

Optical Fluorescence Diagnostic of Wheat Leaf Rust with Laser Scanning Confocal Microscopy

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Received date: March 09, 2018; Accepted date: April 11, 2018; Published date: April 17, 2018

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Abstract

Wheat is the most important grain crop and food source worldwide. The management of diseases and early detection of pathogens is a crucial step in diagnosis programs in wheat. In the primary stage, the symptoms of rust fungus are difficult to identify with visual monitoring and other conventional techniques. In this study, we intended to investigate the early stage leaf rust in wheat crop produced through rust fungus using light fluorescence from laser scanning confocal microscopy (LSCM). The leaf rust and normal samples were analyzed with an excitation of 488 nm wavelength of Ar⁺ laser without any marker or photosensitizer. The small dark pores instead of stomata appears in leaf due to fungus infection and can be observed after two week of leaf tillering. These spots are orange or brown in the beginning and become black, when plants reach maturity. In recent study, the potential of non-invasive techniques for the detection of plant diseases are demonstrated for the development of a rapid and less complex early stage detection procedure that can be utilized to evaluate the infection structures during fungus infection of wheat. The newly developed rapid procedure will be helpful for early stage detection and management fungal infection before proper development during wheat interaction.

Keywords: Wheat leaf rust; Laser scanning confocal microscopy; Fungus infection; Optical sensors

Introduction

Wheat represents one of the most important food crops worldwide and its yields are continuously under threat from plant diseases often caused by pathogenic fungus [1-3]. The wheat leaf fungus *Puccinia triticina* is currently one of the most economically important diseases, and causes premature death of wheat leaves, reducing the ability of plants to capture sunlight, which ultimately reduces their grain production [4,5]. Leaf rust caused by *Puccinia triticina* produces high yield losses up to 20% worldwide [6]. Wheat crop is the richest source of the calories and proteins consumed by humans each day. Wheat production needs to increase dramatically in coming years to meet the needs of a rapidly growing world population, but disease is a continuing threat to current and future yields. Symptoms of leaf rusts include bright yellow, orange or red leaf spots. These leaf spots often produce an abundance of yellow, orange, red, or brown powdery spores that can be easily seen on leaf surface caused by a group of related fungus [7-9].

The optical sensors and laser spectroscopy has become essential approach and latest research fields of applied plant sciences. As a rapid, accurate, non-destructive and economical technology, Laser scanning confocal microscopic study of leaf rust at an early stage of disease provided researchers to gain a better understanding on the overall behaviour and health of plants [10,11]. A microscope image of a leaf epidermis can provide a clear view of epidermal cells, stomata and plant leaf veins. Among these elements, stomata play a major role in protecting the plant against water loss and regulating the gas exchange with the external environment [12]. As a result, the behaviour of stomata provides key information on the water stress level, food

production rate and the overall health of the plant [13,14]. The rust fungus are destructive plant pathogens looked as small-rounded orange or yellow pores on the upper surface of the leaves and reduces both grain yield and weight. A number of fungicides are label to control the disease but scouting and early detection is crucial to time pesticide applications for effective control [4].

Laser scanning confocal fluorescence imaging in plants is unusually challenging because of the large amounts of photosynthetic pigments contained in green plant tissues. For example, chlorophyll can obstruct the penetration of light and has high levels of auto fluorescence at wavelengths that used for fluorescence imaging. Until now, mostly confocal laser scanning microscopes used to overcome these limitations. The rust fluorescence in viral infected wheat leaf recorded by confocal laser scanning microscopy for early stage disease detection [15,16].

Current research activities are towards the development of such technologies to create a practical tool for disease monitoring under field conditions. The spectroscopic and imaging techniques can provide information on disease detection at early stages to control the spread of plant diseases. In LSCM the light collected from point source and detected at sensitive detectors through dichroic mirrors and lenses [17,18].

Materials and Methods

The wheat leaf collected from National Agriculture Research Council (NARC) Islamabad. The sampling done with general laboratories protocol. Several plants were selected randomly to assess disease prevalence and severity we prepared fresh leaf cross sections using freehand sectioning method for optical and laser scanning confocal microscopy [19].

