Hindawi Publishing Corporation
Spectroscopy: An International Journal
Volume 27 (2012), Issue 4, Pages 206–213
doi:10.1155/2012/951064

Chlorophyll Fluorescence Spectra as an Indicator of X-Ray + EMS-Induced Phytotoxicity in Safflower

Jitendra Kumar Pandey,[1] Preeti Srivastava,[2] Ram Singh Yadav,[2] and Ram Gopal[1,3]

[1] *M. N. Saha Center of Space Studies, IIDS, Nehru Science Center, University of Allahabad, Allahabad 211002, India*

[2] *Plant Genetics Laboratory, Department of Botany, University of Allahabad, Allahabad 211002, India*

[3] *Laser Spectroscopy and Nanomaterials Lab, Department of Physics (UGC-CAS), University of Allahabad, Allahabad 211002, India*

Correspondence should be addressed to Jitendra Kumar Pandey, jkpandey18@gmail.com

Abstract. The present investigation deals with the study of in vivo laser-induced chlorophyll florescence spectra (LICF) of safflower leaves (*Carthamus tinctorius* L.) for X-rays + EMS-treated plants. Seeds were treated with different doses of X-ray + EMS (5, 8, 12, 25, and 30 Kr + 0.5% EMS) and were grown in the green house. The effects of the concerned treatment on chlorophyll (Chl) contents and Chl fluorescence were investigated after 7 days of germination. Results obtained revealed that the values of Chl contents, intensity of Chl fluorescence spectra, and fluorescence intensity ratio (FIR) F685/F730 are directly correlated with the treatment doses monitored. The treatment sets of 8, 12, and 25 Kr + 0.5% EMS doses showed an increase in FIR and thereby a decrease in the Chl contents. However, the lowest treatment dose of 5 Kr + 0.5% showed a decrease in FIR and thereby an increase in chlorophyll contents. Safflower seeds treated with 30 Kr + 0.5% EMS were proved to be lethal as they showed no germination. Thus, our study demonstrates early detection of chlorophyll damage caused by various physical and chemical mutagens through the application of LICF spectra.

Keywords: X-ray + EMS treatment, Laser-induced chlorophyll fluorescence, Fluorescence intensity ratio, Photosynthetic pigment contents (chlorophyll *a*, chlorophyll *b*, and carotenoids), Safflower (*Carthamus tinctorius* L.)

1. Introduction

Laser-induced fluorescence (LIF) is a powerful tool for plant investigation, and it can illustrate a lot of information about plant health and identity of plants. Leaf pigments emit fluorescence after irradiation with laser light [1]. The *in vivo* chlorophyll fluorescence spectra of plant leaves shows two fluorescence maxima, one in the spectral region near 685 nm and other in the region near 730 nm [2]. The shape of the fluorescence spectra and the value of the fluorescence intensity ratio (FIR) up to a great extent depend upon the Chl contents and absorbance of the leaves [3]. Fluorescence intensity ratios show a good correlation with pigment contents and pigment ratios [4]. The intensity of the red and far-red chlorophyll

fluorescence is inversely related to the photosynthetic activity. With the decrease in the photosynthesis owing to various stress conditions, the FIR increases. The increase in chlorophyll content in plants results in a decrease in the value of the FIR.

Safflower is one of the world's oldest oilseed crops that have been grown commercially for edible oil and natural dye sources around the world [5]. Safflower petals besides being a source of dye are medicinally important in curing several chronic diseases like hypertension, coronary heart ailments, rheumatism, and male- and female-fertility-related problems [6, 7]. It is an important alternative plant that can be used to increase edible oil sources. It is a highly tolerant crop that can be safely grown under arid and saline sodic conditions [8, 9].

X-rays are nonparticulate electromagnetic radiations with a wavelength of 0.001–10 Å. These are high-energy radiations and consist of photons, that is, small packets of energy. X-rays are produced when very fast moving electrons strike a high-melting-point element like Tungsten in X-ray tubes. X-rays are often referred to as hard (0.001–0.1 Å) or soft (1–10 Å) depending upon their wavelength. X-rays are highly penetrating and sparsely ionizing. Ionizing radiations produce a wide range of effects on DNA through either free radical effects or direct action on DNA. It causes breaks in sugar phosphate backbone of one or both strands of DNA, consequently, leading to the rearrangements through tautomerization, deletions, chromosome loss, and so forth. Mutations are also caused by damage or loss of bases. Sometimes the effect may result in cross-linking of DNA to itself or proteins, breaking of H-bonds of bases, blockage of cell division, organelle failure, or cell death [10–12].

