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Full Length Article

Effect of *Sclerotium rolfsii* on Uptake of Heavy Metal Copper in Pea (*Pisum sativum*)

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Abstract

Pot experiment was designed to assess the effect of southern blight pathogen *Sclerotium rolfsii* Sacc. on growth, yield, qualitative gene expression and metal uptake in pea (*Pisum sativum* L.). Soil was spiked with 25, 50, 75 and 100 ppm of Cu(II) and inoculated with *S. rolfsii*. Growth, biomass and yield of pea plants were significantly declined by 10–70% in treatments inoculated with *S. rolfsii*, and 10–50% due to different concentrations of Cu(II) alone or combined with the pathogen after 30 and 60 days of growth. Cu(II) uptake capacity of pea plants were increased under combine stress of metal and pathogen as compared to metal alone. Higher metal concentration was recorded in roots than in shoots and pods. Expression of defensin like gene was intense due to combined influence of *S. rolfsii* and Cu(II) than in plants under pathogenic stress alone. Metallothionein gene was expressed only in Cu(II)-incorporated treatments. The present study concludes that solitary influence of *S. rolfsii* or Cu was more detrimental to growth and yield of *P. sativum* as compared to combined stress of both the factors.

Keywords: Copper; Defensin; Fungal phytopathogen; Heavy metals; *Sclerotium rolfsii*; Metallothionine

Introduction

Pea (*Pisum sativum* L.) is one of most important legumes extensively cultivated round the world (Rauber *et al*., 2001). Fungal pathogens are regarded as the most devastatingly affecting the growth and yield of peas throughout the world (Nafisa *et al*., 2013). Southern blight caused by *Sclerotium rolfsii* Sacc, often called white mold is a serious and frequent soil-borne fungal disease of many vegetable crops including peas. The fungus is an obligate necrotroph, polyphagous and ubiquitous plant pathogen that can survive as sclerotia in soil for several years even under harsh environmental conditions. Under favorable environmental conditions, the fungus infects susceptible plant cause yellowing and wilting of branches. In case of severe infestation, whole plant wilts and white mycelia can be seen at the soil line around affected plant parts (Punja, 1985).

Generally, fungicides of metal formulation are utilized to manage such devastating fungal diseases. Copper (Cu) containing compounds have long history of successful use as fungicides but now they are being recognized as hazardous pollutant (Peciulyte, 2001). Excessive concentration of Cu in plants consequences in clogging of tolerance mechanism in the root zone that let the surplus Cu to translocate via xylem and phloem to the leaves and catalyzed Fenton-type reactions (FYR). The activation of FYR results in generation of reactive oxygen species (ROS) primarily in chloroplasts that can damage membrane proteins and nucleic acid, and inhibits photosynthetic electron transport (Akhtar *et al*., 2005). The net effect of Cu toxicity on plant biochemistry alters root and shoot growth and reduce yield (Puig *et al*., 2007).

Plants may respond differently due to metal or pathogen stress and can produce various secondary metabolites/proteins (Mittler *et al.*, 2004). These proteins are encoded by many genes belonging to large families (Ooijen *et al*., 2007). In this regard, pathogen related gene namely defensin like gene (Def) accession number: L01578.1 constitute a part of the innate immune system primarily directed against fungal pathogens. Besides, it provides antibacterial, antifungal, proteinase and insect amylase inhibitory activities (Henrik *et al.*, 2009). Metallothionein gene (MTs) accession number: Z23097 gene play key role in intracellular metal sequestration. Plant MTs, are considered to adopt a hairpin structure characteristic Cysteine distribution pattern, where the two domains interact to form a metal containing cluster with a total of four divalent ions (Domènech *et al*., 2006). So far, toxic effects of Cu on green pea with reference to wilt disease are poorly understood. The metal can arrest the growth and multiplication of pathogenic fungi in rhizosphere and in turn limits the growth and various metabolic activities of plant resulting in lower yield (Nafisa *et al*., 2013). So, it is necessary to assess the combined stress of heavy metal and pathogen to address the consequences on plant morphology, growth and yield along with metal

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accumulation in plant. Previously preliminary growth experiments were conducted in Petri plates, to investigate simultaneous effect of *S. rolfsii* and Cu(II) on seed and seedling growth of pea (Nafisa *et al.*, 2013). On the basis of those preliminary results (Nafisa *et al.*, 2013) further elaborated experiment was conducted in pots. Therefore, present research work was aimed to assess the combined stress of *S. rolfsii* and Cu(II) on morphology, growth, yield and metal accumulation along with assessment of Def like and MTs genes expression in pea plants.

