http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 3 (3): 415-422, 2000

Preparation and Evaluation of the Encapsulated Copper Salts or Copper Complexes by Various Types of Vinyl Polymers as Fungicides

Amany L. Kansoh[1], Yosreya M. Abu-Ayana[2] and Fawzia F. Abdel-Mohsen[2]
[1]Microbial Chemistry Department, National Research Center, Dokki, Cairo, Egypt
[2]Polymers & Pigments Department, National Research Center, Dokki, Cairo, Egypt

Abstract: Three formulations of copper salts or copper complexes encapsulated by different kinds of polymers were locally prepared in the Polymers and Pigments Department. The biological activity of these compounds were studied against various types of fungal strains. The dose and the rate of leaching of copper ions were also studied. They were significantly controlled by the type of the polymer film used and the solubility in the medium. The effective doses of the biocide were (0.1- 0.2 mg/ml). The different kinds of used polymers improved the tenacity of the fungicides on the leaf surfaces and improved the dispersion of copper salt suspension. The results provided laboratory support for the concept that the polymers containing chemically bound biocides were useful for controlling microorganisms growth. In field application, the role of polymer film is obviously clear in the protection of most copper salts or complexes that can be used in rainy and windy places to obtain both economic and environmental advantages. The copper uptake by fungal strains were studied to determine their difference in behavior to the biocidal activity of these compounds. The uptake strategy was examined by Transmission Electron Microscope (TEM). In addition, the cytological studies on and in cucumber leaves showed an efficient intracellular diffusion of copper ions. The acute toxicity of these compounds was also studied.

Key words: Encapsulated copper, copper complexes, polymers, copper uptake, fungicides

Introduction

Metal toxicity towards microorganisms is of environmental concern because of possible inhibition of essential microbe-assisted processes. In addition, microorganisms serve as useful models for laboratory-based metal-toxicity studies.

Copper is a potentially toxic metal to most organisms (Somers, 1961) which at low concentrations can act as an essential micronutrient for microbial growth (Stohs and Bagchi, 1995). At toxic concentrations, copper interacts with cellular nucleic acids and enzyme active sites (Ohsumi *et al*., 1988; Cervantes and Gutierrez-Corona, 1994) or absorbed on the cell wall (Tsezos and Volesky, 1982a).

Copper belongs to group 1B in the periodic table, shares many of the characteristics of the transition metals. It forms two series of compounds cuprous and cupric. Both ions show a strong tendency to form complexes by co-ordination with suitable ions or molecules. The complexes so formed being much more effective than the simple copper ions (Hicks, 1971).

The addition of lime to cupric oxychloride improves the adhesion to the leaves of plant and double the activity of copper ions (Kotev and Spasov, 1978). Other several copper salts of organic acids were more effective than the standard copper oxychloride against *Alternaria solani* and *Helminthosporium maydis in vitro.* The highest activity was shown by copper thiosalicylate (Gupta *et al*., 1980).

However, the use of conventional solutions of fungicides have been met with varied degrees of success due to major problems such as difficulties in keeping them in sprayable suspensions, leaching, solubility, evaporation or volatilization, photo-degradation and reaction of the fungicide with other components due to functional groups present in the molecular structure of the fungicide (Noren *et al*., 1979, 1986; Pittman *et al*., 1982; Sondossi *et al*., 1990; Hsiao and Lin, 1995).

In efforts to overcome these disadvantages, copper compounds have been prepared in a highly stable chelated form and dispersed in emulsion polymers or in oil-emulsion forms to be well distributed in a spray mixture with a uniform coverage and adherence on the plant to give active and rain water-resistance fungicides (Telle and Grewe, 1957; Charles, 1966; Evans *et al*., 1966). Copper compounds were successfully encapsulated into polymers to give biologically active ones (Pittman and Lawyer, 1982; Rossmoore, 1990; Sondossi *et al*., 1990; Madan *et al*., 1990; Dyachenko *et al*., 1991; Korsten *et al*., 1997).

The objective of this study was to prepare copper compounds with a variety of hydrolytic propensities (i.e. variable chemical release rates). So the active biocide was slowly hydrolyzed from the polymer at a rate which prevent fungal growth. As a part of our ongoing research work into these fungicidal compounds, it was felt appropriate to delineate a brief presentation of metal-uptake by fungal strains to elucidate the various responses of filamentous fungi. Physical, histological and toxicological examinations were also studied to assure the safety application of these compounds in the field.

Materials and Methods:

Chemicals

Preparation of the test compounds (The prepared compounds were specially formulated for Delta Agro Chemicals Company (Maadi, Cairo - Egypt) as know-how. Thus they are not permitted to be described in full details).

Compound (À) was locally prepared in the Polymers and Pigment Department, National Research Center (NRC). The preparation was based on the chelation of a copper salt with rosin and fatty acid to form a chelated copper complex that can be easily dispersed in a vinyl polymer emulsion. The copper ions and the emulsion polymer concentrations represent 1.3 and 35 percent of the total Formula, respectively.

Compound (A) was prepared by Charles (1966), as a chelated copper compound suspended in mineral oil emulsion. It is a highly stable copper emulsion for controlling plant diseases.

Compound (B) was locally prepared in the Polymers and Pigments Department, NRC. The preparation was based on the solubility of a copper salt in a medium composed of 2 percent water-soluble synthetic polymer and 20 percent natural resin. The copper salt is partially chelated to both the polymer and the resin with a final copper ions concentration equal to 6 percent of the total formula.

