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Response of *Pisum sativum* L. to Southern Blight Disease under Abiotic Stress of Copper

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PAKISTAN

Pea (*Pisum sativum* L.)

- Native to central and southeast Asia
- Annual, self-pollinated, rapid-growing and cool season - *Fabaceae*
  - ✓ low in calories-lose weight.
  - ✓ rich in dietary fiber- avoid from constipation.
  - ✓ iron and vitamin C - strengthen the immune system.
  - ✓ folic acid and vitamin B6 – promote the cardiovascular health.
  - ✓ antioxidants, like vitamin C- keep cancer at bay.
  - ✓ maintain level of cholesterol - aid energy production, nerve function - carbohydrate metabolism- important nutrients for maintaining bone health.

- Ranks forth - on production basis (441.53 thousand tons) among grain legumes - grown on an area of 528.71 thousand hectares in the world (FAO, 2009).
- In ***Pakistan*** – third position- 1689 ha - 83603 tones production - the average per hectare yield is very low (Anonymous, 2010-11).

Factors affecting pea growth and yield

Biotic stress-Pathogens

- Fungal pathogens - most devastating affecting pea growth and yield throughout the world.
- ***Sclerotium rolfsii*** Sacc - an economically important soil-borne plant pathogen
- Attack - 500 cultivated and wild plants species - cause wilt, root rot, stem rot and foot rot
- Southern blight (white mold), southern wilt, collar rot, southern stem rot, sclerotium blight, sclerotium wilt and white mold
- **dark brown lesion on the stem or just beneath the soil level - progressive yellowing and wilting of the leaves- seedling mortality- fall in plant growth and biomass**

- Survival under these conditions demands quick defense responses to inhibit pathogen spread after initial infection and thus to limit disease**.**
- **Plant defensins** are small, highly stable, cysteine-rich peptides that constitute a part of the innate immune system primarily directed against fungal pathogens (Henrik *et al.*, 2009).
- Disrupting microbial membranes - acting as ligands for cellular recognition and signaling
- In addition to pathogen-induction, defensins can also be induced by environmental stress

Abiotic stress- Pollutants

Copper

- Plants - contributor of harmful concentrations of heavy metals in to the food chain
- Essential micronutrients
- Toxic level - in agricultural soils- **wastewater, phosphate fertilizer, fungicides, sewage sludge amendment, poultry manure and wood-preservatives**

Prescribed Limits of Copper

- Drinking-water: 1 mg/L
- Ranges in Pakistani soils: 6.55 to 25.41 mg/L
- Normal range for plants: 5-20 mg/L
- Maximum allowable limits: 40 mg/L

Higher doses inhibits

- **Plant growth, key cellular processes, including photosynthesis and electron transport, lipid peroxidation, disruption of protein functions due to Cu-binding to sulphhydryl groups, Reduced biomass, Stunted growth, Chlorosis, Root malformation**

- Cu - induces the formation of reactive oxygen species (ROS).
- Plants - ROS scavenging systems - prevent or reduce cellular injury - caused by the generation of ROS in response to heavy metal stresses.
- **Metallothioneins** - Cysteine-rich, low molecular weight well-known metal-binding peptides - play a role in Cu tolerance, homeostasis
- Cystein group of MT catch OH·- terminate oxidative stress

OBJECTIVES

The aims of present research work were to:

- ***check the damaging impact of S. rolfsii and Cu(II) on seed and seedling growth of P. sativum***
- ***characterize expression of Defensin like and Metallothionein genes in the test plant***

METHODOLOGY

Pathogenicity test

- *S. rolfsii*, the causal agent of pea's southern blight (Yaqub and Shahzad, 2011)- National Agriculture Research Centre, Islamabad.
- Conidial suspensions of 4.8×10^6 conidia/mL-2 week old cultures of *S. rolfsii*-in sterilized distilled water
- Freshly prepared inoculum of pathogen-mixed with sterilized soil-sterilized *Pisum sativum* L. var. Mateor seeds
- Pots-shaded glasshouse ($25^oC \pm 3$; 12 h photoperiod; soil moisture 50%) in a completely randomized design.
- Symptoms - confirmed on 30^{th} day after germination on the basis of 0-5 scale (Lotunde-Dada, 1993).

  1 = a small number of leaf wilt symptoms in plants,

  2 = slight infection, mycelial mass only on the surface of the soil,

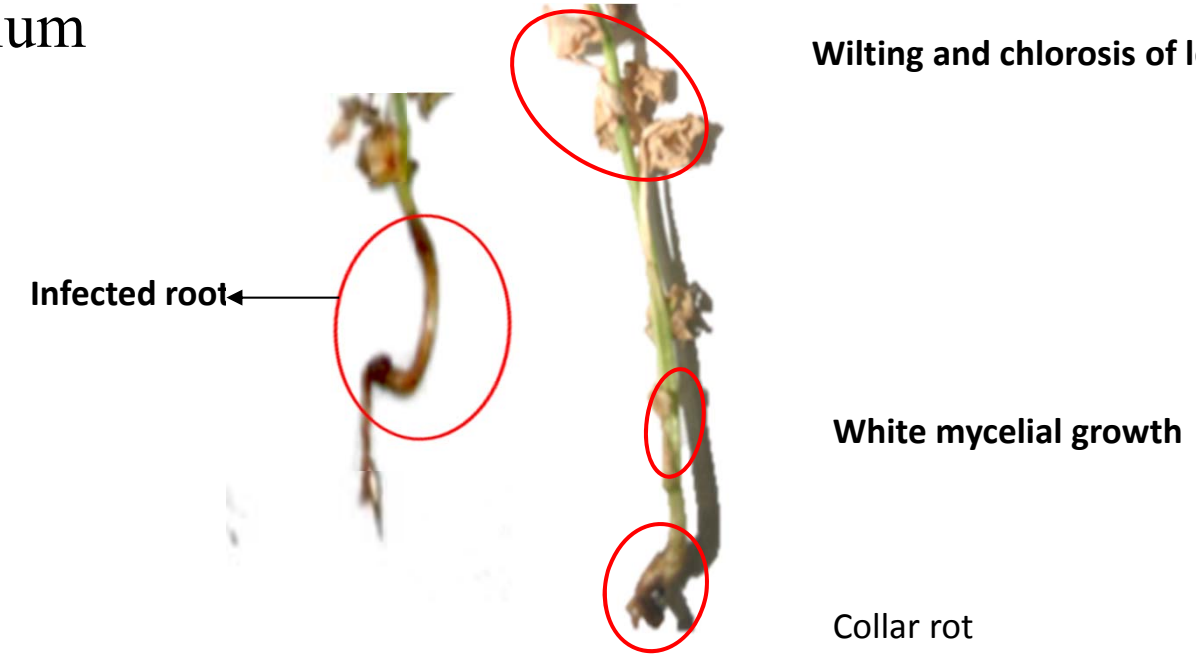
  3 = moderate infection, wilting and blight, mycelial mass around stem,

