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Full Length Research Paper

Role of riboflavin and thiamine in induced resistance against charcoal rot disease of soybean

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Charcoal rots (*Macrophomina phaseolina* (Tassi) Goidanich) of soybean (*Glycine max* (L.) Merr.) is a disease of economic significance throughout the world. Pathogenicity of 14 isolates of *M. phaseolina* was tested on soybean cv. Giza 21 under greenhouse conditions. The obtained data indicated that all the obtained isolates were able to attack soybean plants and caused charcoal rot on the basal stem with various degrees of diseases severity. *M. phaseolina* isolate S13 caused the highest charcoal rot severity (60%) followed by isolates S11 and S8 (57.9 and 56.3%, respectively). The effect of two inducer chemicals, that is, riboflavin (B2) and thiamine (B1) on the induction of systemic resistance in soybean against charcoal rot disease as well as biochemical changes associated with these treatments in soybean plants was investigated. Under greenhouse condition, the dose effect of 0.1 to 15 mM riboflavin and thiamine showed that 2.5 mM of riboflavin and 5 mM of thiamine was sufficient for maximum induction of resistance; higher concentration did not increase the effect. On the other hand, plants treated with riboflavin and thiamine and inoculated with pathogen grew higher than plants treated with sterilized distilled water (SDW) and inoculated with pathogen and increased fresh and dry weight of soybean plants. 10 mM concentration of riboflavin and thiamin recorded the highest dry and fresh weight compared with the control. In time course observation, it was observed that riboflavin and thiamine treated soybean plants induced resistance one day after treatment and reached its maximum level from 5 to 7 days in the case of riboflavin and 6 to 8 days in the case of thiamine and then decreased. Under field conditions, the percentage of damping-off, root rot and/or charcoal rot severity were significantly reduced due to soaking of the seeds in any of riboflavin and thiamine before sowing compared with the control treatment (seed treated with water) in both seasons (2008 to 2009 and 2009 to 2010). Also, these treatments significantly increased nodule numbers per plant, fresh and dry weight of nodules per plant compared with the control in both seasons. Generally, thiamine gave the best results in most cases under greenhouse and field conditions. In physiological studies, activity of defense-related enzymes, including peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), pathogenesis related (PR) protein (chitinase), were increased in the inoculated and non-inoculated plants treated with the thiamine and riboflavin respectively, compared with the control during the experimental period. In general, activity of these enzymes began to accumulate after two days of treatment and reached maximum levels at 8, 6, 8 and 8 days for PO, PPO, PAL and chitinase, respectively then the activities of these enzymes decreased progressively. On the other hand, total phenols and lignin increased in soybean plants inoculated with *M. phaseolina* and treated with thiamine and riboflavin. The highest accumulation of phenols was recorded 6 days after treatment, while lignin recorded the highest level at the 10th day from application. These results suggested that these chemicals mayplay an important role in controlling the soybean charcoal rot disease, through induction of systemic resistance in soybean plants.

Key words: Soybean, charcoal rot, thiamine, riboflavin, induced resistance.

INTRODUCTION

Soybean (*Glycine max* (L.) Merr) is considered as one of

the main oil crops all over the world. It occupies special

importance in Egypt because it contains 20% oil of dry seed weight and is an important source of protein, which reaches 40% of dry weight seed weight along with calcium iron carotene, thiamine and ascorbic acid (El-Abady et al., 2008). Also, soybean plants like many others legumes are capable of fixing and utilizing atmospheric nitrogen through symbiotic relationship with *Rhizobium* bacteria at the root of the crops. The crop thus improve soil fertility and economizes crop production not only for themselves but also for the next crops grown in rotation especially cereal crops (Nassiuma and Wasike, 2002).

Soybean is subjected to many diseases caused by fungi, bacteria, viruses, mycoplasma and nematodes (Sweets, 2008). *Macrophomina phaseolina* (Tassi) Goidanich is an important soil-borne pathogen that causes major economic loss in soybean seed yield (*G. max* (L.) Merr.) (Mengistu et al., 2007 and El-Barougy et al., 2009). The disease charcoal rot, is most evident during the reproductive phases of plant growth, although the fungus can be isolated from plant roots throughout the growing season. Visible symptoms of the disease in the field are most apparent under conditions that reduce plant vigor, such as poor soil fertility, high seeding rates, low soil water, high temperatures, and root injury (Kendig et al., 2000)

The most successful control strategy used for charcoal root rot in soybean has been seed treatment with fungicides (Hewidy et al., 2003). However, there are a series of problems with applying these products such as high cost, environmental pollution, breaking up the ecological balance of the soil (González, 2006). For these reasons, its use is prohibited in Europe and the United States and will be prohibited in developing countries in 2015 according to the montreal protocol (Karliner, 1997).

Therefore, alternative control methods are needed for managing this pathogen. Several alternative measures are being tested. Natural resources such as biological control (El-Barougy et al., 2009), plant extracts and soil solarization (Dubey et al., 2009) chemical inducers (El-Baz, 2007), resistant cultivars (Mengistu et al., 2007), planting dates (Todd, 1993), crop rotation (Francl et al., 1988) have been found to be good and safe means of disease control especially for the soil-borne diseases.

In recent years, the importance of vitamins as nutrients and as control agent for different diseases has been demonstrated (Dong and Beer, 2000). Ahn et al. (2005) demonstrated that thiamine (vitamin B1) induces SAR and vitamin B1 functions as an activator of plant disease resistance. They described that thiamine treated rice, *Arabidopsis thaliana* and vegetable crops showed resistance to fungal, bacterial and viral infections and that thiamine treatment induced the transient expression of PR-genes in rice and other plants through the SA and Ca²⁺ related signaling pathways.

Riboflavin (vitamin B2) produced by plants and microbes acts as a coenzyme in many physiological reaction in plants, microbes and animals (Gastaldi et al.,

1999). It also participates in antioxidation as well as in peroxidation (Packer et al., 1996). In these processes the production of reactive oxygen intermediates (ROIs) results in an oxidative burst and consequently leads to hypersensitive response (Dong and Beer, 2000). Ryals et al. (1996) and Delaney (1997) presented evidence that riboflavin acts as a novel signaling pathway leading to systemic resistance, activating PR-genes in *A. thaliana* and tobacco and induces resistance to pathogens. Our preliminary studies showed that riboflavin causes induced resistance in chickpea against *Fusarium* wilt and charcoal rot diseases (Saikia et al., 2006).

MATERIALS AND METHODS

Isolation, purification and identification of the pathogen

M. phaseolina was isolated from soybean plants infected with charcoal rot disease collected from different locations of Minia, Assuit and New valley governorates. Infected plant tissue showing charcoal rot characteristics and bearing fungal sclerotia were selected. The tissue was cut into 5 mm long and 2 to 3 mm thick pieces. These pieces were surface sterilized with 1% NaOCl solution for about 2 min followed by thorough washing with sterilized water. These surface sterilized pieces were transferred to potato dextrose agar (PDA) medium in 9 cm diameter Petri plates and incubated at 28±2°C for 5 days. Purification of the isolated fungus was carried out using hyphal tip techniques as described by Dhingra and Sinclair (1985). Isolated fungus was identified according to their morphological characters according to Barnett and Hunter (1986). *M. phaseolina* was isolated and the pure culture was maintained in refrigerator at 4°C.

Preparation of fungal inoculum

The inoculum of *M. phaseolina* was prepared from one week old culture grown on 100 ml potato dextrose (PD) broth medium in flask (500 ml) and incubated at 28±2°C according to Muthomi et al. (2007). The content of the flask were homogenized in a blender for one minute. The resulted cultures were used for soil infestation in a greenhouse experiment for studying the pathogenicity test.

