Diagnosis of the Infection of Sunflower by *Orobanche cumana* Using Multicolour Fluorescence imaging

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Abstract

Orobanche cumana is an holoparasite and thus totally dependent on sunflower for fixed carbon. Initial stages of the infection occur in the first weeks after sowing and are critical for the establishment of a continuum between the host and the parasite vascular system. From that moment the parasite obtains its supply of water, mineral nutrients, and assimilates from the host plant. Alterations of plant metabolism can be detected using remote sensing techniques for detection of fluorescence emitted by plants. One of these indirect techniques is multicolour fluorescence imaging. In this work, we assessed the early infection of sunflower by *O. cumana* using multicolour fluorescence imaging and we inferred physiological processes affected in sunflower plants infected by the parasite. Ten germinated seeds of the inbred line NR5 were inoculated with population LP2013 of *O. cumana*. The same number of not inoculated seeds was used as control. Sunflower was planted in pots with soil mixture and grown in greenhouse at 12–22°C for 6 weeks. Multicolour fluorescence imaging was conducted 3, 4, and 5 weeks after inoculation. The two first pairs of fully expanded leaves of each sunflower plant were imaged, and, for each measure date, five fluorescence variables in inoculated plants were compared to those in the control. Three weeks after inoculation, when symptoms of infection were still not observed, decreased levels of blue and green fluorescence and increased far-red fluorescence were observed in leaves of the inoculated plants. At 4 and 5 weeks after inoculation, when inoculated plants

displayed symptoms of infection by *O. cumana*, differences in fluorescence between inoculated plants and the controls were the same and statistically supported. These results are consistent with an increase in total chlorophyll content of sunflower plants infected by *O. cumana*, and a decrease in the accumulation of secondary metabolites, both related to the need of higher photosynthetic activity to supply the parasite with photosynthate. Biochemical mechanisms underlying alterations in photosynthesis must be further investigated. The results obtained showed that multicolour fluorescence imaging can be used to detect fluorescence differences in inoculated sunflower as early as 3 weeks after inoculation. Therefore, this technique can be used as a diagnostic tool for early detection of genotypes of sunflower which are susceptible or resistant to *O. cumana*.

Keywords: early detection; Helianthus annuus L.; multicolour fluorescence; photosynthesis; secondary metabolites

Introduction

Orobanche cumana Wallr. is a parasitic plant that establishes in the root of *Helianthus* spp., including the crop species *H. annuus*. The attachment of seeds of *O. cumana* to the roots of sunflower and the establishment of the nutritional structure or haustorium are critical initial stages of the infection that result in the underground development of bulbous tubercles. During the time these infective processes take place (approximately in the first month after sowing), a continuum between the host and the parasite vascular systems is formed. The parasite then obtains its supply of water, mineral nutrients, and assimilates from the host plant. Because of the absence of functional chloroplasts, *O. cumana* is defined as an holoparasite and is thus totally dependent on sunflower for fixed carbon.

Parasitic plants, including *Orobanche* spp., reduce the growth of their hosts through competition for water and nutrients, C transfer from host to parasite, and physiological dysfunction of the host plants (Stewart and Press, 1990). The rate of transpiration of parasitic angiosperms is often higher than that of host species. This maintains a water potential gradient towards the parasite, facilitating the transfer of organic and inorganic solutes to it (Blamey *et al*., 1997).

The infection of *O. cumana* in sunflower is commonly assessed by visual observation of symptoms, which appear in the crop around flowering and show as non-specific water stress. Prior to that, the infection of sunflower by *O. cumana* is indicated by the presence of tubercles in the roots of the plant or by the emergence of parasite stems aboveground. The development of macroscopically detectable tubercles or, later, of emerged holoparasite stems depends on inoculum density of *O. cumana* and also on environmental conditions. This can take place in 26 d after inoculation (dai) for detection of

underground tubercles (García-Carneros *et al*., 2014) or 35 dai in the case of initial emergence of broomrape stems (Molinero-Ruiz *et al*., 2008).

