Sains Malaysiana 40(2)(2011): 119–124

Is the High Cu Tolerance of *Trichoderma atroviride* Isolated from the Cu-Polluted Sediment Due to Adaptation? An *In Vitro* Toxicological Study

(Adakah Ketahanan *Trichoderma atroviride* yang Diasingkan daripada Sedimen Tercemar Cu disebabkan oleh Adaptasi? Satu Kajian Toksikologi *In Vitro*)

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ABSTRACT

The tolerance of Cu by Trichoderma atroviride*, a tolerant fungus isolated from the drainage surface sediment of the Serdang Industrial Area was investigated under* in vitro *conditions. Only this fungus species can tolerate up to 600 mg/L of Cu on solid medium Potato Dextrose Agar based on the isolation of the most tolerant fungus from the polluted sediment. Toxicity test performed on* T. atroviride*, showed a maximum tolerance at 300 mg/L of Cu concentration when grown in liquid medium Potato Dextrose Broth (PDB). The EC_{50} value of the isolate was 287.73 mg/L of Cu concentration in PDB. The Cu concentration in the drainage surface sediment, where the* T. atroviride *was isolated from, was 347.64 µg/g while the geochemical distributions of the non-resistant and resistant fractions of Cu were 99.6 and 0.4%, respectively. The sediment data indicated that the drainage had greatly received anthropogenic Cu from the nearby industries which are involved in the manufacturing of plastics and electronic products. The present findings indicate that the high Cu tolerance showed by* T. atroviride *could be due to the well adaptation of the fungus to the Cu polluted sediment. Therefore,* T. atroviride *could be a potential bioremediator of Cu pollution in the freshwater ecosystem.*

Keywords: EC_{50}; geochemical distributions of Cu; toxicity test; Trichoderma atroviride

ABSTRAK

Ketahanan Trichoderma atroviride*, sejenis kulat toleran yang telah diasingkan dari permukaan sedimen longkang kawasan perindustrian Serdang, telah dikaji di bawah keadaan* in vitro*. Hanya kulat spesies ini sahaja yang boleh bertahan hingga kepekatan Cu 600 mg/L pada medium pepejal 'Potato Dextrose Agar' berdasarkan kepada pengasingan kulat yang paling tahan dalam sedimen tercemar. Melalui ujian ketoksikan dengan menggunakan* T. atroviride*, didapati bahawa ketahanan maksimum spesies ini dalam cecair medium 'Potato Dextrose Broth'(PDB) adalah pada kepekatan Cu 300 mg/L. Nilai pengasingan EC_{50} bagi kepekatan Cu dalam PDB ialah 287.73 mg/L. Kepekatan Cu dalam sedimen permukaan longkang, di mana* T. atroviride *telah diasingkan, ialah 347.64 µg/g manakala taburan bahagian-bahagian geokimia tak rintang dan rintang Cu masing-masing ialah 99.6 dan 0.4%. Data Cu dalam sedimen menunjukkan bahawa longkang tersebut telah menerima Cu antropogenik dari kawasan-kawasan perindustrian seperti plastik dan produk elektronik. Kajian ini menunjukkan bahawa ketahanan* T. atroviride *terhadap Cu yang tinggi mungkin disebabkan oleh adaptasi kulat tersebut terhadap sedimen yang tercemar dengan Cu. Oleh itu,* T. atroviride *adalah berpotensi sebagai 'bioremediator' bagi pencemaran Cu dalam ekosistem air tawar.*

Kata kunci: Cu, EC_{50}; taburan geokimia; Trichoderma atroviride*; ujian ketoksikan*

INTRODUCTION

Copper is an ubiquitous metal in the environment and it is one of the most common constituent of industrial effluents due to metal processing (Anand et al. 2006) and municipal wastes. This metal is present in rivers and coastal waters and its widespread use has generated much scientific research on its effect in aquatic ecosystems. In Malaysia, wastes of Cu oxide and Cu slag were reported in the late 1990s (Yap et al. 2003). From the biological point of view, Cu is an essential element for all living organisms since it acts as a cofactor for a variety of enzymes. However, the excess of this metal in the environment can be mutagenic and can cause the appearance of highly reactive oxygen radicals in all living organisms (Zapotoczny et al. 2006). According to Aleem et al. (2003), Cu can disrupt the ecological status of the biota and lead to the emergence of heavy metal-resistant microorganisms in the soil and polluted water of industrial regions.