  4 = severe infection, advanced wilt, sclerotia forming around crown

  5= plant dead, 100% rotted

Pathogenecity of *S. rolfsii*

- **40%** reduction in germination rate
- 50% infected plant - wilting, chlorosis and wrinkling of lower leaves
- **Lower stem and upper roots** - dark coloration
- **Roots** - shorter, dark coloration and lateral roots were absent.
- **On infected tissue and soil-** abundant white and branched mycelium



Pathogenecity of *S. rolfsii* against *Pisum sativum*

Treatments used during the experiments

Treatments		
1	C	Control
2	P	*S. rolfsii*
3	M1	25 mg/L of Cu(II)
4	M2	50 mg/L of Cu(II)
5	M3	75 mg/L of Cu(II)
6	M4	100 mg/L of Cu(II)
7	PM1	*S. rolfsii* + 25 mg/L of Cu(II)
8	PM2	*S. rolfsii* + 50 mg/L of Cu(II)
9	PM3	*S. rolfsii* + 75 mg/L of Cu(II)
10	PM4	*S. rolfsii* + 100 mg/L of Cu(II)

The experiment was laid out as a completely randomized design with three replicates of each treatment.

PETRIPLATE BIOASSAYS

Petri plate (9 cm depth) in four sets

one set of treatments received 3 mL sterlized distilled water alone

C

2nd set of treatment was inoculated with 3 mL spore suspension (1.5 ×10⁶) of pathogen alone

P

3rd set of treatment received each of four concentrations of Cu (3 mL)

M1, M2, M3 and M4

0 mg L^{-1}
25 mg L^{-1}
50 mg L^{-1}
75 mg L^{-1}
100 mg L^{-1}

4th set of treatment received each of four concentrations of Cu (1.5 mL) + spore suspension of fungus (1.5 mL)

PM1, PM2, PM3 and PM4

- 20 surface sterilized seeds were placed per Petri plate
- Plates were kept at 25°C ± 3; 12 h photoperiod for 15 days

Harvest

- Germination rate
- Shoot length, fresh and dry weight
- Root length, fresh and dry weigh

Antifungal activity of Cu(II) against *Sclerotium rolfsii*

Metal-amended medium (2 Malt Extract Agar) in the Petri dishes were inoculated aseptically at the centre with 5 mm (diameter) inoculum-disc of test fungus and incubated at 25°C for 7 days.

POT BIOASSAYS

Plastic pots (of 12cm x 10cm (diameter x depth) filled with 5 kg of sandy loam pre sterilize soil in four sets

one set of treatments received water alone

C

2nd set of treatment was inoculated with spore suspension of pathogen alone

P

3rd and 4th sets of treatment received each of four concentrations of Cu, followed by air drying, sieving and aging of soil for a week

0 mg L^{-1}
25 mg L^{-1}
50 mg L^{-1}
75 mg L^{-1}
100 mg L^{-1}

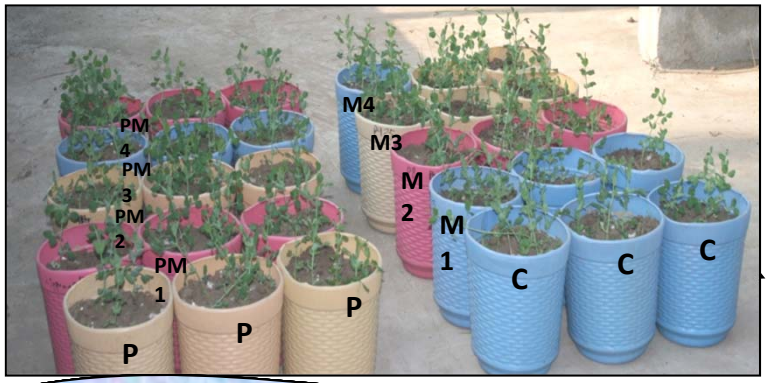
sowing 10 surface sterilized seeds per pot and all pots were placed in a shaded glasshouse at 25°C ± 3; 12 h photoperiod, 50% soil moisture

Metal treated soil in 3rd set was inoculated with spore suspension of pathogen inoculam

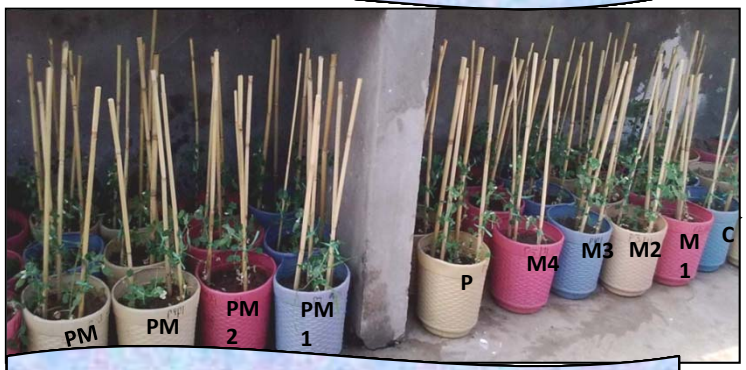
PM1, PM2, PM3 and PM4

4th set of treatments serve as such

M1, M2, M3 and M4



Harvest after 30-days of sowing



Harvest after 60-days of sowing

Harvest after 90-days of sowing

- Morphological studies
  - Root
  - Shoot
- Cu analysis
  - Length
  - Fresh weight
  - Dry weight
- Molecular studies
  - RNA isolation
  - Reverse transcription
  - Polymerase chain reaction

The expression of metallothionein and defensin like gene was checked by using following sets of primers

F- metallothionein 5'ACCATCCTCAGAAGCAGCAC

R- metallothionein 5'TGCAAATGCAACAAGAGGTC

F- Defensin (DEL1) 5'GCTTGTCCTTCCTCCTCCTC

R- Defensin (DER1) 5' TAGTGCACCAACAGCGAAAG

RESULTS

Plate Bioassays

Reduction in germinate rate :

