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Activation of systemic disease resistance by acibenzolar-S-methyl and a non-pathogen *Fusarium oxysporum melonis* (FOM) strain against Fusarium wilt disease in eggplant seedlings*

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ABSTRACT

The plant defence activator acibenzolar-S-methyl (ASM; Actigard 50 WG) and a non pathogenic *Fusarium oxysporum* strain (FOM; *Fusarium oxysporum* f. sp. *melonis*) were assayed on eggplant seedlings for its ability to induce resistance against ***Fusarium*** oxysporum ***f. sp. melongenae***. Pre-treatment of eggplants with ASM and FOM significantly reduced the severity of the disease. The lowest disease ratings were detected at a time interval of 72 h between treatment and pathogen inoculation. With this interval, disease severity in ASM and FOM-treated plants was reduced to 30.1% and 20.1%, respectively, while positive control was 91.0%, at 21th day after inoculation. Microscopical studies showed a strong correlation between the interval of inducer-pathogen inoculation and lignin accumulation in xylem cells undergoing hypersensitive reactions, whereas no staining were observed in negative control plants. Intense lignin accumulation in xylem vessels indicate both treatments are able induce resistance in eggplant against this disease.

Key Words: Fusarium Wilt, Induced Resistance, Non-pathogen *Fusarium oxysporum*, ASM, Lignin, Eggplant

INTRODUCTION

Fusarium wilt, caused by *Fusarium oxysporum* Schlecht. f. sp. *melongenae*, is one of the most important disease of eggplants in especially Mediterranean Region of Turkey which lead to serious yield losses. Under optimal infection conditions, this soilborne pathogen can create necrotic areas by colonizing xylem tissues of susceptible varieties which blocks water and nutrient transfers, resulting death of the plant (Altınok, 2005). Due to the high inoculum density, control of soil-borne plant pathogens are

extremely difficult. Soil disinfestation and fungicide application have been commonly used for controlling these pathogens. There are several studies worldwide on identification of sources of resistance against Fusarium wilt disease on eggplant but currently there are no resistant varieties reported. Pathogen's physiological races are yet to be defined and there is only one record of vegetative compatibility group (VCG) on literature (Katan, 1999). It was reported that vegetative compatibility group (VCG-0320) of Turkey isolates, and were found compatible with European VCGs (Altınok and Can, 2010).

In recent years, many methods have been developed for biocontrol of soil borne plant pathogens by the researchers. Studies on biological control of *F. oxysporum* f. sp. *melongenae* (Fomg) in eggplant are very limited. In plant protection, antibiosis, competition and hyperparasitism are primary antagonistic mechanisms on controlling the diseases with biotic factors. Biological control including beneficial microorganisms is an alternative method for protection of Fusarium wilt diseases (Alabouvette and Couteaudier 1992). Certain microorganisms can be also protected plants by inducing plant defense mechanisms, along with their antagonistic efficiency, for supression of fungal diseases (Kuć, 1982; Matta, 1989). Today, several non-pathogenic bacteria and fungi biopreparations are in use as successful antagonistic agents against many plant diseases. Induced resistance as a natural defense mechanism of plants is one of the ecologically-friendly approaches for plant protection. This inducible defence mechanism as named systemic acquired resistance (SAR) is effective against many virulent plant pathogens including, bacteria, fungi and viruses (Ryals et al., 1994). Some chemicals such as salicylic acid (SA), β-amino butyric acid (BABA), 2,6-dichloroisonicotinic acid (INA) with no direct antimicrobial activity can also induce SAR as well as biotic inducers in plants (Hammerschmidt and Kuć, 1995; Lawton et al., 1996; Oostendorp et al., 2001). SAR is associated with its several cellular defence responses including pathogenesis-related (PR) proteins, synthesis of different defence-related enzymes, rapid and transient production of active oxygen species (AOS), phytoalexins (Benhamou and Belanger, 1998). The plant defence activator acibenzolar-S-methyl (benzo [1,2,3] thiadiazole-7-carbothioic acid-S-methyl ester, ASM) is a systemic compound used for the control of many fungal and bacterial diseases in vegetables (Cole, 1999; Elmer, 2006). The compound has minimum antifungal and bacterial activity, but induces host plant resistance by triggering a natural systemic activated resistance (SAR) response found in most plant species. This chemical inducer may be phytotoxic when applications made in the early stage of plants (Conrath et al., 2001).

