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Contributed Paper

Development of a Novel Biomixture for Pesticide Degradation and Detoxification in Vietnam

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ABSTRACT

A biomixture is a principal element controlling the pesticide degradation efficiency of a biobed. In this study, a biomixture containing peat, as in the original Swedish design (Biomix-1), was compared with two biomixtures containing a spent mushroom substrate (SMS) (Biomix-2) and SMS inoculated with the ligninolytic fungus (Biomix-3) on their degradation of Chlopyrifos, 2,4-D, Cartap, and Cypermethrin and microbial activity. The residual concentration of pesticides was identified by the QuEChERS method. Ligninolytic activity was determined using the MBTH/DMAB assay, and the microbial respiration was assessed by CO_2 evolution via alkaline trap method. Detoxification of Chlopyrifos, 2,4-D, Cartap, and Cypermethrin in the optimal biomixture was also evaluated by the ecotoxicity test. A test for the emergence and growth of higher plants was performed according to ISO 11269-2:2012. The acute toxicity test for earthworms was conducted following ISO 11268-1:2012 and OECD-207:1984. The results showed that the pesticide degradation efficiency and microbial activities of the biomixtures followed the decreasing trend: Biomix-3 > Biomix-2 > Biomix-1. Germination showed a non-significant decreasing trend with increasing applied spent Biomix-3 concentrations, while plant fresh weight values increased significantly up to certain concentrations of spent Biomix-3 compared to the respective control. Similarly, no significant mortality of earthworms was observed between the control and any treatments with spent Biomix-3. However, earthworms in treatments with high spent concentrations (>50 g/kg dry soil) had higher wet biomass reduction rates than earthworms in the controls. Therefore, it was recommended to use Biomix-3 to improve the performance of biobeds and the reuse of spent biomixture in Vietnam due to the availability of substrates.

Keywords: biomixture, ligninolytic fungus, spent mushroom substrate

1. INTRODUCTION

In the last century, pesticides have become an important component of modern global agricultural systems, resulting in a substantial improvement in crop yields by controlling insects and diseases [1]. However, inadequate management of pesticides can lead to contamination of surface and groundwater and large volumes of soil [2], of which, point sources of pesticides, such as accidental spillage during tank filling, packaging disposal, or spraying equipment cleaning after use,

have been identified as the major contamination risks [3]. Given these circumstances, biobeds have been successfully used to reduce point source contamination by pesticides [4]. The original Swedish biobed comprises a clay layer at the bottom, a biomixture layer, and a grass layer on top. The composition of biomixture consisting of soil: peat: wheat straw (1:1:2 by volume) determines the efficiency of the system [2]. In turn, each of the components play an important role in the efficiency of the biomixture: (i) the soil provides sorption capacity and also an active microbial community for pesticide degradation [5]; (ii) peat increases the sorption capacity, improves porosity, regulates the pH in the biomixture, and contributes to moisture control [6]; and (iii) the straw, a lignocellulosic material, is the main substrate for microbial activity, especially by lignin-degrading fungi (e.g., white-rot fungi), producing phenoloxidases (peroxidases and laccases) for pesticide degradation [7]. In fact, the components of biomixtures have been modified from the original design to adapt to be environmentally friendly and based on the availability of substrates in different countries. Mushroom residues (*Pleurotus eryngii, Flammulina velutipes,* and *Lentinus edodes*) and different types of composts have been used instead of peat [2, 8], while maize straw, vine straw, and rice husk have been used instead of wheat straw [9–11]. Additionally, a ligninolytic fungus (*Trametes versicolor*) was employed as the bioaugmentation agent to improve the performance of biomixtures for the elimination of some pesticides [11]. However, the potential use of rice (*Oryza sativa* L.) straw, spent mushroom substrate (*Pleurotus pulmonarius*), and ligninolytic fungus (*Penicillium chrysogenum* N2) has not been studied.

As an agriculture-based countries with about 100,000 tons of annually used pesticides, Vietnam is facing serious challenges concerning environmental pollution related to pesticide use [12]. Due to their low cost, low maintenance, and high efficiency, biobeds are considered a potential approach for residual pesticide disposal. In terms of the availability of substrates for biomixture in Vietnam, about 54 million tons of rice straw is produced for producing about 45 million tons of grain annually [13]. Most of the rice straw is burned on the open fields, causing serious air pollution; thus, re-use of rice straw is essential. However, peat is not environmentally and economically feasible. Meanwhile, the total yield of commercial mushrooms in 2019 was over 270,000 tons, and about 162,000 tons of spent mushroom substrates (SMS) were generated. Most SMS are stacked, impairing the surrounding environment and wasting resources [14].

In this study, biomixtures were prepared with the various compositions, as follows: (i) topsoil, rice (*O. sativa* L.) straw, and peat; (ii) topsoil, rice straw, and SMS (*P. pulmonarius*); and (iii) topsoil, rice straw, and SMS inoculated with the ligninolytic fungus (*P. chrysogenum* N2). This work aimed to determine the pesticide degradation and detoxification efficiencies of these biomixtures to, thereby, develop a feasible and effective biomixture for the performance of biobeds under Vietnam's conditions.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Pesticides

Analytical standard Chlorpyrifos [(O, O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothiate, $C_9H_{11}Cl_3NO_3PS$, CAS No. 2921-89-2], Cartap [(1,3-bis(carbamoylthio)-2-(N,N-dimethylamino) propane hydrochloride, $C_7H_{16}ClN_3O_2S_2$, CAS No. 22042-59-7], 2,4-D [2,4-Dichlorophenoxyacetic acid, $C_8H_6Cl_2O_3$, CAS No. 94-75-7], and Cypermethrin [3-(2,2-dichloroethenyl)-2,2-dimethyl-cyclopropanecarboxylic acid, $C_{22}H_{19}Cl_2NO_3$, CAS No. 52315-07-8] were purchased from Sigma–Aldrich (USA).

