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Effect of inoculation with arbuscular mycorrhizal fungi on the degradation of DEHP in soil

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Abstract: The effect of inoculation with arbuscular mycorrhizal(AM) fungi(*Acaulospora lavis*) on the degradation of di(2-ethylhexyl) phthalate(DEHP) in soil was studies. Cowpea plants(*Pigna sinensis*) were used as host plants and grown in a specially designed rhizobox. The experimental results indicated that, both in sterile and non-sterile soil, mycorrhizal colonization rates were much higher in the mycorrhizal plants than in the non-mycorrhizal plants. Addition of 4 mg/kg DEHP slightly affected mycorrhizal colonization, but the addition of 100 mg/kg DEHP significantly decreased mycorrhizal colonization. DEHP degradation in the mycorrhizosphere (Ms) and hyphosphere (Hs), especially in the Hs, increased after inoculation with *Acaulospora lavis*. It is concluded that mycorrhizal hyphae play an important role in the plant uptake, degradation and translocation of DEHP. The mechanism might be attributed to increased numbers of bacteria and actinomycetes and activity of dehydrogenase, urease and acid phosphatase in the Ms and Hs by mycorrhizal fungi.

Keywords: AM fungi; inoculation; DEHP; concentration; degradation

Introduction

Di (2-ethylhexyl) phthalate (DEHP) is one of the commercial phthalate esters(PAEs) which are important and popular additives in many industrial products including flexible PVC material (as plasticisers), and household products such as paint and glues. With the extensive use of PVC and wide range of other applications, PAEs have been detected in water, air, soil, sediments, fish and crustaceans (Sullivan, 1982). Some of them are suspected to be hepatotoxic(Seth, 1982), mutagenic(Kozumbo, 1982) and carcinogenic(Kluwe, 1982). So PAEs pollution has received extensive attention in recent years. Numerous studies have been reported on the biodegradation of PAEs in natural water (Taylor, 1981; Walker, 1984; Johnson, 1984), wastewater (Nozawa, 1988; Wang, 1996) and soil (Inman, 1984; Wang, 1997).

AM is an association between soil-borne fungi and roots of higher plants, which has many special ecological functions. Various studies showed that AM plays an important role in the degradation of anthracite(Binet, 1998; Leyval, 1998).and crude oil(Cabello, 1997). However, understand on the effect of inoculation with AM fungi on the degradation of PAEs in soil is still poor. In this study, we chose di(2-ethylhexyl) phthalate(DEHP), as a representative of PAEs, and investigated the degradation of DEHP in the mycorrhizosphere(Ms), hyphosphere(Hs) and bulk soil(Bs) after inoculation with AM fungi.

1 Materials and methods

1.1 Soil preparation

The soil used was yellow-brown soil, collected from Qilin Town, Jiangsu Province, with pH 6.95, total N 1.6 g/kg, total P 1.47 g/kg, total K 18.4 g/kg, available P 50.5 mg/kg, and soluble N 239 mg/kg. The soils were passed a 1 mm sieve and artificially contaminated with two doses of DEHP(4 mg/kg and 100 mg/kg) which was diluted with doubled-distilled petroleum ether (at 70—72℃). The moisture level of the soil was adjusted to 60% of its field capacity with sterile distilled water and the soil density in containers was adjusted to 1.3 g/cm^3.

1.2 Experimental device

Special experimental containers were constructed to permit spatial separation of soil zones for root and hyphal growth and soil zones into which neither roots nor hypha had access(Fig.1). The container had two compartments(M and N), the above one(M) for root growth including mycorrhizal structure(root compartment) separated from adjacent one(N) by a stainless net of 30 μm mesh size through which hyphae but not roots could pass. In compartment N, there was a nylon membrane of 0.45 μm pore size 5 mm below the stainless net, which could not be penetrated by AM hyphae. This design produced mycorrhizosphere(Ms) by AM roots, or hyphosphere(Hs) by AM hyphae or root- and hypha-free bulk soil (Bs) in the compartment N. The compartment M contained soils without DEHP and the compartment N contained soils with DEHP.

