Agave durangensis Vinasse as a Biocide for Forest Pest Control

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In forestry practice, the prevention and fight against insect pests and diseases is a priority to preserve the health of these ecosystems. To combat it, insecticides of chemical and biological origin have been used. However, there are alternatives that have not yet been investigated, *e.g.*, the use of agro-industrial waste. In the mezcal distillation process, polluting liquid residues called vinasses are generated, and these can be considered for pest control. In this project, the effect of vinasse from *Agave durangensis* subjected to different treatments was studied to evaluate its effect on forest phytopathogenic fungi. The mezcal vinasse was characterized physicochemically and by its metabolites. Furthermore, the percentage of inhibition *in vitro* of phytopathogenic (causing root wilt) fungi isolated from *Pinus cooperi* seedlings was studied. The fungi inhibition was related to the vinasse concentration. The lower pH and sterile raw vinasse showed a better inhibition effect. Four phytopathogenic strains of *Pinus cooperi* were isolated and identified, which corresponded to the genera *Fusarium*, *Aspergillus*, and *Penicillium*. None of the isolated were able to grow in potato dextrose-mezcal vinasse medium (PDMVM). Therefore, the mezcal vinasse showed fungicide activity *in vitro* against all strains.

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INTRODUCTION

Forest ecosystems provide various environmental services, *e.g.*, clean air and water, conservation of biodiversity, and mitigation against the effects of climate change (MacDicken *et al.* 2016). However, these have been affected by various natural disturbances, *e.g.*, pests (Montagné-Huck and Brunette 2018). There are many concerns about this because pests have an economic impact five times greater than forest fires (Logan *et al.* 2003). In addition, pathogens and pests have a high rate of adaptation, and climate change may favor outbreaks as well as their spread (Rubin-Aguirre *et al.* 2015; Wingfield *et al.* 2015; Jactel *et al.* 2019). In addition, the level of economic loss due to injuries caused by insects and forest pests is multifactorial, since they depend on the type of crop, temporal, and spatial location (Capinera 2020). Zhang *et al.* (2019) mention that diseases and pests endanger forestry. As such, the fight against insect pests and forest diseases is a priority, *i.e.*, pink mealybug (*Hibiscus mealybug*), fungus, and debarker insects (Deschamps-Ramírez 2016).

For pest control, methyl bromide was widely used as a broad-spectrum soil fumigant until 2005 when it was banned (Rai 2020). As such, neonicotinoid pesticides have become the most widely used class of insecticides in the world (Simon-Delso *et al.* 2014). However, they produce considerable environmental impact (Saeed *et al.* 2019). Since most insecticides can be lethal to non-targeted organisms, this leads to the search for chemical and non-chemical alternatives to control pests (Simon-Delso *et al.* 2014). There are various sources of biomolecules, *e.g.*, wastewater; however, the compounds present in wastewater have not been studied in-depth, since reducing pollution is the primary objective (Larif *et al.* 2015). Some residual waters from lignocellulosic biomass, liquid wastes of alcoholic fermentation called vinasses, specifically, are an alternative for preventing and controlling pests. The first reports of the use of vinasse for the control of phytopathogenic fungi dates back to 2008. In addition, it has been reported that wine vinasse showed an efficacy of 100% in terms of suppressing the growth of phytopathogenic fungi (Santos *et al.* 2008). Subsequently, sugar beet vinasse was tested for the control of nematodes in pepper crops as an alternative for disinfecting soil-borne pathogens (Núñez-Zofío *et al.* 2013). Due to this potential, vinasse should be studied as a biocide for the prevention and control of forest pests (Bailón-Salas *et al.* 2021). The raw material for making the biocide is available in Mexico. In addition, the Consejo Mexicano para la Regulación de la Calidad del Mezcal A.C. (COMERCAM) reports that the production of Mezcal in Mexico (2018 to 2019) increased by 40% (COMERCAM 2020). Therefore, its waste or by-products have also increased; in 2019 approximately 107 million liters of vinasse were generated. The vinasses contain various compounds, *e.g.*, alcohols, aldehydes, phenols, and acids (Couallier *et al*. 2006; Freitas *et al.* 2018; Fuess *et al.* 2018). These compounds can participate or assist in the process of the inhibition or control of pests (Santos *et al.* 2008; Núñez-Zofío *et al.* 2013).

