SUMMARY

The bacterium Bradyrhizobium japonicum induces nodulation in Glycine max (soy) plants and other legumes. It is considered of great importance, since it is in the nodules that the bacteria are established, contributing to the biological fixation of N₂. The process is controlled by nitrogenase, an enzyme produced by the nif genes present in the genome of the bacterium. By metabolizing the nitrogenase, the indole acetonitrile transforms it into indole acetic acid (IAA) and releases a nitrogenated molecule. There have been other IAA synthesis routes reported in plants, other genera and species of bacteria, fungi, and algae, named tryptophan-dependent (TRP-D) or tryptophan independent (TRP-I), where this amino acid is the precursor. For TRP-D, there are four pathways to the synthesis of IAA, and only two for TRP-I. The microorganisms may or not have all the routes in their genomes, and the expression of the genes varies with the isolation and the genotype of the host plant. This work reports the results obtained from an B. japonicum soy isolate, cultivated in an enriched LB medium, or alternatively, with tryptophan. With the data obtained, we estimate that B. japonicum uses both TRP-D and TRP-I routes, since in the former type indole acetamide was detected, and in the latter, indole and anthranilic acid were found. Likewise, the presence of TRP in the medium may alter IAA synthesis routes.

KEYWORDS / Biosynthesis / Indole Acetamide / Indole Acetonitrile / Tryptophan-Dependent / Tryptophan Independent /
soybean plant (Ghosh and Basu, 2002; Sprent, 2007; Remans et al., 2008). Five pathways have been proposed for IAA synthesis in bacteria: indole-3-acetamide (IAM), tryptamine (TAM), Trp side-chain oxidase (TSCO), indole-3-pyruvic acid (IPyA) and indole-3-acetonitrile (IaN) pathway; also, one tryptophan-independent pathway from indole-3-glycerol phosphate or indole (Grunenvaldt et al., 2018). When this takes place, B. japonicum, just like other bacterial species in the rhizosphere, uses a metabolic route in which the indole acetonitrile (IaN) is transformed into IAA, a pathway that is mediated by different enzymes, including nitrile hydratase and nitrilase (Rojas et al., 2009; Cassan et al., 2014; Rivera et al., 2018; Duca and Glic, 2020).

In plants, bacteria, fungi, and other organisms, some IAA synthesis routes have been reported to use tryptophan (TRP) as a precursor, which is why they are considered TRP-dependent (TRP-D). In the end, the TRP-D route synthesizes IAA through four different ways: the indole acetic acid (IAM) or ACM route, the pyruvic acid (IPyA) route, the indole acetonitrile (IaN) route and, the tryptamine (TRM) route (Carreño-López et al., 2000; Spaepen et al., 2007; Mano and Nemoto 2012; Cassán et al., 2014). Out of the four routes mentioned, the IPyA route takes place preferably in plants, although it is present in some genera and species of bacteria, such as B. japonicum (Jones et al., 2007), but it is absent in other bacteria studied in our laboratory, such as Trichoderma asperellum and T. koningiiopsis, as the route was not detected using HPLC (Hernández-Mendoza et al., 2008). The TRM and IAM routes have been reported in a wide group of genera and species of bacteria, fungi and plants (Glickman et al., 1998; Spaepen et al., 2007; Naturat et al., 2016).

In these TRP-D routes, the use of TRP has been described as a precursor of the IAM, which stimulates some routes or inhibits others (Kamilova et al., 2006; Idris et al., 2007; Spaepen and Vanderlyden, 2011). In Azospirillum brasilense the presence of TRP in the culture medium in concentrations of 100 and 200ppm, leads to the intensification of a route such as the TRM one and, on the other hand, the route of ACM or IAM become negatively affected in the culture medium (Carreño-López et al., 2000).

Other pathways can also be used by these microorganisms in the synthesis of IAA and they do not use TRP as a precursor (Ona et al., 2005; Idris et al., 2007). They are known as TRP-independent (TRP-I), which begin with the incorporation of anthranilic acid (ANA), which comes from the chorismic acid, to then transform it into phosphoribosyl anthranilate, and from there, to indole (IND) (Cohen 1999; Spaepen et al., 2007; Remans et al., 2008). From this point onwards, there are genes that coordinate the change of this compound into TRP, and thereafter they restart the synthesis of the hormone via the TRP-D routes (Phi et al., 2008). Bacterial genera and species such as A. brasiliense report-edly release ANA and IAA in the culture medium (Hernández-Mendoza et al., 2008). The other route beginning at IND is the change into IAA, where no genes have been described to control this reaction (Idris et al., 2007). An alternate TRP-I route, of which there are scarce references, is the one involving the change from indole-3-glycerol phosphate directly into indole with the action of indole-3-glycerol phosphate ligase/indole synthase.