Greenhouse experiments

Pathogenicity tests

The pathogenicity test of *M. phaseolina* (14 isolates) was carried out at New Valley Agricultural Research Station using soybean Giza 21 cultivar in plastic pots (30 cm diameter) containing sterilized sand loam soil. Soybean seeds were surface sterilized by immersing them in 1% sodium hypochlorite solution for 2 min then washed several times with sterilized water. Ten seeds were sown in a pot and maintained in a greenhouse. After 15 days of sowing, pots were inoculated with 100 ml homogenate culture (10³ CFU/ml) prepared before, with inculcation method described by Saikia et al. (2006). Four pots were used for each isolate as a replicates; each pot contained 5 seedlings. The control was inoculated with the same amount of autoclaved PD medium without fungal inoculum.

Disease severity index was determined after 45 days from inoculation with a diseases scale proposed by Mengistu et al. (2007) and diseases severity (%) was calculated using the following formula:

$$\text{Disease severity (\%)} = \frac{\sum(n \times v)}{5 N} \times 100$$

Where, n= number of plants in each category, v= numerical values of symptoms category, and N = total number of numerical values of symptoms categories.

Effectiveness of thiamine and riboflavin for controlling charcoal rot disease under greenhouse condition

15-day-old seedlings (5 seedling per pot) were injected with 50 μ l of thiamine and riboflavin by sterile syringe at the base of the stem with different concentrations (0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 and 15 mM). Three days after treatment, pots were inoculated with homogenate suspension of *M. phaseolina* isolate (10^3 CFU ml⁻¹, 100 ml pot⁻¹). Four pots were used as a replicates. The whole experiment was designed as complete randomized block design (CRBD). Disease severity (%) was examined for the next 45 days by the formula as given by Mengistu et al. (2007) described earlier. Also, fresh and dry weight of survival plants (g plant⁻¹) was recorded at the end of the experiment.

Time course effect of thiamine and riboflavin

Dose effect of thiamine and riboflavin showed that concentrations of 5 and 2.5 mM, respectively was effective, so this concentration was used for this study. 15-day-old soybean seedlings were injected with 50 μ l of thiamine and riboflavin, individually by sterile syringe at the base of the stem; after 0 to 11 days of treatment, soil of the seedlings were inoculated with the homogenate suspension of *M. phaseolina* isolate (10^3 CFU ml⁻¹, 100 ml pot⁻¹). The disease severity (%) was recorded as mentioned earlier.

Biochemical changes associated with thiamine and riboflavin treatment

To observe the accumulation of peroxidase (PO), polyphenol oxidase (PPO), chitinase, phenylalanine ammonia lyase (PAL) and content each of phenolic compounds and lignin, 15-day-old seedlings were injected with thiamine (5 mM) and riboflavin (2.5 mM) or sterilized distilled water (SDW); 100 μ l plant⁻¹. After 2 days from treatment, pots were inoculated with 100 ml of *M. phaseolina* homogenate suspension per pot. The following treatments were made (1) control, treated with SDW only; (2) pathogens control, treated with *M. phaseolina*; (3) plant treated with thiamine and after 2 days inoculated with the pathogen; (4) plant treated with riboflavin and after 2 days inoculated with the pathogen. The peroxidase (PO), polyphenol oxidase (PPO), chitinase, phenylalanine ammonia lyase (PAL) activities and content of each phenolic compounds and lignin were estimated after 0, 2, 4, 6 and 8 days from inoculation.

One gram of plant tissue was homogenized in 10 ml of ice-cold 50 mM potassium phosphate buffer (pH 6.8) containing 1 M NaCl, 1% polyvinylpyrrolidone, (PVP), 1 mM EDTA and 10 mM β -mercaptoethanol (Biles and Martyn, 1993). After filtration through cheesecloth, the homogenates were centrifuged at 8000 rpm at 4°C for 25 min. The supernatants (crude enzyme extract) were stored at -20°C or immediately used for determination of PO, PPO, chitinase and PAL enzymes activities and total protein. In the case of every enzyme in the investigation, each treatment consisted in four replicates (3 plants/ replicate) and two spectrophotometric readings using Milton Roy spectrophotometer (Milton Roy spectronic1201) were taken per replicate. The experiment for bioassays was repeated twice.

Peroxidase activity

The enzyme activity of PO was determined by a direct spectrophotometrically method (Hammerschmidt et al., 1982) using guaiacol as common substrate for peroxidases. The reaction mixture consisted of 0.2 ml crude enzyme extract and 1.40 ml of a solution containing guaiacol, hydrogen peroxide (H₂O₂) and sodium phosphate buffer (0.2 ml 1% guaiacol+0.2 ml 1% H₂O₂+1 ml 10 mM potassium phosphate buffer), was incubated at 25°C for 5 min and the initial rate of increase in absorbance was measured over 1 min at 470 nm using a spectrophotometer. Peroxidase activity was expressed as units of PO/mg protein (Urbanek et al., 1991).

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Polyphenoloxidase activity

The activity of PPO was determined by adding 50 μ l of the crude extract to 3 ml of a solution containing 100 mM potassium phosphate buffer at pH 6.5 and 25 mM pyrocatechol. The increase of absorbance at 410 nm, for 10 min at 30°C, was measured (Gauillard et al., 1993). One PPO unit was expressed as the variation of absorbance at 410 nm per milligram of soluble protein per minute.

Phenylalanine ammonia-lyase activity

Phenylalanine ammonia-lyase (PAL) activity was determined following the direct spectrophotometric method adapted by Cavalcanti et al. (2007). 200 μ l of the crude enzyme extract previously dialyzed overnight with 100 mM Tris- HCl buffer (pH 8.8), were mixed to obtain a solution containing 200 μ l 40 mM phenylalanine, 20 μ l 50 mM β -mercaptoethanol and 480 μ l 100 mM Tris-HCl buffer (pH 8.8). After incubation at 30°C for 1 h, the reaction was stopped by adding 100 μ l 6 N HCl. Absorbance at 290 nm was measured and the amount of trans-cinnamic acid formed was evaluated by comparison with a standard curve (0.1 to 2 mg trans-cinnamic acid/ml) and expressed as units of PAL min⁻¹ mg protein⁻¹.

Chitinase activity

The chitinase activity was determined using the method described by Wirth and Wolf (1992). High polymeric carbomethyl-substituted chitin labeled covalently to Remazol brilliant violet 5R (CM-Chitin*-RBV. Comp. Loewe Biochemica) was used as the substrate. The reaction mixture was as follows: 0.50 ml 0.01 M Na-acetate buffer (pH 5.2) with 5% (v/v) glycerin, 0.25 ml plant extract and 0.25 ml dye labeled substrate CM*-RBV solution (2 mg/ml). Test samples were incubated in a water bath at 37°C for 120 min. The enzyme reaction was terminated by adding 0.25 ml 2 N HCl. After centrifugation (8000 rpm; 25 min), supernatants containing soluble, dye labeled degradation products were transferred to the cuvet. Absorbance was measured spectrophotometrically at 550 nm, sodium acetate buffer was added to the blanks instead of the plant extract. Enzyme activity was expressed as enzyme unit/mg protein.

Protein concentration

Total protein content of the samples was quantified according to the method described by Bradford (1976).

Determination of phenolic compounds

To assess phenolic content, 1 g fresh plant sample was homogenized in 10 ml 80% methanol and agitated for 15 min at 70°C. One ml of the extract was added to 5 ml of distilled water and 250 μ l of 1 N Folin-Ciocalteu reagent and the solution was kept at

25°C. The absorbance was measured with a spectrophotometer at 725 nm. Catechol was used as a standard. The amount of phenolic content was expressed as phenol equivalents in mg g⁻¹ fresh tissue (Saikia et al., 2006).

