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Biotic Stress as a Defense Mechanism in Soybean (*Glycine max* L.) toward Microbial Pathogen: Biochemical and Physiological Pathways study

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Abstract: In the new global economy, fungal infection has become a central issue for crops. The soil- borne fungus *Fusarium solani*. f. sp. *glycine* roots and leading to the phenomena of sudden death disease. Some facets of the interaction within the soybean/*Fusarium solani. f. sp. glycines*. Pathosystem have been elucidated. The reaction of soybean varieties concerning their resistance has been evaluated against an artificial background infection, prepared from the *Fusarium* fungi characterized by increased pathogenicity. The plant possessing special effective mechanisms to evade or bear the pressures which permit them to defend and acclimatize to the stressful circumstances. This adaptation includes the anatomical, morphological, molecular and biochemical levels. Some of the mechanisms / modifications implemented by plants to acclimatize and defend against the ecological stressful conditions including reactive oxygen species (ROS) signaling, epigenetic memories, molecular crosstalk, synthesis and releasing of plant hormones like ethylene**,** salicylic acid, abscisic acid and jasmonic acid, change inorganic ion fluxes and in redox status, systemic acquired resistance (SAR) and R-gene resistance. The biochemical response of soybean roots to FSG infection, was studied in the current study through matching FSG-inoculated and non-inoculated roots of soybean. This paper contests the claim that the defense response was established by the extraction method for phytoalexins glyceollin from soybean-based on different techniques (TLC, SDS-PAGE, and HPLC) which disclosed that the self-protective response pathway of the tested plants was also proprietary by the induction of peroxidase isozymes production. The incubation temperatures 30C must affect the elicitors to be raised. According to Hypocotyls inoculation, the tested cultivar of Crowford was resistant in comparison with moderate susceptibility in Gizza 22 after *Fusarium* inoculation.

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1. Introduction

Generally speaking, popular infection of plants by pathogenic fungi includes numerous diverse phases. The major phase comprises contact between plant and pathogen encompassing surface recognition and adhesion via hydrolytic degradation of host cell walls, which can be tailed by penetration [1]. First of all, for successful infection formation and preservation inside the host species are additional requests. Secondly, the final stages may rest on various pathogenic determinants that may vary among the different pathogens. These comprise inactivating and overwhelming host defense replies by the fungal agents. Additionally, for some time a nourishing association amid fungus and host plant should be recognized. Special appreciation of the invading fungus may happen in resistant plants, causing activation of defense replies, inhibiting extra development of the pathogen inside the plant [2].

Typically, Plant life has been capable for improving an group of inducible and constitutive methods to defend themselves against pathogens. The most prevalent defense mechanisms in pathogen-challenged plants comprises the hypersensitive response, the production of reactive oxygen species (oxidative eruption), the synthesis of callose and different wall-bound phenolic compounds and the encouraged antimicrobial phytoalexins and increase of pathogenesis-related proteins [3]. These conjoint responses are portion of the unsuited interaction containing a virulent pathogen and resistant plant and are under control of genetic factors.

Under several phytoalexins, principally flavonoids which created by legumes, and known as phenylpropanoid metabolism products. One of the most important components in the host plant's defensive resource are flavonoid and isoflavonoid compounds, but direct evidence of the phytoalexins suggestion is not documented [4].

Additional inducible defense mechanism of plant against pathogens represented in increased lignification at the place of infection which play a role in suppression of the infection process and pathogen growth and is habitual linked with phytoalexins assembly [5].

It is known that the causative agent of sudden death syndrome (SDS) of soybean (*Glycine max* L.) is the *Fusarium solani* (Mart.) Sacc. f. sp. glycines [6]. One of the most important diseases of soybean in the United States is the SDS which has become a mid- to late-season illness [7]. At hand are several of root-invading fungal infection, but for most plant-fungal infections the biochemical activities and root interactions, that arise throughout infection of root are not covered well, either due to some what the difficulty of working with root pathogens and roots. Due to the infection of soybean with SDS many economic losses are result annually [8], which is caused by the soil-borne fungus FSG [9]. The SDS disease due to the fungal infection is restricted mainly in roots and extends to the lower portions of stems and not established in the leaves. Foliar signs have been due to the toxins of the fungus evoked from the roots and transmitted to the leaves [10].

In the USA and other countries, SDS has become distributed widely and reliable problem owing to the shortage of soybean genotypes with enough resistance. Partial resistance was identified as one of causes of SDS syndrome, however even these origins of resistance display merely reduced foliar response, whereas, the roots are remain infected, leading to decreased in plant health. Some investigators found that the hypersensitive kind of resistance has not been established yet [11]. One of the natural barriers against infection with microorganisms is the cell wall of plant. In addition the cell walls of plant comprise several essential constituents such as phenolic mixes which formed from phenylpropanoid units which are present in the form of lignin alcohols and conjugated acids. Phenolic acids are considered as precursors for the production of phenylpropanoid phytoalexins and lignin [12].

During pathogen infection to the plant deposition of phenolic into the cell wall is believed to play an important role in the defense mechanism, which may attributed to a hypersensitive reaction of whole cells or due to local wall reinforcement owing to deposition of papillae [13]. One of the more profuse biopolymers on globe is the lignin which characterized by high strengthening capability that are essential for the defense mechanism of vascular plants [14]. Many investigators found a high association among disease resistance and the degree of lignification in many tests. It was found that the resistant plants precipitate lignin's more speedily and/or display enhanced lignin deposition as matched with susceptible plants [15].

Some researchers suggested in infected tissues of different plant organs that the accumulation of the phenylpropanoid phytoalexins glyceollin was accountable, at least partially, for the resistance of soybean seedlings [16]. It is established that the presence of glyceollin with high quantity in soybean cell suspension cultures treated with *Pseudomonas siringae* pv. *glycinea* in harboring and avirulence gene or with *P. sojae* culture filtrate or cell walls [17]. Also, glyceollin was found to be accumulated in the roots of soybean inoculated with the soybean cyst nematode [18].