EMS is a widely used chemical mutagen that is a nonfunctional agent with one reactive group. It causes ethylation of bases in DNA. EMS is a monofunctional alkylating agent that reacts with DNA at the 7-N and 6-0 positions. Alkylation of ring N causes depurination, which leads to backbone breaks. When 7-ethylguanine is produced, it pairs with thymine to cause G:C $\rightarrow$ A:T transitions. The lethality of EMS is due to alkylation of proteins. Alkylating agents interact with DNA causing changes in its structure. This may result in the loss, addition, or replacement of bases, thus, altering their sequence in the DNA and affecting the fidelity of the genetic message. Relative frequencies of mutation depend on the reactivity of the agents involved. Deletion and insertion leading to producing frameshift mutations. The inactivating alterations include removal of bases, dimmer formation, cross-linking of the two DNA strands, and single or double strand breaks [13, 14].

X-ray and EMS both are highly toxic for plant and animal health. In order to evaluate the mutagenic efficiency of X-rays and EMS on seeds, the present study has been conducted on safflower. Here, we investigate the combined effect of highly toxic chemical mutagen EMS and mutagenic ioning radiation X-ray treatment of seeds on pigment contents and chlorophyll fluorescence response of safflower leaf and to suggest the most appropriate dose for further mutation breeding programs.

2. Material and Methods

2.1. Procurement of Seeds and Chemical

Seeds of safflower (*Carthamus tinctorius* L. var. A1) was obtained from NBPGR, New Delhi, India, and EMS was obtained from Merck, India.

2.2. Plant Growth and X-Ray + EMS Treatment

The dry seeds of safflower were exposed to five different doses of X-ray irradiation, that is, 5, 8, 12, 25, and 30 Kr, respectively. X-ray irradiation was delivered at 230 kV for 84 rad/min at room temperature. X-ray-irradiated seeds were presoaked in 0.5% solution of ethylmethane sulphonate (EMS) for 5 hours. Then, the X-ray + EMS-treated seeds of safflower after washing well in running water were sown in 3 replicates with 10 seeds in each pot. Seeds treated with distilled water were kept as control and were also sown in their respective pot simultaneously in greenhouse conditions to a raise the M_1 generation. All the treated sets except for 30 Kr + 0.5% EMS showed germination, which depicts that this dose is lethal for safflower.

2.3. Determination of Pigment

Plant leaves (20 mg) from control and X-ray + EMS-treated safflower plants were extracted in 3 mL 80% acetone (v/v, in double distilled water), and the extract was used for the measurement of pigment contents. The pigment contents were determined from the transparent, centrifuged acetone extract solution by measuring the absorbance in the region 380–700 nm using the UV/VIS spectrometer (Perkin Elmer lambda 35). The pigment contents were determined according to the method of Lichtenthaler and Wellburn [15].

2.4. Laser-Induced Chlorophyll Fluorescence Spectra

LICF spectra were recorded using computer control Acton 0.5 M triple grating monochromator, Hamamatsu R928 PMT, as a detector, excited with 405 nm violet diode laser (Oxxus CE, made in France, Modal PS-001) light. The beam expander was aligned to obtain 4.0 cm^2 expanded laser light on leaves. The fluorescence radiation was collected on the entrance slit of monochromator.

LICF spectra were recorded in the region of 650–780 nm with 1800 grooves/mm grating blazed at 500 nm wavelength using survey mode of spectra sense software. These spectra were analyzed using GRAMS 32 software with Curve-Fit Array Basic program. Spectral correction was made from the response curve of PMT and grating of monochromator.

2.5. Curve Fitting

Interactive nonlinear curve fitting was made using the Levenberg-Marquardt algorithm method. After choosing the Gaussian spectral function, the individual component peaks were selected. Peak widths were adjusted so as to obtain approximately the line shape of the spectrum. It provides a reasonable matching fit of the spectral data with good F-statistics, standard error for peak amplitude, peak center and bandwidth (full width at half-intensity maximum).

3. Results and Discussion

3.1. Photosynthetic Pigments

Treated the Plants showed better growth than the control plants for 5 Kr + 0.5% EMS treatment as the photosynthetic pigments, that is, Chl *a*, Chl *b*, and carotenoid contents were increased by 7.39,

Table 1: Photosynthetic pigment contents and pigment ratios of control and X-ray + EMS-treated safflower plants.

