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Original scientific paper

The effects of medium nutritional profile on *Bacillus* sp. Par 3 plant-growth promoting and biocontrol activity against *Botrytis cinerea*

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SUMMARY

Substantial agricultural losses resulting from plant diseases caused by different plant pathogens are one of the worldwide challenges today. Among these, *Botrytis cinerea*, responsible for gray mold disease, stands out for its capacity to devastate significant quantities of diverse valuable crops. Utilization of biocontrol agents for suppressing phytopathogens has become imperative, and bacteria from the genus *Bacillus* hold an immense potential due to their rapid replication rate, resistance to adverse environmental conditions, enhanced effectiveness in promoting plant growth and broad-spectrum activity. The objective of this study was to determine the best sources of carbon, nitrogen and phosphorus in cultivation media with the aim of maximizing both antimicrobial activity against *B. cinerea* and plant-growth-promoting (PGP) potential during the early stages of cucumber plant development, exhibited by *Bacillus* sp. isolate Par 3. Antimicrobial activity was tested using the well diffusion method. The influence of *Bacillus* sp. isolate Par 3 on plant germination was tested on cucumber seeds. The largest inhibition zones were achieved in two cases, with 1) sucrose as carbon source, ammonium nitrate as nitrogen source, and diammonium hydrogen phosphate as phosphorus source and 2) glycerol as carbon source, ammonium nitrate as nitrogen source and dipotassium hydrogen phosphate as phosphorus source. Seeds treated with a culture liquid of *Bacillus* sp. isolate Par 3 using the optimized medium exhibited the best results in terms of cucumber germination percentage (100%), root length (53.09 mm) and shoot length (13.26 mm). *Bacillus* sp. Par 3 isolate was identified as *Bacillus subtilis* using 16S rRNA gene sequencing. The results of this study underscore the significance of media optimization for the production of biocontrol agents, taking into account both antimicrobial efficacy and PGP characteristics.

Keywords: *Bacillus subtilis*, *Botrytis cinerea*, cucumber, nutrient medium optimization, plant growth promotion, antimicrobial activity

INTRODUCTION

Rising human population and a need for adequate food supplies present significant challenges for the agricultural sector. Besides the already demanding task of meeting these needs, crop damage caused by pathogens also adds to the complexity. Pathogen infections have been approximated to result in a global food crop loss of 10-16%, equivalent to an economic loss of around 200 million euros each year (Toral et al., 2020).

The necrotrophic fungus *Botrytis cinerea* is a pathogen responsible for significant economic losses, able to attack more than 200 plant species, including tomato, potato, oilseed rape, kiwi fruit, cucumber, and other high-valued and important economic crops (Yang et al., 2020). While this pathogen mainly targets dicotyledonous plants, it can also infect some monocotyledonous plants. *B. cinerea* is the most destructive on mature plant parts or senescent tissue, even though it typically gains entry to such tissues during earlier stages of crop development. After entering tissue, the pathogen remains dormant until changes in the host's environment and physiology trigger sudden rotting. Common symptoms on leaves and soft fruit include soft rot, which manifests as the collapse and water-soaking of parenchyma tissues, followed swiftly by the appearance of gray masses of conidia (Williamson et al., 2007). This phytopathogenic fungus is difficult to control because it has a broad host range, various attack modes, and both sexual and asexual stages to survive under favorable, as well as unfavorable conditions (Hua et al., 2018).

One of the plants most affected by *B. cinerea* is cucumber. Cucumber is one of the most widely used vegetables and a member of the popular Cucurbitaceae family. It holds a vital place in various human diets and is commonly consumed fresh in salads. However, the presence of cucumber gray mold as a severe disease in cucumber cultivation raises concerns about food safety (Soliman et al., 2015).

Currently, chemical fungicides are the most used agents against gray mold caused by *B. cinerea* and they represent about 8% of the global pesticide market. However, the excessive use of chemical pesticides and fertilizers in agriculture has resulted in the accumulation of harmful residues in the environment, which poses risks to human health. These concerns have spurred the exploration of alternative approaches to pest and disease management (Toral et al., 2020).

Biocontrol agents are conducive to sustainable agriculture as they aid in reducing reliance on chemical pesticides. This not only minimizes negative effects on the environment and human health but also prevents the development of pathogen resistance to chemical pesticides. Species belonging to the *Bacillus* genus are widely utilized as biocontrol agents (BCAs) applied as biofertilizers or biopesticides in different crops and against a variety of soil-borne diseases. The widespread use of *Bacillus*-based bioproducts can be attributed to several distinctive traits of this genus, such as rapid replication rate, resistance to adverse environmental conditions, increased efficiency in plant growth promotion, and broad-spectrum activity (Samaras et al., 2021). The aim of this study was to determine the best sources of carbon, nitrogen and phosphorus in cultivation media in terms of maximizing the biocontrol activity against *B. cinerea* and PGP potential in the initial phases of cucumber plant development exhibited by *Bacillus* sp. isolate Par 3.

