

## PIGMENTS, TOTAL SOLUBLE PHENOLS AND LIGNIN LEVELS OF COFFEE SEEDLINGS INOCULATED WITH *Colletotrichum gloeosporioides*

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(Recebido: 26 de agosto de 2010; aceito 6 de outubro de 2011)

**ABSTRACT:** The objective of this work was to study the effects of different isolates of *Colletotrichum gloeosporioides*, as to pigment level (chlorophyll a, b and total), total soluble phenols and soluble lignin in coffee seedlings obtained by artificially inoculated tissue culture. The experiment was carried out in randomized block design in a 2 x 3 factorial scheme (2 genetic materials), 3 isolates (an isolate from mango fruits and another one from coffee plants), with 5 replicates and control (plants without inoculation). The plants were inoculated with a suspension of  $2 \times 10^6$  conidia.ml<sup>-1</sup>. The conidial suspension was sprayed on previously injured leaves. Leaves of inoculated plants were collected at 3 and 7 days after inoculation, except for leaves on the lower part of the plants. Afterwards, samples were prepared for the evaluation of chlorophyll, total soluble phenols and soluble lignin. The results showed that the presence of the pathogen in healthy and diseased plants significantly reduced the amount of chlorophyll. At 7 days after inoculation of healthy and diseased coffee plants higher total soluble phenol and soluble lignin levels were observed in response to the attack by *Colletotrichum gloeosporioides*.

**Index terms:** *Coffea arabica*, *Colletotrichum gloeosporioides*, defense response.

## PIGMENTOS, FENÓIS SOLÚVEIS TOTAIS E LIGNINA EM PLÂNTULAS DE CAFEIRO INOCULADAS COM *Colletotrichum gloeosporioides*

**RESUMO:** Objetivou-se, neste trabalho, estudar os efeitos de diferentes isolados de *Colletotrichum gloeosporioides* sobre os teores de pigmentos (clorofila a, b e total), fenóis solúveis totais e lignina solúvel, em dois diferentes tempos, sobre mudas de café obtidas por cultura de tecidos e inoculadas artificialmente. O experimento foi conduzido em blocos casualizados em esquema fatorial 2 x 3 (2 materiais genéticos), 3 isolados (um obtido de mangueira e 2 isolados obtidos de plantas de café) + 2 (testemunhas sem inoculação), com 5 repetições. As plantas foram inoculadas com uma suspensão de  $2 \times 10^6$  conídios.ml<sup>-1</sup>. A suspensão de conídios foi pulverizada sobre folhas de cafeeiro, previamente feridas. Foram realizadas duas coletas, aos 3 e 7 dias após inoculação, de todas as folhas da muda, exceto as folhas baixas. Em seguida, foi feito o preparo das amostras para avaliação de clorofila, fenóis solúveis totais e lignina solúvel. A partir dos resultados, pode-se concluir que a presença do patógeno, tanto em plantas sadias, como em plantas doentes diminuíram significativamente a quantidade de clorofila. Aos 7 dias após inoculação das plantas de café sadias e doentes, observou-se um maior teor de fenóis solúveis totais e lignina solúvel em resposta ao ataque de *Colletotrichum gloeosporioides*.

**Termos para indexação:** *Coffea arabica*, *Colletotrichum gloeosporioides*, resposta de defesa.

### 1 INTRODUCTION

The state of Minas Gerais stands out as the largest producer of *Coffea arabica* with

approximately 1.15 million hectares (ha) and an estimated production of 15.5 million bags for the 2008/2009 crop, accounting for 45.9% of the national production Companhia Nacional de Abastecimento -

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CONAB (2010). A number of factors may contribute to low coffee productivity, especially diseases. Among them are the blister spot and anthracnose (*Colletotrichum* complex) (PARADELA FILHO et al., 2006), rust (*Hemileia vastatrix*) (CHALFOUN, 1997), Phoma leaf spot (*Phoma* spp.), (CARVALHO et al., 1999) and viruses (CHALFOUN, 1997). Among these, there is a special interest in the study of *Colletotrichum* complex - coffee, which is composed of several species of the pathogen (OROZCO et al., 2003). In Brazil the predominant species is *C. gloeosporioides*, which can cause anthracnose, dieback and blister spot, which is a disease with a low progression in time and space, however, highly deleterious to the infected trees (FERREIRA et al., 2004). For (OROZCO et al., 2003), this pathosystem is complex, dealing with populations of species of *Colletotrichum* that cause various symptoms or colonize plants in an invasive way without overt symptoms, not implying that these isolates are non-pathogenic.

