

The mineral elements like Ca, Cu and Si follow the pattern like $X_1 > X_2 > X_3$ in LIBS results (Table 3) which shows the peak area intensity values of all the three rice samples along with their standard deviations. The same pattern has been observed in the concentrations of all the three rice samples using WDXRF (Table 2). From the WDXRF and LIBS results (Tables 2 and 3), we observed that the mineral elements like Mg, Fe and Cr follow pattern $X_2 > X_1 > X_3$ in LIBS spectra of rice samples and is well correlated with the pattern followed by the same elements in the WDXRF spectra of rice samples. The mineral elements, K and Mn follow the same pattern like $X_3 > X_2 > X_1$ and $X_2 > X_3 > X_1$ in the

Table 1 The XRF energies of the elements detected in the WDXRF spectra of rice grain samples

Elements detected	Energy (KeV)	
	K_{α_1}	K_{β_1}
Oxygen (O)	0.525	–
Magnesium (Mg)	1.254	–
Aluminum (Al)	1.484	1.578
Silicon (Si)	1.740	1.836
Phosphorus (P)	2.015	2.140
Sulfur (S)	2.310	2.465
Chlorine (Cl)	2.625	2.820
Potassium (K)	3.318	3.594
Calcium (Ca)	3.696	4.013
Chromium (Cr)	5.415	–
Manganese (Mn)	5.900	6.49
Iron (Fe)	6.419	7.057
Nickel (Ni)	7.488	–
Copper (Cu)	8.046	8.904
Zinc (Zn)	8.637	9.567
Rubidium (Rb)	13.428	–

Table 2 Concentration of mineral elements quantified in healthy and diseased rice grains using WDXRF spectroscopy

Elements	Healthy rice grains (X_1)		Low infected rice grains (X_2)		Highly infected rice grains (X_3)	
	Concentration (ppm)	$\pm SD^a$	Concentration (ppm)	$\pm SD^a$	Concentration (ppm)	$\pm SD^a$
Calcium (Ca)	400	20	300	15	300	15
Potassium (K)	4400	220	8400	420	9500	475
Chlorine (Cl)	500	25	1300	65	900	45
Magnesium (Mg)	2600	130	3000	150	2400	120
Silicon (Si)	12,700	635	5300	265	4500	225
Phosphorus (P)	4800	240	5100	255	6100	305
Sulfur (S)	1400	70	1600	80	2000	100
Aluminum (Al)	300	15	ND ^b	ND ^b	100	5
Iron (Fe)	100	5	200	10	64	3.2
Chromium (Cr)	11	0.55	46	2.3	ND ^b	ND ^b
Manganese (Mn)	37	1.85	50	2.5	39	1.95
Zinc (Zn)	23	1.15	19	0.95	19	0.95
Copper (Cu)	9	0.45	7	0.35	5	0.25
Nickel (Ni)	8	0.40	18	0.9	9	0.45
Rubidium (Rb)	3	0.15	9	0.45	8	0.40