Determination of lignin

One gram plant tissue from each treatment was mixed with 10 g of trichloroacetic acid (TCA) and incubated at 90°C. Delignification was stopped by cooling the reaction mixture after 240 min of reaction time. The reaction vessel was immersed in cold water and 5 ml of cold acetone were added. Suspension was filtered and liquor was evaporated until dark; high consistency liquid without smell of acetone was obtained. Lignin was precipitated by pouring the liquid in 200 ml cold water. Lignin was filtered and washed with warm water several times. After that, lignin was air-dried overnight at 4°C then weighed (Liken and Perdith, 1999).

Field experiments

The experiment was carried out in the Experimental Farm of New Valley Agriculture Research Station during two successive growing summer seasons; 2009 and 2010, for the control of damping-off and charcoal rot diseases of soybean in naturally infested field by seed soaking in thiamine and riboflavin at concentration 5 and 2.5 mM, respectively for 20 min. In the control treatment, seeds were soaked in water for the same amount of time. The tested treatments were distributed in a complete randomized block design with four replicates, the experimental plot area was 10.5 m² (3×3.5 m) containing five rows; each row was 3.5 m in length and distance between rows was 60 cm. All treatments were sown in hills 20 cm apart on both sides of row ridge and two seeds per hill (plant population = 140,000 plants/fed). All recommended agricultural practices were adopted throughout the two seasons.

Percentage of damping-off was calculated 30 days after planting. Charcoal rot severity was also recorded on a random sample of plants of the plots (20 plants) three months after planting according to Mengistu et al. (2007) as described earlier. At 60 days after cultivation, plants were uprooted (20 plant plot⁻¹) to estimate number, fresh and dry weight of nodules plant⁻¹.

At harvest time, a sample of 20 randomly plants from each experimental unit was taken and the following characteristics were recorded; plant height, number of branches and pods per plant and 100-seed weight. Seed yield (kg fed⁻¹) was determined from the rows of each experimental unit. Seed oil content (%) was estimated by taking seed samples of dried seeds of each plot which were cleaned and ground into very fine powder by grinder to determine seed oil percentage as described by A.O.A.C. (1990) using Soxhelt apparatus and petroleum ether as an organic solvent. The percentage of oil in seeds was calculated by the following formula:

$$\text{Seed oil (\%)} = (A-B)/A \times 100$$

Where, A is the weight of samples before extraction; B is the weight of seed samples after extraction.

Also, seed crude protein percentage was estimated according to A.O.A.C. (1990).

Statistical analysis

All the experiments were performed twice. Analyses of variance were carried out using MSTAT-C program version 2.10 (1991). Least significant difference (LSD) was employed to test for significant difference between treatments at $P \leq 0.05$ (Gomez and Gomez, 1984).

RESULTS

Isolation trails from rotted plants collected from different localities of Minia, Assuit and New Valley governorates yielded a fungus which was identified as *M. phaseolina*.

Results illustrated in Table 1 show that all the obtained isolates (14 isolates) were able to attacked soybean plants and caused charcoal rot on the basal stem with various degrees of diseases severity. *M. phaseolina* isolate S13 caused the highest charcoal rot severity (60%) followed by isolates S11 and S8 (57.9 and 56.3%, respectively) while the other isolates except S4 and S12 caused moderate infection of soybean plants (31.6 to 45.7%). Isolates S4 and S12 were the ones that caused the least amount of infection (8.5 and 12.5%, respectively).

Effectiveness of riboflavin and thiamine for controlling charcoal rot disease under greenhouse conditions

A mark reduction in infection with *M. phaseolina* was observed in riboflavin and thiamine treated soybean plants, while SDW treated plants (control) exhibited heavy infection (Table 1). The disease severity in the treated plants with riboflavin and thiamine was significantly lower than those plants treated with SDW. The dose effect of riboflavin and thiamine showed that the concentration of 2.5 and 5 mM, respectively were most effective and sufficient for induction of resistance to charcoal rot disease (64.1 and 79.0% protection). Concentrations higher than 2.5 and 5 mM in the case of riboflavin and thiamine respectively did not show an increase in resistance. However, less than 2.5 and 5 mM concentration was less effective

On the other hand, plants treated with riboflavin and thiamine and inoculated with pathogen grew higher than plants treated with SDW and inoculated with pathogen, with increased fresh and dry weight of soybean plants. The fresh and dry weight of the treated plants increased along with increasing thiamine and riboflavin concentrations until 10 mM and then dropped a little at higher concentrations. Soybean plants treated with riboflavin and thiamine at 10 mM gave the highest fresh and dry weight; 2.446 and 2.611 g plant⁻¹ fresh weight and 0.729 and 0.750 g plant⁻¹ dry weight, respectively compared with the 0.510 g plant⁻¹ fresh weight and 0.156 g dry weight in SDW treatment (control).

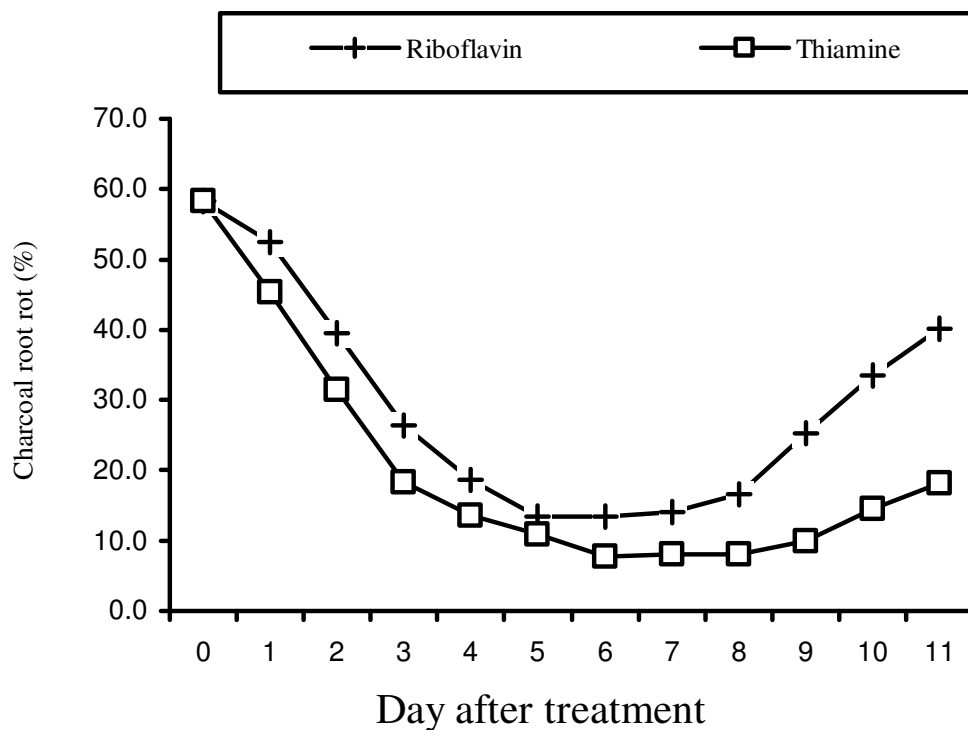
Generally, thiamine gave the best protection against infection with *M. phaseolina* and increased fresh and dry weight of soybean plants than riboflavin at all the concentrations.

Time course effects of riboflavin and thiamine on charcoal rot disease

The time course resistance in riboflavin and thiamine

Table 1. Effect of soybean seedling treatment with different concentrations of inducers resistance riboflavin and thiamine on damping-off and charcoal rot diseases, fresh and dry weights per plant under artificial infection with *M. phaseolina* under greenhouse conditions.