Stress in general can be identified as an influence or a stimulus that is not easy to control by the normal range of homeostatic system in a given organism.

During exposure of the plant to a high stressful condition that is outside the tolerance level, mechanisms are stimulated at physiological, molecular, morphological and biochemical levels. As soon as the stressful condition is stopped, a new physiological condition is recognized and the plant may restore the original condition and by this means, regenerating the homeostasis [19]. Plants are exposed to several adverse environmental conditions including abiotic and biotic factors. In the sequence of evolution, to manage with stressful conditions induced by environment compulsory by nature, plants have developed highly competent and sophisticated strategies. Even however plant species differ in their response and sensitivity to different stressful circumstances, they have settled different acclimatization methods to translate stress perception, gene transcription networks and signaling cascades in reaction to environmental signals and advanced a multitude of defensive methods to acclimatize and survive along the severe unsuitable environmental situations [20].

For protection from the infective microorganisms, plants depend on induced and constitutive biochemical and structural defensive mechanisms. Utilization of induced defensive methods may lead to systemic-induced resistance, previously non-infected parts of a plant can respond to the infection by pathogenic agents and induction of resistance. For several years SIR has been known to occur in many angiosperm plants [21]. Furthermore to the synthesis of phytoalexins, in angiosperms, SIR in its different formulae has also been accompanied with the rise in pathogenesis-related (PR) proteins. Under stressful condition, PR proteins was found to be accumulated, microorganism occurrence, and abiotic stimuli, and are convinced in both SIR and local phenotypes. Though, their definite role in induction to the resistance to pathogens (or insects) remains unclear [22].

In particular, the effect of the biochemical proceedings that happen after infection with FSG in the roots of soybean has not been unexplored. As a consequence, the objective of the present work paper is to investigate the prime induced resistance in soybean plants against the *F. solani f. sp. Phaseoli, F. solani f. sp. glycine, and Colletotrichum lindemuthianum*. The available data will facilitate in understanding the response of plant to FSG infection to improve strategies to increase plant defenses by decreasing the quantity and composition of the plant's phenylpropanoid combinations through genetic engineering.

2. Materials and Methods

To date, various methods have been developed and introduced to measure how fungal infection affects soybeans. In most recent studies, fungal infection has been measured in different ways, in this work the various techniques have been used as TLC, HPLC, and SDS agarose gel. Two types of susceptible soybean cultivar (*Glycine max* L.) were collected from El Rayed city, Kingdom of Saudi Arabia for germination and growing fungal isolates. PDA was prepared according to the procedure used by [6]. The estimation of phytoalexins was done and synthesized by using Chromatographic techniques. This is the typical protocol followed in such experiments.

Fungal Isolate:

F. solani f. sp. Phaseoli and F. solani f. sp. glycine, separated with various levels of violence were designated for use in the current work depending on the findings of former experiments on over 123 isolates (Li *et al*., 2002) at microbiology department of faculty of science, Shaqra University, Kingdom of Saudi Arabia. Fungal cultures were kept on 2% water agar (w/v) at 4^0C or stored in 15% (v/v) glycerol at -80^0C. On another hand, a non-pathogenic to soybean called; *C. lindemuthianum* was also involved for comparative purposes. All pathogen isolates were primarily tested for pathogenicity on soybean hypocotyls and whole grown seedlings under room temperature that was appropriate for successful infection.

Gathering and testing of Root models

In the direction of *F. solani f. sp. glycines* inoculum production, (80 cm^3 of grains were soaked in tap water overnight inside Erlenmeyer flasks (250 ml). Separation of debris and floating seeds from the soaked seeds. Following soaking, the grains were washed three to five times with tap water. Extra washing water was drained, and the grains were autoclaved at 121^0C for 40 min for two successive days. Each flask was individually crawling and isolated by transporting five (4-mm-diameter) plugs from the edge of a 2-week-old *F. solanif. sp. Glycines* culture on water agar. Cultures in flasks were incubated in dark at a temperature of 23^0C and shaken manually every other day to encourage constant fungal growth. Post14 days of incubation, infested seeds were used to inoculate soybean.

Fungal isolates Media Preparation according to the guidelines of Media preparations.

1. Potato dextrose agar medium: Fresh potato slices approximately 169 g of extract have been added to a 20-gram solution of glucose.
2. Czapex Dox medium: Sucrose (23 g) was added to the following, NaNO3(3 g), K2HPO4(1.00 g), MgSO4.7H2O (0.50 g), KCl (0.50 g), FeSO4 (0.01 g) and agar (20.0 g) and then with added distilled water to complete the volume to 1 liter.
3. *Colletotricum spp*. growth medium was prepared by adding 1.8 g to 1.52 g of magnesium sulfate with another 1.78 g of potassium Dihydrogen phosphate.

On one hand, a simple setup has been employed here by growing all tested *Fusarium* isolates on PDA medium, the preferred incubation parameters employed for 7 - 10 days at a temperature of 25 or 30°C. On another hand, the isolate of *C. lindemuthianum* was incubated for 7 to 10 days at 20°C. Stock cultures were consequently kept on PDA slopes and covered with sterilized mineral oil under room temperature or at 4°C, until use. Agar plates were inoculated from the hyphal tips (10 days old cultures) from different fungal isolates. The standard fungal spore suspensions applied for the inoculation of soybean plants/organs were taken from cultures (7 to 10 days old).

