

**Physiological effects of *Pseudomonas fluorescens* CHA0 on tomato (*Lycopersicon esculentum* Mill.) plants and its possible impact on *Fusarium oxysporum* f. sp. *Lycopersici*****Zahra Oraghi Ardebili\*, Narges Oraghi Ardebili, Seyed Mohammad Mahdi Hamdi****Department of Biology, Garmsar Branch, Islamic Azad University, Garmsar, Iran****\*Corresponding author: zoardebili@iau-garmsar.ac.ir; zahraoraghi@yahoo.com****Abstract**

Among biocontrol agents, root-associated fluorescent *Pseudomonas* spp. has received special attention. The aim of the present work was assessment of the effects of *Pseudomonas fluorescens* CHA0 on some physiological parameters in tomato plants and possible interaction with *Fusarium oxysporum* f. sp. *lycopersici*. Seed bacterization resulted in significantly higher vigour index of seedlings, indicating growth promoting effects of *Pseudomonas fluorescens* CHA0. The presence of bacteria in culture medium inhibited the fungal growth to about 50% in comparison with the control plates. Studies on induction of defense mechanisms in present research revealed that *P. fluorescens* CHA0 inoculation significantly induced peroxidase (POX), polyphenoloxidase (PPO) and superoxid dismutase (SOD) activities in root tissues whereas phenylalanine ammonia-lyase (PAL) activity and lignification rates remained unchanged. In conclusion, the accumulation of three key defense enzymes, POX, PPO and especially SOD by this bioagent have contributed to induced resistance in tomato against the pathogen. This bioagent can be used to promote germination and plant growth and to control *Fusarium oxysporum* f. sp. *lycopersici*.

**Keywords:** Biocontrol agent; *Fusarium* wilt; defense mechanism; induced resistance; vigour index; antioxidant enzymes.**Abbreviations:** ISR-induced systemic resistance; PAL-phenylalanine ammonia-lyase; POX-peroxidase; PPO-polyphenoloxidase; SOD-superoxid dismutase.**Introduction**

Plant-growth-promoting rhizobacteria (PGPRs) are used as inoculants for biofertilization, phytostimulation and biocontrol (Bloemberg and Lugtenberg, 2001). Plants can acquire resistance to diseases through various biological agents including necrotizing pathogens, non-pathogens and soil born rhizosphere bacteria and fungi (Van loon et al., 1998). Certain plant growth promoting microorganisms could enhance defensive activity and stimulate plant resistance against soil born pathogens (Whipps et al., 2001). Beneficial micro-organisms that improve plant health through the enhancement of plant resistance/tolerance against biotic stresses include bacteria, such as *Pseudomonas* spp. or *Bacillus* spp. and fungi such as *Trichoderma* sp., *Gliocladium* sp. or mycorrhizal fungi (Pozo et al., 2002). Besides direct interaction with plant pathogens, bioagents reported to induce systemic resistance in plants (Srivastava et al., 2010). Many rhizosphere colonizing bacteria, including *Azotobacter*, *Azospirillum*, *Bacillus*, *Clostridium*, and *Pseudomonas*, typically produce substances that stimulate plant growth or inhibit root pathogens (Vázquez et al., 2000). Among biocontrol agents, root-associated fluorescent *Pseudomonas* spp. has received special attention according to some other studies because of its excellent root colonizing ability, potential to produce a wide variety of anti-microbial metabolites, and its induction of systemic resistance (Erdogan and Benlioglu, 2010). *Pseudomonas* are the most diverse and ecologically significant group of bacteria on the planet (Erdogan and Benlioglu, 2010). Certain strains of fluorescent *Pseudomonas* promote plant growth by secreting auxins, gibberellins and cytokinins and hence they are also called

