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ANTIFUNGAL ACTIVITY OF ETHYL ACETATE SUB-FRACTION OF METHANOLIC EXTRACTS OF *CENCHRUS PENNISETIFORMIS* IN THE PRESENCE OF CR(III) AND CR(VI)

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A B S T R A C T

Fungicidal potential of three different concentrations (3.15, 6.30 and12.5 mg mL^{-1}) of ethyl acetate sub-fraction of shoot and root of *Cenchrus pennisetiformis* (Hochst. & Steud) Wipff was evaluated against *Fusarium oxysporum* f. sp. *lycopersici* under Cr(III) and Cr(VI) toxicity. Ethyl acetate sub-fraction of methanolic root extract exhibited greater antifungal activity than that of shoot extract either alone or combined with the metal ions. Different concentrations of Cr(VI) were found to be more inhibitory to *F. oxysporum* f. sp. *lycopersici* than that of Cr(III). Various concentrations (3.15-12.5 mg mL^{-1}) of ethyl acetate sub-fraction of methanolic root and shoot extracts significantly decreased fungal biomass by 20-70% and 10-50%, respectively over negative control (without Cr ions or plant extracts). Fungal biomass was significantly declined by 60-90% and 40-60% due to different concentrations (100, 200 and 300 ppm) of Cr(VI) and Cr(III), respectively, over negative control. There was up to 100% and 80% reduction in the fungal biomass due to combined effect of ethyl acetate sub-fraction of root and shoot extract, respectively, with various concentrations of Cr(III) or Cr(VI). It was concluded that ethyl acetate sub-fractions of both methanolic shoot and root extracts exhibited significant antifungal potential against *F. oxysporum* f. sp. *lycopersici* in the presence as well as in the absence of Cr ions.

Keywords: Fungicidal potential, grass, heavy metal, Poaceae, wilt pathogen

INTRODUCTION

Fostering concerns on environment and human health due to long term application of synthetic fungicides has diverted the attentions towards natural products based pesticides. Botanical fungicides derived from plants are one of the options to decrease use of synthetic fungicides (Javaid and Akhtar, 2015). Many members of the grass family (Poaceae) have long history of uses as antimicrobial agents (Shah and Bano, 2012). Various species of the wide spread genus *Cenchrus* have been investigated so far for their antifungal activity (Javaid *et al.*, 2012; Singariya *et al.,* 2012). *Cenchrus* is commonly known as buffelgrasses, sandburs and sand spur with features of sharp and spine-covered burrs inflorescences. Amongst its 20-25 species, *C. penni setiformis* has been investigated for its allelopathic (Macias *et al.,* 1992), antifungal and herbicidal potential (Shafique *et al*., 2004; Javaid and Anjum, 2006; Javaid *et al*., 2006). The frequent occurrence of this grass along the roadside in Punjab, Pakistan with considerable antimicrobial activity could effectively be exploited to use as antifungal agent against damaging and difficult fungal pathogens. Ubiquitous soil-borne fungus *Fusarium oxysporum* of division Ascomycota has been enlisted amongst the top ten devastating economically important fungal pathogens that caused massive yield loss in many host plants (Dean *et al.,* 2012). *F. oxysporum* f. sp. *lycopersici* (FOL) is important pathogenic strains that causes vascular wilt disease in tomatoes (Morid *et al.,* 2012). FOL exhibited high degree of host specificity (Sakai, 1998), and is difficult to manage using cultural and chemical methods (Chandel *et al.,* 2010). This fungal pathogen infects and penetrates in healthy plants

