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Research Article

GLYCEROL BIOCONVERSION INTO 1,3-PROPANEDIOL AND 2,3-BUTANEDIOL BY *Lactuca sativa* RHIZOBACTERIA

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Abstract- The present work aimed to isolate *Lactuca sativa* (lettuce) rhizobacteria and assess its capacity to bio-convert glycerol into the added-value compounds 2,3-butanediol and 1,3-propanediol. Six strains were isolated from rhizospheric soil in a selected culture medium and identified by sequencing the 16S rDNA region. The microbial growth pattern was tested in different glycerol concentrations, and the simultaneous production of the compounds of interest was quantified. The species of the *Enterobacteriaceae* family dominated, especially the genus *Enterobacter*. The test of different glycerol concentrations in culture medium suggests a concentration of 20 $g.L^{-1}$ as ideal to promote the fermentation process and grow the isolated rhizobacteria. Three samples were identified as simultaneous producers of the relevant compounds. The best fermenting isolate, *Enterobacter cloacae* (AG3), produced 0.522 $g.L^{-1}$ of 2,3-butanediol and 0.735 $g.L^{-1}$ of 1,3-propanediol. Therefore, lettuce rhizobacteria can produce added-value compounds from the fermentation of glycerol as a sole carbon source.

Keywords- Bioenergy, Fermentation, Byproduct, Carbon source, Industrial microbiology.

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Introduction

Due to increasing demand for fuel and the environmental impact of fossil fuels, biodiesel production has gained prominence in the last years [1]. Brazil stands out as a major world producer, with a biodiesel production of 2.7 million cubic meters in 2012, an increase of around 570% compared to the 2007 production [2]. Its production is essentially made by ethanolic or methanolic trans-esterification of vegetable oils or animal fat, with a substantial co-production of glycerol. Glycerol is a three-carbon compound, and its raw form has contaminants such as methanol, fatty acids, and methyl esters [3]. Due to the increased investment in the biodiesel production chain, the market price of glycerol has fallen; thus, its biotechnological use in fermentation processes for greater added-value compound production has drawn attention [4].

The reduced nature of the glycerol molecule facilitates its metabolization by fermenting microorganisms, to use for bioconverting the compound into raw material with industrial used, such as 1,3-propanediol (1,3-PD), 2,3-butanediol (2,3-BD), ethanol, butanol, ketone bodies, organic acids, hydrogen, and more [5, 6]. The microbial production of these added-value metabolites has been mainly studied in species of the genus *Klebsiella, Enterobacter, Citrobacter*, and *Clostridium* [7]. However, improvement is still necessary; therefore, the exploration of new natural environments has become an important tool for identifying microorganisms with better capacity to ferment glycerol. The rhizosphere, which is the region of soil that is influenced by plant roots, is a potential place to find these microorganisms [8].

Plant growth-promoting rhizobacteria (PGPRs) can use direct or indirect means to facilitate plant adaptation to its environment, which includes the ability to synthesize volatile organic compounds (VOCs) that promote plant growth and provide induced systemic resistance (ISR) [9]. VOCs are a group of more than 30 compounds [10], many of which have high applicability in different industrial sectors, including some compounds targeted for the bioprospection of glycerol fermenting microorganisms, such as 2,3-BD. The microbial production of 2,3-BD is intended for different industrial sectors, such as pharmaceuticals, cosmetics, solvents, and potential use as a fuel [11]. Another exploited compound that results from microbial metabolism of glycerol is 1,3-PD, industrially used in polyester and polyurethane production [12].

Research about glycerol fermenting microorganisms in rhizospheric environment can help identify new lineages to use with this biodiesel industry byproduct to produce 2,3-BD, 1,3-PD, organic acids, and many other added-value compounds. This low-cost, alternate carbon source, which is easily obtainment from fermentation processes, can be used to manufacture industrially interest compounds. In this context, the *Lactuca sativa* (lettuce) rhizosphere can play a role in research about such topic, because it is a minimally explored environment and, thus, passive for bioprospection. The present study is innovative in exploring the biotechnological potential of *L. sativa* rhizosphere to identify vegetable rhizobacteria capable of using glycerol as a sole carbon source, as well as determining the capacity of simultaneous production of 2,3-BD and 1,3-PD.

