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Effect of *Pseudomonas rhodesiae* GRC140 on *Cucumis sativus* L. seedlings with and without Cadmium

Efecto de *Pseudomonas rhodesiae* GRC140 en plántulas de *Cucumis sativus* L. en presencia y ausencia de Cadmio

ROLÓN-CÁRDENAS, Gisela Adelina†´, HERNÁNDEZ-MORALES, Alejandro*´, ARVIZU-GÓMEZ, Jackeline Lizzeta´´ and CARRANZA-ÁLVAREZ, Candy´

´Universidad Autónoma de San Luis Potosí, Facultad de Estudios Profesionales Zona Huasteca. Romualdo del Campo 501, Fraccionamiento Rafael Curiel, CP 79060, Ciudad Valles San Luis Potosí, Mexico.

´´Secretaría de Investigación y Posgrado, Centro Nayarita de Innovación y Transferencia de Tecnología (CENITT), Universidad Autónoma de Nayarit, Tepic, Nayarit, Mexico.

ID 1st Author: *Gisela Adelina Rolón-Cárdenas* / **ORC ID:** 0000-0001-8633-0462, **Researcher ID Thomson:** ABI-7808-2020, **CVU CONACYT ID:** 712240

ID 1st Co-author: *Alejandro, Hernández-Morales* / **ORC ID:** 0000-0002-0412-4946, **Researcher ID Thomson:** P-8441-2014, **CVU CONACYT ID:** 101590

ID 2nd Co-author: *Jackeline Lizzeta Arvizu-Gómez* / **ORC ID:** 0000-0001-7514-6256, **Researcher ID Thomson:** D-1697-2018, **CVU CONACYT ID:** 206695

ID 3rd Co-author: *Candy, Carranza-Álvarez* / **ORC ID:** 0000-0002-6456-3035, **CVU CONACYT ID:** 43169

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Abstract

Pseudomonas rhodesiae GRC140 is an endophytic bacterium isolated from the roots of *Typha latifolia* collected in a Cd-contaminated site. This bacterium has biochemical abilities similar to those exerted by plant growth-promoting rhizobacteria. Moreover, it has been shown that *P. rhodesiae* GRC140 improves the growth of *Arabidopsis thaliana* seedlings in the absence and presence of Cd. The aim of this work was to determine the effect of *P. rhodesiae* GRC140 in *Cucumis sativus* L. growing in nutritive medium with and without Cd. For this, cucumber seeds were superficially disinfected and exposed to a suspension of *P. rhodesiae* GRC140. Inoculated seeds were placed in a nutritive medium with and without Cd, then were incubated at 28 ºC for eight days. After incubation, seedlings were recovered and determined the length of the primary root, the number of roots per plant, hypocotyl length, and the fresh weight. The results showed that *P. rhodesiae* GRC140 negatively affects the growth of *C. sativus* L. seedlings grown in the absence of Cd. On the other hand, in Cd-exposed seedlings, *P. rhodesiae* GRC140 improves the growth of *C. sativus* L. These results suggest that *P. rhodesiae* GRC140 decreases the deleterious effect of Cd in *C. sativus* L.

***Pseudomonas rhodesiae* GRC140, plant growth-promoting rhizobacteria, *Cucumis sativus* L.**

Resumen

Pseudomonas rhodesiae GRC140 es una bacteria endófita aislada de las raíces de *Typha latifolia* recolectadas en un sitio contaminado con Cd. Esta bacteria tiene capacidades bioquímicas similares a las que ejercen las rizobacterias promotoras del crecimiento de las plantas. Además, se ha demostrado que *P. rhodesiae* GRC140 mejora el crecimiento de plántulas de *Arabidopsis thaliana* en ausencia y presencia de Cd. El objetivo de este trabajo fue determinar el efecto de *P. rhodesiae* GRC140 en *Cucumis sativus* L. creciendo en medio nutritivo con y sin Cd. Para ello, las semillas de pepino se desinfectaron superficialmente y se expusieron a una suspensión de *P. rhodesiae* GRC140. Las semillas inoculadas se colocaron en un medio nutritivo con y sin Cd, luego se incubaron a 28 ºC durante ocho días. Después de la incubación, se recuperaron las plántulas y se determinó la longitud de la raíz primaria, el número de raíces por planta, la longitud del hipocótilo y el peso fresco. Los resultados mostraron que *P. rhodesiae* GRC140 afecta negativamente el crecimiento de plántulas de *C. sativus* L. cultivadas en ausencia de Cd. Por otro lado, en plántulas expuestas a Cd, *P. rhodesiae* GRC140 mejora el crecimiento de *C. sativus* L. Estos resultados sugieren que *P. rhodesiae* GRC140 disminuye el efecto deletéreo del Cd en *C. sativus* L.