X-ray + EMS treatment	Chl a (μg/mL)	Chl b (μg/mL)	Total Chl (μg/mL)	Chl a/b	Car (μg/mL)	Chl/Car
Control	8.25 ± 0.06	1.92 ± 0.07	10.17 ± 0.06	4.29 ± 0.12	0.77 ± 0.12	13.14 ± 0.12
5 Kr + 0.5% EMS	8.86 ± 0.05 (7.39)	2.14 ± 0.04 (11.46)	$\mathbf{11.00 \pm 0.05}$ (8.16)	$\mathbf{4.14 \pm 0.07}$ (–3.49)	$\mathbf{0.78 \pm 0.07}$ (1.30)	$\mathbf{14.10 \pm 0.08}$ (7.31)
8 Kr + 0.5% EMS	8.21 ± 0.06 (–0.48)	1.76 ± 0.09 (–8.33)	9.97 ± 0.08 (–1.97)	4.66 ± 0.14 (8.62)	1.07 ± 0.09 (38.96)	9.30 ± 0.08 (–29.22)
12 Kr + 0.5% EMS	7.64 ± 0.08 (–7.39)	1.71 ± 0.07 (–10.94)	9.35 ± 0.08 (–8.06)	4.48 ± 0.13 (4.43)	0.92 ± 0.07 (19.48)	10.21 ± 0.16 (–22.30)
25 Kr + 0.5% EMS	6.69 ± 0.12 (–18.91)	1.22 ± 0.10 (–36.46)	7.91 ± 0.11 (–22.22)	5.47 ± 0.11 (27.51)	1.15 ± 0.24 (49.35)	6.88 ± 0.15 (–47.64)

$\pm$ Values indicate standard deviation (mean $n = 3$). The values in parenthesis show percent decrease/increase over control plant.

11.36, and 1.30%, respectively, over the control plants (Table 1). Except the dose of 5 Kr + 0.5% EMS, with increasing dose of X-ray + EMS treatment, the leaf Chl contents decreased continuously and that decrease was recorded up to 22.22% for 25 Kr + 0.5% EMS as compared to the control plants (Table 1), whereas carotenoid contents increased continuously for all used treatment doses and this increase was up to 49.35% for 25 Kr + 0.5% EMS. The decrease in the Chl *b* content was higher in comparison to the Chl *a* for the doses 8, 12, and 25 Kr + 0.5% EMS; thus the ratio of Chl *a*/*b* increased for these doses and it increased maximally up to 27.51% for 25 Kr + 0.5% EMS. The Chl *a*/*b* ratio decreased for 5 Kr + 0.5% EMS as the increase in the Chl *b* was higher at this dose. As the carotenoids contents increased for all used doses, the Chl/Car ratio decreased for all used doses except the 5 Kr + 0.5% EMS because at this dose the increase in the carotenoid contents was lower than the increase in the Chl contents.

The decrease in the pigment contents for 8, 12,and 25 Kr + 0.5% EMS doses clearly reflects the effect of mutagenic treatment on safflower plants and obviously the maximum inhibition observed at the maximum dose of treatment with minimum Chl contents (or maximum decrease in the Chl contents). The inhibition response decreases with the decrease in the intensity of treatment doses, and it shows a positive response for 5 Kr + 0.5% EMS dose. Lower value of the Chl *a*/*b* indicates the presence of more light-harvesting Chl complexes of LHC2 [16, 17], thus we can assume that a lower number of light harvesting Chl complexes in the case of 8, 12, and 25 Kr + 0.5% EMS-treated plants and it decreases with the increase in the dose of treatment, whereas light-harvesting Chl complexes may be increasing for treatment of 5 Kr + 0.5% EMS dose. Carotenoids are essential constituents of Chl-binding proteins in all higher plants and they have two key roles in plants and algae: firstly they absorb light energy for use in photosynthesis, and secondly they protect chlorophyll from photodamage [18]. Increase in the carotenoid contents may be due to X-ray + EMS-induced damage in photosystem of plant, which has

Table 2: Chlorophyll fluorescence parameters of the curve-fitted spectra of control and X-ray + EMS-treated safflower plants excited by 405 nm violet diode laser.

