Egypt. J. Phytopathol., Vol. **47**, No. 1, pp. 145 - 174 (2019)
ISSN 1110-0230

Induction of Systemic Resistance against Charcoal-Rot of Cowpea Caused by *Macrophomina phaseolina* using Some Inducer Resistance Chemicals

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Sixteen isolates of *Macrophomina phaseolina* were isolated from the roots of cowpea showing charcoal-rot collected from different locations in New Valley Governorate. All the obtained isolates were able to attack cowpea plants (cv. Balady) causing charcoal rot on the basal stem with various degrees of disease severity. Isolate No. 14 was the most aggressive one in this respect causing 85.8% charcoal rot. Potassium silicate (KS), propyl gallate (PG), hydroquinone (HQ) and salicylic acid (SA) at 1, 5 and 10 mM were used in this investigation as promising inducer resistance chemicals (IRCs) for controlling the disease *in vitro* and *in vivo*. Results illustrated that all tested IRCs had little effect on the growth of *M. phaseolina* at different concentrations *in vitro*. In addition, the inhibition of the growth was slightly increased by increasing the IRCs concentrations. Under greenhouse (New Valley Agric. Res. Station) and field conditions (New Valley Agric. Res. Station at El-Kharga and East Al Owainat Res. Station) all the tested IRCs significantly decreased charcoal rot compared to the check treatment (control). Both PG and HQ were the most efficient ones in decreasing the severity of the disease. On the contrary, both SA and KS recorded the lowest protection against charcoal rot severity. Also, all the tested IRCs significantly improved cowpea plant growth parameters, i.e. plant height, No. of branches/plant, fresh and dry weight of plants (kg/feddan) and yield components, i.e. pod length (cm), No. of seeds/pod, the weight of 100 seeds, total seed yield (Kg/ feddan) compared with control during summer season 2017. Cowpea seeds soaked in PG at 5 mM recorded the highest growth and yield components in both locations. While HQ recorded the lowest ones. Analysis of plant mineral compositions showed a significant increase in contents of nitrogen (N), potassium (K), phosphorus (P) and crude protein in cowpea plants raised from cowpea seeds treated with any of the tested IRCs compared with control plants during growing summer season 2017. The highest increase was obtained for these mineral contents when cowpea seeds were soaked in SA except for potassium in East Al Owainat. While, KS treatment recorded the lowest plant mineral contents of estimated minerals in both locations, except for potassium in East Al Owainat.

The activation of peroxidase (PO), polyphenoloxidase (PPO), phenylalanine ammonia-lyase (PAL), pathogenesis-related protein

(chitinase and β-1,3 glucanase) and phenolic contents in inoculated plants with *M. phaseolina* treated with the IRCS were increased compared with untreated inoculated plants and untreated un-inoculated plants. PG recorded the highest levels of oxidative enzymes, pathogenesis-related (PR) protein and phenol contents during all tested periods of determination. In general, enzymes activities of PO, PPO β-1, 3 glucanase and chitinase begin to accumulate after two days of treatment and reached maximum levels at 8 days while PAL reached maximum level at 6 days then decreased progressively. On the other hand, total phenols increased in cowpea plants inoculated with *M. phaseolina* and treated tested IRCS. The highest accumulation of phenols was recorded 6 days after the application.

Keywords: Cowpea, charcoal-rot, induce resistance, plant minerals, oxidative-reductive enzymes, total phenols, growth and crop parameters

Cowpea, *Vigna unguiculata* (L.) Walp. is one of the most important food and forage legumes in the semi-arid regions. It can fix atmospheric nitrogen in the soil at the rate of 56 kg per ha in association with symbiotic bacteria and contributes to soil fertility improvement particularly in smallholder farming systems where little or no fertilizer is used (Kyei-Boahen *et al*., 2017and Mohanapriya *et al*., 2017)

Cowpea provides considerable protein (Singh and Rachie, 1985) and as a food legume it constitutes the natural protein supplement to staple diets, and represents the legume of choice for many populations in Africa (Bliss, 1975). It is often called "meat for poor people", since this protein is the cheapest (Adandonon , 2004).

Cowpea is liable to attacks by many soil-borne fungal diseases (Adegbite and Amusa, 2010). In addition, charcoal-rot caused by *Macrophomina phaseolina* (Tassi) Goid. (the pycnidial state of *Sclerotium bataticola* Taub.) is one of the most destructive diseases in tropic and subtropic areas (Dhingra and Sinclair 1977; Reuveni *et al.,* 1983 and Adegbite and Amusa, 2010).

The information on worldwide losses caused by charcoal rot of cowpea is not available, but there is no doubt that the disease is of increasing importance and has the potential to cause destruction in susceptible cowpea cultivars, especially in the conditions of high temperature and soil water stress. Since the high temperatures and drought stress that accompany climate change will provide more favorable conditions for *M. phaseolina* (Smith and Wyllie, 1999). However, In the Sahelian zone of West Africa, charcoal rot is estimated to cause a yield loss of 10% of cowpea (Ndiaye, 2007).

An investigation for controlling cowpea charcoal rot is considered important, epically because of its prevalence in Egypt, particularly in newly reclaimed land in

the desert. However, the wide host range exhibited by this pathogen complicates management strategies. Soil solarization and chemical control of the disease is difficult and economically not affordable for low-income small scale farmers (Afouda *et al.,* 2012). Even though a few resistant cultivars have been recorded, they often showed partial levels of resistance (Demooy *et al.,* 1989) and are not obtainable to the farmers. Much of the effort to control *M. phaseolina* has focused on chemical usage and soil fumigation (Pearson *et al.,* 1984). Other recommended control practices include biological control of *M. phaseolina*, antagonistic bacteria and fungi that have been investigated (Afouda *et al.,* 2012, Karthikeyan *et al.,* 2015 and Khalili *et al.,* 2016). The reduction in the availability of effectively approved fungicides due to health and environmental concerns and resistance development in target pathogens (Kuck and Gisi, 2007) exhibit the necessity of increasing research to development of novel, effective and sustainable disease control solutions. Therefore, induced resistance could be proposed as an alternative, non-conventional and ecologically-friendly approach for plant protection.

The protection afforded by systemic acquired resistance (SAR) is frequently non-specific and long-lasting (Kessmann *et al.,* 1994). Systemic acquired resistance against pathogens can be induced by several synthetic chemical agents, such as salicylic acid, benzothiadiazole, β-aminobutyric acid, chitosan, and so forth which affect the production of phenolic compounds and increase the activity of various defense-related proteins. (Zakir, 2018). Salicylic acid (SA) was the first synthetic compound shown to induce enhanced activation of a variety of defense responses against major pathogens on various crops (Thakur and Sohal, 2013). Tested antioxidants included propylgallate reduced damping-off, root rot/wilt and area under root rot/wilt progress curve in pepper plants when used as seed soaking, seedling soaking, and soil drench under greenhouse and field conditions (Abdel-Monaim and Ismail, 2010). Moreover, propylgallate as either seed soaking or soil drenching proved sufficient protection against cumin wilt caused by *F. oxysporum* f. sp. *cumini* (Mostafa, 2006). However, Hydroquinone (HQ) is an aromatic organic compound that is a type of phenol, this phenol can act as an antioxidant. It was reported to inhibit some pathogenic fungi as well as improving the growth and yield of peanut (Elwakil, 2003); alfalfa (Al-Askar *et al.,* 2013); Jerusalem Artichoke plants (Al-Askar *et al.,* 2014 & Ezzat *et al.,* 2015). On the other side, silicon (Si) is known to effectively mitigate various environmental stresses and enhances plant resistance against both fungal and bacterial pathogens, silicon has been reported to prevent the incidence of powdery mildew diseases, in several plant species, for example, in barley, cucumber, melon, pumpkin, rose and wheat (Wang *et al.,* 2017). In addition to blast and powdery mildew, the occurrence of soil-borne fungal diseases, such as crown and root rot of cucumber (Chérif *et al.,* 1994); root rot of tomato (Heine *et al.,* 2007); Phytophthora blight of bell pepper (French-Monar *et al.,* 2010); Fusarium crown and root rot of tomato (Huang *et al*., 2011); Phytophthora stem and root rot of soybean (Guérin *et al.,* 2014); root rot and wilt diseases of

fodder beet (Abdel-Monaim *et al*., 2015) and Fusarium wilt of cotton (Whan *et al.*, 2016) were also suppressed by Si application.

