Root characteristics of grafted peppers and their resistance to *Fusarium solani*

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Abstract

Root rot caused by *Fusarium solani*, is one of the most severe diseases in pepper (*Capsicum annuum* L.). Grafting has been attempted as an effective means to control the disease, but little is known about the disease resistance mechanism in grafted pepper. Therefore, we investigated the changes of biomass, cell structure, and the secondary metabolism in roots of control (non-grafted pepper) and grafted peppers using cvs. Weishi and Buyeding as rootstocks and the cv. Xinfeng 2 as a scion. After a manual inoculation, less *F. solani* invaded grafted pepper roots and consequently less serious injury to the root cell ultra-structure compared with the control was found. The roots of grafted pepper infected with *F. solani* exhibited greater biomass production and root activity than the roots of infected controls. Grafting led to an increased content of salicylic acid, benzoic acid, vanillin, lignin, and polyamines, as well as activities of phenylalanine ammonia lyase, polyphenoloxidase, and peroxidase. These results suggest that grafting improved the resistance of peppers to root rot.

Additional key words: *Capsicum annuum*, cell ultra-structure, root rot, secondary metabolism.

Introduction

Pepper (*Capsicum annuum* L.) cultivars are among the most important horticultural crops grown in many countries, where soil-borne diseases arising from continuous cropping are major problems that limit productivity. Root rot is a typical disease caused by *Fusarium solani*, *Verticillium dahliae*, and *Phytophthora capsici*. Root rot incidence is 10 to 30 % in general (Jiang *et al.* 2010). Therefore, improving root rot resistance is a major scientific and economic challenge in pepper.

Grafting has been attempted as an effective means to control soil-borne diseases (Van Steekelenburg 1980, Gisbert *et al.* 2010) and since the fumigant methyl bromide was phased out, it has been more widely used, especially on melons and other plants of S*olanaceae* family (King *et al.* 2008). However, grafting is not as widespread in pepper plants as it is in other vegetable and fruit species (Lee *et al.* 2010, López-Marín *et al.* 2013).

Lee (1994) found that grafting improved growth and disease-resistance and therefore suggested that grafting using tolerant pepper rootstock was a promising strategy to control the problems arising from continuous cropping and soil-borne diseases. Grafted pepper has been shown to increase growth and resistance to *Phytophthora* blight and bacterial wilt caused by *Ralstonia solanacearum* (Santos and Goto 2004, Jang *et al.* 2012). Recently, we identified two pepper cvs. Weishi (WS) and Buyeding (BYD) that have high root rot resistance and might be suitable rootstocks.

Root rot fungi, such as *F. solani*, *V. dahliae*, and *P. capsici*, mostly invade the lenticels and wounds of plant roots. Therefore, the root growth and structural characteristics are closely associated with resistance. In a previous study, we used WS as the rootstock and identified different responses to root rot using manual

inoculation. However, we still do not know how the root structure and other characteristics change after infection with root rot pathogens and whether they are involved in the root rot resistance of grafted peppers. Therefore, in this study, we investigated the changes of the root biomass, cell ultra-structure, and secondary metabolism in grafted or non-grafted peppers before and after inoculation. The results should provide basis for future research on improving the disease resistance of peppers and other horticultural crops.

Materials and methods

Plants and treatments: Based on previous studies, we selected pepper (*Capsicum annuum* L.) cvs. WS and BYD as rootstocks and cv. Xinfeng 2 (XF) as the scion. The pepper seeds were sown in plastic pots filled with sterilized soil (one plant per pot) and grown in a greenhouse at a 14-h photoperiod, an average photon flux density of 300 - 500 μmol m^{-2} s^{-1}, day/night temperatures of 25 - 30/15 - 20 °, and a relative humidity of 75 ± 5 %. When the rootstocks were approximately 30 cm tall (with 5 - 6 leaves), the pepper seedlings were grafted using the cleft grafting method (Lee 1994). The grafted combinations (cultivar/rootstock) were labelled XF/WS and XF/BYD. The rootstock (WS and BYD) seeds are smaller and the seeding stage is longer than those of XF, so XF were sown 2 weeks later than the rootstock plants, and the scion and rootstock were of the same species (XF/XF, control) to obtain plants with a similar biomass to the grafted plants at transplantation time (5 - 6 leaf stage).

