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Bucio-Villalobos, Carlos Manuel; Luna-Olvera, Hugo Alberto; Anguiano-Ruvalcaba, Gloria Laura; Guzmán-de-Peña, Doralinda Asunción

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Effect of Plant Growth Regulators on Mycotoxigenic *Aspergillus* spp. *in vitro*

Carlos Manuel Bucio-Villalobos, Universidad de Guanajuato, Instituto de Ciencias Agrícolas, km 9 Carr. Irapuato-Silao, Ex-hacienda el Copal, Irapuato, Guanajuato, México CP 36820; **Hugo Alberto Luna-Olvera**, Universidad Autónoma de Nuevo León, Instituto de Biotecnología de la Facultad de Ciencias Biológicas, Av. Pedro de Alba y Manuel L. Barragán s/n, Ciudad Universitaria, San Nicolás de los Garza, Nuevo León, México CP 66450; **Gloria Laura Anguiano-Ruvalcaba**, and **Doralinda Asunción Guzmán-de-Peña**, Centro de Investigación y Estudios Avanzados, Departamento de Biotecnología y Bioquímica, Unidad Irapuato, Apdo 629, km 9.6 Libramiento Norte Irapuato-León Irapuato, Guanajuato, México CP 36500. Correspondencia:dguzman@ira.cinvestav.mx

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Abstract. Gibberellic and jasmonic acids and two plant growth regulators (2,4-dichlorophenoxy and naphthalene acetic acids) were evaluated *in vitro* to determine their effect on growth, differentiation, and mycotoxin synthesis in *Aspergillus nidulans* and *A. parasiticus*. Jasmonic acid 1mM had no effect on both fungal species. Gibberellic acid had a stimulatory effect on mycotoxin synthesis in the fungi. Growth factor 2,4-diclorophenoxiacetic acid had no effect in any of the evaluated parameters in *A. nidulans*. However, high concentrations of this compound decreased all the parameters in *A. parasiticus*. High concentration of naphthalene acetic acid decreased growth, sporulation, and sterigmatocystin in *A. nidulans*, while in *A. parasiticus* increased sporulation and decreased aflatoxin synthesis. These results indicate that plant growth regulators modify growth, differentiation, and mycotoxin synthesis.

Additional keywords: *Aspergillus parasiticus*, *Aspergillus nidulans*, gibberellic acid, aflatoxin, sterigmatocystin.

Resumen. Ácido giberélico y jasmónico y dos reguladores de crecimiento de plantas (ácido 2,4-diclorofenoxiacético, y naftalenacético) se evaluaron *in vitro* para determinar su efecto en el crecimiento, diferenciación y síntesis de micotoxinas en *Aspergillus nidulans* y *A. parasiticus*. El ácido jasmónico 1mM no tuvo ningún efecto en las dos especies fúngicas. El ácido giberélico tuvo un efecto estimulatorio en la síntesis de micotoxinas de los hongos. El factor de crecimiento ácido 2,4-dichlorofenoxiacético no presentó ningún efecto en los parámetros evaluados en *A. nidulans*. Sin embargo, altas concentraciones de este compuesto disminuyeron todos los parámetros evaluados en *A. parasiticus*. Altas concentraciones de ácido naftalenacético disminuyeron el crecimiento, esporulación y esterigmatocistina en *A. nidulans*, mientras que en *A. parasiticus* aumentó la esporulación y disminuyó la síntesis de aflatoxina. Estos resultados indican que los reguladores de crecimiento de plantas modifican el crecimiento, diferenciación y síntesis de micotoxinas.

Palabras clave adicionales: *Aspergillus parasiticus*, *Aspergillus nidulans*, ácido giberélico, aflatoxina, esterigmatocistina.

The effect of plant growth regulators on different plant pathogenic fungi has been studied, mainly because alterations in the levels of these compounds are associated with susceptible or resistant reaction in plant pathogen interaction (Singh *et al*., 1997). Early blight of potatoes caused by *Alternaria alternata* (F.:Fr.) Keissl. was under control, when indol acetic acid (IAA), 2,4- diphenol acetic acid (2,4D), and absicic acid (ABA), were exogenously applied (Michniewicz and Rozej, 1988). Auxins such as naphthalene acetic acid ethyl ester (NAA) reduced botrytis blight of cut rose (*Rosa hybrida* L.) flowers caused by *Botrytis cinerea* Pers.:Fr. (Shaul *et al*., 1995). Solutions of NAA in concentration as high as 200 µg/ml decreased micelial growth rate of three isolates of *Sclerotinia sclerotiorum* (Lib.) de Bary *in vitro*, and the same concentration of NAA caused reduced development of *S. sclerotiorum* lesion on cucumber (*Cucumis sativus* L.) detached leaves (Al-Masri *et al*., 2002). ABA and methyl jasmonate caused decreased mycelium growth of *S. sclerotiorum in vitro*. *In vivo*, ABA and gibberellic acid (GA) applied to cucumber plants increased disease development

