REVISTA de la FACULTAD de AGRONOMIA

Identification of indole acetic acid biosynthesis pathways in *Trichoderma asperellum* and *Trichoderma koningiopsis*

Identificación de las vías de biosíntesis de ácido indolacético en *Trichoderma asperellum* y *Trichoderma koningiopsis*

Identificação das vias de biossíntese de ácido indolacético em *Trichoderma asperellum* e *Trichoderma koningiopsis*

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Rev. Fac. Agron. (LUZ). 2025, 42(2): e244229
ISSN 2477-9407
DOI: https://doi.org/10.47280/RevFacAgron(LUZ).v42.n2.XIII.

Received: 26-02-2025
Accepted: 31-05-2025
Published: 02-06-2025

Crop production
Associate editor: Dr. Jorge Vilchez-Perozo
University of Zulia, Faculty of Agronomy
Bolivarian Republic of Venezuela

Keywords:
Indole acetic acid
Tryptophan
Tryptamine
Pyruvic Indole
Pathway IAA

Abstract

Trichoderm spp. produces secondary metabolites associated with plant growth promotion, especially the production of indole acetic acid (IAA), the main plant hormone. The tryptophan-dependent (TRP-D) and tryptophan-independent (TRP-I) production pathways, depending on the precursor involved in IAA synthesis, are well known. The objective of this study was to investigate the tryptophan-dependent (TRP-D) production pathway under *in vitro* liquid culture conditions (Potato Dextrose), supplemented with tryptophan (TRP). The presence of auxinic compounds in TRP-D was quantified using high-performance liquid chromatography (HPLC). Additionally, the morphology of corn seeds was analyzed using scanning electron microscopy (SEM). The interaction of *Trichoderma* spp. with corn seed germination was evaluated under controlled laboratory conditions, conducting the assay in triplicate and performing an analysis of variance (ANOVA). The results showed that the species *T. asperellum* and *T. koningiopsis* can degrade TRP and synthesize IAA through the tryptamine (TRM) and indole acetamide (IAM) pathways. However, IAA synthesis was not detected through the indole pyruvic acid (IPyA) and 3-indole acetonitrile (IAN) pathways. In particular, *T. asperellum* produced significantly higher concentrations of IAA compared to *T. koningiopsis*. Additionally, it was observed that tryptophan supplementation increased IAA production in both species. Finally, *T. koningiopsis* showed a strong relationship with the maize root system, invading the root and establishing a beneficial interaction that could contribute to plant growth and development. These findings suggest that *T. koningiopsis* has significant potential as a biofertilizer in agricultural systems.

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Resumen

El hongo *Trichoderma* spp. produce metabolitos secundarios asociados a la promoción del crecimiento vegetal, especialmente la producción de ácido indol acético (AIA), la principal hormona vegetal. Las vías de producción de triptófano dependiente (TRP-D) y de triptófano independiente (TRP-I), dependiendo del precursor implicado en la síntesis de AIA, son bien conocidas. El objetivo del presente trabajo fue estudiar la vía de producción de triptófano dependiente (TRP-D) en condiciones de medio de cultivo líquido *in vitro* (Papa Dextrosa), suplementado con triptófano (TRP). La presencia de compuestos auxínicos en TRP-D se cuantificó mediante cromatografía líquida de alta resolución (HPLC). Además, la morfología de las semillas de maíz fue analizada utilizando microscopía electrónica de barrido (SEM). La interacción de *Trichoderma* spp. con la germinación de las semillas de maíz se evaluó en condiciones controladas de laboratorio, realizando el ensayo por triplicado y llevando a cabo un análisis de varianza (ANOVA). Los resultados mostraron que las especies *T. asperellum* y *T. koningiopsis* pueden degradar TRP y sintetizar AIA a través de las vías de triptamina (TRM) e indol acetamida (IAM). Sin embargo, no se detectó la síntesis de AIA a través de las vías de ácido indol pirúvico (IPyA) y 3-indol acetonitrilo (IAN). En particular, *T. asperellum* produjo concentraciones significativamente más altas de AIA en comparación con *T. koningiopsis*. Además, se observó que la suplementación con triptófano aumentó la producción de AIA en ambas especies. Finalmente, *T. koningiopsis* mostró una fuerte relación con el sistema radical del maíz, invadiendo la raíz y estableciendo una interacción beneficiosa que podría contribuir al crecimiento y desarrollo de la planta. Estos hallazgos sugieren que *T. koningiopsis* tiene un potencial significativo como biofertilizante en sistemas agrícolas.