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through root tips, root wounds, or lateral roots by its mycelium or germinating spores (Havey, 1995). The fungus after invading host vascular vessels causes disruption in uptake and translocation of nutrients and the disease ultimately ends up with death of host plant (Shalaby and Struckmeyer, 1966). *F. oxysporum* have also been reported to tolerate high concentration (100-1000 ppm) of heavy metal chromium (Amatussalam *et al.,* 2011). Chromium (Cr) is a transition heavy metal and its two oxidation sates viz. Cr(III) and Cr(VI) are the most dominant because of their wide occurrence and stability in natural environment (Pradas *et al.,* 2012). Several industries extensively utilizing Cr and discharging Cr-loaded wastewater to the in vicinity, which causes pollution of water and soil to threatening level (Shanker *et al.,* 2005). Unfortunately, metal loaded wastewater is extensively utilized for growing vegetables in Pakistan (Mahmood and Malik, 2014). It is assumed that when metal laden water is used to irrigate the agricultural fields, these heavy metals interact with phytopathogens present in the respective area. Besides tolerating high concentration of heavy metals, *F. oxysporum* could proliferate by utilizing metal, infect and weaken plant, thus could promote metal uptake in plants as well. The current study was aimed to check the fungicidal potential of ethyl acetate sub-fraction of shoot and root extracts of *C. pennisetiformis* against *F. oxysporum* f. sp. *lycopersici* in the presence of Cr(III) or Cr(VI). This is a new area of research and findings of the this study will be helpful in the management of Fusarium wilt disease of tomato by natural resources especially in soils contaminated by effluents of tannery and other industries.

MATERIALS AND METHODS

Extraction and fractionation of methanolic extract: Crushed dried shoot (stem and leaf) and root materials of *C. pennisetiformis* (200 g each) were soaked in methanol. After 14 days soaked materials were first filtered through cheese cloth, afterwards filtrates were filtered through filter paper (Whatman No. 1), and methanol was evaporated on a rotary evaporator at 45 °C. Methanolic extracts of both root and shoot were partitioned using ethyl acetate in a separating funnel. Solvents were evaporated on a rotary evaporator and 9.14 g and 10.9 g of shoot and root extracts were obtained, respectively.

Metal solutions: Stock solution (1000 ppm) of each Cr(III) and Cr(VI) were made by using potassium dichromate ($K_2Cr_2O_7$) and chromium nitrate [$Cr(NO_3)_3.9H_2O$], respectively.

Preparation of fungal suspension: Pure culture of *F. oxysporum* f. sp. *lycopersici* (FCBP # 1311) was obtained Fungal Culture Bank of Pakistan, University of the Punjab, Lahore, Pakistan. Mycelia/conidia were scraped from the seven-day old culture of the fungus and suspended in 30 mL sterilized water to make 4.8×10^6 conidia mL^{-1}.

Laboratory bioassays: Various concentrations of ethyl acetate sub-fractions of root and shoot were prepared by dissolving weighed amount (1.2 g) of each fraction in 1 mL of DMSO and added to 5 mL of autoclaved malt extract broth. This stock solution was serially double diluted by adding malt extract broth to prepare concentrations of 12.5, 6.25 and 3.125 mg mL^{-1}. Each concentration of the sub-fraction was well-mixed with measured amount of each of Cr(III) and Cr(VI) stock solutions to obtained final metal concentrations of 100, 200 and 300 ppm in the growth medium. Corresponding control treatments of each of the three concentrations of ethyl acetate sub-fractions of shoot and root were prepared by adding various concentrations of the extract in the growth medium separately. For different concentrations of Cr(III) and Cr(VI) ions, corresponding control treatments were made by adding metal ions in growth medium. Negative control was without Cr ions or plant extracts and comprised of growth medium only.

Bioassays were conducted in 10 mL volume glass test tubes each containing 1 mL of the growth medium. Test tubes were inoculated with 15 µL of conidial suspension of *F. oxysporum* f. sp. *lycopersici* aseptically and were incubated at 25°C for 7 days. Each treatment was replicated three times. After incubation period, fungal biomass in each test tube was filtered, dried to constant weight and weighed. Inhibitory effect of ethyl acetate sub-fractions of root and shoot extracts against *F. oxysporum* f. sp. *lycopersici* in the presence of Cr(III) and Cr(VI) were calculated in term of fungal biomass produced in each treatment and compared with fungal biomass in corresponding control treatment.

Statistical analysis: All antimicrobial measurements were made in triplicate and means were calculated with ±SE. Statistical analysis was done by using ANOVA followed by Tukey's HSD at 5% level of significance.