One month after grafting, the grafted and control pepper plants were inoculated with *Fusarium solani* (Mart.) App. *et* Wollenw isolated from a pepper field in Zaozhuang, Shandong, China by the immersion method. The suspension solution was prepared by culturing the pathogen in Martin medium (Jiang *et al.* 2010) and diluting with distilled water to a concentration of 5×10^6 conidia cm^{-3}. The treated plants were transplanted into plastic pots filled with soil + *Vermiculite*. There were 3 replicates per treatment and 50 seedlings per replicate. The roots were sampled for analysis on 0, 5, 10, and 15 d after inoculation.

Investigation of disease incidence and disease index: The disease incidence and disease index of root rot was measured 7 d after inoculation. The presence of root rot was visually rated according to the disease classification assessed on a 0 to 5 degree scale that reflected the condition of the injured plants (0 - no disease; 1 - less than 10 % roots changed colour, no leaves wilted; 2 - 10 to 30 % of the roots were brown, and some leaves started to wilt; 3 - 30 to 50 % roots were brown, and plants were distinctly withered; 4 - more than 50 % of roots were brown, and most leaves withered, except for the heart leaves; 5 - plant dead). The disease index (DI) was calculated according to formula: DI [%] $= \frac{\Sigma \mathrm{di} \times \mathrm{Ii}}{\mathrm{L} \times 5} \times 100$, (where di is disease degree, Ii is the number of injured plants at each degree, L is the total number of samples and 5 is the highest injury degree).

Determination of root biomass, activity, and absorbing area: The root length, root volume, root surface area, and number of root tips and root forks of grafted and non-grafted pepper plants were determined with a root analyzer (*J181A*, Shanghai, China) and analysis software (*WinRHIZO*, USA) on 0, 5, and 15 d after inoculation. The root mass was measured with an electronic balance, and the root activity was assessed by the triphenyltetrazolium chloride (TTC) method (Ruf and Brunner 2003). The root absorbing area and the active absorbing area were measured by methyl-blue absorption method of Zhao *et al.* (2002).

Detection of root cell ultra-structure: Before and after inoculation, the root segments (1 - 2 mm) of grafted and control pepper roots were fixed in 3.5 % (m/v) glutaraldehyde and 1 % (m/v) osmic acid for 2 h. After fixation and dehydration in an ethanol series, the root samples were embedded in *Epon 812* resin and sliced using an *LKB-5* (Stockholm, Sweden) ultrathin slicer. The ultrathin sections were stained with uranyl acetate followed by lead citrate and examined with a *JEM-1200 EX* (*Jeol*, Tokyo, Japan) transmission electron microscope.

Measurement of secondary metabolism substances and activities of related enzymes
The phenolic acid content was analyzed *via* HPLC (*Waters-510* HPLC, *Waters-2487* ultraviolet detector, *Waters*, USA) according to Proestos *et al.* (2005). The lignin content in the pepper roots was determined as described by Iiyama and Wallis (1990) with several modifications according to Syros *et al.* (2004). Polyamines were extracted according to Sharma and Rajam (1995) with several modifications done by Wei *et al.* (2009). The phenylalnineammonia lyase (PAL) activity was determined according to Yuan *et al.* (2001). The polyphenol oxidase (PPO) activity was assayed as described by Rao and Deosthale (1987). The peroxidase (POD) activity was determined by the guaiacol method according to Omran (1980).

Statistical analysis: The data are presented as the mean ± standard deviation (SD) of three replicates. Analysis of variance (*ANOVA*) was performed using *DPS* software. Duncan's multiple range test (DMRT) was applied to analyze differences between measured parameters.

Results

Five days after inoculation, no diseased plants were found in XF/WS. However, the disease incidence in XF/BYD and XF/XF (control) was 26.7 and 43.0 %, and the disease index was 11.7 and 23.3 %, respectively (Table 1). Afterward, the disease incidence and disease index in grafted and control pepper plants gradually increased with time, but the extent of increase varied. Compared to the control, the disease incidence and disease index were significantly lower in grafted plants ($P < 0.05$). Fifteen days after inoculation, the disease incidence and disease index of the control plants increased to 88.7 and 75.7 %, while those of the XF/WS plants only to 23.3 and 19.0 %, and of XF/BYD to 54.0 and 28.3 %, respectively. These data suggest that grafting improved the root rot resistance of pepper.

