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Vinasse as a Substrate for Inoculant Culture and Soil Fertigation: Advancing the Circular and Green Economy

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Abstract

Vinasse is a by-product with a key role in circular economy. In this work, we analyze sugarcane vinasse as culture medium for obtaining single and mixed inoculants. *Trichoderma harzianum* MT2 was cultured in single and sequential co-culture with *Pseudomonas capeferrum* WCS358 or *Rhizobium* sp. N21.2. Fungal biomass in single culture was more than three folds higher in vinasse than in a standard medium, and was higher in co-culture with *Rhizobium* sp. N21.2 than with *P. capeferrum* WCS358. Bacterial growths in vinasse, in particular *P. capeferrum* WCS358, were improved in co-culture with *T. harzianum* MT2. Residual vinasses, obtained after microbial growth, presented almost neutral pH and lower conductivities and toxicity than raw vinasse. Fertigation with residual vinasses modifies characteristics of soil evidenced in the total N, cation exchange capacity, urease and acid phosphatase, and microbial metabolic diversity, in comparison to raw vinasse. In general, soil fertigation with residual vinasse from co-culture with *P. capeferrum* WCS358 is more similar to irrigation with water. Treatment evaluation indicates that vinasse is suitable for the production of mixed inoculants containing *T. harzianum*. The co-culture with *P. capeferrum* WCS358 improves the characteristics of the residual vinasse allowing a fertigation with less detrimental effect in soil in comparison to *Rhizobium* sp. N21.2. Obtaining valuable biomass of single or mixed inoculants in vinasse with lower ecological impact is relevant for the circular and green economy.

Keywords

Fertigation, Inoculant, Vinasse, Trichoderma

1. Introduction

The main effluent generated after ethanol production by the sugar-alcohol industry is vinasse, a dark brown liquid with an acidic pH (3.5-5.0), and high Biological and Chemical Oxygen Demands (54,000 mg L^{-1} and 110,000 mg L^{-1}, respectively). Nearly 13 litres of vinasse are generated on average for each litre of ethanol produced, meaning 10^6 L per year for an average refinery (Christofoletti et al., 2013). Vinasses consist of 93% water and 7% solids, with a high prevalence of potassium, phenolics and melanoidins (de Godoi et al., 2019). Together with the acid pH and the volume, these are the main responsible for the negative ecological impact when vinasses are not properly disposed (Christofoletti et al., 2013). Different strategies have been envisaged to diminish its environmental hazard. Fertigation, i.e., its disposal in soils as fertilizer, is one of the most utilized methods for its management. This practice allows the recycling of water, organic matter and important elements, including nitrogen and phosphorus. However, excessive or improper fertigation leads to acidification (related to the low pH of vinasse) salinization (due to the accumulation of ions, including Na$^+$, K$^+$, Ca^{2+} and Mg^{2+}), and an alteration of the microbial populations in affected soils (Christofoletti et al., 2013). The input of nutrients and biodegradable organic matter through fertigation is also associated to methane, carbon dioxide and nitrous oxide emissions (Fuess et al., 2021).

Several reports analyzed the microbial treatment of vinasse. In these cases, microbial metabolism degrades organic compounds and converts them to CO$_2$ and water, reducing the toxicity of vinasse (Chuppa-Tostain et al., 2020; España-Gamboa et al., 2011). The main drawback to the effective application of these bioremediation processes are the enormous volumes of vinasse generated on a daily basis. Other approaches analyzed the utilization of vinasse as culture medium for producing metabolites (Altenhofen da Silva et al., 2017),

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enzymes (Ahmed et al., 2022) or microbial biomass (Candido et al., 2022; Montalvo et al., 2019). Among the latter, vinasse has been explored for the culture of microalgae (de Carvalho et al., 2022), edible fungi (Nair and Taherzadeh, 2016), or yeasts (Díaz-Vázquez et al., 2022). However, to the best of our knowledge, the production of biofertilizer with vinasse as culture medium has not been previously analyzed. The production of particulate fertilizers containing nutrients from vinasse has been recently reported (Cerri et al., 2020). This strategy clearly shows that vinasse is not necessarily a waste but a by-product. In addition, when vinasse is utilized instead of other sources for the production of these metabolites or for obtaining these biomasses, it becomes a highly valuable by-product with a key role in the future of circular economy (Hoarau et al., 2018; Karp et al., 2022). Circular economy proposes a cyclical production system in which 'wastes' are considered supplies to next processes in a cyclical productive system (Knoess, 1988). The actual and current relevance of circular economy is evidenced in the manner the Sustainable Development Goals of the United Nations are traversed by this concept. At the same time, the concept of 'green economy', i.e., the development of biotechnological processes of economical relevance in a sustainable manner, has also to be taken into account.

In this work, we evaluated vinasse from sugarcane as culture medium for the production of the plant-growth promoter *Trichoderma* (*T.*) *harzianum* MT2, in single and in co-culture with *Pseudomonas* (*P.*) *capeferrum* WCS358 and *Rhizobium* sp. N21.2. *T. harzianum* MT2 is a native isolate obtained from tomato rhizosphere (Malinar, 2020). *T. harzianum* is a relevant biological control agent that promotes plant growth through the mycoparasitism of fungal phytopathogens. In addition, *T. harzianum* also stimulates root and shoot elongation, the uptake of nutrients and increases the stress resistance. *T. harzianum* and related species are at the top of the formulated fungal biofertilizer in a global market estimated at U$S1.66 billion by 2022 (Aloo et al., 2021). Mass production of *T. harzianum* is obtained in solid-state

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fermentations employing seeds of rice, sorghum, and agro-waste products as substrate, or in liquid fermentations in a wide variety of culture media (Dutta et al., 2022).

Mixed inoculants show synergistic potentials for promoting plant growth in contrast to single inoculants (Santos et al., 2019). Mixed bioinoculants containing *Trichoderma* spp. usually include *Bacillus* spp., *Pseudomonas* spp. (Poveda and Eugui, 2022), and rhizobia (Barbosa et al., 2022), among other bacteria. The simplest way for producing mixed inoculants consists in the cultivation of individual strains in axenic cultures that will be mixed in the final commercial products. For a large-scale development, this represents an extra cost of production. Vinasse offers the possibility of obtaining mixed inoculants, as reported previously for other biotechnological applications (Eder et al., 2020; Iltchenco et al., 2020). The simultaneous production of mixed inoculants in vinasse as substrate may present the advantage of generating less-toxic wastes. In co-culture with *T. harzianum* MT2, we analyze in this article two model bacteria with plant growth-promoting potential. *P. capeferrum* WCS358, isolated from potato rhizosphere (Geels and Schippers, 1983), induces the systemic resistance of host plants and secretes pyoverdine siderophore that inhibits phytopathogens (Berendsen et al., 2015). *Rhizobium* sp. N21.2 is a native isolate previously isolated from strawberry rhizosphere. N21.2 strain produces siderophores, solubilizes phosphates, secretes auxins and putatively fixes nitrogen, according to positive PCR amplification of the nitrogenase *nifH* gene (unpublished results).

The aim of this work was to analyze the utilization of the vinasse as culture medium for the production of *T. harzianum* MT2, in single and in co-culture with two model plant growth-promoting bacteria. As a contribution to the circular and green economy, the objective is to obtain valuable biomass in a cost-effective manner from an abundant by-product, reducing at the same time the environmental impact of the residual vinasses, i.e., the vinasse obtained after its utilization as culture medium.