RESULTS AND DISCUSSION

Analysis of variance revealed statistically significant contribution at $P \leq 0.001$of two different metal oxidation

states (MOS), plant parts (shoot and root) (P), extract concentrations (EC) and metal ions concentration (MC) on biomass of *F. oxysporum* f. sp*. lycopersici* (Table 1). The interactions analyzed among the said treatments viz., MOS × EC, MOS × MC, P × EC, P × MC and EC × MC were highly significant (P ≤ 0.001). However, interactive effect of MOS × P was insignificant. Data concerning the effect of three different concentrations (3.15, 6.30 and12.5 mg mL^{-1}) of ethyl acetate sub-fraction of methanolic extract of shoot and root on biomass of *F. oxysporum* f. sp*. lycopersici* under various concentrations (100, 200,300 ppm) of Cr(III) and Cr(VI) is portrayed in Figure 1-4 A and B. Percentage decrease in fungal biomass due to separate and combined application of Cr(III) or Cr(VI) with ethyl acetate sub-fraction is shown in Table 2. The fungal biomass was significantly decreased by 20-70% and 10-50% due to the effect of three different concentrations of ethyl acetate sub-fraction of methanolic root and shoot extracts, respectively over negative control. Separate effect of both oxidation states of Cr was significant as well. Increase in concentration of Cr(VI) reduced fungal biomass more drastically by 60-90% than by 40-60% with Cr(III) over negative control (Figure 1-4 A). When ethyl acetate sub-fraction of each root and shoot extract was combined with different concentrations of Cr(III), fungal biomass was significantly decreased by 80-100% and 70-80%, respectively over negative control (Figure 1 and 2 A). Fungal biomass was declined up to 100% and 60% due to the effect of each root and shoot extract, respectively, combined with Cr(III) concentrations as compared to corresponding concentrations of ethyl acetate sub-fraction only and corresponding Cr(III) concentration only (Figure 1 and 2 A; Table 2). When three concentrations of ethyl acetate sub-fractions of methanolic root and shoot extracts were mixed with each of the three concentrations of Cr(VI), fungal biomass was significantly declined by 100% and 50-100%, respectively over negative control. There was 100% inhibition in biomass of the fungus due to ethyl acetate sub-fraction of methanolic root and shoot extract combined with different Cr(VI) concentrations as compared to corresponding sub-fraction and metal separately (Figure1-4 A; Table 2).

Regression analysis revealed linear relationship between different concentrations of ethyl acetate sub-fraction of methanolic shoot and root extracts of *C. pennisetiformis* and various concentrations of Cr(III) as well as with Cr(VI) ions on biomass *F. oxysporum* f. sp. *lycopersici* (Figure 1-4 B). *C. pennisetiformis* is well-known for its antifungal potential against many fungal species including *Fusarium* spp. (Javaid and Anjum, 2006; Javaid *et al.,* 2006). Existence of steroid, alcoholic, ester along with phenolic compounds have previously been documented in different parts of *Censchrus* spp. with well-known antimicrobial activity (Singariya *et al.,* 2014; Singariya *et al.,* 2015). Antimycotic activity of plant allele chemicals could also be accountable to their inhibition of the extracellular fungal enzymes, cellulase, polygalacturonase, glucosidase and laccase (Macrae and Towers, 1984). Increase in inhibition of fungal growth with increase in concentration of the extracts could be the results of intensification of antioxidant potential of secondary metabolites in the extracts (Pandey *et al.,* 2010).

Reduction in fungal growth due to different concentrations of metal ions might be attributed to disruption in cell function and cell integrity through mutation in biomolecule due to internalization of metal in the cytosol (Pal *et al.,* 2010). Essentiality of Cr(III) for microorganisms could be accountable for its less harmfulness, whereas unavailability of any machinery for Cr(VI) hydrolysis might be attributed to its more injuriousness to the fungus. Besides, Cr(III) toxicity has been reported to occur from antagonism with iron transport (Ramana and Sastry, 1994) and Cr(VI) toxicity was documented to link with its specific antagonism to sulfate uptake in the fungus (Raspor *et al.,* 2003).

More inhibition of the fungal growth under simultaneous effect of plant extract and metal could be due to either their synergistic action. However, various antimicrobial compounds may act as potential sites for binding of Cr ions from the aqueous solution, besides acting as antifungal activity. Nazir *et al*. (2011) reported significant accumulation of different heavy metals in root of *C. pennisetiformis* growing in industrial contaminated areas. Over and above, cycloergost, phytol and β-tocopherol in root extract of different *Cenchrus* spp. might be responsible for greater antifungal and metal scavenging action than shoot (Singariya *et al.,* 2012).

