The Journal of Animal & Plant Sciences, 27(1): 2017, Page: 309-315
ISSN: 1018-7081

THE ROLE OF DSPF, THE TYPE III SECRETION CHAPERONE OF DSPA/E OF *ERWINIA AMYLOVORA*, IN PATHOGENICITY IN HOST AND NON-HOST PLANTS

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ABSTRACT

Erwinia amylovora is a Gram-negative enterobacterium that causes a devastating blight disease on rosaceous plants. In this study, the structure of DspF, a type III secretion system (T3SS) chaperone required for virulence of *E. amylovora*, was investigated in bacterial growth and pathogenesis in host and non-host plants, including apple, strawberry, rose, *Arabidopsis* and tobacco. DspF genes were mutated through homologous recombination and the result was the arrest of disease development on apple, strawberry and rose, and of the hypersensitivity response in tobacco. In addition, bacterial development of the wild type was compared with that of *dspF* mutants in leaves of *A. thaliana* Columbia-5 (Col-5), and the resistant *A. thaliana Landsberg erecta* (Ler-0). It was concluded that DspF is essential for the bacterium to induce hypersensitive responce (HR) disease on host plants (Rosaceous family plant) and non-host plants (*Arabidopsis thaliana*).

Key words: *Erwinia amylovora*, dspA/E, dspF, pathogenesis.

INTRODUCTION

Erwinia amylovora is a Gram-negative enterobacterium bacterium that causes fireblight disease on *Rosaceae* family plants including apple (*Malus* spp.), pear (*Pyrus* spp.), quince (*Chaenomeles* spp.), strawberry (*Fragaria* spp.) and rose (*Rosa* spp.) (Zwet and Keil, 1979; Bradbury, 1986; van der Zwet *et al*., 2012; McNally, 2013). These plants are considered as host plants to *E. amylovora* as they show compatibility and susceptibility to the bacterium invasion. Especially on *Maloideae* subfamily such as apple and pear, *E. amylovora* causes tissue damages in the blossoms, shoots, limbs and entireplant (Ordax *et al*., 2015).

Like many other Gram-negative plant-pathogenic bacteria, *E. amylovora* has a type III secretory system (T3SS) that delivers effector proteins into host plants (Lindgren *et al*., 1986; Kim and Beer, 2000). The T3SS is used to translocate "effector" proteins across the host plasma membrane or secrete "helper" proteins that remain outside the host cell and assist the translocation of the true effectors (Cornelis and van Gijsegem, 2000). The effectors appear to interact directly with host cell components to alter defence pathways (Collmer *et al*., 2002; Innes, 2003).

In phytopathogens, the T3SS is encoded by hypersensitive response (HR), pathogenicity (*hrp*) and *hrp* conserved (*hrc*) genes, and it has been implicated in both the initiation of the disease during compatible interactions and the elicitation of plant defences, leading to resistance in incompatible interactions (Steinberger and Beer, 1988; Barny *et al*., 1990; Bogdanove *et al*., 1996). The hypersensitive response is a process of rapid local cell death at the infection site that restricts bacterial multiplication and is triggered by individual effector proteins in plants carrying a corresponding resistance gene (Dangl and Jones, 2001).

Within the *Erwinia* spp., the necrogenic fireblight pathogen *E. amylovora* has become a model for studying the *hrp* gene cluster. Most *hrp* genes have been found to encode proteins involved in gene regulation or in assembly of the T3SS apparatus (Kim and Beer, 2000; Alfano and Collmer, 2004; Nomura and Whittam, 2004). The proteins secreted by the T3SS of *E. amylovora* are HrpA, HrpN, HrpW and DspA/E. HrpA is the structural protein of the type III secretion pilus (Wei *et al*., 1992; Bogdanove *et al*., 1998a; Bogdanove *et al*., 1998b; Gaudriault *et al*., 1997; Kim and Beer, 1998; Kim and Beer, 2000; Wei *et al*., 2000; Jin *et al*., 2001).

