Role of rock phosphate in alleviation of heavy metals stress on *Fusarium oxysporum*

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ABSTRACT

An endophytic fungus of soyabean (*Glycine max*) roots, *Fusarium oxysporum*, was used to study its activity under heavy metals (Cd^{2+}, Mn^{2+} and Zn^{2+}) stress and the role of rock phosphate (RP) in alleviation of the stress. *F. oxysporum* growth, amino acids and protein were increased by increasing RP concentration (1–6 g/l) after 8 and 14 days. Heavy metals (HM) have a stressing effect on *F. oxysporum* – a significant decrease of amino acids, protein and accumulation of sugar at 1mM/l follows a descending order of $Cd^{2+} > Mn^{2+} > Zn^{2+}$. RP is altering the stressing effects of metal on *F. oxysporum* by a significant growth improvement at 3 and 6 g/l RP, increase of amino acids, protein and decrease of sugar. The solubilization of RP increased under HM application, which may be attributed to an increase of the adsorption of HM by increasing RP concentration. RP has the highest adsorption affinity for Cd^{2+} (81%) followed by Zn^{2+} (71%) and Mn^{2+} (55%).

Keywords: heavy metals; cadmium; manganese; zinc; *Fusarium oxysporum*; rock phosphate

Heavy metals (HM), especially cadmium, manganese and zinc, are released into the environment during agricultural and industrial activities, and may pose a serious threat to the environment and influence the biodegradation in soil (Burkhardt et al. 1993) and plant growth.

Manganese and zinc are essential micronutrients for most living organisms. However, when the concentrations of beneficial metals are high or when metals, e.g. cadmium, with no known essential biological functions are present, they can become toxic (Dedyukhina and Eroshin 1991). Transfer of Cd^{2+} to human happens through the food chain via plant uptake (Brown et al. 1996). Cadmium is one of the most dangerous components of industrial and municipal wastes, because of its phytotoxicity, ecotoxicity and potential carcinogenic and mutagenic effects (Beyersmann 1994).

This toxicity can be overcome either by the exclusion of the HM ion from the cell or by the intracellular sequestration of the HM ion by an inducible binding protein (Failla and Niehaus 1986a, b). Those methods were however ineffective and metal immobilization through precipitation and adsorption is considered a common mechanism to decrease the hazards of HM in contaminated soils (Malakul et al. 1998). Medina et al. (2005) noticed that RP contributes to the improvement of plant growth in a Cd^{2+} contaminated soil by decreasing Cd^{2+} transfer from soil to plant.

This study explains the effect of HM (Cd^{2+}, Mn^{2+} and Zn^{2+}) on primary metabolites of *Fusarium oxysporum*. Also, the role of RP in alleviation of the HM stress on *F. oxysporum* was studied.

MATERIAL AND METHODS

Microorganism. The endophytic fungal species of *Fusarium oxysporum* isolated from soyabean (*Glycine max*) roots (Agriculture Garden of Assiut University) was used throughout the study.

Rock phosphate (RP). RP (purchased from the Geology Department, Assiut University) 84% insoluble in water and 90% P was ground and used throughout the study.

Effect of RP on *Fusarium oxysporum*. RP powder was added to 50 ml medium (g/l: glucose/30, ammonium sulphate/2, KCl/0.5, $MgSO_4$/0.5) in 250 ml Erlenmeyer conical flask, to obtain final concentrations of 1, 3, 6 and 10 g/l. After autoclaving, flasks were inoculated by a disc of 1-week-old *F. oxysporum* and incubated statically at 28°C for 4, 8 and 14 days. Cultures were filtered and the

mycelium was dried and used for determination of the growth mass and some metabolites contents (amino acids, protein and sugar) as described below.

Effect of HM on *F. oxysporum*. HM in form ($CdCl_2$.2.5 H_2O, $MnSO_4$.H_2O, $ZnSO_4$.H_2O) were used. They were added to 50 ml medium (g/l: glucose/30, ammonium sulphate/2, KCl/0.5, $MgSO_4$/0.5 and RP/1) in 250 ml Erlenmeyer conical flask, to obtain final concentrations of 0.1, 0.5 and 1.0mM/l. After autoclaving, flasks were inoculated by a disc of 1-week-old *F. oxysporum* and incubated statically at 28°C for 8 days. Cultures were filtered and the mycelium was dried and used for determination of the growth mass and some metabolites contents (amino acids, protein and sugar) as described below.