***Pseudomonas rhodesiae* GRC140, bacteria promotora de crecimiento vegetal, *Cucumis sativus* L.**

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* Author Correspondence (Email: alejandro.hernandez@uaslp.mx)

† Researcher contributing as first author.

Introduction

Heavy metals emission to the environment is a problem that has been increased in the last years (Mohammed et al. 2011). The presence of high levels of toxic heavy metals in the environment is a consequence of the increase in agricultural, mining, and industrial activities (Covarrubias and Peña-Cabriales 2017). Within the group of heavy metals, Cadmium (Cd) is one of the most studied in soils, sediments, and aquatic environments because it is a highly toxic metal for all biological systems, even at low concentrations (Stout and Nüsslein 2005). In humans, chronic exposure to Cd has been linked to disease development, including cancer of the lung, liver, prostate, breast, and endometrium (Filipič 2012, Hartwig 2010).

To avoid the heavy metals adverse effects on human health and the environment, it has been developed physicochemical methods to reduce their concentrations in the environment. However, these treatments can be inefficient and costly, so it is necessary to explore other environmentally friendly alternatives (Cañizares-Villanueva 2000).

Phytoextraction has been proposed as an efficient and environmentally friendly technique to remove heavy metals from soil or water. This technique uses the capacity of some plants called hyperaccumulators to absorb heavy metals from the soil and accumulate them in their tissues (Marrero-Coto et al. 2012). Some hyperaccumulators plants have low biomass and slow growth when they are exposed to high heavy metals concentrations, thus reducing the efficiency of phytoextraction (Kidd et al. 2007). Nevertheless, to withstand the heavy metal toxic effects, plants establish symbiotic interactions with rhizospheric microorganisms, among these, plant growth-promoting rhizobacteria (PGPR). This bacterial group includes species that contribute to the plant growth through activities such as phosphate-solubilization, siderophore production, indole acetic acid synthesis (IAA), and ACC-deaminase activity (Aeron et al. 2020, Manoj et al. 2020).

PGPR contribute to the adaptation of the plant to environmental conditions and even improve phytoextraction (Ullah et al. 2015). Therefore, the study of plant-bacteria interactions has been taken importance in recent years.

T. latifolia is a cosmopolitan plant that can grow in a great variety of environments. Because of the rapid growth rate, this species tolerates flood, drought, salinity, and the presence of heavy metal (Baldwin and Cannon 2007). Therefore, *T. latifolia* has been extensively studied due to the capacity to tolerate heavy metals and accumulate them mainly in the roots, and to a lesser extent in aerial tissues (Bonanno and Cirelli 2017).

Recently, it has been reported the isolation of *Pseudomonas rhodesiae* GRC140 from the root endosphere of *T. latifolia* that grows in a Cd contaminated environment (Rolón-Cárdenas et al 2020). The biochemical characterization of *P. rhodesiae* GRC140 showed that exerts different activities related to plant growth promotion, including ACC deaminase activity, production of Indole Acetic Acid (IAA) and Phenylacetic acid (PAA) as plant growth regulators, siderophores secretion under Fe-limiting conditions, and solubilization of phosphorus from $Ca_3(PO_4)_2$ (Rolón-Cárdenas et al 2020). These abilities suggest that *P. rhodesiae* GRC140 is a plant growth-promoting rhizobacteria.

In assays with the model plant *Arabidopsis thaliana* Col 0, *P. rhodesiae* GRC140 increases the number and density of lateral roots, the hypocotyl length, and the total fresh weight in *A. thaliana* Col 0 seedlings without Cd exposure. On the other hand, in presence of Cd, *P. rhodesiae* GRC140 increases the number and density of lateral roots of *A. thaliana* Col 0 seedlings, suggesting that bacterium improves the plantlet growth in both conditions (Rolón-Cárdenas et al 2020). This study suggests that *P. rhodesiae* GRC140 can act in a similar manner in the host plant *T. latifolia*.

To determine whether *P. rhodesiae* GRC140 exerts similar effects in other plants, we use cucumber (*Cucumis sativus* L.). This species is an economically important crop widely consumed and has been recommended by US Environmental Protection Agency to perform phytotoxicity studies (Sun et al. 2019). Additionally, it has been demonstrated that *C. sativus* L. plants have a sophisticated system for the detoxification and tolerance of Cd (Ali et al. 2019).

