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Early optical detection of infection with brown rust in winter wheat by chlorophyll fluorescence excitation spectra

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ABSTRACT

Puccinia recondita f. sp. *tritici*, the causative agent of brown rust disease is the reason for major yield losses in winter wheat plants (*Triticum aestivum* L.). Early detection of fungal infections would allow a specific fungicide application. Aim of the current study was to develop an automated and computer based device that differentiate infected and healthy plants non-invasively in an early stage of infection. To achieve the target an optical sensor device has been designed ("MultiDetExc"), which excites chlorophyll fluorescence in discrete wavelengths and detects the induced emissions. Wheat plants were infected artificially with brown rust in a climate chamber experiment to survey the capability of the instrument. The chlorophyll fluorescence excitation spectra of whole wheat plants were measured on several days after infection. As reference methods, HPLC and qPCR analysis were included in the current study to measure the polyphenol content of the leaves and the level of infection.

The recently developed sensor device is an efficient technique to differentiate the infected and not infected wheat plants as soon as four days after inoculation. The measured fluorescence quotients correlate high positive with the polyphenol contents and the relative amount of fungal DNA. An untreated healthy control was measured parallelly, in order to associate the increased synthesis of polyphenols to the fungal infection definitely.

1. Introduction

Fungal infections annually decrease yield and quality of crop plants causing major economic losses. There are numerous fungal diseases in winter wheat; e.g. septoria leaf blotch (*Septoria tritici*), septoria nodorum blotch (*Septoria nodorum*) tan spot (*Drechsleria tritici-repentis*), powdery mildew (*Blumeria graminis*), brown rust (*Puccinia recondita* f. sp. *tritici*), yellow rust (*Puccinia striiformis*) and fusarium diseases. Fungicides are used to keep the crop losses as low as possible and therefore wheat populations are treated preventively with fungicides. Economic losses could be reduced significantly, if the pesticides could be applied as soon as possible (due to early detection) and only the infected ones.

To this end, it would be helpful for plant protection to apply a method that can detect site-specific and noninvasive fungal infection in wheat population, as early as possible and without expensive and time-consuming laboratory tests.

For this purpose, the optical measurement of plant constituents could be suitable. The measuring principle of the sensor device used in this study based on the hypothesis that plants synthesize polyphenols as a defense mechanism in response to fungal infections, because polyphenols act as an antibiotic against microorganism, e.g. fungi (Dixon, 1986). These polyphenols absorb UV radiation and are mainly placed in the epidermis (Cerovic et al., 2002). In order to estimate the polyphenol content optically, the chlorophyll in the leaves is used as an "internal sensor". For this purpose chlorophyll is excited in the intact leaf with radiation of different wavelengths. Subsequently, the emitted fluorescence intensities of chlorophyll are measured. If the leaf is irradiated with UV radiation only a part of it is able to pass through the epidermis in the underlying tissue, because polyphenols absorb UV radiation and are localized in the epidermis. The part of the UV radiation that passes through the epidermis is absorbed by chlorophyll with a specific absorption coefficient. This absorption causes fluorescence. The fluorescence can be detected by a sensor. Visible radiation is used as reference, since it can pass the epidermis almost completely. The fluorescence quotient of UV radiation and visible light induced fluorescence is used as an indicator for the polyphenol content in leaves. Thus the quotient is also a value for fungal infection. The fungus is probably discovered by the host plant using elicitors (Anguelova et al., 1999) - which is triggering defense processes - during the penetration through the closed stomata in the leaf (Börner, 2009).

In 2009, Csefalvay and co-workers were able to detect a *Plasmopara*

Abbreviations: DAI, days after inoculation; F, chlorophyll fluorescence; exc, excitation; ru, relative units; dm, dry mass

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viticola infection in grapevine leaves using chlorophyll fluorescence imaging pre-symptomatically. The measuring area was large enough to include the whole grapevine leaf. However this method does not work under continuous sunlight conditions. Therefore, the chlorophyll fluorescence imaging could not be appropriate for our aim.

The chlorophyll fluorescence excitation technique had applied successfully in various research to make a statement about the polyphenol content of plants leaves of agricultural crops (Bilger et al., 2001; Cartelat et al., 2005). In the research cited a leaf clip with a diameter of 5 mm was used. This technique has a very low measuring distance (several millimeters).

In the present study, a new fluorometer - the MultiDetExc (MultiDetectionExcitation) - was developed, which should also be suitable for precision farming with an online approach. Accordingly, the MultiDetExc has to fulfill special requirements, such as the measurement from a distance and also the measurement at occurring background radiation. Furthermore, the MultiDetExc should be able to measure such a large area that a representative part of the plant is detected.

Sensor devices for site-specific noninvasive detection of various parameters are already in use for precision farming. For example the YARA N-sensor® measures the chlorophyll content of plants, which constitutes a criterion of the nutritional status of the plants (Flowers et al., 2003). Furthermore it is possible to use image analytical procedures, for example an herbicide sensor device that detects weeds and grass weeds (Weis et al., 2008).

The aim of this study was to detect the infection of brown rust noninvasively in an early stage and possibly before the appearance of visible symptoms. Additionally, the sensor must be suitable for remote sensing in precision farming. For this purpose an optical sensor device was developed and tested in a climate chamber experiment.

