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Early detection of nutrient and biotic stress in *Phaseolus vulgaris*

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Prerequisites for optimal, high crop yield are disease-free growth and an equilibrated supply of nutrients. Early signatures of stress-altered physiology, before appearance of symptoms in the visible spectrum, allow timely treatment. Early detection of stress development was carried out on *Phaseolus vulgaris* bean infected with the agriculturally important grey mould pathogen and under conditions of magnesium deficiency, limiting photosynthesis. During stress development, bean plants were monitored by time-lapse imaging with thermal, video and chlorophyll fluorescence cameras, mounted on a gantry robot system. For early detection of grey mould infection, chlorophyll fluorescence imaging proved to be the most sensitive. This technique detected magnesium deficiency at least three days before visual symptoms appeared. Further development of non-contact technology for plant health monitoring will help to achieve optimal productivity in greenhouse and field cultures. Associated establishment of a stress catalogue based on early symptoms will allow swift diagnosis.

1. Introduction

Methods revealing biotic and abiotic stresses in crops at an early stage, and differentiating these stresses, have a clear potential to assist in limiting yield losses in plant production. On a global scale, abiotic stresses reduce crop yield by up to 82% of record yields (Chrispeels and Sadava 2003), whereas biotic stress by fungal, bacterial or viral agents, on average, causes a 15% reduction in yield (Oerke and Dehne 2004). Stress symptoms associated with localized disease outbreaks typically initiate as spots or patches within a crop (Zadoks and Vandenbosch 1994). Such symptoms, and possibly also local nutrient or water shortages within fields could be revealed with non-contact imaging techniques at early time points, when no visible symptoms are yet apparent (Buschmann and Lichtenthaler 1998, Chaerle and Van Der Straeten 2001, Corp et al. 2003, Berger et al. 2004, Jones 2004, Oxborough 2004, Scharte et al. 2005, Oerke et al. 2006). The ability to detect a stress situation at an early stage would allow timely treatment, resulting in limited yield loss and reduced pesticide usage, which would be beneficial, both from an economic and an environmental viewpoint. Moreover, with respect to biotic stress, the build-up of resistance would be avoided or become less likely (Leroux et al. 2002). The foliar fungal plant disease grey rot (also known as grey mould), caused by the common necrotrophic pathogen *Botrytis cinerea*, causes considerable damage in agriculture and horticulture, affecting a wide range of crops (e.g. bean, tomato, cabbage) (Van Kan 2006).

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Among abiotic stresses, nutrient stress is a very common problem, directly linked to soil quality. A proper supply of macro and micro nutrients needs to be guaranteed for optimal plant growth. Hence, early detection of nutrient deficiencies is critical to avoid yield depression. Since $\text{Mg}^{2+}$ is an essential component of functional chlorophyll, its limitation will have a direct impact on photosynthetic capacity (Hermans et al. 2004), and will likely alter chlorophyll fluorescence emission (Lichtenthaler and Miehe 1997, Buschmann and Lichtenthaler 1998, Chaerle and Van Der Straeten 2001). As a consequence of lower photosynthetic assimilation, magnesium deficiency is known to influence plant development (Cakmak et al. 1994, Marschner et al. 1996). Magnesium deficiency was shown to induce interveinal chlorosis followed by necrosis on bean leaves (Marschner and Cakmak, 1989). Pre-visual stages of developing necrosis were previously revealed with high contrast by thermography (Chaerle et al. 2001).

Identification and assessment of mineral deficiencies and pathogen damage are commonly carried out by subjective and time-consuming visual expertise. Non-contact (imaging) techniques are a valid alternative for monitoring stress symptoms in plants, with the additional advantage of objective quantification. Reflectance radiometry was used to monitor lesion formation after Botrytis infection in Vicia faba (field beans), but no pre-visual disease signal detection was possible (Malthus and Madeira 1993). A lower stomatal conductance was, however, measured reproducibly, and linked to a reduction in photosynthetic rate. Hyperspectral crop reflectance imaging was successful in detecting fungal disease severity in wheat (Muhammed and Larsolle 2003, Moshou et al. 2005, Muhammed 2005). In addition to hyperspectral imaging, chlorophyll fluorescence and thermal imaging have shown their potential for revealing stress-related symptoms.