1.3 Plant and AM fungi

Acaulospora lavis was isolated from the Ecological Experimental Station of Red Soil, the Chinese Academy of Sciences, Yingtan, Jiangxi Province, and propagated on

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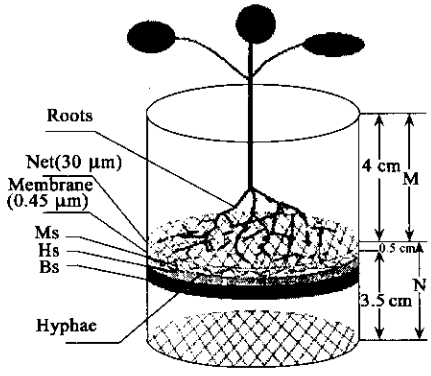

Fig.1 Schematic diagram of experimental system

white clover (*Trifolium repens*) in a greenhouse for 4 months. Colonized clover roots and adhering soil were used as inoculums. Mycorrhizal treatments received 13 g inoculums, and non-mycorrhizal treatments received 13 g sterile inoculums. The inocula were mixed uniformly with the soil of the root compartment. Cowpea (*Pigna sinensis* L.) seeds were disinfected in 10% H_2O_2 for 20 min, washed three times in distilled water and grown for 3 d in vermiculite. Seven seedlings were then transplanted to the container, were thinned to 5 at day 7.

1.4 Experiment design

The DEHP concentrations in soil were 4 mg/kg and 100 mg/kg respectively. There were seven treatments at each DEHP concentration: (1) sterile soil + DEHP(SD); (2) sterile soil + AM fungi(SA); (3) sterile soil + AM fungi + DEHP(SAD); (4) non-sterile(US); (5) non-sterile soil + AM fungi(USA); (6) non-sterile soil + DEHP(USD); (7) non-sterile soil + AM fungi + DEHP(USAD). Each treatment was replicated four times. Plants were kept in a growth chamber (22/18℃ day/night temperature, 75% relative humidity, 15 d, 160 mmol/(s·m^2) PAR).

1.5 Measurement

Mycorrhizal colonization rate was scored according to Koske and Gemma (Koske, 1989). Only the numbers of microorganisms in soil added with 100 mg/kg DEHP were determined by the plate-dilution frequency method of Harris and Sommers (Harris, 1968), and the soil enzymes were determined according to "Activity of soil enzymes" (Zheng, 1986). DEHP was determined by gas chromatography (GC, Hewlett-Packard model 5890 Ⅱ with a flame ionization detector). Briefly, 10 g sample was added to a 100-ml Erlenmeyer flask with ground-glass stopper, to which 60 ml of solvent mixture (acetone: petroleum ether, 1∶1 v/v) was added. The Erlenmeyer flasks were kept static overnight and then vibrated for 2 h. After standing for 2 h, 20 ml of the clear liquid was extracted into a 250 ml separator; this was then supplemented with 100 ml of 6% sodium sulfate solution. The funnel was vibrated for 5 min, and the organic layer was then transferred to a Kuderna Danish apparatus and was allowed to evaporate until the extract volume was reduced to 2 ml. The concentrated sample was cleaned on a column of activated Florist (Florist PR 60/100, SIGMA, USA), then determined.

1.6 Data analysis

ANOVA was used to evaluate the effect of non-mycorrhizal and mycorrhizal plant on DEHP degradation in different microzones. ANOVA was followed by Duncan's test when appropriate.

2 Results and discussion

2.1 Mycorrhizal colonization

No mycorrhizal colonization was observed in the plants grew in the sterile soil (SD treatment). Mycorrhizal plants were well colonized by *Acaulospora lavis* (SA treatment). Due to negative impacts of indigenous microorganisms on AM fungi (Wyss, 1992; McAllister, 1995), colonization rate was lower in the non-sterile soil than in the sterile soil ($P < 0.01$). Compared with no DEHP application, mycorrhizal colonization of cowpea was not significantly affected by the addition of 4 mg/kg DEHP in soil, although there was a slight decrease. However, it significantly decreased ($P < 0.05$) when soil was added with 100 mg/kg DEHP (Fig.2). This implies that there is a level of DEHP, or some other degraded compounds in the soil, which may inhibit initial colonization or sever colonization development of fungi and survival of propagules if colonization was successful. Microscopy revealed more spores in the mycorrhizal roots exposed on 4 mg/kg than those exposed on 100 mg/kg (data and pictures not shown), so propagation of mycorrhizal spores were most likely greatly inhibited by high concentration DEHP.