According to Anand et al. (2006) fungi are a versatile group as they can adapt and grow under various extreme conditions of pH, temperature and nutrient availability as well as at high metal concentrations. For example, some microorganisms have evolved Zn-tolerant ecotypes that can survive in Zn-toxic places, presumably by adapting mechanisms that are in the general homeostasis of Zn (Colpaert & Van Assche 1992). *Trichoderma atroviride* is a potential candidate for bioremediation work owing to its frequent presence in highly polluted areas as had been reported by a few investigations (Lopez & Vazquez 2003). However, the question 'What is the tolerance level of *T. atroviride* to Cu?' needs to be investigated.

In Malaysia, the use of fungi as bioremediators of heavy metal is scarce or non-existence based on the present literature. Therefore, the objective of this study was to determine the tolerance of the fungus *T. atroviride* to Cu toxicity under *in vitro* study and to relate its tolerance to the Cu concentration in the polluted sediment where the fungus was isolated from.

MATERIALS AND METHODS

ANALYTICAL PROCEDURE FOR CU LEVELS IN SEDIMENT SAMPLES

The top 3-5 cm of surface sediments were collected from a drainage receiving metal industrial effluents from Kuyoh River in the Serdang Industrial Area. The sediment samples were placed in acid-wash polyethylene bags and frozen (-10°C) prior to analysis. The sediment samples were later dried at 105°C for at least 16 hour until constant dry weights (Tanner et al. 2000). Afterwards, the dried samples were crushed by using pestle and mortar into powder form and were passed through a 63 μm stainless steel sieve and vigorously shaken to produce homogeneity. The dried samples were then weighted and digested in a combination of concentrated nitric acid (AnalaR grade, BDH 69%) and perchloric acid (AnalaR grade, BDH 60%) in the ratio of 4:1, first at low temperature (40°C) for 1 hour and then at 140°C for at least 3 hours (Yap et al. 2002). The digested samples were then diluted to 40 mL with double distilled water and filtered through Whatman No. 1 filter papers into acid-washed polyethylene bottles, where they were stored until metal determination.

Chemical fractionations of Cu in the sediments were obtained by the modified Sequential Extraction Technique (SET) as described by Badri and Aston (1983) and Yap et al. (2002). The fractions were the easily, freely, leacheable or exchangeable (EFLE), acid-reducible, oxidisable-organic and resistant fractions. The residue used for each fraction was weighted before the next fraction was carried out. The extraction was conducted at constant agitation (150 rpm). The supernatant was removed and the residue was washed with 20 ml double distilled water. They were then filtered through Whatman filter paper No. 1 and the filtrates were stored for Cu determination. For each fraction, a blank was employed using the same procedure as above to check for external contamination. All the prepared samples were determined for Cu by using an air-acetylene flame Atomic Absorption Spectrophotometer (AAS) (Perkin-Elmer AAnalyst 800). The data were presented in μg/g dry weight.

Fungus isolation and culture conditions The dilution method for fungus isolation was carried out primarily based on Lopez and Vazquez (2003) to avoid any colony overlapping. Isolation of fungi was performed on Rose Bengal Agar (1.0 g of KH_2PO_4 + 0.5 g of $MgSO_4•7H_2O$ + 5.0 g of peptone + 10.0 g of dextrose per 1 liter of sterile double distilled water) in 1 liter conical flasks. Then, the solid medium Potato Dextrose Agar (PDA) was used for subculturing. Afterwards, the growths of the fungus in the petri dishes were observed for seven days.

Isolation of the most tolerant fungus on solid medium The solid medium of PDA was used for the fungus isolation procedure despite the fact that agar may affect metal speciation and solubility, because solid media were more appropriate for the observation of fungal growth. Stock solutions of the $CuSO_4•5\ H_2O$ were prepared in double distilled water and were added to PDA solid medium in a series of Cu concentrations (5 - 600 mg/L). The medium was inoculated with one disk of young mycelium from the edge of the stock solid PDA cultures which were cut by using a sterile cork borer, each 0.5 cm in diameter (Zapotoczny et al. 2006).