plant growth promoting rhizobacteria (PGPR) (Haas and Défago, 2005). Fluorescent pseudomonads are non-pathogenic rhizobacteria (Saravanan et al., 2004; Karthikeyan et al., 2006) and several isolates of *Pseudomonas fluorescens*, *P. putida*, *P. aeruginosa* and *P. aureofaciens* suppressed the soil born pathogens through different proposed mechanisms including rhizosphere colonization, antibiosis and iron chelation by siderophore production (Karthikeyan et al., 2006). In plants, certain secondary metabolite pathways are induced by infection with microorganisms (Abdul Jaleel et al., 2009). It is well known that the defense genes are inducible genes and presence of appropriate stimuli or signals like prior application of a biological inducer activate them (Ramamoorthy et al., 2002). Treatment with these rhizosphere bacteria in different plants indicate that this results in sensitize plants to defend themselves against pathogen attack by triggering various defense mechanisms including accumulation of pathogenesis related (PR) proteins, deposition of structural barriers (Benhamou et al., 1996) and induction of synthesis of phenolics (M' Piga et al., 1997; Chen et al., 2000). There are many reports that pretreatment with biological control agents lead to induced systemic resistance (ISR) (Pozo et al., 1996; Pozo et al., 1998; Pozo et al., 1999; Hammerschmidt, 1999; Mohammadi and Kazemi, 2002; Pozo et al., 2002; Thangavelu et al., 2003; Falahian et al., 2007). Inducing the plant's own defense mechanisms by pretreatment of a biological inducer is thought to be a novel plant protection strategy (Ramamoorthy et al., 2002). Approximately, twenty bacterial biocontrol products based on *Pseudomonas*, *Bacillus*, *Streptomyces* and *Agrobacterium*

strains has been marketed yet (Bloemberg and Lugtenberg, 2001). Thus, biological control with *P. fluorescens* provides an effective tool for managing soil borne diseases with a minimization of the ecological impact. In recent years, there are interests in biocontrol and biofertilizers. Many studies have been done on different aspects of interactions between different bioagent species including bacteria, *Pseudomonas* spp., *Bacillus* spp. and fungi, *Trichoderma*, *Gliocladium* and mycorrhizal species, in different varieties of plants. However, the beneficial effects of these bioagents on different plants and their applied mechanisms are varied. Therefore, more study is needed to illustrate different aspects of these interactions. There is not much study about bioagent-induced changes of various defense enzymes, especially PAL and SOD. With the purpose of obtaining a better understanding of the biocontrol process, the present study was carried out to investigate the effects of pretreatment of *Pseudomonas fluorescens* CHA0 on plant growth, germination, and possible interactions with *Fusarium oxysporum* f. sp. *lycopersici*, a major limiting factor in the production of tomato, at Petri and pot levels.

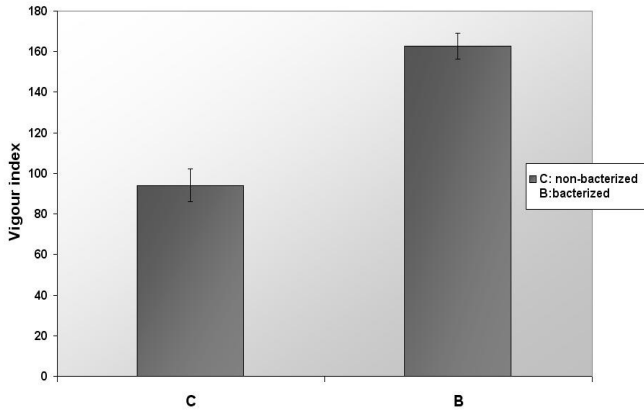
## Results

Study on the effects of applied bacteria on germination and seedling growth rates revealed that seed bacterization improved seed germination and resulted in significantly higher growth rates as shown by higher vigour index (Fig.1). The assessment of inhibiting effects of applied bacteria on the fungal growth illustrated that the presence of bacteria in culture medium significantly inhibited fungal growth. The inhibition percentage of fungal growth on PDA and NA media were 38.75 % and 58.25%, respectively (Fig.2). Studies on induction of antioxidant enzymes, POX, PPO and SOD, revealed that peroxidase activity significantly was induced in both bacterized plants (B and BF) as compared to the control with the highest amount observed in BF whereas there were not any significant differences between pathogen inoculated and control plants (Fig.3). Fourteen days after pathogen inoculation, peroxidase activity of B and BF samples declined although it was still significantly higher than control plants in BF (Fig.3). *Fusarium oxysporum* f.sp. *lycopersici* and/or *Pseudomonas fluorescens* CHA0 inoculation, significantly stimulated the PPO activity in BF, F and B treatment groups at both mentioned times of analysis (seven and fourteen days after pathogen inoculation). The highest PPO activity was found in F and BF respectively on the second time of the analysis (Fig.4). SOD activity significantly increased in three treatment groups including B, F and BF at both times of analysis where stimulating effect of *Pseudomonas fluorescens* inoculation was more than pathogen (Fig.5). Evaluation of the effect of applied treatments on the activity of PAL, the key enzyme of phenyl propanoid metabolism, illustrated that there was no significant difference between treatments at the times of our analysis (Fig.6). *Fusarium oxysporum* f.sp. *lycopersici* and/or *Pseudomonas fluorescens* CHA0 inoculation did not have any significant effects on lignin deposition and it remained unchanged.

