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Enhanced Onion Resistance against Stemphylium Leaf Blight Disease, Caused by *Stemphylium vesicarium*, by Di-potassium Phosphate and Benzothiadiazole Treatments

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In this study, we investigated the induced defense response and protective effects against *Stemphylium vesicarium* by application of benzothiadiazole (Bion®) and di-potassium phosphate salt (K_2HPO_4) to onion. Onion leaves were sprayed with Bion® and K_2HPO_4, then inoculated 2 days later with a virulent strain of *S. vesicarium* under greenhouse conditions. Disease severity and activities of peroxidase (PO), polyphenoloxidase, phenylalanine ammonia-lyase (PAL) and phenol contents were evaluated in the treated leaf tissues. Reduction in the disease severity was observed in plants treated with Bion® and K_2HPO_4. Onion plants treated with Bion® and K_2HPO_4 and inoculated with the pathogen showed significantly higher PAL activity, PO activity, and phenol contents than inoculated water-treated plants 2 days after the treatment. In conclusion, the results of this study provide evidence that application of simple non-toxic chemical solutions as di-potassium phosphate and Bion® can control Stemphylium leaf blight of onion.

Keywords : peroxidase, systemic acquired resistance, polyphenoloxidase, phenylalanine ammonia-lyase, phenol content

Stemphylium vesicarium (Wall.) Simmons is the causal agent of leaf blight in onion (Shishkoff and Lorbeer, 1984) in the main production areas of the world Benzothiadiazole. In Egypt (Assiut Governorate), Stemphylium leaf blight of onion was first reported recently by Hassan et al. (2007).

One potential method to reduce the severity of disease caused by the pathogen is the induction of systemic acquired resistance (SAR). Certain chemicals, such as salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA), potassium salts, amino butyric acid (BABA) and benzothiadiazole, were reported to induce SAR in plants against plant pathogens (Oostendorp et al., 2001).

Benzothiadiazole, known under the commercial name Bion® or Actigard®, is one of the non-toxic synthetic resistance inducers against a broad spectrum of fungal, viral and bacterial pathogens (Anfoka, 2000; Baysal et al., 2005; Ishii et al., 1999). No activity was observed when Bion® was tested *in vitro* on mycelial growth and conidial germination of *Venturia nashicola*, *Botrytis cinerea* and other fungi (Ishii et al., 1999).

Foliar treatments with potassium phosphate salts may simultaneously protect plants against pathogens and provide nutrients as has been previously demonstrated by the use of di-potassium phosphate (K_2HPO_4) (Reuveni and Reuveni, 1997); a single spray of K_2HPO_4 on the lower leaves stimulated growth of cucumber and maize plants and also induced systemic protection. Maize was protected against *Puccinia sorghi* and *Exserohilum turcicum* (Reuveni et al., 1996) and sugar beet against powdery mildew caused by *Erysiphe betae* (Mosa, 2002).

Certain biochemical changes occurring after application of the inducing agents can act as markers for induced systemic resistance (Schönbeck et al., 1980). These include accumulation of phytoalexins, reinforcement of cell wall polymers with deposition of lignin (Thangavelu et al., 2003), and increase in certain enzymes, such as phenylalanine ammonia lyase (PAL) and polyphenoloxidase (PPO), (He et al., 2002). The enzyme peroxidase (PO) has been implicated in the hypersensitive response, the formation of papillae, and the polymerization of lignin from monomeric lignols (Bestwick et al., 1998; Nicholson and Hammerschmidt, 1992).

The aims of this study were to investigate the effect of Bion® and K_2HPO_4 on induction of onion resistance against Stemphylium leaf blight and to characterize the resistance mechanisms and parameters including phenolic content and defense-related enzyme activities, under greenhouse conditions.

Materials and Methods

Host plants. Onion (cv. Giza 6) plants were grown in a greenhouse maintained at 20 ± 2°C during the day and 18 ± 2°C at night. The seedlings were transplanted after 8 weeks into 30 cm-diameter pots containing a mixture of

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sandy loam soil.

Inoculum. Inoculum of *S. vesicarium* isolate No. 1 from a diseased onion in Assiut Governorate, Egypt, in October 2004) was prepared from stock cultures on potato dextrose (PDA) media stored at 4°C. A piece of culture medium containing mycelium (approx. 2 mm) of fungi was placed centrally on PDA agar medium in a 9-cm-diameter Petri dish and incubated at 25 ± 1°C for 15 days under a 12-h photoperiod using a near-ultraviolet light suspended approximately 20 cm above the cultures. Ten ml of sterile distilled water was then added to each plate and colonies were scraped with a sterile needle. The resulting conidial suspension was diluted to 5×10^4 conidia per ml and inoculated onto leaves and seed-stalks of 110-day-old onion plants cv. 'Giza 6' using an atomizer.

