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RESEARCH ARTICLE

Azole antifungal contaminants disrupt mycorrhizal function and risk agricultural sustainability

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Societal Impact Statement

Global food production is increasingly threatened by soil degradation, climate change and the rising costs of synthetic fertilisers. Circular agriculture, which promotes resource reuse, is a promising solution, but using treated wastewater and biosolids in farming introduces risks from emerging contaminants like pharmaceutical residues. Our study examined how common antifungal drugs affect beneficial soil fungi that support plant growth. We found that these contaminants significantly reduced fungal health and impaired nutrient uptake in crops. These findings highlight the urgent need for stronger regulations to protect soil ecosystems, ensuring the long-term sustainability of agriculture and global food security.

Summary

- Circular agriculture promotes waste reduction and resource reuse. However, integrating treated wastewater and biosolids into food production systems introduces emerging contaminants, including pharmaceuticals, with unknown consequences for soil health and function. We examined the impacts of commonly detected azole antifungal pharmaceuticals (clotrimazole, miconazole nitrate, fluconazole) on arbuscular mycorrhizal (AM) fungi, in lettuce and spring onion.
- Spring onion and lettuce were grown in AM fungi-inoculated soil with/without antifungal azoles; isotope tracers (^{15}N, ^{33}P, ^{14}C) were used to quantify nutrient exchange and C flow; AM fungal colonisation and soil hyphae were assessed microscopically; plant tissues were analysed for isotope uptake and biomass; root and soil DNA was sequenced (16S/ITS) and analysed in R for microbial community profiling.
- Azole antifungal exposure significantly impaired AM structures in both species, reducing soil AM hyphal densities by approximately 70% ($P < 0.05$ and $P < 0.001$ in spring onion and lettuce, respectively) and AM fungal root colonisation by approximately 72–82% ($P < 0.001$ for both species). AM function was also negatively impacted, evidenced by a complete shutdown of AM-mediated phosphorus (P) acquisition, in terms of shoot ^{33}P concentration ($P < 0.001$), and

a significant decline in soil fungal diversity in spring onion-AM systems (Shannon's diversity: $P < 0.05$; Simpson diversity: $P < 0.01$). Interestingly, the effects were nutrient-dependent, as only AM-mediated P acquisition (^{33}P concentration) was reduced. As such, the concentration of total P in shoot tissues significantly declined under azole exposure for both plant hosts (spring onion: $P < 0.01$; lettuce: $P < 0.05$), while N was, again, not impacted (spring onion: $P = 0.0798$; lettuce: $P = 0.577$).

- Our findings highlight the context-dependent nature of soil microbial responses to emerging contaminants and underscore the urgent need for further research. Such research is essential to inform improved regulations aimed at mitigating the unintended impacts of these contaminants on soil microbiomes and agricultural sustainability.

KEYWORDS
agriculture, antifungal azoles, arbuscular, biosolids, mycorrhiza, pharmaceutical contamination

1 | INTRODUCTION

Circular agriculture is an innovative approach to sustainable food production that is advocated for in a number of policy initiatives including the UN's Sustainable Development Goals (SDGs) (Schroeder et al., 2019). It aims to create a 'closed-loop' system whereby waste is reduced to a minimum and resources are continually reused (Kirchherr et al., 2023) (Figure 1). For example, by-products from wastewater treatment such as treated sludges (also termed biosolids) and wastewater effluent are particularly valuable potential resources in circular agricultural systems, given their high nutrient and organic matter content (Nguyen et al., 2022). However, despite deployment of advanced water treatment technologies to remove conventional contaminants, wastewater treatment does not routinely remove all potential bioactive chemical contaminants (Miao et al., 2023), allowing inadvertent contamination of wastewater- or biosolid-amended agricultural soils (Carter et al., 2019; Sallach et al., 2021). Emerging evidence demonstrates that, at environmentally relevant concentrations, human-use pharmaceuticals can elicit a suite of sub-lethal responses on plant growth and development (Carter et al., 2015). The wider effects of these bioactive pharmaceuticals on soil health and function - fundamental to the implementation and success of circular agriculture schemes - are largely unknown.

A key component in maintaining soil health is the presence and maintenance of soil microbial communities, which are beneficial to plant growth, among which arbuscular mycorrhizal (AM) fungi play a critical role (reviewed in Fall et al., 2022). AM fungi are a near-ubiquitously occurring (Öpik et al., 2013) group of plant-symbiotic soil microbes which are of particular interest in sustainable/circular agriculture (Thirkell et al., 2017) and receive all their carbon (C) from their host plants (Luginbuehl et al., 2017). By forming mycorrhizal associations in plant roots, AM fungi typically enhance host plant access to soil nutrients such as N and P (Smith & Read, 2008). The function of AMs in terms of symbiotic C-for-nutrient exchange between partners can be affected by a variety of factors, including insect herbivory (Charters et al., 2020; Durant et al., 2023), pathogen attack (Bell et al., 2023) and atmospheric CO_2 concentration (Thirkell et al., 2017, 2019). Furthermore, plant hosts can gain a suite of non-nutritional benefits from associating with AM fungi, such as improved drought and salinity tolerance and resistance (Bowles et al., 2016; Liu et al., 2023), priming of plant defences to reduce impacts of pests and disease (Frew et al., 2021; Song et al., 2015), as well as indirect impacts via alterations to soil properties (Bowles et al., 2016; Cavagnaro, 2016; Rillig & Mummey, 2006; Wu et al., 2014).

In the UK, approximately 3 million metric tons of biosolids are applied each year to between 150,000 and 250,000 ha of land (Black, 2016), while the US Environmental Protection Agency (EPA) reported similar figures, with approximately 2.1 million metric tons applied in 2022 (EPA, 2023). Recent research has confirmed that human-use azole antifungals persist in biosolid-amended soils, showing slow dissipation rates (Chen et al., 2013). These compounds inhibit ergosterol synthesis, a key component of fungal cell membranes, by interrupting the conversion of lanosterol to ergosterol (Herrick et al., 2024), raising concerns for both soil and plant health due to their biological potency and shared biological pathways across fungi, plants and humans (Garduño-Jiménez & Carter, 2024). AM fungi do not contain ergosterol (Olsson et al., 2003), but contain 24-ethyl-cholestrol (Grandmougin-Ferjani et al., 1999) and 24-methyl sterols (Fontaine et al., 2004) as their main cell wall sterols. In addition to ergosterol, lanosterol can also be converted into 24-Ethyl-cholestrol and 24-methyl sterols (Weete et al., 2010) thus, there is clear potential for azole antifungals to negatively impact AM fungi. Previously, the presence of the human-use azole antifungals, clotrimazole, miconazole nitrate and fluconazole, at concentrations found in soils post-biosolid application (ratio of 1:1:1; nominal concentration of 100 ng/g), were found to impair the transfer of soil P from AM fungi to host wheat plants, while root AM colonisation levels remained unchanged (Sallach et al., 2021). However, their effects on AM associations in