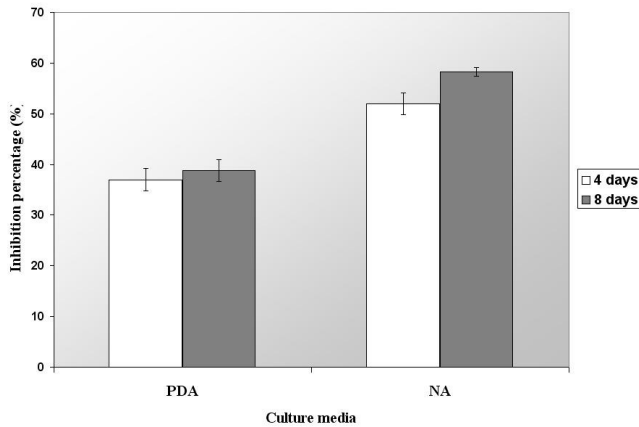
## Discussion

In the present research, seed bacterization resulted in significantly higher growth rates as shown by higher vigour index. This result indicates that *Pseudomonas fluorescens* CHA0 has growth promoting effects. It seems that the enhancing effects of *Pseudomonas fluorescens* CHA0 on

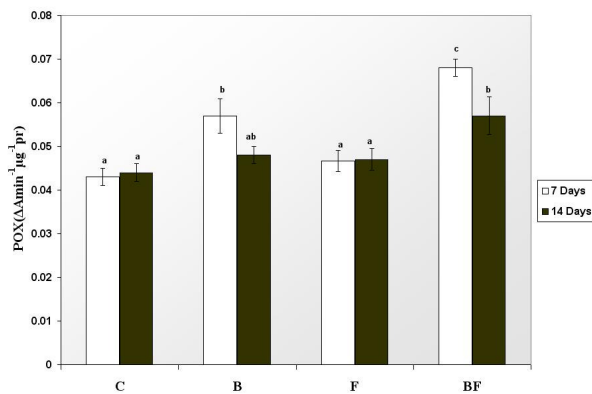
seed germination and seedling growth result from bacterial secreted hormones. These results are in agreements with some other reports about stimulating effects of different plant growth promoting bacteria. Application of *Pseudomonas fluorescens* improved seed germination (Fu et al., 2010; Srivastava et al., 2010) and vigour of seedlings (Nandakumar et al., 2001; Abo-Elyousr and El-Hendawy, 2008). *P. aurantiaca* SR1 colonized the root system of maize and wheat and also showed a significant plant growth promoting effect (Rosas et al., 2009). Pseudomonads promote plant growth by secreting auxins, gibberellins and cytokinins (Thangavelu et al., 2003; Haas and Défago, 2005; Gravel et al., 2007; Yao et al., 2010). Biological seed treatment with antagonistic *Pseudomonas fluorescens* improved seed quality (Umsha, 2006). In this observation, the presence of bacterial bioagent inhibited the fungal growth in culture media, PDA and NA. The inhibition percentages of fungal growth on PDA and NA media were about 38.75 % and 58.25%, respectively. It may be due to nutritional competition, colonization of fungal hyphae by the bacteria and inhibiting compounds secreted by the bacteria. Two *Pseudomonas fluorescens* strains PF1 and PF7 inhibited the mycelial growth of *Rhizoctonia solani* (Nandakumar et al., 2001) and strain Pf 8a produced antibiotics with wide inhibition zone against *Penicillium italicum*. Bolwerk et al. (2003) with working with two *Pseudomonas* biocontrol strains reported that the microbes effectively compete for the same niche, the presence of bacteria negatively affects infection of the tomato root by the fungus, bacteria colonize the hyphae extensively and affect hyphal growth and branching (Bolwerk et al., 2003). Scanning electron microscopy revealed that *P. fluorescens* WCS365 colonizes developing hyphae (Kamilova et al., 2008). In addition to promoting plant growth, *Pseudomonas fluorescens* PF1 as an effective biocontrol agent reduced several plant diseases (Manikandan et al., 2010). Mechanisms of biological control by which rhizobacteria can promote plant growth indirectly, i.e., by reducing the level of disease, include antibiosis, induction of systemic resistance, competition for nutrients and niches (Lugtenberg and Kamilova, 2009). Biocontrol pseudomonads implicated in the suppression of fungal root diseases by various mechanisms including production of antibiotics, toxins, bio-surfactants, or lytic enzymes, competition for colonization sites, nutrients and minerals, and induction of systemic resistance (Erdogan, and Benlioglu, 2010). Siddiqui and Shaukat (2003) suggested that the antibiotic 2, 4-DAPG from *P. fluorescens* CHA0 act as the inducing agents of systemic resistance in tomato roots (Siddiqui and Shaukat, 2003). The production of antifungal metabolites proposed as a main mechanism of antifungal activity of *Pseudomonas fluorescens* against *Fusarium oxysporum* f.sp. *lycopersici* (Kumar et al., 2002; Karkachi et al., 2010). *P. fluorescens* WCS365 efficiently suppressed *Fusarium oxysporum* (Chin-A-Woeng et al., 1998; Dekkers et al., 2000; Bolwerk, 2003). In present research, studies on induction of antioxidant enzymes, POX, PPO and SOD, revealed that peroxidase activities significantly were induced in both bacterized plants (B and BF) with the highest amount observed in BF, whereas there were not any significant differences between pathogen inoculated and control plants. *Fusarium oxysporum* f.sp. *lycopersici* and/or *Pseudomonas fluorescens* CHA0 inoculation significantly stimulated the PPO activity in BF, F and B treatment groups with the highest amount found in F and BF. Significantly increased SOD activities were observed in B, F and BF where stimulating effect of *Pseudomonas fluorescens* inoculation was more than pathogen. The obtained results from present research indicated that the