The present research focuses on studying the effect of salicylic acid, propyl gallate, potassium silicate, hydroquinone as inducer chemicals against the charcoal disease of cowpea either *in vitro* or *in vivo* as well as its influence on plant growth and yield components in the field. Also, biochemical changes associated with the application of these inducer chemicals were assessed.

Materials and Methods

Isolation, purification, and identification of the pathogen:

The fungus was isolated from cowpea plants infected by charcoal rot disease collected from different locations of New Valley Governorate. Infected plant tissues bearing fungal sclerotia were selected and washed under the tap water, then cut into small pieces (5-10 mm) along with a healthy portion. These pieces were surface sterilized by dipping in 1% NaOCl solution for about 2 minutes followed by thoroughly three consecutive washing with sterilized water, then drying with a sterile paper towel. The sterilized pieces were transferred to potato dextrose agar (PDA) medium amended with ampicillin (200 mg / L) then incubated at 28±2 °C for 5 days. Purification of the isolated fungus was carried out on plain agar medium using hyphal tip transfer onto fresh PDA media as described by Dhingra and Sinclair (1985). The isolated fungus was identified according to their morphological characters according to Sutton (1980) and Phillips *et al*. (2013). Subcultures of the obtained isolates were then kept on PDA slants and stored at 4±^{o}C for further studies.

Preparation of fungal inoculum:

A- Solid medium:

The inoculum of *M. phaseolina* was prepared by growing the fungus in glass bottles 500 ml containing sterilized sorghum medium (100 g of sorghum grains and 90 ml of water). The bottles were inoculated with five mycelial plugs (6 mm in diameter) cut from the margin of a 4-day-old colony growing on PDA and incubated at 28 ± 2°C for 18 days until sorghum grain completely colonized with microsclerotia. The incubated bottles were shaken at alternate days for uniform colonization of the grains. The colonized sorghum grains were air-dried for 48 h and ground to coarse particles and then used.

B- Liquid medium:

The inoculum of *M. phaseolina* was prepared from two weeks old culture grown in 100 ml potato dextrose (PD) broth medium in flasks (500 ml) and incubated at 28±2°C according to Muthomi *et al.* (2007). The content of the flask was homogenized with sterile distilled water in a blender. The sclerotial suspension was diluted to contain 2x10^{3}sclerotia/ml water then used for soil infestation.

Greenhouse experiments:

Pathogenicity test:

Pathogenicity test of *M. phaseolina* (16 isolates) was carried out at New Valley Agric. Res. Stat. on cowpea local Balady cultivar. Plastic pots (30 cm in diameter) with a bottom drainage hole were sterilized by dipping in 5% formalin solution for 15 minutes and left for one week until complete formalin evaporation. Pots were filled with steam disinfested sandy clay soil 2:1 (v/v). The inoculum of each isolate was mixed with soil at 2% by weight at the time of sowing. Sterilized un-inoculated sorghum grains were added to the disinfested soil at the same rate for use as healthy control. Cowpea seeds were surface sterilized by immersing them in 1% sodium hypochlorite solution for 2 min then washed several times with sterilized water. Five cowpea seeds were sown in each pot and pots were irrigated directly. Five replicated pots were used for each isolate. All pots were irrigated with tap water as required to maintain sufficient soil moisture, and fertigated two weeks after sowing then every week with 0.1% solution of NPK fertilizer (15:15:15).

Disease-severity rating and incidence:

Forty five days after sowing, cowpea plants were rated for disease severity with a modification of the methods of Mengistu *et al.* (2007). Diseased plants were rated on a scale of 0-5, based on the percentage of foliage yellowing and inspected of longitudinally splitting of the stem and taproot of each plant gently uprooted, in which 0 = no symptoms; 1 = 1-25% chlorosis of leaves and no microsclerotia visible in tissue; 2 = 26-50% chlorosis of leaves and very few microsclerotia visible under the epidermis; 3 = 51-75% chlorosis of leaves, the vascular tissue is partly discolored and little microsclerotia visible in tissue; 4 = more than 75% chlorosis of leaves and vascular tissue is discolored with numerous microsclerotia embedded in the tissue; 5 = completely dead plants. The disease severity (%) was calculated using the following formula:

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

Where:
n= number of plants in each category
v= numerical values of scale symptoms category
N = the total number of numerical values of symptoms categories.

Effect of inducer resistance chemicals (IRCs) on the linear growth of the causal fungus in vitro

The inducer resistance chemicals (IRCs), i.e. propyl gallate, potassium silicate, hydroquinone, and salicylic acid were used separately at concentrations 1, 5 and 10 mM for studying their effect on linear growth of *M. phaseolina*. Mycelium disks (5 mm in diameter) taken from the growing edge of 4-day-old cultures of *M. phaseolina* were transferred to Petri-plates containing 20 ml sterilized Czpeck's solid medium amended with each concentration of each IRCs. IRCs free medium was

served as control. All plates were incubated at 28±2 °C until the Petri-plates in the control treatment were fully covered with mycelial growth (5 days). Four replicates were used for each treatment. The inhibition percentage of radial growth was calculated using the following formula:

$$\text{Inhibition of radial growth (\%)} = (D1 - D2) / D1 \times 100$$

Where:

D1 = Colony diameter in the control

D2 = Colony diameter in treatment.

Effectiveness of IRCs for controlling charcoal rot under greenhouse conditions:

As in the pathogenicity test, sterilized plastic pots were filled with steam disinfested sandy clay soil. The inoculum of highly virulent isolate (No.14) was mixed with soil at 2% inoculum level at the time of sowing. The cowpea seeds were soaked in K- silicate, propyl gallate, hydroquinone and salicylic acid at different concentrations (1, 5 and 10 mM) for 6 hr. In the control treatment, seeds were soaked in water for the same time. Five cowpea seeds were sown in each pot and five replicated pots were used for each treatment. Disease severity (%) was calculated after 45 days from seeding as mentioned before.

