CHEMICAL ENGINEERING TRANSACTIONS
VOL. 65, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Eliseo Ranzi, Mario Costa
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ISBN 978-88-95608-62-4; ISSN 2283-9216
DOI: 10.3303/CET1865119

Differential Sugarcane (*Saccharum x sp*) Biomass Growth Using Long Chain Acyl-homoserine Lactones

Vanessa G. A. Olher[a,b], Alan G. Souza[a], Graciene S. Bido[c], Aline F. Teixeira[c], Wanderley D. Santos[c], Silvana M. O. Santin[a], Osvaldo Ferrarese-Filho[c], Armando M. Pomini[a*]

[a]Universidade Estadual de Maringá (UEM), Departamento de Química, Maringá, Paraná, Brazil
[b]Instituto Federal do Paraná (IFPR), Campus Paranavaí, Paraná, Brazil
[c]Universidade Estadual de Maringá (UEM), Departamento de Bioquímica, Maringá, Paraná, Brazil
ampomini@uem.br

The acyl-homoserine lactones (AHL) are compounds used in intercellular chemical signaling processes by Gram-negative bacteria in a phenomenon known as quorum-sensing. This process makes the microorganisms able to sense and respond to stimuli of individuals of the same species, or even cause physiological changes in the surrounding bacterial populations. In recent years, there is a growing interest in the effect that these compounds might have on higher organisms, such as inter-kingdoms communication mechanisms or physiological and morphological changes in host. In this regard, the study of the effects of these metabolites in plants has gained particular attention. Recently, it was shown that *N*-(3-oxo-octanoyl)-HL is present in the extract obtained from the leaves and stalks of sugarcane cultivated in Brazil, and that this compound can stimulate the growth of roots and shoots of the plant at low concentrations. Interestingly, it was found that both enantiomers (*R*) and (*S*) can accelerate plant growth. Thus, it is reported herein the chemical syntheses of *N*-acyl-HL derivatives with structural variations in the acyl side chain length and presence of carbonyl groups, and their growth-promoting activity evaluation on sugarcane meristems. It was found that all derivatives were able to stimulate plant growth, being *N*-decanoyl-HL the most active compound. Compounds (*R*)-N-decanoyl-HL and its (*S*) enantiomer caused significant increase of growth of roots and shoots in meristems of sugarcane compared to the control. However, the test highlighted the (*S*) enantiomer, which caused an increase in the average weight of dry roots and root length of 90.1% and 92.1%, respectively. A surprising increase in average mass of dry buds and buds length of 134.2% and 390.0% were observed when the stems of sugarcane were treated with this compound. Thus the (*S*) enantiomer has proved to be more active than the (*R*) one. It is believed that these compounds may represent a new frontier for development of plant growth active compounds with application in commercial agriculture.

1. Introduction

Quorum-sensing is a mechanism of intercellular communication between bacterial cells, which makes these microorganisms able to sense and respond to stimuli of individuals of the same species, or even cause physiological changes in the surrounding bacterial populations. In Gram-negative species, the acyl-homoserine lactones (AHL) are the main synthesized signals (Whitehead et al., 2001). In recent years, there is a growing interest in the effect that these compounds might have on higher organisms, such as inter-kingdoms communication mechanisms or physiological and morphological changes in host (Hughes and Sperandio, 2008). In this aspect, the study of the effects of these metabolites in plants has gained particular attention (Hartmann et al., 2014).
One of the first studies on the effects of semiochemicals belonging to the class of AHL in plants was carried out with algae. It was shown that zoospores of algae can detect these compounds in seawater, and use this skill as a microbial biofilms tracing method (Joint et al., 2002). Subsequently, it was found that tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus*) showed resistance to pathogenic organisms when treated with AHL-producing bacteria (Schuhegger et al., 2006; Pang et al., 2009).

