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Partial characterization of a bacterium isolated from tomato with hairy root disease

Caracterización parcial de una bacteria aislada de tomate con la enfermedad de las "raíces peludas"

PACHECO-AGUILAR, Juan Ramiro† & JIMÉNEZ-MU, José Andrés*

Universidad Autónoma de Querétaro, Facultad de Química, Cerro de las campanas s/n, col. Las Campanas, C.P. 76040. Santiago de Querétaro Qro.

ID 1st Author: *Juan Ramiro, Pacheco-Aguilar* / **ORC ID:** 0000-0001-8365-4488, **CVU CONACYT ID:** 87499

ID 1st Co-author: *José Andrés, Jiménez-Mu* / **ORC ID:** 0000-0002-2574-0043

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Abstract

Agrobacterium rhizogenes is a bacterium that in recent years has gained importance for affecting greenhouse hydroponic crops such as tomatoes, peppers and cucumbers, causing excessive root proliferation known as hairy root disease. In the present study, eight rhizospheric bacteria isolates from a tomato crop affected by this disease were characterized. The KOH 3 % test initially made it possible to discriminate the strains, which were later tested for their catalase activity and their ability to grow in selective media such as MG-Te, McConkey and Hofer´s. The results showed that all the strains have catalase activity, but not all of them grew in the selective media, with the exception of the strain named 5(3)4, which was selected to perform *in vitro* potato infection test. Finding 24 days after inoculation, the formation of adventitious roots. This indicates that this isolate could be involved in the rhizogenesis induction of tomato plants, causing the hairy root phenotype.

T-DNA, Rhizogenesis, Semiselective culture medium

Resumen

Agrobacterium rhizogenes es una bacteria que en años recientes ha cobrado importancia por afectar cultivos hidropónicos bajo invernadero tales como tomate, pimiento y pepino, provocando una proliferación excesiva de raíces conocido como la enfermedad de las "raíces peludas". En el presente estudio se aislaron y caracterizaron ocho bacterias rizosféricas provenientes de un cultivo de tomate afectado por dicha enfermedad. La prueba de KOH 3 % permitió inicialmente discriminar las cepas, las cuales después fueron ensayadas en su actividad catalasa y en su capacidad para crecer en medios semiselectivos tales como MG-Te, McConkey y Hofer´s. Los resultados mostraron que todas las cepas poseen actividad catalasa, pero no todas crecieron en los medios selectivos, a excepción de la cepa nombrada como 5(3)4, la cual fue seleccionada para realizar *in vitro*, ensayos de infección de discos de papa, encontrando a los 24 días después de la inoculación, la formación de raíces adventicias. Lo que indica que este aislado podría estar involucrado en la inducción de rizogénesis de las plantas de tomate, provocando el fenotipo de las "raíces peludas".

T-ADN, Rizogénesis, Medio de cultivo semiselectivos

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Correspondence from the author: (E-mail: jmu.andres@hotmail.com)
† Contributing researcher as first author.

Introducción

In recent years, there have been an increasing number of reports of hydroponic greenhouse crops being affected by "hairy root" disease, which is characterised by excessive root proliferation on the plant, affecting crop production and yield (Bosman et al. 2017). The causal agent is *Agrobacterium rhizogenes*, which has been a model for laboratory studies for many years with biotechnological applications. The cause of the "hairy root" phenotype is due to the transfer of bacterial genetic material (T-DNA) into the nucleus of plant cells, which carries genes that control the synthesis of auxins and cytokinins, hormones that promote cell division and differentiation (Nartop, 2018).

For the isolation and characterisation of these bacteria, traditional media for the genus *Agrobacterium* are used, which may not be accurate to discriminate the species, since new molecular tools have allowed the classification of the species causing rhizogenesis into Biovar 1: *Agrobacterium rhizogenes* and Biovar 2: *Rhizobium rhizogenes* (Bosman et al., 2017).

In the present study, eight bacterial isolates from tomato rhizosphere exhibiting the "hairy root" phenotype were characterised by biochemical tests and semi-selective culture media.

Methodology to be developed

Isolation by selective enrichment

Root samples of tomato plants from a production greenhouse, showing the "hairy root" phenotype, were sown in MG-Te liquid medium (Campillo et al., 2012), which contained (g/L): D-mannitol 5.0, L-glutamic acid 2.0, KH_2PO_4 0.5, NaCl 0.2, $MgSO_4 \cdot 7H_2O$ 0.2 g, yeast extract 0.5 g and K_2TeO_3 0.2. This medium is suggested for the isolation of bacteria of the genus *Agrobacterium*. Once the culture medium showed turbidity due to microbial growth, aliquots were taken for two subsequent subcultures, using the same liquid culture medium, in order to enrich the microorganisms growing on this selective medium. Finally, aliquots of the third enrichment culture were sown on nutrient agar to obtain isolates which were classified according to their colonial morphology.

Biochemical tests

KOH test

This is a rapid alternative test used to classify a bacterial strain as Gram-positive or Gram-negative. For this, a bacterial colony from a fresh culture is picked up with a sterile loop and mixed with two drops of 3% KOH on a slide for 30 seconds. Bacterial lysis observed by the formation of a mucoid thread on lifting the loop is considered a positive result for Gram-negative bacteria (Halebian et al., 1981).

Catalase test

This test is performed by transferring a colony of the freshly grown bacterial strain (24 h) to a slide using a sterile loop, then adding 1 drop of 3% H2O2, if the strain contains the enzyme catalase, its activity will produce effervescence (Rahman et al., 2020).