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2. Materials and Methods

2.1 Strains and culture media

T. harzianum MT2 was isolated from tomato rhizosphere and identified after sequencing the ITS region and the D1/D2 region (Genbank No. MT577837) and RNA polymerase subunit II gene (Genbank No. MT641352). *T. harzianum* MT2 was maintained in Yeast-Malt Extract medium (YME) medium (Tavares et al., 2005). *P. capeferrum* WCS358 was maintained in Luria Bertani (LB) medium. *Rhizobium* sp. N21.2, identified after 16S rRNA gene sequencing (Genbank No. OQ349509), was maintained in Yeast Mannitol (YMA) medium (Vincent, 1970). All strains were preserved at -80 °C in 20% glycerol.

2.2 Source and characterization of vinasse

Vinasse obtained directly from the distillery columns was provided by a local sugarcane biorefinery located in Cruz Alta, Tucumán, Argentina during the sugarcane harvest in 2019, and stored at -20 °C. Vinasse was physically and chemically characterized at Estación Experimental Agroindustrial Obispo Colombres (EEAOC, Tucumán, Argentina), following standard procedures: fixed and volatile solids by the Standard Method 2540E (Fixed and Volatile Solids Ignited at 550°C); total solids by the Standard Method 2540C (Total Dissolved Solids Dried at 180 °C); pH and conductivity by the Standard Method 4500 and 2510 with a pH meter and conductivity meter, respectively; chloride by the Standard Method 4500-Cl$^-$-B and sulfide by the Standard Method 4500-S$_2^-$-D; Total Phosphorus by the Standard Method 4500-P-C; Brix by the refractometric method; organic material by gravimetry; Potassium, Calcium, Magnesium and Sodium by Flame Atomic Emission Spectroscopy; total Nitrogen by Kjeldahl method (see supplementary material). Before

utilization, the vinasse was centrifuged at 10.000 g for 5 min to discard the coarse sludge and then autoclaved.

2.3 Fungal and bacterial tolerance to vinasse

Before analyzing the respective growth, the tolerance to vinasse (i.e., the maximal concentration of vinasse that supported the microbial growth) of each strain was determined. Overnight precultures were utilized to inoculate flasks containing 10, 20, 30, 40, 50, 75 and 100% vinasse (dilutions in distilled water). *T. harzianum* MT2 was incubated at 25 °C and 250 rpm. At 0, 48 and 72 h of incubation, dry weights were measured after drying the cell pellets at 105 °C. *P. capeferrum* WCS358 and *Rhizobium* sp. N21.2 were incubated at 30 °C in an orbital shaker at 180 rpm. After 0, 48 and 72 h of incubation, values of CFU mL^{-1} were determined after plating serial dilutions in LB agar or YMA agar for *P. capeferrum* WCS358 and *Rhizobium* sp. N21.2, respectively. For comparison, tolerance values were expressed as percentages considering as 100% the values obtained after the addition of the corresponding inocula.

2.4 Fungal and bacterial single cultures in vinasse

Microbial growths were evaluated at subinhibitory concentrations of vinasse determined as described above. *P. capeferrum* WCS358 and *Rhizobium* sp. N21.2 were grown in diluted vinasse (10 and 20%) and compared with growth in the standard culture media LB and YMA broth, respectively. Cultures were performed aerobically at 30 °C and 180 rpm. Samples were withdrawn during incubations, and the bacterial growths were evaluated through the determination of CFU mL^{-1} after plating serial dilutions in LB agar and YMA agar. *T. harzianum* MT2 growth was evaluated in 10 and 50 % vinasse at 25 °C and 250 rpm and compared to that in YME broth. Samples were also periodically withdrawn, centrifuged and

the cell pellets were dried at 105 °C for the evaluation of the growth by dry weight measurement.

2.5 Sequential co-cultures of *T. harzianum* with bacteria in vinasse

Sequential co-cultures of *P. capeferrum* WCS358+*T. harzianum* MT2 and *Rhizobium* sp. N21.2 +*T. harzianum* MT2 were carried out as follows. First, *P. capeferrum* WCS358 and *Rhizobium* sp. N21.2 were independently cultured in 10 % vinasse for 48 h at 30 °C and 180 rpm. *T. harzianum* MT2 was then inoculated from a 48 h preculture, and pure vinasse was added to achieve a final concentration of 50% (see supplementary material). Incubation was continued at 25 °C and 250 rpm for another 72 h. Bacterial and fungal growths were evaluated through the determination of CFU mL^{-1} and the dry weight method, respectively. Single bacterial and fungal cultures were carried out as controls. At the end of the incubations, complete biomasses were removed by centrifugation at 10.000 *g* for 10 min and the residual vinasses, i.e., the vinasses obtained after the cultures, were analyzed as described below (see supplementary material).

2.6 Physical-chemical analysis and toxicity of vinasses

Acidity was measured with a pHmetrer (Sartorius). Dissolved solids were measured with a brixometer (Arcano) and conductivity was determined with a conductimeter (COM-100, HM Digital). Toxicity of residual vinasse was evaluated by the acute toxicity assay with *Lactuca sativa* seeds. Briefly, seeds were placed in Petri dishes, and 5 mL of residual vinasses or control vinasse (i.e., 50% pure vinasse) all previously diluted in water (1:5) were added. Seeds were also treated with water as control. Plates were incubated in darkness at 25 °C for 5 days. The percentages of germination were determined considering as 100% the number of

seeds germinated with water. The hypocotyl and radicle lengths were measured and values were compared to those obtained with control vinasse.

2.7 Soil fertigation with residual vinasse

Soil not previously fertigated was collected from a local sugarcane farm. Samples were taken from the first 10 cm depth in the furrow zone and nearby the plants. Soil was sieved and then placed in trays for fertigation, as follows. Residual vinasses from single and co-cultures were filtered with gauze to discard the coarse fungal biomass and utilized for the fertigation by aspersion on days 0 and 7 applying 1 L m^{-2} (irrigation sheets- 1 mm) in agreement with local recommendations for fertigation with vinasse (Morandini and Quaia, 2013). A group of trays was irrigated with control vinasse (i.e., 50% pure vinasse) or water as control treatments. All trays were incubated at 25 °C for a total of 14 days, and then soil samples were taken for physical-chemical and biological characterizations. Water contents were daily adjusted to the initial values with distilled water considering the loss of weight.

2.8 Physical-chemical and biological characterization of soil samples

Soil pH and conductivity were determined in at least three independent samples by mixing one volume of soil was mixed with one volume of distilled water and mixed vigorously. After decantation, pH and conductivity were measured in the soil slurries with a pHmeter (Sartorius) and a conductimeter (COM-100, HM Digital), respectively. Soil toxicity was determined in the same soil slurries with lettuce seeds as described previously. For other chemical characteristics, samples of soils from three independent assays were pooled before analysis: Carbonate content by calcimetry; Total Organic Carbon (TOC) by the Walkley-Black method; total Nitrogen by the Kjeldahl method; available Phosphorus by the Bray-Kurtz method; Cation Exchange Capacity (CEC) was calculated from the quantification of