Please cite this article as: Olher V., Souza A., Bido G., Teixeira A., Santos W., Santin S., Ferrarese Filho O., Pomini A., 2018, Differential sugarcane (saccharum x sp) biomass growth using long chain acyl-homoserine lactones, Chemical Engineering Transactions, 65, 709-714 DOI: 10.3303/CET1865119

A pioneer and most comprehensive study on the physiological effects and mechanisms of action of these compounds on plants was carried out for the species *Arabidopsis thaliana* (Ortíz-Castro et al., 2008). It was shown that AHL can cause changes in the pattern of growth of primary roots, accelerating the development of the plant. The compound *N*-decanoyl-HL showed the greatest potential for growth promotion among some structurally similar compounds evaluated. Although the mechanism of action of these compounds in plants is still largely unknown, there are evidences that they can increase the levels of cytokinins and auxins in *A. thaliana* roots (von Rad et al., 2008).
Sugarcane is one of the most important crops in Brazil, used for production of both sugar and ethanol. Our research group recently reported the isolation of *N*-(3-oxo-octanoyl)-HL from an extract obtained from leaves and stems of sugarcane grown commercially in Brazil. This was most likely a compound produced by an endophytic bacterium. It was found that when sugarcane stalks were treated with the same synthetic compound, there was a large change in the biomass of roots and shoots. Both enantiomers were able to stimulate plant growth, causing stretching and other morphological changes in the cells of sugarcane roots (Olher et al., 2016). It was a very interesting result, since improvements of sugarcane crop development and biomass production have direct impact in ethanol and energy production (Ensinas et al., 2013).
Continuing our efforts to understand the activity of AHL compounds in the rooting and sprouting of sugarcane, and more particularly the role of the absolute configuration of these compounds for their biological activities, it is reported herein the synthesis and bioassays with AHL derivatives with different lengths of acyl side chains, and the presence of carbonyl groups in positions 3'. Synthetic compounds were spectroscopically characterized, and were then subjected to biological assays using stalks of sugarcane variety RB96-6928.

2. Materials and methods

2.1 General

The optical rotations were measured on a Perkin-Elmer polarimeter 343 model at 20 °C and 589 nm, with an optical path cell of 10 mm, using EtOAc as solvent. ^{1}H NMR spectra were acquired with a Bruker spectrometer, operating at 300.06MHz. Chemical shifts are reported in ppm with reference to internal TMS (tetramethylsilane, δ = 0.0 ppm). $CDCl_3$ (Aldrich) was used in the NMR analyses. Column chromatography (CC) was performed using silica gel 60 (70-230 mesh) acquired from Merck or Fluka. For thin layer chromatography (TLC), silica gel 60 G (Merck) was employed, with 0.25 mm of stationary phase. All reagents were purchased from Sigma Aldrich (Milwaukee, WI, U.S.A). Dichloromethane was previously distilled and dried by stirring over anhydrous $CaCl_2$. All reagents and solvents had purity grade ACS or higher.

2.2 Syntheses of *N*-(3-oxo-acyl)-HL

In a 50 mL flask, 20 mL of dry CH_2Cl_2 was added under an atmosphere of nitrogen. After, 2.0 mmol of octanoic acid, decanoic acid or dodecanoic acid (288.0; 344.0 and 400.0 mg, respectively) were added. It was also added 2.1 mmol of 4-dimethylaminopyridine (256.0 mg), 2.2 mmol of dicyclohexylcarbodiimide (453.0 mg) and 2.0 mmol of Meldrum's acid (288.0 mg). The solution remained under magnetic stirring at room temperature. After 24 hours, the medium was filtered, and the organic phase evaporated under reduced pressure at 38 °C. The yellow oil obtained was dissolved in 20.0 mL of EtOAc and 5.0 mL of MeOH and extracted with aqueous 2 M HCl (3 x 10 mL) and distilled water (1 x 10 mL). The solution was dried over anhydrous $MgSO_4$. The organic layer was then filtered and evaporated under reduced pressure. The Meldrum´s acylated derivative was stored at -20 °C and used for the next step as soon as possible.
To a solution of Meldrum´s derivative (0.75 mmol of octanoyl-, decanoyl- or dodecanoyl-Meldrum; 202.5; 223.8 and 244.5 mg, respectively) in 22.5 mL of CH_3CN (HPLC grade), 0.75 mmol of (*R*)-α-amino-γ-butyrolactone hydrochloride (103.0 mg) and 1.2 mmol of Et_3N (121.2 mg) were added. The mixture was held under magnetic stirring and reflux for 3 hours. The organic layer was evaporated under reduced pressure and the white solid was dissolved in a mixture of 20 mL of EtOAc and 5 ml of MeOH. The organic solution was extracted with saturated $NaHCO_3$ (3 x 10 mL), 1 M $KHSO_4$ (3 x 10 mL) and saturated aqueous NaCl (3 x 10 mL). The organic layer was then dried over anhydrous $MgSO_4$, filtered and evaporated, yielding a yellowish solid. The products of the reactions were purified by silica gel column chromatography (12 g silica, θ = 2 cm), eluting with n-hexane, CH_2Cl_2 and EtOAc in increasing polarity. Synthetic compounds (**1**-**3**) were obtained as white solids with 40 %, 62 %, and 70 % yield respectively.