Nonpathogenic cultures for comparison with pathogenic isolates

Technically speaking, the *Fusarium* sp. isolated from infected tomato has been applied in the current work for assessment of the pathogenic isolates to soybean. It was observed that an isolate of *F. solani* was not capable for induction of infection to the non-host soybean plants. The isolate of Fusarium tomato was applied to inoculate the integral hypocotyls of soybean after inoculation of the hypocotyls at 30°C for 4 h for soybean tests, followed by inoculation at the same places of infection with the pathogenic isolates of fungi (*F. solani f. sp. Glycine, F. solani f. sp. Phaseoli* and *C. lindemuthianum*). Once the hypocotyls injected merely with the pathogenic isolates, this system known as positive control or "cultivar-pathogen/ susceptible (non-induced) combination" or. Nonetheless, at what time the hypocotyls inoculated solitary with the non-pathogenic isolate, this combination called "mock control".

Detection of disease through fungal inoculation

Six contamination places were tried for each fungal disengage and control planning each time point

determined per test; three recreate tests were carried out. The segments were regularly reserved for each fungal vaccinated-hypocotyl a ways off at any rate 0.5 mm separated and dissected. Comparative response types were picked to permit a definite assessment of both of the host tissue and the parasitic disconnect in the contemplated blend. Distracting and longitudinal segments were cut off at various immunization destinations of the hypocotyls, 0.0, 4, 6, 8, 12, 24, 36 and 48 h after vaccination. Hypocotyls were recolored with a 1% methylene blue for 2 min. what's more, were seen under the light magnifying lens. Likewise some of the time 10 µl of toluidine blue stain (0.25% w/v) was utilized for 4 min. Over the top toluidine and methylene blue were assimilated from the slide, and the areas were cleared and mounted in lacto phenol. This method was utilized to recognize parasitic material in plant tissues. On account of serious staining of necrotic cells, contagious hyphae could be barely seen. Weaken arrangement used to wash off overabundance color. Naturally cut areas were mounted after 3 min in NaOH (0.1 N) and analyzed.

Scientific assessments of phytoalexins

1. Phytoalexins glyceollin extraction process from soybean

Glyceollin was extricated and evaluated by a changed strategy for the extraction of phytoalexins that was completed as follows. Hypocotyl tissue was macerated with 8 ml of 85 % ethanol per g crisp load in a blender for 5 min, and left in the dissolvable for 1 hour in obscurity. The concentrate was separated through a Buchner pipe and the plant material washed with a further equivalent volume of 80% ethanol. The ethanolic removal has vanished under vacuum at 40 ^{0}C to 1/5 of its underlying volume. The pH of this watery arrangement was acclimated to pH 3.0 with 1N HCl. The fermented remove was then shaken multiple times with ethyl acetic acid derivation in an isolating channel. The consolidated natural dissolvable stages were dissipated in a revolving evaporator at 40 ^{0}C. The separated material, got by this methodology, were kept in brisk fit cylinders in the profound cooler until use.

2. Chromatographic Practices

TLC (Thin layer chromatography) technique

Thin-layer chromatography (TLC) was utilized to isolate glyceollin from different mixes. Glyceollin was isolated from different mixes inside the concentrate utilizing TLC with the dissolvable blend 95:8, toluene: methanol. The glyceollin containing band (Rf = 0.14) was segregated and seen under UV radiation at 254 nm and afterward, glyceollin eluted with 95% ethanol [23].

HPLC (High-performance liquid chromatography) technique

Reversed-phase HPLC was achieved to glyceollin study where glyceollin concentration in each sample was resolute using the same HPLC system but then again the absorbance was measured at 286 nm [24] using a gradient of acetonitrile and acidified water (Milli-Q water at pH 3.0 with phosphoric acid) with the ratio of 1:1. Glyceollin was a molecular weight of 310.3. The identification of the phenolic compounds was achieved by comparing their retention times with those from authentic standards.

SDS-PAGE for peroxidase iso-enzymes (Protein)

Sodium dodecyl sulphate poly acrylamide gel (SDS-PAGE) electrophoresis of proteins was carried out in 11.25% polyacrylamide gels, under conditions that ensure dissociation of proteins into their polypeptide subunits and that minimizes aggregation. The strong anionic detergent Sodium dodecyl sulphate (SDS) was used after mixing with B-Mercaptoethanol (2ME) as reducing agent and heat to disconnect the proteins before they were loaded onto the gel. The complex of denatured polypeptide and SDS become negatively charged by bounding SDS to the backbone of the polypeptide chain. When proteins were treated with SDS and 2ME, the polypeptides become rods of negative charges with equal charge unit per length. Here, a discontinuous PAGE system was used as described by [25].

The chemicals are as follows:

Stock solutions:

Monomer solution (Acrylamide: bisacrylamide / 30:0.8%) acrylamide (30 g) and N-N-methylene bisacrylamide (0.8 g) were dissolved in 60 ml distilled water and made up to 100 ml. The monomer solution was stored in an amber color bottle at 4 ℃.

Resolving / Separating gel buffer [pH 8.8]

This solution was prepared by dissolving 22.7 gm. Tris base in 60 ml of distilled water and adjusted the pH. The final volume was made up to 100 ml with distilled water and stored in an amber color bottle at 40C.

Sodium Dodecyl Sulphate 10% (SDS 10%)

SDS (10 g) was dissolved in distilled water (100 ml).

Stacking gel buffer [pH6.8]

The stacking gel buffer was prepared by adding 7.2gm of Tris dissolved in distilled water (60 ml). The final volume (100ml) was completed with distilled water, then the pH 6.8 was adjusted and finally stored in an amber color bottle at 40C.

Sample loading buffer:

It was prepared as Tris/HCl (0.1M), 6.8 pH, glycerol (20%,W/V), 4% SDS, 1% (V/V) DTT and 0.2% (V/V) bromophenol blue.