**Fig 1.** The mean of vigour index in bacterized (B) and non-bacterized (C) seeds. The vertical bars indicate standard errors of three replications.

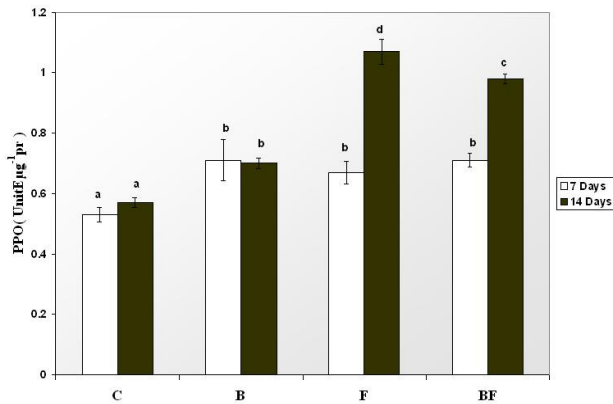


**Fig 2.** The fungal inhibition percentage by *P. fluorescens* CHA0 (four and eight days after interactions) in two culture media, PDA and NA. The vertical bars indicate standard errors of three replications.

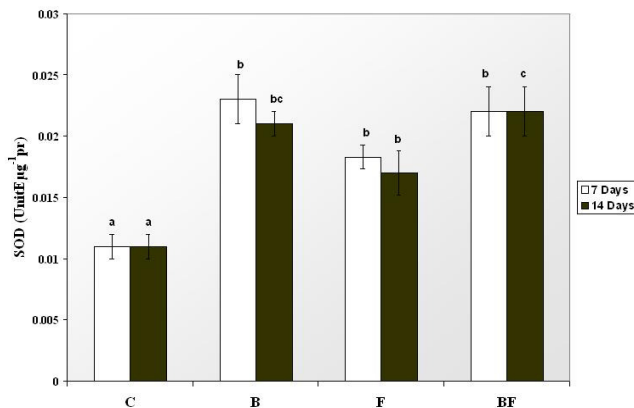


**Fig 3.** Changes in peroxidase (POX) activity induced by *P. fluorescens* CHA0 in tomato roots challenged with or without the pathogen *F. oxysporum lycopersici* at seven and fourteen days after pathogen inoculation. The vertical bars indicate standard errors of three replications. Four treatment groups were control (C), bacterium inoculated (B), fungus inoculated (F) and bacterium-fungus treated (BF) plants.