MATERIALS AND METHODS

Microorganisms

The antagonistic microorganism used in this study was *Bacillus* sp. isolate Par 3, isolated from tomato rhizosphere by using the selective medium HiCrome *Bacillus* agar (HiMedia Laboratories, India). The procedure was as follows: 1 g of soil sample was mixed with 9 ml of saline solution, subjected to heat treatment (100 °C, 8 min), serially diluted (10 and 100-fold), and placed on the surface of a selective medium (100 µl, HiCrome *Bacillus* agar, Himedia Laboratories, India), followed by incubation at 28 °C for 48 h and selection of single colonies. Colony selection and incubation steps were repeated until visually pure cultures were obtained. The isolate was kept on a nutrient agar slant (4 °C). Biochemical characterization of the isolate *Bacillus* sp. Par 3 was done using a VITEK2 device and BCL cards according to the manufacturer's instructions (Biomerieux, France). The isolate was identified by the 16S rRNA gene sequencing (Macrogen, Netherlands) of PCR (polymerase chain reaction) products obtained using the primers 27f and 1492r. The PCR procedure and genomic DNA isolation were previously described (Pajčin et al., 2020). The 16S rRNA gene sequence (1411 bp) was deposited in the NCBI GenBank database under accession number OR690892 and compared to the NCBI GenBank database sequences using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/).

Botrytis cinerea isolate R9, which was used as a test pathogenic microorganism in this research, was isolated from cucumbers with symptoms of gray mold and stored on SMA (Sabouraud maltose agar, Himedia India) at a temperature of 4°C. Identification was confirmed by polymerase chain reaction by amplifying and sequencing

the amplified region using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG - 3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') (White et al., 1990). Gene sequences were analyzed using the BLASTn algorithm online at http://blast.ncbi.nlm.nih.gov/ with already deposited sequences in the NCBI GenBank database. The sequence was deposited in the NCBI GenBank database under the accession number OR644880.

Screening of plant growth promotion traits

For better understanding the PGP potential of the producing strain *Bacillus* sp. isolate Par 3 PGP, parameters such as the ability to produce surfactin and indole acetic acid were screened as described below. *Bacillus* sp. Par 3 was cultivated in Erlenmeyer flasks, using nutrient broth medium (Himedia Laboratories, Mumbai, India), for 48 h on a rotary shaker (170 rpm) at 28 °C. L-Trp (1.02 g/l) was added to the media for the experiments aimed at indole components quantification. To obtain a cell-free supernatant of the cultivation broth for the mentioned experiments, bacterial biomass was separated by centrifugation (12000 × g, 10 min, 25°C, Z 326 K, Hermle LaborTechnik GmbH, Germany).

Surfactin production

Surfactin concentration in the culture supernatant of *Bacillus* sp. Par 3 was determined using the CPC-BTB (cetylpyridinium chloride-bromothymol blue) method (Yang et al., 2015). In this method, bromothymol blue (BTB) and the mediator cetylpyridinium chloride combine to form a green-colored complex. When surfactin is introduced, it forms a colorless complex with the CPC, releasing BTB molecules into the medium, resulting in a detectable color change that can be measured spectrophotometrically. The quantification process involved mixing 300 µl of supernatant sample with 2.4 ml of the CPC-BTB reagent and incubating the mixture at 25°C for 5 minutes. Finally, the absorbance at 600 nm was measured using a UV1800 spectrophotometer (Shimadzu, Japan), and surfactin concentration was determined on the basis of standard curve prepared using the surfactin standard (Sigma-Aldrich, Burlington, MA, USA) (Valenzuela-Ávila et al., 2020).

Indole acetic acid production

Determination of IAA concentration in the supernatant of *Bacillus* sp isolate Par 3 was performed with a slight modification of the colorimetric method described by Syed-Ab-Rahman et al. (2018). In brief, 1 ml of *Bacillus* sp. Par 3 cultivation broth supernatant was mixed with 2 ml of Salkowski reagent (1.2% [w/v] $FeCl_3$ in 7.9 M H_2SO_4) and incubated in a dark place at room temperature for 30 minutes. Pink color development indicates the microorganism's ability to produce IAA. After the 30-minute incubation period, spectrophotometric measurements were taken at a wavelength of 535 nm (UV 1800, Shimadzu, Japan). Distilled water was used as a blank sample, and the calibration curve was prepared using the indole acetic acid (IAA) standard (Sigma-Aldrich, Burlington, MA, USA).

