Indian Journal of Experimental Biology
Vol. 59, December 2021, pp. 884-890

Mucor assisted degradation of sulfosulfuron in irrigation water

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Received 31 December 2018; revised 03 September 2020

Sulfosulfuron is a widely used wheat herbicide, which has two major environmental issues, *viz*. carryover effect to succeeding crops, and groundwater and surface water contamination. The safer aquatic environment requires the degradation of the herbicide. In this present investigation, we have attempted to isolate an efficient fungus naturally abundant in water, which can degrade sulfosulfuron in aqueous phase. *Mucor* sp., a fungus isolated from irrigation water was characterized as a sulfosulfuron-degrading microorganism. It survived in the minimal broth having sulfosulfuron at a level of 2000 mgL^{-1}. It was able to degrade entire amount of sufosulfuron applied to water within 27 days. Two major routes of degradation were established. One route involved the cleavage of sulfonylurea bridge resulting in the formation of a couple of major metabolites, *viz*. 2-amino-4,6-dimethoxypyrimidine (I) and 2-ethylsulfonylimidazo{1,2-a}pyridine-3-sulfonamide (II). The other route was the cleavage of sulfonylamide linkage, which formed the metabolite *N*-(4,6-dimethoxypyrimidin-2-yl)urea (III). Three other metabolites, *N*-(4,6-dimethoxypyrimidin-2-yl)-*N*'-hydroxyurea (IV), *N*,*N*'-bis(4,6-dimethoxypyrimidin-2-yl)urea (V) and *N*-(4,6-dimethoxypyrimidin)-*N*'-(4-hydroxy-6-methoxy pyrimidin-2-yl)urea (VI) were also identified. The survival of *Mucor* sp. at a very high concentration of sulfosulfuron and its ability to degrade the herbicide through various biochemical mechanisms showed its potential in the bioremediation process of sulfosulfuron contaminated water.

Keywords: Biodegradation, Bioremediation, Herbicides, Microbial degradation, Sulfonylurea herbicide

Sulfosulfuron, 1-(4,6-dimethoxypyrimidin-2-yl)-3-(2-ethylsulfonylimidazol [1,2-*a*]pyridine-3-yl) sulfonylurea, is a selective and systemic pyrimidinylsulfonylurea herbicide. It controls grassy and broad leaf weeds in wheat at an application rate of 25 g/ha[1]. Two obnoxious weeds in wheat, *viz*. *Phalaris minor* and *Avena ludoviciana* are being managed by the application of this herbicide[2]. At the same time, the residues of sulfosulfuron in the soil can damage many crops like maize (*Zea mays*), sorghum (*Sorghum bicolor*), barley (*Hordeum vulgare*), oats (*Avena sativa*) and sunflower (*Helianthus annuus*)[3]. In general, the half-life (DT_{50}) of it is within the range of 20-60 days, depending upon the nature of soil and climate[4]. The physical displacement of herbicide through leaching down or runoff may lead to the groundwater or surface water contamination. The solubility of sulfosulfuron in water at an ambient temperature (25°C) is nearly 18 mgL^{-1} at pH 5, whereas at pH 7, it is around 1630 mg/L[4]. Therefore, the chance of leaching or runoff of sulfosulfuron is high when it is applied on soil with agronomic pH, i.e. pH within the range of 6.5 to 7.5. Lafontaine *et al*.[5] have shown sulfonyl urea herbicides including sulfosulfuron in the surface water collected from the St. Lawrence River, Canada. In acidic aqueous phase, sulfonylureas mainly degrade chemically as was observed in many members of this group, *viz*. sulfometuron-methyl[6], chlorsulfuron[7], metsulfuron-methyl[8], rimsulfuron[9], chlorimuron-ethyl[10], nicosulfuron[11], sulfosulfuron[12], flazasulfuron[13] and pyrazosulfuron[14]. However, in agronomic pH, sulfonylureas are quite stable and do not undergo pH-dependent chemical hydrolysis of sulfonylurea bridge. In this situation, degradation occurs mainly by photolysis or by microbial enzymatic processes. The enzymes or enzyme consortia released by microorganisms present in the natural water bodies govern the type of biochemical reaction to degrade the herbicide molecule. In the case of sulfonylurea herbicides, the first step of degradation is mostly the abiotic cleavage of sulfonylurea bridge mediated by water, pH and/or sunlight. However, as observed in