Toxicity test The liquid medium PDB was prepared with river water at Kuyoh River filtered by using ultra filtration (micro pore filter paper 0.45 μm). Appropriate amounts of stock solutions of Cu were added to the liquid medium (vol/vol) of each conical flask (250 ml) to reach Cu concentrations of 25, 50, 75, 100, 200 and 300 mg/L, respectively. Afterwards, the Cu concentrations in all the treatments were determined by AAS in order to measure their exact Cu concentrations. The medium was inoculated with three disks of young mycelium each 0.5 cm in diameter from the edge of the stock solid PDA cultures were cut by using a sterile cork borer (Zapotoczny et al. 2006). The temperature used in all the media treatments on the shaker was 27.27ºC. The light density was not measured since it is known not an important factor in the growth of fungus.

After seven days of toxicity test, the EC_{50} value (the effective metal concentration required to reduce biomass growth by 50% when compared to the growth of the control) (Yap et al. 2004) of the most tolerant species was calculated by using prohibition analysis (PC STAT) in the range of 25 – 300 mg/L of Cu concentrations in liquid medium PDB.

RESULTS AND DISCUSSION

CU CONCENTRATIONS IN THE SURFACE SEDIMENT OF KUYOH RIVER

The mean total Cu concentration in the drainage sediment of Kuyoh River in the Serdang Industrial Area was 347.6 ± 10.7 μg/g dry weight. Since there is no reported sediment quality guideline for Cu concentrations in the sediment from Malaysia, the present results are compared with the reported sediment quality guidelines for Cu polluted sediment (>54 μg/g) set by the Hong Kong Environmental Protection Department (Liang &Wong 2003). The comparison indicates that the Cu concentration which we found was considered as 'seriously contaminated'.

The mean Cu concentrations and the percentages of the four geochemical fractions of the sediment are presented in Table 1. From Table 1, it is apparent that the 'oxidisable-organic' fraction made up the most (90.8%) portion of the total Cu of the Kuyoh River Industrial Area. This indicated that the absorption of Cu was mainly related to the organic matter. This might be due to high affinities of Cu to the humic substances that consisted of organic matter which are chemically very active in complexing Cu (Forstner & Wittmann, 1981). Based on the high percentage (90.8%) of the 'oxidisable-organic' fraction in the sediment, the Cu may be associated with various forms of organic matter such as living organisms, detritus or coating on mineral particles. In addition, an elevated level of Cu is found in the form of stable complexes and metal sulphide particles (Tokalioglu et al. 2000).

The 'Non-resistant' fractions (summation of EFLE, acid-reducible, oxidisable-organic fractions) recorded significantly ($P < 0.05$) higher levels of Cu (99.6%) when compared to the 'resistant' fraction (0.4%). This indicated that anthropogenic Cu in the Serdang Industrial Area could have contributed to an elevation of the total Cu concentration in the sediment. The 'non-resistant' fraction of Cu in the sediment is certainly of great concern from the ecotoxicological point of view.

In general, a comparison of the Cu concentration of the drainage sediment from the Serdang Industrial Area with Malaysian studies shows that the present finding is higher than those for most of the other rivers in Malaysia, as shown in Table 2, except for the Sepang Besar River (Saed, 2001). When compared to other regional studies (Table 2), the present finding was within the Cu ranges of the Kaohsiung River (Taiwan) and the Shing Mun River (Hong Kong) but higher than those of the Pearl River (China) and the Singapore River.

Cu tolerance by T. atroviride *on solid medium* From this *in vitro* study, *T. atroviride* was found to be able to grow up to 600 mg/L of Cu on solid medium while Anand et al. (2006) who conducted an experimental study on *Trichoderma viride* demonstrated the Cu toxicity test to be in the range of 1000-5000 mg/L on agar medium. In another study Zapotoczny et al. (2006) reported the Cu concentration to be in the range of 100-600 mg/L on malt agar for *Acremonium pinkertoniae*. These different ranges of heavy metal tolerance on solid media could be attributable to metal binding properties of various agar media or to be diverse abilities of different fungi. Lopez and Vazquez (2003) stated that when the mycelia were transferred to medium without dextrose, *T. atroviride* was more accumulative of Cu ions.