2.1.2 Biomixtures

Soil was collected from the topsoil layer (0−20 cm) of an arable field in Ha Dong-Hanoi (Vietnam), air-dried at room temperature, and

sieved through 2 mm mesh. Commercial garden peat was obtained from a local flower market in Bac Tu Liem-Hanoi (Vietnam). Rice (*O. sativa* L.) straw was collected from a rice field in Ninh Binh (Vietnam) and chopped into 2−3 cm pieces. SMS was collected from an oyster mushroom (*P. pulmonarius*) growing farm in the Agricultural Genetics Institute (Bac Tu Liem-Hanoi-Vietnam). The ligninolytic fungal strain identified as *P. chrysogenum* N2 was previously isolated from soil in Thua Thien Hue (Vietnam) using potato dextrose agar (M096, Himedia, India) [15]. The colonies of *P. chrysogenum* N2 on Sabouraud dextrose agar (SAB) were green (dark green to black on the reverse side of the agar plate) with a velutinous texture. The 28S rRNA nucleotide sequence of *P. chrysogenum* N2 (Figure 1) had 100% identity with that of *P. chrysogenum* IF2SW-F5 and was deposited in the DDBJ/EMBL/GenBank database with accession number MW412637. The phylogenetic tree constructed by the Neighbor-Joining method is shown in Figure 2. The ligninolytic activity of the extracellular crude enzymatic extract produced by *P. chrysogenum* N2 after 48 h of incubation in Czapek Dox broth (M076, Himedia, India) supplemented with 1% (w/v) lignin was 173.6 U/L. For inoculum preparation, *P. chrysogenum* N2 was used to inoculate a 500 mL Erlenmeyer flask containing 200 mL of potato dextrose broth (M403, Himedia, India) and incubated on a shaker (New Brunswick, Innova 44R, Eppendorf, Germany) at 150 rpm at 30 °C for 72 h. Subsequently, the wet biomass was harvested and served as the bioaugmentation agent.

2.1.3 Spent biomixtures

After a 30 day pesticide treatment, the representative composite samples from the biomixture having the highest degradation efficiency for pesticides (Biomix-3) contaminated with Chlopyrifos, 2,4-D, Cartap, and Cypermethrin were collected, named SB-Chlor, SB-2,4D, SB-Car, and SB-Cyp, respectively, and used for plant growth tests and acute toxicity tests.

2.2 Methods

2.2.1 Experimental setup and sampling

The three biomixtures were prepared by thoroughly mixing topsoil, rice straw, and either i) peat (Biomix-1), ii) SMS (Biomix-2), or iii) SMS inoculated with 5% (w/v) of the ligninolytic fungus *P. chrysogenum* N2, in volumetric proportions of 1:2:1. The biomixtures (2.5 kg each) were placed in PVC containers (45 cm length × 32 cm width × 28 cm depth), irrigated with distilled water to 60% water-holding capacity (WHC), and pre-incubated in the dark at 25 ± 1 °C for 15 days. This pre-incubation condition was recommended for

Figure 1. Colonial morphology of *Penicillium chrysogenum* N2.

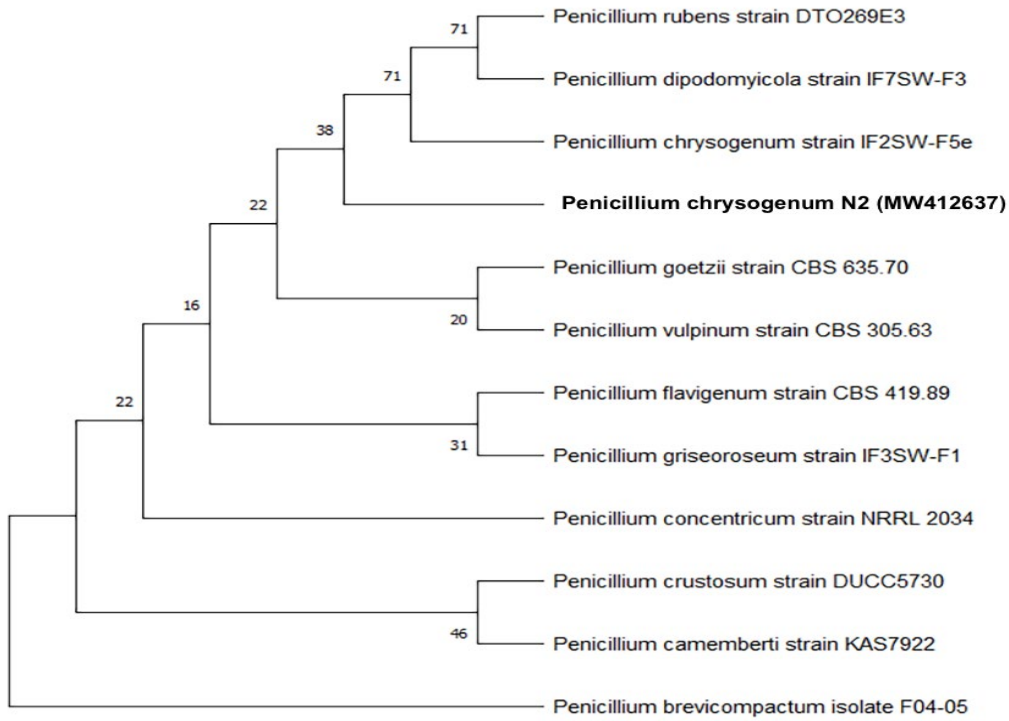

Figure 2. Neighbour-joining phylogenetic tree using Mega-X [30], Tamura-Nei substitution model, transitions/transversions with gamma distribution. Bootstrap test (2000 replicates).

the dissipation of most pesticides and activity of ligninolytic fungi [16]. Then, one bulk sample of the biomixtures was separated into sub-samples (10 g dry weight) contained in glass flasks (500 mL). The sub-samples were individually contaminated with Chlorpyrifos, Cartap, Cypermethrin (100 mg/kg^{-1}), and 2,4-D (10 mg/kg) and incubated in the dark at 25 ± 1 °C. The initial WHC of sub-samples was maintained through the periodic addition of distilled water. The representative composite samples were collected at days 5, 10, 15, 20, 25, and 30 of incubation, stored at -20 °C, and analysed for the residual concentration of pesticides and microbial activity (microbial respiration and ligninolytic activity).