Treatment	Conc. (mM)	% Charcoal rot	% Protection	Fresh weight (g plant ⁻¹)	Dry weight (g plant ⁻¹)
Riboflavin	0.1	55.3	12.5	1.125	0.328
	0.25	47.0	25.6	1.561	0.492
	0.5	35.0	44.6	1.735	0.543
	1.0	28.9	54.3	1.998	0.594
	2.5	22.7	64.1	2.343	0.705
	5	22.7	64.1	2.370	0.713
	10	22.7	64.1	2.446	0.729
	15	25.3	60.0	2.329	0.689
	Thiamine	0.1	50.2	20.6	1.562
0.25		41.9	33.7	1.786	0.538
0.5		35.7	43.5	1.780	0.542
1.0		25.3	60.0	2.182	0.664
2.5		18.4	70.9	2.440	0.742
5		13.3	79.0	2.561	0.726
10		13.3	79.0	2.611	0.750
15		14.5	77.1	2.532	0.730
Control		63.2	-	0.510	0.156
LSD at 0.05		5.07	-	0.316	0.052

**Figure 1.** Time course effect of riboflavin (2.5 mM) and thiamine (5 mM) on *M. phaseolina* infection.

treated soybean plants became apparent after one day from application and reached maximum level at 5 to 7 days after treatment with riboflavin and 6 to 8 days after

treatment with thiamine (Figure 1). Charcoal rot resistance declined quickly after the 8th day in the case of treatment with riboflavin and in the case of thiamine

Table 2. Effect of seed soaking in riboflavin (2.5 mM) and thiamine (5 mM) on damping-off and charcoal rot diseases, number of nodules, fresh and dry weight of nodules per plant during summer seasons (2009 and 2010) under field conditions.

Treatments	% Damping-off ⁽¹⁾	% Root rot/charcoal rot ⁽²⁾	% Survival plants	Number of nodules per plant	Fresh weight of nodule (mg/plant ⁻¹)	Dry weight of nodule (mg plant ⁻¹)
Summer season 2009						
Riboflavin	6.45	9.25	84.30	17.23	235.50	69.26
Thiamine	3.23	5.25	91.52	14.05	248.57	73.01
Control	14.33	26.59	59.08	11.18	168.47	51.76
LSD at 0.05	1.72	2.95	7.85	2.48	12.14	6.19
Summer season 2010						
Riboflavin	6.96	12.74	80.3	15.01	228.32	65.60
Thiamine	4.12	6.05	89.83	12.93	237.82	68.44
Control	16.41	28.24	55.35	10.48	160.70	48.96
LSD at 0.05	0.86	1.48	8.27	1.98	11.42	5.37

¹Damping-off were recorded after 30 days from planting as follows: Damping-off (%) = (Pre-emergence+ post emergence / No. of planted seeds) x100; ²charcoal rot was recorded according to Mengistu et al. (2007) based on 0 to 5 scale according to percentage of foliage yellowing or necrosis (0 = 0%, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, 4 = up to 76%, 5 = completely dead plants).

Table 3. Plant height, number of branches, pods per plant, weight of 100 seeds, seed yield, protein and oil percentages as affected by soybean seed soaking in riboflavin (2.5 mM) and thiamine (5 mM) during summer season of 2009 and 2010 under field conditions.

Treatment	Plant height (cm)	Number of branches per plant	Number of pods per plant	Weight of 100 seeds	Seed yield (Kg fed ⁻¹)	% Protein in seed	% Oil in seed
Summer season 2009							
Riboflavin	69.54	3.14	73.14	15.80	1485.54	37.48	19.65
Thiamine	74.25	3.24	79.47	16.27	1589.11	39.99	20.84
Control	57.23	2.72	58.14	13.10	1035.07	34.29	17.59
LSD at 0.05	5.87	0.07	6.04	1.77	63.21	2.46	1.23
Summer season 2010							
Riboflavin	65.99	3.04	68.54	15.59	1401.80	37.00	19.04
Thiamine	70.45	3.14	77.12	15.97	1515.09	39.16	19.37
Control	53.36	2.59	52.71	12.95	998.17	33.64	16.51
LSD at 0.05	4.44	0.10	5.93	1.53	60.83	2.42	1.17

treatment, it was reduced after the 9th day. The reduction of resistance to charcoal rot disease was higher in the case of riboflavin than in thiamine.

Field experiments

Effect of riboflavin and thiamine on damping-off and charcoal rot diseases and nodulation of soybean under field condition

The effect of riboflavin (2.5 mM) and thiamine (5 mM) on incidence of damping-off and charcoal rot diseases and nodulation of soybean under field is shown in Table 2.

The percentage of damping-off, root rot and/or charcoal rot severity were significantly reduced due to soaking of the seeds in any of riboflavin and thiamine before sowing compared with the control treatment (seed treated with water) in both seasons. In this regard, thiamine was the highest effective in the reduction of damping-off and charcoal rot severity than riboflavin treatment; thiamine recorded 3.23, 4.12% damping-off and 5.25, 6.05% charcoal rot and riboflavin had 6.45, 6.96% damping-off and 9.25, 12.74% charcoal rot compared with 14.33, 16.41% damping-off and 26.59, 28.24% charcoal rot in the control in both seasons, respectively.

Also, the data present in Table 3 summarized that soybean seeds soaked in thiamine and riboflavin resulted

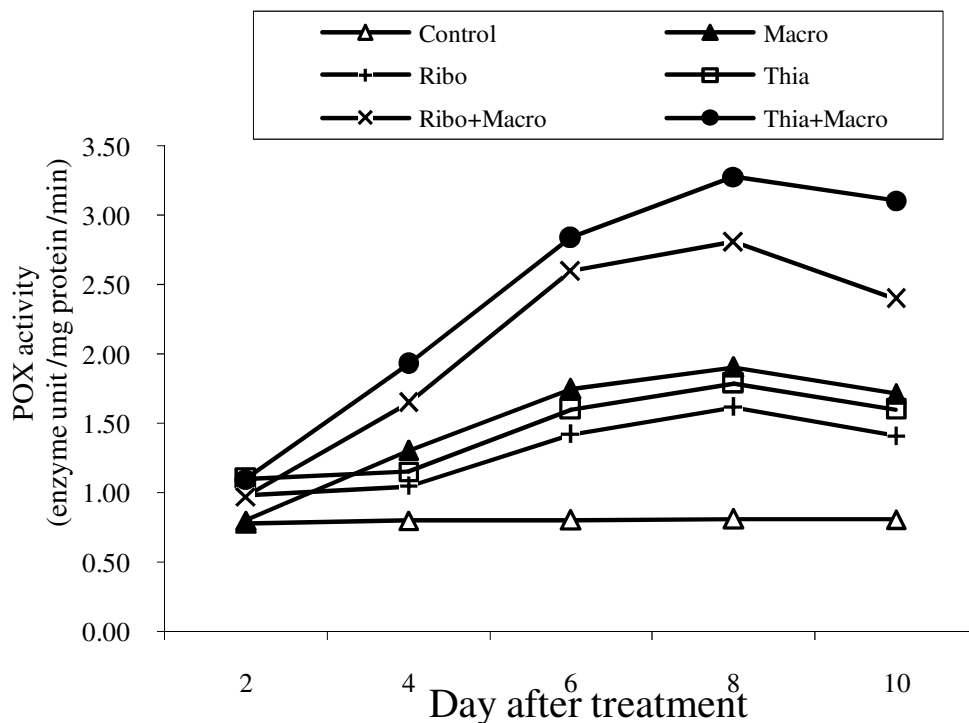


Figure 2. Effect of riboflavin (2.5 mM) and thiamine (5 mM) on activity of peroxidase (POX) in inoculated and non-inoculated soybean plants. The samples were collected from both inoculated and non-inoculated plants after 2, 4, 6, 8 and 10 days after treatment.

in significant increase of nodule numbers per plant, and fresh and dry weight of nodules per plant compared with the control in both seasons. Riboflavin treatment increased nodule numbers per plant (17.23 and 15.01 nodule plant⁻¹) than thiamine treatment (14.05 and 12.93 nodule plant⁻¹); in contrary thiamine treatment increased the fresh and dry weights of nodules per plant than the riboflavin treatment in both seasons.

