

Investigation of Fire Blight Susceptibility and Iron Homeostasis of Pear (*Pyrus communis* L.) Following Invasion of Tissues by hrpW⁻, hrpN⁻ and dspA/E⁻ Mutants of *Erwinia amylovora*

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Abstract

Erwinia amylovora, the causal agent of fire blight disease in rosaceous plants contains type III secreted effector proteins including DspA/E, HrpN and HrpW which are secreted into host plants during the pathogenicity stages. In order to investigate the role of these effector proteins in the interaction with the host plants, susceptible (Bartlett), tolerant (Harrow Sweet) and resistant (Dargazi) pear cultivars were inoculated with wild-type and mutant strains of *E. amylovora* (hrpW⁻, hrpN⁻ and dspA/E⁻) under *in vitro* conditions. Based on the results, HrpW protein may be involved in pathogenicity in Dargazi cultivar. Different levels of pathogenicity were observed by dspA/E⁻ mutant in cultivars. The results showed the key role of HrpN, in the defense mechanisms of Dargazi cultivar, and its pathogenic role in Harrow Sweet and Bartlett cultivars. An increase in ferritin levels was observed in all cultivars inoculated with the wild type strain, but resistant and tolerant cultivars showed higher ferritin levels and a decrease in Fe²⁺ was observed only in these cultivars. The obtained data show that the HrpW protein does not affect iron homeostasis. Inoculation of Harrow Sweet and Dargazi cultivars with all strains increased ferritin, which was associated with a decrease in Fe²⁺. Based on the results, it is not possible to associate any of the effector proteins with changes in ferritin and Fe²⁺. In general, the ability of resistant pear cultivars to increase ferritin levels and regulation of iron can be one of the reasons for their resistance to fire blight. According

38 to the results, different mechanisms are employed by pear cultivars to respond to the causative
39 agent of fire blight.

40 **Key words:** Bartlett, Harrow sweet, Dargazi, *hrpW*⁻, *hrpN*⁻, *dspA/E*⁻

41

42 **Introduction**

43 One of the most important destructive diseases of pear fruits in the world is fire blight, which is
44 caused by the bacterial agent *Erwinia amylovora* (Abdollahi *et al.*, 2004). This bacterium is a type
45 of rod-shaped bacterium, and so far, no disease management approach has been definitively
46 effective. (Vanneste, 2000). The use of antibiotics and copper-based compounds, pruning of
47 infected tissues, and the use of resistant cultivars are the most important methods of disease control.
48 (Gusberti *et al.*, 2015). According to studies, the most effective and economical method of fire
49 blight disease management is the use of resistant or tolerant cultivars (Vanneste, 2000). **The**
50 **improvement of fire blight resistance on *Cydonia oblonga* using the hybridization breeding showed**
51 **that the fire blight resistance genes in quince are recessive and Transferring resistance to hybrids**
52 **is more successful using resistant varieties as pollinators (Shahin *et al.*, 2020).**

53 In non-host plants such as tobacco and resistant hosts plants, the reaction to *E. amylovora*
54 infection is incompatible Which leads to hypersensitive reaction (HR) (Venisse *et al.*, 2001). Also,
55 in host plants, the reaction is compatible and leads to infection (Holtappels *et al.*, 2018).
56 investigations have shown that in both compatible and incompatible reactions, the production of
57 reactive oxygen species (ROS) and oxidative bursts are the main responses against the attack of *E.*
58 *amylovora* (Wang *et al.*, 2019). *E. amylovora* produces three types of effector proteins including
59 HrpN, DspA/E and HrpW during pathogenicity in the host plants (Narayanasamy, 2008). A gene
60 cluster in the bacterial genome with a length of about 62 kb, which contains the *hrc*, *hrp* and *dsp*
61 genes, is responsible for producing these effector proteins. Meanwhile, two effector proteins, HrpN
62 and HrpW, are produced by *hrp* genes, and DspA/E is produced by *dsp* genes (Oh and Beer 2005).
63 DspA/E and HrpN proteins have been cited as the main options for compatible interaction in the
64 host, and HrpW protein appears to play a negligible role in this interaction (Taheri Shahrestani *et*
65 *al.*, 2020). According to recent research, the presence of active chloroplasts is necessary for the
66 pathogenicity of HrpN protein in the host (Taheri Shahrestani *et al.*, 2020; Abdollahi *et al.*, 2015).
67 Hypersensitive reaction, degradation of mitochondrial function and consequent programmed cell
68 death are the results of HrpN protein function in non-host plants (Xie and Chen 2000). The DspA/E
69 effector protein is an essential pathogenicity factor of *E. amylovora* because *dspA/E*⁻ mutant strains

70 did not cause disease in the host plants (Taheri Shahrestani *et al.*, 2020; Gaudriault *et al.*, 1997;
71 Bogdanove *et al.*, 1998). Oxidative burst seems to be essential for successful bacterial infection in
72 a compatible interaction (Venisse *et al.*, 2001). The report of Venisse *et al* (2003) indicates the
73 combined role of two effector proteins, HrpN and DspA/E, in causing oxidative burst in the
74 interaction of *E. amylovora* with host plants. According to their results, the *dspA/E* mutant does
75 not cause any symptoms, while the *hrpN* mutant is still able to cause some fire blight symptoms.
76 On the other hand, the *dspA/E* mutant had a greater ability to activate antioxidant enzymes than
77 the *hrpN* mutant (Venisse *et al.*, 2003). Azarabadi *et al* (2016) also reported that tolerance to fire
78 blight is associated with changes in the production pattern of ROS and especially the effect of two
79 major species of hydrogen peroxide (H₂O₂) and hydroxyl (OH⁻) radical in host tissues. Therefore,
80 considering the key role of DspA/E in the formation of ROS, the levels of effect of this effector
81 protein, and the reaction of the organelles involved in the production of reactive oxygen species,
82 can determine the level of host resistance to fire blight.

83 *E. amylovora* elicits a rapid oxidative burst in host plants (Venisse *et al.*, 2001, 2003). According
84 to Abdollahi *et al.*, (2015), oxidative burst in resistant genotypes of apples and pears inoculated
85 with *E. amylovora* triggered earlier than in susceptible genotypes. Large amounts of ROS such as
86 singlet oxygen (O²), superoxide anion (O^{2*}), H₂O₂ and OH⁻ are produced as one of the primary
87 responses of plant cells under various abiotic and biotic stresses (Sharma *et al.*, 2012). Resistance
88 to disease and destructive activities are among the different roles of ROS in cellular functions. The
89 production and removal of ROS must be tightly controlled in the cell to prevent oxidative damage.
90 Also, due to the numerous roles that ROS have, they should not be completely removed. The
91 expression of disease resistance genes by H₂O₂ has been proven (Hassani *et al.*, 2015; Radwan *et*
92 *al.*, 2010; Radwan *et al.*, 2006). Another role of H₂O₂ is to act as a substrate for oxidative cross-
93 linking in cell walls (Smirnov and Arnaud 2018). Therefore, one of the strategies of plants to
94 prevent the spread of pathogens is fine-tuned H₂O₂ generation. Thus, H₂O₂ accumulated by the
95 plant is toxic to pathogens but is not toxic to the plant itself. Thus, toxic levels of H₂O₂ act in two
96 ways to limit infection. On the one hand, it directly leads to the elimination of the pathogen, and
97 on the other hand, it induces genes related to plant defense (Dat *et al.*, 2000; Smirnov and Arnaud
98 2018). Another mechanism of resistance to pathogens is HR, in which plant resistance genes
99 identify pathogenic proteins (Balint-Kurti, 2019). The formation of very high levels of H₂O₂ during
100 the HR response induces programmed cell death and pathogen elimination (Abdollahi *et al.*, 2015).