It is time to change this paradigm, stop seeing vinasse supplies only as waste and treating them as by-products, as well as revaluing the “waste” and giving them another type of value *via* the sustainable management of these materials (Ordaz-Díaz *et al.* 2019).

In addition, Mexico ranks tenth worldwide in total forest area. The state of Durango is the entity with the highest forest production in the country (SRNyMA 2006; SRNyMA 2015), whose most prominent genera are *Pinus* located in the coniferous forest (SRNyMA 2015). This genus is threatened by pine wilt disease (PWD), which is responsible for environmental and economic losses (Proença *et al.* 2017). Since fungal infection can cause the transplanted young pine seedlings to fail to establish, alternatives are needed to fumigate the soil substrate and control the disease (Gordon *et al.* 2015).

In this study the objectives were to determine the effect of the metabolites and microbial biomass contained in mezcal vinasse on fungal strains related to forest pests.

EXPERIMENTAL

Sampling and Physicochemical Characterization

The raw vinasse samples were taken from a mezcal factory in Nombre de Dios, Durango, collected in high-density polyethylene containers, and conserved at a temperature of 4 °C (Fig. S1a). The digested vinasse was taken from an anaerobic digester at the Universidad Politecnica de Durango (Fig. S1b).

Several parameters were used to characterize the mezcal vinasses; the total dissolved solids (TDS), electrical conductivity (EC), and pH were determined *in situ* with an HQ40d portable device (Hach Company, Loveland, CO). The settling solids (SS) were analyzed gravimetrically. The biochemical oxygen demand (BOD_5) was determined *via* the manometric-respirometric method with a BODTrak ™ II apparatus (Hach Company, Loveland, CO). Measurement of the chemical oxygen demand (COD) was determined using the closed reflux method (colorimetric), and the turbidity was determined using the spectrophotometric method (Hach DR 5000 Spectrophotometer), to analyze the water and wastewater (Rice *et al.* 2012). Different analytes, *i.e.*, Cd, Fe, Ca, K, Mg, Na, Cr, Cu, As, Pb, Ni, and Zn, were analyzed *via* atomic absorption spectrometry (EAA) (AAnalyst™, PerkinElmer, Waltham, MA) according to standard NMX-AA-051 (2016). All determinations were carried out in triplicate.

Metabolites Identification

The melanoidins quantification was carried out using an absorbance curve *vs.* the concentration of synthetic melanoidins obtained from the glucose-glycine model, with concentrations of 18, 2, 35, 47, 60, and 70 mg/L, *via* spectrophotometry at 340 nm. For the determination of phenols, the HI3864 aminoantipyrine method (Hanna Instruments) was determinate, in site immediately after sampling according to WPCF (2005).

Phytopathogenic Fungi Strain Isolation and Identification

Young *Pinus cooperi* seedlings samples at the nursery that presented fungal disease were collected (Fig. S3a through S3c). Soil, root, trunk, and foliage samples of *P. cooperi* were sown on PDA agar (Difco ™) (Fig. 3Sd through 3Sg) and incubated at a temperature of 28 °C ± 0.5 °C. The seedlings were donated by the Facultad de Ciencias Forestales y Ambientales nursery of the State of Durango. Morphological identification was made according to the methodology outlined by Leslie and Summerell (2008), Pitt and Hocking (2009), Houbraken *et al.* (2011), and Visagie *et al.* (2014). Colonies were visibly differentiated taking into consideration their shape, colony margins, color, and surface characteristics.

Bioassays

A multifactorial experimental design with multiple levels was carried out. The selected factors were as follows: type, concentration, sterile (yes/no), and pH with levels of 3, 5, 2, and 2, respectively (Table 1). The response variable was the diameter of the inhibition zone (mm).

Table 1. Factors and Levels Analyzed for the Vinasse Samples Against Isolated *Agave durangensis* Fungus

		Factors			
	No.	Vinasse Type	Concentration (%)	Sterile	pH
Level	1	Raw vinasse	0, 25, 50,75 and 100	Yes	Neutral
	2	Dry vinasse		No	Initial
	3	Digested vinasse		-	-

For the development of the treatment, the pH of the vinasse needed to be adjusted using calcium hydroxide, and the concentrations were diluted in distilled water. The characterization of some parameters, *i.e.*, the pH, TDS, and EC, are shown in Table 2.

