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Pseudocercospora griseola Causing Angular Leaf Spot on *Phaseolus vulgaris* Produces 1,8-Dihydroxynaphthalene-Melanin

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Abstract *Pseudocercospora griseola* is the causal agent of angular leaf spot of common bean (ALS). It has undergone parallel coevolution with its host and two major groups have been defined, "Andean" (*P. griseola* f. *griseola*) and "Mesoamerican" (*P. griseola* f. *mesoamericana*). The aim of this study was to analyze the nature and the level of the dark pigment synthesized by the representatives of each group. After 21 days of incubation on potato dextrose agar medium, *P. griseola* f. *griseola* isolate S3b developed colonies with diameters of 17.5 ± 1.3 mm and concentric rings of pigmentation. Isolate T4 of *P. griseola* f. *mesoamericana* presented smaller colonies (9.9 ± 0.3 mm) with a uniform dark-gray color. Both isolates, S3b and T4, produced the same pigment, a 1,8-dihydroxynaphthalene-melanin, although different in quantity and structural features as suggested by the IR spectrum. The *P. griseola* f. *griseola* isolate S3b had a higher growth rate and melanin content as well as smaller sensitivity to melanin synthesis inhibitors compared to the isolate T4 of *P. griseola* f. *mesoamericana*. These results suggest a possible link between melanin and growth in *P. griseola*.

Keywords Melanin · *Pseudocercospora griseola* · Phytopathogen · Fungus · Dark pigment · Angular leaf spot · *Phaseolus vulgaris* · "Mesoamerican" group · "Andean" group · Variability

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Introduction

Pseudocercospora griseola (Sacc.) Crous & U. Braun is the causal agent of angular leaf spot (ALS), a disease of common bean (*Phaseolus vulgaris* L.), that causes yield losses in many countries around the world [1–3]. It is an anamorph of *Mycosphaerella*, whose mycelium and conidia are pigmented [4]. This pathogen has undergone a parallel coevolution with its host in two major groups, intraspecific formae *P. griseola* f. *griseola*, the "Andean" group and *P. griseola* f. *mesoamericana*, the "Mesoamerican" one [5]. Silvera [6], Stenglein [7], Crous et al. [5], and Fermoselle et al. [8] found considerable levels of diversity among isolates of *P. griseola* from those two groups, which might be reflected either by their cultural requirement and/or characteristics when cultured in vitro.

When fungi are cultured in vitro, the synthesis of pigments might be used as a diagnostic trait that may provide additional information regarding phylogenetically related fungi, including those belonging to the genus *Mycosphaerella* [9]. In this sense, *M. africana*, *M. pseudafricana*, and *M. pseudocryptica* differ in their ability to produce pigments [10].

Several pigments and chromophore molecules have been described in fungi; though the biological role of most of them remains obscure [11–14]. In preliminary experiments, we observed that cultures of *P. griseola* isolates grown on potato dextrose agar (PDA) medium developed pigmented colonies. The pigments responsible for the black (dark) color showed remarkable resistance to temperature and the combination of it and acids (Saparrat, M. and Balatti, P. unpublished results).

Melanins are an important group of pigments synthesized by fungi. They are resistant polymeric substances that protect fungi from environmental stresses such as UV-light, predation, desiccation as well as metals [11, 14–18]. Additionally they might be involved in triggering plant defense mechanisms [15]. The role of melanins in pathogenesis is controversial but even though they may not be directly involved in the process, they can improve the survival ability of several organisms under stress conditions [13, 18–20]. Regarding this, we hypothesized that the dark pigments synthesized by *P. griseola* might have an ecological and/or biological role such as virulence and/or survival. In this article, we report the nature and the level of pigment synthesized by representative isolates of the *P. griseola* f. *griseola* and f. *mesoamericana*.

Materials and Methods

Isolates

Monosporic isolates of *P. griseola* representing the Andean (*P. griseola* f. *griseola* isolate S3b) and Mesoamerican (*P. griseola* f. *mesoamericana* isolate T4) group were used in this study [21]. They were kept at −20°C on filter papers impregnated with a conidium–mycelium suspension, and cultures of them were grown on PDA medium and maintained at 4°C.