Pre-treatment of susceptible plants with avirulent pathogens may induce resistance to pathogen attack (Kuć, 1982). Several reports have documented the inducible mechanism to Fusarium wilt by using nonpathogenic strains of *F. oxysporum* (Fuchs et al., 1997; Bora and Özaktan, 1998) or formae speciales of *F. oxysporum* such

as f. sp. *melonis* in cucumber (Gessler and Kuć, 1982) and f. sp. *dianthi* in tomato (Kroon et al., 1991). Induced resistance requires that the plant be exposed to the inducing agent prior to the attack by the pathogen. The time needed for development of induced resistance is in the range of one to a few days in the case of Fusarium wilt diseases (Matta, 1989). The role of pathogen-induced lignins and related polymers has been closely correlated with the defence responses of several plants. Some of the most extensive work has dealt with lignification in graminaceous plants including reed canary grass and wheat (Vance et al., 1976). Lignin or the lignification process may role in plant defence against infection by mechanical barriers to pathogen attack, increasing the resistance of plant cell walls to the diffusion of toxins from the pathogen and plant cell wall to be more resistant pathogen cell-wall degrading enzymes (Ride, 1978).

The objective of this work was to test the plant defence activator Acibenzolar-S-methyl (benzo [1,2,3] thiadiazole-7-carbothioic acid- S-methyl ester, ASM; Actigard 50 WG, Syngenta Crop Protection, Inc., Basel Switzerland) and *Fusarium oxysporum* formae speciales nonpathogenic on eggplant (FOM; *Fusarium oxysporum* f. sp. *melonis*) and for its ability to induce resistance in eggplants against Fusarium wilt in climatize conditions.

MATERIALS AND METHODS

Plant material

Eggplant seedlings (*Solanum melongena* L. **cv**. "Pala**"**) with four fully expanded leaves were used for pot experiment. Plants were grown in pots (8.5 cm diam) in a soil mix containing sand, perlite, and peat compost in the greenhouse and kept in growth chambers (25°C, 60-70% RH, 12-h photoperiod, 50 to 60 Klux m^{-2}). Seedlings were watered daily and fertilized with NPK (15:15:15).

Application of ASM

ASM was dissolved in distilled water to obtain a concentration of 0.2 mg ml^{-1} and then sprayed twice to eggplant seedlings. *Fomg*10 (*Fusarium oxysporum* f. sp. *melongenae*) as the most virulent isolate by means of DS in a former study were selected to pathogen inoculation (Altınok and Can, 2010). In order to determine the optimum time interval for SAR induction, seedlings were first treated with ASM and then inoculated with the pathogen (*Fomg*10) suspension 24 h, 48 h, 72 h or 96 h after treatment using the root-dip assay modified from that of Biles and Martyn (1989). Pathogen inoculum consisted of spore suspension obtained from one-week-old culture on Potato Dextrose Agar (PDA; Merck, Germany) and each seedling with wounded roots was submerged for 10 min, with 100 ml of the conidial suspension (1×10^6 conidia ml^{-1} in sterile H_2O), while control plants were sprayed with sterile distilled water instead of ASM.

Inoculation of non-pathogen *Fusarium oxysporum*

A *Fusarium oxysporum* formae speciales nonpathogenic on eggplant (FOM; *Fusarium oxysporum* f. sp. *melonis*) was used as a biotic inducer in pot experiments. Pathogen and inducer fungus were cultured on PDA and *Fusarium* minimal medium (FMM); for 7 days in the dark at 25 °C (Nelson et al., 1983). Eggplants were inoculated with the pathogen (Fomg10) 24 h, 48 h, 72 h or 96 h after FOM (10^6 spore ml^{-1}) treatment. Control plants were dipped with sterile distilled water instead of FOM suspension. After pathogen inoculation the seedlings were transplanted into plastic pots and kept in a growth chambers as described above.