Compound (C) was locally prepared in the Polymers and Pigments Department, NRC. This preparation was based on the suspension

Table 1: Comparison between the effect of the concentrated copper compounds against different fungal strains

Compounds	Inoculation time (days)	Inhibition zone diameters (mm)*					
		Rizoctonia solani	*Alternaria solani*	*Fusarium oxysporium*	*Botrytis* sp.	*Aspergillus niger*	*Deplodia ohryzae*
B	1	70	71	67	56	48	57
	6	52	55	40	39	30	53
	13	39	41	20	25	-	-
A	1	30	35	26	25	35	30
	6	60	65	55	47	45	48
	13	85	89	74	66	59	65
À	1	-	-	-	-	-	-
	6	53	60	48	41	40	48
	13	74	76	65	62	56	62
C	1	-	-	-	-	-	-
	6	71	73	68	60	50	58
	13	75	78	71	63	53	61

*Inhibition zone diameters were measured in millimeters (mm).
B, A, À and C are different preparations of copper compounds (see Materials &Methods), at high concentrations.
(-) no inhibition zone, but no growth on the compound sample.
The copper compounds' concentration is 60 mg/ml as copper ions.

of partially soluble copper salt in a vinyl polymer emulsion. The copper ions and the emulsion polymer concentrations represent 3 and 50 percent of the total formula, respectively.

Microbiology

Organisms and growth conditions: The fungal strains Rhizoctonia solani and *Alternaria solani* were obtained from the Plant Pathology Institute, Giza. *Fusarium oxysporium, Botrytis* sp., *Aspergillus niger* and *Deplodia ohryzae* fungal strains were obtained from the National Research Center (NRC) culture collections. The cultures were grown on Czapeck's dox agar medium (Difco, 1984) and incubated at 28°C for 72 hs. They were routinely maintained and stocked at 4°C.

For studying the kinetics of biosorption, the test organism was grown in 50-ml culture broth of Czapeck's dox medium in 250 Erlenmeyer flasks, incubated on a rotary shaker (180 r.p.m) at 28°C for 72 hs.

Experimental Procedures: The antifungal potentialities of the prepared compounds (A, À, B and C) were determined by the agar diffusion technique (Brown *et al.*, 1986). Each compound and its serial dilutions based on the weight of the biocide and not on the basis of total weight of the polymer added, were tested against the test fungal strains.

To determine the variable chemical release rate of each compound, a spore suspension of the test organism was applied to a plates containing Czapeck's dox agar medium-at time intervals (1-13 days) according to the formulation of the test compound. Growth inhibition zones were also measured after 48 hs of incubation at 28°C.

The minimum inhibitory concentrations of each compound were determined. To study, whether these compounds act as Fungicides and/or Fungistatic agents. The plates of actively growing mycelia of the fungal strains were sprayed with the compounds at their diluted concentrations used in the field and incubated for 7 days at room temperature. Non-sprayed plates were taken as controls. Organism samples from each sprayed plate were chosen randomly, recultured on biocide free media and incubated for a week at 28°C. The growth of these organisms was observed and recorded.

Copper uptake: To study the copper uptake, *Alternaria solani* and *Deplodia ohryzae* fungal strains were used as biosorbents for copper ions. The mycelia were developed in the culture broth, (incubated on a rotary shaker - 180 r.p.m - at 28°C for 72 hs) separated by centrifugation, washed twice with distilled water and suspended in saline solution amended with 300 ppm copper. The mycelia used for the uptake experiments were prepared to provide 0.55 gm dry weight. Sodium azide (65 µg/ml) was added to the mycelial suspension as an inhibitor of metal ion uptake (Volesky and May-Philips, 1995). The mixture was kept shaken at 28°C for 24 hs. Samples were withdrawn at time intervals (0.5-24 hs) and analyzed for residual copper concentrations using Atomic Absorption Spectrophotometer (Varian.Spectr.AA220). Experimental samples with no added cells were used as controls. Copper uptake was calculated as Cu mM / g.d.w.(Q) according to the equation of Volesky (1990).

Electron Microscopy: Transmission electron microscopy (TEM) has been found to be a useful tool to determine the subcellular localization of the absorbed copper metal (Tsezos and Volesky, 1982b). Two samples were prepared, the first was used for the absorption of copper and the second was used as a blank. The samples were immediately fixed with 2.5 percent glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) and prepared according to the procedure of (Glauert, 1965). Semi-thin and Ultra-thin sections were prepared by LKB ultratome and a diamond knife, and stained with uranyl acetate and lead citrate. The micrographs were taken by EM 10 Carl-Zeiss Transmission Electron Microscope.

Histology: The behavior of the compounds (A) and (B) as fungicides on and in cucumber leaves was carried out by Diab *et al*. (1999) at the Central Laboratory of Pesticides, Agricultural Research Center, Giza - Egypt.

The physical and cytological examinations of the surface and the cross sections of cucumber leaves were studied. These sections were prepared for microscopic examinations and stained with rubeanic acid according to the method of Holczinger (1959). These micrographs showed the compounds' behavior in and on the cucumber leaves.

Toxicology: The toxicity of compound (B) was carried out by Nabila (1999) at Pesticide Chem. and Toxicology Lab. Faculty of Agriculture, Alexandria University. Female rats, approximately 100-150 g, were obtained from the Faculty of Medicine, Alexandria University. All rats were examined for health status and acclimatized at laboratory conditions for 2 weeks prior to use.

