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Preservation of Food Sugar Beet via the Control of *Rhizoctonia solani* AG 2-2IIIB by Extreme Factors

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Abstract: Sugar beet (*Beta vulgaris* L.) is a valuable sugar-providing crop accounting for around 25% of global white sugar production. It is an integral part of the food industry. Several factors cause a decrease in the yield of this food crop. The infection of phytopathogenic fungi is one of the most hazardous. Though several methods to control *Rhizoctonia solani* have been developed, the problem is still not solved. Thus, the goal of this work was to investigate the effect of abiotic factors (pH, Eh, presence or absence of O_2) and biotic ones (microbial community) on the fungal pathogen *R. solani* AG 2-2IIIB for its control and sugar beet protection. Microbiological methods were used to conduct the research. Thermodynamic calculations were applied to determine the conditions necessary for suppression of *R. solani* AG 2-2IIIB. As a result, a comparison of the effect of abiotic and biotic mediated factors on the efficiency of the growth of *Rhizoctonia solani* AG 2-2IIIB was carried out. Obtained data showed the following: mainly the abiotic factors provided via chemical buffers or removal of oxygen caused the inhibition of *R. solani;* the values of $5.5 \leq$ pH ≤ 8.5, Eh ≤ -200 mV, and oxygen absence inhibited its growth. The maintenance of these factors via microbial communities enhanced the suppressive effect, bringing about the death of mycelium. The study of microbial strains revealed the inhibitory effect of microbial metabolites and provided more effective control of *Rhizoctonia solani* AG 2-2IIIB.

Keywords: sugar beet; pathogenic fungus suppression; thermodynamic prediction; extreme factors; microorganisms

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1. Introduction

Human population growth requires an increase in crop yields to increase the amount of food produced. However, the productivity of fields depends on many factors: climate conditions, application of fertilizers, pathogens, etc. [1].

Fungi play an important role in soil: they decompose organics, transport nutrients, and interact with plants and the microbiome [2]. However, fungal pathogens are among the most hazardous to crops. They cause disease in different crop species and contribute to harvest loss on a large scale. *Rhizoctonia solani* is one of the most widespread pathogens, infecting more than 200 species of valuable agricultural crops [3]: sugar beets [4], potatoes [5], rice plants [1,3], maize, cotton [3], etc. For example, *R. solani* threatens to destroy up to 45% of the rice crop worldwide because of a serious rice sheath blight disease [3]. In the period from 2003 to 2015, about 19% of potato yields were destroyed by *R. solani* in the Castilla y León region (Spain's largest autonomous region) [5]. In general, about 5–10% of the sugar beet area of Europe and the United States is affected or threatened by this pathogen [6]. The infection of sugar beets by *R. solani* can result in a loss of up to 60% of yield. Moreover, stored sugar beets can be destroyed by it [7]. About 5% (70,000 ha) of the sugar beet cropping area in Europe is affected by *R. solani* [8]. Sugar beet is essential for

agriculture and industry, since it is one of only two plants providing sucrose [7]. It covers about 35% of the global white sugar harvest [9]. Therefore, the project of reducing the impact of *R. solani* and inhibiting its growth is of great interest to those wishing to prevent the loss of crop yields.

Different strategies have been developed to fight against this plant pathogen. The cultivation of sugar beet varieties resistant to *R. solani* is one of the most important among them [10,11]. However, only partially resistant varieties are known. They require additional control and provide lower yields [8]. Suppression of the pathogen in soil is another strategy. Formerly, the application of fungicides was a widespread method. However, their long-term use caused the formation of resistance, as well as pollution of the environment [1,12]. The appearance of new strains frequently caused a loss in the efficiency of existing fungicides [13]. Therefore, other approaches are of great interest. Nowadays, the method of suppression of *R. solani* growth by microorganisms or by some organic compounds is popular [1,5,8]. For example, *Trichoderma harzianum* was shown to be antagonistic to *R. solani* via growth on the mycelium, penetration into cells, and production of chitinase and β-(1,3)-glucanase [12]. Microbial species *Pseudomonas stutzeri* was reported to inhibit the growth of *R. solani* via lipase production for the degradation of fungal cell wall lipids [1]. *Bacillus* sp. was shown to be a commonly used control agent [14]. The application of different organic amendments was also revealed to be promising. The inhibition of *R. solani*, together with plant growth promotion, was shown via the application of compost [5]. Chitin and keratin amendments have been shown to suppress pathogenic fungi [8]. Brassica tissues (mustard oilcake) were also reported to inhibit *R. solani*. The thioglucoside compounds that they contain were hydrolyzed to isothiocyanates, providing fungi suppression [12]. These methods are promising. However, they are often unpredictable, since there is not enough data to regulate and control the process [8,15,16]. The creation of unfavorable growth conditions for *R. solani* is the general purpose of each one of these methods. However, they approach the problem in a limited way, considering the interaction of certain compounds or biological control agents with *R. solani* separately. That is their weakness.

Several anastomosis groups of *R. solani* are involved in sugar beet and potato disease, including AG 1 to AG 5. The isolates belonging to AG 2 and AG 4 were identified as the most frequently occurring in sugar beet production, and AG 3 in potato cultivation [17,18]. Crown and root rot, as well as the damping off of sugar beets, are mostly caused by two intraspecific groups of *R. solani:* AG 2-2 IIIB and AG 2-2 IV [17,19–21]. AG 2-2 IIIB is more virulent to sugar beets than AG 2-2 IV [19]. Both of these are found worldwide, but AG 2-2 IIIB seems to be more common in Europe, especially in the fields where corn and sugar beets are cultivated alternately [22]. In Europe, more than 36,000 ha are affected by *R. solani* AG 2-2 IIIB each year [23,24]. The productivity of valuable food plants strongly depends on the control of this phytopathogenic agent.

Therefore, the goal of the work was to investigate the effect of abiotic factors (pH, Eh, presence or absence of O_2) and biotic ones (microbial community) on the fungal pathogen *R. solani* AG 2-2IIIB for its control and sugar beet protection.

2. Materials and Methods

2.1. Thermodynamic Background of the Growth Conditions of Rhizoctonia solani AG 2-2IIIB

R. solani AG 2-2IIIB (strain ID 105) culture was obtained from mature sugar beet root and stored on wheat grains at −30 °C. In short, it was grown on a PDA medium (BioMaxima S.A., Lublin, Poland). The strain was identified predominantly based on morphological features, and then with anastomosing tests with testers and ITS1-ITS2 sequence [25]. The ITS1-ITS2 sequence of *R. solani* ID 105 was deposited in the GeneBank database with the accession number KX810067.

