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Research Article

Arbuscular mycorrhizas accelerate the degradation of colour containing organic pollutants present in distillery spent wash leachates

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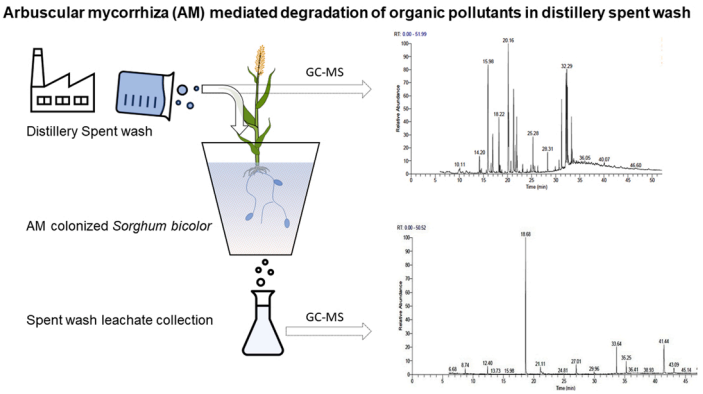
HIGHLIGHTS

- Mycorrhizal degradation of organic pollutants in spent wash was studied.
- Mycorrhizal and non-mycorrhizal *Sorghum bicolor* were fertilized with spent wash.
- Raw spent wash contained 65 complex coloured organic compounds.
- Non-mycorrhizal plants reduced complex coloured organic compound number to 42.
- Mycorrhizal plants reduced the number to only 26 colourless organic compounds.

GRAPHICAL ABSTRACT



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ABSTRACT

Distillery spent wash (DSW) from molasses-based distilleries is being used as a low-cost alternative to chemical fertilizers in countries like India and Brazil. However, using DSW as a fertilizer substitute causes organic pollutant leaching, including melanoidins and caramel colourants that turn bodies of water dark brown. This study investigated the arbuscular mycorrhiza (AM) mediated degradation of organic pollutants in DSW.

Mycorrhizal and non-mycorrhizal *Sorghum bicolor* were grown in microcosms for 16 weeks. The plants were fertilized with either raw DSW or Hoagland solution. Leachates draining from the microcosms after fertilization were collected three times in 30-day intervals. Each 30-day collection was preceded by two fertilizations. A gas chromatography-mass spectrometry comparative analyses of raw DSW with leachates of the third collection from mycorrhizal and non-mycorrhizal microcosms was made. Sixty-five and 42 complex organic compounds were detected in raw DSW and leachate collected from the non-mycorrhizal pots respectively. Only 26 compounds were detected in leachate collected from mycorrhizal pots. Absent from leachate of the mycorrhizal pots were: colour-containing organic compounds diacetone alcohol; 3-amino-2-cyano-6-methyl-6,7-dihydrothieno[2,3-*b*] pyrazine S-oxide; cyclohexane; 1,2-benzenedicarboxylic acid, butyl 8-methylnonyl ester; 2-pyrrolidinone; and

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acetic acid, dodecyl ester present in raw DSW. The results indicate that AM fungi can degrade organic pollutants in DSW.

1. Introduction

Molasses based ethanol producing distilleries are important affiliated industries of agriculture that contribute significantly to the socio-economic development of many countries [27]. However, they are water intensive and generate large volumes of wastewater in the form of distillery spent wash (DSW). For instance, 8 – 15 litres of DSW is generated as waste effluent for every litre of alcohol produced [39].

Molasses based DSW has high biological and chemical oxygen demands of 45,000–55,000 mg L^{-1} and 80,000–120,000 mg L^{-1} respectively [20]. It contains melanoidins, caramel colorants, total dissolved solids (10,480 mg L^{-1}), phenolics (510 mg L^{-1}), sulphates (3786 mg L^{-1}) and phosphates (739 mg L^{-1}; [10,15,53]). The melanoidin and caramel colorants generated when the sugarcane molasses are processed and distilled leave the DSW with a dark brown colour. [15,64,9].

DSW has been used as a source of organic carbon and plant nutrients in agroecosystems [25,26,29]. High concentrations of N, P, K and Ca in spent wash applied to fields at doses of 125 m^3 Ha^{-1} or lower significantly improve growth and yield of crops, but at higher doses (> 250 m^3 Ha^{-1}) it can be harmful to plant growth and adversely affect soil properties [10,29,39,45,9].

Distillery spent wash has become a low-cost alternative to chemical fertilizers in countries such as India and Brazil, as it is readily available in large quantities [23,26,27]. However, using it as a fertilizer substitute can lead to potentially toxic organic compounds accumulating in groundwater and water bodies by leaching, which can impact the endocrine and reproductive systems in animals [19,49,59,60]. The water in wells and streams contaminated with DSW leachates turn dark brown, which makes them unesthetic and unfit for human consumption. Moreover, high nutrient load causes eutrophication of water bodies and the coloured water poses threats to aquatic life by blocking out sunlight and inhibiting photosynthesis, thereby reducing oxygenation [39].

DSW leaching into groundwater is amongst the largest causes of soil and groundwater pollution in agroecosystems [62]. While the European Union has prohibited application of distillery sludge on agricultural land (EU 86/276/EEC), the Indian Central Pollution Control Board has issued directives to distilleries to achieve zero liquid discharge [14]. In the Indian state of Maharashtra, distilleries have been ordered to provide potable water to affected villages where water has been polluted by DSW leachates [5]. Indeed, Indian environmental tribunals have ordered offending distilleries to pay damages in the range of US$ 23,000 – 825,000 to restore the bodies of water polluted by DSW leaching (*Ashok Gabaji Kajale v M/s Godhavari bio-refineries limited* NGT 68/2014; *Goa Foundation v Department of Mines and Geology & Ors* NGT 107/2019; [11]). There is, therefore, an urgent need to find biological solutions to prevent groundwater pollution caused by DSW seepage and other agricultural inputs, and to decontaminate DSW polluted soil.

Degradation of DSW by physical or chemical methods such as ozonation, chemical decolourization, flocculation treatment, activated carbon absorption and activated sludge treatment have high costs and generate secondary pollutants [9,65]. Conventional biological treatment methods are rendered ineffective because the antioxidant properties of melanoidins make them toxic to aquatic macro- and microorganisms [15,34,54,55]. However, fungal species of *Coriolus*, *Aspergillus*, *Phanerochaete* as well as bacterial species of *Pseudomonas*, *Bacillus*, *Alkaligenes* and *Lactobacillus* are known to degrade and decolourize melanoidins [33,34,42,47,64]. While treating DSW biologically using fungal and bacterial species has achieved varying levels of success in degradation, their role in prevention or reduction of DSW leaching into groundwater is not yet studied.

Arbuscular mycorrhizal (AM) fungi are important constituents of the soil microflora in agroecosystems [63] and serve as an interface between plant roots and the abiotic and biotic components of the soil [35,51]. AM fungi are widespread in terrestrial ecosystems and form symbiotic associations with two-thirds of terrestrial plant species [56]. Through their extensive mycelial network in soil, AM fungi assimilate and transmit soil nutrients to the plant host in exchange for photosynthetic C [57,67,68]. Importantly, these fungi reduce leaching losses [13,7]. They can reduce P leaching by 50% and N leaching by up to 40% [37]. It is hypothesized that the AM fungal characteristic of exploring and acquiring nutrients in the soil [58] could be exploited to reduce leaching of melanoidin and organic pollutants in DSW into groundwater.

