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Induction and detection of toxin in *Cercospora zeina* and *Cercospora sorghi* f. sp. *maydis*

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ABSTRACT

Cercosporin is a phytotoxin with toxic activity against large number of organism, it is produced by hypha in the culture medium. This way, the objective of this study was to induce the production of Cercosporin in *Cercospora zeina* (CZ) and *Cercospora sorghi* f. sp. *maydis* (CS) in 16 culture media, and to verify which medium is most suitable for detection of the toxin. Agar plugs were extracted and they were put in KOH for 16 hours. Cercosporin concentration was determined by using a molar extinction coefficient of 23300 L mol $^{-1}$. Under the conditions in which the fungi were cultured, it was not possible to induce the toxin in CZ, which reinforces the hypothesis that the toxin is only produced in planta. CS produced a high concentration of toxin in coconut water Sococo® medium with yeast extract, 177.34 nmol plug^{-1}. Only the M1D medium did not produce the toxin. There was a difference in the production of toxin depending on the brand and concentration of PDA medium. It is concluded that the media with coconut water Sococo® are efficient in the induction of Cercosporin in vitro by CS, which are promising for research in this area. Furthermore, CZ did not produce the toxin in the studied conditions.

Key words: Cercosporin, maize gray leaf spot, *Zea mays* L.

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RESUMO

A cercosporina é uma fitoxina com atividade tóxica contra um amplo espectro de organismos, produzida pelas hifas em meio de cultura. O objetivo deste estudo foi induzir a toxina em *Cercospora zeina* (CZ) e *Cercospora sorghi* f. sp. *maydis* (CS) em 16 meios de cultura e averiguar qual o meio mais adequado para se detectar a toxina. Discos miceliais foram imersos em KOH por 16 horas e a concentração de toxina foi calculada utilizando-se o coeficiente de 23.300 L mol $^{-1}$. Nas condições em que foram submetidos os fungos não foi possível induzir a toxina em CZ, fato que reforça a hipótese de que a toxina é produzida apenas em planta. Em CS, a produção de toxina foi intensa nos meios com água de coco da marca Sococo®, principalmente quando foi adicionado extrato de leveduras, apresentando 177.34 nmol disco^{-1} enquanto o meio M1D não foi capaz de induzir a toxina. A diferença na produção de toxina em BDA é devida à concentração e às marcas comerciais diferentes. Conclui-se que os meios com água de coco Sococo® são eficientes na indução da Cercosporina in vitro por CS, o que constitui uma substância promissora para estudos dessa linha de pesquisa; além disto, CZ não produziu a toxina nas condições estudadas neste trabalho.

Palavras-chave: Cercosporiose do milho, *Zea mays* L., Cercosporina

Introduction

Many plant-pathogenic fungi produce secondary metabolites of low molecular weight, which have a significant role in interaction with the host plant (Yoder, 1980; Wolpert et al., 2002). This is the case with many species of Cercospora (Assante et al., 1977; Fajola, 1978; Lynch & Geoghegan, 1979) that produce the toxin Cercosporin.

However, there are highly destructive and virulent pathogenic species that do not produce the toxin in culture medium, and this is true of *Cercospora sojina* and *Cercospora zeina*. Carson et al. (2002) and Brunelli et al. (2008) previously observed that some isolates from the former group II, now *C. zeina*, did not produce Cercosporin.

The most commonly accepted hypothesis to explain this observation is that these species produce the toxin only *in planta,* because the appropriate conditions for inducing the toxin were not provided. Furthermore, both contain the gene that confers self-protection against the toxin's action, which reinforces the hypothesis that they are indeed toxin producers *in vivo* (Goodwin & Dunkle, 2010).

Cercosporin is a plant toxin harmful to a wide range of organisms, so it is not selective or specific. It is produced by hyphae in culture medium, resulting in an accumulation of Cercosporin crystals around the hyphae and on the surface of the culture medium (Daub & Chung, 2009). It presents reddish color that turns green in alkaline conditions (in the presence of KOH), exhibiting greatest absorption at a wavelength of 480 nm (Jenns et al., 1989). It belongs to the perylenequinone group, so it contains photosensitizing molecules that are able to absorb light and generate reactive oxygen species, which damage the cellular membrane, present color and absorb visible light (Daub & Chung, 2009).

The production of the toxin *in vitro* via polyketide pathway varies with several factors such as: culture medium, temperature, light and C:N ratio; it also varies between species and isolates from a single species under different cultures, temperatures and light regimes (Jenns et al., 1989). Light is the most important factor for activating the toxin and for biosynthesis, its absence stops Cercosporin production, while the slightest exposure to light induces production (Daub & Chung, 2009). In addition, other factors are associated with biosynthesis of Cercosporin, such as calcium/calmudolin, protein G and MAP kinase (Chung, 2003; Shim & Dunkle, 2003).