Thermal infrared imaging (thermography) visualizes temperature differences over the leaf surface. Deviation of leaf temperature over time and/or place indicates disturbed evaporation, caused by physiological changes upon infection, abiotic stress, or mechanical destruction of the leaf tissue (local wounding). Fluorescence imaging measures the fluorescence conversion rate of blue light (excitation maximum 500 nm) absorbed by chlorophyll to re-emitted red light (emission maximum 700 nm). A higher output of red light indicates a lower conversion of light energy by the photosynthetic apparatus. Lower activity of photosynthetic assimilation, as reflected by chlorophyll fluorescence measurements, can be related to the influence of stress factors (Rolfe and Scholes 1995, Chaerle and Van Der Straeten 2001, Karavaev and Polyakova 2002).

The power of an imaging approach is illustrated here by presymptomatic monitoring of Botrytis fungal infection and magnesium deficiency in common beans. As it is very sensitive to Botrytis infection, Phaseolus vulgaris was chosen as a test plant. The disease progress was followed by means of time-lapse thermographic (far infrared 8 to 12 $\mu$m), chlorophyll fluorescence, and a video image series. Magnesium deficiency also leads to clearly visible symptoms and was followed using the same procedure.

2. Material and methods

2.1. Plant growth and treatments

Bean (Phaseolus vulgaris); seeds were soaked in water for one hour, and sown in vermiculite. After germination, plants were grown hydroponically on a standard
half strength (0.5x) Hoagland solution. The hydroponic set-up consisted of a floating square piece of polystyrene (8 × 8 cm) in a slightly larger growth container. The plant stem was put through a central hole and fastened with a Rockwool plug. An aquarium air pump provided the necessary aeration to guarantee sufficient oxygen availability in the nutrient solution. The plants first stayed in a controlled plant growth chamber and were then transferred to the imaging room, which was operated under the same controlled conditions. The growth and monitoring settings were a photosynthetic photon flux density (PPFD) of 60 μmol m$^{-2}$ s$^{-1}$ with a 16 h light, 8 h dark regime, a temperature of 21 ± 0.5°C and 50 ± 5% relative humidity.

Fourteen days after germination, plants were transported into the robotized imaging room, where infection was carried out. At this stage, the two primary leaves were fully expanded. For efficient *Botrytis* infection, at least one day of very high humidity (95 to 100%) was needed. By placing a plastic foil enclosure over the plants, humidity reached approximately 95%. Infection with *Botrytis cinerea* R16 was carried out with a suspension containing 65 × 10$^3$ spores ml$^{-1}$ in a solution of 0.01 M glucose and 0.0067 M KH$_2$PO$_4$ solution (phosphorous promotes infection) (Audenaert et al. 2002). On each leaf, 10 droplets of 10μl were applied with a micropipette. In an additional experiment, a 10 fold higher concentration was used: 0.1 M glucose and 0.067 M KH$_2$PO$_4$ solution. The effect of droplet size was also tested where 5μl was used instead of 10μl.

Magnesium deficiency was studied by comparing leaf growth and physiology of plants growing on a control Hoagland solution (+ Mg) with plants developing on a magnesium free solution (− Mg). Seven days after initiating the hydroponics culture, plants were transferred to the imaging room.

### 2.2. Imaging setup

Three cameras were positioned sequentially above the plant leaves, by means of a gantry (XYZ) robot system as shown in figure 1 (Chaerle et al. 2003). The system was programmed to acquire thermal, fluorescence and video images of selected leaves every 30 min. These three types of images were subsequently stored and processed on a Linux workstation. The different images were converted to an image

![Figure 1](image_url)
sequence (mpeg), in order to easily evaluate time related changes induced by pathogen infection or nutrient deficiency.