101 The precursor for the formation of OH[•] radicals during the Fenton or Haber-Weiss reaction is H₂O₂.
102 It has been observed that during the infection of resistant pear cultivars with *E. amylovora*, the
103 conversion rate of H₂O₂ to OH[•] radical is low, which can be due to the activity of the non-enzymatic
104 antioxidant system of the host cells (Azarabadi *et al.*, 2016).

105 Ferritin is one of the most important metal binding proteins and can stores metal ions (Fe³⁺ and
106 Cu²⁺) in its core and prevents the formation of OH[•] from H₂O₂ (Halliwell and Gutteridge 2015).
107 Iron, as an essential element for plants, firstly participates in the redox reactions and structure of
108 many intracellular enzymes such as peroxidase (POD), catalase (CAT) and superoxide dismutase
109 (SOD), and secondly, through the Fenton or Haber-Weiss reaction, produces ROS (Dat *et al.*,
110 2000). Thus, Fe²⁺ may cause the formation of harmful OH[•] radicals from the H₂O₂ precursor
111 through the Fenton reaction. The most important cause of necrosis is OH[•] radicals and because
112 they are very toxic to macromolecules, their production must be controlled. According to these
113 statements, it is necessary to regulate the iron content of the cell. In this regard, creating transgenic
114 plants expressing ferritin genes has increased plant resistance against stresses (Yadav *et al.*, 2017;
115 Xi *et al.*, 2011). In this regard, transgenic tobacco plants expressing ferritin produced more ferritin
116 and showed greater resistance to cold stress (Hegeduse *et al.*, 2002). Iron also regulates virulence-
117 related functions in *E. amylovora*. The siderophore desferrioxamine (DFO) is produced by this
118 plant pathogen to sequester iron during the infection process. Also, the protective role of DFO for
119 bacteria during the oxidative burst induced by the defense response of the host plant has been
120 proven (Pandey, 2023). Therefore, the host and the pathogen have developed different and complex
121 strategies to compete with each other for iron resources. So far, there have been no detailed
122 investigations on iron homeostasis in host plants after the attack of the disease agent. However, in
123 our previous study, the levels of active iron in pear cultivars decreased after inoculation with a
124 wild-type strain of *E. amylovora* (Maleki *et al.*, 2021). In this research, we tried to investigate the
125 role of *E. amylovora* effector proteins in iron homeostasis and defense mechanisms of pear
126 cultivars.

127 **Materials and Methods**

128 **Bacterial strain**

130 The characteristics of *E. amylovora* strains used in this study are given in Table 1. The effect of
131 each of the effector proteins of HrpW, HrpN and DspA/E proteins on the pathogenicity of *E.*
132 *amylovora* was investigated using *hrpW*⁻, *hrpN*⁻ and *dspA/E*⁻ mutants, respectively and compared

133 with the wild-type strain. The bacterial strains were cultured in LB (Luria-Bertani) liquid medium
134 at 28°C. For the pathogenicity assay of the bacteria, each bacterial strain was cultured overnight
135 then turbidity of the bacterial inoculum was measured via the spectrophotometer at $\lambda 600\text{nm}$ and
136 adjusted to OD=1 using sterilized potassium phosphate buffer (pH = 7) and used for inoculation of
137 *in vitro* shootlets of pear cultivars (Abdollahi *et al.*, 2004).

138 139 **Plant material and growth conditions**

140 Three pear **cultivars** with different susceptibility levels to fire blight, including Bartlett
141 (susceptible), Harrow Sweet (tolerant) and Dargazi (resistant), were used for *in vitro* inoculation.
142 Establishment and proliferation of pear cultivars were done on QL medium (Quoirin and Lepoivre
143 1977) enriched with 1 mg/L BAP, 1 mg/L 2ip, and 0.1 mg/L NAA (pH=5.7). For all media, 8 g/L
144 agar, 30 g/L sucrose and 5 g/L pectin were used. **The presence of a carbon source in the culture**
145 **medium in *in vitro* conditions causes the inactivation of the electron transport chain (ETC) of**
146 **chloroplast (Yabuta *et al.*, 2007; Oswald *et al.*, 2001; Fuentes *et al.*, 2005). Therefore, in all**
147 **experiments, the ETC activation was perform by removing sucrose from the culture medium.** Pear
148 shootlets were grown *in vitro* at a constant temperature of 23 ± 1 °C under 16 h light photoperiod
149 using white fluorescent lamps (Sylvania, Germany) at $40 \mu\text{molm}^{-2} \text{s}^{-1}$ photon flux and subcultured
150 every 45 days (Abdollahi *et al.*, 2015). Pear shootlets with an approximate length of 3 cm were
151 used for inoculation using 200 microliters of each bacterial strain (Abdollahi *et al.*, 2004). **for this**
152 **purpose, basal inoculation was carried out by adding 200 μl of the overnight grown bacterial**
153 **suspension (OD= 1) on the surface of the proliferation medium. Subsequently, 4–5 mm of the basal**
154 **ends of the shootlets were removed, and transferred to the test tubes.** Five replications were
155 considered for each treatment. The percentage of shootlets necrosis was calculated using the
156 following formula: % necrosis = necrosis length/shootlets length \times 100.

157 158 **Electrolyte leakage determination**

159 Electrolyte leakage assay was used to evaluation of membrane stability of pear cultivars. In this
160 method, 0.1 g pear shootlets were rinsing with distilled water and cut into 5 mm disks. Then it was
161 transferred to 50 ml tubes and 10 ml of sterilized distilled water was added to them and incubated
162 at room temperature for 24 hours in a shaker (110 rpm). Using an EC meter (WalkLAB
163 conductivity pro meter), the electrical conductivity of distilled water containing suspended sample
164 discs was read as EC1. EC2 was measured after immersing the test tubes for 45 min in a boiling

165 water bath (110 °C). the relative electrolyte leakage (EL) was calculated using the following
166 equation (Sairam and Srivastava, 2001):

$$167 \quad EL\% = [EC1/EC2] \times 100$$

168
169 **Ferritin levels measurement**
170 The ferritin assay kit (Eliza kit, Pishtaz Teb, Tehran, Iran) was used to measure the ferritin levels
171 of pear cultivars according to the manufacturer's protocol. First, 1 gram of the pear shootlets was
172 ground in the ice-cold extraction buffer (100mM sodium chloride, 10 mM sodium phosphate
173 buffer, 1mM PMSF and 2% W/V PVP, pH = 7.2) and then centrifuged at 15,000g for 4 minutes at
174 4°C (Lukac *et al.*, 2009). The supernatant was used to measure ferritin using the kit. Finally, the
175 absorbance of the samples was read using an ELISA reader (Stat Fax 2100, Awareness) at 450 nm.

