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Full Length Research Paper

Change of some defense compounds of cucumber treated with *Bacillus cereus* and salicylic acid against *Meloidogyne javanica*

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In this research, the effects of antagonist bacterium (*Bacillus cereus*) and salicylic acid (SA) as chemical inducer alone and in combination against root knot nematode (*Meloidogyne javanica*) as the most important nematode in Iran, on cucumber roots (cv. superdominus) was investigated. The used concentrations were 10^9 cfu/ml for bacteria and 5 mM for SA. In greenhouse experiments some parameters were evaluated including nematode and plant growth criteria in treated plants with the two above materials. Results of this experiment showed that combination of biocontrol agent and chemical inducer decreased galling, egg masses and egg numbers on roots of cucumber. In biochemical analysis, also, some of defense compounds such as hydrogen peroxide and peroxidase activities were measured. Results showed that hydrogen peroxide level and peroxidase activity increased after inoculation until 4th and 5th days respectively and then decreased gradually. Soil drenching of *B. cereus* and SA caused maximum induction in roots.

Key words: *Bacillus cereus,* salicylic acid, *Meloidogyne javanica,* peroxidase, hydrogen peroxide.

INTRODUCTION

Root knot nematodes *Meloidogyne* spp. are the second identified nematode and one of important plant pathogens. They have a wide range host including more than 2000 plant species (Gugino et al., 2008). Peroxidase oxidizes a wide range of substrates and is implicated in various physiological processes including pathogen defense, stress response, oxidation of phenolics and lignin polymerization (Criquet et al., 2001). Sometimes peoxidase biosynthesis by pathogen infection, induce resistance in plants. Anionic peroxidases are related with cell wall and polimerize cinnamyl alcohols *in vitro* and contribute in lignin formation. Peroxidase activity and lignification process in related with anionic cell wall peroxidases (Zacheo et al., 1993). There is a direct relation between peroxidase increasing and resistance to *Meloidogyne incognita* in tomato cultivars (Zacheo et al., 1993). Hammerschmidt et al. (1995) showed that peroxidase change hydroxyprolin protein cell wall compounds which affect cell wall resistance to pathogen destructor enzymes. *Trichoderma* induced resistance and phytoalexine production; also, it can increase peroxidase and chitinase in cucumber roots (Yedidia et al., 1999). Williamson (1996) identified genes which induce peroxidase, lipoxygenase and chitinase in tomato roots infected by *Meloidogyne* spp.

Active oxygen species (AOS) usually are few produced, due to electron transmission in chloroplast, mitochondria and enzymatic activity in other involved parts in cell oxidation-reduction processes. Some active oxygen species especially hydrogen peroxide also are generated in plants in response to biotic or abiotic stresses (Desikan et al., 2000). Plant cells need H_2O_2 as a substrate in several cell wall-located reactions. Most of these reactions are catalyzed by peroxidases (Barcelo, 1998). They are induced in response to some microbial and

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chemical stimulators in plants (Desikan et al., 2000). H_2O_2 has an intensive trend to proteins, lipids and nucleic acids and scientists believe that its effect mechanism is decomposition of related tissues.

Effect of cadmium (Cd) on hydrogen peroxide and superoxide anion accumulation in leaves of *Phaseolus aureus* and *Vicia sativa* was investigated. Results showed that concentrations of 100 µM of cadmium significantly increased both of them (Zhang et al., 2009). Adam et al. (1989) showed that *Pseudomonas syringae* PV. *Syringae* infection in tobacco plants induced HR reaction which follows by an increasing in O^{2-} production and lipid peroxidation (Adam et al., 1989). Antimicrobial activity of active oxygen species such as H_2O_2 was proved by Alamillo et al. (2001) *in vitro* and by Garcia-Olmedo et al. (2001) *in vivo* conditions. AOS play a dual role in defense: 1) direct role by antibiotical activity at pathogen attraction site 2) indirect role as mediator of activation of other defense compounds (Baker et al., 1995) which act as signal in systemic acquired resistance and induce defense genes. H_2O_2 is also necessary to phytoalexine synthesis (Kuzniak et al., 1999). Active oxygen species produce aerobic metabolism products so are toxic but act as regulator of some biological processes and pleiotropical effects creation by its signal transduction role and regulate programmed cell death (Gadjev et al., 2008).