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Because of the tolerance of cucumber to Cd, we study how *P. rhodesiae* GCR140 influences the growth of *C. sativus* L. seedlings in the presence and absence of Cd. The results with this plant model could be used to extend our knowledge in the role of *P. rhodesiae* GRC140 in *T. latifolia* exposed to Cd.

Methodology

P. rhodesiae GRC140 revitalization

P. rhodesiae GRC140 was inoculated in LB broth and incubated at 28 ^{o}C overnight. Ten µl was inoculated and streaked in LB agar medium plus 5.34 µM, the maximum tolerable concentration of Cd (as $CdCl_2$) that bacterium tolerates (Rolón-Cárdenas et al. 2020). Plates were incubated at 28 ^{o}C for 24 h to obtain separated colonies.

Bacterial suspension preparation

A single *P. rhodesiae* GRC140 colony was picked and inoculated in LB Broth and incubated at 28 ^{o}C overnight. Then, a bacterial suspension in saline solution (NaCl 0.85 %) was prepared, adjusting samples of the overnight culture to an OD of 0.2 at 600 mm.

Disinfection of *C. sativus* L. seeds

C. sativus L. seeds (American cucumber, Hostaflor brand) were immersed in 5.0 ml of a disinfectant mixture (50 % commercial hypochlorite supplemented with 0.2 % Triton X-100) for 30 min. Then, seeds were washed three times with sterile distilled water for 5 min. The water of the last rinse was inoculated in LB agar plates to ensure that the disinfection process was successful. Disinfected seeds were air-dried in the laminar flow hood.

Plant-bacteria interaction assays

Fifteen disinfected seeds were placed in 50 ml conical tubes and then 3 ml of the *P. rhodesiae* GRC140 suspension adjusted to 0.2 were added. Inoculated seeds were incubated for 30 min at room temperature.

To determine the effect of *P. rhodesiae* GRC140 in cucumber, five seeds were placed in culture flasks with a nutritive medium containing 1 mM KNO_3, 50 mM KCl, 1 mM Ca $(NO_3)_2$, 8.31 mM $FeSO_4$, and 1.3 mM $ZnSO_4$ (Rodríguez-Hernández et al. 2015) adjusted to pH 5.7 with 3.5 mM Buffer MES supplemented with 1.0 % commercial sucrose and 0.7 % bacteriological agar. Flasks were incubated in darkness at 30 °C for 72 h until germination. Once germinated, they were placed under fluorescent light with a photoperiod of 16 h light:8 h dark for eight days. After incubation, the length of primary roots, the number of lateral roots per plant, hypocotyl length, and the fresh weight were determined with concern to non-inoculated cucumber seedlings.

Regarding the effect of *P. rhodesiae* GRC140 in seedlings exposed to Cd. Inoculated *C. sativus* L. seedlings were placed on a nutritive medium supplemented with 20 mg L^{-1} of Cd. The flasks were incubated in the same conditions and the growth of the seedlings was compared with non-inoculated plants.

Statistical analysis

All experiments were performed in triplicate. Statistical analysis was performed using GraphPad Prism Version 5.01. Analysis of variance (ANOVA) was performed with Tukey's method to compare the means between treatments ($p < 0.05$).

Results

P. rhodesiae GRC140 negatively affects the growth of *C. sativus* L. seedlings

Previously, it has been demonstrated that *P. rhodesiae* GRC140 promotes the growth of *A. thaliana* seedlings, being the main effects observed at the root system and the length of hypocotyl, which in turn increases the total fresh weight in plantlets (Rolón-Cárdenas et al. 2020). This agrees with other studies that demonstrated that *P. rhodesiae* strains promote the growth of *Solanum lycopersicum* (Romero et al. 2016), *C. aconitifolius*, *Phaseolus vulgaris*, and *Bacopa monnieri* (Jimtha and Radhakrishnan 2018). The beneficial effects described for some *P. rhodesiae* strains have been mainly observed at the level of the root system, increasing the length of the primary root, and promoting secondary roots generation.

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Because of this, the effects of *P. rhodesiae* strains have been attributed to IAA produced by the bacterium (Romero et al. 2016, Jimtha and Radhakrishnan 2018, Rolón-Cárdenas et al. 2020).

Conversely, when *P. rhodesiae* GRC140 was inoculated on *C. sativus* L. seedlings, it was observed a decrease in the general plant growth with respect to non-inoculated plantlets (Figure 1B). *P. rhodesiae* GRC140 decreases significantly ($p < 0.05$) the primary root length (Figure 2A), the hypocotyl length, and thus the total fresh weight (Figure 3A, B). No significant differences were observed in the number of lateral roots per plant (Figure 2B). Therefore, in the conditions of this study, the results indicated that *P. rhodesiae* GRC140 exerts a negative effect in *C. sativus* L. seedlings.