It was concluded that various concentrations of ethyl acetate sub-fractions of both the extracts proved the most effective both in the presence or absence of Cr (III) and Cr (VI) exhibited up to 100% inhibition in growth of the target fungal pathogen.

Table 1. Analysis of variance (ANOVA) for the effect ethyl acetate sub-fraction of methanolic extracts of shoot and root of *Cenchrus pennisetiformis* on biomass of *Fusarium oxysporum* f. sp. *lycopersici* under Cr(III) and Cr(VI) stress.
* significant at P ≤ 0.001

Sources of variation	df	SS	MS	F-values
Metal oxidation states (MOS)	1	15400	15400	187*
Plant parts (P)	1	17741	17741	215*
Extracts conc. (EC)	3	113343	37781	459*
Metal conc. (MC)	3	57928	19309	234*
MOS × P	1	104	103	1.26^{ns}
MOS × EC	3	12278	4092	50*
MOS × MC	3	6786	2262	28*
P × EC	3	7290	2430	30*
P × MC	3	4107	1368	17*
EC × MC	9	19061	2117	26*
Error	161	13245	82	
Total	191			

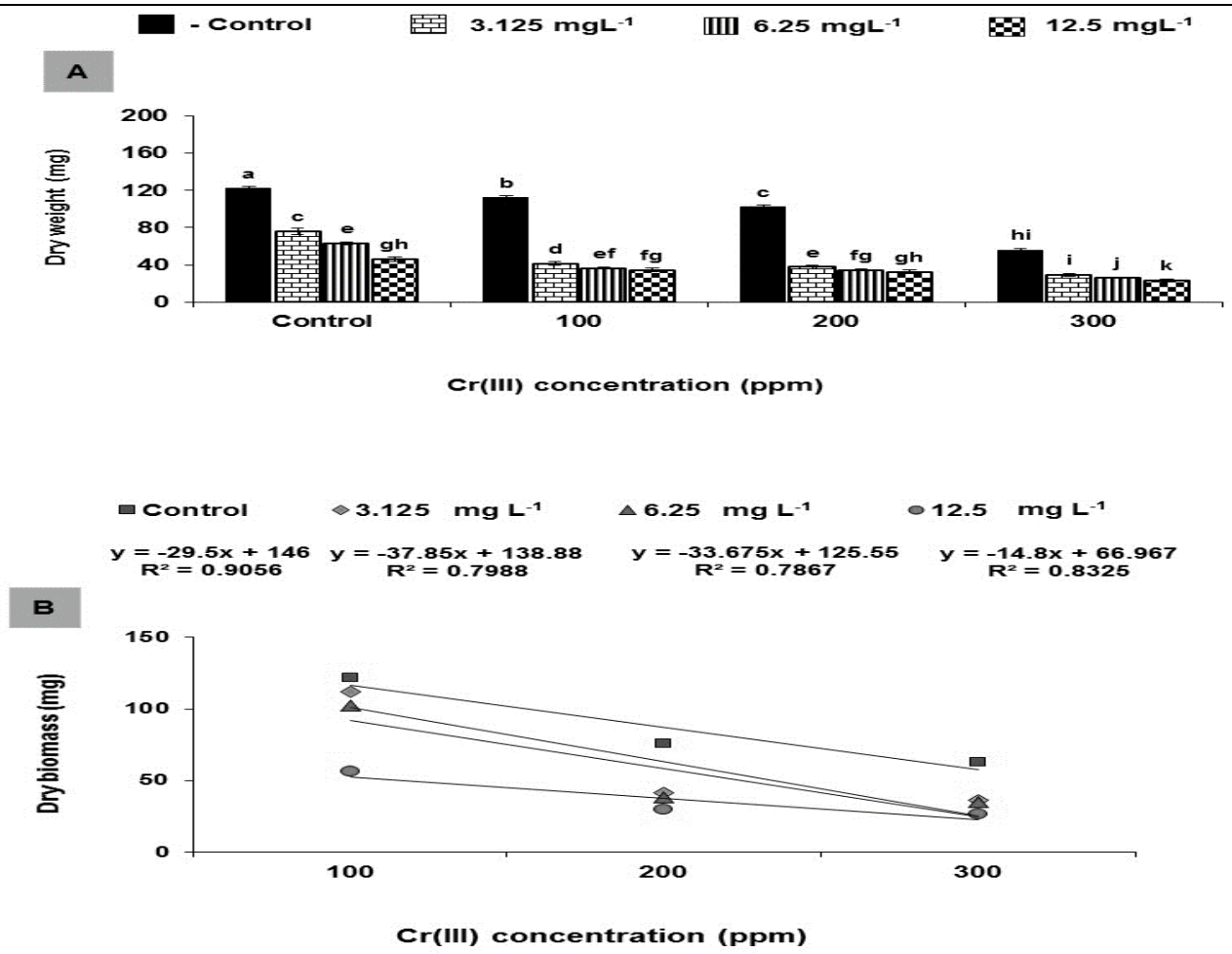

Figure 1. Effect of different concentrations of ethyl acetate sub-fraction of methanolic shoot extract of *Cenchrus pennisetiformis* on growth of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) under Cr(III) stress. A: Effect on fungal biomass; B: Regression analysis for the relationship between different concentrations of Cr(III) (along with different concentrations of root extracts of *C. pennisetiformis*) and biomass of FOL. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P ≤ 0.05) as determined by Tukey's HSD test.

Table 2. Percentage decrease in fungal biomass due to separate and combined application of Cr(III) and Cr(VI) with ethyl acetate sub-fraction of *Cenchrus pennisetiformis.*