Table 2: Minimum inhibitory concentrations of the copper compounds (as apper ions) against different fungal strains

Microorganisms	Compounds	Inoculation time (days)	Inhibition zones (mm) of different cu- concentrations						
			60 (mg/ml)	1.2 (mg/ml)	0.6 (mg/ml)	0.3 (mg/ml)	0.2 (mg/ml)	0.15 (mg/ml)	0.1 (mg/ml)
	B	1	71	52	46	35	24	23	22
Alternaria solani	A	1	35	-	-	-	-	-	-
		6	65	46	36	30	22	22	22
		13	89	58	44	40	25	25	22
		1	-	-	-	-	-	-	-
	À	6	60	40	32	23	22	-	-
		13	76	55	40	28	24	22	-
	C	1	-	-	-	-	-	-	-
			73	55	45	35	23	22	-
		13	78	58	50	36	24	23	-
	B	1	70	50	42	37	29	23	-
Rhizoctonia solani	A	1	30	-	-	-	-	-	-
		6	60	53	50	48	46	44	37
		13	85	71	69	55	54	50	43
		1	-	-	-	-	-	-	-
	À	6	53	45	43	40	25	-	-
		13	74	60	55	50	30	-	-
	C	1	-	-	-	-	-	-	-
		6	71	50	43	40	30	23	-
		13	75	55	48	41	30	24	-
Fusarium oxysporium	B	1	67	43	36	30	25	23	-
	A	1	26	-	-	-	-	-	-
		6	55	42	37	34	30	28	22
		13	74	49	45	45	38	31	25
		1	-	-	-	-	-	-	-
	À	6	48	36	30	28	25	22	-
		13	65	45	40	38	31	28	23
	C	1	-	-	-	-	-	-	-
		6	68	43	40	29	25	22	-
		13	71	45	40	32	26	23	-
Botrytis sp.	B	1	56	42	35	26	22	-	-
		1	25	-	-	-	-	-	-
	A	6	47	40	40	32	30	22	21
		13	66	51	49	39	36	30	23
		1	-	-	-	-	-	-	-
	À	6	41	36	31	25	23	-	-
		13	62	45	36	29	25	-	-
	C	1	-	-	-	-	-	-	-
		6	60	40	30	23	20	-	-
		13	63	45	36	25	23	-	-
Deplodia ohryzae	B	1	48	38	31	24	22	-	-
	A	1	35	-	-	-	-	-	-
		6	45	30	27	25	30	25	22
		13	59	50	46	41	37	31	26
		1	-	-	-	-	-	-	-
	À	6	40	28	22	22	21	-	-
		13	56	41	31	25	25	24	-
	C	1	-	-	-	-	-	-	-
		6	50	35	28	25	22	-	-
		13	53	39	34	25	23	-	-
Aspergillus niger	B	1	57	41	33	25	23	-	-
	A	1	30	-	-	-	-	-	-
		6	48	35	26	25	25	22	22
		13	65	48	40	37	35	32	27
		1	-	-	-	-	-	-	-
	À	6	48	35	29	26	25	23	22
		13	62	46	37	35	35	30	29
	C	1	-	-	-	-	-	-	-
		6	58	40	32	27	22	22	-
		13	61	42	32	29	25	23	-

*Inhibition zone diameters were measured in millimeters (mm).

B, A, À and C are different preparations of copper compounds (see Materials & Methods), at different concentrations (0.1 - 60 mg/ml)

Table 3: Acute Oral Toxicity of $CuSO_{4\cdot5}H_2O$ via Gavage, in Female Rats

Dose mg/kg	Mortality		
	No.	%	LD_{50}
0	0/5	0	520 mg/kg
400	0/5	0	
500	2/5	40	
600	3/5	60	
750	4/5	80	

Table 4: Acute Oral Toxicity of compound B (Delcup 6%) via Gavage, in Female Rats

Dose mg/kg	Mortality		
	No.	%	LD_{50}
0	0/5	0	290 mg/kg
100	1/5	20	
250	2/5	40	
400	3/5	60	
500	4/5	80	
750	5/5	100	

Table 5: Acute Subcutaneous Toxicity of $CuSO_{4\cdot5}H_2O$ in Female Rats

Dose mg/kg	Mortality		
	No.	%	LD_{50}
0	0/5	0	430 mg/kg
100	0/5	0	
250	1/5	20	
400	2/5	40	
500	3/5	60	
600	4/5	100	

Table 6: Acute Subcutaneous Toxicity of compound B (Delcup 6 %) in Female Rats

Dose mg/kg	Mortality		
	No.	%	LD_{50}
0	0/5	0	100 mg/kg
50	1/5	20	
100	3/5	60	
250	4/5	80	
400	5/5	100	

The acute toxicity was carried out according to Balazs (1984). Linear relationship usually exists between the logarithms of the doses and the toxic responses, therefore, doses were chosen so that the logarithm of the dose increases by equal increments.

To test the oral acute toxicity, animals were housed in stainless-steel cages (one animal/cage) and given standard diet and water ad liabitum throughout the study. Groups, each of 5 female rats, were given $CuSO_4$ as technical and formulated forms by gavage at dose levels of 0 (distilled water), 400, 500, 600, and 750 mg/kg for technical form, and 100, 250, 400, 500, and 750 mg/kg for the formulated form.

The administration volume of each dose is 4 ml/kg body weight and the volume of each dose was changed according to the dose level.

The subcutaneous acute toxicity test was carried out by subcutaneous administration. The tested doses were 0, 100, 250, 400, 500, 600 mg/kg for technical form, 0,50, 100, 250, 400 mg /kg for the formulated form.

Following administration, animals were observed for the appearance of signs of toxicity and the number of deaths during the course of the study that were calculated as mortality percent. The test was terminated two weeks after dosing.