Pigment Extraction

The pigment was extracted from 10-day-old cultures grown on PDA at 25°C according to Gadd [22].

Characterization of Pigments

Qualitative diagnostic tests for fungal melanins were conducted as described by Gadd [22]. The pigment was purified by acid hydrolysis [23], while a part of it was dissolved in KOH 1 M [24] to analyze the UV–visible absorbance spectrum and another part was spread on a ZnSe window for infrared (IR) spectroscopy analysis.

Treatment with Melanin Synthesis Inhibitors

Aliquots of stock solutions (10,000 μg ml^{-1}) of tricyclazole (Ultra Scientific Analytical Solutions, United States, in ethanol) or Kojic acid (Parafarm, Argentina, in water) were added to autoclaved PDA medium to make concentrations of 1, 10, or 100 μg ml^{-1} [25]. Inoculation was performed by placing a 7-day-old monosporic colony (2 mm diameter) grown on PDA. Plates were incubated in the dark at 24 ± 2°C for a 21-day period. Colonies were analyzed for pigmentation and growth, which was estimated by measuring the diameters of the colonies on four replica plates selected at random, using as positive controls plates free of inhibitors. The differences in the colony diameters among

treatments were assessed using the Tukey Test at 5% (Statistics 5.0).

Melanin and Enzyme Activity in Liquid Cultures

Hundred milliliters of potato-dextrose (PD) broth was inoculated and incubated in non-agitated conditions at 24 ± 2°C under a 12-h photoperiod. After 28 days of incubation, the cultures were harvested by centrifuging at 20,000*g* for 10 min at 4°C. While laccase/peroxidase activity was measured in the supernatant according to Saparrat et al. [26], melanins were measured on the pellets, which were dried at 80 ± 5°C. Twenty-five and 50 mg samples of dried fungal biomass were used to extract melanin, which was quantified by measuring the optical density at 280, 430, and 460 nm in an alkaline solution [27–29]. The experiments were designed completely at random with three replicates, considering a fixed effects model. A three-way ANOVA test was performed considering the source of fungal origin (isolate), the size of the sampled biomass, and wavelength as the three main factors and their double and triple interactions. Then, a two-way ANOVA was used to analyze differences between isolates and biomass-sample size within each wavelength, and a Tukey Test at 5% was applied for mean comparisons. In order to establish the relative importance of each source, it was determined the percentage of variation for each effect as the ratio between the sum of squares of the source and the sum of squares of treatments. A correlation coefficient of Pearson (*r*) was applied to measure the association between the absorbance level and the categorical variables.

Enzyme Activity on Agar Plates

Extracellular oxidative enzyme activity was estimated on the modified Czapek Dox agar (2%, w/v) basal media with low (1.1 mM) and high (10.9 mM) ammonium tartrate level, supplemented with guaiacol (1 mM), whose oxidation was estimated by the appearance of a reddish-colored halo around colonies. The ability of the isolates to decolorize the Poly R 478 synthetic dye (Sigma, 0.01%, w v^{-1}), which was supplemented to the modified Czapek Dox agar basal media with low and high ammonium tartrate level, was evaluated [30].

Results and Discussion

P. griseola f. *griseola* (S3b) developed colonies with diameters of 17.5 ± 1.3 mm and concentric rings of pigmentation after 21 days of incubation on PDA medium; however, the colonies of *P. griseola* f. *mesoamericana* (T4) were smaller (9.9 ± 0.3 mm) and presented a uniform dark-gray color. Silvera [6], Stenglein [7] and Fermoselle et al. [8] have already reported differences among isolates of each group, including conidiation and pigmentation of the colonies.

The pigments present in cultures of S3b and T4 were dark gray in solid phase and turn to a dark-brown color after heating them at 100°C for 2 h in 1 M KOH solution and were insoluble in 3 N HCl as well as in water and organic solvents such as acetone, chloroform, or ethanol. The pigments were decolorised with H_2O_2 and resulted in a reddish orange and gray precipitate when they reacted with $FeCl_3$ (polyphenol test) and an ammoniacal silver nitrate solution, respectively. Furthermore, the pigments absorbed a wide UV–visible spectrum even though they had an absorption peak at 230 nm; they also presented a gradient of log 10 absorbance (230–800 nm) versus wavelength plots of −0.0029 and −0.0037 for the S3b and T4, respectively. All these findings together suggest that the pigments produced by these isolates are melanins [31, 32].