Treatment of X-ray + EMS	Curve-fitted chlorophyll fluorescence parameters							
	F_{685}				F_{730}			
	Peak (nm)	Height (arb)	Width (nm)	Area (arb)	Peak (nm)	Height (arb)	Width (nm)	Area (arb)
Control	682.87	3198.59	22.40	80911	727.39	1249.70	47.92	53306
5 Kr + 0.5% EMS	683.12	1565.35	21.35	35533	727.14	675.36	49.46	35365
8 Kr + 0.5% EMS	683.64	4533.64	23.44	112898	727.14	1585.76	55.73	92891
12 Kr + 0.5% EMS	683.38	6479.01	23.48	161588	725.32	1715.12	52.07	94431
25 Kr + 0.5% EMS	683.65	9349.17	22.91	227651	723.50	2893.03	54.69	167043

similar symptoms like photodamage in plant, in response to the fact that the carotenoid contents of the plant increase and the increase was at its maximum in the case of 25 Kr + 0.5% EMS-treated plants. Increase in the carotenoid contents was comparatively lower than the increase in the Chl *a* and *s* for 5 Kr + 0.5% EMS dose, which further shows better physiological condition of the plant at this dose.

3.2. Laser-Induced Chlorophyll Fluorescence Spectra

The LICF spectra of the control and X-ray + EMS-treated plants exhibit two fluorescence maxima in red (F_{685}) and far-red (F_{730}) regions (Figure 1). The curve-fitted fluorescence parameters such as peak position, peak height, band width, and band area are given in the Table 2. These spectra indicate that the intensities of the Chl fluorescence (red and far-red) are much affected by the X-ray + EMS treatment. The variation in the Chl fluorescence intensity at F_{685} is markedly different than the fluorescence intensity at F_{730}. The intensity of the fluorescence emission was much increased at 685 nm for inhibitory doses of X-ray + EMS treatment. The fluorescence intensity at 685 nm is much higher than at 730 nm as presented in Figure 1 and Table 2. Similarly, the decrease in the fluorescence intensity at 685 nm was much higher than the decrease at the 730 nm for 5 Kr + 0.5% EMS dose. FIR (F_{685}/F_{730}) was calculated from curve-fitted LICF spectra (Table 3). The FIR showed a decrease of 7.26% for 5 Kr + 0.5% EMS dose. The FIR increased significantly with increase in the dose of treatment. It increased up to 48.04% for 25 Kr + 0.5% EMS treatment.

The intensity and shape of the Chl fluorescence emission spectrum of leaves at room temperature are primarily dependent on the concentration of the fluorophore Chl *a* and to a lower degree also on the leaf structure, the photosynthetic activity, and leaf optical properties. The later determine the penetration of excitation light into the leaf as well as the emission of Chl fluorescence from different depths of the leaf. The fluorescence intensity near 685 nm increases with the decrease in the fluorophore Chl *a*. The increase in the short-wavelength red fluorescence with the decrease in the Chl contents is due to the reduction of the reabsorption of the emitted red Chl fluorescence by the Chl absorption band. In the

Table 3: Fluorescence intensity ratio (F_{685}/F_{730}) of control and X-ray + EMS-treated safflower plants excited by 405 nm violet diode laser.

X-ray + EMS treatment	Fluorescence intensity ratio
Control	1.79 ± 0.02
5 Kr + 0.5% EMS	1.66 ± 0.02 (–7.26)
8 Kr + 0.5% EMS	2.00 ± 0.01 (11.73)
12 Kr + 0.5% EMS	2.26 ± 0.04 (26.26)
25 Kr + 0.5% EMS	2.65 ± 0.02 (48.04)

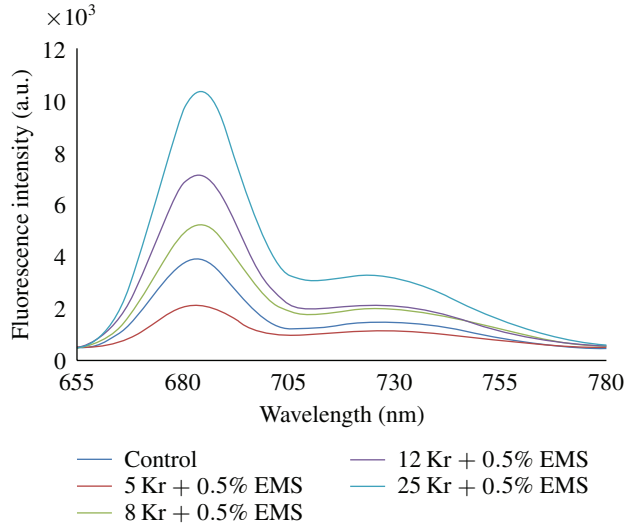

Figure 1: Gaussian curve-fitted laser-induced chlorophyll fluorescence spectra of control as well as X-ray + EMS treated safflower plant leaves excited with 405 nm violet diode laser.