The image capture area was 50 × 50 mm (identical for the three cameras). The primary leaf size of the *Phaseolus* bean is typically 10 × 8 cm. Primary and first trifoliate leaves were imaged. In every experiment, six bean plants, representing 12 primary leaves and six trifoliate leaves, were monitored. Each plant occupied approximately 15 × 15 cm.

For every plant position, two fluorescence images were taken in sequence. The first image (indicated by Fs) was obtained after 1 second of illumination with non saturating blue light (PPFD = 250 μmol m⁻² s⁻¹), comparable to the light intensity present in the growth chamber (indicated by Fₛ, 250 μmol m⁻² s⁻¹ PAR). Fₛ is the steady-state value of fluorescence upon photosynthesis. The second image (indicated by Fm) was taken after one second of illumination with saturating blue light (PPFD = 1000 μmol m⁻² s⁻¹). Fₘ is the maximum fluorescence-emission intensity attainable before the leaf adapts to the higher light intensity.

The video camera provided a standard visual image, taken at the same time and location as the images described above.

The ImageJ software package (http://rsb.info.nih.gov/ij) was used to register the displayed images (i.e. adjust for the difference in field of view and magnification between the three imaging sensors) by means of the TurboReg plugin (Thevenaz et al. 1998). The 8-bit fluorescence images were normalized for optimal visualization, by selecting the same range of pixel intensities over all images (but separately for Fₘ and Fs images) across all time points of a single experiment. The colour images were adjusted for optimal contrast using the same procedure by procesing the RGB channels separately.

Leaf temperature data was obtained with the AGEMA Research software. Identical temperature ranges were selected for images from one experiment, based on the optimal contrast of image details, and visualized by the same colour palette.

3. Results and discussion

3.1. Botrytis infection

The combination of video, fluorescence and thermal information makes it possible to follow and interpret the presymptomatic evolution of an infection. Twenty-one hours after droplet application of the fungus (*Botrytis cinerea*), the leaf surface showed no traces of infection in the video image as shown in figure 2. The thermographic and fluorescence images both show three spots. These spots correspond to the locations of applied droplets (see figure 2, lower row of images +21 h). The two spots indicated by black arrows reveal the start of the infection process. The thermal image shows two distinct spots with a higher temperature of 18.7°C compared to 18.4°C of the surrounding unaffected tissue. This can be explained by stomatal closure, resulting in a lower evaporation rate. The fluorescence image shows two brighter zones where a lower conversion of light energy by the photosynthetic apparatus is most likely causing a higher fluorescence emission level. Both phenomena are provoked by the influence of infection on transpiration and photosynthesis.

Mechanical, localized wounding of a leaf surface remains visible by thermography for at least half a day (Chaerle et al. 2002). Wounded tissue loses water by destruction of cell integrity. This results in a lower temperature due to evaporation.
The spot indicated with a white arrow was wounded with a micropipette tip ($T = 18.2\, ^\circ\text{C}$). The fluorescence image at 21 h post infection shows the same response for both the wounded spot and the progression of infection. By comparison, the thermal image displays a clear difference (lower versus higher temperature for the infected spots). The combination of both thermal and fluorescence imaging thus provides a discriminating method for presymptomatic visualisation of injury and infection. The wounding treatment caused only superficial abrasion damage, without leading to complete loss of chlorophyll fluorescence at the site of treatment. The increase in $F_s$ fluorescence at the wounded spot is reminiscent of the early response of the tissue around crushing injuries (Quilliam et al. 2006).

The leaf depicted in the upper three images in figure 2 was treated with a control solution on the left half, and with spore suspension on the right leaf half, both however without glucose and $\text{KH}_2\text{PO}_4$ additives. In these images, no spots are discernable. This indicates that to obtain a successful infection, a spore suspension containing water only is inadequate and also illustrates the necessity of phosphate and glucose (or a carbon source in general). This solution helps achieve maximum infection efficiency, without leaf wounding. The use of a helping solution mimics the leaf exudates upon minor or superficial tissue damage under natural growing conditions.