P: 30%

M-M4: 6-20%

PM1-PM4: 6-26%

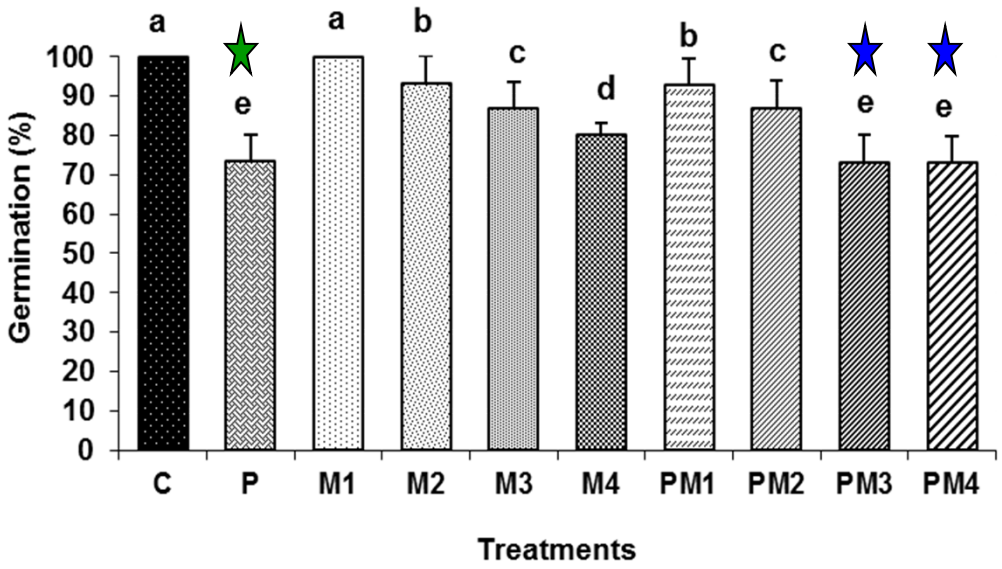


Fig. 1: Effect of *Sclerotium rolfsii* and Cu(II) on germination % age of *Pisum sativum*.

C: Control; **P:** Treatments inoculated with pathogen; **M1, M2, M3 & M4:** Treatments treated with 25, 50, 75 and 100 mg/L of Cu(II), respectively**; PM1, PM2, PM3 and PM4:** Treatments inoculated with pathogen combine with metal in each of four concentration 25, 50, 75 and 100 mg/L of Cu(II), respectively.

Reduction in shoot growth and biomass :

P: 15-40%

M: 2-31%

PM: 7-31%

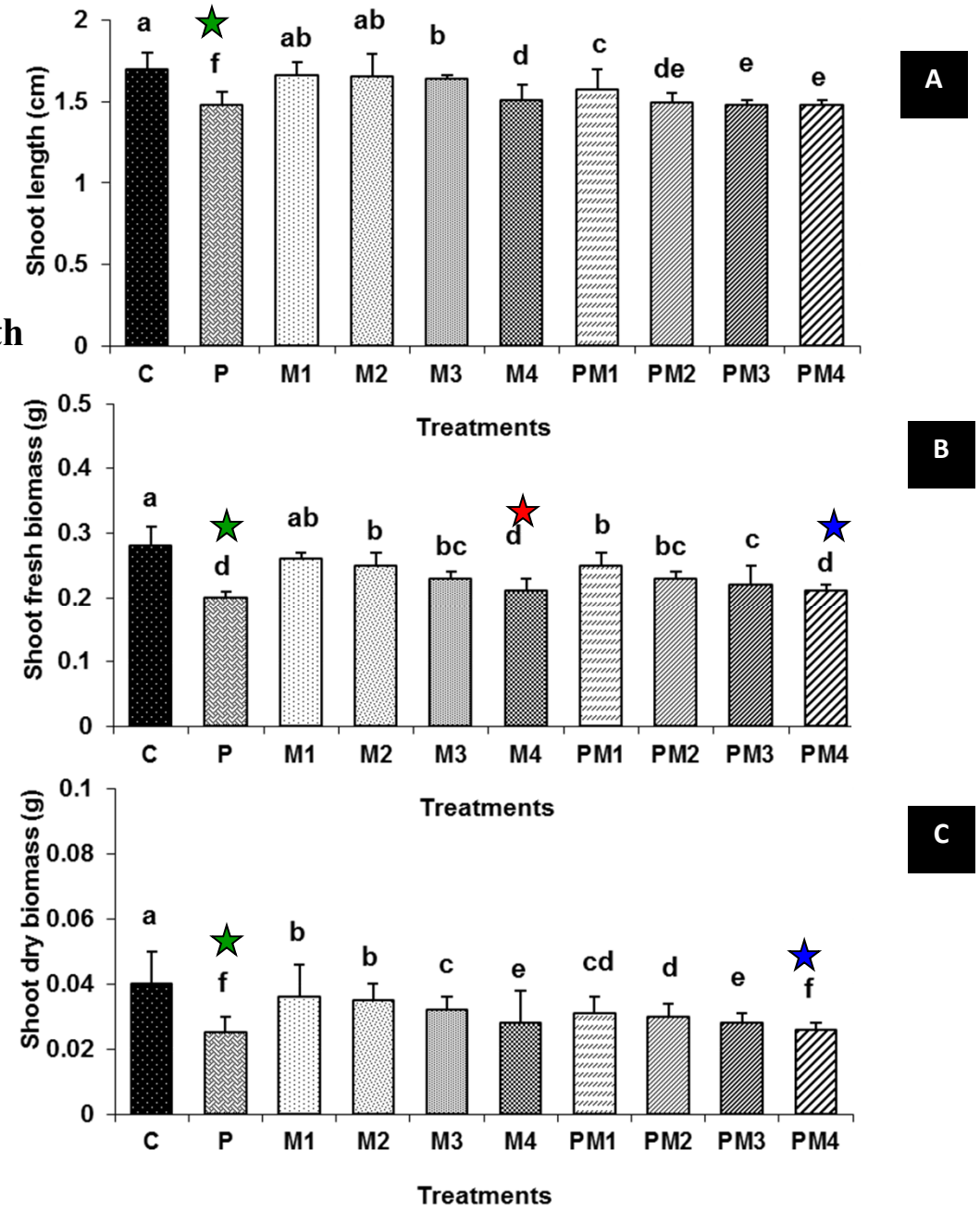


Fig. 2: A-C: Effect of *Sclerotium rolfsii* and Cu(II) on shoot growth of *Psium sativum*.