To evaluate the growth conditions of *R. solani* AG 2-2IIIB, and to have the opportunity to regulate them, the Pourbaix diagrams were used [26,27]. A field of water stability (i.e., the zone where water remains in the liquid state) determined by pH and redox

potential (Eh) was built. The effect of pH and Eh on the growth of *R. solani* AG 2-2IIIB was assessed.

2.2. Factors Studied to Control Rhizoctonia solani AG 2-2IIIB

The following factors were studied to control *R. solani* AG 2-2IIIB: abiotic (pH, Eh, O_2 content created via chemical buffers and air removal via its replacement by argon) and biotic (microbial community of the compost). Their influence on *R. solani* AG 2-2IIIB was investigated.

2.3. pH Maintenance via Abiotic Factors to Control Rhizoctonia solani AG 2-2IIIB

To cultivate the fungus, peptone broth (BioMaxima S.A., Poland) with yeast extract (BioMaxima S.A., Poland) was used. The medium was prepared in accordance with the instructions of the manufacturer.

To maintain pH of the culture medium, buffer solutions at a concentration of 10 g/L were used (NaH_2PO_4, Na_3PO_4, and their mixture). The solution of NaH_2PO_4 was characterized by pH = 4.43. The buffer Na_3PO_4 provided pH = 12.1. The mixture of NaH_2PO_4/Na_3PO_4 at the ratio 1.6:1 resulted in pH = 7.09. The Na_3PO_4/NaH_2PO_4 mixture with the ratio 1.2:1 provided pH = 8.45. The heating during the sterilization of the solutions did not significantly change the pH values. To study the efficiency of the fungus growth, the following values of pH of culture medium were studied: 5.5; 7.0; 8.5. To reach the required pH, the culture medium was mixed with the corresponding buffer solution at the ratio 1:1. In the medium, the solution of NaH_2PO_4 provided pH = 5.3; the mixture NaH_2PO_4/Na_3PO_4 provided pH = 7.0; the mixture Na_3PO_4/NaH_2PO_4 provided pH = 8.5. The culture medium without buffers was twice diluted by distilled water and was used as the control for *R. solani* AG 2-2IIIB growth. The cultivation was conducted in 150 mL flasks where 50 mL of medium was added. The inoculation of *R. solani* AG 2-2IIIB was carried out via introduction of an agar block with a three-day culture. The block diameter was 7 mm. The cultivation was conducted at 25 °C. The experiment was carried out in five replicates. Growth activity was evaluated via the measurement of the length of mycelium from the agar block to its distal part.

2.4. Eh Maintenance via Abiotic Factors to Control Rhizoctonia solani AG 2-2IIIB

To evaluate the effect of the redox potential of the culture medium and oxygen on the growth of the fungus, the following procedure was carried out. The cultivation of *R. solani* AG 2-2IIIB was conducted in 150 mL flasks with 50 mL of peptone broth. *R. solani* AG 2-2IIIB inoculation was carried out via the introduction of the agar block (7 mm in diameter) with the three-day culture. The conditions created for the cultivation are described in Table 1.

Table 1. Conditions for the growth of *Rhizoctonia solani* AG 2-2IIIB.

Conditions	Factors		
	O_2	Argon	Fe(II)
Aerobic	+ [1]	− [2]	−
Anoxygenic	−	+	−
Anaerobic	−	+	+

[1] Presence of factor; [2] absence of factor.

For aerobic conditions, the cultivation was conducted in flasks with cotton stoppers to ensure that air was present. For anoxygenic conditions, the removal of air was ensured via its replacement with inert gas (argon). For this purpose, the flask was purged with argon at a flow rate of 0.5 L/min for 10 min. To create anaerobic low potential conditions, air was replaced by argon, and the solution of Fe(II) citrate (2 mL) was added to create low potential conditions (−200 . . . −180 mV) in the medium.

The stock solution of low potential buffer Fe(II) citrate at the concentration of 20 g/L was prepared as follows. To prepare 100 mL of the solution, 50 mL of distilled water was added to a 200 mL flask, closed with a cotton stopper, and purged with argon at a rate of 0.5 L/min for 10 min. After that, 17.34 g of trisubstituted sodium citrate was added into the flask under a stream of argon. After the dissolution of sodium citrate crystals, 8.66 g of $FeSO_4 \times 5H_2O$ crystals were added to the flask. To obtain pH = 7.0, Na_2CO_3 was added. The pH value was controlled potentiometrically. To reach the volume of 100 mL of solution, the remaining amount of distilled water was added after the pH adjustment. The resulting mixture was purged with argon for 5 min and hermetically closed with a rubber plug and metal shutter. The solution was sterilized in a boiling water bath for 20 min.

The cultivation was conducted at 25 °C. The experiment was carried out in triplicate. The growth activity was evaluated via the measurement of the length of mycelium from the external edge of the agar block to its distal part.

2.5. Verification of Rhizoctonia solani AG 2-2IIIB Growth after Unfavorable Anoxygenic and Anaerobic Cultivation Conditions

To evaluate whether anoxygenic and anaerobic conditions cause the death of *R. solani* AG 2-2IIIB, the following procedure was conducted. After 7 days of exposure under such conditions (see Section 2.3), the agar blocks with the fungus that served as the inoculum were transferred to 150 mL flasks with 50 mL of peptone broth. The cultivation was carried out in aerobic conditions at 25 °C. The growth was observed via the measurement of the length of mycelium from the agar block to its distal part.

2.6. Maintenance of Unfavorable Growth Conditions for Rhizoctonia solani AG 2-2IIIB via Biotic Factors

To study the pathway to control *R. solani* AG 2-2IIIB via biotic factors, a microbial community from compost was used. Plant residues were used for composting. The compost sample was taken from the countryside in Opole region. Before the investigation, it was stored in the refrigerator at +5 °C. The species content of its microbiome was not identified, since there was no need to analyze it. The compost served as the source of microbial community. Though the composition of species in it can differ, their general function is the same: degradation of organic substrate reducing Eh and O_2 content in the medium [28,29]. Creation of such conditions is necessary to suppress *R. solani* AG 2-2IIIB.