exchange cations (Ca^{2+}, Mg^{2+} and K^+), which were determined by the ammonium acetate method, except for Na^+ that was determined by the Saturated Paste method. Soil enzymatic activities were determined in at least three independent samples as follows. Hydrolysis of fluorescein diacetate (FDA; µg fluorescein g^{-1} h^{-1}), acid phosphatase (AP; µg *p*-nitrophenol g^{-1} h^{-1}) and urease (UA; µg $N-NH_4$ g^{-1} h^{-1}) activities were quantified by spectrophotometry employing published protocols (Adam and Duncan, 2001; Jastrzębska, 2011), with modifications (Raimondo et al., 2019). Catalase activities (CAT; mmol H_2O_2 consumed g^{-1} h^{-1}) were determined by titration (Jastrzębska, 2011), with modifications (Raimondo et al., 2019). Quantification of heterotrophic microorganisms was performed in at least three independent samples in Plate Count Agar (PCA) by plating serial dilutions of soil supernatants on PCA in triplicate and incubating at 30 °C for 120 h. Results were expressed as CFU g^{-1} of soil. Microbial functional diversity was analyzed by studying patterns of carbon source utilization with Biolog EcoPlates. Microorganisms were extracted from 5 g of soil from each treatment after shaking at 200 rpm at 25 °C for 45 min with 45 mL of 0.9 % NaCl solution. The coarse particles were allowed to decant for 30 min at room temperature and 150 µL of these suspensions were utilized to seed wells of Biolog EcoPlates. Microplates were incubated at 25 °C for 7 days and the absorbance was daily measured at 590 nm (A_{590}). The average metabolic response (AMR) for each treatment was calculated as the mean difference between the A_{590} of wells containing a carbon source (A_{590CS}) and the control well with water (A_{590W}): AMR=Σ(A_{590CS}-A_{590W})/95 (Konopka et al., 1998). Principal Component Analysis was performed with data obtained after 72 h of incubation when larger differences in the AMR among treatments were determined.

2.9 Statistics analysis

All assays were performed independently at least in triplicates. Vinasses were analyzed with ANOVA followed by Dunnett´s post hoc test at $P<0.05$ considering the control vinasse as control.

Conductivity, pH, toxicity, counts of heterotrophic microorganisms and enzyme activities in soil were analyzed with ANOVA followed by Dunnett´s post hoc test at $P<0.05$ considering the soil treated with control vinasse as control. Statistics were performed with Minitab 19 software.

3 Results and Discussion

3.1 Vinasse as growth medium for single cultures

The main limitation for the exploitation of vinasse as culture medium is the toxicity of this by-product. Tolerance determinations showed that 30% vinasse was inhibitory for both bacteria (see supplementary material). As presented below, tolerances were lower than fungal tolerance, but comparable to others bacteria (Ventorino et al., 2019). *Rhizobium* sp. N21.2 and *P. capeferrum* WCS358 growths were then evaluated in 10% and 20% vinasse, and compared with those in the standard media YMA and LB broth, respectively. *Rhizobium* sp. N21.2 showed an 8 h-lag phase in 10% vinasse and then attained $3.03 \cdot 10^8$ CFU mL^{-1}, lower than in YMA medium ($1.90 \cdot 10^9$ CFU mL^{-1}) (Fig. 1a). CFU mL^{-1} decreased after 8 h of incubation in 20% vinasse, reaching $7.03 \cdot 10^3$ CFU mL^{-1} after 72 h (Fig. 1a). *P. capeferrum* WCS358 grew similarly in 10% vinasse and LB medium attaining $2.50 \cdot 10^9$ CFU mL^{-1} after 24 h (Fig. 1b). In contrast, CFU mL^{-1} counts decreased in vinasse 20% during the first 24 h, but then slowly increased reaching $4.03 \cdot 10^8$ CFU mL^{-1} after 72 h (Fig. 1b). Inhibition of microbial growth in vinasse has been mainly attributed to phenolics like luteolin, tricin, apigenin, and naringenin (Rodrigues Reis et al., 2020). *Pseudomonas* spp. and *Rhizobium*

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spp. possess different catabolic pathways for these compounds, which could be related to the different behaviors in vinasse (Rao and Cooper, 1994).

T. harzianum MT2 tolerated higher vinasse concentrations in comparison to both bacteria, even resisting 100% pure vinasse (see supplementary material). This tolerance is in agreement with the reported high fungal tolerance to vinasse (Rodrigues Reis et al., 2020). Two dilutions were then utilized to compare the fungal growth to that in YME broth: 10% vinasse, in which bacteria showed the better growth, and 50% vinasse that allowed the better fungal growth. Growth in 10% vinasse was slower than in YME, in which the stationary phase was attained after 24 h with a biomass of 0.73 mg mL^{-1} (Fig. 1c). In contrast, biomass in 10% vinasse did not exceed 0.44 mg mL^{-1}. After a 12 h-lag phase, *T. harzianum* MT2 growth markedly increased in 50% vinasse reaching biomass values of 2,49 mg mL^{-1}, more than three folds higher than in YME (Fig. 1c). These results show that *T. harzianum* can be cultured in sugarcane vinasse for fungal mass production with better results than a standard culture medium. The analysis of the fungal growth suggests that *T. harzianum* MT2 first utilizes readily metabolizable nutrients, and then compounds derived from complex components. In agreement, when *Aspergillus niger* grows in vinasse, monosaccharides are first accumulated from the degradation of complex molecules with a relatively weak increase in biomass. After glucose and fructose exhaustion, *A. niger* utilizes mannitol with a sudden increase in biomass (Chuppa-Tostain et al., 2018). A lack of biphasic growth in *P. capeferrrum* WCS358 or *Rhizobium* sp. N21.2 could be related to a minor capacity to degrade complex compounds, which contrasts with the recognized lytic enzyme production in *T. harzianum* (Schuster and Schmoll, 2010).

3.2 Vinasse as growth medium for sequential co-cultures

Considering the relevance for agriculture of mixed bioinoculants and the possible improvements in the characteristics of the residual vinasse, *P. capeferrrum* WCS358 and *Rhizobium* sp. N21.2 were evaluated in sequential co-cultures with *T. harzianum* MT2 (see supplementary material). Growths were evaluated during the second stage after the inoculation of *T. harzianum* MT2 and the supplementation with vinasse. No significant differences were found between the growth of *T. harzianum* MT2 in single and in co-cultures with *P. capeferrum* WCS358, which attained 3.64 mg mL^{-1} and 3.30 mg mL^{-1}, respectively, 48 h after vinasse supplementation. Fungal biomasses then decreased to 2.53 mg mL^{-1} and 1.99 mg mL^{-1} (Fig. 2a). Growth of *T. harzianum* MT2 was lower in co-culture with *Rhizobium* sp. N21.2 attaining 1.64 mg mL^{-1} after 48 h of supplementation, but with values of 3.12 mg mL^{-1} after 72 h (Fig. 2a). In conclusion, sequential co-culture in vinasse with *P. capeferrrum* WCS358 is not detrimental for *T. harzianum* MT2 growth. Although the co-culture with *Rhizobium* sp. N21.2 slows down *T. harzianum* MT2 growth, higher fungal biomass can be obtained.

