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FUNGICIDAL POTENTIAL OF ALLELOPATHIC WEED *Cenchrus pennisetiformis* ON GROWTH OF *Fusarium oxysporum* f. sp. *lycopersici* UNDER CHROMIUM STRESS[1]

Potencial Fungicida de Grama Alelopática ***Cenchrus pennisetiformis*** *no Crescimento de* ***Fusarium oxysporum*** **f. sp.** ***lycopersici*** *sob Estresse de Cromo*

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ABSTRACT - The present study was conducted to assess antifungal potential of the allelopathic grass *Cenchrus pennisetiformis* Hochst. & Steud. against the fungal plant pathogen *Fusarium oxysporum* f. sp. *lycopersici* (cause of tomato wilt disease) under chromium stress. Laboratory experiments were performed in 10 mL volume glass test tubes each containing 1.0 mL of malt extract broth with seven concentrations (0, 50, 100, 150, 200, 250, 300 and 350 ppm) of each of Cr(III) and Cr(VI), and two concentrations (5% and 6%) of methanolic leaf, stem or root extract of *C. pennisetiformis*. A metal + weed extract amended medium was inoculated with the pathogen and incubated for 7 days at 25 C. Different concentrations of Cr(III) and Cr(VI) proved equally inhibitory resulting in 10-84% and 18-89% reduction in fungal biomass, respectively. Methanolic leaf, stem and root extracts of the weed reduced fungal biomass by 12-25%, 14-23% and 46-50%, respectively, over negative control. In combined application of methanolic extracts of different parts of *C. pennisetiformis* and metal solutions, root extract in combination with either Cr(III) or Cr(VI) showed the highest inhibitory potential against the fungus followed by leaf and stem extracts. In combination with methanolic root, leaf and stem extracts, different concentrations of Cr(III) and Cr(VI) significantly reduced fungal biomass by 54-99%, 14-99% and 9-95%, respectively, over negative control. Such studies have not been carried out previously. Results of the present investigation suggest that *F. oxysporum oxysporum* f. sp. *lycopersici*, the cause of Fusarium wilt disease in tomato, can be managed by application of extracts (or alternatively biomass) of *C. pennisetiformis* in chromium contaminated soils.

Keywords: grass, heavy metal, *Poaceae*, wilt pathogen.

RESUMO - O presente estudo foi conduzido para avaliar o potencial antifúngico da grama alelopática ***Cenchrus pennisetiformis*** *Hochst. & Steud. contra o fungo patógeno de plantas* ***Fusarium oxysporum*** *f. sp.* ***lycopersici*** *(causador da doença murcha-de-fusário) sob estresse de cromo. Experimentos laboratoriais foram realizados em tubos de ensaio de vidro com volume de 10 mL, cada um contendo 1,0 mL de extrato de caldo de malte em sete concentrações (50, 100, 150, 200, 250, 300 e 350 ppm), para cada Cr(III) e Cr(VI), e duas concentrações de extrato de folha, caule ou raiz metanólica (5% e 6%) de* ***C. pennisetiformis****. Um meio alterado de metal + extrato de grama foi inoculado com o patógeno e incubado por sete dias em uma temperatura de 25 ºC. Concentrações diferentes de Cr(III) e CR(VI) demonstraram resultados igualmente inibitórios em reduções de 10-84% e 18-89% na biomassa do fungo, respectivamente, em relação ao controle. Extratos metanólicos de folha, caule e raiz de grama reduziram a biomassa do fungo em 12-25%, 14-23% e 46-50%, respectivamente, quando comparados ao controle. Em aplicações combinadas de extratos metanólicos de partes diferentes de* ***C. pennisetiformis*** *e soluções metais, extratos de raiz combinados tanto com Cr(III) quanto com Cr(VI) mostraram os maiores potenciais inibitórios contra o fungo, seguidos pelos extratos de folha e caule. Na combinação com extratos metanólicos de raiz, folha e caule, concentrações diferentes de Cr(III) e Cr(VI) reduziram significativamente a biomassa do fungo em 59-99%, 14-99% e 9-95%, respectivamente, comparando-se ao controle. Esses estudos não foram*

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realizados anteriormente. Os resultados desta pesquisa sugerem que ***F. oxysporum*** *f. sp.* ***lycopersici****, causador da murcha-do-fusário em tomates, pode ser manejado pela aplicação de extratos (ou, alternativamente, biomassa) de* ***C. pennisetiformis*** *em solos contaminados com cromo.*

Palavras-chave: grama, metal pesado, *Poaceae*, patógeno de murcha.