(*R*)-*N*-(3-oxo-decanoyl)-HL (1) $[\alpha]_D^{20}$ +7.0 (*c* 1.0, EtOAc). ^{1}H NMR ($CDCl_3$, 300.06 MHz) δ 0.88 (3H, t, *J* 6.0 Hz, H-10'), 1.29 (8H, m, H-6'-H-9'), 1.56 (2H, m, H-5'), 2.23 (1H, m, H-4a), 2.74 (1H, m, H-4b), 2.53 (2H, t, *J* 6.0 Hz, H-4'), 3.47 (2H, s, H-2'), 4.48 (1H, ddd, *J* 10.8, 9.3, 6.6 Hz, H-5), 4.28 (1H, ddd, *J* 9.0 and 1.8 Hz, H-5), 4.58 (1H, dd, *J* 9.3 and 10.8 Hz, H-3).
(*R*)-*N*-(3-oxo-dodecanoyl)-HL (2) $[\alpha]_D^{20}$ +10.0 (*c* 1.0, EtOAc). ^{1}H NMR ($CDCl_3$, 300.06 MHz) δ 0.86 (3H, t, *J* 6.0 Hz, H-12'), 1.24 (12H, m, H-6'-H-11'), 1.55 (2H, m, H-5'), 2.24 (1H, m, H-4a), 2.69 (1H, m, H-4b), 2.51 (2H,

t, J 6.0 Hz, H-4'), 3.45 (2H, s, H-2'), 4.45 (1H, ddd, J 10.8, 9.3, 6.6 Hz, H-5), 4.27 (1H, ddd, J 9.0 and 1.8 Hz, H-5), 4.59 (1H, dd, J 9.3 and 10.8 Hz, H-3).
(*R*)-*N*-(3-oxo-tetradecanoyl)-HL (3) $[\alpha]_D^{20}$ +10.0 (*c* 1.0, EtOAc). ^{1}H NMR ($CDCl_3$, 300.06 MHz) δ 0.76 (3H, t, J 6.0 Hz, H-14'), 1.14 (16H, m, H-6'-H-13'), 1.46 (2H, m, H-5'), 2.16 (1H, m, H-4a), 2.55 (1H, m, H-4b), 2.51 (2H, t, J 6.0 Hz, H-4'), 3.75 (2H, s, H-2'), 4.37 (1H, ddd, J 10.8, 9.3, 6.6 Hz, H-5), 4.18 (1H, ddd, J 9.0 and 1.8 Hz, H-5), 4.48 (1H, dd, J 9.3 and 10.8 Hz, H-3).