Reduction in root growth and biomass :

P: 50-60%

M: 15-50%

PM: 15-50%

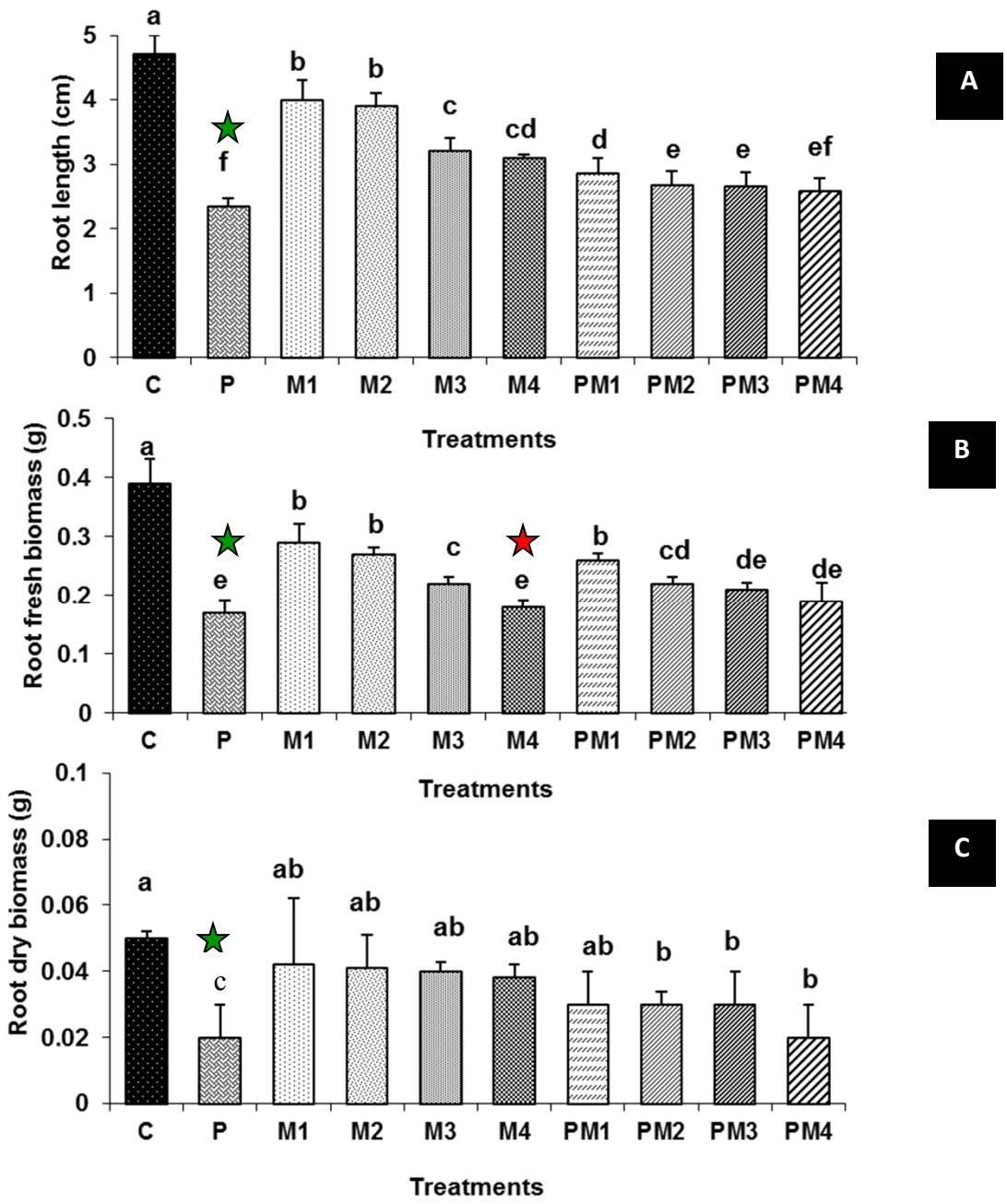


Fig. 3: A-C: Effect of *Sclerotium rolfsii* and Cu(II) on root growth of *Pisum sativum*.

Antifungal activity of Cu

Fungal growth (cm) suppressed by 10%, 18%, 22% and 50% at concentration of 25, 50, 75 and 100 mg/L, respectively

