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INDUCTION OF RESISTANCE IN PEANUT PLANTS AGAINST ROOT ROT DISEASES UNDER GREENHOUSE CONDITIONS BY SOME CHEMICAL INDUCERS

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ABSTRACT

The effectiveness of three chemical inducers resistance, hydrogen peroxide (H_2O_2) at 0.25, 0.5 and 1%, Bion (benzo 1,2,3)thiodiazole-7-carbothioic– methlye ester) and salicylic acid at 2, 4 and 8 mM were tested in greenhouse under artificial inoculations. all tested inducers significantly reduced damping-off, wilt and root rot incidence, Salicylic acid at 4 mM and Bion at 8 Mm followed by Hydrogen peroxide at 0.25 % gave the highest effect on all parameters of disease incidence and consequently increasing percentage of healthy survival plants. Increasing concentration of salicylic acid up to 4 mM and hydrogen peroxide up to 0.25 % led to a decrease in their effect on reducing the disease incidence. While the effect of Bion on reducing of diseases incidence increased with increasing of their concentration. This study indicated that, there is a correlation between induced resistance and some biochemical changes in roots tissues like increased the activity of oxidative enzymes (peroxidase and polyphenoloxidase) and accumulation of phenol compounds. Salicylic acid at 4 mM followed by Hydrogen peroxidase at 0.25% and Bion at 8 mM recorded the highest content of phenol compounds and highest increase of activity of oxidative enzymes (peroxidase and polyphenoloxidase) in roots of peanut plants.

INTRODUCTION

Peanut, (*Arachis hypogaea* L.) is one of the most export and locally consumed crops in Egypt. Damping – off, wilt and root rot disease are among the most destructive diseases attacking peanut in Egypt (Yehia *et al.*, 1979). Induced disease resistance can be defined as the process of active resistance dependent on the host plants physical or chemical barriers activated by biotic or abiotic agents (Kloepper *et al.*, 1992). There are numerous reports demonstrating that resistance can be systemically induced by a chemical substrates (Aly and Afifi, 1989, Harfoush and Salama, 1992, Reuveni *et al.*, 1992 and Gamil 1995 a,b).

Various chemicals have been discovered that seem to act at various points of the defense–activating network. Some compounds *e.g.*, salicylic acid (SA); Hydrogen peroxide (H_2O_2) and benzo 1,2,3)thiodiazole-7-carbothioic– methlye ester (Bion) have been shown to induce resistance in plants (Atta–Aly *et al.*, 1991, Abd-El-Kareem *et al.*, 1993, Gorlach *et al.*, 1996, Gusui *et al.*, 1997, Colson, 1998 and Mosa, 2002). In peanut Meena *et al.*, (2001) showed that, foliar application of SA at a concentration of 1 mM significantly reduced late leaf spot disease intensity and increased the pod yield under

greenhouse conditions. While Mahmoud (2004) found that, various chemical inducers treatment of peanut plants as a seed soaking were effective in reducing pod rot diseases caused by *R. solani, M. phaseolina, S. rolfsii,* and *Aspergillus* spp., salicylic acid at 4 mM and cobalt at 1 ppm followed by hydrogen peroxide at 0.25 % gave the highest effective on all types of pod rot incidence.

Inducer of systemic resistance sensitizes the plant to respond rapid after infection. These responses include phytoalexin accumulation, phenols, lignifications and activation of peroxidase, polyphenol oxidase and chitinase (Ebel and Hahlbrock, 1982, Kuc, 1982, Boller, 1985, Metraux and Boller, 1986, Meena, *et al.*, 2001 and Mahmoud, 2004). In addition, many investigators reported that inducers of systemic resistance accumulated or enhanced new proteins in systemically protected leaves (Gianinazzi *et al.*, 1980 and Tuzun and Kloepper 1994).

The aim of this research is an attempt to study the effect of some chemical inducers in reducing of peanut root rot diseases.