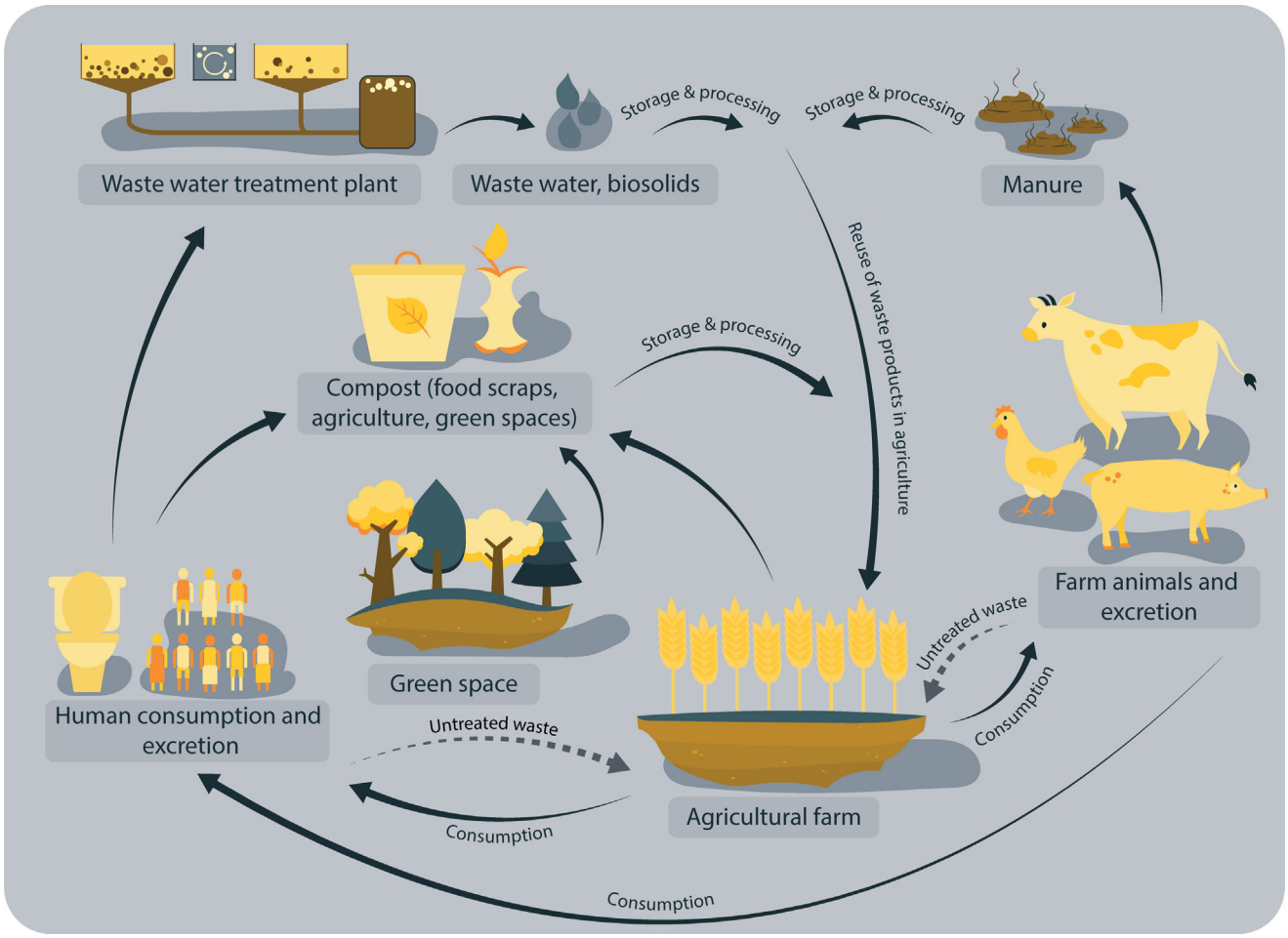

FIGURE 1 Circular agriculture feedback loop illustrating the reuse of human, animal and plant waste products to meet nutrient and irrigation demands of food production. Dashed arrows represent untreated human or animal waste; solid lines represent the movement of treated waste (e.g. through composting or wastewater treatment). Emerging contaminants enter the system following human consumption or veterinary use (e.g. farm animals and excretion) and can remain in resources such as biosolids, wastewater or manure following treatment and storage, thereby providing a pathway back into the agricultural soils following resource reuse.

other food crops remain unknown. Given the context dependency and species specificity of mycorrhizal functioning (Camuy-Velez et al., 2025), it is critical that the ubiquity of azole antifungal impacts on mycorrhizal function is investigated across food crop classes.

We tested the hypotheses that the presence of three azole-based antifungal pharmaceuticals, fluconazole, miconazole nitrate and clotrimazole, in environmentally relevant concentrations in soil would impair AM fungal-to-plant transfer of soil nutrients and plant-to-fungal C transfer, simultaneously driving substantial shifts in microbial soil communities by reducing the diversity of fungi relative to bacteria. We investigated AM fungal function in economically important leafy green (*Lactuca sativa*) and bulb (*Allium fistulosum*) salad crops grown in agricultural soil spiked with environmentally relevant concentrations of a mix of commonly detected human-use azole antifungal contaminants using a combination of stable and radio-isotope tracers. We assessed wider impacts of azole antifungal contamination on the soil microbiome via DNA (ITS and 16S) sequencing of both plant root and soil communities.

2 | METHODS

2.1 | Plant material, growth conditions and experimental treatments

Spring onion (*A. fistulosum* cv. '(Spring) White Lisbon') and lettuce (*L. sativa* cv. Capitata) seeds were planted in air-dried and sieved agricultural field soil collected from the University of Leeds Farm in June 2021 (pH 6.86; organic C content 2.05%; 33% clay, 59% silt; 8% sand) in pots that measured 11.5 cm tall and 10.5 cm for both length and width. One plant was grown per pot. Spring onion and lettuce were selected to represent globally popular mycorrhiza-forming salad crops of economic importance (Guo et al., 2023). These species, representative of other soil-grown crops such as leeks and other non-brassicaceous leafy greens, are cultivated in countries where the reuse of wastewater (both treated and untreated) is routine, allowing farmers to cultivate higher-value, water-intensive vegetable crops (Thebo et al., 2017). Wastewater reuse in Pakistan, for instance, is

used to meet spring onion irrigation requirements (Ahmad et al., 2017). Each pot was filled with 1 kg substrate (1:1 soil: sand) mixed with 25 g of a commercially available mixed-species AM fungal inoculum comprising species *Funneliformis mosseae*, *Funneliformis geosporus*, *Claroideoglomus clarodeum*, *Rhizophagus intraradices*, *Glomus micoraggregarum*, *Diversispora spp*, in a clay particulate carrier matrix (Plantworks, UK). Spring onion and lettuce seeds were sown in soil spiked with commercially prescribed human-use antifungal azoles comprising a 1:1:1 mixture of clotrimazole, miconazole nitrate and fluconazole ($n = 14$ spring onion, $n = 12$, lettuce) or control soils without antifungals ($n = 16$ spring onion, $n = 12$ lettuce). Antifungals were applied at an environmentally relevant rate (100 ng/g) (as per Sallach et al., 2021). At the time of planting, two nylon mesh-windowed cores were inserted into each pot and filled with bulk substrate (as per Field et al., 2012). The pore size (35 μm) of the mesh covering the cores allowed penetration by AM fungal hyphae but excluded plant roots. An additional mesh-covered windowed core in each pot was filled with glass wool and later used to monitor the below-ground respiration of ^{14}C during labelling. Plants were maintained in glasshouse conditions (16 hour day, 18°C day, 16°C night, 60% humidity) and watered between 2 and 3 times a week as needed. At around 6 weeks, the roots from two additional pots set up at the same time but without cores were cleared in KOH and stained with acidified ink using methods described below (Vierheilig et al., 1998) to confirm colonisation by AM fungi in both species examined.

