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Acetoine and chitinase production by native rizobacteria from Western Paraná

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Abstract
The objective of this study was to investigate 28 strains of native rhizobacteria from the west region of Paraná, evaluating the acetoin and chitinase production capacity, because of their role in biocontrol. The bacteria isolates were submitted to the modified Voges-Proskauer (VP) assay in Clark & Lubs liquid medium for glucose fermentation. Metabolite production was evaluated in a spectrophotometer, and it showed a wide variation in concentrations (0,476 to 1,865) for different isolates. Strains 241, 320, 326, and 273 showed higher values than the others tested. According to positive results in the qualitative test of VP, 11 isolates were chosen for the tests in minimal medium containing chitin as the only source of carbon. Isolates that presented the degradation halo formation were considered as chitinolytic bacteria, evidencing chitinase activity. Among these strains, two belonged to the genus *Bacillus* spp. (56 and 121) and two to the genus *Enterobacter* spp. (292 and 151). Therefore, the present work was able to identify native strains with potential for biocontrol studies allowing to continue investigations of other metabolites produced by those bacteria in this collection regarding plant protection.
Keywords: biocontrol; chitin degradation; PGPB.

Produção de acetoína e quitinase por rizobactérias nativas do Oeste do Paraná

Resumo
O objetivo deste estudo foi investigar 28 cepas de rizobactérias nativas da região oeste do Paraná, avaliando a capacidade de produção de acetoína e quitinase, devido ao seu papel no biocontrole. Os isolados de bactérias foram submetidos ao ensaio Voges-Proskauer (VP) modificado em meio líquido Clark & Lubs para fermentação de glicose. A produção de metabólitos foi avaliada em espectrofotômetro e apresentou grande variação nas concentrações (0,476 a 1,865) para os diferentes isolados. As estirpes 241, 320, 326 e 273 apresentaram valores superiores aos das demais testadas. De acordo com os resultados positivos no teste qualitativo de VP, 11 isolados foram escolhidos para os testes em meio mínimo contendo quitina como única fonte de carbono. Isolados que apresentaram formação de halo de degradação foram consideradas bactérias quitinolíticas, evidenciando atividade quitinase. Dentre essas cepas, duas pertenciam ao gênero Bacillus spp. (56 e 121) e dois para o gênero Enterobacter spp. (292 e 151). Portanto, o presente trabalho foi capaz de identificar cepas nativas com potencial para estudos de biocontrole permitindo continuar as investigações de outros metabólitos produzidos por essas bactérias nesta coleção no que diz respeito à proteção de plantas.
Palavras-chave: biocontrole; degradação de quitina; BPCP.

Introduction

In agricultural production systems, the improper utilization of pesticides has gradually promoted the loss and alteration in the diversity of plants, insects and natural microorganisms. This has led to functional imbalance and the selection of genetic resistance that promote establishing pests and diseases in commercial

plants (BETTIOL; MORANDI, 2009; BRASIL, 2012; SILVA *et al*., 2015).

The sustainable cultivation of food has been motivating more researches that aim to develop knowledge about the control of phytopathogens to reduce the use of fungicides in the crops of economic importance. In this context, biological control consists of one alternative and advantageous means for the control of some plant diseases. Microorganism diversity is a tool to understand antagonistic relationships among or between the species, and essential to identify individuals with biotechnological potential for biocontrol (ZHANG *et al*., 2017).

In rhizosphere, plant growth-promoting bacteria (PGPB) act beneficially in the interaction with plants due to the ability to improve the plant performance. The PGPB demonstrated production of phytohormones, phosphate solubilization, nutrient mineralization and biological nitrogen fixation (BNF). Additionally, the PGPB presented indirect mechanisms by helping the population control of pathogenic microorganisms through the ecological relations, like competition and predation, in addition to the synthesis of compounds that promote the induction of systemic resistance (ISR) (HUNGRIA; ARAÚJO, 1994; HUNGRIA *et al*., 2010; FILGUEIRAS; MENESES, 2015; SPOLAOR *et al*., 2016). The microorganisms can use several mechanisms to inhibit the growth of pathogens. Among them, the production of antimicrobial substances, such as siderophores, β- 1, 3 glucanase, chitinases and antibiotics (MAKSIMOV *et al*., 2011; VIANA *et al*., 2013; PEREIRA; OLIVEIRA, 2016; CHANDRASEKARAN *et al*., 2017).

