Annals of Agricultural & Crop Sciences

Open Access

Austin Publishing Group

Special Article - Sugarcane Sustainable Production

Vinasse Waste from Sugarcane-Based Bioethanol Production Plants Kills *Moniliophthora Perniciosa*, the Causative Agent of Cacao *Witches' Broom Disease*

Ferraz P[1,2], Amorim-Rodrigues M[1,2], Cassio F[1,2] and Lucas C[1,2] *

[1]Institute of Science and Innovation for Bio-Sustainability (IB-S), University of Minho, Campus de Gualtar, Portugal

[2]Department of Biology, Centre of Molecular and Environmental Biology (CBMA), University of Minho, Campus de Gualtar, Portugal

***Corresponding author:** Lucas C, Institute of Science and Innovation for Bio-Sustainability (IB-S), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Received: February 18, 2021; **Accepted:** March 16, 2021; **Published:** March 23, 2021

Abstract

Bioethanol production based on sugarcane juice fermentation yields *vinasse*, a dark, dense liquid waste high in potassium. In Brazil, one of the world's biggest bioethanol producer, *vinasse* is used for fertirrigation of the sugar-cane fields, replacing mineral soil amendments. Nevertheless, the production largely exceeds this application, prompting exacerbated usage, unduly long-term storage and even illegal dumping. *Vinasse* thus progressively became an environmental hazard, damaging soils and superficial and ground waters, adding a negative burden to a supposedly green-fuel industry. The solution resides in decreasing production yields and/or using it for yet other economically interesting applications. This work focuses on the latter. *Vinasses* from three bioethanol plants from Brazil were tested for their ability to contain the proliferation of *Moniliophthora perniciosa*, the aggressive filamentous fungus responsible for cacao *Witches' Broom Disease* (WBD). This disease caused a severe economic fall-out in South American cacao producing regions, particularly seriously in Brazil. Immersing or spraying the mycelium with *vinasse* either kills the fungus or impedes its proliferation at varying time/dosage. Identically testing another genetically unrelated phytopathogen, showed this effect is not that of a generalized biocide/fungicide. Results suggest that *vinasse* could be used to contain/revert the prevalence of cacao's WBD to manageable levels. *Vinasse* would thus shift from industrial waste with disposal-associated costs, to being a tool for the agronomic sustainability and revival of the South American regional cacao-dependent socio-economies.

Keywords: *Vinasse*; Bioethanol; Cacao; *Witches' broom disease*; *Moniliophthora perniciosa*

Introduction

Brazil produces bioethanol mostly from yeast fermentation of sugarcane juice. The process involves the physical extraction of the juice from the cane which is then fermented by yeasts. After each fermentation cycle, the must is centrifuged, and the yeast biomass separated and recycled. The subsequent distillation process yields a dark liquid waste called *vinasse* [1]. Brazilian industry generates an average 12L of *vinasse* per litre of ethanol produced [2], meaning that each year the country generates around 300-400 billion litres of *vinasse* [1]. This residue has been used for more than 50 years for fertirrigation (fertilization + irrigation) of the sugarcane fields [1,3]. This appears to be a virtuous cycle, since *vinasse* is rich in potassium and other minerals [4], avoiding the need for chemical fertilization [5]. In the long run, *vinasse* is apparently beneficial for soil quality and sugarcane production yield [4,6-9]. But in reality, the amounts of *vinasse* produced per year are far bigger than the fields can absorb, leading to saturation. Additionally, the *vinasse's* low pH (3.5-5.0) and the corrosive nature [10] are promoting undesirable changes of soil composition and physicochemical properties [3,11], and serious contamination of ground water [12]. These problems are exacerbated by inappropriate long-term storage and illicit discharges.

The most studied possibilities of *vinasse* utilization relate with its reuse in the energy equation of the ethanol plant [4,13]. Nevertheless, *vinasse* is rich in minerals and organic matter [1,14,15], around 100-130 g/L (COD) [14], and includes sugars, organic acids, ethanol and glycerol [1,15], which suggests *vinasse* should be fit for microbial growth. Studies proposing this possibility include the cultivation of economically promising filamentous fungi such as the edible *Rhizopus oligosporus* [15], or the biotechnology relevant *Aspergilus oryzae* [16,17] and *Neurospora intermedia* [18]. In the present work, we explored yet another possibility, that of using *vinasse* for controlling the proliferation of the cacao phytopathogen *Moniliophthora perniciosa*, while used for fertirrigation of the cacao fields, as it happens with sugarcane.