Before inoculation, XF/WS (Fig. 1*A*) and XF/BYD (Fig. 1*B*) plants had more fine roots and root hairs than the control plants (Fig. 1*C*). After infection with *F. solani*, the roots of grafted and the control pepper were damaged, as shown by the decrease in number of lateral roots and root hairs. However, less damage was observed in grafted plants compared with control plants, especially in XF/WS (Fig. 1).

The fresh mass, total length, volume, surface area, and number of tips and forks of XF/WS and XF/BYD roots were significantly greater than those of the control

Table 1 Disease incidence [%] and disease index [%] of root rot in grafted (XF/WS or XF/BYD) and control (XF/XF) peppers. Means ± SDs, n = 3, different letters indicate that means are significantly different ($P < 0.05$).

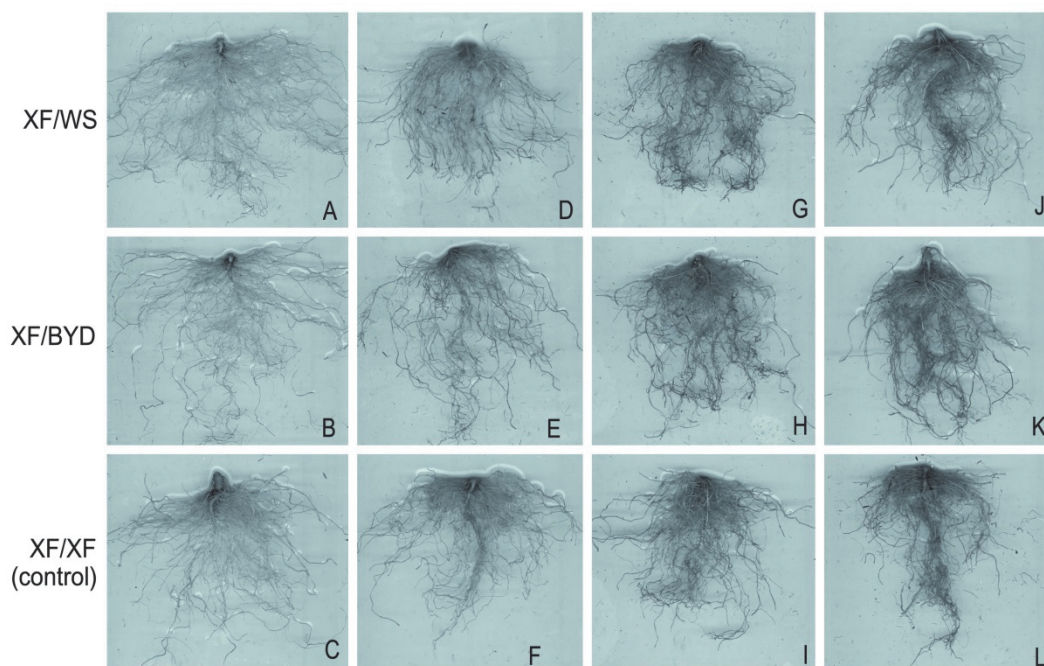| Treatments	5 d after inoculation		10 d after inoculation		15 d after inoculation	
	disease incidence	disease index	disease incidence	disease index	disease incidence	disease index
XF/WS	0 c	0 c	10.7 ± 1.2 c	8.7 ± 0.6 c	23.3 ± 1.5 c	19.0 ± 0.1 c
XF/BYD	26.7 ± 1.5 b	11.7 ± 0.6 b	32.3 ± 1.5 b	19.3 ± 1.2 b	54.0 ± 2.0 b	28.3 ± 1.5 b
XF/XF	43.0 ± 2.0 a	23.3 ± 0.6 a	60.3 ± 2.1 a	46.7 ± 2.5 a	88.7 ± 1.5 a	75.7 ± 3.1 a