INTRODUCTION

Chromium (Cr) is an important multipurpose metal extensively utilized in different industries including metallurgy, refectory, plating, tannery, wood preservation and pigment manufacturing etc. Cr(III) and Cr(VI) are the two most stable oxidation forms of Cr in the environment (Smith et al., 2002). Cr(III) mainly exists as hydroxides, oxides or sulphates and small amounts of it are vital for humans, animals and microorganisms. However, its essentiality for plants is debatable (Sharma et al., 2003). The divalent oxyanions of Cr(VI) (such as CrO_4^{-2}, or $Cr_2O_7^{-2}$) are 100 to 1,000 times more toxic, more soluble in water and more motile and strong oxidizing agent than Cr(III) (Iqbal et al., 2011). Therefore, Cr(VI) has been declared lethal for all living beings (Cardenas-Gonzalez and Acosta-Rodrýguez, 2010). So far, it is imperative to assess the impact of both oxidation states of chromium metallic ions on the growth of microorganisms including fungi (Kurshied et al., 2014). Fungi have been reported to cause over 70% of plant diseases. Such fungal phytopathogens exhibit potential to accumulate heavy metals as well. It has also been documented that growth, sporulation and pathogenicity of fungi are considerably limited by some heavy metal ions, especially at high concentrations (Jaworska and Gorczycaa, 2004). However, variation in spectrum of interaction between fungi and heavy metal has been reported (Kurshied et al., 2014). Many fungal species of the genera *Alternaria, Fusarium, Trichoderma, Fusarium, Aspergillus* and *Penicillium* are enlisted as metal-tolerant fungi (Nazina et al., 2002). Tkaczuk (2005) reported that strong pollution of soil by Cr^{3+} could be a restrictive factor for development and pathogenicity of fungi in the environment. In contrast, Hasan (2007) found significant growth improvement of *F. oxysporum* under Cd^{2+}, Mn^{2+} and Zn^{2+} stress.

Fusarium oxysporum f. sp. *lycopersici* (Sacc.) Snyd & Hans, pathogen of Fusarium wilt is a soil-borne fungus that damages field and greenhouse-grown tomato plants globally in warmer areas (Abdel-Monaim, 2012). The fungus grows preferentially at 28-29 °C in dry soil and the proliferation of the pathogen inside the plant is favored by moist soil (Nelson et al., 1981). Seedlings infected by this fungus show yellowing of the lower leaves, often only on one side of the plant followed by reduced growth and eventually death of entire plant (Kumar et al., 2008). Control of wilt diseases of tomato depends mainly on chemical pesticides. However, chemical control for soil-borne diseases is usually unsuccessful due to high tolerance of wilt pathogens in diverse environments and having extremely wide host range. In addition, due to health and environmental risks associated with the use of synthetic chemicals, scientists are focusing on alternate cultural and biological options for disease management like field sanitation, crop rotation, bioactive compounds extracted from antagonistic fungi, bacteria or allelopathic plants and use resistant varieties (Javaid and Rauf, 2015).

Plant products may serve as a novel alternative source of pesticides to overcome fungal diseases under metal-stressed conditions along with metal remediation potential (Prasad, 2012). Antimicrobial activity of extracts, oils or volatile materials of many plant species including weeds has been well documented (Iqbal and Javaid, 2012). *Cenchrus pennisetiformis* is a summer growing, drought tolerant, and extremely valuable perennial fodder grass of *Poaceae* found predominantly in the world vegetation (Hall, 2008). This genus has 22 species in tropical and warm temperate regions of Africa and America; while 5 species occur in Pakistan (www.eflora.org). Various *Cenchrus* spp. have variations in contents of malondialdehyde, proline, and carbon isotope that classified these as potential biological control agent (Chandra and Dubey, 2010). Few

preliminary studies conducted so far revealed that extract of *C.pennisetiformis* has antifungal and herbicidal potential (Shafique et al., 2004; Javaid et al., 2006). However, simultaneous impact of metal stress and plant extract on pathogen growth needs to be explored. The present study was, therefore, planned to study antifungal activity of methanolic extracts of different parts of *C. pennisetiformis* for management of *F. oxysorum* under abiotic stress of chromium.

MATERIALS AND METHOD

Isolation, culturing and identification of *F. oxysporum* f. sp. *lycopersici* (FOL): *F. oxysporum* f. sp. *lycopersici* was aseptically isolated from roots of tomato plants suffering from wilt disease. The fungus was sub-cultured and maintained on a pantachloronitrobenzene (PCNB) agar medium and identified on the basis of morphological characters (Ignjatov et al., 2012).

Preparation of metal solutions: Cr(III) and Cr(VI) stock solutions were prepared by dissolving 2.82 g of potassium dichromate ($K_2Cr_2O_7$) and 7.69 g of chromium nitrate [$Cr(NO_3)_3$. $9H_2O$] (Merk, Germany in 1,000 mL of double distilled water, respectively. Further dilutions of 50, 100, 150, 200, 250, 300 and 350 ppm were made with distilled water to make final volume 48 mL of each metal concentration of each of the two salts. Malt extract (0.6 g) was added in each 6 mL metal aliquots in pre-sterilized glass test tubes. Tubes were sealed with sterilized cotton plugs and autoclaved.