Disease Assessment

Disease symptoms development was assessed at 7^{th}, 11^{th}, 14^{th}, 17^{th} and 21^{th} day after inoculation (DAI) with a Fusarium yellow rating of 0 to 4, in which 0 = no lesions, 1 = slight leaf chlorosis and necrosis, 2 = vein clearing on outer leaflets, 3 = yellowing and dropping of leaves, 4 = dead plant. Plants were evaluated individually and a mean percent disease severity index (DSI%) was calculated for each assessment day based on the scale values, according to Townsend-Heuberger formula below (Townsend and Heuberger, 1943).

$$P = \Sigma \frac{n \times v}{Z \times N} \times 100$$

In the formula, P; Percentage of disease severity, n; Number of plants in the disease scale, v; Numerical value of disease score, Z; Highest score number, N; Total number of plants. The data were subjected to an analysis with Levene's homogenity of variance test then grouped by Duncan's multiple range test ($P \geq 0.05$) contained in the SPSS software (SPSS Inc., Chicago, IL, USA). Both ASM and FOM experiments were conducted with three replicates of 10 seedlings and repeated twice, representative result of one experiment for each is presented.

Staining for lignin

Microscopic examination of infected vascular bundles with pathogen was carried out on a microscope (Nikon Optiphot), equipped with differential interference contrast (DIC). Photomicrographs were taken using Kodak Gold 200 ISO print film and developed commercially.

General tissue clearing

Infected vascular bundles were detached from plant then transferred to 100% methanol, and incubated overnight at room temperature, to remove chlorophyll, followed by soaking in a aqueous solution saturated with chloral hydrate (2.5 g ml^{-1}) for

12-24 h, to soften and clear the tissue (Soylu, 2006). Finally the vascular bundles were mounted in 50% glycerol and a cover-slip placed over the samples to produce semi-permanent preparations.

Lignified structures were visualized using the phloroglucinol/HCl test. Infected vascular bundles were incubated in a solution of 1% phloroglucinol in 100% methanol overnight. Following further incubation of cleared tissues in chloral hydrate, they were subsequently mounted on slides, a few drops of concentrated hydrochloric acid were added and finally the tissues were covered with a cover slip. After ~10 min, lignified xylem appeared reddish-orange, but colour faded within ~2-4 h, therefore, preparations were examined immediately (Gahan, 1984; Vallet et al.,1996; Soylu, 2006).

RESULTS AND DISCUSSION

The effects of the ASM and FOM treatments to Fusarium wilt disease

The time between initial treatment with ASM and FOM significantly protected the efficacy of induced resistance against damage from subsequent inoculation with *Fomg*10. All interval times drastically reduced the DS, but the greatest efficiency by means of disease suppression was achieved with ASM (Figure 1) and FOM treatment (Figure 2) 72 h before inoculation. In induced resistance experiment to both inducers and control plants, the initial symptoms appeared one week after inoculation as yellowing of the older leaves. The mean DS in control plants was 11.2% (ASM) and 12.3% (FOM) at 7 DAI. The systemic progress of the disease in control plants increased with time and by 21 DAI, browning areas were observed in the xylem of infected stems. Eventually, most of the plants collapsed and died. In 72 h inoculations, the mean DS in control plants reached 91% whereas ASM and FOM-treated seedlings were only 30.1% and 20.1%, respectively. Resistance induced in eggplant seedlings by ASM and FOM can be distinguished in Figure 1 and Figure 2. Since the lowest disease ratings were detected at a time interval of 72 h between treatment and pathogen inoculation, this interval was taken into consideration in order to detect lignin deposition in both of the pot experiments.

The determination of phenolics and lignin compounds that accumulate during infection has been based mainly on microscopy and histochemistry as described by Nicholson and Hammerschmidt (1992), lignin deposition in infected vascular bundles was demonstrated histochemically in this study as well. The stain was used here (phloroglucinol) in the presence of HCl, reacts with aromatic aldehydes, such as cinnamaldehyde, present in lignins and exhibits a bright reddish-pink colour in infected plant tissue. Results indicate a strong correlation between the timing and extent of cell death and high levels of lignin accumulation in cell-walls and cytoplasm of cells undergoing HR, whereas no staining of lignin-like material was observed during the

compatible interaction. The results showed that a significant proportion observed in eggplant vascular bundles undergoing HR is associated with rapid accumulation of lignin-like compounds indicated with phloroglucinol-HCl staining at sites by 11 DAI (Figure 3).