2.3 Syntheses of *N*-acyl-HL

In a 5 ml flask, Et_3N (0.11 mmol; 11.1 mg), (*R*) or (*S*)-α-amino-γ-butyrolactone hydrochloride or hydrobromide (0.11 mmol; 15.1 and 20.0 mg respectively) and a solution of decanoic or dodecanoic acid (0.16 mmol; 27.5 and 32.0 mg respectively) in 2.5 mL of ultrapure water (Milli-Q) were added. It was further added 1.0 mg of NaOH only in the reaction pot containing dodecanoic acid. Then, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.16 mmol; 24.8 mg) was added. The reaction mixture was held under magnetic stirring at room temperature. After 24 hours, it was extracted with EtOAc (3 x 10 mL) and the organic layer was extracted with aqueous 5 % $NaHCO_3$ (2 x 6 mL), 1M $NaHSO_4$ (1 x 6 mL) and saturated aqueous NaCl (1 x 6 mL). The solvent was evaporated under reduced pressure, yielding a white solid. Then, the obtained material was purified by chromatography on a silica gel column, prepared with a small upper layer containing silica gel macerated with NaOH (5% by mass) (12 g silica, θ = 2 cm), and eluted with n-hexane, CH_2Cl_2 and EtOAc in increasing polarity. This procedure gave 12 fractions, and the fractions eluted with 100% EtOAc gave white crystals, yielding the products (**4**-**6**) with yields of 48 %, 50 %, and 45 % respectively.

(*R*)-*N*-(decanoyl)-HL (4) $[\alpha]_D^{20}$ +9.0 (*c* 1.0, EtOAc.^{1}H NMR ($CDCl_3$, 300.06 MHz) δ 0.87 (3H, t, J 6.0 Hz, H-10'), 1.25 (12H, m, H-4'-H-9'), 1.62 (2H, m, H-3'), 2.24 (C-2'), 2.15 (1H, m, H-4a), 2.80 (1H, m, H-4b), 4.45 (1H, ddd, J 10.8, 9.3, 6.6 Hz, H-5), 4.28 (1H, ddd, J 9.0 and 1.8 Hz, H-5), 4.57 (1H, dd, J 9.3 and 10.8 Hz, H-3).
(*R*)-*N*-(dodecanoyl)-HL (5) $[\alpha]_D^{20}$ +3.0 (*c* 1.0, EtOAc). ^{1}H NMR ($CDCl_3$, 300.06 MHz) δ 0.87 (3H, t, J 6.0 Hz, H-12'), 1.25 (16H, m, H-4'-H-11'), 1.62 (2H, m, H-3'), 2.24 (C-2'), 2.15 (1H, m, H-4a), 2.80 (1H, m, H-4b), 4.45 (1H, ddd, J 10.8, 9.3, 6.6 Hz, H-5), 4.28 (1H, ddd, J 9.0 and 1.8 Hz, H-5), 4.57 (1H, dd, J 9.3 and 10.8 Hz, H-3).
(*S*)-*N*-(decanoyl)-HL (6) $[\alpha]_D^{20}$ -22.0 (*c* 1.0, EtOAc). ^{1}H NMR ($CDCl_3$, 300.06 MHz) δ 0.88 (3H, t, J 6.0 Hz, H-10'), 1.27 (12H, m, H-4'-H-9'), 1.62 (2H, m, H-3'), 2.25 (C-2'), 2.14 (1H, m, H-4a), 2.84 (1H, m, H-4b), 4.47 (1H, ddd, J 10.8, 9.3, 6.6 Hz, H-5), 4.29 (1H, ddd, J 9.0 and 1.8 Hz, H-5), 4.57 (1H, dd, J 9.3 and 10.8 Hz, H-3).

