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Production of siderophores by the bacterium *Kosakonia radicincitans* and its application to control of phytopathogenic fungi

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Production of siderophores by *Kosakonia radicincitans* and its application to control of phytopathogenic fungi

Abstract

The present work aimed to characterize and to improve the production of the siderophore produced by *Kosakonia radicincitans* and apply it to control of phytopathogenic fungi. The siderophore, characterized by liquid chromatography coupled to tandem mass detection (LC-MS/MS), was identified as enterochelin. The improvement of its production was carried out using different conditions. The higher production of enterochelin (45 mg g^{-1} dry weight biomass) was reached in a medium with lactose as the carbon source, 0.82 µmol.L^{-1} of iron, at 28°C and 200 rpm. The siderophore at 60 mg L^{-1} of concentration inhibited the conidia germination and the mycelial growth of *Penicillium expansum*, *Botrytis cinerea*, *Rhizopus sp*., *Alternaria sp*. and *Cladosporium cladosporoides*. Biocontrol of *Botrytis cinerea* and *Penicillium expansum* were performed in apple fruit using *Kosakonia radicincitans* and enterochelin, alone or in a mixture. The mixture was the more effective. In this case, the reduction of decay provoked for *B.cinerea* reached 52%.

Keywords: Biocontrol; Enterochelin; Siderophores; *Kosakonia radicincitans*, Biofungicide

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1. Introduction

Biological control is an environmentally friendly alternative to combat the post-harvest diseases in fruits caused by phytopathogenic fungi. Also, some phytopathogenic fungi are dangerous for human health because they are producers of mycotoxins. Competition for iron has an essential role in the biocontrol of phytopathogenic microorganisms, overall when the biological control agent is a microorganism producer of siderophores (Droby et al., 2009; Sharma et al., 2009; Spadaro and Droby, 2016). These compounds sequester the iron, producing inhibition of the growth of the pathogen or its metabolic activity (Calvente et al., 1999; Sansone et al., 2005; Tortora et al., 2011). Moreover, bacteria which are producers of siderophores play an essential role as plant growth promoters (Katiyar and Goel, 2004, Numan et al., 2018).

Kosakonia radicincitans is a promoter of plant growth not only for wheat or maize but also for tomato and radish (Berger et al., 2017 and 2018). While the capacity to produce siderophores by this bacterium was proved by means of the CAS assay (Bergottini et al., 2015), until now the molecule was not characterized, and its production was not optimized either.

Taking into account the role of siderophores in the biocontrol interactions the objectives of this work were: 1) to identify the siderophore produced by *K. radicincitans*, 2) to improve its production by changing of the growth conditions of the bacterium, and 3) to examine the effectiveness of the siderophore for controlling phytopathogenic fungi.

2. Materials and Methods

2.1 Microorganisms

Kosakonia radicincitans was isolated from the skin of apples. For the biochemical identification were used API 20 E and API 50 CHE systems (bioMérioux, France). For molecular identification, Macrogen (Korea) amplified the 16S rRNA sequence using the following bacterial-specific primer set: 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT

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CMT TTR AGT TT) 3'. The analysis of 16S rDNA sequence revealed that the isolated (accession number: NR_117704.1) was closely related (99%) with *K. radicincitans*.

Penicillium expansum, *Botrytis cinerea, Rhizopus sp*., *Alternaria sp*. and *Cladosporium cladosporioides* were used in the control assays. *P. expansum* was a gift from INTA (National Institute of Agricultural Technology, Alto Valle, Rio Negro, Argentina). All the other fungi were purchased from CEREMIC (Mycological Reference Center, Rosario, Argentina)

2.2 Culture media

K. radicincitans was maintained on a synthetic medium (YGM) containing: Glucose 10 g L^{-1}, Yeast extract 5 g L^{-1} and Agar 20 g L^{-1}. Phytopathogenic fungi were maintained on Potato Dextrose Agar (PDA).

A primary medium for siderophore production "Vitamin free medium" (VF) was used. This medium was prepared with glucose 10 g, $(NH_4)_2SO_4$ 3.5 g, L-asparagine 1.5 g, L-methionine 0.02 g, L-histidine 0.010 g, L-tryptophane 0.020 g, KH_2PO_4 1 g, $MgSO_4$ 0.5 g, NaCl 0.5 g. The solution was made up to 1000 mL with distilled water, and the pH was adjusted to 7.00 $\pm$ 0.2. Iron measured by Atomic absorption spectrometry was 0.82 μmol L^{-1}.