Water stress and alterations of plant metabolism caused by either pathogen infection or drought could be analysed by using remote sensing approaches, such as detection of autofluorescence emitted by plants. One of these techniques, known as multicolour fluorescence imaging, is based on the detection of fluorescence in the blue, green, red, and far-red regions of the spectrum and, although indirect, it is a highly sensitive, non-destructive, and non-subjective tool to study mainly the activity of the secondary metabolism. Many compounds in plants emit autofluorescence when illuminated with UV light. Secondary metabolites, such as phenolics, emit mainly in the blue/green regions and have a broad range of activities, mainly related to stress defence. On the other hand, chlorophyll, the most abundant pigment in plants, emits in red and far-red regions of the spectrum. Analyses of multicolour fluorescence imaging and multispectral imaging have been successfully applied for the detection of pathogen infection in plant species (Dammer *et al*., 2011; Calderón *et al*., 2013; Pérez-Bueno *et al*., 2014). The aim of this work is to assess the infection of sunflower by *O. cumana* in early stages using multicolour fluorescence imaging and to infer physiological processes affected in the infected sunflower plants.

Materials and methods

The population LP2013 of *O. cumana* was inoculated onto the susceptible sunflower inbred line NR5 following previous methodology (García-Carneros *et al*., 2014). Ten seedlings were transplanted into soil mixture infested with*O. cumana* at an inoculum density of 0.02 mg of parasite seeds/g soil. Ten germinated seeds were transplanted to un-infested soil mixture and used as controls. Plants were grown in glasshouse at 12–22°C for 6 weeks and watered as needed. At the end of the experiment, sunflower plants were uprooted to visually assess the presence of nodules of the parasite in the roots.

Multicolour fluorescence imaging was conducted starting at horizontal development of the first pair of true leaves in the plants, which occurred 3 weeks after inoculation (wai). Thereafter measurements were taken 4 and 5 wai. In each plant, all upper true leaves were simultaneously imaged using an Open FluorCam FC 800-O and the images were analysed using the Fluorcam7 software (Photon Systems Instruments, Brno, Czech Republic) according to Pérez-Bueno *et al*. (2014). Images were captured always approximately at the same daytime and with plants having similar water status. Each measure day, and prior to imaging, plants were visually assessed. Control and inoculated plants were confirmed to have the same number of similarly developed true leaves. For each measure date, mean values of fluorescence at 440 nm (blue), 520 nm (green), and 740 nm (red) as well as the ratios

440/740 (blue normalized to far-red) and 520/740 (green normalized to far-red) were considered. When clear trends of the variables were observed, the robustness of the data was statistically assessed by analysis of variance (ANOVA) according to a complete randomized design. The effect of treatment (control or inoculation) was analysed for individual pairs of true leaves with one measurement per leaf (replicate); between four and eight replicates were considered.

Results

No significant differences on fluorescence emission were observed between the two pairs of fully expanded leaves present in the plants. Therefore, and although all the pairs were analysed, only results for the first pair of leaves are presented. Three weeks after inoculation none of the inoculated sunflower plants displayed visual symptoms of infection. One week later, 4 wai, symptoms consisting of reduced height of plants, and small size of leaves, appeared in inoculated plants. The time course of the five fluorescence variables measured in control vs inoculated plants is presented in Figure 1.

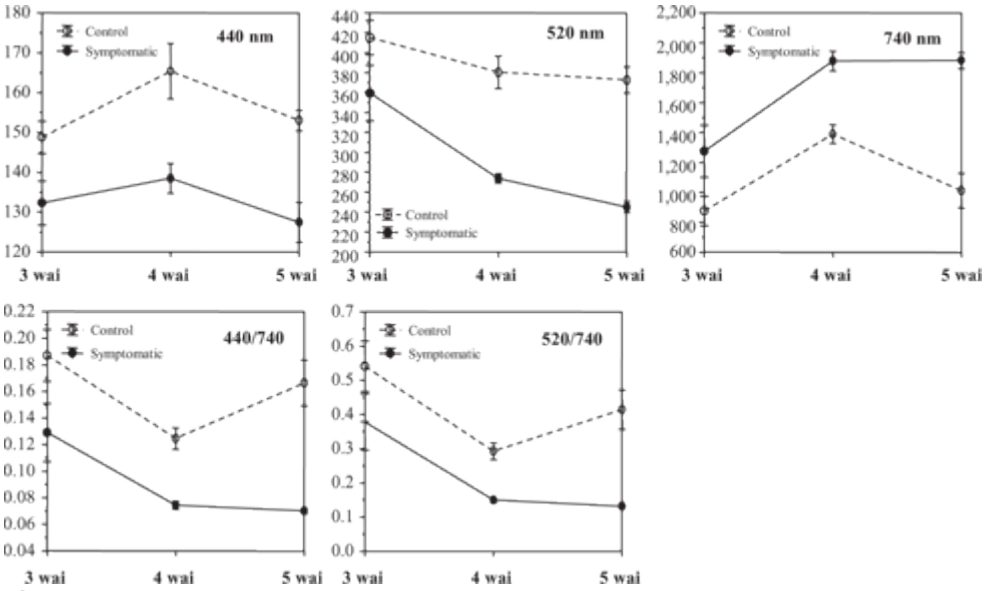

Figure 1

Time course of fluorescence variables measured in the first pair of leaves of control plants and symptomatic plants of sunflower infected by *Orobanche cumana*

