Colegio de Postgraduados

3-oxo-C12-HSL and C4-HSL promote root system development in *Solanum lycopersicum* L. *in vitro*

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ABSTRACT

Objective: To evaluate the effect of the synthetic C4-HSL and 3O-C12-HSL molecules produced by wild strains of *P. aeruginosa* as promoters of the root system of *Solanum lycopersicum* L *in vitro*.

Design/methodology/approach: Wild and control strains were studied, in which crude extraction in ethyl acetate and quantification of both molecules were performed. Different concentrations of synthetic 3O-C12-HSL and C4-HSL (10, 12, 24, 48, 96, and 192 μM) were used. The ability to elongate the primary root, as well as the length and density of root hairs under conditions were evaluated. Laboratory-controlled studies.

Results: The results obtained showed no beneficial effect on PAO1 in the crude extracts. However, a positive effect was observed when using the synthetic molecules 3O-C12-HSL and C4-HSL on primary root elongation at a concentration of 24 μM and on root hair density at a concentration of 96 μM, respectively, with a statistically significant difference ($p<0.05$) compared to the controls.

Findings/Conclusions: This study reports positive effects on primary root elongation and root hair density using the synthetic molecules C4-HSL and 3O-C12-HSL in *S. lycopersicum*.

Keywords: AHL, crude extract, 3O-C12-HSL, C4-HSL, *P. aeruginosa*, *S. lycopersicum*, *in vitro*.

INTRODUCTION

Currently, agriculture faces a high demand for the production of high-quality food; however, achieving this involves overcoming several challenges, including the loss of soil fertility, water scarcity, land-use change and climate change, the overuse of agrochemicals,

high costs, and low yields, which together make food production an unsustainable system. Microorganisms may represent an alternative to increase agricultural production in small spaces while producing organic food that benefits environmental and human health. Numerous studies have reported the use of Plant Growth-Promoting Bacteria (PGPB). *P. aeruginosa* is considered a PGPB, but it is also an opportunistic bacterium, which limits its use in agriculture. However, it has been reported that autoinducer molecules (AHLs) of the Quorum Sensing (QS) system modify root architecture and increase biomass in *Cicer arietinum* and *Triticum aestivum* (Gahoi *et al*., 2021; Ibal *et al*., 2021) when C8-, C10-, and C12-HSL are added, respectively. In *P. aeruginosa*, the QS system is well studied, in which two AHLs (autoinducers) have been described: N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL). These molecules bind to their receptor proteins LasR and RhlR to activate the transcription of genes involved in virulence and persistence in diverse environments (Mukherjee *et al*., 2017). However, studies suggest that the agricultural use of these AHLs can promote germination, increase plant fresh and dry weight, and induce root elongation in *Arabidopsis thaliana* and *Lactuca sativa* L. (Von Rad and Ortiz Castro, 2008; Ortiz J. *et al*., 2024). It has also been reported that *P. aeruginosa* strains promote the growth of *Solanum lycopersicum* L. (Hariprasad *et al*., 2013; Adesemoye *et al*., 2008). This vegetable is important to study because it is part of the Mexican diet (Martínez-Rodríguez *et al*., 2017). Therefore, the objective of this study was to determine the effect of the synthetic C4-HSL and 3O-C12-HSL molecules and crude extracts produced by wild strains of *P. aeruginosa* on promoting root growth of *Solanum lycopersicum* L. under *in vitro* conditions, in order to evaluate their potential as a viable alternative for the development of tomato cultivation in current agriculture.

MATERIALS AND METHODS

Biological material: Wild strains of *P. aeruginosa* were isolated and identified using the automated VITEK system (bioMérieux®) from different environments in the state of Guerrero, and control strains were included (Table 1).