After a decrease of two log units, in presence of *T. harzianum* MT2 and a concentration of 50% vinasse, *P. capeferrum* WCS358 rapidly recovered attaining similar values (2.90·10^8 CFU mL^{-1}) to the initials when the culture was supplemented (Fig. 2b). In single cultures only supplemented with vinasse, no colonies of *P. capeferrum* WCS358 were obtained after 24 h (Fig. 2b). In contrast, *Rhizobium* sp. N21.2 in co-culture remained constant at 10^9 CFU mL^{-1} for 48 h before it decreased to 2.70·10^6 CFU mL^{-1} in the next 24 h (Fig. 2c). When *Rhizobium* sp. N21.2 was alone in 50% vinasse, CFU mL^{-1} constantly declined. After 72 h, no colonies were obtained (Fig. 2c). The improvement in the bacterial growth in co-culture could be related to the degradation of phenolics by *T. harzianum* MT2. Previous reports showed that co-cultures of bacteria and fungi have advantages in terms of resistance to toxic compounds (Losa and Bindschedler, 2018). The differences between both bacteria in co-

culture are in agreement with the lower growth of *Rhizobium* sp. N21.2 in single culture, and could also be related to the response to phenolics (Rao and Cooper, 1994). These results indicate that mixed inoculants containing *T. harzianum* can be obtained after culturing in sugarcane vinasse, and suggest that the bacterial combination could have an influence in the final product. Additional essays are required to evaluate the performance of *T. harzianum* MT2, *P. capeferrum* WCS358 and *Rhizobium* sp. N21.2 for plant-growth promotion after being cultured in vinasse medium. The residual vinasse as a component in the formulation of these bioinoculants could decrease the production costs. However, further experiments are required to evaluate the stability of the microorganisms in residual vinasse and the plant-growth promotion activities *in planta* when residual vinasse is present. To note, in this work vinasse was sterilized previously to the inoculation. The microbial diversity of vinasse is expected to be low but variable, which includes members of *Bacillota, Pseudomonadota, Bacteroidota* and *Actinomycetota* phyla (Cassman et al., 2018). It is expectable that these accompanying microorganisms compete with strains to be cultured. However, in the search of less expensive and more sustainable technologies, future experiments should evaluate the effect of these microorganisms and the possibility of utilizing non-sterilized vinasse for the production of bioinoculants.

3.3 Microbial growth enhances the characteristics of residual vinasse

From a perspective of circular economy, the results of microbial growths indicate the possibility of exploiting sugarcane vinasse for producing the valuable biomass of *T. harzianum.* Single or mixed inoculants containing *T. harzianum* can be obtained for agricultural practices in an inexpensive manner reducing the production costs related to the culture medium. However, from a perspective of a green economy, the characteristics of the wastes generated after utilizing vinasse as culture medium, and their ecological impact should

also be considered. Then, the characteristics of the residual vinasses obtained from the single culture of *T. harzianum* MT2 and the sequential co-cultures were analyzed and compared with 50% vinasse (control vinasse). *T. harzianum* MT2 and *P. capeferrum* WCS358+*T. harzianum* MT2 decreased the acidity of vinasse with respect to the control reaching values close to neutrality (pH=6.47 and pH=6.90, respectively). Lower values were obtained with vinasse from co-culture of *Rhizobium* sp. N21.2+*T. harzianum* MT2 (pH=6.08) (Table 1). Several studies already reported that microbial growth in vinasse decreases its toxicity (Ahmed et al., 2022; Rulli et al., 2020). The neutralization, for instance, is a valuable feature, considering that that the acidity is a serious concern for fertigation. The lower acidity with residual vinasse from *T. harzianum* MT2 could be related to the consumption of organic acids generated from the metabolism of carbohydrates, as reported previously for *A. niger* (Chuppa-Tostain et al., 2018). The even higher pH in vinasse from *P. capeferrum* WCS358+*T. harzianum* MT2 co-culture could also be related to the well-known utilization of organic acids by *Pseudomonas* spp. (Lynch and Franklin, 1978). Vinasse conductivities also diminished in comparison to the control (9.47 dS m^{-1}), with significant differences with the single culture of *T. harzianum* MT2 (9.22 dS m^{-1}). No differences were observed between both co-cultures (Table 1). Cultivation also reduced the dissolved solids in all residual vinasses in comparison to control (4 °Bx) (Table 1). More marked values were obtained with the single culture of *T. harzianum* MT2 and with the co-culture *P. capeferrum* WCS358+*T. harzianum* MT2 (2,13 °Bx) (Table 1). Both cases are in agreement with the decreases in the acidity determined in the corresponding residual vinasses. Rhizobia also utilize organic acids with a preference over carbohydrates (Iyer et al., 2016). However, the survival of *Rhizobium* sp. N21.2 was reduced in 50% vinasse, even in presence of *T. harzianum* MT2, which could explain the lower neutralization and the higher amount of dissolved solids in these co-cultures.

Considering that preliminary assays showed no germination of lettuce seeds with pure vinasses, samples were first diluted 1:5 in water before toxicity tests were performed. No differences were observed in the germination percentages with vinasse from single culture of *T. harzianum* MT2, in comparison to the control vinasse. Slightly higher values were measured with vinasse from *P. capeferrum* WCS358+*T. harzianum* MT2 (23.60%), but lower with *Rhizobium* sp. N21.2+*T. harzianum* MT2 (17.74%). Hypocotyl lengths were longer with *T. harzianum* MT2 (2.49 cm) in comparison to control (1.40 cm). Similar values to the single culture were obtained with *P. capeferrum* WCS358+*T. harzianum* MT2 (2.40 cm), and longer (2.66 cm) with *Rhizobium* sp. N21.2+*T. harzianum* MT2 (Table 1). Length of radicles tended to increase with residual vinasse from *T. harzianum* MT2 (1.11 cm) with respect to the control vinasse (0.71 cm) (Table 1), and with no significant differences between co-cultures. Overall morphology of the seedlings showed the clearest difference in the toxicity. Seedlings obtained with residual vinasse developed morphologies similar to water treatment. In contrast, seedlings with control vinasse were abnormal with twisted forms (Fig. 3). The probable degradation of phenolics by *T. harzianum* MT2 in both single and co-culture could also explain the decrease in vinasse toxicity evidenced with lettuce seeds. Other factors, like the decrease in the conductivity of residual vinasses, cannot be ruled out. For instance, high concentrations of soluble salts have also been implicated in the phytotoxicity of effluents (Wang, 1991). Microbial production of plant-growth regulators or alleviators of stress during growth in vinasse can also impact on lettuce seedlings. In conclusion, residual vinasses from single and co-cultures present better characteristics than raw vinasse, which suggest a lower ecological impact when utilized in fertigation. Further experiments are required to compare the effect of residual vinasse with pure non-sterilized vinasse, which contains a variable microbiota (Cassman et al., 2018) that could degrade organic compounds.

3.4 Fertigation with residual vinasses is better than with control vinasse for physical-chemical and toxicity characteristics of soil

Soil samples were fertigated with residual vinasses obtained from single fungal culture and co-cultures, and then the short-term impact was determined. In our work, two applications of 1 L m^{-2} (irrigation sheets=1 mm) with 50% diluted vinasse is equal to 520 m^3 ha^{-1} year^{-1}, or 260 m^3 or pure vinasse ha^{-1} year^{-1}. This is comparable to local recommendations that suggest fertigation of 150 m^3 ha^{-1} year^{-1} for a sustainable utilization of vinasse (Morandini and Quaia, 2013). The analysis of the soils showed that the impact of the residual vinasses on the physical-chemical and chemical characteristics of soils is comparable with control vinasse. The pH of soils fertigated with residual vinasses was similar (pH 7.13-7.19) to that with control vinasse (pH=7.12), and slightly higher than with water (pH=6.82) (Table 2). The fertigation with vinasses augmented the conductivity in comparison to the irrigation with water (0.35 dS m^{-1}). In comparison to control vinasse (0.67 dS m^{-1}), treatments with residual vinasses from *T. harzianum* MT2 and *Rhizobium* sp. N21.2+*T. harzianum* MT2 showed lower values (0.63 dS m^{-1}) and, though not statistically significant, even lower with *P. capeferrum* WCS358+*T. harzianum* MT2 (0.59 dS m^{-1}). In comparison to the irrigation with water, these results suggest a salinization process at short-term, possibly related to the higher Mg^{2+} contents determined after fertigation with residual vinasses (Table 2). Soluble salts, including magnesium salts, are the main factors determining soil conductivity, which reflects soil salinity (Haj-Amor et al., 2022). However, factors like organic matter from vinasses could also influence the soil salinity (Haj-Amor et al., 2022).