M. perniciosa is a filamentous fungus that causes a devastating disease in the cacao plant (*Theobroma cacao*) and fruit known as *Witches' Broom Disease* [19]. This was responsible for the steep production fall-out in South and Central America countries, mainly in Brazil, where it posed a serious social-economic crisis in the cacao producing region. The Brazilian production fell 70% in a period of 10 years after the onset of the disease in 1989 [20,21]. This scenario had a huge impact on the regional and national economy, leading the country to shift from being the 2nd world producer to becoming a net importer of cacao beans [21,22].

Citation: Ferraz P, Amorim-Rodrigues M, Cassio F and Lucas C. *Vinasse* Waste from Sugarcane-Based Bioethanol Production Plants Kills *Moniliophthora Perniciosa*, the Causative Agent of Cacao *Witches' Broom Disease*. Ann Agric Crop Sci. 2021; 6(2): 1073.

M. perniciosa is a very aggressive fungus, virtually impossible to eradicate. Its exceptional virulence is intrinsically related to its ability to infect all the pant tissues in all the stages of the cacao plant life-cycle [20,23,24]. *M. perniciosa* is a hemibiotrophic fungus, which infectious cycle has two distinct phases: a biotrophic and a saprotrophic. After the initial infection, a series of cell death events occurs in the infected tissues causing these to become necrotic and to form a particular structure called dry broom [20]. Those necrotic or dead plant cells are then colonized by the fungus, and pink-coloured basidiocarps are produced. In alternating periods of drought and humidity, each basidiocarp can produce 2 to 3.5 million spores [25]. The spores are released mainly at night and are locally dispersed by water and over long distances by wind, and can remain latent in the soil or inside pruned plant branches for long periods of time [20,23].

Classical chemical fungicides, usually azole or copper-based compounds, are not effective against *M. perniciosa* [26]. Presently, cacao *Witches' Broom Disease* has one single functional management technique, which consists in spraying the affected plantations with Tricovab®, a *Trichoderma stromaticum* live suspension (reviewed by [24]). This other fungus is very efficient in antagonizing *M. perniciosa*, but it has to be multiplied in the surface of rice grains, being supplied lyophilized, as a service on demand, by an agro-support governmental organization (www.ceplac.gov.br). This is therefore a very expensive and unsustainable solution, only possible since it is almost fully subsidized by the Brazilian government. Alternatives could consist in using other microbes, easier and cheaper to cultivate, as biofungicides (reviewed by [24]), or possibly implementing a sustainability-prone solution controlling the fungus with an inexpensive agro-friendly industrial waste that is produced in very high amounts, and that might cumulatively contribute with mineral amendments for the cacao plants.

Three different *vinasses* were tested against two strains of the fungus originating from Brazil *via* a credited international culture collection. The fungi did not grow on *vinasse*. Accordingly, both died upon immersing or spraying with two of the *vinasse* batches, although at varying time/dosage, and were inhibited from proliferating by the third batch. This killing effect of *vinasse* was not shared by another genetically distant phytopathogenic fungus, suggesting specificity. Results support the use of *vinasse* for fertirrigation of cacao plantations as a means to, in time, clean the resident fungus inoculum. If not eradicating WBD, *vinasse* should at least reduce it to manageable levels. On the other hand, this new utilization would contribute to the alleviation of the negative unsustainable burden *vinasse* associates to the bioethanol industry.