Effect of riboflavin and thiamine on vegetative growth and yield parameters of soybean under field conditions

The data present in Table 3 demonstrated that both treatments significantly improved plant height and increased number of branches and pods per plant, weight of 100 seeds, total seed yield, dry seed contain of protein and oil compared with the control treatment during the two experimental seasons (2009 and 2010). In this respect, soaking seeds in thiamine increased all the studied characters than those treated with riboflavin in both seasons; plant height was from 57.23 and 53.36 in the control treatment to 74.25 and 70.45 cm in both seasons, respectively, number of branches per plant increased from 2.72 and 2.59 to 3.24 and 3.14 and increased pods number per plant from 58.14 and 52.71 to 79.47 and 77.12 in both seasons.

Also, thiamine treatment increased each of the 100-seed weight compared with the control treatment (from 13.10 and 12.95 to 16.27 and 15.97 g), increased total seed yield (from 1035.07 and 998.17 to 1589.11 and 1515.09 kg fed⁻¹) and increased percentage of protein in dry seeds from 34.29 and 33.64% in the control treatment to 39.99 and 39.16% and oil contain in dry seeds from 17.59 and 16.51 to 20.84 and 19.37% in both seasons, respectively.

Biochemical changes associated with riboflavin and thiamine treatments

Accumulation of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) enzymes and pathogenesis related (PR) protein (chitinase), phenolic compounds and lignin in plants inoculated with *M. phaseolina* or non-inoculated plants treated and untreated with riboflavin and thiamine were studied.

Peroxidase activity

The data in Figure 2 showed that PO activity of the inoculated and non-inoculated soybean plants treated with riboflavin and thiamine was higher than that of the untreated plants after all time from application. Inoculated

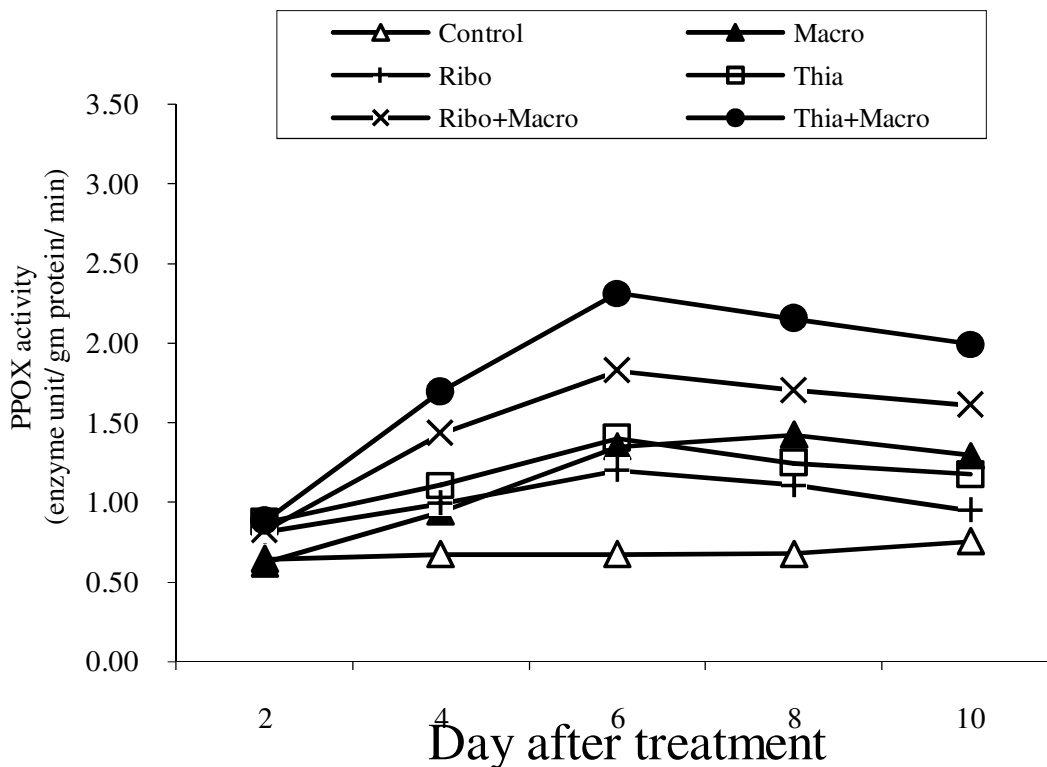


Figure 3. Effect of riboflavin (2.5 mM) and thiamine (5 mM) on activity of polyphenol oxidase (PPOX) in inoculated and non-inoculated soybean plants. The samples were collected from both inoculated and non-inoculated plants after 2, 4, 6, 8 and 10 days after treatment.

plants caused highly PO activity than the non-inoculated plants whether treated and untreated especially after 4 days from treatment with thiamine and riboflavin. Thiamine treatment recorded high enzyme activity than riboflavin. The highest levels of PO were determined 8 days after treatment in all cases. The highest of PO activity was recorded in soybean plants inoculated with the pathogen and treated with thiamine at the 8th day from application ($3.280 \text{ enzyme unit mg}^{-1} \text{ protein min}^{-1}$). In general, the enzyme activity rapidly increase until 8 days after application then decreased progressively.

Polyphenol oxidase activity

In general, a significant increase in the activity of PPO was observed in both non-inoculated and inoculated soybean plants following treatment with riboflavin and thiamine more than the control treatment (Figure 3). PPO accumulated more markedly in plants treated with thiamine than riboflavin especially in inoculated plants. Data also showed that PPO activity increase as days after application of treatment increased until the 6th day then decreased progressively. Maximum levels of PPO were recorded at the 6th and 8th days from application of treatments, respectively in all cases. Soybean plants

treated with thiamine and inoculated with the pathogen recorded the highest level of PPO activity at the 6th day from application ($2.312 \text{ enzyme unit mg}^{-1} \text{ protein min}^{-1}$) followed by the 8th of application of the same treatment ($2.151 \text{ enzyme unit mg}^{-1} \text{ protein min}^{-1}$). On the other hand, PPO activity in the inoculated plants increased markedly than the non-inoculated plants in all tested periods.

Phenylalanine ammonia lyase activity

The data in Figure 4 show that the levels of PAL activity in the inoculated plants were highly increased than in the non-inoculated control plants until the 6th day from inoculation and then decreased approximately equal in activity in the 8th day from inoculation. On the other hand, the data showed that PAL activity was highly significantly increased in the inoculated plants and treated with thiamine or riboflavin than the inoculated plants only. Also, the activity of PAL increased by increasing time after application until the 8th day from application then the activity decreased. The higher activities of PAL were determined in the inoculated soybean plants on the 8th day from treatment with thiamine and riboflavin (2.877 and $2.651 \text{ enzyme unit mg}^{-1} \text{ protein min}^{-1}$, respectively).