T3SS chaperones presumably target their substrates on conserved region of the T3SS, such as the cytoplasmic ATPase that is associated with the secretory apparatus (Büttner and He, 2009). DspF aspecific chaperone for DspA/E involves in DspA/E stability and secretion into the medium (Gaudriault *et al*., 1997, 2002). The disease-specific effector DspA/E is a type III secreted effector protein (198 kDa) required for pathogenicities in the apple and pear by pathogen *E. amylovora*. A DspA/E homolog, AvrE, of *Pseudomonas syringae* pv. *tomato* is recognized as an avirulence protein in the soybean plant (Wei *et al*., 1992; Barny,

1995; Lorang and Keen, 1995; Bogdanove *et al*., 1998a, 1998b).

In the current study, we investigated the activity of the *dspA/E* gene and its chaperone DspF as a pathogenicity factor of *E. amylovora* and how the *dspA/E* and *dspF* gene knockout affects the disease processes in host plants (apple, strawberry, rose, and tobacco and non-host plants (*Arabidopsis thaliana*).

MATERIALS AND METHODS

Bacterial strains and culture properties The research was conducted in the laboratories of the institute of Warwick Horticultural Research International, University of Warwick, UK during june, 2009 to july 2010**.** Wild type strain G83 of *E. amylovora* from our collection was used in this experiment. The wild strain was grown on nutrient glucose agar (NGA) at 26-28 ºC. Mutant's *dspA/E* and *dspF* obtained from the wild type strain of *E. amylovora* were grown on NGA with kanamycin (Km) at 28ºC (Ashmawy *et. al.,* 2015). The *Escherichia coli* (Bioline/Bio-85038) used for cloning consisted of electroSHOX competent cells (Bioline). Competent cells of *E. coli* were grown in Luria-Bertani (LB) medium at 37°C.

PCR amplification: Purified DNA template was analyzed by touch-down PCR targeting the *dspA/E* and *DspF* genes of *E. amylovora*. Primers amplifying fragment of *dspA/E* and *dspF* genes were designed using the Primer3 program (version 0.4.0), and their sequences were checked for specificity in the GenBank database using BLAST (*http://blast.ncbi.nlm.nih.gov*). All primers used are listed in Table 1.

PCR amplifications were carried out in a 100 µl reaction mixtures containing 100 ng genomic DNA; 5 µl of 10-fold-concentrated buffer, 0.2 µM each primer, 0.25 mM µM each deoxynucleoside triphosphate, and 2.5 U of *Taq* polymerase (Invitrogen). The assays were performed in a programmable heat block (model MyCycler™ thermal cycler, BioRad, USA). Thermal cycling consisted of preliminary denaturation for 4 min at 95°C. And then 10 cycles using a touch-down strategy (an initial cycle of 94 ºC for 30 sec, 65ºC for 30 sec, 72ºC for 2 min, then lowering the annealing temperature for each cycle by 1ºC during the following 9 cycles), followed by 25 cycles at 95ºC for 30 sec, 56ºC for 30 sec and 72ºC for 2 min. Five microliters of the reaction product was mixed with 1 µl of loading buffer (60% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanol), and then the mixture was run on a 1,5% horizontal agarose gel (containing ethidium bromide at 1 µg/ml) at 100 V for 50 min in 50 mM Tris-borate-EDTA buffer (pH 8.0). The bands were visualized with a UV transilluminator and photographed with a charge-coupled-device camera.