Preparation of methanolic extract: Crushed dried root, stem and leaf materials of *C. pennisetiformis* (200 g each) were soaked in methanol for 14 days. Afterwords, the soaked materials were passed through cheese cloths to separate debris. Filtrates were filtered through Whatman No. 1 paper filter and methanol was evaporated on a rotary evaporator at 45 °C.

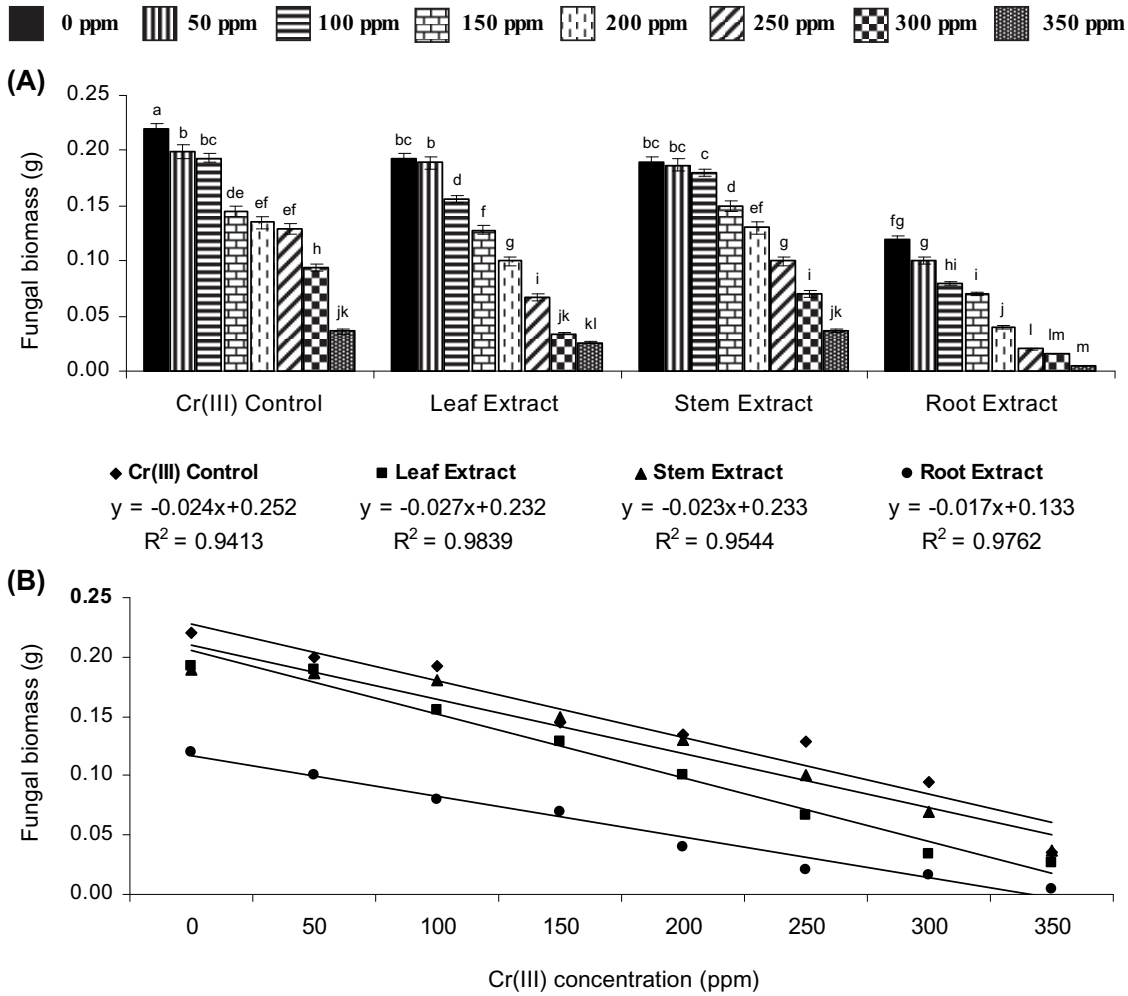
Laboratory bioassays: There were two sets of treatments. In the first set, different concentrations of Cr(III) were separately combined with 5% and 6% concentrations of methanolic leaf, stem and root extracts of *C. pennisetiformis.* Malt extract at 2 g 100 mL^{-1} of different concentrations of Cr(III) was added and autoclaved at 121 C and 103 kPa pressure for 30 minutes. The medium was cooled and appropriate quantities of methanolic extracts of different parts of *C. pennisetiformis* were added to achieve 5% and 6% final concentrations of these extracts in the growth medium. In a similar way, the growth medium in the second set of treatments was prepared by using solutions of different concentrations of Cr(VI). Different concentrations of Cr(III) and Cr(VI) were also used without addition of methanolic extracts to serve as control. Both sets of the bioassays were carried out in 5 mL volume test tubes each containing 1.0 mL growth medium. Each treatment was replicated three times. Tubes were inoculated with 15 µL conidial suspension of *F. oxysporum* f. sp. *lycopersici* and incubated at 28 ± 2 C for 7 days. Thereafter, materials were filtered using pre-weighed paper filters, dried at 70 °C and weighed. All the data were analyzed by analysis of variance followed by Tukey's HSD test using computer the software Statistics 8.1. The relationship between Cr(III) Cr(VI) concentrations and fungal biomass was calculated by using MS Excel.

