Original Research Article

Production of Acetic Indole Acid in *Macrophomina phaseolina.*

ABSTRACT:

Aims: The fungus *Macrophomina phaseolina* is a phytopathogen of great importance attacking various crops and causing severe losses especially in sorghum, beans, soybeans and others. Since acetic indole acid (AIA) is a hormone that some authors associate with the pathogenic power of other fungi such as *Fusarium oxysporum*, it was decided to perform this work to detect whether *M phaseolin*a isolated from beans is layers of producing AIA and which synthesis pathways are involved in its production.

Study design: In vitro with 3 repetitions.

Place and Duration of Study: Instituto Politécnico Nacional. Centro de Biotecnología Genómica. Laboratorio de Biotecnología Experimental. Blvd del Maestro s/n esq. Elías Piña, Col Narciso Mendoza 88710 Reynosa, Tamaulipas, México. between November 2019 and June 2020.

Methodology: Analyses performed using HPLC.

Results: showed the production of indole acetamide (ACM) that occurs in the first 60 hours of incubation. Another compound produced is Indole (IND) which is released after 60 hours when ACM production has ceased. Similarly, the fungus can metabolize AIA in the culture medium and tryptophan, the main precursor of AIA, apparently has no effect on the amount of AIA synthesized.

Conclusion: The phytopathogenic *fungus Macrophomina phaseolina* can produce indole acetic acid, a hormone that stimulates plant growth through the TRP-D pathway via indole acetamide, which occurs mainly in the first 60 hours of growth of the fungus in the culture medium. In addition, the presence of indole was detected, which allows us to estimate that the fungus uses another little explored route that is TRP-I and that from this compound the AIA can be synthesized directly or transformed into TRP.

*Keywords: [*Biosynthesis, Tryptophan pathway, HPLC, auxinic compounds*)*

1. INTRODUCTION

Macrophomina phaseolina (Tassi) Goid is a fungal pathogen that attacks more than 500 species of plants such as beans, sorghum, corn, cotton, soybeans and sunflower among others (Dinakaran and Mohammed, 2001; Mayek-Pérez *et al*., 2001, 2008; Khan, 2007; Kaur *et al*., 2012). *M phaseolina* causes carbonaceous rot (Mayek-Pérez *et al*., 1997; 1999) taking this name from the dark color of the accumulation of sclerotia on the root surface of infected plants, where they can remain up to 15 years even with water deficiencies and temperatures above 40 ° C (Mayek-Pérez *et al*., 1999; 2001; Wrather *et al*., 2001; Leyva *et al*., 2015; Márquez *et al*., 2021). In the petri dish, the colonies of *M. phaseolina* present dense mycelial growth, with a hairy appearance, at the beginning of dark gray color (1 to 6 days) and later of black coloration (Mayek-Pérez *et al*., 2001; Márquez *et al*., 2021). In advanced stages of infection *M phaseolina* destroys the aerenchyma, leaving only the intact conductive tissues inside the stem (Marquez *et al*., 2021) (Figure 1A) contributing this to the fall of plants (Figure 1B).

Various species of fungi, bacteria and plants can produce AIA, the main plant growth hormone. In the production process of this hormone, the routes called Tryptophan Dependent (TRP-D) and Independent Tryptophan (TRP-I) have been described, depending on the use of the amino acid as a growth cofactor (Östin *et al* 1999; Mano and Nemoto, 2010; Uribe-Bueno *et al*., 2019). Of the TRP-D route, 4 pathways are known which are tryptamine (TRM), Indole-3-pyruvic (IPyA), Indole acetamide (ACM or IAM) and indole acetate nitrile (IAN). As far as the TRP-I pathway is concerned, it starts from anthranilic acid to indole and from there can pass to AIA or TRP (Prinsen, 1993; Sitbon *et al*., 2000; Mano and Nemoto, 2012; Uribe-Bueno *et al*., 2020).

The importance of knowing the synthesis pathways is that one of them, the indole acetonitrile pathway when transformed into AIA, a nitrogenous molecule is released giving rise to biological nitrogen fixation (FBN). This step is the responsibility of nitrogenase, an enzyme controlled by nit genes (Licea-Herrera *et al*., 2021). FBN has been extensively studied in bacteria such as *Azospirillum brasilense, Bradyrhizobium japonicum* and other agents used as inoculants in grasses and legumes respectively (Licea-Herrera *et al*., 2021; González *et al*., 2021; Ahmad *et al*., 2005; Tsavkelova *et al*., 2007; Vega-Celedon *et al*., 2016).