^aSD standard deviation

^bND not detected

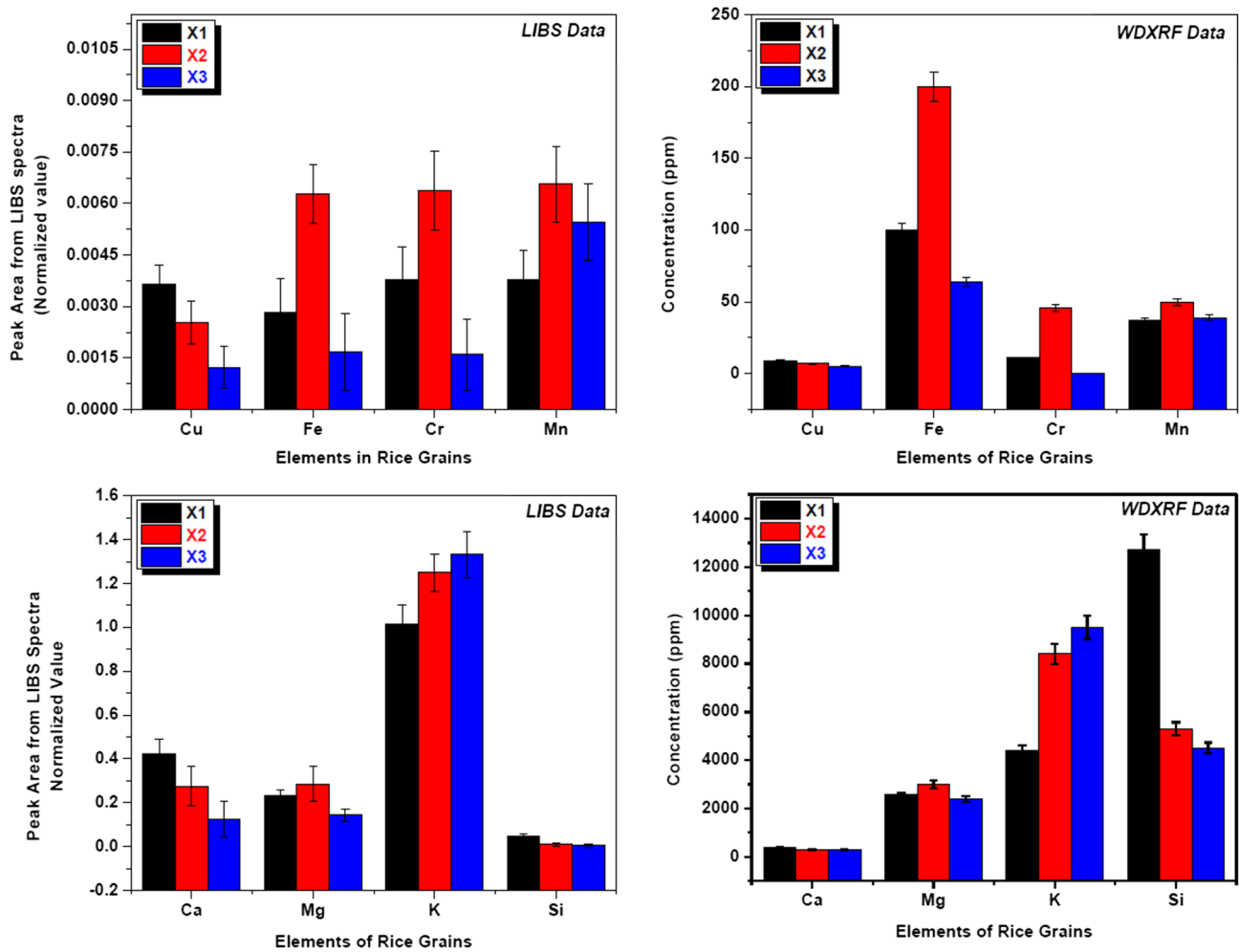


Fig. 7 Bar diagram of comparison of intensity variation of mineral elements from the LIBS spectra and the concentration variation of minerals elements by WDXRF spectroscopy of rice grains (X_1 , X_2 , and X_3)

Table 3 Peak area of the atomic lines of the elements detected in the LIBS spectra of rice grains (healthy, low infected and highly infected)

Elements	Healthy rice grains (X_1)		Low infected rice grains (X_2)		Highly infected rice grains (X_3)	
	Peak area (LIBS)	\pm SD ^a	Peak area (LIBS)	\pm SD ^a	Peak area (LIBS)	\pm SD ^a
Ca (393.36 nm)	0.42294	0.06922	0.27511	0.09132	0.12483	0.08412
Mg (279.55 nm)	0.2315	0.02561	0.28558	0.08094	0.14284	0.02938
K (766.48 nm)	1.01706	0.08799	1.24929	0.08487	1.33136	0.10335
Si (288.15 nm)	0.04517	0.00999	0.00975	0.00869	0.0042	0.00928
Cu (327.35 nm)	0.00364	0.000551	0.00254	0.000633	0.00123	0.000615
Fe (238.40 nm)	0.00284	0.000984	0.00628	0.000855	0.00168	0.00111
Cr (425.43 nm)	0.00377	0.000964	0.00639	0.00115	0.0016	0.00104
Mn (403.07 nm)	0.00378	0.000864	0.00657	0.0011	0.00546	0.00112

^aSD standard deviation

spectra of rice samples obtained by LIBS and WDXRF. Concluding the results, we observed that LIBS and WDXRF are the complementary techniques which can be used together to

investigate the changes in mineral elements of rice samples infected by false smut disease and hence may be further explored for other plant diseases.

