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ACCUMULATION OF PHYTOALEXINS AND THEIR ROLE IN RESISTANCE OR SUSCEPTIBILITY OF POTATO TUBER SLICES INOCULATED WITH *FUSARIUM SOLANI* INCUBATED AT TWO REGIMES OF TEMPERATURE

Maha H. Mohamed* and Mostafa H. Mostafa

Plant Pathology Department, Faculty of Agriculture, Ain Shams University, 1124 Cairo, Egypt.

***Corresponding Author: Maha H. Mohamed**
Plant Pathology Department, Faculty of Agriculture, Ain Shams University, 1124 Cairo, Egypt.

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ABSTRACT

The present investigation was aimed to study the role of phytoalexins accumulated in potato tuber slices inoculated with *Fusarium solani* caused the potato dry rot on disease resistance or susceptibility. Potato tuber slices inoculated with *F. solani* were incubated at two different regimes of temperature *i.e.*, 16°C or 25°C. Disease severity on slices incubated at 25°C was very higher than that found on slices incubated at 16°C. This observation was opposite to phytoalexins accumulated at 16°C whereas the intensity of phytoalexin spots separated by TLC was very intensive than that accumulated at 25°C. The role of phytoalexins accumulated in potato tuber slices inoculated with *F. solani* incubated at 16°C was studied by inhibiting of biosynthesis of them in inoculated slices by HMG-CoA reductase (3-hydroxy-3-methylglutaryl-CoA reductase) inhibitor. This treatment led to increase disease severity of *Fusarium* infection accompanied by disappearance of phytoalexin spots on TLC and benzene extracts of such slices had no effect on spore germination and germ tube length when spores were germinated in it. These results strongly indicated the role of phytoalexins in disease resistance especially at 16°C.

KEY WORDS: Potato dry rot, phytoalexins, temperature, HMG-CoA reductase, rosuvastatin, TLC.

INTRODUCTION

Potato dry rot causing by *Fusarium* spp. is devastating disease of tubers during storing conditions. It could cause complete loss of tubers either in storage or during their cultivation **(Hooker, 1981; Turkensteen 2005; Wale 2008).** Since this phenomenon has very bad impact on agricultural economics, therefore, many studies were carried out on this disease around the world **(Seppanen, 1983; Theron and Holz, 1991; Cullen *et al.*, 2005; Daami-Remadi *et al.*, 2012)**.

One aspect of these studies was focused on the effect of temperature degree of storing of potato tuber on disease incidence and severity. It was found that the best temperature of incubation ranges from 10-35 ˚C, and below this degrees disease severity reduces due to that cold temperature has a harmful effect on *Fusarium* growth and its sporulation **(Daami-Remadi *et al.*, 2006a, and b)**.

It is well established in many laboratories that potato tuber tissues infected by *Fusarium* spp. accumulate sesquiterpenoid stress metabolites SSM (phytoalexins) in infected tissues, therefore the role of phytoalexins in resistance of potato tuber to *Fusarium* infection became doughty process **(Varns *et al.*, 1971; Jadhav *et al.*, 1991; Mostafa, 2018).**

In the present study, a comparison between to regimes of temperature of incubation of inoculated potato tuber slices by *Fusarium solani*, the causal agent of potato dry rot in relation to accumulation of phytoalexins in inoculated tissues was studied.

Moreover, phytoalexin biosynthesis was inhibited in inoculated potato tuber tissues using specific inhibitor of HMG-CoA reductase (3-hydroxy-3-methylglutaryl-CoA reductase) enzyme which consider the key enzyme in phytoalexin synthesis in relation to resistance or susceptibility of inoculated potato tuber tissues inoculated by *F. solani* and this effect was studied on such phenomenon.

MATERIALS AND METHODS

Potato tubers and preparation of slices

Potato tubers cv. Balmoral were kindly obtained from Institute of Vegetable Research, Agriculture research center, Giza, Egypt and stored for one month under refrigerator condition. Upon used, tubers were rinsed in tap water to remove any adherent soil particle then left to dry. Before using, tubers were surfacely sterilized by

$NaClO_3$ (5%) for 5 min. then washed several times by sterilized distilled water (SDW) and left to dry at room temperature. Tubers were sliced into slices (1 cm thick) with exclusion of top and basal parts, and then washed several times by SDW to remove the residual parts of starch; left to dry then transferred to large Petri dishes (15 cm in diameter) contained wetted filter paper and every dish contained 4 slices.