Materials and Methods

Experimental Details

Procurement of pathogen and pathogenicity test: Pure culture of *S. rolfsii* was procured from National Agriculture Research Centre, Islamabad, Pakistan. The fungus was sub-cultured and maintained on 2% malt extract agar (MEA). To confirm the Koch's pathogenicity postulate, soil was sterilized with formalin. Pea seeds were sown in pre sterilized soil inoculated with 4.8×10^6 conidia 50 mL^{-1} of pathogen in 4.5 kg of soil. Pots were monitored for disease development. Symptoms caused by *S. rolfsii* on pea seedlings were confirmed 30 days after germination on the basis of disease rating 0–5 scale (Latunde-Dada, 1993). Where, 1 = a small number of leaf wilt symptoms in plants, 2 = slight infection, mycelial mass only on the surface of the soil, 3 = moderate infection, wilting and blight, mycelial mass around stem, 4 = severe infection, advanced wilt, sclerotia forming around crown, 5 = plant dead, 100% rotted.

Further confirmation of *S. rolfsii* was done by re-isolating, inoculating and culturing the fungus from inoculating roots. Four sections of root approximately 0.5 cm in length were then cut from similar sized roots in each replicate and placed onto MEA petri plates. Fungal colonies emerged from the inoculated roots were re-identified on morphological basis and confirmed as *S. rolfsii.*

Metal solution: Copper sulphate ($CuSO_4.5H_2O$) (MERCK) was used for preparation of Cu(II) solution during whole experimental trials. A stock solution of 1000 ppm of metal was prepared and further concentrations of 25, 50, 75 and 100 ppm were prepared by diluting stock solution with double distilled water.

Pot experiment: Soil utilized was sandy loam (sand: 76%, silt: 15%, clay: 9) having pH 8.0, electrical conductivity 2.81 mS L^{-1}, organic matter 0.87%, organic carbon 0.5%, nitrogen 0.044%, available phosphorous 15 mg kg^{-1}, available potassium 180 mg kg^{-1}, chloride 142 mg kg^{-1}, biocarbonates, 6 meq L^{-1}, sulphate, 18.1 meq L^{-1}, calcium, 6 meq L^{-1}, magnesium 7 meq L^{-1} and sodium 12 meq L^{-1}. Iron, copper and zinc found were 7.5, 1.35, 5.1 ppm, respectively. Soil was fumigated with 10% formalin. Fumigated soil was spiked with each of 25, 50, 75 and 100 ppm of Cu(II) following method of Zhang *et al.* (2011). Five kilograms of metal-spiked soil was filled in each plastic pot (12 × 10 cm) and was artificially infected by pouring 100 mL of conidial suspensions (4.8×10^6) of pathogen in the upper 5 – 15 cm layer in each pot. The infected soil in each pot was left for 3 days under natural environmental conditions for the establishment of the pathogen. In each pot, 15 surface sterilized seeds of pea var. Meteor were sown. The pots were placed in glass house (25 ± 3ºC; 12 h photoperiod and 70% relative humidity) and moisture level was maintained to field capacity by irrigating occasionally with tap water. Control treatment was neither spiked nor inoculated, while soil in one set of pots was inoculated with *S. rolfsii* conidia only and other was spiked with each of four concentrations of Cu(II). Each treatment of whole experiment was replicated thrice in a completely randomized design and the complete experiment was repeated.