AHL extraction and elucidation: A pre-inoculum of the strains of interest was prepared in LB (Luria-Bertani) medium and grown overnight at 37 °C with shaking at 225 rpm. Subsequently, 30 mL of PPGAS broth (NH_4Cl 0.02 M, 1.069 g/L; KCl 0.02 M, 1.49 g/L; Tris-HCl 0.12 M, 18.91 g/L; peptone 1%, 10 g/L; glucose 0.5%, 25 mL/L; $MgSO_4$ 0.0016 M, 3.2 g/L) were inoculated with an appropriate volume of the pre-inoculum to reach an initial absorbance of 0.05 at 600 nm, and incubated at 37 °C for 24 h with shaking at 225 rpm. Cultures were centrifuged at 14,000 rpm for 10 min at 4 °C, and

Table 1. *P. aeruginosa* strains used in this study.

Origen	Cepa
Rizósfera de jitomate	MAZ 0105
Rizósfera de maíz	TIX 0303
Aislado clínico	MCD
Control	PAO1-UW
Mutante experimental	PAO1-Δ*rhlI*/Δ*lasI*

the supernatant was transferred to a clean 50 mL polypropylene tube. Then, 5 mL of supernatant were mixed with 5 mL of acidified ethyl acetate (1000 mL ethyl acetate + 100 μL acetic acid) in a 15 mL polypropylene tube and manually shaken vigorously for 15 min. Subsequently, the mixture was centrifuged at 3,500 rpm at 4 °C.

Once the phases were separated, the upper organic phase was collected, and 5 mL of acidified ethyl acetate were again added to the lower phase, repeating the procedure to obtain the organic extract. The upper-layer fractions were combined and evaporated in a fume hood until a volume of 1 mL was reached, transferred to a 1.5 mL Eppendorf tube, and allowed to evaporate completely in the hood. After this process, 50 μL of methanol were added to recover the extract, which was stored at −20 °C until use (Grosso-Becerra *et al.*, 2014). For C4-HSL confirmation, thin-layer chromatography (TLC) plates silica gel 60 F254 (MERCK® 105554) were used. Using a 1 μL micropipette, up to 5 μL of the sample were applied to the spotting point. Synthetic C4-HSL (SIGMA® 09945) and N-hexanoyl-L-homoserine lactone (SIGMA® 56395) were used as standards. Methanol-water (60:40) was used as the mobile phase. Detection was performed using the CV026 biosensor, incubated at 30 °C for 16 h (Grosso-Becerra *et al.*, 2014). Quantitative analysis of the TLC plates was carried out using ImageJ software (Phattanawasin *et al.*, 2016). For the detection of 3O-C12-HSL, a modification of the method reported by Morales *et al.* (2017) and Pearson *et al.* (1997) was used.

In vitro* culture conditions of *S. lycopersicum: The experiment was carried out in a plant growth chamber. A total of 700 *S. lycopersicum* seeds were obtained from the brand Vita®. The disinfection process was performed following the protocol of Rangel-Estrada *et al.* (2015). After the disinfection treatment, seeds were placed in groups of 10 in Petri dishes containing 1.5% agar and allowed to germinate in darkness at 25 °C for 72 h. Subsequently, only germinated seeds were selected and transplanted to Petri dishes containing 1.5% bacteriological agar, to which the treatment to be evaluated was added before gelation (50 °C) to reach the final AHL concentrations (Ortiz-Castro *et al.*, 2008).

The experiment was conducted in an *in vitro* plant culture incubation room under a photoperiod of 16 h of light at an intensity of 200 ± 50 μmol m^{-2} s^{-1}, temperature of 25 ± 2 °C, and relative humidity of 50-60%, followed by 8 h of darkness at 25 °C (Schwarz *et al.*, 2014).

Treatments used in the bioassay with *S. lycopersicum*: The treatments used in this study were as follows: negative control; solvent control (ethyl acetate); crude ethyl acetate extracts of AHLs from wild and control strains; and different final concentrations (10, 12, 24, 48, 96, and 192 μM) of the synthetic AHL molecules C4-HSL (SIGMA® 09945) and 3O-C12-HSL (SIGMA® 56395), respectively, for evaluation. Ten seeds were used per treatment, and three independent experiments were performed in triplicate.