Fig. 1. Roots of grafted (XF/WS, XF/BYD) and control (XF/XF) peppers before and after inoculation: *A,B,C* - before inoculation, *D,E,F* - 5 d after inoculation, *G,H,I* - 10 d after inoculation, *J,K,L* - 15 d after inoculation.

before inoculation ($P < 0.05$). During the experiment, the above parameters all decreased, but XF/WS had the highest values and the control the lowest values from beginning to end. Fifteen days after inoculation, the root mass, length, volume, surface area, and number of tips and forks of XF/WS were 58.5, 52.7, 62.9, 55.3, 39.3 and 29.8 % higher than those of the control, respectively, and the values for XF/BYD were 31.7, 37.9, 16.9, 44.4, 21.1, and 14.6 % higher, respectively, compared with the control (Fig. 2).
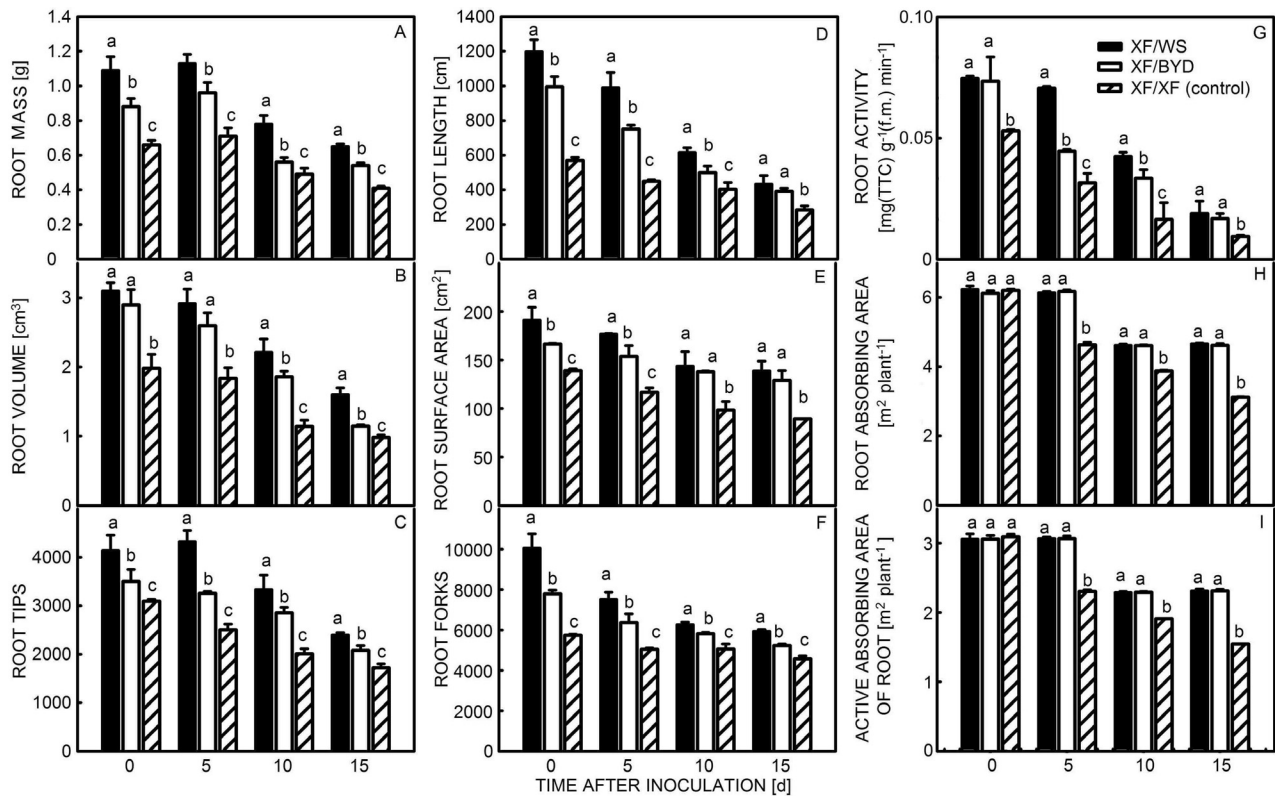
Fig. 2. The root growth, activity, and absorbing area of grafted (XF/WS and XF/BYD) and control (XF/XF) peppers: root fresh mass (*A*), root volume (*B*), number of root tips (*C*), root total length (*D*), root surface area (*E*), number of root forks (*F*), root activity (*G*), root absorbing area (*H*), and active absorbing area of root (*I*). Means ± SDs, *n* = 3, different letters indicate that means are significantly different (*P* < 0.05).
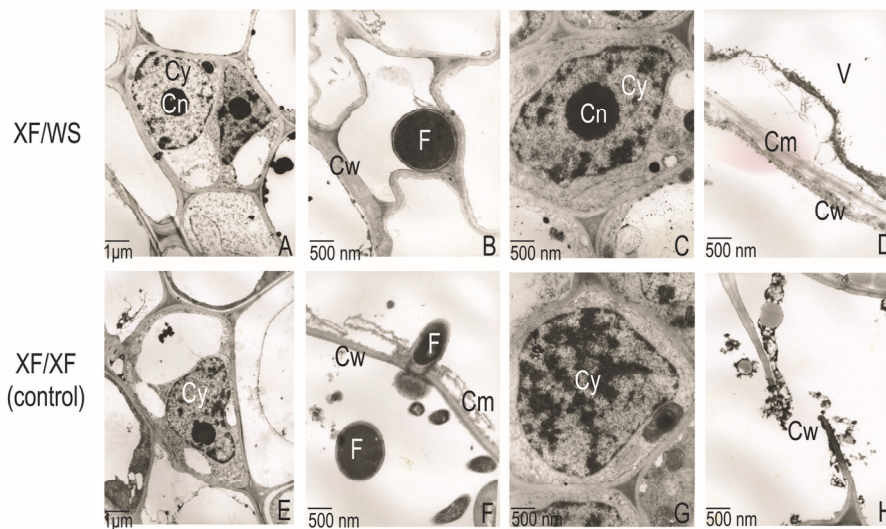