green leaves, about 90% of the emitted Chl fluorescence at 685 nm reabsorbed by the Chl molecules of the leaf and the reabsorption is caused by the overlapping of short-wavelength range of the Chl fluorescence emission spectrum with the long-wavelength range of the Chl absorption spectrum. Since the red Chl fluorescence maximum near 690 is more strongly affected by the reabsorption than the long-wavelength maximum near 730–740 nm, the ratio F_{685}/F_{730} increases with decreasing Chl content and *vice-versa*. Thus FIR is strongly influenced by variation in Chl content and photosynthetic activity of the leaf, and in various plants the ratio F_{685}/F_{730} is an inverse indicator of the Chl contents of the plant leaves [3, 19–25].

4. Conclusion

The effect of treatment with X-ray + EMS can be distinctly observed by *in vivo* Chl fluorescence spectroscopy. The intensities of the Chl fluorescence at short-wavelength red fluorescence (near 685 nm) and long-wavelength far-red fluorescence (near 730 nm) and the ratio of fluorescence intensities at 685

and 730 nm (FIR) depend upon the dose of X-ray + EMS treatment. The FIR is the lowest (1.66) in the case of 5 Kr + 0.5% EMS-treated set, which depicts that this dose has biostimulatory effect on plant and thus could be safely employed for breeding purposes as compared to control plants where it was recorded to be 1.79. The applied FIR method has several advantages. It is a nondestructive/*in vivo* and noncontact/remote sensing technique, and the plant leaves remain intact during the measurement because cutting induces additional stress. Additional measurements can be performed with the same plant at any time. It can be used with chlorophyll-fluorescence LIDAR techniques for remote monitoring of vegetation and damage assessment.

Acknowledgment

P. Srivastava and J. K. Pandey are thankful to UGC, New Delhi, for granting fellowship in order to carry out the research work.

References

[1] R. Maurya, S. M. Prasad, and R. Gopal, "LIF technique offers the potential for the detection of cadmium-induced alteration in photosynthetic activities of *Zea Mays* L," *Journal of Photochemistry and Photobiology C*, vol. 9, no. 1, pp. 29–35, 2008.

[2] J. K. Pandey and R. Gopal, "Laser-induced chlorophyll fluorescence: a technique for detection of dimethoate effect on chlorophyll content and photosynthetic activity of wheat plant," *Journal of Fluorescence*, vol. 21, no. 2, pp. 785–791, 2011.

[3] C. Buschmann, "Variability and application of the chlorophyll fluorescence emission ratio red/far-red of leaves," *Photosynthesis Research*, vol. 92, no. 2, pp. 261–271, 2007.

[4] R. Maurya and R. Gopal, "Laser-induced fluorescence ratios of Cajanus cajan L. under the stress of cadmium and its correlation with pigment content and pigment ratios," *Applied Spectroscopy*, vol. 62, no. 4, pp. 433–438, 2008.

[5] H. Baydar and O. Y. Gökmen, "Hybrid seed production in safflower (Carthamus tinctorius) following the induction of male sterility by gibberellic acid," *Plant Breeding*, vol. 122, no. 5, pp. 459–461, 2003.

[6] S. D. More, C. V. Raghavaiah, D. S. Hangarge, B. M. Joshi, and A. S. Dhavan, "Tolerant genotypes and management for alleviation of salinity stress in safflower (Carthamus tinctorius L.) in India," in *Proceedings of the 6th International Safflower Conference*, E. Esendal, Ed., pp. 180–186, Istambul, Turkey, June 2005.

[7] Z. Ekin, "Resurgence of safflower (*Carthamus tinctorius* L.) utilization: a global view," *Journal of Agronomy*, vol. 4, no. 2, pp. 83–87, 2005.

[8] N. Camas, A. K. Aryan, and C. Cirak, "Relationships between seed yield and some characters of safflower (*Carthamus tinctorius* L.) cultivars grown in the Middle Black Sea Conditions," in *Proceedings of the 6th International Safflower Conference*, E. Esendal, Ed., pp. 193–198, Istambul, Turkey, June 2005.