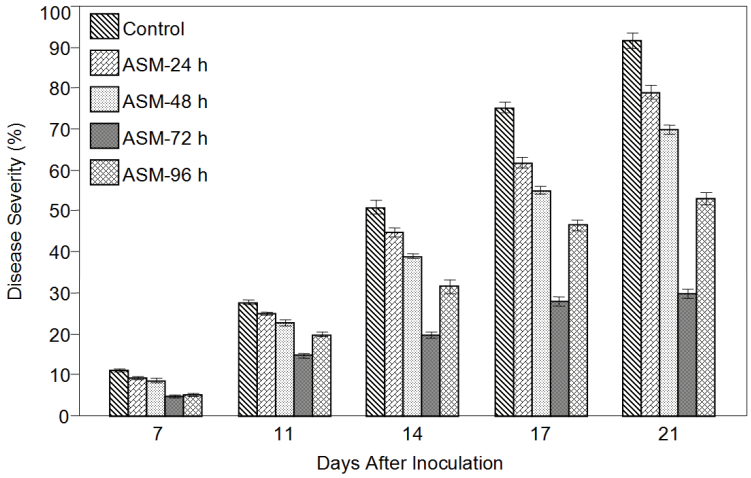

Figure 1. Effect of acibenzolar-S-methyl (ASM) treatment on the severity of Fusarium wilt disease caused by *Fusarium oxysporum* f. sp. *melongenae* (*Fomg*). After treatment with ASM or water (control), eggplant seedlings were inoculated 24, 48, 72, 96 h later with the *Fomg*10 isolate. Inoculated seedlings were scored at 7, 11, 14, 17 and 21th day after inoculation using 0-4 scale. A mean disease severity was calculated from each treatment. Error bars indicate ±1 standard error of the mean.

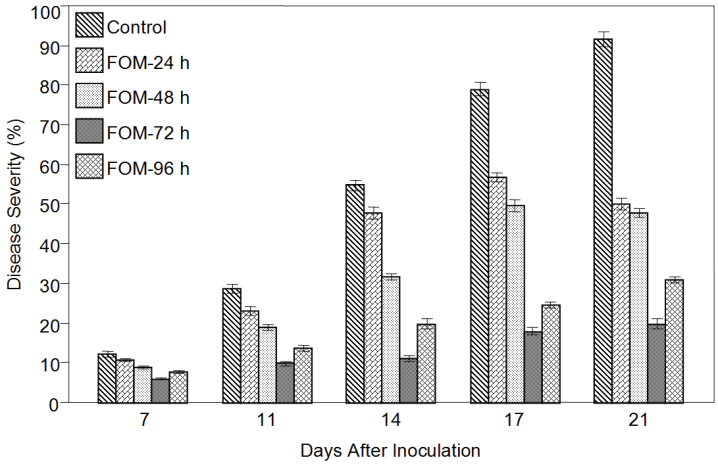

Figure 2. Effect of non-pathogen *Fusarium oxysporum* (FOM) treatment on the severity of Fusarium wilt disease caused by *Fusarium oxysporum* f. sp. *melongenae* (*Fomg*). After treatment with FOM or water (control), eggplant seedlings were inoculated 24, 48, 72, 96 h later with the *Fomg*10 isolate. Inoculated seedlings were scored at 7th, 11th, 14th, 17th and 21th DAI using 0-4 scale. A mean disease severity was calculated from each treatment. Error bars indicate ±1 standard error of the mean.