Running buffer

Tris (50mM), glycine (384 mM) and SDS (0.1%, W/V), all adjusted to 8.3pH. It was prepared by

dissolving 3 g Tris, 14.4 gm. glycine, 10% (W/V) SDS in one liter of distilled water.

10% ammonium persulphate (APS) was freshly prepared.

TEMED, used as supplied as a yellow reagent always kept in dark bottles

3. Results

In this study, an amalgamation of the morphological method, light microscopy, histochemistry, and biochemical analysis has revealed that:

Induced changes (phenotypic reactions induced) on cellular and morphological levels in the tested cultivars,

- No visible reactions
- Macroscopically visible reactions

Table (1): Constituents of 12.5% separating and 4% stacking gels for SDS-PAGE.

Stock solution	Resolving gel	Stacking gel
Acrylamide solution	4 ml	650 µl
1.5 M Tris-HCl pH 8.8	2.7 ml	–
0.5 M Tris-HCl pH 8.8	–	1.25 ml
Distilled water	3.8 ml	3.05 ml
10% SDS	125 µl	50 µl
10% APS	83.3 µl	25 µl
TEMED	13.3 µl	8 µl
Total volume	10.5 ml	5 ml

Subsequent, the induced reactions were explored in more detail using light microscopy. Hypocotyls tissue of soybean was analyzed at 0.0, 4, 6, 8, 12, 24, 36 and 48 hours after inoculation. The first morphological variations were only observed at 6 hours at 30 ^{0}C which further increased within 24h.

To auxiliary analyze the cellular changes induced by the isolates of fungal pathogens under study, the histochemical analysis was also performed to inspect cell wall reactions in the form of possible lignification. **Figures 1, 2, 3 and 4** give an impression of the lignified cell wall visualized within a plant cell using toluidine blue, cellular changes are accompanied by hypersensitivity. Cell death was observed **(i.e. macro and microscopically visible cell death)**.

The microscopic analysis is an excellent technique to uncover potential triggering defense mechanisms that are operating early in the resistance induced in the studied pathosystems combinations of this study. Representative example photographs are shown.

Induced resistance of soybean counter to the target pathogens *F. solani f.sp. Phaseoli, F. solani f.sp. Glycine* and *C. lindemothianum*.

The following reaction types, induction as a result of inoculating the selected CVS. of soybean hypocotyls (intact) with the nonpathogenic *Fusarium* tomato isolate, that is served as an abiotic elicitor in this study, and 4 hours later inoculated with each of the pathogenic isolates of the three pathogens i.e. *F. solani f.sp. Phaseoli, F. solani f.sp. glycine and C. lindemothianum*. Both of the tested isolates (the original and the reisolated one) for all the three target pathogens behaved similarly, **Table (2).**

Table (2): Cultivars reactions to each of the tested isolates *F. solani f.sp. phaseoli, F. solani f.sp. glycine and C. lindemothianum* (The original and the reisolated one) at 30^{o}C temperature.

Fungal isolate	Incubation temperature (C)	Cultivars of *Glycine max.* L.	
		Crowford	Giza 22
A	30	R	Is
B	30	R	Is

*= intact hypocotyls (excised) of 10 days old seedlings of different cultivars of soybean. A = the original isolate.
B = the reisolated isolate.
R = resistant, hypersensitive flecks only.
Is = intermediate reaction tending towards susceptibility.

From the above results, it is obvious that the two isolates behaved the same. It was clear as revealed by the size of necrotic lesions was reduced visibly on the induced hypocotyls (Fig.1) indicating that the nonpathogenic isolate (*Fusarium* tomato isolate) could be effectively used in protecting the Fusarium diseases of soybean of this study. Also has the ability to induce resistance against further infection by the pathogenic *Colletotrichum lindemothianum*.

Infection of soybean intact hypocotyl by *F. solani*

f.sp. Phaseoli **(the causal pathogen of root and hypocotyl rot of soybean):**

Experiments were carried out to check the resistance and susceptibility in soybean cultivars at temperature, 30^{0}C. The gained culture of the pathogen along with the reisolated one was each inoculated onto hypocotyls when plants were 9-10 days old.

Infection sites were locked resistant when hypersensitive flecks were seen 1-2 days after inoculation and infection progressed no further. They were classed susceptible when longitudinal spreading streaks accompanied by collapse and rotting of hypocotyl tissue were observed**.** More details can be assumed as follow:

At 30^{O}C:

Macroscopic observations exhibited a few brown necrotic spots in the inoculation sites,1 day after inoculation of hypocotyls of cv. Crowford, these flecks remained unchanged and very few number of hypocotyls became moderately rotted also the root system became rotted with brown exudates **(Figure 1).**

On the other hand, the inoculated hypocotyls of cv. Giza 22 developed numerous brown spots within 1 day after inoculation. One day later, these flecks elongated forming streaks then these hypocotyls became rotted and brown **(Figure 3)**.

Table (3): Reactions of different cultivars of soybean to inoculation with *F. solani* f.sp *phaseoli* at 30 C.

Symptoms									**Final Reaction type**
Cultivar	**Time (hours) after inoculation**								
	0	**4**	**6**	**8**	**12**	**24**	**36**	**48**	
Crowford	N	F+	F+	F+	F++	F++	F++	$F^{++}/B^{+}/rot^{-}$	R
Giza 22	N	F++	F++	F+++	F+++	st	st	st/B^{++}/r ot++	Is

N = no reaction.
F+ = very few brown flecks are observed at the inoculation sites. F++ = few brown flecks are observed at the inoculation sites.
F+++ = numerous brown flecks are observed at the inoculation sites. St = brown streaks are observed at the inoculation sites.
B = browning of hypocotyls tissue; represent grades of browning intensity as follows:
B+ = Light brown, B++ = Moderate brown. Rot- = hypocotyls are not rotted.
Rot+ = hypocotyls are slightly rotted. Rot++ = hypocotyls are moderately rotted.
R = resistant, flecks and slight browning of hypocotyls without rotting.
Is =intermediate reaction tending towards susceptibility, the presence of limited streaks accompanied by moderate browning and moderate rotting of hypocotyls.