Qualitative Gene Expression

RNA from the leaves of 60-days old plant was isolated using Trizol reagent (Wang *et al*., 2009). Leaves were taken for RNA isolation, because RNA is very sensitive and start degrading very fast. Beside, leaves are easily accessible part of the plant getting less time to pick and sample. Ground-frozen tissue was mixed with Trizol reagent in 1:1 ratio and kept in ice for 5 min followed by centrifugation at 13,200 rpm for 15 min. Ice chilled isopropanol was added to the supernatant and it was centrifuged again for 10 min at 13,200 rpm. Resulting RNA pellet was washed twice with 75% ethanol, air dried and dissolved in 50 µL of RNase free water. Quality of extracted RNA was checked through agarose gel electrophoresis. A pair of primers (forward and reverse) was used to amplify a single gene. The sequences of forward and reverse primers used for the amplification of MTs were: 5'ACCATCCTCAGAAGCAGCAC and 5'TGCAAATGCAACAAGAGGTC, respectively and for Def were 5'GCTTGTCCTTCCTCCTCCTC and 5' TAGTGCACCAACAGCGAAAG, respectively. cDNA was synthesized by using TOPscript™ cDNA synthesis kit (Enzynomics, Korea). Amplicon size was 140 bp for Mts and 186 bp for Def-like gene.

The expression of MTs and Def like gene was checked by PCR reaction using PCR machine (TECHNE TC-412) programmed with following conditions; initial denaturation at 95ºC for 2 min; followed by 40 cycles denaturation at 95ºC for 30 s, annealing at 55ºC for 1 min and primer extension at 72ºC for 1 min coupled with final extension at 72ºC for 5 min. The reaction was terminated at 4ºC. Size of amplified DNA fragment was analyzed by agarose gel electrophoresis.

Disease Rating and Harvesting

Symptoms of disease were appeared 45 days after germination on stem, leaves and collar region of plant. Disease was rated on the basis of 0–5 rating scale

(Latunde-Dada, 1993). Disease incidence (DI) was determined using following formula.

Disease incidence (%) = (number of infected plants/total number of plants) × 100.

Disease rating and incidence was recorded at 45 and 60 days of post-emergence, however different growth parameters of the plants were taken 30 and 60 days after germination. For growth parameters shoots, roots and pods were separated. Length (cm) and fresh weight (g) of the plants were recorded. For dry weight, materials were oven dried at 80°C for 24 h and weighed.

Determination of Cu(II) Concentration

Dried powdered samples (0.5 g) of roots, shoots and pods were digested with 10 mL of HNO_3 for about 20 minutes. Digested samples were analyzed on atomic absorption spectrophotometer (Z-5000 Polarized Zeeman) for remaining Cu(II) concentration.

Statistical Analysis

Data obtained from different treatments were compared through mean values. All means were tested for a significant difference by applying Duncan's Multiple Range Test using computer software COSTAT (Steel and Torri, 1997).

Results

Morphological, Growth and Yield Assays

Disease incidence was up to 50% and 80% after 45 and 60 days of emergence, respectively. The infected plants showed wilting, chlorosis and wrinkling of lower leaves at both growth stages. However, rotting of stem and roots near the soil line along with white fungal mycelia and brown sclerotia were observed on infected soil after 60 days of germination. However, soil infestation with *S. rolfsii* exhibited the maximum reduction of 42% in germination as compared to control. Shoot length, and fresh and dry weight were significantly declined by 20–30, 20–40 and 30–70%, and that of roots by 20–40, 30–40 and 40–50%, respectively over control after 30 and 60 days of growth. Number of pods was not significantly declined, whereas both fresh and dry weights of pods were declined by 40% due to disease (Figs. 1–4).

Plants under Cu(II) stress alone exhibited chlorosis, shortened internodes and stem, stunted, curled and fragile roots along with absence of lateral roots and root hairs, Increasing concentration of Cu(II) from 25 to 100 ppm resulted in progressive and significant reduction in germination by 4–35%, whereas shoot length and fresh weight were significantly suppressed by 15–40% both after 30 and 60 days as compared to control. Shoot dry weight was non-significantly different after 30 days, while significantly decreased by 30–50% after 60 days