Biochemical changes in cowpea plants treated with the inducer resistance chemicals:

The activity of peroxidase, polyphenoloxidase (PPO), phenylalanine ammonia-lyase (PAL), pathogenesis-related protein (chitinase and β-1,3 glucanase) and phenolic content was estimated. As in the pathogenicity test, sterilized plastic pots were filled with steam disinfested sandy clay soil. Surface sterilized cowpea seeds were sown and fifteen days later, seedlings were injected with 50 µl/plant of K-silicate, propyl gallate, hydroquinone, salicylic acid at 5 mM and sterilized distilled water (SDW) by sterile syringe at the base of the stem (Saikia *et al.*, 2006). After 2 days of treatments, pots were inoculated with 100 mL of *M. phaseolina* homogenate suspension $2x10^3$sclerotia/ml water per pot. The following treatments were made (1) Control- treated with SDW only; (2) Control treated with *M. phaseolina*; (3) Treatment with K- silicate; (4) Treatment with propyl gallate; (5) Treatment with hydroquinone and (6) Treatment with salicylic acid. The activity of the enzymes and phenolic content was estimated after 0, 2, 4, 6, 8 and 10 days post inoculation

Assessment the activity of oxidative and pathogenesis-related protein enzymes:

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^{o}C for 25 min. The supernatants (crude enzyme extract) were stored at −20^{o}C or immediately used for determination of PO according to to Chakraborty & Chatterjee (2007), PPO (Gauillard *et al*., 1993), PAL (Cavalcanti *et al*., 2007), and pathogenesis-related protein, chitinase, and β- 1,3-glucanase enzymes activities

according to Wirth and Wolf (1992) and Pan *et al*., (1991), respectively. In the case of every enzyme under investigation, each treatment consisted of four replicates (3 plants/ replicate) and two spectrophotometric readings using Milton Roy Spectrophotometer (Milton Roy Spectronic 1201) were taken per replicate.

Estimation of phenolic contents:

The total phenol contents were quantified by the Folin-Ciocalteau phenol reagent according to the protocol of Xu and Chang (2007). The standard curve was prepared using known concentrations of gallic acid (GAE). The total phenol content in the test samples was calculated from the Gallic acid standard curve. The amount of phenolic content was expressed as phenol equivalents in mg/ gm fresh tissue.

Field experiments:

The experiment was carried out in the Exp. Farm of New Valley Agric. Res. Stat., at El-Khargaand East Al Owainat Stat.ARC during growing summer season 2017 for controlling charcoal rot disease of cowpea in a naturally infested field where both locations have a back history of natural infestation with *M. phaseolina*.

Cowpea seeds (cv. Balady) were soaked in the tested inducer resistance chemicals at 5 mM for 6 hr. In the control treatment, seeds were soaked in only water for the same period. The disinfected cowpea seeds were sown in the field on May 3&4 2017 for both locations, respectively. The treatments were distributed in a randomized blocks design with four replicates; the experimental plot area was 12 m^2 (4×3 m) containing 10 rows of 3 m length and 40 cm width. All recommended agricultural practices were adopted throughout the two locations. Charcoal rot severity was also recorded on a random sample of plants of the plots two months after planting as mentioned before. Also, the averages of plant height, and the number of branches/plant were assessed for twenty randomly plants/ experimental plot. At the same time, half of the plot area was cutting to determine fresh forage yield (ton /feddan) then dry forage yield (ton/feddan) was determined as follows: subsamples of 250gm each were dried at 70°C to constant weight and dry matter percentage was estimated. The dry forage yield (ton /feddan) was calculated by multiplying fresh forage (ton /feddan.) with dry matter percentage DM% (Abdel-Aziz *et al*., 2008). The other half of the experimental plot was left to maturity and the following measurements were estimated; pod length (cm), No. of seed /pod, the weight of 100 seeds and total seed yield (Kg /feddan).

Chemical constitutes:

The dry plant samples were ground and prepared for wet digestion using H_2SO_4 and H_2O_2 methods as described by Page *et al*., (1982). The digests were then subjected to the measurement of nutrients Nitrogen (N), Phosphorus (P), and Potassium (K) (Cottenie *et al*., 1982). The previously determined nitrogen of dry forage was used for calculating total crude protein (TCP %) by multiplying N-values by 6.25.

Statistical analysis

Completely randomized design (CRD) and randomized blocks design (RBD) were implemented in the greenhouse experiment and field experiment, respectively. The obtained data were subjected to computer statistical software (ASSISTAT) originated by Silva & Azevedo (2009). Data analyzed using analysis of variance (ANOVA), and mean values were compared using the least significant difference (LSD) at a significance level of $P \leq 0.05$.

Results

Isolation of the causal fungus:

Isolation trails from cowpea rotted roots with charcoal-rot collected from different localities of New Valley governorates yielded one fungus only. The fungus was identified as *Macrophomina phaseolina* (Tassi) Goid (the pycnidial state of *Sclerotium bataticola* Taub).

Pathogenicty test of sixteen M. phaseolina isolates:

Results illustrated in Fig. **(**1) show that all the obtained isolates (16 isolates) were able to attack cowpea plants (cv. Balady) causing charcoal rot on the basal stem with various degrees of disease severity,45 days after inoculation. Isolate 14 caused the highest charcoal rot severity (85.8%) followed by isolates 4, 10, 9 and 1, being 76.4, 75.4, 72.4 and 64.6% disease severity, respectively). While the other isolates lower incidence and severity showed.

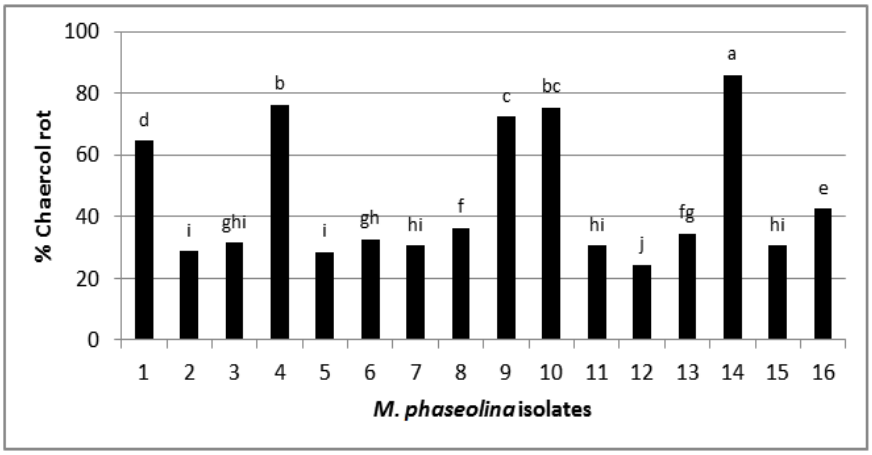

Figure (1): Pathogenicity test of sixteen *M. phaseolina* isolates isolated from naturally diseased cowpea plants. Columns with the same letter represent values that are not significantly different according to the LSD test ($p \leq 0.05$).

Effect of IRCs on linear growth of M. phaseolina in vitro.

Data presented in Table (1) indicate that all tested IRCs, *i.e.* HQ, KS, PG and SA had little effect on suppression of the linear growth of the causal fungus at different concentrations *in vitro*. The percentages of linear growth inhibition of *M. phaseolina* were slightly increased, being 8.53, 10.48 and 12.4 % by increasing the concentration of the inducer resistance chemicals, *i.e.* 1, 5 and 10 mM, respectively. HQ recorded the highest inhibitory effect, being 16.17% followed by SA10.13%. Both PG and KS recorded the lowest inhibition percentages, being 7.6% and 7.97%, respectively.

Table (1): Effect of the tested IRCs at different concentrations on the mycelial growth of *M. phaseolina in vitro*, 5 days after incubation at 28± 2 ° C.

