Plant Growth-Promoting Ability and *N*-acyl-homoserine Lactones Production by Siderophore-Producing Rhizobacteria

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ABSTRACT

Eighty bacterial isolates were obtained from the rhizosphere of sugarcane, corn, chili and watercress. Approximately 18% (15 of 80) of the tested isolates produced sideroderphore units in the range 31.8–79.9% when tested using chrome azurol sulphonate assay. The siderophore-producing bacteria were also tested for their ability to produce plant growth-promoting factors including auxin, gibberellic acid and the ability to solubilize phosphate. All of these isolates were able to solubilize tricalcium phosphate ($Ca_3(PO_4)_2$) in the range 56.8–270.3 $\mu g.mL^{-1}$ and were able to produce auxin and gibberellin in the range 2.4–22.9 $\mu g.mL^{-1}$ and 185.5–246.4 $\mu g.mL^{-1}$, respectively. Isolates Su04, Su09, and Wa65 produced the greatest amount of siderophore units. Sequence analysis of 16S rRNA gene from the three best bacterial isolates (Su04, Su09 and Wa65) indicated that the strains were *Burkholder cepacia, Pseudomonas boreopolis* and *Agrobacterium tumefaciens*, with 99, 98 and 98% sequence similarity, respectively. Fifteen siderophore-producing bacteria were able to produce *N*-acyl-homoserine lactones (AHLs) in the range 62.9–660.9 Miller units, with isolate Su04 producing the greatest amount of AHLs (660.9 Miller units). AHLs production by isolates Su04, Wa63 and Wa65 rapidly increased when the culture was grown to the late log phase, after about 18 hr of incubation, and increased until the late stationary phase after about 36 hr. The study identified a series of siderophore-producing rhizobacteria able to solubilize phosphate and produce plant growth-promoting factors and AHLs which have potential for application as plant growth-promoting agents in agriculture.

Keywords: siderophere-producing rhizobacteria, phosphate solubilization, production of plant hormones, *N*-acyl-homoserine lactones

INTRODUCTION

Iron is an essential element for almost all living organisms since it is involved in electron transfer, biocatalysis and small molecule storage and transport processes (Aisen *et al*., 2001). While iron is the fourth most abundant element on Earth and is present in abundance in various forms, it generally remains unavailable to plants due to differences in its valence state, solubility and

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bioavailability (Crowley, 2006). Iron-deficiency-induced chlorosis is a major agricultural problem resulting in diminished crop yields in an estimated 30% of calcareous soils worldwide (Mori, 1999). When the amount of iron available to plants is inadequate for growth, the leaves become pale green, yellow or white, and eventually brown (Romheld and Marschner, 1986). However, microorganisms in soils can produce siderophores which acquire iron (III) from their surrounding environment (Pinton *et al*., 2007). Siderophores are low molecular weight, extracellular organic compounds, produced by a variety of bacteria and fungi growing under low iron stress (Liles and Cianciotto, 1996). They act as chelating agents with high binding affinity and specificity for iron (III). Siderophore-producing bacteria chelate iron and make it available to plant roots. Moreover, siderophores are thought to play a role in the biocontrol of phytopathogenic microorganisms by sequestering iron, and thereby inhibiting pathogen growth or metabolic activity (Siddiqui, 2006).

Rhizosphere bacteria that are capable of aggressively colonizing plant roots and promoting plant growth are generally referred to as plant growth-promoting rhizobacteria or PGPR (Tsavkelova *et al*., 2007). PGPR can benefit plant development through multiple mechanisms of action, either directly through the production of plant growth-promoting substances and increasing nutrient availability in soil, or indirectly by the suppression of rhizosphere-dwelling plant pathogens (Pinton *et al*., 2007). Many bacterial species are capable of producing plant growth-stimulating compounds, including auxins, ethylene, gibberellins and cytokinins. PGPR activity has been reported in strains belonging to several bacterial genera, including *Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Burkholderia* and *Bacillus*.

Cell-to-cell communication is widely spread in bacteria and controls a broad range of activities that result in an altered bacterial phenotype to environmental conditions during growth (Turovskiy *et al*., 2007). Quorum sensing (QS), the term introduced in 1994 by Fuqua *et al*. (1994), is an example of cell-to-cell communication and depends on the production, secretion and response to small, diffusible signal molecules also called autoinducers (Kaplan and Greenberg, 1985). The QS mechanism depends on the synthesis and release of chemical signals into the environment and on the detection of these signals as a function of cell population density (Fuqua and Greenberg, 2002).