Accordingly to observations of the infection type monitoring 10-15 days after inoculation with *F. solani* f.sp. *phaseoli.*

Fig. 1. Reaction of hypocotyls of cv. Crowford resistant (R), 1 day after inoculation with *F. solani* f.sp. *phaseoli* at 30^{o}C. Note the few brown necrotic flecks in the inoculation sites

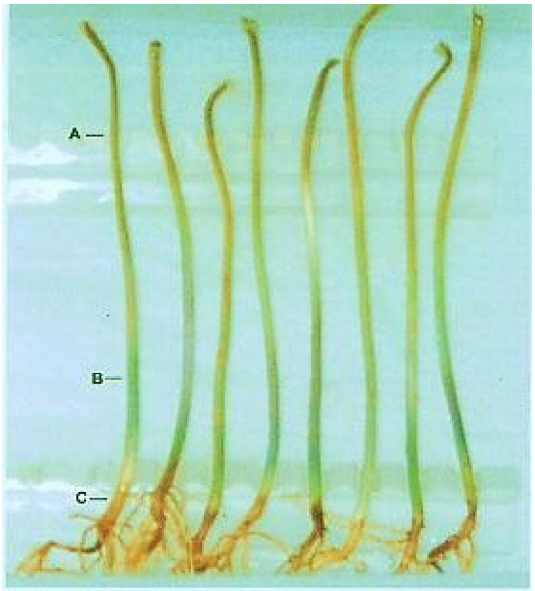

Fig. 3. Reaction of hypocotyls of cv. Giza 22 (intermediate Susceptible), 1 day after inoculation with *F. solani* Susceptible), 1 day after inoculation with *F. solani*

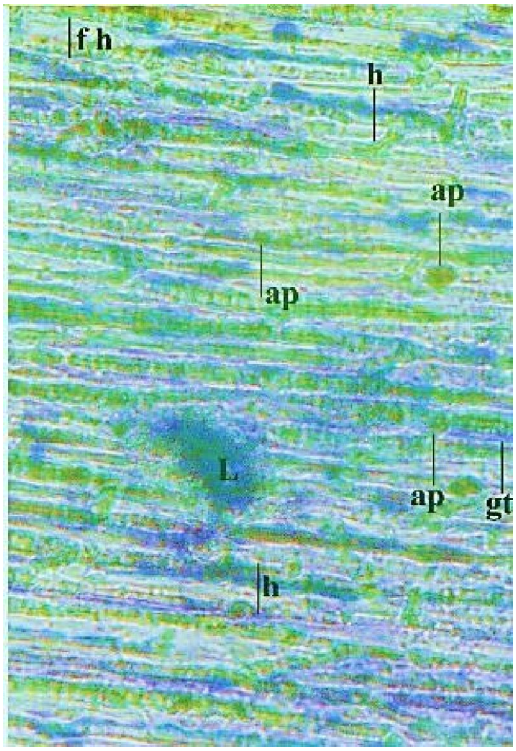

Fig. 2. Surface view of cv. Crowford (resistant) hypocotyl, 1 day after inoculation with *F. solani* f.sp. *phaseoli* at 30°C

Microscopic observations of the inoculation sites of cv. Giza 22 (intermediate susceptible) hypocotyls showed that 1 day after inoculation, fungal hyphae penetrated and extended longitudinally and intercellular **(Fig. 4)**. The germ tubes of macro conidia formed a hyphal network on the surface of the hypocotyls. After penetration, hyphae extended in all directions. They are observed clearly within the epidermal and cortical cells, 1 day after inoculation. The browning of the infected cells and aggregation of fungal hyphae were also observed.

On the other hand in cvs. Crowford resistant hypocotyls, after 1 day of inoculation there was no more development in germinating of the fungal hyphae within the hypocotyl tissue (Figure. 2). And the reaction was characterized by a restriction of the majority of fungal hyphae within the stomata leading to closing them. These regions were stained with blue color then dark blue also no more progressing in germination of penetrating hyphae was observed.

Phytoalexins production

Phytoalexins production, namely glyceollin was monitored at 24 h after inoculation of soybean cultivars (cvs. Giza 22 and Crowford) with the pathogenic *F. solani* f.sp.*phaseoli,* after challenged with the nonpathogenic fungal pathogen i.e. non related Fusarium pathogen, namely Fusarium tomato isolate. Healthy cultivars were also subjected to similar analysis. Total content of glyceollin was determined. The concentration of glyceollin was 135740 and 37886 gg^{-1} biomass dry wt, as evaluated by the area peak of glyceollin at retention time in the range of 20.56-20.72, in healthy cv. Giza 22 and cv. Crowford respectively. But with the induced systems, the concentration of glyceollin was 294814 and 482243 gg^{-1} biomass dry wt respectively.