To investigate the effect of microbial community on *R. solani* AG 2-2IIIB, the agar blocks with the fungus were inoculated in aerobic conditions into 150 mL flasks with 50 mL of peptone broth and 1 g of compost. The flasks were hermetically closed with silicon stoppers. The cultivation was carried out for 7 days at 25 °C. The fungus growth was observed via the measurement of the length of the mycelium. The cultivation of *R. solani* AG 2-2IIIB and microbial community was also conducted in separate flasks under the same conditions. The values of pH and Eh were measured potentiometrically via Elmerton pH/conductivity meter CPC-411, electrode pH EPS-1, and electrode redox ERS-2, respectively.

To confirm the death of mycelium after cultivation, the agar blocks with Rhizoctonia were sequentially transferred from the flasks to peptone agar in Petri plates after each day of cultivation. For this purpose, the agar block was ground in the sterile mortar. The mixture was transferred onto peptone agar and spread over the surface with a Dryhalsky spatula.

To confirm the effect of microbial community on *R. solani* AG 2-2IIIB, cultivation on a solid substrate imitating the structure of soil was conducted. To model it, glass balls with a diameter of 2 mm were used. Petri plates were used as cultivators. The Petri plates were filled with 3 layers of glass balls and nutrient broth. To create aerobic conditions, glass balls were placed into the bottom of Petri plates that were covered with a lid to provide oxygen access. To create anoxygenic and anaerobic conditions, balls were placed into the lid and covered with the bottom, used upside down. To create anaerobic conditions the solution of Fe(II) citrate (1 mL) was added to peptone broth. The limitation of oxygen access was achieved by filling the gap between the lid and the bottom with a mixture of petroleum

jelly and paraffin in a ratio of 1:1 after the inoculation. The separate growth of *R. solani* AG 2-2IIIB and the microbial community as well as their joint cultivation were studied. The agar blocks (7 mm diameter) with the pre-cultivated Rhizoctonia were placed in the center of the plates and used as the growth control. To study the effect of microbial community, a suspension of compost was prepared. For this, 1 g was diluted in 99 mL of peptone broth. This suspension (0.1 mL) was used to inoculate Petri plates. The cultivation took 7 days at 25 °C. The fungus growth was observed via the measurement of the length of the mycelium from the agar block to its distal part. To indicate the pH and redox potential of the medium in Petri plates, the indicator 0.01% bromothymol blue (BTB) was used. It changes color depending on the conditions: pH > 7—blue, pH = 7—green, 5 < pH < 6—yellow, and pH < 5—bright yellow. As the redox indicator, it turns colorless at Eh ≤ -200 mV (pH = 7.0–7.8). Sodium resazurin solution (0.01%) was also used to control redox potential. At the potential above -50 mV, it is purple. At the potential range of -50 to -100 mV, it is pink, and at less than -100 mV, the colorless form is observed. The solutions of the indicators were added to the media until the color was visually detectable.

R. solani AG 2-2IIIB growth on its natural substrate (sugar beets) was also studied with reference to the effect of microbial community. For this purpose, a sugar beet tuber was sliced with a 5 mm width and 5–7 cm diameter. One slice was placed on filter paper, with one per Petri plate. Petri plates with sugar beets were sterilized in an autoclave at 1.0 atm., 120 °C for 20 min. The inoculation was conducted via the placement of 5 agar blocks with the pre-cultivated *R. solani* AG 2-2IIIB at an equal distance from each other. The experimental Petri plates contained Rhizoctonia and 5 mL of the suspension of microbial community in sterilized tap water. In the control plates, *R. solani* AG 2-2IIIB and the microbial community were cultivated separately. The indicators BTB and resazurin sodium salt were used. The length of the grown mycelium was measured. The treatments were conducted in triplicate at 25 °C for 10 days.

2.7. Isolation of the Best Performing Antagonistic Strains against Rhizoctonia solani AG 2-2IIIB

The isolation of strains from microbial community was conducted via the investigation of its effect on *R. solani* AG 2-2IIIB in 150 mL flasks in peptone broth. After 7 days of cultivation, the aliquot (0.1 mL) of the culture liquid was transferred to nutrient agar and spread on the surface via the spatula. The grown microbial colonies were reisolated 3 times to obtain pure cultures.

2.8. Study of the Antagonistic Properties of the Isolated Strains against Rhizoctonia solani AG 2-2IIIB

The antagonistic properties of the isolated microbial strains were investigated via two methods. The first one provided the placement of the agar block with the pre-cultivated *R. solani* AG 2-2IIIB in the center of nutrient agar in the Petri plate, with the creation of the stripe of microbial culture in the distal part of the plate. Here, the indirect interaction between the fungus and the strain could be studied. The second method was conducted as follows. The strains were inoculated on nutrient agar at an equal distance from each other, creating a spot with a diameter of 2 cm. The agar blocks with the pre-cultivated *R. solani* AG 2-2IIIB were placed in the center of each spot. In this way, the direct interaction of the fungus and microorganisms could be observed. Sodium resazurin added to the medium was used as the indicator of metabolic activity.

2.9. Data Analysis

Data analysis was conducted using the statistical platform of Microsoft Excel. Graphs were constructed via OriginLab software, version 8.5.1. Mean values and standard deviations (SDs) were determined with a 95% confidence level. The values were presented as the mean $\pm$ SD.

3. Results

3.1. Thermodynamic Background for Control of Rhizoctonia solani AG 2-2IIIB

To develop the approach of effective control of the phytopathogenic fungus *Rhizoctonia solani* AG 2-2IIIB, a method of thermodynamic prediction was applied to consider the key conditions of the environment that affect its growth. The field of water stability in the coordinates of pH and Eh was studied to highlight the optimal conditions as well as those causing inhibition and death of the fungus. We applied classical Pourbaix diagrams adapted for biological systems [26,30]. In biology, microbiology, and biochemistry, standard potentials (E_o') are calculated for pH = 7.0 at a one-molar concentration of reacting components and a temperature of 25 °C [31] (Figure 1). Under these conditions, the zone of water stability is in the range of standard redox potentials from $E_o' = -414$ mV (lower limit) to $E_o' = +814$ mV (upper limit). Water has the properties of a binary redox buffer. At the values of redox potential $E_o' > +814$ mV, water oxygen (O^{2-}) acts as a reducing agent and is oxidized to molecular oxygen O_2:

$$2H_2O = O_2 + 4H^+ \text{ (reaction No. 1).}$$

Clearly, the redox potential of water returns to its original value, i.e., to $E_o' = +814$ mV. At the values of redox potential $E_o' < -414$ mV proton (H^+) serves as the oxidizer. If the redox potential of the solution is less than −414 mV, two protons ($2H^+$) will be reduced to H_2 and the potential will return to initial value $E_o' = -414$ mV:

$$2H^+ + 2e = H_2 \text{ (reaction No. 2).}$$

That is why the whole diversity of bioenergetic reactions are possible only within the zone of the thermodynamic stability of water, i.e., in the E_o' range from −414 to +814 mV.