Many compounds produced by secondary metabolic pathways are formed after the occurrence of infection by plant pathogens, providing greater resistance to disease. Compounds such as phytoalexins, phenols and flavonoids may accumulate around sites of infection, reducing the colonization of the pathogen (MALKIN and NIYOGI, 2000).

Lignin, in addition to the functions inherent in plant physiology, presents itself as a physical and chemical defensive barrier, hindering the penetration of fungi, bacteria and insect consumption, in order to protect plants against biotic and abiotic factors, arising out of the environment (DAVIN and LEWIS, 1995). Lignified cell walls could also be a barrier, preventing the movement of nutrients to the pathogen. Lignin precursors themselves may exert a toxic effect on the pathogen or also lead to lignification of the structures of the pathogen (HAMMERSCHMIDT and KUC, 1982).

Given the above, the objective of this work was to study the effects of different isolates of *C. gloeosporioides* in coffee seedlings from embryo culture obtained from plants with and without symptoms of blister spot and evaluate this effect on pigment level (chlorophyll a, b total), total soluble phenolics and soluble lignin.

## 2 MATERIAL AND METHODS

### Obtaining plants from zygotic embryo tissue cultures

Fruits of coffee (*C. arabica*), cultivar Red Catucaí, with and without symptoms of blister spot at the green-cane stage were harvested, washed and disinfected with 70% alcohol for 1 min and 2% sodium hypochlorite for 15 minutes, and then washed three times with sterile distilled water. Subsequently, the embryos were excised and placed in test tubes with 15 ml of MS medium (MURASHIGE and SKOOG 1962), plus  $\text{gL}^{-1}$  of activated carbon, 300  $\text{mg L}^{-1}$  ascorbic acid and 2.5  $\text{mg.L}^{-1}$  of gibberellin adjusted to pH 5.8 and solidified with 6  $\text{g.L}^{-1}$  agar (RIBEIRO et al., 2003). The embryo cultures were kept in a growth room at  $27 \pm 1$  °C and a photoperiod of 16 hours. Transference of the seedlings to other tubes with the same culture medium occurred every couple of months, until these seedlings were suitable for acclimatization. Acclimatization was performed according to Carvalho et al. (1999). Where seedlings with approximately 2-3 cm of length were removed from the test tubes and transferred to polypropylene trays with 11 x 5 x 5 cm cells containing vermiculite. They were placed in a growth chamber with approximately 90% relative humidity, mean temperature of 25 °C, automatic misting system and natural lighting. The supply of nutrients was done with a weekly application of 1 ml of Hoagland and Arnon (1950) nutrient solution per seedling, starting on the day of planting and extending to the inoculation period.

### Collection of isolates and inoculations

The isolates were derived from the experimental field of the Federal University of Lavras. These isolates were obtained from mango leaves with anthracnose (I1), stem tips of trees without blister spot symptoms, but with dieback (I2) and trees with symptoms of blister spot (I3). Parts of infected tissue were surface sterilized with 50% alcohol for 30 seconds and 1% sodium hypochlorite for one minute, washed with water and dried outdoors. Then they were transferred to Petri dishes with 2% MEA (malt extract and agar) culture medium and chloramphenicol. The dishes were incubated for seven days in BOD at 22° C and a photoperiod of 12 hours. The purified colonies were used to obtain spore

cultures. Isolates of *C. gloeosporioides* were maintained and grown in 2% MEA culture medium. Conidial suspensions were prepared by scraping conidia with the aid of a Drigalsky handle and sterile distilled water (SDW) followed by filtration in sterile gauze. Suspensions for inoculation were calibrated to the concentration of  $2 \times 10^6$  conidia mL<sup>-1</sup>.

The inoculation was performed on fully expanded young leaves of coffee plants, from fruits with and without blister spot symptoms. Three days before inoculation, these plants were subjected to humid chamber conditions, carried out with the aid of plastic bags and kept in a growth chamber at a temperature of 30 °C. The conidial suspension was sprayed on leaves of the seedlings with the aid of a 500 ml manual atomizer. The leaves were previously injured with the help of entomological needles at 0.5 mm equidistant totaling an area of 1.3 cm in diameter. After inoculation, the seedlings were kept in a greenhouse and in the first 24 hours after inoculation they were submitted to a moist chamber made with the help of plastic bags.