Materials and Methods

Moniliophthora perniciosa strains CBS 441.80 and 442.80, and *Colletotrichum gloeosporioides* CBS 100471 were purchased from CBS-KNAW Collections, The Netherlands. They were maintained at 4ºC on MEA (20g/L malt extract w/ 20g/L agar), and cultivated in the same medium at 30°C. *Vinasse* was obtained from Fermentec Lda (https://www.fermentec.com.br/), and originated from three different bioethanol producing plants in the State of São Paulo, Brazil: Usina Alta Mogiana (http://www.altamogiana.com.br/), Usina Batatais (http://www.usinabatatais.com.br/) and Usina da Pedra (https://www.pedraagroindustrial.com.br/). *Vinasse* was stored at 4ºC. Prior to utilization it was centrifuged for 30min at 13.000xg and 4°C to eliminate particles in suspension, and autoclaved (121ºC, 1 atm, 20min). Growth assays were performed inoculating (i) 20mL of *vinasse* (100% *vinasse*); (ii) 10mL of *vinasse* +10mL of up$H_2$0 (50% *vinasse*); or (iii) 5mL of *vinasse* +15mL of up$H_2$0 (25% *vinasse*) - in a glass tube (Ø 3cm; 13cm height) with a one-week ME-grown fungus agar plug of approximately 0.8x0.8 cm. Tubes were incubated at 30°C and 200rpm orbital shaking. Cultures identically inoculated and incubated in liquid ME medium were used as control. Growth was assessed after 10 days, decanting the culture supernatant, and checking for mycelium development. The putative viability of any remaining fungal cells was assessed incubating this agar plug in MEA at 30ºC for 1 week. These assays were repeated in the same manner, using *vinasse* supplemented with 2% of malt extract as a supplementary nutrient source. Death along incubation time in *vinasse* was assessed using a fully-grown fungal culture previously grown in liquid ME medium in a glass tube (until the formation of a globular mycelia), which was then transferred to a glass tube containing 20mL of *vinasse*, under the same conditions described above. Each sample/incubation time corresponding to an independent tube identically inoculated from fully-grown fungal cultures. The incubation period varied between 1 and 10 days (T1 to T10), and at increasing time points, the mycelia was taken from the *vinasse*, gently wash with sterile water and its viability assayed in MEA at 30°C for 1 week. The death-inducing effect without immersing the cells in *vinasse* was performed by spraying *vinasse* on a MEA plate containing fully grown mycelia. The assay was performed for 10 days, applying a single spray at T0, or repeating it once each 24h. The plates were then incubated at 30°C for 3 days. All assays were performed at least in three independent replicates (n ≥3).

Results and Discussion

The *vinasse* used in this work originated from three bioethanol plants from the State of São Paulo in Brazil, all of which identically producing bioethanol from a sugarcane juice fermentation process. According to the literature, the composition and physical properties of *vinasses* do not differ significantly (e.g. [14,15]). The three *vinasses* used in this work have ±6 % solids in suspension (mostly ashes), pH ≈ 4.5, 0.115 ± 0.060 % (w/v) sugars and 0.9% (w/v) organic acids (information kindly supplied by Fermentec, Lda. (https://www.fermentec.com.br)), values that fall within published data.

Does *vinasse* inhibit mycelium development?

The pH of *vinasse* is adequate to support the development of *M. perniciosa* which optimal growth occurs at pH between 4.5 and 5.5 (our unpublished results), and the presence of carbon source (totaling ± 0.5% (w/v)) should allow some fungal multiplication. Therefore, *vinasse* was centrifuged, to remove the solids in suspension, and the remaining liquid fraction was sterilized and used to inoculate the two *M. perniciosa* strains. After 10 days (Figure 1a) there was no visible growth of either fungus. Microscopic inspection confirmed the absence of hyphae associated with the agar plugs used as inocula. To check whether there were still residual viable mycelia, the fungal plugs were taken from the *vinasse* (Figure 1a), re-inoculated in solid MEA medium, and incubated for a further 10 days at 30°C. As can be seen in Figure 1b, the plugs of the *vinasses* from Alta Mogiana and Batatais did not develop any growth, suggesting the fungus was killed. The fungal plug taken from the *vinasse* from Pedra developed mycelia,