RESULTS AND DISCUSSION

There was a significant reduction of 10-83% in fungus growth due to different concentrations (50-350 ppm) of Cr(III) over negative control (without Cr ions or plant extracts). When leaf, stem and root extracts of *C. pennisetiformis* were added in the growth medium combined with different concentrations of Cr(III), the reduction of fungal biomass was more pronounced with root extract. Whereas, 6% concentrations of all the extracts showed a more significant reduction in fungal biomass than 5% concentrations. The fungal biomass was significantly declined by 24-88%, 23-83%, 57-98% due to 5% and by 40-90%, 40-88% and 65-99% due to 6% concentration of leaf, stem and root extracts in combination with different concentration (50-350 ppm) of Cr(III), respectively, over the negative control. The leaf, stem and root extracts of *C. pennisetiformis* alone significantly inhibited fungal biomass by 12%, 14% and 45% due to 5% and by 25%, 22% and 50% due to 6%

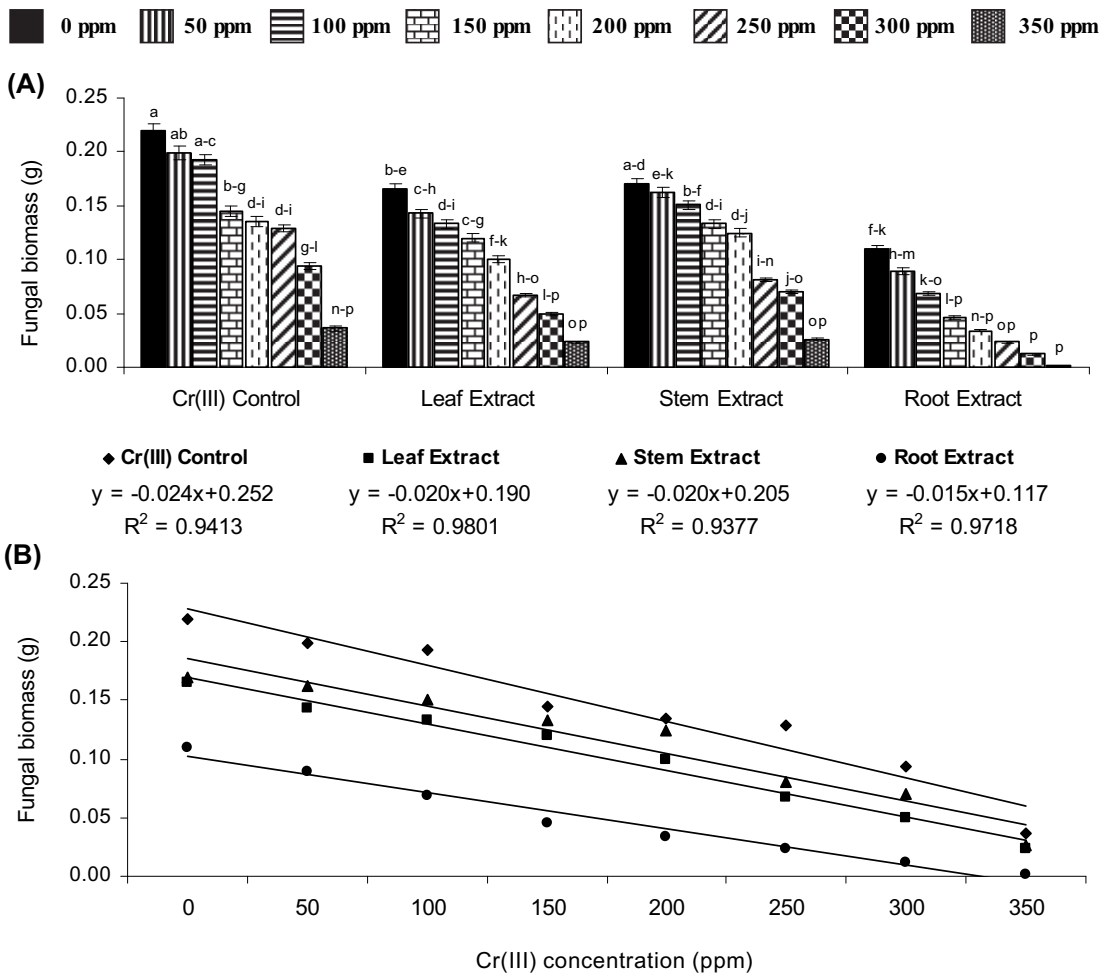
concentration, respectively, as compared to negative control. However, methanolic extract of leaf, stem and root in combination with different concentrations (50-350 ppm) of Cr(III) considerably reduced fungal biomass by 2-86%, 2-81% and 17-96% due to 5% and 14-86%, 5-85% and 20-98% due to 6% concentrations, respectively over their corresponding methanolic extracts control treatments. Whereas, 5% concentration of leaf, stem and root extract in combination with different concentrations of Cr(III) suppressed the fungal biomass by 12-28%, 0-26% and 45-86%, respectively as compared to corresponding metal concentration alone. While, the fungal biomass was decreased by 30-50%, 10-40% and 50-94% due to simultaneous effect of 6% concentration of leaf, stem and root extract, respectively and different concentrations of Cr(III), respectively over corresponding metal concentration alone (Figure 1 and 2, Table 1).