MATERIAL AND METHODS

1. Isolation and purification of the causal organism (s):

Peanut plants showing symptoms of root rot disease were collected from different locations namely Beni-Suef, Giza, Ismailia and Nobaria at . The infected roots were washed thoroughly with tap water, cut into small pieces (1 cm.) each surface disinfested with sodium hypochlorite 2 % for two min., re- washed several times with sterilized water, dried between folds of sterilized filter paper, and were placed onto potato dextrose agar plates (PDA) supplemented with streptomycin-sulfate (100 µg/ml). Petri dishes were incubated at 28°C for five days. The growing fungi were purified using the hyphal-tip and single spore techniques (Brown, 1924 and Hawker, 1960)

2. Identification of causal organism (s):

Identification of the isolated fungi was carried out based on taxonomic criteria for these fungi as described by Barentt and Hunter (1977) for the genera of imperfect fungi, Ellis (1976) for *Macrophomina phaseolina*, Booth (1977) for *Fusarium* spp. Maren and Johan (1988) for *Aspergillus* spp. and Sneh *et al.*, (1992) for *Rhizoctonia solani.*

3. Preparation of fungal inoculum:

Inocula of isolates of *F. solani*, *F. oxysporium M. phaseolina*, *R. solani*, *Sclerotium rolfsii* were prepared using sorghum - coarse sand - water (2:1:2 v/v) medium. The ingredients were mixed, bottled and autoclaved for 2 hours at 1.5 air pressure. The sterilized medium was inoculated using agar discs, obtained from the periphery of 5-day-old colony of each of the isolated fungi. The inoculated media were incubated at 28°C for 15 days and were then used for soil infestation.

4. Soil Infestation:

Inoculum of each isolate of *F. solani, F. oxysporium, M. phaseolina, R. solani* and *S. rolfsii* was mixed thoroughly with the soil

surface of each pot, at the rate of 2% w/w , and was covered with a thin layer of sterilized soil. The infested pots were irrigated and kept for 7 days before sowing.

5. Disease assessment

Disease assessment was recorded as percentage of damping-off (pre- and post emergence) after 15 days from planting using the following formula =

$$\% \text{ damping - off} = \frac{\text{pre- emergence + post emergence}}{\text{No. of planted seeds}} \times 100$$

The wilted plants and root rot plants were recorded after 45 days and during the harvest time, healthy survival plants were recorded using the following formula:

% Healthy survival plants =

100 - (% damping-off + % wilted plants + % root rot plants)

6. Experiments implementation:

The experiments were carried out at Agriculture Research Center, Giza. Peanut seeds, cv. Giza 5, were used for sowing in 50 cm-diameter pots containing soil previously infested with mixture of *R. solani*, *F. solani*, *F. oxysporium S. rolfsii* and *M. Phaseolina* (2% w/w). Ten seeds were sown per each pot. Five replicate pots were used for each experiment. Disease assessment was recorded as percentage of damping- off, wilt, root rot and survival plants at 15, 45 days and during the harvesting time as previously mentioned.

7. Effect of chemical inducers:

The effectiveness of three chemical inducers resistance, hydrogen peroxide (H_2O_2), bion and salicylic acid on incidence of root rot and wilt of peanut was tested in greenhouse under artificial inoculations. Each chemical inducer was used, as seed soaking treatments, at three different concentrations *i.e.* 2, 4 and 8 mM. for bion and salicylic acid; 0.25, 0.5 and 1% hydrogen peroxide. Peanut cv. Giza 5 Seeds were soaked in the chemicals inducers for 4 hours.

Treated seeds were then allowed to dry for 24 hours before sowing. Seeds treated with sterilized water used as control. Any of the treated and untreated peanut seeds was sown in pots (50 cm. diam.) contained soil infested with mixture of pathogenic fungi at the rate of 2 % (w /w) as mentioned before, ten seeds/pot and 5 pots for each treatment were used. The disease assessments were carried out as previously mentioned.

8. Specific biochemical changes associated with chemical inducers treatments:

A study was conducted to identify some biochemical changes that associated with induced resistance by various chemical inducers treatments. Activity of oxidative enzymes, Peroxidase (PO) and Polyphenoloxidase (PPO), as well as Phenolic compounds were determined in the 15-day-old roots of treated and untreated peanut plants.