2.2.2 Determination of physicochemical properties

The pH in 1:5 (w/v) 1N KCl extract was measured with a pH meter ISO 10390:2005. Water content was determined by the gravimetric method (ISO 11465:1993). Bulk density was measured with a soil sampler of known volume to collect intact cores, which were oven-dried and weighed [17]. Organic carbon (OC) and total carbon (C) after dry combustion was determined according to ISO 10694:1995. Total nitrogen (N) was determined by the modified Kjeldahl method (ISO 11261:1995). Cellulose and lignin were estimated by the detergent extraction method [18]. WHC of the biomixtures was measured as suggested by Castillo and Torstensson [6].

2.2.3 Ligninolytic enzyme activity

The analysis was performed using the 3-methyl-2-benzothiazolinone hydrazone (MBTH)/3-(dimethylamino) benzoic acid (DMAB) assay according to the method described by Castillo et al. [19]. To extract the enzymes, samples of the biomixtures (10 g dry weight) were weighed in Erlenmeyer flasks and 50 mL of 100 mM succinate-lactate buffer (pH 4.5) was added to each sample. The flasks were shaken at 100 rpm for 2 h, and 10 mL of the supernatant was collected and centrifuged at 1610 × g for 20 min and filtered through a 0.45 μm membrane. The reaction mixture contained 300 μL 6.6 mM DMAB, 100 μL 1.4 mM MBTH, 30 μL 20 mM $MnSO_4$, 10 μL 10 mM H_2O_2, and 1560 μL of the sample in a 100 mM succinic/lactic acid buffer (pH 4.5). The reaction was followed at 590 nm (ε= 0.053 μM/cm) in a spectrophotometer (UV-1800, Shimadzu, Japan). This assay is normally used for the measurement of manganese peroxidase (MnP) activity, but high lignin peroxidase (LiP) and laccase activity can interfere with the measurements. Because no correction was made for the possible presence of LiP and laccase activity, this measurement may represent the sum of MnP, LiP, and laccase activities [6].

2.2.4 Microbial respiration

The CO_2 produced in biomixtures was evaluated through incubation experiments according to the method described by Iannotti et al. [20]. The biomixture (50 g) was placed in a 1 L flask with a vial containing 10 mL 0.5 M NaOH and a vial with 10 mL distilled water for maintaining a humid atmosphere. The flask was hermetically sealed and incubated at 25 °C. The CO_2 captured in the NaOH solution was determined by titrating the remaining alkali with 0.1 M HCl after precipitation of carbonate with 0.1 M $BaCl_2$. Respiration was expressed as accumulated mg CO_2/100 g biomixture.

2.2.5 Extraction and quantification of pesticides

The residual concentration of pesticides in a biomixture was determined by the QuEChERS method (EN 15662:2008) [21]. Briefly, 10 g of homogenised biomixture in a 50 mL centrifuge tube was added with 10 mL acetonitrile and mixed by vortex and ultrasonic bath for 5 min. Then, the sample was added with 4 g $MgSO_4$, 1 g NaCl, 1 g $Na_3Citrate{\cdot}2H_2O$, and 0.5 g $Na_2HCitr{\cdot}1.5H_2O$, shaken vigorously by hand for 2 min and centrifuged at 3,000 rpm for 5 min. Later, a 3 mL aliquot of the supernatant was transferred to a 15 mL dSPE centrifuge tube containing 0.2 g PSA, 1 g $MgSO_4$, and 5 mg GCB, shaken for 5 min and centrifuged at 3000 rpm for 5 min. Next, a 2 mL aliquot of the supernatant was transferred to a 15 ml centrifuge tube, and 0.1 mL formic acid was added. Chlopyrifos, Cartap, and Cypermethrin were analysed by GC-FPD (Shimadzu 2010 GC), LC-MS/MS (Agilent 6410 Triplequad), and GC-ECD (GC Varian 3800 GC), respectively. The residual concentration of pesticides was calculated as a percentage of the initial concentration of pesticides.

2.2.6 Plant growth tests

Tests for the emergence and growth of higher plants were performed according to ISO 11269-2:2012 [22]. Two species of higher plants (*Zea mays* L., monocotyledon, and *Phaseolus aureus* Roxb, dicotyledon) were used for testing. Their seeds were purchased from commercial supplies. The tests were carried out in plastic pots (9 cm diameter × 10 cm height) containing 450 g (ca. 10 cm soil depth) of un-amended soil (control) or amended with the different concentrations of spent biomixtures, randomly distributed. Soil moisture content was maintained at 80%. Ten uniform seeds of each species were sown at a depth of 5 mm and equidistant from each other. The average number of germinated seeds (G) per pot was recorded after 7 days. Subsequently, only five representatives germinated seeds were kept

per pot. Pots were then kept for 14 days (counted after 50% germination was observed in the control treatment) at room temperature (28 ± 2 °C), with a light intensity of 7,000−8,000 lx at the soil surface and photoperiod of 16:8 h (light: dark). Average plant fresh weight (FW) per pot was recorded after 14 days. Seed germination (G; % of the control) and plant growth (FW, % of the control) were calculated as follows:

G (%)
= (Average number of seeds germinated per pot in the spent biomixture ÷ Average number of seeds germinated per pot in the control) × 100

FW (%)
= (Average plant fresh weight per pot in the spent biomixture ÷ Average plant fresh weight per pot in the control) × 100

2.2.7 Acute toxicity tests

Acute toxicity tests were conducted following ISO 11268-1:2012 [23] and OECD-207:1984 [24]. Adult earthworms (*Eisenia fetida*), about 2 months old, with a clitellum, and a wet mass between 300 and 600 mg were purchased from the earthworm-rearing farm in Trau Quy-Hanoi (Vietnam) for testing. The earthworms were fed cow manure in the fertile soil and acclimated in an environment-controlled chamber at 20 ± 1 °C in the dark with 70% relative humidity. The earthworms were depurated on moist filter paper for 24 h and rinsed with deionised water, before the toxicity tests. Later, earthworms were individually placed into 500 g dry weight soil amended with the treatment concentrations of spent biomixtures in a 1 L high-density polyethylene container in the environment-controlled chamber. The relative humidity, temperature, and light intensity were adjusted to 70%, 20 ± 1 °C, and 400−800 lx, respectively. To prevent the escape of the earthworms from the soil, plastic containers were covered with a plastic film having several tiny holes to maintain ventilation and minimise moisture evaporation. All treatments were conducted in quadruplicate, with 10 earthworms per quadruplicate. The soil moisture content was adjusted twice a week by replenishing weight loss with the appropriate amount of deionised water. During 14-day exposure, the dead earthworms, which did not respond to gentle needle probing in the tail, were immediately removed and their number was recorded. The earthworms that survived at the end of exposure were collected, depurated on a moist filter paper for 24 h, rinsed with deionised water, and weighed for wet biomass determination [25].