In order to calculate the LD_{50} values for the compound in both forms, and doses which are expected to give mortalities between 20 and 90 percent were applied. Regression lines were plotted on log-dose probit scale, then form the corresponding Ld-p line and the LD_{50} values were determined for both forms.

Results and Discussion

To answer the question of, whether or not a polymer-anchored biocide can be active against microorganisms growth, evaluations of the biological activity of these compounds were studied.

The results summarized in Table 1, show the effect of high concentrations of various compounds (B, A, À, & C) against different fungal strains.

Compound (B) initially showed inhibition zones that decreased by time of incubation. This is due to the fast copper release from the compound as its copper salt is completely soluble, highly ionized in the medium and encapsulated by a water-soluble polymer. So, the inhibition zones decreased by time as the concentration of the biocide was diluted by diffusion throughout the agar medium in the plates.

Compound (A) showed slight inhibition zones against all fungal strains at initial time while compounds (À & C) showed none. However, all these compounds (A, À, & C) after 6 days of biocide release showed a noticeable inhibition zones that increased with time up to the 13 th day. This phenomena is due to the slow release rate of the biocide, so by time, almost a constant level of the biocide was maintained that was not affected by dilution due to diffusion. An example of these data was shown in Fig. 1.

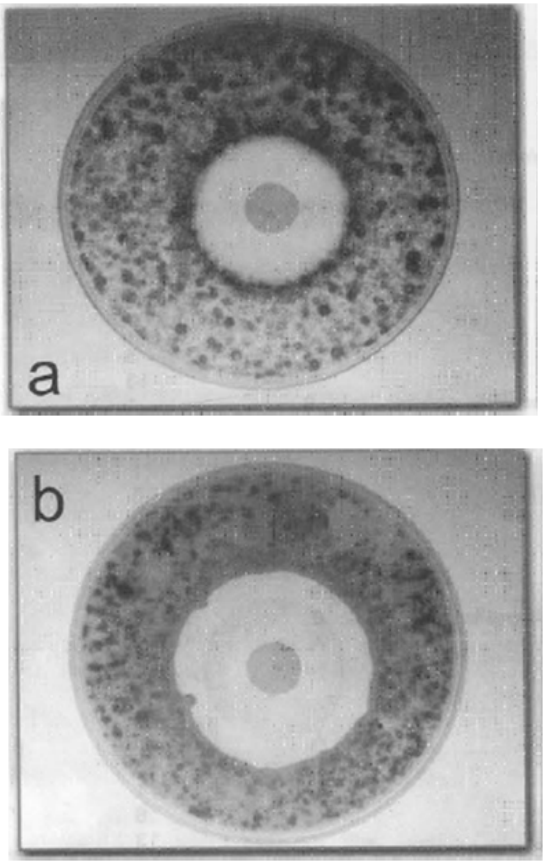

Fig 1: The biocide release rate of a compound (A), as an example, after 6 days (a) and 13 days (b)

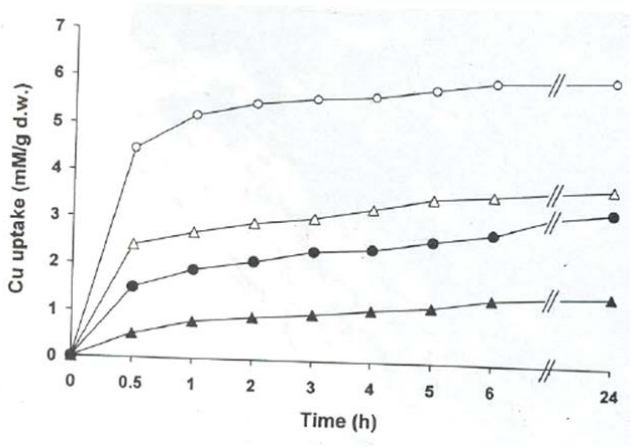

Fig. 2: Copper uptake by *Alternaria solani mycelium* (◦) parent cells, (•) parent cells amended with Na azide and *Deplodia ohryzae* mycelium (△) parent cells, (▲) parent cells amended with Na azide

This difference in behavior suggests that biocide release rates could be influenced by the polymers compositions as well as the functional group used to attach it. In compound (À) copper ions was chelated and encapsulated by mineral oil emulsion and this lead to slow ionization and slow release of copper from the oil film. However, the chelated copper in compound (A) was covered by a micronized film of a vinyl polymer emulsion that lead to faster release of copper than compound (À) and so a slight growth inhibition was observed with compound (A) at early times.

Copper salt in compound (C) is sparingly soluble in a medium encapsulated by a polymer emulsion so its free copper ions will be more lower than compound (B). However the release rate of copper ions from the polymer emulsion film of compound (C) was almost the same as compound (A). This showed a high biocidal activity after 6 days, with a slight increase up to 13 days. The biological activity of polymers with chemically bound biocides has been reported by (Pittman and Lawyer, 1982; Noren *et al.*, 1986; Sondossi *et al.*, 1990).

In general, the compounds (A, À, B, and C) are biologically active against all the tested fungal strains however, their responses to these compounds are variable. *Alternaria solani* and *Rhizoctonia solani* are more sensitive organisms than the other strains. The minimum inhibitory concentrations (MIC) of these compounds were studied for economising their use.