Cercosporin can be detected *in vitro* culture extracts two days after the culture has been transferred to the medium (Jeens et al., 1989) or from damaged tissues in the infected plants (Daub & Ehrenshaft, 2000). *In vitro* Cercosporin is synthesized only in culture media that favors vegetative growth, and is repressed under nutritional conditions that induce the process of microcyclic conidiation, such as in V8 juice medium (Daub & Chung, 2007). This suggests that fungal development and secondary metabolism are antagonistic.

The objective of this study, therefore, was to induce toxin production in two species of Cercospora, from maize in 16 culture media and to verify which medium is the most suitable for detecting the toxin.

Material and Methods

Isolates of *Cercospora zeina* LV-23, IN-5 and BA-150 were used, from three locations: Lavras, Indianápolis and Bambuí,

Table 1. Components of the culture media used to induce Cercosporin by two maize's Cercospora species

Culture media	Abbreviations	Components of culture media	pH
Water-agar	AA	20g of agar	6.42
Water-agar with Yeast	AA+Y	20g of agar + 1.5g of yeast extract	9.14
Potato Dextrose Broth from Himédia®*	PDA-H	20g of PDB + 20g of agar	9.15
Potato Dextrose Broth from Himédia®* with Yeast	PDA-H + Y	24g of PDB + 20g of agar + 1.5g of yeast extract	9.00
0.2 de Potato Dextrose Broth from Himédia®*	0.2 PDA-H	4.8g of PDB + 20g of agar	8.97
0.2 Potato Dextrose Agar from Himédia ®** with Yeast	0.2 PDA-H +Y	5g of PDB + 20g of agar +1.5g of yeast extract	8.99
Potato Dextrose Agar from Merck®**	PDA-M	39g of PDA	5.32
Potato Dextrose Agar from Merck ® **with Yeast	PDA-M + Y	39g of PDA + 1.5g of yeast extract	5.25
0.2 Potato Dextrose Agar from Merck ®**	0.2 PDA-M	7.8g of PDA	9.00
0.2 de Potato Dextrose Agar da Merck ®** with yeast	0.2 PDA-M +Y	7.8g of PDA + 12g of agar + 1.5g of yeast extract	8.98
Coconut Water	CW	1000mL of "Sococo®" coconut water + 20g of agar	5.14
Coconut Water from Sococo ® with Yeast	CW+Y	100 mL of coconut water, 1.5g yeast extract, 20g of agar	8.98
Coconut Water with magnesium chloride, manganese and chloride and calcium chloride	CW+S	Coconut water with stock solution at 1% of $MgCl_2$ 20.3g; $CaCl_2$ 10.2g; $MnCl_2$ 1.0g for 100mL of coconut water	7.20
Coconut Water from Sococo ® with magnesium chloride, manganese chloride and calcium chloride and Yeast	CW+S+ Y	Coconut water with stock solution at 1% of $MgCl_2$ 20.3g; $CaCl_2$ 10.2g; $MnCl_2$ 1.0g for 100mL of coconut water and 1.5g of yeast extract	8.52
Modified M1D	M1D	$Ca(NO_3)_2$, 1.2 mM; KNO_3, 0.79 mM; KCl, 0.87 mM; $MgSO_4$, 3.0 mM; NaH_2PO_4, 0.4 mM; sucrose, 87.6 mM; Ammonium tartarate 27.1 mM; FeC1 $_3$, 7.4,uM; MnSO4, 30,uM; $ZnSO_4$, 8.7,uM; H_3BO_3, 22,uM; KI, 4.5 1mM, Inositol (5g/l); Thiamine (0.5 g l^{-1}); Biotina (0.5 g l^{-1}) e Coconut water (12 ml l^{-1}).	5.50
Seasoned Tomato Juice	STJ	200 mL Seasoned Tomato Juice "Superbom"; 20 g of agar; 3,2 g de $CaCO_3$	6.59

Potato Dextrose Broth* from Himédia® - Do not contain Agar in the composition; Potato Dextrose Agar** from Merck® or Himédia® - Contain Agar in the composition

respectively, and *Cercospora sorghi* f. sp. *maydis*, from Lavras LV- 2. All localities are in the state of Minas Gerais, Brazil and they were grown in Seasoned Tomato Juice medium, then transferred to 16 culture media as shown in the Table 1.

All the media was sterilized by autoclave and their pH(s) were measured and, when necessary, adjusted with NaOH or KOH (Table 1) according to the protocol of each medium.