2.2 | Quantification of C for nutrient exchange between plants and AM fungal partners

Eight weeks after germination, a 100 μl aqueous solution containing 1 MBq of ^{33}P orthophosphoric acid (0.297 ng per pot; 111TBq $mmol^{-1}$ Sp Act; Hartmann Analytic, Brunswick, Germany) and 1 mg/ml of ^{15}N ammonium chloride (0.1 mg per pot; Sigma Aldrich, UK) was introduced into one of the mesh-covered, soil filled cores within each pot via a pierced capillary tube (as in Field et al., 2012). Immediately before isotope addition and every 48 hours after, one core in each pot was gently rotated to sever AM fungal hyphal connections between the core contents and the host plant while the second soil-filled core remained static. In half of the pots for each treatment, the static core was supplied with 100 μl ^{33}P and ^{15}N solution, while in the other half of the pots for each treatment, ^{33}P and ^{15}N were added to the rotated core. To quantify AM fungal transfer of ^{33}P and ^{15}N to host plants, the amount of $^{33}P/^{15}N$ detected in plant shoots grown in pots where the rotated core was labeled was subtracted from the equivalent values in plants where the static core was labelled. Plant shoots were monitored every 48 hours with a handheld Geiger counter until the accumulation of radioactivity plateaued for 2 weeks. The tops of all cores were sealed and 1 MBq of ^{14}C-$NaCO_3$ (1.62 GBq $mmol^{-1}$ Sp Act; Perkin Elmer, USA) was added into cuvettes in each pot. The pots were enclosed within gas-tight chambers and 2 ml 90% lactic acid injected into the cuvette, releasing a pulse of $^{14}CO_2$ into the chamber headspace. Above- and below-ground gas samples were assessed by mixing 1 ml of air taken from the chamber headspace or the glass wool-filled mesh core with 10 ml CarbonTrap CarbonTrap (Meridian Biotechnologies Ltd., UK) and 10 ml of the scintillant CarbonCount CarbonTrap (Meridian Biotechnologies Ltd., UK) throughout the $^{14}CO_2$ labelling period to monitor assimilation of ^{14}C by plants and belowground respiration. Activity of samples was quantified by scintillation counting (TriCarb 4910TR, Perkin Elmer, USA). Plants were maintained within chambers for a 16-hour photoperiod.

2.3 | Plant harvest

Plant roots, shoots and soils were separated and frozen at −20°C before freeze drying (Scanvac Coolsafe freeze-drier, LaboGene A/S, Denmark; Vacuum pump RZ 2.5, Vacuubrand GMBH+CO KG, Germany). Sub-samples of roots (approximately 1 g) and bulk soil (approximately 5 g) were taken for assessment of AM fungal colonisation of host roots and soil hyphal lengths. Total dry biomass was recorded for all components.

2.4 | AM fungal colonisation of roots and soil

Roots were cleared with KOH and stained using acidified ink as detailed in Vierheilig et al. (1998). Briefly, roots were cleared in 10% KOH at 70°C for 50 minutes for both spring onion and lettuce, stained in ink-vinegar for 24 hours at room temperature and left at room temperature to clear in 1% acetic acid for 1 week. Roots were then mounted on slides using polyvinyl lacto-glycerol (PVLG), dried for 48 hours at 70°C and visualised at 100x magnification under a light microscope. Mycorrhizal colonisation was assessed as the frequency of arbuscules, hyphae and vesicles as per McGonigle et al. (1990), and the presence of these structures was generalised during assessment to give a total root colonisation measure.

To assess fungal hyphal lengths in soil, approximately 5 g of soil from each pot was mixed with 500 ml water. A total of 200 ml was decanted, and a 10 ml sample was taken. This was split into two 5 ml replicates that were vacuum filtered onto 0.45 μm filters and stained with ink-vinegar stain. The filters were placed on slides using PVLG, and hyphal lengths were quantified under 100x magnification using the gridline-intersect method (Brundrett et al., 1994)

2.5 | Fungal acquired ^{33}P and ^{15}N, and plant fixed ^{14}C analyses

To quantify ^{33}P assimilation by plants, between 5 and 45 mg plant shoot and root tissues were weighed, in triplicate, into acid-washed test tubes for acid digestion as per Field et al. (2012). In brief, 1 ml of concentrated sulphuric acid was added to each sample and incubated for 2 hours. These were heated at 350°C (BT5D heat block, Grant

Instruments [Cambridge], Ltd.) for 15 minutes, and 100 µl of hydrogen peroxide was added to each tube before being returned to the heat block. Samples were diluted up to 10 ml with dH_2O. 2 ml of the diluted sample was added to 10 ml of Emulsify-safe (Perkin Elmer, USA) scintillant, ^{33}P activity was determined via liquid scintillation counting (Packard Tri-Carb 4910TR, Perkin Elmer, Beaconsfield, UK) and calculated using Equation S1.

^{15}N in plant shoots was quantified by continuous-flow isotope ratio mass spectrometry (IRMS) (PDZ 2020 IRMS; Sercon Ltd.) using 2–5 mg of shoot and root tissues weighed into tin capsules (Sercon Ltd.). Background ^{15}N content of plant material was quantified using plant materials not labelled with ^{15}N. The IRMS detector was regularly calibrated to a commercially available reference gas, with air used as the reference standard. Equation S2 was used to calculate ^{15}N transfer to plants by AM fungi (Thirkell et al., 2019).

^{14}C in plant and soil samples was quantified using sample oxidation (307 Packard Sample Oxidizer, Perkin Elmer) and liquid scintillation counting. A total of 10–170 mg of freeze-dried and homogenised plant tissue or soil samples were weighed in duplicate into combustocones (Perkin Elmer, USA), before complete combustion in oxygen. $^{14}CO_2$ was captured in 10 ml CarbonTrap and mixed with 10 ml of the liquid scintillant, CarbonCount (Meridian Biotechnologies Ltd.) and sample activity assessed via liquid scintillation counting (Packard Tri-Carb 4910TR, Perkin Elmer, Beaconsfield, UK). Total plant-fixed C (^{12}C and ^{14}C) transferred to AM fungi was calculated using Equation S3 and Equation S4 as per Field et al., 2012.

2.6 | Total P and N analyses

Spectrophotometry was used to quantify the amount of total P assimilated into shoot tissues by the plants across their lifetimes. A total of 0.5 ml of acid digested shoot tissue was combined with 0.5 ml developer solution (4.8 g of ammonium molybdate ($[NH_4]_6Mo_7O_{24}.4H_2O$) and 0.1 g of antimony potassium tartrate ($C_6H_4O_7SbK$) dissolved in 250 ml 2 M H_2SO_4), 0.2 ml 0.1 M L-ascorbic acid ($C_6H_8O_6$) and 2.6 ml dH_2O in cuvettes. Samples were incubated in the dark for 45 minutes, and their optical densities were measured at 882 nm on a spectrophotometer (Jenway® 6,300 Visible Spectrophotometer; Cole-Parmer), and P quantified using a standard curve (made by measuring the optical density of a series of standard P solutions and a no-plant blank). Total N acquired through the plants' lifetimes in shoot tissues was quantified by IRMS (see ^{15}N quantification methods above).