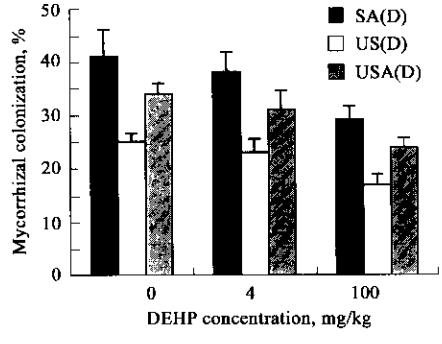

Fig.2 Frequency (%) of mycorrhizal roots of cowpea

2.2 DEHP degradation

In the sterile and non-sterile soil added with 4 mg/kg or 100 mg/kg DEHP, after inoculation with *A. lavis* (SAD and USAD), the remaining concentrations of DEHP were significantly lower than those in the non-inoculation treatments (SD and USD) (Table 1 and Table 2). In each treatment, the remaining concentrations of DEHP in the Ms and Hs were lower than those in the Bs, especially in the Hs, and the difference was significant at $P < 0.01$. It could be seen that DEHP degradation was promoted by inoculation with AM fungi. Because the remaining

concentrations of DEHP in the Hs of SD and USD were higher than those in the SAD and USAD, this suggests that hyphae may play an important role in the degradation and translocation of DEHP. Our results are in broad similar to those of Binet *et al*. (Binet, 1998), who found that the degradation of anthracene in mycorrhizal *Lolium perenne* rhizosphere was more than in non-inoculated plants.

Table 1 Effect of inoculation with AM fungi on the degradation of DEHP when soil was added with 4 mg/kg DEHP

Treatments	Remaining DEHP concentration, mg/kg			
	SD	SAD	USD	USAD
Ms[1]	7.62[a2]	5.59[**3a]	5.22[a]	4.30[**a]
Hs	7.91[a]	5.39[**b]	4.77b	3.77[**b]
Bs	7.83[a]	7.25[*c]	5.26[a]	4.49[*c]

Notes: 1. Ms: mycorrhizosphere; Hs: hyphosphere; Bs: bulk soil.; 2. different letters in a column indicate significant difference between Ms, Hs and Bs in each treatment at $P = 0.01$ by Duncan's multiple range tests; 3. * $P < 0.05$; ** $P < 0.01$. Indicates significant differences between inoculation and non-inoculation in each treatment by ANOVA

Table 2 Effect of inoculation with AM fungi on the degradation of DEHP when soil was added with 100 mg/kg

Treatments	Remaining DEHP concentration, mg/kg			
	SD	SAD	USD	USAD
Ms[1]	70.07[a2]	45.06[**3a]	30.41[a]	20.97[**a]
Hs	72.64[a]	37.05[**b]	29.73[a]	19.24[**a]
Bs	73.42[a]	59.85[**c]	32.94[b]	25.26[**b]

Notes: as for Table 1

2.3 Soil microbes

There is more DEHP degradation in the non-sterile soil than in the sterile soil (Table 1, Table 2), as metabolic breakdown of PAEs by microorganisms is considered to be one of the major routes of environmental degradation for those widespread pollutants due to their low rate of the hydrolysis and photolysis (Giam, 1984). Application with 100 mg/kg DEHP decreased numbers of bacteria and actinomycetes in the Ms, Hs and Bs, especially bacteria, which maybe were killed by DEHP. Inoculation with AM fungi, however, increased the numbers of bacteria and actinomycetes in the Ms and Hs (Table 3). Mycorrhiza maybe selectively influenced persistence of bacterial inoculates as well as other native bacteria (Andrade, 1998), or changed numbers of specific mycorflora in the rhizosphere of mycorrhizal roots through root exudates (Bansal, 1994) and hyphosphere through external mycelium (Schreiner, 1997). In addition, some fungi could mutate spontaneously and form new enzyme system under the environmental stress (Gramass, 1999). Such changes may be helpful to the degradation of DEHP in soil. AM may play a positive role in the microorganisms mutation and adaptation. The relationship of AM and adaptability of indigenous microorganisms to environmental stress still need further study.

Table 3 Changes of numbers of bacteria, actinomycetes and fungi in the Ms, Hs and Bs when soil was added with 100 mg/kg DEHP and inoculated with AM fungi