The most studied groups with potential for biocontrol are some species belonging to the genus *Pseudomonas* spp. and *Bacillus* spp., because of their capacity to produce antibiotics. Other bacteria of the genus *Enterobacter* spp. and *Klebsiella* also presents the potential of production of volatile organic compounds such as acetoin, involved in the induction of resistance against phytopathogens (HUNGRIA; ARAÚJO, 1994; RUDRAPPA *et al*., 2010; EGAMBERDIEVA *et al*., 2015). Pérez-Portuondo *et al*. (2017) verified that the production of acetoin by isolates of rhizosphere bacteria; it was demonstrated that the presence of this compound is involved in the induction of plant resistance.

Chitinase is a importance metabolite, an enzyme produced by microorganisms that promote the degradation of chitin, leading to breaking down the cell wall of fungi, by this way inhibit their growth (FAHEEM *et al*., 2015). Biologically active oligosaccharides, produced by chitin breaking bacteria, are compounds of a commercial interest with biotechnological potential as a resistance vegetable elicitor (EL HADRAMI *et al*., 2010; HAMID *et al*., 2013).

Research such as the Yasir *et al*. (2009), demonstrated the presence of chitinolytic activity for bacterial isolates present in the vermin compost substrate, which showed antifungal activity against *Rhizoctoniasolani, Colletotrichumcoccodes, Pythium ultimum, Phytophthoracapsici* and *Fusariummoniliforme.*

Therefore, contributes to the development of sustainable agriculture, the present study had as objective to evaluate native rhizobacteria to acetoin and chitinase production capacity in the soil of the west region of Paraná, using a modified Voges-Proskauer (VP) quantitative test, working with microvolumes.

Material and methods

Obtention of isolates

The strains were isolated from soil samples of 17 areas under different managements practices in the west region of Paraná (Table 1) and belong to the bacteria collection of the Grupo de Pesquisas em Fixação Biológica de Nitrogênio (FIXTEC) from Universidade Federal do Paraná (UFPR) – Setor Palotina. The determination of the genera of the isolates was carried out through partial sequencing of the 16S rRNA gene (SILVA, 2016).

Table 1. Description of management type and collection site of soil samples from which the strains were isolated.

Isolated	Source	Soil type
188	Native forest	Eutrophic Red Oxisoil
10	Agropastoral system	Dystrophic Red Latosoil
317	Sugarcane monoculture with mineral fertilization	Eutrophic Red Argisoil
265	Sugarcane monoculture with vinasse fertilization	Eutrophic Red Argisoil
320	Succession corn - convetional soybean	Eutrophic Red Nitosoil
127	Fallow with previous cultivation and sugarcane, corn and soybean	Dystrophic Red Latosoil
300	Native forest	Eutrophic Red Argisoil
302	Crotalaria	Eutrophic Red Argisoil
81	Pasture	Eutrophic Red Nitosoil
15	Agropastoral system	Eutrophic Red Latosoil
56	Agropastoral system	Eutrophic Red Latosoil
102	Corn - soybean succession	Eutrophic Red Nitosoil
121	Native forest	Eutrophic Red Oxisoil
57	Fallow with previous cultivation and sugarcane, corn and soybean	Dystrophic Red Latosoil
203	Crotalaria	Eutrophic Red Argisoil
299	Rotation with sugarcane, soybean and corn	Eutrophic Red Argisoil
326	Fallow with previous cultivation and sugarcane, corn and soybean	Dystrophic Red Latosoil
273	Sugarcane monoculture with vinasse fertilization	Eutrophic Red Argisoil
220	Corn - soybean succession	Eutrophic Red Oxisol
142	Succession corn - organic soybean	Eutrophic Red Oxisol
208	Sugarcane monoculture with mineral fertilization	Eutrophic Red Argisole
152	Native forest	Eutrophic Red Argisoil
130	Succession corn - wheat	Eutrophic Red Oxisoil
194	Rotation with sugarcane, soybean and corn	Eutrophic Red Argisoil
241	Succession corn - convetional soybean	Eutrophic Red Nitosoil
24	Sugarcane monoculture with vinasse fertilization	Eutrophic Red Argisoil
151	Native forest	Eutrophic Red Argisoil
292	Crotalaria	Eutrophic Red Argisoil

Qualitative and quantitative acetoin assays

The detection of acetoin production was carried out utilizing the Barrit alpha-naphthol reagents, according to Clark and Lubs method (STELATO, 2011). Results interpretation was based on the culture medium coloration changes, pink to red for a positive result and copper colour for the negative one. The quantitative test was carried out following Voges-Proskauer's modified test (VP) (ROMICK; FLEMING, 1998). To obtain the acetoin standard curve, serial dilutions from the stock solution of pure acetoin (Sigma ≥ 96 %) were prepared 0,1; 0,3; 0,5; 1; 2; 3 e; 4 mmol/L, in triplicate with a volume of 2 µL. The acetoin production was recorded by reading in a Nanodrop2000 (Thermo Fischer ^{TM}) at the wavelength of 490 nm. The quantitative results of acetoin production were used for a cluster analysis using the WARD algorithm with the software Bionumerics (Applied Math) and a frequency analysis with software excel (Microsoft).