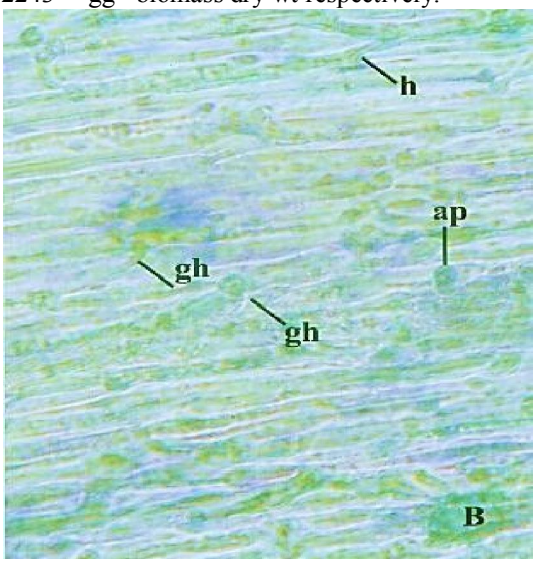

Fig. 4. Surface view of cv. Giza 22 intermediate susceptible (Is) hypocotyl, 1 day after inoculation with *F. solani*

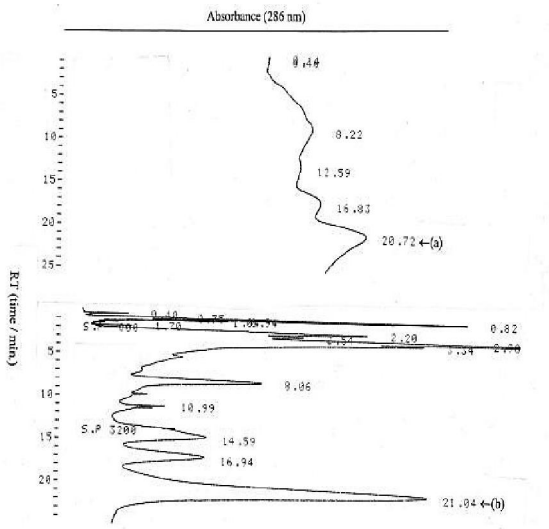

Fig. 5. HPLC-chromatograms of phytoalexin glyceollin extracted from healthy hypocotyls of soybean cv. Crowford (a) with absorbance area 378860 gg^{-1} biomass dry wt and retention time (RT) 20.72, and glyceollin extracted from cv. Crowford / *F. solani* f.sp. *phaseoli* induced combination, 1 day after inoculation at 30 C (b) with absorbance area 482243 gg^{-1} biomass dry wt. Absorbance at 286 nm and retention time (RT) 21.04.

Table (4): Phytoalexins production, glyceollin was measured after inoculation of cultivars of soybean at 30 C with the nonpathogenic fungal pathogen (i.e. Fusarium tomato isolate) for 4 h, then challenged with the pathogenic *F. solani* f.sp. *phaseoli* for 24 h.

	Soybean (*Glycine max* L.)			
	Control cultivars (healthy)		Cultivar-pathogen (induced) combination	
	Crowford	Giza 22	*F. solani* f.sp. *phaseoli*	
			cv. Crowford	cv. Giza 22
Flow rate (ml /min.)	1	1	1	1
Absorbance (nm)	286	286	286	286
Retention time (RT)	20.72	20.56	21.04	21.63
Absorbance area (Conc.)	378860	135740	482243	294814
Absorbance area (%)			78.5	46
Potential source as an inducer			+++	+
Chemical class	Petrocarpenoids (Glyceollin)			

* represented samples out of three replicates

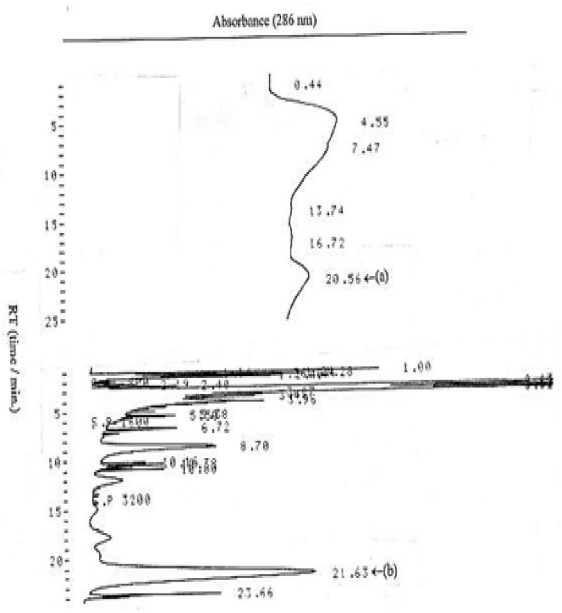

Fig. 6. HPLC-chromatograms of phytoalexin glyceollin extracted from healthy hypocotyls of soybean cv. Giza 22 (a) with absorbance area 135740 gg^{-1} biomass dry wt and retention time (RT) 20.56, and glyceollin extracted from cv. Giza 22 / *F. solani* f.sp. *phaseoli* induced combination, 1 day after inoculation at 30 C (b) with absorbance area 294814 gg^{-1} biomass dry wt. Absorbance at 286 nm and retention time (RT) 21.63.