The aim of this study is investigation of antagonistic effect of *Bacillus cereus*, induction effect of salicylic acid and integration of them on root knot nematode *Meloidogyne javanica* on cucumber (var. *superdominus*) as the most important plant parasitic nematode in Iran under greenhouse conditions and evaluation of some defense compounds.

MATERIALS AND METHODS

Preparation of materials

Preparation of nematode inoculums

The samples of root knot nematode were prepared from infested fields, single egg mass was reared on susceptible tomato cultivar (Early Urbana). After that, females were identified according to perennial pattern technique (References).

Preparation of antagonist bacterium (B. cereus)

The bacterium was obtained from plant pathology laboratory of Aboureyhan Campus, University of Tehran. The declared isolate was stored in sterile distilled water, and was cultured in nutrient agar medium. Density of 10^9 colony forming unit (cfu) per milliliter of water of 24 h bacterium culture was prepared by serial dilution (10^1-10^9) in 590 nm (Zhang et al., 2002).

Preparation of salicylic acid

SA (Merck) was prepared and was dissolved in ethanol and water according to results obtained from other studies, concentration of 5 mM of SA has no effect on antagonist bacteria (*B. cereus*), so in this experiments, to evaluate the effect on nematode, concentrations of 0, 1, 3, 5, 7 and 10 mM also were prepared (Siddiqui and Shaukat, 2003).

Standard protein

Total protein of plant extract was measured by Bradford (1976).

Antagonistic effect of *Bacillus cereus* and induced resistance on cucumber against *M. javanica* by SA alone and in combination with each other were used in greenhouse before nematode inoculation

In this experiment disinfested seeds of cucumber (cv. superdominus) were planted in 1 kg pots filled with sterile soil (sand, field soil, humus 1:1:1) in 24-27°C under greenhouse conditions. Four to six leaves of seedlings were treated with 25 ml of the above concentrations of bacterial suspension and SA, at 2 continuous days and both of them used alone and in combination as soil drenching, but SA were used in both soil drenching and foliar spray methods. Next day, 2000 J_2 of root knot nematode per plant were inoculated. Control plants only treated with distilled water only instead of bacterium and SA. After 45 days, the number of galls and egg masses per plant and the number of eggs per individual egg mass were measured. The experimental design was completely randomized design with 6 treatments with 5 replications (Each replication includes one seedling).

Defense compounds and enzymes extraction

To evaluate the level of some plant defense compounds induced by *Bacillus cereus* and salicylic acid, H_2O_2 and peroxidase levels in plant were measured. Seeds of cucumber were planted in 1 kg pots filled soil (field soil, humus and sand 1:1:1) at 24 to 27°C in greenhouse. Treatments were: sterile distilled water-treated plant, inoculated plant with nematode, Bacillus and salicylic acid alone and in combination. The seedlings were inoculated at 6 leaves stage as follows:

1) Soil drenching of 30 ml of bacterial suspension alone and in combination with SA at first day (to better contact with root and less loss of suspension, soil drenching method was selected) Soil drenching of 30 ml of SA alone and in combination with *B. cereus* at the second day.
2) Inoculation of all of seedlings (these plants were not treated by bacterium or SA and must be inoculated with nematode as control) by 1000 J_2 of nematode at third day.

To evaluate the response of defense system of plant, root samplings were taken at 7 continuous days (which started at the next day after nematode inoculation). At each day, 20 plants were sampled for the analysis. After washing and removing of soil from roots, they were transported to laboratory for extraction.