Laser scanning confocal microscope (LSCM) Carl Zeiss 510, Jena, Germany was used to visualize fungal structures by their light and laser fluorescence imaging. To analyses the growth of fungus structures of leaf rust in wheat leaves, the maximum length of the fungal colonies parallel to the length of the leaf measured. Microscopic chlorophyll fluorescence intensity of the axial leaf surfaces are captured with the CLSM system (40X water emulsion and 60X oil emulsion objective) at 488 nm wavelength of Argon ion laser of 30 mW power. The leaf specimen set on a cover glass and fluorescence image taken using Ar⁺ laser and He-Ne lasers. The excitation light with the excitation wavelengths of 488 nm passed through 450-490 nm dichroic mirrors down to the sample by 510 nm long pass filter and emitted fluorescence by emission wavelength of 510-565 nm to the PMT tubes. The images plotted by Zen software of Carl Zeiss, Germany [20,21].

Results and Discussion

The entrance of microbial species into host plant tissues is a first step toward infection. In plants, the stomata of plants regulate gas exchange and water transpiration in response to changing environmental conditions. The stomata also have play an important role in host defence for infections and close upon detection of potential microbial pathogens to prevent the infection of the leaf interior [22]. The stomata is also important in Photosynthesis process of plants for food production with the help of sunlight, carbon dioxide and water. In case of leaf rust, the pathogen inters through stomata and start destruction of stomata cells, due to which the leaf stops Photosynthesis process, and with time appears brown, yellow or blackish. The immune system of plant initiated by stomata and with the destruction of stomata immune system of the plant stops working [23,24]. The wheat plant field along with fresh leaves of day 14, 24, 37 and 48 are illustrated in Figure 1. It can be seen that by visual observation no symptom of rust on leaves can be observed before day 37. But in case of laser scanning confocal microscopic imaging rust stomata destruction can be observed from day 18 of wheat tillering. In our study we have taken different samples of wheat plant leaf from second weak to onward and observed with laser scanning confocal microscopy setup describe above. When a fresh leaf illuminated with Ar⁺ laser of 488 nm laser using a laser scanning confocal microscope (LSCM), the chlorophyll fluorescence is collected and appears green under the confocal microscope because chlorophyll absorbs 488 nm laser energy and gives off fluorescence between 510 nm to 550 nm wavelength. In Figure 2a and 2b the differential interference contrast (DIC) and fluorescence images of fresh wheat leaves on day 18 are shown and rust appears after day 18 can be observed in Figure 2c and 2d for DIC and fluorescence.

The microscopic images of the infection in leaves and intracellular movement of the fungus through vascular bundles of xylem and phloem recorded. The confocal micrograph of infected leaves showed the dark pores appears after stomata infection. Once the fungi colonize and infect the tissues, they produce mycotoxins, which spread to the surrounding tissue, and can move to the rest of the plant through xylem and destroy immune system of the stomata to plant defence [25]. The stomata become dark after infection and up till day 48 the rust appears on the surface of wheat leaf. Fungus enters the plant via the stoma and host and fungal membranes allows the transport of nutrients from the host to the fungus. The initial symptoms of rust starts in leaf and small chlorotic spots on the leaves appear from day 18 and cannot seen with necked eye. We can see that in Figure 1, it cannot observed before day 48, but with laser fluorescence it can be seen at day 18 as shown in Figures 2c, 2d, 3 and 4. The dark pores start

developed after day 14 and appears on leaf surface from day 35. The rust dark pores on baby plant are observed round and mature leaves most often are shaped irregularly, long and narrow as can be seen in Figures 4a-4d.