One of the most prominent characteristics of melanins is the IR spectrum [28, 33] and because of this it was used to identify the pigment produced by *P. griseola* (Fig. 1). The analysis resulted in a repeatable and conserved pattern of absorption at 2,917–2,850, 1,707, 1,625, 1,628–1,623, 1,500, and 1,280–1,250 cm^{-1}, which correspond to –O–CH_3 and –CH_2– groups, C=O ketones, carboxylic acids esters, and conjugated double bonds (C=C and C=O; C=O in the composition of secondary amines and C–C bonds conjugated with benzene rings), including N–H bending and aromatic CC bonds, as well as acid, ester, and phenol groups, respectively. Briefly, the pigment has a typical conjugated quinoid structure as well as a melanoprotein component, which are both characteristic features of melanins. Melanins from other fungi such as *Inonotus obliquus* and *Synchytrium endobioticum* presented a similar pattern of chemical groups and/or bonds [28, 33, 34]. Although the IR spectra of melanin, either from S3b or T4, had

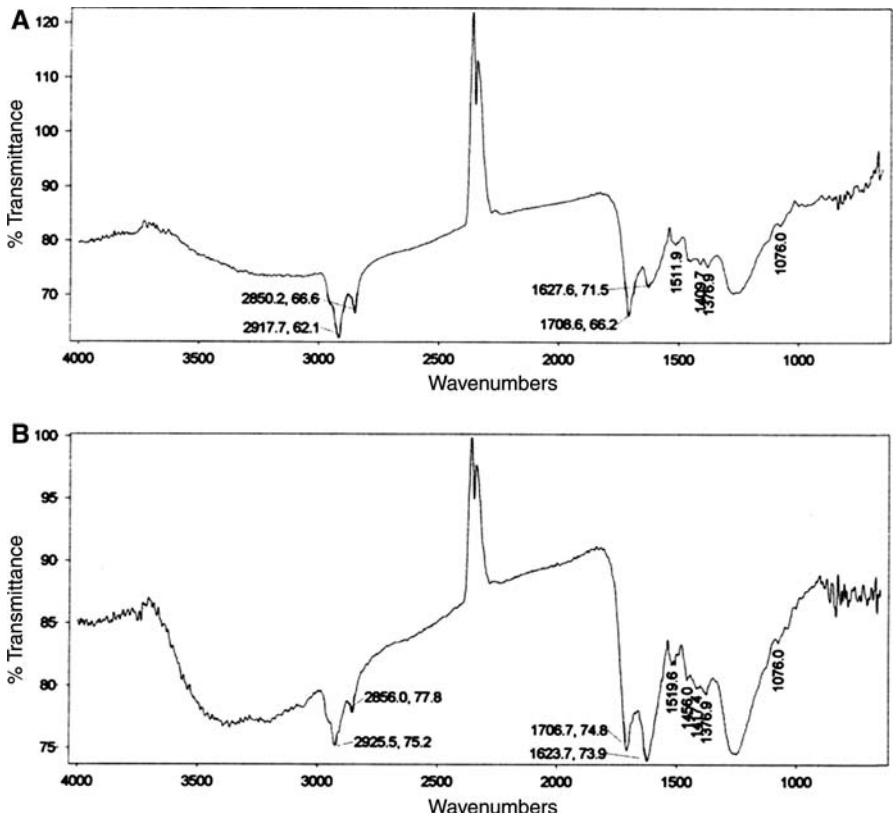

Fig. 1 IR spectrum of pigments synthesized by *P. griseola* f. *griseola* (**A**) and f. *mesoamericana* (**B**)

similar absorption bands, a higher ratio between those peaks associated to C=O group (around 1,707 cm^{-1}) versus C–O one (at 1,250 cm^{-1}) was found in the melanin of isolate S3B compared to the T4 one, which incidentally have a different color. Regarding this, Babitskaya et al. [28] established a correlation between the color intensity of melanins and their content in COOH and C=O groups as well as other oxidized functional groups, which may therefore explain the differences in pigmentation of the representatives of each group in *P. griseola*.