When the fungus was grown in growth medium containing different concentrations (50-350 ppm) of Cr(VI), its growth was significantly declined by 18-89% in comparison to negative control. Cr(VI) was proved slightly more toxic to fungal growth than Cr(III). Lower



(A) Effect on fungal biomass; **(B)** Regression analysis for the relationship between different concentrations of Cr(III) (along with 5% leaf, stem and root extracts of *C. penisetiformis*) and biomass of FOL. Vertical bars show standard errors of means. Values with different letters at their top show significant difference ($p \leq 0.05$) as determined by Tukey's HSD Test.

Figure 1 - Effect of different concentrations of Cr(III) and 5% methanolic leaf, stem and root extracts of *Cenchrus pennisetiformis* on biomass of *Fusarium oxysporum* f. sp. *lycopersici* (FOL).



(A) Effect on fungal biomass; **(B)** Regression analysis for the relationship between different concentrations of Cr(III) (along with 6% leaf, stem and root extracts of *C. pennisetiformis*) and biomass of FOL. Vertical bars show standard errors of means. Values with different letters at their top show significant difference ($p \leq 0.05$) as determined by Tukey's HSD Test.

Figure 2 - Effect of different concentrations of Cr(III) and 6% methanolic leaf, stem and root extracts of *Cenchrus pennisetiformis* on biomass of *Fusarium oxysporum* f. sp. *lycopersici* (FOL).

concentration (5%) of methanolic leaf and stem extracts along with different concentrations of Cr(VI) suppressed fungal biomass by 24-90% and 23-86%, respectively, over negative control. However, 5% extract of root along with seven concentrations of Cr(VI) showed significant reduction of 57-99% in fungal biomass in contrast to negative control. In case of 6% extracts of leaf, stem and root along with different concentrations of Cr(VI), there was significant decline of 39-99%, 36-94% and 65-99% in fungal biomass over negative control. The fungal biomass was significantly decreased by 12%, 14% and 45% due to 5% concentration and 25%, 22% and 50% due to 6% concentration of leaf, stem and root extracts as compared to negative control. When the two different concentrations of the three extract were mixed with seven different concentrations of Cr(VI), biomass of the fungus was reduced up to 99% in comparison to their respective methanolic extract control treatment. When combined effect of plant extract and different concentration of Cr(VI) was assessed against their corresponding metal concentrations, inconsistent variation in fungal biomass were observed. The 5% concentration of leaf and stem extracts either increased fungal biomass by 1-30% or decreased by 2-40% over