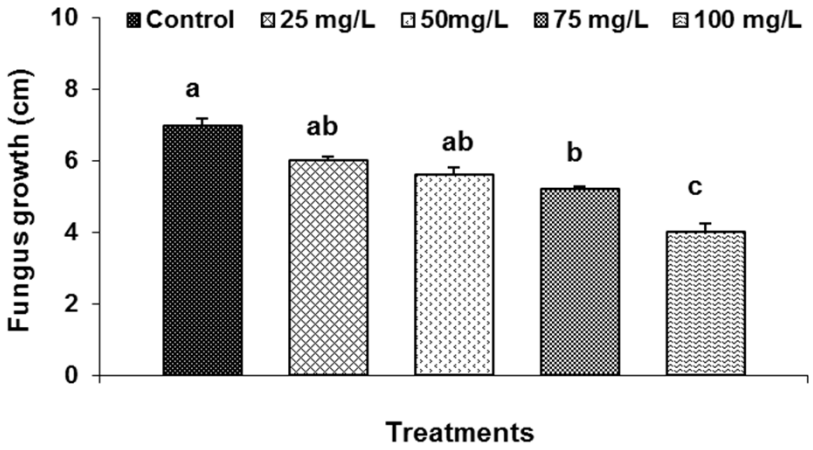
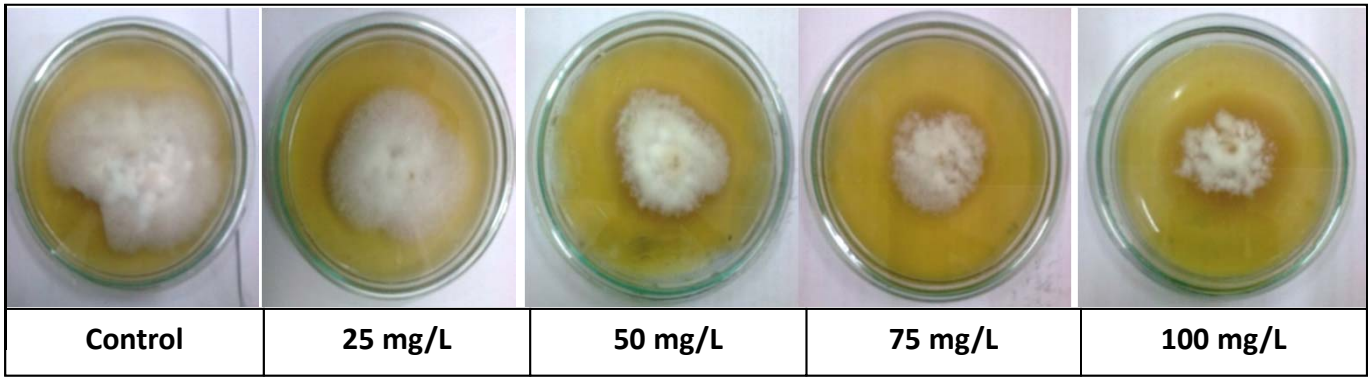
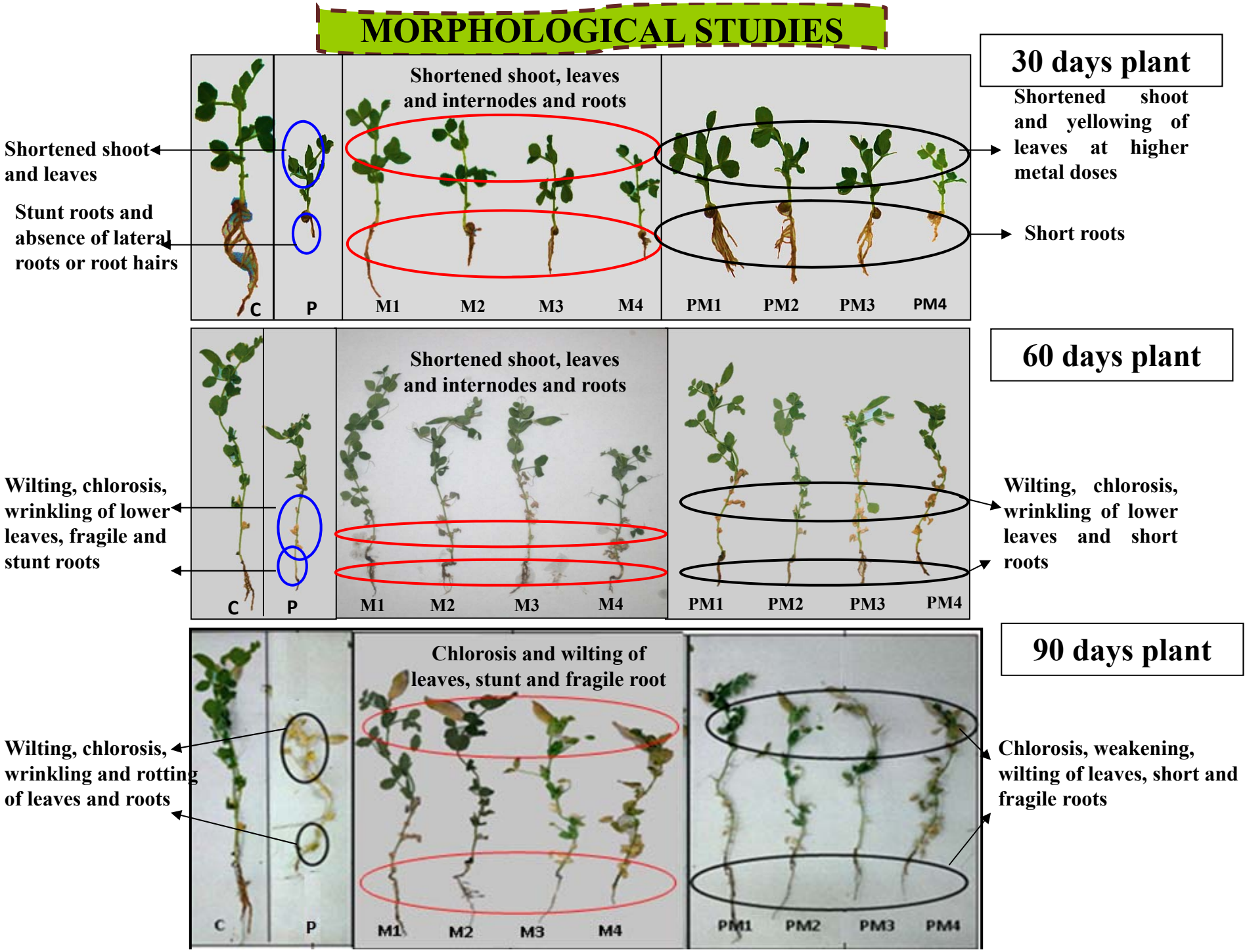


Fig. 4: Effect of different concentrations of Cu(II) on mycelial growth of *Sclerotium rolfsii* after 7th day of inoculation



Influence of different concentration of Cu(II) on growth of *Sclerotium rolfsii* after 7^{th} d of inoculation.