Inhibitors of melanin synthesis might be helpful tools to elucidate their biochemical pathway and as a consequence of this, the chemical nature/structure of the pigment can be studied [35]. Tricyclazole (5-methyl-1,2,4-triazolo[3,4-b]benzothiazole) and kojic acid are inhibitors of 1,8-dihydroxynaphthalene (DHN) and 3,4-dihydroxyphenylalanine (DOPA) melanin synthesis, respectively. Colonies of S3b and T4 presented a similar pigmentation and cultural characteristics, whether they were grown in the presence or absence of Kojic acid.

Tricyclazole inhibited growth and melanin production in both isolates (Fig. 2) and this effect became more important as the incubation period increased (data not shown). These findings suggest that S3b and T4 synthesize, like most Ascomycota anamorphs, a 1,8-dihydroxynaphthalene (DHN)-type of melanin. Although growth of isolate S3b occurred at a higher rate than T4 ($F = 1397.6$, $P < 0.001$), no difference in isolates growth was found at 100 μg ml^{-1} of tricyclazole (Fig. 3). However, at low concentration of tricyclazole (1 and 10 μg ml^{-1}), the isolates (S3b and T4) behaved significantly different (Tukey, $P < 0.05$).

Growth of T4 in the presence of 1 or 10 μg ml^{-1} tricyclazole was similar to that of the control (Fig. 3). Isolate S3b, in the presence of 10 μg ml^{-1}, responded in a completely different way, since colonies were larger and significantly different to those of the controls and those grown on media supplemented with 100 μg ml^{-1} tricyclazole (Tukey test, $P < 0.05$). So, S3b and T4 differed in their sensitivity to tricyclazole, suggesting a relationship between

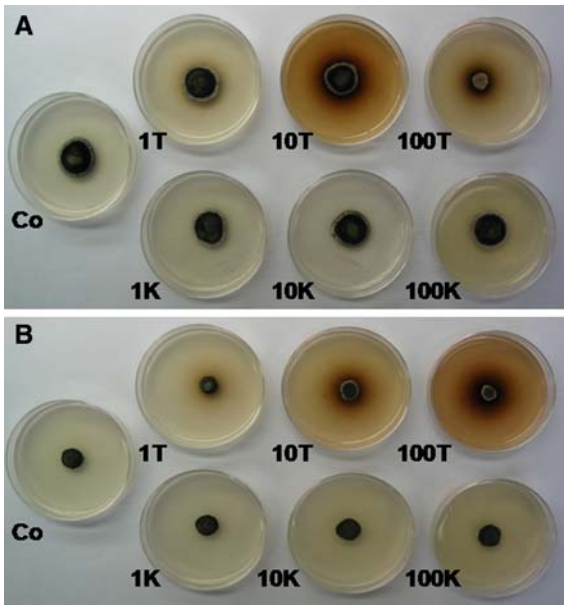

Fig. 2 Colonies of *P. griseola* f. *griseola* (**A**) and f. *mesoamericana* (**B**) grown on PDA in absence (control, Co) and presence of 1, 10 and 100 μg ml^{-1} of tricyclazole (T) or kojic acid (K) for 21 days of incubation

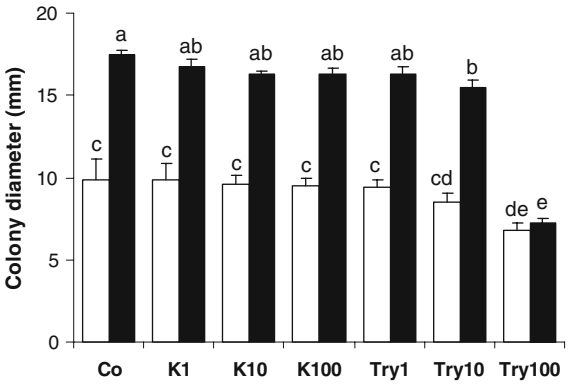

Fig. 3 Growth of colonies of *P. griseola* f. *griseola* isolate S3b (*black bars*) and *P. griseola* f. *mesoamericana* isolate T4 (*white bars*) on PDA (control, Co) and in the presence of kojic acid (K) or tricyclazole (Try) at 1, 10 and 100 μg ml^{-1} after 21 days of incubation. Values are means of four replicates. Error bars correspond to standard deviation. Bars with the same letter are not significantly different (Tukey test, $P < 0.05$)

melanin and fungal growth. Brush and Money [36] found that the inhibition of melanin synthesis was accompanied by a reduction in growth of *Wangiella dermatitidis* (Ascomycota).