Fig. 3. The cell ultrastructure of roots of in grafted (XF/WS) and control (XF/XF) pepper plants: *A* - uninfected XF/WS; *B,C,D* - XF/WS infected by *F. solani* for 5 d; *E* - uninfected XF/XF; *F,G,H* - XF/XF infected by *F. solani* for 5 d. F - fungus, Cm - cell membrane, Cn - cell nucleus, Cy - cytoplasm, Cw - cell wall, V - vacuole.

Grafting led to a remarkable increase in root activity and showed no significant difference between XF/WS and XF/BYD before inoculation (Fig. 2*G*). The root activities of grafted and control plants declined gradually following inoculation, but the decline was the lowest in XF/WS and the highest in control (*P* < 0.05). Therefore, a

higher root activity in the grafted pepper plants probably played an important role in the enhancement of root rot disease resistance.

Before inoculation, no differences were observed in the absorbing area (Fig. 2*H*) and the active absorbing area (Fig. 2*I*) of roots between grafted and control plants. Five days after inoculation, the absorbing area and active absorbing area significantly decreased in the control plants, but no change was detected in the grafted plants. Afterward, the root absorbing area and active absorbing area decreased gradually in grafted and the control plants, but the decrease were remarkably less in grafted plants than in control plants ($P < 0.05$).

To further evaluate the relationship between the root characteristics and the root rot resistance in grafted peppers, we determined the changes in the root ultra-structure of grafted XF/WS and control plants 5 d after inoculation (Fig. 3). The results showed that invading conidia of *F. solani* were observed in both XF/WS and control root cells (Fig. 3*B,F*), but the density was significantly lower in grafted roots than in control roots. The control root cells were seriously damaged, as evidenced by membrane injury, a disaggregation of the nucleus and cytoplasm (Fig. 3*G*), and a rupture of the cell wall (Fig. 3*H*). However, the ultra-structure of the grafted pepper roots was mostly normal, except for some plasma membrane injury (Fig. 3*C*) and plasmolysis (Fig. 3*D*).