This study investigated whether inoculation with AM fungi could (i) reduce leaching of melanoidin and organic pollutants to groundwater; (ii) affect physio-chemical properties of DSW; (iii) degrade organic compounds in molasses-based DSW; and (iv) be developed as an effective bioremediation tool for agricultural soils contaminated with DSW.

2. Materials and methods

2.1. Plant and fungal materials

Sorghum bicolor var. Maldandi M-35–1 (Maharashtra State Seeds Corporation Limited) was used as host plant. A consortium of mycorrhizal inoculum was prepared by mixing *Rhizophagus irregularis* MUCL 41833 pot culture in a 1:1 ratio with rhizosphere of *S. bicolor* growing in agricultural fields at Sakarwadi, Maharashtra, India (28°30'47.3"N, 77°18'51.5"E). This inoculum consortium contained 24 spores g^{-1} and included AM fungal phylotypes Glo1 – 7 (Supplementary S1.1; S1.2).

2.2. Experimental Design

Locally collected black cotton soil was air dried and autoclaved at 121 °C for two hours. Microcosms were prepared by transferring 7.5 Kg portions of autoclaved black cotton soil to individual plastic pots (10-inch diameter). Each pot was provided with a ~1 cm hole at the bottom. The hole was covered with a 0.45 mm mesh that allowed water to pass through but not the black cotton soil particles. Fifty grams of viable or autoclaved (121 °C for 2 h) mycorrhizal inoculum was added to each pot and overlain with autoclaved soil giving each a final weight of 8 Kg. Pots that received viable mycorrhizal inoculum were labelled as mycorrhizal and those that received autoclaved mycorrhizal inoculum as non-mycorrhizal. Each non-mycorrhizal pot received 10 mL inoculum wash to correct for possible differences in bacterial and non mycorrhizal fungal communities [31]. The inoculum wash was prepared by suspending mycorrhizal inoculum in sterile deionized water at a ratio of 1:6 and running it through a series of sieves, the smallest of which had a pore size of 8 μm.

S. bicolor seeds were washed with liquid detergent and surface sterilized with 1.25% sodium hypochlorite for five minutes. Surface sterilized seeds were rinsed with sterile deionized water and sown in trays containing 0.8% agar. Germinated seedlings were transferred to microcosms after five days. Each microcosm received five seedlings. Prepared microcosms were placed on a raised stand.

S. bicolor plants were subjected to four treatments: (i) treatment I, non-mycorrhizal plants fertilized with Hoagland solution [28]; (ii) treatment II, mycorrhizal plants fertilized with Hoagland solution; (iii) treatment III, non-mycorrhizal plants fertilized with molasses based raw DSW; and (iv) treatment IV, mycorrhizal plants fertilized with molasses based raw DSW. With 10 replications for each treatment, there were a total of 40 pots. Molasses based DSW was obtained from M/s Godavari Biorefineries Limited, Maharashtra. The physico-chemical

characteristics of the DSW used in this study are presented in Supplementary S2.

2.3. Plant growth conditions

Plants were grown at ambient temperature (25 – 40 °C) with a day/night cycle of 16 – 8 h. Soil moisture in the pots was maintained at 15 – 20% of the pot weight. Fifteen days after transplanting seedlings, pots of treatments I and II were fertilized with 10 mL of Hoagland solution and pots of treatments III and IV were fertilized with 10 mL of raw DSW respectively. This was followed with fertilization every 15 days until the end of the experiment. Pots were randomised weekly.

2.4. Collection of leachates

Prior to fertilization, a beaker was placed below each microcosm, to collect the leachate. After 30 days, leachate in each beaker was transferred to a dark coloured bottle, and the beaker was again placed below the microcosms. The 30-day cycle was repeated twice more, giving a total of three leachate collections from each microcosm, and labelled leachate I, leachate II and leachate III respectively. During each 30-day collection cycle, microcosms received two fertilizations and were watered ten times with double deionized water.

2.5. Harvest

Plants were harvested after 16 weeks. Roots were washed with tap water to remove the adhering soil particles. Fresh weights of shoots and roots were measured separately. One subsample of the roots was set aside to determine AM fungal colonization. A second subsample of roots was stored at −20 °C (until analysis) for the extraction of genomic DNA and amplification of the AM fungal 18 S ribosomal RNA gene to identify AM fungal phylotypes (Supplementary S1.1; S1.2). Shoots and roots were dried at 70 °C for five days and weighed again to determine the dry weight. Dry weights of root subsamples were corrected to account for the removed subsamples.

2.6. AM fungal root colonization

Plant roots were cleared in 10% KOH and stained with 0.05% trypan blue. Percent AM fungal colonization in the roots was estimated following the line intersection method [38]. Mycorrhizal dependency [48] of the plants was calculated using the equation

$$\left(\frac{(biomass\ of\ mycorrhizal\ plant) - (biomass\ of\ non\ mycorrhizal\ plant)}{biomass\ of\ mycorrhizal\ plant}\right) \times 100$$

Dried shoot and root tissues were acid digested using the H_2SO_4−peroxide digestion [1]. Phosphorous (PO_4−P) and nitrogen (NO_3−N) concentrations in root and shoot tissues were determined following the molybdenum blue and indophenol blue methods [12,16] respectively.

2.7. Physico-chemical analyses of leachates

NO_3−N and PO_4−P concentrations in leachates collected from mycorrhizal and non-mycorrhizal pots were estimated as above.

Absorbance of leachates was measured at 475 nm using a Thermo Fisher Scientific Gensys 10-S spectrophotometer (USA). Total dissolved solids (TDS) and electrical conductivity of leachates were measured following the potentiometric method [2].

2.8. Gas chromatography – mass spectrometry (GC-MS) analyses of leachates

2.8.1. Liquid–liquid extraction

Raw DSW (used for fertilization) obtained from the distillery and leachate III collected from non-mycorrhizal and mycorrhizal microcosms of treatments III and IV respectively, were analysed for organic compounds. Samples were extracted following Bharagava & Chandra, [8]. A 10 mL aliquot of sample was acidified (pH < 2.0) with 35% HCl and transferred to a separatory funnel. An equal volume of ethyl acetate was then added, and the mixture was shaken continuously for 5 h with intermittent pause for liquid-liquid extraction. This was repeated three times until all organic pollutants were extracted from the leachate. The separated organic layers from the sample were kept together and concentrated on a rotatory evaporator (Rotavapor RE 120, Buchi, Flawil, Sweden) at ≤ 40 °C until the solvent completely evaporated. A 3 mL aliquot obtained from the concentrate was dissolved in 2 mL of 99% methanol and filtered using 0.22 μm filters (Millipore Ltd., Bedford, MA, USA).

2.8.2. Derivatization of extracted samples

A 300 μL volume of extracted samples was transferred to gas-chromatograph vials and dried with nitrogen. Pyridine (50 μL) was added to the dried samples followed by silylation with 80 μL trimethylsilyl (TMS); N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) and trimethyl chlorosilane (TMCS). The mixture was then heated at 70 °C for 30 min with periodic shaking to dissolve the residues.