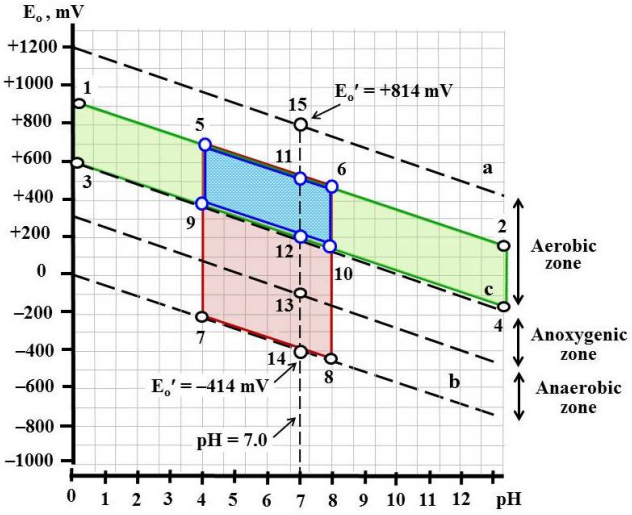

Figure 1. Thermodynamic calculations of zones compatible and incompatible with the life of *Rhizoctonia solani* AG 2-2IIIB: upper (a) and lower (b) limits of water stability in the E_o' range from +814 mV to −414 mV; c—separation of aerobic and anoxygenic zones. Upper limit of water stability is described by reaction $2H_2O = O_2 + 4H^+$ and equation $E_o = 1.228–0.0591 \times pH—0.0295 \times lgPH_2$, where PH_2 is partial pressure of hydrogen. Lower limit is described by reaction $2H^+ = 2e = H_2$ and equation $E_o = 0.000–0.0591 \times pH \times lgPH_2$.

Microorganisms efficiently use redox reactions in a wide range of water stability potentials to obtain energy. Thus, microorganisms use reaction No. 1 for both aerobic metabolism ($C_{organic} + 2O_2 + 4H^+ = CO_2 + 2H_2O$) and photosynthesis ($CO_2 + 2H_2O + h\eta = C_{organic} + 2O_2 + 4H^+$). Reaction No. 2 is used by microorganisms, for example, for hydrogen fermentation ($C_6H_{12}O_6 + 4H^+ = 2H_2 + CO_2 + CH_3COOH$) and methanogenesis ($4H_2 + CO_2 = CH_4 + 2H_2O$).

The application of thermodynamic prediction to suppress the growth of microorganisms, in fact, is the calculation of zones that are incompatible with the life of this organism depending on pH and Eh. To achieve the suppression or death of the investigated strain, it is necessary to create corresponding conditions using physicochemical factors, biological factors (microbiome, etc.) or combination of them. Therefore, we calculated the thermodynamic conditions taking into account the physiological characteristics of *Rhizoctonia solani* AG 2-2IIIB (Figure 1).

R. solani AG 2-2IIIB belongs to obligate aerobic microorganisms with optimal pH values varied between 4.0 and 8.0. The considerations of its metabolism in relation to aerobic conditions, expressed in the pH-Eh dependence, are as follows. The aerobic zone is in the pH range from 0.0 to 13.0 and covers the area of 600 mV in the range of E_o' from +200 mV (point No. 12) to +814 mV (point No. 15). However, taking into account the fact that air content is not 100% O_2 (i.e., is not 1 M) but only 21%, the value of the standard redox potential (E_o') will not be equal +814 mV, but only +500 mV. Thus, aerobic conditions in the expression of redox potential, are in the range of E_o' from +200 to +500 mV (points No. 12 and No. 11, respectively). Considering that *R. solani* AG 2-2IIIB has pH limitations, a suitable zone for its growth is limited by the points No. 5, 6, 9, 10. In other words, *R. solani* AG 2-2IIIB can grow only under aerobic conditions in the pH range varied between 4.0 and 8.0 and in the Eh range from +200 to +500 mV. Its existence outside of this specified zone is impossible. However, it should be taken into account that Rhizoctonia must be suppressed in soil ecosystems. Therefore, it is obvious that strong acidic, strong alkaline, and high potential conditions are not suitable, because they will lead to drastic changes in the soil microbiome. Consequently, we chose suppressive extreme factors that provide slightly alkaline, anoxygenic (absence of O_2), and anaerobic (absence of O_2 and Eh ≤ -200 mV) conditions. Such suppressive factors belong to physicochemical or biological categories. Physicochemical suppressive conditions can be created as follows. Slightly alkaline conditions can be created by phosphate buffers (mixture of Na_2HPO_4 and Na_3PO_4) and anoxygenic ones—due to the replacement of air with argon and anaerobic conditions—by the application of argon and low-potential reducing agents, such as Fe(II) citrate or Ti(III) citrate). Biological systems can simultaneously generate all three mentioned suppressive factors. The soil or compost microbiome is a good example. The natural microbiome is diversified and consists of aerobic, facultative, and obligate anaerobic microorganisms capable of fermenting a wide range of organic substrates. To see the effect, there is no need to identify the species content in microbial community. The conditions of the microbial community's growth are of most interest. Clearly, when soil is flooded (a high layer of water) and some organic substrates (carbohydrates, proteins, etc.) are introduced into the soil, the overall microbial metabolism will shift towards anoxygenic and then deep anaerobic low-potential conditions. Thus, aerobic microorganisms will create anoxygenic conditions due to the consumption of O_2, and then anaerobic microorganisms should create low-potential ones (Eh = $-300 \ldots -200$ mV) via the fermentation of organic compounds. During fermentation, due to the accumulation of alkaline end metabolites (S^{2-}, NH_3 etc.), an increase in pH is also expected. To verify the proposed approach, it is advisable, first of all, to test the effect of each physicochemical suppressive factor, and then their combined action on Rhizoctonia using a diversified microbiome. Finally, it is assumed that soil flooding and organic amendments are necessary and sufficient conditions for the strong suppression or death of *Rhizoctonia solani* AG 2-2IIIB.