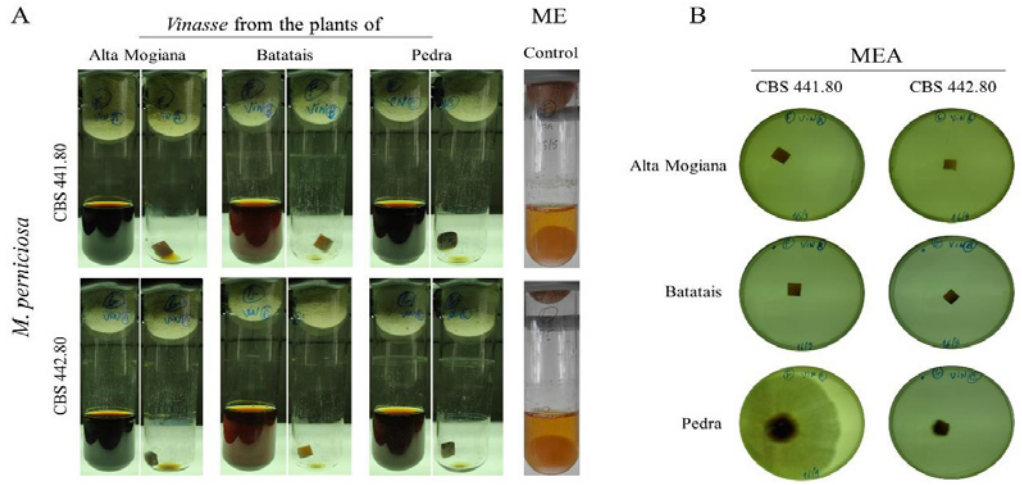

Figure 1: Two *M. perniciosa* strains originating from contaminated cacao fruits and trees in Brazil were grown in unsupplemented *vinasse* originating from three Brazilian bioethanol plants. (A) Results were scored after a 10 days incubation at 30ºC, before and after decanting the *vinasse* showing the inoculum agar plug. Control growth in identical period and temperature in MEA showing the fungal biomass ball. (B) The plugs from (A) were inoculated in MEA solid medium. Only the plugs originating from the incubation in *vinasse* from Pedra showed mycelia growth.

showing that it rather strongly inhibited mycelium development. These results show that *vinasse* from different plants, and possibly also different batches, has different effects over the *Witches' broom* fungus, either killing it or just inhibiting its development.

Are the effects dependent on dose?

To verify if lower concentrations of *vinasse* were sufficient to exert these effects, the assays were repeated using the same 3 batches of *vinasse* (i) undiluted (100% *vinasse*); (ii) diluted 1:2 (50% *vinasse*) or (iii) diluted 1:4 (25% *vinasse*). Results are presented in Table 1. The *vinasse* from Pedra plant only inhibit the growth of either fungi undiluted, while the other two *vinasses* were effective against each of the fungal strains at different dilutions. The further assessment of the viability of residual mycelia was made as above. No fungal development was observed indicating that the concentrations of *vinasse* from Alta Mogiana and Batatais that were able to inhibit fungal growth also killed the fungus. This ability was therefore shown to be dose-dependent, suggesting it relies on a specific component in the *vinasse*, and depends on the relative amounts towards those of mycelium. This points to expect that differences in strength may possibly be found from batch to batch.

Is there a fungal growth-associated bias?

Considering that the carbon source present in *vinasse* might not be sufficient to allow the fungal to develop generously, and/or that the death of mycelia could occur at such different speed that the 10 days contact with the *vinasse* would not be enough to detect death of the mycelia, two different assays were performed. The first consisted in increasing the amount of carbon source to support survival and growth in *vinasse* by adding malt extract 2% (w/v) and repeating the incubation in the same conditions used in the previous assay. Results were identical to the previous ones (not shown), indicating that the growth inhibition observed when inoculating the fungal plugs in *vinasse* is not related with nutrient deprivation. The second approach aimed to establish how fast *M. perniciosa* was dying on *vinasse*. The assay consisted in having ME-grown mycelia and replacing ME with