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some cases, microbes can also cleave this chemical bond. Sharma *et al*. [15] revealed the enzymatic sulfonylurea bridge cleavage of chlorimuron by *Aspergillus niger* isolated from the soil. The degraded products, after sulfonylurea bridge cleavage, undergo extensive enzymatic degradation as seen in the case of orthosulfamuron by *Aspergillus niger*[16], chlorsulfuron and metsulfuron-methyl by *Streptomyces griseolus*[17], triflusulfuron-methyl by *Streptomyces griseolus*[18], primisulfuron by *Phanerochaete chrysosporium* and *Trametes versicolor*[19], chlorimuron-ethyl by *Aspergillus niger*[15] and *Sporobolomyces* sp.[20], and nicosulfuron by *Plectosphaerella cucumerina*[21].

The transformation of sulfosulfuron in water by any axenic microorganism is not yet well studied. However, as it is already established that sulfosulfuron is a potential member to contaminate ground as well as surface water, it is necessary to understand the interaction between the herbicide and microbes in water and the fate of it therein. In this study, we have made an attempt to isolate sulfosulfuron degrading fungus from irrigation water and investigated its impact on degradation of the herbicide.

Materials and Methods

Chemicals

All chemicals, media and reagents were procured from Merck Ltd., Mumbai. All solvents were distilled and dried before use. HPLC-grade solvent and reagents were used during chromatographic and spectroscopic analysis. Deionised water was obtained from the Milli-Q SP Reagent water system (Millipore, Bedford, MA, USA).

Samples of technical sulfosulfuron (90.5%) and analytical sulfosulfuron (99.9%) were obtained from Indofil Chemicals Company, Mumbai. Technical sulfosulfuron was further purified by recrystallization until a constant melting point of 201°C was achieved. It is a white amorphous solid. The purity was checked by thin layer chromatography (solvent system – chloroform: acetonitrile: 2:3, v/v; R_f =0.35).

Irrigation water

The irrigation water samples were collected in sterile bottles from six different locations in the field of the research farm of the Directorate of Weed Research, Jabalpur during the month of February following a standard method[22]. Samples were stored at 4°C until processing. The sources of irrigation water were bore wells installed at different locations in the farm. The pH value of the irrigation water measured by digital pH-meter was 7.5±0.2. The daily average temperature during the sample collection period was 20°C. There was no rainfall during the sample collection period.

Isolation and identification of sulfosulfuron-degrading fungus from irrigation water

The collected water sample was enriched with sulfosulfuron (5 mg in 100 g of water) and incubated for a weak at 30°C. The method described by Sharma *et al*.[15] was followed for the isolation and identification of sulfosulfuron-degrading fungus. For the selection of fungi as a suitable sulfosulfuron-degrading agent, serial dilution following agar plating of incubated water was done. Fungi that appeared on PDA plates (prepared from 200 g of potato, 20 g of dextrose, 20 g of agar and 1000 mL of water) after 5 days of incubation were further plated for obtaining pure cultures. The fungi screened from the sulfosulfuron-enriched water were again incubated for 7 days in the minimal PDA broth (prepared from 10 g of potato, 20 g of dextrose, and 1000 mL of water) containing different levels of sulfosulfuron, *viz*. 25, 50, 100 and 200 mg per 100 mL of broth. The most efficient fungus was screened out on the basis of their growth and was further inoculated on potato dextrose agar (PDA) plates. After two to three days of incubation, the colony morphology of the isolate was examined. The fungus was characterized on the basis of its colony morphology and microscopy of spore structures.

Degradation of sulfosulfuron by *Mucor* sp.