On the other hand, phytopathogens such as *F oxysporum* and other endophytes can colonize plant tissues without causing apparent signs of infection (Pavithra *et al*., 2020) in addition to producing compounds that stimulate the development of colonized plants (Spaepen *et al*., 2007; Kazan *et al*., 2009; Shih-Feng *et al*., 2015; Rana *et al*., 2020). In the case of *M phaseolina*, strains that produce AIA by the ACM and IAN pathways have been described (Suebrasri *et al*., 2020). On the other hand, the production of acetic indole acid has been associated with the beginning of the pathogenic process, so it is of high interest to elucidate whether *M phaseolina* can produce AIA and the synthesis pathways used in it, as this would impact on the knowledge of the host parasite relationship.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

The *Macrophomina phaseolina* strain was provided by the Plant Biotechnology Laboratory of the Center for Genomic Biotechnology of the National Polytechnic Institute and was isolated from soil on Palos Island, Veracruz. The isolation and maintenance of the strain was used YEM agar and for the tests, falcon tubes of 50 mL were prepared with 20 mL of liquid culture medium added or not with 100 ppm of tryptophan, which were inoculated with a disc of 5 mm from the growth zone of the fungus. Subsequently, the tubes were incubated for 96 h at 150 rpm and 37° C. The tests were done in triplicate and samples were taken every 12 hours until 96 h after inoculation. The samples were centrifuged at 3000 rpm for 15 min and the supernatant was recovered, which was then filtered with 0.45µ membranes and deposited in vials to be analyzed by HPLC

For the determination of the compounds, standards of the key auxiliary compounds were used in the determination of the synthesis pathways. The compounds evaluated were, Tryptophan (TRP, Sigma-Aldrich™), acetic indole acid (AIA, Fulka™), Tryptamine (TRM, Aldrich™), Indole 3 acetonitrile (IAN, Aldrich™), Indole acetamide IAM, Aldrich™), pyruvic indole acid (IPyA, Sigma™) anthranil acid (AAN) and Indole (IND). The running conditions were mobile phase of 80:20 (phosphates: acetonitrile) pH 3.1, wavelength of 300 nm, a flow of 1 mL/min, an injection volume of 20 µL in an Ultrasphere C-18™ column 150*4.6 MM 300C (Hernández-Mendoza *et al*., 2008).

Prior to the determination of the samples, a gradual series of each compound was made to construct a calibration curve with coefficient of determination. Only for detected compounds are these calibration curves shown in this article.

3. RESULTS AND DISCUSSION

Calibration curves were performed for all the compounds mentioned in order to confirm the concentrations and in this case only the results for tryptophan (TRP), acetic indole acid (AIA), indole (IND) and indole acetamide (ACM) are shown since they were the compounds detected (Figure 2). The curves and their respective coefficients of determination are shown in Figure 2. The retention times allow to differentiate its presence, since the AIA is observed at 1. 4568 min, the TRP at 1.739 min, the ACM at 2.9782 and finally the IND at 4.6412 min.

On the other hand, the analyses carried out show that *M phaseolina* P05 does have the ability to produce acetic indole acid as part of its metabolism and its production is apparently not influenced by the concentration of TRP since the amounts detected are similar in the culture media added or not with the amino acid

Regarding the determination of the presence of TRP in culture media, 36 hours after the start of the trial, the concentrations are similar in both culture media and the TRP added at the beginning is metabolized before 36 h (Figure 2B).

When analyzing the auxin compounds in the HPLC results, indole acetamide (ACM or AMI) and indole were identified as the only two intermediate auxin compounds between TRP and AIA. In this case, the first compound corresponds to one pathway of the TRP-D synthesis pathways and the presence of Indole is

associated with the TRP-I pathway. This could be interpreted as a change in metabolism influenced by a high concentration of AIA at the beginning and after 60 h of incubation the metabolism of the fungus changes to an alternate route where the IDN participates.

The concentrations detected of AIA by Mahmoud and Mostafa (2017) fluctuate between 11 and 110 ppm, while those detected in this work by the Mpo05 strain of *M phaseolina* vary between 0.6 to 5 pp. In the case of *Trichoderma koningiopsis* strain NRRL50190 and *T asperellum* strain NRRL50191, concentrations fluctuate between 1 and 6 ppm (Uribe-Bueno *et al.,* 2020).