Selection of carbon, nitrogen and phosphorus sources – Media composition and cultivation conditions

Substrates for cultivation were prepared according to a full experimental design by varying the basic components of the medium - sources of carbon, nitrogen and phosphorus. The effect of all components and their interactions on the outcome was investigated, where all combinations of independent variables were included in the full experimental design. Glycerol and sucrose were used as carbon sources (5 g/l). Potassium nitrate, ammonium nitrate and ammonium sulfate were used as nitrogen sources (1 g/l). Dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ammonium dihydrogen phosphate, and diammonium hydrogen phosphate were used as sources of phosphorus (1g/l). The effects of variations in medium composition were investigated by testing the *in vitro* antimicrobial effect of *Bacillus* sp. Par 3 cultivation broth on the growth of *B. cinerea* pathogen. The cultivation broth sample that exhibited the best results in terms of suppressing the phytopathogen *B. cinerea* was used to determine its effect on the germination of cucumber seeds, as well as the cultivation broth sample produced on the synthetic commercial medium (nutrient broth, Himedia Laboratories, India) under the same cultivation conditions. Cultivation of the *Bacillus* isolate was carried out on a rotary shaker at 28 °C and 170 rpm, under spontaneous aeration, during 96 h, with 10% (v/v) inoculum prepared using nutrient broth (Himedia Laboratories, India).

Antimicrobial activity assay

The suspension of the test microorganism *B. cinerea* isolate R9 was prepared by adding fungal spores into sterile saline. Sabouraud maltose agar

Table 1. Biochemical characteristics of *Bacillus* sp. isolate Par 3

BXYL	+	LysA	–	AspA	(–)	LeuA	(+)	PheA	+	proA	–
BGAL	+	PyrA	+	AGAL	+	AlaA	(–)	TyrA	+	BNAG	–
APPA	–	CDEX	–	dGAL	+	GLYG	–	INO	–	Mdg	+
ELLM	+	MdX	–	AMAN	–	MTE	+	GlyA	–	dMAN	–
dMNE	+	dMLZ	+	NAG	+	PLE	+	IRHA	–	BGLU	+
BMAN	–	PHC	–	PVATE	–	AGLU	+	dTAG	–	dTRE	+
INU	+	dGLU	–	dRlB	–	PSCNa	–	NaCl 6.5%	+	KAN	–
OLD	–	ESC	+	TTZ	+	POLYB_R	+				

media (Himedia Laboratories, India) were melted and tempered (50±1 °C) and, before pouring into Petri plates, inoculated with 1 ml of previously prepared spore suspension (10^5 spores/ml). The well diffusion method was employed in triplicate tests to evaluate the antimicrobial activity of the cultivation broth samples (100 µl) obtained after 4 days of cultivation of the producing microorganism, *Bacillus* sp. Par 3, against the phytopathogenic isolate. Incubation was performed at 26 °C for 96 h and followed by inhibition zone diameter measurements. Sterile distilled water was used as a negative control.

Plant germination assay

The influence of *Bacillus* sp. Par 3 isolate on plant germination was tested on cucumber (*Cucumis sativus* L.) seeds. Seeds used in this assay were surface sterilized by chlorine bleach solution (6% (v/v), 1 min) and thoroughly washed with sterile distilled water for 5 min. After drying, fifty cucumber seeds were placed in each Petri plate containing filter paper and then soaked with 1 ml of optimized cultivation broth and 5 ml of sterile tap water. The Petri plates were then incubated at 25 °C for 7 days. After the incubation period, the length of cucumber roots and shoots was measured and compared to the negative control, which used tap water, and cultivation broth produced by using nutrient broth as cultivation medium (Himedia Laboratories, India).

Experimental data analysis

Statistical analysis of the experimental data was performed using the Statistica 13.3 software (Dell Technologies, TX, USA). Duncan's multiple range test was performed to establish homogenous groups of variances of the dependent variables. All statistical analyses were performed at the significance level of 95%.

RESULTS

Identification and biochemical characterization of *Bacillus* sp. isolate Par 3

Biochemical characterization of the *Bacillus* sp. isolate Par 3 was conducted using VITEK2 biochemical tests, and the results are shown in Table 1. Based on the BLASTn results, *Bacillus* sp. isolate Par 3 was identified as a member of the *Bacillus subtilis* group with 97.45% sequence similarity with several other *B. subtilis* strain sequences of the 16S rRNA gene available in the NCBI GenBank database.

PGP traits of the producing strain *Bacillus* sp. Par 3

The capacity of *Bacillus* sp. isolate Par 3 for promoting plant growth was investigated in terms of its ability to produce surfactin and IAA. The results shown in Table 2 represent mean values and standard deviations of concentrations of produced surfactin and IAA obtained in 3 repeated tests.

Table 2. Screening results of PGP traits from cultivation broth of *Bacillus* sp. isolate Par 3

	Concentration (mg/l)
Surfactin production	440.67±0.67
IAA production	7.96±0.02

Analysis of effects of different nutrients on antimicrobial activity of *Bacillus* sp. Par 3 against phytopathogenic *B. cinerea*

Table 3 presents the effects of carbon, nitrogen and phosphorus sources used in the medium for *Bacillus* sp. Par 3 cultivation, and their interaction, on antimicrobial activity against *B. cinerea* based on the analysis of variance. Statistically significant effects are bolded in Table 3. It can be inferred from the data presented in Table 3 that the antimicrobial activity of *Bacillus* sp. Par 3 against the phytopathogen *B. cinerea* is influenced significantly by all nutrients provided except the carbon source. Among the combinations of independent variables tested, only the combination of carbon source and nitrogen source did not display a statistically significant effect on antimicrobial activity.