2.8.3. GC-MS analysis

Dissolved residues were analysed as TMS derivatives [40] using GC-MS (Thermo Scientific, FL, USA). Derivative samples (2.0 μL) were injected into a DB-5 MS capillary column (30 m × 0.25 μm I.D. × 0.25 mm film thickness) containing 5% phenyl and 95% methylpolysiloxane. Helium at a flow rate of 1.1 mL min^{-1} was used as the carrier gas. Organic compounds were conducted in full-scan mode using a mass range of 45–800 amu. Selected peaks were identified using the mass spectral database library of the National Institute of Standard and Technology (NIST), USA v1.0.0.12 provided with the instrument.

2.9. Statistical Analyses

Differences in the means were analysed with a one-way analysis of variance (ANOVA) followed by a Tukey's (HSD) post-hoc test. Significance was at $p \leq 0.05$. Statistical analyses were done using R version 4.2.0.

3. Results

3.1. Plant biomass and AM fungal root colonization

Mycorrhizal and non-mycorrhizal *S. bicolor* fertilized with DSW had significantly higher shoot and root biomasses ($F_{7,\ 72} = 435.65$; $p < 0.0001$) compared to plants fertilized with Hoagland solution. Moreover, shoot and root biomasses of mycorrhizal plants fertilized with DSW (treatment IV) or Hoagland solution (treatment II) were higher than those of the corresponding non-mycorrhizal plants from treatments I and III respectively (Fig. 1). Percent root AMF colonization of *S. bicolor* was similar in mycorrhizal plants fertilized with DSW or Hoagland solution (Fig. 2a). However, plants fertilized with Hoagland solution had higher mycorrhizal dependency compared to plants fertilized with DSW ($F_{1,\ 18} = 6.34$; $p = 0.02$; Fig. 2b).

3.2. Nutrient content

Mycorrhizal and non-mycorrhizal plants fertilized with DSW had higher shoot and root NO_3−N content compared to plants fertilized with

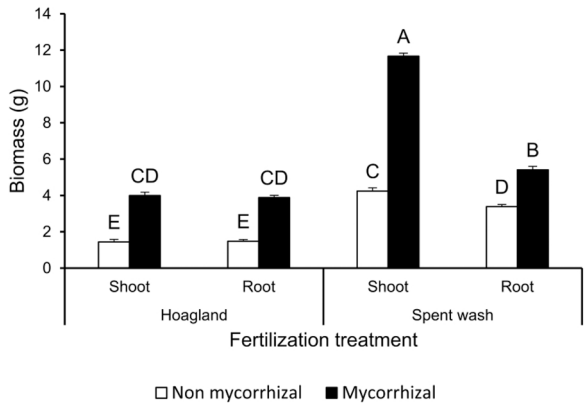


Fig. 1. Shoot and root biomasses of mycorrhizal and non-mycorrhizal *S. bicolor* plants fertilized with Hoagland solution or distillery spent wash. Values are average of 10 replicates. Error bars represent standard error of the means. Values that do not share an alphabet are significantly different ($p \leq 0.05$) according to Tukey's HSD post-hoc test.

Hoagland solution ($F_{7,\ 72} = 844.13$; $p < 0.0001$). Shoot and root tissues of mycorrhizal plants fertilized with DSW (treatment IV) or Hoagland solution (treatment II) had higher NO_3−N than the corresponding non-mycorrhizal plants of treatments I and III respectively (Fig. 3a).

Shoot and root PO_4−P content was higher in mycorrhizal and non-mycorrhizal plants fertilized with DSW compared to plants fertilized with Hoagland solution ($F_{7,\ 72} = 2378.40$; $p < 0.0001$). Mycorrhizal plants of treatment II and treatment IV had higher shoot and root PO_4−P content than the non-mycorrhizal plants (treatments I and III respectively) (Fig. 3b).

3.3. Physico-Chemical analyses of Leachates

3.3.1. N and P content in leachates

NO_3−N and PO_4−P concentrations were higher in leachate collected from microcosms fertilized with DSW compared to those fertilized with Hoagland solution (Fig. 4a, b). In both fertilization regimes, NO_3−N and PO_4−P concentrations were highest in leachate I and lowest in leachate III respectively. AM fungi significantly reduced NO_3−N and PO_4−P content in the leachates. No differences were found in NO_3−N and PO_4−P concentrations in leachate I collection between the mycorrhizal and non-mycorrhizal microcosms. The NO_3−N and PO_4−P concentrations in leachates II and III were lower in mycorrhizal pots compared to non-mycorrhizal pots ($F_{11,\ 108} = 3374$, $p < 0.0001$ and $F_{11,\ 108} = 13191$, $p < 0.0001$ respectively). In leachate II and III, AM fungi reduced NO_3−N concentration by 16% and 33% and 14% and 41%, respectively, where leachates were from pots fertilized with Hoagland solution or DSW. PO_4−P concentration in leachate II and III from mycorrhizal pots fertilized with Hoagland solution or DSW was 11% and 47% and 13% and 32% lower respectively, compared to those collected from the non-mycorrhizal pots. Leachate volume in leachate III was lower from mycorrhizal pots than in the non-mycorrhizal pots ($F_{11,\ 108} = 699$, $p < 0.0001$) for both fertilization regimes (Fig. 4c).

3.3.2. Pigments, TDS and electrical conductivity

Absorbance, TDS and electrical conductivity of leachate collected from mycorrhizal as well as non-mycorrhizal plants decreased over time from leachate I to leachate III. Leachate III had lower absorbance, TDS and electrical conductivity compared to leachate I (Fig. 5a−c). Leachates of microcosms fertilized with DSW were more optically dense compared to leachates of microcosms fertilized with Hoagland solution ($F_{11,\ 108} = 249.75$; $p < 0.0001$). Arbuscular mycorrhizas accelerated decolourization of DSW leachate. Leachate I from mycorrhizal and non-mycorrhizal microcosms had similar absorbances. However, leachates II and III from treatment IV had significantly lower absorbance than those from treatment III respectively. Indeed, leachate III from mycorrhizal plants fertilized with DSW had absorbance similar to that of mycorrhizal plants fertilized with Hoagland solution (Fig. 5a).

TDS levels were also higher in the leachates collected from plants fertilized with DSW compared to those fertilized with Hoagland solution ($F_{11,\ 108} = 820.65$; $p < 0.0001$). No differences were observed in TDS in leachate I collection of mycorrhizal (treatment IV) and non-mycorrhizal (treatment III) microcosms. But leachates II and III from treatment IV had lower TDS than plants of treatment III. TDS content in leachate III from mycorrhizal plants fertilized with DSW was similar to TDS content of leachate III collected from mycorrhizal plants fertilized with Hoagland solution. However, in the case of non-mycorrhizal plants, TDS content of leachate III collected from plants fertilized with DSW was higher than plants fertilized with Hoagland solution (Fig. 5b).