Mycelia plugs of each species of *Cercospora*, 4 mm in diameter, were placed in the culture medium and incubated for 10 days under continuous light at 27 ºC.

To extract and quantify the Cercosporin the methodology described by Chung (2003) was used, with some modifications. Three mycelial plugs, 4 mm in diameter, were placed in 8 mL of KOH (5 mol.L^{-1}) for 16 hours at a temperature of 25 ^{o}C in the dark. Absorbance readings were done with a Beckman DU-600 spectrophotometer. The concentration of Cercosporin was calculated using the molar extinction coefficient of 23300 L mol^{-1} for readings of A_{480} nm (Jenns et al., 1989). To determine the Cercosporin concentration per plug, the result was divided by 3.

The experiment was conducted two times. The experimental design was completely randomized, with 16 treatments and three repetitions, so that for each Petri dish three toxin absorbance readings were taken. The means were compared by Scott-Knott test at 5% probability.

Results and Discussion

It was possible to induce and detect Cercosporin only on *C. sorghi* f. sp. *maydis*, and not on *C. zeina*.

Among the 16 culture media used (Figure 1) it was only in modified M1D that Cercosporin could not be detected, although this treatment apparently presented all characteristics of toxin. It showed the typical reddish color, and the hyphae turned pinkish, a characteristic seen in isolates that produce large quantities of toxin. However, when the mycelial plugs grown in M1D medium were placed in an alkaline medium, KOH 0.5 N, the medium turned orange and not green, as occurs with Cercosporin (Jenns et al., 1989). It was therefore concluded that this was not Cercosporin, but rather another substance with visible characteristics in the culture medium that were similar to those of the studied toxin. This is an example of how qualitative analysis based on graded scales for color intensity in culture media can lead to errors if a chemical analysis is not also carried out. Therefore, it is important to carry out chemical analysis as well as qualitative analysis, to achieve the necessary precision.

The concentration of the toxin in the media varied from 177.34 to 2.73 nmol $plug^{1}$ (Table 2). The media with coconut water produced the most Cercosporin especially when enriched by yeasts and/or salts (S), such as: $MgCl_2$ $CaCl_2$ and $MnCl_2$. CW+Y presented the highest concentration of Cercosporin, 177.34 nmol $plug^{-1}$, followed by CW+Y+S, CW+S; PDA+Y (H) and CW.

Coconut water is very rich in nutrients, such as sugars and other glycides, amino acids and their starches, and other metabolites. These nutrients together with yeast extract, which contains small quantities of calcium necessary for the induction