At 23.5 h after infection, the fluorescence image proved to be more sensitive than thermography to visualize the infection progress (figure 3). At the locations indicated by black arrowheads, fluorescence symptoms appeared in the transition from 21 h to 23.5 h. In contrast, in the thermal image no additional local temperature changes could be discerned at these locations. At 23.5 h after infection, photosynthetic inhibition was clearly evident in the fluorescence image, whereas at the same time, the leaf’s surface had a normal appearance in the video image. Two
applied control water droplets were visible as cool spots in the thermal image (white arrow heads). No increase in fluorescence intensity was apparent at these locations.

When comparing the thermal image of figure 4 (59 h after infection) with that in figure 3 (23.5 h), the temperature of the wounded spot (indicated by a white arrow)
has increased, indicating wound healing. Also, the water droplets have completely evaporated. In the fluorescence image of figure 4, a black pinpoint (indicated by a black arrow) appeared in the first white spot. This high intensity spot was already visible 21 h after infection (see figure 3, black arrow).

Depending on the period of saturating humidity (20 h for figures 2 to 4 versus 24 h for figure 5 below), and the strength of the helping solution, disease progression was markedly affected. In figures 2 to 4, small isolated spots with little expansion over time were observed in the fluorescence images. Under the conditions used for the experiment in figure 5 (ten fold higher concentration of helping solution), a more prominent growth of the fungus caused clearly discernable visual symptoms, which were also detected with high (internal) contrast in the chlorophyll fluorescence images.

Twenty-seven hours after the droplets were applied, the fluorescence picture visualized three distinct zones at the infection sites (figure 5, +27 h). The core of the infection zone was black, corresponding to the visual lesions and complete chlorophyll breakdown, hence resulting in a complete lack of fluorescence emission. The expanding grey zone around these spots indicates tissue invaded by the pathogen. In the visual and in the thermal images precipitation of solutes from the infection solution mask these zones. The zone of lower fluorescence emission

Figure 5. *Botrytis* infection in *Phaseolus vulgaris* bean using a ten fold more concentrated helper solution than that used for previous figure images. Upper row: 27 h after infection; lower row: 5 d after infection. In the chlorophyll fluorescence picture, three zones are discernable: a central black zero fluorescence zone, a surrounding grey low intensity zone and a whitish halo with fluorescence higher than the unaffected leaf tissue. The reflection, visible as bright white in the visual and bright yellow in the thermal picture, is due to precipitation of solutes from the concentrated helper solution. In the thermal picture at +27 h, white arrowheads indicate mechanical wounding at the time of infection. After 5 d of infection, the black zone in the fluorescence images has expanded. The surrounding grey and white zones have expanded outward. The brownish necrotic dead regions in the visual picture correspond to the lower temperature border in the thermal image (blue border and dark blue spots, one example indicated by black arrowhead). The mechanical wounding is visible in both video and fluorescence images, and has increased in temperature (white arrowheads). Temperature range of the thermal images is 2°C.
indicates the start of tissue degradation. Around the grey zone, a bright shining zone ('halo') was visible. This higher fluorescence intensity results from lower energy consumption by the photosynthetic apparatus, due to partial inhibition. Under such conditions, dissipative light energy conversion (heat release and fluorescence) increases at the expense of photosynthesis. In the chlorophyll fluorescence images, the symptoms of Botrytis infection evolve from an early white, high intensity zone to a grey, and finally to a black spot. This can be explained by a gradual deterioration of plant tissue. At the first stage, the photosynthetic apparatus is affected, so a higher percentage of absorbed light energy is re-emitted as fluorescence rather than stored in energy-rich chemical bounds, leading to the white, high intensity zone. At the second stage, other cell components and structures are degraded leading to a chlorophyll breakdown, so the cell gradually looses its capacity to convert light energy. This results in a fluorescence rate lower than that of the surrounding tissue, and is observed as a grey zone. Finally, upon cell death, fluorescence ceases due to the absence of functional chlorophyll. Five days after infection, the black central zone in the fluorescence image expanded beyond the grey zone visible at 27 h after infection (figure 5, +5 d). Grey and white border zones are still visible, indicating further growth of the fungus in the leaf tissue. This is corroborated by the low temperature (dark blue) spots at the lesion borders in the thermal picture (one example is indicated: lower left of +5 d thermal picture, black arrowhead), which show recent damage of the tissue. This temporal evolution of Botrytis cinerea-induced symptoms from early symptoms to dead tissue, as recorded by continuous time-lapse imaging, is comparable to the gradual expansion of cell death during the resistant tobacco-TMV interaction (Chaerle et al. 2004). Wounding applied at the time of infection is indicated by white arrowheads. The dark fluorescence zone is completely desiccated, hence its high visible reflectance and a high temperature.