2.7 | DNA extraction, library preparation and sequencing

DNA was extracted from freeze-dried and homogenised samples containing 0.25 g (fresh weight; ± 10%) soil and 0.1 g (fresh weight± 10%) roots from both the spring onion ($n = 5$ 'no drug', $n = 5$ 'drug') and lettuce experiments ($n = 4$ 'no drug', $n = 4$ 'drug) according to the instructions of the Qiagen DNeasy® PowerSoil® Pro Kit. Then, amplicon PCR reactions were carried out (16S: 1 cycle of 95°C for 1 minute; 30 cycles of 95°C for 30 seconds, 51°C for 30 seconds, 72°C for 30 seconds; 1 cycle of 72°C for 10 minute. ITS: 1 cycle of 95°C for 15 minute; 30 cycles of 94°C for 30 seconds, 55°C for 1 minute, 72°C for 30 seconds; 1 cycle of 72°C for 10 minute), followed by library preparation for Illumina sequencing. The 16S primers used were F515 and R806 as described in Caporaso et al. (2010) for bacterial amplification, and ITS primers reverse_newITSR (Table 1) and forward_1624F (Hadziavdic, et al., 2014) were used for fungal DNA amplification (Table 1). The set of root and soil samples were subjected to next-generation sequencing using 2 × 250 bp Illumina (Illumina, USA) MiSeq paired-end reads by the NERC Environmental Omics Facility (NEOF) at the Centre for Genomic Research (University of Liverpool, Liverpool, UK). DNA sequences were uploaded to the NCBI Sequence Read Archive and can be found under the BioProject accession number PRJNA1197164.

2.8 | Read processing and data analysis

16S sequences were analysed using the paired-end read DADA2 pipeline (https://github.com/khmaher/HPC_dada2), which includes removing primer sequences, trimming sequences based on their quality throughout the reads, inspecting error plots, dereplicating reads, performing DADA denoising, merging reads, removing chimeras and finally assigning taxonomies to the ASVs. The ITS sequences were run

TABLE 1 Nucleotide sequences of primers used for DNA sequencing.

16S primers	
5151F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA
806R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT
ITS primers	
ITS_Forward_1624	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNCCTTTGTACACACCGCCCGTCG
Reverse_new_ITSR	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCAAGAGATCCRTTGYTRAAA

Note: The table includes the full sequences of primers used in amplicon PCR for sequencing microbial DNA. Each sequence consists of three components: red indicate *NEOF adapters*, which are required for compatibility with Illumina sequencing platforms; green represent *unique molecular identifiers (UMIs)*, which help track and eliminate duplicate reads during data processing; unhighlighted sections are the *target-specific primer sequences* that bind to bacterial (16S rRNA gene) or fungal (ITS region) DNA.

using the same paired-end read DADA2 pipeline; however, the read merging step was skipped so the sequences could be analysed as single-end reads. This was done because of a lack of overlap for the forward and reverse reads of the ITS region. 16S sequences were aligned to the SILVA database file silva_nr99_v138.1_wSpecies_train_set.fa.gz (Quast et al., 2013; Yilmaz et al., 2014; Callahan et al., 2016; https://zenodo.org/records/4587955), and ITS sequences were aligned to the UNITE database file sh_general_release_dynamic_25.07.23.fasta (Abarenkov et al., 2023; https://doi.plutof.ut.ee/doi/10.15156/BIO/2938067). ITS sequences were largely unidentified following the standard protocol, so the BLAST (Altschul et al., 1990) tool was used to align the sequences against the NCBI database of uncultured eukaryotic fungi accession list ((uncultured) AND "uncultured eukaryote" [porgn:__txid100272] OR "uncultured fungus" [porgn:__txid175245]). The results from this were uploaded to MEGAN 6.25.9 (Huson et al., 2016) and alignments with multiple matches were narrowed to provide a lowest/least common ancestor. The MEGAN output was exported and merged with that from DADA2. Further analysis was performed in R studio following the alpha and beta diversity workshop from NEOF (https://neof-workshops.github.io/Metabarcoding_6xxzqz/Course/11-Further_analysis.html).

2.9 | Data analysis

Statistics were performed with R studio (2020) and GraphPad Prism 9.0.0 for Windows (2020). Graphs were made using GraphPad Prism 9.0.0 for Windows48. For % colonisation, hyphal length density, shoot and root biomass, shoot [P], shoot [N] and 16S and ITS alpha diversity data were analysed using a Welch two-sample *t*-test. Outliers were removed if they were more two standard deviations away from the mean and are as follows: Spring onion colonisation (drug) had one outlier, lettuce hyphal density (no drug) had one outlier, spring onion shoot biomass (drug) had one outlier, spring onion [P] (no drug and drug) had two outliers each and spring onion [total N] (drug) had three outliers.

For hyphal densities, hyphal extractions were performed on only eight replicates for spring onion. [^{33}P], [^{15}N] and ^{14}C datasets were analysed using Mann–Whitney *U* tests, as they did not satisfy the assumptions of a one-way ANOVA, regardless of transformations. For spring onion [^{33}P] analysis, one outlier was removed from the 'Drug' treatment as it was greater than two standard deviations from the mean, and one value was excluded from the 'No Drug' treatment of lettuce [^{15}N] analysis as it was an outlier. During spring onion total C analyses, one value was removed as an outlier from the 'No Drug' treatment, two were removed from the 'Drug' treatment and one value was removed as an outlier from lettuce total C analyses for the 'Drug' treatment. Lastly, NMDS (non-metric dimensional scaling) plots were generated using Bray-Curtis distances, and the data was analysed using PERMANOVAs, comparing between plant groups (spring onion vs. lettuce) and between treatment groups ('no drug' vs. 'drug').

3 | RESULTS

3.1 | AMF colonisation and hyphal lengths in soil

Both percent root length colonisation and hyphal densities in soil were significantly reduced in plants exposed to antifungal pharmaceuticals in both spring onion (Figure 2a, $P = 2.48 \times 10^{-5}$; Figure 2b, $P = 0.0318$; Table S1) and lettuce (Figure 2c, $P = 6.20 \times 10^{-7}$; Figure 2d, $P = 1.30 \times 10^{-4}$; Table S1). Mean percent colonisation for spring onion decreased by 72.6% and by 82.4% for lettuce (Figure 2a,c). Mean fungal hyphal densities in soil decreased by 71.8% for spring onion and by 70.0% for lettuce (Figure 2b,d).

