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Adding value to leachate from grape pomace composting through its use as a culture medium and component of biofertilizer

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ABSTRACT
Industrial activities produce substantial amounts of waste and by-products, posing significant environmental risks. Composting emerges as a vital strategy for organic waste decomposition and enhancement. However, composting generates leachate, which can pollute soil and water. Thus, our study aimed to assess the potential of grape pomace composting leachate as a component of a culture medium for *Bacillus subtilis* F62, a plant growth-promoting bacterium, and as a fertilizer for strawberry plants. Initially, we evaluated the physicochemical properties of the leachate and its impact on bacterial growth at various dilutions. Subsequently, we tested the leachate alone and in combination with the bacterium on micropropagated strawberry plants. Results revealed that despite low carbon and nitrogen concentrations, the leachate facilitated bacterial growth similar to LB medium at 10% and 25% dilutions. Furthermore, both leachate application methods, alone and combined with the bacterium, yielded vigorous strawberry plants, particularly with the biofertilizer containing *B. subtilis* F62. In conclusion, leachate from grape pomace composting shows promise for bacterial growth and fertilizer development to enhance strawberry plant growth and development.

KEYWORDS*:* *Bacillus subtilis* F62. Growth promotion. Organic fertilizer. Strawberry plants.

1 INTRODUCTION

Various forestry, agricultural, and agro-industrial activities yield substantial waste and by-products (FERJANI et al., 2019). Within this scope, the wine industry contributes significantly to waste generation along the grape processing chain, discarding more than 20% of its total weight as pulp, stems, and other by-products (MUHLACK; POTUMARTHI; JEFFERY, 2018; ŠUNJKA; MECHORA, 2022). Despite its nutrient-rich composition, this waste cannot serve directly as fertilizer due to inherent physicochemical characteristics, such as high acidity and electrical conductivity (ALCARAZ et al., 2018).

Traditionally, these residues have been regarded as liabilities, with their improper disposal resulting in severe environmental impacts, including biodiversity loss, soil and water pollution, and the emission of greenhouse gases (FERJANI et al., 2019; SANTIAGO BADILLO et al., 2021; AHMAD et al., 2020). However, when subjected to appropriate treatments, wastes possess untapped potential a source of bioactive compounds and for the production of biopesticides, biofertilizers, biochars, and bioenergy (ROY et al., 2018; SEBASTIÁN-NICOLÁS et al., 2020; AJENG et al., 2020; DA SILVA et al., 2022; ŠUNJKA; MECHORA, 2022).

One strategy employed to reduce the volume and weight of these residues is composting (GUTIÉRREZ-MICELI et al., 2017). This process involves the decomposition of organic waste by mesophilic and thermophilic microorganisms in the presence of oxygen, resulting in a stable compound suitable for agricultural fertilizer (SAYARA et al., 2020). However, composting leads to the release of a leachate or percolate, with high biochemical oxygen demand and potential toxic compounds, which may require special treatment before final disposal (HE et al., 2015; MOKHTARANI; YASROBI; GANJIDOUST, 2015; ROY et al., 2018). Although these treatments are costly, they encompass oxidative, physicochemical, and biological processes, with the potential for cost reduction through percolate conversion into high-value-added products (ROY et al., 2018).

Among the potential uses of composting leachate, its role in organic fertilizer production and as a nutrient source for bacterial growth is noteworthy (GUNJAL; KAPADNIS; PAWAR, 2018; MORA et al., 2022). Unlike conventional culture media for microbial cultivation, which are resource-intensive and generate waste, leachate utilization offers a sustainable alternative, minimizing environmental impacts and promoting more sustainable practices while enhancing the value of these by-products (YÁNEZ-MENDIZÁBAL et al., 2012; BALASUBRAMANIAN; TYAGI, 2017).

Bacillus subtilis, a Gram-positive and spore-forming bacterium (RABBEE et al., 2019), has garnered attention for its pivotal role in enhancing plant growth, inducing resistance mechanisms, and controlling diseases (MENENDEZ; GARCIA-FRAILE, 2017; MORALES-CEDEÑO et al., 2021). Its growth-promoting capacity is attributed to the establishment of positive interactions with plants, production of secondary metabolites and phytohormones, improvement in nutrient absorption, and protection against pathogens and environmental stresses (SHARMA et al., 2019; LEGEIN et al., 2020; MORALES-CEDEÑO et al., 2021). Additionally, *B. subtilis* exhibits a remarkable ability to colonize plant tissues through biofilm formation, further contributing to its persistence and effectiveness as a growth-promoting agent (DIMKIC et al., 2022).