Before inoculation, 13 phenolic acids were isolated from control pepper roots (Table 2). Among them, chlorogenic acid was present in the highest amount, followed by gallic acid, caffeic acid, and benzoic acid (51.52 μg g⁻¹ DW). The same phenolic acids were isolated from XF/WS roots. Gallic acid was present in the highest amount in XF/WS roots, followed by chlorogenic acid, caffeic acid, and benzoic acid, with others in lower amounts. In comparison with control, grafted pepper roots had a higher content of caffeic acid, benzoic acid,

phloretin, salicylic acid, phlorizin, and P-hydroxybenzoic acid, but lower content of chlorogenic acid, gallic acid, cinnamic acid, and ferulic acid. No differences were observed in content of vanillin, catechinic acid and quercetin. After inoculation, gallic, chlorogenic, and catechinic acid decreased gradually in both XF/WS and the control roots, and many others increased during the first 5 or 10 d after infection but subsequently decreased, except for salicylic acid, which increased with time of infection. XF/WS roots showed a higher content of salicylic acid, benzoic acid, and vanillin but a lower content of gallic acid, chlorogenic acid, and quercetin, compared with control roots.

After inoculation, the lignin content increased gradually with time, but the extent of increase was remarkably less in XF/WS and XF/BYD than in the control ($P < 0.05$) (Fig. 4*A*); however, 15 d after inoculation, the lignin content in XF/WS and XF/BYD increased by 105.9 and 71.5 %, respectively, but in the control only by 40.9 %.

Before inoculation, XF/WS and XF/BYD roots showed a higher putrescine (Put) content ($P < 0.05$) compared with the control roots, but no distinct difference was found in spermidine (Spd) content (Fig. 4*B,C*). The XF/BYD roots revealed higher spermine (Spm) content than the control ($P < 0.05$) (Fig. 4*D*), whereas XF/WS had a similar Spm content to the control. After inoculation, Put, Spd, and Spm content firstly increased and then decreased, but was highest in XF/WS and lowest in the control ($P < 0.05$).

The enzymes closely associated with plant disease resistance are PAL, PPO, and POD. The PAL activity in roots was dramatically elevated after infection *F. solani* for 10 d, being 1.17 - 1.83-fold higher than before inoculation, but subsequently declined. Compared with the control, the grafted roots showed higher PAL activity ($P < 0.05$) throughout the experiment (Fig. 4*E*). PPO

Table 2. Changes of phenolic acids [μg g⁻¹(d.m.)] in roots of grafted (XF/WS) and control (XF/XF) peppers before and after inoculation. Means ± SDs, $n = 3$, different letters indicate that means are significantly different ($P < 0.05$).

Phenolic compounds	Before inoculation		5 d after inoculation		10 d after inoculation		15 d after inoculation	
	XF/WS	XF/XF	XF/WS	XF/XF	XF/WS	XF/XF	XF/WS	XF/XF
Gallic acid	224.5 b	294.3 a	179.0 b	282.5 a	153.2 b	277.9 a	8.0 b	254.7 a
Chlorogenic acid	122.4 b	334.0 a	74.7 a	37.3 b	56.7 a	37.3 b	45.8 a	32.9 b
P-hydroxybenzoic acid	24.2 a	14.1 b	24.4 a	27.4a	22.6 a	26.0 a	20.7 a	22.1 a
Ferulic acid	20.0 b	30.9 a	36.6 a	41.0 a	62.6 a	33.7 b	18.6 b	33.8 a
Benzoic acid	68.3 a	51.5 b	162.7 a	72.3 b	134.6 a	57.0 b	118.9 a	59.8 b
Cinnamic acid	7.5 b	18.3 a	19.3 b	26.4 a	21.9 a	23.2 a	20.4 a	8.7 b
Vanillin	8.4 a	7.2 a	39.0 a	10.8 b	15.7 a	5.5 b	9.3 a	5.5 b
Catechin	32.2 a	35.6 a	21.4 b	34.8 a	19.0 a	11.5 b	13.1 a	8.3 b
Caffeic acid	91.8 a	76.1 b	63.2 b	299.9 a	60.5 b	86.4 a	58.4 a	25.3 b
Phlorizin	33.3 a	21.7 b	11.6 b	27.5 a	45.7 b	78.4 a	92.7 a	18.3 b
Salicylic acid	36.5 a	6.6 b	46.9 a	31.6 b	87.0 a	36.5 b	91.1 a	9.1 b
Quercetin	26.5 a	29.9 a	4.8 b	34.0 a	11.4 b	102.5 a	14.6 b	60.4 a
Phloretin	38.4 a	29.2 b	14.3 b	22.7 a	15.5 b	31.3 a	26.6 a	24.5 a