Fungal isolate

A pure culture of *Fusarium solani* (Mart.) Sacc which previously isolated from potato tubers showed dry rot symptoms and proved its ability to cause severe rot on potato tuber slices was subculture on slants of potato sucrose agar medium (PSA) for 8 days. Spore suspension in sterilized distilled water from these slants was prepared and adjusted to be $5X10^5$ spores/ml then used as inoculums.

Inoculation of potato tuber slices

Slices in Petri dishes were inoculated by 2 ml of fungal spore suspension and gently spread on slice surface, then incubated at either 16 ^oC or 25 ^oC in the dark for 72 h then disease severity was determined on each slice. Three dishes every one contained 4 slices were used for each particular treatment and every experiment were replicated 3 times.

Determination of disease severity

After incubation of inoculated slices for 72 h, disease severity was determined by two parameters : i) by counting of spores produced on inoculated slices and expressed as No. of spores/$cm^2$of inoculated surface, and ii) by determining the percentage of area slices covered with fungal mycelium.

TLC separation of sesquiterpenes accumulated in potato tuber slices (phytoalexin)

Equal areas of the upper surface of inoculated slices was removed (2 mm thick) then dipped in benzene for several days to extract of sesquiterpenes accumulated in potato tuber tissue due to fungal infection. Benzene phase was collected in clean beaker then left to dry at reduced pressure till dryness. The residues were dissolved in 1 ml of chloroform. 25 µl of chloroform was spotted on silica gel plate (Fluka layer thickness 0.2 mm). Different separation system was trialed: Chloroform: acetone 7:3; MeOH-$CHCL_3$ (1:19), MoOH-ET_2O (1:19); Et_2 O **(Stoessl *et al.*,1976).**

Chromatograms were stained either by vanillin (1% in 50% aq. phosphoric acid) at 110^oC, or phosphomolybdic acid (5% in alcohol) or they were subjected to UV lamp.

Effect of incubation of inoculated potato tuber slices under two levels of temperature on disease severity and phytoalexin accumulation in potato tuber slices

Slices of potato tuber were inoculated by *Fusarium solani* spore suspension then divided into two groups; the first was incubated at 16^oC and the others at 25^oC for three days. After incubation the percentage of surface covered by fungal mycelium mats was estimated and photographed, then each slice was divided into 2 equal portions; the first was used to estimation of sporulation and the second for extraction of phytoalexins.

Effect of HMG-CoA reductase (3-hydroxy-3-methylglutaryl-CoA reductase) inhibitor on accumulation of phytoalexins in potato tuber slices inoculated with *Fusarium solani*

Potato tuber slices were treated before inoculation with *Fusarium solani* by the compound rosuvastatin which known medically as Crestor. Tested compound was grinded then suspended in SDW and prepared in three tested concentrations *i.e.* 5, 10 or 20 mg/100 ml in distilled water. The upper surface of slices was dipped in particular concentration of the tested compound for one hour, left to dry then inoculated with the fungal spore suspension. Slices in Petri dishes were incubated at 16^oC for 3 days. After this period, inoculated slices were photographed and the percentage of inoculated surface covered by fungal mycelium mats was estimated. Every slice was divided into 2 equal portions; one of them was used for determination of sporulation and the other for extraction of phytoalexins in inoculated slices.

Effect of compounds extracted by benzene from inoculated potato tuber slices on spore germination of *F. solani*

The organic component of the upper 2 mm of Inoculated potato tuber slices by *F. solani* were extracted by benzene as previously mentioned after they had been incubated at 16 ^oC for 72 hr from inoculation. As mentioned above, inoculated slices either treated by phytoalexin inhibitor or not were extracted. Benzene extract was evaporated under reduced pressure then the residues were dissolved in 100 µl acetone thereafter, the final volume was completed to 1 ml by sterilized distilled water. Half ml. of this suspension was mixed with 0.5 ml *Fusarium solani* spore suspension ($5X10^5$ spores/ml.) then distributed on glass slides previously sterilized in Petri dishes and incubated at 25 ^oC for 24 h. Spore germination and germ tube length was examined microscopically and photographed.