2.3 Culture procedure

K. radicincitans was grown in 500 mL Erlenmeyers flasks with baffles containing 125 mL of the VF medium, which were inoculated with 10 mL of a 24 h actively growing culture. The flasks were incubated at 28°C and shaken at 140 rpm in a rotatory shaker placed into a stove room. After 24 h and 48 h, the cells were removed by centrifugation at 11000 x g for 10 min in a Sorvall SS-3 (DuPont Instruments). Immediately, biomass (dry weight) and siderophore concentration in supernatants were determined. All assays were made in triplicate.

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2.4 Detection of siderophores in culture supernatants

In preliminary assays, two colorimetric methods, Arnow and ferric perchlorate assays (Payne, 1994) were used for detecting siderophores in the cultures supernatants. Subsequently, at the stage of production, determination of siderophores was carried out by the Arnow assay, using as standard Enterochelin solution (HPLC grade, E3910, analytical standard obtained from Sigma, Steinheim, Germany)

2.5 Siderophore characterization by TLC and UHPLC-(+)ESI-MS/MS

Siderophore was extracted from supernatants of culture media by a three times extraction procedure with ethyl acetate, the organic phase was concentrated by evaporation to dryness and then reconstituted with methanol. Subsequently, the methanolic extract was seeded on TLC plates (SIL G/UV254 POLYGRAM ®) As solvent was used 200 mL of a mixture of benzene, acetic acid, and water, in the following proportion: 125:72:3. As standard was used enterochelin solution (HPLC grade, E3910, obtained from Sigma, Steinheim, Germany). Ferric chloride was used for revealing.

Mass spectrometry analyses were performed on a Quattro PremierTM XE Micromass MS Technologies triple quadrupole mass spectrometer with a ZSprayTM electrospray ionization source (Waters, Milford). Detection was carried out in multiple reaction monitoring (MRM) mode of selected ions at the first (Q1) and third quadrupole (Q3).

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2.7 Production of enterochelin

2.7.1 Growth kinetics

The kinetics of growth of the microorganism and its relation to the production of enterochelin was evaluated using VF medium, at 28°C and 140 rpm. For these assays, 500 mL Erlenmeyer flasks with baffles, containing 125 mL of the medium, were inoculated with 10 mL of a 24 h culture came from a VF medium. Determination of the siderophores and biomass was carried out every three h in a 40 h period. Experiments were done in triplicate.

2.7.2 Effect of the iron concentration

To study the influence of the iron concentration on enterochelin production, the VF medium, was supplemented with various amounts of iron ($FeSO_4$). The total iron concentration in the medium was determined before inoculation by atomic absorption spectrometry. Final concentrations were: 0.82 µmol L^{-1}, 3.32 µmol L^{-1}, 5.82 µmol L^{-1} and 20.82 µmol L^{-1}. Assays were performed at 28°C and 140 rpm. Experiments were done in triplicate. Enterochelin concentration was expressed as mg enterochelin g^{-1} dry weight biomass.

2.7.3 Effect of the carbon source, temperature and agitation rate

To investigate the effect of the carbon sources and the temperature on the production the enterochelin and biomass, glucose of VF medium, was replaced by glycerol (10 g L^{-1}) or lactose (10 g L^{-1}). In all cases, the relation between carbon / nitrogen was maintained constant. The assays were performed at 10°C and 28°C. The agitation rate was maintained at 140 rpm. Experiments were done in triplicate. Enterochelin concentration was expressed as mg enterochelin g^{-1} dry weight biomass.

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The assays for studying the effect of the agitation rate were performed at 140 rpm, 200 rpm, and 240 rpm. VF media with glucose, glycerol or lactose were used in these assays. Assays were performed at 28°C. Experiments were done in triplicate. Enterochelin concentration was expressed as mg enterochelin g^{-1} dry weight biomass.

2.8 Effect of enterochelin on conidial germination and mycelial growth of phytopathogenic fungi

The effect of enterochelin on conidial germination of phytopathogenic fungi was studied using microcultures assays using an aqueous solution of the siderophore. The siderophore was extracted from the culture medium as was described before, but it was reconstituted with water. 60 µL of Potato Dextrose Broth (PDB), 30 µl of enterochelin in aqueous solution (60 mg L^{-1}) or distilled water (as control) and 30 µL of conidial suspension (10^6 conidia mL^{-1}) were put on appropriate slides. The slides were maintained in a wet chamber at 28°C, for 7 h. After this time, slides were observed with a light microscope (Olympus model CH30RF200, Olympus Optical CO, Japan) at a magnification of *x400*. Conidial germination was assessed counting at least 100 conidia per slide. The percentage of conidia inhibition of phytopathogens was calculated according the following expression: % Germination inhibition = [(germinated conidia in the control – germinated conidia in the sample) / germinated conidia in the control] x 100.