Soil toxicity parameters tested in lettuce seeds, particularly evidenced in the hypocotyl and radicle lengths, diminished with co-cultures. No significant differences were observed in the percentages of germination, with values between 93.3% (fertigation with vinasse from *T. harzianum* MT2 culture) and 98% (irrigation with water) (Table 2). Interestingly, hypocotyl

lengths were higher with control vinasse (2.42 cm) and with vinasses from *T. harzianum* MT2 and *P. capeferrum* WCS358+*T. harzianum* MT2 cultures (2.45 cm), that the irrigation with water (2.18 cm). Radicle lengths were higher with control (2.61 cm) and with vinasse from *T. harzianum* MT2 culture (2.63 cm). In contrast, the shortest lengths were obtained with *Rhizobium* sp. N21.2+*T. harzianum* MT2 and *P. capeferrum* WCS358+*T. harzianum* MT2: 2.23 cm and 2.22 cm, respectively (Table 2). It is plausible that *T. harzianum* MT2, *P. capeferrum* WCS358 and *Rhizobium* sp. N21.2 produce plant-growth regulators in vinasse, as mentioned previously.

3.5 Fertigation with residual vinasses improves biological characteristics of soil

Some biological parameters also showed that fertigation with residual vinasses are less detrimental to soil quality than fertigation with raw control vinasse. Quantification of enzymatic activities in fertigated soils showed that UA after treatment with residual vinasses from *T. harzianum* MT2 culture (21.08 µg N-NH$_4$ g^{-1} h^{-1}) was lower than with control vinasse (27.62 µg N-NH$_4$ g^{-1} h^{-1}). Even lower values were determined with *P. capeferrum* WCS358+*T. harzianum* MT2 (19.73 µg N-NH$_4$ g^{-1} h^{-1}), very similar to water (19.30 µg N-NH$_4$ g^{-1} h^{-1}). Intermediate activity (24.56 µg N-NH$_4$ g^{-1} h^{-1}) was determined with *Rhizobium* sp. N21.2+*T. harzianum* MT2 (Fig. 4a). Urease activity (UA) is a relevant indicator of soil quality, mainly related to the N cycle and highly influenced by soil disturbances (Adetunji et al., 2020). For instance, sewage sludge increases UA causing the release of nitrogen available for plants. The comparison of UA and total N values (Table 2) suggest that residual vinasse from *T. harzianum* MT2 contributes to the overall N cycle lowering the enzymatic activity and supplying N to the soil. To note, the efficiency is better with vinasse from *P. capeferrum* WCS358+*T. harzianum* MT2 co-culture.

Residual and control vinasses induced similar AP activities in comparison to water. The exemption was the vinasse from *P. capeferrum* WCS358+*T. harzianum* MT2 co-cultures (213.56 µg g^{-1} h^{-1}), which caused a reduction in comparison to water (291.63 µg g^{-1} h^{-1}) and to control (268.69 µg g^{-1} h^{-1}) (Fig. 4b). Phosphatases participate in the P cycle releasing phosphate from phosphate monoester that can be biologically uptaken (Adetunji et al., 2020). The decrease after fertigation with vinasse from *P. capeferrum* WCS358+*T. harzianum* MT2, suggests a negative effect on P cycle. In sights of a putative fertigation with residual vinasses from co-cultures, these results suggest that proper amendments would be required, even if the values of available P in soil were higher that with control vinasse. Noteworthy, other factors should also be considered in the interpretation of these results. For instance, length treatment may influence the impact of vinasse fertigation on soil biological characteristics. In this work, two fertigations were performed on days 0 and 7, and analysis were performed at short term on day 14. More marked differences in enzyme activities, including urease and dehydrogenase, may be determined when soil is analyzed several months after vinasse fertigation, as described elsewhere (Tejada et al., 2007). In contrast, AP also remained unaltered after long treatment with vinasse fertigation (Tejada et al., 2007). Soil type is also relevant in the impact of vinasse fertigation (Narvaez Castillo et al., 2010).

No differences were determined in FDA and CA, regardless of the irrigation used (Fig. 4c and d). Heterotrophic microbial population after fertigation with residual vinasses was also not modified significantly, except for vinasse from *Rhizobium* sp. N21.2+*T. harzianum* MT2, attaining 1,96·10^7 CFU g^{-1}, two fold higher than with control vinasse (9,32·10^6 CFU g^{-1}) and water (8,15·10^6 CFU g^{-1}) (Fig. 4e). The constant values of the fluorescein diacetate hydrolysis, together with the increase in the heterotrophic microorganisms after fertigation with vinasse from *Rhizobium* sp. N21.2+*T. harzianum* MT2 indicate that this treatment decrease, in relative terms, the microbial activity in soil (Green et al., 2006).

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The metabolic diversity of the microbial community in the fertigated soils was assessed using Biolog Ecoplates. The evaluation of the Average Metabolic Response (AMR) showed two groups of treatments that differed from water irrigation. One group, including the treatment with vinasses from *T. harzianum* MT2 culture and *P. capeferrum* WCS358+*T. harzianum* MT2 co-culture, showed slower increases in the AMR with maximal values of 0.20 and 0.18 after 120 h, respectively (Fig. 5a). *P. capeferrum* WCS358 and *T. harzianum* MT2 may produce beneficial compounds with a positive effect on the soil. However, it should also be taken into account the presence of fungal propagules and bacterial cells in the residual vinasses. To note, in an approach to industrial developments that require non-expensive methods, in this work residual vinasses were not sterilized before fertigation. A second group with a faster increase in the AMR and higher final values (0.22 and 0.23) included the fertigation with control vinasse and vinasses from *T. harzianum* MT2+*Rhizobium* sp. N21.2 co-cultures. Soil irrigated with water showed the slowest increase in AMR throughout this study, presenting final values of 0.13 (Fig. 5a).

Employing the Principal Component Analysis, the separation on the PC1 axis in the two groups mentioned above could be clearly distinguished, though no major differences were observed on the PC2 axis (Fig. 5b). The first group (residual vinasses from *T. harzianum* MT2 and *P. capeferrum* wCS358+*T. harzianum* MT2) together with water irrigation was located towards the negative PC1 values, due to the influence of the carbon sources Glucose-1-phosphate, D-Xylose and L-Phenylalanine (Torres et al., n.d.) (Fig. 5b). Within this group, the most distant and dispersed treatment was the soil irrigated with water. The second group (control vinasse and vinasse from *Rhizobium* sp. N21.2+*T. harzianum* MT2 co-culture), on the contrary, was located towards the positive side of PC1, mainly due to the utilization of D-Mannitol, L-Arginine and L-Asparagine (Torres et al., n.d.) (Fig. 5b).

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4 Conclusions

The actual vision considers vinasse as a by-product with broad potential. Vinasse can be utilized for the production of biomass valuable for agricultural practices at low cost, taking into account that large volumes of this by-product are produced and discarded on a daily basis. Considering the enormous volumes produced per year, the percentage of vinasse utilized in the manner explored in this work could be relatively marginal. However, it is the value of the biomass obtained what counts, considering the global market of biofertilizer. In addition, the residual vinasse generated shows less negative impact on the environment. Toxicity can be diminished by the fungal growth, allowing later fertigation. The sequential co-culture with plant growth-promoting bacteria permits to obtain a mixed bioinoculant enhancing the characteristics of the residual vinasse. However, it is important to properly select the bacterium for the co-culture. These results are relevant in terms of circular and green economy considering that an agroindustrial by-product can be utilized for the production of inoculants for agriculture, generating residual vinasse of lower ecological impact.