Statistical analysis: Standard deviation of the averages of three distict experiment was calculated according to Ghahramani (2000).

RESULTS

Effect of temperature degree of incubation on potato tuber slices infection with *F. solani* in relation of phytoalexin accumulation with resistance or susceptibility

Potato tuber slices cv. Balmoral was prepared for inoculation by spore suspension of *F. solani*. Inoculated slices or not were incubated either at 16^oC or at 25^oC for 72 h. Data of this study are presented in Figs (1, and 2) illustrated by Photo (1). It is clearly shown from this Data that temperature of incubation plays considerable

role in the ability of *F. solani* for infection of potato tuber slices. The higher degree of incubation led to very severe infection as it causes great incidence of the percentage of slice surface colonized by mycelium mats and considerable increase of sporulation of the pathogen on colonized tissues in comparison to slices incubated at 16°C.

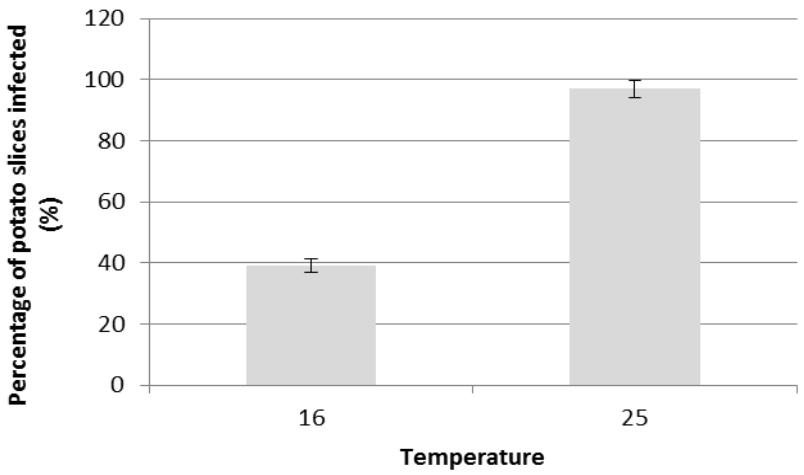

Fig. (1): Percentage of potato tuber slices area covered by mycelia of *Fusarium solani* due to inoculation by fungal spores incubated either at 16°C or 25°C for 72 h.(average of three distinct experiments)

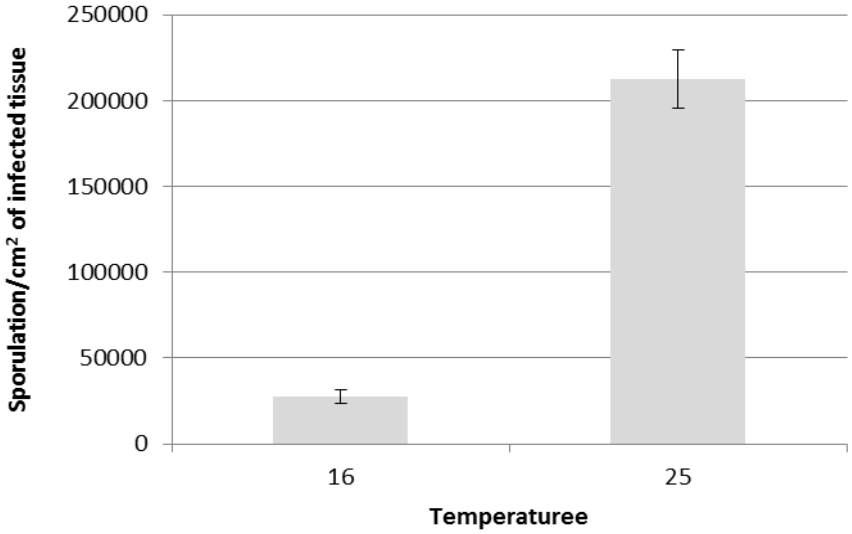

Fig. (2): Spore count of *Fusarium solani* on inoculated surface of potato tuber slices incubated at 16°C or 25°C for 72 h. (average of three distinct experiments)

Photo (1): Comparative between potato tuber slices inoculated by spore suspension of *Fusarium solani* incubated either at 16°C or 25°C for 72 h.