Treatments	Cr (III) conc. (ppm)	Extract conc.(%)	Decrease over negative control (%)	Decrease over corresponding ethyl acetate extracts(%)	Decrease over corresponding metal (alone) concentration (%)
Cr(III)	0	0 (-control)	-	-	-
	100	0	38	-	-
	200	0	48	-	-
	300	0	62	-	-
Shoot extract	0	3.125	10	-	-
	100	3.125	66	63	46
	200	3.125	70	68	42
	300	3.125	72	16	25
	0	6.25	16	-	-
	100	6.25	69	63	50
	200	6.25	69	66	45
	300	6.25	73	14	29
	0	12.5	54	-	-
	100	12.5	76	47	61
	200	12.5	76	53	58
	300	12.5	81	22	50
Root extract	0	3.125	20	-	-
	100	3.125	70	60	50
	200	3.125	100	100	100
	300	3.125	100	100	100
	0	6.25	30	-	-
	100	6.25	78	70	64
	200	6.25	78	100	100
	300	6.25	100	100	100
	0	12.5	70	-	-
	100	12.5	84	50	74
	200	12.5	84	100	100
	300	12.5	100	100	100
Treatments	Cr (VI) conc. (ppm)	Extract conc. (%)	Decrease over negative control (%)	Decrease over corresponding ethyl acetate extracts (%)	Decrease over corresponding metal (alone) concentration (%)
Cr(VI)	0	0 (-control)	-	-	-
	100	0	60	-	-
	200	0	75	-	-
	300	0	90	-	-
Shoot extract	0	3.125	10	-	-
	100	3.125	46	40	-40
	200	3.125	62	59	-60
	300	3.125	72	58	-183
	0	6.25	29	-	-
	100	6.25	96	95	90
	200	6.25	96	95	80
	300	6.25	96	100	60
	0	12.5	54	-	-
	100	12.5	96	91	90
	200	12.5	96	100	100
	300	12.5	100	100	100
Root extract	0	3.125	20	-	-
	100	3.125	100	100	100
	200	3.125	100	100	100
	300	3.125	100	100	100
	0	6.25	30	-	-
	100	6.25	100	100	100
	200	6.25	100	100	100
	300	6.25	100	100	0
	0	12.5	70	-	-
	100	12.5	100	100	100
	200	12.5	100	100	100
	300	12.5	100	100	100