Quorum sensing is common among Gram-negative, plant-associated bacteria and regulates several physiological traits and behaviors associated with plant–bacterial interactions (Dong *et al*., 2007). In general, Gram-negative bacteria use acylated homoserine lactones as autoinducers. These processes include symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation and biofilm formation (Miller and Bassler, 2001). Both the legume-nodulating rhizobia and the pseudomonads suppressing the growth of other microorganisms use quorum-sensing gene regulation in relation to their stimulation of plant growth (Gonzalez and Marketon, 2003).

With the rising international concern for improving food and environmental quality, the use of PGPR has been suggested as a means to reduce chemical inputs into agriculture and increase crop productivity (Herman *et al*., 2008). Isolation of native PGPR adapted to the local environment and their study may contribute to the formulation of improved inoculants for use on regional crops. Based on this need, the current study isolated native, siderophore-producing rhizobacteria from the rhizosphere of local Thai plants and evaluated their ability to produce chemical factors known to be involved in plant growth promotion.

MATERIALS AND METHODS

Soil sample collection and isolation of rhizobacteria

Soil samples were collected in plastic bags from the rhizosphere of sugarcane, corn, chili and watercress grown in calcareous soil. The plants were taken out of the bag without disturbance and the plant roots were shaken softly to remove the root-zone soils. Then, the soil adhering to the root surrounds were collected. Soil samples were serially diluted in saline solution (0.85% weight per volume NaCl) and 100 µL aliquots of dilutions were spread-plated, in triplicate, onto the surface of nutrient agar (NA). Plates were incubated at 28 °C for 2 d and well-isolated colonies were further purified by re-streaking. Pure cultures were stored at -80 °C in 25% glycerol for further studies.

Screening for siderophore production

Siderophore production by bacterial isolates was detected through the formation of orange halos surrounding the bacterial colonies cultured on the Chrome Azurol S (CAS) agar plates after incubation at 30 °C for 48 hr. The CAS agar plates were prepared according to the procedure described by Alexander and Zuberer (1991). Quantitative assay of the siderophore production was carried out using 5 mL of CAS broth and 250 µL of bacterial cultures (OD_{600} = 1.0). Following inoculation, CAS broth was incubated at 30 °C for 48 hr, with shaking at 180 revolutions per minute. Bacterial cells were removed from the culture medium by centrifugation at 10,000×g for 5 min, and 0.5 mL of supernatant was added to 0.5 mL of CAS indicator solution. This mixture was incubated at 25 °C for 30 min before determining the absorbance of the mixture at 630 nm. For controls, CAS broth was processed through the same procedure and under the same conditions, but without inoculation with bacteria. Siderophore units in the solution were calculated as described by Payne (1994).

Polymerase chain reaction amplification of 16S rRNA and DNA sequencing

The gene-encoding 16S rRNA was amplified from selected strains using the polymerase chain reaction (PCR) technique and bacterial universal primers 8F (Brosius *et al.*, 1981) (forward) (5′-AGA GTT TGA TCA TGG CTC AG -3′) and 1492R (Lane, 1991) (reverse) (5′-GGTTACCTTGTTACGACTT-3′). The PCR mixture consisted of deoxynucleotides at 10 mM each, 10 mM of each primer, 25 mM 10X buffer, and 5 U of *Taq* DNA polymerase (Denville Scientific Inc., Metuchen, NJ, USA.). Target DNA was obtained using a suspension of cells obtained from a fresh colony on NA plates and resuspended in dH_2O. PCR was performed using the following cycle conditions: 94 °C for 5 min, followed by 35 cycles of 94 ºC for 1 min, 53.5 °C for 1 min and 72 °C for 1.5 min and a final extension step at 72 °C for 7 min. The PCR products were purified from agarose gels using a QIA quick PCR purification kit (QIAGEN, Valencia, CA, USA) and DNA was sequenced at the University of Minnesota Genomics Center. Sequences were compared to the NR sequence database at the National Center for Biotechnology Information (NCBI) using the BLASTN algorithm (http://blast.ncbi.nlm. nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_ TYPE=BlastSearch&LINK_LOC=blasthome).