2.2.8 Statistical analyses

Experiments were conducted with three independent replicates. Data were statistically analysed by one-way analysis of variance (ANOVA), and the averages were compared by Duncan multiple range test at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Physicochemical Properties of Single Substrates and Biomixtures

Some differences in the physicochemical characteristics of the single substrates and biomixtures were observed between the SMS and peat (Table 1). The pH of the peat was 5.5, while that in the SMS was 8.12. The OC content was 36.5% in SMS and 29.65% in peat. Total N content was 1.74% in peat and 1.82% in SMS. The C/N ratio was 20.05 in SMS but was lower in peat (17.04). However, higher contents of cellulose (33.6%) and lignin (21.55%) were found in peat, while lower contents of cellulose (22.12%) and lignin (14.5%) were found in SMS. Additionally, the pH of the biomixtures varied from 6.23 to 6.85. The OC content (20.55%) in the peat-based biomixture (Biomix-1) was higher than that in SMS-based biomixtures (Biomix-2 and Biomix-3), despite the lower OC content in peat compared with SMS. The higher density of the peat (0.38 g/cm^3) resulted in its higher gravimetric amount being present in Biomix-1. Similarly, the

Table 1. Physicochemical properties of single substrates and biomixtures.

Material	pH 1:5 KCl	Dry matter (%)	Density (g/cm^3)	OC (%)	N (%)	C/N ratio	Cellulose (%)	Lignin (%)	g in 100 g biomixture
Soil	6.21	72.57	1.32	1.45	0.09	14.36	nd	nd	
Rice straw	6.56	85.61	0.06	34.24	0.69	49.32	38.06	15.51	
Peat	5.5	69.30	0.38	29.65	1.74	17.04	33.6	21.55	
SMS	8.12	43.50	0.19	36.5	1.82	20.05	22.12	14.5	
Soil: peat: rice straw (Biomix-1)	6.23	nd	nd	20.55	0.98	20.57	nd	nd	75: 21.6: 3.4
Soil: SMS: rice straw (Biomix-2)	6.85	nd	nd	15.25	0.65	23.06	nd	nd	84.1:12.1:3.8
Soil: SMS: rice straw + fungal biomass (Biomix-3)	6.65	nd	nd	17.62	0.73	24.12	nd	nd	84.1:12.1:3.8 + 5 g biomass

OC: Organic carbon; SMS: Spent mushroom substrate.

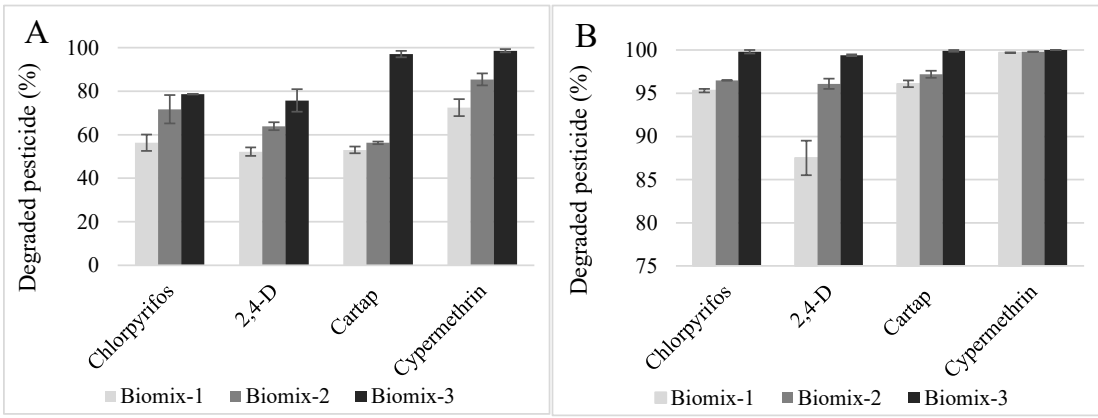

Figure 3. Pesticide degradation in biomixtures on days 15 (A) and 30 (B) of incubation.

lower OC content of Biomix-2 (15.25%) was considered a result of the low density of the SMS substrate (0.19 g/cm^3). Total N content of Biomix-1, Biomix-2, and Biomix-3 were 0.98, 0.65, and 0.73%, respectively. The C/N ratio fluctuated between 20.57 and 24.12. The biomixture is a key factor controlling the efficiency of the biobed [7]. As mentioned, the original Swedish biomixture was designed to promote the growth and activity of lignin-degrading fungi, by adding a lignocellulosic substrate and by keeping the pH at around 5. In this study, the results showed that the peat can be replaced by SMS in the components of the biomixture to minimise the operational cost and adapt the biobed to Vietnam's conditions.