Cloning and gene knockout of *dspA/E* and *dspF*: The purified PCR fragments of the wild type *E. amylovora* containing *dspA/E* and *dspF*, were cloned into a vector pCR®4 unit using a TOPO TA Cloning®Kit (Invitrogen), and was transformed into electroSHOX competent *Escherichia coli* cells (Bioline) by following the manufacturer's instructions. The plasmid containing *dspA/E* and *dspF* were isolated by using a plasmid DNA with a midiprep kit (Qiagen). The presence of the *dspA/E* and *dspF* genes was confirmed by running in 1.5% w/v ultrapure™ agarose gel. Sequencing of the purified plasmids was done in automated DNASTAR software (Madison, WI, U.S.A.). The purified pCR4-TOPO vector/*dspA/E* and pCR4-TOPO vector/*dspF* plasmids were prepared from transformed *E. coli* strains by method stated by Sambrook *et al.* (1989). The purified pCR4-TOPO vector/*dspA/E* and pCR4-TOPO vector/*dspF* plasmid was digested with excess *Eco*RV to perform deletion of *dspA/E* and *dspF genes*. The effectiveness of the digestion procedure was monitored with gel electrophoresis. Then, digested pCR4-TOPO vector/a*dspA/E* and pCR4-TOPO vector/*dspF* plasmids were transfomed into competent *E. amylovora* and electroporated with a Bio-Rad™ Micro Pulser unit. Transformation of digested pCR4-TOPO vector/a*dspA/E* and pCR4-TOPO vector/*dspF* were assayed by using competent *E. amylovora* cells prepared with the following procedure; 2 µl of digested pCR4-TOPO vector/a*dspA/E* and pCR4-TOPO vector/*dspF* were mixed with 25 µl of competent *E. amylovora*. Transformation was completed by using standard procedures (Bauer, 1990) and the cells were streaked onto NGA supplemented with kanamycin in order to observe the transformed colonies (kanamycin resistance, Km^r).

Pathogenicity tests: Pathogenicity assays were performed with the wild type strain G83, mutant a*dspA/E* and a*dspF* strains of *E. amylovora* on host and non-host plants. The wild type G83 strain of *E. amylovora* was grown on NGA, and the a*dspA/E* and a*dspF* mutant strains of *E. amylovora* were grown on NGA with added kanamycin (Km) at 28°C. The isolates were then suspended in steril distilled water at 10^7 cfu ml^{-1} (OD_{600} = 0.05). Sterile distilled water was used as a negative control on plant leaves. Bacterial suspensions were injected with a 1-ml syringe without needle into leaves through stomatal pores on the abaxial surface. In this assay, wild type G83, a*dspA/E* and a*dspF* mutant strains of *E. amylovora* were inoculated on leaves of apple, strawberry and rose cultivars for pathogenicity testing and inoculated on leaves of *Nicotiana benthamiana*, *Arabidopsis thaliana* Columbia-5 (Col-5, resistant variety), *Landsberg erecta* (Ler-0, sensitive variety) to elicit and test for HR.Following this, bacterial colonies were counted for the presence of wild type mutants

strains of *E. amylovora* at 0, 24, 48 and 72 h after inoculation in leaves of *N. benthamiana*, Col-5 and Ler-0 to determine the role of the *dspA/E* gene and its chaperone *dspF* in pathogenicity. For counting of bacterial colonies, infected *N. benthamiana*, Col-5 and Ler-0 leaves were harvested using a cork-borer (10 mm diameter). Leaf disks were washed with sterile distilled water and each leaf disk was placed into the microfuge tube (2 ml) with steril with sterile distilled water (100 µl) and samples were macerated with plastic pestles. Samples were then serially diluted with sterile distilled water and plated on NGA and NGA with Km at 28°C. The Colony Forming Units (CFU) per unit area of tissue (cm^2) was counted three days after inoculation on NGA and NGA with Km at 28°C. The experimental design in the plant pathogenicity tests consisted of three replicates of four plants per treatment. The pathogenicity assays were performed twice.

Statistical analysis: Logarithmic transformation of CFU was used to normalize the data distribution. All data were analyzed using the Two-way ANOVA procedure of the SPSS 15.0 statistical software package (SPSS Inc., Cary, NC, USA). Means were separated by using Tukey's multiple comparison test and significances were evaluated at $P \leq 0.05$ for all tests.

RESULTS

.**Gene knockout of *dspE* and *dspF*:** Amplification of *dspA/E* and *dspF* regions of *E. amylovora* by PCR method had successfully produced a single-band product. The purified PCR fragments of the wild type *E. amylovora* containing *dspA/E*, was successfully cloned in a vector pCR®4 unit and transformed into electroSHOX competent *Escherichia coli* cells. The presence of the *dspA/E* and *dspF* genes inserts was confirmed by separation on agarose gel and also by restriction digestion. Plasmid with the *DspA/E* and *DspF* regions subjected to EcoRV digestions were analysed and sequenced. The sequences of the mutant a*dspA/E* and a*dspF* strains of *E. amylovora* were compared with that of the wild type. Samples of undigested and digested plasmids were separated by electrophoresis and the restriction of the large plasmids present in the wild type of *E. amylovora* showed a band profile different from that of a*dspA/E* and a*dspF* strains *E. amylovora*. The analysis of the plasmid contents of mutant a*dspA/E* and a*dspF* strains *E. amylovora* transformed with the TOPO vector showed that the mutant strains were maintained after repeated culturing on NGA supplemented with Km.

