ISSN No:-2456-2165

Microscopic Analysis and Study of Some Pathogenic Agents by Fluorescence Spectroscopy

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Abstract:- Optical spectroscopy applied in agriculture remains a challenge in developing countries for a better monitoring of agricultural productivity. The phytopathological approach by fluorescence spectroscopy makes it possible to detect viral and bacterial diseases in particular in real time in order to early follow the evolution of pathogens and consider preventing their spread. To illustrate this work, a multi-spectral microscope has been designed specifically for the representation of fluorescence images on a portion (leaf blade) of a plant without using a fluorescent molecule. Through the obtained microscopic images, the corresponding fluorescence spectrum has been reconstructed in order to distinguish a healthy leaf from a diseased leaf in terms of contrast. The main contribution is the development of the method of fluorescence spectroscopy by multispectral imaging at the microscopic scale for the early diagnosis of bacterial wilt due to *Ralstonia solanacearum* in tomato plants, two symptoms caused by the African cassava mosaic virus in a cassava field, and Cucumber mosaic virus following its inoculation to some zucchini plants without the use of a fluorescent marker. The results obtained show the effectiveness of the proposed technique, in order to facilitate the identification of these pathogens present in tomato, cassava and cucumber plants respectively by biological analysis laboratories.

Keywords:- *Multispectral Imaging, Pathogens, Optical spectroscopy, Fluorescence.*

I. INTRODUCTION

Light microscopy and multimodal spectroscopy developed these last years are essential and its applications have seen the day in a lot of multidisciplinary field in physics [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11]. In other disciplines, the technique of light microscopy allowed to do numerous biological, studies on cell morphology, nucleus and complex molecules. Serological methods (such as Enzyme linked Immunosorbent assay-ELISA) coupled with electron microscope observations can be used to identify virus particles [12, 13, 14, 15, 16, 17, and 18]. In addition, optical microscopy has been developed in fluorescence imaging because of the possibilities to detect plant cells which cannot be visualized with conventional optical microscopy. Otherwise, fluorescence imaging was also used to reveal the presence of symptoms of pathogens [19, 20, 21, 22, 23, and 24]. A research study also relied on chlorophyll fluorescence for sorting the quality of soybeans [25]. Recently, the effect of temperature on the optical properties of vegetable oil has been studied using laser scanning fluorescence spectroscopy, UV and Visible [26]. These technological advances in imaging harmless and extremely fast allow to allow you to see a blood cell in a vessel. Currently, our fluorescence imaging technique for the detection of viral and bacterial diseases in real time could be one of them. This spectroscopic research topic applied to phytopathology for the diagnosis of certain plant diseases is of capital importance. The study of these pathogens (Bacterium *Ralstonia solanacearum*, African cassava mosaic virus and Cucumber mosaic virus) constitutes a constraint to the development of these crops causing significant economic damage in Mali [27].

ISSN No:-2456-2165

These pathogens respectively affect different plant species such as nightshades (tobacco, potato, tomato, eggplant, pepper and banana [28], cassava, cucumber and zucchini. In this work, we are interested in the spectral characterization of three pathogens causing significant yield losses due to the poorly controlled threats of *Ralstonia solanacearum*, African cassava mosaic virus and Cucumber mosaic virus in some fields.

Thus, the application of optical fluorescence spectroscopy as a means of analysis and study of pathogens remains a multidisciplinary approach which is not too much developed by scientific researchers in phytopathology. In this article, the fluorescence technique was developed in multispectral imaging for the early detection of *Ralstonia solanacearum*, African cassava mosaic virus and Cucumber mosaic virus in the field conditions.

Thus, the application of optical fluorescence spectroscopy as a means of analysis and study of pathogens remains a multidisciplinary approach very little developed by scientific researchers in phytopathology. In this article, the fluorescence technique was developed in multispectral imaging for the early study of *Ralstonia solanacearum*, African Cassava Mosaic Virus and African Cucumber Mosaic in the field of plant cultivation.