Effect of HM/RP interaction on *F. oxysporum*. The previous medium was supplied with HM (0.5 and 1.0mM/l) and different concentrations of RP (1, 3 and 6 g/l). The medium was sterilized and inoculated with a disc of 1-week-old *F. oxysporum*, and incubated statically at 28°C for 8 days. Cultures were filtered and the mycelium was dried and used for determination of the growth mass and some metabolites contents (amino acids, protein and sugar) as described below. The insoluble P residue of RP was determined by the gravimetric method.

Determination of metabolites content. The mycelia of different treatments were oven-dried at 80°C for 24 h. The dried materials were grounded and used for extraction of free amino acids, soluble and total protein, and soluble and total sugar. The soluble metabolites were extracted by boiling 50 mg ground materials in 10 ml water in test tubes. Total protein of mycelia was extracted with 1M/l NaOH and total sugars were extracted with 1.5M H_2SO_4. The extracted samples were cooled, completed to a known volume and filtered before assaying the metabolic contents. Free amino acids were assayed by using ninhydrin/$SnCl_2$ reagent at 570 nm (Lee and Takahashi 1966). Protein was assayed by Folin-Ciocalteau reagent at 700 nm (Lowry et al. 1951). Sugar was assayed by Anthrone reagent at 620 nm (Toennies and Kolb 1964).

Adsorption of HM on RP. A 50 ml of 2 g/l $NaNO_3$ containing 1.0mM/l HM were equilibrated with 1, 3 and 6 g/l RP powder in 250 ml Erlenmeyer conical flasks. The slurries were autoclaved and shaken on a rotatory shaker at 30 rpm at 28°C for 24 h. The supernatant was separated by filtration and centrifugation. Samples without RP (controls) in 50 ml of 2 g/l $NaNO_3$ were included. The amount of adsorbed HM was taken as the difference between the amount added initially and the amount remaining in solutions after equilibration. Metal concentrations in the filtrates were anylysed using the Atomic adsorption spectrophotometer (Buck Scientific, Model 210 VGR).

Statistical analysis. Triplicate data of each experiment were analysed statistically at 5% level.

RESULTS AND DISCUSSION

Effect of RP and HM on *Fusarium oxysporum*

The effect of different concentrations (1–10 g/l) of RP on *F. oxysporum* growth and metabolic contents was studied. The mycelium growth increased with increasing RP concentration from 1 to 6 g/l after 8 and 14 days of incubation, but decreased at 10 g/l (Figure 1). Also, amino acids and protein were accumulated in mycelium of *F. oxysporum* with increasing rock phosphate from 1 to 6 g/l (Figure 2). Previously, it was found that the increase of phos-

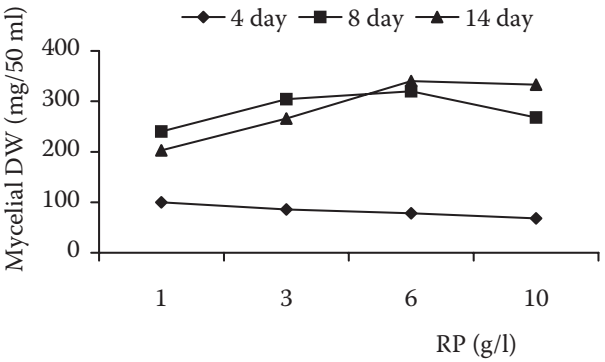

Figure 1. Effect of rock phosphate on growth (mg dry weight/50 ml medium) of *F. oxysporum* in liquid medium

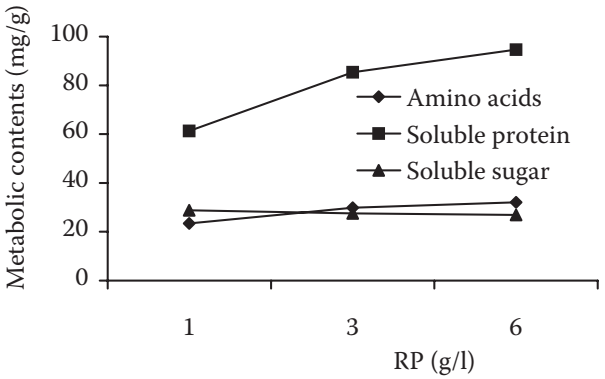

Figure 2. Effect of rock phosphate on metabolic contents (mg/g dry weight) of *F. oxysporum* after 8 days of incubation

Table 1. Effect of heavy metals on *F. oxysporum* metabolites at 28°C in liquid medium[a]

HM	Concentration (mM/l)	Growth biomass (mg/50 ml)	Amino acids (mg/g dry wt)	Soluble proteins (mg/g dry wt)	Soluble sugars (mg/g dry wt)
Control	0	190	23.4	61.3	28.8
	0.1	188	21.4	62.0	28.0
Zn^{2+}	0.5	192	18.3	66.0	28.3
	1.0	200	13.5*	54.0	31.8*
	0.1	186	23.3	62.3	30.0
Mn^{2+}	0.5	172	18.2	70.0	33.9*
	1.0	165*	16.8*	60.0	36.9*
	0.1	180	18.3	68.6	30.1
Cd^{2+}	0.5	168*	8.0*	55.0	36.1*
	1.0	160*	6.0*	46.0*	53.8*

[a]control and all treatments contain 1 g RP/l liquid medium, *means a significant effect compared to control

phorus in the form of KH_2PO_4 from 35 to175mM/l in the medium stimulated the mycelial growth of *Aspergillus parasiticus* and *A. terreus* (Hasan and Issa 1993).