Primary root growth and root hairs: The roots of *S. lycopersicum* were analyzed using an SMZ 10 stereoscopic microscope (NIKON®) after seven days of exposure. Root hairs were counted at 3x magnification, and micrographs were captured using a Motic Cam 5 5.0 MP camera (MOTIC®). The results were interpreted following the methodology described by Ortiz-Castro *et al.* (2008), and root hair density was calculated using the protocol of Ortiz *et al.* (2024).

Statistical analysis: The significance of the estimated concentrations of the main AHLs produced by *P. aeruginosa* strains was determined by one-way ANOVA with Tukey's *post hoc* test. The significance of the treatments in the *in vitro* bioassay with *S. lycopersicum* was determined by one-way ANOVA at a significance level of 0.05 ($p<0.05$), followed by Dunnett's *post hoc* test. The following software packages were used: SigmaPlot v16 for data tables, GraphPad Prism v8 for graph preparation, and ImageJ2 for quantitative measurement of TLC plates.

RESULTS AND DISCUSSION

Three environmental *P. aeruginosa* strains were isolated, selected, and identified, originating from the rhizosphere of maize and tomato, along with one clinical isolate. In addition, two control strains were included in this study, namely the PAO1 strain and the double mutant of the autoinducer synthase genes, as shown in Table 1. To strengthen the study, final concentrations of 10, 12, 24, 48, 96, and 192 μM of the synthetic AHL molecules C4-HSL and 3O-C12-HSL, respectively, were also included.

Regarding the concentration of AHL molecules in the extracts of each strain, PAO1 showed a concentration of 11.32 μM for 3-oxo-C12-HSL and 0.70 μM for C4-HSL, whereas no AHLs were detected in the mutant strain. In the wild strains, differential concentrations of both AHL molecules were detected, as shown in Figure 1.

Primary root growth and root hair density. Figure 2 shows the effect of crude ethyl acetate extracts from wild strains and the double mutant of *P. aeruginosa*, which caused a clear decrease in primary root length and root hair density, with statistical significance ($p<0.05$). In addition, strain MAZ 0105 inhibited primary root elongation and root hair density by 32%, with statistical significance ($p\leq0.05$).

In contrast, stimulation with synthetic AHLs at different concentrations produced an increase in primary root length at concentrations of 12, 24, and 48 μM for C4-HSL and 12 μM for 3O-C12-HSL, whereas a decrease was observed when roots were exposed to 3O-C12-HSL at a concentration of 192 μM (Figure 3).

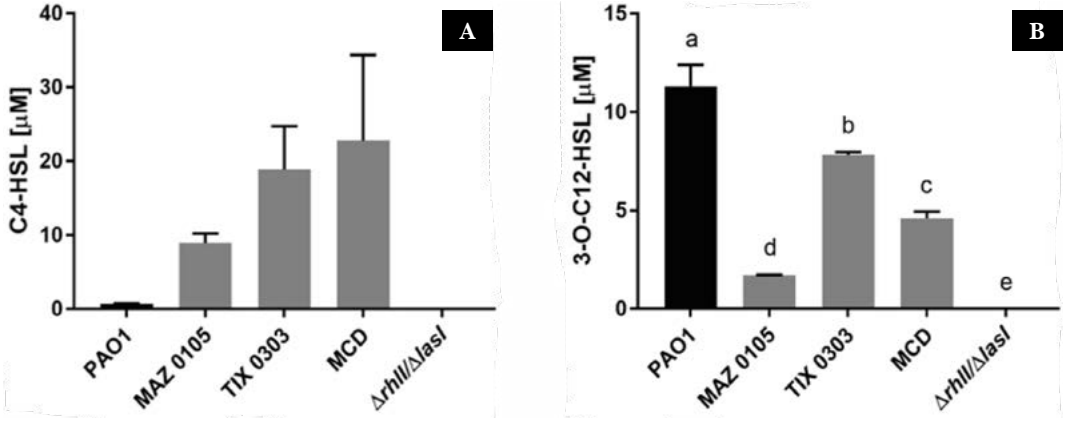

Figure 1. Estimation of the concentrations of the main AHLs produced by *P. aeruginosa* strains. (A) C4-HSL. Bars represent the mean ± SD (n=2). (B) 3O-C12-HSL. Bars represent the mean ± SD (n=3). Different letters indicate statistically significant differences ($p<0.05$).