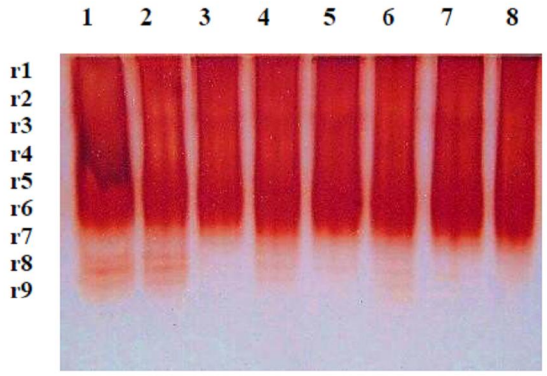

Fig. 7. Electrophoretic banding profile (zymogram) of peroxidase isozyme in soybean cvs. Giza 22 and Crowford inoculated with Fusarium tomato isolate for 4h, then inoculated with *F. solani* f.sp. *phaseoli, F. solani* f.sp. *glycine* and.
C. lindemothianum separately (i.e. cultivar-pathogen / resistant (induced) combination) for 24 hours at 30°C.
Lanes:
1 = *Glycine max* L. cv. Giza 22 inoculated with Fusarium tomato isolate (mock control).
2 = *Glycine max* L. cv. Crowford inoculated with Fusarium tomato isolate (mock control).
3 = *Glycine max* L. cv. Giza22 inoculated with Fusarium tomato isolate for 4 h, then inoculated with *F. solani* f.sp. *Phaseoli*
4 = *Glycine max* L. cv. Crowford inoculated with Fusarium tomato isolate for 4 h, then inoculated with *F. solani* f.sp. *Phaseoli* 5 = *Glycine max* L. cv. Giza22 inoculated with Fusarium tomato isolate for 4 h, then inoculated with *F. solani* f.sp. *glycine.*
6 = *Glycine max* L. cv. Crowford inoculated with Fusarium tomato isolate for 4 h, then inoculated with *F. solani* f.sp. *glycine.*
7 = *Glycine max* L. cv. Giza22 inoculated with Fusarium tomato isolate for 4 h, then inoculated with *Colletoterichum lindemothianum*
8 = *Glycine max* L. cv. Crowford inoculated with Fusarium tomato isolate for 4 h, then inoculated with *Colletoterichum lindemothianum*

Peroxidase induction in soybean (*Glycine max* L.) in response to microbial challenge: Soybean (*Glycine max* L.) cultivars

Figure (7) and Table (6) presented the zymogram of banding pattern of peroxidase isozymes and the existence vs. lack of Electrophoretic bands for two soybean genotype inoculated with Fusarium tomato isolate for 4 h, then inoculated with *F. solani* f.sp. *glycine, F. solani* f.sp. *phaseoli,* and *C. lindemothianum* (i.e. cultivar-pathogen / resistant (induced) combination) at 30^0C for 24 hrs compared to healthy cultivars. Three bands No. r7, r8 and r9 were established in each of induced and healthy cultivars. Also these bands (r8 and r9) were present in cv. Crowford that inoculated with *F. solani* f.sp. *glycine* and *F. solani* f.sp. *phaeoli* whereas, not found with the rest of cultivars indicating the resistance of cultivar Crawford. So these bands can be deliberated as a possible marker linked with cv. Crawford only when that inoculated with *F. solani* f.sp. *glycine* and *F. solani* f.sp. *phaseoli.* R3 and r6 were specifically associated with cv. Crowford regardless the pathogen involved. It is interesting that r3 and r4 characterized the induced resistance developed in all of cultivar Crawford tested interactions. All soybean combinations were regarded as indicators for the induction of peroxidase isozymes as a result of the resistance induced against the tested fungi. The results were summarized in Tables (5 and 6).

Table (5): (1) and (0) presence and absence of band in the position corresponding to peroxidase isozymes of soybean (*Glycine max.* L.) cvs Giza 22 and Crowford inoculated with Fusarium tomato isolate for 4 h, then inoculated with *F. solani* f.sp. *phaseoli*, and *F. solani* f.sp. *glycine* and *C. lindemothianum* separately (i.e. cultivar-pathogen / resistant (induced) combination) for 24 hours at 30^oC.

Rows (No. of bands)	Soybean (*Glycine max.* L.)							
	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
r1	1	1	1	1	1	1	1	1
r2	1	1	1	1	1	1	1	1
r3	0	1	0	1	0	1	0	1
r4	0	0	1	0	1	0	0	0
r5	0	0	1	0	1	0	0	0
r6	0	1	0	1	0	1	0	1
r7	1	1	1	1	1	1	1	1
r8	1	1	0	1	0	1	0	0
r9	1	1	0	1	0	1	0	0

Table (6): (+) and (-), presence and absence of band in the position corresponding to peroxide isozyme of cultivar-pathogen / resistant (induced) combination of soybean, 24 hours after inoculation with *F. solani* f.sp. *phaseoli, F. solani* f.sp. *glycine* and *C. lindemothianum* at 30 C for soybean.

No. of Row	**Soybean (*Glycine max* L.)**							
	Control		**Cultivar-pathogen (induced) combination**					
	cv. Giza 22 (healthy) (lane 1)	cv. Crowford (healthy) (lane 2)	*F. solani* f.sp. *phaseoli*		*F. solani* f.sp. *glycine*		*C. lindemothianum*	
			cv. Giza 22 (lane 3)	cv. Crowford (lane 4)	cv. Giza 22 (lane 5)	cv. Crowford (lane 6)	cv. Giza 22 (lane 7)	cv. Crowford (lane 8)
r1	+	+	+	+	+	+	+	+
r2	+	+	+	+	+	+	+	+
r3	-	+	-	+	-	+	-	+
r4	-	-	+	-	+	-	-	-
r5	-	-	+	-	+	-	-	-
r6	-	+	-	+	-	+	-	+
r7	+	+	+	+	+	+	+	+
r8	+	+	-	+	-	+	-	-
r9	+	+	-	+	-	+	-	-

4. Discussion

Plants depend on constitutive and prompted mechanical and biological defensive systems for safeguard from infective pathogens. Distribution of induced defensive system may lead to systemic-induced resistance.

In this study, it was experiential that in direct cuticular penetration of the tested pathogenic fungi of hypocotyls, hyphae raise sub cuticular before attacking the rest of the tissues intercellular. Plentiful chlamydospores are molded on the surface and inside the outer cortex of the necrotic lesions. That lesion tissue of soybean (cv. Giza 22) hypocotyls and roots which are infected with *F. solani f.sp. phaseoli*, covers cell wall degrading enzymes which cause tissue softening and protoplast death.

Resistance in the plants are commonly principal to susceptibility, and in an avirulence pathogen is leading to virulence. If a host privations a specific resistance gene, the corresponding a virulence gene cannot be noticed and a resistance response is not originated [26]. The changes amid incompatible and compatible interactions are obsessed by the continuous replacement of new avirulence and new resistance genes, leading to an arms race among host and pathogen.