For degradation studies, 25 mg of sulfosulfuron was added to 100 mL of sterile dextrose-minimal broth (prepared from 100 g of potato, 10 g of dextrose and 1000 mL of water) in 250 mL flask. The sulfosulfuron was allowed to dissolve overnight on shaker before use. The pH of the sulfosulfuron-spiked broth was maintained at 7.0. Twenty such flasks were incubated with isolated *Mucor* sp. in the dark at 25°C for 27 days in BOD incubator. Three flasks with minimal broth and sulfosulfuron, and without the incubation with *Mucor* sp. were kept in dark as control. In a similar way, another set of experiment was carried out in sterilized irrigation water instead of dextrose minimal broth.

Degraded products were extracted by partitioning in chloroform from the broth as well as irrigation water sampled at different time intervals, *viz*. 3, 9, 15, 21 and 27 days of incubation. The solvent was then evaporated under low pressure in a rotary vacuum evaporator to obtain a crude mixture of products. The presence of sulfosulfuron was checked by TLC and

LC-MS/MS after suitable dilution of the crude extract. The sampling was discontinued after 27 days as the last samples did not show any spot of sulfosulfuron on TLC plate and the abundance of the total ion at m/Z 473 (M+1) for sulfosulfron was found insignificant in Q1 TIC of LC-MS/MS. Products were purified by the preparative thin-layer chromatography and characterized by the spectroscopic techniques.

Liquid chromatography-mass spectroscopy

An API 3200 Qtrap mass spectrometer (AB Sciex) was used for mass characterization of degraded products. The parameters were set following the mass spectrometric method developed by Yadav & Choudhury[3] for the analysis of sulfosulfuron and its metabolites. Mass spectrometric analysis was performed with electrospray ionization (ESI) in positive (5500 eV) mode for each sample. The nebulizer gas and heater gases were adjusted at 30 and 55 psi, respectively. The ion source temperature was set at 500°C. Each sample was injected by infusion technique at the rate of 10 μLs^{-1}.

Preparation of major metabolites

Acid hydrolysis of sulfosulfuron: Sulfosulfuron undergoes acid hydrolysis on its urea bridge as observed in many other sulfonylurea herbicides. The procedure for this reaction was standardized following the acid hydrolysis of chlorimuron described by Choudhury *et al*.[23]. A 200 mg portion of sulfosulfuron was added to 100 mL of distilled water. The pH of the solution was adjusted to 2.5 by addition of 2 mL concentrated hydrochloric acid. The solution was stirred magnetically at 32°C and the reaction was monitored by thin layer chromatography and continued till the disappearance of the spot of sulfosulfuron, which took 48 h. The products formed were separated by preparative thin layer chromatography, purified by crystallization from benzene and characterized by spectroscopic methods. Compounds were structurally assigned as 2-amino-4,6-dimethoxypyrimidine (I) and 2-ethylsulfonyl imidazo {1,2-a} pyridine-3-sulfonamide (II) with the help of mass-spectra (Figs. 2 and 3).

Results and Discussion

Isolation and characterization of sulfosulfuron-degrading fungus

The sulfosulfuron-degrading fungus isolated from irrigation water was allowed to grow in the minimal media having sulfosulfuron as a carbon and nitrogen source. *Mucor* sp., a widely distributed fungus in water, survived and grew in that media with sulfosulfuron at the level as high as 2000 mgL^{-1}. This organism was characterized on the basis of its morphological characters (Fig. 1A and 1B). Colonies of this fungus were found as a dark brown mass grew to several centimeters in height filling Petri dish at 20°C within 5 days. Mycelia are branched and coenocytic. Diameters of sporangiophores were measured from 12.5 to 38.5 µm. Tall sporangiophores were unbranched and covered with droplets at the basal part, whereas the short sporangiophores were branched sympodially. Sporangia were globose, pale yellow or gray-brown when young and black-brown at maturity, 90-220 µm in diameter. These morphological features were in agreement with the descriptions of *M. piriformis*[24].