(Al-Masri *et al*., 2002), while methyl jasmonate decreased mycelial growth rate of *S. sclerotiorum* and reduced mould disease severity on cucumber plants. The effect of the plant regulator varies according to the concentration used, and the inhibitory effect can change when higher concentration of the hormones is used (Al-Masri *et al*., 2002). The antifungal activity was clearly illustrated in *Neurospora crassa* Shear and Dodge (Nakamura *et al*., 1978; Tomita *et al*., 1984) and their use to control phytopathogenic fungi and disease development has been analyzed in different systems. Even though *Aspergillus flavus* (Link:Fr). and *A. parasiticus* Speare grow as saprophytes in soil and they can cause disease in the field (Scheidegger and Payne, 2003; Widstrom, 1996), they are of great concern because they can cause considerable economic losses during production of corn (*Zea mays* L.) and nuts [*Carya illinoensis* (Wangenh.) K. Koch] (Cardwell *et al*., 2001), mainly because its capacity to produce potential carcinogenic substances known as mycotoxins (Payne and Brown, 1998) which can get in the alimentary chain (Task Force Report, 2003). Thus, the need to control *A. flavus* and *A. parasiticus*, their growth, sporulation and mycotoxin production in corn, nuts and other commodities has been aimed and hundred of substances have been analyzed. Substances such as cinnamon, pepper, vanillin (López-Malo *et al*., 1997; Zaika and Buchanan, 1987), caffeine (Buchannan and Lewis, 1984), also fungicides such as dichlorvos or insecticides (Bennett and Christensen, 1983), plant compounds such as glycosides, flavonoids, carotenoids (Norton, 1997) have some inhibitory effect mainly because they affect fungal growth. The effect of plant growth regulator over the synthesis of aflatoxin by *A. flavus* or *A. parasiticus* has been analyzed. The methyl jasmonate added at different concentrations to media inhibited aflatoxin synthesis *in vitro*, but fungal growth was unaffected, spore germination was delayed, and viability was not affected (Goodwrich-Tanrikulu *et al*., 1995). These studies suggested the potential use of this plant regulator during storage of commodities to reduce the level of aflatoxin contamination. However, because growth and spore viability are unaffected, the use of this growth regulator has some restriction in the application, since favorable environmental conditions could trigger aflatoxin synthesis. Therefore, it is necessary to find a substance that could inhibit fungal growth, sporulation, and mycotoxin synthesis, to avoid reactivation of the fungus under favorable environmental conditions. The search of such substance could be facilitated using a fungal model system such as *Aspergillus nidulans* (Eidam) G. Wint. The objective of this work was to analyze the effect of plant hormones and two plant regulators on growth, differentiation, and mycotoxin synthesis in *A. parasiticus* and *A. nidulans* as a part of the effort to control the synthesis of aflatoxin and sterigmatocystin by these mycotoxigenic fungi.

MATERIALS AND METHODS

Strains. *A. parasiticus* NRRL 16992 was obtained from the American Type Culture Collection (ATCC) and *A. nidulans* from the Fungal Genetic Stock Center (FGSC). They were maintained and periodically transferred on slants or plates containing potato-dextrose-agar (PDA, Difco).

Media and culture conditions. *A. parasiticus* cultures were incubated in Erlenmeyer flasks (250 mL) containing 50 mL of Hsieh and Mateles medium (1971) with 5% glucose as carbon source. *A. nidulans* was cultured in 250 mL flasks containing 50 mL of Käfer (1977) medium with 2% sucrose in order to induce sterigmatocystin production. Media were inoculated with 10^7 spores per 50 mL of medium. After the addition of hormones or growth regulators, *A. parasiticus* cultures were incubated at 28°C and *A. nidulans* cultures were incubated at 37°C. Growth, spore number, and aflatoxin (AFB) or sterigmatocystin production were determined after 6 days of growth.