Palabras clave: ácido indol acético, triptófano, triptamina, indol pirúvico, vía IAA.

Resumo

O microorganismo *Trichoderma* spp. produz metabólitos secundários associados à promoção do crescimento vegetal, especialmente a produção de ácido indolacético (AIA), o principal hormônio vegetal. As vias de produção dependentes de triptofano (TRP-D) e independentes de triptofano (TRP-I), dependendo do precursor envolvido na síntese de AIA, são bem conhecidas. O objetivo deste estudo foi investigar a via de produção dependente de triptofano (TRP-D) em condições de cultura líquida *in vitro* (Batata Dextrose), suplementada com triptofano (TRP). A presença de compostos auxínicos em TRP-D foi quantificada usando cromatografia líquida de alta eficiência (HPLC). Além disso, a morfologia das sementes de milho foi analisada usando microscopia eletrônica de varredura (SEM). A interação de *Trichoderma* spp. com a germinação das sementes de milho foi avaliada em condições controladas de laboratório, conduzindo o ensaio em triplicado e realizando uma análise de variância (ANOVA). Os resultados mostraram que as espécies *T. asperellum* e *T. koningiopsis* podem degradar TRP e sintetizar AIA através das vias de triptamina (TRM) e indol acetamida (IAM). No entanto, a síntese de AIA não foi detectada através das vias de ácido indol pirúvico (IPyA) e 3-indol acetonitrilo (IAN). Em particular, *T. asperellum* produziu concentrações significativamente mais altas de AIA em comparação com *T. koningiopsis*. Além disso, observou-se que a suplementação com triptofano aumentou a produção de AIA em ambas as espécies. Finalmente, *T. koningiopsis* mostrou uma forte relação com o sistema radicular do milho, invadindo a raiz e estabelecendo uma interação benéfica que poderia contribuir para o crescimento e desenvolvimento da planta. Esses achados sugerem que *T. koningiopsis* tem um potencial significativo como biofertilizante em sistemas agrícolas.

Palavras-chave: ácido indol acético, triptofano, triptamina, indol pirúvico, via IAA.

Introduction

The microorganism *Trichoderma* spp. is widely distributed throughout the world; it can be isolated from soil, decomposing organic matter or agricultural waste, and is associated with the control of agents causing root diseases in wild and cultivated plants (Braithwaite *et al.,* 2017; Lopez *et al*., 2016). *Trichoderma* parasitizes phytopathogenic fungi (Yao *et al.,* 2023), and it has also the ability to set up symbiotic relationships with plants, promoting their development and growth. It secrets secondary metabolites such as auxins, antibiotics, and phytoalexins, as well as compounds involved in the defense system of plants such as salicylic and jasmonic acids (Guzmán-Guzmán *et al.,* 2017; Lubna *et al.,* 2018). Furthermore, it releases enzymes that degrade cell walls and contribute to the bioavailability of nutrients (Ghasemi *et al*., 2020).