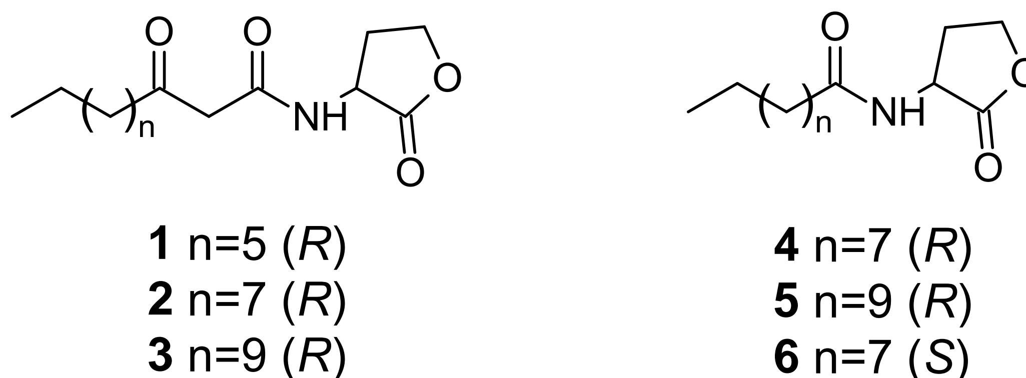

Figure 1. Synthesized compounds.

2.4 Bioassays with sugarcane stems

The bioassays with sugarcane (*Saccharum x sp*, variety RB96-6928) stems were carried out as described in the literature (Olher et al., 2016). Stems (n = 6) with approximately 3.0 cm diameter were sterilized with 70 % EtOH, incubated on vermiculite and kept in a growth chamber at 30 °C for 8 days with a photoperiod cycle 12 : 12 h light : dark. Artificial irrigation was performed every two days with control solution (100 µL of DMSO and 1000 mL of H_2O) or a solution of synthetic compounds **1**-**6** dissolved in the described control solution at a concentration of 2 µM. After the incubation period, the lengths of shoots and roots were measured, and the

mass of dry roots and shoots were estimated after oven drying at 80 °C. All measured parameters were divided by the diameter of the individual stems. All data were subjected to Dunnett´s statistical tests.

3. Results and discussion

The AHLs were synthesized according to a procedure described in the literature (Pomini et al., 2009). The compounds (*R*)-*N*-(3-oxo-decanoyl)-HL (**1**), (*R*)-*N*-(3-oxo-dodecanoyl)-HL (**2**), (*R*)-*N*-(3-oxo-tetradecanoyl)-HL (**3**), (*R*)-*N*-(decanoyl)-HL (**4**), (*R*)-*N*-(dodecanoyl)-HL (**5**), and (*S*)-*N*-(decanoyl)-HL (**6**) were obtained. All synthesized compounds (Figure 1) were characterized by spectroscopic techniques of nuclear magnetic resonance and polarimetric analysis.