Protein extraction

Half g. of root tissues were ground and homogenized with liquid nitrogen in porcelain mortar (which has been cold in refrigerator before) and 1 ml sample phosphate buffer 0.1 M (pH=6) was added and mixed. The mortar were on ice basin all of stages. They were poured in to 2 ml microtubes and they were centrifuged at 4°C for 20 min at 13000 g. Supernatants were separated and were kept at -

40°C (Bradford, 1976).

POX evaluation

Two millimeter of reaction mixture including some extraction (which have 40 µg protein), 20 µl guaiacol and adequate citrate phosphate buffer 25 mM (pH 5.4) were poured in to micro tubes until total volume reached 2 ml and were used as blank in spectrophotometer at 475 nm. Then 10 µl hydrogen peroxide 30% was added to complex and absorbance was measured for 1 min by 10 seconds intervals (Sahebani, 2003; Reuveni and Bothma, 1995).

Statistical analysis

All data were analyzed by SAS.9. Comparison of means of squares was performed by Duncan's multiple range tests ($P \leq 0.05$). The experiments design were factorial design in 5 treatments (nematode, SA, *B. cereus*, combination of them and plant treated only with distilled water) with 4 replications at 7 sampling days (7 days continuously after the last treatment).

Hydrogen peroxide level measurement

About 0.3 g of root tissues were ground and homogenized with liquid nitrogen in porcelain mortar (which had cooled in refrigerator before) and 3 ml TCA (trichloro acetic acid) 0.1% was added to them and after complete mixing, they were transformed in to 2 ml microtubes. Then samples were centrifuged at 4°C for 15 min at 12000 g. Supernatants were transformed to new microtubes. 0.5 ml of supernatants were mixed with 1 ml 1M KI buffer and were poured in to new microtubes (Velikova et al., 2000). The samples were kept in room temperature for 5 min, the absorbance was measured spectrophotometrically against a healthy plant root extract blank at $\lambda = 390$ nm.

RESULTS

Antagonistic effect of *Bacillus cereus* and induced resistance on cucumber against *M. javanica* by SA alone and in combination under greenhouse conditions before nematode inoculation

The results of this experiment showed that treatments were significantly ($p \leq 0.05$) different compared to control in the numbers of galls. According to Table 1, the treatment 6 (soil drenching of bacteria +SA) had a significant ($p \leq 0.05$) difference with control and with other treatments. The lowest numbers of galls were found in this treatment. Treatment 2 (soil drenching of bacteria) and 5 (soil drenching of bacteria + spraying of SA), treatment 3 (spraying of SA) and 4 (soil drenching of SA) had no significant ($p \leq 0.05$) differences (Table1).

All treatments were significantly ($p \leq 0.05$) different compared to control in the numbers of egg masses. Treatment 6 (soil drenching of bacteria +SA) had significant ($p \leq 0.05$) difference compared with control and other treatments and the lowest numbers of egg masses were measured in it. There were no difference between treatment 2 (soil drenching of bacteria) and 5 (soil drenching of bacteria and spraying of SA), 3 (spraying of SA) and 4 (soil drenching of SA) (Table 1).

All treatments were significantly ($p \leq 0.05$) different compared to control in the numbers of eggs per egg mass. Treatment 6 (soil drenching of bacteria +SA) had the lowest numbers of eggs. Treatments 3 (spraying of SA) and 4 (soil drenching of SA), also treatments 5 and 6 had no differences (Table 1).

POX measurement

The results showed that there are significant ($p \leq 0.05$) differences among treatments and sampling days. Also, among their reciprocal effect on changes of peroxidase activity, significant ($p \leq 0.05$) difference was observed. Enzyme activity in all treatments and in control (nematode only) began at first day after nematode inoculation, and reached to maximum level at 5th day, then decreased. All treatments have significant ($p \leq 0.05$) difference compared with control and others. Combination of bacteria and SA, and SA alone produced maximum induction and increased peroxidase activity. At 6th day after nematode inoculation in all treatments, POX activity decreased (Figure 1).