II. MATERIAL AND METHODS

1. Biological sample

The present work required the selection of three endemic plants in West Africa having a high consumption rate, namely tomato, cassava and zucchini. The plant samples were mainly collected during the internship of the practical realization of my thesis in Yamoussoukro in Ivory Coast (Fig.1). For sampling, we collected some tomato plants withered by *R. solanacearum* in a garden located in the district of 80 housing units in Yamoussoukro in Ivory Coast, without going through the inoculation stage. Then, we got some cassava leaves infected by African cassava mosaic virus (ACMV) transmitted by the white fly *Bemissia tabaci* in a cassava field located in the north of the Yamoussoukro INPHB in Ivory Coast. Thus, two infected leaves by the virus have been selected one of which was distorted and the other was discolored with a pale green color type mosaic. Finally, we used a banana plant infected by Cucumber mosaic virus (CMV) whose symptoms have disappeared (masked symptom: invisible to the naked eye) due to high temperature; this disease has been inoculated mechanically on zucchini plants in order to perform a spectroscopic analysis. This experiments took place at the Plant Pathology and Biology Laboratory at INPHB) in Yamoussoukro in Ivory Coast. From each of these tropical plant diseases, a healthy leaf had been chosen for each collection as the reference sample before starting the phase of their acquisition to obtain significant results compared to the pictures which were taken.

Fig.1. Targeted locality for collecting samples in the fields

2. Data acquisition method

The spectral detection of the selected pathogens was thus carried out by a multispectral fluorescence microscope (Fig.2); through the images acquired by samples of the leaves of diseased and healthy plants. Its Operating principle is similar to the one of the conventional fluorescence microscope. The excitation light is filtered by an excitation filter by selecting a band of wavelength. A dichroic mirror specifically reflects this wavelength band to send it towards the sample. The excitation light is focused on the sample by the objective, allowing to collect the light emitted by the sample, in a higher wavelength range. This sample show is done in all directions, but alone the rays captured by the lens passing through the dichroic mirror. This emitted light is so filtered by an emission filter before reaching the sensor [29]. The resulting image is formed by fluorescence ratios contrasting the healthy and diseased areas [30]. The final image is registered through the help of a digital EMCCD camera (8x8µm) equipped with specific software for image acquisition.

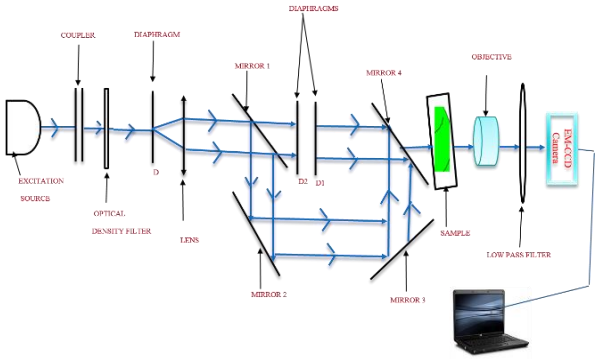

Fig.2. Laser-induced fluorescence imaging device.

For the experiment, three laser sources were used for excitation of sample types (infected and healthy leaves); producing imaging in blue (at 450 nm), green (at 532 nm) and red (at 638 nm) for the diagnosis of the health of these plants. We provided the fluorescence emission through two different low pass filters that block wavelengths greater than 550 nm and 650 nm in order to recover fluorescence ratios. A total of five (5) images were acquired for each sample (Figs. 3, 4 and 5).

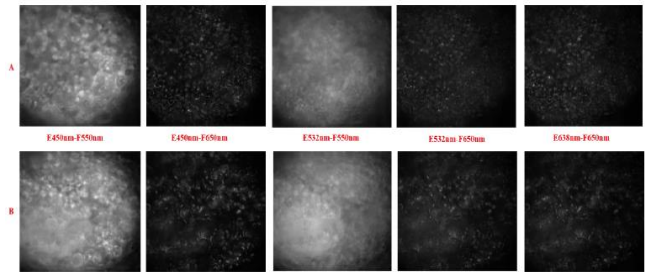

Fig.3. Image of healthy tomato leaves (A), and infected by *R. solanacearum* (B) under excitation of 3 lasers and 2 low-pass filters.

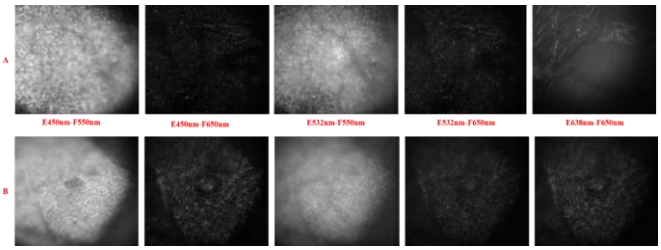

Fig.4. Images of healthy zucchini leaves (A) and infected (B) by AMV under excitation of 3 lasers and 2 low-pass filters.