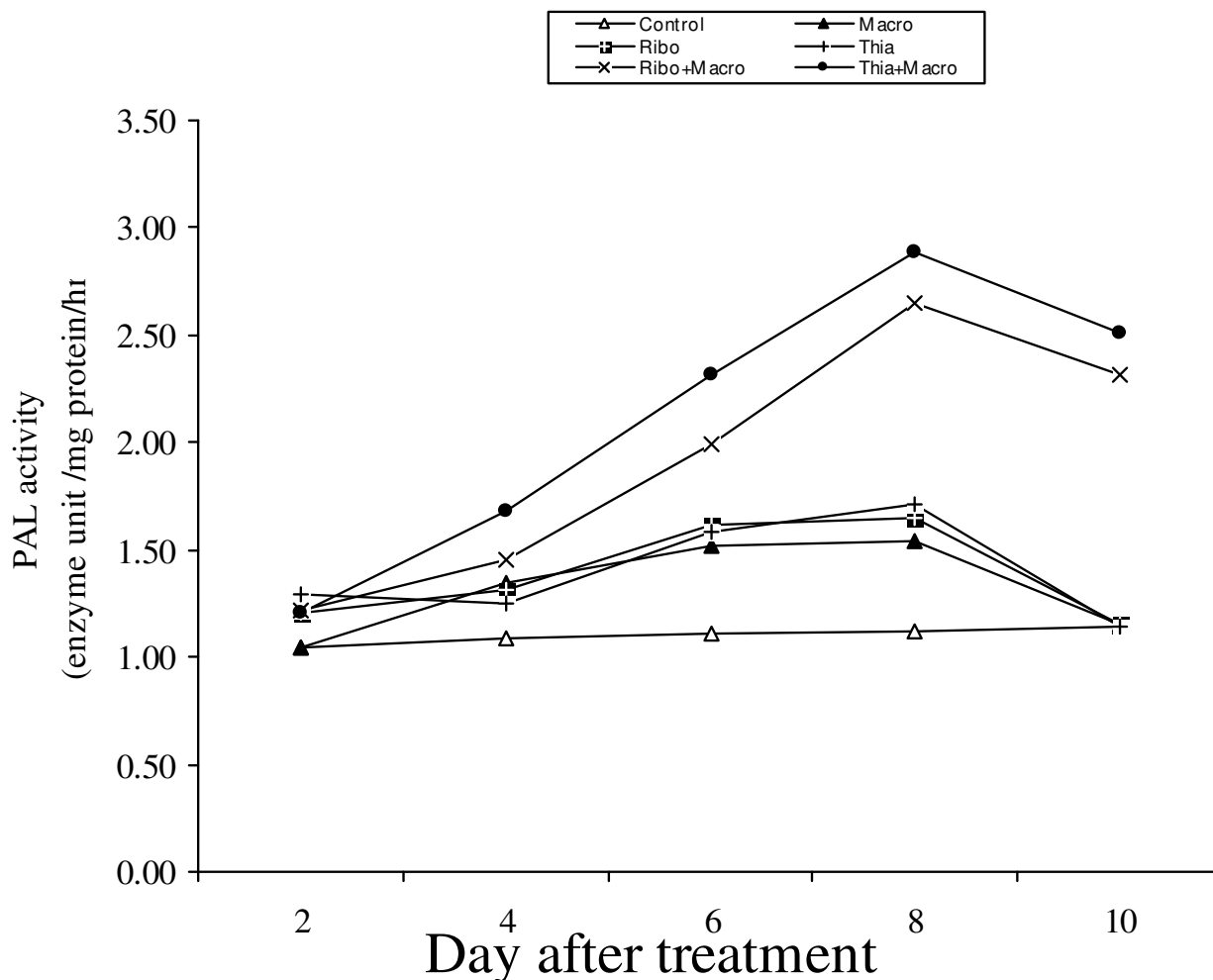


Figure 4. Effect of riboflavin (2.5 mM) and thiamine (5 mM) on activity of phenylalanine ammonia lyase (PAL) in inoculated and non-inoculated soybean plants. The samples were collected from both inoculated and non-inoculated plants after 2, 4, 6, 8 and 10 days after treatment.

Chitinase activity

The non-inoculated soybean plants data presented in Figure 5 indicated that plants treated with thiamine and riboflavin exhibited enzyme activity higher than that of the untreated control after all the tested periods of enzyme determination especially at 8 days after application. The inoculated plants data showed that the treated plants caused significant increase of chitinase activity than that of the untreated plants after all the tested periods of determination. In general, soybean plants treated with riboflavin caused higher enzyme activity than thiamine. The enzyme activity was increased at 2, 4, 6 and 8 days after application and then decreased at 10 days from application. Maximum enzyme activity was recorded after 8 days from the treatment with riboflavin (7.442 enzyme unit mg^{-1} protein min^{-1}) in the inoculation plants followed by riboflavin treatment in the inoculated plant at the 10th day from application (7.002

enzyme unit mg^{-1} protein min^{-1}).

Total phenol content

The total phenols were measured in the inoculated and non-inoculated soybean plants treated and untreated with thiamine and riboflavin (Figure 6). It was obvious that riboflavin and thiamine did not show significant role to accumulation of phenolic compounds in the non-inoculated plants. However, thiamine or riboflavin pre-treated soybean plants challenge inoculated with the pathogen showed rapid increase in the accumulation of phenol compounds. The accumulation of phenols in the inoculated plants was highly increased than the non-inoculated plants during all the tested determination periods. On the other hand, the phenols content were exhibited at the 6th day from application in the inoculated and treated plants with both compounds then decreased

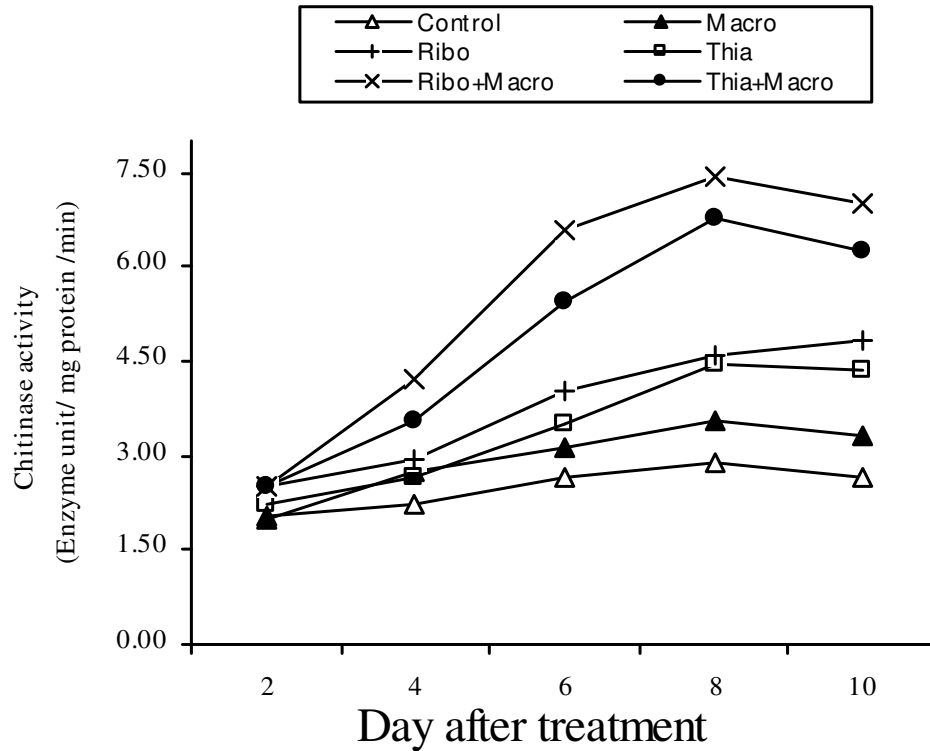


Figure 5. Effect of riboflavin (2.5 mM) and thiamine (5 mM) on activity of chitinase in inoculated and non-inoculated soybean plants. The samples were collected from both inoculated and non-inoculated plants after 2, 4, 6, 8 and 10 days after treatment.

progressively thereafter.

Lignin content

Data in Figure 7 indicate that lignin content was increased in soybean plants treated with thiamine and riboflavin. Plants inoculated with the pathogen contained high level of lignin than non-inoculated control plants. The accumulation of lignin increased with increasing determination periods after the treatment application and/or inoculation with the pathogen. Plant treated with thiamine caused increase in the lignin content than plants treated with riboflavin in inoculated or non-inoculated plants. Also, riboflavin and thiamine treatments increased the lignin content in non-inoculated plants than the inoculated untreated plants. The highest lignin content was recorded by thiamine treatment in inoculated plant after 10 days (0.257 mg/g dry weight) from application followed by riboflavin treatment (0.239 mg/g dry weight) in the same period of treatment application.