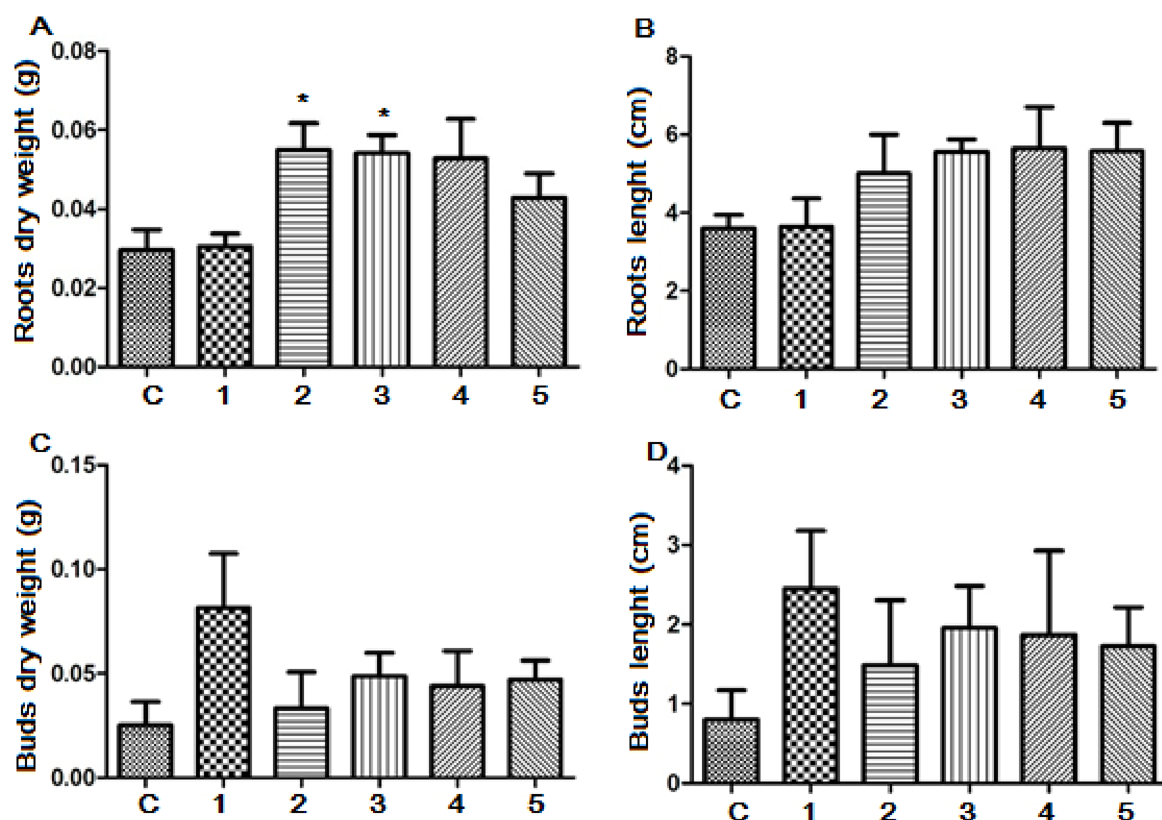

*Figure 2. Effect of control solution (C; H_2O/DMSO), and compounds **1-5** (concentration 2 μM), in the growth of roots and shoots in meristems. (A) roots dry weight; (B) roots length; (C) buds dry weight; (D) buds length. Standard deviations are also shown (n = 6). Results were subjected to Dunnett's statistical tests and means significantly higher than the control ($p \leq 0.05$) are marked *.*

Biological assays were performed to access the effects of synthetic compounds on the growth of sugarcane meristems of the cultural variety RB96-6928. The effects of the synthesized compounds for changes in dry mass and length of roots and shoots were evaluated (Figure 2).

The tested compounds caused changes in the length and mass of roots (Figure 2), especially the compound **2**, which stimulated the increase of the mass of roots by 97.8% when compared to the control. Regarding the average length and mass of dry buds, compounds **1** and **3** were the most active. Noteworthy, compound **3** significantly stimulated the growth of both roots and shoots. Compounds **4** and **5** were also significantly active. Considering the great activity observed for compound **4** for the growth of roots, we also decided to investigate the activity of its enantiomer (*S*)-*N*-decanoyl-HL (**6**) (Figure 3).

Compounds (*R*)-*N*-decanoyl-HL (**4**) and its (*S*) enantiomer (**6**) caused significant increase in the growth of roots and shoots of meristems of sugarcane when compared to the control. However, the test highlighted the (*S*) enantiomer, which caused an increase in the average weight of dry roots and root length of 90.1% and 92.1%, respectively. A surprising increase in average mass of dry buds and buds length of 134.2% and 390.0% were observed when the stems of sugar cane were treated with the compound (*S*)-*N*-decanoyl-HL (**6**). Thus the (*S*) enantiomer has proved to be more active than the (*R*) one, in the same manner as previously

observed for the enantiomers of *N*-(3-oxo-octanoyl)-HL (Olher et al., 2016). Moreover, this study shows that despite structural variations, the (*R*) enantiomers of various AHL are also able to regulate the growth of sugarcane meristems. However, it is important to point that several assays had high standard deviations, which led to restricted results after Dunnett's statistical tests (Figures 2 and 3). This may be explained by the genetic instability and somaclonal variations reported for sugarcane meristem clones (Zucchi et al., 2002). This phenomenon certainly deserves further investigation at the molecular level.

