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Research article

Eucalypt plants are physiologically and metabolically affected by infection with *Ceratocystis fimbriata*

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ABSTRACT

Ceratocystis wilt, caused by *Ceratocystis fimbriata,* is currently one of the most important disease in eucalypt plantations. Plants infected by *C. fimbriata* have lower volumetric growth, lower pulp yields and reduced timber values. The physiological bases of infection induced by this pathogen in eucalypt plant are not known. Therefore, this study aims to assess the physiological and metabolic changes in eucalypt clones that are resistant and susceptible to *C. fimbriata*. Once, we evaluated in detail their leaf gas exchange, chlorophyll *a* fluorescence, water potential, metabolite profiling and growth-related parameters. When inoculated, the susceptible clone displayed reduced water potential, CO_2 assimilation rate, stomatal conductance, transpiration rate, photochemical quenching coefficient, electron transport rate, and root biomass. Inoculated resistant and susceptible clones both presented higher respiration rates than healthy plants. Many compounds of primary and secondary metabolism were significantly altered after fungal infection in both clones. These results suggest that, *C. fimbriata* interferes in the primary and secondary metabolism of plants that may be linked to the induction of defense mechanisms and that, due to water restrictions caused by the fungus in susceptible plants, there is a partial closure of the stomata to prevent water loss and a consequent reduction in photosynthesis and the transpiration rate, which in turn, leads to a decrease in the plant's growth-related. These results combined, allowed for a better understanding of the physiological and metabolic changes following the infectious process of *C. fimbriata*, which limit eucalypt plant growth.

1. Introduction

A global expansion of clonal plantations of *Eucalyptus* spp. has occurred in recent years to meet the growing demand for wood fiber. Single species or clones cover large areas, which has led to increased incidence of pests and diseases. Ceratocystis wilt, caused by the fungus *Ceratocystis fimbriata* Ellis & Halsted, is one of the most common and severe diseases on eucalypt plantations (Alfenas et al., 2009; Ferreira et al., 2013). The pathogen mainly infects the parenchyma, phloem and xylem vessels, promoting radial browning and wilting in infected plants (Ferreira et al., 2006). The disease can lead to reductions in volumetric growth of 87% and losses of up to 13.7% in the cellulose yield, as well as reducing timber quality for sawmill (Mafia et al., 2013). Genetic resistance is the most efficient and feasible way to control the disease in eucalypt.

Pathogens causing foliar diseases can affect the physiology of a plant by losing healthy leaf area, which has a negative impact on its ability to exchange gases or reducing the efficiency of photosynthesis related processes (Shtienberg, 1992). Reductions in photosynthetic rate can be also caused by drought stress, which induces stomata closure,

Abbreviations: d.a.i., days after inoculation; h.a.i., hours after inoculation; Ψ_w, water potential; Ψ_{am}, predawn water potential; Ψ_{md}, water potential close to noon; *A*, net carbon assimilation rate; g_s, stomata conductance; C_i/C_a, relationship between the internal and external leaf CO_2 concentrations; *E*, transpiration rate; R_d, dark respiration; (F_v/F_m), maximum quantum yield of photosystem II; q_p, photochemical *quenching*; NPQ, non-photochemical *quenching*; ETR, rate of linear electron transport; GC–MS, Gas chromatography–mass spectrometry; RGR, relative growth rates; RN, resistant clone, not inoculated; RI, resistant clone, inoculated; SN, susceptible clone, not inoculated; SI, susceptible clone, inoculated; ANOVA, analysis of variance; DT, darkened tissue

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limiting the availability of CO_2 in the chloroplasts, which has a direct effect on the biochemical step of photosynthesis (Bowden et al., 1990; Saeed et al., 1999; McElrone and Forseth, 2004; Pascual et al., 2010). Considering that vascular infection of *C. fimbriata* may limit the translocation of water and nutrients in the xylem, it is important to understand the implications of the pathogen's presence in the conducting vessels to plant physiology as a whole. In this sense, photosynthetic characteristics such as gas exchange and chlorophyll *a* fluorescence have been considered indicators for the photosynthetic apparatus when it is under stress, including under infection by pathogens (Pinkard and Mohammed, 2006; Berger et al., 2007; Domiciano et al., 2009; Pascual et al., 2010; Alves et al., 2011).

Studies have shown that infections caused by foliar pathogens lead to a decrease in the photosynthetic rate (Chou et al., 2000; Berger et al., 2007; Domiciano et al., 2009), changes in the photosynthetic apparatus (Lichtenthaler and Miehé, 1997) and the capture, use and dissipation of light (Baker and Rosenqvist, 2004; Bonfig et al., 2006; Alves et al., 2011). However, the consequences of a vascular pathogen's infection on the physiological processes of forest species are little known. In eucalypt plants, for example, few studies of physiological changes due to pathogens causing foliar diseases (Pinkard and Mohammed, 2006; Alves et al., 2011) and in the defence responses of eucalypt to *C. fimbriata* infection (Pimenta et al., 2017). Furthermore, less is known about the effects of pathogen infection on plant primary metabolic pathways and their role in plant defenses.