According to the above information, B. japonicum stimulates the growth of soybean plants and, therefore, the aim of the present work is to analyze the TRP-D or TRP-I routes for the synthesis of IAA used by B. japonicum BjBV-05 isolated from soybean and grown in culture media, enriched or not, with tryptophan.

Materials and Methods

The B. japonicum BjBV-05 strain was isolated from nodules of the vernal variety of soybean and provided by the Plant Biotechnology Laboratory, Genomic Biotechnology Center, Instituto Politécnico Nacional, Mexico. The culture medium used to activate the strain and prepare the inoculation was yeast-extract mannitol (YEM) incubated at 30°C for 72h at 200rpm. The tests to synthesize metabolites were carried out in 50ml Falcon tubes, with 20ml of LB medium, enriched and non-enriched with 100ppm of tryptophan. Each treatment was carried out in triplicate, incubated in the same medium not enriched with trypto

Other compounds found in the culture medium with TRP enrichment (Figure 2) are ACM and indole acetonitrile (IAN), which maintained a constant production during incubation and the highest point of synthesis was after 72h. In turn, in the same medium enriched with TRP, compounds of route TRP-I, which are key steps in the synthesis for the

Results

The population of B. japonicum in the culture medium without TRP (Figure 1) increases rapidly and reaches the peak of growth after 48h, to slowly decrease thereafter. In the culture medium enriched with TRP, a peak was observed after 48h, to decrease slightly after 72h and, from there, begin an exponential growth phase for B. japonicum. According to the estimations of population growth by optic density, after 48h of incubation, the B. japonicum population was slightly higher, but with no significant statistical difference.

The strain of B. japonicum only synthesized IAA in the culture medium enriched with TRP (Figure 2), which confirms that TRP is an enhancement factor for the formation of this plant hormone, since in the culture medium that was not enriched with tryptophan, the hormone was not detected.

When B. japonicum was grown in a culture medium without TRP enrichment (Figure 3), the indole aceto

Other compounds found in the culture medium with TRP (Figure 2) are ACM and indole acetonitrile (IAN), which maintained a constant production during incubation and the highest point of synthesis was after 72h. In turn, in the same medium enriched with TRP, compounds of route TRP-I, which are key steps in the synthesis for the
generation of IAA, were identified. Anthranilic acid (ANA) reached the peak of production after 72h and indole (IND) was generated during all of the B. japonicum incubation period.

In the case of the auxinic compounds found in the culture medium without TRP (Figure 3) the compounds detected in the amounts generated were observed to be different to those found in the culture medium with TRP. Here, although TRM, ACM and IAN were found, IAA was not. In this case, as in the media with TRP, TRM was found in small amounts and IAN showed no variations during the entire time of incubation. The results of the chromatograms of the analyzed samples did not indicate the presence of indole pyruvic acid, and it is therefore estimated that, at least with this methodology, this pathway may not exist in B. japonicum.

Regarding the synthesis of IAA with the route of TRP-I present in B. japonicum, this was confirmed to be possible, since it was detected by HPLC, anthranilic acid (ANA) and indole. Indole was detected in the first 12h and it remained present in the same levels up to 96h, while ANA showed a peak of high production at 72h of incubation, to later disappear.

Some kinetics are described in particular ways, as they are considered more important in the studies performed. Figure 4, for example, shows that B. japonicum did not synthesize IAA in the absence of TRP, confirming that TRP acts a promoter in this genus and species of bacteria.

On the other hand, IAN production is related to the biological fixation of nitrogen, and the data obtained show that B. japonicum did not synthesize more IAN when the culture medium did not contain TRP; yet, after 48h of incubation, concentrations were practically similar (Figure 5).

Regarding indole-3-hydroxyethyl (tryptophol), it was observed that its concentration was low in the absence of precursor TRP in the culture medium (Figure 6) and its synthesis began in the first hours of incubation, whereas in the presence of the precursor, the formation increased until it reached 35ppm after 82h.

Based on the data presented, Figure 7 outlines the routes used by B. japonicum towards the synthesis of IAA, both the tryptophan-dependent and the tryptophan-independent ones, since the auxinic compounds involved in both routes were detected in this study.
Rhizobacteria synthesize indol acetic acid (IAA) among the compounds that they release into the growth culture medium, and in the case of *B. japonicum* high amounts of IAA are produced, since these bacteria are important synthesis centers (Bashan et al., 2014; Ona et al., 2005; Tsavkelova et al., 2007). *B. japonicum* modulates the soybean plant roots to carry out the biological fixation of nitrogen, where it synthesizes IAA, the main plant growth hormone.

In this study, tryptophan (TRP) was used for enrichment of the LB culture medium, as it can be naturally found among the compounds that make up the radicular exudates (Kravchenko et al., 2004) and it has been reported to be a precursor in the synthesis of IAA (Ghosh and Basu, 2002; Idris et al., 2007; Guruprasad et al., 2011; Mohite, 2013). The results obtained show that the media in which TRP was added is where the highest amount of IAA was produced.