Materials and Methods

Methods Rhizobacteria Isolation

To isolate the rhizobacteria, three samples of *L. sativa* rhizospheric soil were collected in March 2012 from an organic garden located in a rural area of the municipality of Goiânia, GO (16º42'34.51"S 49º20'23.21"O) and sent to the Environmental Microbiology and Biotechnology Laboratory from the Tropical

Pathology and Public Health Institute from the Federal University of Goiás (LAMAB/IPTSP/UFG). The samples were initially dried in an oven at 50 °C for 24 hours, and the dried material was later sieved in a 2.0 mm granulometric sieve (Grunutest®). A 1.0 g sieved soil sample was added to cultural vials containing 9.0 mL of a fermenting medium consisting of (g/L): 3.4g K2HPO4; 1.3 g KH2PO4; 2.0g (NH4)2SO4; 0.2g MgSO4.7H2O; 0.02 g CaCl2.2H2O; 2.0 g CaCO3; 1.0 g yeast extract; 20.0 g glycerol; 1.0 ml trace element solution; and 2.0 ml Fe solution. The composition of the Fe solution per liter was 5 g FeSO4.7H2O and 4 ml HCl (37%). The trace element solution per liter consisted of 70 mg ZnCl2; 0.1g MnCl2.4H2O; 60 mg H3BO3; 0.2g CoCl2.2H2O; 20 mg CuCl2.2H2O; 25 mg NiCl2.6H2O; 35 mg Na2MoO4.2H2O; and 0.9 ml HCl (37%) according by Günzel et al. [13]. The inoculated tubes were incubated in an oven at 30 °C for 48 hours. After this time, a 100 µL aliquot was taken from each tube and transferred to a new fermentation tube and incubated again. This step was repeated three times. The serial dilution method was used to isolate the microorganisms and to differentiate the bacterial strains, BIOLOG's GEN III MicroPlateTM panel was used. Isolated microorganisms were identified by the initials AG followed by a differentiation number, and they were stored at -20 °C in Nutrient Broth (HIMEDIA®) supplemented with 25% (m/m) of glycerol.

Molecular Identification of isolated bacteria

The molecular identification of isolates was made by a partial sequencing of the 16S rDNA region. The genomic DNA was extracted with the method described by Rodrigues et al. [14]. The amplification of the 16S rDNA gene region was done by PCR with the use of the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3'). For the reaction, 35.5 µL of ultra-pure water (miliQ®), 5.0 µL of sample buffer (10X) (Ludwig Biotec LTDA), 1.5 µL of MgCl2(50 mM) (Ludwig Biotec LTDA), 1.0 µL solution of each primer (10 mM) (InvitrogenTM), 4.0 µL of dNTP solution (2.5 mM) (Ludwig Biotec LTDA), 1.0 µL of Taq polymerase (5 U) (Ludwig Biotec LTDA), and 1.0 µL of DNA (50 ng) were used; totaling a final volume of 50 µL. PCR reaction was conducted in a thermal cycler (VeritiTM 96-Well Thermal Cyclers) under the following conditions: 3 min initial denaturing at 94 °C and 30 denaturing cycles at 94 °C/ 1 min, annealing at 55 °C/ 30 s and extension at 72 °C/ 30 s, final extension at 72 °C/ 10 min. The PCR product was purified with the E.Z.N.A.® Cycle Pure Kit (Omega). The sequencing was done in the ABI 3130xl Applied Biosystems® platform, using the primers 27F, 1541R, 926F (5'-AAACTYAAAKGAATTGACGG-3'), 530F (5'-TGACTGACTGAGTGCCA GCMGCCGCGG-3'), 519R (5'–GTNTTACNGCGGCK GCTG –3') and 907R (5'–GTNTT ACNGCGGCKGCTG –3'). The sequences obtained were evaluated for their quality, united in the CodonCode Aligner (CodonCode Corporation) software, and compared to the available sequences in the GenBank NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and RDP (http://rdp.cme.msu.edu/) database. To better identity and similarity patterns, the identification followed the adapted methodology of Carvalho-Neto et al. [15]. In this approach, a phylogenetic tree was built with type strains of the best hits, the sequences were aligned by CLUSTAL W, the tree was built by the neighbor-joining method (Jukes-Cantor model with a 1000 repetition bootstrap in the MEGA7 program), and *Thermococcus coalesces* (NR 040968.1) was used as an external group. The isolated and sequenced samples of 16S rDNA were deposited at the GenBank NCBI with the access numbers KX061930, KX061931, KX061932, KX061933, KX061934, and KY271050.