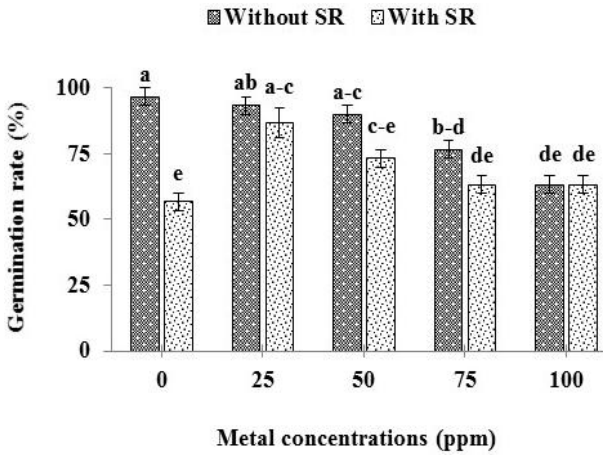

Fig. 1: Effect of *Sclerotium rolfsii* (SR) and Cu(II) on germination % age of *Pisum sativum*. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference (*P≤0.05*) as determined by Duncan's Multiple Range Test

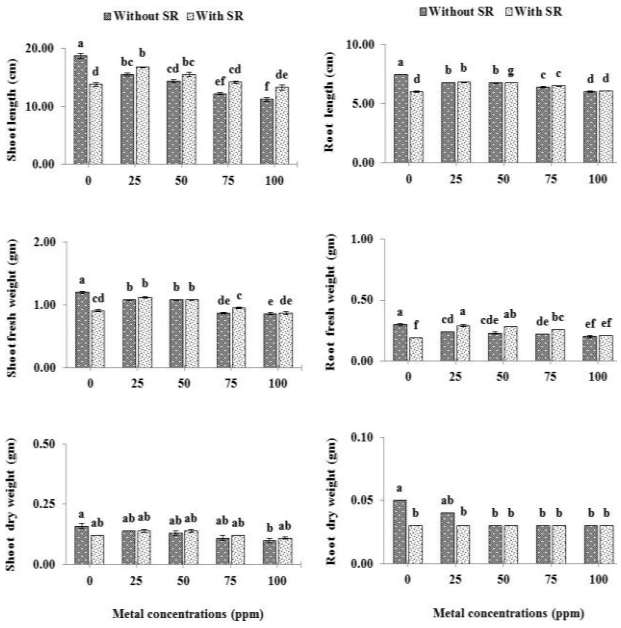

Fig. 2: Effect of *S. rolfsii* (SR) and Cu(II) on growth parameters of pea after 30 days. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference (*P≤0.05*) as determined by Duncan's Multiple Range Test

growth with increase in metal concentrations over control. Root length and weight was declined up to 20–40% after 30 and 60 days with different concentration of Cu(II). Number as well as fresh and dry weight of pods was declined by 20–35% due to metal concentrations of 25 to 100 ppm (Figs. 1–4).

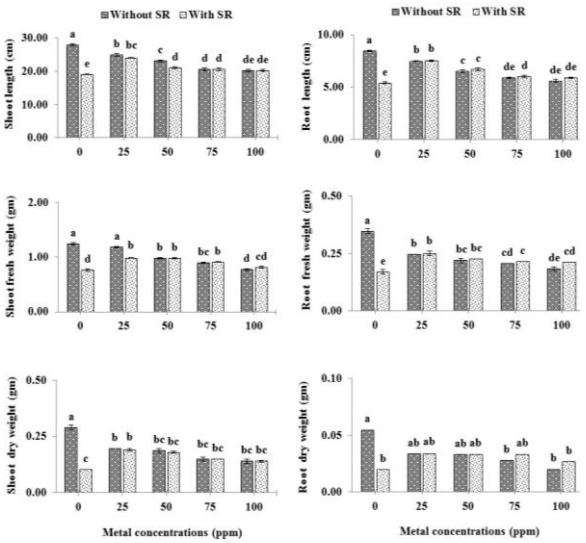

Fig. 3: Effect of *S. rolfsii* (SR) and Cu(II) on growth parameters of pea after 60 days. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference (*P≤0.05*) as determined by Duncan's Multiple Range Test