It has been shown that PGPR exert both beneficial and deleterious effect in plants (Tsukanova et al. 2017). The beneficial effect has been widely described and highlights for applications in agricultural practices to improve productivity (Noumavo et al. 2016). Regarding adverse effects, it has been recognized that plant-associated bacteria could act as weak biotrophic pathogens that cause costs to their host plants. Thus the beneficial or detrimental effects on the plant are highly context-dependent (Partida-Martínez and Heil 2011). In this study, *P. rhodesiae* GRC140 did not promote the growth of cucumber seedlings, which could be due to the inoculum quantity used in the assay.

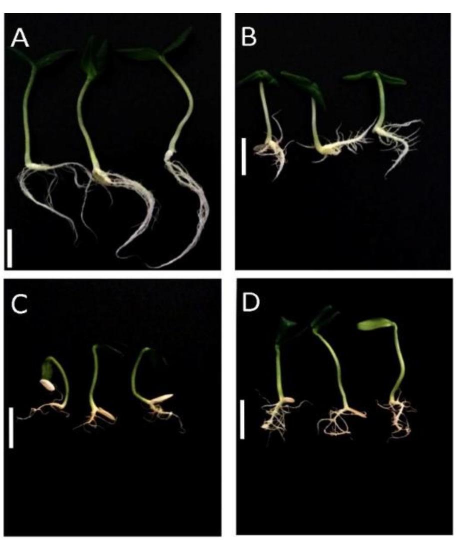

Figure 1 *P. rhodesiae* GRC40 effect on *C. sativus* L. seedlings. Seedlings not exposed to Cd A) Non-inoculated, B) Inoculated with *P. rhodesiae* GRC140. Seedlings exposed to Cd C) Non-inoculated, D) Inoculated with *P. rhodesiae* GRC140. Bar = 2 cm

P. rhodesiae GRC140 improves the growth of *C. sativus* L. seedlings exposed to 20 mg L^{-1} of Cd

It has been demonstrated that Cd affects the general growth of *C. sativus* L. seedlings in a concentration-dependent manner. Cd decreases the growth of the primary root and increases the number of secondary roots (Rolón-Cárdenas et al. 2015). Similar effects have been observed in *A. thaliana* seedlings exposed to 2.5 mg L^{-1} of Cd, being the length of the taproot the most affected parameter (Rolón-Cárdenas et al. 2020). However, beneficial effects have been observed when *P. rhodesiae* GRC140 is inoculated in *A. thaliana* Col 0 seedlings exposed to 2.5 mg L^{-1} of Cd. It was reported that the bacterium increases the number and density of lateral roots, and the length of the hypocotyl. Thus indicating that *P. rhodesiae* GRC140 exerts protective effects in *A. thaliana* seedlings exposed to 2.5 mg L^{-1} of Cd. These effects have been attributed to the IAA and PAA synthesized by *P. rhodesiae* GRC140 (Rolón-Cárdenas et al. 2020).

To determine the effect of *P. rhodesiae* GRC140 in *C. sativus* L. exposed to Cd, seeds were immersed in bacterial suspension and then placed in media plus to 20 mg L^{-1} of Cd. The results showed that Cd inhibits the general growth of non-inoculated cucumber seedlings, reducing the length of primary root (Figure 1C), whereas, in the plants inoculated with *P. rhodesiae* GRC140, it was observed a better general growth of plantlets exposed to Cd (Figure 1D), suggesting that the bacterium alleviates the toxic effects generated by Cd exposure.

P. rhodesiae GRC140 increases significantly ($p < 0.05$) the primary root length of *C. sativus* seedlings exposed to 20 mg L^{-1} of Cd (Figure 2A). Despite that it was observed an increase in the number of lateral roots in inoculated seedlings, there are no statistical differences with respect to non-inoculated plants (Figure 2B). These results differ from those observed in *A. thaliana* seedlings where *P. rhodesiae* GRC140 increases the number of lateral roots but not exert a beneficial effect on the length of the primary root (Rolón-Cárdenas et al. 2020).