H_2O_2 measurement

In this experiment, there are significant ($p \leq 0.05$) differences among treatments and sampling days compared with control. Soil drenching of salicylic acid alone and in combination with Bacillus cereus has significant ($p \leq 0.05$) differences compared with control and induced the highest level of H_2O_2 in plant. Nematode induced the minimum H_2O_2 production.

The results showed that, at 4th day, a peak was observed and then it decreased. It was found that reciprocal effects of treatments and sampling days have significant differences ($p \leq 0.05$). Soil drenching of bacteria +SA induced the highest level of hydrogen peroxide at 4th day in cucumber root (Figure 2).

DISCUSSION

To evaluate root knot nematode pathogenicity in plant, gall numbers, eggmasses and egg per eggmass were measured. Sahebani (2003) measured eggmass numbers and egg per eggmass too. Soil drenching of bacteria suspension and salicylic acid decrease gall, eggmass and egg numbers, Nandy et al. (2003) found that salicylic acid decreased gall and egg numbers of *Meloidogyne incognita* on okra and cowpea. Our results showed that salicylic acid can suppress nematode density especially in combination with *B. cereus*. As a result, the antagonist bacterium increased plant growth,

Table 1. Average of gall number, egg mass per plant and egg per individual egg mass evaluation in "effect of *Bacillus cereus* and SA on cucumber against *M. javanica* before nematode infection in greenhouse experiment"

treat	Average of gall number	Average of egg mass number per root	Average of egg number per egg mass
control	252.2^{a}	290.4^{a}	400^{a}
soil drenching of bacteria	96^{c}	108^{bc}	272^{b}
spraying of SA	146^{b}	120^{b}	140^{c}
soil drenching of SA	124^{bc}	104^{bc}	103^{ac}
soil drenching of bacteria and spraying of SA	78^{de}	70^{ca}	80^{ac}
soil drenching of bacteria +SA	50^{e}	37.6^{u}	50.4^{u}

*Data are means of 5 replications. The treatments with the same letters have no significantly different according to Duncan's multiple range Test at p≤0.05.