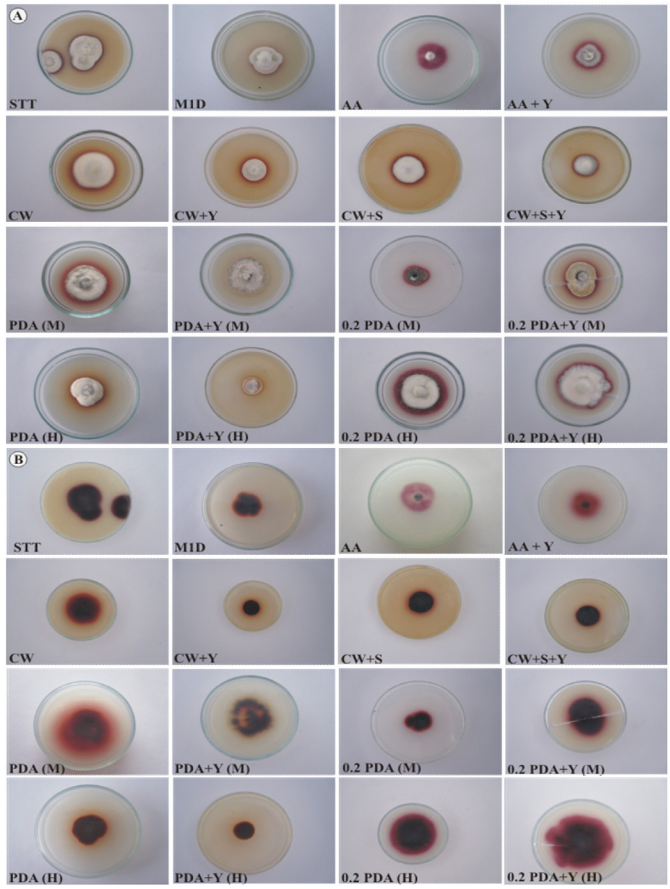

Figure 1. Production of Cercosporin by *Cercospora sorghi* f. sp. *maydis* in various culture media incubated under continuous light at 27 ^{o}C (A): front (B): reverse. See the red color around the fungal colonies; it indicates the possible production of toxin. STJ- Seasoned tomato juice; M1D- Modified M1D; AA- Water-agar; AA+Y -Water-agar + yeast; CW- Coconut water; CW+Y- Coconut water with yeast; CW+S- Coconut water with magnesium chloride, manganese chloride and calcium chloride; CW+Y+ S - Coconut water with magnesium chloride, manganese chloride, calcium chloride and yeast; PDA (M)- Merck® brand Potato Dextrose Agar; PDA + Y(M)- Merck® brand Potato Dextrose Agar with yeast; 0.2 PDA+Y (M)- 0.2 of Potato Dextrose Agar from Merk with yeast; PDA(H) -Himédia® brand Potato Dextrose Agar; PDA + Y(H)- Himédia® brand Potato Dextrose Agar with yeast; 0.2 PDA(H)- 0.2 of Potato Dextrose Agar from Himédia® and 0.2 PDA +Y (H)- 0.2 of Potato Dextrose Agar from Himédia® with yeast

of the toxin (Chung, 2003), lead to a high concentration of Cercosporin.

You et al. (2008) observed that yeast extract may or may not boost toxin production, depending on the culture medium used, without altering the expression of the genes involved in biosynthesis, which indicates the existence of other mechanisms that may be involved. In this medium, yeast extract and other components, such as salts, provided an increase in Cercosporin production, although the difference between the toxin concentrations in media was not significant: the differences between CW and CW+Y+S and between CW and CW+S were 21.28 and nmol plug $^{-1}$, respectively.

In this study, the salts contributed little to increasing toxin concentration, and yeast extract was the compound that most boosted it. However, when yeast and salts were added simultaneously, the production of toxin fell in comparison to CW+Y medium. This probably occurred due to an excess of

Table 2. Production of Cercosporin in various culture media by *Cercospora sorghi* f. sp. *maydis*

Culture media	Concentrations (nmol plug^{-1})
CW+Y	177.34 a
CW+Y+S	106.84 b
CW+S	89.96 b
PDA+Y(H)	87.96 b
CW	85.56 b
PDA (M)	66.01 c
PDA (H)	15.46 d
STJ	9.99 d
0.2 PDA (H)	6.52 d
0.2 PDA (M)	5.25 d
0.2PDA+ Y (H)	4.31 d
AA+Y	4.08 d
AA	4.01 d
0.2 PDA+ Y (M)	3.70 d
PDA+Y (M)	2.73 d

Coconut water with yeast (CW+Y); Coconut water with magnesium chloride, manganese chloride, calcium chloride and yeast (CW+Y+ S); Coconut water with magnesium chloride, manganese chloride and calcium chloride (CW+S); Potato Dextrose Agar from Himédia® with yeast (PDA + Y(H)); Coconut water (CW); Merck® Potato Dextrose Agar (PDA(M)); Himédia® Potato Dextrose Agar (PDA(H));Seasoned tomato juice (STJ); 0.2 of Potato Dextrose Agar from Himédia® (0.2 PDA(H)); 0.2 of Potato Dextrose Agar from Merck® (0.2 PDA(M)); 0.2 of Potato Dextrose Agar from Himédia® with yeast (0.2 PDA +Y (H)); Water-agar + yeast (AA+Y); Water-agar (AA); 0.2 of Potato Dextrose Agar from Merk with yeast (0.2 PDA +Y (M)) and Merck® Potato Dextrose Agar RA with yeast (PDA + Y(M)). Results obted by *Cercospora sorghi* f. sp. *maydis* (LV-2).

nutrients, principally calcium, because this is present in both yeast extract and in the salts.

The salts used in the CW+Y+ S and CW+S media are sources of Ca^{2+}, Mg^{2+} and Mn^{2+}, and Ca^{2+} and Mn^{2+} ions, producing a stimulatory effect on the production of the toxin in *C. nicotiane*. Ca^{2+} is involved in initial translation signals that may activate the expression of genes involved in regulation and biosynthesis of Cercosporin (Chung, 2003).

The medium STJ, which is used as much as V8 medium juice for sporulation, induced toxin production at 9.99 nmol plug^{-1}, a much lower quantity than with CW+Y, which produced 177.34 nmol plug^{-1}.