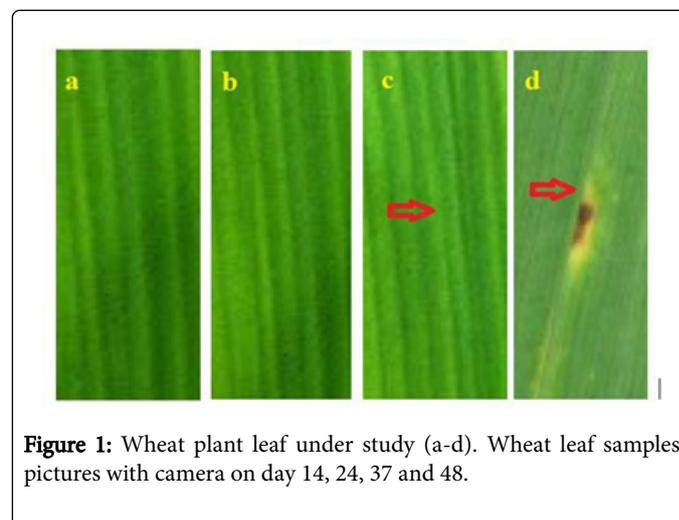


Figure 1: Wheat plant leaf under study (a-d). Wheat leaf samples pictures with camera on day 14, 24, 37 and 48.

In this study, the biomarker used is the auto fluorescence coming by chlorophyll or green fluorescence protein (GFP) at excitation of 488 nm laser energy and gives off fluorescence between 510 nm to 550 nm. Chlorophyll has an absorption band in the green region of the visible spectrum, which produces a significant amount of fluorescence at wavelengths 510-565 nm when excited with wavelengths of 488 nm of Ar⁺ laser. The light spectrum of normal leaf at day 14 and rusted wheat can be seen in Figure 5. The fluorescence from normal shows continues spectrum as compared to the rusted, in which fluorescence suddenly drops at dark pores.

The wheat leaf study with ultraviolet radiations and light microscopy was conducted by different scientists in past and the work [26] applies UV radiation to leaves under blue fluorescence. The plant leaves are then captured using a fluorescent microscope and the resulting images are filtered and segmented to extract stomata and guard cells. Even though this method produces reliable results, it requires a relatively featureless background as well as methods of applying UV radiation to the leaf. In addition, the work presented uses image-processing techniques on microscope images to classify different stomata structure [27]. A watershed technique employed to extract a single stoma from a nearly featureless background. However, the proposed method would not perform well in the presence of multiple stomata and a feature-rich background. In our previous research, we have used different optical methodologies to characterize viral and infectious diseases and in recent study we extended it to an agriculture [28-35].

Conclusion

In plant and animals, the early detection of pathogens is a crucial step in diagnosis and management programs. The failure to adequately identify and detect plant pathogens using conventional, culture based morphological techniques has led to the development of nucleic acid-based molecular approaches. At the microscopic level, confocal fluorescence imaging of leaves infected by pathogens can be easily detected at an early stage to manage the disease effectively. Recent results confirm several previous findings based on traditional

techniques but also provide new leaf rust detection mechanism in plants that is much more dynamic and flexible than previously used to study fungal infection process in plants.

In this study, the wheat leaf rust associated with leaf death due to fungus entrances via stomata presented using laser scanning confocal microscope. The fungus pathogen enters through stomata and start destruction of stomata cells, due to which the leaf stops photosynthesis process, and with time appears brown, yellow or blackish. The immune system of plant is initiated by stomata against fungus with the destruction of stomata immune system of the plant stops working. The wheat plant field along with fresh leaves of day 14, 24, 37 and 48 are studied and it can be seen that by visual observation no symptom of rust on leaves can be observed before day 24. But in case of laser scanning confocal microscopic imaging rust stomata destruction can be observed from day 18 of wheat tillering. This technique can developed a rapid and effective way to early detection of wheat leaf tissue infected with the fungal pathogen.

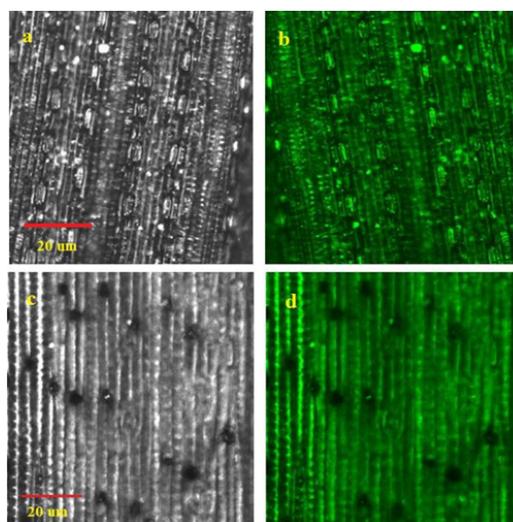


Figure 2: Wheat leaf normal and rust fungus images at wheat tillering stage of plant specimens examined under Ar⁺ laser of 488 nm and 30 mW power of laser scanning confocal microscope for the disease progress of rust fungus. (a,b) DIC and luorescence wheat leaf sample image at 40x water emulsion objective on day 14 for normal study, (c, d) DIC and luorescence images of the rusted sample at 40x magnification on day 24 for rust study.