MORPHOLOGICAL STUDIES



Pot Bioassays

	Treatments	Shoot length (cm)	Shoot fresh wt (g)	Shoot dry wt (g)	Root length (cm)	Root fresh Wt (g)	Root dry wt (g)	No of pods/plant	Fresh wt of pods (g)	Dry wt of pods (g)
30 days after sowing	C	18.7±0.4a	1.19±0.02a	0.16±0.01a	7.43±0.3a	0.30±0.03a	0.05±0a	0	0	0
	P	13.8±0.3d	0.92±0.01cd	0.12±0.0ab	6.0± 0.3d	0.19±0.03f	0.03±0b	0	0	0
	M1	15.5±0.2bc	1.08±0.01b	0.14±0 ab	6.78±0.54b	0.24±0.01cd	0.04±0ab	0	0	0
	M2	14.3±0.3cd	1.08±0.01b	0.13±0.01ab	6.75±0.26b	0.23±0.03c-e	0.03±0b	0	0	0
	M3	12.2±0.2ef	0.87±0.01de	0.11±0.02ab	6.38±0.45c	0.22±0.02de	0.03±0b	0	0	0
	M4	11.2±0.3f	0.86±0.02e	0.1±0.0b	6±0.17d	0.2±0.01ef	0.03±0b	0	0	0
	PM1	16.8±0.1b	1.12±0.01b	0.14±0.02ab	6.81± 0.3b	0.29±0.03a	0.03±0b	0	0	0
	PM2	15.5±0.3bc	1.08±0.02b	0.14±0.01ab	6.8± 0.12b	0.28±0.08ab	0.03±0b	0	0	0
	PM3	14.2±0.2cd	0.95±0.01c	0.12±0.01ab	6.5±0.3c	0.26±0.04bc	0.03±0b	0	0	0
	PM4	13.3±0.4de	0.87±0.0de	0.11±0.01ab	6.07±0.41d	0.21±0.03ef	0.03±0b	0	0	0
60 days after sowing	C	28±0.3a	1.237± 0.01a	0.29±0.01a	8.5±0.06a	0.253±0.01a	0.04±0a	2±0.5a	1.3±0.02a	0.45±0.01a
	P	19±0.26e	0.76±0.03d	0.1±0.029c	5.4±0.12e	0.17±0.01e	0.02±0b	2±0.5a	0.66±0.01f	0.23±0.0f
	M1	25±0.39b	1.19±0.07a	0.2±0.02b	7.5±0.06b	0.25±0.0b	0.034±0ab	2±0a	1.12±0.01b	0.39±0.01b
	M2	23±0.35c	0.978±0.05b	0.185±0.01bc	6.65±0.12c	0.221±0.01bc	0.033±0ab	1±0ab	1.07±0.01bc	0.37±0.0bc
	M3	20.6±0.49d	0.893±0.02bc	0.15±0bc	5.9±0.06de	0.21±0.0cd	0.026±0b	1±0ab	0.92±0.02de	0.32±0.0d
	M4	20±0.29de	0.772±0.07d	0.14±0.02bc	5.6±0.15de	0.18±0.01de	0.025±0b	0±0b	0.89±0.01de	0.31±0.01de
	PM1	24±0.09bc	0.984±0.01b	0.19±0.01b	7.51±0.06b	0.251±0.01b	0.035±0.01ab	2±0.5a	1.09±0.02bc	0.38±0.01bc
	PM2	21± 0.25d	0.978±0.05b	0.18±0.01bc	6.7±0.11c	0.223±0.0bc	0.033±0.01ab	2±0.3a	1.04±0.01c	0.36±0.01c
	PM3	20.7± 0.44d	0.91±0.09b	0.15±0.01bc	6±0.12d	0.215±0c	0.033±0.01ab	1±0ab	0.93±0.01d	0.31±0.01de
	PM4	20.5±0.47de	0.81±0.05cd	0.14±0bc	5.9±0.06de	0.214±0.0cd	0.027±0b	1±0ab	0.86±0.01e	0.3±0.0e
90 days after sowing	C	30±0.72a	2.23±0.04a	0.45±0.03a	15.5±0.28a	0.27±0.01a	0.06±0a	5±0.5a	2.0±0.1a	0.7±0.0a
	P	20.9±0.6e	1.4±0.02g	0.23±0.01c	10.8±0.61g	0.19±0.0c	0.02±0b	5±0a	1.14±0.01e	0.4±0.01g
	M1	24.4±0.33cd	1.8±0.12c	0.37±0.04ab	14.51±0.44b	0.25±0.01b	0.04±0ab	4±0.5ab	1.74±0.05b	0.61±0.01b
	M2	23.4±0.32cd	1.78±0.11cd	0.35±0.03a-c	13.35±0.47cd	0.237±0.02bc	0.036±0b	3±0b	1.51±0.01cd	0.53±0.0d
	M3	22.5±0.26de	1.45±0.04g	0.31±0.02bc	13±0.44d	0.215±0.02 bc	0.03±0b	3±0b	1.45±0.05cd	0.51±0.01d
	M4	21±0.18e	1.42±0.05g	0.3±0.02bc	11.1±0.4fg	0.2±0.01 bc	0.025±0b	3±0b	1.4±0.01d	0.49±0.0e
	PM1	26.5±0.32b	2.01±0.05b	0.39±0.02ab	14.23±0.29b	0.221± 0.03 bc	0.034±0b	4.5±0.3ab	1.63±0.00bc	0.57±0.01c
	PM2	25.5±0.36bc	1.65±0.09de	0.34±0.03a-c	14±0.17bc	0.22±0.01 bc	0.034±0.0b	4±0.5ab	1.48±0.01cd	0.52±0.01d
	PM3	24.4±0.45cd	1.62±0.04ef	0.33±0.01a-c	12.6±0.21de	0.21±0.02 bc	0.03±0b	4±0ab	1.37±0.01d	0.48±0.0e
	PM4	23±0.33de	1.51±0.05fg	0.33±0.02a-c	11.8±0.72ef	0.21±0.02 bc	0.03±0c	3.3±0ab	1.31±0.01de	0.46±0.01f

For each harvest separately values with different letters in a column are significantly different according to Tukey's HSD Test ($P \leq 0.05$). ± show standard errors of means of three replicates. Values with different letters show significant difference ($P \leq 0.05$) as determined by

C: Control; P: Seeds inoculated with pathogen; M1, M2, M3 & M4: Seeds treated with 25, 50, 75 and 100 mg/L of Cu(II), respectively; PM1, PM2, PM3 and PM4: Seed incubated under combine stress of pathogen and 25, 50, 75 and 100 mg/L of Cu(II), respectively.