Microbial tolerance of sulfosulfuron at different concentration

Mucor sp., isolated from the sulfosulfuron-enriched water could tolerate the herbicide while the fungus was incubated in the minimal media spiked with sulfosulfuron at the level of 5 to 200 mg per 100 mL. In all the concentrations of sulfosulfuron, i.e. 25, 50, 100 and 200 mg per 100 mL *Mucor* sp. grew remarkably indicating its capacity to degrade sulfosulfuron as a source of nutrients for growth. There is no report available on the tolerance of *Mucor* to any sulfonyl urea herbicides including sulfosulfuron. However, Aluffi *et al*.[25] recently observed that *Mucor* spp. could survive in the pesticide contaminated soil and it had good growth performance in the media fortified with glyphosate after a variable acclimation period; and Seo *et al*.[26] reported that *Mucor* sp. could grow in carbofuran contaminated water and degrade the pesticide more than a decade ago.

Isolation and characterization of degradation products

The analysis of samples drawn after a different duration of incubation of *Mucor* sp. in water spiked

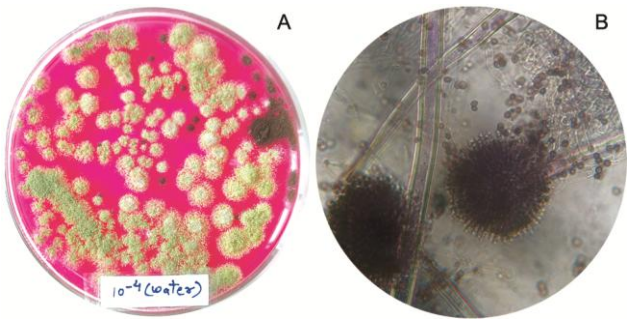

Fig. 1 — (A) Growth of fungi on PDA plates after serial dilution of irrigation water; and (B) Spores, sporangiophore and mycelia of *Mucor piriformis*

with sulfosulfuron (2000 mg L^{-1}) revealed that the fungus utilized the entire amount of the herbicide within 27 days. The incubation of *Mucor* sp. in minimal media and water led to a major degradation of the compound. The degradation products were extracted, cleaned up and analyzed by LC-MS/MS. Product ion mass analyses of different peaks available in the Q1 TIC on the injection of purified samples facilitated in constructing the possible structures of six key metabolites, which give the direction of degradation pathways. The structures are further confirmed by mass spectra of the synthesized metabolites and related literatures.

By computing the fragments given in Fig. 2(I) and (II), the structures of two degradation products are assigned as 2-amino-4,6-dimethoxypyrimidine (I) and 2-ethylsulfonyl imidazo {1,2-a} pyridine-3-sulfonamide (II), which are further confirmed by the spectra of synthesized products given in section 2.6 and also by the spectra of previously reported compounds[12,27]. The fragmentation pattern described in Fig. 2(III) clearly leads to assign the metabolite III as *N*-(4,6-dimethoxypyrimidin-2-yl)urea. The same product was also formed during *Aspergillus*-assisted degradation of orthosulfamuron in rice soil[16]. A similar kind of metabolite was also observed during the microbial degradation of chlorimuron-ethyl[15]. The investigation of the fragmentation pattern in Fig. 2(IV) shows a molecular ion peak at m/Z 214. A fragment of m/Z 182 is produced by the loss of 22 amu (H_2ON). The -NH-OH (hydroxylamine) group is the likely fragment for H_2ON. The presence of the peaks at m/Z 155 and 139 indicates that the molecule consists of a dimethoxy pyrimidinyl ring. Hence, the peak at 182 is due to one carbonyl group added to dimethoxy pyrimidinyl amine. Therefore, from this fragmentation pattern the structure of the compound is assigned as *N*-(4,6-dimethoxypyrimidin-2-yl)-*N*'-hydroxyurea (IV). The critical analysis of the fragmentation patterns given in Figs. 3(V) and (VI), metabolites V and VI are assigned as *N*,*N*'-bis(4,6-dimethoxypyrimidin-2-yl)urea and *N*-(4,6-dimethoxy pyrimidin)-*N*'-(4-hydroxy-6-methoxy pyrimidin-2-yl)urea, respectively. The structure of the metabolite V elucidated from the spectrum given in the Fig. 2(V) is also supported by the previously reported one[3].