activities of antioxidant enzymes, as an important part of defense mechanisms, were induced by applied *Pseudomonas fluorescens* CHA0 as a bioagent. The enhanced activities of defense enzymes may contribute to bioprotection of plants against pathogens. Enhanced activities of SOD may result in increased  $H_2O_2$  concentrations which it could act as a signaling agent, inducer of other defense genes. Therefore, stimulated activity of SOD may lead to induced systemic resistance. Other important parts of defense mechanisms are phenylpropanoid metabolism and lignifications of cell walls. Study on the activity of PAL, the key enzyme of phenylpropanoid metabolism, illustrated that there were not any significant differences of PAL activity between treatments. It is probable that changes in PAL occurred earlier and declined to the control level at the time of our analysis. It is also possible that induction of PAL is a long term response and may be found later. Fungal and bacterial treatments did not result in increased lignifications levels of root cell walls. It is possible that improved lignifications, as a part of defense mechanism, is a long term response and may be observed later. It is likely that induced systemic resistance (ISR) triggered by *P. fluorescens* WCS365 is the underlying mechanism of biocontrol of *Fusarium oxysporum* (Dekkers et al., 2000). Ultrastructural observations confirmed that *P. fluorescens* strain 63-28 may function as an inducer of plant disease resistance (Mpiga et al., 1997). Thangavelu et al. (2003) suggested that the enhanced activities of defense enzymes may contribute to bioprotection of Banana plants against *Fusarium oxysporum*. It is now clear, that any stress condition or significant change in environment is associated with up- or down-regulation of hundreds of genes (Cheeseman, 2007). Induction of defense proteins positively correlates with defense against pathogen invasion in different plants (Ramamoorthy et al., 2002). POX is part of the pathogenesis related protein - 9 (PR-9) families (Chen et al., 2000). The activity of POX is a useful marker for localized and systemic acquired responses in plants challenged by a pathogen (Martinez et al., 2000; Mohammadi et al., 2002). Various abiotic and biotic inducers of resistance stimulated activity of POX and PAL in treated plants (Ruiz et al., 1999). PPO is involved in the oxidation of poly phenols into quinons (antimicrobial compounds) and lignification of plant cells during microbial invasion, and also may participate in the responding defense reaction and hypersensitivity by inducing plant resistance against fungi (El- Khallal, 2007). Accumulation of PAL, POX, and PPO by *P. fluorescens* in roots contributed to induced resistance in banana against *Fusarium oxysporum* f.sp. *cubense* (Saravanan et al., 2004). Bacterization with *Pseudomonas* sp. strain GRP3 elevated POX activity in roots (Sharma et al., 2003). Inoculated *Pseudomonas* sp. DW1 had stimulating effect on SOD activity in the leaves of eggplants (Fu et al., 2010).  $H_2O_2$  is produced indirectly by spontaneous or SOD-mediated dismutation (Cheeseman, 2007). It is well established that oxidative metabolism, and particularly  $H_2O_2$ , is involved in a wide variety of reactions and signaling cascades necessary for all aspects of plant growth (Cheeseman, 2007).  $H_2O_2$  generation have direct antimicrobial activity inhibiting germination of spores of many fungal pathogens and participate in the formation of phenoxy-radicals during phenol-polymerization within the plant cell wall (El- Khallal, 2007).  $H_2O_2$ , in addition to being a toxic agent, has been regarded as a signaling molecule and a regulator of the expression of some genes encoding antioxidants, defense proteins, and signaling proteins in cells (Hung et al., 2005).

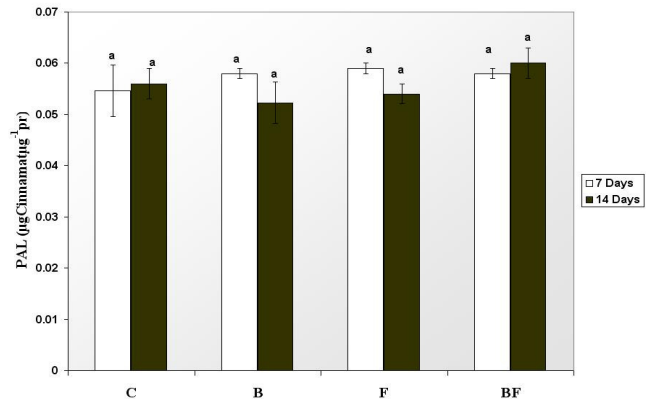


**Fig 4.** Induction of polyphenoloxidase (PPO) activity by *P. fluorescens* CHA0 in tomato roots challenged with or without the pathogen *F. oxysporum lycopersici* seven and fourteen days after pathogen inoculation. The vertical bars indicate standard errors of three replications. Four treatment groups were control (C), bacterium inoculated (B), fungus inoculated (F) and bacterium-fungus treated (BF) plants.