Acknowledgement

Data availability

Dataset related to Ecoplates can be found at doi: 10.17632/kmp62j6kpd.1, an online data repository hosted at Mendeley Data (Torres et al. 2022). Other data are available on request.

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Figure 1. Vinasse as medium for the pure culture of plant-growth promoters. *Rhizobium* sp. N21.2 (a) and *P*. *capeferrum* WCS358 (b) were grown in 10% vinasse and 20% vinasse, and compared to growth in YMA and LB broth, respectively. *T*. *harzianum* MT2 (c) was grown in 10% vinasse and 50% vinasse and compared to growth in YME broth. Error bars represent standard deviations.

Figure 2. Vinasse as medium for the sequential co-culture of plant-growth promoters. Growth of *Rhizobium* sp. N21.2 (a) and *P*. *capeferrum* WCS358 (b) after the addition of *T*. *harzianum* MT2 and the supplementation with vinasse, in comparison with single cultures after vinasse supplementation. Growth of *T*. *harzianum* MT2 (c) after been added to *Rhizobium* sp. N21.2 or *P*. *capeferrum* WCS358 single cultures, in comparison to single culture. Error bars represent standard deviations.

Figure 3. Morphologies of lettuce seedlings. Seeds were germinated in residual vinasses from single culture of *T. harzianum* MT2 (a), sequential co-cultures of *P. capeferrum* WCS358+*T. harzianum* MT2 (b) and *Rhizobium* sp. N21.2+*T. harzianum* MT2 (c) are compared with control vinasse (d) and water (e).

Figure 4. Biological characteristics of fertigated soil. Urease activities (a), acid phosphatase (b), fluorescein diacetate hydrolysis (c), catalase (d) activities, and heterotrophic microorganisms (e) were determined after irrigation with water or fertigation with control vinasse, residual vinasses from single culture of *T. harzianum* MT2, sequential co-cultures of *P. capeferrum* WCS358+*T. harzianum* MT2 and *Rhizobium* sp. N21.2+*T. harzianum* MT2. Error bars represent standard deviations. Values significantly different from the control vinasse (ANOVA followed by Dunnett's post hoc test, *P*<0.05) are indicated with *.