This study confirms the synthesis of acetic indole acid by *Macrophomina phaseolina* and is the first report of the presence that this hormone is produced by *M phaseolina* in a Mexican strain of this phytopathogen. This strain was isolated from beans and several studies have been carried out with it (Mayek-Pérez *et al*., 97, 99, 2001), however, in this fungus no studies have been carried out on the production of this hormone or any auxin compound (Mayek-Pérez *et al*., 1997; Leyva-Mir *et al*., 2015).

With this it can be concluded that the microorganism produces AIA on its own without the need for enrichment with TRP in the culture medium as is the case of other fungi such as *T asperellum, T koningiopsis*, bacteria such as *A brasilense* or *B japonicum* (Uribe-Bueno *et al*., 2020; González *et al*., 2021).

In the samples where there was an addition of the TRP it is estimated that the metabolite potentiates the production of the AIA and the TRP achieving a greater concentration in a shorter time of hours unlike the samples that were not added TRP (Sitbon *et al*., 1997; Uribe-Bueno *et al*., 2020; González *et al*., 2021). In this sense, the TRP is like the samples that were added with TRP. From these results where TRP is added to growth medium of *M phaseolina,* this does not increase its concentration since they do not change in media supplemented with the amino acid.

For the TRP-independent pathway, which is the primary route for AIA production in some plants and microorganisms has an efficacy of up to 90% and is one that is a pathway influenced by environmental factors including light, cold and stress.

4. CONCLUSION

The phytopathogenic *fungus Macrophomina phaseolina* can produce indole acetic acid, a hormone that stimulates plant growth through the TRP-D pathway via indole acetamide, which occurs mainly in the first 60 hours of growth of the fungus in the culture medium. In addition, the presence of indole was detected, which allows us to estimate that the fungus uses another little explored route that is TRP-I and that from this compound the AIA can be synthesized directly or transformed into TRP. This compound is synthesized after 60 hours, once the ACM is no longer synthesized.

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Figure 1. Sorghum stem showing healthy tissue (1A) and damaged with numerous black sclerotia of *Macrophomina phaseolina* (1A below). Right damage in sorghum culture caused of *M phaseolina* on sorghum culture (1B). (image 1A J Luis Hernández- Mendoza. 1B. Ing José Luis Machuca).

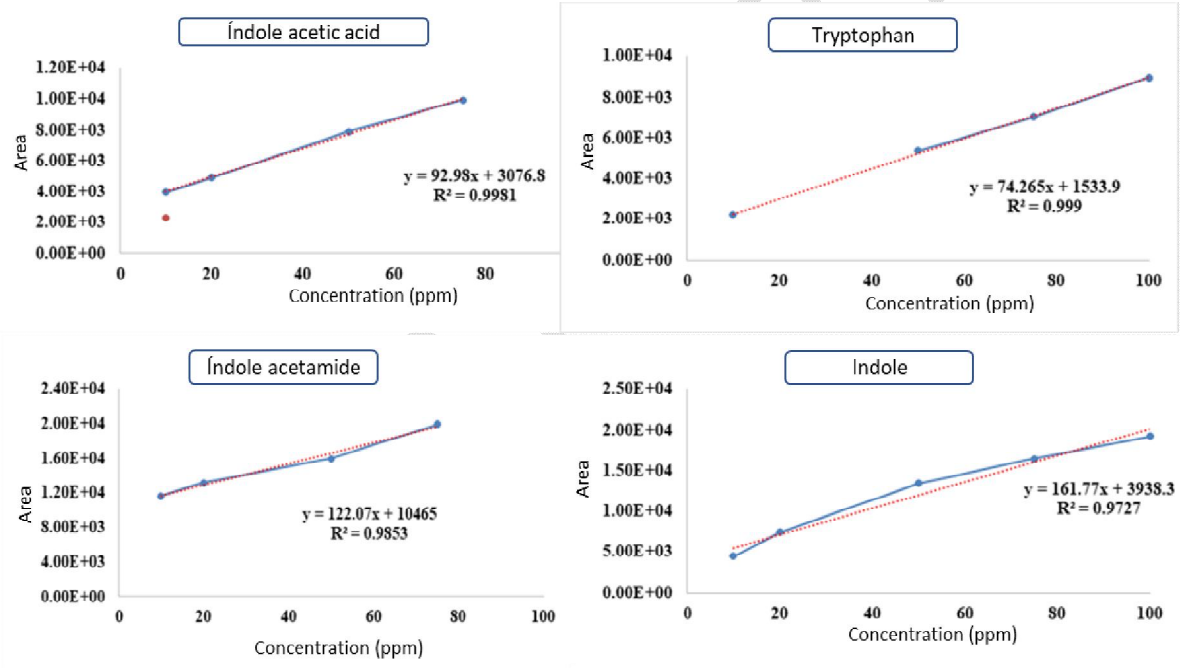

Figure 2. Graphs made with the concentrations and areas obtained in order to estimate the concentrations of these auxin compounds in the samples of the growth of *M phaseolina* in culture medium.