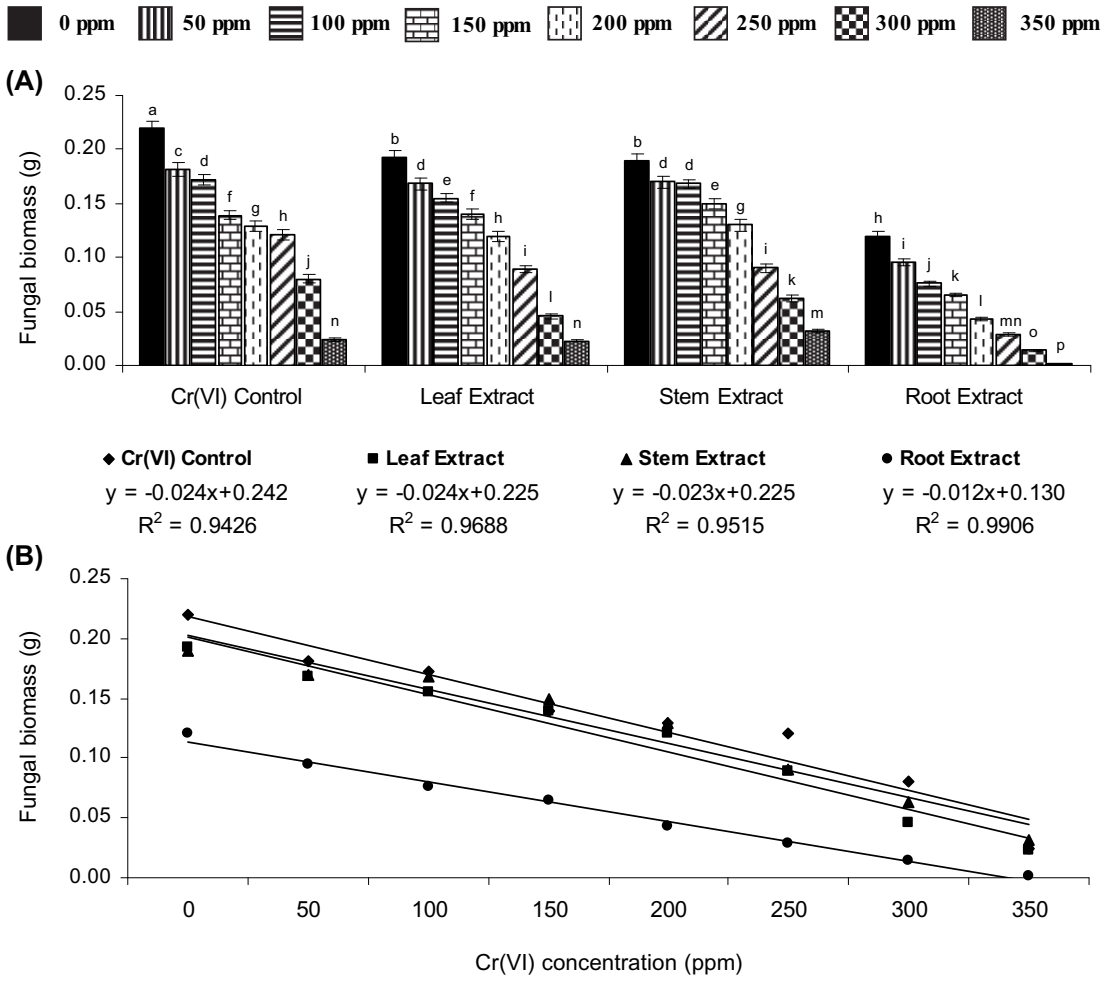
Table 1 - Percentage of the decrease in fungal biomass due to separate and combined application of Cr(III) and methanolic extracts of *Cenchrus pennisetiformis*

Treatment	Cr (III) conc. (ppm)	Extract conc. (%)	Decrease over negative control (%)	Decrease over corresponding methanolic extracts (%)	Decrease over corresponding metal (alone) concentration (%)
Cr (III)	0	0 (-control)	-	-	-
	50	0	10	-	-
	100	0	12	-	-
	150	0	34	-	-
	200	0	39	-	-
	250	0	41	-	-
	300	0	57	-	-
	350	0	83	-	-
Leaf extract	0	5	12	-	12
	50	5	14	2	5
	100	5	29	20	19
	150	5	42	34	12
	200	5	55	47	26
	250	5	70	65	48
	300	5	85	83	64
	350	5	88	86	28
	0	6	25	-	25
	50	6	35	14	28
	100	6	40	20	31
	150	6	45	28	20
	200	6	55	40	26
	250	6	70	60	48
	300	6	78	70	47
	350	6	90	86	36
Stem extract	0	5	14	-	14
	50	5	15	2	6
	100	5	18	5	7
	150	5	42	21	-3
	200	5	41	35	4
	250	5	55	47	22
	300	5	68	63	26
	350	5	83	81	-3
	0	6	23	-	23
	50	6	26	5	18
	100	6	32	11	22
	150	6	45	21	8
	200	6	43	27	7
	250	6	63	53	37
	300	6	68	60	26
	350	6	88	85	28
Root extract	0	5	46	-	45
	50	5	55	17	50
	100	5	64	33	58
	150	5	68	42	52
	200	5	82	67	70
	250	5	91	83	85
	300	5	93	87	83
	350	5	98	96	86
	0	6	50	-	50
	50	6	60	20	55
	100	6	69	40	65
	150	6	79	60	68
	200	6	85	70	75
	250	6	90	80	82
	300	6	94	90	87
	350	6	99	98	94

- Sign indicates increase in fungal biomass.