Table 3. Analysis of variance of the influence of nutrients choice in cultivation medium on antimicrobial activity of the production microorganism *Bacillus* sp. Par 3 against the phytopathogen *B. cinerea*

Effect	SS	DF	MS	F	*p*-value
Intercept	**218240.2**	**1**	**218240.2**	**5505.710**	**<0.0001**
C	72.0	1	72.0	1.816	0.1841
N	**1694.7**	**2**	**847.3**	**21.377**	**<0.0001**
P	**725.0**	**3**	**241.7**	**6.097**	**0.0013**
C*N	70.1	2	35.0	0.884	0.4197
C*P	**883.2**	**3**	**294.4**	**7.427**	**0.0003**
N*P	**2193.1**	**6**	**365.5**	**9.221**	**<0.0001**
C*N*P	**1425.0**	**6**	**237.5**	**5.992**	**0.0001**
Error	1902.7	48	39.6		

Based on the results of Duncan's multiple range test (Table 4), the largest inhibition zone diameter was achieved through nutrient combinations numbered 23 and 24.

Table 4. The results of Duncan's test for the influence of nutrient choice on antimicrobial activity of the production microorganism *Bacillus* sp. Par 3 against *B. cinerea*. Data represent mean values and standard deviations of three independent experiments.

	Carbon source	Nitrogen source	Phosphorus source	Inhibition zone diameter (mm)
1.	Glycerol	Ammonium sulfate	Potassium dihydrogen phosphate	34.00 ± 5.29^{a}
2.	Sucrose	Ammonium sulfate	Dipotassium hydrogen phosphate	36.33 ± 5.13^{ab}
3.	Sucrose	Potassium nitrate	Ammonium dihydrogen phosphate	37.00 ± 1.00^{ab}
4.	Sucrose	Ammonium sulfate	Potassium dihydrogen phosphate	37.67 ± 4.04^{ab}
5.	Glycerol	Ammonium sulfate	Ammonium dihydrogen phosphate	46.33 ± 7.77^{bc}
6.	Glycerol	Ammonium sulfate	Diammonium hydrogen phosphate	49.00 ± 11.53^{cd}
7.	Glycerol	Potassium nitrate	Diammonium hydrogen phosphate	51.00 ± 4.58^{cde}
8.	Glycerol	Ammonium nitrate	Ammonium dihydrogen phosphate	52.66 ± 12.86^{cdef}
9.	Glycerol	Potassium nitrate	Ammonium dihydrogen phosphate	53.00 ± 13.45^{cdef}
10.	Glycerol	Ammonium nitrate	Diammonium hydrogen phosphate	56.33 ± 7.09^{cdef}
11.	Sucrose	Ammonium nitrate	Potassium dihydrogen phosphate	56.33 ± 14.15^{cdef}
12.	Glycerol	Ammonium nitrate	Potassium dihydrogen phosphate	56.66 ± 6.67^{cdef}
13.	Sucrose	Ammonium sulfate	Diammonium hydrogen phosphate	59.33 ± 1.15^{def}
14.	Glycerol	Potassium nitrate	Potassium dihydrogen phosphate	59.66 ± 0.58^{def}
15.	Glycerol	Ammonium sulfate	Dipotassium hydrogen phosphate	60.33 ± 4.16^{def}
16.	Sucrose	Potassium nitrate	Potassium dihydrogen phosphate	63.00 ± 1.00^{ef}
17.	Sucrose	Ammonium nitrate	Ammonium dihydrogen phosphate	63.00 ± 1.00^{ef}
18.	Sucrose	Potassium nitrate	Dipotassium hydrogen phosphate	63.00 ± 1.00^{ef}
19.	Sucrose	Potassium nitrate	Diammonium hydrogen phosphate	63.66 ± 0.58^{f}
20.	Sucrose	Ammonium nitrate	Dipotassium hydrogen phosphate	64.00 ± 0.00^{f}
21.	Sucrose	Ammonium sulfate	Ammonium dihydrogen phosphate	64.33 ± 0.58^{f}
22.	Glycerol	Potassium nitrate	Dipotassium hydrogen phosphate	64.66 ± 0.58^{f}
23.	Glycerol	Ammonium nitrate	Dipotassium hydrogen phosphate	65.00 ± 1.00^{f}
24.	Sucrose	Ammonium nitrate	Diammonium hydrogen phosphate	65.00 ± 0.00^{f}

Notably, the final six combinations in Table 4, exhibiting the most substantial inhibition zones, showed no statistically significant distinctions. They were grouped within the same statistical significance level, forming a homogeneous cluster. Consequently, any of these combinations can be applied with equal importance and it can be assumed that they will provide approximately the same value of inhibition zone diameter against the phytopathogen *B. cinerea*. On the other hand, the smallest inhibition zone diameter against the phytopathogen *B. cinerea* was noted in the case of nutrient combination comprising glycerol as carbon source, ammonium sulfate as nitrogen source, and potassium dihydrogen phosphate as phosphorus source. The inhibition zone diameter achieved by applying the cultivation broth sample obtained using nutrient broth as a medium was 43.50±0.50 mm.