3.2 Pesticide Degradation in the Biomixtures

Pesticide degradation in the biomixtures is shown in Figures 3 and 4. The efficiency of pesticide degradation depends on the type of pesticide, period of incubation, and composition of the biomixture. The degradation of Chlorpyrifos, 2,4-D, Cartap,

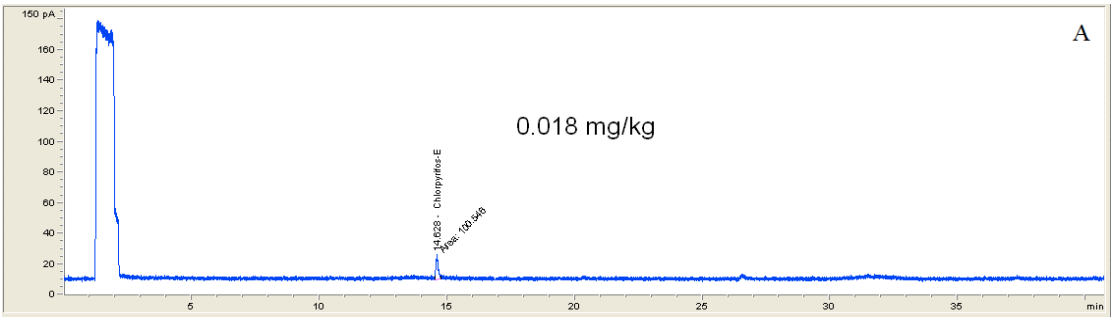

Figure 4. Chromatogram of Chlorpyrifos residue in Biomix-3 on day 30 of incubation.

and Cypermethrin on day 30 compared with day 15, increased from 56.3 to 95.3%, 52.2 to 87.5%, 53 to 96.1%, and 72.5 to 99.7%, respectively, in Biomix-1, from 71.7 to 96.5%, 63.9 to 96.1%, 56.3 to 97.2%, and 85.4 to 97.8%, respectively, in Biomix-2, and from 78.6 to 99.8%, 75.7 to 99.4%, 97.1 to 99.9%, and 98.6 to 100%, respectively, in Biomix-3. In the biomixtures, the degradation efficiency for Cypermethrin fluctuated between 72.5 and 100% and was higher than that for Cartap (53−99.9%), Chlorpyrifos (56.3−99.8%), and 2,4-D (52.2−99.4%). The efficiency of pesticide degradation in the biomixtures followed the decreasing trend Biomix-3 > Biomix-2 > Biomix-1 with statistically significant differences between these types of biomixtures. Meanwhile, pesticide dissipation was not significantly different between three biomixtures containing different SMS (*P. eryngii*, *F. velutipes,* and *L. edodes*) and biomixtures containing peat [2], and bioaugmentation with the ligninolytic fungus *T. versicolor* did not improve the pesticide removal capacity of the biomixture [12]. Here, the higher pesticide degradation efficiency of Biomix-3 could be accounted for by the higher pesticide degrading capacity of ligninolytic fungi, which is linked to the production of extracellular lignin-modifying enzymes and intracellular enzymatic complexes, such as cytochrome P450 [26].

3.3. Microbial Activity in the Biomixtures

3.3.1 Microbial respiration

Microbial respiration (CO_2 production) of the biomixtures contaminated with pesticides was presented in Figure 5. The CO_2 production was significantly higher in Biomix-3 compared with Biomix-1 and Biomix-2. This result could be attributed to inoculation with the lignin-degrading fungal strain *P. chrysogenum* N2 as a start inoculum that increased the proliferation of microorganisms in the biomixture during pre-incubation and the subsequent pesticide treatment period by the degradation of the components of the biomixture, which provided nutrients and readily available C sources, hence, higher CO_2 production. Additionally, there was a significant difference ($p>0.05$) in the production CO_2 between Biomix-1 and Biomix-2, probably since the replacement of peat with SMS in biomixture composition affected the microorganisms in their respiratory activity. This agrees with the comment that microbial respiration activity was higher in biomixtures containing SMS (*P. eryngii*, *F. velutipes,* and *L. edodes*) than the original type [2]. More readily available C and active mycelium and spores from the cultured mushroom remaining in the SMS can explain the higher activities. Besides, the microbial respiration (Figure 5) and pesticide degradation (Figure 4) in different biomixtures followed a similar behaviour,

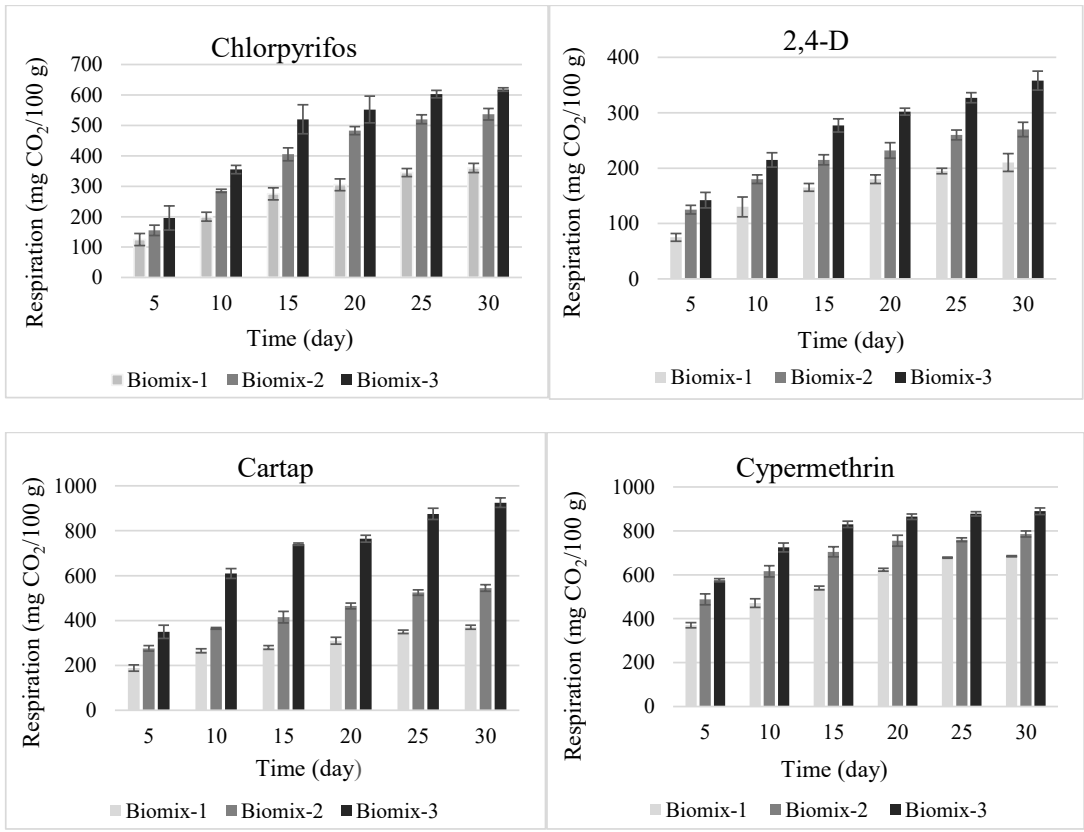

Figure 5. Microbial respiration (mg CO_2/100 g) in biomixtures during the treatment period.

obtaining both the highest values with Biomix-3. The respiration rate has been used previously as an indicator of pesticide degradation in biobeds, where the dissipation rates of some pesticides were correlated with basal respiration in 13 different biomixtures [6].