Hormones and growth regulators. The hormones and growth regulators used in this study were: Jasmonic acid, gibberellic acid, 2.4-dichlorophenoxy acetic acid, and naphthalene acetic acid in different concentrations. All were obtained from Sigma Chemical Co. and were dissolved separately in water and sterilized by filtration through millipore (0.45 µm, 25 mm, HAWP). The initial concentrations used were based on those utilized for plant-tissue culture (Hall, 1999). Hormones were added at the time of inoculation.

Determination of growth, sporulation, and mycotoxin synthesis. Mycelium was recovered from the culture media by filtration, resuspended in 10 mL of distilled water containing 0.01% Triton X100, transferred to Falcon culture tubes, and gently shaken to free the spores. Samples of this suspension were taken and spore concentration was determined using a hemocytometer. Spores and mycelium were transferred back to the culture medium and subjected to chloroform extraction to recover aflatoxins, after which, the mycelium was filtered and washed with distilled water, and dried at 70 to 80ºC to constant weight.

Determination of aflatoxins. Cultures treated as described previously, were extracted with 30 mL of acetone for 30 min and with 30 mL of chloroform for 30 min (Keller *et al*., 1994). Extracts were passed by 3 g of anhydrous sodium sulphate, resuspended in 1 mL of methanol (grade HPLC), and purified by extract-clean cartridge C-18 (Alltech, Co.). Quantitative determination of the mycotoxins was achieved by HPLC at 364 nm using a Zorbax LC18 column (DuPont). The mobile phase was a mixture of water-acetonitrile-methanol (45:15:40 by vol.) (Guzmán-de-Peña and Ruiz-Herrera, 1997). Standard solutions of aflatoxins B_1, B_2, G_1, and G_2 were run under the same conditions in order to identify and calculate the concentration of aflatoxins in the samples. Data are expressed per total aflatoxins in 50 mL culture medium. Total amounts, types of aflatoxins, and their ratios varied among experiments. Each experiment was repeated at least three times with duplicate or triplicate samples.

Determination of sterigmatocystin. Sterigmatocystin (ST)

was extracted with acetone and chloroform (Keller *et al*., 1994). Its concentration in the extracts was determined by HPLC using a C_{18} reverse-phase column with a solvent system of deionized water and methanol with the following elution program: from 0 to 5 min, 60% methanol, and from 5 to 20 min, 70% methanol (Guzmán-de-Peña *et al*., 1998). Absolute amounts of ST varied among different experiments, because the intrinsical variability of fungi behavior, but the reported differences observed among samples or treatments were reproducible. All experiments were repeated at least twice with duplicate or triplicate samples.

Experimental design and statistical analysis. The experiments were performed with five replicates per treatment and each experiment had three replicates. The experimental unit was a 250 mL Erlenmeyer flask containing 50 mL of medium. Data were transformed to log (X+1) and analysis of variance and Tukey's test were applied to data using SAS (version 6.12; SAS Institute, Cary, NC, USA).

RESULTS

Jasmonic, gibberellic, 2,4-dichlorophenoxyacetic and naphthalene acetic acids were utilized at various concentrations to evaluate their effects upon the mycotoxigenic capabilities of *A. parasiticus* and *A. nidulans*. Growth, sporulation, and mycotoxin synthesis were not affected when 1 mM jasmonic acid was added to the *A. nidulans* culture. A stimulatory effect on ST synthesis was observed when gibberellic acid was added to *A. nidulans*, even though growth and sporulation was not affected (Table 1). Giberellic acid increased aflatoxin production in *A. parasiticus*, even though the difference was not statistically significant. The growth factor 2,4-dichlorophenoxyacetic acid (2,4D) did not affect any of the evaluated parameters in *A. nidulans* (Table 2), whereas in *A. parasiticus* increased aflatoxin production. When 2,4D concentration was increased (5 and 50 mM), different results were observed in *A. parasiticus* (Table 3): growth was diminished, sporulation decreased dramatically at 5 mM, and aflatoxin was also decreased at 5 and 50 mM, respectively. The results obtained with naphthalene acetic acid (NAA) are interesting. Low concentrations (0.5 mM) diminished sporulation of *A. nidulans* with respect to the control (Table 4) and also

Table 1. Effect of gibberellic acid on mycotoxins synthesis by *Aspergillus nidulans* and *Aspergillus parasiticus*.

Organism	Gibberellic acid (mM)	Growth dry weight (mg/50 ml)	Spore number (x 10^7/50 ml)	Mycotoxin mg/50ml	Mycotoxin ng/mg mycelium
A. nidulans				Sterigmatocystin	
	0	263 a	2.8 a	65 b	247 b
	0.01	301 a	3.6 a	145 a	485 a
	0.10	261 a	2.9 a	158 a	606 a
A. parasiticus				Aflatoxin B_1	
	0	567 a	12 a	448 a	791 b
	0.01	492 a	10 a	504 a	1026 a
	0.10	505 a	18 a	740 a	1467 a

Each value is the mean of five replicates.
Values with different letters in a column are statistically different (Tukey, p = 0.05).