activity was significantly increased by inoculation in both grafted and the control roots (Fig. 4*F*) and 15 d after inoculation, the PPO activities in XF/WS and XF/BYD were 78.5 and 33.1 % higher than in the control, respectively. POD activity increased significantly during

the first 5 d following inoculation but subsequently decreased (Fig. 4*G*). The grafted roots showed an obviously higher POD activity than the control roots ($P < 0.05$).
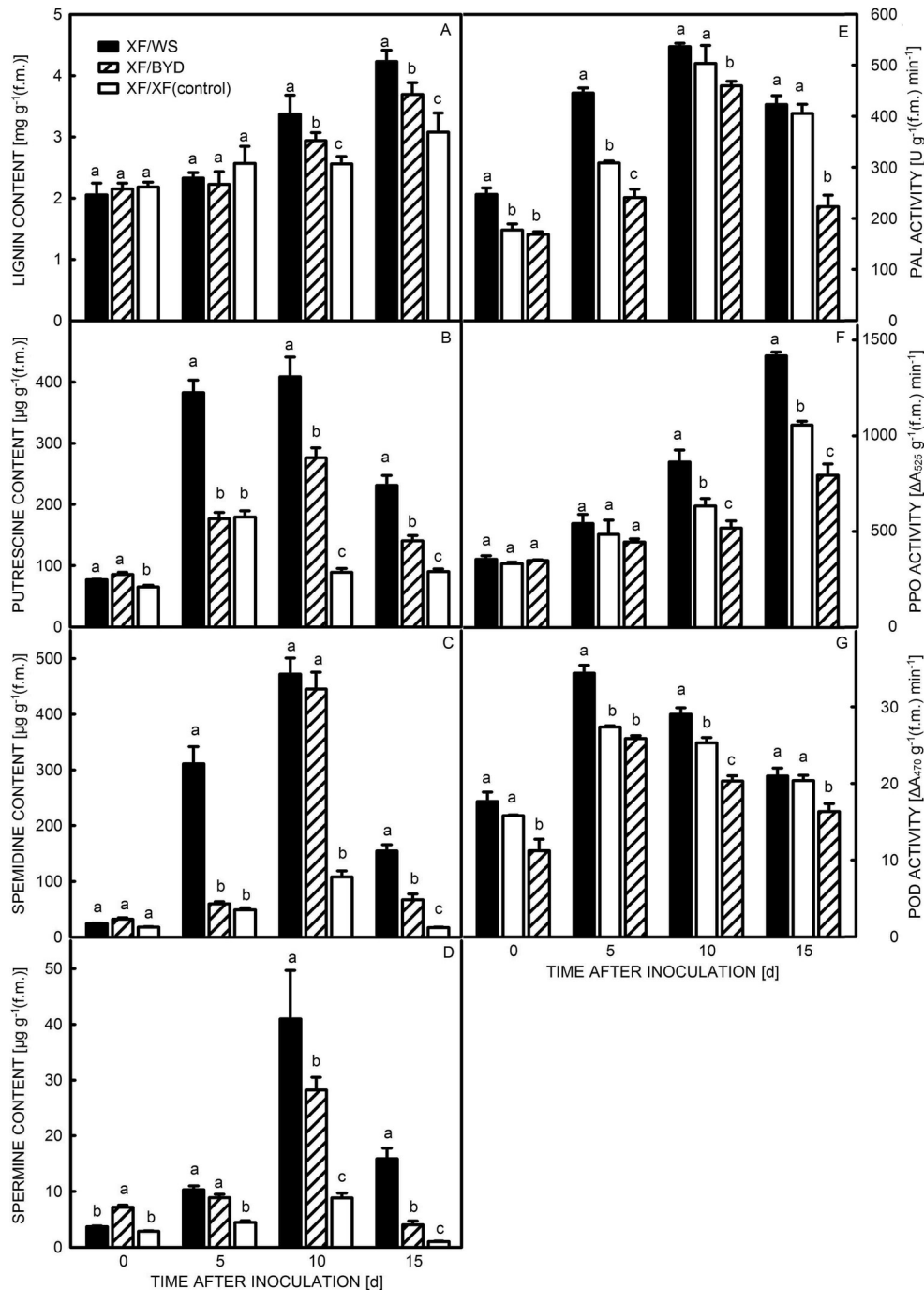