When T4 was cultured in increasing concentrations of tricyclazole, it developed colonies with concentric circles, which was probably related to pigment synthesis. When S3b was cultured in the presence of 100 μg ml^{-1} of tricyclazole, it presented differences in colony pigmentation, which was of a uniformly orange-light brown color (Fig. 2), probably due to the accumulation of 2-hydroxyjuglone and flaviolin. They might be shunt products from the autoxidation of 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) and 1,3,8-trihydroxynaphthalene (1,3,8-THN), respectively, since tricyclazole block the reduction step of 1,3,6,8-THN and 1,3,8-THN, respectively, via inhibition of enzymes (reductases) of the DHN-melanin biosynthesis pathways as was observed for *Colletotrichum lagenarium*, *Pyricularia oryzae*, and *Verticillium dahliae* [37, 38].

Melanin is a complex macromolecule, whose physico-chemical characteristics are a function of the source of origin of the pigment, therefore its isolation and quantification might vary. The amount of melanin synthesized by S3b and T4 was analyzed on 28-day-old liquid cultures. The statistical analysis showed that the estimation of melanin content by absorbance is dependent on the fungal isolate, the size of the sample, and wavelength ($P < 0.001$), the latter being the largest source of variation (Table 1). Furthermore, a significant linear relationship between biomass-sample size and absorbance level ($r = 0.26$, $P < 0.03$) and between wavelength and absorbance level ($r = -0.78$, $P < 0.001$) was observed, which was higher at 280 nm (12.4) than at 430 and 460 nm, which reached levels of 1.4 and 1.0, respectively (Tukey, $P < 0.01$). Due to this reason, we standardized

Table 1 ANOVA for melanin content from 25 and 50 mg de biomass of liquid cultures of *P. griseola* f. *griseola* isolate S3b and *P. griseola* f. *mesoamericana* isolate T4 measured at 280, 430 and 460 nm: mean square significance

Source of variation	d.f.	MS	R^2
Wavelength	2	832.80***	79.40
Isolate	1	13.32***	0.63
Weight	1	170.56***	8.13
Wavelength × isolate	2	7.33***	0.70
Wavelength × weight	2	97.42***	9.29
Isolate × weight	1	3.04*	0.14
Wavelength × isolate × weight	2	1.72 ns	0.16
Error	50	0.645	

d.f. degree of freedom, *MS* means square

* $P < 0.05$, *** $P < 0.001$, *ns* not significant

the procedure to estimate melanin content by measuring absorbance levels at 280 nm of alkaline solutions generated from 50 mg of biomass samples. In this sense, the isolate S3b had higher content of melanin (absorbance of 18.34 ± 2.28) than T4 (absorbance of 14.88 ± 1.06, $P < 0.01$).

DHN melanin synthesis in many fungi involves polymerization mediated by the activity of extracellular laccases [18, 20, 37, 39, 40]. Although *P. griseola* had extracellular oxidative activity on agar cultures supplemented with guaiacol and/or on the Poly R dye, no laccase and/or peroxidase activity was detected in the supernatants of liquid cultures (data not shown). This might be due to the fact that enzymes are localized and/or immobilized in fungal walls [18, 41]. Immunolocalization studies have detected phenol-oxidases including laccases in fungal walls and associated polysaccharide sheaths as was observed for *Trametes versicolor* [15, 42].

Based on our findings, we conclude that the dark pigment synthesized by cultures of *P. griseola* is a DHN melanin, whose quantity and IR spectrum possibly varies whether the fungus belongs to the Andean or Mesoamerican group. In this sense, S3b, a representative isolate of *P. griseola* f. *griseola*, had a higher growth rate, melanin, and chromophore content as well as smaller sensitivity to tricyclazole compared to T4, a *P. griseola* f. *mesoamericana* isolate. These results suggest a possible link between melanin and growth in *P. griseola*, and future studies should be aimed at assessing the role of melanin in pathogenicity.

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