### Collection of plant tissue

Collections of all the seedling leaves were made, except for the lowest, three and seven days after inoculation. During harvesting, the leaves were protected with aluminum foil, placed in a polystyrene box containing liquid nitrogen and brought to the laboratory where they were kept frozen at -80° until the implementation of biochemical analysis.

### Determination of total, a and b chlorophyll

From the stored plant material, 0.2 g of leaf tissue from each treatment were macerated and added with 5 mL of 85% acetone and stored for 24 hours, protected from light, for pigment extraction. Subsequently, the material was centrifuged at 8000g for 15 min and the supernatant was collected for quantification of chlorophyll a, b and total using a spectrophotometer, according to the formulas proposed by Arnon (1949).

- Chlorophyll a (mg/g) =  $12.7 (663 \text{ nm}) - 2.69 (645 \text{ nm}) \times V (\text{ml}) / 1000 \times W (\text{g})$

- Chlorophyll b (mg/g) =  $22.9 (645 \text{ nm}) - 4.68 (663 \text{ nm}) \times V (\text{ml}) / 1000 \times W (\text{g})$

- Chlorophyll total (mg/g) =  $20.2 (645 \text{ nm}) + 8.02 (663 \text{ nm}) \times V (\text{ml}) / 1000 \times W (\text{g})$  Where: W= 200mg fresh leaf, and V= 5ml of 85% acetone

### Evaluation of lignin and total soluble phenols

Leaf tissue stored at -80° C was ground in liquid nitrogen with mortar and pestle to obtain a fine powder. Subsequently, the samples were lyophilized for 16h. An aliquot of 30 mg of lyophilized material was transferred to a 2 mL micro tube, homogenized with 1.5 mL of 80% methanol and kept under agitation, for 16h on a rotary shaker protected from light at room temperature. The suspension was centrifuged at 12,000 g for 5 min. The supernatant was transferred to a new micro tube, and with which total soluble phenolic compounds was determined, while the solid residue was used for determination of soluble lignin.

### Determination of total soluble phenols

The total phenolics were determined by adding 150 mL of methanol extract, mixing 150 mL of Folin-Ciocalteu 0.25 N and kept at room temperature for 5 min, then adding 150 mL of 1M Na<sub>2</sub>CO<sub>3</sub>, and homogenized and maintained for 10 min at room temperature. The mixture was then homogenized with 1 mL of distilled and deionized water and kept at room temperature for one hour. The phenolic was determined at 725 nm (SPANOS and WROLSTAD, 1990).

### Determination of lignin

1.5 mL of water was added to solid residue obtained above, homogenized and centrifuged at 12,000 for 5 min. The supernatant was discarded and the residue was dried at 65°C for 15 h. The dry residue, insoluble in alcohol, containing lignin and esterified phenolic acids from cell wall, was used for the lignin determination. For this, a volume of 1.5 mL of thioglycolic acid and containing 2M HCl (1:10) was added to the residue. The tubes were gently agitated to hydrate the residue and then placed in a water bath at 100°C for 4 h. Afterwards, they were placed on ice to cool for 10 min and then centrifuged at 10,000g for 10 minutes. The supernatant was discarded and the precipitate washed with 1.5 mL of distilled and deionized water and again centrifuged at 10,000g for 10 min. After this period the supernatant was discarded and the pellet was resuspended in 1.5 mL 0.5 M NaOH, and the mixture shaken in a rotary shaker for 15 h at room temperature. The mixture was centrifuged at 10,000g for 10 min and the

supernatant was transferred to a new tube, then 200 mL of concentrated HCl was added to the supernatant and kept cold (4°C) for 4 h to allow precipitation of lignin linked to the mercaptoacetic acid. The mixture was then centrifuged at 10,000g for 10 min, the supernatant discarded and the pellet resuspended in 2 mL of 0.5 M NaOH. The absorbance of this solution was determined at 280 nm and values calculated based on the lignin curve and expressed as mg of lignin per mg of dry tissue (DOSTER and BOSTOCK, 1988).