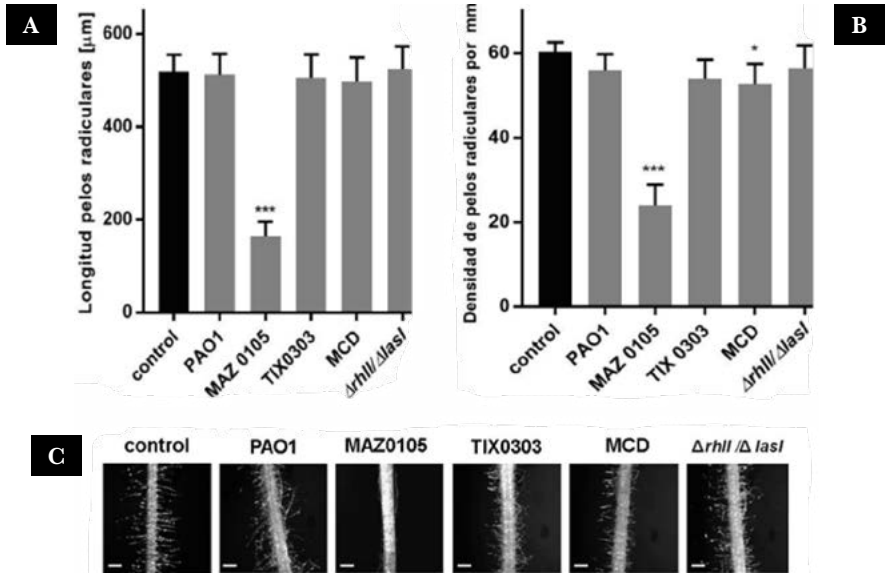

Figure 2. Primary root length and root hair density of *S. lycopersicum* exposed to crude ethyl acetate extracts from wild and control strains. (A) Root length. Bars represent the mean ± SD (n=30). (B) Root hair density per mm^2. Bars represent the mean ± SD (n=9), with statistically significant differences: ***=p≤0.05. (C) Micrographs at 3x magnification of seedlings from the strains; white bars correspond to 200 μm.