8.1. Determination of phenolic compounds:

A known amount of 15-day-old of healthy peanut roots were cut into small portions, immediately plunged into 95% boiling ethanol for 10 min., in order to kill the tissues then extracted for 10-12 hours in soxhlet units using 75% ethanol till the percolate was colorless. The combined ethanol extracts were filtered and rotary evaporated to nearly dryness at 60°C. The dried residues were re-dissolved in a known volume of 50% isopropanol alcohol. The later isopropanol extracts were used for determining free, total and conjugated phenols using folin and ciocatalteus reagent as described by Snell and Snell, (1953). Phenolic compounds were calculated as milligrams equivalent of catechol/1 g fresh weight of peanut root.

8.2. Determination of oxidative enzyme activates:

Two grams of 15-day-old of healthy peanut roots samples were cut into small portions and were grounded in a mortar in presence of purified sand and 4 ml. of 0.1 M sodium phosphate buffer ph 7.1 as described by Goldschmidt *et al.*, (1968). The homogenate was strained through four layers of cheesecloth then the filtrates were centrifuged at 3000 rpm for 20 min. at 6°C.

The obtained supernatant fluids (enzyme extracts) were used for assaying the oxidative enzymes peroxidase (PO) and polyphenoloxidase (PPO) using a spectrophotometer at 425 and 495 mm, respectively. Change in absorbance was recorded automatically every 30 sec. intervals for 180 sec. Enzyme extract was replaced by distilled water in control blank cuvette .

8.2.1. Peroxidase activity:

Peroxidase assay (based on oxidation of pyrogallol to purpurogallin in the presence of H_2O_2) was determined according to the method described by Allam and Hollis, (1972). The reaction mixture contained 0.5 ml of 0.1 M sodium phosphate buffer solution at pH 7.0; 0.3 enzyme extracts; 0.3 ml 0.05 M pyrogallol and 0.1 ml 1.0 % H_2O_2. The mixture was completed with distilled water up to 3 ml. The absorbance of 1 ml. was recorded and peroxidase activity was expressed as the change in absorbance/minute/1.0 g fresh weight.

8.2.2. Polyphenoloxidase activity:

The activity of polyphenoloxidase was measured as described by Matta and Dimond, (1963). The reaction mixture consisted of 0.3 ml. sample (1.0 ml. sodium phosphate buffer pH 7, and 1.0 ml 10^{-3} M catechol) and completed with distilled water to 6.0 ml. The polyphenol oxidase activity was assayed as mentioned above and expressed as the change in absorbency/ minute/1 g fresh weight.

9. Statistical analysis:

The data were statistically analyzed by analysis of variance (ANOVA) using the statistical Analysis System (SAS Institute, inc, 1996). Means were separated by Duncan's Multiple Range Test at $P \leq 0.05$ levels.

RESULTS

1. Effect of induced resistance:

The effectiveness of three chemical compounds *i.e.* Bion, Saliylic acid (SA) and Hydrogen peroxidase (H_2O_2) to induce resistance against damping-off, wilt and root rot pathogens were studied under greenhouse conditions in artificially infested soil. Data in Table (1) indicated that, all tested chemical inducers at the tested concentrations showed significant reducing incidence of damping-off compared to non-treated control. Salicylic acid at 4 mM followed by hydrogen peroxide at 0.25% was the most effective treatments. Bion at 8 mM followed by salicylic acid at 4 mM recorded highly significant effect in reducing wilt and root rot compared to other treatments, also both treatments gave the highest survival percentage of plants.

Table (1): Effect of chemical inducers, as seed soaking treatment, on damping-off, wilt, and root rot of peanut cv. Giza 5 under greenhouse conditions in artificially infested soil [x].