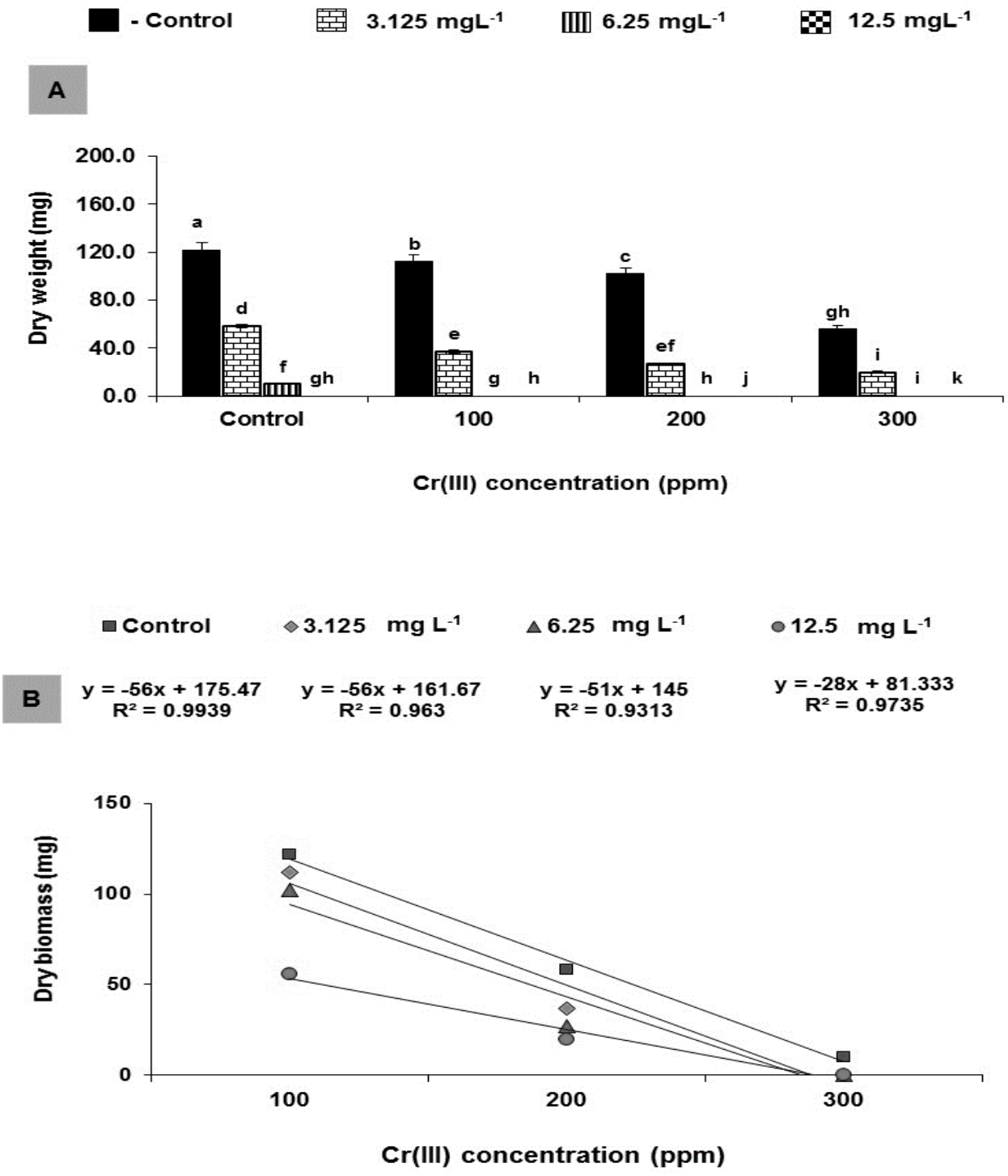

Figure 2. Effect of different concentrations of ethyl acetate sub-fraction of methanolic root extract of *Cenchrus pennisetiformis* on growth of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) under Cr(III) stress. A: Effect on fungal biomass; B: Regression analysis for the relationship between different concentrations of Cr(III) (along with different concentrations of root extracts of *C. pennisetiformis*) and biomass of FOL. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by Tukey's HSD test.