Sesquiterpene stress metabolized was extracted either from healthy or inoculated slices incubated at 16^{O}C or 25^{o}C by benzene then its components were separated by TLC using different systems of immobilization. It was found that chloroform: acetone (7:3 v/v) was the best system which gives the best resolution of benzene extract component. Separated bands were visualized either by exposure to UV light, phosphomolybdic acid in methanol or vanillin phosphoric acid to insure that separated bands belong to sesquiterpene compounds. Separated bands were photographed as indicated in Photo (2).

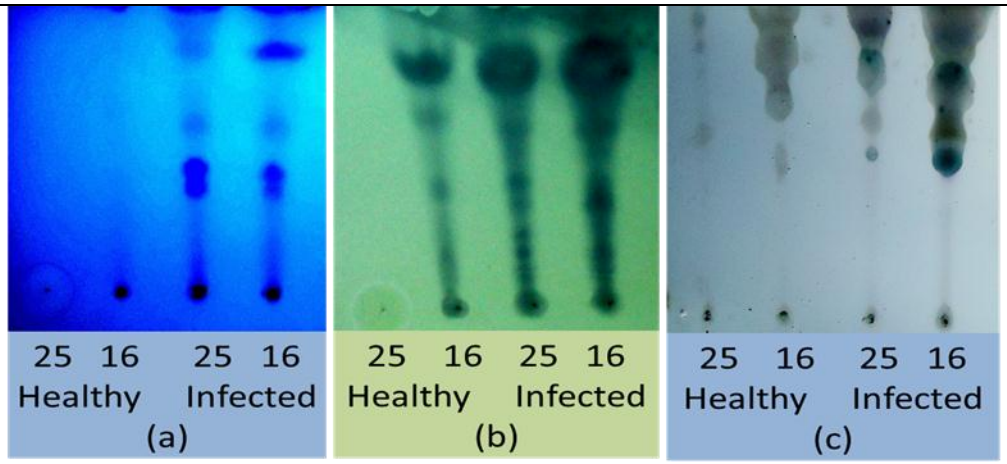

Photo (2): Visualization of potato tuber sesquiterpene stress metabolites either by exposure to UV light (a), phosphomolybdic acid (b), or vanillin phosphoric acid (c) separated by TLC extracted from healthy or inoculated potato tuber slices incubated at 16^{o}C or 25^{o}C.

It is clearly shown from this photos that exposure of silica gel contained separated compounds to UV light gave the best resolution of separated compounds in comparison to phosphomolybdic acid in ethanol or vanillin phosphoric acid. The last two reagents gave positive result with compounds isolated from healthy non inoculated potato tuber slices. Therefore, in further study UV light was used to visulate separated compounds.

As shown from photo (2a) four spots were observed with Rf values 0.50, 0.57, 0.74 and 0.88. Pattern of separated compounds from slices inoculated with *F. solani* is approximately similar but the intensity of all spots from slices incubated at 16^{o}C especially the compound No.4 with Rf 0.88 was very intensive in comparison with compounds isolated from slices incubated at 25^{o}C.

Effect of the inhibitor of SSM synthesis on disease severity due to *F. solani* inoculation on inoculated potato tuber slices incubated at 16^{o}C

In this study, potato tuber slices before inoculation was treated with the compound known as SSM inhibitor (rosuvastatin) at three tested concentrations *i.e* 5, 10 or 20 mg/100 ml water in addition to control. Data obtained illustrated by Figs (3 and 4) and photo (3) indicate clearly that inhibition of SSM accumulation in inoculated tissues led to great increase of disease severity and such increase is proportionally correlated with the increasing of inhibitor concentration.