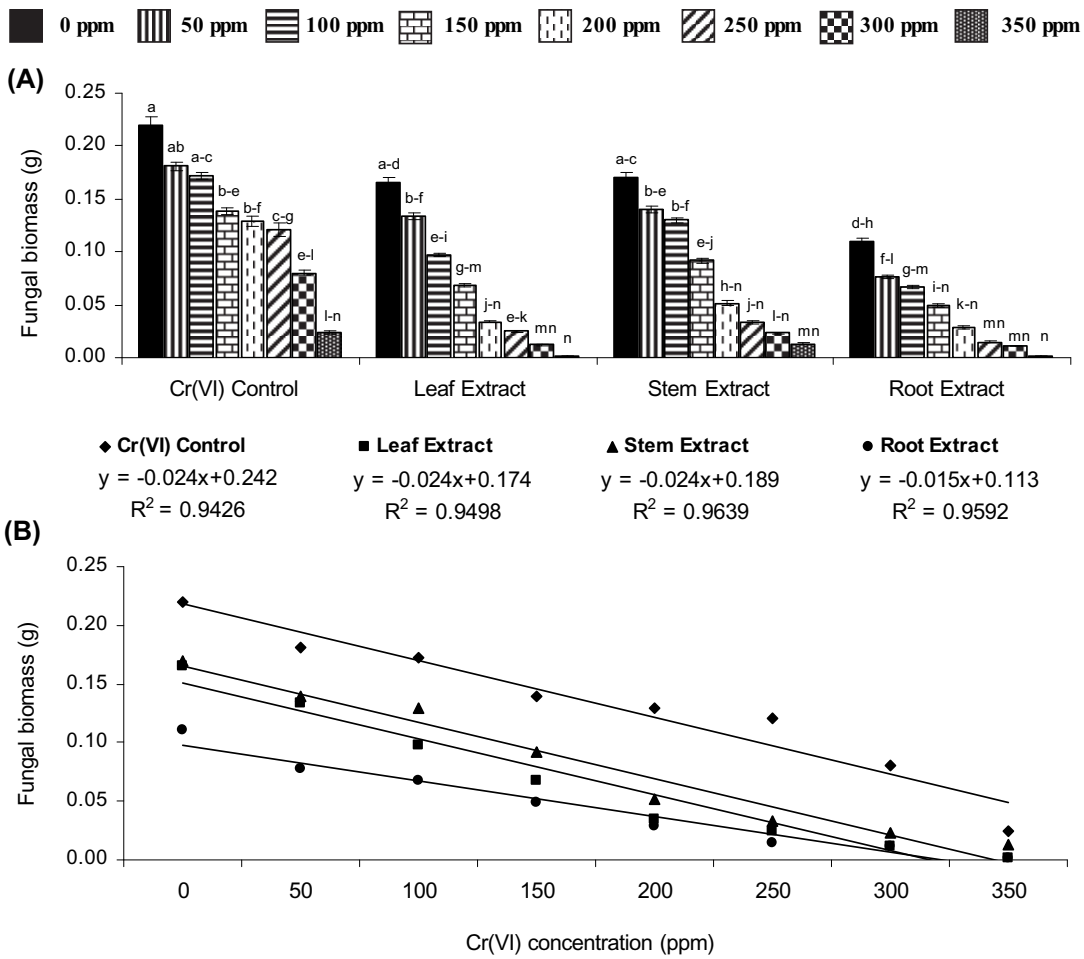
corresponding metal concentration. However, 5% concentration of root extract along with different concentrations (50-350 ppm) of Cr(VI) consistently decreased fungal biomass by 45-92%, that of 6% of each of leaf, stem and root reduced fungal biomass by 26-92%, 23-45% and 60-95%, respectively over corresponding metal concentration (Figure 3 and 4, Table 2).

The 50% growth of *F. oxysporum* in medium incorporated with 50-250 ppm of either Cr(III) or Cr(VI) exhibits its metal-tolerant behavior and thereafter reduction and absence of growth indicated toxic effect of the metal (Amatussalam et al., 2011). Impairment of cell function and loss of cell integrity due to internalization of the metal in the cytosol likely to cause reduction in fungal biomass (Pal et al., 2010). Absence of fungal growth at the highest metal ions concentrations could be attributed to non-sporulation under extreme toxicity level or prolongation of lag phase (Anahid et al., 2011). More harmfulness of hexavalent than trivalent Cr to *F. oxysporum* could be due to essentiality of Cr(III) for microorganisms in minute amounts whereas no machinery is available for Cr(VI) hydrolysis in fungi. Cr(VI) toxicity was reported to link with its specific antagonism to sulfate uptake,



(A) Effect on fungal biomass; **(B)** Regression analysis for the relationship between different concentrations of Cr(VI) (along with 5% leaf, stem and root extracts of *C. pennisetiformis*) and biomass of FOL. Vertical bars show standard errors of means. 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 Cr(VI) and 5% methanolic leaf, stem and root extracts of *Cenchrus pennisetiformis* on biomass of *Fusarium oxysporum* f. sp. *lycopersici* (FOL).