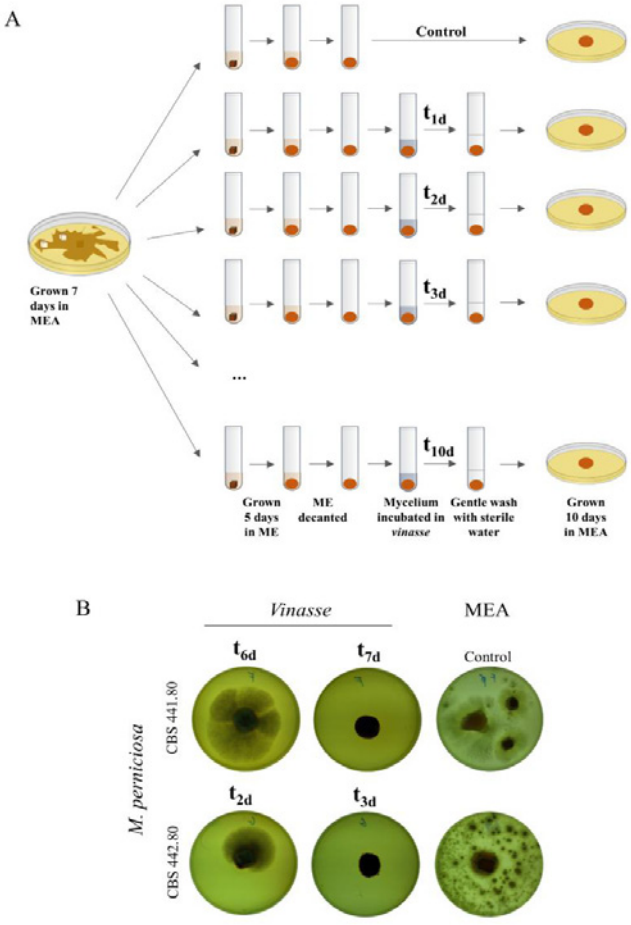

Figure 2: (A) Scheme of the procedure used to assay the effect of *vinasse* on the viability of the *M. perniciosa* along time. (B) Representative results of the turning point in the loss of viability which occurs at different time points for each fungal strain. The controls show the growth of the mycelium ball fragmented, which occurs whenever the inoculum is large.

vinasse (Figure 2a). At increasing time points, the mycelia were taken from the *vinasse* and their viability was assayed in solid MEA medium as before. This was done using the *vinasse* originating from Alta Mogiana plant, for having proved able to kill the two fungal strains. Results showed that the complete death of the mycelia from the two fungal strains indeed occurred when the fungi were immersed in *vinasse*, but at different times. The strain CBS 441.80 lost full viability between day 6 and day 7 (Figure 2b), while the strain CBS 442.80 lost full viability faster, between day 2 and day 3 (Figure 2b). *Vinasse* has thus a different time/dosage effect on each of the two strains of *M. perniciosa*, which must be considered for the putative application in the field.

Immersing or spraying?

To check whether the death-inducing effect could be obtained without the need to immerse the mycelium in *vinasse*, this was used to spray MEA fully grown mycelia. The spray was applied once every 24h for 10 days and the evolution of the mycelium was photographed (Figure 3). This time the three *vinasses* were used. Mycelium progressively shrunk, becoming darker coloured. *Vinasses* from the different plants had different time/dosage effect. The *vinasse* from Batatais killed the two strains faster than the one from Alta Mogiana, and Pedra's presented the weaker inhibitory effect. Moreover, it was also possible to verify that each *vinasse* had a different time-effect on each of the *M. perniciosa* strains, being CBS 442.80 more sensitive since the mycelium disappeared between days 5 and 10 (except when using the *vinasse* from the Pedra plant). These results are consistent with the ones obtained by immersing mycelium in *vinasse*. Results further indicate that the spraying has to be repeated over time in order to observe the desired effect, corroborating the hypothesis that the continuity of the treatment could contribute to clean the field of hidden mycelium over time.