176 **Estimation of Active iron (Fe⁺²)**

177 The method of Katyal and Sharma (1980) with slight modifications was used to estimate the
178 amount of active iron in pear cultivar shootlets. First, one gram of fresh pear shootlets was taken,
179 washed by distilled water and the moisture was removed by absorbent papers. Then the leaves were
180 separated and chopped into fine bits. The samples were treated with 0.4 g/L ortho-phenanthroline
181 extract (pH 3.0) for 20 hours then centrifuged at 5000 rpm and room temperature. The supernatant
182 was used to estimation of Fe⁺² by reading the transmittance at 510 nm by spectrophotometer.

183 184 **Statistical analyses**

185 Comparison of all data was done using Microsoft Excel (Microsoft, USA-Version 2007) by
186 drawing curves and Microsoft SigmaPlot (Sigma-Aldrich, USA-Version 11.5) was used to one-
187 way analysis of variance (ANOVA) with Duncan's Multiple Range Test (DMRT).

188 189 **Results and Discussion**

190 **Necrotic lesion development**

191 In this study, our aim was to investigate the role of *E. amylovora* effector proteins including
192 HrpN, DspA/E, and HrpW in interaction with different pear cultivars including susceptible
193 (Bartlett), tolerant (Harrow Sweet) and resistant (Dargazi) cultivars. The necrosis assay was
194 successful in all shootlets of *in vitro* pear cultivars inoculated with mutant and wild-type strains of
195 *E. amylovora*, while no disease symptoms appeared in any of the non-inoculated shootlets (Fig. 1).
196 The effect of plant cultivars and bacterial strains on disease development was evaluated as

197 significant ($P < 0.01$). The appearance time and progression rate of necrosis in pear cultivars
198 inoculated with mutant strains of *E. amylovora* were different from those inoculated with the wild-
199 type strain (Fig. 1). Inoculation of resistant pear cultivar (Dargazi) with wild-type and *hrpW*⁻ strains
200 of *E. amylovora*, showed slower necrosis progress and a lower percentage of necrosis compared
201 with those of susceptible and tolerant cultivars (Fig. 1 and Fig. 2). It indicates that the susceptibility
202 behavior of cultivars to *E. amylovora* can be evaluated using *in vitro* system (Abdollahi *et al.*,
203 2004). Harrow Sweet and Bartlett cultivars showed signs of necrosis three days after inoculation
204 with the wild-type strain of *E. amylovora* and the appearance of symptoms in Dargazi cultivar was
205 delayed for up to seven days post-inoculation (Fig. 1 and Fig. 2a). On the other hand, the
206 development rate of necrotic lesions in Harrow Sweet cultivar was slightly lower than Bartlett.
207 Interestingly, the first signs of necrosis in Dargazi cultivar were observed after 7 dpi and the final
208 percentage of necrosis lesions in this cultivar was completed after 13 dpi. The late appearance and
209 slow progress of the disease in the resistant cultivar Dargazi is consistent with the previous results
210 of Abdollahi *et al.* (2004). Therefore, the delay in the appearance and progression of disease
211 symptoms is one of the signs of disease resistance *in vitro* condition. In other words, in *in vitro*
212 conditions, due to the absence of wood tissues and lignin barriers of tissues, as well as high
213 humidity and favorable conditions for the growth of the disease agent, resistance to the disease
214 manifests itself as a delay in the development of the disease. Almost, the appearance and
215 progression rate of necrosis lesions in pear cultivars inoculated with the *hrpW*⁻ mutant strain were
216 similar to those of pear cultivars inoculated with the wild-type strain. Accordingly, in the necrosis
217 assay, no significant difference ($P > 0.05$) was observed in the pathogenicity of the *hrpW*⁻ mutant
218 and the wild-type strain (Fig. 1). Based on these results and the results of previous studies (Taheri
219 *et al.*, 2020; Kim and Beer 1998; Venisse *et al.*, 2003), it is concluded that this protein has no effect
220 on the pathogenicity of *E. amylovora*. The ROS produced in the host plant during the pathogenesis
221 of *E. amylovora* causes lipid peroxidation, resulting in electrolyte leakage from the cells (Foyer *et*
222 *al.*, 1994; Venisse *et al.*, 2001). Mock-inoculated *in vitro* shootlets showed electrolyte leakage of
223 about 10% (Fig. 1 and 4). A small percentage of electrolyte leakage has already been reported in a
224 number of healthy plants (Krasuska and Gniazdowska 2012; Filek *et al.*, 2012; Brisset and Paulin
225 1991). The efficiency of using the two indicators of the appearance of disease symptoms as well
226 as the necrosis progression to evaluate the resistance of different pear cultivars was not exactly
227 corresponded to previous research (Abdollahi and Salehi 2017; Abdollahi *et al.*, 2015). In our

228 study, the appearance and progression of necrosis occurred with a delay of several days, depending
229 on the variety. According to our experiments in active chloroplast condition and the results of
230 previous reports in this regard (Abdollahi *et al.*, 2015; Taheri *et al.*, 2020), the delay in the
231 appearance and progression of the disease could be due to the interaction of bacterial effector
232 proteins with host cell chloroplasts and the key role of chloroplasts during systemic acquired
233 resistance (SAR) (Debroy *et al.*, 2004).

234 **Electrolyte Leakage**

236 *E. amylovora* elicits an oxidative burst in host plants during plant defense responses in which
237 ROS are produced (Shetty *et al.*, 2008). Lipid peroxidation and consequent electrolyte leakage
238 from cells are the results of ROS activity (Venisse *et al.*, 2001). In this study, electrolyte leakage
239 was measured as the main indicator of the severity of disease damage to cells. The studied cultivars
240 significantly differed in their electrolyte leakage values ($P < 0.01$). Electrolyte leakage of all *in*
241 *vitro* shootlets of the studied cultivars before inoculation with *E. amylovora* strains was estimated
242 to be about 10% (Fig. 1 and 3) which is consistent with previous researches (Filek *et al.*, 2012;
243 Krasuska and Gniazdowska 2012). Based on the results, after the appearance of necrosis symptoms
244 in pear cultivars, the electrolyte leakage rate was estimated to be more than 70%. Harrow sweet
245 and Bartlett cultivars showed the first major changes in electrolyte leakage after inoculation with
246 *E. amylovora* (Fig. 3). Despite the electrolyte leakage of these two cultivars from the first days
247 after inoculation, the progression of electrolyte leakage in Harrow sweet genotype was slightly
248 faster than Bartlett genotype. Considering these results and comparing them with the results of
249 necrosis, it is found that Harrow sweet cultivar, despite more fire blight resistance, has less
250 membrane stability than Bartlett cultivar. Unlike susceptible and tolerant cultivars, electrolyte
251 leakage in the resistant cultivar started about two days post inoculation with wild-type strain of *E.*
252 *amylovora* and then progressed at a slower rate. According to the results of this study, membrane
253 damage in resistant cultivar (Dargazi), does not start from the first days after inoculation, unlike
254 the sensitive and tolerant pear cultivars (Fig. 3a). As a result, membrane stability in Dargazi cultivar
255 is higher than other cultivars and also Bartlett cultivar has higher membrane stability than Harrow
256 sweet cultivar (Fig. 3a). Therefore, it seems that the membrane stability of tolerant cultivar (Harrow
257 sweet) could not be the reason for its relative resistance to disease.