Screening for plant growth-promoting attributes
Determination of tricalcium phosphate solubilizing ability

The ability of each bacterial isolate to solubilize tricalcium phosphate was determined through the formation of transparent halos surrounding bacterial colonies on the National Botanical Research Institute agar (NBRIP) medium (Nautiyal, 1999). This medium contained 5 g of $MgCl_2.6H_2O$, 0.25 g of $MgSO_4.H_2O$, 0.2 g of KCl, 0.1 g of $(NH_4)_2SO_4$, 5 g of $Ca_3(PO_4)_2$ and 10 g of glucose.

Quantitative determination of tricalcium phosphate solubilization was determined using NBRIP broth. A 250 µL aliquot of bacterial culture (OD_{600}=1) was inoculated in 5 mL

of NBRIP broth and incubated at 30 °C for 48 hr, with shaking at 180 revolutions per minute. The phosphorus content in cultures was determined using the vanadomolybdophosphoric acid method (Greenberg *et al.*, 1992). Bacterial cells were removed from the culture medium using centrifugation at 10,000×*g* for 5 min and 3.5 mL of supernatant was added to 1 mL of vanadate-molybdate reagent. A control consisted of broth without inoculation of bacteria. The amount of phosphate solubilization was expressed as micrograms per milliliter compared with the control.

Determination and quantification of indole-3-acetic acid production

The production of indole acetic acid (IAA) by each of the 15 isolates was determined using the Salkowski method (Glickmann and Dessaux, 1995). The bacteria were inoculated in the nutrient broth containing 5 mM L-tryptophan. After 24 hr of growth, the bacterial culture was centrifuged at 8,000×*g* for 5 min, and 1 mL of supernatant was mixed with 1.5 mL of Salkowski's reagent (15 mL 0.5 M $FeCl_3$, 300 mL concentrated H_2SO_4 and 500 mL H_2O). The reaction mixture was incubated at room temperature for 30 min and absorbance of the solution at 535 nm was determined (Glickmann and Dessaux, 1995). The concentration of IAA was determined by comparing with the standard curve processed using the same procedure and conditions. The amount of IAA produced was expressed as micrograms per milliliter.

Determination of gibberellic acid

Production of gibberellic acid (GA) by each of the 15 isolates was determined using the method of Paleg (1965). Bacteria were individually inoculated into nutrient broth containing 5 mM L-tryptophan and grown for 24 hr. A 2.5 mL aliquot of supernatant, produced as above, was added to 0.2 mL of 0.5M zinc acetate. After 2 min, 0.2 mL of 0.5M potassium ferrocyanide was added, the

solution was centrifuged at 8,000×*g* for 10 min and 5 mL of 5% HCl was added to 5 mL of supernatant. The sample was incubated at 20 °C for 75 min and the absorbance of the sample was measured at 254 nm using a spectrophotometer. The amount of GA present was calculated from a standard curve.

Production of acyl homoserine lactones by siderophore-producing bacteria

Agrobacterium tumerfaciens NTL4 [pCF218] [pCF372] (Zhu *et al.,* 1998) was used as the reporter strain for the detection of AHLs. Cells were grown at 30 °C in AT minimal medium (Tempé *et al.*, 1997) supplemented with tetracycline (4.5 µg.mL^{-1}) and spectinomycin (50 µg.mL^{-1}). AT medium is a common laboratory medium used to support growth of *Agrobacterium tumefaciens*. *A. tumefaciens* KYC6 (Fuqua *et al.,* 1994), a positive control strain, was grown at 30 °C in AT minimal medium with tetracycline (4.5 µg.mL^{-1}) and kanamycin (100 µg.mL^{-1}). *Escherichia coli* HB101 was grown in Luria broth (Bertani, 1951) and served as the negative control strain for AHLs production.