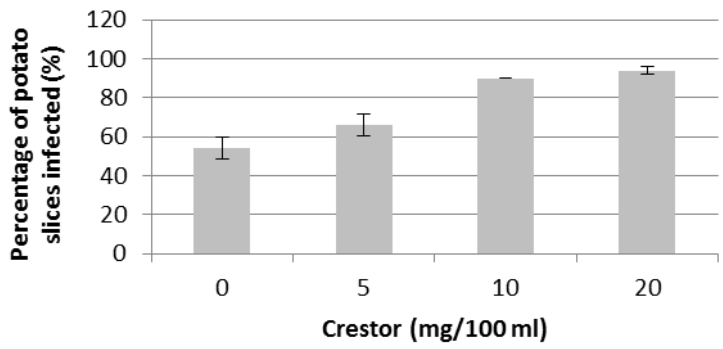

Fig. (3): Effect of different concentrations of phytoalexin inhibitor on severity of infection of potato tuber slices by *Fusarium solani* determined as the percentage of slice surface covered by fungal mycelia. (average of three distinct experiments).

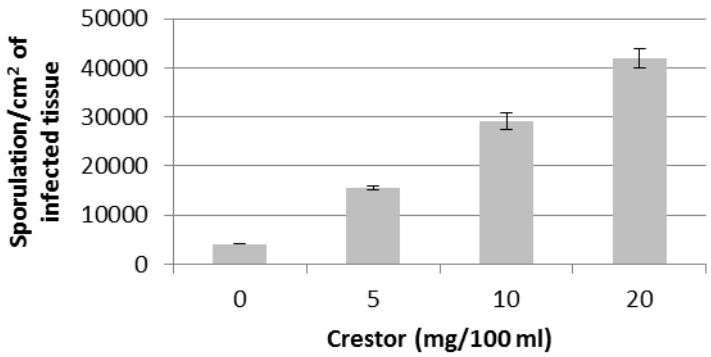

Fig. (4): Effect of different concentrations of phytoalexin inhibitor on severity of infection of potato tuber slices by *Fusarium solani* determined as No. of spores/cm^2 formed on slice surface. (average of three distinct experiments).

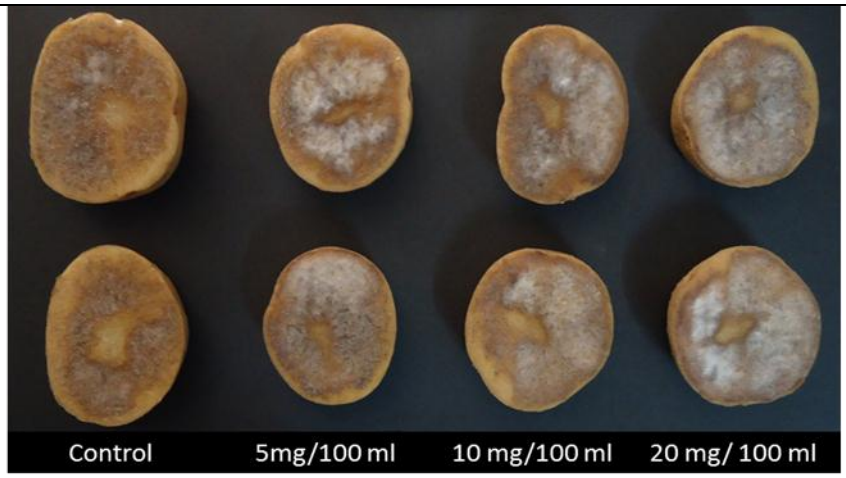

Photo (3): Effect of different concentrations of phytoalexin inhibitor on showing infection type of slices inoculated with *Fusarium solani* the causal of potato dry rot.

Separation of SSM compounds by TLC clearly indicated the disappearance of separated compounds from inoculated by *F. solani* slices due to the treatment with phytoalexin synthesis inhibitor especially at the higher concentration of rosuvastatin.