258 **Interaction of pear cultivars with *hrpN*⁻ strain**

260 appearance of necrosis was observed in Dargazi, Bartlett and Harrow sweet pear cultivars after
261 4, 7 and 14 days after inoculation with *hrpN*⁻ mutant strain, respectively (Fig. 2b). Thus, in the
262 Harrow sweet and Bartlett cultivars, symptoms appeared later than when they were inoculated with
263 the wild-type strain of *E. amylovora*. On the other hand, symptoms of necrosis appeared earlier in
264 the resistant cultivar (Dargazi). Thus, Dargazi cultivar acted like a sensitive cultivar after
265 inoculation with *hrpN*⁻ mutant strain. These results indicate the role of HrpN on induction of plant
266 defense mechanisms in Dargazi cultivar and as pathogenicity factor in Harrow sweet and Bartlett
267 cultivars. Also, the rate of necrosis progression in all studied cultivars did not show a significant
268 difference with the control ($P > 0.05$). initiation of electrolyte leakage after inoculation of cultivars
269 with *hrpN*⁻ mutant strain was observed first in Dargazi cultivar, then in Bartlett and Harrow sweet
270 (Fig. 3b). However, electrolyte leakage in Dargazi and Bartlett cultivars progressed rapidly but
271 lasted up to 21 days in Harrow sweet cultivars (Fig. 3b). These results were consistent with the
272 results of necrosis studies (Fig. 1). Previous studies have shown that HrpN protein has two roles,
273 including induction of the defense mechanisms and pathogenicity factor in the host tissue (Dong
274 *et al.*, 1999; Taheri *et al.*, 2020; Norliza *et al.*, 2018; Qiu *et al.*, 1997). Early appearance of necrosis
275 symptoms and electrolyte leakage in Dargazi cultivar indicates that the role of HrpN effector
276 protein in induction of defense mechanisms was more likely than its pathogenic role in this cultivar.
277 Unlike Dargazi cultivar, the pathogenic role of HrpN was more prominent in Harrow sweet and
278 Bartlett cultivars, because a significant delay in electrolyte leakage and necrosis symptoms
279 appearance were observed ($P < 0.01$). Dong *et al* (1999) Showed that HrpN induces pathogenesis-
280 related (PR) protein genes in plants, and also in Arabidopsis transgenic plants, which had lost their
281 ability to accumulate salicylic acid, HrpN protein neither elicited resistance nor activated SAR gene
282 expression. Therefore, HrpN protein induces resistance through the SAR signal transduction
283 pathway in a SA-dependent manner.

284 **Interaction of pear cultivars with *hrpW*⁻ strain**

286 Symptoms of necrosis were observed in Dargazi, Harrow sweet and Bartlett cultivars 9, 3- and 2-
287 days post-inoculation with the *hrpW*⁻ strain, respectively (Fig. 2c). The rate of necrosis progression
288 in Bartlett cultivar was higher than other cultivars and was completed after three days. Harrow
289 sweet and Dargazi cultivars showed complete necrosis at a slower rate. The results of electrolyte
290 leakage in pear cultivars inoculated with *hrpW*⁻ mutant strain were almost consistent with the
291 results of inoculation with non-mutant strain (Fig. 1 and 3). These results are consistent with a

292 previous report by Venisse *et al* (2003). The results of necrosis and electrolyte leakage experiments
293 in Dargazi cultivar inoculated with wild type strain and *hrpW*⁻ mutant strain show slight
294 differences. Therefore, it seems that the effector protein HrpW may have slight effect on the
295 pathogenicity of *E. amylovora* in Dargazi cultivar and no effect on Harrow sweet and Bartlett
296 cultivars. According to previous results using the *hrpW*⁻ mutant strain, the HrpW protein had no
297 effect on induction of hypersensitive reaction and pathogenicity of *E. amylovora*. However,
298 according to previous reports by Taheri *et al.*, (2017) And Abdollahi (2003) this effector protein
299 may have little effect on induction of plant defense mechanisms, which requires further research.

300

301 **Interaction of pear cultivars with *dspA/E*⁻ strain**

302 Symptoms of necrosis were appeared in Harrow sweet and Dargazi pear cultivars 12 and 9 days
303 after inoculation with *dspA/E*⁻ mutant strain, respectively and the rate of necrosis progression was
304 higher in Harrow sweet cultivar than Dargazi cultivar. (Fig. 1 and 2d). However, Bartlett cultivar
305 did not show Symptoms of necrosis even after 30 days (Fig. 2d). Electrolyte leakage initiated later
306 in pear cultivars inoculated with the *dspA/E*⁻ mutant strain (Fig. 3d). Also, electrolyte leakage in
307 these cultivars reached 100% in a longer period of time. Harrow sweet cultivar started electrolyte
308 leakage before Dargazi cultivar but reached maximum electrolyte leakage in a longer period of
309 time. In Bartlett cultivar, even after 30 days from inoculation, no significant increase in relative
310 electrolyte leakage was observed (**P < 0.01**). It seems that the increase in relative electrolyte leakage
311 of this cultivar after 30 days to about 27% was due to plant stresses in *in vitro* condition. According
312 to these results, the effector protein DspA/E has a significant effect on the pathogenicity of *E.*
313 *amylovora*, so that in Bartlett cultivar, even after 30 days post-inoculation, no symptoms of necrosis
314 and significant electrolyte leakage were observed (Fig. 2 and 3). These results confirm the previous
315 results regarding the non-pathogenicity of *dspA/E*⁻ mutant strain in pear (Bogdanove *et al.*, 1998 ;
316 Gaudriault *et al.*, 1997). Thus, according to the results of electrolyte leakage and necrosis
317 experiments, the effector protein DspA/E can be considered as the main pathogenicity factor of *E.*
318 *amylovora*.

319

320 **Ferritin levels**

321 In this study, by measuring Fe²⁺ and plant ferritin, we investigated the role of iron in the resistance
322 of different pear cultivars to fire blight. Inoculation of pear cultivars by wild-type strain of *E.*
323 *amylovora* caused significant differences (P < 0.01) in ferritin level in all pear cultivars (Fig. 4).