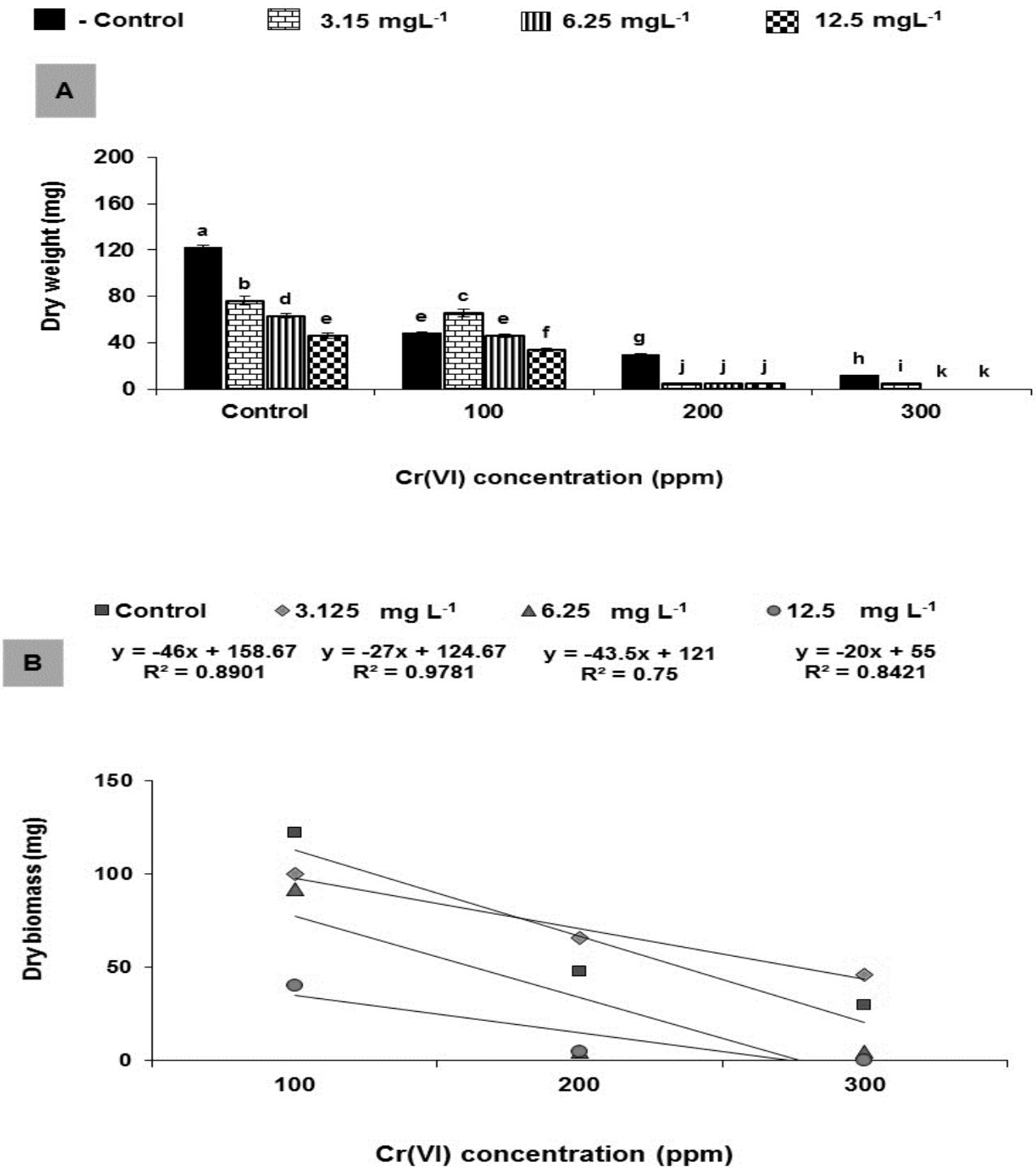

Figure 3. Effect of different concentrations of ethyl acetate sub-fraction of methanolic shoot extract of *Cenchrus pennisetiformis* on growth of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) under Cr(VI) stress. A: Effect on fungal biomass; B: Regression analysis for the relationship between different concentrations of Cr(VI) (along with different concentrations of root extracts of *C. pennisetiformis*) and biomass of FOL. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by Tukey's HSD test.