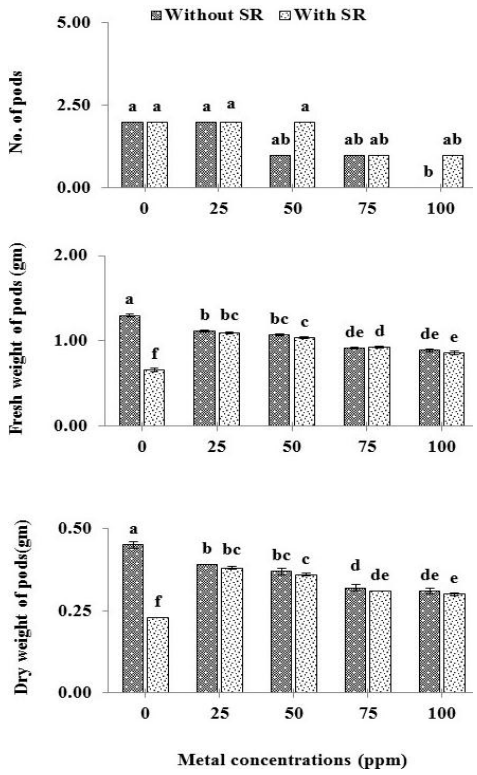

Fig. 4: Effect of *S. rolfsii* (SR) and Cu(II) on yield of pea after 60 days. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference (*P≤0.05*) as determined Duncan's by Multiple Range Test

Simultaneous application of the pathogen and the metal resulted in yellowing and wilting of leaves, fragile, short roots and shoots, while crown region of plants remained unaffected. Disease incidence was 30% and 50% at 45 and 60 days of germination, respectively. Combined application of the metal solution and the pathogen exhibited slightly less drastic effects on pea growth and yield after 30 days and almost same after 60 days as was recorded due to Cu(II) alone (Figs. 1–4). Germination was significantly suppressed by 13–35% over control. Length, fresh and dry weight of shoot was significantly declined by 5–30% after 30 days and 15–50% after 60 days of growth. Root length was significantly dropped by 10–30% at both harvests. Dry biomass of root was significantly declined by 40% after 30 days and non-significantly different after 60 days as compared to control. Different concentrations of Cu(II) combined with *S. rolfsii* significantly reduced number, fresh and dry weight of pods by 15–30%.

Qualitative Gene Expression

Def like gene was induced in pea by all treatments. However, the gene expressed more pronouncedly in the treatments under combined action of *S. rolfsii* + Cu(II) as compared to rest of the treatments (Fig. 5).

Treatments provided either with Cu(II) alone or in combination with *S. rolfsii*, showed intense MTs gene expression, whereas this gene did not express in the treatments inoculated with pathogen only. Under combined stress of pathogen + metal, MTs gene was expressed only at higher concentrations of metal solution i.e., 75 and 100 ppm as compared to synchronized MTs expression in treatments provided with four different concentration of Cu only (Fig. 6).

Remaining Cu(II) Concentration (ppm)

The pea plant uptake a total of 30–50% Cu(II) after 30 days and 30–60% of Cu(II) after 60 days of growth from soil amended with four different concentrations of metal solution alone or combined with the pathogen (Table 1). Besides, the uptake of metal was constantly declined with increasing concentrations of the metal solution. Whereas treatments with simultaneous stresses of the pathogen and the metal showed 2–4% more concentration of Cu(II) than in treatments provided with metal solution alone. About 75% of the metal was accumulated in roots and 25% in shoots during 30 days of growth. Whereas Cu(II) accumulation was found in ratio of 70:23:7% in root: shoot: pods after 60 days of sowing (Fig. 7).