3.2 | Plant biomass and total P and N

Spring onion shoot (Figure 3a) and root (Figure 3b) biomass was not altered by exposure to antifungal pharmaceuticals (shoot: $P = 0.577$; root: $P = 0.648$; Table S1), while lettuce biomass was significantly greater (shoot: $P = 8.99 \times 10^{-6}$; root: $P = 7.60 \times 10^{-5;}$ Table S1). Lettuce shoots nearly doubled in size while root biomass increased by approximately 229%. Exposure to the antifungal pharmaceuticals resulted in a significantly reduced concentration of total P (mg g^{-1}) in the shoots in both crops (spring onion: Figure 4a, $P = 0.00800$; Table S1, lettuce: Figure 4c, $P = 0.0122$; Table S1). However, the concentration of N in the shoots was unaffected by the presence of azole antifungals in the soil in both spring onion (Figure 4b, $P = 0.0798$; Table S1) and lettuce (Figure 4d, $P = 0.577$; Table S1). Interestingly, when the total P and N content was not normalised for biomass (i.e. mg per plant), it was only in the lettuce shoots that a significant difference in N content was observed, where the shoot N content increased under antifungal treatment (Figure S1d; Table S1). For the remaining treatments, no significant differences were observed relative to control treatments (Figure S1a,b,c; Table S1).

3.3 | AM C-for-^{33}P/^{15}N exchange

The concentration of AM fungal-acquired ^{33}P ([^{33}P]) in the shoots of both spring onions and lettuce was lower when grown in soils contaminated with azole antifungals; however, this reduction was statistically significant only in spring onions (Figure 5a, $P = 0.0007$; Figure 5d, $P = 0.272$; Table S2). Similarly, although total ^{33}P content in shoot tissues were lower in antifungal-treated pots compared to controls (Figure S2a,c), the difference was significant only for spring onions ($P = 0.0107$; Table S2). In contrast, neither AM fungal-acquired [^{15}N] (Figure 5b,e) nor total ^{15}N (Figure S2b,d) in the shoots of either crop were affected by azole antifungal exposure (Figure 5b, $P = 0.326$; Figure 5e, $P > 0.999$; Table S2).

Allocation of plant-fixed C to extraradical AM fungal mycelium was lower for both crops when azole antifungal pharmaceuticals were present in the soil compared to control treatments (Figure 5c,f; Table S2). In spring onion, C allocation to AM fungi was reduced by 92.9% (Figure 5c, $P = 0.312$) and by 69.28% by lettuce (Figure 5f, $P = 0.0568$).

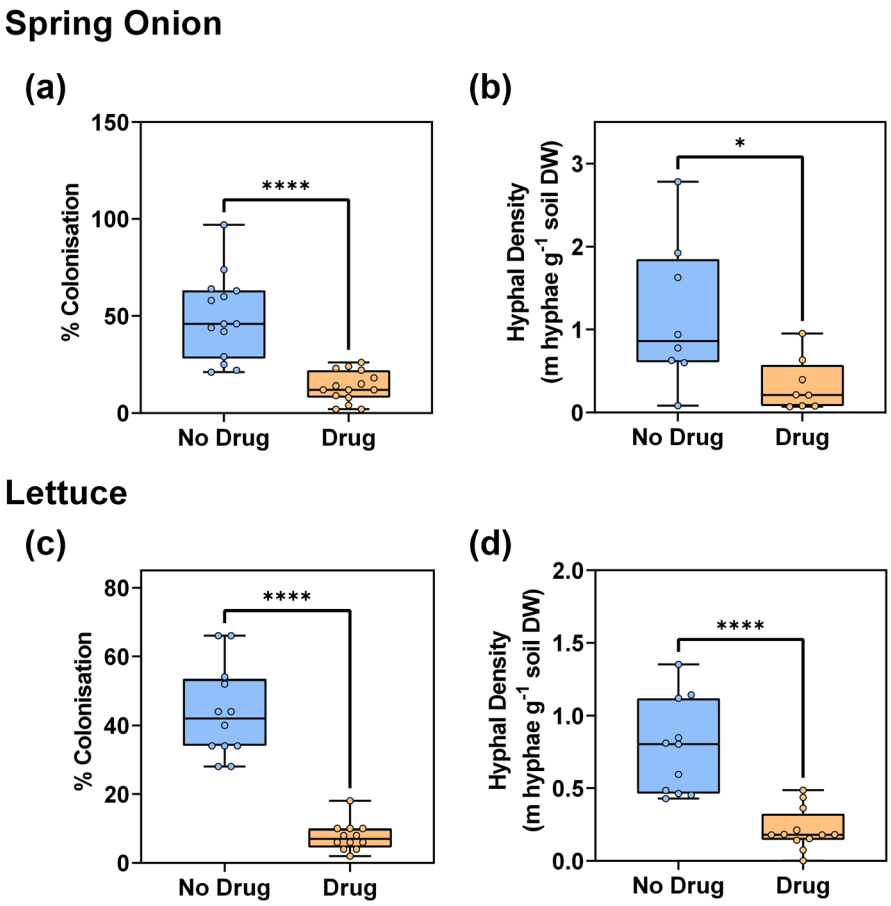

FIGURE 2 (a) Percent colonisation of spring onion roots. (b) Soil hyphal lengths for spring onion. (c) Percent colonisation of lettuce roots. (d) Soil hyphal lengths for lettuce. Blue boxes denoted as 'no drug' represent treatments that were not exposed to antifungal azoles. Orange boxes denoted as 'drug' represent treatments that were exposed to antifungal azoles. (a) $n = 14$ (no drug), $n = 15$ (drug). (b) $n = 8$ (no drug), $n = 8$ (drug). (c) $n = 12$ (no drug), $n = 12$ (drug). (d) $n = 11$ (no drug), $n = 12$ (drug). Boxes extend from the 25th to the 75th percentile, and median values are represented by lines within the boxes. Whiskers extend to minimum and maximum data points. Significant differences are denoted with stars (Welch two-sample *t*-test). *$P < 0.05$, ****$P < 0.0001$.

3.4 | Fungal (ITS) root and soil diversity

Within spring onion roots, fungal (ITS) alpha diversity was reduced when the soil was treated with antifungals for both Shannon's diversity index (Figure S3a, $P = 0.0174$) and Simpson diversity index (Figure S3b, $P = 0.00851$), whereas both indices were only slightly reduced in lettuce (Figure S3a,b, $P = 0.136$ and $P = 0.121$, respectively). However, soil fungal diversity for both spring onion (Figure S3c,d, Shannon: $P = 0.0945$; Simpson: $P = 0.0842$) and lettuce (Figure S3c,d, Shannon: $P = 0.817$, Simpson: $P = 0.401$) was unaffected by antifungal treatment.

NMDS plots were generated to compare the differences in fungal (ITS) beta diversity in roots and soil between plant types and antifungal treatments vs. control (Figure S4a,b). In roots, fungal community diversity was not significantly different between control ('no drug') and antifungal ('drug') treatments (Figure S4a; $P = 0.188$) but was between spring onion and lettuce roots ($P = 0.001$). This pattern holds true for fungal community diversity in the soil (Figure S4b), where antifungal treatment did not affect diversity ($P = 0.141$), but plant type did ($P = 0.045$).