Table 2. Effect of 2,4-dichlorophenoxyacetic acid on mycotoxin synthesis by two species of *Aspergillus*.

Organism	2, 4-D acid (mM)	Growth dry weight (mg)	Spore number (x 10^7/50 ml)	Mycotoxin mg/50 ml	Mycotoxin ng/mg mycelium
A. nidulans				Sterigmatocystin	
	0	309 a	3.7 a	207 a	672 a
	0.02	321 a	3.7 a	221 a	691 a
	0.20	298 a	3.5 a	222 a	746 a
A. parasiticus				Aflatoxin B_1	
	0	559 ab	13 a	821 b	1469 b
	0.02	575 a	18 a	1552 ab	2700 ab
	0.20	492 b	25 a	2221 a	4516 a

Each value is the mean of five replicates.
Values with different letters in a column are statistically different (Tukey, p = 0.05).

sterigmatocystin synthesis was affected negatively. Different behavior of *A. nidulans* was observed when the concentration of NAA was increased to 50 mM (Table 5): growth was decreased, while spore production was totally inhibited, and mycotoxin synthesis was dramatically reduced. When *A. parasiticus* was cultured with 0.5 mM of NAA, growth was affected negatively, whereas sporulation increased three fold and aflatoxin synthesis remained unaffected (Table 6). When the concentration of NAA was increased to 50 mM, growth and aflatoxin synthesis by *A. parasiticus* were greatly diminished, whereas sporulation increased three fold (Table 7).

DISCUSSION

The objective of this study was to determine if hormones or growth regulators have an effect upon growth, development, and mycotoxin production in two *Aspergillus* species. Plant pathogenic fungi have shown to be affected by such plant compounds (Al-Masri *et al*., 2002; Gooddrich-Tanrikulu *et al*., 1995; Michniewicz and Rozej 1987; Shaul *et al*., 1995; Tomita *et al*., 1984) *in vitro* or in plant. It has been clearly shown how disease symptoms can be decreased when plant regulators are applied to the plants. Also, the antifungal activity of gibberellic acid on *Neurospora crassa* was reported several years ago (Nakamura *et al*.,1978). In this study, it was found that 2,4-dichlorophenoxy acetic acid and naphthalene acetic acid, as well as the plant hormone, gibberellic acid affected growth and toxin production on the fungal species tested. Gibberellic acid, for example, did not affect *A. nidulans*, but it stimulated aflatoxin production in *A. parasiticus*. These results are different from those found by Al-Masri *et al*. (2002), who reported that GA reduced mycelial growth rate of *S. sclerotiorum in vitro*. The possible mode of action is by changing the pH of the medium from 4.1 to 3.74. However, Michniewcz and Rozej (1987) reported that GA at 10^{-7} and 10^{-5} M, stimulated growth, sporulation,

Table 3. Effect of 2, 4-dichlorophenoxyacetic acid on aflatoxin synthesis by *Aspergillus parasiticus*.

2,4D acid (mM)	Growth dry weight (mg/50 ml)	Spore number (x 10^7/50 ml)	aflatoxin mg/50 ml	aflatoxin µg/mg mycelium
0	335 a	19 a	752 a	2.2a
5	226 b	6 b	182 b	0.8b
50	219 b	13 a	64 b	0.2b

Values are the mean of five replicates for each treatment.
Values with different letters in a column are statistically different (Tukey, p = 0.05).

Table 4. Effect of low concentrations of naphthalene acetic acid upon *Aspergillus nidulans* FGSC 26 in Käfer's medium.

NAA (mM)	Growth dry weight (mg/50 ml)	Spore number (x 10^7/50 ml)	Sterigmatocystin mg/50 ml	Sterigmatocystin µg/mg mycelium
0	288 a	12.95 a	271 a	0.9a
0.05	283 a	11.05 a	178 ab	0.6ab
0.50	245 b	2.94 b	135 b	0.5b

Values are the mean of five replicates for each treatment.
Values with different letters in a column are statistically different (Tukey, p = 0.05).

Table 5. Effect of concentration of naphthalene acetic acid upon *Aspergillus nidulans* FGSC 26 in Käffer's medium.