324 Thus, two days after inoculation with wild-type strain of *E. amylovora*, the ferritin content of
325 Bartlett, Harrow sweet and Dargazi cultivars increased by 27%, 47% and 46%, respectively (Fig.
326 4). Resistant and tolerant cultivars in our experiment had higher levels of ferritin even before
327 inoculation with *E. amylovora*. According to the results, all cultivars used in this experiment have
328 the ability to increase ferritin levels, but the rate of this increase is much higher in resistant and
329 tolerant cultivars. The results of changes in ferritin content in pear cultivars inoculated with wild-
330 type and *hrpW*⁻ mutant strains of *E. amylovora* were consistent with each other (Fig. 4). These
331 results indicate that HrpW protein has no effect on increasing the expression of ferritin genes in
332 the cultivars used in our study. Inoculation of Harrow sweet cultivar with *dspA/E*⁻ mutant strain
333 increased ferritin levels, which could indicate the possible role of DspA/E protein in inhibiting
334 ferritin gene expression. In Bartlett cultivar, *hrpN*⁻ and *dspA/E*⁻ mutant strains reduced ferritin
335 content compared to the time of inoculation with wild-type strain of *E. amylovora* (Fig. 4). This
336 indicates that the increase in ferritin observed in this cultivar is due to the interaction of two effector
337 proteins, HrpN and DspA/E. Ferritin is one of the important proteins that is considered during
338 various stresses in plants (Briar *et al.*, 2010). it can store and oxidize up to 4,500 Fe²⁺ atoms in its
339 core, thus preventing the formation of destructive free radicals OH[•] during the Fenton reaction
340 (Ong *et al.*, 2006). Recent research has shown that the expression of exogenous ferritin genes in
341 transgenic plants has led to resistance to pathogens and abiotic stresses (Yadav *et al.*, 2017; Malnoy
342 *et al.*, 2003; Xi *et al.*, 2011; Xang *et al.*, 2017; Deak *et al.*, 1999). In view of the above, it seems
343 that one of the characteristics of resistant and tolerant pear cultivars used in this study, is their
344 ability to increase ferritin levels after infection with *E. amylovora*. Therefore, the sensitive cultivar
345 Bartlett lacks sufficient ability in this regard.

346 347 **Active iron (Fe²⁺)**

348 The concentrations of Fe²⁺ in all pear cultivars before inoculation with wild-type strain of *E.*
349 *amylovora* was not significantly different (**P > 0.05**) (Fig. 5). Two days after inoculation with wild-
350 type strain of *E. amylovora*, the amount of Fe²⁺ in Dargazi and Harrow sweet cultivars decreased
351 by 28% and 33%, respectively, and no significant change was observed in Bartlett cultivar (**P >**
352 **0.05**). The results of variation in Fe²⁺ concentration in pear cultivars inoculated with the *hrpW*⁻
353 mutant strain and the wild type strain of *E. amylovora* were almost similar (Fig. 5). This also
354 indicates that HrpW effector protein has no effect on pathogenicity or induction of defense
355 mechanisms of pear cultivars. Inoculation of Dargazi cultivar using wild-type strain and *hrpW*⁻ and

356 *hrpN*⁻ mutant strains, reduced Fe²⁺ concentration. However, the use of the *dspA/E*⁻ mutant strain
357 to inoculate the Dargazi cultivar did not cause a significant change in Fe²⁺ concentration (**P > 0.05**)
358 (Fig. 5). Prior to this experiment, inoculation of Dargazi cultivar with all strains of *E. amylovora*
359 had increased ferritin levels. **Thus, ferritin is not an essential regulator of iron homeostasis in**
360 **Dargazi cultivar and DspA/E effector protein play a key role in the control of iron by other**
361 **pathways.** Inoculation of Harrow sweet cultivar with each strains of *E. amylovora* reduced Fe²⁺
362 concentration almost equally. Therefore, changes in Fe²⁺ in this cultivar cannot be attributed to any
363 of the effector proteins of *E. amylovora*. However, in the previous experiment, inoculation of this
364 cultivar with all strains of *E. amylovora* increased ferritin levels. Thus, it is possible that the
365 interaction of the *E. amylovora* effector proteins caused regulation of iron in this cultivar. Unlike
366 Dargazi and Harrow sweet cultivars, inoculation of Bartlett cultivar with wild-type and *hrpW*⁻
367 mutant strains did not cause significant change in active iron concentration (**P > 0.05**). Based on
368 the Fig. 5, it can be concluded that in Bartlett cultivar, the interaction of two effector proteins,
369 HrpN and DspA/E, prevented the change of iron content, but the separate effect of each of these
370 two proteins led to a decrease in active iron. According to the results of the ferritin test, this
371 decrease in active iron is not related to ferritin. Because in similar conditions ferritin has decreased.
372 **Therefore, the decrease in the amount of active iron in this cultivar could be due to other iron**
373 **storage proteins or other cell methods to regulation of iron. As a result, this genotype does not have**
374 **the ability to control and regulation of iron in the face of wild-type strain of *E. amylovora*.**

375 Previously, the role of iron in the virulence of plant pathogens in only a limited number of
376 pathogens has been investigated. However, **so far**, no information is available on the role of effector
377 proteins in plant iron homeostasis. The issue of iron homeostasis in plants is a very complex issue
378 that is affected by many factors. In our recent study in greenhouse conditions, depending on the
379 susceptibility of pear cultivars, fire blight spread to a certain part of the stem length and then
380 stopped (Maleki *et al.*, 2021). In this regard, Aznar *et al.*, (2015) Showed that strong iron depletion
381 occurs in leaf tissues colonized by *D. dadantii*, while ahead of colonial areas, healthy plant cells
382 still have accumulated ferritin and iron. On the other hand, the production of ferritin and
383 siderophores during infection in host tissues by *E. amylovora* complicates the competitive situation
384 much more. Zhao *et al.* (2005) found that the Ftn gene encoding ferritin is induced in *E. amylovora*
385 during infection in pear tissues. Siderophores are the virulence factors of *E. amylovora* that are
386 produced in iron-limited environments and enable the pathogen to overcome the condition of iron

387 limitation (Franza and Expert 2013). They can also protect bacteria against reactive oxygen species
388 produced by the Fenton reaction (Venisse *et al.*, 2003). Several reports have shown that
389 siderophores can trigger plant defense responses (Aznar *et al.*, 2014; Dellagi *et al.*, 2009). Thus,
390 iron starvation by the production of siderophores leads to the accumulation of antimicrobial
391 compounds and other plant defense responses. Together these data show that iron deficient plants
392 may be more resistant to *E. amylovora* than non-deficient plants. For instance, iron starved *A.*
393 *thaliana* plants were more resistant to the *Dickeya dadantii*. Given the conditions of this study in a
394 culture medium with sufficient amounts of iron, competitive iron conditions may show other
395 interesting results.