3.3.2 Ligninolytic activity

High ligninolytic activity was detected in all biomixtures during the pesticide treatment with a peak at day 15 and then a constant rate (data not shown). On day 30, the ligninolytic activity in each biomixture contaminated with Cartap and Cypermethrin was higher than those contaminated with Chlorpyrifos and 2,4-D (Figure 6). Besides, the ligninolytic activity in peat-based Biomix-1 fluctuated between 3.6 and 5.2 U/kg and was significantly lower ($p<0.05$) than the SMS-based Biomix-2 (5.8−10.2 U/kg). However, the SMS and lignin-degrading fungal strain-based Biomix-3 had higher ligninolytic activity (10.7−16.3 U/kg) compared with SMS-based Biomix-2 (Figure 6). In addition to the ligninolytic fungus *P. chrysogenum* N2, it may be due to the ligninolytic activity possessed by the oyster mushroom (*P. pulmonarius*) cultivated on the SMS used in this study. The ligninolytic enzymes (peroxidases and laccases) produced by white-rot fungi are nonspecific and can degrade a wide range of pesticides [7, 27]. Similar to the microbial respiration (Figure 5), the ligninolytic activity (Figure 6) and pesticide degradation (Figure 3) in the different biomixtures followed

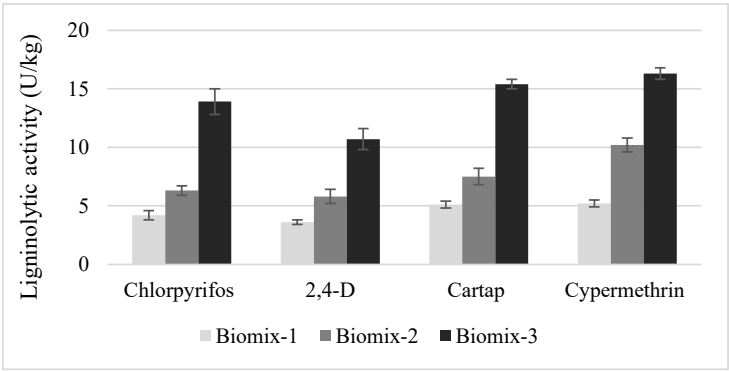

Figure 6. Ligninolytic activity in biomixtures after 30 days of pesticide treatment at 25 °C.

a similar behaviour, obtaining both the highest values in Biomix-3. This is consistent with some previous studies that reported that ligninolytic activity was correlated to the degradation of several pesticides [6, 8, 27].

3.4 Effect of Spent Biomixtures on the Emergence and Growth of Higher Plants

The spent biomixtures had effects on the emergence and growth of *Z. mays* L. and *P. aureus* Roxb after 14-day exposure. Germination showed a non-significant decreasing trend with increasing spent biomixture concentration ($p>0.05$). Nevertheless, in general, the plant fresh weight showed an inverse pattern to germination. The fresh weight of *P. aureus* Roxb and *Z. mays* L. significantly increased up to 200 and 500 g/kg, respectively. The spent biomixture concentration that resulted in the highest plant fresh weight depended on the types of spent biomixtures and test plants. The fresh weight of *Z. mays* L. was highest (106%) in the 500 g/kg treatment of SB-2,4-D (Figure 7), and *P. aureus* Roxb was highest (102%) in the 200 g/kg treatment of SB-Cryp (Figure 8) compared to the control. Increased plant fresh weight could be related to the plant-available nutrients in the spent biomixture substrates, particularly N, P, and K.

3.5 Effect of Spent Biomixtures on Acute Toxicity to Earthworms (*Eisenia fetida*)

In the controls of acute toxicity tests, the mortality and wet biomass reduction of adult *E. fetida* were below 10 and 20%, respectively (Figure 9), satisfying the ISO (1993) requirements of bioassay validation. No significant mortality of the earthworms was observed between the controls and any treatment ($p>0.05$). Meanwhile, the significant wet biomass reduction of the earthworms was found between the controls and some treatments ($p<0.05$). Earthworms in the 10 g/kg treatment of the SB-Car and SB-Cyp after 14 days had lower wet biomass reduction rates than earthworms in the controls. A possible explanation could be that low application rates of spent biomixtures can be used as an extra source of food for microbiota and could benefit earthworm growth. Some authors have reported similar results and attributed the phenomenon to a hormetic response, i.e., the ability of a substance to be toxic at high concentrations but a stimulant at low concentrations [28]. Conversely, earthworms in the treatments with high spent biomixture application rates (>50 g/kg dry soil) had higher wet biomass reduction rates than earthworms in the controls. The wet biomass reduction rate was highest (326%) in the 200 g/kg treatment of SB-