Treatments	% Mycelial growth inhibition at (mM)			
	1 mM	5 mM	10 mM	Mean
Potassium silicate	6.5	8.5	8.9	7.97
Propyl gallate	6.5	7.4	8.9	7.60
Hydroquinone	12.5	15.6	20.4	16.17
Salicylic acid	8.6	10.4	11.4	10.13
Mean	8.53	10.48	12.40	-
LSD at 0.05				
Treatments (A) =		1.16		
Concentrations (B) =		1.00		
Interactions (A×B) =		2.00		

Effectiveness of IRCs at different concentrations for controlling charcoal-rot under greenhouse conditions:

Data presented in Table (2) show that all tested IRCs at the different concentrations significantly decreased charcoal-rot caused by *M. phaseolina* in pots compared with control. All the tested IRCs at 5 mM concentration were more effective for decreasing charcoal-rot severity than the lower concentration (1 mM) or higher concentration (10 mM). PG recorded the highest protection against charcoal rot severity followed by (HQ) and (SA), while (KS) gave the lowest protection in this respect. Generally, PG at 5 mM followed by HQ at 5 mM were the highest effective for decreasing charcoal rot severity, while KS at 1 mM caused the lowest decrease of charcoal rot severity in pots.

Effect of the tested IRCs on charcoal-rot severity under field conditions:

Results illustrated in Fig. (2) reveal that all tested IRCs significantly decreased charcoal rot disease compared to the control treatment (check) under field conditions in El-Kharga and East Al Owainat Stations during the growing season 2017, respectively. PG and HQ resulted in the lowest charcoal rot severity compared with control treatment in the two locations. However, PG resulted in 26.24 and 19.33%

charcoal-rot severity and HQ recorded 27.67 and 22.28% charcoal-rot severity compared with 45.67 and 38.67% in control in both locations, respectively. On the contrary, SA and KS recorded the lowest protection against charcoal rot disease in both locations.

Table (2): Effect of the tested IRCs as seed treatment on the severity of charcoal-rot of cowpea plants grown in artificially infested soil with *M. phaseolina* under greenhouse conditions, 45 days after sowing.

Treatments	% Charcoal-rot severity at (mM)			
	1 mM	5 mM	10 mM	Mean
Potassium silicate	45.4	25.6	29.9	33.63
Propyl gallate	30.5	11.2	23.4	21.70
Hydroquinone	35.1	14.8	16.5	22.13
Salicylic acid	42.5	16.5	28.7	29.23
Mean	38.38	17.03	24.63	-
Control		82.6		
LSD at 0.05				
Treatments (A) =		1.99		
Concentrations (B) =		1.31		
Interactions (A×B) =		2.62		

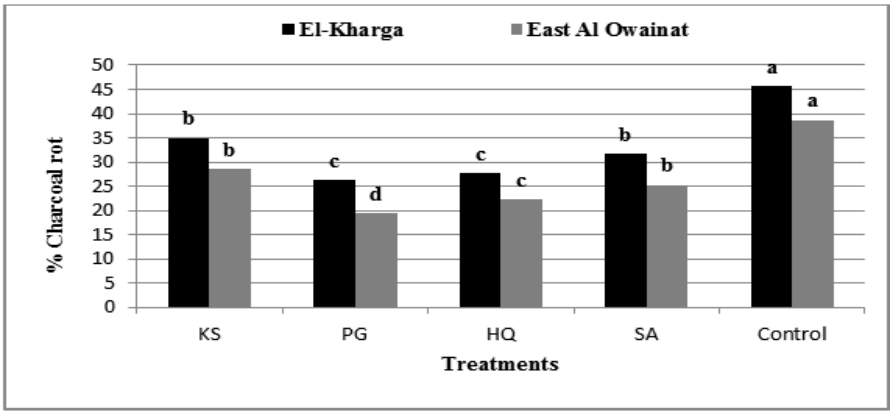

Figure (2): Effect of IRCsas seed treatments on charcoal-rot severity of cowpea plants grown under field conditions (natural infection) at El-Kharga and East Al Owainat Stations during summer growing season 2017.

Effect of the tested IRCs on some growth and crop parameters under field conditions:

The effect of soaking cowpea seeds in the tested IRCs on the growth and crop parameters under field conditions at El-Kharga and East Al Owainat Stations during summer season 2017 were assessed. Data in Table (3) indicate that all the tested IRCs significantly improved cowpea plant height (cm), the number of branches /plant, fresh and dry weight of plants (ton/feddan) compared control. soaking cowpea seeds in PG at 5 mM was the most effective treatment, it recorded the highest plant height (90.0 and 89.64 cm) and the number of branches (4.8 and 5.6 branch/plant) compared with control treatment which recorded 53.25 and 52.34 cm and 2.5 and 2.7 (branch/plant) in both locations, respectively. Also, PG treatment recorded the highest fresh and dry weights (ton/ feddan), that increased fresh weight from 7.07 and 7.27 ton/ feddan in control treatment to 14.65 and 14.25 ton/ feddan in both locations, respectively. Also, dry weight was increased from 2.15 and 2.12 ton/ feddan in control treatment to 3.75 and 3.72 ton/ feddan in both locations, respectively. On the contrary, soaking cowpea seed soaked in HQ and SA at 5 mM showed no significant differences and were less effective treatments in both locations compared to other rest treatments.

Table (3): Effect of the tested IRCs as seed treatments on some growth parameters of cowpea plants grown under field conditions (natural infection) at El-Kharga and East Al Owainat Stations during summer growing season 2017.

Treatments	Plant height (cm)	No. of Branches/ plant	Fresh weight (Ton / feddan)	Dry Weight (Ton/ feddan)
El-Kharga				
Potassium silicate	83.15	4.0	13.63	3.34
Propyl gallate	90.00	4.8	14.65	3.75
Hydroquinone	75.25	4.1	12.23	2.97
Salicylic acid	77.25	4.5	11.48	2.88
Control	53.25	2.5	7.07	2.15
LSD at 0.05	4.37	0.44	1.54	0.39
East Al Owainat				
Potassium silicate	81.49	5.2	13.43	3.28
Propyl gallate	89.64	5.6	14.25	3.72
Hydroquinone	72.48	4.8	11.86	2.84
Salicylic acid	75.69	5.0	11.00	2.76
Control	52.34	2.7	7.27	2.12
LSD at 0.05	4.84	0.57	2.23	0.30

Data presented in Table (4) indicate that all tested IRCs significantly increased weight of 100 seeds and total seed weight Kg / feddan compared with control in both locations during the growing season 2017. Cowpea seeds soaked in PG at 5 mM recorded the highest value of all yield components in both locations. While HQ treatment was recorded the lowest seed yield at the two locations.

Table (4): Effect of the tested IRCs as seed treatments on some crop parameters of cowpea plants grown under field conditions (natural infection) at El-Kharga and East Al Owainat Stations during summer growing season 2017.

Treatments	Pod length (cm)	No. of seed/ pod	Weight of 100 seeds	Total seed yield (Kg/ feddan)
		El-Kharga		
Potassium silicate	18.2	12.8	7	589.6
Propyl gallate	18.6	13.2	7.4	625.5
Hydroquinone	17.1	12.4	6.8	536.8
Salicylic acid	17.4	12.5	6.9	555.4
Control	15.7	10.1	5.2	302.14
LSD at 0.05	2.27	1.76	1.56	22.24
		East Al Owainat		
Potassium silicate	17.9	12.4	7.2	558.8
Propyl gallate	17.5	13.0	7.5	602.0
Hydroquinone	16.8	12.0	6.9	513.6
Salicylic acid	16.5	11.9	6.8	542.2
Control	14.5	9.6	5.1	288.3
LSD at 0.05	3.09	2.62	1.40	17.85

Mineral contents:

Analysis of plant mineral compositions showed a significant increase in nitrogen (N), potassium (K), phosphorus (P), and crude protein in plants raised from cowpea seeds treated with any of the tested IRCs compared with control plants in both locations (Table 5). The highest increase was obtained for these mineral contents when cowpea seeds were soaked in SA except for K% in East Al Owainat. While, KS treatment recorded the lowest plant mineral contents of all tested minerals in both locations, except for K% in East Al Owainat which ranked the second.