Plant germination assay

Figure 1 presents data regarding the germination and length of roots and shoots of cucumber seeds treated with the cultivation broth of *Bacillus* sp. isolate Par 3 based on an optimized medium (nutrient combination number 24), in comparison to the negative control and seeds treated with the cultivation broth of *Bacillus* sp. isolate Par 3 based on a commercial medium - nutrient broth. Seeds treated with the culture liquid of *Bacillus* sp. isolate Par 3 in nutrient broth exhibited a slightly higher germination percentage (98%) than seeds treated with tap water (96%), yet still lower than seeds treated with cultivation broth based on the optimized medium (100%). Treating seeds with cultivation broth derived from the optimized medium has proven to be highly successful, considering the following mean values for root and shoot length: 53.90±14.90 mm and 13.26±4.88 mm, respectively, with corresponding maximum values of 82 mm and 24 mm. The substantial growth enhancement, achieved by treating seeds with culture liquid from the optimized medium, is most prominently demonstrated in root growth.

DISCUSSION

Members of the genus *Bacillus* are extensively researched examples of PGP rhizobacteria. *Bacillus* species hold significant potential due to their capacity to generate

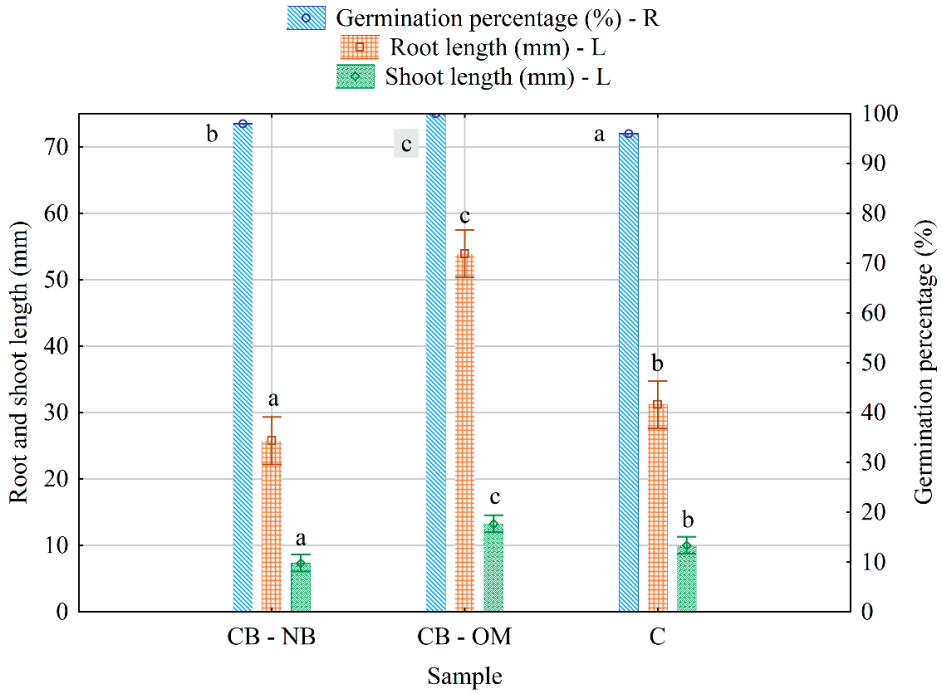

Figure 1. The effects of *Bacillus* sp. Par 3 cultivation broth based on nutrient broth (CB-NB) and optimized medium with previously selected carbon, nitrogen and phosphorus sources (CB-OM) on cucumber seed germination, root and shoot length in comparison to control (tap water - C). Means and standard deviations of root and shoot lengths are given together with homogenous group designations (a, b, c) according to Duncan's multiple range test.