After accidental wounding in combination with high humidity, cellular exudates can substitute for the helping agents and enable successful fungal infection. Conditions within a crop canopy typically are more humid, which could also favour fungal development (Muckenschnabel et al. 2003). Rapid detection of the early stages of infection with broad host range pathogens, as Botrytis cinerea, would enable timely countermeasures as well as limit the use of phytosanitary products. Glasshouse and open field applications of early disease detection systems are proposed.

3.2. Magnesium deficiency

3.2.1. Primary leaves. Although no visible symptoms could be distinguished after seven days (see video image in +7 d/−Mg row in figure 6; image modalities from left to right are thermal, visual, F_s, F'_m) brighter spots became apparent between the veins in the F_s fluorescence image (third image from the left).

The first necrotic spots became visible on the primary leaf of a bean plant ten days after the plants were placed in the hydroponic solution (figure 6 +10 d/−Mg, as indicated by black and red arrows). However, these necrotic spots did not correspond to the location of the brighter spots observed in the chlorophyll fluorescence images captured three days earlier (figure 6, +7 d/−Mg), but matched the presymptomatic spots of higher temperature indicated by a black arrow. At the ten-day time point, increases in chlorophyll fluorescence emission are apparent in the tissue along the main veins (figure 6, +10 d/−Mg). At this stage, visual symptoms are already clearly visible as chlorotic zones corresponding to the pattern seen in the fluorescence images. Only a few necrotic spots developed in the upper left
Figure 6. Magnesium deficiency in common bean primary leaves. From left to right: thermographic, video and chlorophyll fluorescence images captured at low and high light excitation respectively. At the 7d time point, the thermal image visualizes an emerging necrotic lesion (black arrow), which co-localizes with visible necrosis at the 10d time point (black arrow). A red arrow indicates the black region in the +10d chlorophyll fluorescence image corresponding to the lesion seen in the video image. In the thermal image at +10d, the border of the lesion (black arrow) is colder than the surrounding tissue. At the 7d time point, the F'_s chlorophyll fluorescence image visualizes spots of increased fluorescence between the main veins. Three days later (+10d), an inverse pattern emerged, with higher fluorescence associated with the main veins. The F'_m image indicates that in the interveinal regions fluorescence emission decreased, this is associated with loss of chlorophyll. Temperature range of the thermal images is 1.5°C. At the 7d time point, the field of view of the video camera was not well-aligned with that of the other cameras. The missing visual image parts are not essential at this time point, since the accompanying fluorescence images do not reveal any damage in that area; chlorophyll fluorescence imaging always monitors cell death earlier than video imaging (Chaerle et al. 2004). In order to illustrate matching of the image types, the missing image parts were filled by the corresponding areas of the F'_m fluorescence images (see also the +10d/-Mg images).
part of the visual image. These observations indicate that chlorophyll fluorescence imaging and thermography visualize different early symptoms of magnesium deficiency, a general chlorophyll fluorescence increases preceding interveinal chlorosis and the random appearance of necrotic spots.

In the +10 d/+Mg Fs image small higher intensity spots appeared. Although conditions of temperature and humidity were kept stable throughout the imaging period, this is probably related to transient stomatal patchiness (West et al. 2005). The higher intensity spot pattern in the +7 d/+Mg Fs images is however unambiguously stable, and evolves into the pattern apparent in the +10 d image. In primary leaves, chlorophyll fluorescence thus detected symptoms three days before yellowing became obvious in the visual images.