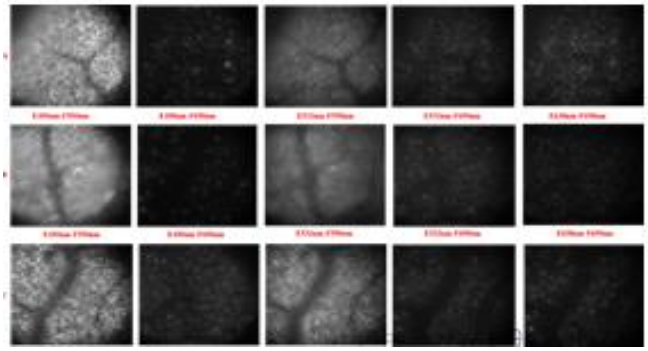

Fig.5. Images of healthy cassava leaves (A), deformed (B) and bleached by AMV under excitation of 3 lasers and 2 low-pass filters.

However, optical images always contain always noise (parasitic information) which may be due to the working environment, electronic systems, etc. These noises combine random details digitally to the content of the pixel and cause the correlation of images inducing the repetition of certain information from one spectral plane to another. In addition, whatever the type of measurement mode, it is necessary to perform the measurements in the dark or in a suitable place to obtain a usable image in order to proceed diagnosis of these three parasitic diseases: *Ralstonia solanacearum*, African cassava mosaic virus and Cucumber mosaic virus.

3. Data analysis

Treatment consists to analyze fluorescence images by optical spectroscopy for obtaining additional information on the samples to be studied. The representation of the spectrum of these five acquired images does not allow to better discrimination the leaves of a plant (healthy and infected). For this purpose, we proceeded to a method of combining the images acquired using the formula of Michelson (1927):

$$M=\frac{Lmax-Lmin}{Lmax+Lmin}$$

M: Michelson contrast or modulation contrast;
Lmax: Maximum luminance of light bands;
Lmin: Minimum luminance of the dark bands.

The acquired fluorescence images hasn't been sufficient for the reconstruction of their corresponding signature to study spectrally the pathogens. Indeed, using the algorithmic procedure, the images obtained have been combined two by two to form a succession of ten fluorescence images from the Michelson formula; with a database of vectors consisting of the average intensities of pixels. Finally, the average spectrum was reconstructed through an area of each of the combined images to better represent their corresponding spectral signature. This signal obtained translates the number of combinations of fluorescence images in function of contrast. This method of analysis was thus applied to viruses and bacteria selected in order to discriminate them.

III. RESULTS AND DISCUSSION

Results in fluorescence mode (Figures 6, 7 and 8) were obtained by illuminating the healthy and infected leaves of tomato, cassava and zucchini with blue, green and red lasers with filters allowing only wavelengths around 550 and 650 nm to pass in order to characterize spectrally by acquiring images of the leaves infected with *R. solanacearum*, African cassava mosaic virus and Cucumber mosaic virus. Therefore, when the leaf is infected with these pathogens, we see that in fluorescence this one is differentiated by a spectral deformation (See representative in figs. 6, 7 and 8).

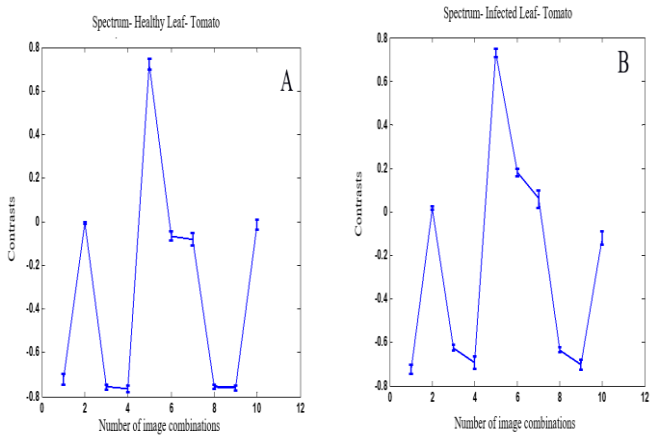

Fig.6. Spectral comparison of healthy tomato leaves (A) and infected (B) by *R. solanacearum* in fluorescence under excitation of 3 lasers and 2 low-pass filters.