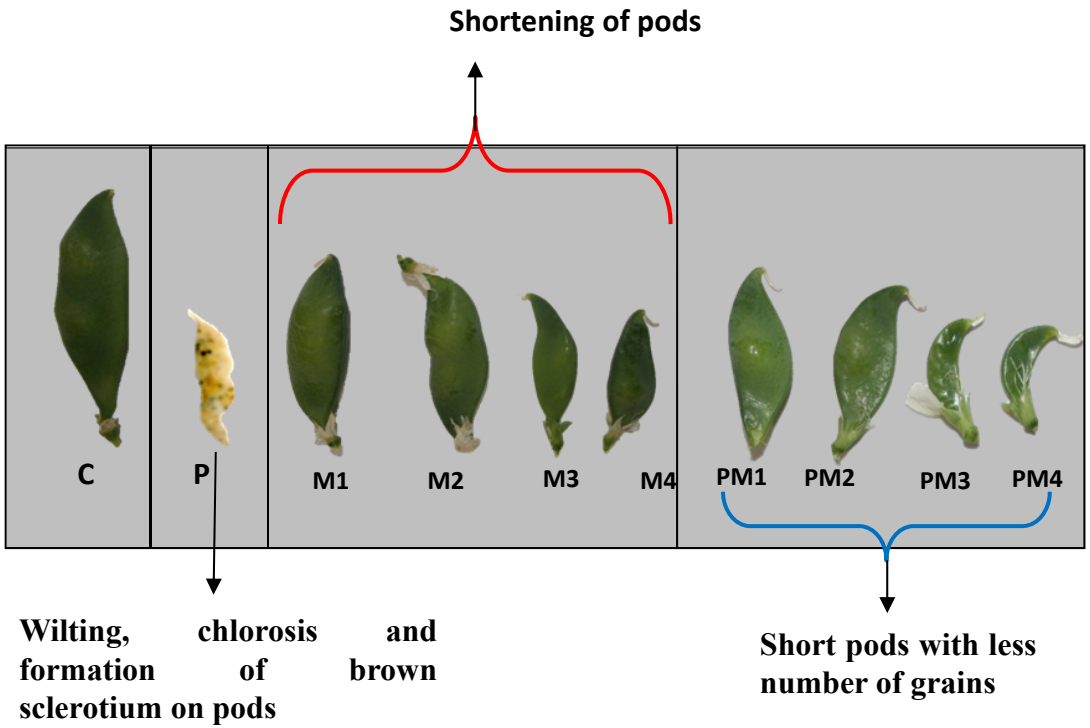
Reduction rate in plant growth and biomass due to different treatments at three growth stages

Treatments	Germination rate	Shoot length cm	Shoot fresh weight gm	Shoot dry weight gm	Root length cm	Root fresh weight gm	Root dry weight gm	Plant Death rate
Pathogen	42%	26-40%	22-65%	25-40%	20-30%	40-50%	50-60%	70%
M1-M4	4-35%	20-40%	5-40%	15-50%	10-25%	20-50%	40-50%	25%
PM1-PM4	20-35%	10-40%	10-40%	10-50%	10-30%	10-50%	10-50%	40%

- P > M ≥ PM
- P > M4 > M3 ≥ PM4 > M2 ≥ PM3 > PM2 ≥ M1 ≥ PM1 > C

Symptoms on plant due to different treatments

1. **Pathogen:** wilting, chlorosis, wrinkling of lower leaves, rotting of stem and roots near the soil line along with abundant white branched mycelium and brown sclerotia
2. **Metal:** chlorosis, shortened internodes and stem, stunt, curled and fragile roots along with absence of lateral roots or root hairs
3. **Metal + Pathogen :** yellowing and wilting of leaves, fragile, short roots and shoots

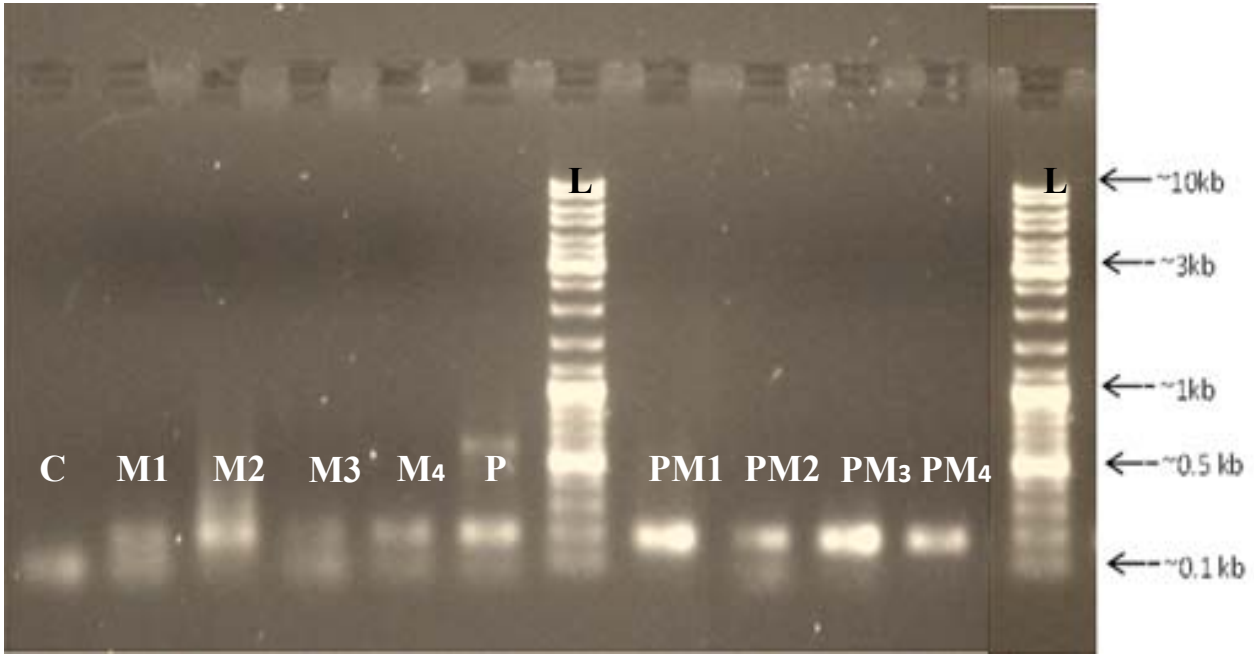


- **Pathogen: 40-50% reduction in fresh and dry weights-Wilting, chlorosis and sclerotium were also observed**
- **Metal alone or combined with Pathogen: 20%, 20-35% and 15-30% reduction in the number of pods, fresh and dry weight, respectively**