Degradation pathways

The first degradation step of any sulfonylurea urea herbicide is generally the cleavage of sulfonylurea bridge, which is pH-dependent. In all most all cases, the pH should be moderately to highly acidic. However in the present experiment, the pH of the media was neutral and that of the irrigation water was neutral to slightly alkaline (pH 7.5±0.2). The incubation of the sulfosulfuron spiked media and water without the fungus did not show any significant degradation of the herbicide. In the presence of the fungus *Mucor* sp. in media and irrigation water, 2-amino-4,6-dimethoxypyrimidine (I) and 2-ethylsulfonyl imidazo {1,2-a} pyridine-3-sulfonamide (II) were detected. The cleavage of sulfonylurea bridge can only cause the formation of those two products. It was a decarboxylation reaction of sulfonylurea bridge and a decarboxylase type of enzyme catalyzed the reaction. This type of decarboxylation reaction was also noticed during the degradation of chlorimuron by *Trichoderma* sp.[15], and nicosulfuron by *Plectosphaerella*. *Mucor* sp., probably with the help of a hydrolase type of enzyme, also degraded sulfosulfuron through the hydrolysis on sulfonyl amide linkage with the formation of the metabolite *N*-(4,6-dimethoxypyrimidin-2-yl)urea (III). An oxidative hydroxylation, perhaps catalyzed by a monooxygenase type of enzyme, on the terminal nitrogen of urea moiety present in the product III led to the formation of *N*-(4,6-dimethoxypyrimidin-2-yl)-*N*'-hydroxyurea (IV). *Trichoderma* sp. and *Aspergillus niger* could also exhibit a similar oxidative hydroxylation reaction forming *N*-hydroxyurea derivative during the degradation of chlorimuron and sulfosulfuron, respectively[3, 15]. *N*,*N*'-bis(4,6-dimethoxypyrimidin-2-yl)urea (V) was formed by the addition of two units of 4,6-dimethoxypyrimidinyl derivatives. There was a fair possibility of the union of products I and IV, where the terminal amino and hydroxy groups involved in the condensation process catalyzed by the dehydrolase enzyme. The product *N*-(4,6-dimethoxypyrimidin)-*N'*-(4-hydroxy-6-methoxypyrimidin-2-yl)urea (VI) was generated from the product V by dealkylation of one methoxy group attached to pyrimidinyl ring. From these modes of degradation and resulting metabolites, a scheme on the *Mucor*-assisted degradation pathways of sulfosulfuron has been proposed (Fig. 3).

It was observed that *Mucor* could perform efficiently both oxidative and hydrolytic pathways for the degradation of endosulfan[28]. A stereo selective oxidative transformation of aldrin to its trans dieldrin could also been mediated by a species of *Mucor*[29]. An efficient transformation of thebaine, an opiate alkaloid, into northebaine by *Mucor piriformis* has

also been observed during a biotransformation process in artificial media[30]. A co-immobilized consortium of *Mucor* sp. and a bacteria *Bacillus* sp. on vermiculite by physical adsorption could degrade benzo[a]pyrene, a polycylic aromatic hydrocarbon pollutant very efficiently[31]. Therefore, the synergistic effect of it in consortium with other microorganisms for the degradation of sulfonyl urea herbicides