The *in vitro* inhibition effect of *A. durangensis* vinasse against fungal strains was analyzed because the main problem found in the studied nursery was root wilt. After the puncture inoculation and growing of the isolated fungal colonies from the root in the plates, 100 µL of mezcal vinasse was added and after incubation for 24 h, the zone of inhibition was measured through diameter inhibition zone (mm).

Table 2. Physicochemical Characterization of the Raw Stillage at a Neutral pH (adjusted) and the Anaerobically Digested Vinasse

Vinasse Type	ID	pH	SDT (ppt)	EC (mS/cm)
Raw	Initial	5.93	1.82	3.64
	Final	7.2	2.54	5.07
Digested	-	8	1	2

Another experiment was performed in which the inhibition of the soil, trunk, and foliage isolates was evaluated using potato dextrose-mezcal vinasse medium (PDMVM). The PDMVM contained PDA agar (Difco^{TM}) dissolved in 1 L of mezcal vinasse. The plates were incubated at a temperature of 28 °C ± 0.5 °C up to 24 h and 48 h. Afterward, the growth was observed in comparison with the control. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Physicochemical Characterization

The physicochemical and metabolites characterization is shown in Table S2 and metal and metalloid characterization is shown in Table S1 (see Appendix). Mezcal vinasse is safe in terms of not generating additional problems due to contamination by heavy metals and metalloids. According to the results shown in Table S1, mezcal vinasse is free of various heavy metals, *e.g.*, chromium, cadmium, lead, and nickel, and in addition, arsenic was not detected. Furthermore, mezcal vinasse is rich in calcium (575.33 mg/L), iron (15.04 mg/L), and magnesium (96.58 mg/L) (Table S1). Calcium in the soil contributes to the stabilization of organic carbon (Wan *et al.* 2021). In addition, it is widely known that the union between iron and organic carbon in the soil allows for its stabilization and storage (Yu *et al.* 2017). It has been shown that magnesium fertilizers can improve soil quality, specifically red soil (Nan *et al.* 2006). In addition, magnesium increases the availability of N and P (Velescu *et al.* 2021). Mezcal vinasse shows a copper concentration of 358.6 mg/L (Table S1). The presence of this ion is very positive for fungal control since it is a component of some biocides that protect wood from fungal attack (Schilling and Inda 2011). It is also part of commercial pesticide formulations applied in agriculture (Willis *et al.* 2016).

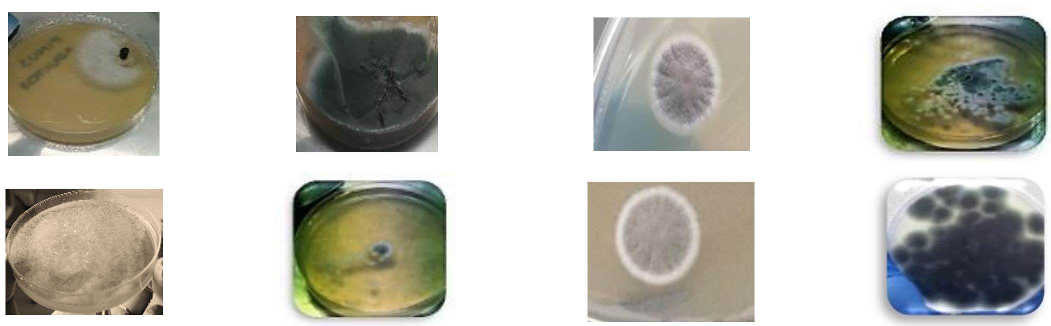
A characteristic low pH of 5.90 ± 0.05 was reported. An EC of 2.40 mS/cm ± 0.02 mS/cm was found, which was near the value reported by Mejía-Rivas *et al.* (2021). The color (4.355 ± 63.64 (Pt-Co) and turbidity (357 ± 24.88) NTU, were both related to the presence of melanoidins and polyphenols (Fitzgibbon *et al.* 1995). These results were also similar to those of Mejía-Rivas *et al.* (2021) in terms of *A. durangensis* vinasse. The biodegradable organic material fraction was very low, with a BOD_5 of 719 mg/L ± 4.24 mg/L and a COD of 19410 mg/L ± 70.71 mg/L. This left a high concentration of non-biodegradable organic matter.