ISSN No:-2456-2165

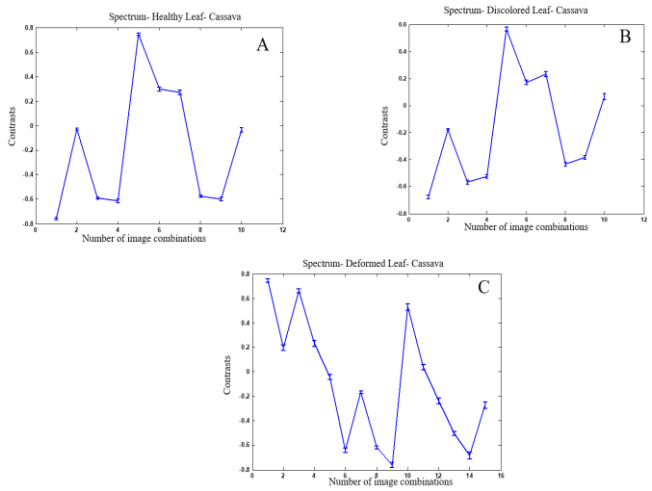

Fig.7. Spectral comparison of healthy cassava leaves (A) and bleached (B) and deformed (C) by fluorescence AMV under excitation of 2 lasers and 3 low-pass filters.

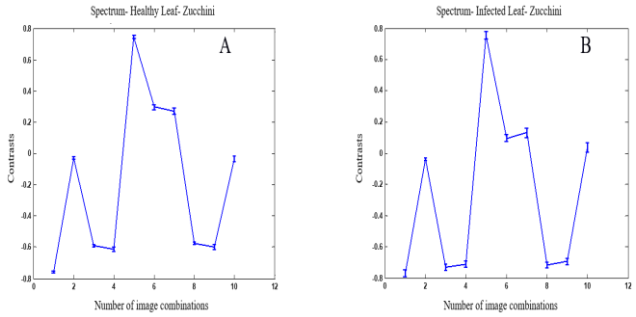

Fig.8. Spectral comparison of healthy zucchini leaves infected with CMV in fluorescence under excitation of 3 lasers and 2 low-pass filters.

There was not enough data available on the spectral detection of plant leaf diseases at the time of initiation of this research work, using the multispectral and multimodal microscope. It therefore appeared necessary to question, to describe and analyze the spectral behavior of early infection of *R. solanacearum*, African cassava mosaic virus on cassava and zucchini leaves in the field for a fluorescence study.

In addition, our fluorescence spectroscopic analysis shows that at the infection stage (early or mature) of each disease, regardless of the state of the leaf of a chlorophyll plant (healthy or stressed), they can fluoresce at a higher contrast sensitivity. But at lower contrast sensitivity, the infected leaf absorbs insufficient light and fluoresces less; by causing a spectral modification which may be due to a reduction (or degradation) of the content of chlorophyll or other nutrients.

Our fluorescence results also reveal that the effect of the leaf infected with these pathogens (*R. solanacearum*, AMV and CMV) can cause a drop in energy intensities at the spectral gap band before their fluorescence stage. Indeed, we then deduce that a decrease of the chlorophyll concentration contained in the stressed leaf can be used as a function of the contrast. The excitation of the chlorophyllian pigments by visible light (Red, Blue and Green) produced an increase in intensity chlorophyll fluorescence from leaves infected with *R. solanacearum*, AMV and CMV; producing the opposite effect at certain points of the spectrum. These viral and bacterial infections can therefore often cause stunted growth and reduced agricultural production.