Detection of chitinase production

The culture medium utilized was described by Hsu e Lockwood (1975), it presents the chitin as only carbon's source. The chitin source utilized in this study was obtained from the purification of shrimp (MOURA *et al*., 2006). For homogeneous growth of colonies was used germinating paper that contained a liquid medium for bacterial growth previously sterilized in the autoclave. The paper was inserted in the centre of the plate that contained chitin medium. The test was realized in duplicate for each isolate and the plates were incubated in B.O.D for eight days at 27°C. The strains that showed a halo of degradation around the disc were considered producers of the chitinase enzyme (HSU; LOCKWOOD, 1975).

Results and discussion

From the total of 28 strains submitted to the acetoin quantification method, 25 of them produced positive results for the presence of this compound (Table 2). Between those, four presented reddish colour of high intensity, all of them belong to the genus *Enterobacter* (10, 127, 203 and 151 strains). According to Stelato (2011) and Sánchez *et al*. (2015), this is because the *Enterobacter* genus *is* typically used as a positive control for this test because of the high capacity of acetoin production, although some isolates in this work are not able to produce such a compound, as isolates 265, 152 and 142. The objective of the qualitative test was to confirm the presence of acetoin as a form of screening to send the samples to the quantitative test because it reduces the cost to realize a more complex method (FIGURE 1).

The quantitative results of acetoin of reduction are described in Table 2. Cluster analysis by ward method resulted in 6 classes regarding acetoin production (APPENDIX 1). The classes formed and the frequency of each class are summarized in table 3.

Table 2. Results of qualitative and quantitative test for the acetoin and chitinase production by the strains of native rhizobacteria from the west region Paraná.

GÊNUS	ISOLATED	CHITINASEHALO[1]	ACETOÍN[2]	[C] ACETOÍN (mM)[3]
Paenibacillus sp.	188	-	+	1,560
Enterobacter sp.	10	-	+	1,226
Enterobacter sp.	317	-	+	0,802
Enterobacter sp.	265	-	-	-
Enterobacter sp.	320	-	+	1,841
Enterobacter sp.	127	-	+	1,365
Enterobacter sp.	300	-	+	1,413
Enterobacter sp.	302	-	+	0,825
Falsibacillus sp.	81	-	+	1,627
Falsibacillus sp.	15	-	+	0,905
Bacillus sp.	56	+	+	1,508
Bacillus sp.	102	-	+	1,103
Bacillus sp.	121	+	+	1,333
Enterobacter sp.	57	-	+	0,738
Enterobacter sp.	203	-	+	1,302
Enterobacter sp.	299	-	+	0,964
Enterobacter sp.	326	-	+	2,142
Enterobacter sp.	273	-	+	1,706
Enterobacter sp.	220	-	+	0,440
Enterobacter sp.	142	-	-	-
Enterobacter sp.	208	-	+	0,539
Enterobacter sp.	152	-	-	-
Enterobacter sp.	130	-	+	0,857
Enterobacter sp.	194	-	+	1,674
Enterobacter sp.	241	-	+	1,865
Enterobacter sp.	24	-	+	1,468
Enterobacter sp.	151	+	+	1,380
Enterobacter sp.	292	+	+	0,476

[1]+ presence and - the absence of the halo of degradation according to report by Hsu and Lockwood (1975).
[2] + production – non-production of acetoin according to the Clark and Lubs method (STELATO, 2011).
[3] [C] the concentration of acetoin in millimolar (mM) using the modified Voges-Proskauer (VP) test (ROMICK; FLEMING, 1998).

Table 3. Frequency of classes of acetoin-producing bacteria.