Determination of microbial tolerance to different glycerol concentrations

The microbial tolerance to different glycerol concentrations was determined by culturing rhizobacteria in Nutrient Broth for 48 hours at 30 °C. After the growth, each sample was inoculated in culture vials at a 10% proportion of the total volume of the medium present in each vial. The Bushnell Haas Broth medium [16] contained (g/L): 1.0 g KH2PO4; 1.0 g K2HPO4; 1.0 g NH4NO3; 0.2 g MgSO4.7H2O; 0.05 g FeCl3; and 0.02 g CaCl2.2H2O, added to 0.1% of yeast extract and incubated at 30 °C. The medium was supplemented with different glycerol concentrations (2.0%; 4.0%; 6.0%; 8.0%; 10%; 12%; and 15% m/v) as a sole carbon source. The vials were then incubated at 30 °C and microbial growth assessed by turbidity spectrophotometry (OD600nm) (BioRad®) at 0, 24, 36, 48 and 72 hours. The experiment was carried out in triplicate.

Rapid triage method determination of 2,3-butanediol-producing rhizobacteria

The determination of 2,3-butanediol-producing microorganisms was performed by the Voges-Proskaueh (VP) test with modifications [17]. The isolated bacteria were cultured in two series of Bushnell Haas Broth [16] at 30 °C for 24 hours with each series containing a different sole carbon source: glycerol (2.0% m/v) or glucose (0.5% m/v). After the incubation period, the culture mediums were centrifuged at 3000 rpm for 10 min and the supernatant was separated to determine the presence of 2,3-BD. Then, 70 µL of the supernatant were added to 50 µL of Barritt I reagent (5.0% alpha-naphtol alcoholic solution) and to 17 µL of Barritt II reagent (4.0% KOH aqueous solution); the solution was then stirred for 15 min at 100 rpm. The positive result was seen by coloration that varied from red to light pink, with no color alteration in the solution for a negative result.

Glycerol fermentation and fermenting products determination

An inoculum corresponding to 10% (v/v) final volume of the fermentation was added to a 25 mL fermenting medium [13] in Schott type vials with a 50 mL capacity. The inoculated vials were incubated at 30 °C for 48 hours and without stirring. Then, 3.0 mL aliquots were extracted at the times 0 and 48 hours of incubation and were filtered with a Millipore©type of filter (pore size of 0.25µm). The supernatant was separated to determine both initial and final glycerol, 2,3-BD, and 1,3-PD concentration. The determination was performed in an Agilent Technologies 1200 series chromatograph with a Hamilton PRP-X300 column and a RID-6A refractive index detector. The following operational conditions were used: 20 µL sample volume, ultrapure water mobile phase (miliQ®), 0.8 mL/min flow rate, and temperature at 50 °C. The resulting data reflected the procedure in triplicate.

Statistical Analysis

To analyze the averages of the fermenting products in the determination test, the Analysis of Variance (ANOVA) statistical technique was used by means of the Scott-Knott clustering test ($p<0.05$) with the help of Sisvar 5.3 Build 77 program.

Results and Discussion

Isolation and identification of morphospecies

Due to biological interaction, the microbial density present in the rhizosphere is greater than that present in soil which is uninfluenced by vegetable roots, thus revealing the complexity of the microbial community in this environment [8]. The rhizospheric micro-biodiversity of *L. sativa* has not been well acknowledged or explored for its biotechnological capacity. The few studies that report the isolation and identification of *L. sativa* rhizobacteria show the following rhizobacteria genus and species: *Enterobacter cloacae*, *Burkholderia* sp., *Chryseobacterium formosense*, *Rhizobium leguminosarum*, and *Pseudomonas mendocina* [18-20].

In this study, six morphospecies from *L. sativa* rhizospheric soil samples were isolated and discriminated by biochemical tests. From these isolates, five were characterized as gram-negative and one as gram-positive; the probable species are shown in [Table-1].