Figure 5. Metabolic diversity of fertigated soils. Biolog EcoPlates were utilized to evaluate the Average Metabolic Response (AMR) of soils after irrigation with water or fertigation with control vinasse or residual vinasses. Values of AMR were obtained at different incubation times of the Ecoplates (a). Principal Component Analysis (b) was performed with data obtained after 72 h of incubation.
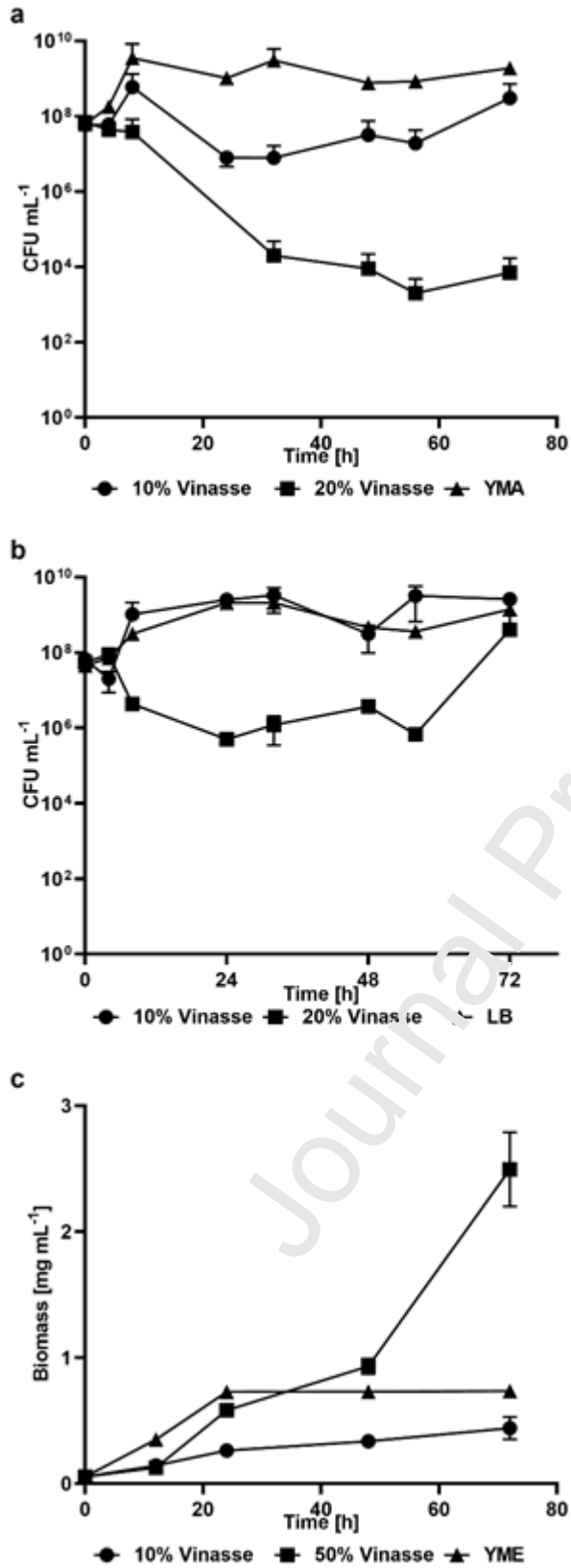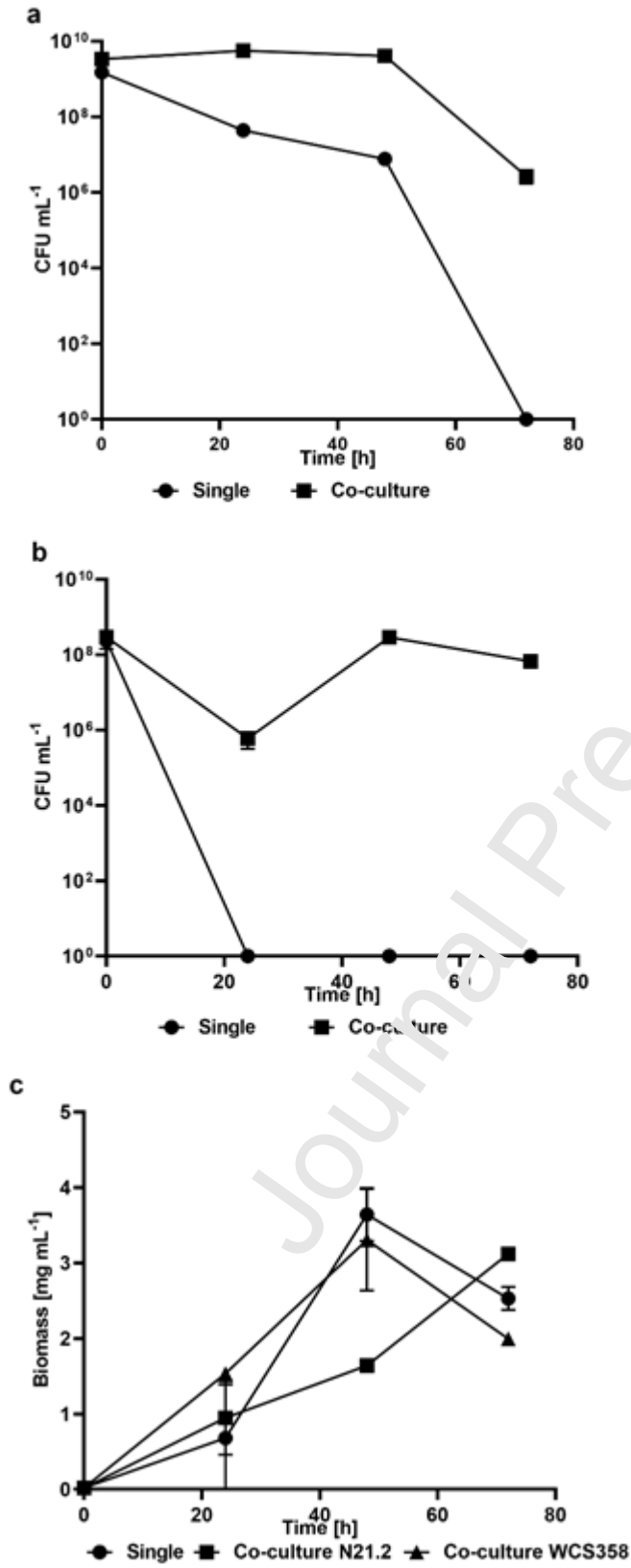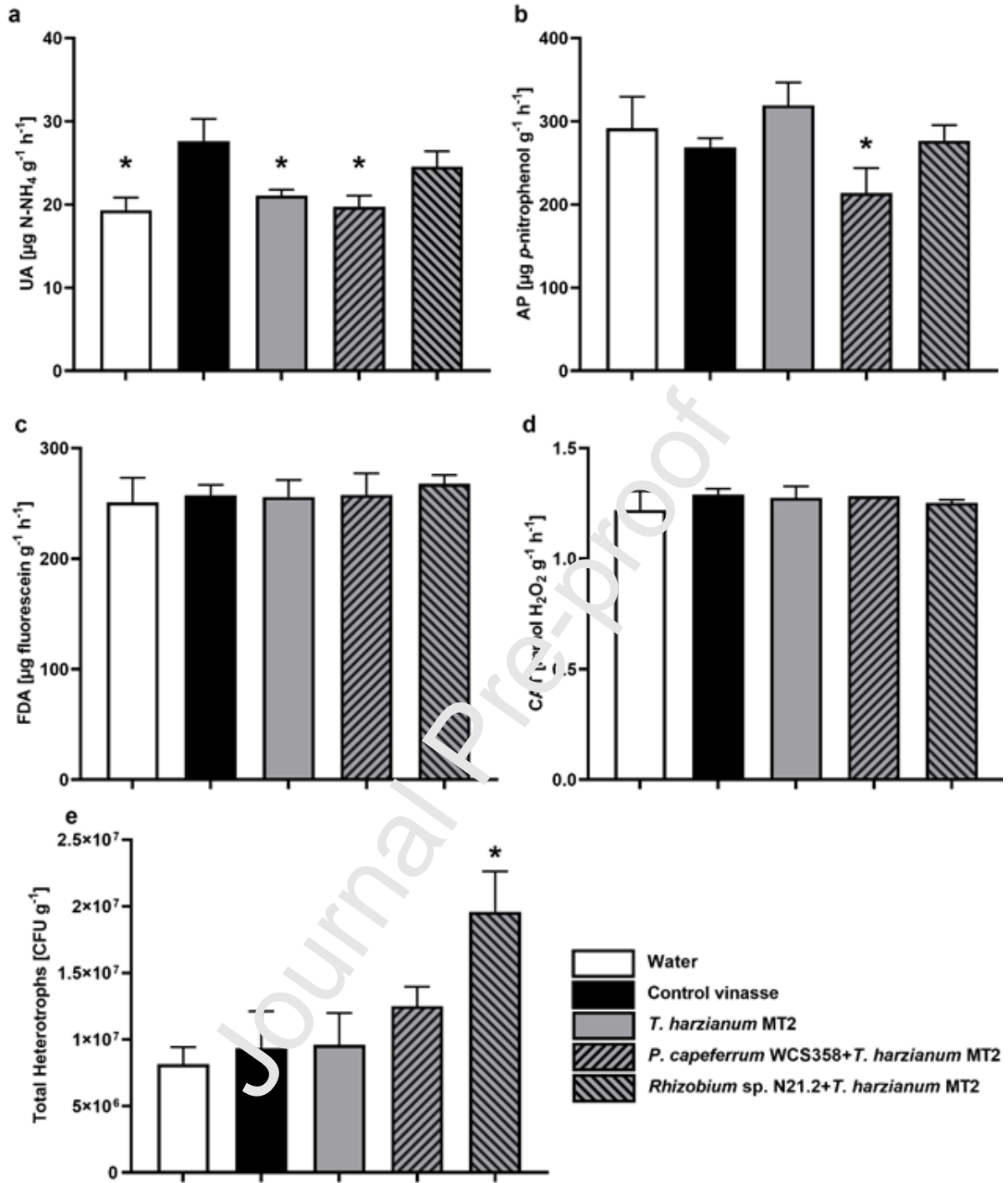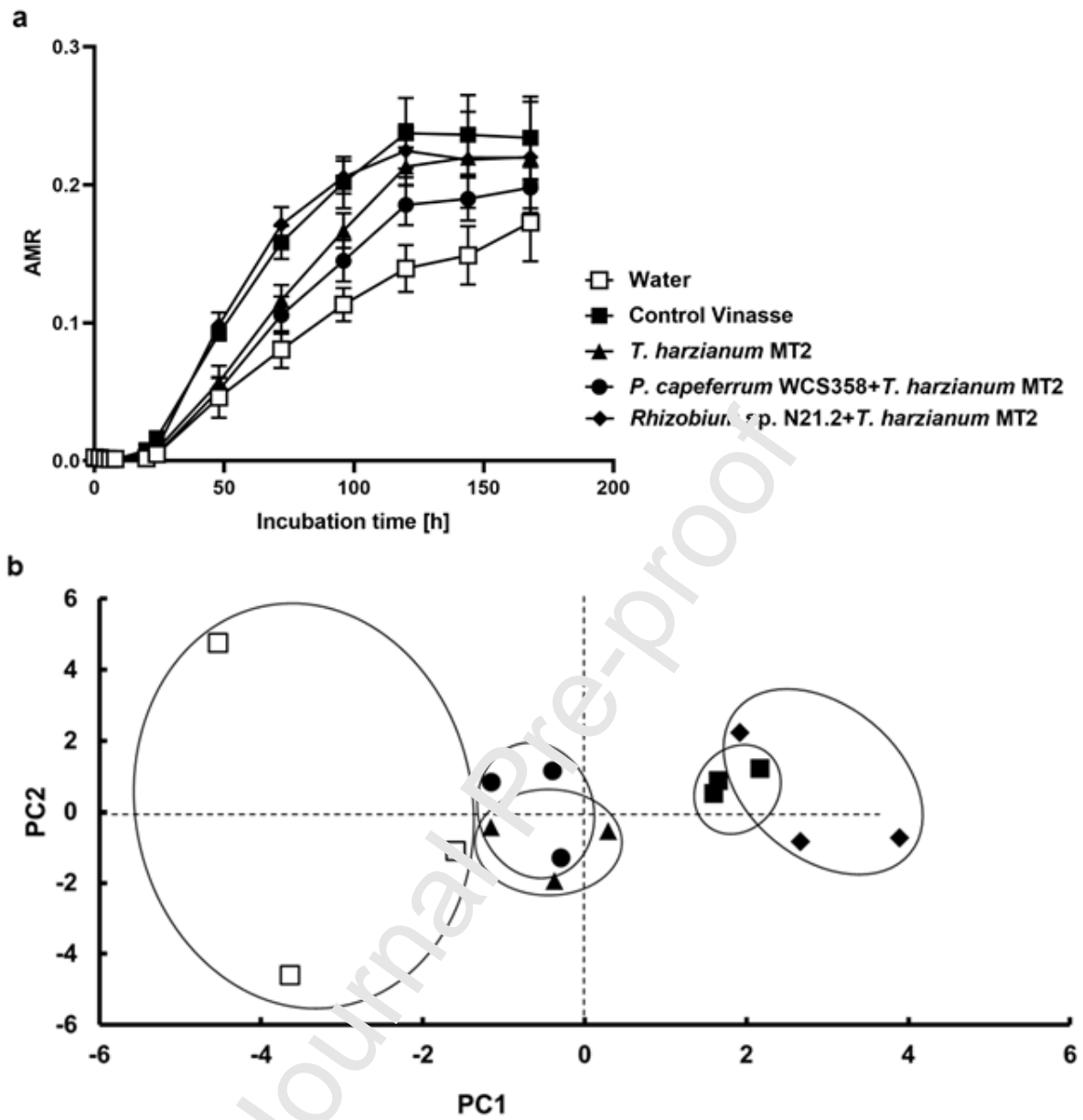
Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Table 1. Physical-chemical and chemical characterization and toxicity of control vinasse and residual vinasses[a]