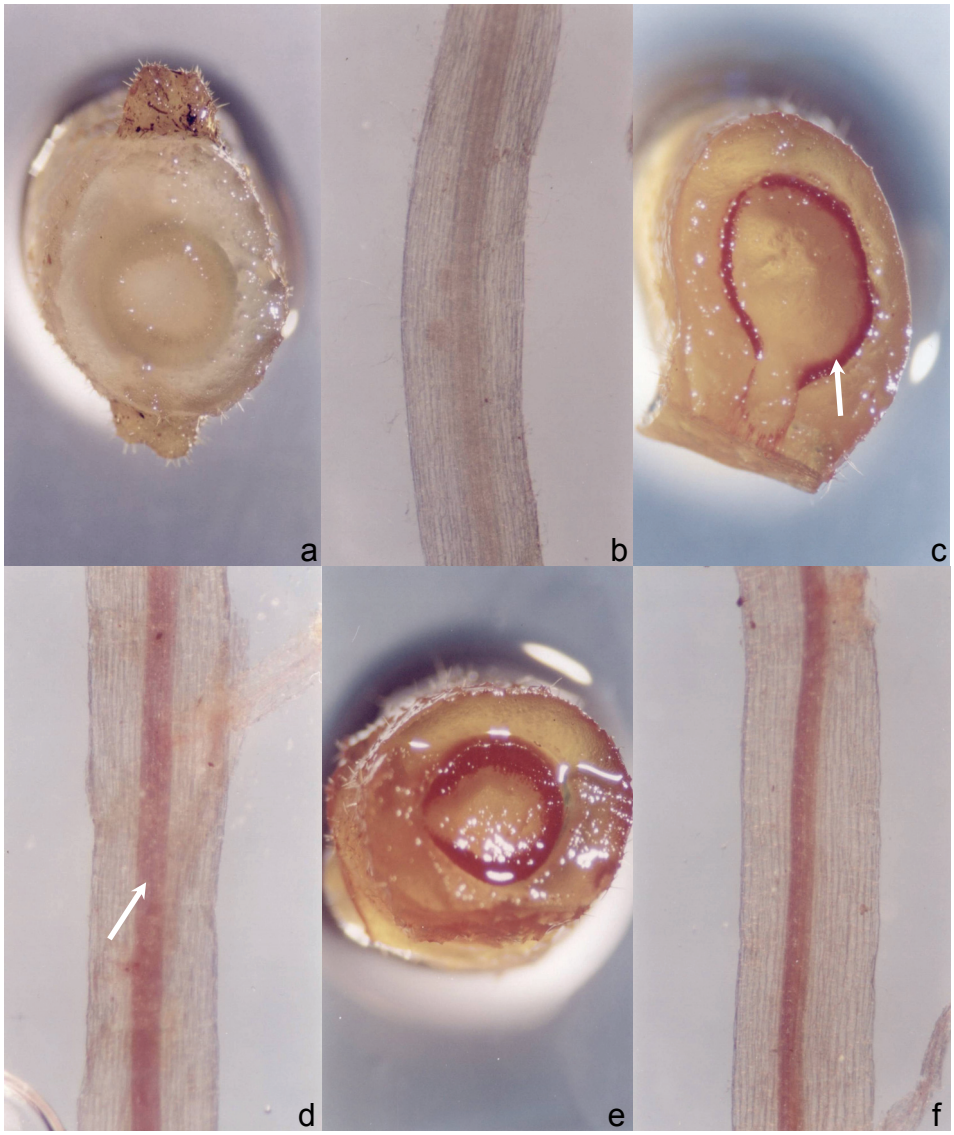

Figure 3. Localization of lignin-like compounds in the xylem of inoculated plants at 11th DAI. The eggplant seedlings inoculated with the *Fomg*10 isolate 72 h after ASM and FOM treatments. In (a) and (b), no staining in vascular bundles (control plant). The site of lignification seen as reddish-orange coloration, is localized only in xylem vessels (arrow) of ASM-treated plants (c and d). Similarly, accumulation of lignin-like compounds in xylem of FOM-treated plants (e and f).

The results show the inhibitory effects of the plant activator ASM and non-pathogenic *Fusarium oxysporum* strain on the disease development by *Fomg*. Both inducers induced important levels of disease resistance in eggplant seedling. Similarly, a

nonpathogenic strain of *F. oxysporum* protected cucumber against Fusarium wilt (Mandel and Baker, 1991). Induction of resistance by ASM has also been recorded in many plants (Cole, 1999; Narusaka et al., 1999; Elmer, 2006). In a resistance inducing experiment on eggplant against *Ralstonia solanacearum*, role of chitosan, salicylic acid, methyl salicylate and methyl jasmonate elicitors on cell wall strengthening and activation of defense enzymes were investigated. After elicitor applications, significant increase in total phenolic substance content were observed at roots. Peroxidase activity were found highest 24 h after CHT and SA treatments (Mandal, 2010).