various advantageous elements, including indole acetic acid (IAA), hydrocyanic acid (HCN), siderophores, hydrolytic enzymes, antimicrobial compounds, along with their capabilities for phosphate solubilization and nitrogen fixation (Vlajkov et al., 2023). The *Bacillus* isolate employed in this research was identified using 16S rRNA sequencing, revealing its affiliation with *Bacillus subtilis* species with identification accuracy of 97.45%, while biochemical characterization was done using the VITEK2 device and BCL cards (Biomericux, France). Biochemical characterization, shown in Table 1, included 46 tests, encompassing the examination of carbon source utilization, enzymatic activities, inhibition by 6.5% NaCl, and resistance to antibiotics. Previous research had proved that *B. subtilis* has a potential for use as a biocontrol agent. Bu et al. (2021) investigated the influence of *B. subtilis* L1-21 isolate on the suppression of *B. cinerea* phytopathogen in postharvest tomatoes, resulting in 86.57% control efficacy. In a study conducted by Touré et al. (2004), the investigated *B. subtilis* isolate GA1 demonstrated high effectiveness in reducing gray mold incidence within the initial 5 days after pathogen inoculation, and it maintained a protection level of 80% over the subsequent 10 days. Furthermore, it has been determined that in addition to its significant antagonistic effect, *B. subtilis* holds great importance due to its PGP properties. Strain RH5, examined by Jamali et al. (2020), exhibited a range of PGP attributes (indole acetic acid, siderophore, hydrogen cyanide production and phosphate, Zn, K solubility), hydrolytic enzymatic (chitinase, protease, cellulase, xylanase) activity, and presence of antimicrobial peptide biosynthetic genes (bacylisin, surfactin, and fengycin), which support the strain in efficient colonization of hyphae and pathogen inhibition. *B. subtilis* also enhances stress tolerance in plant hosts by inducing the expression of stress-response genes, phytohormones, and stress-related metabolites (Hashem et al., 2019).

Commercially available media, frequently utilized during preliminary investigation phases of bioprocess development for manufacturing biocontrol agents, are considered inadequate for the production scale-up stages. The rationale behind this lies in their prohibitively high cost, which constrains the potential transition to large-scale industrial production and subsequent product commercialization. This underscores a need for additional efforts to identify nutrient sources that can support particular isolates' growth and metabolic activity. The discovery of waste streams rich in suitable nutrients for microbial growth and metabolic activity is a step further in the creation of complex media based on natural components (Vlajkov et al., 2022).

It is crucial to note that the nutritional requirements of microorganisms are highly dependable on a particular strain. Among the pivotal elements shaping the traits and metabolic functioning of these strains is their ability to effectively process particular nutrients in both qualitative and quantitative terms. Carbon is the most significant component of a medium since it provides microorganisms with energy, aids their growth, and helps them produce primary and secondary metabolites. The creation of biomass and/or primary or secondary metabolites can frequently be affected by the rate at which a carbon source is digested (Singh et al., 2017). The carbon sources used in this study were picked to act as exemplars of typical industrial waste streams. These sources might be taken into account as parts of culture medium in further studies to direct research towards integration of the circular economy principles in the production of biocontrol agents.

Sucrose was chosen as one of carbon sources, considering that it is the main component of molasses, the by-products of sugar beet and sugar cane processing. Molasses have been mainly used as cultivation media for biosurfactant production by different *Bacillus* strains (Saimmai et al., 2011; Al-Bahry et al., 2013). Furthermore, it was found that *Bacillus* cultivation on molasses could lead to the production of diverse types of enzymes and other valuable products (Gojgic-Cvijovic et al., 2019; Shikha et al., 2007; Chaijamrus & Udpuay, 2008). The main factors behind extensive use of molasses as substrates are their affordable pricing, as compared to other carbon sources, and the existence of various additional substances besides sucrose. These include vitamins, minerals, and organic substances, all of which are crucial for the fermentation process (Saimmai et al., 2011). Glycerol was used as the second carbon source. A notable volume of glycerol is obtained as a by-product from the continually expanding biodiesel sector. This has garnered considerable scientific attention for its potential conversion into various value-added products through microorganisms. For example, crude glycerol derived from biodiesel production was employed as a carbon source in the cultivation medium for biosurfactant production by *Bacillus* strains (Sousa et al., 2012; de Sousa et al., 2014). Potassium nitrate, ammonium nitrate, and ammonium sulfate were observed as the most promising nitrogen sources to promote antifungal activity against *B. cinerea*. Dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ammonium dihydrogen phosphate, and diammonium hydrogen phosphate were observed as the most potent sources of phosphorus. The selection of these nutrient sources was conducted not only to

support growth, antifungal activity and PGP traits of the producing strain *Bacillus* sp. Par 3, but also to be additional plant nutrient sources, considering that cultivation broth usually contains small residual amounts of initially added nutrients which were not used by the producing microorganism during cultivation (Pajčin et al., 2020).

Factorial ANOVA was employed to determine whether there were significant differences between inhibition zone diameters obtained by assaying cultivation broth samples of *Bacillus* sp. Par 3 against *B. cinerea*, depending on different carbon, nitrogen and phosphorus sources in cultivation medium. ANOVA results showed that the effect of phosphorus inorganic compounds from phosphorus and nitrogen sources, as well as their interaction, were significant (p-values less than 0.05), while the effect of carbon source selection showed less effect even in combination with nitrogen and phosphorus sources.