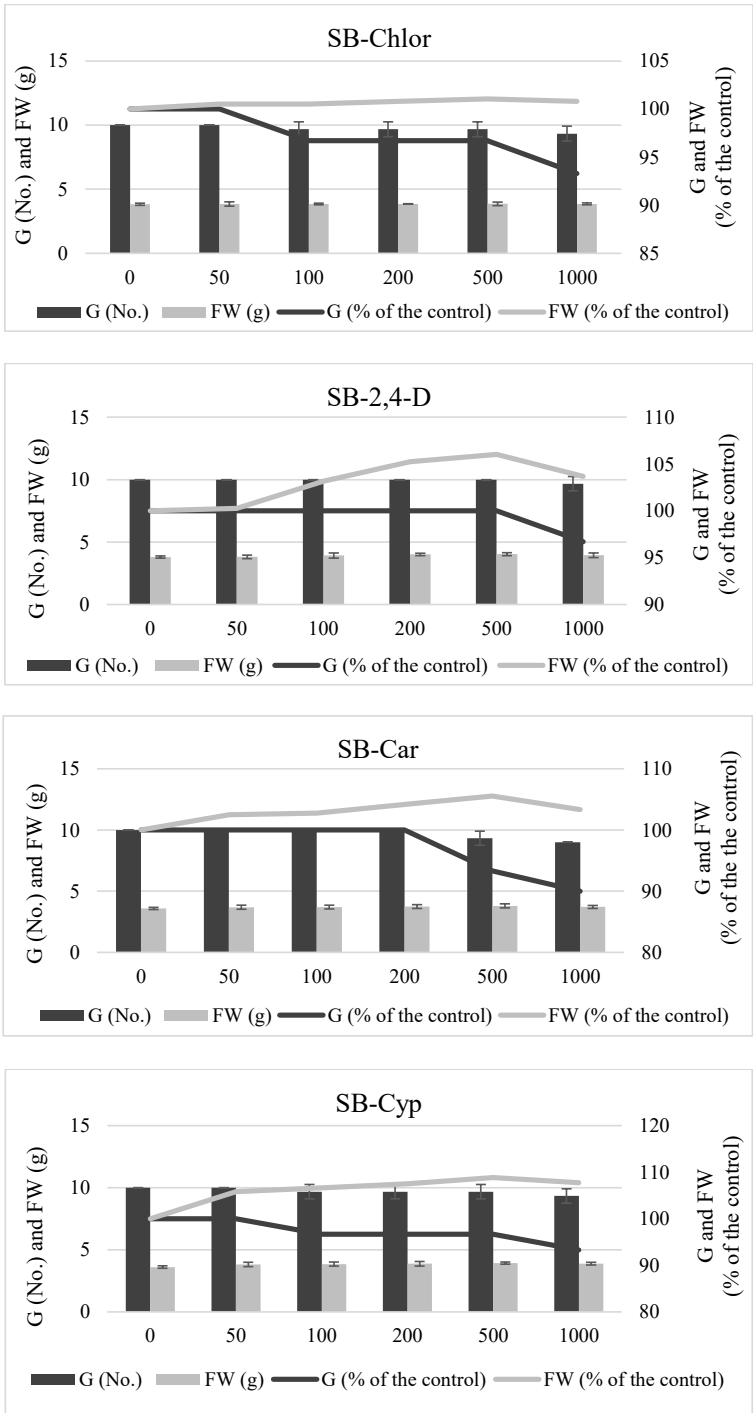

Figure 7. Germination (G) and fresh weight (FW) values (% of the control) of *Zea mays* L. exposed to spent biomixture concentrations (0, 50, 100, 200, 500, and 1000 g/kg dry soil).

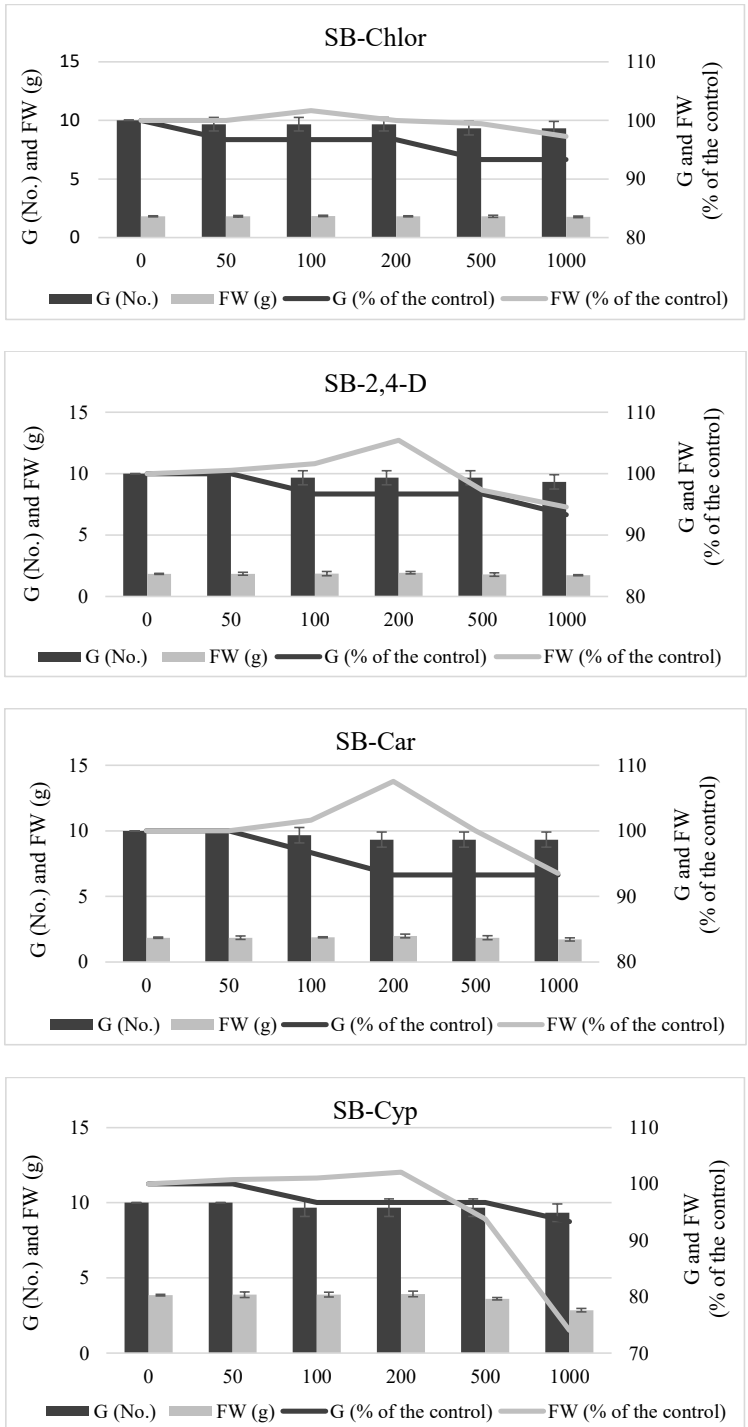

Figure 8. Germination (G) and fresh weight (FW) values (% of the control) of *Phaseolus aureus* Roxb exposed to spent biomixture concentrations (0, 50, 100, 200, 500, and 1000 g/kg dry soil).