NAA (mM)	Growth dry weight (mg)	Spore number* (x 10^7/50 ml)	Sterigmatocyistin mg/50 ml	Sterigmatocyistin µg/mg mycelium
0	220 a	13.9 a	588 a	2.6a
5	178 a	8.4 b	569 a	3.1a
50	25 b	0.0 c	15 b	0.6 b

*Total amount from culture/unit.
Each value is the mean of five replicates.
Values with different letters in a column are statistically different (Tukey, p = 0.05).

Table 6. Effect of concentration of naphthalene acetic acid upon *Aspergillus parasiticus* ATCC 16992 in synthetic medium.

NAA (mM)	Growth dry weight (mg/50 ml)	Spore number* (x 10^7)	Aflatoxin mg /50ml	Aflatoxin µg/mg mycelium
0	614 a	12.58 a	462 a	0.7a
0.05	610 a	15.08 a	654 a	1.0a
0.50	423 b	38.31 b	406 a	0.9a

*Total amount from culture/unit.
Each value is the mean of five replicates.
Values with different letters in a column are statistically different (Tukey, p = 0.05).

Table 7. Effect of concentrations of naphthalene acetic acid (NAA) upon *Aspergillus parasiticus* ATCC16992 in synthetic medium.

NAA (mM)	Growth dry weight (mg/50 ml)	Spore number* (x 10^7/50 ml)	Aflatoxin mg/50 ml	Aflatoxin µg /mg mycelium
0	512a	12.6 a	771 a	1.5 a
5	231b	10.4 a	235 b	1.0 a
50	145c	37.4 b	25 c	0.1 b

*Total amount from culture/unit.
Each value is the mean of five replicates.
Values with different letters in a column are statistically different (Tukey, p = 0.05).

and spore germination of *Fusarium culmorum* (Wm. G. Sm.) Sacc. These authors speculate that GA acts as a nitrogen and carbon regulator during metabolism. The stimulatory effects of low molarities (0.02 and 0.2 mM) of 2,4-dicholorophenoxyacetic acid on aflatoxin production, during the cultivation of *A. parasiticus*, is interesting due to the wide use of this compound as an herbicide. These results are similar to those of Bean and Southall (1983) who studied the effects of pyridazinone herbicides on growth and aflatoxin production by *A. parasiticus*. They found that three concentrations of the compound stimulated synthesis of aflatoxin by *A. parasiticus*. Similar results were obtained when several fumigants were applied to wheat (*Triticum* spp.) cultivated *in vitro* and inoculated with *A. parasiticus*. In those studies, 25% increase in aflatoxin production was obtained due to the addition of the fumigants (Draughon, 1983). Interestingly, in this study, it was found that 50 mM of 2,4-D inhibited aflatoxin synthesis in *A. parasiticus* by 91%. Similar results were found by Damann and Tubajija (2002). They indicated that a high rate of Liberty™ herbicide applied in the field reduced aflatoxin contamination by 70 to 84%. Naphthalene acetic acid had no striking effects at low concentrations (0.05 and 0.5 mM), whereas at 50 mM, growth, sporulation, and mycotoxin synthesis were inhibited in *A. nidulans*. A morphological change was observed associated with these inhibitions, by the fluffy colony type. This type of growth is typical of mutants with deletion on *flb*A and *flu*G genes in *A. nidulans*; these genes are involved in the regulation of growth, sporulation, and regulation of mycotoxin synthesis (Yu *et al*., 1996; Wieser *et al*., 1997). In *A. parasiticus*, low concentrations (0.05 and 0.5 mM) of NAA increased sporulation, while aflatoxin synthesis remained unaffected. However, high concentration of NAA (50 mM) inhibited growth and aflatoxin synthesis up to 97%, but sporulation showed a three fold increase. Super sporulation was described by Calvo *et al*. (1999) when linoleic acid and two of its lipoxygenase derivatives stimulated morphological differentiation in *A. nidulans*, *A. flavus*, and *A. parasiticus*. These authors suggested that fatty acids in the seed interfered with signals that regulate fungal sporogenesis, but they did not analyze the level of mycotoxin (ST) produced by the fungus under those conditions. The effect of plant growth regulators observed in this study, is in agreement with the statement of Al-Masri *et al*. (2002) "plant regulators action varies according to the concentration used and some of the inhibitory or stimulatory effect can change". Therefore, studies are conducted to find out the optimal concentration of NAA that will cause total inhibition of *A. nidulans* and *A. parasiticus* (growth, sporulation, and toxigenic activity) in synthetic culture media and in different grains and nuts.

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