Inducers	Conc.	Disease incidence (%)			Survival (%)
		Damping-off	Wilt	Root rot	
Bion [y]	2 mM	24 b [z]	8 de	20 ab	48 d
	4 mM.	20 c	8 de	18 bc	54 c
	8 mM.	20 c	6 e	14 d	60 b
SA	2 mM.	22 bc	10 c	16 dc	52 cd
	4 mM.	16 d	6 e	10 e	68 a
	8 mM.	18 cd	8 de	14 d	60 b
H_2O_2	0.25%	12 e	10 c	16 dc	62 b
	0.50%	16 d	12 bc	18 bc	54 c
	1.00%	20 c	14 ab	20 ab	46 e
Control		26 a	18 a	22 a	34 f

x) Soil in each pot was infested with a mixture of pathogenic fungi (F. solani, F. oxysporium M. phaseolina, R. solani, Sclerotium rolfsii) at the rate of 2% (w/w).
y) Chemical inducers were used as seed soaking treatment for 4 hr. before sowing.
z) Means in each column with the same letter are not significantly different according to Duncan's Multiple Range Test (P = 0.05).

Data in Fig. (1) showed that, there is a relation between chemical inducers concentration and their effect on the incidence of the different studied disease parameters. Data clearly indicated that, increasing the concentration of bion led to increase its effect in reducing the incidence of the disease while, increasing concentration of salicylic acid up to 4 mM led to a decrease in its effect in reducing the disease incidence. It was obvious that increasing of hydrogen

peroxide concentrations had minor effect in reducing damping-off, wilt and root rot.

2. Biochemical changes associated with chemical inducers:

The effect of certain chemical inducers, as seed soaking treatment, on various biochemical changes *i.e.* phenol content and activity of peroxidase (PO) and polyphenolodixidase (PPO) in peanut plants, grown under greenhouse conditions in artificially infested soil with various root rot pathogens (*F. solani*, *F. oxysporium M. phaseolina*, *R. solani*, *Sclerotium rolfsii*), was studied.

2.1. Effect of chemical inducers on phenol contents in roots (15-day-old):

Results presented in Table (2) indicated that, phenol contents including the free, conjugated and total phenols were obviously higher in roots of plants grown from seeds treated with either chemical inducer than the untreated control. The highest phenol contents were induced by salicylic acid at 4 mM and bion at 8 mM. Data clearly indicated that, phenol contents were affected by three tested concentrations of each chemical inducer. The higher concentration of H_2O_2 and SA led to decrease of phenols content in roots while, increase of bion concentration caused an increase of phenol contents in peanut roots.

Table (2): Effect of chemical inducer on phenol contents in healthy roots (15-day-old) of peanut plants cv. Giza 5 [y].

Chemical [z]	Conc.	Phenol content		
		Free	Conjugate	Total
Bion	2.00 mM	7.12	3.69	10.81
	4.00 mM	7.44	4.72	12.16
	8.00 mM	7.82	6.32	14.13
Hydrogen peroxide (H_2O_2)	0.25 %	9.53	4.12	13.65
	0.50 %	7.64	2.77	10.41
	1.00 %	5.92	2.99	8.91
Salicylic acid (SA)	2.00 mM	7.79	3.63	11.42
	4.00 mM	9.19	6.11	15.30
	8.00 mM	6.51	3.16	9.67
Control		5.42	2.31	7.72

y) Soil in each pot was infested with mixture of fungi (*F. solani*, *F. oxysporium M. phaseolina*, *R. solani*, *Sclerotium rolfsii*) at the rate of 2% (w/w).

z) Chemical inducers were used as a seed soaking treatment for 4 h. before sowing.