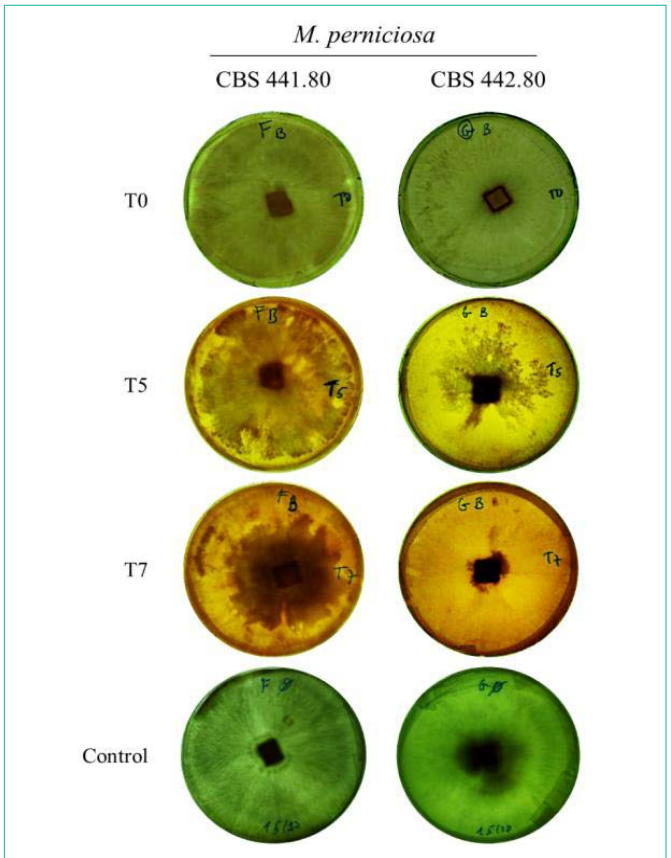

Figure 3: Chosen examples of the effect of spraying *vinasse* on the top of fully-grown mycelia on MEA once every 24h. Arrows indicate shrunk residual mycelium after 7 applications of *vinasse*.

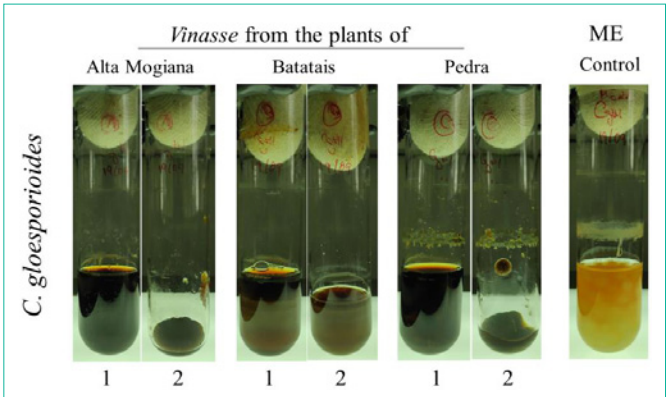

Figure 4: *C. gloeosporioides* grown in unsupplemented *vinasse* originating from three different bioethanol plants from Brazil. Inoculum was done using a fully-grown mycelia agar plug as used for *M. perniciosa*. Results were scored after 10 days incubation at 30°C (1), and after decanting the remaining *vinasse* showing the fungal mycelium that was formed (2). Control growth was performed in MEA in identical period and temperature. *C. gloeosporioides* did not form a mycelium ball, instead it spread eventually filling the full liquid volume available.

Is the effect over *M. perniciosa* specific?

Finally, as an attempt to understand whether the ability of *vinasse* to kill filamentous fungi was specific for *M. perniciosa* and/or the correspondent taxa, the assays of fungal growth in *vinasse* for 10 days were repeated using another phytopathogenic fungus, *Colletotrichum gloeosporioides* [27]. This is one of the causative agents of olive anthracnose, a disease causing serious economic losses in the Mediterranean region [28] and it was chosen because it is a very resilient fungal species. Results (Figure 4) showed that *C. gloeosporioides* was able to grow without the need for nutrient supplementation, fully occupying the volume of *vinasse* with mycelium which thus became altogether a hard-solid mass. These results indicate that the ability of *vinasse* to kill *M. perniciosa* does not correspond to a generalized antifungal activity, and therefore depends on some specific trait of *M. perniciosa* biology.

Vinasse chemical composition is very complex [13]. Future approaches might allow the identification of the compounds or chemical groups in *vinasse* that are active against *M. perniciosa*, eventually enabling the creation of an engineered simpler and more efficient solution which considers the environmental effects in the long run. Moreover, the evaluation of the potential spectrum of *vinasse* application as a specific fungicide will have to be addressed experimentally. In particular, there is one possibility that stands out, which is that *vinasse* might affect the proliferation of *M. roreri* [29], another phytopathogenic filamentous fungus genetically very close to *M. perniciosa* also causing a severe and economically threatening disease in cacao fruits known as *moniliasis* or frosty pod rot [30].