Pathogenicity: The apple, strawberry and rose leaves were inoculated with wild type G83, mutant a*dspA/E* and a*dspF* strains of *E. amylovora* for pathogenicity testing. No symptoms were observed on apple (Figure 1A), strawberry (Figure 1B) and rose (Figure 1C) leaves when they were inoculated with mutant a*dspA/E* and a*dspF* strains of *E. amylovora*. In contrast, wild type G83 stran of *E. amylovora* caused necrosis on the apple (Figure 1A), strawberry (Figure 1B), and rose leaves (Figure 1C). The necrotic spots on apple, strawberry and rose leaves developed in the infiltrated area after three days incubation at 20 °C and 70% relative humidity. These spots gradually increased in size until seven days after inoculation. Sterile distilled water used as a control on the apple, strawberry and rose leaves did not cause any necrotic symptoms.

In order to elicit HR, the wild type G83, mutant a*dspA/E* and a*dspF* strains of *E. amylovora* were inoculated on tobacco leaves of two varieties that ie; resistant variety Col-5 and sensitive variety Ler-0.. For wild type G83 of *E. amylovora*, HR elicitation was observed in tobacco (Figure 2A) and Col-5 leaves (Figure 2B) and it caused necrosis on the leaves of Ler-0 (Figure 2B) after three days at 20 °C and 70% relative humidity. In contrast, no symptoms were observed on Col-5 and Ler-0 tobacco leaves inoculated with mutant a*dspA/E* and a*dspF* strains of *E. amylovora*.

Bacterial density: The growth of wild type G83, mutant a*dspA/E* and a*dspF* strains of *E. amylovora* after inoculation on leaves were also analysed. Refering to data on Table 2 and also on Figure 3, there was a significant growth difference observed between wild and the *E. amylovora* mutant strains ($P<0.05$). As shown on Figure 3, in Ler-0 leaves, at 24 h after inoculation, the growth of the wild type G83 was at $1.0 \times 10^{6.41}$ CFU/ml and this was higher than that of either mutant strains; 1.0 x 105.93 CFU/ml for a*dspA/E* strain and $1.0 \times 10^{6.37}$ CFU/ml for a*dspF* strain. Although the growth of wild type G83 was higher than that of a*dspA/E* and a*dspF* mutant strains in the leaves of Ler-0, the growth of the wild type strain was however depleted in the resistant leaves Col 5. Since the plant immune system was not stimulated by the effectors of the mutant strains, the mutant a*dspA/E* and a*dspF* strains grew well in both Col-5 and Ler-0 tobacco leaves.