For plants, primary metabolites like sugars, amino acids, organic acids and fatty acids, among others, are up or downregulated during stress responses (Krasensky and Jonak, 2012; Rojas et al., 2014). These compounds are directly or indirectly involved in the induction of defense responses, by preventing or stopping the proliferation of a potential pathogen (Rojas et al., 2014). While the regulation of defense responses has been heavily studied for decades, less is known about the effects of pathogen infection on plant's primary metabolic pathways and their role in plant defense (Rojas et al., 2014). In this study we tested the hypothesis that by colonizing the vascular eucalypt system, *C. fimbriata* reduce the water flow to the leaves which interfere in the photosynthetic machinery and metabolism limiting plant growth. Thus, the aim of this work was to evaluate the physiological and metabolic changes that result from infecting two commercial eucalypt clones that have different levels of resistance with *C. fimbriata*.

2. Materials and methods

2.1. Plant material and experimental conditions

Two commercial eucalypt clones that differing in resistance to Ceratocystis wilt were used: the CLR236 hybrid clone of *Eucalyptus grandis* x *E. urophylla* (resistant) and the CLR240 clone of *Eucalyptus grandis* (susceptible), previously evaluated by artificial inoculation at *Clonar Resistência a Doenças Florestais*®. The rooted cuttings with ninety days were transplanted into 5 L capacity pots containing MecPlant® substrate enriched with superphosphate (6 kg m^{-3}) and Osmocote® (NPK 19:06:10 in 1.5 kg m^{-3}) and maintained in a greenhouse until the inoculation point was reached. The plants were kept in a greenhouse under ambient temperature and average humidity conditions of 25.2 °C and 78.9%, respectively, while the experiments were performed. In each pot was added 1 L of water per day.

2.2. Inoculum

The fungal isolate (SBS-1), used in this study, obtained from the mycological collection of the Forest Pathology Laboratory of the Universidade Federal de Viçosa (UFV) was identified by morphological and molecular characteristics and it is routinely used in plant resistance studies in our laboratory (Guimarães et al., 2010; Rosado et al., 2010). The fungus was grown in Petri dishes (9 cm in diameter) containing MEYA medium (2% malt extract, 0.2% yeast extract and 2% agar) at 28 ± 1 °C, a photoperiod of 12 h and a light intensity of 40 μmol photons m^{-2} s^{-1} for ten days. To collect the spores (conidia, ascospores and aleuroconidia), sterile water containing 1% Tween 20 was added to the plates, and, then, the surface of the colony was scraped of with the aid of a sterile Drigalski handle. The spore suspension was filtered through a double layer of gauze and adjusted to 2.0×10^6 spore ml^{-1}.

2.3. Inoculation

The plants were inoculated 60 days after transplanting. A longitudinal cut approximately 2 cm in length and approximately 0.2 cm deep was made with a scalpel in the stems of the plants at a height of 5 cm above the collar, where 500 μl of the spore suspension was deposited. The wound was covered with plastic wrap to prevent drying and the entry of other microorganisms. As a control, the same incision was made in plants of the same age, but only a sterile solution containing water plus 1% Tween 20 was deposited inside the longitudinal section.

2.4. Determination of the leaf water potential

Measurements were made on the third fully expanded leaves (from the branch's apex to its base) in the middle third of the plants. An evaluation was performed before the inoculation of the plants and 14, 28 and 35 days after inoculation (d.a.i.). The water potential of each plant was determined in individual leaves predawn (Ψ_{am}) and at noon (Ψ_{md}) using the pressure chamber technique (PMS Pressure Chamber, PMS Instrument Company, Oregon, USA). Each leaf was cut at the petiole base with a scalpel and immediately inserted into the pressure chamber.

2.5. Determination of the gas exchange parameters

Gas exchange analyses were performed at the beginning of the light period, from 08:00 to 12:00h, using a portable open-flow gas exchange system (Li 6400XT, Li-Cor, Inc., Lincoln, NE, USA). The net carbon assimilation rate (A, μmol CO_2 m^{-2} s^{-1}), stomata conductance (g_s, mol H_2O m^{-2} s^{-1}), relationship between the internal and external leaf CO_2 concentrations (C_i/C_a) and the transpiration rate (E, mmol H_2O m^{-2} s^{-1}) were determined under CO_2 concentration of 400 ppm, light intensity of 1000 μmol photons m^{-2} s^{-1} and ambient temperature of 27 °C. The evaluations were performed before fungus inoculation, 17 and 25 days after inoculation (d.a.i.). For the gas exchange measurements, only in the third fully expanded leaves (from the branch's apex to its base) on the third branch (counted from the plant's apex) were used. The dark respiration ($R_{d,}$ μmol CO_2 m^{-2} s^{-1}) was determined after 2 h of darkness, from 21:00 to 00:00, with a fixed CO_2 concentration of 400 ppm on the same leaf previously used for gas exchange analysis.

2.6. Determination of the chlorophyll a fluorescence parameters

The chlorophyll *a* fluorescence was determined using a light-modulated fluorometer coupled to an IRGA with the same leaf used for measuring the gas exchange. After adapting to the dark for 1 h, the leaf tissues were exposed to a weak pulse of infrared light (1–2 μmol of photons m^{-2} s^{-1}) to determine its initial fluorescence (F_0). Then, a 1 s saturating light pulse with an irradiance of 6000 μmol photons m^{-2} s^{-1} was used to estimate the maximum emitted fluorescence (F_m). The minimum fluorescence of light-adapted leaves (F_0') and the maximum chlorophyll fluorescence (F_m') were determined using the same equipment, and the gas exchange data were concomitantly collected. This procedure also allowed the estimation of other fluorescence-related parameters, such as the maximum quantum yield of photosystem II (F_v/F_m), quantum yield of photochemical *quenching* (q_p), quantum yield of