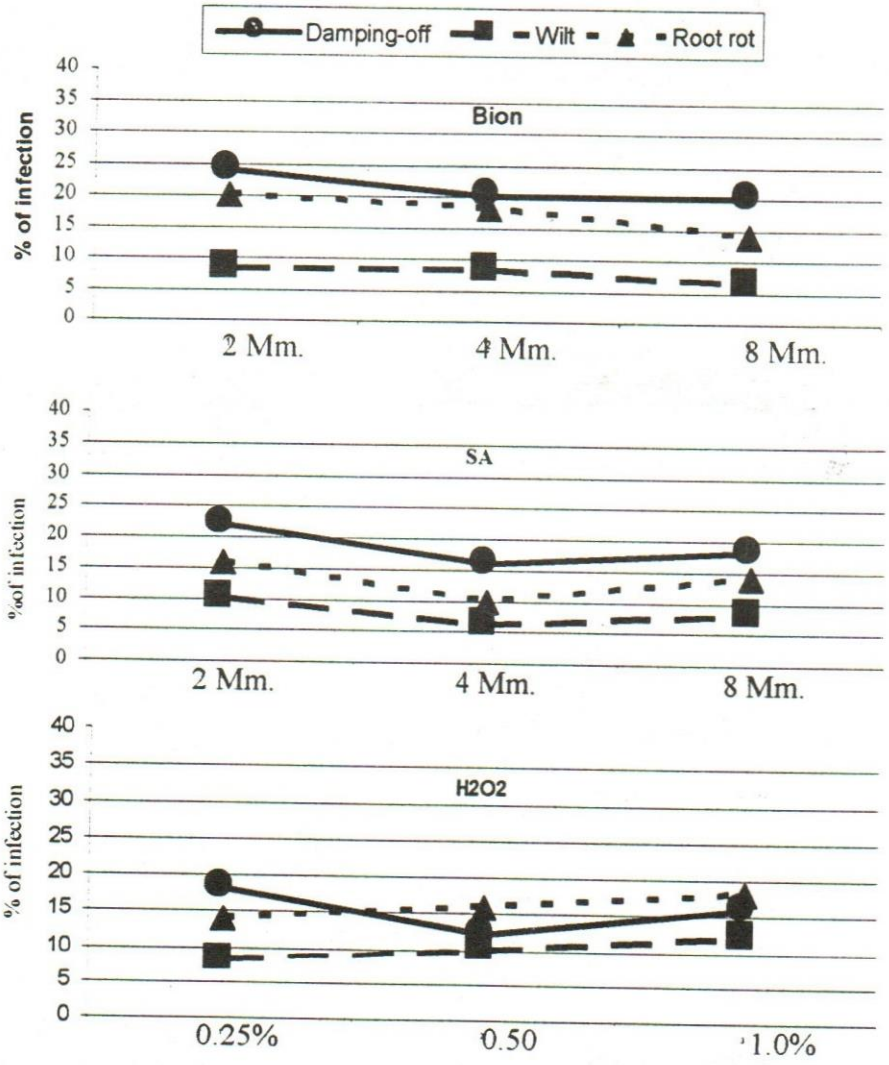

Fig (1): Relationship between different concentrations of chemical inducers and incidence of damping-off, wilt, and root rot of peanut cv. Giza 5 grown under greenhouse conditions in artificially infested soil with *F. solani*, *F. oxysporium M. phaseolina*, *R. solani*, *Sclerotium rolfsii*.

2.2. Effect of chemical inducers on peroxidase (PO) and polyphenoloxdase (PPO) activities in roots (15-day-old).

Data in Table (3) showed that all tested chemical inducers increased the activity of oxidative enzymes *i.e.* peroxidase and polyphenoloxidase in peanut roots compared to those grown from untreated seeds (control). In this respect, activity of PO showed the

highest increase when salicylic acid was used at 4 mM followed by hydrogen peroxidase at 0.25% and bion at 8 mM. The same trend was recorded for activity of PPO. Salicylic acid at 4 mM induced the higher level of (PPO) activity followed by hydrogen peroxidase at 0.25% and bion at 8 mM. Data showed also that, higher concentration of salicylic acid and H_2o_2 was accompanied by a decrease of the activates of the tested enzymes, while the increase of bion led to increase the activity of the same enzyme.

Table (3): Effect of chemical inducers on peroxidase and polyphenoloxidase activity in roots (15-day-old) of peanut plants cv. Giza 5 [x].

Chemical [y]	Conc.	Enzyme activity [z]	
		Peroxidase (PO)	Polyphenoloxidase (PPO)
Bion	2.00 mM	0.656	0.492
	4.00 mM	0.860	0.641
	8.00 mM	0.980	0.811
Hydrogen peroxide (H_2O_2)	0.25 %	1.020	0.712
	0.50 %	0.710	0.631
	1.00 %	0.506	.0320
Salicylic acid (SA)	2.00 mM	0.871	0.792
	4.00 mM	1.110	0.913
	8.00 mM	0.602	0.514
Control		0.501	0.211

x) Soil in each pot was infested with mixture of fungi (*F. solani, F. oxysporium M. phaseolina, R. solani, Sclerotium rolfsii*) at the rate of 2% (w/w), four replicates for each treatment.

y) Chemical inducers were used as a seed soaking treatment for 4 h. before sowing.

z) Activities of peroxidase, and polyphenoloxidase enzymes were expressed as the change in absorbance / minute/1.0 g fresh weight.