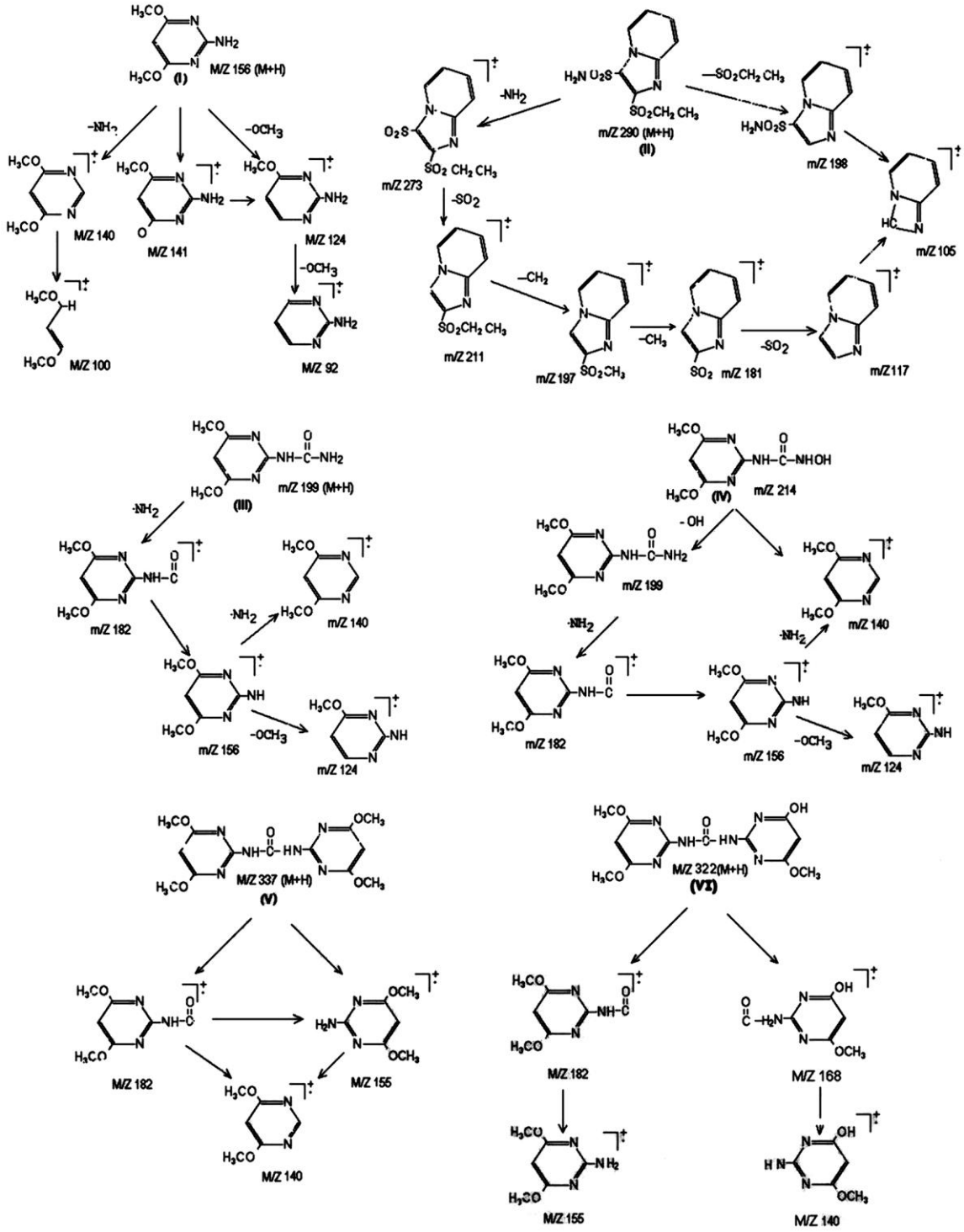

Fig. 2 — Fragmentation pattern of the metabolites 2-amino-4,6-dimethoxypyrimidine (I), 2-ethylsulfonyl imidazo {1,2-a} pyridine-3-sulfonamide (II), *N*-(4,6-dimethoxypyrimidin-2-yl)urea (III), *N*-(4,6-dimethoxypyrimidin-2-yl)-N'-hydroxyurea (IV), *N*,*N*'-bis(4,6-dimethoxypyrimidin-2-yl)urea (V) and *N*-(4,6-dimethoxy pyrimidin)-*N*'-(4-hydroxy-6-methoxy pyrimidin-2-yl)urea (VI)