### Statistical design and data analysis

The experimental design was randomized blocks in 2 x 3 (2 genotypes (Red Catucaí cultivar seedlings obtained from seeds of plants with blister spot symptoms (MOPCS) and without (MOPSS)), 3 isolates of *C.gloeosporioides* and two additional treatments (injured controls of each genetic material)) with 5 replications. The data was subjected to ANOVA and means were compared by Tukey test ( $P \leq 0.05$ ) and comparisons with additional treatments were performed by Dunnett's test using the statistical software (SAS INSTITUTE, 1992).

## 3 RESULTS AND DISCUSSION

### Chlorophyll a, b and total

There was no effect of the interaction between the isolates I1, I2 and I3, and the seedling type, (MOPCS) and (MOPSS) at 3 or 7 days after

inoculation (DAI) for the levels of chlorophyll a, b and total (Table 1). Comparing the levels of pigment in plants inoculated with different strains in each type of seedling (MOPCS and MOPSS), and only injured plants, which was the inoculation method, there was no effect of the isolates (Table 1). Probably, this fact was due to the short time between inoculation and the collection of leaves, only a maximum of seven days.

The main function of chlorophyll is to absorb energy of the photons of light emitted by the Sun, thus contributing to an electron transport chain that culminates in the hydrolysis of water molecules and the production of ATP, needed for the biochemical phase of photosynthesis where carbohydrates are synthesized from carbon dioxide (PAZZA, 2010).

This function can be prevented when there is degradation of the chlorophyll level in plants, which may be happening in this study. The greatest damage to the plant occur after the absorption of light, with the reduction of chlorophyll in photosynthetic cells and tissues. This reduction can be caused by a variety of factors including the lack of chlorophyll synthesis due to the degradation of chloroplasts, necrosis of tissues and photosynthetic cells, reduced photosynthetic surface of organs and tissues, and finally, loss of leaves (SCHOLETS, 1992). Although we have seen a significant degradation of chlorophyll in studies of genetic material over time, we can say that this fact is linked to the loss of photosynthetic area, because until now the presence of the pathogen

**TABLE 1** – Average levels of chlorophyll (Cl) a, b, and total (Cl T) (mg g<sup>-1</sup> MF<sup>-1</sup>), in coffee leaves from seedlings from Red Catucaí coffee seeds with (MOPCS) and without (MOPSS) symptoms of blister spot, 3 and 7 days after inoculation (DAI) with three isolates of *C. gloeosporioides* (anthracnose in mango leaves - I1, stem tips of trees without blister spot symptoms, but with dieback - I2 and trees with symptoms of blister spot - I3) through injury compared to a control with additional injury.

Isolate	3 DAI						7 DAI					
	MOPCS			MOPSS			MOPCS			MOPSS		
	Cl a	Cl b	Cl T	Cl a	Cl b	Cl T	Cl a	Cl b	Cl T	Cl a	Cl b	Cl T
Mango(I1)	0.79	0.60	1.50	0.83	0.75	1.58	0.48*	0.23*	0.71*	0.50*	0.22*	0.72*
Coffee (I2)	0.82	0.84	1.65	0.78	0.73	1.51	0.51*	0.24*	0.75*	0.54*	0.23*	0.77*
Coffee (I3)	0.81	0.82	1.64	0.83	0.65	1.48	0.48*	0.24*	0.72*	0.52*	0.24*	0.76*
Injured Control	0.89	0.98	1.86	0.80	0.92	1.72	0.49	0.24	0.73	0.50	0.22	0.72

\* not significant.

outside the tissue has not been seen. Perhaps it may have only interfered internally with the degradation of chloroplasts. Lopes and Berger (2001) state that healthy areas of leaves with symptoms of anthracnose have their photosynthetic rate decreased, because the effect of anthracnose extends beyond the visual area of the lesion, which, according to Bastiaans (1991) can be defined as a virtual injury. For some pathosystems involving necrotrophic organisms, the virtual lesion may be the result of the production and dissemination of toxins around the symptomatic area of tissue, thus interfering in photosynthetic activity. This fact may be related to the results obtained in this study.