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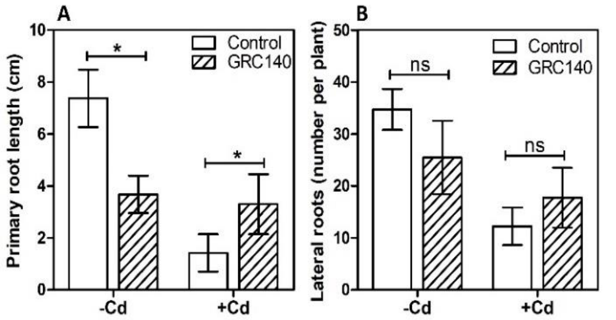

Figure 2 *P. rhodesiae* GRC40 effect on A) Primary root length, B) Lateral roots of *C. sativus* L. seedlings. Control indicated non-inoculated plants, whereas GRC140 inoculated plants. Absence of Cd (-Cd), Presence of Cd (+Cd).

On the other hand, *P. rhodesiae* GRC140 increases significantly ($p < 0.05$) the total fresh weight of *C. sativus* seedlings exposed to 20 mg L^{-1} of Cd (Figure 3A). Despite that it was observed an increase in the hypocotyl length in inoculated seedlings, there are no statistical differences with respect to non-inoculated plants (Figure 3B). It has been shown that *P. rhodesiae* GRC140 did not improve the total fresh weight and hypocotyl length in *A. thaliana* seedlings exposed to 2.5 mg L^{-1} of Cd (Rolón-Cárdenas et al. 2020).

The results showed that *P. rhodesiae* GRC140 increases the length of the primary root and the total fresh weight, suggesting that it exerts protective effects in cucumber seedlings exposed to 20 mg L^{-1} of Cd. This indicated that bacterium contributes to the Cd tolerance in non-host plant *C. sativus* L.

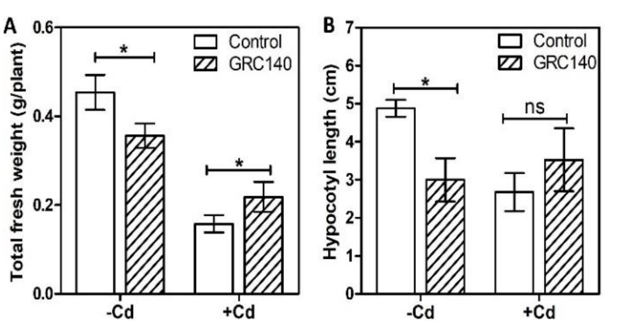

Figure 3 *P. rhodesiae* GRC40 effect on A) Total fresh weight, B) Hypocotyl length of *C. sativus* L. seedlings. Control indicated non-inoculated plants, whereas GRC140 inoculated plants. Absence of Cd (-Cd), Presence of Cd (+Cd).

Discussion

P. rhodesiae GRC140 is an endophytic bacteria isolated from inside of the roots *T. latifolia* collected from a site contaminated with Cd (Rolon-Cárdenas et al. 2020). This bacterium can grow in LB media supplemented with 5.34 µM of Cd, showing higher Cd-tolerance than the reported for *Cupriavidus metallidurans* CH34 (3.5 mM) (Mazhar et al. 2020). These findings suggest that *T. latifolia* selects Cd-tolerant bacterial populations that promote the growth and confer tolerance to Cd. This agrees with the reported for *Spartina maritima, S. densiflora, and Sulla coronaria* whose roots are colonized by heavy metal tolerant PGPR that promote growth in these conditions and increase tolerance to heavy metals (Paredes-Páliz et al. 2016, Paredes-Páliz et al. 2017, Chiboub et al. 2016).

P. rhodesiae GRC140 inoculation in *C. sativus* seedlings exposed to Cd improves the root system growth, suggesting that bacterium confers tolerance to Cd. It has been shown that PGPR isolated from *S. densiflora* roots reduce the stress caused by As, Cu, Pb, and Zn; they promote plant growth and increase the phytoaccumulation of heavy metals in its roots (Paredes-Páliz et al. 2016, Paredes-Páliz et al. 2017). Moreover, the inoculation of *Pseudomonas* spp. and *Rhizobium sullae* in *S. coronaria* plants improve the plant growth under Cd stress (Chiboub et al. 2017). These effects are attributed to microbial activities, including nitrogen fixation, ACC deaminase activity, siderophore production, phosphate solubilization, IAA synthesis (Kong and Glick 2017).

Regarding *P. rhodesiae* GRC140, it has been shown that synthesized IAA and PAA, which could be involved in the growth and tolerance of *C. sativus* L. seedlings to Cd. Currently, we work on understanding the role of *P. rhodesiae* GRC140 in its host plant, *T. latifolia.*

Conclusion

P. rhodesiae GRC140 improves the growth of *C. sativus* L. seedlings exposed to 20 mg L^{-1} of Cd, suggesting that bacterium contributes to the Cd-tolerance. However, in absence of Cd, bacterium did not show beneficial effects in cucumber plantlets.

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