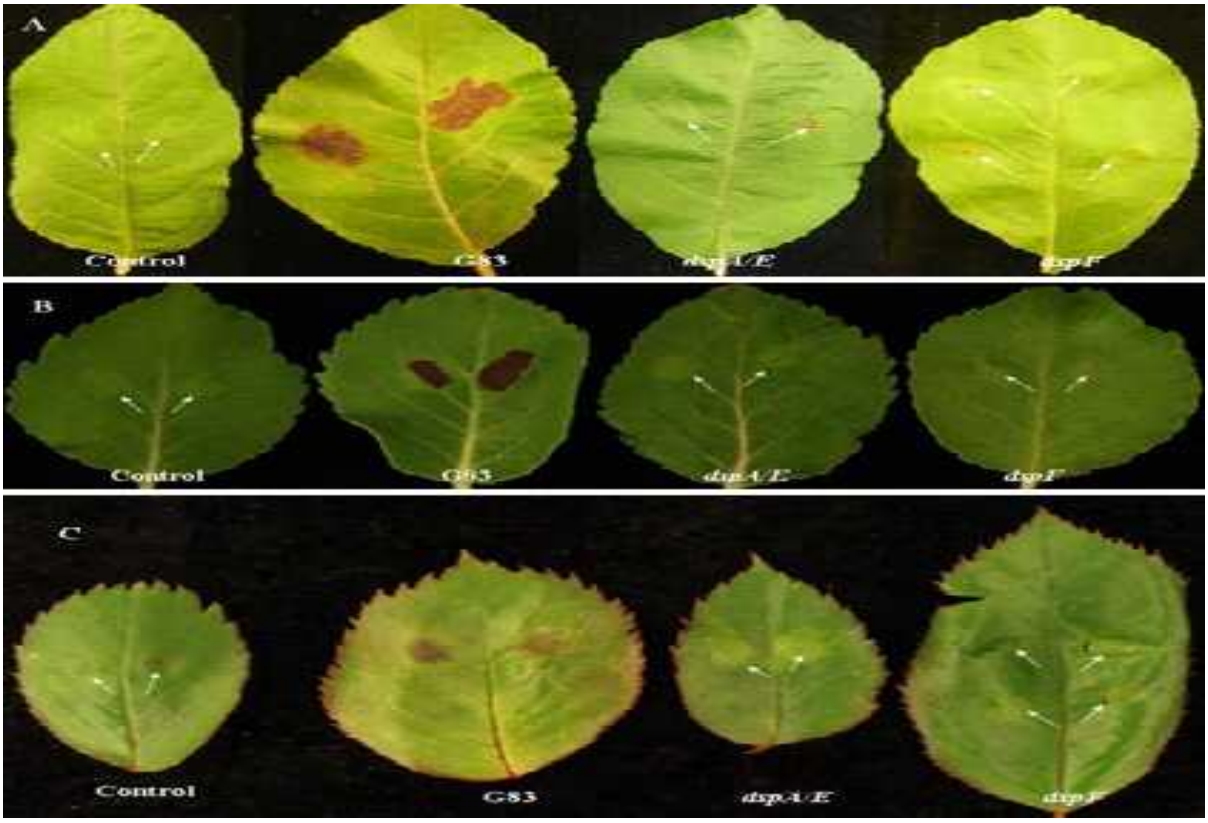


Figure 1. *E. amylovora* growth in apple leaves (A), strawberry leaves (B), rose leaves (C) infected with wild type G83, mutant a*dspA/E and* a*dspF* strains of *E. amylovora* at 20 îC with 70% relative humidity conditions: No symptoms were observed on apple (Figure 1A), strawberry (Figure 1B) and rose (Figure 1C) leaves when they were inoculated with the mutant strains of a*dspA/E* and a*dspF*. In contrast, wild type G83 strain of *E. amylovora* caused necrosis on the apple (Figure 1A), strawberry (Figure 1B), and rose leaves (Figure 1C). Sterile distilled water was used as a control.