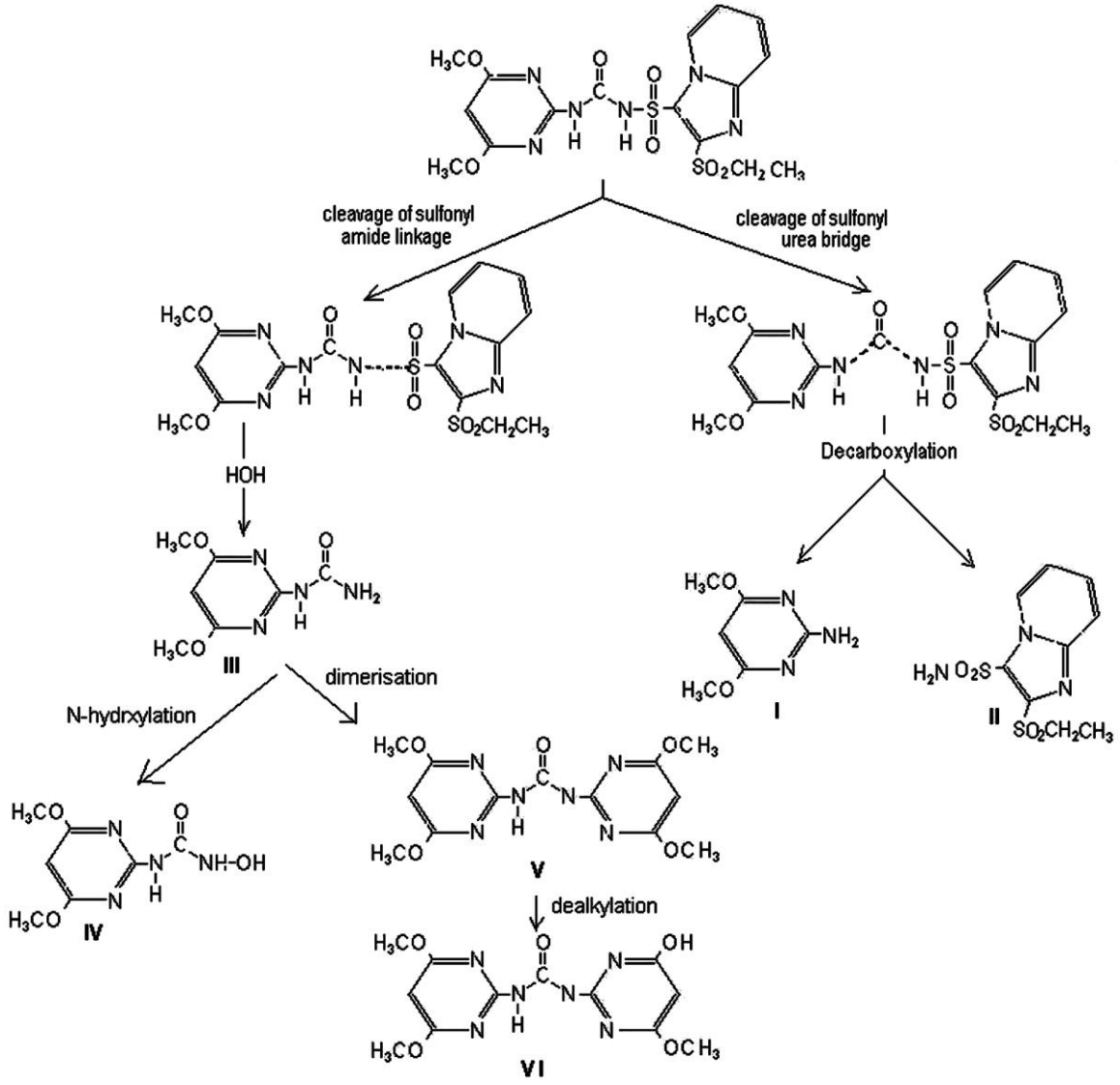

Fig. 3 — The plausible pathways of the *Mucor* mediated degradation of sulfosulfuron in water

including sulfosulfuron is required to be investigated in future. Thus, along with the wide spectrum of its biochemical performance, our research findings on *Mucor piriformis* may be explored further for the bioremediation of substrates like water and soil contaminated with sulfosulfuron and other related compounds.

Conclusion

Results from the above study have demonstrated that the fungus *Mucor* sp., found from the irrigation water could survive in the minimal media containing sulfosulfuron at the level of 2000 mg/L. Its growth during the incubation in minimal media revealed its capacity to abstract energy, carbon, nitrogen and sulfur by degrading sulfosulfuron enzymatically. Different biochemical reactions including the cleavage of sulfonylurea bridge and hydrolysis of sulfonyl amide linkage involved in the *Mucor* assisted degradation process of sulfosulfuron provide better understanding of *Mucor* sp.

Conflict of interest

Authors declare no conflict of interest.

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