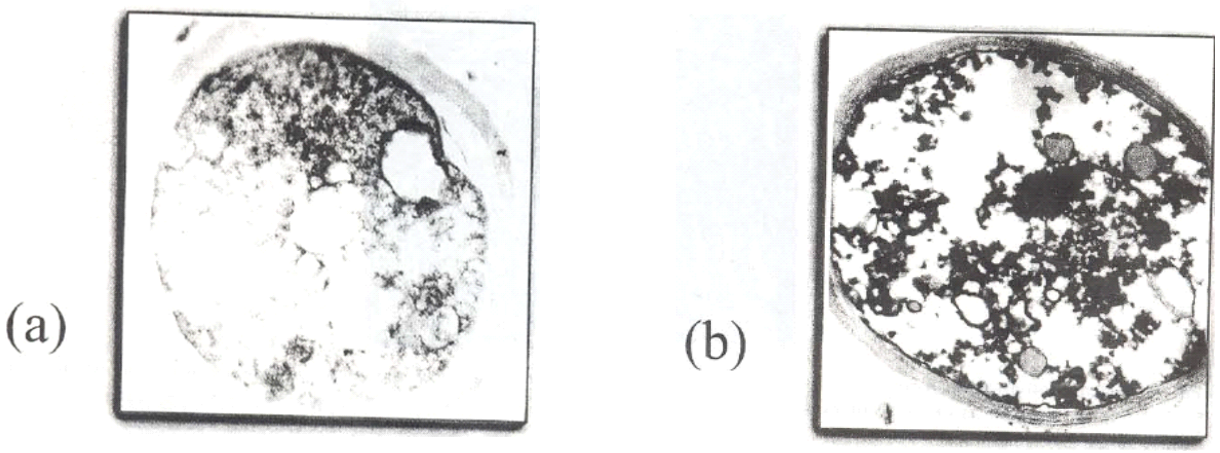

Fig. 3: TEM photograph of *Alternaria solani* (a) before and (b) after copper uptake X40,000. Copper ions are located within the cell wall and inside the cytoplasm

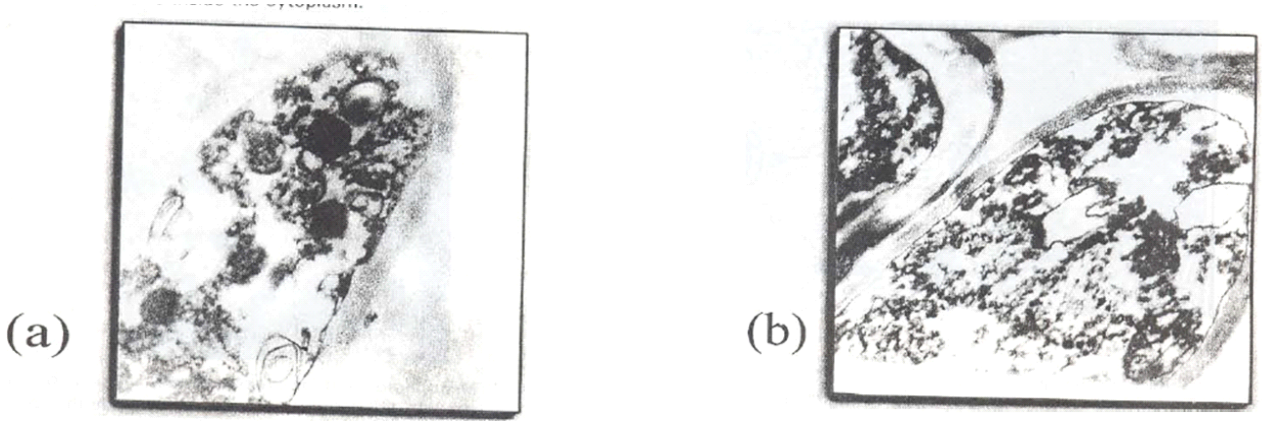

Fig. 4: TEM photograph of *Deplodia ohryzae* (a) before and (b) after copper uptake X40,000. Copper ions are located within the cell wall with less transport into the cell