Fig. 4. Content of secondary metabolites and activities of related enzymes in roots of grafted (XF/WS and XF/BYD) and control (XF/XF) peppers: lignin (*A*), putrescine (*B*), spermidine (*C*), spermine (*D*), PAL (*E*), PPO (*F*), and POD (*G*). Means ± SDs, *n* = 3, different letters indicate that means are significantly different ($P < 0.05$).

Discussion

Soilborne pathogens often infect and damage plant roots, and, as a result, plant nutrient and water uptake can be negatively affected. Therefore, root system size and vigor may be associated with resistance to soilborne diseases. In this study, we demonstrate that the root rot resistance can be improved by grafting robust rootstocks, based on a decrease in disease incidence, disease index, and the quantity of *F. solani* that invaded pepper roots. We found that *F. solani* invasion significantly reduced growth in both grafted and non-grafted pepper roots. However, the grafted peppers had remarkably more root hairs, a greater root volume and absorption area, and higher root activity (Figs. 1, 2). Therefore, the grafted pepper roots, which were less seriously injured by *F. solani,* retained stronger absorption ability and maintained higher physiological functionality during the experiment. This is an important cause of the enhancement of disease resistance in grafted peppers.

Plant secondary metabolism encompasses all physiological and biochemical facets of "secondary products" including the functional and evolutionary aspects (Iriti and Faoro 2009). Phenolic acids are important secondary metabolites common in higher plants and exhibit strong allelopathy (Classen *et al.* 1990). They are involved in defense against biotic and abiotic stresses and contribute significantly to antioxidant activity in plant tissues (Ferrat *et al.* 2003, Pourcel *et al.* 2007, Bharti *et al.* 2013). Anwar *et al.* (1995) reported that the composition of phenolic acids in maize and sorghum leaves change after the *Peronosclerospora sorghi* invasion. Among the compounds detected in our study, XF/WS roots showed a significant increase in salicylic acid, benzoic acid, and vanillin over the control roots both before and after inoculation, which suggested that these compounds played a major role in improving the disease resistance of grafted pepper plants.

Enhanced activities of secondary metabolism-related enzymes in plants are usually caused by pathogen invasion. The PAL is a key enzyme in the phenyl-propanoid pathway. Thus, the formation of lignin, sinapic acid esters, and flavonoids all depend on PAL activity (Olsen *et al.* 2008). Yin *et al.* (2013) found that PAL activity, consistent with transcripts of *PAL* gene, is significantly induced in apples by *Diplocarpon mali* inoculation, and inoculated plants of a resistant cultivar have greater PAL activity than those of the the susceptible cultivar. The PPO is involved in the oxidation of polyphenols into quinones (antimicrobial compounds) and the lignification of plant cells during microbial invasion. Previous studies indicated that PPO activity is related to the extent of disease resistance demonstrated by wheat heads following inoculation with *Fusarium graminearum* conidia (Mohammadi and Kazemi 2002). Enhanced POD activity has been also correlated with improved disease resistance in wheat (Mohammadi and Kazemi 2002) and apple (Yin *et al.* 2013). Lignins are aromatic compounds that are deposited in the cell wall and play an important role in preventing pathogen infection. Considering the overall results of this study, we noticed that PAL, PPO, and POD activities increased in both grafted and non-grafted peppers after inoculation, and lignin content increased with an increase in those enzyme activities. These results indicated that infected plants accelerated secondary metabolism and formed more phenolic acids and lignin. Moreover, higher PAL, PPO, and POD activities were observed in grafted plants than in control plants, suggesting that they improves the effectiveness of the cell wall barrier to prevent or retard the spread of a pathogen. This finding might explain why grafted plants exhibited an increase in lignin content especially at a later time after inoculation compared to control plants which contributed to the inhibition of the *F. solani* conidial invasion. Therefore, we suggest that PAL, PPO, and POD activities in pepper roots are positively correlated with the degree of resistance against *F. solani*.

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