DISCUSSION

Soybean (*G. max* (L.) Merr) is one of the most important legumes plants. Soil borne diseases including root rot,

wilt and charcoal rot cause important considerable losses in yield. Charcoal rot is a widespread root and stem disease of soybean caused by the soil-inhabiting fungus *M. phaseolina*. The disease is more damaging in years with extended periods of hot. Dry weather isolation trails from rotted soybean plants yielded 14 isolates of *M. phaseolina* conforming to other reports (Wrather et al., 2001; Mengistu et al., 2007; El-Barougy et al., 2009). Pathogenicity test demonstrated that all the obtained isolates able to infection of soybean plants caused typical charcoal rot symptoms with different percentage of disease severity (Figure 8).

The use of fungicides to control soil borne diseases of economically important crops has been used in agriculture for many years. However, recently the use of chemical has been reduced for several reasons, including pollution of environment, particularly ground water and food supplies. Recently, an increasing desire to reduce the use of fungicides is seen through the attempts to develop integrated pest managements approaches, where natural resources are put to maximum use. Chemically induced resistance (IR) is a suitable strategy to utilize natural defenses of the plant to control pathogens. This phenomenon has been studied at the molecular level and has proved to be mediated by salicylic acid and associated with a number of defense responses and genes (Ton et al., 2005). Induced

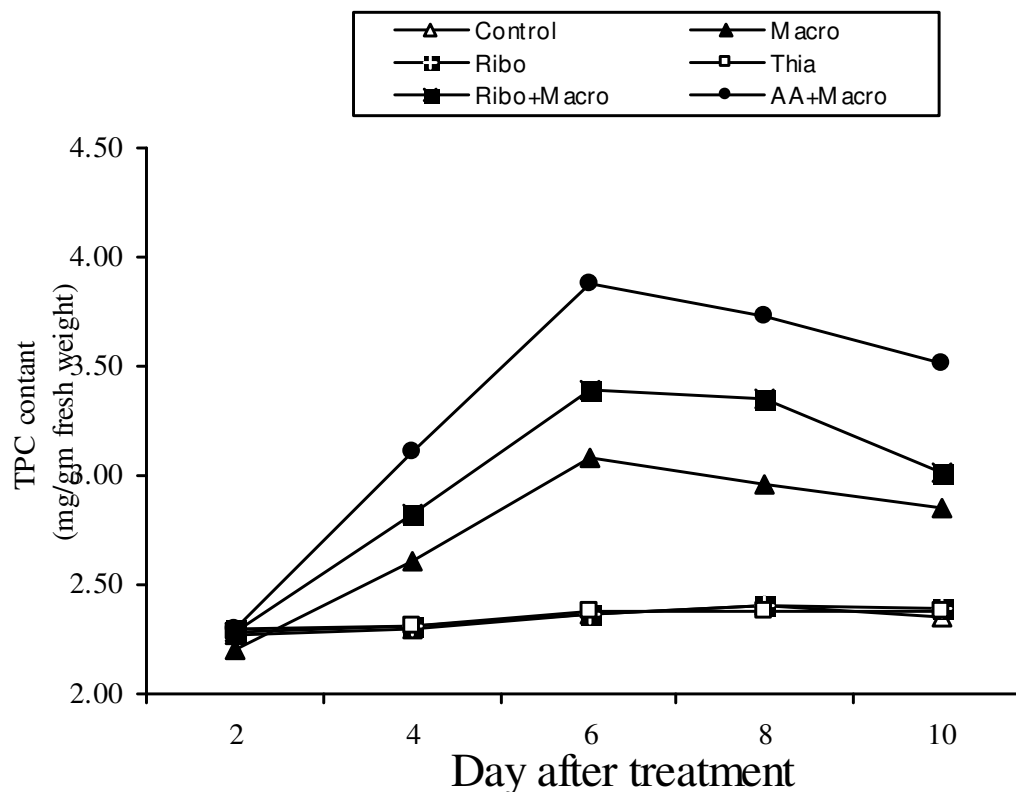


Figure 6. Effect of riboflavin (2.5 mM) and thiamine (5 mM) on total phenol content (TPC) in inoculated and non-inoculated soybean plants. The samples were collected from both inoculated and non-inoculated plants after 2, 4, 6, 8 and 10 days after treatment.

resistance was reported to be activated by exogenous application of thiamine and riboflavin (Ahn et al., 2005; Dong and Beer, 2000; Saikia et al., (2006). Thiamine and riboflavin are promoted as a safe, reliable and non phytotoxic plant protection agent. It was recently identified by scientists as a novel disease- control compound. Application of thiamine and riboflavin to a variety of plants before challenge with the pathogens triggered a set of plant defense reactions that resulted in the creation of a fungitoxic environment, which protect them by different (physical and/or chemical means) mechanisms (Sierra and Vidal-Valverde, 1999).

The obtained results in this work indicated that both thiamine and riboflavin reduced DSI caused by artificial infection with *M. phaseolina* and increased fresh and dry weights of the resulted plants. The efficiency of riboflavin and thiamine was varied with various concentrations. The dose effect of 0.1 to 15 mM riboflavin and thiamine showed that 2.5 and 5.0 mM concentration was sufficient for maximum induction of resistance, in the case of riboflavin and thiamine, respectively; higher concentration did not increase the effect. In time course observation, it was observed that riboflavin and thiamine treated soybean plants induced resistance one day after treatment and reached its maximum level from 5 to 7 days after treatment with riboflavin and 6 to 8 days after treatment

with thiamine.

Similar results were reported by Saikia et al. (2006) who found that riboflavin at 1.0 mM caused induction of systemic resistance in chickpea against *Fusarium* wilt and charcoal rot diseases. At this concentration, riboflavin neither caused cell death of the host plant nor directly affected the pathogen's growth. In time course observation, it was observed that riboflavin treated chickpea plants induced resistance 2 days after treatment and reached its maximum level from 5 to 7 days and then decreased. Also, some studies observed that foliar application of riboflavin effectively controlled several diseases of tobacco (Dong and Beer, 2000).

Ahn et al. (2005) demonstrated that thiamine induces SAR and vitamin B1 functions as an activator of plant disease resistance. They described that thiamine treated rice, *A. thaliana* and vegetable crops showed resistance to fungal, bacterial and viral infections.

Under field condition, thiamine (2.5 mM and 5 mM thiamine) significantly reduced damping-off, root and/or charcoal rot severity and increased nodule numbers, fresh and dry weights of nodules per plant compared with the untreated plants. Also, these treatments increased vegetative growth and yield parameters; improved plant height and increased number of branches and pods per plant, weight of 100 seeds, total seed yield, dry seed