We also note that the study of fluorescence gives almost the same information as that of the studies of transmission, reflection and scattering. Some food crops required humidity and high temperature for their growth, thus, the study of fluorescence is indeed a complementary diagnostic study of plant diseases. Otherwise, it has been shown that the red fluorescence was caused by excitation of chlorophyll pigments by visible light between 660 and 780 nm, and blue fluorescence by excitation of ultraviolet radiation [31]. The data resulting from this work have been at the base hence the identification of CMV in a zucchini plant. However, researchers have shown that tomato resistance to bacterial wilt is not universal in crop fields [32]. Some major components must also be taken into account to control bacterial wilt [33, 34, and 35]. According to the culture environment, tomato growers may not feel the effect of *R. solanacearum* under intense rainfall. Soil moisture (excess water) and elevated temperature above 25 ° C also more often promote survival and progression of *R. solanacearum* [36, 37, and 38]. In the same order of ideas, abiotic (insufficient water, lack of nutrients, etc.) or biotic factors, varietal resistance can harbor this pathogen without showing symptoms. Our spectroscopic results also can also be used to consider means of control or alternative control precaution for *R. solanacearum* in real time before the contamination of the entire tomato crop field. In addition, the results we obtained then indicate that the leaves of tomato plants contain a remarkable amount of chlorophyll substance whether they are healthy or diseased. Therefore, despite the attack of phytobacteriosis on the tomato plant; we show that all these leaves participate to photosynthesis in their own way to meet the nutritional needs of the plant. Across Mali, a comparative analysis of four sub-phenotypes of *R. solanacearum* in race 1 was realized on the greenhouse tobacco plant, and contributed to the differentiation of three groups of subphenotypes. Each of these strains is therefore able to infect Solanaceae in the open field as well. But, two of these strains tested have similar effects which are not aggressive on Solanaceae [9, 10, and 11]. This essay put in evidence the strong virulence and aggressiveness of certain emerging Malian strains. However, Malian strains (DR3b and DR4 sub-phenotypes) appear to be more aggressive than emerging strains from Ivory Coast. These results obtained are taken into account in our discussion for the understanding of our results. In Africa, particularly in Mali, these strains constitute a new challenge for breeders who develop Solanaceae cultivars. A similar study was carried out in 2012 in Guyana where the experimentation protocol does not conform to the one we have undertaken. These researchers have succeeded in genetically characterizing certain strains of *R. solanacearum* and showing their influence on the cucumber plant. Their results prove that there is a similarity between the strains of Guyana and those of Martinique [39]. The studies of these various strains are not equally comparable to those we have studied. This suggests that the detection of our strains can not

only be a criterion for the identification (or early characterization) of this bacterium in transmission but also in reflection and diffusion. In general, phytopathogenic bacteria are more numerous than pathogenic bacteria from animals. The fluorescence spectroscopic study on bacterial wilt is to understand how a Solanaceae plant can function with early infection of pathogens. *Ralstonia solanacearum* can be better studied and characterized in Solanaceae. Otherwise, microscopic and spectroscopic analysis shows that the onset of infection of this type of bacterium on a Solanaceous leaf systematically plays the remarkable role like that of the normal plant, normally playing the role of photosynthesis to bring of the necessary food for the plant. Cucumber mosaic virus has been isolated and then identified in Ivory Coast by Fauquet and Thouvenel (1987) producing mosaics on cucumber and zucchini, but their yield losses in pistachio (*Lagenaria siceraria*) are not estimated [40]. At the early stage, when CMV remains dormant in the zucchini plant until senescence, it is probable that it is out of danger without being influenced by the effect of the virus. In this case, the plant can be saved by giving a good harvest. However, according to the culture environment and mostly cool temperatures, the zucchini plant can quickly manifest the infection producing severe leaf symptoms and making their fruits difficult to consume.

Finally for the spectroscopic study of ACMV in fluorescence, the different samples tested allowed to bring some additional information on the Cassava mosaic in Ivory Coast. In addition, the researchers made a comparison of the African cassava mosaic virus from Ivory Coast and the Indian mosaic virus showing that these two viruses have distinct strains with different effects on the cassava plant [41].

IV. CONCLUSION

At the end of this spectroscopic study, the results obtained during this research work with new microscope and new analysis strategy adopted, aims to characterize or early identify *Ralstonia solanacearum*, AMV and CMV in the leaves of tomato plants, Cassava and Zucchini by Laser Scanning Fluorescence Spectroscopy. These results can help limit the damage caused by parasitic diseases. In addition, this spectroscopic information can be an effective means for agronomists to better control this bacterium and viruses and to facilitate the monitoring of these crop plants in real time. Thus, the multispectral imaging method reveals that these bacterial and viral diseases are detectable by optical fluorescence spectroscopy in order to study the evolution of leaves stressed by them. In addition, this detection technique provides a powerful means to characterize leaf samples from infected plants in a wide variety of applications. The analyzes developed during this work were the object of the characterization, identification and classification of the selected parasitic diseases. The spectral study of infected plants by fluorescence imaging also provided us with innovative information on the basis of the appropriate method. Thus, the analysis of optical spectra has led to a conclusion that multispectral imaging or in particular multispectral fluorescence microscopy is a very effective method for the early study of these infectious diseases.

ACKNOWLOGMENTS

The authors especially thank ISP for the financial contributions. We would like to thank Prof. S. Svanberg, Dr. M. Brydegaard, Mr. A. Merdasa for their technical support in carrying out this work and the people who helped to review the article.

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