3.2.2. Trifoliate leaves. In trifoliate leaves, the earliest symptoms appeared about one week later than in primary leaves. In figure 7, a series of dark spots can be seen in the F’m image, 13 days after transfer to the hydroponics solution (panel +13 d/-Mg, white arrow), and these spots correspond to the locations of later necrosis (see panel +17 d/-Mg, video image). The thermal image also shows co-localized spots of higher temperature (white arrow), although a non-correlating warmer region is also present on the other leaf-half. Fourteen days after transfer to the hydroponic solution (figure 7, panel +14 d/-Mg), the necrosis-linked symptoms in the fluorescence images had further expanded. Visible chlorotic effects and the associated symptoms in the fluorescence images became clearly apparent. The left half of the thermal image now appears to depict higher leaf temperature, however this is possibly due to leaf inclination. The plants growing on the magnesium deficient solution had a significantly higher temperature of 0.5°C (see the temperature scales), presumably due to magnesium deficiency induced stress. Seventeen days after the bean plants were transferred to a magnesium deficient growth solution, the necrotic effects on the trifoliate leaves became visible (figure 7, panel +17 d/-Mg). The entire leaf blade was now chlorotic. Severe inhibition of leaf growth was apparent after 17 days of magnesium deficiency. Using chlorophyll fluorescence imaging, early precursor signs of necrosis and chlorosis were detected in trifoliate leaves approximately four days before the visible symptoms could be discerned.

4. Conclusions

A non-contact imaging system with several sensors can be used to assess the effects of specific plant-pathogen and nutrient deficiency impacts on plant leaves. In addition to previous research with this multi-sensor imaging set-up (Chaerle et al. 1999, 2004), these tests prove pre-visual detectability of biotic stress, allowing farmers to take timely measures to prevent pathogen spread. This data indicates that with the combination of thermal and fluorescence imaging an accurate presymptomatic visualization of a fungal infection can be achieved. For early detection of the Phaseolus vulgaris–Botrytis cinerea plant-pathogen interaction, chlorophyll fluo-
Figure 7. Magnesium deficiency in common bean trifoliate leaves. From left to right: thermographic, video and chlorophyll fluorescence images captured at low and high light excitation respectively. After 13 d of growing on a magnesium free solution, dark spots were observed in the F’_m image. Co-localized spots of higher temperature were visible in the
cence imaging proved to be most sensitive. Importantly, thermal imaging clearly discerned fungal infection from wounding, supporting the power of a multi-sensor imaging approach.

In primary and secondary bean leaves, presymptomatic detection of leaf chlorosis associated with magnesium deficiency was possible with chlorophyll fluorescence imaging, while thermography could detect necrosis-associated symptoms. In trifoliate leaves, the difference in leaf expansion already indicated severe growth inhibition upon prolonged and severe magnesium deficiency. The experiments described focused on the development of magnesium deficiency symptoms and the ability to detect them at an early stage based on the appearance of patterns or necrotic spots. In a more gradual build-up of deficiency, as could be encountered during cultivation of plants in glasshouse or field conditions, detection of necrosis or chlorosis patterns could be complemented by quantification of chlorophyll fluorescence of the whole leaf blade, as compared to reference values (see figure 7). This approach might enable an earlier detection of subtle symptoms, before growth retardation becomes apparent.

A combination of different imaging techniques has the potential for early detection (and ultimately identification) of stresses. This could be achieved by building a knowledge database of reaction-patterns specific for each stressor, validated by multi-sensor laboratory plant-stress imaging experiments. The observed effects could be extrapolated to field scale, but limitations of imaging techniques should be taken into account. Changing weather conditions limit the applicability of thermography, whereas Chl-FI at field scale is hampered under conditions of high solar irradiation. Assessment with autonomous vehicles could be a viable solution. Quantification of an early detectable threshold will be needed to realize an effective early-warning system. The described imaging approach is also amenable to screen plant cultivars or mutants for stress resistance. Upscaling from growth room to glass house and nursery applications will become feasible in the near future.

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