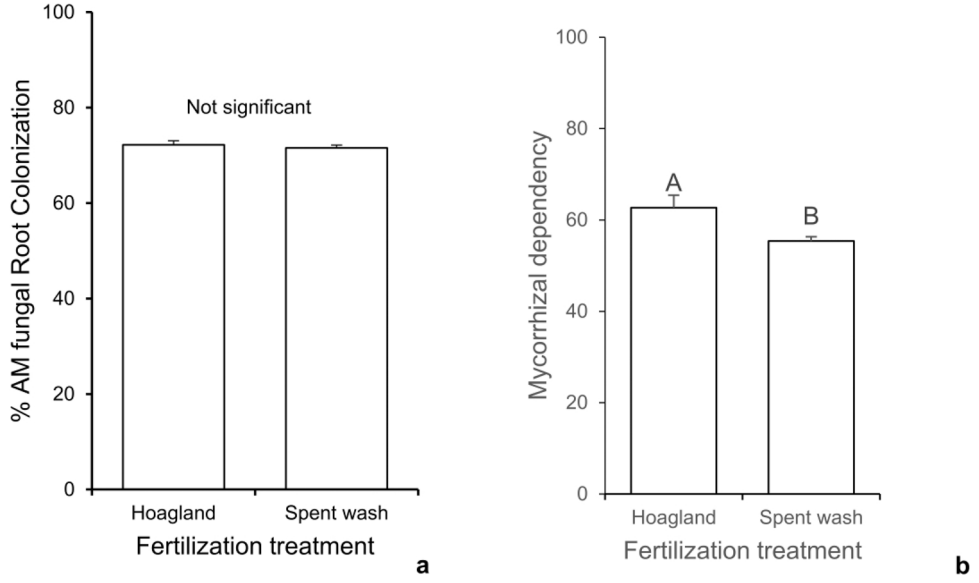


Fig. 2. Percent root AM fungal colonization in *S. bicolor* plants fertilized with Hoagland solution or distillery spent wash (a); and mycorrhizal dependency of *S. bicolor* plants fertilized with Hoagland solution or distillery spent wash (b). Values are average of 10 replicates. Error bars represent standard error of the means. Values that do not share an alphabet are significantly different ($p \leq 0.05$) according to Tukey's HSD post-hoc test.

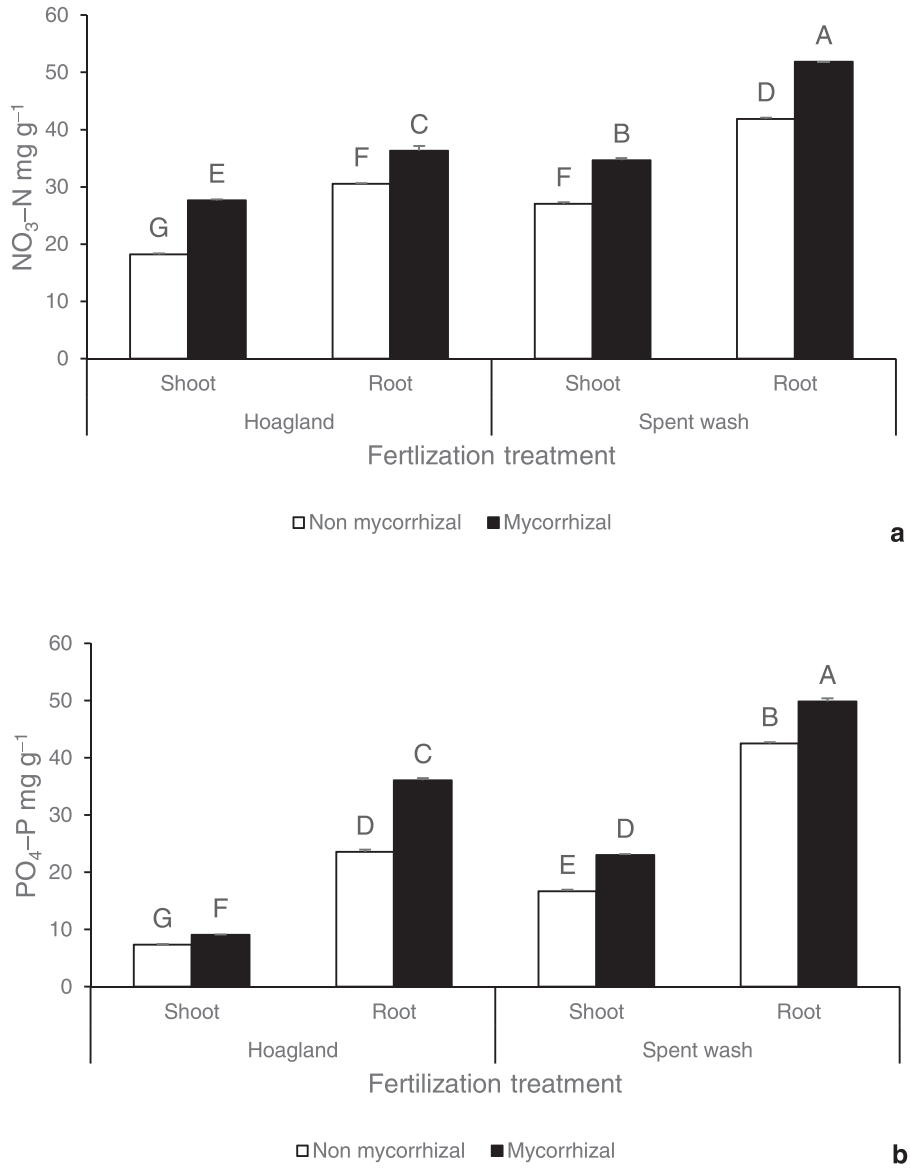
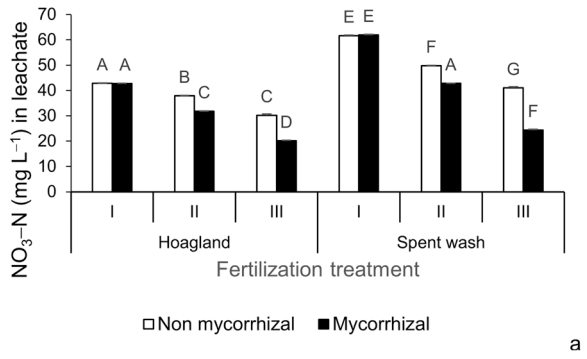


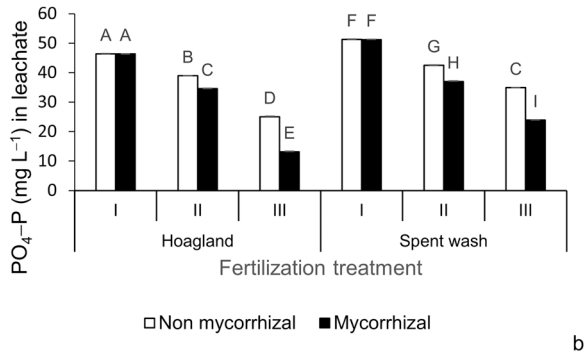
Fig. 3. NO_3–N (a) and PO_4 –P (b) concentrations in shoots and roots of *S. bicolor* plants fertilized with Hoagland solution or distillery spent wash. Values are average of 10 replicates. Error bars represent standard error of the means. Values that do not share an alphabet are significantly different ($p \leq 0.05$) according to Tukey's HSD post-hoc test.

Leachate collections of plants fertilized with DSW had higher electrical conductivity than those of plants fertilized with Hoagland solution ($F_{11,\ 108} = 905$; $p < 0.0001$). Electrical conductivities of leachates from mycorrhizal and non-mycorrhizal plants were similar in leachate I (Fig. 5c). Leachates II and III from mycorrhizal microcosms had lower electrical conductivity compared to those from non-mycorrhizal microcosms respectively. Like leachate absorbance and TDS content, the electrical conductivity of leachate III from mycorrhizal plants fertilized with DSW was similar to that of plants fertilized with Hoagland solution. However, leachate III from non-mycorrhizal plants fertilized with DSW had a higher electrical conductivity compared to that of non-mycorrhizal plants fertilized with Hoagland solution (Fig. 5c).