4 Discussion

The FTIR analysis suggests the changes in the content of proteins, starch and lipids in the diseased rice samples after the infestation of false smut disease with respect to healthy rice plant sample. The increasing pattern of proteins observed in the diseased rice plant samples can be related to one of the immunity responses [24] that plants activate during the invasion of pathogens. Starch is a major component of the rice plant which serves as a storage of carbohydrates to support the growth and the metabolism in the plants needed when photosynthesis is not possible like in nights. Amylases are the enzymes that are produced by various microorganisms like fungi, yeast and bacteria in the plant's body. The spores or cells produced by the fungus in the plant's body are mainly responsible for the starch reduction in the spikelets of the rice plants as they act as starch sinkers that suck out all the important nutrients including starch from the plant's body parts and make it more deficient of basic nutrients and starch. Thus, we can conclude that fungus attack on the rice plant totally degrades the quality of the rice spikelets by changing the nutrient content of all the active biomolecules of the plant.

The changes in the elemental composition of the rice plant samples due to the rice false smut disease are assessed with the help of two elemental analysis methods, LIBS and WDXRF. Both techniques show that the concentration values of some major and minor elements changes with the association of false smut disease in rice plant. It has been reported that the production of mechanical barriers, induction of inhibitory compounds and activation of defense mechanism all are related with the continuous availability of the essential nutrients in the plants [25]. In our results, we have found that the elements like K, P, and S show an increasing pattern and the reason behind their increasing pattern can be related to induction of the inhibitory compounds for the activation of defense mechanism within the plant during the invasion of pathogen. After analyzing the WDXRF spectra, we have observed that the concentrations of K and P increase with the presence of yellow and black smutty balls on the spikelets of the rice plant and follows the pattern like $X_3 > X_2 > X_1$. It has been also reported that the rapid mobilization and accumulation of some mobile mineral elements like K and P around the infection sites slows down the further movements of fungus growth in the plants [25]. The increasing pattern of elements K and P was also observed in corn crop which is also from the poaceae family after the inoculation of *G. mosseae* (now, *Funneliformis mosseae*) and *G. intradices* (now *Rhizogonus intraradices*) [26]. Sulphur (S) uptake in plants is directly proportional to the availability of some special compounds like salicylic acid, jasmonic

acid, auxin, glucosinolates, and methyl transferases that involves in defense mechanisms [27]. The content of S rises from 1400 ppm in healthy rice plant to 2000 ppm in diseased rice samples in order to activate the defense mechanism in plant's body.

Apart from these elements, we have observed little increasing pattern of Cl and Mn and was also observed by various researchers during the study of infected plant materials [19, 28]. Mn is an essential plant nutrient which plays many physiological processes like biosynthesis of phenols, photosynthesis, and biosynthesis of lignin and also several defense mechanisms [29]. The increasing pattern of Mn was also observed in the barley plant and the authors concluded that the content of Mn generally stimulated with the presence of microbes in the rhizosphere of the soil [30]. The increasing content of Cl can be considered as one of the defense responses that plant activates against the invasion of fungal spores [29]. The same increasing patterns of K, P, S, Mn, Cl, and Ni were also observed in wheat gall nematode using WDXRF spectroscopy [28].

We have also observed decreasing pattern of some elements like Ca, Mg, Si, Al, Fe, Zn, and Cu after the infestation of false smut disease on rice spikelets. Ca and Mg are major nutrients in the rice plants which play a crucial role in maintaining the outer structure of the cell walls and membranes in plant's body during the invasion of the pathogens. In addition to providing a physical barrier by modifying the cell walls against pathogens, Ca also helps to regulate the passage of sugar and amino acids from cells to intercellular spaces in the plant tissues and thus maintain a level between sugar and amino acids [14]. The content of Ca and Mg shows a decreasing pattern (Table 2) with the association of fungus or smutty balls on the rice spikelets. The same decreasing pattern of Mg was also observed by Bateman [31] in the bean plant infected by fungi *Rhizotonia Solani*. The decreasing content of Ca and Mg in diseased rice samples can be related with the indication of softening and weakening of the cell walls and the leakage of the sugars and amino acids from the plant tissues which may attract the attention of fungal spores. In a study by Meyer and Hirose [32], the authors reported the appearance of GSFR (green stem and foliar retention) disease symptoms due to the unbalance of the macro nutrients Ca, K and Mg. Recently, Ranulfi et al. [33] used LIBS technique in order to show the higher amount of Ca and Mg and lower amount K in healthy leaves of soybean plant than that of diseased plants caused by GSFR. Our study is well supported by Ranulfi et al. [33], as we have also observed the same trends of higher content of Ca, Mg and lower content of K in healthy rice samples than that of diseased rice samples caused by false smut.