At this time, histological studies signposted that hyphal growth of *F. solani* f.sp. *phaseoli* and *F.solani* f.sp.*glycine* is repressed early in resistant soybean cultivar Crowford but not in susceptible cv.Giza22. Hypersensitivity was found to be common in a extensive range of plant host-pathogen interactions. Likewise, hypersensitivity as a host incompatibility response involves rapid death of plant cells accompanied by restriction of growth and spread of the pathogen whether the pathogen be a fungus, a bacterium or a virus [27].

From the above results it was obvious that the two isolates (the original pathogen and reisolated one) behaved the same.

It was clear as revealed by the size of necrotic lesions was reduced visibly on the induced hypocotyls (Fig.1) indicating that the nonpathogenic isolate (Fusarium tomato isolate) could be effectively used in protecting the Fusarium diseases of soybean of this study [28]. Also has the ability to induce resistance against further infection by the pathogenic *Colletotrichum lindemothianum*. Experiments were carried out to check the resistance and susceptibility in soybean cultivars at temperature, 30 C. The obtained culture of the pathogen as well as the reisolated one was each inoculated onto hypocotyls when plants were 9-10 days old [29].

The current results show infection sites were closed resistant when hypersensitive flecks were seen 1-2 days after inoculation and infection progressed no further. They were classed susceptible when longitudinal spreading streaks accompanied by collapse and rotting of hypocotyl tissue were observed [30]. [31]have shown that host cell death is associated with an accumulation of phytoalexins which have a role in disease resistance.

[32] reported that phytoalexins are low molecular weight antimicrobial compounds synthesized by plants de novo upon infection. They represent an extremely diverse group of secondary metabolic compounds including isoflavonoid, pterocarpans, stilbenes and saponins. In some cases, accumulation of phytoalexins has been shown to be instrumental in disease resistance.

[33] pointed out that the host-inflectional formation of phytoalexins and the observation that they are not normal constituents of non-infected plant tissues indicates that they are products of metabolism arising from host-parasite interactions. In general, all parts of the soybean plant (Root, Stem, cotyledons, and true leaves) are capable of producing phytoalexins although the specific phytoalexins, and its amount can vary with the plant part and the conditions to which it is subjected [34].

[16] reported that, glyceollin is phenylpropanoid-derived phytoalexins and it is formed as part of a general defensive response of the plants. Many workers reported that phenolic compounds are found at high levels in resistant cultivars of soybean plant compared to susceptible ones. From the above mentioned results, in soybean, the HPLC analysis showed production of glyceollin in both healthy and the induced systems. I.e. Fusarium pathogen and the non-pathogen, both have the ability to induce phytoalexins (glyceollin) production in soybean systems (cvs. Crowford and Giza 22) studied [35]. However, a remarkable accumulation was shown with the induced systems as compared with healthy ones (Figs. 5 and 6). Further, the soybean cultivar Crowford was proved to exhibit a potential source for phytoalexins production (with respect to glyceollin) among the other tested cultivars of soybean.

Signaling pathways activate a series of defense responses that curb or eliminate the pathogen. These responses include the hypersensitive response (HR), up regulation of phenylalanine ammonium lyase (PAL), a key enzyme in plant defense, deposition of cell wall reinforcing materials, and synthesis of a wide range of antimicrobial compounds including pathogensis related (PR)-proteins and phytoalexins [36].

Peroxidases can be induced locally in response to infection but enhanced activity has also been associated with induced systemic responses.

Peroxidase activities have been correlated with plant resistance and responses to pathogens and are most likely involved in the oxidation of phenolic residues into cell wall polymers in pathogen-infected tissues. This includes the substrates used for polymerization of lignin and suberin [37].

The above results of SDS peroxidase iso-enzymes electrophoresis results demonstrate that, many bands are characteristic for the tested cultivars and others are induced as a result of the host- pathogen interaction systems. Three bands no. r7, r8 and r9 were found in each of healthy and induced cultivars [38]. But these bands (r8 and r9) were found in cv. Crowford that inoculated with F. *solani f.sp. phaeoli* and *F. solani f.sp. Glycine* while absent with the rest of cultivars indicating the resistance of cultivar Crawford. So these bands can be considered as a potential marker associated with cv. Crawford only when that inoculated with *F. solani f.sp. phaseoli* and *F. solani f.sp. glycine.* R3 and r6 were specifically associated with cv. Crowford regardless the pathogen involved. This indicates that they were associated with pathogenesis especially in case of cv.Crowford. It is interesting that r3 and r6 characterized the induced resistance developed in all of the cultivar Crawford interactions. All soybean combinations were regarded as indicators for the induction of peroxidase isozymes as a result of the resistance induced against the tested fungi [39]. The current results show the presence of three common bands (r1, r2 and r7) in the tested cultivars (control). Also were found in the induced combinations, indicating that all of them are not related with pathogenesis.

The obtained results explain that the bands r8 and r9 were observed in both of the soybean cvs. Crowford and Giza 22 (healthy), but they were detected in the cv. Crowford/ *F. solani f. sp. Phaseoli* and cv. Crowford/ *F. solani f. sp. Glycine* induced systems. Its absence from the other tested combination systems, pointed to its possible involvement in the induced resistant interaction mentioned above. Whether these bands are representing a proteins related pathogenesis of defense types, is not clear since the susceptible contributions were not subjected in this study for this type of analysis. The induced bands r3 and r6 for only cv. Crowford/ (*F. solani f. sp. Phaseoli* and *C. Lindemothianum*) induced resistant system, indicating that the host plant (soybean) reactivated the pathogensis of these isolates regardless its origin. The current results of SDS peroxidase iso- enzymes analysis indicated that the specific bands of each soybean cultivar and the elicited one as a result of the interaction between the host and the pathogen [40].

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