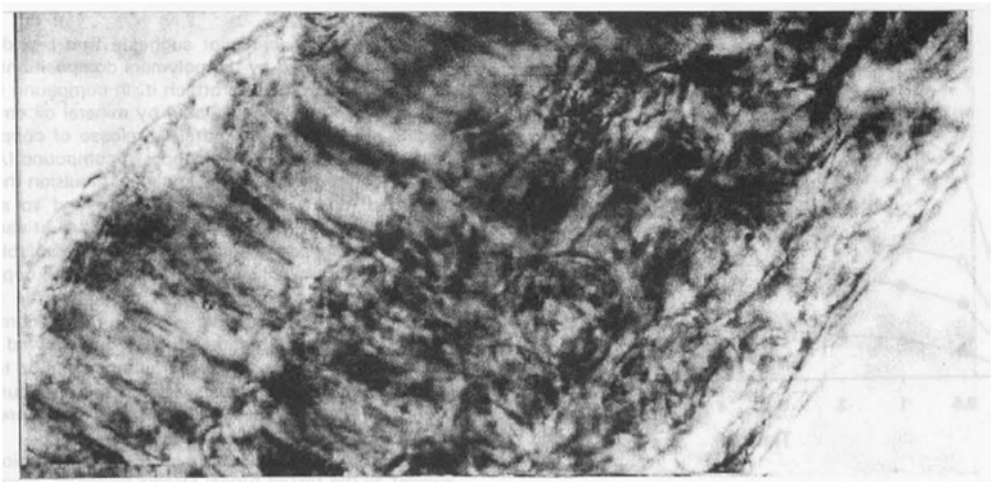

Fig. 5: Micrograph of a cross section of unsprayed cucumber leaf as a control

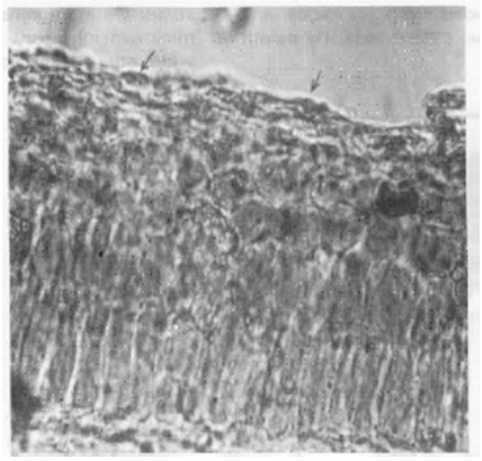

Fig. 6: Microphotograph of a cross section of cucumber leaf stained with rubeanic acid showing the presence of compound (A) on the plant surface as greenish black spots, X400

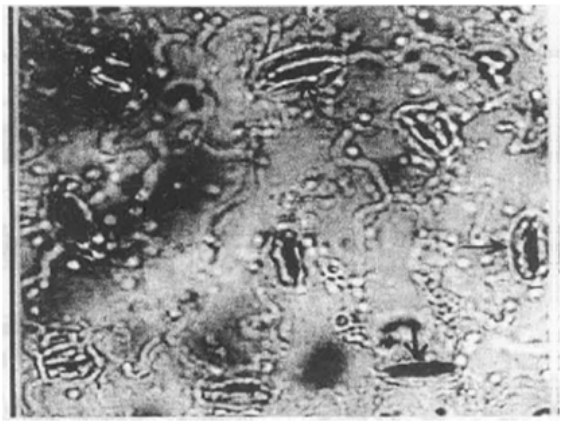

Fig. 7: Microphotograph of a section of surface film of cucumber leaf stained with rubeanic acid showing the presence of compound (A) (greenish black) inside the stomata (arrows), X100

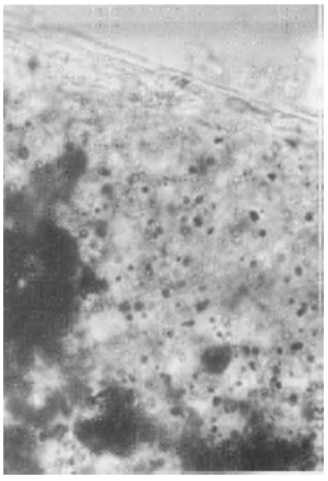

Fig. 8: Microphotograph of cross section in cucumber leaf showing penetration of compound (B) inside the spongy layer and underneath the epidermal layer stained with rubeanic acid stain (greenish black) X1000

The MIC values were based on the weight of the biocide in each compound and not on the total weight added. Data in Table 2 showed that compound (B) was effective at a concentration of 0.15 mg/ml (≅ 1: 400 aqueous dilution) against *Rhizoctonia solani, Alternaria solani* and *Fusarium oxysporium,* while concentration of 0.20 mg/ml (≅ 1: 300 aqueous dilution) was quite active against *Botrytis* sp., *Aspergillus niger* and *Deplodia ohryzae.* Compound (À) was most effective at a concentration of 0.10 mg/ml (≅ 1:600 aqueous dilution) against all the tested organisms. Compound (A) was active at a concentration of 0.20 mg/ml against *Rhizoctonia solani, Alternaria solani, Botrytis* sp. and *Aspergillus niger* while a concentration of 0.15 mg/ml was active against *Deplodia ohryzae* and *Fusarium oxysporium.* Compound (C) was active at a concentration of 0.15 mg/ml against *Rhizoctonia solani, Alternaria solani, Fusarium oxysporium* and *Deplodia ohryzae* while a concentration of 0.20 mg/ml was most effective against *Botrytis* sp. and *Aspergillus niger.* Although the highly diluted solutions of the compounds have low copper concentrations, they showed a noticeable growth inhibition zones. This may be due to the completely soluble biocide at high dilutions with an efficient spread. These results revealed the most effective and most economical dilutions that can be used in the field.

These results provide also, a laboratory support for the concept that binder polymers containing chemically bound biocides can be useful for controlling microbial growth. In addition, these compounds proved to be fungicidal to *Alternaria solani* and *Rizoctonia solani.* These microorganisms were treated with the diluted concentrations that used in the field for one week and failed to grow again when transferred to biocide free media. On the otherhand, these compounds proved to be fungistatic against *Fusarium oxysporium, Deplodia ohryzae, Botrytis* sp. and *Aspergillus niger* as they began to grow weakly when subcultured on the biocide free media (data were not shown).

As a general conclusion, we can suggest that compounds (A & C) can be used in rainy and windy places, as their biocides were encapsulated by polymer emulsion film that protects the biocide from the severe weather conditions and gives a suitable release rate of biocide for a long time. Although the compound (A) have the same properties as the compounds (A & C), it can't be used in the same weather as its oily emulsion film gives less protection than the polymer emulsion one. Compound (B) is only suitable for use in dry or mild weather, as the hydrolytic cleavage of its active agent occurs too rapidly with fast release and this need successive dosing.