The assays for assessing the effect of enterochelin on the mycelial growth were performed in Eppendorf tubes in which were put 500 µL of PDB, 200 µL of an aqueous solution of enterochelin (60 mg L^{-1} or 120 mg L^{-1}) and 300 µL of conidial suspension (10^6conidia mL^{-1}). Distilled water was used as controls. The tubes were incubated at 28°C for 120 h. After this time, the mycelium was separated by filtration and dried in a stove at 50°C until constant weight. The

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inhibition (%) of mycelial growth was calculated as % Growth Inhibition = [Growth of the control - Growth of the sample) / Growth of the control] x100

2.9 Effect of enterochelin in the biocontrol of *Botrytis cinerea* and *Penicillium expansum*

The effect of enterochelin in combination with *K. radicincitans* was evaluated on Red delicious apple fruit. Before the assays, firmness, sugars, and pH were evaluated, and the maturity parameters determined on fruit were 9.7 ± 0.91 Kg cm^{-2} of firmness, 15.48 ± 0.48 ° Brix and pH 3.7 ± 0.07. Washed and disinfected apples were wounded on three opposite sides, with the tip of a disinfected 10-penny nail (3 mm diameter, 3 mm deep). For the biocontrol assays, 20 µL of *K. radicincitans* suspension (10^6cells mL^{-1} according to Mac Farland scale), alone or in mixture with enterochelin (60 mg L^{-1}) was put into each wound site. Also, enterochelin alone (60 mg L^{-1}) was applied, and sterile distilled water was used as a control. Two hours later, 20 µL of a suspension of *B. cinerea* or *P. expansum* (10^5conidia mL^{-1}) were inoculated. After ten days at 15 ºC, the wounds were examined, and diameters (ϕ) of the rots were measured. There were three replicates of three apples per treatment, and the experiment was repeated twice. The results were expressed as follow: % reduction in decay severity = [(ϕ control - ϕ treatment) / ϕ control] x 100.

2.10 Data analysis

The data were analyzed using the analysis of variance (ANOVA) using Statistical Software InfoStat, 2008. The statistically significant differences (at $p < 0.05$) were analyzed by the Test of Multiple Range of Duncan.

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3. Results and Discussion

3.1 Siderophore characterization

Preliminary assays were directed to know what type of siderophore produces *K. radicincitans.* Arnow test and Ferric perchlorate assay were used for the determination of siderophore in the supernatant of the culture medium. The chelating agent was detected by the Arnow assay useful for the detection of catecholate while the Ferric perchlorate assay, commonly used to identify hydroxamates, gave negative results.

Firstly identification of the siderophore using TLC was carried out using enterochelin as standard. The Rf values obtained for samples and standard were coincident. Thus, the compound produced by the bacterium *K. radicincitans* was tentatively identified as enterochelin.

These results were later corroborated and validated by high-resolution liquid chromatography coupled to tandem mass spectrometry. *K. radicincitans,* as other bacteria belongs to the Enterobacteriaceae family, produces enterochelin, a siderophore type catechol. Also., UPLC MS / MS, allowed us to verify that it only produces enterochelin, unlike *Escherichia coli* that produces four siderophores among them two hydroxamate-type (Valdebenito et al., 2006).

3.2 Improvement of the production of enterochelin

3.2.1 Growth kinetics

With the objective to associate the different growth phases of *K. radicincitans* with the enterochelin production, the kinetics of growth and the production of the siderophore were evaluated in the VF medium, (Figure 1), at 28°C and 140 rpm. The adaptation phase was not observed because the inoculums consisted in a culture in the exponential period of growth came from an iron-deficient medium. Stationary phase was reached at 24 h. The siderophore was detected in the culture medium since the beginning, and an increase of its production occurred

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through the stage of growth. These results showed the character of metabolite associated with the growth of this siderophore. Then, the end time of the process was set at the beginning of the stationary phase. According to the results of the kinetics of growth and production, enterochelin only was produced in the growth phase, and there was no production in stationary phase as in other cases, for example, *Bacillus megaterium* (Santos et al., 2014). The stress of the stationary phase did not seem triggered an increase in the production of the siderophore, as could be expected if it is taken into account the role of enterochelin as stress protector (Adler et al., 2014).