Here, we highlighted the key conditions affecting the growth of *R. solani* AG 2-2IIIB. They are as follows: pH, Eh, and presence of O_2. According to thermodynamic calculations, control of *R. solani* AG 2-2IIIB growth can be achieved via the creation of the described conditions. In this regard, two directions of the investigation were conducted. The first one created the specific growth conditions via the application of abiotic factors, namely pH and Eh buffers, oxygen absence, etc. Another pathway was to create such conditions via the application of microbial communities in order to regulate the pH, Eh, and O_2 content of the medium caused by their growth.

3.2. Application of Abiotic Factors for Control of Rhizoctonia solani AG 2-2IIIB

3.2.1. Effect of pH on *Rhizoctonia solani* AG 2-2IIIB Growth

The response of *R. solani* AG 2-2IIIB depending on the pH of the culture medium was investigated. Peptone broth with yeast extract was used as the cultivation medium. The pH values (5.5; 7.0; 8.5) were maintained via the buffer solutions. The experiment resulted in the conclusion that alkaline conditions strongly suppressed the fungus growth (Figure 2). A visually detectable mycelium was observed only on the fourth day of cultivation. No changes were seen after 7 days of study. However, the alkaline conditions did not cause the death of the fungus. The recovery of mycelium growth was observed after the transfer of the agar block with mycelium onto nutrient agar on Petri plates. It evidenced that slightly alkaline conditions did not cause the devastation of *R. solani* AG 2-2IIIB.

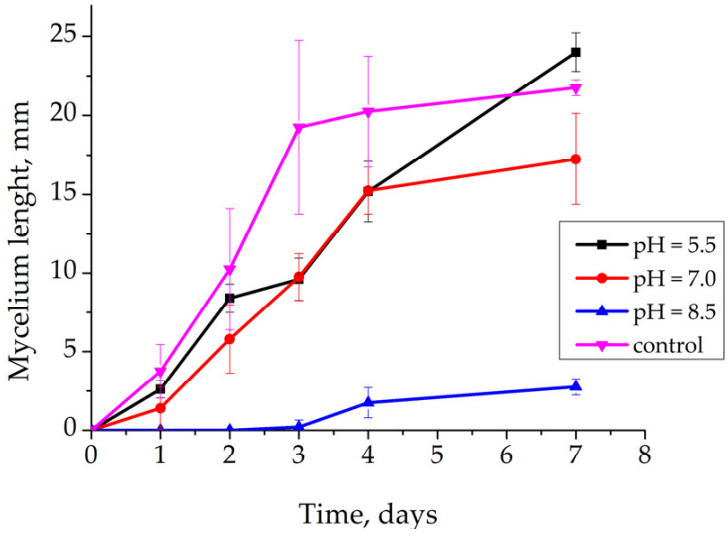

Figure 2. Effect of pH on *Rhizoctonia solani* AG 2-2IIIB growth: maintenance of pH = 5.5 (black line); maintenance of pH = 7.0 (red line); maintenance of pH = 8.5 (blue line); no pH maintenance (pink line).

Neutral and acidic conditions were suitable for the growth. Although the growth dynamics were similar, the increase in mycelium biomass was more intense at pH = 5.5. The lower growth efficiency compared to the control without pH regulation may have been caused by the higher ionic power of the solution with buffers. Thus, the thermodynamic calculations of the effect of pH on control of *R. solani* AG 2-2IIIB were confirmed. The conclusion that unfavorable pH conditions cause only growth inhibition without the death of mycelium was the key point of the study.

3.2.2. Effect of Eh and Presence of O_2 on the Growth of *Rhizoctonia solani* AG 2-2IIIB

Aerobic conditions were shown to provide the effective growth of *R. solani* AG 2-2IIIB. Under such conditions, the growth of mycelium was active, resulting in a length of 29.3 ± 1.2 mm after 6 days of cultivation (Figure 3). The pH value ranged from 6.8 to 7.2. Eh slightly decreased from +255 mV to +238 mV. Thus, there was no inhibition of Rhizoctonia growth.

The investigation of growth showed its inhibition under anoxygenic and anaerobic conditions. Very little mycelium growth under anoxygenic conditions was observed after only 6 days (1.7 ± 1.5 mm). The pH was similar under anoxygenic conditions and ranged from 6.7 to 7.1, but Eh reduced from +126 mV to +103 mV. Such conditions inhibited growth. Complete absence of mycelium growth came about under anaerobic conditions. In this case, the pH was 6.8–7.1. Eh remained at −200 ± 10 mV providing unfavorable conditions for *R. solani* AG 2-2IIIB. Thus, the presence of oxygen is necessary for *R. solani* AG 2-2IIIB growth. It provides high redox potential of the medium. Together, these factors maintained

the metabolic activity of the fungus. The absence of oxygen and low redox potential did not support the growth of the fungus.

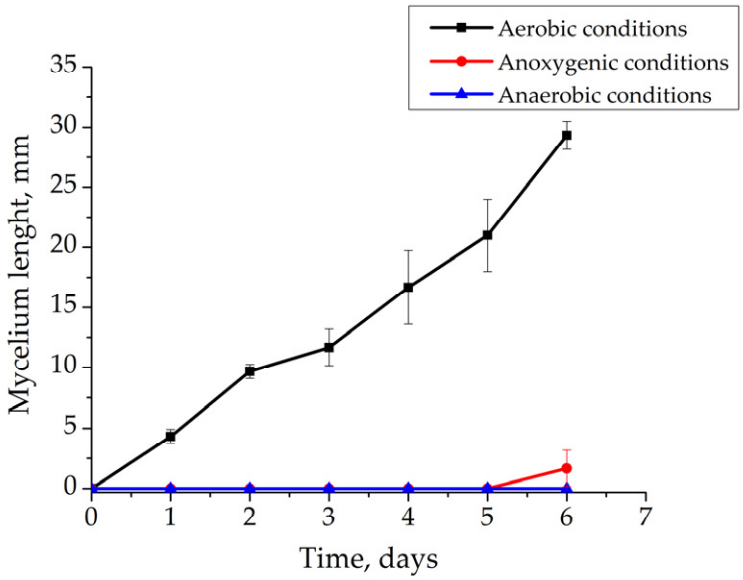

Figure 3. Effect of Eh and presence of O_2 on *Rhizoctonia solani* AG 2-2IIIB growth: aerobic conditions (black line); anoxygenic conditions (red line); anaerobic conditions (blue line).