At the Cu exposure of 200-600 mg/L, deep bluish mycelia were clearly visible which was indicative of the

TABLE 1. Concentrations (μg/g ± standard deviation dry weight) of Cu in the geochemical fractions of the surface sediment collected from the Kuyoh River in Serdang Industrial Area

EFLE	Acid-reducible	Oxidisable-organic	Resistant	Non-resistant	Total (100%)
25.89 ± 3.32 (7.46)	4.71 ± 0.81 (1.36)	315.21± 12.5 (90.8)	1.33 ± 0.04 (0.38)	345.81 ± 8.57 (99.62)	347.15 ± 10.7 (100)

Note: The values in parentheses represent the fraction in percentages (%). EFLE= easily, freely, leacheable or exchangeable. Non-resistant fraction was summation of EFLE, acid-reducible and oxidisable-organic fractions

TABLE 2. Total concentrations (μg/g dry weight) of Cu in sediment reported in regional and Malaysian studies

	Locations	Concentrations	Reference
Regional studies	Singapore River, Singapore	10–80	Sin et al. (1991)
	Kaohsiung River, Taiwan	40–998	Chen and Wu (1995)
	Pearl River, China	8.70–140	Cheung et al. (2003)
	Shing Mun River, Hong Kong	207 – 1660	Sin et al. (2001)
Malaysian studies	Pasir River	2–23	Lim and Kiu (1995)
	Rambai River	31–144	Lim and Kiu (1995)
	Terengganu River	0.21–1.70	Mushrifah et al. (1995)
	Juru River	14.00–72.00	Lim and Kiu (1995)
	Sarawak River	1.00– 10.20	Lau et al. (1996)
	Tengi River	BDL–139	Sahibin et al. (2000)
	Sepang Besar River	10.6–573.4	Saed (2001)
	Sepang Kecil River	51.17–57.86	Saed (2001)
	Kg. Pasir Puteh (Intertidal site)	103.4	Yap et al. (2004)
	Kuyoh River Industrial Area	347.6	This study

Note: BDL= below detection limit

presence of Cu ions. Subramanyam and Gupta (1986), Venkateswerlu et al. (1989), Anand et al. (2006) and Zapotoczny et al. (2006) also reported the formation of blue colored mycelia in the presence of Cu by *Neurospora crassa*, *Cunninghamella blackesleeana*, *Acremonium pinkertoniae* and *Trichoderma viride*, respectively. They suggested that the blue color of the mycelia was caused by the binding of the Cu ions to the cell walls of the fungus mycelium.

Cu tolerance by T. atroviride *in liquid medium* It was found that *T. atroviride* could survive and tolerate Cu exposure until 300 mg/L. The present results are strongly supported by a study reported by Lopez and Vazquez (2003). They found that *T. atroviride* survived at concentrations of 0 - 300 mg/L of Cu. However, there was a dramatic decrease in the fungal growth at 350 mg/L of Cu exposure. They reported no growth for this fungus at 400 mg/L whereas a little biomass at this Cu exposure concentration was observed in our study.

Other authors had also reported almost similar Cu concentrations as the maximum tolerance to what was found in similar to our results. Garcia-Toledo et al. (1985) found that *Rhizopus stolonifer* and *Cunninghamella blakesleeana* ceased to grow at 450 and 500 mg/L of Cu exposures, respectively. Tsekova and Todorova (2002) also reported a similar level of Cu tolerance by *Aspergillus niger* B-77 strain where 300 mg/L of Cu inhibited the growth of the fungus.

The difference of the inhibitory capacity to the growth of *T. atroviride* between the solid and the liquid media was due to the difference of Cu availability between the two media (Anand et al. 2006). On the other hand, the differences between the inhibitory effects to fungus growth and the levels of Cu removal in liquid medium were due to differences of the properties and components of the liquid medium. For example, the lethal Cu concentration of the present study (400 mg/L) differed from the result of Lopez and Vazquez (2003) (350 mg/L). This is because PDB liquid medium was used in the present study while they used the Sabouraud liquid medium (Scharlau) that consisted of 1% peptone and 2% dextrose. They established peptone as being responsible for this, rather than any other nitrogen-containing organic substrates, because of its comparatively low metal binding affinity. Thus, the metal availability of the liquid medium increased and the lethal concentration of their experiment limited to 350 mg/L when compared to the present result.