Discussion

S. rolfsii inoculation significantly decreased seed germination, shoot and root growth and biomass of pea.

Table 1: Effect of *S. rolfsii* (SR) on copper uptake by pea plants

Cu(II) given to the soil (ppm)	Cu(II) uptake by plant (ppm)	
	1st harvest	2nd harvest
25	12.6±0.02f (50%)	13.86±0.02g (55%)
50	21.4±0.03e (43%)	23.53±0.01e (47%)
75	26.19±0.02c (35%)	28.18±0.03c (38%)
100	27.07±0.02b (28%)	30.06±0.01b (31%)
SR + 25	12.6±0.02f (50%)	14.65±0.08f (59%)
SR + 50	23±0.02d (46%)	24.91±0.03d (50%)
SR + 75	28±0.06b (37%)	30.26±0.01b (40%)
SR + 100	33.57±0.02a (33%)	35.04±0.02a (35%)

Values in parenthesis shows percentage of metal uptake by plant over corresponding metal concentration. Values with different letters in each column show significant difference (*P≤0.05*) as determined by Duncan's Multiple Range Test

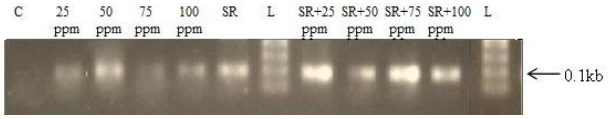

Fig. 5: Expression of Def like gene in pea under different treatments

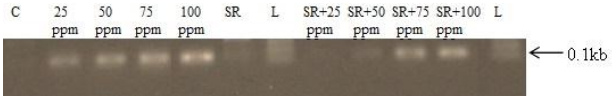

Fig. 6: Expression of metallothionein gene in pea under different treatments

The pathogenicity of *S. rolfsii* probably be correlated with production of variety of enzymes by the fungus (Karaman and Matavuly, 2005; Azmat and Khan, 2014), that might exhibit inhibitory action on different physiological and metabolic functions of the plant through disturbing working of oxidative enzymes, consequently plant growth and biomass declined with drying of whole plant within few days after infection (Southerton and Deverall, 1990). Disease infected plants showed wilting, chlorosis, wrinkling of lower leaves, rotting of stem and roots near the soil line along with abundant white branched mycelium and brown sclerotia. Disease incidence was up to 80% and infected plants showed severe infection wilting, sclerotia formation around crown as determined by disease rating scale (Anahosur, 2001).

The plant grown under Cu(II) stress alone exhibited significant and progressive reduction in germination, growth and biomass of vegetative and reproductive parts with increasing Cu(II) concentrations. Current results are supported by previous findings on different plant seedlings or adult plants with Cu(II) and other metals (Radoviciu *et al*., 2009; Houshmandfar and Moraghebi, 2011; Nafisa *et al*., 2013). The reduction in germination, growth and yield parameters is attributed to toxic concentration of metal as a dominant factor affecting the plant development (Adriano, 1986). Oxygen depletion at higher metal concentration could be another possibility of stunted root growth.

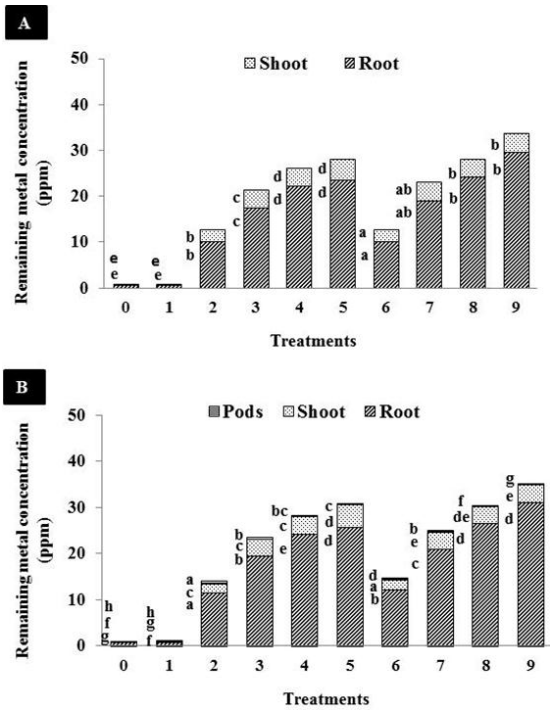

Fig. 7: Influence of *S. rolfsii* (SF) on copper accumulation by pea plant after 30 (A) and 60 (B) days of growth. 0: Control, 1: *S. rolfsi* (SR), 2: 25 ppm, 3: 50 ppm, 4: 75 ppm, 5: 100 ppm, 6: SR+25 ppm, 7: SR+50 ppm, 8: SR+75 ppm, 9: SR+100 ppm