Metabolites Identification

Figure S2 shows the curve *vs.* the concentration of synthetic melanoidins. The equation shows an adjustment coefficient of 0.99. The melanoidin concentration was 473.75 mg/L ±14.50 mg/L, while the phenol concentration was 1.30 mg/L ± 0.08 mg/L (as shown in Table S2). Phenolic compounds can inhibit microorganisms and are the responsible for the mortality of some pests (Larif *et al.* 2013; Freitas *at al.* 2018). It is suggested in the future to investigate and identify biologically active ingredients with a potential fungicidal or fungistatic effect.

Phytopathogenic Fungi Strain Isolation and Identification

Four strains were isolated in total; 1 from the root, 1 from the trunk, 2 from the foliage, and 3 from the soil. The isolates correspond to the *Fusarium*, *Penicillium*, and *Aspergillus* genera (Fig. 1). The fungal strain isolated from the root showed macroconidia and hyphal coils. Furthermore, white cottony aerial mycelium was present (Fig. 1). According to Leslie and Summerell (2008), this strain corresponds to the specie *Fusarium circinatum*. This pathogen has been reported to affect *Pinus* species and some other conifers in Mexico. *Fusarium circinatum* is one of the most important pathogens of *Pinus spp.*, as it causes pitch canker (Wingfield *et al.* 2008).



(a) (b) (c) (d)

Fig. 1. The identified strains: a) *Fusarium circinatum*; b) *Aspergillus sp.*; c) *Penicillium sp.*; and d) *Aspergillus sp.*

Table 3. Morphological Characterization of the Isolate Strain in PDA Culture Media

ID	Size	Color	Form
a	Unlimited (covers all medium)	White	Dry, flat, and velvety
b	Unlimited (covers all medium)	Green, and white mycelial halo	Dry, flat, and velvety
c	Unlimited (covers all medium)	Grey, and white mycelial halo	Dry, edges within the colony, and velvety
d	Unlimited (covers all medium)	Green, and white mycelial halo	Dry, flat, and velvety

These three genera (Fig. 1) are of importance, since they are pathogens that decrease the viability of *Pinus cooperi* seedlings. Furthermore, the *Aspergillus* genus is the second most pathogenic fungus that affects the germination of *Pinus* seeds, followed by *Fusarium* (Ishtiaq *et al*. 2015). In addition, the three fungi isolated are fungi that have been found in the seeds of *Pinus* species and all cause a decrease in quality (López *et al*. 2021). Considering what was reported by other authors, it was decided to carry out the tests only on the fungus *Fusarium circinatum*, considered the most pathogenic for the selected pine species.

Bioassays

The inhibition percentage determination analysis is shown in Fig. 2. *Fusarium circinatum* was inhibited by raw vinasse treatment at a pH of 5.9. It did not show inhibition with the other three treatments, *i.e.*, raw vinasse with neutral pH, dry vinasse, and digested vinasse. This can be attributed to the fact that the raw vinasse with neutral pH was adjusted with calcium hydroxide, which precipitated the compounds that are involved in the inhibition. Calcium hydroxide exerts the precipitation of melanoidins in *A. durangensis* vinasse (Mejía-Rivas *et al*. 2021). As for the dry vinasse, this may be due to the evaporation of all the phenolic compounds present in the sample, leaving only the effect of the melanoidins. Therefore, the melanoidins present in the mezcal vinasse do not have a fungicidal effect. Only coffee melanoidins have been reported to have a bactericidal effect (Rufián-Henares and Cueva 2009). In addition, the digested vinasse has been anaerobically biotransformed and no longer contains the inhibitory compounds to the fungus.