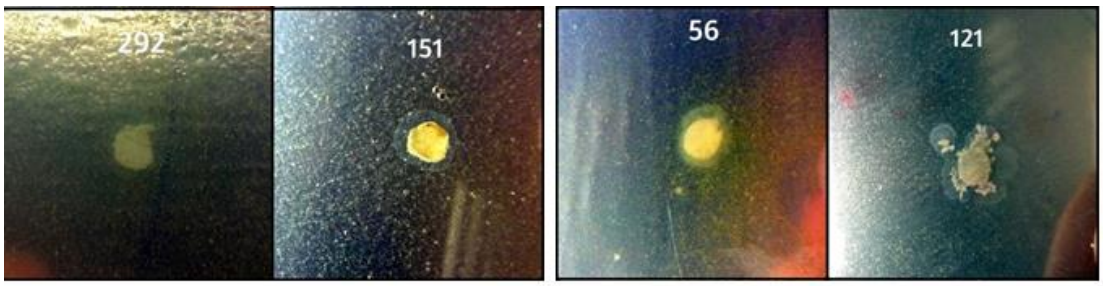
Produção de Acetoína (mM)	Frequencia total	Frequencia de Enterobacter
0-1	12	11
1100-1200	1	0
1201-1450	7	6
1450-1800	5	2
1801-1900	2	2
1901-2200	1	1

Lee *et al*. (2012), reported good results in induced systemic resistance (ISR) in plants of the genus *Arabidopsis* with the rhizobacteria strain *Paenibacillus*, where the production of 0.008 mM of acetoin was an effective for biocontrol. The strain 326 showed the highest value in the production of acetoin (2.142 mM), becoming a potential candidate or future studies of biocontrol.

The use of rhizobacteria in studies of induction of resistance in plants has been associated to reducing the damage caused by pathogens attack (VIANA *et al*., 2013). In the work of Rudrappa *et al*. (2010), it was observed a decrease in the severity of the attack of *Pseudomonas syringae* pv. tomato on *Arabidopsis thaliana* when inoculated with one isolate of *Bacillus subtilis* that can produce acetoin. In the opposite side, mutant strains with lower acetoin production, in that study, resulted in less control of the disease.

Eleven strains with higher production of acetoin were chosen for the chitin degradation assay. The strains that stood out as good candidates for chitinase production belonged to genus *Bacillus* (56 and 121) and *Enterobacter* (292 and 151).

Figure 1. Production of chitin degradation halo by the chitinase production in strains 151 and 292 belonging to the genus Enterobacter and in strains 56 and 121 belonging to the genus Bacillus of rhizobacteria from Western Paraná.



Several authors have already reported the production of chitinolytic enzymes by bacteria of those genera (WEN *et al*., 2002; SHALI *et al*., 2010; VELUSAMY; KIM, 2011). Liu *et al*. (2013) reported the genomic analysis of the strain *Enterobacter cloacae* subsp. *cloacae* ENHKU01, which is a PGP bacterium, allowed to demonstrate the presence of several genes related to mechanisms of antagonism to microorganisms, as bacteriocins, production of siderophores and chitinase, this last one found in strains in this work.

Use of *Bacillus* genera for biocontrol of plants diseases have been reported previously. Li *et al*. (2010) and Huang *et al*. (2012), reported the use of the strain of *Bacillus pumilus* for control of the pathogen *Rhizoctonia solani* in cucumbers. Ji *et al*. (2013) demonstrated the ability of the *Bacillus amyloliquefaciens* strain to control a wide variety of fungi, including: *Alternaria panax*, *Botrytis cinera*, *Colletotrichum orbiculare*, *Penicillium digitatum*, *Pyricularia grisea* and *Sclerotinia sclerotiorum*.

According to studies, bacteria of the genus *Bacillus* present a large capacity for the production of compounds with antagonistic activity (MONTEIRO *et al*., 2005; HAN *et al*., 2015). Thus, isolates 151, 56, 292 and 121 can be applied in the biocontrol because of the capacity of forming halos of chitin degradation with ample

reports of inhibition of the growth of phytopathogenic fungi (KEMPKA *et al*., 2008; SURYANTO *et al*., 2012; RAMPINO *et al*., 2013; ZALILA-KOLSI *et al*., 2016).

Conclusions

The modified Voges-Proskauer (VP) quantitative test proved to be a proper screening for the authentication of acetoin producing strains revealing the presence of the molecule in 25 of the 28 strains analyzed;

Among the 25 strains positive for the presence of acetoin, four isolates belonging to the *Enterobacter* genus (326, 241, 320 and 273) presented higher production of this metabolite, representing good candidates for ISR study in the biocontrol;

Isolates 151, 292, 56 and 121 belonging to the *Enterobacter* genus and isolates 56 and 121 belonging to the *Bacillus* genus were the only strains that presented halo of chitin degradation and could be better studied for use in the biocontrol of phytopathogens by the antagonistic attack.

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APPENDIX 1
Cluster analysis of acetoin-producing bacteria using the WARD method.