DISCUSSION

The results of this study indicated that resistance to peanut damping-off, wilt and root rot diseases could be induced by chemical treatments. In greenhouse all tested chemical inducers significantly reduced damping-off, wilt and root rot incidence. This is agreement with many investigators (Abd El-Kareem *et al.*, 1993, Gorlach *et al.*, 1996, Gusui *et al.*, 1997, Colson, 1998 and Mahmoud, 2004) who studied the effect of these chemical inducers on induction of plants against soilborne fungi. Salicylic acid at 4 mM and Bion at 8 Mm followed by hydrogen peroxide at 0.25 % gave the highest effective on all parameters of disease incidence and consequently increasing percentage of healthy survival plants. While, Bion at 2 mM and hydrogen peroxide at 1% gave the lowest effect. Increasing the concentration of Bion compound led to enhancing induced peanut resistance to damping-off, wilt and root rot diseases. Although Bion compound has no direct antimicrobial activity against many fungal and

bacteria pathogens (Gorlach *et al.*, 1996) but, it enhanced activities of the defense related enzymes chitinase and peroxidase (Siegrist *et al.*, 1997; Abou-Taleb, 2001 and Mosa 2002). While, in many plants investigated so far, Bion treatment is associated with increases in activities of many classes of pathogenesis-related protein (Gorlach et al., 1996 and Abou-Taleb, 2001).

Peanut seed treatment with hydrogen peroxide (H_2O_2) at the rate of 0.25 % caused reduction to damping-off, wilt and root rot. This may be due to the role of hydrogen peroxide in activating an array of host defense mechanisms including induced the appearance of a large amount of the enzyme as peroxidase and chitinase. This was accompanied by a significant increase in the lignin and suberin content (Gusui *et al.*, 1997). Hydrogen peroxide positively influences the local and systemic accumulation of SA that is correlated with the enhancement of peroxidase activity (Martinez *et al.*, 2000). Moreover, hydrogen peroxide inhibits pathogens directly, and/or it may generate other reactive free radicals that are antimicrobial (Peng and Kuc, 1992). This study provides further evidence that; hydrogen peroxide at 0.25 % concentration enhancement the activity of oxidative enzymes besides increasing the content of phenols compounds. On the other hand, increasing tne concentration of hydrogen peroxide led to decrease its effect on damping-off, wilt and root rot diseases, this may be due to the role of hydrogen peroxide in rapid generation of active oxygen species (AOS) called the oxidative burst (Lamb and Dixon, 1997). That may be given opposite effect on the physiological processes in the plants when increased its concentration especially the role of hydrogen peroxide in accumulation of salicylic acid (Martinez *et al.*, 2000).

Effect of salicylic acid (SA) in induced resistance of peanut to damping-off, wilt and root rot diseases increased with increasing its concentration from 2 to 4 mM, which cause increase the activity of oxidative enzymes and content of phenol compounds. This is in agreement with Klessig *et al.*, (1999); Martinez *et al.*, (2000) and Mahmoud (2004), who stated that, there is significant increase in the total peroxidase activity after treated with salicylic acid This may be due to the role of salicylic acid in generation of the oxidative burst in incompatible interactions by inducing a rapid transient generation of O_2 which is responsible on regulation of peroxidase activity (Mur *et al.*, 1996 and Rao *et al.*, 1997). On the other hand, the effect of salicylic acid on damping-off, wilt and root rot incidence decreased with increasing its concentration from 4 to 8 Mm. That paralleled with decreased in the activity of oxidative enzymes and content of phenols. This is may be due to the salicylic acid have damage effects at high concentration on the plants physiological processes, which includes inhibited phosphorus uptake and potassium absorption (Glass, 1974 and Harper and Balke, 1981), and caused the collapse of the transmembrane electrochemical potential of mitochondria, which had effect on ATP-production (Glass, 1974 and Macri *et al.*, 1986), and

reduced of transpiration by effect on stomatal behavior (Larque-Saavedra, 1978 and 1979).