Fifteen selected siderophore-producing bacteria were evaluated for their ability to produce AHLs. These bacteria were streaked onto NA plates, grown at 30 °C for 48 hr and then one pure colony was picked into 5 mL of nutrient broth (NB) and incubated at 30 °C for 48 hr, with shaking at 180 revolutions per minute. Cells were subsequently harvested using centrifugation at 8,000×*g* at 4 °C for 10 min. The supernatants were discarded and the pellets were washed with 0.85% NaCl three times and then diluted to OD_{600} near 1.0 in 0.85% NaCl. A 0.25 mL aliquot of culture was added to 10 mL NB, and incubated at 30 °C with shaking at 180 revolutions per minute for 36 hr. The cells were removed by centrifugation at 8,000×*g* and 4 °C for 10 min. The supernatants were subsequently extracted with an equal volume of ethyl acetate (Zhu *et al.*, 1998). The solution was mixed thoroughly by inversion for 10 s and then placed on ice for 2 min. A 100

µL aliquot of the upper layer was transferred to a new tube containing 1 mL of a *A. fumerfacien* NTL4 [pCF218] [pCF372] suspension grown in 10 mL of AT minimal medium (10%v/v) to OD_{600} readings of 0.5. Cultures were incubated until OD_{600} reached 1.0 and production of AHLs was quantified by measuring β-galactosidase activity using o-nitrophenyl-β-D-galactopyranoside as substrate (Miller, 1972).

N-acyl-homoserine lactones production at different growth phase

In order to elucidate whether growth phase influenced AHLs production, a study was conducted using the three bacterial isolates producing the greatest amount of AHLs (Su04, Wa63 and Wa65). Bacteria were grown in NB and incubated at 30 °C, with shaking at 180 revolutions per minute. AHLs production was quantified by measuring the β-galactosidase activity at 0, 6, 12, 18, 24, 30, 36, 42 and 48 hr after incubation.

Effect of spent culture supernatant on N-acyl-homoserine lactones production

To study the influence of spent culture supernatant on AHLs production, isolates SU04, Wa63 and Wa65 were grown in NB at 30 °C, with shaking at 180 revolutions per minute until the stationary phase of growth (approximately 36 hr). Cells were harvested using centrifugation at $8,000 \times g$ and the spent medium was filtered through a 0.45 µm filter and stored at -20 °C until needed. Subsequently, bacteria were subcultured into sterile fresh AT minimal medium at 30 °C, allowed to grow until the early log phase ($OD_{600} = 0.2$) and cultures were amended with final concentrations of 0, 10, 20, and 40 % spent medium. Production of AHLs was determined as described above after 18 hr of incubation.

RESULTS AND DISCUSSION

Eighty bacterial isolates were obtained from rhizosphere soils of four different plant species. A total of 24, 18, 19 and 19 isolates were obtained from sugarcane, corn, chili and watercress, respectively. Of these, only 15 isolates produced orange halo zones against a dark blue background on CAS medium, indicating that these isolates produced siderophores. Siderophore-production ability (units) varied between 31.8 and 79.9% (Table 1) irrespective of the plant species from which the isolates were obtained. Isolates Su04, Su09 and Wa65 showed the greatest siderophore-production ability, making about 79 % siderophore units.

Sequence analysis of the 16S rRNA PCR product from each bacterium indicated that these 15 isolates represented members of the genera *Burkholderia, Pseudomonas*, *Staphylococcus, Stenotrophomonas*, *Microbacterium*, *Cupriavidus*, *Bacillus*, *Arthrobacter*, *Agrobacterium* and *Promicromonospora*. Sequence analysis of the 16S rRNA PCR products from isolates Su04, Su09 and Wa65 were 99, 98 and 98%, respectively, identical to the same region in *Burkholderia cepacia, Pseudomonas boreopolis and Agrobacterium tumefaciens*, respectively. These 15 isolates also showed the ability to solubilize tricalcium phosphate and produce IAA, gibberellic acid, and AHLs.

Phosphorus is one of the major nutrients being second only to nitrogen in requirement for plants (Vassileva *et al*., 1998). Most of the phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by the plants (Pradhan and Sukla, 2005). The ability of bacteria to solubilize tricalcium phosphate has been of interest to soil microbiologists as it can enhance the availability of phosphorus for plant growth. In the current experiments, phosphate-solubilizing ability varied between 56.8 and 270.3 µg.mL^{-1} (Table 1). Isolate Wa65 showed a maximum phosphate-solubilizing ability of 270.3 µg.mL^{-1}. Interestingly, bacterial isolates that were able to solubilize tricalcium phosphate also produced high levels of siderophores. This may be related to the fact that some phosphate-

solubilizing organic acids secreted by bacteria also have siderophore-like functions (Vassilev *et al.*, 2006**)** and are released in response to iron stress (Machuca *et al.*, 2001).