Indirectly, *Trichoderma* spp. can alter the microflora present in the soil. In greenhouse and field, it has been proved that *Trichoderma* spp. stimulates growth and production of biomass in *Arabidopsis*, tomato, lettuce, pepper, papaya, passion fruit and beans, among others (SAS, 2004). *Trichoderma* spp. stimulates plant growth through changes in the concentration of plant hormones such as IAA, gibberellic acid, cytokinin, and ethylene (Cai *et al*., 2015; Lubna *et al*., 2018). It has also been reported that when the culture medium contains indole 3 ethanol (IE) the microorganism can use it to synthesize IAA from tryptophan (TRP) via tryptamine (TAM) and tryptophol (TIF) (Leontovyčová *et al*., 2020). When *Trichoderma* spp. is grown in a medium supplemented with IE uses it to synthesize IAA from TRP through the synthesis pathway of TAM and TIF.

In plants, IAA induces the formation of lateral roots and increases the number of root hairs, which helps with nutrient absorption, embryogenesis, growth, and the development of many organs, including roots, hypocotyls, leaves, stems, and flowers; it also contributes to tropism and apical dominance, including the regulatory responses of the plant against environmental changes (Fu *et al*., 2015; Yao *et al.,* 2023). The mechanisms of IAA synthesis are described as TRP-Dependent (TRP-D) or TRP-Independent (TRP-I) pathways. The TRP-I pathway starts with chorismic acid, which is transformed into anthranilic acid (AA) and then to indole 3-glycerol phosphate or indole (Fu *et al*., 2015; Uribe-Bueno *et al*., 2020).

The TRP-D pathway is made up of 4 large pathways: 1) the indole pyruvic acid (IPyA) pathway; 2) the tryptamine (TRM) pathway; 3) the 3-indole acetonitrile (IAN) pathway; 4) the indole-3-acetamide (IAM) pathway (Tariq & Ahmed 2022). It has been found that IAA can be stored in plants such as corn, *Arabidopsis* and *Lotus japonicus*, by conjugating with amino acids such as N-β-D-glucopyranosyl indole-3-acetic acid, and then recovered through a metabolic pathway (Yin *et al.,* 2021; Yao *et al.,* 2023). Of the pathways, the IAN pathway has great importance, since when this molecule converts to IAA, a nitrogenous group is lost, remaining in the soil in the form of nitrogen assimilable by plants. In other cases, IAN is an alternate pathway to camalexin, a substance associated with the resistance of plants to phytopathogens (Abu-Zaitoon *et al*., 2016). The presence of TAM, IAN, IPyA and IAM as pathways for the synthesis of IAA does not automatically translate into an increase for IAA generated, since this compound has a complex regulatory system and can be transformed into similar metabolites such as Indole-3-butyric Acid, 4-chloroindole-3-acetic acid, or associate with amino acids (Zuo *et al*., 2019).

The present study analyzed by HPLC the synthesis pathways of IAA in the fungal species *Trichoderma asperellum* and *T. koningiopsis*, under liquid culture media conditions supplemented with tryptophan as well as interacting with corn seeds.

Materials and methods

Biological material

Pure cultures of native strains of *Trichoderma asperellum* (NRRL50191) were used, originating from the rhizosphere of sunflower (Helianthus annuus), and Trichoderma koningiopsis (NRRL50190), collected from soils cultivated with sorghum (Sorghum bicolor) in northern Mexico. Both *T. asperellum* and *T. koningiopsis* were independently grown in Potato Dextrose Broth (PDB) medium, supplemented with 100 ppm of TRP, and inoculated at a concentration of $1x10^6$ spores.mL^{-1} for each fungus. PDB medium without TRP was used as a control for each treatment. The cultures were incubated for 72 h at 25 °C with continuous stirring at 200 rpm. Samples of 3 mL were taken every 24 h for a total of 120 h. These samples were centrifuged at 10,000 rpm for 10 minutes, and the supernatants were stored at -20 °C until analysis.