[9] S. Kizil, Ö Çakmak, S. Kirici, and M. Inan, "A comprehensive study on safflower (*Carthamus tinctorius* L.) in semi-arid conditions," *Biotechnology and Biotechnological Equipment*, vol. 22, no. 4, pp. 947–953, 2008.

[10] F. J. De Serres, "X-ray induced specific-locus mutations in the ad-3 region of two-component heterokaryons of Neurospora crassa. VI. Induction kinetics of gene/point mutations, multilocus deletions and multiple-locus mutations," *Mutation Research*, vol. 231, no. 2, pp. 109–124, 1990.

[11] L. J. Stadler and H. Roman, "The effect of X-rays upon mutation of the gene A in maize," *Genetics*, vol. 33, no. 3, pp. 273–303, 1948.
[12] F. H. Ssobles, "Peroxides and the induction of mutations by x-rays, ultraviolet, and formaldehyde," *Radiation Research*, vol. 3, supplement, pp. 171–183, 1963.
[13] G. R. Hoffmann, "Genetic effects of dimethyl sulfate, diethyl sulfate, and related compounds," *Mutation Research*, vol. 75, no. 1, pp. 63–129, 1980.
[14] G. A. Sega, "A review of the genetic effects of ethyl methanesulfonate," *Mutation Research*, vol. 134, no. 2-3, pp. 113–142, 1984.
[15] H. K. Lichtenthaler and A. R. Wellburn, "Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents," *Biochemical Society Transactions*, vol. 11, pp. 591–592, 1983.
[16] H. K. Lichtenthaler, G. Kuhn, U. Prenzel, C. Buschmann, and D. Meier, "Carotenoid composition of chlorophyll-carotenoid-proteins from radish chloroplasts," *Zeitschrift für Naturforschung*, vol. 37, pp. 464–475, 1982.
[17] H. K. Lichtenthaler, "Chlorophylls and carotenoids—pigments of photosynthetic membranes," in *Methods in Enzymology*, S. P. Colowick and N. O. Kaplam, Eds., vol. 148, pp. 350–382, Academic Press, San Diego, Calif, USA, 1987.
[18] G. A. Armstrong and J. E. Hearst, "Genetics and molecular biology of carotenoid pigment biosynthesis," *FASEB Journal*, vol. 10, no. 2, pp. 228–237, 1996.
[19] H. K. Lichtenthaler and U. Rinderle, "The role of chlorophyll fluorescence in the detection of stress conditions in plants," *CRC Critical Reviews in Analytic Chemistry*, vol. 19, supplement 1, pp. S29–S85, 1988.
[20] A. A. Gitelson, C. Buschmann, and H. K. Lichtenthaler, "Leaf chlorophyll fluorescence corrected for re-absorption by means of absorption and reflectance measurements," *Journal of Plant Physiology*, vol. 152, no. 2-3, pp. 283–296, 1998.
[21] R. Gopal, K. B. Mishra, M. Zeeshan, S. M. Prasad, and M. M. Joshi, "Laser-induced chlorophyll fluorescence spectra of mung plants growing under nickel stress," *Current Science*, vol. 83, no. 7, pp. 880–884, 2002.
[22] F. Babani and H. K. Lichtenthaler, "Light-induced and age-dependent development of chloroplasts in etiolated barley leaves as visualized by determination of photosynthetic pigments, CO_2 assimilation rates and different kinds of chlorophyll fluorescence ratios," *Journal of Plant Physiology*, vol. 148, no. 5, pp. 555–566, 1996.
[23] A. A. Gitelson, C. Buschmann, and H. K. Lichtenthaler, "The chlorophyll fluorescence ratio $F_{735}F_{700}$ as an accurate measure of the chlorophyll content in plants," *Remote Sensing of Environment*, vol. 69, no. 3, pp. 296–302, 1999.
[24] K. B. Mishra and R. Gopal, "Detection of nickel-induced stress using laser-induced fluorescence signatures from leaves of wheat seedlings," *International Journal of Remote Sensing*, vol. 29, no. 1, pp. 157–173, 2008.
[25] J. K. Pandey and R. Gopal, "Laser-induced chlorophyll fluorescence and reflectance spectroscopy of cadmium treated *Triticum aestivum* L. plants," *Spectroscopy*, vol. 26, no. 2, pp. 129–139, 2011.