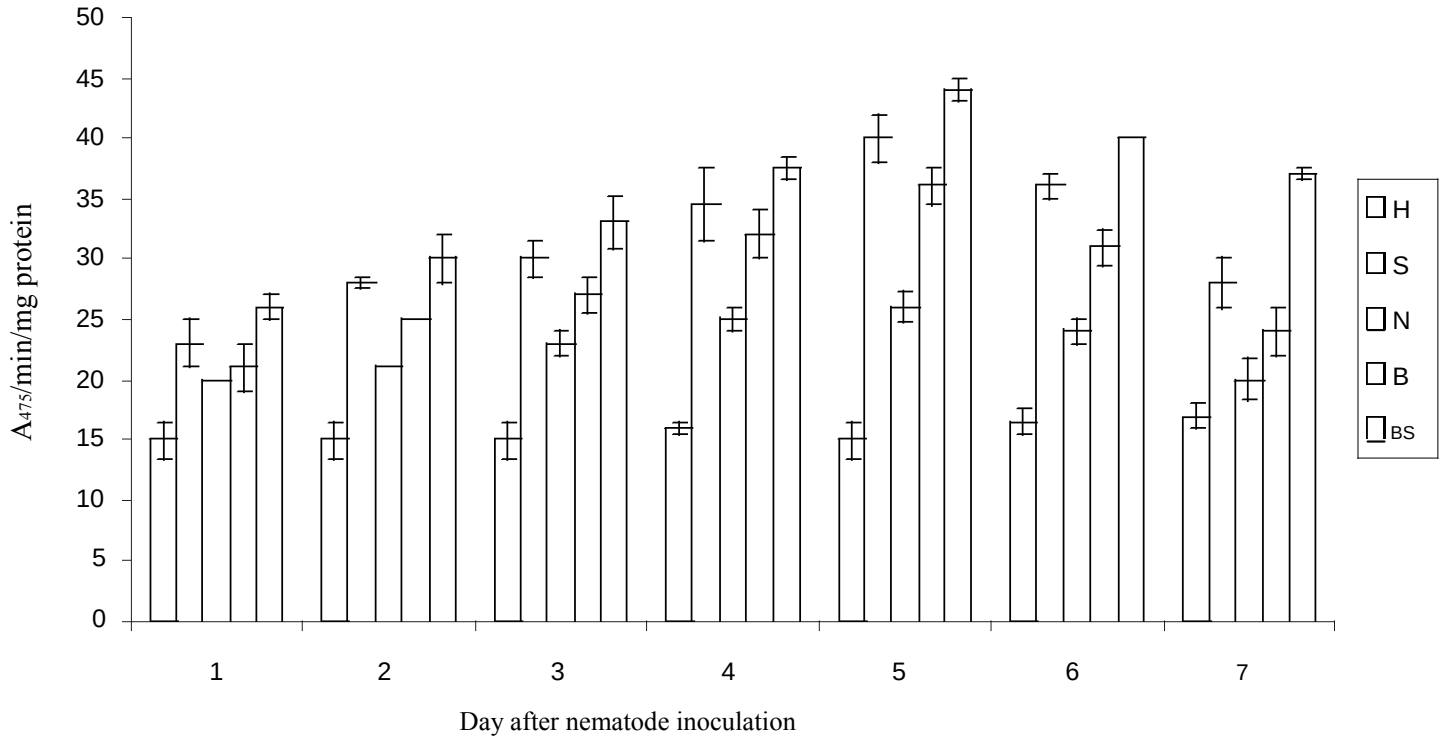

Figure 1. Changes of peroxidase activity at1 to 7 days after nematode inoculation. Columns with the same letters have no significant (p≤0.05) differences, H: Distilled water, S: SA, N: Nematode, B: Bacteria, BS: Bacteria +SA. Each number is mean of 4 replications.