Table (5): Effect of the tested IRCs as seed treatments on nitrogen (%), potassium (%), phosphorus (%), and crude protein (%) of cowpea plants grown under field conditions (natural infection) at El-Kharga and East Al Owainat Stations during summer growing season 2017.

Treatments	% Contents of			
	N	K	P	Protein
El-Kharga				
Potassium silicate	4.10	2.11	0.26	25.63
Propyl gallate	4.32	2.23	0.33	27.00
Hydroquinone	4.21	2.32	0.28	26.31
Salicylic acid	4.35	2.36	0.36	27.19
Control	3.01	1.09	0.18	18.81
LSD at 0.05	0.40	0.23	0.05	2.50
East Al Owainat				
Potassium silicate	3.88	2.21	0.24	24.25
Propyl gallate	4.11	2.05	0.27	25.69
Hydroquinone	4.25	2.29	0.29	26.56
Salicylic acid	4.32	2.11	0.39	27.00
Control	2.96	1.02	0.18	18.50
LSD at 0.05	0.30	0.14	0.05	1.86

Biochemical changes in cowpea plants treated with the tested IRCs:

The activity of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL) and pathogenesis-related (PR) protein (chitinase and β-1,3-glucanase) and phenolic compounds in plants treated with the inducers chemical resistance inoculated with the pathogenic fungus were assessed.

Peroxidase activity:

Data in Fig. (3) show that PO activity in cowpea plants treated with the tested inducer resistance chemicals (KS, PG, HQ and SA) and inoculated with *M. phaseolina* was higher in the tested inducer resistance chemicals than in untreated plants either inoculated with the pathogenic fungus or non-inoculated (control), ten days after the application. Cowpea plants treated with PG recorded the highest levels of PO activity followed by HQ, while plants treated with KS recorded the lowest increase , 10 days after the of application. Inoculated untreated plants recorded high PO activity more than non-inoculated untreated plants (control). The highest levels of PO was noticed 8 days after treatment in all cases then decreased progressively.

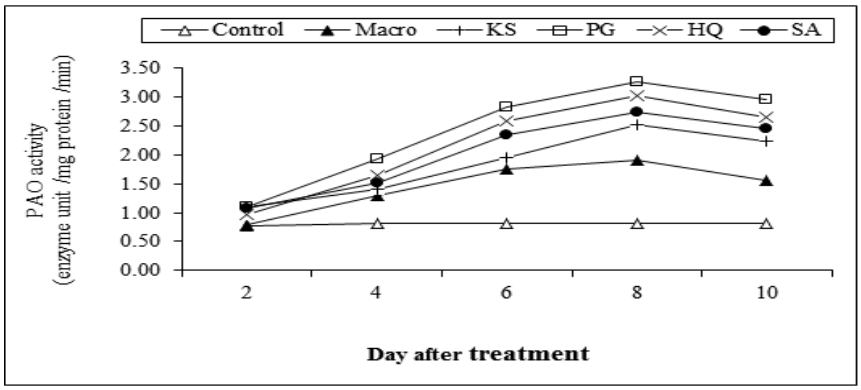

Figure (3): Activity of peroxidase (PO) in cowpea plants treated with the tested IRCs and inoculated with *M. phaseolina* using untreated plants either inoculated with the tested fungus or non-inoculated plant (control).

Polyphenoloxidase (PPO) activity:

Data illustrated in Fig. (4) show that all the tested IRCs increased the level of PPO activity in cowpea plants inoculated with *M. phaseolina*, ten days after the application compared with untreated plants either inoculated with the pathogenic fungus or non-inoculated plants (control). The maximum increase was recorded by all treatments eight days after treatment then decreased. PG treatment recorded the highest level followed by SA during all the detection periods, while KS and HQ, gave the lowest activity. On the other hand, in all cases, untreated cowpea plants and inoculated with the pathogenic fungus recorded high levels of PPO activity more than uninoculated untreated (control).

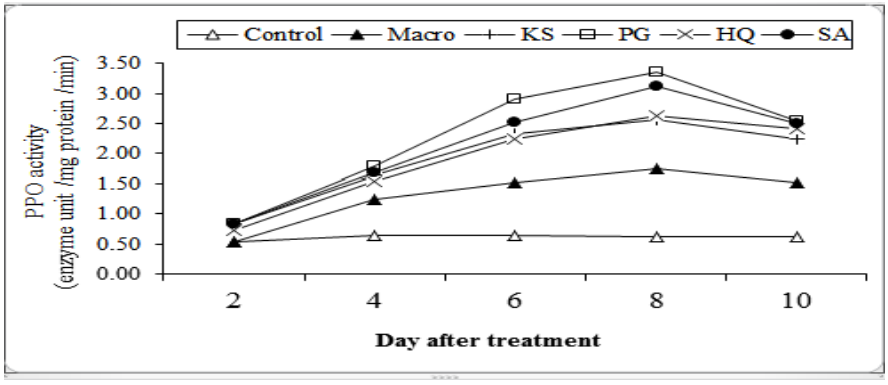

Figure (4): The activity of polyphenoloxidase (PPO) in cowpea plants treated with the tested IRCs and inoculated with *M. phaseolina* using untreated plants either inoculated with the tested fungus or non-inoculated plants (control).

Phenylalanine ammonia-lyase activity:

Data illustrated in Fig. (5) reveal that the levels of PAL activity in treated inoculated plants with the tested IRCs were increased than non-inoculated untreated plants (control), ten days after the application. The highest increase of the PAL activity was achieved by all tested IRCs, six days after application then decreased gradually. On the other hand, the highest increase in PAL activity was occurred in inoculated plants and treated with PG followed by SA compared with the other two treatments. Also, untreated plants and inoculated with the pathogenic fungus recorded high PAL level more than non-inoculated untreated (control), ten days after the application.

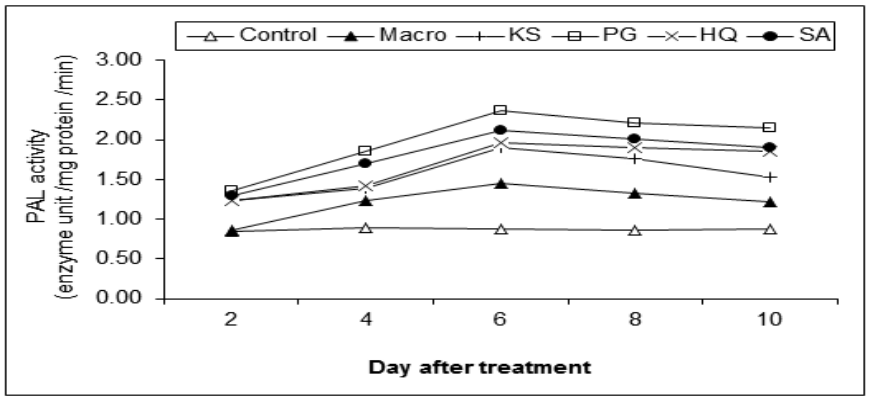

Figure (5): Activity of phenylalanine ammonia-lyase (PAL) in cowpea plants treated with the tested IRCs and inoculated with *M. phaseolina* using untreated plants either inoculated with the tested fungus or non-inoculated plant (control).