For the spring onion root and soil bacterial communities, both Shannon's diversity index (Figure S5a,c, $P = 0.0197$, $P = 0.00116$, respectively) and Simpson diversity index (Figure S5b,d, $P = 0.0201$, $P = 0.0447$) decreased when soils were contaminated with the antifungal azoles compared to not contaminated, indicating a decrease in bacterial diversity. For lettuce roots, both Shannon's (Figure S5a, $P = 0.0497$) and Simpson (Figure S5b, $P = 0.0205$) diversity indices increased under antifungal exposure, indicating greater bacterial diversity in plant roots in azole antifungal-treated pots than the controls. However, this was not mirrored in the soil bacterial community diversity, as both indices were unaffected by antifungal presence (Figure S5c,d, Shannon's $P = 0.561$; Simpson $P = 0.809$).

To compare the difference in bacterial diversity between plant type and control, and antifungal treatments, NMDS plots were generated using Bray-Curtis distances (Figure S6a,b) for beta diversity. In both root and soil samples, the bacterial communities of spring onion and lettuce are different from one another (root: $P < 0.001$; soil: $P < 0.001$; PERMANOVA). However, antifungal azole treatment had no impact on the diversity of the bacterial communities in the roots ($P = 0.688$; PERMANOVA) or soil ($P = 0.351$; PERMANOVA).

4 | DISCUSSION

We investigated how common azole antifungal pharmaceuticals affect microbial function and diversity in soils supporting two economically important salad crops. Our results indicate that these chemicals, commonly found in biosolids applied to agricultural soils (Chen et al., 2013), selectively impair the ability of AM fungi to support soil

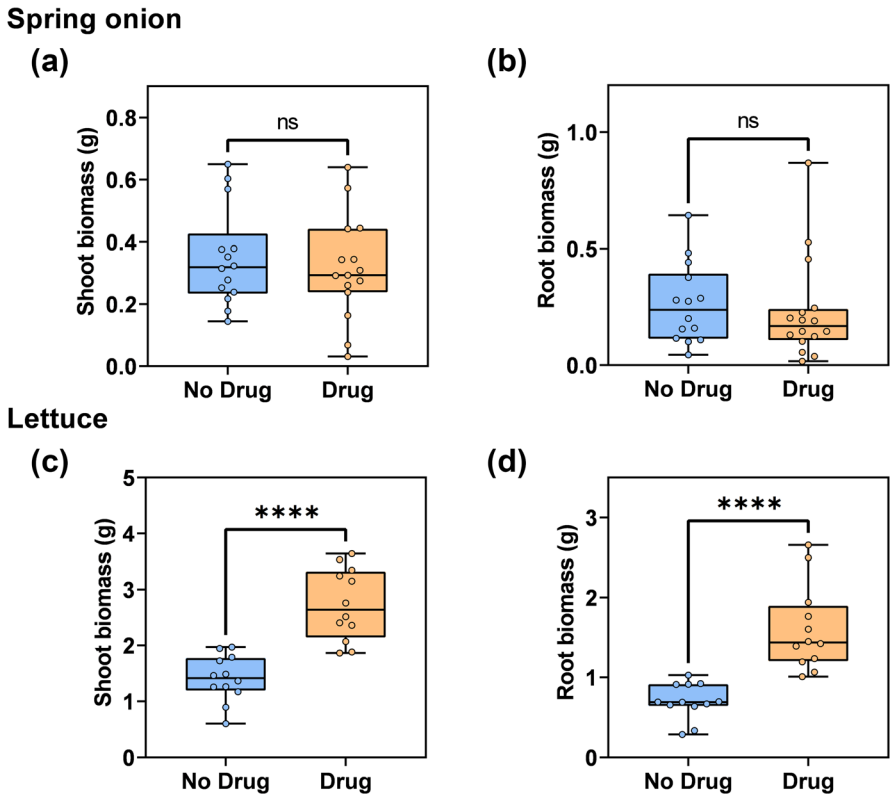

FIGURE 3 (a) Spring onion shoot and (b) root biomass. (c) Lettuce shoot and (d) root biomass. Blue boxes denoted as 'no drug' represent treatments that were not exposed to antifungal azoles. Orange boxes denoted as 'drug' represent treatments that were exposed to antifungal azoles. (a) $n = 14$ (no drug), $n = 15$ (drug). (b) $n = 14$ (no drug), $n = 16$ (drug). (c) $n = 12$ and (d) $n = 12$. Boxes extend from the 25th to the 75th percentile, and median values are represented by lines within the boxes. Whiskers extend to minimum and maximum data points. Significant differences are denoted with stars (Welch two-sample *t*-test). ****$P < 0.0001$. 'ns' refers to a non-significant difference.

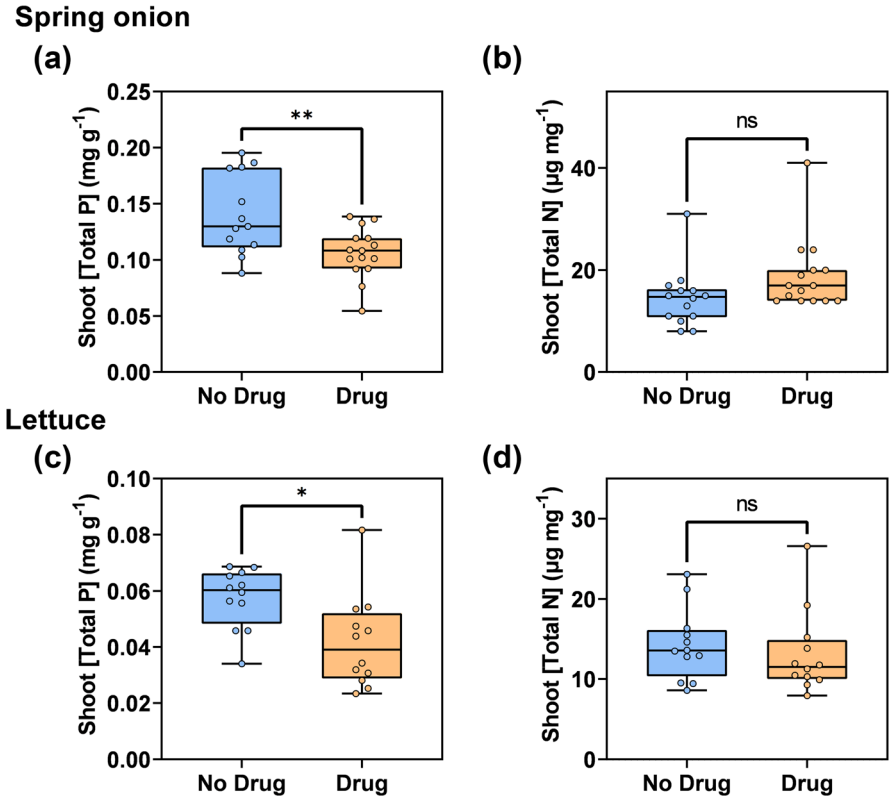

FIGURE 4 Concentration of total P in spring onion (a) and lettuce (c) shoots and concentration of total N in spring onion (b) and lettuce (d) shoots. Blue boxes denoted as 'no drug' represent treatments that were not exposed to antifungal azoles. Orange boxes denoted as 'drug' represent treatments that were exposed to antifungal azoles. (a) $n = 12$ (no drug), $n = 14$ (drug). (b) $n = 14$ (no drug), $n = 13$ (drug). (c) $n = 12$ (no drug), $n = 12$ (drug) and (d) $n = 12$ (no drug), $n = 12$ (drug). Boxes extend from the 25th to the 75th percentile, and median values are represented by lines within the boxes. Whiskers extend to minimum and maximum data points. Significant differences are denoted with stars (Welch two-sample *t*-test). *$P < 0.05$, **$P < 0.01$. 'ns' refers to a non-significant difference.