McConkey Agar

This medium is used for the identification of enteric Gram-negative bacteria, which has also been used for the characterisation of Agrobacterium isolates (Asbin et al., 2017). The culture medium contains (g/L): meat peptone 1.5, gelatin peptone 17.0, casein peptone 1.5, lactose 10.0, bile salts 1.5, NaCl 5.0, neutral red 0.03, crystal violet 0.001 and agar 13.5, which is prepared and poured onto plates, where the study strains are then streaked. After 48 h of growth, the pinkish-reddish colouration of the colonies indicates lactose fermentation, while non-fermenting colonies are colourless.

Hofer's medium

This selective medium is recommended for the isolation of *Agrobacterium* species (Gupta et al., 2012), which consists of (g/L): Mannitol 10.0, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.1, yeast extract 1.0, thymol blue 0.016 and agar 15.0. Strains are streak-seeded on this medium and those showing growth with a colour change to yellow are considered positive for Agrobacterium.

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MG-Te medium

This culture medium is prepared as described in the section on isolation by selective enrichment, except that 18 g/L agar is added to the liquid medium to solidify it. The strains are then streaked and incubated at 28 °C for 48 h, bacterial growth accompanied by a black precipitate is considered positive for this biochemical test.

Infection test on potato discs

The strain was inoculated in 10 mL of Luria Bertani broth and placed in constant agitation for 16 h at 28 °C. The culture was then centrifuged at 4000 rpm to recover the cell pellet, which was resuspended in 10 mL of 0.85 % NaCl and centrifuged again to remove any remaining culture medium. Finally, it was resuspended in a minimum volume (2 mL) of saline. Cell counting was carried out in a Neubauer chamber, adjusting the density to 10^8 cells/mL. Then, 100 µL of this suspension were inoculated onto disinfected potato discs (Gaafar, 2015) placed on sterile towels in a Petri dish that served as a humid chamber, which was incubated at 28 °C for 24 days.

Results

Obtaining bacterial isolates

From the enrichment culture on MG-Te selective medium, eight bacterial isolates were obtained that presented differences in their morphologies, these were named as: Cj 1, Cj 3, Cj 5, 4(3)1a, 5(3)3H, 5(3)3a, 5(3)3-1 and 5(3)4.

Biochemical tests

Table 1 shows in summary the results of the tests performed on the bacterial isolates, we can find that only two of them were Gram positive, which were discarded, because the species of the genus *Agrobacterium* or *Rhizobium* causing rhizogenesis are Gram negative (Hooykas and Hooykas, 2021). Bosman et al. (2017) report that it is common for bacterial isolates from hydroponic cultures to exhibit catalase activity, which allows them to resist hydrogen peroxide (H2O2), which is widely used for disinfection of irrigation systems.

The resistance of rhizogenesis-inducing bacteria is not only limited to biocides, but also to grow at extreme pH acidic (3) and alkaline (11), the latter was evaluated with Hofer's medium, where five isolates were positive, this test has also been routinely used for screening *Agrobacterium rhizogenes* as reported by Murugesan et al. (2010).

	KOH	Cat	MCky		MHof	MG-Te
Cj 1	-	+	-		+	+
Cj 3	-	+	-		+	-
Cj 5	-	+	+		+	-
4(3)1a	+	+	-		-	-+
5(3)3H	+	+	-		-	-+
5(3)3a	-	+	-		+	-
5(3)3-1	-	+	-		-	-+
5(3)4	-	+	+		+	+
KOH 3%: - Gram negative, + Gram positive, + or -: positive or negative for the test, -+: not defined. Cat: catalase, MCky: McConkey, MHof: Hofer's medium and Mg-Te: Tellurite medium.						

Table 1 Results of biochemical tests performed on bacterial isolates

Finally, the selective MG-Te medium was not only used for the initial screening of microbial populations related to the genus Agrobacterium, but also to verify them once pure isolates were available, whose colonial phenotype showed a black precipitate (Figure 1), characteristic of tellurite reduction (Mougel et al., 2001).

Figure 1 Isolate 5(3)4 growing on MG-Te agar plate, showing black colonies, characteristic of tellurite reductio.

In summary, biochemical tests and semi-selective media allowed us to better describe and differentiate the isolates, as well as discriminate between them, finding that at least one strain, 5(3)4, had most of the characteristics related to rhizogenesis-promoting bacteria. It was therefore selected for the potato disc root induction assay.

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Root induction on potato discs

In Figure 2, we can observe the result of 5(3)4 inoculation in potato discs after 24 days, where the formed roots were best observed with the use of a stereoscope (Figure 2-B). This root induction could probably be due to the transfer of the Ri plasmid from the bacterium to the potato cells, indicating that the 5(3)4 isolate could be a strain of *Agrobacterium rizhogenes* or *Rhizobium rhizogenes* (Moehninsi & Navarre, 2018), which could be corroborated by amplification and sequencing of the 16S rRNA gene (Moehninsi & Navarre, 2018), which could be corroborated by amplification and sequencing of the 16S rRNA gene (Moehninsi & Navarre, 2018).

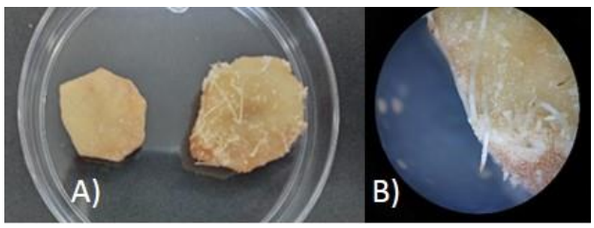

Figure 2 Root induction test on potato slices. A) Left: uninoculated control, Right: inoculated with strain 5(3)4. B) Image of roots through a stereoscope

Conclusions

The bacterial isolate named 5(3)4 exhibits reported biochemical properties related to the genus *Agrobacterium*, including the induction of adventitious roots in potato discs. Therefore, this strain may be one of the strains responsible for the induction of "hairy roots" in tomato crops.

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