Cercosporin is preferentially synthesized in media that stimulate vegetative growth, such as PDA, and it is repressed when grown in media that favor sporulation, such as V8 juice medium (200 ml V8 vegetable juice®, 3g CaCO, 20g agar, 800 mL distilled water) and STJ, because the nutritional conditions needed for each process are antagonistic (Daub & Chung, 2009). Therefore, this is the first report of toxin production in culture media that induces the sporulation process, although the two processes are not observed concomitantly. It is thus possible to infer that there is another mechanism activating the toxin in this medium, as yet unknown. This new mechanism may be a new gene that activates the metabolic pathways that were already involved in this process or another new pathway that still needs to be elucidated.

The AA medium is the poorest in nutrients, only containing water and agar, which has small quantities of minerals, such as P, Fe, K, Cl, I and Ca, as well as cellulose, anhydrogalactose and proteins, which were enough to induce toxin production at 4.01 nmol plug^{-1}, a quantity very close to that of the treatment enriched with yeast, AA+Y, at 4.08 nmol plug^{-1}. Therefore, in this medium the yeast extract did not help to increase toxin concentration.

Most of the PDA media presented a lower concentration than the treatments with coconut water, except for PDA+Y(H), which elicited a production of 87.96 nmol plug^{-1}, a value which was not statistically different from those of the treatments CW, CW+S and CW+S+Y, but was significantly greater when compared to the other treatments containing PDA, especially when compared to PDA+Y (M), which produced a concentration of 3.70 nmol plug^{-1}.

The difference in the results is exclusively due to the composition of each medium, varying by brand. This points to the extreme importance of mentioning the brand when conducting experiments which produce Cercosporin; if this is not done, the result may not be reproducible.

Jenns et al. (1989) stated that the best medium for toxin production was PDA. Later, Chung (2003) observed that the best condition for *C. nicotianae* to produce Cercosporin was under light with a fine layer of 15 mL PDA medium (Difco® Detroit, MI, USA), in a 9-cm Petri dish, while for *C. zeae maydis* 0.2 PDA from Difco® is frequently used. In this case, production is maximized in media that are poor in nutrients, such as diluted PDA, and very little or no toxin appears in rich media (Shim & Dunkle, 2003). However, in this work the opposite was observed for *C. sorghi* f. sp. *maydis*, because PDA (H) and (M) media induced more toxin production, 66.01 and 15.46 nmol plug^{-1}, than did dilute media with 0.2 PDA (H) and (M), which produced 6.52 and 5.25 nmol plug^{-1}. Furthermore, the addition of yeasts, in the diluted PDA media of the brands Merck® and Himédia®, did not help in the production of toxin, while in the CW medium, the yeast extract helped toxin induction. It is not known why yeast extract can increase or reduce Cercosporin production (You et al. 2008).

In regard to calcium, You et al. (2008) succeeded in inducing the toxin when they used a quantity of 10-100 mmol L^{-1} in PDA medium (PDA EM Science®). However, the authors suggested that maintaining endogenous Ca^{2+} in homeostasis is better for obtaining biosynthesis of the toxin in *C. nicotianae*. Therefore, when excessive doses of calcium are added, the process occurs later. Mg^{+2} does not present significant contributions for biosynthesis of Cercosporin.

For the two *C. zeina* isolates it was not possible to induce and detect Cercosporin, probably because of the lack of essential substances that are present in maize for the initiation and production of this toxin. This result supports the hypothesis that production only occurs *in planta,* because the pathogen contains the gene that confers self-protection against the toxin (Goodwin & Dunkle*,* 2010).

However, there is huge difficulty in obtaining conclusive results *in planta.* Furthermore, studies with wall-degrading enzymes should be carried out in an attempt to clarify which mechanisms permit this pathogen to be more aggressive than the other species of *Cercospora* in maize that do not produce the toxin *in vitro*, which helps in the pathogenesis process.

Based on the above considerations, it is important to highlight that the fungi, *C. zeina,* does not produce toxin in vitro in the media studied in this work, this does not mean that it does not produce Cercosporin in other media and/or conditions in vitro.

In this work it can be seen that media made with coconut water is highly conducive to Cercosporin production, making this medium a new alternative for producing the toxin.

Furthermore, the toxin may be produced by *C. sorghi* f. sp. *maydis* in media that stimulate sporulation. Although the pathogenicity of this species of Cercospora has not been proven (Carson & Goodman, 2006), these unprecedented results may contribute to future biochemical and gene expression investigations.

Conclusion

It may thus be concluded that the best media in which to induce the toxin production in *C. sorghi* f. sp *maydis* were those made of coconut water Sococo®, especially when supplemented with yeast or with yeast and chlorides of magnesium, manganese and calcium. Production of Cercosporin in STJ is also possible, being visible and detectable by spectrometry. Only the medium M1D did not produce the toxin, but it did produce the latter's typical red pigmentation. Besides, *C. zeina* did not produce Cercosporin under studied conditions.

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