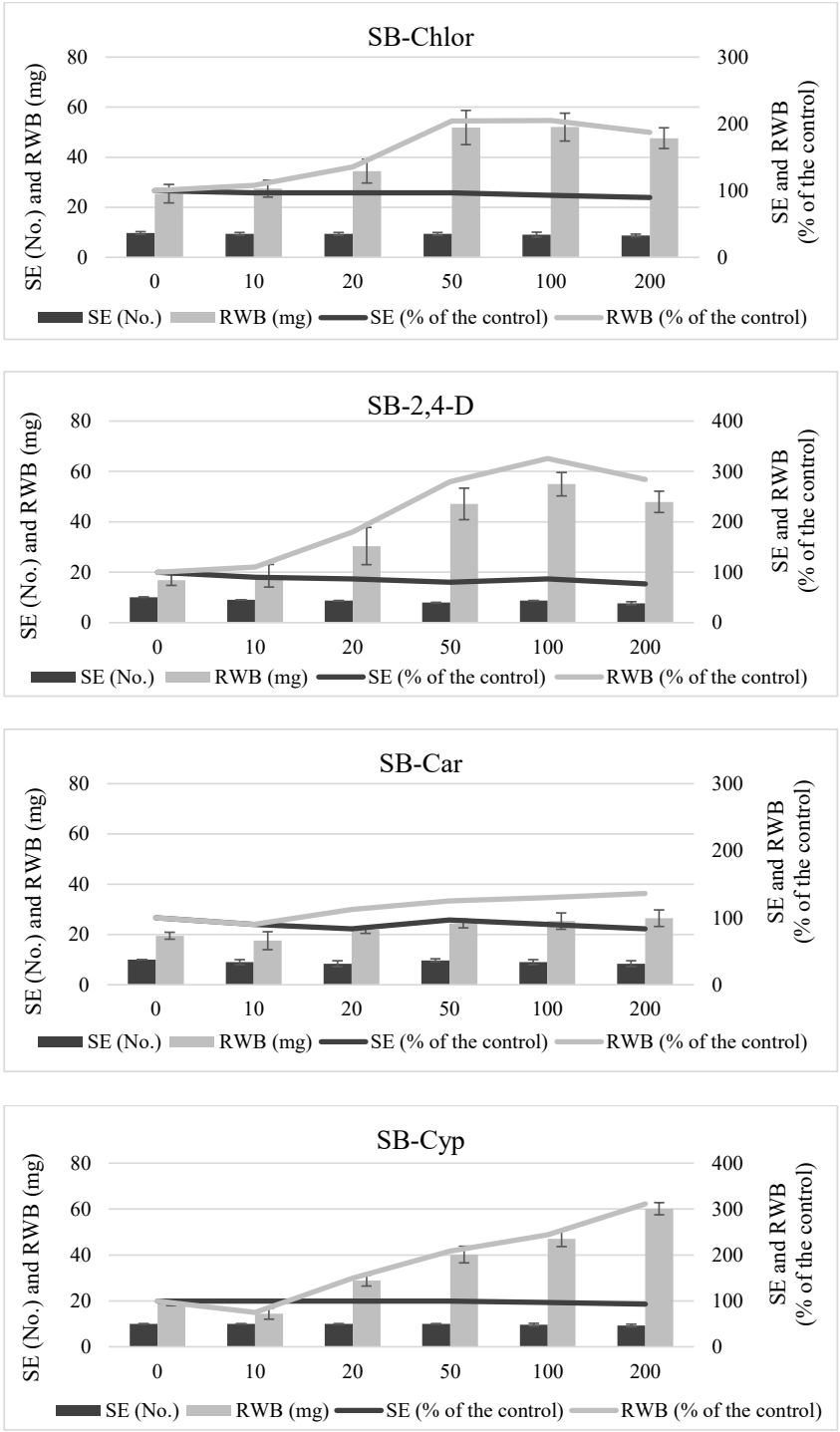

Figure 9. Surviving earthworms (SE) and reduced wet biomass (RWB) values (% of the control) of earthworms (*Eisenia fetida*) exposed to spent biomixture concentrations (0, 10, 20, 50, 100, and 200 g/kg dry soil).

2,4-D compared to the control (Figure 9). Biomass reduction may reflect the reduction in feeding and physiological changes in the earthworms [29]. The differences in biomass reduction rates of the earthworms were mostly attributable to the differences in substrate composition of spent biomixtures, including the breakdown products of pesticides.

4. CONCLUSIONS

The composition of biomixtures was determined not only by the function of the components but also by the local availability of the materials. In Vietnam, soil and rice straw are widely available, but peat is not an option for farmers from either an environmental or economic perspective. Meanwhile, SMS is widely produced and available to every farmer. The results of this study showed that the pesticide degradation efficiency and microbial activities (ligninolytic activity and microbial respiration) in SMS-based biomixtures were higher than those in the peat-based biomixture; thus, SMS is a suitable substitute for peat. Moreover, it is recommended to use the SMS (*P. pulmonarius*)-based biomixture inoculated with the ligninolytic fungus (*P. chrysogenum* N2) for improved performance of the biomixture. However, although no statistically significant differences about the germination of higher plants and mortality of earthworms were observed between the controls and treatments of the spent SMS-based biomixture inoculated with the ligninolytic fungus *P. chrysogenum* N2, further studies of ecotoxicity are essential before it can be reused as biofertiliser in agriculture.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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