3.2.3. Recovery of *Rhizoctonia solani* AG 2-2IIIB Growth after Unfavorable Anoxygenic and Anaerobic Cultivation Conditions

Cultivation conditions affect the efficiency of growth. Aerobic conditions were shown to support the growth of *R. solani* AG 2-2IIIB. On the other hand, slight growth was observed in the case of anoxygenic conditions, and its absence was visualized in anaerobic ones. However, to test whether such conditions cause growth inhibition or death of *R. solani* AG 2-2IIIB, the inoculated agar blocks were transferred from anoxygenic and anaerobic conditions to the fresh peptone broth, and cultivation took place in aerobic conditions. Therefore, it was necessary to evaluate it after unfavorable growth conditions.

The following results were obtained. The recovery of the active fungus growth was predictable in aerobic conditions since the slight growth of mycelium was observed under anoxygenic ones. The length of mycelium was 6.7 $\pm$ 0.3 mm after one day of cultivation. One week of cultivation resulted in obtaining mycelium of 38.3 $\pm$ 2.1 mm in length. Thus, anoxygenic conditions can be concluded to cause only the suppression, but not the death of *R. solani* AG 2-2IIIB. After the resumption of optimal conditions, growth was recovered. However, the death of mycelium was evidenced in the case of pre-cultivation under anaerobic conditions. The recovery of growth was absent. Thus, creation of strict anaerobic low-potential conditions provided the death of fungal mycelium. Such an approach can be used to control its growth. Since the creation of such conditions via chemical and physical methods is complicated, microbiological methods are considered to be more promising.

3.3. Application of Biotic Factors for Control of Rhizoctonia solani AG 2-2IIIB

Alkaline pH, absence of O_2, and low Eh created via abiotic factors are the key conditions shown to suppress *R. solani* AG 2-2IIIB. The application of microbial communities possessing a wide range of metabolic activity is also suitable as an effective tool to create such unfavorable conditions. Moreover, suppression can be enhanced due to the antagonistic properties of microbial exometabolites and even bacterial consumption of the dead mycelium as the nutrient source. In this regard, the application of microbial communities can provide effective control and irreversible suppression of *R. solani* AG 2-2IIIB. The co-cultivation of microbial communities from compost with *R. solani* AG 2-2IIIB revealed the following results (Figure 4). The control growth of *R. solani* did not significantly change

pH (6.9–7.2) and Eh (+223 . . . +257 mV). The length of mycelium reached 31 ± 0.5 mm after 7 days. However, the growth of microbial community affected the medium pH and Eh. No significant difference was observed between the growth of microbial community and its cultivation together with *R. solani* AG 2-2IIIB. The pH decreased from 7.2 to 6.1, and Eh dropped from +251 to −125 mV during 7 days.

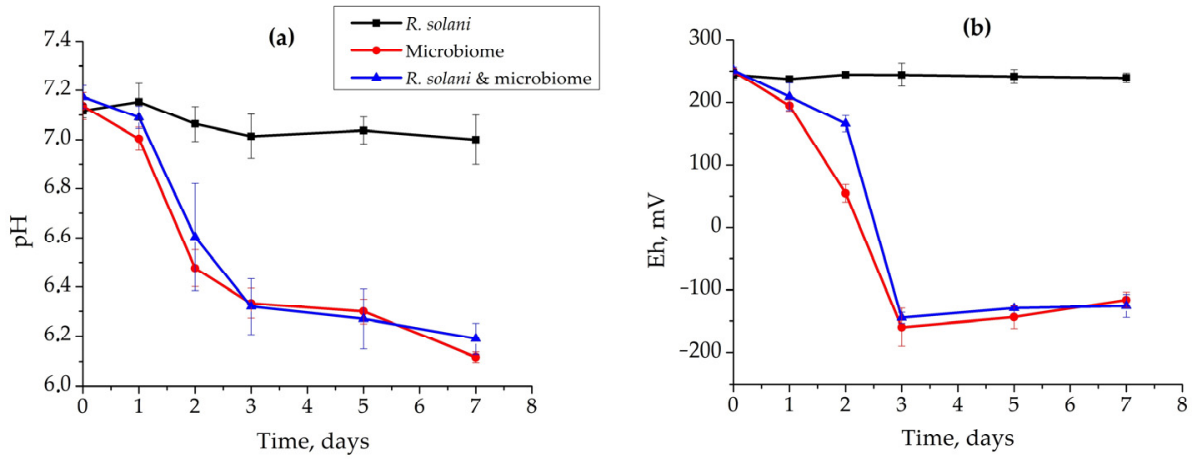

Figure 4. Dynamics of pH (**a**) and Eh (**b**) during the growth of *R. solani* AG 2-2IIIB and microbial community: separate cultivation of *R. solani* AG 2-2IIIB (black line); separate cultivation of microbiome (red line); co-cultivation of *R. solani* AG 2-2IIIB and microbiome (blue line).

Under these conditions, *R. solani* AG 2-2IIIB growth in the presence of microbial community was not observed. Since pH was favorable for *R. solani* AG 2-2IIIB, it was suggested that low redox potential and oxygen absence were the key conditions suppressing it. Moreover, the following transfer of the agar block, used as the inoculum of Rhizoctonia to peptone agar, confirmed complete death of mycelium during the cultivation with microbial community. Even one day of co-cultivation was enough to completely suppress *R. solani* AG 2-2IIIB. Therefore, a microbial community can be effectively used to control *R. solani* AG 2-2IIIB. This happens due to the consumption of oxygen, the creation of low redox potential (−150 mV and lower), and possibly production of antagonistic exometabolites during the growth of microbial community.

Similar patterns of *R. solani* AG 2-2IIIB growth interacting with the microbial community were confirmed during the cultivation on solid substrate (glass balls). Aerobic conditions allowed for the growth of mycelium, which reached 23 ± 2.1 mm length after 7 days. Anoxygenic and anaerobic conditions inhibited *R. solani* AG 2-2IIIB. The microbial community actively grew in all variants (aerobic, anoxygenic, and anaerobic). Moreover, after 1 day of cultivation, even in aerobic conditions, it reduced redox potential to −200 mV and lower, which was indicated by the discoloration of sodium rezasurin and BTB. Under such conditions *R. solani* AG 2-2IIIB growth in the presence of the microbial community was absent.

The following study of the opportunity to recover the growth of *R. solani* AG 2-2IIIB after 7 days of cultivation via its transfer to peptone agar was also confirmed. The mycelium started to grow on the second day after anoxygenic conditions. After anaerobic conditions, the growth recovery was absent. However, no growth of *R. solani* AG 2-2IIIB was observed after its joint cultivation with a microbial community under aerobic, anoxygenic, and anaerobic conditions. Therefore, microbial community plays a major role in not only inhibition, but also in the death of *R. solani* AG 2-2IIIB.