Zn^{2+} stimulated the growth biomass of *F. oxysporum* at 1mM/l, but Mn^{2+} and Cd^{2+} had a stressing effect resulting in lowering the growth by 13 and 16%, respectively (Table 1). It is proved that Zn^{2+} stimulates the growth of microorganisms but Mn^{2+} and Cd^{2+} are generally considered to be toxic to microorganisms (Failla and Niehaus 1986a, b, Dedyukhina and Eroshin 1991). Lopez and Vazquez (2003) found that Cd^{2+} and Zn^{2+} reduced the growth of *Trichoderma atroviride* by 50% at 125 and 200 mg/l, respectively.

Zn^{2+} and Mn^{2+} at 0.5mM/l and Cd^{2+} at 0.1mM/l raised protein contents (Table 1). This may be attributed to the stimulation of nitrate reductase, malate dehydrogenase and acid phosphatase. However, at a higher concentration (1mM/l), HM significantly inhibited amino acids and protein contents. This may be attributed to the toxic action of metals on the enzymatic reactions responsible for protein biosynthesis. However, sugars were significantly increased in mycelium of *F. oxysporum* by using HM, especially Cd^{2+} and Mn^{2+} at 0.5 and 1.0mM/l, respectively (Table 1). Generally, HMs have a stressing effect on *F. oxysporum* resulting in a decrease of amino acids, protein and accumulation of sugars following a descending order of $Cd^{2+} > Mn^{2+} > Zn^{2+}$ (Table 1). Tsekova et al. (2000) found that Cd^{2+} is toxic for *A. niger* and this is accompanied by a decreased production of DNA and protein as well as an increased production of lipids and polysaccharides. They concluded that Cd^{2+} probably influences the lipid composition of the membranes.

Effect of HM/RP interaction on *F. oxysporum*

The role of supplemental RP in altering the stressing effects of HM on *F. oxysporum* was studied. RP at 3 and 6 g/l significantly alleviates heavy metal stress in *F. oxysporum;* it enhances the growth mass and the accumulation of amino acids and soluble proteins (Table 2). Soluble sugars were decreased when RP concentration increased at 3 and 6 g/l (Table 2). This may be a result of inhibited cell division that allowed wall components to be diluted by being divided.

By studying the effect of HM/RP interaction on insoluble metabolites it was found that RP, when added at 3 and 6 g/l, decreased 1mM/l HM stress significantly increasing insoluble protein and decreasing accumulation of insoluble sugar, too (Table 3). RP thus seems to indirectly minimize the toxic effect of HM by increasing the biosynthesis of protein, amino acids and by decreasing sugars. Medina et al. (2005) noticed that RP contributes to the improvement of plant growth in a Cd^{2+} contaminated soil by decreasing Cd^{2+} transfer from soil to plant.

RP may be a competitive inhibitor of HM uptake by adsorption on its surface. This leads to an increased solubilization of RP by lowering insoluble P in culture of *F. oxysporum* (Figure 3). This is consistent with the hypothesis that HM

Table 2. Effect of heavy metals (HM) on soluble metabolites in *F. oxysporum* under rock phosphate (RP) treatment at 28°C in liquid medium after 8 days

HM	HM:RP (mM/l:g/l)	Growth biomass (mg/50 ml)	Amino acids (mg/g dry wt)	Soluble protein (mg/g dry wt)	Soluble sugars (mg/g dry wt)
Zn^{2+}	0.5:1	192	18.3	66.0	28.3
	0.5:3	250*	29.8*	105.0*	27.5
	0.5:6	293*	31.9*	110.0*	25.0
	1:1	200	13.5	54.0	31.8
	1:3	299*	28.1*	89.8*	26.3
	1:6	312*	34.9*	100.5*	24.7
Mn^{2+}	0.5:1	172*	18.2	70.0	33.9
	0.5:3	298*	26.6*	77.0	31.4
	0.5:6	294*	26.8*	80.0*	29.4
	1:1	165*	16.8	60.0	36.9
	1:3	282*	29.8*	88.9*	25.7
	1:6	235*	31.4*	104.0*	25.0*
Cd^{2+}	0.5:1	168	8.0	55.0	36.1
	0.5:3	211*	16.2*	62.6	30.0
	0.5:6	290*	24.4*	66.1*	26.8*
	1:1	160	6.0	46.0	53.8
	1:3	300*	27.0*	78.0*	32.5*
	1:6	270*	24.0*	86.0*	29.2*

*means a significant effect compared to control

retention by RP happens through the dissolution of RP (release P into solution) and formation of metal-phosphate complex (consumption of P) as explained previously by Cao et al. (2004) in their study with Pb retention mechanism by RP.