The partial sequencing of the 16S rDNA gene and the phylogenetic tree construction with type strains of the best identity microbial kinds found mostly the *Enterobacteriaceae* family (AG1, AG3, AG4, AG5, and AG7). Four isolates had great similarity with the *Enterobacter* genus (AG1, AG3, AG5, and AG7) and one with the *Bacillus* genus (AG6) [Fig-1]

Table-1 *Most probable microorganisms identified from the partial 16S rDNA gene sequence*

Isolate	Closest species	Identity	Access number
AG1	*Enterobacter ludwigii*	99%	NR042349.1
AG3	*Enterobacter cloacae*	99%	NR117679.1
AG4	*Escherichia hermannii*	99%	NR104940.1
AG5	*Enterobacter cancerogenus*	94%	NR044977.1
AG6	*Bacillus* sp.	100%	NR074453.1
AG7	*Enterobacter ludwigii*	99%	NR042349.1

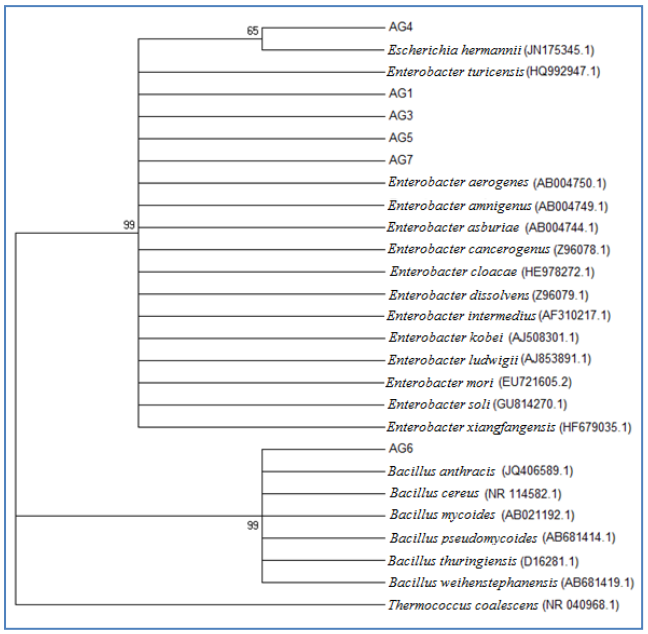

Fig-1 Phylogenetic relation between isolated rhizobacteria and type strains with greater similarity. The Neighbor-joining method was used in the analysis, a Jukes-Cantor model and bootstrap values are based on 2000 repetitions. *Thermococcus coalescens* was used as an external group

Bacteria of the genus *Enterobacter* are considered PGPR, which is why their representatives can be easily found in rhizospheric environments. They present lineages that can solubilize inorganic phosphate; produce indole acetic acid, hydrocyanic acid (HCN), and siderophores; fixate nitrogen; produce antimicrobial compounds, VOCs, among other direct and indirect plant growth promoting factors [21]. Furthermore, the prospection of environmental strains is important to develop research about glycerol fermentation, because during the metabolization process of glycerol, they produce 1,3-PD and acetate as primary products, in addition to ethanol [7, 22].

The AG6 isolate showed greater similarity to strains of the genus *Bacillus*, which can convert glycerol into different relevant commercial products [23, 24]. Nevertheless, different bacteria of the *Enterobacteriaceae* family are also described as capable of using glycerol as a sole carbon source. Dharmadi et al. [25] isolated an *Escherichia coli* sample capable of anaerobic fermentation of glycerol; Sathianachiyar and Devaraj [26] isolated bacteria of the genus *Pseudomonas,* and different strains of the *Klebsiella* were reported by Almeida et al. [5], all with the same metabolic capability. Among the isolated kinds, the *Enterobacter* has been the most commonly described and with the greatest glycerol reduction potential [7, 22, 27].

Different glycerol concentration growth analysis

Fermentation bioprocesses operate in high substrate concentrations to increase the production yield and reduce costs with product separation. For this reason, determining the best growth range and influence of the carbon source in the fermentation process is import. In this study, microorganisms grown in mediums with different glycerol concentrations suggest that high glycerol concentrations inhibit microbial growth [Fig-2]. Glycerol concentration is characterized as an important limiting factor in the fermentation process, because the compound interferes significantly in the osmotic potential of the fermenting medium [28].