This high tolerance to Cu observed in *T. atroviride* could be attributed to the fact that the fungus was well adapted to the Cu-contaminated sediment. Indeed, several authors have established *in vitro* adaptation of fungi to heavy metals (Lopez & Vazquez 2003).

Toxicity studies of Cu (EC_{50}) The EC_{50} value calculated from the prohibition analysis of *T. atroviride* for Cu^{2+} was 287.73 mg/L (Probit line: Y=1.6805X + 0.8675; 95% confidence interval: 2.1 – 4.5). When compared with other studies of other microorganisms (Table 3), it was clear that the EC_{50} values for Cu^{2+} of the other species are lower than that for *T. atroviride*. However, *Pisolithus tinctorius* recorded a similar (200 mg/L) to *T. atroviride* (287.73 mg/L). The comparison of the EC_{50} values among the different species indicated differences of sensitivity of the different species to Cu toxicity. For example, *Scleroderma flavidum* was shown to exhibit less sensitivity to Cu and Ni than *Lactarius rufus* while *Amanita muscaria* appeared to have the less sensitivity to Cd than a range of other ectomycorrhiza fungal species (Hartley et al. 1997).

When compared with other EC_{50} values reported for other microorganisms (Table 3), it is clear that the EC_{50} of this study is higher. This indicated that *T. atroviride* is less sensitive when compared to other microorganisms. The high tolerance of *T. atroviride* indicates that this species is a good bioremediator of Cu^{2+}.

It seems that there is a connection between the elevated Cu concentration in the sediment of Kuyoh River in the Serdang Industrial Area and the EC_{50} of the isolated fungus, *T. atroviride*. The high level of EC_{50} in *T. atroviride* could be due to the adaptation of this isolate

TABLE 3. Comparisons of EC_{50} values for Cu concentrations with other reported studies.

No.	Organisms	EC_{50} value (mg/L)	Microorganism	Authors
1.	*Pisolithus tinctorius*	200	Fungus	Hartley et al. (1997)
2.	*Thelephora terrestris*	10.0	Fungus	Hartley et al. (1997)
3.	*Cenoccum geophilum*	10.0	Fungus	Hartley et al. (1997)
4.	*Chlorella vulgaris* Beijerinck- WB strain	0.28	Algae	Nacorda et al. (2007)
5.	*Chlorella vulgaris* Beijerinck- SB strain	0.680	Algae	Nacorda et al. (2007)
6.	*Vibrio fischeri*	0.397	Bacterium	Rosen et al. (2008)
7.	*Lingulodinium polyedrum*	0.090	Dinoflagellate	Rosen et al. (2008)
8.	*Ceratocorys horrida*	0.166	Dinoflagellate	Rosen et al. (2008)
9.	*Pyrocystis noctiluca*	0.185	Dinoflagellate	Rosen et al. (2008)
10.	*Trichoderma atroviride*	287.7	Fungus	This study

to its habitat with high Cu contamination. It is known that microorganisms isolated from natural environment contaminated by heavy metals often exhibit tolerance to multiple pollutants because of their ecological and physiological adaptations to such polluted environments (Lopez & Vazquez 2003).

CONCLUSION

From the present work, it was found that *T. atroviride* was the only species able to grow in 600 mg/L of Cu on PDA solid medium. *In vitro* assays with *T. atroviride* confirmed its high tolerance to Cu^{2+} by surviving until 300 mg/L of Cu^{2+} concentration in PDB liquid medium. When compared with other studies, it was clear that the Cu^{2+} EC_{50} values of other species are lower than that of *T. atroviride*. The high EC_{50} value of Cu indicated that *T. atroviride* is less sensitive to Cu. The high tolerance to Cu^{2+} observed in *T. atroviride* could be attributed to the fact that the tolerant fungus has adapted well to the Cu-polluted sediment. The present findings suggest that *T. atroviride* is a potential bioremediator of Cu^{2+}. However, further studies are needed to confirm its practical use as a bioremediating agent under field conditions.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the financial support provided through the Research University Grant Scheme (RUGS), [vot nos. 91230 and 91986], by Universiti Putra Malaysia.

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Received: 5 March 2009
Accepted: 9 July 2010