2 OBJECTIVES

This study aimed to valorize composting leachate by utilizing it as a culture medium to cultivate the plant growth-promoting bacterium *B. subtilis* F62, and to evaluate its potential as a biofertilizer for enhancing the growth of strawberry plants under greenhouse conditions.

3 METHODOLOGY

3.1 Plant growth-promoting rhizobacterium

The bacterium *B. subtilis* F62 was obtained from the collection of the Laboratory of Biological Control of Plant Diseases, at the University of Caxias do Sul (UCS), located in Caxias do Sul, Rio Grande do Sul State, Brazil. Molecular identification was conducted through the sequencing of the *16S rDNA* gene, resulting in a complete match with an existing sequence in the GenBank database for *B. subtilis*, accession number NR 102783.2.

3.2 Composting leachate

The stabilized leachate, free from toxic compounds, was provided by Beifiur Ltda., located in Garibaldi, Rio Grande do Sul State, Brazil. This liquid effluent was derived from grape pomace composting, conducted in a covered structure over a three-year period, with leachate recirculation. After collection from the stabilization pond, the leachate was placed in 250 mL flasks and autoclaved at 121 °C, 1.5 bar for 1 hour, to inactivate present microorganisms.

Subsequently, physicochemical analyses were performed on samples of the composting leachate and Luria-Bertani (LB) liquid medium (Lenoxx®). The commercial medium was dissolved in sterile water and prepared according to the manufacturer's instructions. The concentrations of C and N were determined using a thermal conductivity/infrared detector and converted into percentage contents of the respective elements. Additionally, the C/N ratio, pH, and electrical conductivity (mS cm^{-1}) were evaluated. The analyses were performed in triplicate.

3.3 Bacterial growth in leachate-containing medium

The pre-inoculum was prepared by adding a single bacterial colony to a flask containing 10 mL of LB liquid medium (1% peptone, 0.5% yeast extract, and 0.5% sodium chloride), with the pH adjusted to 7.0. Thereafter, the pre-inoculum was placed in an orbital shaker at 130 rpm and 28 °C. After 24 hours, this culture was transferred to a flask containing 90 mL of liquid medium. This medium was prepared using compost leachate at various dilutions (10%, 25%, 50%, 75%, and 100%). These concentrations were achieved by diluting the leachate in sterile water, followed by pH adjustment to 7.0. The control consisted of LB liquid medium.

Following this, bacterial cultures were incubated in an orbital shaker under the same conditions mentioned above for 24 hours. Bacterial counting was performed by serial dilutions on LB medium plates, supplemented with 1.5% agar. After incubation at 37 °C for 24 hours, the number of colony-forming units per milliliter was determined and expressed as CFU mL^{-1}. The assay was performed in triplicate, using a completely randomized design.

3.4 Plant material

Strawberry runners cv. Albion (*Fragaria × ananassa* Dusch.) were collected from one-year-old plants and individualized into nodal segments, each containing a bud. These explants underwent surface disinfection in a laminar air-flow cabinet. Initially, the nodal segments were treated with a 70% ethanol solution (v/v) for 1 minute, followed by immersion in a 1% NaOCl solution (v/v) with 2 drops of Tween 20 for 20 minutes. Subsequently, they were rinsed three times with sterile water and inoculated into flasks containing 15 mL of MS medium (Murashige and Skoog), supplemented with 6 g L^{-1} agar, 30 g L^{-1} sucrose, and 0.5 mg L^{-1} 6-benzylaminopurine (BAP). The pH of the culture medium was adjusted to 5.8. Explants were transferred to fresh culture medium at 30-day intervals for a period of 90 days.

Rooting was then induced in MS medium supplemented with 6 g L^{-1} agar, 15 g L^{-1} sucrose, and 1 mg L^{-1} indole-3-butyric acid (IBA). Incubation was carried out in a growth chamber at a temperature of 25 ± 2 °C, with a photoperiod of 16 hours of light provided by 20 W LED lamps, and a light intensity of 72 µmol m^{-2} s^{-1}. Plants with four leaves and a well-developed root system were transferred from the culture flasks to plastic cups containing 200 mL of autoclaved substrate. For the experiment, a commercial substrate consisting of a mixture of peat and vermiculite in a ratio of 9:1 was used. Acclimatization was carried out at 25 ± 2 °C, a relative humidity of 80%, and a photoperiod of 16 hours of light with a light intensity of 350 µmol m^{-2} s^{-1}.