Conclusions

Vinasse is the main waste product from sugarcane bioethanol

Table 1: Growth (+) or absence of growth (-) of the *M. perniciosa* strains CBS 441.80 and CBS 442.80 when incubated in *vinasse* from the plants of Alta Mogiana, Batatais and Pedra, undiluted (100% *vinasse*) and in 2 different dilutions, 50% and 25% *vinasse*. Results presented were identical in three independent replicates.

M. perniciosa	Alta Mogiana			Batatais			Pedra		
	100%	50%	25%	100%	50%	25%	100%	50%	25%
CBS 441.80	-	-	+	-	+	+	-	+	+
CBS 442.80	-	-	-	-	-	+	-	+	+

production process. It has a very high fertilization ability due to its high N, P and K contents and has therefore been largely used to fertirrigate the sugarcane fields for decades. Nevertheless, if applied in excess it becomes a critical problem for soils and groundwater. Many suggestions in the literature regard solutions that promote the reduction of the amount of *vinasse* produced per litre of ethanol, its concentration, clearing or neutralization. Other suggestions regard the use of *vinasse* for biotechnological applications, including the growth of some economically interesting microorganisms or aerobic/anaerobic digestion. For now, the most cost-effective application for *vinasse* keeps being fertirrigation, in time improving soil quality and crop productivity [3,5], provided it is done carefully and responsibly, guaranteeing the control over all the environmental implications.

The utilization of *vinasse* has associated pollution problems that only recently are being recognized [3], but altogether it appears to be beneficial [6-9]. Our results clearly show the potential of *vinasse* to control the filamentous fungus *M. perniciosa*, cacao *Witches' Broom Disease* causative agent. The impact of the introduction of *vinasse* in the cacao agro-ecosystem needs to be studied, not only in terms of chemical balances, but also in terms of the long-term effect on the associated soil microbiota. The experience from sugarcane fields shows that the resident soil microbial community is very resilient [31,32], which comes into agreement with our finding that the inhibition of WBD by *vinasse* is not a generalized biocidal effect, which would be detrimental for the environment in the long run. The problem resides in the huge amounts of *vinasse* produced in Brazil every year, and the fact that this country was the most affected by the disease, the utilization of *vinasse* for its containment is an expressive possibility. There may be logistic and cost problems hindering the utilization of *vinasse* in the short-term deriving from the distances separating the bioethanol plants from the cacao fields, associated with this waste physicochemical properties, in particular its pH and corrosive nature. Nevertheless, these problems should in time be technically overcome in view of the advantage of contributing to solve two serious and urgent problems with a single procedure: introducing a new alternative for the disposal of high amounts of *vinasse*, reducing the serious danger to the environment and groundwater of unlawful discharges and precarious storage, and the containment of cacao *Witches' Broom Disease*. Together, the procedure should impact positively in the overall economical equation and contribute significantly to the socio-economic recovery of the regions of Brazil that most suffered with the crisis caused by cacao production fall-out in the last decades.

Acknowledgements

We thank the researchers and technicians at Fermentec Lda, for kindly supplying the batches of *vinasse* from the three ethanol plants in Brazil, and sharing valuable information on *vinasse* and the industrial bio-ethanol production process. This work was supported by the strategic programme UID/BIA/04050/2013 (POCI-01-0145-FEDER-007569) funded by national funds through the FCT I.P. and by the ERDF through the COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI), and the project EcoAgriFood (NORTE-01-0145-FEDER-000009) from Norte Portugal Regional Operational Programme (NORTE 2020) under the PORTUGAL 2020 Partnership Agreement through the European Regional Development Fund (ERDF). PF and MA-R are PhD students of the Doctoral Programmes in Applied and Environmental Microbiology (DP_AEM), (FCT grant PD/BD/113814/2015) and in Molecular and Environmental Biology (PDBMA), (FCT grant PD/BD/145354/2019), respectively.

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