3.3.3. Characterization of organic pollutants in raw spent wash and leachate III collected from treatments III and IV

Ethylene acetate-extracted sample of raw DSW was composed of 65 complex organic compounds. The organic compounds that contributed colour to DSW were: (1S-cis)-Ethynyl-2-methylbicyclo[3.1.0]hexane; diacetone alcohol; acetic acid, dodecyl ester; cyclohexane; 1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester; 2-Pyrrolidone; and 3-amino-2-cyano-6-methyl-6,7-dihydrothienol[2,3,-*b*]pyrazine S-oxide, with retention time (RT) values of 6.72, 7.97, 18.49, 23.86, 26.93 and 32.29 respectively. (Table 1a). The ethylene acetate-extraction of leachate III collected from the non-mycorrhizal plants (treatment III) contained 42 complex organic compounds, but these were different from those detected in raw DSW and had altered RT peaks (Fig. 6a, b). Cyclohexane, 1,4-dimethyl-2-octadecyclohexane and 3-[4′-(t-Butyl) phenyl]furan-2,5-dione with respective RT values 12.59 and 23.06 (Table 1b) were the colour containing compounds (Table 1b). The ethylene acetate-extracted leachate III collected from mycorrhizal plants (treatment IV) contained only 26 simplified organic compounds (Fig. 6c; Table 1c). No colour containing organic compounds were detected in the third leachate collection from the mycorrhizal plants. Most RT peaks detected in raw DSW and leachate III from non-mycorrhizal plants were absent in leachate III collected from the mycorrhizal treatment. (Fig. 6c; Table 1c).





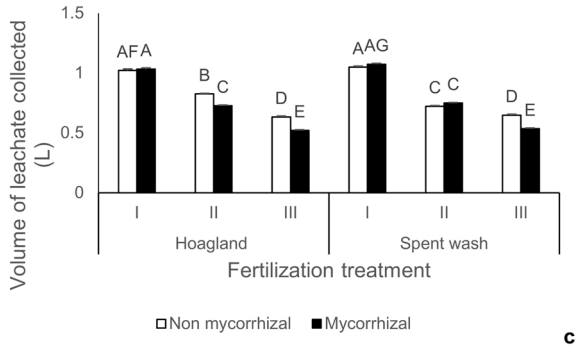
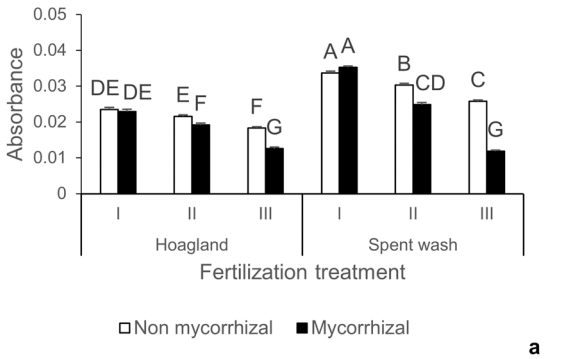
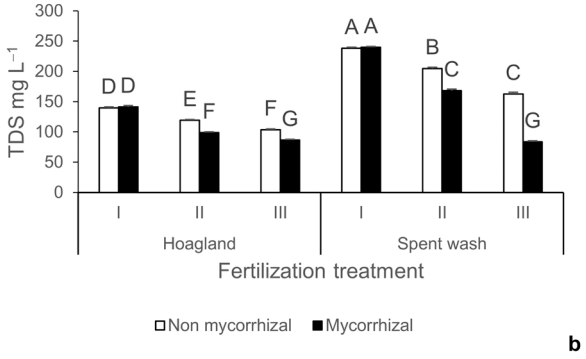


Fig. 4. NO_3–N (a) and PO_4–P (b) concentrations in leachates I, II and III; and volume of leachate collected (c) from mycorrhizal and non-mycorrhizal *S. bicolor* plants fertilized with Hoagland solution or distillery spent wash. Values are average of 10 replicates. Error bars represent standard error of the means. Values that do not share an alphabet are significantly different ($p \leq 0.05$) according to Tukey's HSD post-hoc test.





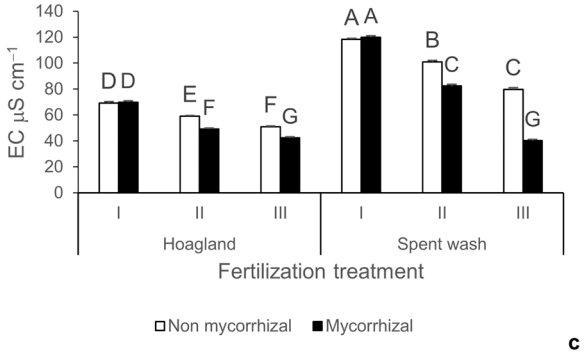


Fig. 5. Absorbance (a); TDS (b); and electrical conductivity (c) of leachates I, II and III from mycorrhizal and non-mycorrhizal *S. bicolor* plants fertilized with Hoagland solution or distillery spent wash. Values are average of 10 replicates. Error bars represent standard error of the means. Values that do not share an alphabet are significantly different ($p \leq 0.05$) according to Tukey's HSD post-hoc test.

4. Discussion

AM fungi are well documented for their role in enhancing shoot and root biomass of host plants [17,41,44,56]. They facilitate growth of host plants primarily through enhanced uptake of nutrient ions, particularly P, from the soil solution [17,21,6].

N and P concentrations in DSW are generally higher than that of commercially available chemical fertilizers [22]. The available N and P contents in the DSW used in this experiment were 177% and 420% higher, respectively, than Hoagland solution. Mycorrhizal plants in treatments II and IV respectively had higher biomass than non-mycorrhizal plants of either fertilization regime (Fig. 1). However, plants fertilized with DSW had lower mycorrhizal dependency compared to those fertilized with Hoagland solution (Fig. 2b). Mycorrhizal dependency is a measure of the degree of dependence of plants on AM fungal colonization for their maximum growth and yield at a particular level of soil fertility [30,48,61]. Soil nutrient levels influence mycorrhizal dependency of host plants [43]. The lower mycorrhizal dependency of plants fertilized with DSW could be an outcome of substantially higher N and P levels in DSW. In both fertilization treatments mycorrhizal plant tissues had higher N and P concentrations compared to non-mycorrhizal plants indicating that AM fungal hyphae promoted a more efficient uptake of nutrient ions than plant roots.

A continuous application of DSW in split doses results in increased levels of organic-C and other nutrients such as N and P introduced to the soil [29]. Whereas N and P inputs to soil may facilitate plant growth, organic-C can promote growth of bacterial and fungal microflora including mycorrhizas [32,36,52]. Indeed, AM fungi form symbioses with plants for uptake of organic carbon [57]. Yet, despite higher

Table 1a
Organic compounds detected in GC-MS analysis of ethylene acetate-extract of raw spent wash and their retention time (RT) values. (1S-cis)− 1-Ethynyl-2-methylbicyclo[3.1.0]hexane; diacetone alcohol; acetic acid; cyclohexane; 1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester; 2-Pyrrolidinone; 3-amino-2-cyano-6-methyl-6,7-dihydrothieno[2,3-*b*]pyrazine S-oxide contribute colour to distillery spent wash.