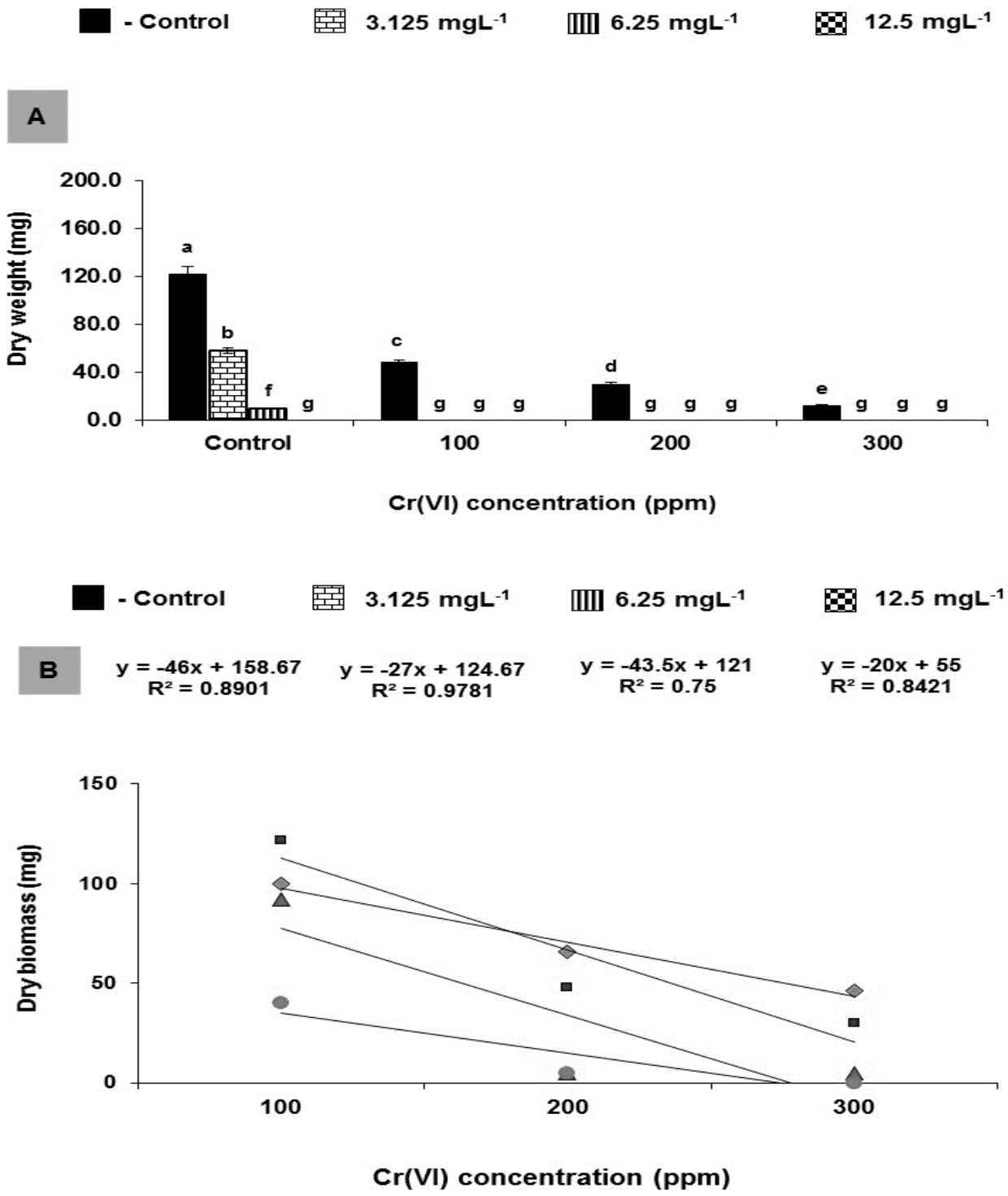

Figure 4. Effect of different concentrations of ethyl acetate sub-fraction of methanolic root extract of *Cenchrus pennisetiformis* on growth of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) under Cr(VI) stress. A: Effect on fungal biomass; B: Regression analysis for the relationship between different concentrations of Cr(VI) (along with different concentrations of root extracts of *C. pennisetiformis*)and biomass of FOL. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by Tukey's HSD test.

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