3.2.2 Effect of iron concentration on enterochelin production

A study was carried out in VF medium (0.82 μmol L^{-1} iron concentration) supplemented with higher amounts of iron, to verify the sensitivity to this metal in the enterochelin production by *K. radicincitans*. The production of the siderophore in VF medium without the addition of iron reached a concentration of 30.27 mg g^{-1}biomass (d.w.), but there was a considerable reduction in the production at an iron concentration of 3.32 μmol L^{-1}. At this concentration, the enterochelin production only reached 3.85 mg g^{-1} biomass (d.w.), however, there were no significant changes in biomass production.

The production of the siderophore by *K. radicincitans* seemed more affected by the concentration of the iron added in culture medium other bacteria. Tailor et al. (2012) showed that the production of siderophore by *Pseudomonas fluorescens* was repressed at the iron concentration higher than to 5 μmol L^{-1}. In our case, the iron concentration higher than 0.82 μmol L^{-1} suppressed the production. This data is not so relevant if it is a production process carried out under controlled conditions, but it becomes critical if *K. radicincitans* is used as a promoter of plant growth, taking advantage of its capacity to produce siderophores. In this case, the pH of the soil and the iron concentration available in it would condition the ability of this bacterium to produce enterochelin “in situ”.

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3.2.3 Effect of the carbon source, the temperature, and the agitation rate

Taking into account the results of the API 50 CHE assay, glycerol and lactose were selected as alternative carbon sources. On the other hand, it also was reported the production of siderophores by *Pseudomonas fluorescens* ATCC 13525 at 25°C and 10°C (Katiyar and Goel, 2004). Taking into account these facts we design an experiment with the three carbon sources (glucose, glycerol, and lactose) at 28°C and 10°C. Results are shown in Figure 2A. There can be seen that glycerol and lactose provoked an increase in the siderophores production at the two temperatures assayed. There were no significant differences between the production at 28°C with glucose and the production in glycerol or lactose at 10°C. The highest production of enterochelin (40.5 mg g^{-1} d.w) was reached at 28°C in a medium with glycerol. Lactose not only stimulated the siderophore production but also favored the growth of the microorganism. The evaluation of the effect of the carbon source on the production of siderophore had the objective of selecting the carbon source that not only increased the production but also was cheap and easy to obtain. Then, the glycerol and the lactose are suitable substrates not only taking consider the biochemical profile of *K. radicincitans* but also because the former is a byproduct of biodiesel production and the latter is a component of whey. The low temperature decreased the production of siderophore and biomass, however at 10 ° C lactose was the most appropriate carbon source. This fact indicates that the bacterium would produce siderophore at low temperatures and this would be very important if the bacterium is applied as biofungicide for use in biocontrol of postharvest to cold storage temperature.

With the intention to force the aerobic metabolism and to increase the need for iron of the microorganism, experiments were carried out at different agitation rates. The highest productions of siderophore were reached in the medium with lactose at 200 rpm (45 mg g^{-1}d.w) and with glycerol

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at 240 (44.5 mg g^{-1}d.w) (Figure 2B). The increase in the agitation rate favored the increment of biomass in media with lactose and glucose, but no in glycerol medium.

3.4 Effect of enterochelin on conidial germination and mycelial growth of phytopathogenic fungi

The phytopathogenic fungi chosen to test the antimicrobial capacity of enterochelin were those indicated by the bibliography as a cause of significant economic losses, for example, *B.cinerea* or those that in addition to producing losses can affect the health of consumers due to the production of toxins such as *P. expansum* (Murray, et al. 2012).

Conidial germination of the phytophatogenic fungi was reduced in the presence of enterochelin (Figure 3A). Of the five fungi assayed, *Rhizopus* sp was the most inhibited. In this case, inhibition of 92% was observed when enterochelin was added to incubation medium at a concentration of 60 mg L^{-1}. Mycelial growth was also reduced in the presence of enterochelin, and except in the case of *Alternaria*, there were significant differences when the enterochelin level was increased to 120 mg L^{-1} (Figure 3B). Enterochelin from *K. radicincitans* showed in-vitro antifungal activity against critical phytopathogenic fungi such as *P. expansum* or *B. cinerea*.