Interaction of *Trichoderma* spp. with corn seed germination

Ten seeds of corn (*Zea mays*, Pioneer 30P49) were placed in a 60 mm Petri dish containing 10 mL of a spore suspension (1×10^6 spores.mL^{-1}) for each strain: MTES (Corn control), MTaTES (Corn + *T. asperellum*), MTaTRP (Corn + *T. asperellum* + Tryptophan), MTkTES (Corn + Control), MTkTRP (Corn + *T. koningiopsis* + Tryptophan), and MTkIAA (Corn + *T. koningiopsis* + Indole-3-acetic acid). The assays were conducted in triplicate. Samples of 1 mL were taken every 12 h up to 96 h post-inoculation. These samples were centrifuged, filtered, and analyzed by High-Performance Liquid Chromatography (Hewlett Packard-Agilent™, model 1100; Waldbronn, Germany). The concentration of IAA and other auxinic compounds was determined for each treatment. Analysis of variance (ANOVA) was performed using Tukey's test to compare significant means of treatments, utilizing SAS38 software, version 8 for Windows.

Analysis by HPLC

The supernatants collected from the culture media and corn seeds inoculated with *Trichoderma* spp. were filtered through nylon membranes (0.45 µm, Millipore™; Cork, Ireland) and injected into a High-Performance Liquid Chromatography (HPLC) system using a C18 ultrasphere column (150 x 4.6 mm) (Beckman Ultrasphere™; Fullerton, USA). The mobile phase consisted of 80/20 (deionized water-acetonitrile) with a pH of 3, at a flow rate of 1 mL.min^{-1}. Detection was performed using a UV detector (G1314A, Hewlett-Packard Agilent™; Waldbronn, Germany) at a wavelength of 220 nm (Hernandez-Mendoza *et al*., 2010). The obtained data were compared with runs performed using commercial standards (figure 1).

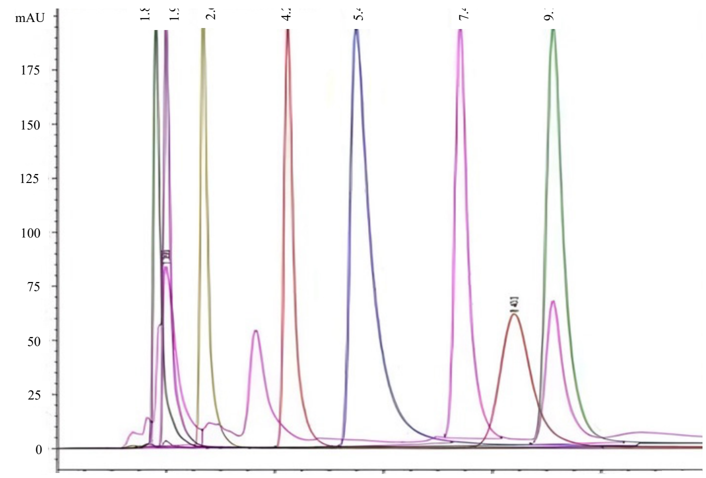

Figure 1. Chromatogram where the standads of the auxinic compounds that were evaluated in this study are integrated. Retention time for Kyn = 1.881 min; L-tryptophan TRP = 1.999 min; tryptamine TRM = 2.681 min; indole acetamide IAM = 4.237 min; indole 3 acetonitrile IAN = 5.496 min; indole-3-pyruvic acid IPyA = 7.408 min; indoleacetic acid IAA = 9.117 min.

Scanning electron microscopy (SEM)

The morphology of corn seeds was analyzed using scanning electron microscopy (SEM) with a Jeol JSM-820 microscope (Jeol Mexico SA de CV, Mexico). The corn samples were recovered from trays and dried before being sent for scanning.

Results and discussion

Synthesis pathways of IAA in *Trichoderma* spp.