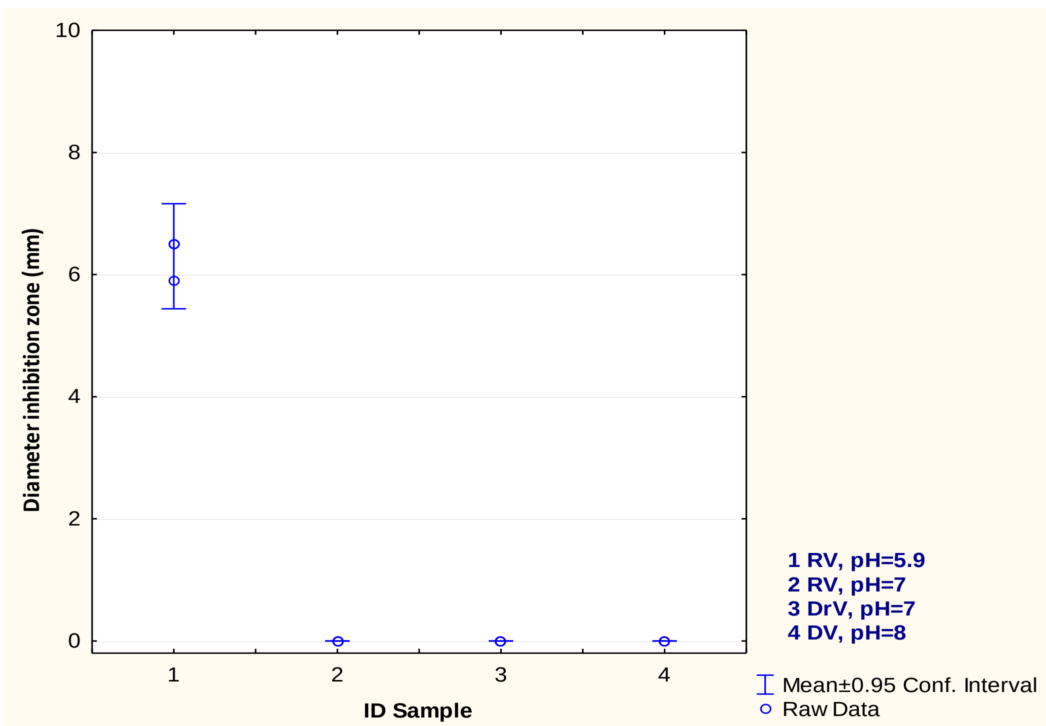


Fig. 2. *In vitro* control effect of *F. circinatum* with 100 µL at 24 h of 1) raw vinasse at a pH of 5.9; 2) raw vinasse at a neutral pH; 3) dry vinasse; and 4) digested vinasse

The effect of the mezcal vinasse concentration against fungal strain showed that the higher the concentration, the greater the inhibition (Fig. 3). However, this was observed at concentrations greater than 75%.

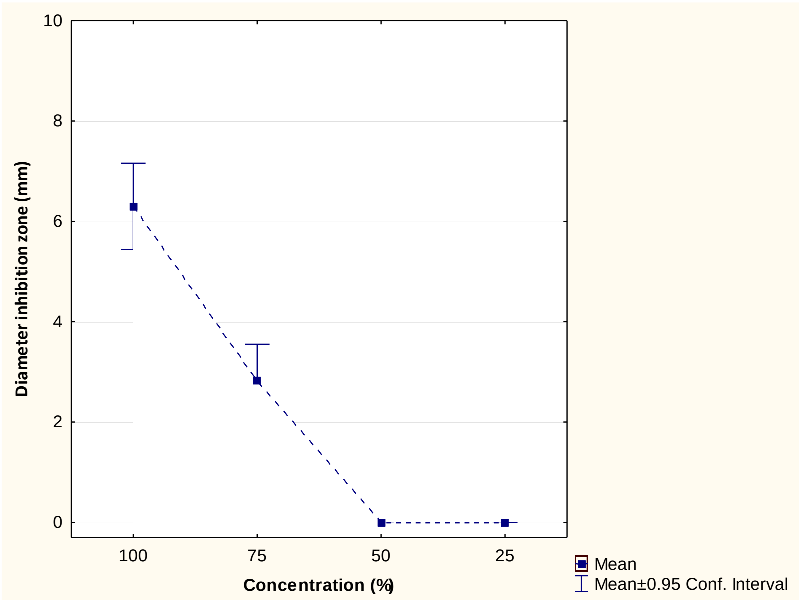


Fig. 3. *In vitro* control effect of *F. circinatum* with 100 µL at 24 h at different concentrations of mezcal vinasse

The sterile raw vinasse showed a better inhibition effect against *Fusarium*. This can be attributed to the fact that the sterile vinasse does not contain active microbial biomass that can grow in the medium (Fig. 4).