3.5 Effect of enterochelin in the biocontrol of *Botrytis cinerea* and *Penicillium expansum*

In assays carried out on apple fruits, the mixture of *K. radicincitans* and enterochelin was the more effective against both phytopathogens. However, *B. cinerea* was best controlled (Figure 4), perhaps due to enterochelin inhibit its attack enzymes as it was previously reported (Sansone et al., 2011).

For *B. cinerea*, reduction of decay severity reached 30% when *K. radicincitans* was used alone *K. radicincitans*, but the mixture of the bacterium and the siderophore improved the biocontrol. In the last case reduction of decay severity was 52%. In the case of *P. expansum* the

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mixture of bacterium and siderophore also enhanced its control, but in general, this phytopathogen was less controlled than *Botrytis*.

4. Conclusions

In this work, we demonstrated that *K. radicincitans* produce enterochelin as a metabolite associated with the growth of the bacterium. Its production was influenced by environmental factors such as growth temperature, carbon source, iron level, and agitation rate. Lactose and glycerol improved bacterial growth and enterochelin production and could be suitable substrates for large-scale production of biomass and the siderophore. Enterochelin from *K. radicincitans* showed "in vitro" antifungal activity against critical phytopathogenic fungi such as *P. expansum* or *B. cinerea*. Results also demonstrated that this siderophore help to the control of these fungi in apple fruit when it is used in mixture with *K. radicincitans.*

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Conflict of Interest

The authors state that there are no conflicts of interest. The present work is original, has not been published before, is not under consideration by another journal, and all authors approve the submission.

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References

Adler, C., Corbalan, N.S., Peralta, D.R., Pomares, M.F., de Cristóbal, R.E., Vincent, P.A., 2014 The alternative role of enterobactin as an oxidative stress protector allows *Escherichia coli* colony development. PLoS One. 9(1): e84734. doi:10.1371/journal.pone.0084734.

Berger, B., Baldermanna, S., Ruppel, S. 2017 The plant growth-promoting bacterium *Kosakonia radicincitans* improves fruit yield and quality of *Solanum lycopersicum*. J. Sci. Food. Agric. 97, 4865-4871.

Berger, B., Patz, S., Ruppel, S., Dietel, K., Faetke, S., Junge, H., Matthias, B., 2018. Successful Formulation and Application of Plant Growth-Promoting *Kosakonia radicincitans* in Maize Cultivation. BioMed Res. Int. https://doi.org/10.1155/2018/6439481.

Bergottini, V.M., Revord, M., Junier, P., Otegui, M.B., Zapata, P.D., Sosa, D.A., Mulot, M., Zopfi, J., Wiss, F., Benrey, B., 2015. Bio-inoculation of yerba mate seedlings (Ilex paraguariensis St. Hill.) with native plant growth-promoting rhizobacteria: a sustainable alternative to improve crop yield. Biol. Fertil. Soils 51, 749-755.

Calvente, V., Benuzzi, D., Sanz de Tosetti, M.I., 1999. Antagonistic action of siderophores from *Rhodotorula glutinis* upon the postharvest pathogen *Penicillium expansum*. Int. Biodeterior. Biodegrad. 43,167–172.

Droby, S., Wisniewski, M., Macarisin, D., Wilson C., 2009. Twenty years of postharvest biocontrol research: Is it time for a new paradigm? Postharvest Biol. Technol. 52, 137–145.

Katiyar, V., Goel, R., 2004. Siderophore mediated plant growth promotion at low temperature by mutant of fluorescent pseudomonad. Plant Growth Reg. 42, 239.

Murray, R., Candan, A., Vazquez, D., 2012. Manual de Poscosecha de Frutas. Manejo Integrado de Patógenos. Ediciones INTA, Buenos Aires. ISNB N° 978-987-679-274-5

ACCEPTED MANUSCRIPT

Numan , M., Bashir, S., Khan,Y., Mumtaz, R., Khan Shinwari, Z., Latif Khan, A., Khan, A., AL-Harrasib, A., 2018. Plant growth promoting bacteria as an alternative strategy for salt tolerance in plants: A review. Microbiol. Res. 209, 21-32.

Payne, S., 1994. Detection, isolation, and characterization of siderophores. Meth. Enzymol. 235, 329-344.