In order to determine which combination of carbon, nitrogen and phosphorus sources is the most suitable for producting bioactive agents effective against *B. cinerea* by *Bacillus* sp. Par 3 isolate, the well diffusion method was applied. The largest inhibition zones were achieved in two cases, with 1) sucrose as carbon source, ammonium nitrate as nitrogen source, and diammonium hydrogen phosphate as phosphorus source, and 2) glycerol as carbon source, ammonium nitrate as nitrogen source and dipotassium hydrogen phosphate as phosphorus source (Table 4). Based on the findings presented in Tables 3 and 4, it can be deduced that biocontrol agents that play crucial roles in suppressing the phytopathogen *B. cinerea* mainly depend on the source of phosphorus, followed by the source of nitrogen. However, carbon source did not show a statistically significant effect on diameters of inhibition zones against the phytopathogen *B. cinerea*. According to the results shown in Table 4, it can be inferred that ammonium nitrate demonstrated superior performance as nitrogen source, possibly due to two different nitrogenous ions as separate nitrogen sources for microbial growth. Antimicrobial activity of *Bacillus* species is largely attributed to the action of a wide spectrum of secondary metabolites, among which metabolites of lipopeptide nature have a significant impact. Production of these secondary metabolites with antimicrobial activity is considered as an indirect mechanism of plant growth promotion due to an ability to suppress pathogens, thus providing more favorable conditions for plant growth and development (Soni & Keharia, 2021). Surfactin, a cyclic lipopeptide, is one of the most important biosurfactants because of its strong activity as a surfactant and its antimicrobial activity (Janek et al., 2021). In a study conducted by Zhou et al. (2023), the concentration of produced surfactin under optimized fermentation conditions was 1.82 g/l. Our research showed that *Bacillus* sp isolate Par 3 has the ability to produce surfactin in a concentration of 440.67±0.67 mg/l, which might be accelerated through optimization of the medium and process parameters. According to previous research, the possibility of producing surfactin by cultivating *Bacillus* strains on waste streams of biodiesel, dairy and wine industries has been proven (Sousa et al., 2012; De Andrade et al., 2016; Dmitrović et al., 2022). Previous studies have also shown an important role of surfactins produced by *Bacillus* species in facilitating biofilm formation and plant root colonization (Debois et al., 2014; Aleti et al., 2016).

To investigate the effect of cultivation broth of *Bacillus* sp. isolate Par 3 on cucumber seed germination, the medium with sucrose as carbon source, ammonium nitrate as nitrogen source, and diammonium hydrogen phosphate as phosphorus source was chosen for further research due to a wider availability of molasses in these areas.

Besides antimicrobial activity, species belonging to the *Bacillus* genus have numerous benefits in stimulating plant growth. They synthesize a multitude of secondary metabolites that influence the environment and enhance the availability of nutrients to plants. Some species within this genus produce plant hormones such as cytokinins, gibberellins or indole acetic acid. Additionally, they can generate siderophores, ACC deaminase, various enzymes (proteases, pectinases, cellulases, lipases, etc.), and have an ability to decompose diverse organic and inorganic compounds of phosphorus, potassium and zinc, thereby augmenting the accessibility of these crucial elements to plants (Hajnal Jafari et al., 2020). The production of indole acetic acid (IAA), which falls under the category of phytohormones, is one of the most significant benefits for plants. It influences various physiological activities in plants, including cell enlargement, cell division, root growth initiation, growth rate, phototropism, geotropism, and apical dominance (Chrouqi et al., 2017). Previous research had confirmed that three root-colonizing *Bacillus* strains, including *B. amyloliquefaciens, B. subtilis* and *B. tequilensis* possess the capacity to produce IAA, and *in vivo* experiments have substantiated the role of IAA-producing strains in promoting plant growth (Shahid et al., 2021; Khan et al., 2021). The concentration of IAA produced by the isolate *Bacillus* sp. BioSol021 was 15 mg/l (Vlajkov et

al., 2023), while *Bacillus* sp. E25 and *Bacillus* sp. CR71, investigated by Rojas-Solis et al. (2020), produced IAA in concentrations ranging from 20.46±1 to 31.18±1.5 μg/ml, depending on saline stress. In the present study, the demonstrated capability of IAA production by the tested *Bacillus* sp. isolate Par 3 in a concentration of 7.96±0.02 mg/l makes it a promising candidate for development of biotechnological products for agricultural applications, with a possibility to direct medium optimization towards maximization of IAA production. In a study conducted by Kumar et al. (2012), all seven tested *Bacillus* isolates from the rhizosphere showed IAA production, while maximal IAA production was recorded in *Bacillus* sp. BPR7 cultivation broth (17 μg/ml).