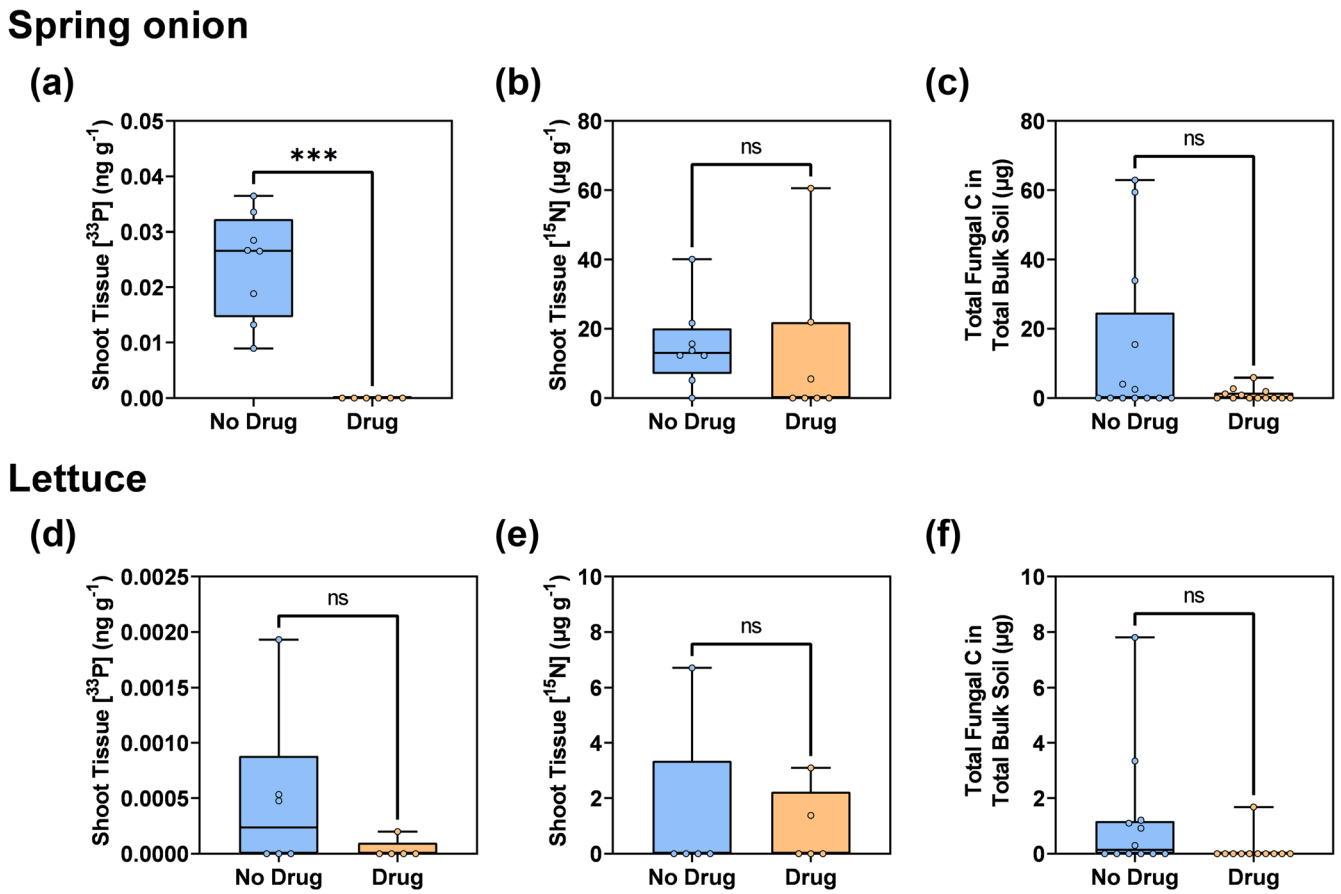

FIGURE 5 Nutrient transfer between plants and AM fungi. (a) Concentration of fungal-acquired [^{33}P] (a) and [^{15}N] (b) in spring onion shoot tissues and spring onion-acquired fungal C in total bulk soil (c). (b) Concentration of fungal-acquired [^{33}P] (d) and [^{15}N] (e) in lettuce shoot tissues and lettuce-acquired fungal C in total bulk soil (f). Blue boxes denoted as 'no drug' represent treatments that were not exposed to antifungal azoles. Orange boxes denoted as 'drug' represent treatments that were exposed to antifungal azoles. (a) $n = 8$ (no drug), $n = 6$ (drug); (b) $n = 8$ (no drug), $n = 7$ (drug); (d,e) $n = 6$ (no drug), $n = 5$ (drug); (c) $n = 13$. (f) $n = 12$ ('no drug'), $n = 11$ ('drug'). Boxes extend from the 25th to the 75th percentile and median values are represented by lines within the boxes. Whiskers extend to minimum and maximum data points. Significant differences are denoted with stars (Mann–Whitney *U* test). $P < 0.001$. 'ns' refers to a non-significant difference.

P assimilation in certain crops, highlighting an urgent need to more broadly assess and mitigate the unintended consequences of pharmaceutical contamination in agricultural soils.

4.1 | Effects of azole antifungals on AM fungi and crop nutrient assimilation

In our experiments, exposure to soil-borne azole antifungal pharmaceuticals reduced the total P assimilated by both crops over their lifetime via plant and AM fungal assimilation pathways (Figures 4 and 5). Interestingly, although the total P content in lettuce shoots remained consistent across both treatments, shoot biomass nearly doubled in plants exposed to antifungals. This increase may be attributed to a reduction in C allocation to AM fungi under antifungal exposure, allowing more plant-fixed C to be redirected toward shoot growth. Consequently, the unchanged total P content combined with increased biomass suggests a dilution effect, where P uptake did not keep pace with growth, leading to a lower tissue P concentration. In contrast, neither shoot biomass or total shoot P content in spring onions was significantly affected by antifungal treatment. Given that total P availability was consistent across all treatments, observed differences in shoot P content and biomass suggest each crop has a distinct capacity for P uptake, potentially relying on AM fungi to different extents.

Our ^{33}P tracer analysis provides further insights into this. Azole antifungals exposure caused a near-collapse of transfer of AM fungal-mediated ^{33}P transfer to spring onion, both in terms of total ^{33}P transferred and tissue [^{33}P], corroborating similar findings in a cereal crop, wheat (*Triticum aestivum* L. cv. 'Skyfall') (Sallach et al., 2021). However, AM-acquired ^{33}P detected in lettuce shoots was not affected by the same antifungal treatment. In control treatments, spring onion plants assimilated significantly more ^{33}P via AM fungi than lettuce, demonstrating a clear contrast in their AM dependencies. This is important because it suggests that the sensitivity of AM-mediated nutrient uptake in response to pharmaceutical contamination is not uniform across crop species, but the impact on nutritional benefits rather depends on their inherent mycorrhizal dependency

(Tawaraya, 2003). Crops like spring onion, which rely heavily on AM fungi for P acquisition, may be at greater risk of nutrient deficiency under antifungal exposure compared to crops like lettuce that appear less dependent on these symbiotic relationships. The observed decline in AM function between control and antifungal-treated soils could stem from decreased diversity of fungal communities in the roots, rather than the soil, as shown when plants were grown in antifungal-treated soils compared to control soils (Figure S3). Since AM fungal richness promotes plant P uptake (van der Heijden et al., 1998), the loss of key fungal species in response to antifungal exposure may have driven the observed declines in P transfer.