- **The pathogenicity of *S. rolfsii* probably be correlated:**
  - **with production of variety of enzymes by the fungus - exhibit inhibitory action on different physiological and metabolic functions of the plant through disturbing level of oxidative enzymes.**
- **Cu(II) stress in plant may lead to:**
  - **oxygen depletion at higher metal concentration**
  - **damaged vascular bundle due to inhibition of enzymes involved in photosynthetic reaction, conduction of water molecules and desired nutrients from roots to aerial parts**
- **Simultaneous occurrence of pathogen and metal may cause drastic effect on plant growth due to following reasons:**
  - **In the presence of Cu, it might be expected that fungus would not either grow or flourish.**
  - **detrimental effect of Cu(II) on plant**
  - **puncturing and penetration of roots outer cell layer by growth of *S. rolfsii* thus providing more absorption sites in roots**
  - **utilization of Cu(II) and rest of soil nutrients by the fungus for its own growth**

Expression due to Def like gene

Expression of Def like gene was more pronounced in the treatments under combined action of *S. rolfsii* + Cu (II) followed by solitary action of *S. rolfsii* and Cu(II), respectively

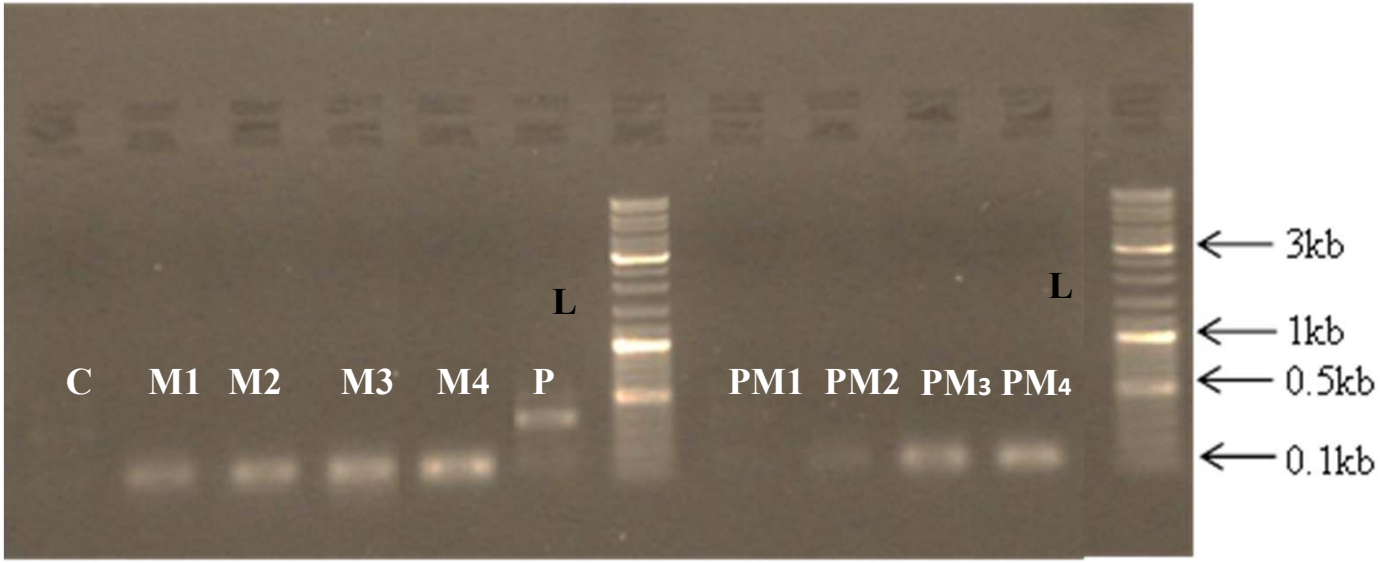


Production of plant defensin gene expression under biotic and abiotic stresses was confirmed by the previous findings:

- **Phytoalexin and lignin biosynthesis are key responses to pathogen attack**
- **Response to pathogen and Cu confirms its role as an abiotic elicitor.**
- **Participate in management of Cu stress, or signal transduction - shared by the stress-response systems.**

Expression due to Metallothionein

Treatments provided with either Cu(II) alone or combined with *S. rolfsii*, showed intense and highly conserved gene expression, whereas MTA gene did not expressed in treatments inoculated with pathogen and control.



Expression of MTA gene in *P. sativum* indicated its role:

- Preventing or reducing cellular injury caused by the generation of ROS in response to Cu stresses
- Keeper of metal homeostasis through chelating, effluxing or sequestering Cu ions

Remaining Cu(II) concentration in the *P. sativum*

Treatments	Cu(II) given to the soil (mg/L)	Cu(II) uptake by plant (mg/L)	
		1st harvest	2nd harvest
C	0	0.69±0.01g	0.71±0.01g
P	0	0.88±0.01g	0.9±0.01g
M1	25	12.5±0.02f	13.8±0.02f
M2	50	21.5±0.03e	23.2±0.01e
M3	75	26.36±0.02c	28.1±0.03c
M4	100	27.83±0.02bc	29.97±0.01b
PM1	25	12.5±0.02f	14.53±0.08f
PM2	50	23±0.02d	24.45±0.03d
PM3	75	28±0.06b	30.15±0.01b
PM4	100	33.81±0.02a	35±0.02a

Data are the mean values of n=3 in a column values with different letters show significant difference (P≤0.05) as determined by Tukey's HSD test.

- ***P. sativum*** **uptake a total of 20-40% at 30 d and 30-50% at 90 d of growth.**
- **The Cu uptake tendency was detected in order of: soil > root > shoots at 30 d whereas the order was of soil > root > pods ≥ shoots at 90 d.**
- **The ranges of Cu content were 0.75-2.1 µg/kg in treatment supplied with Metal alone and 0.65-2.3 µg/kg in treatments under combine stress of Metal and Pathogen.**

Recommended values of 0.005-0.02 µg/kg in plant (DW) (Adriano, 1986)

CONCLUSIONS

- ✓ *The maximum reduction in plant growth, biomass and yield was evidenced in treatments **inoculated with 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 50-100 mg/L. The uptake preference of Cu was found in order of: **soil > roots > shoots > pods.***
- ✓ *A considerable expression of **defensin like (Def) and metallothionein (MTA) genes** in the P. sativum revealed gene roles in handling both biotic and abiotic stresses.*

The study will be helpful in providing important information to agriculturalists particularly to plant pathologists, agronomists, food technologist and environmentalists regarding limitations in crop productivity, affecting food production and yield quality due to detrimental soil borne fungal pathogens and metal pollution.

The study suggests to prevent extensive use of Cu-based pesticides to control plant diseases and Cu-loaded wastewater for irrigation that in turn result in land degradation along with food safety issues.

THANKS