Through the production of enzymes, such as cellulases, proteases, pectinases, lipases and other, the breakdown of complex compounds into simpler forms metabolizable by bacteria is facilitated. This leads to an increase in the diversity/biodiversity of soil microflora, which enhances soil fertility and availability of easily accessible nutrients (Hashem et al., 2019; Mohandas et al., 2018). In this study, the influence of the cultivation broth of *Bacillus* sp. Par 3 isolate was investigated on cucumber seeds. It was found that medium optimization for *Bacillus* cultivation has a significant effect on the improvement of germination, i.e. elongation of cucumber roots and stems, which underscores the importance of medium optimization in the production of biological preparations. This is a significant finding in comparison with the commercial medium (nutrient broth), considering the possibility of reducing significantly the production cost related to cultivation medium by using formulated medium with widely available and cost-efficient components, which also affects market price of the biocontrol product and its market competitiveness (Ortiz & Sansinenea, 2023). Further techno-economical analyses will be required to assess the cost-effectiveness of the proposed medium composition on a large scale, which is possible to achieve by using bioprocess simulation tools. Further research is also required to determine the specific PGP traits of the isolate *Bacillus* sp. Par 3, as well as mechanisms involved in cucumber growth promotion.

CONCLUSION

Based on the results obtained in this study, it can be concluded that *Bacillus* sp. isolate Par 3, identified as a member of the *Bacillus subtilis* group, has a significant potential for application as a biocontrol and PGP agent. Proven production of surfactin and IAA in significant concentrations is crucial for its use as a biocontrol and PGP agent in agriculture. By selecting suitable sources of carbon, nitrogen and phosphorus, antimicrobial activity of the isolate was notably enhanced, along with its capacity to promote plant growth. The highest antimicrobial activity with inhibition zones of 65 mm was observed when using media with the following composition: 1) sucrose as carbon source, ammonium nitrate as nitrogen source, and diammonium hydrogen phosphate as phosphorus source, and 2) glycerol as carbon source, ammonium nitrate as nitrogen source and dipotassium hydrogen phosphate as phosphorus source. Treating cucumber seeds with the isolate multiplied on the sucrose-based optimized medium resulted in higher germination rate and significantly faster and greater root and shoot development, compared to the cultivation broth based on commercial medium and negative control. The ability of *Bacillus* sp. Par 3 isolate to utilize sucrose and glycerol as carbon sources opens a possibility for utilization of waste streams from the sugar industry and biodiesel production as substrates for producing this biocontrol agent. Subsequent research would focus on using these waste streams for *Bacillus* sp. Par 3 cultivation and determining optimal process conditions to maximize the beneficial properties of this biocontrol and PGP agent.

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Uticaj sastava medijuma na sposobnost podsticanja rasta biljaka i biokontrolna svojstva izolata *Bacillus* sp. Par 3 protiv fitopatogena *Botrytis cinerea*

REZIME

Jedan od globalnih problema današnjice predstavljaju značajani gubici u poljoprivredi usled biljnih bolesti koje izazivaju različiti patogeni. Među ovim patogenima, bitnu ulogu ima plasan *Botrytis cinerea*, koja kao uzročnik sive truleži, nanosi veliku štetu različitim važnim usevima. Upotreba biokontrolnih agenasa za suzbijanje fitopatogena postala je imperativ, pri čemu bakterije iz roda *Bacillus* imaju veliki potencijal zbog brzog razmnožavanja, otpornosti na nepovoljne uslove okoline, sposobnosti promovisanja rasta biljaka i širokog spektra delovanja. Cilj ovog istraživanja bio je da se utvrde najbolji izvori ugljenika, azota i fosfora u medijumu za kultivaciju *Bacillus* bakterija, sa namerom da se postigne što veća antimikrobna aktivnost protiv fitopatogena *B. cinerea* i podstakne brži rast krastavca u različitim fazama razvoja, primenom izolata *Bacillus* sp. Par 3. Da bi se utvrdila najpogodnija kombinacija izvora ugljenika, azota i fosfora za proizvodnju bioaktivnih agenasa koji efikasno deluju protiv *B. cinerea* od strane izolata *Bacillus* sp. Par 3, primenjena je metoda bunarića. Uticaj izolata *Bacillus* sp. Par 3 na klijanje biljaka testiran je na semenkama krastavca. Najveće zone inhibicije postignute su pri upotrebi sledeća 2 medijuma: 1) saharoza kao izvor ugljenika, amonijum nitrat kao izvor azota i diamonijum hidrogenfosfat kao izvor fosfora, i 2) glicerol kao izvor ugljenika, amonijum nitrat kao izvor azota i dikalijum hidrogenfosfat kao izvor fosfora. Semena tretirana kultivacionom tečnošću izolata *Bacillus* sp. Par 3 korišćenjem optimizovanog medijuma pokazala su najbolje rezultate u pogledu procenta klijanja (100%), dužine korena (53,09 mm) i dužine izdanka (13,26 mm) krastavca. *Bacillus* sp. Par 3 izolat je identifikovan kao *Bacillus subtilis* metodom sekvenciranja 16S rRNA gena. Rezultati ovog istraživanja ističu značaj optimizacije medijuma za proizvodnju biokontrolnih agenasa, uzimajući u obzir kako antimikrobnu efikasnost, tako i karakteristike promocije rasta biljaka.

Ključne reči: *Bacillus subtilis*, *Botrytis cinerea*, krastavac, optimizacija medijuma, podsticanje rasta biljaka, antimikrobna aktivnost