The unconditional role of the microbial community in the death of *R. solani* mycelium was also confirmed by the cultivation on its natural substrate (sugar beets) (Figure 5).

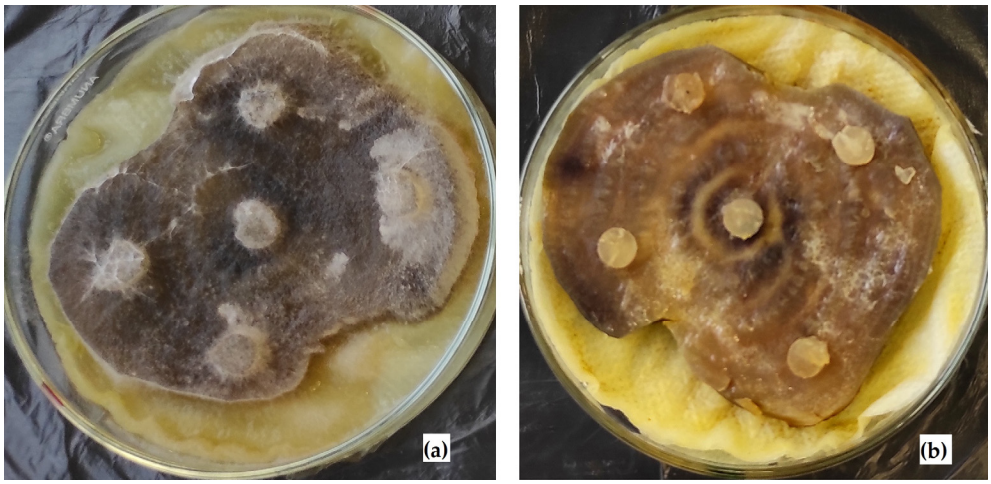

Figure 5. Cultivation of *R. solani* AG 2-2IIIB on sugar beet slices: growth without microbial community (**a**); absence of growth with microbial community (**b**).

The sole cultivation of *R. solani* AG 2-2IIIB did not cause growth suppression. The green color of BTB indicated aerobic conditions and pH equal to 6.8–7.2. Under these conditions, the mycelium length reached 17.1 $\pm$ 1.3 mm. According to the yellow color of BTB, the growth of the microbial community provided acidification to pH = 5.0–6.0 during the first day of cultivation. The following BTB discoloration showed the creation of low redox potential (Eh ≤ -200 mV) in the liquid phase. Under such conditions, the absence of *R. solani* AG 2-2IIIB growth in the presence of the microbial community was predictable. The negative effect of the microbiome was so strong that, even after 10 days of cultivation, there was no growth of Rhizoctonia on the surface of sugar beet slices. The following transfer of agar blocks to peptone agar confirmed the *R. solani* AG 2-2IIIB growth absence, indicating the key role of the microbial community in the death of the fungus culture.

3.4. Antagonism of the Isolated Microbial Strains against Rhizoctonia solani AG 2-2IIIB

Since the microbial community was shown to be the best performing tool to control *Rhizoctonia solani* AG 2-2IIIB, the study of the antagonistic properties of its components was of great interest. For this, seven dominant strains were isolated. They showed different levels of inhibition of the growth of *Rhizoctonia solani* AG 2-2IIIB after 4 days of cultivation (Figure 6).

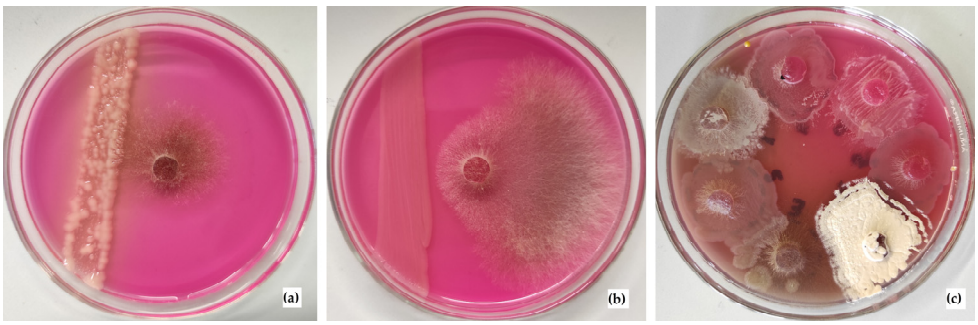

Figure 6. Antagonistic activity of the isolated strains against *R. solani* AG 2-2IIIB: no growth suppression by the strain A.3 (**a**), growth suppression by the strain A.5 (**b**), and direct interaction of microorganisms and fungal mycelium — fungus and individual microorganism were inoculated at the same point (**c**).

Two methods of observing the antagonism showed similar results (Table 2). Both of them revealed that strains A.4, A.5, and A.7 most effectively suppressed mycelium growth. The first method, where *R. solani* AG 2-2IIIB was placed in the center of the nutrient agar

in the Petri plate, was cultivated with the stripe of microbial culture. It revealed that some microbial metabolites of A.4 and A.5 strains could be responsible for the growth suppression. The strain A.7 probably synthesizes fewer inhibitory metabolites. However, direct contact also caused the suppression of *R. solani* AG 2-2IIIB. It is noteworthy that the A.4 strain turned out to be very active and not only inhibited the growth of mycelium, but also completely displaced the fungus (Figure 6c—the white colony, which completely overgrew *R. solani* inoculum).

Table 2. Efficiency of suppression of *Rhizoctonia solani* AG 2-2IIIB by microbial strains.

Strains	Indirect Interaction	Direct Interaction
A.1	– [1]	–
A.2	+ [2]	+
A.3	–	–
A.4	+++ [4]	+++
A.5	+++	++
A.6	++ [3]	++
A.7	++	+++

[1] "–" no suppression; [2] "+" weak suppression; [3] "++" middle suppression; [4] "+++" strong suppression.