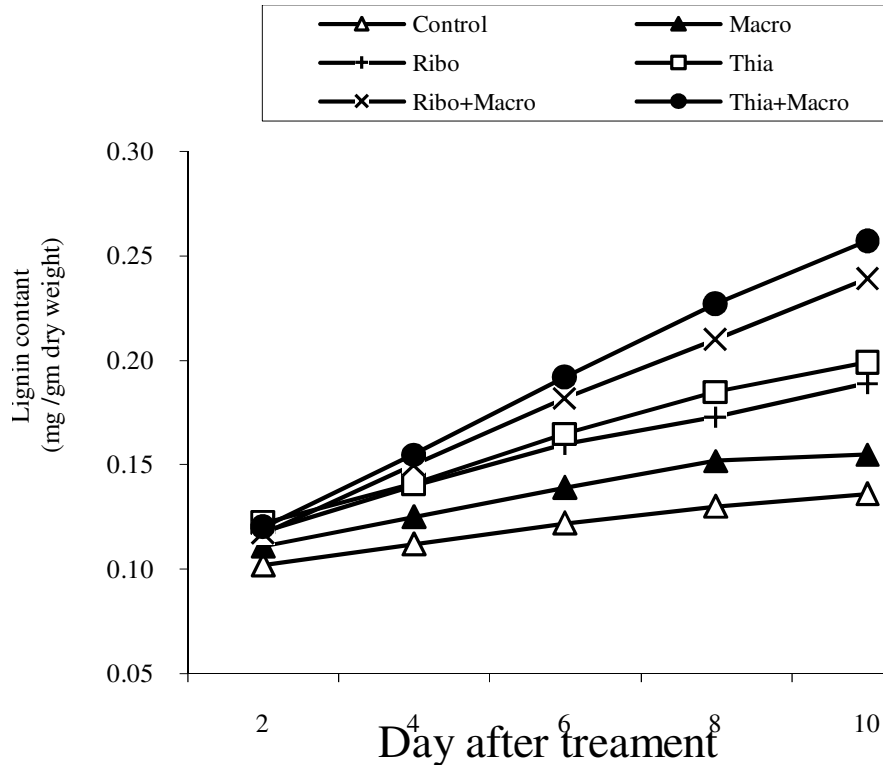


Figure 7. Effect of riboflavin (2.5 mM) and thiamine (5 mM) on lignin content in inoculated and non-inoculated soybean plants. The samples were collected from both inoculated and non-inoculated plants after 2, 4, 6, 8 and 10 days after treatment.

contain of protein and oil compared with the control treatment during the two experimental seasons (2009 and 2010). Thiamine treatment was better than riboflavin in all the cases except for nodule numbers per plant where, riboflavin was the best.

Several mechanisms that mediate the disease protection induced by different chemicals have been demonstrated, including blocking of disease cycle, the direct inhibition of pathogen growth (Thompson et al., 2000) and the induction of resistance to plant against pathogen infection (Ahn et al., 2005).

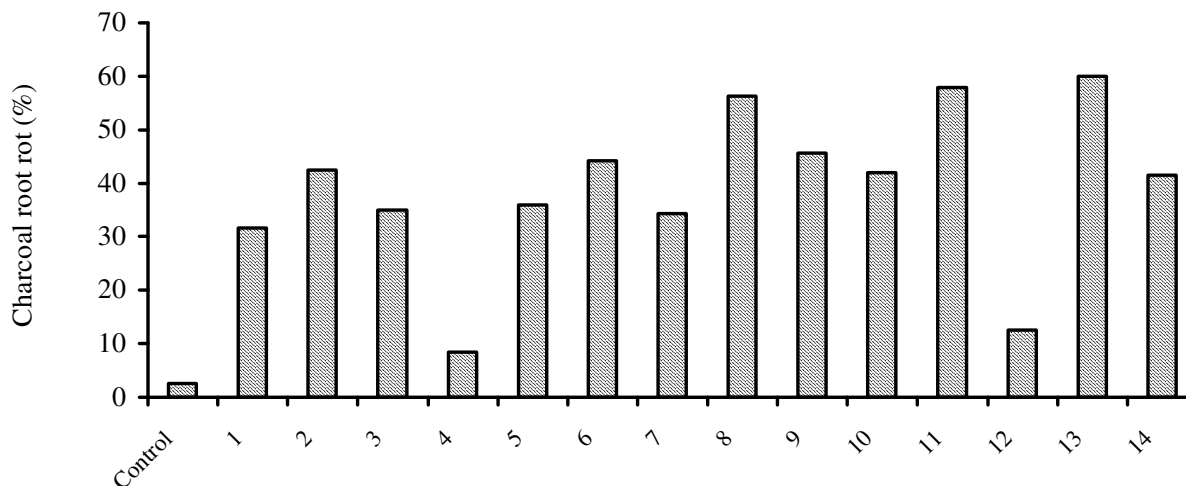
Thiamine confers systemic acquired resistance (SAR) on susceptible plants through priming, leading to rapid counterattack against pathogen invasion and perturbation of disease progress (Ahn et al., 2005). Priming reduces the metabolic cost required for constitutive expression of acquired resistance. Thiamine treatment and subsequent pathogen invasion triggered hydrogen peroxide accumulation, callose induction and PR1/PAL1 transcription activation in *Arabidopsis* mutants insensitive to jasmonic acid (*jar1*), ethylene (*etr1*) or abscisic acid (*abi3-3*), but not in plants expressing bacterial NahG and lacking regulation of SAR (*npr1* (nonexpressor of PR genes 1) (Ahn et al., 2007).

Recent studies of riboflavin indicate the function of the compound in mediating resistance signal transduction. Riboflavin is an antioxidant that induces resistance in

plants against pathogen (Packer et al., 1996). Riboflavin is a cofactor of enzyme flavoproteins, some of which catalyze lipid peroxidation a main process in producing ROIs that serve as a signaling network in plant immune responses (Alvarez et al., 1998). The role of riboflavin in peroxidation is antagonistic to its role in antioxidation (Dong and Beer, 2002). Balance between both reactions should be a part of the signaling mediation and may affect whether programmed cell death occurs. Glycosylated forms of riboflavin, which are considered unimportant in plant (Sierra and Vidal-Valverde, 1999), may serve as a signal-storage compound. This function may be similar to that of calmodulins and glycosylated SA, which function in the Ca^{2+} and SA signal storage, respectively. Finally, the universal existence of flavin kinases, required to activate flavoproteins may be linked with protein kinase cascades, which are a typical mode of signal transduction. Therefore, there is a reasonable basis for riboflavin to mediate a distinct signal transduction pathway.

Zhang et al. (2008) showed that riboflavin induced pathogen resistance in *A. thaliana* against infection by *Pseudomonas syringae* pv. tomato DC3000 (Pst) through the expression of defense response genes and cellular defense events, including H_2O_2 burst, hypersensitive cell death (HCD) and callose deposition in the plant.

On the other hand, Saikia et al. (2006) reported that



Macrophomina phaseolina isolates

Figure 8. Pathogenicity tests of *M. phaseolina* isolates isolated from natural diseased soybean plants. Charcoal rot (%) was recorded after 45 days from inoculation according to Mengistu et al. (2007) based on 0 to 5 scale according to percentage of foliage yellowing or necrosis (0= 0%, 1 = 1 to 25%, 2 = 26 to 50%, 3= 51 to 75%, 4= up to 76%, 5= completely dead plants) and disease severity (%) = $\sum(n \times v)/5 N \times 100$ (LSD at 0.05= 3.67).

accumulation of phenols and greater activities of phenylalanine ammonia lyase (PAL) and peroxidase enzymes may be one of the basic ways to participate in the action of thiamine and riboflavin in inducing resistance in chickpea plants against *Fusarium* wilt and charcoal rot diseases.

In this study, it was showed that the plants showed a significant increase in PO, PPO, PAL and chitinase activities in the inoculated and non- inoculated plants with *M. phaseolina* and these components begin to accumulate 2 days after treatment and reached maximum levels at the 8, 6, 8 and 8 days for PO, PPO, PAL and chitinase, respectively then the activities of these enzymes decreased progressively. Thiamine treatment increased the activities of PO, PPO, PAL and chitinase in the inoculated and non-inoculated plants than the riboflavin treatment. Also, comparatively least disease severity was found at 6 to 8 days and 5 to 7 days after treatment with thiamine and riboflavin respectively; this indicated that thiamine and riboflavin mediated induced resistance in soybean plants was related with the increase in PO, PPO, PAL and chitinase activities.

On the other hand, total phenols and lignin increased in the soybean plants inoculated with *M. phaseolina* and treated with thiamine and riboflavin. The highest accumulation of phenols was recorded the 6th day from the treatment applications, while lignin recorded the highest level at the 10th day from application.

In conclusion, this study provides further evidence that may facilitate the application of simple non-toxic chemicals as thiamine and riboflavin for controlling charcoal rot diseases in soybean. Their low cost, low toxicity to man and environmental pollution make them ideal for seed

soaking for disease control under field conditions and also for increased seed yield and seed content from oil and protein.

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