The results obtained show that both *T. asperellum* and *T. koningiopsis* (figures 2A and 2B), when grown in PDB medium alone or supplemented with TRP, were able to metabolize TRP and synthesize IAA, IAM, and TRM. Neither IPyA nor IAN were detected in the HPLC analysis, suggesting that the microorganisms under study lack the capability to synthesize these compounds. This indicates that, among the reported pathways of IAA synthesis in different organisms, *T. asperellum* and *T. koningiopsis* utilize only two active pathways: IAM and TRM. The IAM pathway has been described in both bacteria and plant species (Tang *et al.,* 2023).

When *T. asperellum* was grown in media without TRP, it produced up to 61 ppm of IAA at 96 h. In contrast, *T. koningiopsis* synthesized only 16 ppm of IAA in the same period. This increase in IAA synthesis can be attributed to the strain. When TRP was added to the medium, both microorganisms showed an increase in IAA production. These results are consistent with previous findings in *P. veronii*, *P. fluorescens*, *P. putida*, and *Rhizobium* spp. (Bajguz *et al*., 2023; Peñafiel-Jaramillo *et al*., 2016; Nieto-Jacobo *et al*., 2017).

Based on the different amounts of TRP detected over time in media with or without the addition of TRP, we estimated that its availability is determined by the conditions of the medium and the metabolism of the organism itself (Hirayama & Mochida, K., 2022; Peñafiel-Jaramillo et al., 2016; Saleem *et al*., 2024). The Tukey test detected significant differences ($p<0.05$) in the production of IAA, which was higher when the microorganism was grown in media supplemented with TRP (figure 2B).

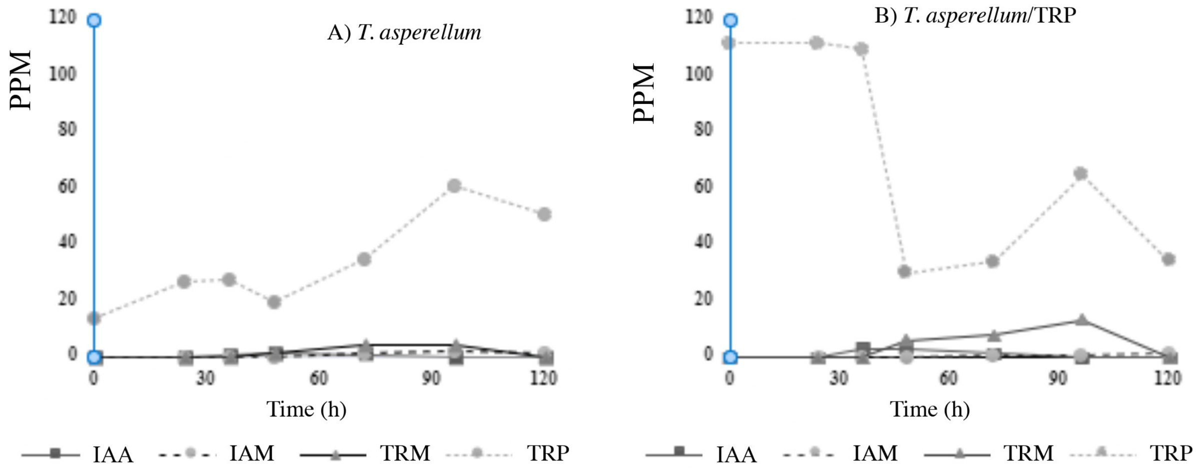

Figure 2. Kinetics of production of auxinic compounds in *T. asperellum* and in culture medium alone (A) and medium enriched with TRP (B).

Interaction of corn seeds with *Trichoderma* spp.

The analysis of auxin compounds in corn germination, both alone and in interaction with *T. asperellum* and *T. koningiopsis* (figures 3A, 3B, and 3C), did not reveal the presence of IAA. These results are consistent with previous studies on *Trichoderma* spp. strains, where IAA synthesis depended on the culture conditions and the physiological state of the seeds (Nieto *et al*., 2017). It is possible that IAA was not detected because it is intracellularly linked with sugars, amino acids, and/or peptides in the seeds, as previously reported.