**Fig 5.** Induction of superoxid dismutase (SOD) activity by *P. fluorescens* in tomato roots challenged with or without the pathogen *F. oxysporum* f.sp. *lycopersici* at seven and fourteen days after pathogen inoculation. The vertical bars indicate standard errors of three replications. Four treatment groups were control (C), bacterium inoculated (B), fungus inoculated (F) and bacterium-fungus treated (BF) plants.

Several of the biocontrol bacterial strains of *Pseudomonas fluorescens* provided significant control of different types of diseases in radish (Leeman et al., 1995; Raaijmakers et al., 1995), tomato (Raupach et al., 1996; M Piga et al., 1997; Larkin and Fravel, 1998; Siddiqui et al., 2001; Ramamoorthy et al., 2002; Guo et al., 2004; Campbell et al., 2006), chickpea (Vidhyasekaran and Muthamilan, 1995; Ramandeep et al., 2007), alfalfa (Quagliotto et al., 2009), olive (Mercado-Blanco et al., 2004), cucumber (Liu et al., 1995; Raupach et al., 1996), eggplants (Malandraki et al., 2008), carnation (Van Peer et al., 1991), banana (Raguchander et al., 1997; Saravanan et al., 2004), wheat (Weller and Cook, 1983), cotton (Erdogan and Benlioglu, 2010) and potatoes (Uppal et al., 2008). Mycorrhizal fungi increased biocontrol potential of *Pseudomonas fluorescens* against *Gaeumannomyces graminis* (Siasou et al., 2009). Bautista et al. (2007) recommended that the utilization of *P. fluorescens* for the control of *Rhizoctonia solani* is a suitable



**Fig 6.** Amounts of phenylalanine ammonia-lyase (PAL) activity in bioagent and/or pathogen treated roots. The vertical bars indicate standard errors of three replications. Four treatments were control (C), bacterium inoculated (B), fungus inoculated (F) and bacterium-fungus treated (BF) plants.

strategy for the management of the disease in field conditions (Bautista et al., 2007).

## Materials and methods

### The preparation of Bacterial and fungal inoculums

Microorganisms used were prepared from Iranian Research Institute of Plant Protection. *Pseudomonas fluorescens* CHA0 was cultured on KB agar slants (King et al., 1954). *Pseudomonas fluorescens* CHA0 was multiplied in NB for 24 h and bacterial cells were collected by centrifugation and population was adjusted to  $3 \times 10^8$  colony forming units (cfu). *Fusarium oxysporum* f. sp. *lycopersici* causing wilt disease was maintained on sterilized sandy soil mixed with maize powder at 19:1 w/w. The concentration of microconidia was adjusted to  $10^7$  conidia mL<sup>-1</sup>.

### Efficacy of *Pseudomonas fluorescens* CHA0 on plant growth-promotion under laboratory conditions

#### Seed bacterization

Seeds of tomato (*Lycopersicon esculentum* Mill.) were surface-sterilized. Ten ml of bacterial inoculum containing  $3 \times 10^8$  cfu mL<sup>-1</sup> was added to Petri plates. Then, 100 ml of 1% carboxy methyl cellulose was used as adhesive material and seeds were soaked in the bacterial suspension for 12 h. Then, the bacterial suspension was drained off and the seeds were dried overnight in sterile Petri plates.

#### Plant growth-promotion

Plant growth-promoting activity of *Pseudomonas fluorescens* CHA0 was assessed based on the seedling vigour index in Petri plates as described by Ramamoorthy (2002). The vigour index was calculated by using the following formula

$$\text{Vigour index} = (\text{Mean root length} + \text{Mean shoot length}) \times \text{Germination}(\%)$$

### **Determination of inhibition percentage of the fungal growth**

Inhibition percentage of the fungal growth in plate was determined in two NA (nutrient agar) and PDA (potato dextrose agar) media. *P. fluorescens* CHA0 were cultured four sides of Petri plates and 24h after culture of bacteria, discs of the four-day old mycelium of *Fusarium oxysporum* f. sp. *lycopersici* were placed in the center of plates. The percentage of growth inhibition of *Fusarium oxysporum* f. sp. *lycopersici* was assessed four and eight days after culture in comparison with the control plates.