According to literature, short chain AHL (*N*-butanoyl-HL and *N*-hexanoyl-HL) showed no significant effects on the development of *A. thaliana* roots (Ortíz-Castro et al. 2008; von Rad et al. 2008). On the other hand, long-chain homologues were active, highlighting the *N*-decanoyl-HL, with the highest growth activity for *A. thaliana*. In this work the same compound also showed significant biological activity, stimulating the growth of sugarcane roots, with biomass increases even higher than those observed for *N*-(3-oxo-octanoyl)-HL found in the extract of the plant and recently published by our research group (Olher et al. 2016).

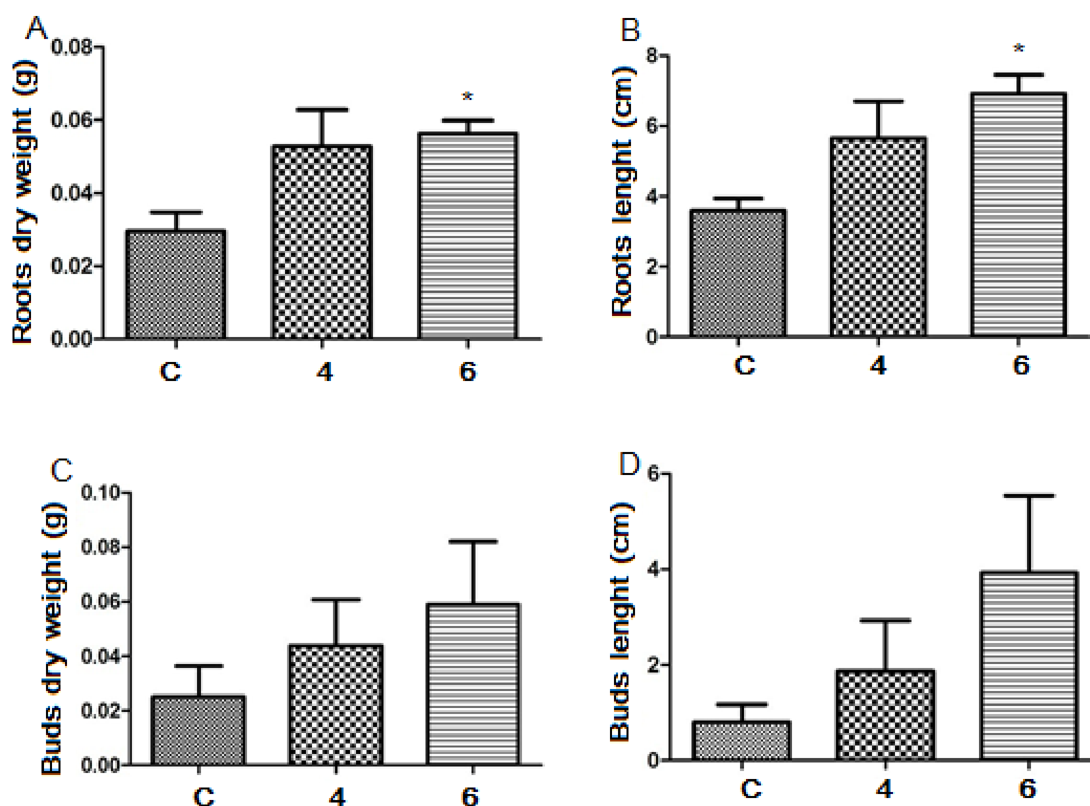

*Figure 3. Effect of control solution (C; H_2O/DMSO), and compounds **4** and **6** in the growth of roots and shoots in meristems. (A) roots dry weight; (B) roots length; (C) buds dry weight; (D) buds length. Standard deviations are also shown (n = 6). Results were subjected to Dunnett's statistical tests and means significantly higher than the control ($p \leq 0.05$) are marked *.*

4. Conclusion

Herein, the biological activity of six acyl-homoserine lactones were investigated on sugarcane growth, and *N*-decanoyl-HSL was especially active. It has a simple chemical structure and may be synthesized from a simple, one step procedure from commercially available building blocks. It is believed that acyl-homoserine lactones may represent a new frontier for development of plant biomass growth regulator products. However, large scale field trials are necessary for better understanding the effects of these compounds in crop production of sugarcane.

Acknowledgements

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação Araucária.

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