Pathogenesis related (PR) proteins:

The activity of the pathogenesis-related (PR) proteins (chitinase and β-1,3-glucanase) in cowpea plants treated with the tested IRCs and inoculated with *M. phaseolina* were estimated. Data presented in Figs. (6 & 7) show that all the tested inducer resistance chemicals increased the activity of chitinase and β-1, 3-glucanase enzymes in cowpea plants inoculated with the tested fungus compared with untreated plants either inoculated with the pathogenic fungus or non-inoculated (control), ten days after the application. Cowpea plants treated with PG and SA treatments recorded more activity of both enzymes than hydroquinone and potassium silicate treatments. However, the highest activity of both enzymes was shown 8 days after the application of the tested inducer resistance chemicals then decreased gradually. Also, the activity of both enzymes recorded high levels in cowpea plants inoculated with the pathogenic fungus more than non-inoculated untreated (control).

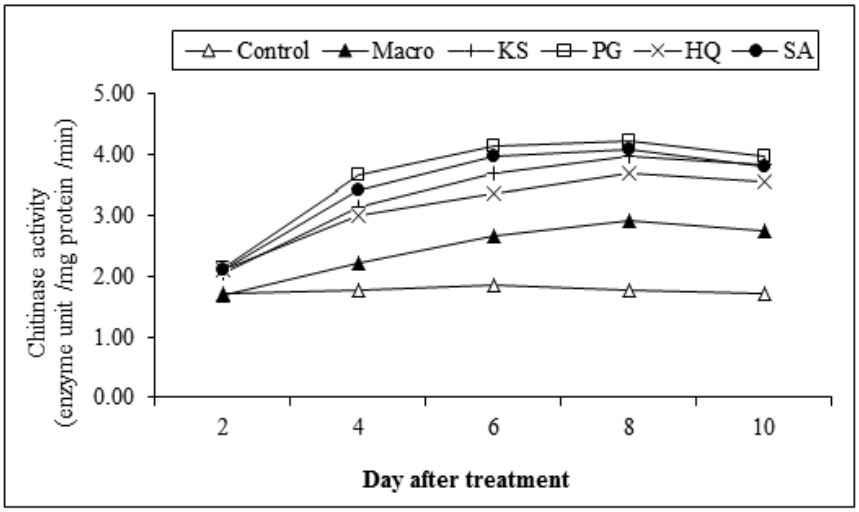

Figure (6): Activity of chitinase in cowpea plants treated with the tested IRCs and inoculated with *M. phaseolina* using untreated plants either inoculated with the tested fungus or non-inoculated plant (control).

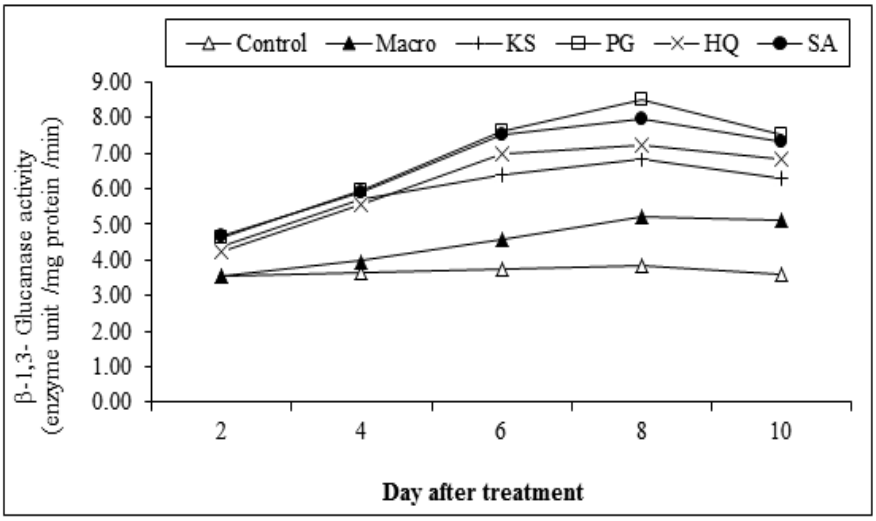

Figure (7): Activity of β-1, 3- glucanase in cowpea plants treated with the tested IRCs inoculated with *M. phaseolina* using untreated plants either inoculated with the tested fungus or non-inoculated plant (control).

Total phenol content:

Data presented in Fig. (8) reveal that all the tested inducer resistance chemicals increased the accumulation of total phenol compounds in cowpea plants inoculated with *M. phaseolina* more than untreated cowpea plants either inoculated with the tested pathogen or non-inoculated (control). Also, untreated cowpea plants inoculated with the pathogenic fungus recorded high content of total phenol compounds compared with non-inoculated (control). PG followed by SA recorded the highest contents of total phenol compounds more than HQ and KS, ten days after the application. Moreover, the maximum level of phenolic compounds was recorded, six days after the application then decreased gradually.

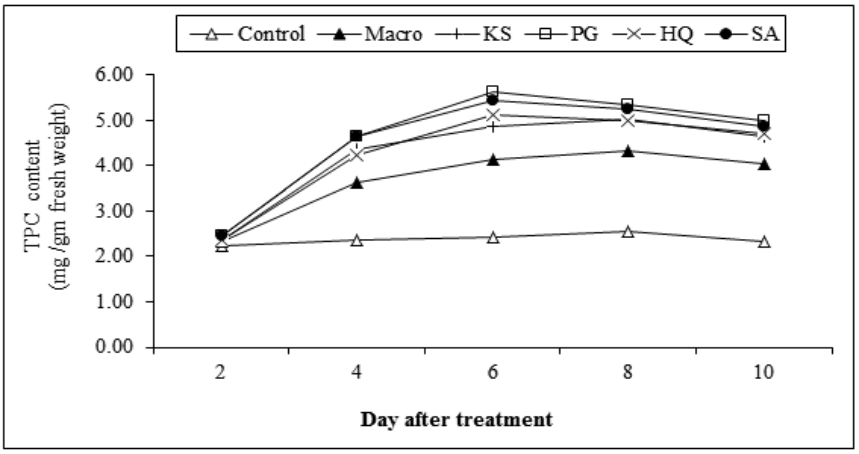

Figure (8): Total phenols content in cowpea plants treated with the tested IRCs and inoculated with *M. phaseolina* using untreated plants either inoculated with the tested fungus or non-inoculated(control).

Discussion

Cowpea (*Vigna unguiculata* (L.) Walp.) is one of the most famous members of family Fabaceae worldwide. It is of major importance to the livelihoods of millions of relatively poor people in less developed countries of the tropics (Singh *et al.*, 1997).It is an important grain legume widely consumed in Egypt as a cheap source of high-quality protein and other important nutrients (Da Silva *et al.*, 2018) Great losses occur as a result of seed decay and seedling damping-off caused by some pathogens including *M. phaseolina* (El-Mohamedy *et al*., 2006). The fungus is a soil- and seed-borne plant pathogen with a very wide host range that attacks more than 500 crop species including some of the most important crops such as soybean, cotton, and corn (Gupta *et al.*, 2012). The wide host range and the primary inoculum source of the pathogen (microsclerotia) which can survive up to 15 years depending on environmental conditions complicate management schemas (Cook *et al.*, 1973;

Papavizas, 1977; Short *et al.*, 1980). Microsclerotia in soil, infected seeds or host tissues serve as primary inoculum (Abawi and Pastor-Corrales, 1990). Root exudates induce germination of microsclerotia and root infection of hosts. The infective hyphae enter into the plant through root epidermal cells or wounds. During the initial stages of pathogenesis, the mycelium penetrates the root epidermis and is restricted primarily to the intercellular spaces of the cortex of the primary roots. As a result, adjacent cells collapse and heavily infected plantlets may die. At flower onset, the fungal hyphae grow intracellularly through the xylem and form microsclerotia that plug the vessels (Mayek-Pérez *et al.*, 2002) and disrupt host cells. The infected plants show necrotic lesions on stems, branches, and peduncles.