Since *Alternaria solani* and *Deplodia ohryzae* showed high and low responses to the biocidal activity of these compounds, therefore a comparison between the two organisms according to the process of copper uptake was studied. Results show that *Alternaria solani* cells featured higher copper uptake capacity (6.0 m MCu/g D.W.) than *Deplodia ohryzae* cells (3.8 mM Cu/g D.W.) under the same conditions. The plotted data of copper uptake by fungal strains (Fig. 2) show an initial rapid uptake up to two hs., followed by gradual uptake, with equilibrium attained after 24 hs. The initial, rapid, phase of metal adsorption onto the cell wall involves extracellular binding, while the second, slower phase is due to the exchange of metal ions across the cell wall and this process depend on the cell metabolism. Addition of sodium azide as a metallic inhibitor at the start of the uptake experiments reduced the copper uptake. This may be due to the fact that a phase of the accumulation processes is indicative of an energy-dependant activity. Such observations have also been noticed by Stokes and Lindsay (1979), Tsezos and Volesky (1982a), Gadd and White (1985), Volesky and May-Philips (1995) and Philip *et al*. (1995). It is of great importance to determine whether the metal ions coordinated or not in order to define the uptake strategy.

Transmission Electron Microscope (TEM) examination of the sorbent cells before and after up-take gives the most direct evidence of this effect. The TEM photographs of *Alternaria solani* (Fig. 3a, b) represent the mycelia before and after copper uptake, respectively. Fig. 3b indicate that the substantial portion of the metal is adsorbed onto the cell wall and a part of the metal ions has been transported into the cell and this appear as an electron-dense areas in the cytoplasm. Fig. 4a, b of *Deplodia ohryzae* before and after copper uptake, respectively, revealed strong electron dense areas on the outer surface of the cells. This indicates that the cells retained the biosorped copper ions on the outer surface of the cells with less transport into the cell. This up-take of metals ions was observed in other studies, as biosoorption of uranium and thorium by *Rhizopus arrhizus* (Tsezos and Volesky, 1982a, b). This study provides a useful tool for the elucidation of uptake and the various responses of filamentous fungi to heavy metal ions.

Of all these fungicidal compounds (A, B, & C) that were locally prepared in our laboratory, two different compounds (A & B) were chosen to be used in the field. The behavior of these compounds (A & B) on and in cucumber leaves were examined physically and cytologically (Diab *et al.*, 1999). The physical examination showed that both compounds were dissolved easily in the water, sprayed well on the plant leaves and dried out after one hour of spraying. Also, no phytotoxicity symptoms were appeared on the plants. The cytological examinations of the cucumber leaves sprayed with the compounds (A) or (B) were also, studied. The copper ions that stained with rubeanic acid (Holczinger, 1959) appear as greenish black spots in electron micrographs. A microphotograph of a cross section of unsprayed cucumber leaf (Fig. 5) was taken as a control. Figure 6 and 7 showed the presence of the compound (A) on the plant surface and inside the stomata, whereas the compound (B) penetrated the spongy layer and underneath the epidermal layers of the leaves as shown in Fig. 8. The deeper diffusion of the compound (B) through the plant tissue was due to the complete solubility of copper salt with fast release.

Since compound (B) has the ability to penetrate the leaves tissues and contains the highest copper ions concentration that is equal to 6 per cent of the total formula. This made us study the toxicity of this compound.

Acute toxicity is a term that signifies the toxic effects produced by a single dose of a compound. Knowledge of the acute toxicity of a compound is of great importance when either the case of an accidental poisoning with a chemical or the therapeutic trial of drug is considered, and also used for planning chronic toxicity studies. Acute toxicity test might reveal effects that can not be detected in multiple-dose tests due to the lower doses administered in the latter, or to tolerance.

In the toxicological studies (Nabila, 1999), the acute oral toxicity tests showed that the median lethal doses (LD_{50}) of the $CuSO_4$ in the technical form as Copper Sulfate Pentahydrate ($CuSO_4 \cdot 5H_2O$)purity 98.5 percent was 520 mg /kg, while the oral LD_{50} of its formulated form Delcup 6 percent (compound B) was 290 mg /kg (metallic Cu) 1133 mg /kg ($CuSO_4 \cdot 5H_2O$ 23.57 %). Data are shown in Table 3 and 4, respectively.

The subcutaneous LD_{50} of the Copper Sulfate Pentahydrate ($CuSO_4 \cdot 5H_2O$) purity 98.5 per cent was 430 mg/kg and the LD_{50} of Delcup 6 per cent (compound B) was 100 mg/kg (metallic Cu) or 396.3 mg/kg ($CuSO_4 \cdot 5H_2O$ 23.57%), as shown in Table 5 and 6, respectively. This compound showed no erythyma or corrosion on the skin after treatment for 3 days.

Acknowledgment

Thanks to the members of the Medical Analysis Department of Research Institute of Ophthalmology for preparing the semi-thin and ultra-thin sections of the mycelial samples.

References

Balazs, T., 1984. Measurement of Acute Toxicity. In: Methods in Toxicology, Payet, G.E. (Ed.). Blackwell Scientific Publications, Oxford, Edinburgh, pp: 49-56.

Brown, D., M.J.M. Hitchcock and E. Katz, 1986. Purification and characterization of kynurenine formamidase activities from *Streptomyces parvulus*. Can. J. Microbiol., 32: 465-472.

Cervantes, C. and F. Gutierrez-Corona, 1994. Copper resistance mechanisms in bacteria and fungi. FEMS Microbiol. Rev., 14: 121-138.

Charles, D.E., 1966. Stable copper emulsion for controlling plant diseases. United States Patent Application No. US3262846 A, July 26, 1966.

Diab, M.M., M.M. Abu-Zaid, M. Fawzy and S. Abd El-Salem, 1999. Technical report of delta agency and Trade: Behavior of the fungicides Fungicup 1.3% (compound A) and Delcup 6% (compound B) on and in cucumber leaves. Central Laboratory of Pesticides, Agricultural Research Center, Giza, Egypt.