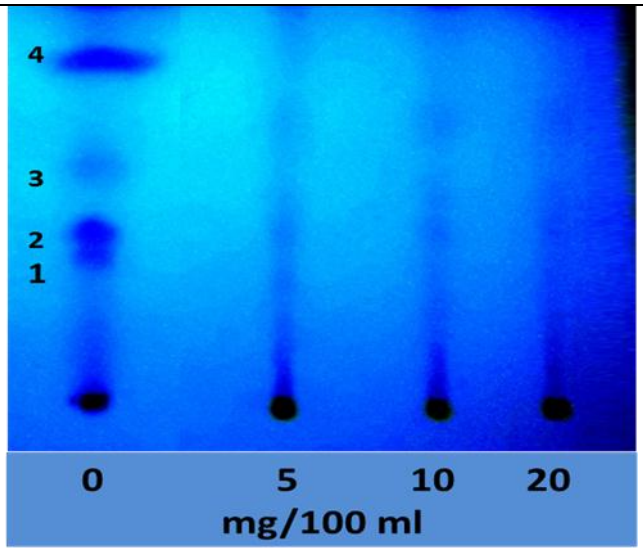

Photo (4): TLC analyses of sesquiterpenes isolated from potato tuber slices inoculated with *F. solani* either treated or not by sesquiterpene biosynthesis inhibitor rosuvastatin. Chloroform: acetone 7:3 v/v was used as immobile phase.

Effect of components of benzene extract from inoculated potato tuber slices by *F. solani* on spore germination and germ tube length of *F. solani in vitro*

SSM from potato tuber tissues treated or not with SSM inhibitor and inoculated with *F. solani* was extracted by benzene. Benzene extract was evaporated under reduced pressure till dryness and the residue was dissolved in 100µl acetone and the final volume was completed by SDW to one ml. Half ml. of such suspension was mixed with 0.5 ml of *F. solani* spore suspension and left to germinate. Data obtained illustrated by Figs (5 and 6) and Photo (5) indicate clearly that component of benzene extract of control slices have very sever toxic effect on spore germination. Inhibition of SSM accumulation in infected tissues reflexes their effect whereas spore germination and germ tube length was slightly affected.

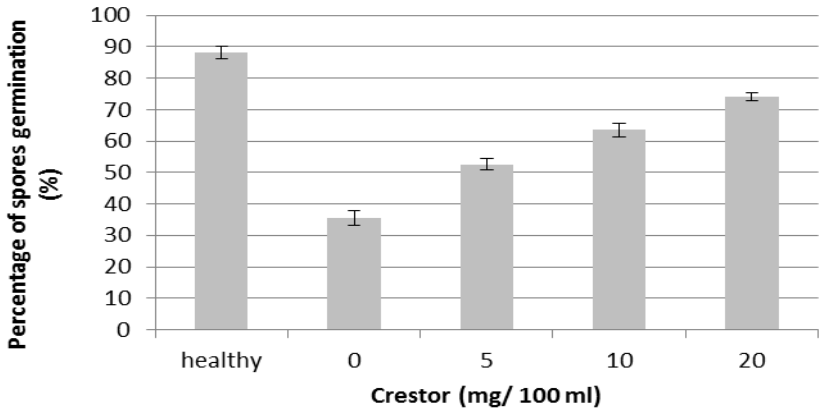

Fig. (5): Effect of benzene components of potato tuber slices treated or not with phytoalexin synthesis inhibitor inoculated with *Fusarium solani* on spore germination of it. (average of three distinct experiments).

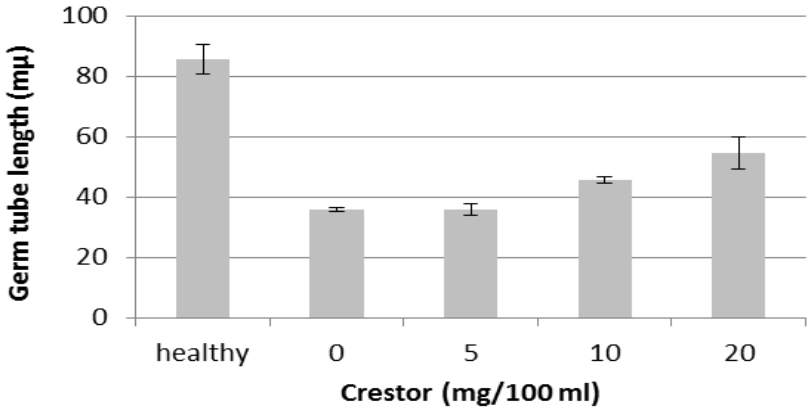

Fig. (6): Effect of benzene components of potato tuber slices treated or not with phytoalexin synthesis inhibitor inoculated with *Fusarium solani* on germ tube length of the pathogen. (average of three distinct experiments).