The use of fungicides to control the charcoal disease of economically important crops has been proved in agriculture for many years. However, a growing concern is increased for human safety and public perception that pesticides are harmful to human health and the environment (Gullino & Kuijpers, 1994; Carvalho, 2017). So, recent research priorities preferred disease control programs that are safe and compatible with sustainable agriculture.

Isolation trials from rotted roots of cowpea roots showing charcoal-rot symptoms yielded 16 isolates of *M. phaseolina* conforming to other reports (El-Mohamedy *et al*., 2006 & Amusa *et al*., 2007). Pathogenicity test demonstrated that all the obtained isolates were able to infect cowpea plants caused typical charcoal rot symptoms with different percentages of disease severity.

The results indicated that all the tested IRCs, *i.e.* potassium silicate (KS), hydroquinone (HQ), propyl gallate (PG) and salicylic acid (SA) were able to decrease charcoal rot caused by *M. phaseolina* either in pots or under field conditions. Propyl gallate recorded the highest protection against charcoal rot severity followed by Hydroquinone, while K- silicate and salicylic acid gave the lowest protection. On the other hand, all the tested IRCs increased the tested growth parameters (plant height, number of branches, fresh and dry weight) and yield components (pod length, number of seeds/pods, the weight of 100 seeds and total seed yield Kg /feddan) during summer season 2017 under field conditions. Induced resistance was reported to be activated by exogenous application of KS, PG, HQ and SA (Mostafa, 2006; Abdel-Monaim and Ismail, 2010; Al-Askar *et al.*, 2013; Thakur & Sohal, 2013; Abdel-Monaim *et al*., 2015 ; Ezzat *et al.*, 2015; and Wang *et al.*, 2017).

Abdel-Monaim and Ismail (2010) reported that propyl gallate at 200 ppm was more efficient in reducing infection with damping-off, root rot and wilt diseases in pepper plants as well as increasing the seedling fresh weight, dry weight, plant height, plant branching, number of pod/ plant and pod yield/ plant. Also, tuber treatment by Arbuscular mycorrhiza fungi+ HQ was the most effective in increasing the survival of Jerusalem Artichoke seedlings infected by *Sclerotinia sclerotiorum*

or *Rhizoctonia solani* as compared to the infected controls without treatment and was the most effective treatment in reducing disease incidence of tuber rots and plant mortality (Ezzat *et al.,* 2015). Also, the treatment with HQ recorded positive effect on disease parameters caused by *S. sclerotiorum* and *R. solani* (Al-Askar *et al.,* 2013). Additionally, Elwakil (2003) found that HQ improved the growth of peanut and raised the yield by up to 50 %. However, HQ as an antioxidant is a molecule capable of inhibiting the oxidation of other molecules, which delay or inhibit oxidative damage to target molecules such as lipids, proteins, nucleic acids and carbohydrates (Anbudhasan *et al*., 2014).

The mode of action of Si-induced resistance is acting as a physical barrier and as a modulator of host resistance to pathogens. Silicon deposited beneath the cuticle and forms a cuticle-Si double layer to protect the plant from pathogen penetration, therefore decreasing disease incidence (Ma and Yamaji, 2006& 2008). Silicon-induced resistance is associated with the density of silicified epidermal cells, the double cuticular layer, the intensified Si-cellulose membrane, and complexes formed with organic compounds in cell walls that support plants mechanically. The physical barriers decrease pathogen penetration and plant cells become resistant to enzymatic degradation by the fungal pathogen during invasion (Inanaga *et al.,* 1995; Fauteux *et al.,* 2005; Datnoff *et al.,* 2007; Van *et al.,* 2013).

Treatment with SA and its derivative induced expression of pathogenesis-related (PR) proteins (Malamy *et al.,* 1990 and Gaffney *et al.,* 1993). Salicylic acid plays an important role in the induction of plant defense against several plant pathogens (Kumar, 2014). Also, it may affect different biochemical processes in plants, including seed germination, ion uptake, and permeability of membrane (Dolatabadian *et al*., 2009). It regulates the activities of various enzymes such as, peroxidase (POD), polyphenoloxidase PPO, phenylalanine ammonia-lyase (PAL) which are the major components of induced plant defense against biotic and abiotic stresses (Idrees *et al*., 2011).

It has been found that, all the tested IRCs increased cowpea contents of nitrogen (N), potassium (K), phosphorus (P), and crude protein compared with control. SA recorded the highest levels of all tested mineral contents and crude protein, while KS treatment recorded the lowest cowpea plant contents from all tested minerals during the growing season at two locations. In this respect, many investigations reported the use of some chemical inducers to increase plant resistance, growth parameters and mineral contents in several crops (Chandra *et al.,* 2007; Maity and Bera, 2009; Khan *et al.,* 2010 and Ezzat *et al*., 2015). The tested IRCs may encourage some defense mechanisms *i.e.* oxidative enzymes, and phenolic compounds (Abdel-Monaim *et al*., 2015). In this study, all tested IRCs increased activity of defense-related enzymes, including peroxidase, polyphenoloxidase, phenylalanine ammonia-lyase, pathogenesis-related (PR) protein (chitinase and β-1,3- glucanase) in cowpea plants inoculated with *M. phaseolina* compared with control plants during the

experimental period. In general, the activity of these enzymes begin to accumulation two days of treatment and reached maximum levels at 6 days for PAL and 8 days for PO, PPO, chitinase and β-1,3- glucanase, respectively, then the activities of these enzymes were decreased progressively. In this respect, defense enzymes such as peroxidase, polyphenoloxidase, phenylalanine ammonia-lyase, chitinase, and β-1,3glucanase are related to induced resistance inducement in plants (Gajanayaka *et al*., 2014; Seneviratne *et al.*, 2014; Prasannath and De Costa, 2015). Peroxidases are a class of PR proteins that belong to PR-9 and induced in host plant by pathogen infection. They are expressed to limit the cellular extending of infection by the establishment of structural barriers or producing reactive oxygen species (Passardi *et al*., 2005). Moreover, peroxidases have been implicated in a range of defense-related processes, including the hypersensitive response, lignification, cross-linking of phenolics and glycoproteins, suberization and phytoalexin production (Nicholson & Hammerschmidt, 1992; Wojtaszek, 1997). However, polyphenol oxidases or tyrosinases (PPOs) are a group of copper-containing enzymes that catalyze the oxidation of hydroxy phenols to their quinone derivatives, which have antimicrobial activity (Chunhua *et al*., 2001). Quinones are effective inhibitors of SH (Sulfhydryl enzymes) group of enzymes which may inhibit the pathogens (Goodman *et al.*, 1967). A significant increase in PPO activity in response to host-pathogen interactions has been found in several plant species. Also, several studies reported a positive correlation between PPO expression and resistance/tolerance to biotic stresses (Taranto *et al.*, 2017). Meantime, Phenylalanine ammonia-lyase (PAL) catalyzes the non-oxidative deamination of phenylalanine into trans-cinnamic acid and ammonia which is the initial step in the biosynthesis of phenolic compounds. PAL which can be induced by some biotic and abiotic stresses is one of the important enzymes in plants due to the synthesis of various phenolic compounds as well as anthocyanin that are responsible for the resistance of plant pathogens (Dixon and Paiva, 1995). Other defense enzymes include pathogenesis-related proteins (PRs) such as β -1, 3-glucanases PR-2 and chitinases PR-3, which are responsible for the hydrolysis of cell wall components in sequence such as chitin and β-1,3-glucans (Ebrahim *et al.*, 2011). β-glucanases participate in the decomposition of glucans like callose which occurs in plant tissues as one of the components of wall modifications involved in resistance responses (Smart, 1991). While, chitinases improves plant defense against chitin containing plant pathogens (Jalil*et al.*, 2015). B-1, 3-glucan and chitin, a polymer of N-acetylglucosamine are major cell wall components of many fungi. Since β-1, 3 glucanase and chitinases are capable of attacking the cell wall of fungal pathogens, these enzymes have been proposed as direct defense enzymes of plants (Abeles *et al*., 1970). Also, Mauch *et al*., (1988) reported that in combination, chitinase and β-1, 3glucanaseact synergistically to inhibit fungal growth. The mode of action of chitinase is relatively simple. They decompose the cell wall chitin polymers in situ, resulting in a weakened cell wall and showing fungal cells osmotically sensitive (Jach *et al*., 1995).