3.5 Growth promotion assay

The effect of the plant growth-promoting bacterium *B. subtilis* F62 was assessed in an experiment with strawberry plants obtained through micropropagation. After a 30-days acclimatization period, the strawberry plants were transplanted into 1 L pots filled with the same substrate described in Section 3.4. At this stage, the number of leaves and the height of each plant were measured to assess the variation in these responses throughout the trial.

The plants underwent to the following treatments: control (sterile water), leachate (applied at concentrations of 10% and 25%), leachate + bac (10% leachate broth containing 6.3 × 10^7 CFU mL^{-1} of *B. subtilis* F62). The strawberry plants were maintained in a greenhouse at a temperature of 23 ± 2 °C for 60 days, with daily irrigation at approximately 50% of the maximum water-holding capacity. After 60 days, the plants were removed from the substrate and washed. Subsequently, the variation in the number of leaves, variation in plant height, root length, plant dry weight, and root dry weight were determined. The dry weight was determined after drying the plant material to a constant weight at 60 °C. The experiment followed a completely randomized design, with three replicates of 20 plants per treatment. The assay was repeated twice.

3.6 Statistical analysis

The normality of the data was assessed using the Shapiro-Wilk test, and the homogeneity of variances was evaluated using Levene's test. Parametric data underwent one-way ANOVA, followed by Tukey’s test. Non-parametric data were evaluated using the Kruskal-Wallis test and compared using the Dunn-Bonferroni test. Statistical significance was set at $p < 0.05$. All analyses were carried out using SPSS software version 22 for Windows.

4 RESULTS AND DISCUSSION

4.1 Composting leachate

The results of the physicochemical analysis of the leachate and LB medium Lennox® are presented in Table 1. LB medium exhibited a higher percentage of C and N compared to the composting leachate, whereas the C/N ratio was similar in both products.

Table 1 – Physicochemical characteristics of Luria-Bertani (LB) medium and leachate from grape pomace composting

Characteristics	LB medium Lennox®	Composting leachate
C (%)	0.622	0.340
N (%)	0.305	0.165
C/N	2.04	2.06
pH	7.00	9.34
Conductivity (mS cm^{-1})	3.16	3.02

Source: Author, 2024.

4.2 Bacterial growth in leachate-containing medium

Following the assessment of physicochemical characteristics of the composting leachate, the effect of various dilutions of this effluent on the growth of *B. subtilis* F62 was evaluated. Results demonstrated that media containing leachate at concentrations of 10% and

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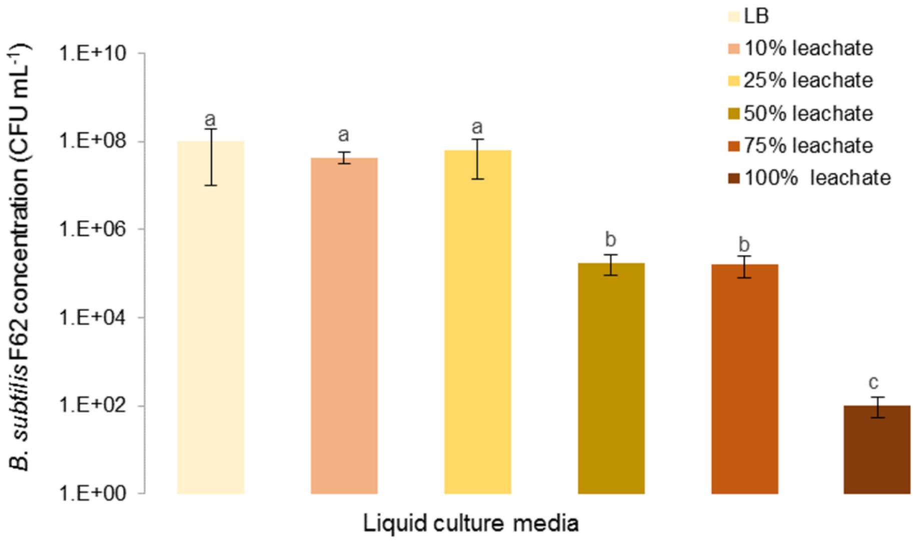
25% promoted bacterial growth comparable to the control (LB medium), with counts ranging from 4.3×10^7 to 6.3×10^7 CFU mL^{-1}. Conversely, leachate concentrations exceeding 50% exhibited an inhibitory effect on bacterial growth compared to the control (Figure 1).

Figure 1 - Effect of various dilutions of leachate in water as a culture medium for *Bacillus subtilis* F62. Different letters indicate statistically significant difference according ANOVA, followed by Tukey's test (p < 0.05)



Source: Author, 2024.