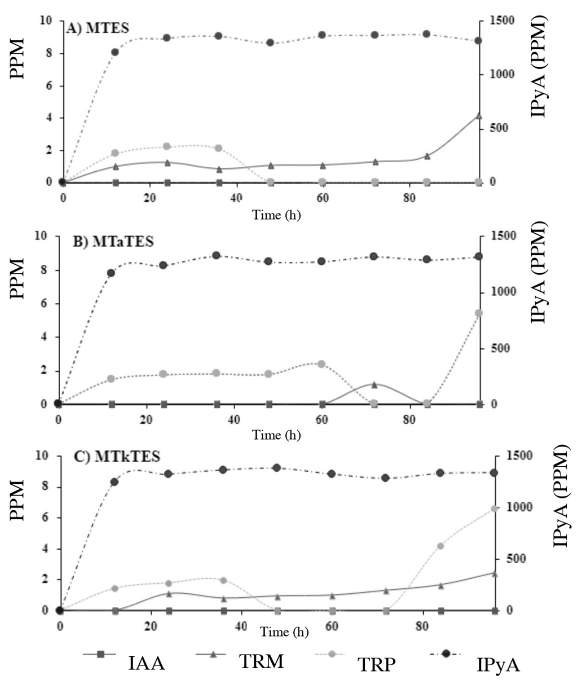

Figure 3. Detection of metabolites in corn germination (Pionner 30P49). A) Corn seeds; B) Corn seed interacting with T. asperellum (Ta); C) Corn seed interacting with T. koningiopsis (Tk). (IAA= Indole acetic acid; TRM= Tryptamine; TRP= Tryptophan; IPyA= Indole Pyruvic acid).

A high production of IPyA (between 1199 and 1313 ppm) was observed in the three control treatments at the end of the kinetics. In the Corn-Control treatment (Figure 3A, MTES), there was an increase in TRM from 1 to 4 ppm after 12 h, with a tryptophan production of 2 ppm between 12 and 36 h. In contrast, in the *T. asperellum*-control treatment (figure 3B, MT a TES), there was a lower production of TRM (2 ppm) at the end of the kinetics, and the concentration of TRP was 7 ppm. In the *T. koningiopsis*-control treatment (Figure 3C, MTkTES), TRM production was 1 ppm at 72 h, with a concentration of 5 ppm at the end of the kinetics.

Regarding auxin compounds, the tryptamine pathway (TRM) is not the major precursor of IAA, as high accumulated levels of tryptamine do not cause any change in IAA levels (Sztein *et al*., 2002). As for IPyA, it is an important pathway for IAA biosynthesis in many microorganisms and plants (Feng *et al*., 2024; Pirog *et al.,* 2022; Morffy & Strader, 2020), but it was not detected in the assays conducted here.

The presence of IAA was detected in the treatments where TRP was added to the corn seeds (Figures 4A and 4B). Furthermore, the IAA synthesis pathway through tryptamine remained active, with an increased production of this auxin in treatment B (MTkTRP). The IAA accumulated due to the availability of TRP. Similar responses have been seen in *T. atroviride* IMI206040, *T. virens* Gv29.8, *T. atroviride* B LU132, and *T. reesei* QM6a, when TRP was added to the culture medium (Etesami & Glick, 2024; Nieto-Jacobo *et al*., 2017). Our data indicated that the indole-3-pyruvic acid (IPyA) pathway was stimulated in treatments with inoculated corn (Figure. 4). In other cases, the production of IAA was influenced by the abiotic factors affecting the plants and microorganisms, including the content of nutrients, humidity and pH (Etesami & Glick, 2024). This work demonstrates the chemical interaction of *Trichoderma* spp. with corn seeds as been reported in earlier works (Naveed *et al*., 2015; Dam & Bouwmeester, 2016), by detecting synthesis of IAA in *T. asperellum* (NRRL50191) at 36 h; the concentration of IAA increased until it reached a maximum of 6 ppm at 60 h and decreased later to 3 ppm.