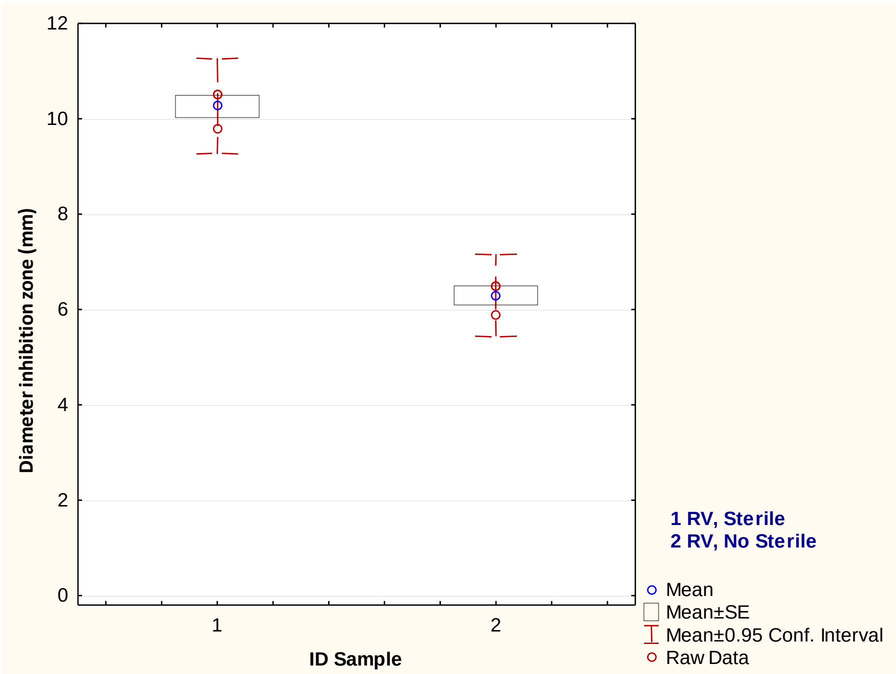


Fig. 4. *In vitro* control effect of *F. circinatum* using sterile raw vinasse and non-sterile vinasse

None of the four isolated fungi were able to grow in PDMVM (Table S3). Therefore, vinasse could be used as an alternative to heat treatment in commercial nurseries, as suggested by Berbegal *et al.* (2015).

CONCLUSIONS

1. Four fungi were identified in *Pinus cooperi*, which correspond to the genera *Fusarium*, *Penicillium sp.*, and *Aspergillus*. It was decided to carry out the tests only on the fungus *Fusarium circinatum,* considered the most pathogenic for the selected pine species.
2. The percentage of inhibition of the fungus *Fusarium circinatum* is directly proportional to the vinasse concentration.
3. A lower pH favors fungi inhibition. It is necessary to consider the effect of long-term stillage application on soil and plants.
4. Sterile raw vinasse showed a better inhibition effect. All isolates were unable to grow on potato dextrose-mezcal vinasse medium (PDMVM), which was supplemented with vinasse.
5. The melanoidins present in the mezcal vinasse do not have a fungicidal effect.

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APPENDIX

Supplemental Material

Fig. S1. Sampling of the a) storage pit; and b) anaerobic digester of the mezcal vinasses

Table S1. Metal and Metalloid Characterization

Parameter	Sample	Unit	QL
Cd	ND	mg/L	0.5
Fe	15.04	mg/L	0.5
Ca	575.33	mg/L	20
Mg	96.58	mg/L	10
K	1690	mg/L	10
Na	134.22	mg/L	20
Cr	ND	mg/L	0.5
Cu	358.6	mg/L	10
As	ND	mg/L	0.1
Pb	ND	mg/L	1
Ni	ND	mg/L	4
Note: ND: Not detectable quantification limit; and QL: quantification limit			