Traditionally, culture media used for bacterial cultivation are considered complex, lacking a well-defined chemical composition (TEIXIDÓ; USALL; TORRES, 2022). For instance, LB medium comprises carbon and nitrogen sources, including tryptone and yeast extract. Moreover, these media present disadvantages such as high cost, lack of standardization, and low result reproducibility (ELISASHVILI; KACHLISHVILI; CHIKINDAS, 2019; TEIXIDÓ; USALL; TORRES, 2022). Hence, the development of media utilizing industrial waste or lignocellulosic substrates offers an alternative for microbial cultivation (ELISASHVILI; KACHLISHVILI; CHIKINDAS, 2019). Although these alternative media also possess a complex composition, they have low production costs and contribute to waste recycling.

In this study, the utilization of leachate from grape pomace composting enabled the growth of *B. subtilis* F62 to a level similar to that observed in LB medium, despite differences in their physicochemical composition. It was noted that leachate exhibited lower concentrations of carbon and nitrogen and require pH adjustment to facilitate bacterial development. According to Chen et al. (2019), the low nutrient concentration in the leachate is attributed to the composting process and the microbial-driven mineralization of organic compounds. Furthermore, the chemical composition of the leachate is also influenced by the characteristics of the composted biomass, composting technology, and climatic conditions throughout this process (ROY et al., 2018). Consequently, the accumulation of compounds recalcitrant to microbial decomposition, such as humic and fulvic acids, may hinder bacterial growth in subsequent applications (MULLANE et al., 2015).

In addition, the use of undiluted leachate as a culture medium resulted in a low rate of bacterial proliferation, due to the presence of inhibitory substances. However, diluting the leachate in sterile water facilitated bacterial multiplication, thereby minimizing the harmful effect of these compounds. Similarly, Santiago Badillo et al. (2021) observed reduced growth of *Bacillus subtilis* when using undiluted leachate. In this regard, Sanadi et al. (2019) emphasize the importance of diluting composting effluents to reduce the concentration and phytotoxicity of heavy metals, ammonia, and low molecular weight organic compounds.

Nevertheless, leachate dilution also significantly decreases the concentration of essential macronutrients, such as carbohydrates and proteins. This dilution may have affected the growth rate of *B. subtilis* F62, preventing the bacterial population from reaching concentrations higher than those observed in LB medium. Therefore, it may be beneficial to combine leachate with other industrial residues containing high nutrient concentrations to mitigate the impact of dilution. In this context, Santiago Badillo et al. (2021) evaluated the addition of co-substrates like whey permeate and yeast from beer fermentation to composting leachate. This supplementation led to increased rates of multiplication and sporulation of *Bacillus megaterium* and *B. subtilis*.

The initial autoclaving step served to eliminate the naturally occurring microbial community in the leachate. This ensured the inactivation of any undesirable microorganisms or those potentially pathogenic, enhancing the proliferation of *B. subtilis* F62. As noted by Mora et al. (2022), microbial populations in leachate are often highly diverse, encompassing both beneficial and potentially harmful microorganisms. Therefore, comprehending the microbial communities in leachates or managing them is crucial to mitigate any adverse effects on plant crops and human health (BORTHONG et al., 2018).

4.3 Growth promotion assay

The effect of compost leachate at concentrations of 10% and 25%, as well as the presence of the bacterium *B. subtilis* F62 cultured and stored in this effluent, was evaluated in the growth of strawberry plants (Table 2). The results showed that applying leachate at concentrations of 10% and 25% resulted in increased plant and the root dry weight compared to the control. However, diluted leachate at 10% led to a higher number of leaves in strawberry plants compared to the control. Regarding plant height, no significant difference was observed among the tested treatments (Table 2).

Conversely, utilizing compost leachate at 10% containing the growth-promoting bacterium resulted in a statistically significant increase in the number of leaves, root length, plant dry weight, and root dry weight compared to the control (Table 2 and Figure 2).