(A) Effect on fungal biomass; **(B)** Regression analysis for the relationship between different concentrations of Cr(VI) (along with 6% leaf, stem and root extracts of *C. pennisetiformis*) and biomass of FOL. Vertical bars show standard errors of means. 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 Cr(VI) and 6% methanolic leaf, stem and root extracts of *Cenchrus pennisetiformis* on biomass of *Fusarium oxysporum* f. sp. *lycopersici* (FOL).

because Cr(VI) oxyanions have high homology with the fungus sulphate ions. Sulfate can serve as an electron acceptor in anaerobic respiration and can also be reduced for the formation of organic compound in fungi. It is stated that Cr transported into the cell by sulphate permease, and promotes three-step sulphate reduction, which cause higher toxicity to the fungal cells by formation of reactive oxygen species in the cell (Raspor et al., 2003). Whereas Cr(III) toxicity resulted from antagonism with iron transport (Ramana and Sastry, 1994).

When leaf, stem and root extracts of *C. pennisetiformis* were added in metallic ions containing growth medium, fungal biomass was declined more profoundly as compared to presence of metal ions alone. The *C. pennnisetiformis* is well-known for its antifungal potential against many fungi including *Fusarium* spp. (Javaid et al., 2006; Javaid and Naqvi, 2012). The existence of steroid compounds has previously been documented in different parts of *Censchrus* spp. (Singariya et al., 2012, 2014). Therefore, the presence of different bioactive steroid compounds could be speculated in *C. pennnisetiformis* as well that probably confer resistance against *F. oxysporum*. These compounds may serve as potential sites for binding of chromium ions from the aqueous

Table 2 - Percentage of the decrease in fungal biomass due to separate and combined application of Cr(VI) and methanolic extracts of *Cenchrus pennisetiformis*

Treatments	Cr (III) conc. (ppm)	Extract conc. (%)	Decrease over negative control (%)	Decrease over corresponding methanolic extracts (%)	Decrease over corresponding metal (alone) concentration (%)
Cr (VI)	0	0 (-control)	-	-	-
	50	0	18	-	-
	100	0	22	-	-
	150	0	37	-	-
	200	0	41	-	-
	250	0	45	-	-
	300	0	64	-	-
	350	0	89	-	-
Leaf extract	0	5	12	-	12
	50	5	24	13	7
	100	5	30	20	10
	150	5	36	28	-0.7
	200	5	45	38	7
	250	5	60	53	26
	300	5	79	77	43
	350	5	90	88	4
	0	6	25	-	25
	50	6	39	19	26
	100	6	56	42	43
	150	6	69	59	52
	200	6	85	81	74
	250	6	89	85	79
	300	6	95	93	85
	350	6	99	98	92
Stem extract	0	5	14	-	14
	50	5	23	11	6
	100	5	24	12	2
	150	5	36	21	-8
	200	5	41	32	-0.8
	250	5	59	53	26
	300	5	72	68	22
	350	5	86	84	-33
	0	6	22	-	23
	50	6	36	18	23
	100	6	40	23	24
	150	6	69	46	34
	200	6	77	70	60
	250	6	85	81	73
	300	6	90	86	71
	350	6	94	98	45
Root extract	0	5	45	-	45
	50	5	57	20	48
	100	5	66	37	56
	150	5	71	47	53
	200	5	81	65	67
	250	5	87	76	76
	300	5	94	88	83
	350	5	99	98	92
	0	6	50	-	50
	50	6	65	30	58
	100	6	70	40	61
	150	6	78	56	64
	200	6	87	74	78
	250	6	93	86	88
	300	6	95	90	86
	350	6	100	99	95

- Sign indicates increase in fungal biomass.

solution, therefore may help in reducing metal toxicity in the soil. Nazir et al. (2011) reported significant accumulation of different heavy metal in root of *C. pennisetiformis* growing in industrial contaminated areas. The highest fungicidal potential of methanolic root might be related with occurrence of diversity of sterols, such as cycloergost, phytol and β-tocopherol in root extract of different *Cenchrus* spp. (Singariya et al., 2012).

The present study concludes that both Cr(III) and Cr(VI) ions, especially in high concentrations (150-350 ppm), are toxic to the *F. oxysporum* f. sp. *lycopersici* growth. Their toxic effects on the fungal growth were significantly enhanced when these ions are present in combination with root extract of *C. pennnisetiformis.*

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