Sansone, G., Rezza, I., Calvente, V., Benuzzi, D., Sanz de Tosetti, M., 2005. Control of *Botrytis cinerea* strains resistant to iprodione in apple with rhodotorulic acid and yeasts. Postharvest Biol. Technol. 35, 245–251.

Sansone, G., Rezza, I., Calvente, V., Fernandez, G., Benuzzi, D., Sanz, M.I.,2011. Inhibitors of polygalacturonase and laccase of *Botrytis cinerea* and their application to the control of this fungus. Int. Biodeterior. Biodegr. 65, 243-247.

Santos, S., Neto, I., Machado, M., Soares, H., Soares, E., 2014. Siderophore production by *Bacillus megaterium*: effect of growth phase and cultural conditions" Appl. Biochem Biotechnol. 172,549–560.

Sharma, R.R., Singh, D., Singh, R., 2009. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. Biol Control 50, 205–221.

Spadaro, D., Droby, S., 2016. Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists. Trends Food Sci. Technol. 47, 39-49.

Tailor, A., Joshi, B. 2012. Characterization and optimization of siderophore production from *Pseudomonas fluorescens* strain isolated from sugarcane rhizosphere. J. Environ. Res. Develop. 6, 688-694.

Tortora, M.L., Díaz-Ricci, J.C., Pedraza, R.O., 2011. *Azospirillum brasilense* siderophores with antifungal activity against *Colletotrichum acutatum*. Arch Microbiol. 193, 275-86.
ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Valdebenito, M., Crumbliss, A., Winkelmann, G., Hantke, K., 2006. Environmental factors influence the production of enterobactin, salmochelin, aerobactin, and yersiniabactin in *Escherichia coli* strain Nissle 1917. Int. J. Med. Microbiol. 296, 513-520.

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Legends to Figures

Fig. 1: Growth kinetics of *Kosakonia radicincitans* and production of enterochelin (n=3), in VF medium with glucose, at 28°C and 140 rpm.

Fig.2: A) Effect of the temperature, and the carbon source on enterochelin production

Assays were carried out in VF medium with glucose, glycerol or lactose at 10°C and 28°C, and at 140 rpm. Samples were taken at the beginning of the stationary phase. Measurements with the same letter did not show statistically significant differences (95% confidence level, n=3).

B) Effect of the agitation rate and the carbon source on enterochelin production

Assays were carried out in VF medium with glucose, glycerol or lactose at 140 rpm, 200 rpm, and 240 rpm, and at 28°C. Samples were taken at the beginning of the stationary phase. Measurements with the same letter did not show statistically significant differences (95% confidence level, n=3).

Fig. 3: A) Effect of enterochelin on the conidia germination of phytopathogenic fungi

60 µl of Potato Dextrose Broth (PDB), 30 µl of enterochelin in aqueous solution (60 mg L^{-1}) or distilled water (as control) and 30 µl of conidial suspension (10^6 conidia mL^{-1}) were put on appropriate slides. The slides were maintained in a wet chamber at 28°C, for 7 h. Measurements with the same letter did not show statistically significant differences (95% confidence level, n=3).

B) Effect of enterochelin on the mycelial growth of phytopathogenic fungi

In Eppendorf tubes were put 500 mL of PDB, 200 mL of an aqueous solution of enterochelin (60 mg L^{-1} or 120 mg L^{-1}) and 300 mL of conidial suspension (10^6conidia mL^{-1}). Distilled water was used as controls. The tubes were incubated at 28°C for 120 h. Measurements with the same letter did not show statistically significant differences (95% confidence level, n=3).

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Fig. 4: Effect of enterochelin in the biocontrol of *Botrytis cinerea* and *Penicillium expansum*

For the biocontrol assays, Red delicious apple fruits were wounded in three sites and, 20 mL of *Kosakonia radicincitans* suspension (10^6cells mL^{-1}), alone or in mixture with enterochelin (60 mg L^{-1}) was put into each wound site. Also, enterochelin alone (60 mg L^{-1}) was applied, and sterile distilled water was used as a control. Two hours later, 20 mL of a suspension of *B. cinerea* or *P. expansum* (10^5conidia mL^{-1}) were inoculated. After ten days at 15 ºC, the wounds were examined, and diameters (f) of the rots were measured. There were three replicates of three apples per treatment, and the experiment was repeated twice.

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Highlights

Kosakonia radicincitans produced a siderophore identified as enterochelin

Production of enterochelin was influenced by environmental factors.

Enterochelin showed antifungal activity against phytopathogenic fungi.