Rice plant is well reported high Si accumulator from the soil [34]. This high accumulation of Si is deposited as a 2.5 μm layer on the cell walls makes the leaf blades of the rice so

hard that it protects plant from the outer biotic stress like grazing of animals, pests, pathogens and thus maintain the outer safety of the rice plant [35]. From Table 2, we observed the deficiency of Si content in diseases samples (4500 ppm) than that of health rice samples (12,700 ppm), which may be one of the reasons of fungal infection of false smut disease in rice. Mineral element Si along with Ca and Mg are generally associated with the maintenance of the outer cell walls safety and decreasing concentration can be related as the indication of the weakening of the outer membrane of the cell walls [14].

The elements such as Fe, Zn, Al, and Cu shows a decreasing pattern in the rice samples with the infection of rice false smut disease whereas the content of Cr element increases with the infestation of rice false smut disease on rice plant samples. Al and Cr are not considered as essential elements for plants get entered into the plant from the soil and water. Rice has been considered as the most Al-tolerant cereal crop and can be withstand with higher concentration of Al than other crops [36]. The content of Al falls down from 300 ppm (healthy rice) to 100 ppm (diseased rice samples). Cr is a toxic environmental pollutant and cause deleterious effect on plant physiological processes like uptake of water nutrients, photosynthesis, induced oxidative stress by generating ROS signals and cause cell death in plants [37]. Cr toxicity generally starts from 5 ppm in agronomic plants [37]. In our study, the concentration of Cr has been increased with the occurrence of smutty balls on the rice spikelets and raised from 11 ppm (healthy rice samples) to 46 ppm (diseased rice samples). The accumulation of Cr in diseased rice grains may be considered as one of the consequences that happened due to the growth of fungus and smutty balls on the rice plant spikelets. Cr toxicity was also reported by Singh et al. [28] in wheat seeds infected by nematode and fungus and by Sharma et al. [19] in okra plant infected by nematode and fungus.

It has been reported that the pathogens after invading the host plant developed strategies to bypass the host defenses and deprived them for certain elements while host plant developed ways to limit the movement of pathogens by increasing the concentration of some minerals in the plant [38]. Therefore, the decreasing concentration of the elements like Ca, Cu, Zn, Mg, Si, and Fe and the increasing concentration of the elements like Cr, K, P, and S with the appearance of smutty balls and the growth of the fungal spores on the rice plant may be considered as the one of the consequences of the rice false smut disease which ultimately affects the quality of rice grain.

5 Conclusion

Modern food and crop production practices are turning towards the spectroscopic methods to monitor the crop health as it becomes one of the most used non-invasive

methods to detect the plant diseases before the symptoms are visible. For the first time, advanced spectroscopic techniques FTIR, LIBS and WDXRF have been employed to study the rice plants infected by false smut disease. In this study, we have successfully assessed the spectral changes that appeared in the elemental composition and molecular structures of the rice grains due to the infestation of false smut disease by using spectroscopic methods, FTIR, LIBS and WDXRF. FTIR study revealed the changes appeared in the internal structure of various bio compounds like starch, protein, lipids, and fatty acids of the rice grains due to false smut disease. Concentration changes associated with the mineral nutrients of the rice grains due to the false smut disease have been investigated and quantified using elemental analysis techniques LIBS and WDXRF. The results obtained from the spectroscopic techniques in diseased rice grains have been compared with the results of healthy rice grains and significant observations have been made. Thus, we concluded that the spectroscopic techniques such as FTIR, LIBS and WDXRF provide a good understanding in order to assess the change in concentrations of mineral nutrients and to assess the role of complex bio-molecules in the diseased plant samples which may further help in diagnosing the various plant diseases.

Acknowledgements Financial support from DST, Govt of India to Ms. Neha Sharma, SRF and Inspire Fellowship (No. DST/INSPIRE Fellowship/2016/IF160893) is duly acknowledged. Authors are also grateful to CIL and SAIF, Punjab University, India to provide experimental facilities of FTIR and WDXRF spectroscopy. Mr. Tejbir Singh (SAIF, Punjab University) is gratefully acknowledged for providing the details on the technical aspects of WDXRF spectrometry to discuss the results throughout the manuscript.

Author contributions The two authors K and PKR contributed equally to the final version of the paper.

Compliance with ethical standards

Conflicts of interest The authors Neha Sharma, Kamni, Virendra K. Singh, Sandeep Kumar, Yonghoon Lee, Piyush K. Rai, and Vivek K. Singh declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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