The results suggest that the glycerol concentration of 2.0% (m/v) is the best for isolate growth and consequently for the fermentation process. The *Enterobacter ludwigii* isolate (AG7) could tolerate high glycerol concentrations, with a maximum value of $OD_{600nm}$of 1.391 in the lower concentration tested and 0.307 in the higher concentration. Ito et al., found similar data [29], who observed that an *Enterobacter aerogenes* strain presented the best rate in the production of relevant compounds (H_2, acetate, ethanol, lactate) in the lower glycerol concentrations tested (0.5 at 1.0% m/v), and that increased concentration of the carbon source reduced the production yield.

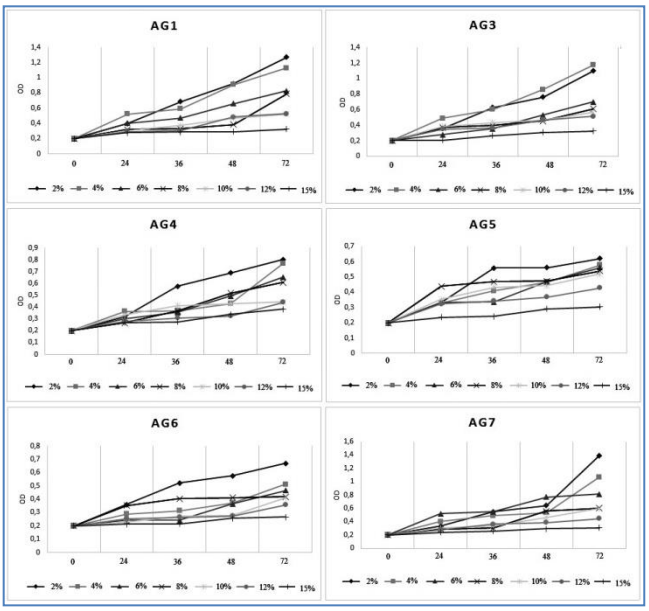

Fig-2 Growth curve of rhizobacteria isolates during 72 hours in culture medium containing different concentrations of glycerol (OD600nm)

Dabrock et al. [30] argue that because they are primary metabolites, the production capacity of industrial interesting products such as propanediol and butanediol is directly related to increased microbial density, that is, the fermenting microorganisms produce these compounds during their exponential growth stage and the decrease of this stage alters their bioconversion performance.

Production screening of 2,3-butanediol and fermentation process

Except for the *Escherichia hermannii* isolate (AG4), the isolated rhizobacteria were positive when cultured in a glucose medium. This result for the AG4 is in accordance with the documented literature, because all species of genus *Escherichia* are negative in the VP test [31]. Four isolates (AG1, AG3, AG5, and AG7) were positive when cultured in a glycerol medium; the AG6 sample, positive in the glucose test, was negative with glycerol. This result is because the release of glycerol in the oxidative metabolism involves additional enzymatic steps, and transforming it into an energetic fuel for metabolic consumption is more complex than glucose, which is promptly metabolized by the microbial glycolytic pathway [32].

The Voges-Proskauer test (VP) is used to identify microorganisms that possess the butanedioic metabolic pathway. This pathway has as a final product of 3-hydroxy-2-butanone (acetoin), which is the same producing metabolic pathway as 2,3-butanediol [17]. The acetoin produced by the microorganisms reacts with the atmospheric oxygen and KOH forming diacetyl, which reacts with alpha-naphthol, producing a colored complex that confirms the positive test [33]. Mariotto [34] indicated that the acetoin production is not equivalent to the 2,3-BD production, as the first is a precursor of the second and many factors may influence the concentration of one or the other during the fermentation process. Therefore, the standard identification test of the acetoin metabolic compound, precursor of 2,3-BD, allows verification of the possible morphospecies capable of producing the compound of interest. However, the test may not indicate the produced concentration of 2,3-BD due to its limit of detection, but only the capacity of the microorganism to produce the compound of interest, i.e., the test is just a qualitative screening for both glucose and glycerol mediums. This triage test can provide better characterization of microorganisms after isolation, which allows better selection criterion of the samples in fermentation processes with the purpose of producing 2,3-BD.