### Lignin

For the lignin, there was no interaction effect between the three isolates of *C. gloeosporioides*, I1, I2 and I3 used and the type of seedlings, MOPCS

and MOPSS at 3 or 7 DAI. At 3 DAI, there was also no effect observed in the seedlings, nor in the type of isolate (Table 2). However, at 7 DAI, the lignin concentration was not influenced by the type of seedling, but were influenced by the type of isolate. The isolate *C. gloeosporioides* of coffee (I2 and I3) provided greater lignification, not differ among themselves, but differing from the mango *C. gloeosporioides* isolate (I1) (Table 2). As the inoculation was performed by injury, we used an additional control only with the injury and without the presence of the fungus. At 3 DAI no differences were observed in lignin concentration between injured control and the isolates in the two types of seedlings. At 7 DAI, it was observed that in MOPSS inoculated with coffee isolates (I2 and I3), there was greater lignification than the injured control, This was not observed for the mango isolate (I1) (Table 3). This effect was also not observed at 7 DAI in MOPCS

**TABLE 2** – Average lignin level  $\mu\text{g (mg MS)}^{-1}$ , of coffee leaves from seedlings from ‘Red Catucaí’ coffee seeds with (MOPCS) and without (MOPSS) blister spot symptoms, 3 and 7 days after inoculation (DAI) with three isolates of *C. gloeosporioides* (anthracnose in mango leaves - I1, tree stem tips without symptoms of blister spot, but with dieback - I2 and the trees with symptoms of blister spot - I3) through injury.

Isolates	3 DAI			7 DAI			
	MOPCS	MOPSS	Average	Isolates	MOPCS	MOPSS	Average
Mango (I1)	23.1	18	23.1 a	Mango (I1)	15.9	13.6	14.7 b
Coffee (I2)	21.2	16.2	21.2 a	Coffee (I2)	18.8	18.9	18.9 a
Coffee(I3)	22.7	20.1	22.7 a	Coffee (I3)	18.4	21.7	20.1 a
Average	22.3 A	18.1 A		Average	17.7 A	18.06 A	

Averages with same letter in uppercase and lowercase line in the column do not differ by Tukey test ( $P \leq 0.05$ ).

**TABLE 3** – Average lignin level  $\mu\text{g (mg MS)}^{-1}$ , of coffee leaves from seedlings from ‘Red Catucaí’ coffee seeds with (MOPCS) and without (MOPSS) blister spot symptoms, 3 and 7 days after inoculation (DAI) with three isolates of *C. gloeosporioides* (anthracnose in mango leaves - I1, tree stem tips without symptoms of blister spot, but with dieback - I2 and the trees with symptoms of blister spot - I3) through injury compared to additional injured control.

Isolates	3 DAI		7 DAI	
	MOPCS	MOPSS	MOPCS	MOPSS
Mango (I1)	18.0 <sup>ns</sup>	23.1 <sup>ns</sup>	15.9 <sup>ns</sup>	13.6 <sup>ns</sup>
Coffee (I2)	16.0 <sup>ns</sup>	21.2 <sup>ns</sup>	18.8 <sup>ns</sup>	18.9 <sup>*</sup>
Coffee (I3)	20.1 <sup>ns</sup>	22.7 <sup>ns</sup>	18.4 <sup>ns</sup>	21.7 <sup>*</sup>
Injured Control	21.7	18.2	16.8	12.74

\* = significant, by the Dunnett test ( $P \leq 0.05$ ). <sup>ns</sup> = not significant.

inoculated with the same isolates when compared with the control of the same plant material with injury (Table 3). This may be due to sampling (collection) being carried out with the leaves not yet fully expanded. According Botelho et al. (2009) and Nojosa, Resende and Resende (2005) the lignin may be present with greater intensity in older leaves than in younger leaves.

Lignin is an important component in plant resistance to pathogens, thus limiting their action by forming a physical barrier Botelho et al. (2005).

In This study the cumulative tendencies basically followed a pattern similar to the (MOPSS) in collection 1 that did not differ and showed an amount of lignin equal to (MOPCS) when inoculated with the fungus or even when not inoculated as in the case of the control.

### Determination of Total Phenols

There was no significant interaction between the types of seedlings and isolates and not even among these factors isolatedly, to 3 DAI (Table 4). The same behavior was observed at 7 DAI, except for the type of seedlings (MOPCS and MOPSS) because MOPSS showed higher levels of total soluble phenols, also observed in controls with injury and without inoculation (Table 5).

Comparing the total soluble phenolic levels in each seedling type inoculated with isolates of *C. gloeosporioides* and its injured control (Table 5), there was no effect of the isolates on these levels at any assessment time.