Treatments	Bacteria, cfu g^{-1} soil			Actinomycetes, cfu g^{-1} soil			Fungi, cfu g^{-1} soil		
	Ms[1]	Hs	Bs	Ms	Hs	Bs	Ms	Hs	Bs
SD	1.08×10^2	2.02×10^2	1.64×10^2	2.29×10^2	2.22×10^2	2.04×10^2	2.98×10^1	2.78×10^1	2.45×10^1
SAD	1.45×10^4	0.88×10^4	0.92×10^4	1.94×10^3	1.62×10^3	1.02×10^3	3.84×10^1	3.71×10^1	2.01×10^1
US	4.31×10^6	2.38×10^6	2.57×10^6	6.70×10^5	5.22×10^5	3.46×10^5	7.61×10^4	6.08×10^4	5.47×10^4
USA	6.64×10^7	2.43×10^7	0.69×10^7	3.65×10^6	2.55×10^6	1.46×10^6	8.43×10^4	7.30×10^4	5.53×10^4
USD	5.50×10^5	4.42×10^5	7.36×10^5	5.74×10^4	4.81×10^4	4.45×10^4	1.19×10^3	1.13×10^3	1.29×10^3
USAD	8.91×10^6	4.10×10^6	1.78×10^6	4.11×10^5	7.52×10^5	2.57×10^5	1.95×10^3	1.43×10^3	1.06×10^3

Note: 1. Ms: mycorrhizosphere; Hs: hyphosphere; Bs: bulk soil

Table 4 Changes of activity of dehydrogenase, urease and acid phosphatase in the Ms, Hs and Bs when soil was added with 100 mg/kg DEHP and inoculated with AM fungi

Treatments	Dehydrogenase, mg TPF/(g soil·24 h)			Urease, mg NH_3/(g soil·24 h)			Acid phosphatase, mg PNP/(g soil·24 h)		
	Ms[1]	Hs	Bs	Ms	Hs	Bs	Ms	Hs	Bs
SD	1.21[a2]	1.05[b]	1.02[b]	0.38[a]	0.32[b]	0. 29[b]	0.28[a]	0.22[b]	0.18[b]
SAD	1.34[*3a]	1.24[*b]	1.07[c]	0.46[*a]	0.44[*a]	0.32[b]	0.35[*a]	0.36[*a]	0.27[b]
US	1.53[a]	1.35[b]	1.31[b]	0.62[a]	0.52[b]	0.47[b]	0.61[a]	0.49[b]	0.38[b]
USA	1.61[a]	1.48[*b]	1.33[c]	0.65[a]	0.58[ab]	0.52[b]	0.65[a]	0.42[b]	0.39[b]
USD	1.30[a]	1.27[a]	1.10[b]	0.58[a]	0.49[b]	0.47[b]	0.48[a]	0.43[a]	0.28[b]
USAD	1.42[*a]	1.37[*a]	1.18[b]	0.60[a]	0.54[ab]	0.48[b]	0.55[*a]	0.51[*a]	0.29[b]

Notes: 1. Ms: mycorrhizosphere; Hs: hyphosphere; Bs: bulk soil; 2. different letters in a line indicate significant difference between Ms, Hs and Bs in each treatment at $P = 0.01$ by Duncan's multiple range tests; 3. * $P < 0.05$. Indicates significant differences between inoculation and non-inoculation in each treatment by ANOVA

2.4 Soil enzymes

As indicated in Table 4, the activity of enzymes (dehydrogenase, urease and acid phosphatase) decreased as a result of soil sterilization and DEHP application, which maybe resulted from DEHP inhibition and the decrease of microorganisms that secreted some soil enzymes. Both in the sterile and in the non-sterile soil, inoculation with AM fungi significantly increased activity of dehydrogenase and acid phosphatase in the Ms and Hs compared with non-inoculation, especially in the Hs. However, urease activity

only significantly increased in the Ms and Hs in the sterile soil, and slightly changed in the non-sterile soil. Dehydrogenase and acid phosphatase activity increased after inoculation with AM fungi, which was mainly affected by the increased microorganisms. Also, AM hyphae and mycorrhizal roots can secrete phosphatase (Jayachandran, 1992). In this experiment, it was found that hyphae seemed to secrete dehydrogenase from marked difference in dehydrogenase activity between inoculation and non-inoculation in the Hs (Table 4). Therefore, AM may promote plant growth and improve rhizosphere environment where lived many microorganisms by altering activity of soil enzymes. Those change possible affected DEHP degradation. In a word, AM indirectly enhanced the degradation of DEHP by increasing soil enzymes activity.

3 Conclusions

AM could promote the degradation of both low and high concentrations of DEHP added to soil, especially in the hyphosphere, though, to some extent, mycorrhizal colonization was inhibited by high concentration of DEHP. Hyphae played an important role in degrading and transferring DEHP. Activity of soil microbes and enzymes in the Ms and Hs increased after inoculation with AM fungi, which are also helpful to promote degradation of DEHP.

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