The glycerol fermentation at 2.0% (m/v) for 48 hours of the AG3 and AG7 isolates, previously identified as acetoin producers, indicated that they were capable of

simultaneously producing both compounds of interest, 2,3-BD and 1,3-PD [Table-2]. The AG3 isolate presented the highest simultaneous production of the compounds, though no significant statistical difference was seen compared to the other producing isolates. Although the molecular identification found that the AG1 and AG7 samples are both *Enterobacter ludwigii* sp, both isolates clearly belong to distinct microbial lines, as discriminated by a biochemical analysis through GEN III MicroPlate™ panel. Moreover, AG1 was incapable of producing 1,3-propanediol, whereas AG7 was not.

Table-2 *Production and yield of 20 g.L^{-1} glycerol fermentation*

Isolate	2,3-BD		1,3-PD	
	Product Concentration (g.L^{-1})	Yield (g.g^{-1})	Product Concentration (g.L^{-1})	Yield (g.g^{-1})
AG1	0.501a	0.0211	-	-
AG3	0.522a	0.0219	0.735a	0.0308
AG5	0.459a	0.0191	-	-
AG6	0.357a	0,0149	0.842a	0.352
AG7	0.414a	0.0172	0.349a	0.0145

The same letters in the columns show no significant differences ($p<0.05$) according to the Scott-Knott test.

The AG6 sample, negative for the qualitative test, presented a lower productive yield of the compound; however, it stood out in 1,3-PD production. The obtained results for the AG6, AG3, and AG7 isolates suggest two distinct tendencies of glycerol metabolization: reductionist pathway metabolism, preferred by AG6 sample, and oxidative pathway metabolism, observed in the *Enterobacteriaceae* family isolates. In the reductionism process, there is the glycerol molecule dehydration action by the dehydratase glycerol enzyme, and in the end the reaction product, 3-hydroxypropionaldehyde is reduced by the enzymatic action of 1,3-propionaldehyde oxidoreductase into 1,3-propanediol [27]. In the oxidative process, the glycerol, through an enzymatic action of dehydratase glycerol, is converted into dihydroxyacetone, a product used in the metabolic glycolytic pathway and subsequently in the production of fermenting metabolite compounds or, when in aerobic condition, oxidation in the cellular respiratory route for energy production [22].

The 2,3-BD and 1,3-PD production was lower than the results the obtained by different authors who used glycerol as a sole carbon source [7, 24, 35]. However, these authors made different modifications to the culturing conditions than the tests described here, such as pH fluctuation and control, manipulation of the regeneration of bacterial growth cofactors, and carbon flux, stirring and aeration of the fermentation process.

The lack of tools to maximize the production of the compounds of interest was the main limitation of this study. The main tools used to increase microbial metabolite production from glycerol conversion are genetic improvement or other molecular technique, such as gene therapy and fermentation medium optimization to meet the specific needs of natural microbial lines [4, 36]. Yen et al. [37] observed that the fermentation process for the simultaneous production of 2,3-BD and 1,3-PD when subjected to higher diluted oxygen concentrations results in better yields, and that lower concentrations contribute to an increased production of 1,3-PD. Sattayasamitsathit et al. [38], after varying the concentration of some fermenting medium compounds, observed through a mathematical model, that the variation of the medium components influence the compound production. These modifications permitted an equivalent production of 13.54 g.l^{-1}and 6.61g.l^{-1}of 1,3-PD and 2,3-BD, respectively, through the analyzed microorganism. Therefore, the maximization production experiments are in the standardization stage for further research.

Conclusion

Isolated rhizobacteria can grow in a glycerol as the sole carbon source in the medium, thus enabling their use in fermenting processes for producing compounds of interest. The triage method for 2,3-BD production was shown to be efficient in evincing producing microorganisms and two isolated rhizobacteria were capable of simultaneously producing 2,3-BD and 1,3-PD. Therefore, this study is an important step to encourage the biotechnological development of new fermenting technology and its sustainable application.

Abbreviations: 1,3-propanediol (1,3-PD); 2,3-butanediol (2,3-BD); plant growth-promoting rhizobacteria (PGPR); volatile organic compounds (VOCs); induced systemic resistance (ISR); hydrocyanic acid (HCN).

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Conflict of Interest: The authors declare that they have no competing interests.

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