The present investigation indicated that, there is a correlation between induced resistance and some biochemical changes in plant tissues like increase in the activity of enzymes and accumulation of phenols compounds. This is in agreement with Meena, *et al.*, (2001) who stated that, foliar application of salicylic acid at a concentration of 1 mM in peanut led to changes in the activates of phenylalnine ammonialyase, chitinase, beta-1.3- glucanase, peroxidase, polypheroloxidase and in the contents of phenolic compounds. While Mahmoud (2004) found that, the chemical inducers showed changes in the activity of oxidative enzymes and phenolic contents in primordial pods of peanut. This biochemical changes became a marker to induce resistance (Reuveni *et al.,* 1992). This is due to the role of peroxidase activity in disease development that has been correlated with the expression of resistance in different host – pathogen system (Hammerschmidt and Kuc, 1982; Coffey and Cassidy, 1984 and Cadena-Gomez and Nicholson, 1987). Peroxidases have several functions, which could have an effect on the resistance of a plant such as lignin production (Hammerschmidt and Kuc, 1982 and Edreva, 1989), phenylalanune ammonia lyase activity, and phenol accumulation (Tena and Valbuena, 1983). Another possible role for peroxidase is the oxidative cross~linking of pre-existing hydroxyproline-rich structural proteins in the cell wall, making the cell wall more resistant to degradation by microbial enzymes (Bradley *et al.,* 1992). In addition, peroxidases are implicated in an oxidative defense mechanism in elicitor treated (Apostol *et al.,* 1989), and generated hydrogen peroxide which, consider an antimicrobial agent (Peng and Kuc, 1992). Thus, peroxidase could not only participate in the biosynthesis of antimicrobial compounds and lignin but also serve as a regulator for the entire metabolic process (Peng and Kuc, 1992). While, phenol compounds play an important role in plant defense such, phenols are essential for the biosynthesis of lignin, which consider an important structural component of plant cell walls (Hahlbrock and Scheel, 1989). Phenol compounds in peanut seeds have been conducted concerning the effects of elicitors, such as chitosan. (Ebel and Hahlbrock, 1982).

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إستخدام بعض المستحثات الكيماوية في إستحثاث المقاومة لأمراض أعفان جذور الفول السوداني تحت ظروف الصوبة

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في تجارب الصوبة أعطت معاملة الفول السوداني بالكيماويات المستحثة للمقاومة تأثيراً معنوياً في تقليل نسبة الإصابة بموت البادرات و أعفان الجذور والذبول في الفول السوداني، وقد أعطي كلاً من حمض السلسيلك عند تركيز ٤ ملليمول تلاه فوق أكسيد الهيدروجين بتركيز ٠,٢٥ % والبيون عند ٨ ملليمول أعلي تأثيراً في خفض نسبة الإصابة. أوضحت الدراسة أن زيادة تركيز حمض السلسيلك أعلي من ٤ ملليمول يؤدي إلي إنقاص قدرته علي خفض الإصابة وكذلك الحال عند زيادة تركيز فوق أكسيد الهيدروجين عن ٠,٢٥ % ، بينما تزيد قدرة البيون في خفض نسبة الإصابة بزيادة تركيزه. أظهرت الدراسة أن هناك علاقة بين معاملة بذور الفول السوداني بالكيماويات المستحثة للمقاومة وحدوث تغيرات كيموحيوية في أنسجة الجذور حيث زاد نشاط الإنزيمات المؤكسدة(بيروكسيديز و البولي فينول أوكسيديز) وكذلك محتواها من الفينولات. كانت أفضل المعاملات تأثيراً في زيادة نشاط الإنزيمات المؤكسدة(بيروكسيديز و البولي فينول أوكسيديز) وكذلك محتوى أنسجة الجذور من الفينولات هي المعاملة بحمض السلسيلك عند تركيز ٤ ملليمول تلاه كلا من فوق أكسيد الهيدروجين بتركيز ٠,٢٥ % والبيون عند ٨ ملليمول.