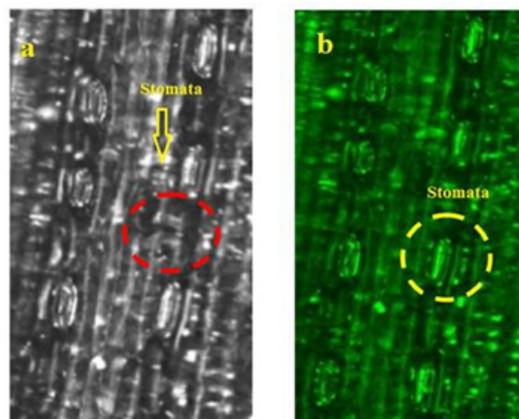


Figure 3: Laser scanning confocal microscopic images for normal tillering stage on day 14. (a) DIC and (b) fluorescence at 488 nm excitation and 510 nm-560 nm fluorescence with 60X oil emulsion objective.

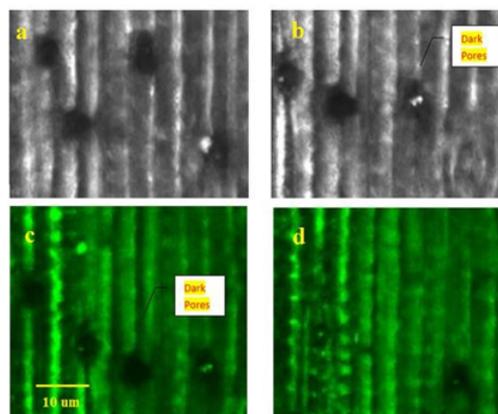


Figure 4: Wheat leaf rust images with dark pores plant specimens examined under Ar⁺ laser of 488 nm and 30 mW power of laser scanning confocal microscope with 30 μm dimension for the disease progress of rust fungus. (a,b) DIC wheat leaf sample image with dark pores (5 μm) at 60x oil emulsion objective on day 24, (c,d) Fluorescence rusted sample at 60x magnification on day 24 for rust study.

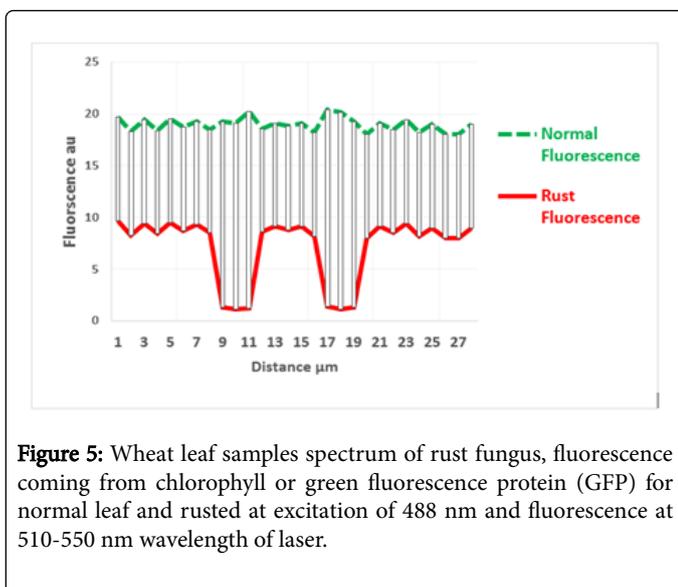


Figure 5: Wheat leaf samples spectrum of rust fungus, fluorescence coming from chlorophyll or green fluorescence protein (GFP) for normal leaf and rusted at excitation of 488 nm and fluorescence at 510-550 nm wavelength of laser.

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