### **Experimental design**

The pot experiment was carried out in a complete randomized design with four treatments and three replications. Potting soil, sandy loam mixed with pit, was autoclave-sterilized and placed in plastic pots. Untreated seeds served as control (in half of the pots, bacterized seeds were planted). Thirty-day-old seedlings were treated. There were four treatments including control (C), bacterium (B), fungus (F) and bacterium-fungus (BF) inoculated plants. In B and BF treatments, 10 ml of bacterial suspension ( $10^8$  cfu mL<sup>-1</sup>) was added to each pot. Bacterized plants were divided into two treatments. In BF treatment, bacterized plants were inoculated with *F. oxysporum* f. sp. *lycopersici*, one day after application of bacteria, 50 ml of conidial suspension ( $10^7$  microconidiam<sup>-1</sup> pot<sup>-1</sup>). Plants without prior treatment of *P. fluorescens* were inoculated with the pathogen (F). Plants were harvested at seven and fourteen days after the pathogen inoculation for the biochemical analysis.

### **Preparation of enzyme extracts and quantification of protein content**

Enzymes were extracted at four centigrade degree from 1g FW root tissue using phosphate buffer (0.1 M pH7) as an extraction buffer. Protein content of the enzyme extracts were measured according to the procedure of Bradford (1976) using BSA as the standard.

### **Determination of peroxidase (POX) activity**

Peroxidase activity was assayed as described by Abeles and Biles (1991). The peroxidase activity was expressed as an increase in absorbance per min per microgram protein ( $\Delta A_{min}^{-1} \mu g^{-1} pr$ ).

### **Assessment of polyphenol oxidase (PPO) activity**

PPO activity was measured as described by Raymond et al., (1993). The reaction mixture consisted of 2.6 ml of 0.2 M phosphate buffer PH 7.6 and 200 $\mu$ l of pyrogallol as a substrate. The rate of increase in the absorbance at 430nm was measured. A unit of enzyme activity is defined as the amount of enzyme causing an absorbance increase of 0.01 units per min at 430 nm as described by Shi et al., 2001, previously. The activity was expressed as unit enzyme per micro gram protein (UnitE $\mu$ g<sup>-1</sup>pr).

### **Measurement of superoxid dismutase (SOD) activity**

SOD activity was assayed by the photochemical method described by Giannopolitis and Ries (1977). One unit SOD activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT (nitro blue

tetrazolium) reduction measured at 560 nm. The activity was expressed as unit enzyme per micro gram protein (UnitE $\mu$ g<sup>-1</sup>pr).

### **Determination of phenylalanine ammonia lyase (PAL) activity**

PAL activity was measured according to the procedure described by Beaudoin-Eagan and Thrope (1985). PAL activity was assessed by measuring the amount of cinnamic acid produced at 290nm and is expressed as microgram of cinnamate per microgram of protein ( $\mu$ gcinnamate $\mu$ g<sup>-1</sup>pr).

### **Estimation of lignin deposition**

The Pg-HCl test was performed using a method described by Redman (1999). Roots were decolorized in 70% ethanol for 24 hours, washed with distilled water and exposed to 1% phloroglucinol for one hour. The roots were then exposed to 6M HCl until a red color developed. The rate of red colour was compared.

### **Statistical procedure**

Analysis of variance was performed on all data sets. Duncan test with probability of 0.05 was used to show significant differences between treatments. All data are presented as mean  $\pm$  SE.

### **Conclusions**

From these results we concluded that accumulation of three key defense enzymes, POX, PPO and especially SOD by *P. fluorescens* CHA0 in tomato roots contributed to induced resistance in tomato against *F. oxysporum* f.sp. *lycopersici*. The *Pseudomonas fluorescens* CHA0 has strong potential to promote seedling growth and inhibit *F. oxysporum* f.sp.*lycopersici* in tomato plants through induced resistance against pathogen probably by inducing defense enzymes or directly inhibiting the fungal growth by producing its own chitinase or antibiotic substances.

### **Acknowledgements**

This study is supported by the Islamic Azad University, Garmsar branch. Authors would like to thank Dr. Rastegar, Dr. Jabbarpoor, and Iranian Research Institute of Plant Protection for providing the living microorganisms.

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