Difco, 1984. Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures. 10th Edn., Difco Laboratories, USA.

Dyachenko, Y.K., L.A. Obozova, K.N. Lyubomirova and N.V. Razukrantova, 1991. Complex compounds of benzo-2, 1, 3-selenadiazole and their derivatives containing copper salts of fatty acids: Synthesis and biological activity. Ind. Applied Microbial, 24: 37-40.

Evans, E., J.R. Cox, J.W.H. Taylor and R.L. Runham, 1966. Some observations on size and biological activity of spray deposits produced by various formulations of copper oxychloride. Ann. Applied Biol., 58: 131-144.

Gadd, G.M. and C. White, 1985. Copper uptake by Penicillium ochro-chloron: influence of pH on toxicity and demonstration of energy-dependent copper influx using protoplasts. Microbiology, 131: 1875-1879.

Glauert, A.M., 1965. The Fixation and Embedding of Biological Specimen. In: Techniques for Electron Microscopy, Key, D.H. (Ed.). Davis Co., Philadelphia, pp: 166-212.

Gupta, K.C., R.A. Singh and B. Das, 1980. Antifungal activity of some organic copper compounds against some plant pathogens. Pesticides, 14: 29-30.

Hicks, J., 1971. Comprehensive Chemistry. Macmillan, London, Basingstoke, Pages: 564.

Holczinger, L., 1959. Histochemical detection of free fatty acids. Acta Histochem., 8: 167-175.

Hsiao, W.F. and Y.C. Lin, 1995. Effects of fungicides on the entomopathogenic fungus, *Beauveria bassiana* Bals. Chin. J. Entomol., 15: 295-304.

Korsten, L., E.E. De Villiers, F.C. Wehner and J.M. Kotze, 1997. Field sprays of *Bacillus subtilis* and fungicides for control of preharvest fruit diseases of avocado in South Africa. Plant Dis., 81: 455-459.

Kotev, S. and S. Spasov, 1978. Use of copper-oxychloride lime in non-stake cultivation of tomatoes. Gradinarska i Lozarska Nauka, 15: 166-174.

Madan, V.K., J.P.S. Yadav, A.D. Taneja and V.P. Kudesia, 1990. Fungicidal activity of some new carboxy alkyl substituted thioureas and their COI), Zn (II), Cd (II) and HOD complexes. Indian J. Microbial, 30: 173-178.

Nabila, B., 1999. Technical report of delta agency and trade: Determination of acute toxicity in female rats of the formulated fungicide Delcup 6% (compound B). Pesticide: Chemistry and Toxicology Department, Faculty of Agriculture, Alexandria University, Egypt.

Noren, G.K., G.K. Korpi and G.J. England, 1979. Release rate characteristics of microencapsulated 2,3,5,6-tetrachloro-4-methylsulfonylpyridine. J. Applied Polym. Sci., 24: 2369-2374.

Noren, G.K., M.F. Clifton and A.H. Migdal, 1986. Investigation of microencapsulated fungicides for use in exterior trade sales paints. J. Coatings Technol., 58: 31-39.

Ohsumi, Y., K. Kitamoto and Y. Anraku, 1988. Changes induced in the permeability barrier of the yeast plasma membrane by cupric ion. J. Bacteriol., 170: 2676-2682.

Philip, L., L. Lyengar and C. Venkobachar, 1995. Biosorption of copper(II) by *Pseudomonas aeruginosa*. Int. J. Environ. Pollut., 5: 92-99.

Pittman, Jr. C.U. and K.R. Lawyer, 1982. Preliminary evaluations of the biological activity of polymers with chemically-bound biocides. J. Coatings Technol., 54: 41-46.

Pittman, Jr. C.U., K.S. Ramachandran and K.R. Lawyer, 1982. Synthesis of fungicidal monomers, polymers and latices. J. Coatings Technol., 54: 27-40.

Rossmoore, H.W., 1990. The interaction of formaldehyde, isothiazolone and copper. Int. Biodeterior., 26: 225-235.

Somers, E., 1961. The fungitoxicity of metal ions. Ann. Applied Biol., 49: 246-253.

Sondossi, M., V.F. Riha and H.W. Rossmoore, 1990. The potentiation of industrial biocide activity with Cu^{2+}. I. Synergistic effect of Cu^{2+} with formaldehyde. Int. Biodeterioration, 26: 51-61.

Stohs, S.J. and D. Bagchi, 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radic. Biol. Med., 18: 321-336.

Stokes, P.M. and J.E. Lindsay, 1979. Copper tolerance and accumulation in penicillium ochro-chloron isolated from copper-plating solution. Mycologia, 71: 796-806.

Telle, O. and F. Grewe, 1957. Fungicidal copper spray with increased activity and high resistance to water. German Patent No. 1,088,285 (Cl. 451). April-June, 1957.

Tsezos, M. and B. Volesky, 1982a. The mechanism of thorium biosorption by *Rhizopus arrhizus*. Biotechnol. Bioeng., 24: 955-969.

Tsezos, M. and B. Volesky, 1982b. The mechanism of uranium biosorption by *Rhizopus arrhizus*. Biotechnol. Bioeng., 24: 385-401.

Volesky, B. and H.A. May-Philips, 1995. Biosorption of heavy metals by *Saccharomyces cerevisiae*. Applied Microbiol. Biotechnol., 42: 797-806.

Volesky, B., 1990. Biosorption of Heavy Metal. CRC Press, Boca Ration, USA., pp: 7-44.