396 Based on the presented results, it seems that DspA/E has the most role in pathogenicity of *E.*
397 *amylovora* and the role of HrpN in induction of plant defense mechanisms is more important and
398 HrpW has little effect on the pathogenicity of *E. amylovora* in Dargazi cultivar. Also, regardless
399 of the possible role of DspA/E effector protein in iron homeostasis in Dargazi cultivar, it seems
400 that iron homeostasis in pear cultivars is the result of the interactions of effector proteins, especially
401 HrpN and DspA/E. Previously, Venisse *et al.* (2003) showed that the elicitation of oxidative burst
402 in the interaction of *E. amylovora* and pear is the result of the combined action of two effector
403 protein DspA/E and HrpN. However, in general, the ability of resistant pear cultivars to increase
404 ferritin and iron homeostasis can be one of the reasons for their resistance to fire blight. According
405 to the results, refraining from excessive consumption of iron sources before and after the attack of
406 the disease agent can prevent severe damage.

407

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410

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Table 1 Strains used in this work.

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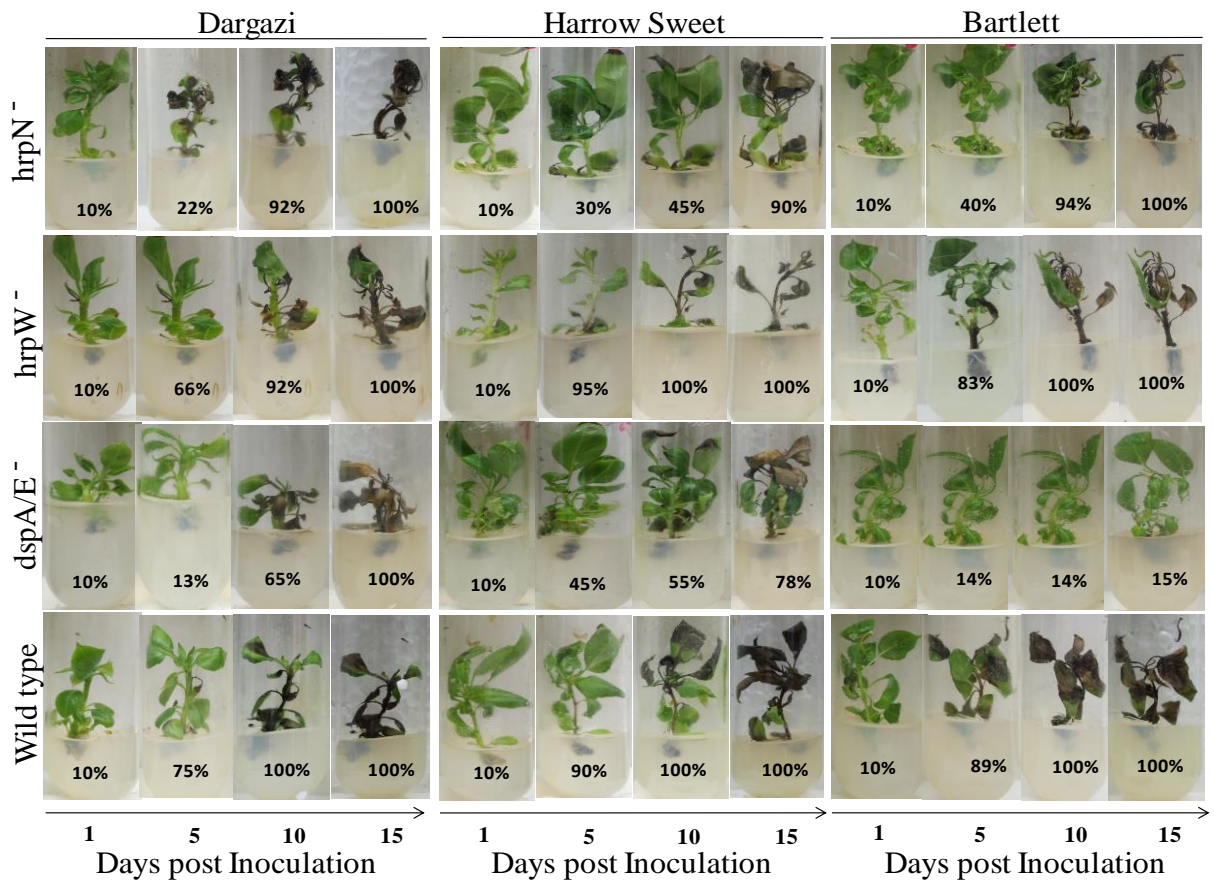
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Designation	Mutated gene	Relevant characteristics
CFBP ^a 7956	<i>hrpN</i> ⁻	Tn3-gus-km ^R
CFBP7980	<i>hrpW</i> ⁻	Mvd 11734-km ^R
CFBP7981	<i>dspA/E</i> ⁻	<i>dspA/E</i> 605: vidA-kan- Expressed a b,glucuronidase fusion
ATCC ^b 49,946	Wild-type	Wild

606 ^a CIRM-CFBP: International Centre for Microbial Resources-French.607 ^b American type culture collection.

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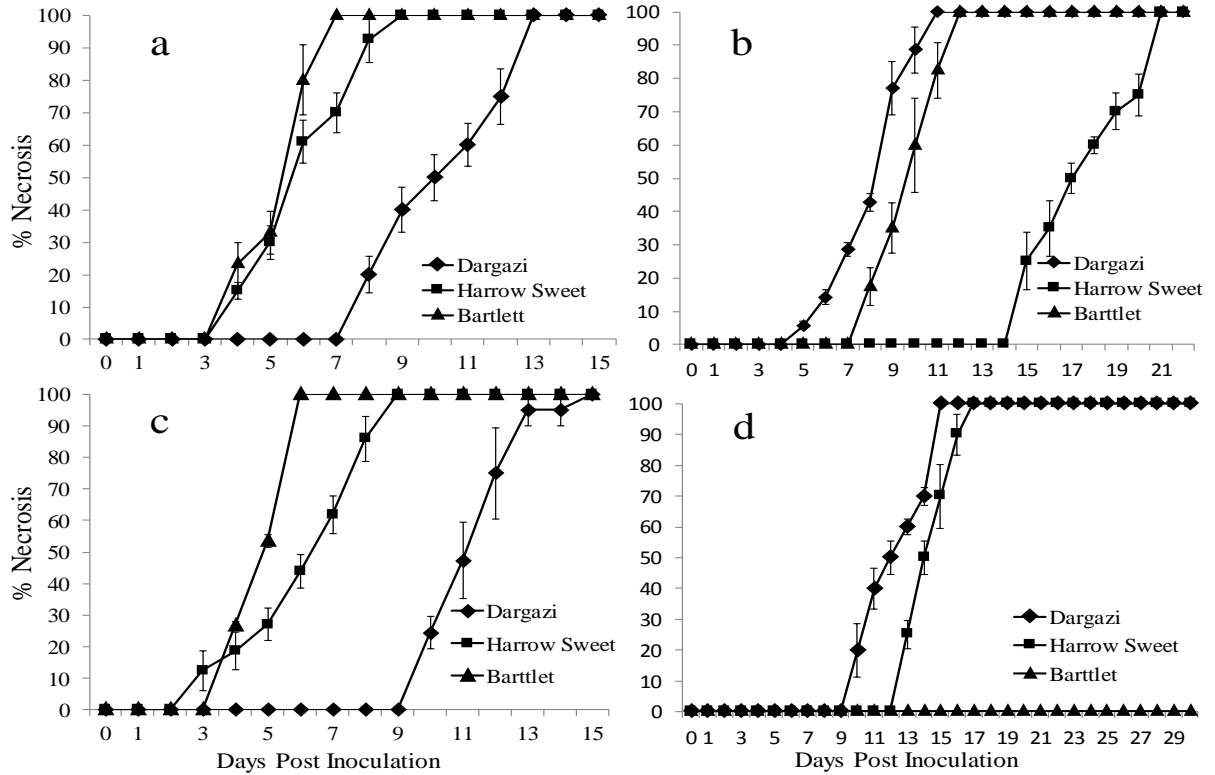