As in the case of lignin, probably, no significant differences and correlations for the concentrations of total soluble phenolic compounds were due to the fact that the sampling (collection) was carried out with the leaves not yet fully expanded. Salgado et al. (2008) argues that the new leaves have lower levels of total phenols when compared to older leaves. Phenolic compounds serve as a natural defense against herbivores and pathogens, a correlation having been found between the levels of this substance and the plant resistance (GOODMAN; KIRAL; WOOD, 1986; MISAGHI, 1980).

Therefore, the level of plant infestation or infection can be attributed to differences in the concentrations of these compounds in plants Salgado et al. (2008).

In general, no increase or decrease in pigment caused by *C. gloeosporioides* isolates in MOPCS as well as in MOPSS. Probably 7 days after inoculation was not sufficient for the coffee *C. gloeosporioides* to begin to induce symptoms of chlorosis in plants. The kind of seedlings did not affect the levels of lignin, however coffee isolates induced an increase in their levels. This fact is probably the recognition of pathogens by plants, and was not observed in seedlings inoculated with the isolate of mango *C. gloeosporioides*. More studies should be done related to increasing the time between inoculation and the collection of the material and the verification of the symptoms caused by bacteria.

**TABLE 4** – Average level of total soluble phenols,  $\mu\text{g}$  catechol  $(\text{mg MS})^{-1}$ , in coffee leaves from seedlings obtained from seeds from ‘Red Catucaí’ coffee with (MOPCS) and without (MOPSS) blister spot symptoms, 3 and 7 days after inoculation (DAI) with three isolates of *C. gloeosporioides* (anthracnose in mango leaves - I1, tree stem tips without symptoms of blister spot, but with die off - I2 and the trees with symptoms of blister spot - I3) through injury.

Isolate	3 DAI			7 DAI		
	MOPCS	MOPSS	Average	MOPCS	MOPSS	Average
Mango(I1)	2.61	2.68	2.64 a	2.6	3.15	2.88 a
Coffee (I2)	2.63	2.98	2.81 a	2.59	3.18	2.89 a
Coffee (I3)	2.71	2.65	2.68 a	2.54	2.7	2.62 a
Average	2.65 A	2.77 A		2.58 B	3.01 A	

Averages with same uppercase letter on the line and lowercase in the column, do not differ by the Tukey test ( $P \leq 0.05$ ).

**TABLE 5** – Average levels of total soluble phenols, µg catechol (mg MS)<sup>-1</sup>, in coffee leaves from seedlings from ‘Red Catucaí’ coffee seeds with (MOPCS) and without (MOPSS) blister spot symptoms, 3 and 7 days after inoculation (DAI) with three isolates of *C. gloeosporioides* (anthracnose in mango leaves - I1, tree stem tips without symptoms of blister spot, but with die off - I2 and the trees with symptoms of blister spot - I3) through injury, compared to an additional control with injury.

Isolate	3 DAI		7 DAI	
	MOPCS	MOPSS	MOPCS	MOPSS
Mango (I1)	2.7 <sup>ns</sup>	2.7 <sup>ns</sup>	2.6 <sup>ns</sup>	3.1 <sup>ns</sup>
Coffee (I2)	2.6 <sup>ns</sup>	3.0 <sup>ns</sup>	2.6 <sup>ns</sup>	3.2 <sup>ns</sup>
Coffee (I3)	2.6 <sup>ns</sup>	2.6 <sup>ns</sup>	2.5 <sup>ns</sup>	2.7 <sup>ns</sup>
Injured Control	2.8	2.9	2.5	2.9

\* = significant, by the Dunnett test(P=0,05). <sup>ns</sup> = not significant.

#### 4 CONCLUSIONS

The amount of chlorophyll decreased from first to second harvest in both pathosystems studied.

Higher levels of total soluble phenolics and lignin was observed at 7 days after inoculation of *C. gloeosporioides* in coffee seedlings obtained from seeds of plants with and without symptoms of blister spot.

#### 5 ACKNOWLEDGEMENTS

To the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for granting the scholarship to the first author to complete the Master’s program in Plant Pathology at Federal University of Lavras, and to the other authors for their performance in the realization of this work; in particular to Claudio Ogoshi by dedication and Professor Mario for all his teaching.

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