	H₂O	Control Vinasse	*T. harzianum* MT2[b]	*P. capeferrum* WCS358 +*T. harzianum* MT2[b]	*Rhizobium* sp. N21.2 +*T. harzianum* MT2[b]
pH		4.73±0.04	6.47±0.14*	6.90±0.21*	6.08±0.13*
Conductivity [dS m⁻¹]		9.49±0.02	9.22±0.10*	9.34±0.08	9.36±0.0929
Dissolved solids [°Bx]		4±0	2.13±0.11*	2.13±0.15*	2.63±0.231*
Vinasse toxicity					
Germination [%]	100±20*	19.35±3.22	20.43±8.12	23.60±6.71	17.74±2.28
Hypocotyl [cm]	2.65±0.36*	1.40±0.54	2.49±0.55*	2.40±0.75*	2.66±0.48*
Radicle [cm]	4.00±0.86*	0.71±0.29	1.11±0.20*	0.92±0.46	0.98±0.32

[a] Mean values are presented with ±Standard Deviation

Values significantly different to the control vinasse (ANOVA followed by Dunnett´s post hoc test at $P<0.05$) are indicated with *.

[b] Residual vinasses obtained after the indicated culture.

Table 2. Physical-chemical and chemical characterization and toxicity of soils after treatments with control vinasse, residual vinasses or water[a]

	H$_2$O	Control Vinasse	*T. harzianum* MT2[b]	*P. capeferrum* WCS358 +*T. harzianum* MT2[b]	*Rhizobium* sp. N21.2 +*T. harzianum* MT2[b]
pH	6.82±0.05*	7.12±0.02	7.13±0.04	7.19±0.02	7.17±0.08
Conductivity [dS m^{-1}]	0.35±0.07*	0.67±0.08	0.63±0.16	0.59±0.06	0.63±0.13
CO$_3$$^{2-}$ [%]	<0.20	<0.20	<0.20	<0.20	<0.20
TOC [%]	3.86	3.94	4.11	4.16	4.13
Total N [%]	0.241	0.247	0.251	0.260	0.258
Available P [ppm]	13.5	13.0	13.2	13.4	13.4
CEC [mEq 100 g^{-1}]	16.10	15.71	16.43	16.22	16.07
Exchange Cations					
Ca^{2+} [mEq 100 g^{-1}]	10.50	10.56	10.55	10.74	10.71
Mg^{2+} [mEq 100 g^{-1}]	2.95	3.10	4.15	3.94	3.78
K$^+$ [mEq 100 g^{-1}]	1.28	1.30	1.28	1.28	1.26
Na$^+$ [mEq 100 g^{-1}]	<0.25	<0.25	<0.25	<0.25	<0.25

Soil toxicity

Germination [%]	98.89±1.96	96.63±3.89	94.38±3.37	97.75±3.37	91.01±12.15
Hypocotyl [cm]	2.18±0.42*	2.42±0.39	2.45±0.44	2.45±0.53	2.06±0.45*
Radicle [cm]	2.34±0.82	2.61±0.69	2.63±0.67	2.22±0.80*	2.24±0.67*

[a] Mean values are presented with ±Standard Deviation

Values of pH, conductivity and soil toxicity significantly different to the control vinasse (ANOVA followed by Dunnett´s post hoc test at $P<0.05$) are indicated with *.

[b] Residual vinasses obtained after the indicated culture.

Soil toxicity

CRediT author statement

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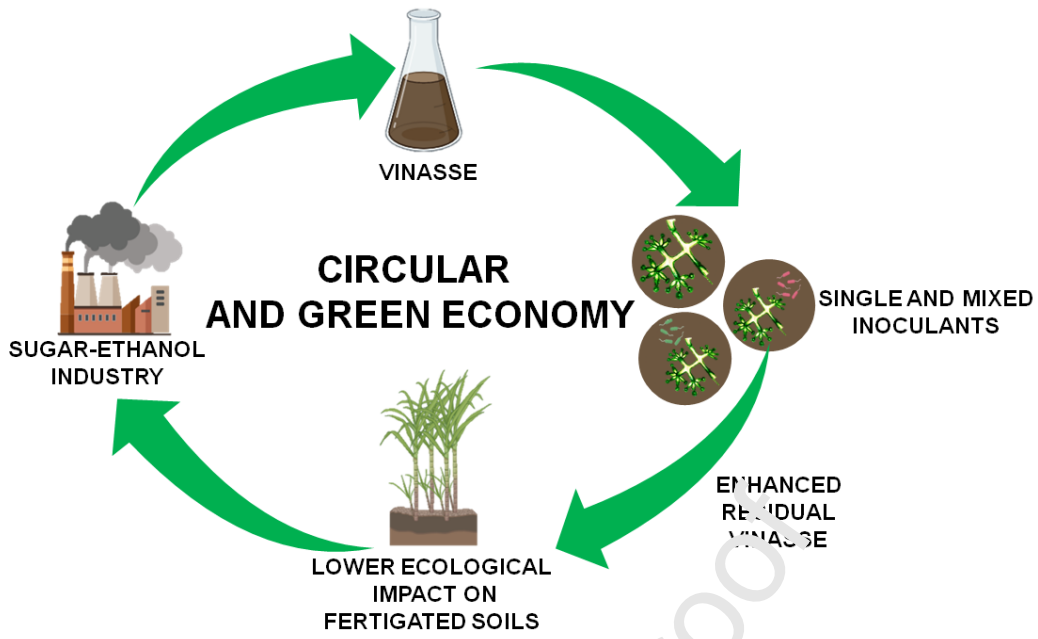
Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Graphical abstract

Highlights

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Vinasse is suitable to produce inoculants containing *Trichoderma harzianum*.

T. harzianum can be co-cultured with *Pseudomonas capeferrum* and *Rhizobium* sp.

Co-cultures enhance the characteristics of residual vinasses.

Fertigation with residual vinasses improves soil characteristics.

The utilization of vinasse as culture medium contributes to circular and green economy.

Highlights