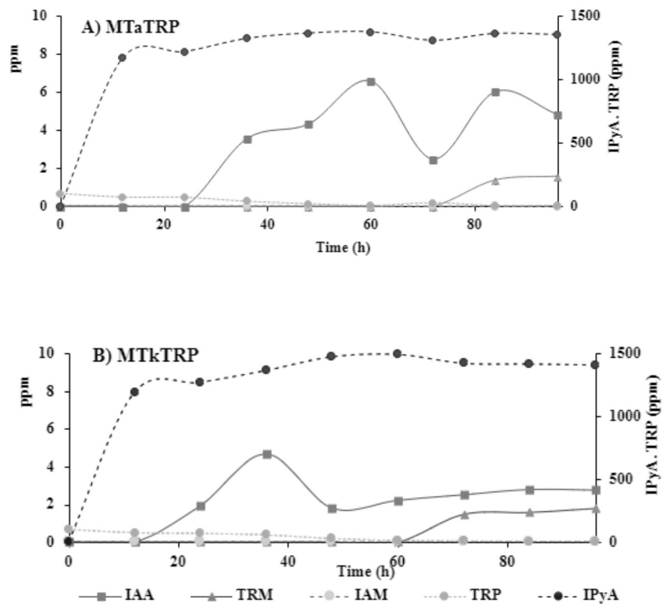

Figure 4. Detection of auxin metabolites in corn seeds germinating (Pionner 30P49) in a medium supplemented with TRP (Tryptophan). A) Interacting with T. asperellum (Ta); B) Interacting with T. koningiopsis (Tk). (IAA= Indole acetic acid; TRM= Triptamine; IAM= Indole acetamide; TRP= tryptophan; IPyA= Indole Pyruvic acid).

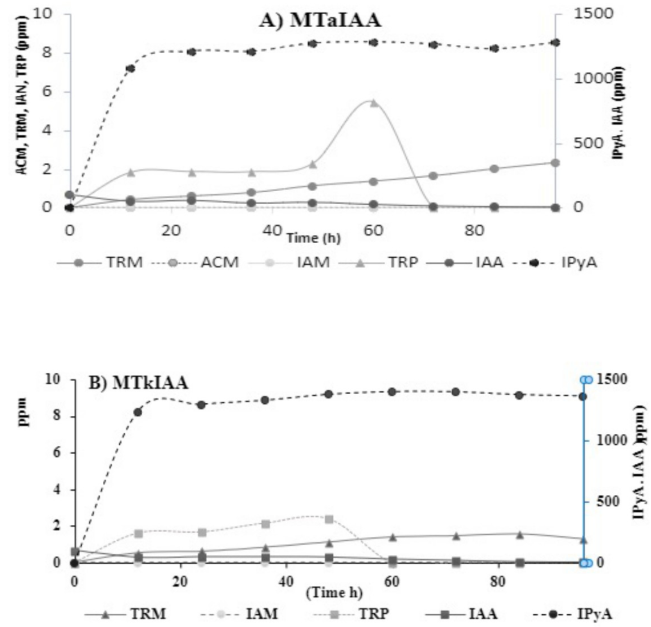

Figure 5. Detection of auxins in corn seeds germinating (Pionner 30P49) in a medium supplemented with IAA (indole acetic acid) and interacting with: A) T. asperellum (Ta); B) T. koningiopsis (Tk). (ACM=Indole acetonitrile; TRM= Triptamine; IAM=Indole acetamide; TRP=tryptophan; IAA= Indole acetic acid; IPyA= Indole Pyruvic acid).