The obtained data revealed that total phenols were increased in cowpea treated plants and inoculated with *M. phaseolina*. The highest accumulation of phenols was recorded at 6 days from application. These results suggested that these chemicals may play an important role in controlling the cowpea charcoal rot, though they have induction of systemic resistance in cowpea plants. In this regard, the role of phenolic compounds in disease resistance was postulated by Nicholson and Hammerschmidt (1992). The phenolic compounds may contribute to enhance the mechanical strength of the host cell wall and may also inhibit the fungal growth, as phenolics are fungi toxic in nature. Altering the level of phenolic compounds in plants has been demonstrated to change disease susceptibility (Yao *et al.,* 1996).

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(Received 09/03/2019;
in revised form 23/03/2019)

استحثاث المقاومة الجهازية لمرض العفن الفحمى فى اللوبيا المتسبب عن الفطر *Macrophomina phaseolina* باستخدام المستحثات الكيميائية

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تؤدي الإصابة بمرض العفن الفحمي فى اللوبيا المتسبب عن الفطر *Macrophomina phaseolina* إلى خسائركبيرة في المحصول. تم عزل ستة عشرعزلة من الفطر *M. phaseolina* من نباتات لوبيا مصابة من مواقع مختلفة في محافظة الوادي الجديد. كانت جميع العزلات التي تم الحصول عليها قادرة على إصابة نباتات اللوبيا (صنف بلدي) مسببة العفن الفحمي على قاعدة الساق بدرجات مختلفة من شدة الاصابة. وكانت العزلة رقم 14 الأكثر شراسة ، حيث سببت عفن فحمي بنسبة ٨٥,٨ ٪ .تم استخدام سيليكات البوتاسيوم (KS) ، بروبيل جالات (PG) ، هيدروكينون (HQ) وحامض السلسليك (SA) بتركيزات ١ , ٥ و ١٠ مللى مول/لتر كطرق واعدة لمكافحة هذ المرض في المعمل وعلي النباتات. أوضحت النتائج أن جميع المستحثات الكيميائية التي تم اختبارها لم يكن لها تأثير يذكر على نمو *M. phaseolina* بتركيزاتها المختلفة في المعمل. وقد زاد تثبيط نمو الفطر *M. Phaseolina* زيادة طفيفة عند زيادة تركيزات المستحثات الكيميائية. أما تحت ظروف الصوبة (محطة بحوث الوادي الجديد) والحقل (محطة بحوث الوادي الجديد بالخارجة ومحطة بحوث شرق العوينات) قللت المستحثات الكيميائية معنويا العفن الفحمي مقارنة بالنباتات الغير معاملة. كما خفض المركبان بروبيل جالات و الهيدروكينون شدة الاصابة بالعفن الفحمي بينما كان حامض السلسليك وسليكات البوتاسيوم اقلهم تاثيرا.

كذلك ، فإن جميع المستحثات الكيميائية المختبرة قد حسنت بشكل ملحوظ مقاييس النمو لنباتات اللوبيا ،ومنها طول النبات ، عدد الفروع / النبات ، الوزن الطازج والجاف للنباتات (كجم / فدان) وكذلك مكونات المحصول ومنها طول القرن (سم) ، عدد البذور / القرن ، وزن ١٠٠ بذرة ، وكذلك وزن المحصول (كجم / فدان) مقارنة مع النباتات الغير معاملة خلال الموسم الصيفي ٢٠١٧. وقد سُجلت أعلي قيم لمكونات المحصول في كلا الموقعين عند إستخدام مادة البروبيل جالات كمعاملة نقع للبذور بتركيز 5 مللي مول /لتر وكان مركب الهيدروكينون أقلهم تاثير.

أظهر تحليل العناصر النباتية زيادة معنوية في محتويات النيتروجين ، البوتاسيوم ، الفوسفور والبروتين الخام في نباتات اللوبيا المزروعة من بذور اللوبيا المعاملة بأي من المستحثات الكيميائية مقارنة مع النباتات الغيرمعامله خلال الموسم الصيفي ٢٠١٧. تم الحصول على أعلى زيادة

لهذه العناصر عند نقع بذور اللوبيا في حامض السلسليك باستثناء عنصر البوتاسيوم في موقع شرق العوينات. بينما سجلت معاملة سليكات البوتاسيوم أدنى مستوى للعناصر في كلا الموقعين باستثناء عنصر البوتاسيوم في شرق العوينات.

لوحظت زيادة في نشاط إنزيمات البيروكسيديز ، البولي فينول اكسيديز ، وفينيل ألانين الأمونيا ليز ، والبروتينات المرتبطة بالعدوى (الشيتينيز و بيتا ١-٣ جلوكانيز) ومحتوي الفينولات في النباتات المعداه بفطر *M. phaseolina* والمعاملة بالمستحثات الكيميائية مقارنة بالنباتات المعداه والغير معاملة و النباتات الغير المعداه والغير معاملة. وكانت أعلى مستويات الإنزيمات المؤكسدة ،والبروتينات المرتبطة بالعدوى ومحتوى الفينول خلال جميع فترات الاختبار المحددة ناتجة من المعاملة بالبروبيل جالات .

بشكل عام ، يبدأ نشاط إنزيمات البيروكسيديز ، البولي فينول اكسيديز ، وفينيل ألانين الأمونيا ليز ، والبروتينات المرتبطة بالعدوى (الشيتينيز و بيتا ١-٣ جلوكانيز) في الزيادة بعد يومين من المعاملة ثم يصل للمستويات القصوى بعد ٨ أيام بينما انزيم فينيل ألانين الأمونيا ليزيصل إلى المستويات القصوى بعد ٦ أيام ثم تنخفض أنشطة هذه الأنزيمات بشكل تدريجي. من ناحية أخرى ، زاد محتوي الفينولات في نباتات اللوبيا المعداه بالفطر *M. phaseolina* والمعاملة بالمستحثات المختلفة, وسُجلت أعلى نسبة تراكم للفينولات بعد 6 أيام من المعاملة.