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610 **Fig. 1** Comparison of *in vitro* necrosis progression in Dargazi (resistant), Harrow sweet (tolerant),
 611 and Bartlett (susceptible) pear cultivars after inoculation with wild-type strain (a) and three mutants
 612 of *Erwinia amylovora* (*hrpN*⁻, *hrpW*⁻ and *dspA/E*⁻). The percentages expressed in the lower part of
 613 each cell represent the mean electrolyte leakage of the pear cultivars after inoculation with the
 614 wild-type strain of *Erwinia amylovora*.

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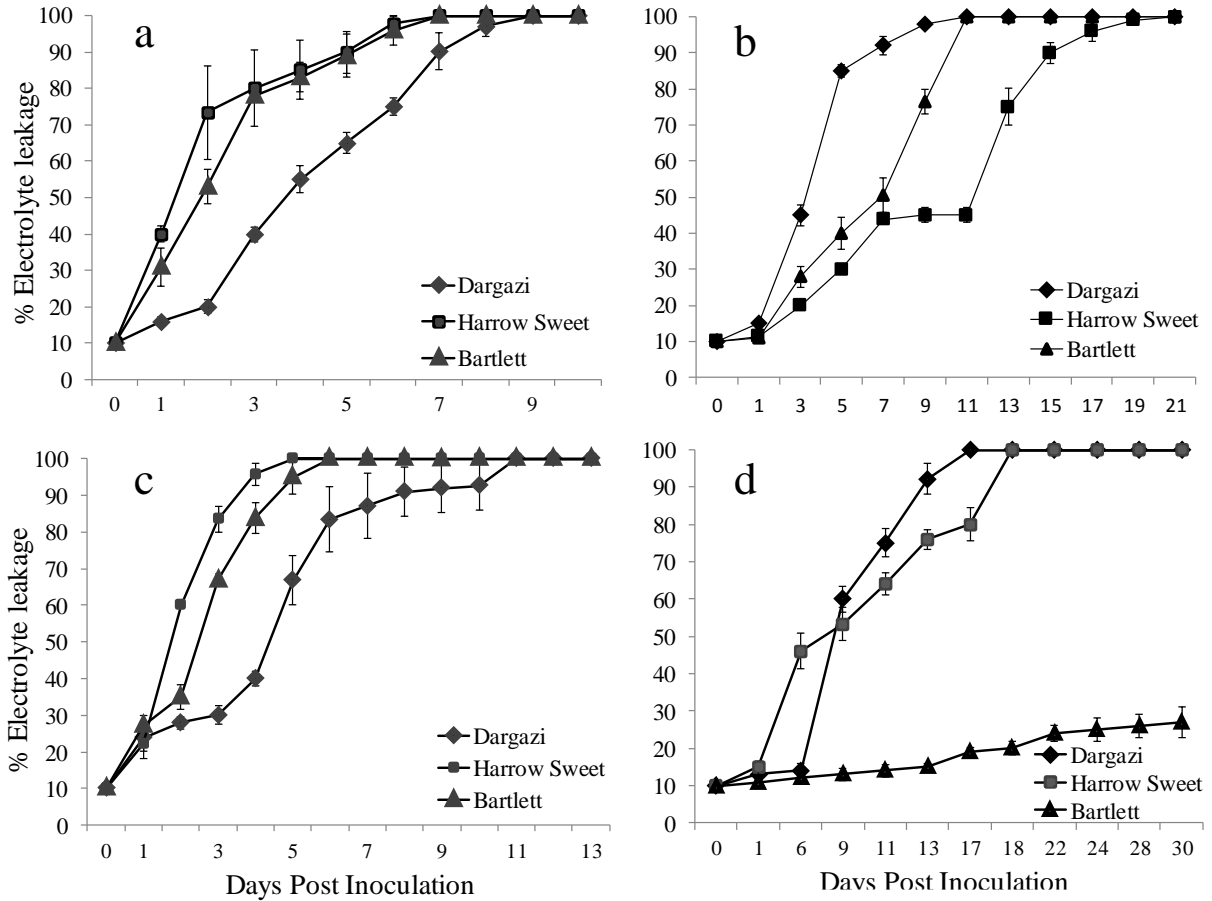
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618 **Fig.2** Comparison of necrosis development in Dargazi (resistant), Harrow sweet (tolerant), and
 619 Bartlett (susceptible) pear cultivars after inoculation with the wild-type strain (a) and three mutants
 620 of *Erwinia amylovora* including *hrpN*⁻ (b), *hrpW*⁻ (c), and *dspA/E*⁻ (d). The values are the mean of
 621 five replications and the bars are means \pm standard errors.

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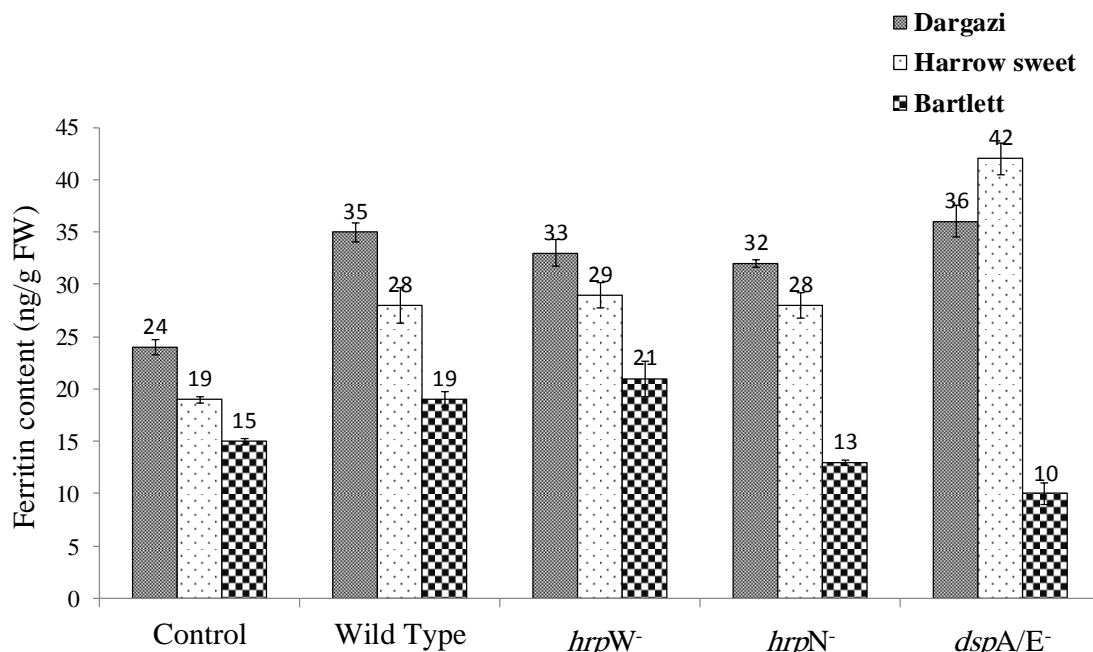