Table 3. Effect of 1mM/l HM on insoluble metabolites in *F. oxysporum* under RP treatment at 28°C in liquid medium after 8 days

HM	HM:RP (mM/l:g/l)	Insoluble protein (mg/g dry wt)	Insoluble sugars (mg/g dry wt)
Zn^{2+}	1:1	55.0	90.0
	1:3	99.2*	80.5
	1:6	101.5*	79.3
Mn^{2+}	1:1	65.0	92.0
	1:3	104.1*	80.7
	1:6	78.0*	73.5*
Cd^{2+}	1:1	50.0	100.0
	1:3	99.0*	73.8*
	1:6	69.0*	72.8*

*means a significant effect compared to control

Adsorption of HM on RP

By studying the ability of RP in adsorption of HM it was found that RP has a high adsorption affinity to all HM and the adsorption of HM increased with increasing RP concentration (Table 4). RP has the highest adsorption affinity for Cd^{2+} followed by Zn^{2+} and Mn^{2+}. They were adsorbed on RP on average by 81, 71 and 55%, respectively.

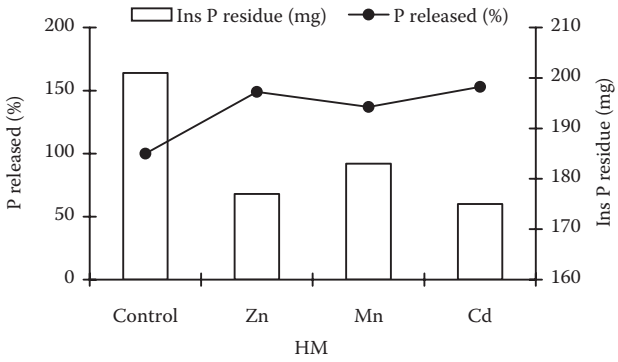

Figure 3. Solubilization of rock phosphate in *F. oxysporum* culture medium under heavy metals treatment after 8 days of treatment

Table 4. Adsorption of HM on RP

HM	HM:RP (mM/l:g/l)	HM non-adsorbed		HM adsorbed		% average
		ppm*	%	ppm	%	
Zn^{2+}	1.0:0	65	100	0	0	71
	1.0:1	42.5	65.4	22.5	34.6	
	1.0:3	12.0	18.5	53.0	81.5	
	1.0:6	1.8	2.8	63.2	97.2	
Mn^{2+}	1.0:0	55	100	0	0	55
	1.0:1	40.4	73.5	14.6	26.5	
	1.0:3	22.6	41.1	32.4	58.9	
	1.0:6	11.0	20.0	44.0	80.0	
Cd^{2+}	1.0:0	112	100	0	0	81
	1.0:1	50.6	45.2	61.4	54.8	
	1.0:3	8.6	7.7	103.4	92.3	
	1.0:6	3.1	2.8	108.9	97.2	

*all of RP data are significant compared to 1mM/l HM

Singh et al. (2001) investigated the effectiveness of phosphatic clay, a by-product of the phosphate mining industry, for immobilizing HM (Pb^{2+}, Cd^{2+}, and Zn^{2+}) from aqueous solutions. They found that the amounts of metals sorbed onto phosphatic clay decreased in the order $Pb^{2+} > Cd^{2+} > Zn^{2+}$. Fayiga and Ma (2005) found that RP significantly reduced Cd^{2+} from soil by about 13 mg/kg and was effective in decreasing metal uptake by *Pteris vittata* and thus can be used as an economic amendment for metal polluted soils. They also indicated that Ca and P are major constituents of RP. Medina et al. (2005) found that RP contributes to the improvement of plant growth in a Cd^{2+} (5 mg/kg) contaminated soils by decreasing its transfer from soil to plant.

RP thus seems to directly minimize: (1) the toxic effect of HM by adsorption on its surface and (2) the formation a metal-phosphate complex compound that prevents the fungus from HM uptake. So, the application of RP that adsorbed HM outside the cells represents the practical method to overcome HM toxicity.

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