Lignin deposition of infected vascular bundles was demonstrated histochemically within papilla and nearby walls in several plants (Aist, 1983). The detection of phenolics and lignin that accumulate during infection has been based mainly on fluorescence microscopy and histochemistry as discussed by Nicholson and Hammerschmidt (1992). In particular, histochemical stains have been used for localization of induced changes in cell wall polymers, which are insoluble and thus more difficult to quantitate by conventional means. The multifunctionality of lignins permits them to react with many different histochemical reagents to produce coloured products (Vance et al., 1976). Many studies agree that the accumulation of phenolics like lignin may be associated with cell death, thus being the first step in plant defence mechanisms in infected plants (Cohen et al., 1990). Lignin-like materials were localized in cells undergoing HR has been implicated in highly cultivar specific resistance expressed by wheat to the rust fungus *Puccinia recondita* (Southerton and Deverall, 1990). Although most of the studies on plant lignin deposition are based on fungal pathogens, bacterial infections may also lead to lignin accumulation (Soylu, 2006).

This is the first study on induction of resistance against the wilting agent, *Fusarium oxysporum* f. sp. *melongenae*, on eggplant. The results of the study demonstrate that susceptible eggplants enhance a systemically induced resistance to *Fomg* infection in response to ASM and FOM application. Similar observations have highlighted the ASM as a commercial product in activating SAR in tobacco (Friedrich et al., 1996) and tomato (Benhamou and Belanger, 1998).

In conclusion, the plant defence activator ASM seems to be a useful tool for induced resistance studies in eggplant as observed in other plant species. Cell-wall lignification at the reaction sites would be involved in resistance to non-pathogen *Fusarium* strains. A good knowledge on the mechanisms responsible for plant defense should be examined under commercial conditions. Further studies will be necessary to determine the association of enhanced pathogenesis-related (PR) proteins, especially chitinases and β-1,3-glucanases with systemic resistance.

ÖZET

ACIBENZOLAR-S-METHYL VE NON-PATOJEN *FUSARİUM OXYSPORUM MELONİS* (FOM) TARAFINDAN PATLICAN FİDELERİNDE FUSARİUM SOLGUNLUK HASTALIĞINA KARŞI SİSTEMİK DAYANIKLILIĞIN TEŞVİKİ

Bitki aktivatörü acibenzolar-S-methyl (ASM; Actigard 50 WG) ve patojenik olmayan bir *Fusarium oxysporum* (FOM; *Fusarium oxysporum* f. sp. *melonis*) streyninin patlıcan fidelerinde ***Fusarium oxysporum f. sp. melongenae***'ya dayanıklılığı teşvik etme yetenekleri araştırılmıştır. ASM ve FOM ile patlıcandaki ön uygulamalar hastalık şiddetini önemli derecede düşürmüştür. Hastalık şiddetindeki en büyük düşüş, uygulamadan 72 sa sonra patojen inokulasyonu verildiğinde elde edilmiştir. Bu aralık kullanıldığında, inokulasyondan sonraki 21. günde, pozitif kontrol bitkilerinde hastalık şiddeti %91.0, ASM ve FOM uygulanan bitkilerde ise sırasıyla %30.1 ve %20.1 olarak belirlenmiştir. Mikroskopi çalışmaları, teşvik edici-patojen inokulasyonu arasındaki süre ile hipersensitif reaksiyon gösteren ksilem hücrelerindeki lignin birikimi arasında güçlü bir bağlantı olduğunu göstermiştir. Ksilemde yoğun lignin birikiminin gözlenmesi, her iki uygulamanın da patlıcanda bu hastalığa karşı dayanıklılığı teşvik ettiğini göstermektedir.

Anahtar Kelimeler: Fusarium Solgunluğu, Uyarılmış Dayanıklılık, Non-patojen *Fusarium oxysporum*, ASM, Lignin, Patlıcan

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