2. Materials and methods

2.1. Experimental design and pathogen inoculation

Plants of the winter wheat cultivar *Triticum aestivum* L. “Ritmo” were used for the current experiment. This cultivar was selected, because it is regarded as susceptible to brown rust. The resistance degree of the cultivar Ritmo against brown rust is eight, in a defined range from one (resistant) to nine (susceptible) (Bürling et al., 2011).

The winter wheat plants were grown by vernalized wheat seeds and the seedlings were raised in the climate chamber. During the vernalization the wheat grains were in the dark on moist sand for eight weeks at 4 °C. The seedlings were planted in 9 × 9 × 9.5 cm^3 plastic pots filled with standard soil (Type-T, Terrau Professional GEPAC Einheitserde, Einheitserde und Humuswerke Gebr. Patzer GmbH & Co. KG, Sinntal-Jossa, Germany). After six weeks, each plant was treated with 5 ml of respectively growth regulator (Stabilan, Nufarm GmbH & Co. KG, Linz, Austria; dilution factor: 1:400) and fertilizer (Wuxal, Wilhelm Haug GmbH & Co. KG, Pfäffingen, Germany; dilution factor: 1:1000) by application in the pot. The conditions of the climate chamber are given in Table 1.

In the current experiment two variants were tested, plants which were inoculated with brown rust spores and healthy control plants. From a total of 300 plants, 120 plants were inoculated with uredospores of *Puccinia recondita* f. sp. *tritici* (received from BASF, Ludwigshafen, Germany) and 120 plants were used as healthy controls. The remaining 60 plants were used as controls for the days minus one and minus three. The inoculation began eight weeks after potting when the plants were in the growth stage 31 to 32 according to Zadoks (Zadoks et al., 1974).

Table 1
Conditions of the climate chamber.

Temperature [°C]	Air humidity [%]	Light intensity [µmol photons m^{-2} s^{-1}]	Day length [h]
20 °C ± 1 °C	70	280–300	16

The spores were mixed with distilled water containing 0.01% Tween-20 (Roth, Karlsruhe, Germany) (Anguelova et al., 1999) and sprayed on the plants (5 ml per plant, 10^5 spores/ml). Afterwards, the plants were enveloped in plastic foil for 72 h and placed in a climate cabinet with the same conditions as the climate chamber (Table 1). The control plants were sprayed with a mixture of distilled water containing 0.01% Tween-20 and also wound in plastic foil for 72 h. In this way, all plants were treated identically, except that controls did not come into contact with fungal spores.

The measurements were usually performed every two days and lasted for 21 days. On each test day 15 plants per variant were taken at random out of a set of 150 plants per variant. No plant was measured twice. New plants were used for every measurement day. All measurements (described in 2.2–2.4) were performed before and after artificial inoculation with brown rust. The chlorophyll fluorescence was measured with the MultiDetExc. Furthermore, the polyphenol content of the leaf material was quantified by HPLC and the amount of fungal DNA of the leaf material was determined by quantitative PCR (qPCR).

Sixteen individual sets of measurements over the whole plant were done per plant and day with the MultiDetExc. After the noninvasive optical measurements, six leaves of the same plants were frozen in liquid nitrogen and stored at −85 °C for the reference measurements. Therefore, the top three leaves were selected from two shoots of each plant. The leaves of all fifteen plants per variant and measurement day were combined to collective samples. The collective samples were freeze dried and grounded. Three extractions of the pulverized leaf material were done for polyphenol content’s measurements with the HPLC. Therefore, the sample size was three per variant and test day. One extraction of the same pulverized leaf material was done for fungal DNA’s measurements with the qPCR. Thus, the sample size was only one per variant and test day. Table 2 gives an overview of the performed measurement.

2.2. Evaluation of chlorophyll fluorescence

The noninvasive chlorophyll fluorescence measurements were performed in this study with a fluorometer which was newly designed and developed at the Institute of Agricultural Engineering of the University of Kiel, the MultiDetExc (MultiDetectionExcitation, Fig. 1). The application process of a patent is currently pending (Patent No. 14815682.1-1554). The instrument has eight excitation wavelengths; one of these is used as reference wavelength. In addition the device has two detection wavelengths.

The excitation radiation is generated from pulsed (1.8 kHz) LEDs in the range of 625–365 nm (Table 3), wherein the 625 nm serves as reference radiation for normalizing. Four LEDs are used for every wavelength and these LEDs are homogeneously distributed on a metal disc.

In the center of the metal disc of the device, a collecting optic (Auto Exakter 135 mm/1:2.8) is fitted. The optic transmits the emitted radiation from the measuring object through a y-fiber optics to two diodes (S3071 8 J, Hamamatsu Photonics, Hamamatsu, Japan). These diodes absorb radiation between 320 nm and 1060 nm and have their maximum sensitivity at 920 nm. In order to detect only the two peaks of the chlorophyll fluorescence at 685 nm and 735 nm (Stober and Lichtenthaler, 1993), a band-pass filter (Laser Components, Olching, Germany) was placed in front of each diode. These filters were custom-made components. One filter has its transmittance peak at 685 nm, the other one at 735 nm (Table 3). Between the optical system and the y-fiber optics, a long-pass filter is inserted (cut of wavelength 645 nm). The diodes are connected to amplifiers (DLPCA-200, Femto, Berlin, Germany) and the signal of the amplifiers is routed to an oscilloscope (DSC-X 2004A, Agilent Technologies, Santa Clara, California) which indicates the fluorescent signal in digital data. The measuring area of