Auxin production varied between 2.4 and 22.9 $\mu g.mL^{-1}$ (Table 1). Isolate Chi60 showed maximum auxin production of 22.9 $\mu g.mL^{-1}$. Gibberellins production varied between 185.5 and 246.4 $\mu g.mL^{-1}$ and isolate Wa65 showed maximum gibberellins production of 246.42 $\mu g.mL^{-1}$.

AHLs production was also assayed in the 15 siderophore-producing bacterial isolates using an *A. tumefaciens* reporter strain (Zhu *et al.*, 1998). The results showed that these isolates produced differing quantities of AHLs, within the range 62.9–660.9 Miller units (MU) as shown in Table 1. The highest quantity of AHLs produced was observed in isolate Su04 (660.9 MU), followed by isolates Wa65, and Wa63 producing 389.9 and 335.3 MU, respectively. Furthermore,

the production may be controlled by growth phase, strain, culture medium and environment, as well as population density (Phunpruch and Baebprasert, 2003). Consequently, isolates capable of high AHLs production and other plant growth-promoting attributes were selected for further investigation of the effect of growth phase on AHLs production**.**

AHLs production over time was determined to better understand the influence of growth phase on AHLs production. The results in Figure 1 show that AHLs production started to rapidly increase when cultures grew to the late log phase, about 18 hr after incubation, and a maximum quantity was produced at the late stationary phase (about 36 hr), and thereafter the production decreased (Figure 1). Among the tested strains, isolate Su04 produced the greatest amount of AHLs (up to 615.0±13.0 MU at 36 hr), followed by strains Wa65 and Wa63 producing 389.0±9.0

Table 1 Percentage (±SD) of siderophore units, phosphate-solubilizing ability and auxin, gibberellin and acylhomoserine lactone production by 15 rhizosphere bacteria isolated from a variety of plants.

Isolate name	Percent siderophore units (%)	Phosphate-solubilizing ability ($\mu g.mL^{-1}$)	Auxin production ($\mu g.mL^{-1}$)	Gibberellin production ($\mu g.mlL^{1}$)	Acylhomoserine lactone production (Miller units)
Su04	79.9±0.4	255.0±10.0	12.9±0.2	189.3±11.2	660.9±43.9
Su09	79.6±0.3	114.3±7.5	0.0±0.0	205.1±11.9	64.8±11.4
Su11	64.1±3.1	175.7±2.1	8.9±0.0	198.9±9.4	80.1±6.7
Su18	64.4±3.5	117.0±36.0	5.3±0.5	214.4±0.0	77.5±11.9
Su20	31.8±2.0	183.4±7.4	12.6±0.4	198.9±9.0	57.3±13.2
Cor25	76.3±0.5	99.2±11.5	5.0±0.5	197.3±11.8	62.9±16.4
Cor26	66.9±2.5	207.6±10.3	10.5±0.2	207.4±5.4	120.2±7.9
Cor38	63.4±8.4	56.8±3.5	7.4±0.1	205.1±8.4	96.7±17.2
Chi44	56.5±3.6	94.7±12.0	8.3±0.6	208.9±15.8	78.9±5.5
Chi60	55.3±3.1	223.4±24.1	22.9±0.7	193.4±7.0	98.3±10.3
Wa63	61.3±1.7	135.8±15.7	11.9±0.4	185.5±2.3	335.3±13.2
Wa64	65.5±1.4	130.8±11.4	6.6±0.4	197.5±10.3	88.8±5.9
Wa65	79.5±0.5	270.3±1.6	17.3±0.7	246.4±14.4	389.0±9.0
Wa70	57.5±5.5	57.2±8.1	2.4±0.1	202.2±10.9	94.9±12.5
Wa72	67.3±4.7	193.3±7.9	3.1±0.0	202.4±9.8	100.4±11.3

and 335.3±13.2 MU, respectively. This is in accordance with quorum-sensing phenomenon (Dumenyo *et al.*, 1998). A lower level of AHLs was detected during the death phase due to increasing media pH and AHLs hydrolysis (Yates *et al.*, 2002).