Table S2. Physicochemical and Metabolites Characterization

Parameter	Sample				
	1	2	3	Mean	SD
pH	5.9	5.9	5.8	5.90	0.05
EC (mS/cm)	2.4	2.42	2.4	2.40	0.02
TDS (ppt)	1.2	1.18	1.2	1.20	0.01
Turbidity (NTU)	384	335	352	357	24.88
COD (mg/L)	19460	19360	-	19410	70.71
BOD_5 (mg/L)	722	716	-	719	4.24
Color (Pt-Co)	4310	4400	-	4355	63.64
TS (mg/L)	26200	25220	-	25710	692.96
TVS (mg/L)	21690	21160	-	21425	374.76
TFS (mg/L)	4510	4060	-	4285	318.19
Melanoidins (mg/L)	463.5	484.0		473.75	14.50
Phenols (mg/L)	1.25	1.4	1.25	1.30	0.08

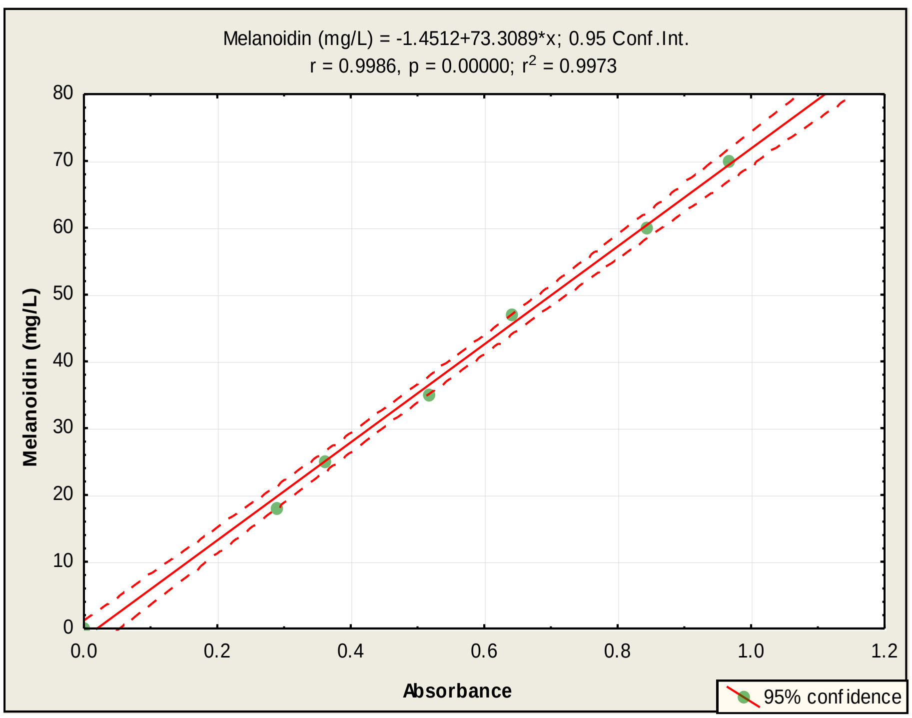


Fig. S2. Absorbance *vs* concentration of the synthetic melanoidins

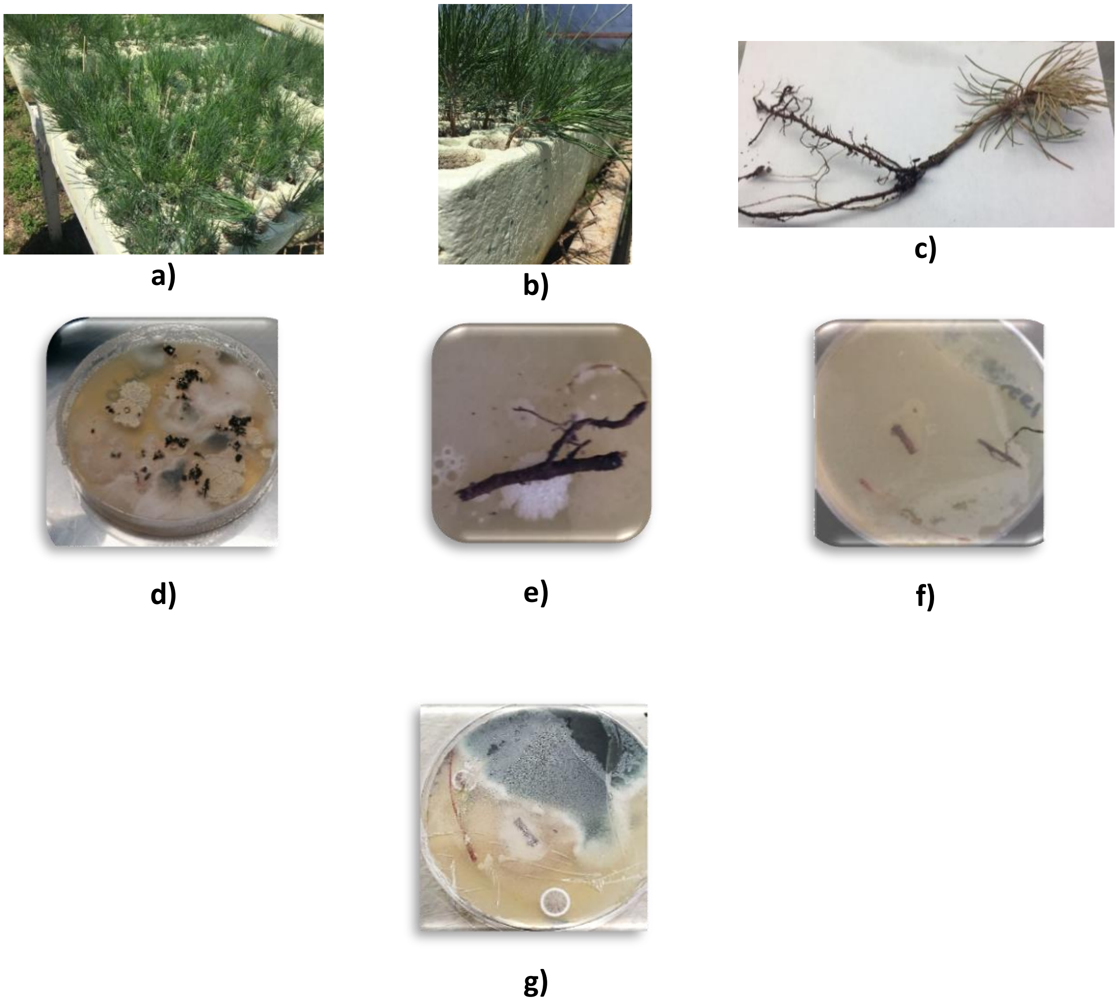
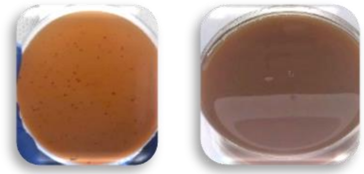
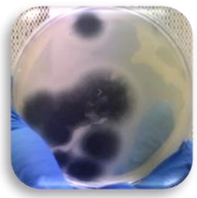
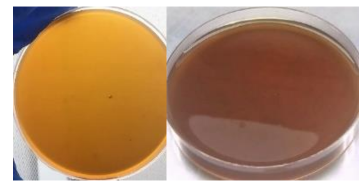
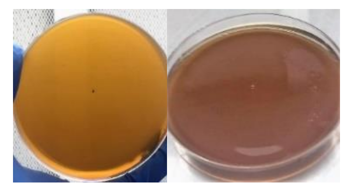


Fig. S3. Sampling of the infected young pine seedlings with isolated fungi *Pinus cooperi*: a) Healthy *Pinus cooperi* seedlings; b) Diseased *Pinus cooperi* seedlings; c) Root wilt; d) soil sample in PDA medium at 48 h of incubation; e) root sample in PDA medium at 24 h of incubation; and sample of the trunk root and aerial part in PDA medium at f) 24 h; and g) 48 h of incubation

Table S3. Evaluation of the Inhibitory Effect of *A. durangensis* Vinasse Against 4 Isolated Fungal Strains of *P. cooperi* at 24 h and 48 h of Incubation

Strain ID	24 h		48 h	
	Control	*A. durangensis* vinasse effect	Control	*A. durangensis* vinasse effect
a (*Fusarium circinatum*)				
b (*Aspergillus sp.*)				
c (*Penicillium sp.*)				
d (*Aspergillus sp.)*