S. No	RT	Compound Name
1.	6.52	Benzaldehyde
2	6.72	(1S-cis)− 1-Ethynyl-2-methylbicyclo[3.1.0]hexane
3	7.36	Pregan-20-one, 2-hydroxy-5,6-epoxy-15-methyl-
4.	7.97	diacetone alcohol
5.	9.89	trans-13-Octadecenoic acid
6.	10.11	1-Octene, 2,6-dimethyl-
7.	10.74	1,1-Bis(methylthio)− 2-methyl-2-phenyl-1,2-dihydroazeto[2,1-*b*] quinazoline
8.	11.43	2-Cyclohexylpiperidine
9.	12.18	4,5,6,8-PTetramethoxy-2,3-dihydroindeno[1,2,3-ij] isoquinolin-9-ol
10.	13.09	Pentasiloxane
11.	14.20	1b,4a-Epoxy-2 H-cyclopenta[3,4]cyclopropa[8,9]cycloundec[1,2-*b*]oxiren-5(6 H)-one, 7-(acetyloxy)decahydro-2,9,10-trihydroxy-3,6,8,8,10a-pentamethyl-
12.	14.57	Dodecanal
13.	15.98	Propanenitrile, 2-oxo-
14.	16.60	2-tert-Butyl-4-isopropyl-5-methylphenol
15.	16.92	Benzoic acid, 4-ethoxy-, ethyl ester
16.	18.22	Erythritol
17.	18.49	Acetic acid, dodecyl ester
18.	18.79	Hexadecamethylcyclooctasiloxane
19.	20.16	2-Butene
20.	20.69	2,4-Diphenyl-1-butene
21.	21.25	2,6-Bis(tert-butyl)phenol
22.	21.57	3-Chloropropionic acid
23.	21.92	1-Nonadecene
24.	23.15	1 H-Indene, 1-(phenylmethylene)-
25.	23.86	1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester
26.	24.07	EICOSAMETHYLCYCLODECASILOXANE
27.	24.40	Alpha-phenyl-alpha-tropylacetaldehydetosylhydrazone
28.	24.65	1,2-Benzenedicarboxylic acid, dibutyl ester
29.	24.84	Hexadecanoic acid
30.	25.28	1-Docosene
31.	25.64	Propanoic acid, 3-mercapto-, dodecyl ester
32.	25.88	2,2-dideutero octadecanal
33.	26.25	5,5-Dimethyl-3-methylene-1-phenyl-7-azabicyclo[4.1.0] heptanes
34.	26.93	2-Pyrrolidinone
35.	27.04	Lucenin 2
36.	27.27	9,12-Octadecadienoic acid (*Z,Z*)-,2,3-bis[(trimethylsilyl)oxy]propyl ester
37.	27.48	Docosane
38.	27.93	Cyclic octaatomic sulphur
39.	28.31	Behenic alcohol
40.	29.62	Docosane
41.	29.89	Cyclohexane
42.	30.67	n-Tetracosanol-1
43.	31.22	Benzene, 1,1′-(3-methyl-1-propene-1,3-diyl)bis-
44.	32.11	2-Bromooctanal
45.	32.29	3-amino-2-cyano-6-methyl-6,7-dihydrothieno[2,3-*b*]pyrazine S-oxide
46.	33.23	1,3,5-Benzetriol
47.	33.43	N,N-Dimethyl-N-phenylbutyl-4-ammonio-1-butanesulfonate
48.	33.73	Octadecane, 3-ethyl-5-(2-ethylbutyl)-
49.	34.31	Docosane
50.	34.62	Octadecane, 3-ethyl-5-(2-ethylbutyl)-
51.	35.30	Nonacosane
52.	35.63	Octadecane, 3-ethyl-5-(2-ethylbutyl)-
53.	36.05	Lucenin 2
54.	36.82	Tetratetracontane
56.	37.05	Lucenin 2
57.	37.81	Lupan-3-ol
58.	38.26	acetate
59.	38.39	2,6-di-tert-butyl-4-[4-(4-chloro-phenyl)-phthalazin-1-ylsulfanyl]-phenol
60.	38.66	2,4-Octadienoic acid,9a-(acetyloxy)− 1a,1b,4,4a,5,7a,7b,8,9,9a-decahydro-4a,7b-dihydroxy-3-(hydroxymethyl)− 1,1,6,8-tetramethyl-5-oxo-1 H-cyclopropa[3,4]benz[1,2-*e*]azulen-9-yl ester,[1aR [1aà, 1bá, 4aá, 7aà, 7bà, 8à,9á (2Z,4E),9aà]]-
61.	40.07	2,4-bis(Ethylmercapto)− 7-(3′-methyl-5′-oxo-1′-phenyl-2′-pyrazolin-4′-yl)− 5-phenylpyrido[2,3-*d*]pyrimidine
62.	41.00	2,4-Dihydroxybenzoic acid
63.	42.14	Oleic acid, eicosyl ester
64.	46.60	1-Octacosanol, 24-Ethyl-ë (22)-coprostenol
65.	49.25	á-Sitosterol, Tris(2,4-di-tert-butylphenyl) phosphate

organic-C in DSW, no differences were found in root AM fungal colonization in the two fertilization regimes (Fig. 2a). The absence of differences in the two fertilization regimes seems to suggest that the higher C-content of DSW did not influence AM fungal colonization (Fig. 2a).

AM fungi and plant roots have been shown to alter soil chemistries by removing or degrading complex organic compounds in the soil solution [18,46,66]. This ability of AM fungi may have a role in degrading organic compounds in spent wash leachates.

Reductions in leachate absorbance and electrical conductivity (Fig. 5a, c) indicate plant roots and AM fungal hyphae may be altering spent wash chemistry. While DSW leachate absorbance was reduced in both mycorrhizal as well as non-mycorrhizal pots, the reduction was significantly higher in mycorrhizal pots. Absorbance of DSW leachate of mycorrhizal and non-mycorrhizal pots was similar in leachate I. However, absorbance of DSW leachate II collected from mycorrhizal plants was less than that collected from non-mycorrhizal plants and the absorbance of DSW leachate collected in leachate III from mycorrhizal plants was similar to leachate from plants fertilized with Hoagland solution (Fig. 5a). Likewise, electrical conductivity of leachates from both mycorrhizal and non-mycorrhizal pots reduced with time (Fig. 5c). Lower electrical conductivity of leachates collected from mycorrhizal plants compared to non-mycorrhizal plants indicates a higher extraction of nutrient ions by AM fungi from the soil solution (Fig. 5c). A similar pattern was observed with respect to nutrient leaching losses. While leaching losses of mycorrhizal and non-mycorrhizal plants were similar at first leachate collection, at the third leachate collection, nutrient loss was significantly reduced in leachate from mycorrhizal plants compared to non-mycorrhizal plants (Fig. 4a, b). AM fungi have been shown to reduce loss of nutrients like P and N by leaching to groundwater [3,7,37]. This is achieved by retaining greater concentrations of soil nutrients in the fungal and host biomass [24,50]. The results have also confirmed N and P losses to leaching were indeed lower in mycorrhizal plants than non-mycorrhizal plants.