To investigate whether culture supernatant from each isolate could induce quantitative changes in production of AHLs, spent culture medium was prepared from isolates Su04, Wa63, and Wa65 grown to the stationary phase. Cultures were grown in the presence or absence of spent culture supernatant supplementation. The results shown in Figure 2 indicate that spent culture was capable of inducing AHLs production in all the tested isolates. In all the tested isolates, the
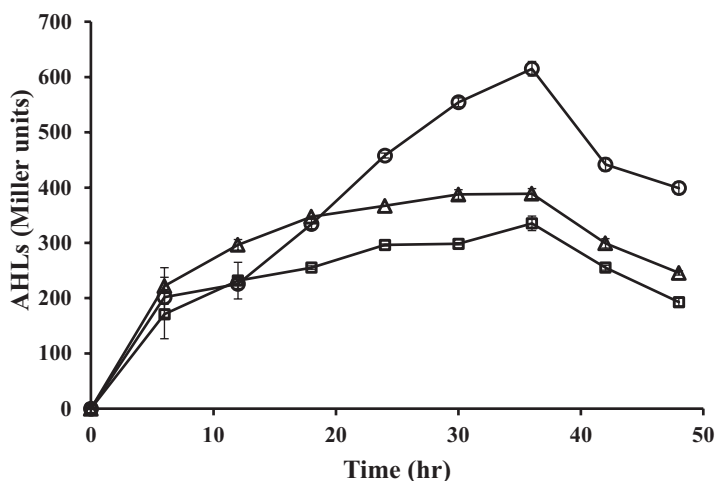

Figure 1 *N*-acyl-homoserine lactones (AHLs) production of isolate *Burkholder cepacia* strain Su04, *Pseudomonas boreopolis* strain Wa63 and *Agrobacterium tumefaciens* strain Wa65 at different growth phases. ○ = Su04; □ = Wa63; Δ = Wa65. Error bars indicate ±SD.
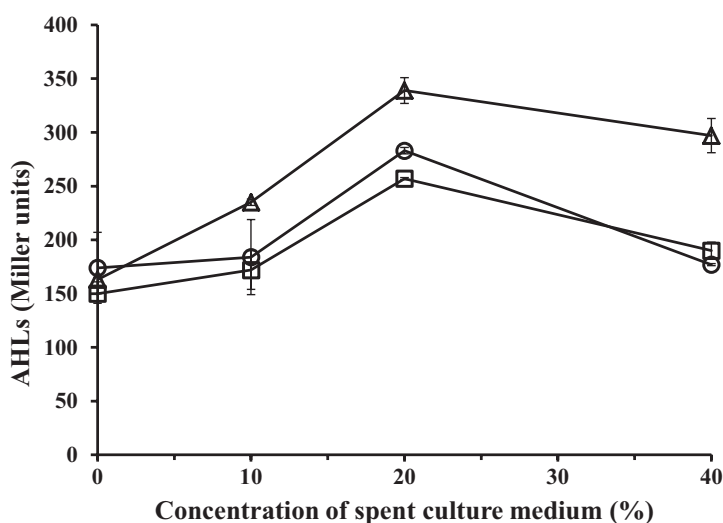

Figure 2 *N*-acyl-homoserine lactones (AHLs) production (Miller unit) by isolates Su04, Wa63 and Wa65 in addition of spent culture medium with various concentrations. ○ = Su04; □ = Wa63; Δ = Wa65. Error bars indicate ±SD.

maximum induction of β-galactosidase activity occurred with 20% spent culture supernatant. Isolate Wa65 showed the greatest β-galactosidase activity (339±12 units) followed by strains Su04 and Wa63, producing approximately 283±3 and 257±1 units, respectively. The β-galactosidase activity decreased with 40% spent culture supernatant in all tested isolates. The spent culture medium may result in quantitative changes in the production of AHLs. Allison *et al*. (1998) also reported the effects of spent culture medium on biofilm formation—*Pseudomonas fluorescens* B52 biofilms grown on glass coverslips in spent medium were reduced in total biomass compared to biofilms grown in fresh medium.

CONCLUSION

The results confirmed the existence of siderophore-producing bacteria in the rhizosphere of sugarcane, corn, chili and watercress grown in calcareous soil. Moreover, this study revealed that siderophore-producing rhizobacteria have characteristics which suggest the potential to promote plant growth. These siderophore-producing bacteria could be useful as plant growth regulators in eco-friendly and sustainable agricultural practices. The most promising isolates will be further tested in pot trials for their ability to antagonise pathogens. In addition, the involvement of siderophore and AHLs production in these siderophore-producing rhizobacteria will be investigated.

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