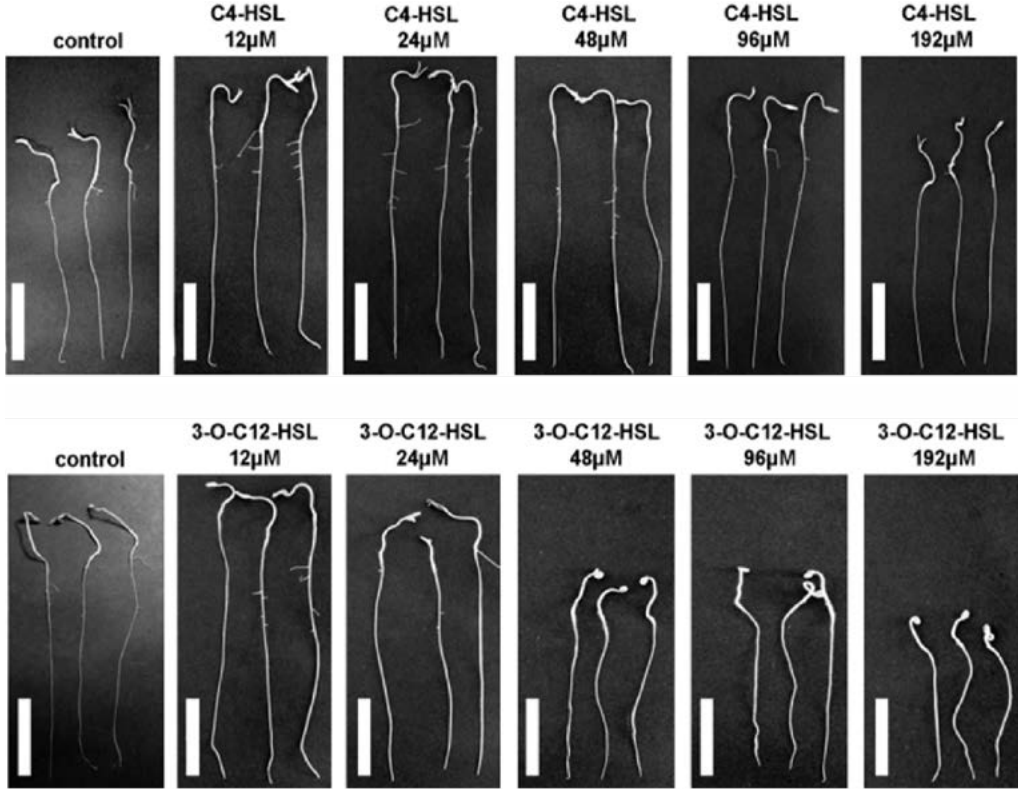

Figure 3. Images of seedlings exposed to different concentrations of synthetic AHLs. The upper panel shows the effects of C4-HSL and the lower panel shows those of 3O-C12-HSL. White bars correspond to 3 cm.

Plants treated with 3O-C12-HSL at a concentration of 24 μM showed an increase in root hair length, and at concentrations from 48 to 192 μM, both root hair length and density increased, with statistical significance ($p<0.05$). For C4-HSL, an increase in root hair length was observed only at 24 μM, whereas an increase in root hair density was observed at concentrations of 96 μM and 192 μM, with statistically significant differences compared to the control ($p<0.05$) (Figure 4).

The search for new strategies to promote plant growth and to produce food with higher nutritional value in a sustainable and low-cost manner has led us to investigate whether QS system molecules from *P. aeruginosa* can enhance primary root development and increase root hair density under *in vitro* conditions, thereby providing evidence for their potential use in agricultural production models. However, in this study, the results demonstrate that crude ethyl acetate extracts obtained from wild and control *P. aeruginosa* strains are not able to promote root elongation or root hair density under *in vitro* conditions in *S. lycopersicum* (Figure 2), suggesting that their concentrations may be too low to exert an effect. In contrast, strain MAZ0105 significantly inhibited root and root hair development, which further confirms that these molecules are present at very low concentrations and are insufficient to stimulate primary root and root hair development. Therefore, we confirmed a favorable inductive effect of both synthetic AHLs on root growth at a concentration of 24 μM and on root hair density at concentrations of 96 μM, respectively (Figure 4). In contrast, a concentration of 192 μM of 3O-C12-HSL reduced primary root growth but promoted root hair development in both length and density, in agreement with what has