Application of Bion® and K_2HPO_4. Bion® and K_2HPO_4 were dissolved in distilled water to give 125 ppm and 10 mM, respectively, and then sprayed onto whole plants (50 ml per seedling) two days before inoculation with the pathogen. After treatment, plants were maintained in a greenhouse as described above. For controls, the plants were inoculated or unionoculated but sprayed with water. The experiment was repeated twice under greenhouse conditions in 2004 and 2005. Autoclaved pots (20 cm in diameter) were filled with autoclaved clay soil and each pot planted with two seedlings. Three replicates were used, and each replicate consisted of four pots.

Symptom severity of Stemphylium leaf blight was evaluated at 5, 10 and 15 days after inoculation using a 0-4 scale (0=no symptom, 1=1-25% seed-stalk area symptom, 2= 26-50% seed-stalk area symptom, 3=51-75% seed-stalk area symptom, 4=76-100% seed-stalk area symptom). Disease severity was averaged over the 12 plants per treatment.

Biological assay. Each inducer was added to PDA medium at the tested concentration and then poured in Petri dishes (9 cm in diameter) and seeded in the center with disks (5 mm diameter) from 10-day-old cultures of *S. vesicarium*. Plates were incubated at 27°C until the fungus had completely covered the surface of the control plates. The diameter of radial growth was measured and the percentage of inhibition growth was calculated compared with control plates. The experiment was repeated twice and eight plates were used for each treatment.

Preparation of samples for determining enzyme activities. Onion plants were assigned in equal numbers to six treatment groups. Plants in the first and second group were treated with Bion® and K_2HPO_4 and inoculated with a fungal suspension 2 days after treatment. Plants in the third group were treated with water and inoculated with a fungal suspension 2 days after treatment (control). Plants in the fourth to sixth groups were treated with water, Bion®, or K_2HPO_4 but not inoculated with a fungal suspension. Duplicate samples of leaf tissue (1 g fresh weight) for enzyme extraction were harvested at 0, 2, 4, 6, 8, and 10 days after application for each treatment, weighed and immersed in liquid nitrogen. The frozen leaf segments for each sample were homogenized (1:5 w/v) in an ice-cold mortar using 50 mM potassium phosphate buffer (pH 7.0) containing 1 M NaCl, 1% polyvinylpyrrolidone, 1 mM EDTA and 10 m M β-mercaptoethanol. The homogenates were centrifuged at 17,000 g for 20 min at 4°C. The supernatant (crude enzyme extract) was collected and divided into 1.5 ml portions. Protein concentrations were determined using bovine serum albumin as a standard according to Bradford (1976). The extract was then used to determine the activities of PO, PPO and PAL.

Peroxidase (PO) activity: Fifty µl of the crude enzyme extract was taken in 1 ml of 10 mM potassium phosphate buffer and mixed with 1 ml of 100 mM pyrogallol and 1 ml of 1% H_2O_2. The initial rate of increase in absorbance was measured over 1 min at 470 nm using spectrophotometer (Spectronic^R 20 Genesys^{TM}, Schutt labortechink). PO activity was expressed as units of PO/mg protein (Urbanek et al., 1991).

Polyphenoloxidase (PPO) activity: Two hundred µl of the crude enzyme was added to 700 ml of potassium phosphate buffer (pH 6.0). One hundred ml of 0.2 M catechol was added and the absorbance determined at 420 nm (rate of increase in absorbance for 1 min) and compared with the standard. The enzyme activity was expressed as µg PPO/ mg protein (Gauillard et al., 1993).

Phenylalanine ammonia-lyase (PAL) activity: PAL activity was assayed according to Nagarathna et al. (1993). PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.0) and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. The amount of trans-cinnamic acid synthesized was calculated. Enzyme activity was expressed as nmol trans-cinnamic acid min mg protein^{-1}.

Estimation of total phenol content: Phenol content was estimated according to Malick and Singh (1980). Onion leaves were immersed in liquid nitrogen, homogenized in 80% methanol (1 g plant material in 10 ml) and stored in

the deep-freezer (–20°C). Later, the homogenate was centrifuged at 15,000 g for 30 min at 4°C. Residue was re-extracted five times with the volume of 80% ethanol, and centrifuged; supernatants were pooled. The pooled supernatant was evaporated to dryness; residue was dissolved in 5 ml distilled water and used to estimate the phenols. Reaction mixture contains 2 ml of extract mixed with 1 ml of distilled water and 0.5 ml of Folin-Ciocaulteau's reagent. After 3 min, 2 ml of 20% sodium carbonate solution was added. Contents were mixed thoroughly and placed in a boiling water bath for exactly 1 min. Contents were cooled, and absorbance was read at 650 nm using spectrophotometer (Spectronic^{R} 20 Genesys^{TM}, Schutt labortechink) against the reagent blank. Standard curve was prepared using different concentrations of gallic acid. Phenol content of the extract was expressed as mg phenol/gm material. The experiment was conducted in four replicates and repeated twice.