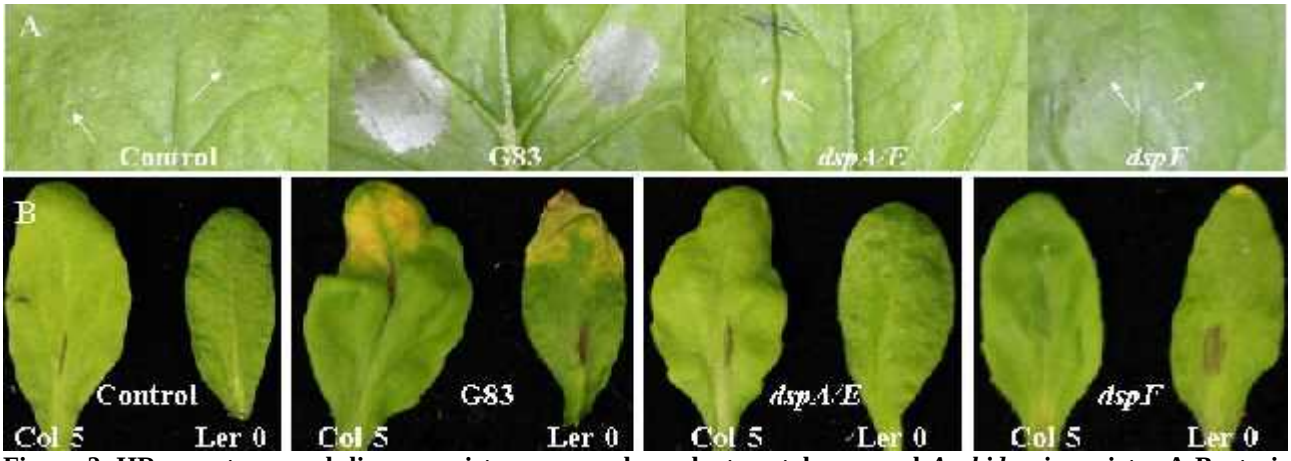
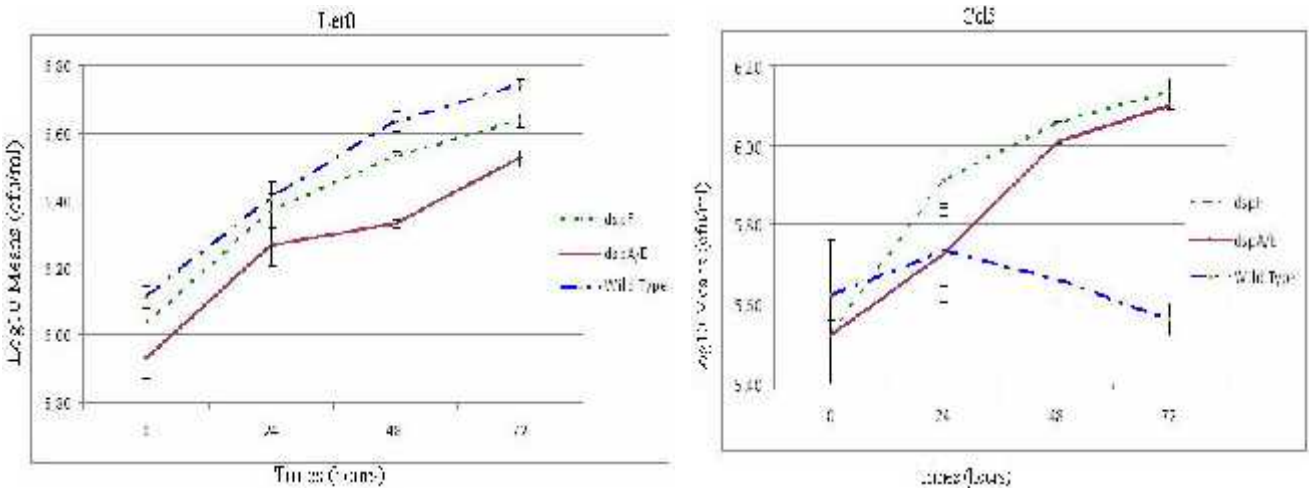


Figure 2. HR symptoms and disease resistance were dependent on tobacco and *Arabidopsis* variety; A Bacterial growth in tobacco leaves; B:. Bacterial growth in *Arabidopsis* leaves; Col-5 and Ler-0 leaves (B) 3 d after wild type G83, mutant a*dspA/E and* a*dspF* strains of *E. amylovora* inoculations (OD600=0.05) at 20 îC with 70% relative humidity conditions: No symptoms were observed on *N. benthamiana* and *A. thaliana* when they were inoculated with mutant a*dspA/E and* a*dspF* strains of *E. amylovora*. In contrast, wild type G83 of *E. amylovora* caused necrosis on *N. benthamiana* and *A. thaliana.* Sterile distilled water was used as a control.



A **B**

Figure 3. *E. amylovora* growth in leaves of *A. thaliana* depends on day-time of the isolates of *E. amylovora* inoculation (OD_{600} = 0.05). a) The growth of wild type G83 strain of *E. amylovora* was higher than that of mutant a*dspA/E and* a*dspF* strains of *E. amylovora* in Ler-0 leaves at 24 h after inoculation. b) In contrast, the growth of wild type G83 strain was lesser than that the mutant a*dspA/E and* a*dspF* strains of *E. amylovora* in the leaves of Col-5 after inoculation (P<0.05). In other words, the resistant variety Col-5 showed resistance to the growth of wild type G83 strain.