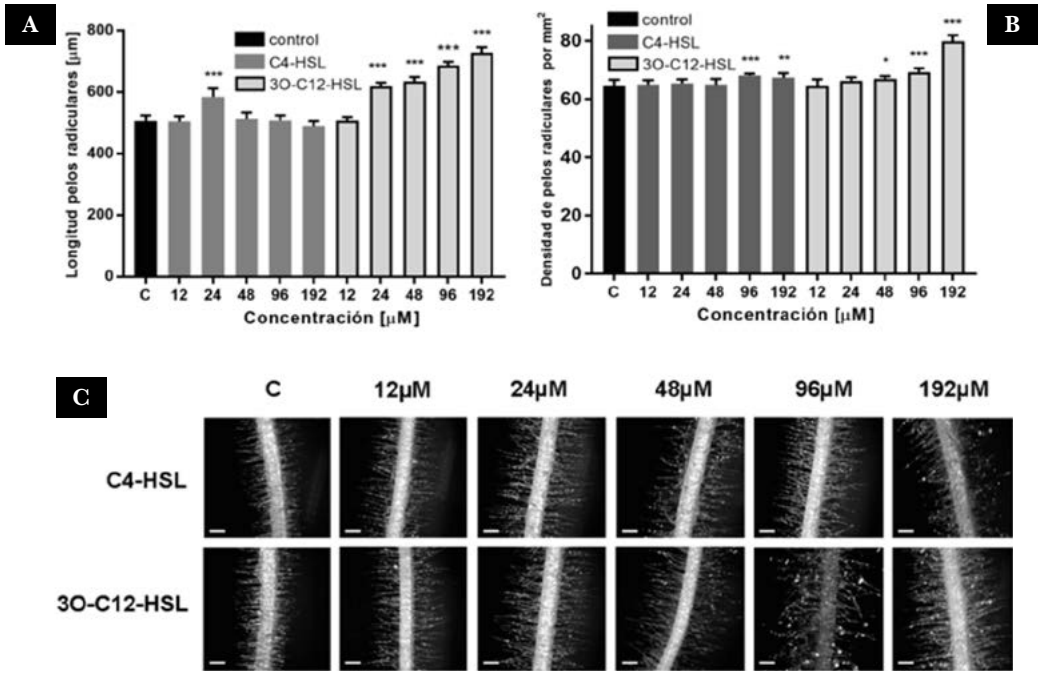

Figure 4. Determination of root hair length and density. (A) Root hair length and (B) root hair density of *S. lycopersicum* exposed to synthetic C4-HSL and 3O-C12-HSL, respectively. Bars represent the mean ± SD, with statistically significant differences: ***=$p\leq0.05$. (C) Micrographs at 3x magnification of plants grown with synthetic AHLs. White bars correspond to 200 μm.

been reported for *A. thaliana* (Von Rad *et al*., 2008; Ortiz-Castro *et al*., 2008). Several studies have demonstrated the influence of AHLs on the development of different plant species by modifying the root system and increasing biomass 30 days after application in *Cicer arietinum* and *Triticum aestivum*, as reported by Gahoi *et al*. (2021). In another study, Ibal *et al*. (2021) described the promotion of shoot and root growth when C8-, C10-, and C12-HSL were used, respectively. However, caution should be exercised in the use of these molecules, as they may alter the substrate microbiome and generate undesirable biological processes. Another study reported that the use of AHL-producing microorganisms is capable of promoting germination and increasing dry weight, shoot development, and root length in lettuce (*Lactuca sativa*) (Ortiz J. *et al*., 2024).

Although the use of AHL molecules to improve root development, plant height, and biomass production is very promising, it has been reported that treatments of *C. equisetifolia* with 3O-C10-HSL, 3O-C12-HSL, and 3O-C14-HSL significantly affect all developmental parameters under continuous monoculture, and that this effect may be due to the fact that these molecules promote the development of microbial communities in the soil and, in turn, through their enzymatic effects on soil nutrients, inhibit their availability, thereby negatively impacting plant development (Zhang, 2025). This indicates that we must be cautious in regulating AHL concentrations in agriculture, as they may enhance or ultimately impair biomass production.

The use of microbial molecules opens the possibility of promoting root and root hair development in various plant species, which may improve adaptation to environmental and soil conditions where they are cultivated (Tron *et al*., 2015). Our results in *S. lycopersicum* show that, although the AHLs studied share some effects at certain concentrations, C4-HSL tends to enhance longitudinal growth of the primary root, whereas 3O-C12-HSL more strongly promotes the growth and development of root hairs. Another advantage of using these AHLs as plant growth promoters in *S. lycopersicum* is that 3O-C12-HSL has been reported to induce systemic resistance against some pathogens (Hartmann *et al*., 2021). Nevertheless, further studies are still needed to demonstrate that these AHL molecules are beneficial and safe for agricultural applications.

CONCLUSIONS

The use of PGPB in agriculture represents an alternative to increase plant production; however, studying microorganisms that produce signaling molecules such as AHLs is a promising area for enhancing root development, biomass, and fruit production. In this study, we found that synthetic C4-HSL and 3O-C12-HSL in *S. lycopersicum* exhibited in vitro effects on primary root growth at a concentration of 24 μM and on root hair density at concentrations of 96 μM, respectively, whereas the concentrations obtained in crude ethyl acetate extracts were unable to generate a positive effect.

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