4.2 | Mechanisms of AM fungal inhibition by azole antifungals

Azole antifungals disrupt fungal physiology by inhibiting 14-demethylase, a cytochrome P450 enzyme responsible for converting lanosterol into ergosterol, which is a crucial sterol in fungal cell membranes (Akins, 2005; Merk and Mukhtar, 1989). However, studies have shown that AM fungal structures do not contain ergosterol (Olsson et al., 2003) but rather 24-ethyl cholesterol (Grandmougin-Ferjani et al., 1999) and 24-methyl sterols (Fontaine et al., 2004) as their main cell wall sterols. Importantly, lanosterol is a precursor to these sterols in addition to ergosterol (Weete et al., 2010), and 14-demethylase is involved in their formation. Without 24-ethyl cholesterol and 24-methyl sterols, AM fungal membranes lose integrity, reducing hyphal function and growth, thereby reducing AM fungal access to nutrients and their ability to transfer these to host plants. In our study, exposure to fluconazole, clotrimazole and miconazole nitrate may have driven the observed reduction in root colonisation by AM fungi and shortened extraradical hyphal length density, thereby limiting fungal ability to access distant P pools. It may also have resulted in reduced arbuscule formation, directly impairing the bidirectional nutrient exchange between the fungus and its host plant.

The decline in root colonisation and hyphal development observed suggests that these antifungals impair fungal viability at multiple stages of their life cycle, reducing the overall density and effectiveness of AM fungal networks. The observed decline in ^{33}P assimilation via AM fungi in spring onions could be due to the reduced mycorrhizal hyphal networks in antifungal-treated soils. AM fungi play a key role in mobilising poorly soluble inorganic P, such as calcium-phosphate complexes, by releasing organic acids and phosphatases (Smith & Read, 2008). With reduced hyphal length and reduced colonisation, the ability of AM fungi to access and solubilise organic and inorganic P pools would be diminished, and transport of P to host plants would be disrupted. Given the low mobility of P in soils compared to N (Tian et al., 2024), plants often depend on AM fungi to extend their nutrient absorption beyond the root zone. The impaired AM hyphal networks in antifungal-exposed soils likely constrained this process, disproportionately affecting crops with high mycorrhizal dependency - such as spring onions.

Unlike P, N is more mobile in soils and can be taken up directly by plants in dissolved forms such as nitrate (NO_3^-) and ammonium (NH_4^+). Although AM fungi can assist in N assimilation, their role is less critical than in P uptake (George et al., 1995). In our experiments, ^{15}N transfer via AM fungi was unaffected by antifungal exposure. This suggests that while the density of AM fungal networks was reduced, N's greater soil mobility allowed sufficient interaction with the remaining fungal hyphae for effective uptake. The unaltered N uptake in both species tested may indicate that the antifungals specifically target sterol-dependent fungal processes rather than directly affecting the plants' direct N acquisition mechanisms. Alternatively, the remaining extraradical AM fungal network, despite being less dense following exposure to azole antifungals, retained enough functionality to support N transfer. Our findings align with previous research showing that fungicide-induced reduction in mycorrhizal P uptake do not necessarily correspond with similar disruptions in mycorrhizal N transfer (Edlinger et al., 2022; Schweiger & Jakobsen, 1998).

As obligate biotrophs, AM fungi rely entirely on host plants for C in exchange for nutrients. Under normal conditions, plants direct 20–30% of their photosynthetically fixed C toward AM fungi to sustain the symbiosis (Smith & Read, 2008). In antifungal-treated soils, we observed reduced C allocation to AM fungal mycelium, suggesting plants "invested" less resource in symbionts that were performing poorly. We also observed increased biomass of lettuce in antifungal-treated pots, indicative of a shift in C allocation from AM fungal partners to plant growth instead. This aligns with a "reciprocal rewards" model of symbiosis (Kiers et al., 2011), where plants preferentially allocate resources to symbionts that provide more resources. However, it could equally be that the reduced extent of the fungal network itself lowered fungal demand for plant resources, leading to decreased C transfer to AM fungi.

4.3 | Implications for sustainable farming and future research

Maximising soil health and function, including the nutritional benefits conferred on host plants by AM fungi, is a high priority for sustainable farming (George & Ray, 2023). While wastewater and biosolid application provide organic matter and enhance the fertility of agricultural soils (Eden et al., 2017), the benefits may be offset by pharmaceutical contamination. Given that antifungals accumulate in soils with repeated biosolid amendments (Chen et al., 2013), their long-term effects on microbial communities warrant further investigation. Our controlled experiments highlight the potential for widespread pharmaceutical contamination to negatively impact AM fungal symbiosis and crop nutrient acquisition. However, real-world agricultural systems involve additional stressors, such as pesticides, microplastics and climatic variability that may further influence these interactions.

Furthermore, other pharmaceuticals, including different antifungals, build up and persist in soils following repeated biosolids amendment (Chen et al., 2013). Despite the necessary simplification, our experiments suggest the commonly used compounds applied to the soils in

our experiments have a particularly detrimental impact on root-associated fungal communities, with subsequent negative effects on fungal-acquired P assimilation in certain crop species. It remains undetermined as to the extent that co-occurring factors would influence the fate of antifungals in the soil environment and subsequently their bioavailability for crop uptake and potential effects. Similarly, the effects of repeated exposure and build-up of antifungals in soils require further exploration. It is now critical that the impacts of other and combined emerging contaminants, as well as build-up and repeated exposure, on soil microbial diversity and function are properly assessed before application of biosolids and wastewater is advocated for. Simultaneously, exploration of technological solutions for the removal of contaminants from biosolids prior to application should also be prioritised.

In summary, our research demonstrates that azole antifungals in soil have significant effects on AM fungi-crop symbioses, particularly impacting P transfer while leaving N transfer largely intact. Spring onions, which appear to rely on AM fungi more strongly than lettuce, experienced greater reductions in fungal-mediated nutrient uptake than lettuce. The persistence of antifungals in biosolid-amended soils and their selective effects on nutrient acquisition highlight the urgent need for crop-specific risk assessments and targeted management strategies. As biosolid use continues to rise, mitigating pharmaceutical contamination must be prioritised to safeguard soil health and sustainable food production.

AUTHOR CONTRIBUTIONS

L.C., J.B.S. and K.J.F. conceived and designed the experiments, which were set up by E.D., K.J.F. and L.C. E.D. conducted lab work, data collection and data analysis. E.D., K.J.F., J.B.S. and L.C. harvested the experiments and discussed the results. A.W. and S. M. assisted E.D. with sequencing analysis. All authors, led by E.D. and L.C., contributed to writing and approving the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

K.J.F. is an editor at *Plants, People, Planet* but has played no part in the handling, review or decision-making processes associated with this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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