In the case of *T. koningiopsis* (NRRL50190) the production of IAA started at 24 h and reached its maximum (5 ppm) at 36 h. This is a significant difference ($p<0.05$) with the inoculation of corn seeds with *Trichoderma*. Both isolates stimulate germination by activating biochemical and transcriptional processes more quickly through chemiosmotic reactions, favored by the pH gradient in the plant cells and the excretion of exudates, which are captured by the microorganisms, increasing the synthesis of IAA (Li *et al*., 2015; Peñafiel-Jaramillo *et al*., 2016). There were no significant differences (Tukey 0.05) in the production of IAA between corn seeds interacting with *T. asperellum* in media supplemented with TRP and between the treatments MTaTRP (*T. asperellum* average 2.288889a) and MTkTRP (*T. koningiopsis* average 1.833333ab), but there were significant differences between these treatments and the control (MTaTES, average 1.011111ab and MTkTES, average 0.844444b) (Table 2). In the assays where the interaction of *Trichoderma* with corn seeds was evaluated in a medium rich in IAA (figures 5A and 5B), no significant differences ($p<0.05$) in the synthesis of IAA and IPyA were observed between the two microorganisms, nor when compared with the control. Furthermore, regarding the synthesis of TRP, the highest concentration (5 ppm) was detected in the treatment Corn-*T. asperellum*-IAA. With respect to the degradation of IAA, there were no significant differences ($p<0.05$) between treatments.

The interaction between the corn radicle and *Trichoderma* spp. was confirmed by the colonization of the root. Scanning electron microscopy showed hyphal penetration and the formation of haustoria next to the site where the mycelium enters the radicular cortex (figure. 6). In this case, the most important thing is that the hyphal cell that penetrates the cortex has a cell wall with distinct characteristics than the rest of the mycelium, which remains outside. This hyphal cell has a thinner and more flexible cell wall, while the hyphal cells that remain outside have a thicker and more solid cell wall.

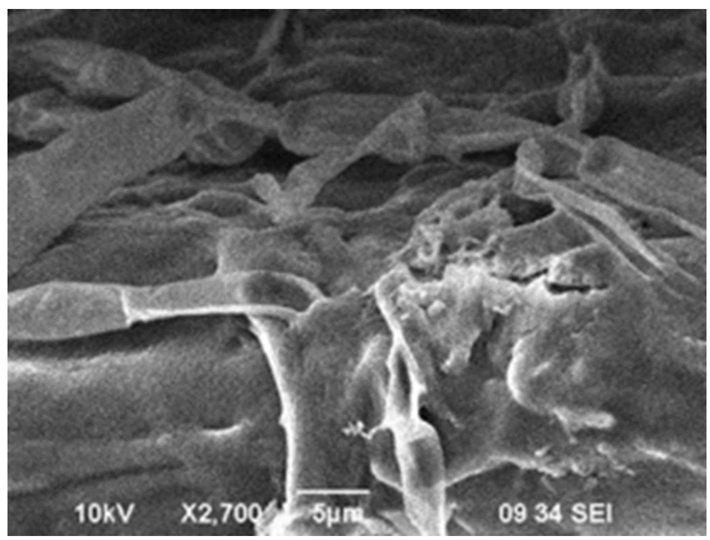

Figure 6. Hyphae of *T. koningiopsis* penetrating the root cortex of a corn seedling (30P49 Pioneer) six days after germination.

Conclusions

The fungi *T. asperellum* NRRL50191 and *T. koningiopsis* NRRL50190 produce IAA through the TRM and IAM pathways, but they are unable to convert IAN and IPyA into IAA. The production of IAA changed when corn seeds were inoculated with spores of *Trichoderma*, forming a close association due to the fungal hyphae penetrating the rootlets of the seedlings.

Acknowledgments

Romero-Domínguez had a CONACYT scholarship during his Master of Science studies (B091484). Hernández-Mendoza, Quiroz-Velásquez, and Hernández-Delgado are EDI-IPN. Hernandez-Delgado is COFAA-IPN. Hernández-Mendoza and Quiroz-Velásquez are SNI.

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