No suppression of *R. solani* AG 2-2IIIB was shown by the strains A.1 and A.3 (Figure 6a). The strains A.2 and A.6 showed weak and middling effects, respectively. Though mycelium started to grow, direct contact with the microbial biomass suppressed the subsequent growth of the fungus. Thus, the investigation of the effect of the separate microbial strains on the growth of *R. solani* AG 2-2IIIB showed significant differences in the efficiency of the fungus suppression, as well as different pathways via direct contact or the synthesis of inhibitory metabolites. Further study of the best performing strains and the patterns of *R. solani* AG 2-2IIIB control is of great importance. It was assumed that the integration of the antagonistic properties of individual strains in the microbial community provided not only suppression of *R. solani* AG 2-2IIIB, but the death of the mycelium. Therefore, the further optimization of the regulation of microbial metabolism can become an effective tool to control the hazardous sugar beet pathogen *R. solani* AG 2-2IIIB in order to increase the yield of this valuable food plant, as well as to preserve it under storage.

4. Discussion

Sugar beet diseases caused by Rhizoctonia have been known for more than a century. However, despite many studies, the economic losses of the crop yield have reached up to 2% in the USA [32]. The loss of the production of sugar beets caused by *Rhizoctonia solani* AG 2-2 can reach, in some cases, more than 25% in the United States [33].

The conditions of *R. solani* growth have been studied since it was reported as the most widespread and hazardous crop pathogen. Attention to growth conditions has been primarily associated with the study of the pathways to suppress pathogens and prevent plant diseases [34]. Different approaches have been used to control this phytopathogenic fungus to protect sugar beets and increase its yield. The strategies were based on the development of resistant varieties of beets [33], application of fungicides [13], and treatment with bacterial strains [1] or some organic compounds [5]. However, these possess some drawbacks. For example, Rhizoctonia-resistant varieties lose 10–15% of their productivity [33], or they are not sufficiently adapted to the climate [6]. The application of chemicals causes the development of resistance to fungicides and leads to hazardous contamination of the environment [12].

Herein, a comprehensive approach based on thermodynamic calculations was suggested to evaluate a general view of the conditions that promote and suppress *R. solani*. This background was used to create conditions to control phytopathogenic *R. solani*. The approach suggested in the study to control *R. solani* AG 2-2IIIB via abiotic and biotic factors corresponds to the data in literature. However, the available data show that the investiga-

tion of growth conditions was conducted sporadically, without the use of a generalized background. The generalized patterns affecting *R. solani* AG 2-2IIIB were shown. Based on thermodynamic calculations, it was found that unfavorable growth conditions can be created via both pathways. The first one is the application of abiotic factors (pH and redox buffers, presence or absence of O_2). The second pathway is to use biotic factors such as microbial communities to create the appropriate conditions not only to inhibit *R. solani* AG 2-2IIIB growth, but also to cause the death of mycelium.

The value of pH is one of the important factors affecting the activity of Rhizoctonia and pathogenicity [35,36]. A wide variation of pH (6.0–8.0) was shown for optimum growth and sclerotia production [36]. Other reported data revealed the optimum growth of fungus in the pH range 5.5–7.0 [35]. The absence of oxygen, together with low redox potential, is another important condition to suppress *R. solani*. Anaerobic soil disinfestation implemented via the addition of easily degradable organics with water was shown to be effective in the control of a wide range of plant pathogens, including *R. solani* [37].

The application of microorganisms, conducted in our research, was shown to be an effective and up-to-date approach to control *R. solani* AG 2-2IIIB. It is one of the most promising ways to provide the suppression of the fungus as well as to prevent environmental pollution. Moreover, some microbial strains such as *Bacillus* sp. [33] and yeasts (*Candida valida*, *Rhodotorula glutinis*, and *Trichosporon asahii*) [38] described in literature were shown not only to suppress *Rhizoctonia solani*, but also to increase the yield of sugar beets. *Bacillus amyloliquefaciens* SB14 strain was shown to decrease diseases of sugar beet caused by *R. solani* AG-4 and *R. solani* AG 2-2 [9,39]. Other bacterial strains such as *Serratia plymuthica* 3Re4-18, *Pseudomonas fluorescens* L13-6-12, and *Pseudomonas trivialis* RE*1-1-14, and fungal species (*Trichoderma gamsii* AT1-2-4, *Trichoderma velutinum* G1/8) were also shown to be promising to control *Rhizoctonia solani* [40]. The antagonistic mechanisms can be different: competition for substrate and space, production of inhibitory metabolites, cell wall degrading enzymes, etc. [38,41].

The conducted research confirmed that microorganisms were able to inhibit or even kill *R. solani* AG 2-2IIIB. The study showed that the creation of anaerobic conditions (absence of air and low redox potential) by the microbial community of compost was the most effective to control *R. solani* AG 2-2IIIB. The investigation of the antagonistic properties of the isolated microbial strains was also shown to affect the fungus. The collected data confirmed that the application of microbial communities was the most promising approach. It provided the integration of the suppressive environmental conditions created by microorganisms with the antagonistic properties of the strains. These experimental data correspond to those described in literature, such as the synergistic effect of the combination of several strains such as *Gliocladium virens* and *Burkholderia cepacia* [38,42]. Another study also confirmed the increase in the antagonistic effect of combining several microbial strains. Moreover, microorganisms could contribute to plant health via the stimulation of root growth, germination rates, biomass yield, resistance to stress, etc. [40].

Although the approach of *Rhizoctonia solani* control via the application of microorganisms is promising, there are still a lot of gaps in the knowledge of the effective regulation of this process. The conducted research contributed to the understanding of the patterns of the application of microbial communities instead of single strains. Microbial communities were shown to be a promising tool, since they possess integrated mechanisms against *R. solani* AG 2-2IIIB, providing the simultaneously acting different pathways to protect a valuable food source: sugar beets.

5. Conclusions

The application of thermodynamic prediction provided the background for studying the conditions for *Rhizoctonia solani* AG 2-2IIIB control. Obtained data showed the following. The abiotic factors provided via the chemical buffers or removal of oxygen mainly caused the inhibition of *R. solani*. The values of $5.5 \leq \text{pH} \leq 8.5$, $\text{Eh} \leq -200$ mV, and oxygen absence inhibited its growth. The maintenance of these factors via microbial communities enhanced

the suppressive effect, which provided the death of mycelium. The study of microbial strains revealed the inhibitory effect of microbial metabolites and provided more effective control of *Rhizoctonia solani* AG 2-2IIIB. Agreeing with the thermodynamic prediction, the main negative factors inhibiting *R. solani* AG 2-2IIIB growth are anaerobic conditions and a diversified microbial community. The simultaneous combination of these factors leads to the suppression of metabolism and death of *R. solani* AG 2-2IIIB. The proposed approach is promising for further development, since it provides environmentally friendly control of phytopathogenic fungi to prevent the disease of sugar beets: a valuable food crop.

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