Photo (5): Morphological feature of *Fusarium solani* germ tube germinated in benzene components of potato tuber slices treated or not with phytoalexin synthesis inhibitor inoculated by it. a; control non treated non inoculated slices, b; non treated inoculated slices, c; slices treated by 5 mg/ 100 ml water, d; slices treated by 10 mg/100 ml water, e; slices treated by 20 mg/100 ml water.

DISSCUSION

Since **Muller and Burger (1940)** have adopted the phytoalexin concept, many published scientific works was focused on phytoalexins of many plant species published from many laboratories all over the world. These works firstly focused on phytoalexin compounds from potato tuber tissue inoculated by incompatible races of *Phytophthora infestans*, the causal agent of potato and tomato late blight. Their intensive research resulted in isolation and identification of many phytoalexin compounds. **Stoessl *et al*. (1976)** have summarized these compounds in a very excellent review. Potato phytoalexins were found that they belong chemically to sesquiterpene compounds and they included rishitin, lubimin, phytoberin, rishitanol, hydroxylubimin, rishitanol, anhydro-ß-rotunol and solavetivone **(Tomiyama *et al.*, 1968; Katsui *et al.*, 1971&1974 and Coxon *et al.*, 1974).** The type of phytoalexin produced by a given plant greatly depends upon the pathogen used in a study and the variety of potato tuber (**Stoessl *et al.*, 1976).**

Kuc *et al.*, (1979) intensively studied the effect of temperature of incubation of potato tuber slices inoculated by incompatible races of *Phytophthora infestans* and by other non pathogenic fungi on elicitation of phytoalexins in inoculated tissues. They revealed that accumulation of phytoalexins in inoculated tissues was higher at 19^{o}C than at 14^{o}C or 25^{o}C.

In the present study potato tuber slices inoculated by *F. solani* were incubated either at 16^{o}C or 25^{o}C for 72 h. It was observed that slices incubated at 25^{o}C showed very severe infection comparing with slices incubated at 16^{o}C which showed slight infection accompanied by hypersensitive like reaction. Extraction and separation by TLC of phytoalexins associate with both types of infection indicated the presence of four compounds in both cases, but the intensity of these compounds was very higher in case of resistance. There are not any study was carried out in relation to accumulation of phytoalexins in potato tuber slices inoculated by *Fusarium* spp. in resistance of potato tuber slices to infection which may refer to incubation of inoculated tissue usually carried out at 23-25^{o}C and under this condition, slices induced very low amount of phytoalexins **(Kuc *et al.*, 1979).**

It was found that phytoalexins of potato tuber are considered sesquiterpene compounds **(Stoessl *et al.*, 1976)** and the key step in biosynthese of such compounds is synthesis of mevalonic acid and the later is synthesized by action of HMG-CoA reductase on HMG-CoA (ß-hydroxy-ß-methylglutaryl-CoA) **(Bach *et al.*, 1990).** This is the key step in biosynthesis of sesquiterpene in plants or animals.

In the present study a compound known as Rosuvastatin (medicaly known as Crestor) the specific inhibitor of HMG-CoA reductase **(Mc Taggart *et al.*, 2001)** was used in order to inhibit this key compound in biosynthesis of potato phytoalxins. The compound was tested in three concentrations *i.e.*, 5, 10 or 20 mg/100 ml distilled water by dipping potato tuber slices in particular concentration for one hour, then inoculated by *F. solani* spore suspension and incubated at 16^{o}C for 72 h in the dark. Observation led to speculate that rosuvastatin inhibitor severely caused severe infection of inoculated slices in comparison to non-treated inoculated slices which showed very slight infection accompanied by hypersensitive reaction. Moreover, phytoalexin bands were disappeared specially at 20 mg/100 ml. water. In this respect **Tomma *et al.*, (1999)** found that deficiency in phytoalexin production caused enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria bassicicola*. This finding indicated that synthesis of phytoalexin by plant during fungal pathogenesis consider the key stone of plant disease resistance.

Benzene extract of inoculated potato tuber slices non-treated with this inhibitor caused inhibitory effect when tested on spore germination of the pathogen, meanwhile extract of treated slices by inhibitor eliminate its toxic effect on *F. solani* spore germination *in vitro*.

The obtained results clearly indicated that phytoalexin accumulation in potato tuber slices special at 16^{o}C play an important role in resistance of potato tubers to *Fusarium* spp. infection.

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