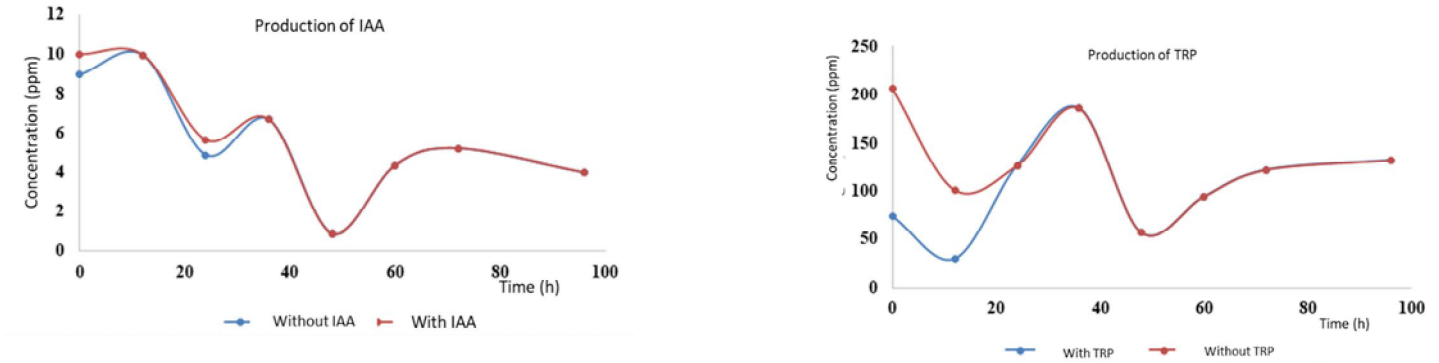

Figure 3. Production kinetics of indole acetic acid by *Macrophomina phaseolina* in a YEM culture medium enriched or not with IAA or TRP.

Figure 4. Production of indole acetamide (ACM) and Indole by *Macrophomina phaseolina* in a YEM culture medium enriched or not with TRP.

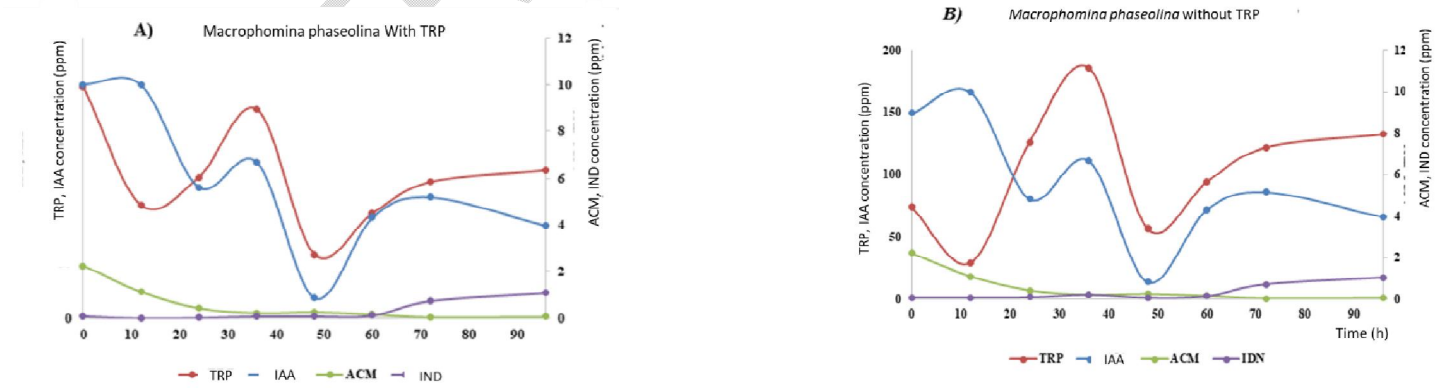

Figure 5. Kinetics of production of auxin compounds (Indole, ACM and IAA) in *M. phaseolina* in YEM medium added (A) or not with tryptophan (B).