Values with different letters at their top show significant difference (*P≤0.05*) as determined by Duncan's Multiple Range Test

Reduction in plant growth and biomass due to Cu(II) can also be correlated with damage to vascular bundle due to inhibition of enzymes involved in photosynthetic reaction, conduction of water molecules and desired nutrients from roots to aerial parts (Azmat *et al*., 2006). Moreover, Cu toxicity resulted in shortened internodes, short, fragile shoots and roots, curling in roots, absence of lateral roots or roots hairs, due to mobility of metals in the plants (Sheldon and Menzies, 2005).

Different stress factors occurring in combination of the metal and the pathogen may be considered additive or interactive on pea growth and biomass (Niinemets, 2010). It seems that under combine stress, Cu(II) and *S. rolfsii* kept competing with each other probably due to antifungal action of Cu that likely to inhibit spore germination and mycelial growth rate of the fungus (Nagahashi *et al*., 1996), and ultimately reduced the infection level in pea plants. Furthermore, pea plants exhibited yellowing, wilting of leaves from lower side, weak and shorten roots and shoots along 40% plant mortality.

Both Def and MTs genes responded distinctively to different treatments. Plant defensin gene expression under biotic and abiotic stresses was supported by the previous findings (Lay and Anderson, 2005; Sudo *et al*., 2008;

Bahramnejad *et al.*, 2009). The antifungal action of plant defensin could be linked with synthesis of phytoalexin and lignin due to induction of systemic resistance in plants. Plant defensin are pathogenesis-related proteins that are well-known to act as inhibitor against pathogen due presence of proteinase, polygalacturonase-inhibitors and lectins. These inhibitors can interfere with pathogen nutrition and retard their development, thus contributing to disease resistance. Through production of number of antifungal compounds inculding phytoalexins and lignin (Guest and Brown, 1997). Our results showed that defense-related genes were prominently expressed with the maximum Cu-concentration. The acquired findings are logical by assuming role of Cu as an abiotic elicitor that induces resistance against pathogen attack (Graham, 1980). Defense-related genes could be effective targets for increasing tolerance to Cu. Alternatively, the role of Cu as an antifungal agent may act in part by inducing defense-response genes (Sudo *et al*., 2008).

Expression of MTs gene in pea indicated its role in preventing or reducing cellular injury caused by the generation of reactive oxygen species in response to Cu stresses (Lombardi and Sebastiani, 2005). Role of MTs could also be attributed as keeper of metal homeostasis through chelating, effluxing or sequestering Cu ions (Hall, 2002). Recently, distinct MTA or Cu-responsive genes expression profiles due to Cu increasing concentrations suggested MTA role as indicators of various environmental pollutants (Lettieri, 2006). This study therefore revealed the additional potential of using MTs and Def like genes as biomarkers for biotic and abiotic stresses because of their acute sensitivity.

Cu uptake tendency was detected in order of: root > shoots at 30th day, whereas the order was of root > shoots > pods at 60th days of sowing. These results were similar to reported in previous literature that among the plant parts, a very large amount of metal retained in roots compared to its content in shoots and grains (Abedin *et al*., 2002). It has been stated that a large amount of Cu absorbed by the plants is bonded with root, and only small fraction of metal was translocated to shoots and pods. Therefore, Cu level in roots is related to its concentration in soil, whereas levels in shoots did not reflect the Cu concentration in the soil (Jarvis and Whitehead, 1981).

Conclusion

The current study concludes that growth and yield of pea plants were considerable declined due to *S. rolfsii* alone in comparison to the treatments provided with metal alone or combined with pathogen. The adverse influence of Cu(II) on the test plant was increased with elevating metal concentrations in the range of 25–100 ppm. Root of pea uptake more concentration of Cu as compared of shoot and pods. A considerable expression of Def like and MTs genes express in pea seedlings under disease and meal stress.

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