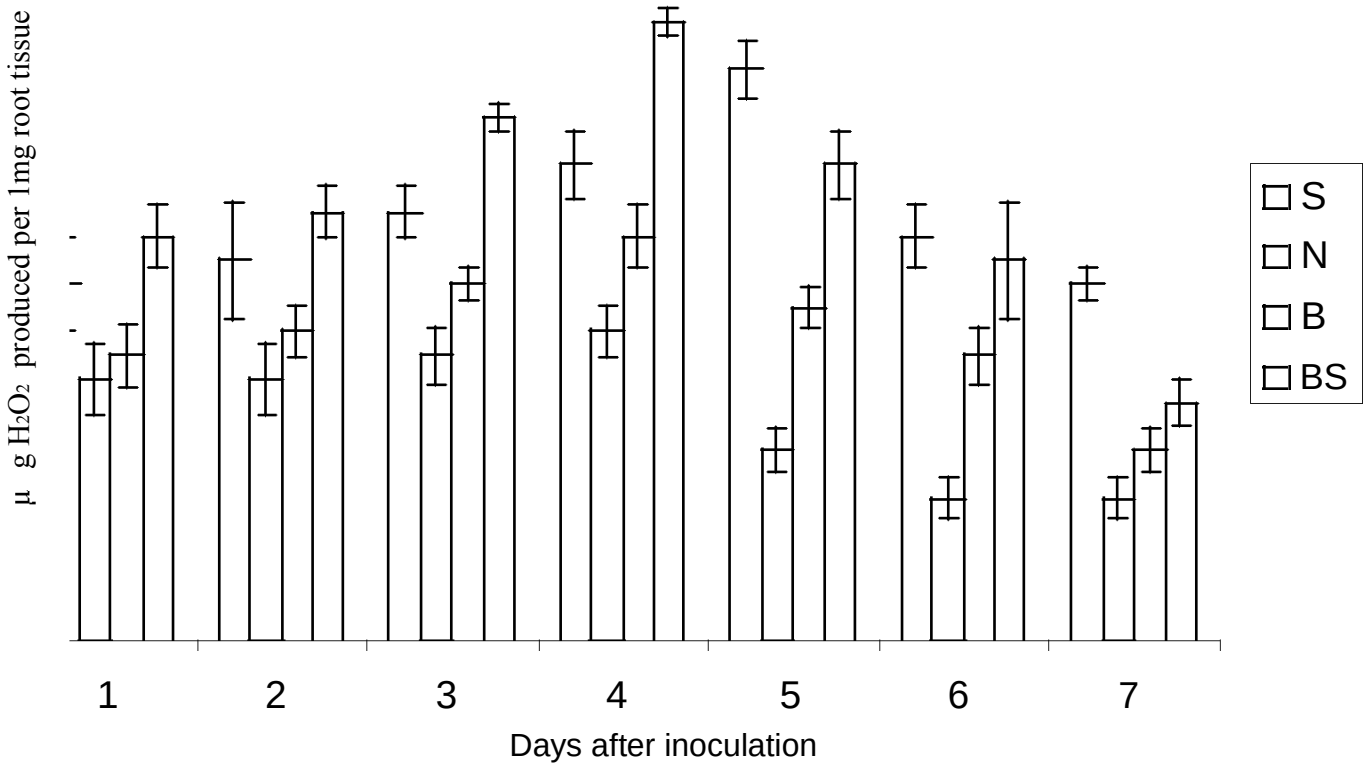

Figure 2. H_2O_2 level produced in cucumber seedlings at 1 to 7 days after nematode inoculation. Columns with the same letters have no significant differences (P≤0.05). S: SA, N: Nematode, B: Bacteria, BS: Bacteria +SA. Each number is mean of 4 replications.

and SA induced plant defense. Anionic peroxidases are related with cell wall lignifications and cationic peroxidases play a key role in defense mechanisms against oxidative stresses (Penel and Castillo, 1991). Harman et al. (2004) affirmed that *Trichoderma harzianum* induced systemic resistance in corn against fungi pathogens.

Premachandran et al. (1983) reported that peroxidase (POX) has an effective role in resistance to *Meloidogyne* spp. Change of POX activity is a marker of biochemical processes in plants which are part of resistance reaction.

Evaluation of hydrogen peroxide showed that stimulation of plant defensive system with applicated inducer, H_2O_2 level increased gradually and reached to maximum amount at 4th day, then decreased gradually. In a study by Sahebani and Hadavi (2009), effect of β-minobutyric acid (BABA) and *Pseudomonas fluorecens* CHAO on inducing hydrogen peroxide and related enzymes level in roots of infected tomato with root knot nematode (*M. javanica*) was investigated which showed more enhancement than control and the highest amount was measured at 5th day after treating. Mokhtari et al. (2007) used combination of biocontrol factors *Pseudomonas fluorescens* and *Trichoderma harzianum* against root knot nematode (*M. javanica*) on tomato roots. They found that foliar spraying of *P. fluorescens* CHAO caused an induction of hydrogen peroxide in roots. Soil drenching of fungi and spraying of bacteria increased H_2O_2 more than others.

Conclusion

In conclusion, chemical inducer (salicylic acid) in combination with biocontrol agent (*Bacillus cereus*) stimulated plant defense system and increased H_2O_2 level. At the same trend, Zhang et al. (2010) showed that copper induced hydrogen peroxide production in *Elsholtzia haichowensis* leaves. Also, 100 µM of Cu significantly increased H_2O_2 accumulation and superoxide dismutase and other antioxidant enzymes.

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