IAA synthesis pathways are divided depending on the use or not of TRP as a precursor. One of them, TRP-D, depends on this amino acid, and four routes are known for it, according to the key auxinic compounds generated: the route of indole pyruvic acid (IPyA), the indole acetamide (IAM or ACM) route, the route of tryptamine (TRM) and the route of indole acetonitrile (IAN) (Normanly, 2010; Mano and Nemoto, 2012; Nonhebel, 2015; Naturatat et al., 2016; Li et al., 2018). Out of these, routes of IAM, ACM and IAN were detected in this work and they correspond to the TRP-D pathway.

The TRM route has previously been described in genera and species of *Bacillus cereus* and *Azospirillum brasilense* (Hartman et al., 1983; Remans et al., 2007), and is absent in other genera such as Arthrobacter pascens strain ZZ21 (Li et al., 2018). In yeast such as *Rhodospiridiobolus flaviales* DMKU-CP293 and *R. paludigenum*, this route is also reported in a wide range of organisms that used TRM for the synthesis of IAA. Also, fungi such as *Fusarium graminearum* use this same route (Luo et al., 2016; Bunsangiam et al., 2019). The route of indole acetamide (ACM or IAM) has been described in *B. japonicum* (Spaepen and Vanderlryden, 2011; Glickmann et al., 1998) and in other microorganisms; this compound was detected in the present study.

Pyruvic acid (IPyA) was not present among the compounds detected in this work, and we therefore assume that the strain used does not use this path of the synthesis of IAA. This TRP-D
route has been reported in plants and fungi such as *Neurospora crassa*, where it seems to be one of the main routes for IAA synthesis (Phi et al., 2008; Sandar and Kempken 2018).

Indole (IND) and AAN, reported as precursors of IAA, were detected in the present study. Therefore, we estimate that they are produced by the studied strain which, along with fungi and plants, has the ability to produce IAA using TRP-I pathways (Normanly, 2010; Nonhebel, 2015; Naturatat et al., 2016; Li et al., 2018).

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ESTUDIO DAS VIAS DE BIOSSÍNTESE DO ÁCIDO INDOLE-ACÉTICO EM Bradyrhizobium japonicum BBJV-05

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RESUMO

A bactéria Bradyrhizobium japonicum induz a nodulação em plantas de Glycine max (soja) e outras leguminosas. Isto é considerado de grande importância, já que é nos nódulos onde as bactérias se estabelecem, contribuindo para a fixação biológica do N₂. O processo é controlado pela nitrogenase, uma enzima produzida pelos genes nif presentes no genoma da bactéria. Ao metabolizar a nitrogenase, a indol acetamida é o precursor. Para TRP-D, há quatro vias para a síntese de IAA e apenas duas para TRP-I. Os microorganismos podem ter ou não todas as rotas em seus genomas, e a expressão génica varia com o isolamento e o genótipo da planta hospedeira. Este trabalho relata os resultados obtidos a partir de uma cepa de B. japonicum isolada de soja, cultivada em um meio LB enriquecido, ou alternativamente, com triptófano. Com os dados obtidos, estimamos que B. japonicum utiliza ambas rutas TRP-DR e TRP-I, já que na primeira via se detectou indol acetamida, e na segunda, indol e ácido antranílico. Assim, a presença de TRP no meio pode alterar as rotas de síntese de IAA.

RESUMEN

La bacteria Bradyrhizobium japonicum induce la nodulación en plantas de Glycine max (soya) y otras legumbres. Esto es considerado de gran importancia, ya que en los nódulos donde las bacterias se establecen, contribuyendo a la fijación biológica de N₂. El proceso es controlado por la nitrogenasa, una enzima producida por los genes nif presentes en el genoma de la bacteria. Al metabolizar la nitrogenasa, el indol acetamida se transforma en ácido indol acético (IAA) y libera una molécula nitrogenada. Se han reportado otras rutas de síntesis de IAA en plantas y en otros géneros y especies de bacterias, hongos y algas, denominadas dependientes de triptófano (TRP-D) o independientes de triptófano (TRP-I), donde este aminoácido es el precursor. Para TRP-D, hay cuatro vías para la síntesis de IAA y solo dos para TRP-I. Los microorganismos pueden tener o no todas las rutas en sus genomas, y la expresión de los genes varía con el aislamiento y el genotipo de la planta huésped. Este trabajo reporta los resultados obtenidos a partir de una cepa de B. japonicum aislada de soya, cultivada en un medio LB enriquecido, o alternativamente, con triptófano. Con los datos obtenidos, estimamos que B. japonicum utiliza ambas rutas TRP-DR y TRP-I, ya que en la primera vía se detectó indol acetamida, y en la segunda, indol y ácido antranílico. Asimismo, la presencia de TRP en el medio puede alterar las rutas de síntesis de IAA.