Moreover, the volume of leachate collected from the mycorrhizal pots was lower than that collected from non-mycorrhizal plants (Fig. 4c). AM fungi have been shown to significantly reduce water losses from the soil by retaining moisture in the fungal mycelium and host biomass [4,70]. AM fungal mycelium expands the absorptive surface area of roots [17,41]. The reduced volume of leachate in the third collection could be an outcome of the increased root and fungal mycelial biomass (Fig. 4c).

The reduced absorbance and electrical conductivity in leachates from mycorrhizal plants, compared to non-mycorrhizal plants, were reflected in the differences observed in organic compound composition of raw DSW and leachate III from non-mycorrhizal and mycorrhizal plants subject to treatments III and IV respectively (Fig. 5a, c; Table 1). The exact chemical structure of melanoidin ($C_{17–18}H_{26–27}O_{10}N$) is not known but it is assumed that its elemental composition and chemical structure is majorly dependent on the nature and molar concentrations of parent reacting compounds and reaction conditions [9]. Diacetone alcohol; (1S-cis)− 1-Ethynyl-2-methylbicyclo[3.1.0]hexane; 3-amino-2-cyano-6-methyl-6,7-dihydrothieno[2,3-*b*]pyrazine S-oxide; cyclohexane; 1,2-benzenedicarboxylic acid, butyl 8-methylnonyl ester; 2-Pyrrolidinone; and acetic acid, dodecyl ester detected in raw DSW

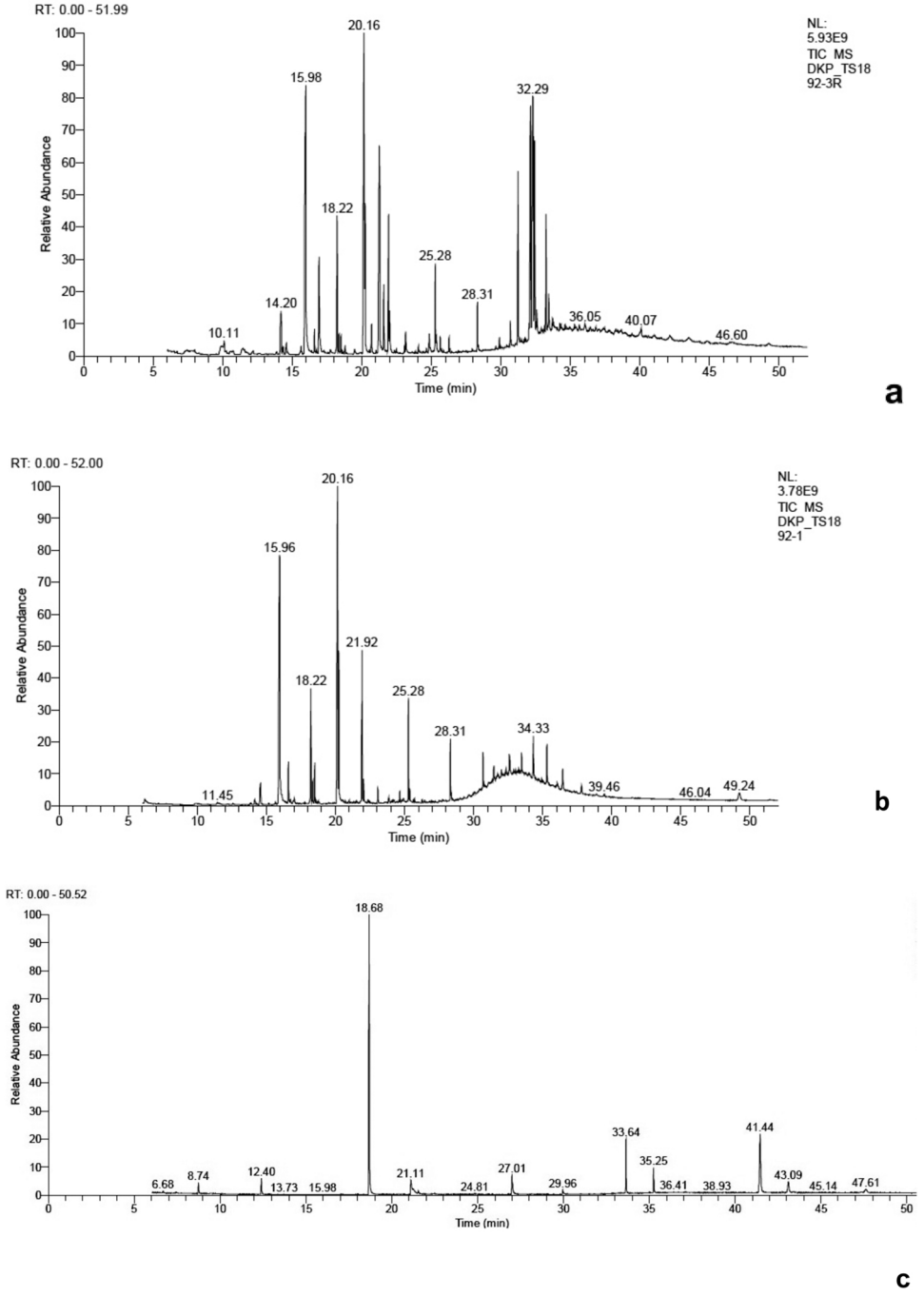


Fig. 6. Gas-chromatography and mass spectrometry profiles of organic compounds extracted from raw distillery spent wash (a); leachate III from non-mycorrhizal *S. bicolor* (b); and leachate III collected from mycorrhizal *S. bicolor* (c). Retention time (RT) peaks were identified using the mass spectral database library of the National Institute of Standard and Technology, USA v 1.0.0.12.

(Table 1a) and the trace organic compounds: cyclohexane, 1,4-dimethyl-2-octadecylohexane; and 3-[4′-(t-Butyl)phenyl]furan-2,5-dione detected in leachate from non-mycorrhizal pots (Table 1b) are believed to be constituents of melanoidin [69,9]. These organic compounds that colour DSW were not detected in leachate III collection from mycorrhizal plants (treatment IV). This may explain the complete decolourization of DSW leachate at third collection from mycorrhizal plants. Several of the organic compounds detected in raw DSW were also not detected in leachate III from non-mycorrhizal plants (treatment III) (Table 1a, b; Fig. 6a, b). This indicates that plant roots and AM fungal hyphae both degrade or bio-transform the organic constituents of distillery spent wash, but the AM fungi are doing this at a faster rate.

5. Conclusions

Biological remediation methods using bacterial and fungal species have reported varying levels of success in decolourizing melanoidin. Yet AM fungi may offer other benefits. For instance, results from this study have shown that AM fungi contribute to increased host plant biomass and better nutrient uptake. They also reduce the nutrient and organic pollutants present in DSW leaching into groundwater. The numbers of organic compounds (26) detected in leachate III from mycorrhizal plants were significantly lower than the 42 and 65 organic compounds in leachate III from non-mycorrhizal plants and raw DSW respectively. This indicates that AM fungi accelerate the degradation of organic

Table 1b
Organic compounds detected in GC-MS analysis of ethylene acetate-extract of leachate III collected from non-mycorrhizal plants with their retention time (RT) values. Cyclohexane; 1,4-dimethyl-2-octadecylohexane; 3-[4′-(t-Butyl)phenyl] furan-2,5-dione are colour containing organic compounds.