Table 1. Primers used for PCR in this study.

Gene	Primers	Oligonucleotide sequence (5'-3')	Amplicon size (bp)
dspA/E	DspA/E/iF (forward) DspA/E/iR (reverse)	ATGGAATTAAAATCACTGGGAACTGAA, TTAGCTCTTCATTTCCAGCCCTTCCTT	820
DspF	dspF/F (forward) dspF/R(reverse)	ATGACATCGTCACAGCAGCGGGTTGAA TTATGCCGCGCTACTCTCGTCTAA	670

Table 2. Statistical analysis of bacterial growth in leaves of *A. thaliana* depends on the day-time of the isolates of *E. amylovora* inoculation (OD_{600}= 0.05)

Isolates	Times (hours)	Log10 Means ± Std. Deviation(cfu/ml)		N
		Ler-0	Col-5	
a***dspF***	0	6.03 ± 0.08 **ab**	5.52 ± 0.07**a**	3
	24	6.37 ± 0.09 **cd**	5.91 ± 0.12 **bc**	3
	48	6.53 ± 0.02 **e**	6.06 ± 0.09 **c**	3
	72	6.64 ± 0.03 **ef**	6.14 ± 0.05 **c**	3
a***dspA/E***	0	5.93 ± 0.10 **a**	5.52 ± 0.24 **a**	3
	24	6.27 ± 0.12 **c**	5.73 ± 0.21 **ab**	3
	48	6.33 ± 0.02 **cd**	6.00 ± 0.09 **c**	3
	72	6.53 ± 0.04 **e**	6.10 ± 0.02 **c**	3
G83	0	6.11 ± 0.06 **b**	5.62 ± 0.24 **a**	3
	24	6.41 ± 0.08 **d**	5.73 ± 0.15 **ab**	3
	48	6.63 ± 0.05 **ef**	5.66 ± 0.10 **ab**	3
	72	6.74 ± 0.03 **g**	5.56 ± 0.07 **a**	3

DISCUSSION

In the current study, the role of the DspA/E and its chaperone protein DspF of *E. amylovora* was evaluated in bacterial growth and pathogenesis in host and non-host plants. The induced cell death observed may be attributable to the *dspA/E* gene and its chaperone protein DspF, which are known to induce HR in *N. benthamiana* and *A. thaliana* (Barny *et al.,* 1990; Gaudriault *et al.*, 1998; Kim and Beer, 1998; Wei *et al.*, 1992: Degrave *et al.*, 2013; Buonaurio *et al.*, 2015;

McNally *et al.*, 2016). Similarly, this study shows that DspA/E and its chaperone DspF locus of *E. amylovora* are essential for *E. amylovora* pathogenicity in host and non-host plants. In this work, the mechanisms of pathogenicity in host and non-host plants can be understand by using the wild type and mutant strains a*dspA/E* and a*dspF* of *E. amylovora*. The wild type G83 of *E. amylovora* caused severe disease common symptoms when inoculated into the leaves of apple, strawberry and rose. This strain also was positive for HR induction on the leaves of *N. benthamiana* and *A. thaliana*. In contrast, the mutant strains *DspA/E* and *dspF* were neither HR positive nor virtually virulent in plant pathogenicity tests (Figs. 1 and 2). The results of the current study suggest that the mutant strains may positively regulate R protein-mediated plant resistance in host plants, and that the role of mutant strains in limiting pathogen growth is understood. It is highly suggested that the putative protein or molecules interacting with DspA/E and its chaperone DspF in plants and their biochemical functions should be subjected to future studies in understanding plant-pathogen interaction.

Acknowledgements: We would like to thank the Scientific and Technical Research Council of Turkey (TÜBITAK-BIDEB) and Ondokuz Mayis University – AGED for the financial support of the research. We also like to thank Assoc. Dr. Soner Cankaya of Ondokuz Mayis University, Samsun, Turkey for statistical analysis.

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