At 3 wai, a decrease in the blue and green fluorescence was observed in the inoculated plants (132 and 360 respectively) as compared to the controls (149 and 415 rel. units, respectively). Conversely, they showed higher far-red fluorescence than the controls (1,275 and 875 rel. units, respectively). The same trends than those of blue and green fluorescence were obtained for the ratios 440/740 and 520/740 (Figure 1).

One week later, differences of fluorescence parameters between symptomatic plants and the controls were clear and statistically supported. Leaves of symptomatic plants presented a significant lower fluorescence than the one of the control plants at 440 nm (138 rel. units, $p \leq 0.0032$) and 520 nm (274 rel. units, $p<0.0001$). Far-red fluorescence of leaves was significantly higher ($p<0.0001$) in symptomatic sunflower than in the controls (1,879 and 1,389 rel. units, respectively) (Figure 1). Similar results were obtained at final time; significant ($p<0.0001$) decreases of blue and green fluorescence were measured in the first pair of leaves in symptomatic plants (127 and 245 rel. units, respectively) as compared to control plants (153 and 373 rel. units, respectively). Leaves fluorescence at 740 nm was significantly higher in symptomatic plants than in the controls: 1,282 and 1,011 rel. units, respectively. Differences between symptomatic plants and the controls were confirmed at both dates (4 and 5 wai) by normalized measures: 440/740 and 520/740 (Figure 1).

Discussion

Low levels of blue and green fluorescence were detected early in inoculated plants and remained lower than those in the controls along the time of study. A decrease in blue and particularly green fluorescence intensity suggests a decrease in the accumulation of secondary metabolites in the host plant (see review by Buschmann and Lichtenthaler, 1998). In parasitized plants, the quantitative modification of secondary metabolites can be the result of a decrease in metabolic activities in favour of an increased photosynthesis (Barón *et al*., 2012). An increase in total chlorophyll content was suggested by the increase in far-red fluorescence (emitted by chlorophyll *a*) which was early detected in inoculated sunflower. We suggest the increase in total chlorophyll content might be related to an increase in the content on photosystems in the thylakoid, possibly elicited by the need of higher photosynthetic activity in order to supply the parasite with photosynthate.

Therefore, our results show that *O. cumana*, which depends on the host plant for carbohydrates, affects sunflower secondary metabolism, and very likely primary metabolism, probably by manipulating the source–sink interactions in the plant (Barón *et al*., 2012). In tomato plants infected by *O. aegyptiaca*, an increased photosynthetic surface area relative to the non-photosynthetic biomass was observed by Barker *et al*. (1996). Increased carbon allocation and delayed leaf senescence in normal-sized leaves occurred in tobacco plants infected by *O. cernua* (Hibberd *et al*., 1998). The effect of the infection of *O. cumana* on the growth and development of sunflower has been explained by reductions in biomass, shoot dry weight, height, and head diameter, but significant reductions in biomass have not been observed until initiation of parasite emergence aboveground (Alcántara *et al*., 2006). The effects of infection on the regulation of the photosynthetic machinery of sunflower plants during initial stages of parasitism by *O. cumana*must be further investigated.

Multicolour fluorescence imaging allowed us to discriminate infected from non-infected plants at an early time (3 wai), when symptoms of the infection by *O. cumana* were not visually detected in sunflower. Our work is not only the first application of the multicolour fluorescence imaging for the analysis of how the parasitism of *O. cumana* inhibits secondary metabolism of sunflower, but it also constitutes the evidence that this technique can be used as a non-invasive tool for early diagnosis of infection of the host by the holoparasite in the absence of symptoms. Because early stages of infection are the most efficient targets for genetic resistance of *H. annuus* against the root parasite (Serghini *et al*., 2001) and genetic resistance remains the most important measure for controlling sunflower broomrape (Fernández-Martínez *et al*., 2010), one of the applications of this technique might be the early diagnosis of resistance and/or susceptibility of genotypes of sunflower to *O. cumana*.

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