S. No	RT	Compound Name
1.	6.20	4-bromopyrazole-3(5)-carboxylic acid
2.	7.22	Benzene, 1,4-Dichlorobenzene
3.	7.74	Docosane
4.	9.89	2,2,3,4-Tetramethyl-2 H-thiete
5.	10.09	1-Tetradecanol
6.	10.75	3-Methoxycarbonylmethyl-5-(4-methoxyphenyl)− 2-phenylimino-3,6-dihydro-2 H-1,3,4-thiadiazinium bromide
7.	11.45	Caprolactam
8.	12.19	Dimethyl endo-6-(dibromomethyl)− 6-methyl-5-oxobicyclo[2.2.2] octa-2,7-diene-2,3-dicarboxylate
9.	12.59	Cyclohexane, 1,4-dimethyl-2-octadecylohexane
10.	13.36	2-(4′-pentenyl)cyclohexanone
11.	13.85	2-(4′-Nitrobutyryl)cycloheptanone
12.	14.16	1-Undecene, 8-methyl-
13.	14.56	Ecgonine
14.	15.96	(E)− 5,5-Difluoro-4-hydroxy-3-methyl-2-dodecen-6-one
15.	16.60	2-tert-Butyl-4-isopropyl-5-methylphenol
16.	17.02	3-Octen-2-one, 7-methyl-
17.	18.22	1-Hexadecene
18.	18.49	Dodecan-1-yl acetate
19.	20.16	Epi-9-angeloyloxy-10,11-epoxy-10,u1-dihydrobrickecliol
20.	20.99	Heptacosane
21.	21.14	Pentacosane
22.	21.92	1-Nonadecene
23.	23.06	3-[4′-(t-Butyl)phenyl]furan-2,5-dione
24.	23.86	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
25.	24.23	2,6-Dibromo-N,N,4-trimethylbenzamide
26.	24.65	9,9-Dimethyl-8,10-dioxapentacyclo[5.3.0.0(2,5).0(3,5).0(3,6)] decane
27.	25.28	1-Octadecene
28.	26.02	Docosanoic acid, 8,9,13-trihydroxy-, methyl ester
29.	26.26	(R)-(-)-N-Octyl-2-methylbutyramide
30.	26.47	5-Oxohexanethioic acid, S-t-butyl ester
31.	28.31	n-Tetracosanol-1
32.	30.68	1-Docosene
33.	31.46	Hexadecanoic acid, 3-[(trimethylsilyl)oxy]propyl ester
34.	32.60	1-Methoxycarbonyl-6-methyl-2-octyl-1,2,3,4-tetrahydropyridine
35.	33.47	Heptacosane
36.	34.33	Pentacosane
37.	35.31	Pentacosane
38.	36.44	Tetratetracontane
39.	37.81	Heptacosane
40.	39.46	Rhodopin
41.	46.04	2,11,13,22,23,25-Hexaoxa-1,12(1,3,2)-dibenza-24(2,9)− 1,10-phenanthrolinabicyclo[10.10.3] pentacosaphane
42.	49.24	Stearic acid, 3-(octadecyloxy)propyl ester

Table 1c
Organic compounds detected in GC-MS analysis of ethylene acetate-extract of leachate III collected from mycorrhizal plants and their retention time (RT) values.

S. No.	RT	Identified Compounds
1.	6.68	Silanamine
2.	8.74	3-Oxaoct-4-en-2-imine, N-trimethylsilyl-4-trimethylsilyloxy
3.	10.41	1,4-Dimethyl-3-(((tert-butyldimethylsilyl)oxy)methyl)− 3-methoxy-2,5-piperazinedione
4.	12.40	Trimethylsilyl ether of glycerol
5.	13.73	N-(2-Hydroxy-2-phenylethyl)acetamide
6.	15.98	4-Pentamethyldisilyloxyhexadecane
7.	16.11	1,1,3,3,5,5,7,7,9,9-Decamethyl-pentasiloxane
8.	18.68	Silane, (dodecyloxy)trimethyl- (CAS)
9.	19.12	cis-5,6,6a,12c-Tetrahydrodibenzo[*a*,*i*]biphenylen-12c-o
10.	20.78	(4-Ethynylphenyl)diphenylmethoxymethane
11.	20.88	5,6à-Epoxy-5à-cholestan-3à-ol
12.	21.11	2-Propenoic acid, oxybis(methyl-2,1-ethanediyl) ester
13.	24.81	7,9-di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2, 8-dione
14.	27.01	Hexadecanoic acid, trimethylsilyl ester
15.	29.29	(2 S,3 S,6 R,7 R)− 2,3-Epoxy-6,7-O-isopropylidene-1,6,7-nonadecantriol
16.	29.39	(23 S,24 S)− 3á-Acetoxy-23-hydroxy-24-methyl-5àcholestan-6-one
17.	29.96	Octadecanoic acid
18.	32.20	2,3-Bis(3′-Methoxy-2′-nitrophenylimino)− 2 H-indoLe
19.	33.64	1,3-Bis(2-methoxy-3-nitrophenyl)tetrahydro-2-pyrimidinone
20.	35.25	1,2,3,4,5-Pentaisopropylbis(cyclopentadienyl)cob alticimium
21.	36.41	1-Methyl-3-(3,4-dimethoxyphenyl)− 6,7-dimethoxyisochromene
22.	38.93	2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl) bromobenzene
23.	41.44	Dithioerythritol, O,O′,S,S′-tetrakis(trimethylsilyl)-
24.	43.09	1,8-Diphenyl-3,4,10,11-tetrahydro[1,4]dioxino[2,3-g:5,6-g′] diisoquinoline
25.	45.14	Chalcone
26.	47.61	5,11,17,23-Tetra-t-butyl-25,26,27,28-tetrahydroxycalix-4-arene

compounds in DSW and can be employed as effective bioremediation tools for agricultural soils contaminated with DSW. Moreover, in this study AM fungi indigenous to soils of the affected area were employed along with *R. irregularis* as inoculum. *S. bicolor* (used as the host plant) is a commonly grown crop in the affected area. A similar remediation model using local crops and indigenous AM fungal flora could be tested in other areas where DSW has polluted soils and groundwater. However, it must be noted that the results presented in this study are from a controlled microcosm experiment. Further studies in field conditions are needed to test the ability of AM fungi to degrade organic pollutants in DSW.

CRediT authorship contribution statement

Vikrant Goswami: Pot experiments, sample analyses. **Sharma Deepika:** Molecular analyses of mycorrhizal inoculum, writing. **Ram Chandra:** GC-MS analyses. **C.R. Babu:** Conceptualization, writing. **David Kothamasi:** Conceptualization, Supervision, statistical analyses, writing.

Environmental Implication

Leaching of spent wash generated from molasses-based distilleries causes soil and groundwater pollution in agroecosystems. Organic pollutants in the spent wash leachate render waters unfit for human consumption. Melanoidin and caramel colorants that are generated during processing and distillation of sugarcane molasses add a dark colour to distillery spent wash. High nutrient load in the spent wash causes eutrophication and the darkened water poses threats to aquatic life by blocking out sunlight and inhibiting photosynthesis. This paper analyses arbuscular mycorrhiza mediated degradation of colour containing organic pollutants, including melanoidins, in distillery spent wash.

Declaration of Competing Interest

DK received Research funding from the Central Pollution Control Board, India and logistic support from M/s Godavari Biorefineries Ltd.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.131291.

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