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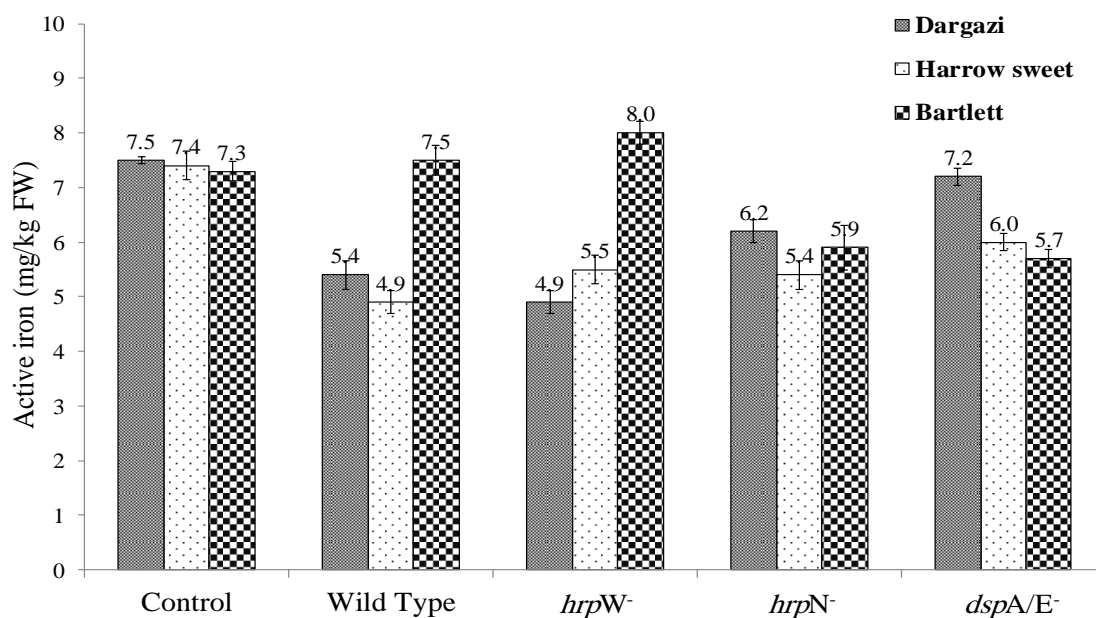
626 **Fig. 3** Comparison of electrolyte leakage changes during post-inoculation of Dargazi (resistant),
 627 Harrow sweet (tolerant), and Bartlett (susceptible) pear cultivars with the wild-type strain (a) and
 628 three mutants of *Erwinia amylovora* including *hrpN*⁻ (b), *hrpW*⁻ (c), and *dspA/E*⁻ (d). The values
 629 are the mean of five replications and the bars are means \pm standard errors.

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 633 **Fig. 4** Changes in ferritin contents in the Dargazi (resistant), Harrow sweet (tolerant), and Bartlett
 634 (susceptible) pear cultivars before inoculation and after 2 days post-inoculation with wild type and
 635 mutant strains of *Erwinia amylovora*. The values are mean of 3 replications and the bars are mean
 636 \pm standard errors.
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 639 **Fig. 5** Changes in Fe²⁺ contents in Dargazi (resistant), Harrow sweet (tolerant), and Bartlett
 640 (susceptible) pear cultivars before inoculation and after 2 days post-inoculation with wild type and
 641 mutant strains of *Erwinia amylovora*. The values are mean of 3 replications and the bars are means
 642 \pm standard errors.
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645 پروتئین های موثره باکتری *Erwinia amylovora* شامل HrpN ، HrpW و DspA/E قبل از ایجاد ضایعه
646 نکروز باعث تغییر محتوای آهن و فریتین در گلابی شدند

647

648 ر، مالکی. ح، عبداللهی. س، پیری و ک، پهلوان افشاری

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650 چکیده

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652 *Erwinia amylovora* دارای پروتئین های موثره HrpW، DspA/E و HrpN است که طی مراحل بیماری زایی از
653 طریق مسیر ترشحی نوع 3 به داخل سلول های گیاهان میزبان ترشح می شوند. به منظور بررسی اثر متقابل این پروتئین
654 های موثره با گیاهان میزبان، ارقام گلابی مقاوم (درگزی)، متحمل (هارو سویت) و حساس (بارتلت)، در شرایط درون شیشه
655 ای با سویه های نوع وحشی و جهش یافته *E. amylovora* (hrpN-، dspA/E- و hrpW-) تلقیح شدند. بر اساس
656 نتایج، احتمال تاثیر پروتئین HrpW در بیماری زایی رقم درگزی وجود دارد. سطوح مختلف بیماری زایی توسط پروتئین
657 موثره DspA/E در ارقام گلابی مشاهده شد. نتایج نشان داد پروتئین موثره HrpN در سیستم دفاعی اکتسابی رقم مقاوم
658 درگزی نقش کلیدی و در رقم هاروسونیت نقش بیماری زایی دارد. علیرغم افزایش فریتین در تمامی ارقام گلابی پس از تلقیح
659 با سویه نوع وحشی، ارقام مقاوم و متحمل گلابی سطوح فریتین بالاتری را نسبت به رقم حساس نشان دادند. همچنین کاهش
660 $+Fe_2$ تنها در رقم مقاوم و متحمل مشاهده شد داده های به دست آمده نشان می دهد که پروتئین HrpW تاثیر در تغییرات
661 میزان آهن ندارد. تلقیح رقم درگزی و هاروسونیت با همه سویه ها باعث افزایش فریتین و کاهش $+Fe_2$ همراه بود. بر
662 اساس نتایج، امکان ارتباط جداگانه هر یک از پروتئین های موثره با تغییرات فریتین و $+Fe_2$ وجود ندارد. به طور کلی می
663 توان نتیجه گیری کرد، توانایی رقم گلابی مقاوم در افزایش میزان فریتین و کنترل آهن می تواند یکی از دلایل مقاومت آن
664 به بیماری آتشک باشد. با توجه به این یافته ها، مسیرهای مختلفی توسط ارقام گلابی برای پاسخ به عامل بیماری آتشک
665 استفاده می شود.

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