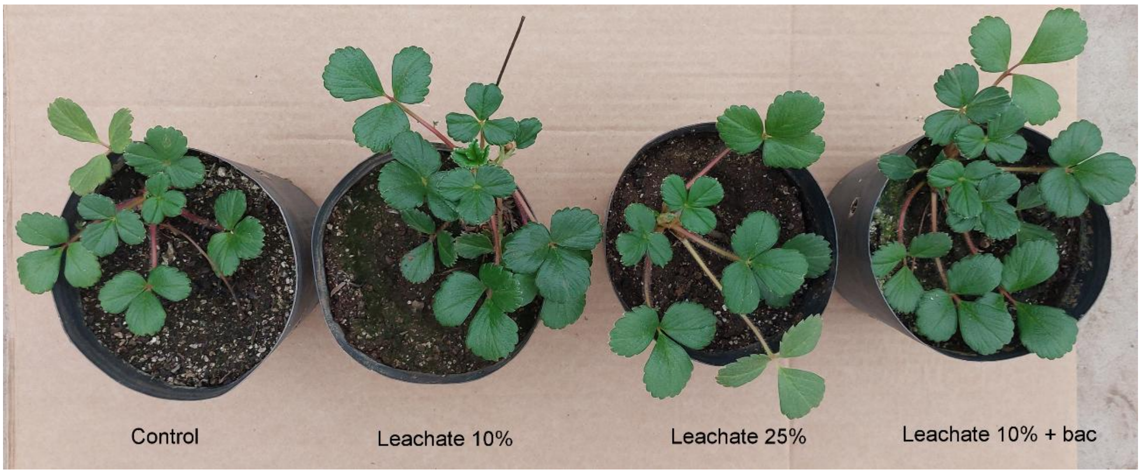
Table 2 - Growth responses in strawberry plants subjected to the following treatments: control (sterile water), leachate (at concentrations of 10% and 25%), and leachate + bac (culture broth with 10% leachate and 6.3×10^7 CFU mL^{-1} of *Bacillus subtilis* F62). Different letters indicate statistically significant differences according to the Kruskal-Wallis test, followed by the Dunn-Bonferroni test ($p < 0.05$)

Treatments	Δ Number of leaves	Δ Plant height (cm)	Root length (cm)	Plant dry weight (g)	Root dry weight (g)
Control	2.0 ± 0.4 c	4.6 ± 1.2	2.8 ± 0.8 b	0.82 ± 0.7 c	0.75 ± 0.7 b
Leachate 10%	3.2 ± 0.6 b	5.2 ± 0.5	3.4 ± 0.9 ab	0.98 ± 0.5 b	0.94 ± 1.1 a
Leachate 25%	2.8 ± 0.7 bc	5.4 ± 0.9	4.1 ± 0.3 ab	0.95 ± 0.4 b	0.99 ± 0.6 a
Leachate 10% + bac	5.0 ± 0.5 a	4.9 ± 0.7	4.6 ± 0.5 a	1.23 ± 1.1 a	1.03 ± 0.9 a

Source: Author, 2024.

Next, in Figure 2, the effect of different treatments on the vigor and number of leaves in strawberry plants can be observed, highlighting the synergistic action between the leachate at a concentration of 10% in combination with the bacterium *B. subtilis* F62.

Figure 2 - Effect of leachate (10% and 25%) and leachate 10% + bacteria (*Bacillus subtilis* F62) on the vigor and number of leaves in strawberry plants



Source: Author, 2024.

Regarding the plant growth promotion and development, it was observed that the composting leachate had a positive effect on the strawberry plants. Similarly, Čabilovski et al. (2023) reported that applying vermicompost leachate over three years to field-grown strawberries led to an increase in total soluble solids, total anthocyanins, and antioxidant activity, while reducing total acidity in strawberries. Singh et al. (2010) also found that foliar application of vermicompost leachate benefited strawberry plants, increasing leaf area, plant dry matter, and strawberry yield. Furthermore, using leachate from bovine manure composting with plant residues in a 1:2 ratio resulted in fewer occurrences of albinism and malformed fruits, as well as a reduced incidence of gray mold in strawberries.

The use of composting leachate as a carrier for *B. subtilis* F62 significantly enhanced the development of the strawberry plants, underscoring the importance of this microorganism in biofertilizer production. Likewise, Gunjal, Kapadnis, and Pawar (2018) investigated the use of leachate from sugarcane bagasse fermentation as a fertilizer and substrate for the plant growth-promoting bacterium *Bacillus circulans*. They found that maize plants treated with this formulation exhibited increased root growth. Mora et al. (2022) also evaluated the application of Orgaon®, a commercial product derived from composting agricultural residues diluted at a 1/512 ratio, in association with the bacteria *Pseudomonas agronomica* and *B. pretiosus*. Their findings demonstrated that treating alfalfa (*Medicago sativa*) with this product increased germination rate, seed viability, and plant dry weight.

5 CONCLUSIONS

1. Applying diluted leachate from grape pomace composting supported the growth of *B. subtilis* F62 comparably to the standard culture medium.
2. Treatments with leachate in combination with *B. subtilis* F62 stimulated the vegetative development of strawberry plants, resulting in greater plant growth and vigor compared to using leachate alone.
3. The combination of composting leachate and *B. subtilis* F62 can contribute to waste valorization and the development of biological formulations aimed at more sustainable agricultural production.

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