Enterochelin helped to the biocontrol of *P. expansum* and *B. cinerea* in apple fruit.

This siderophore could be used for a biofungicide formulation.

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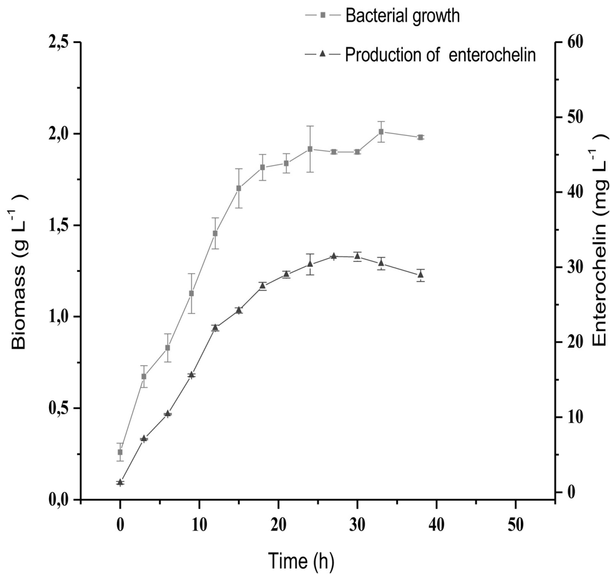


Figure 1

A

Gluc
Glyc
Lac

Enterochelin mg g^{-1} biomass (dw)

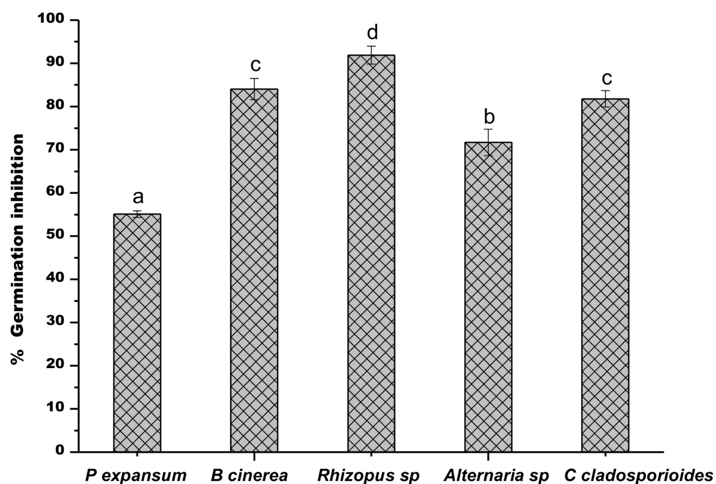
Temp (^{0}C)

B

Enterochelin mg g^{-1} biomass (dw)

Agitation rate (rpm)

Figure 2



B

Enterochelin 60 mg L^{-1}
Enterochelin 120 mg L^{-1}

% Growth Inibition

40
30
20
10
0

a cd a e bc d e e ab e

P expansum
B cinerea
Rhizopus sp
Alternaria sp
C cladosporioides

Figure 3

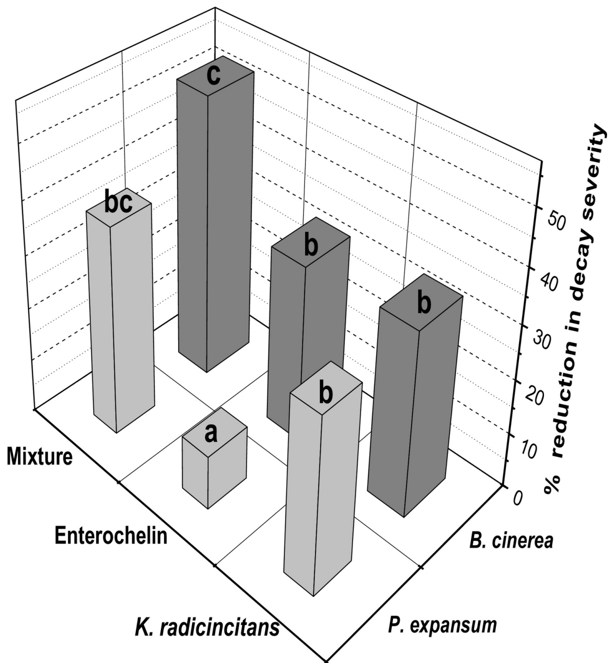


Figure 4