Statistical analysis. All greenhouse experiments were arranged in a completely randomized split-plot design with three replicates of 12 plants for each treatment and repeated twice. Each sample of extract was measured twice in each replicate, and at least three replications were performed per analysis. The significance of differences between mean values was determined. Analysis of variance (ANOVA) was carried out, and the significance of differences among the treatments was determined according to Least Significant Difference (LSD) (Gomez and Gomez, 1984).

Results

Biological assay. There was no significant difference in radial growth when *S. vesicarium* was grown on PDA media amended with the chemical inducers compared with PDA alone (Table 1).

Effect of Bion® and K_2HPO_4 on symptom development. The severity of symptoms caused by *S. vesicarium* was reduced in onion plants pretreated with Bion® and K_2HPO_4. Application of either Bion® or K_2HPO_4 on onion plants led to significant reduction in disease severity (Fig. 1) at 5, 10 and 15 days. In the control plants, disease index of onion

Table 1. Effect of chemical inducers on mycelia growth of *S. vesicarium*

Chemical inducers	Mean of growth diameter (cm)	Mycelia growth inhibition (%)
Bion®	7.23±1 a	0.9
K_2HPO_4	7.30±1.2 a	0.0
Control	7.30±1.1 a	0.0

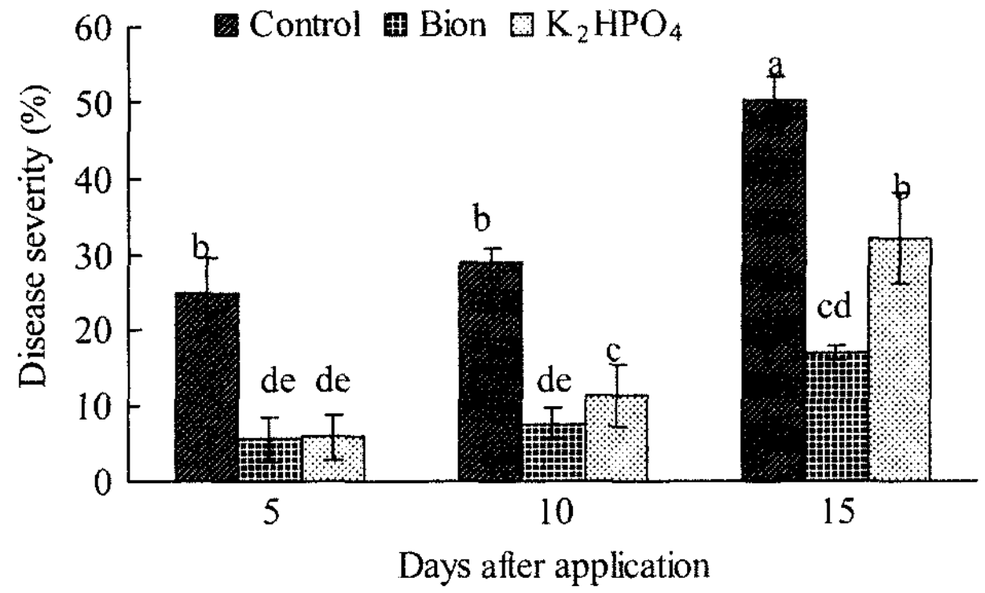

Fig. 1. Effect of Bion® and K_2HPO_4 treatment on the diseases severity of Stemphylium leaf blight of onion. Different letters indicate significant differences between treatments according to L.S.D. test (P=0.05).

was 50% compared with 17% and 32% for Bion® and K_2HPO_4 treated plants at 15 days, respectively. The severity of symptoms increased with time. At 15 days, most of inoculated control plant leaves had developed severe blight.

PO activity. Four days after application of Bion® and K_2HPO_4 to inoculated and uninoculated plants peroxidase (PO) activity was significantly higher than water-treated plants. The course of PO activity in inoculated plants was different from that in uninoculated plants (Fig. 2). Eight and ten days after treatment, compared with water controls, PO activity was significantly higher in both K_2HPO_4-treated inoculated plants and Bion®-treated inoculated plants, but activity in Bion®-treated plants was significantly higher than in the K_2HPO_4-treated plants. The activity increased to 68% greater than water treated plants (control) in Bion®-treated plants at 6 days after treatment, and was significantly higher than in control plants. The activity in control plants remained on the same level up to ten days after treatment. In Bion®-treated plants the highest activity (155% more than control) was observed at ten days after treatment.

PPO activity. Increased activity of PPO were observed 2 days after treating onion plants with Bion® alone and when inoculated with the pathogen when compared with all other treatments and remained at higher levels throughout the experimental period. The activity reaches the maximum level on ten days after application (Fig. 3). Untreated plants (water treatment) did not show any significant fluctuation in PPO activity in the study period.

PAL activity. Both Bion® and K_2HPO_4 induced significant quantities of PAL in the treated onion plants over all time intervals, while untreated plants did not show any change in

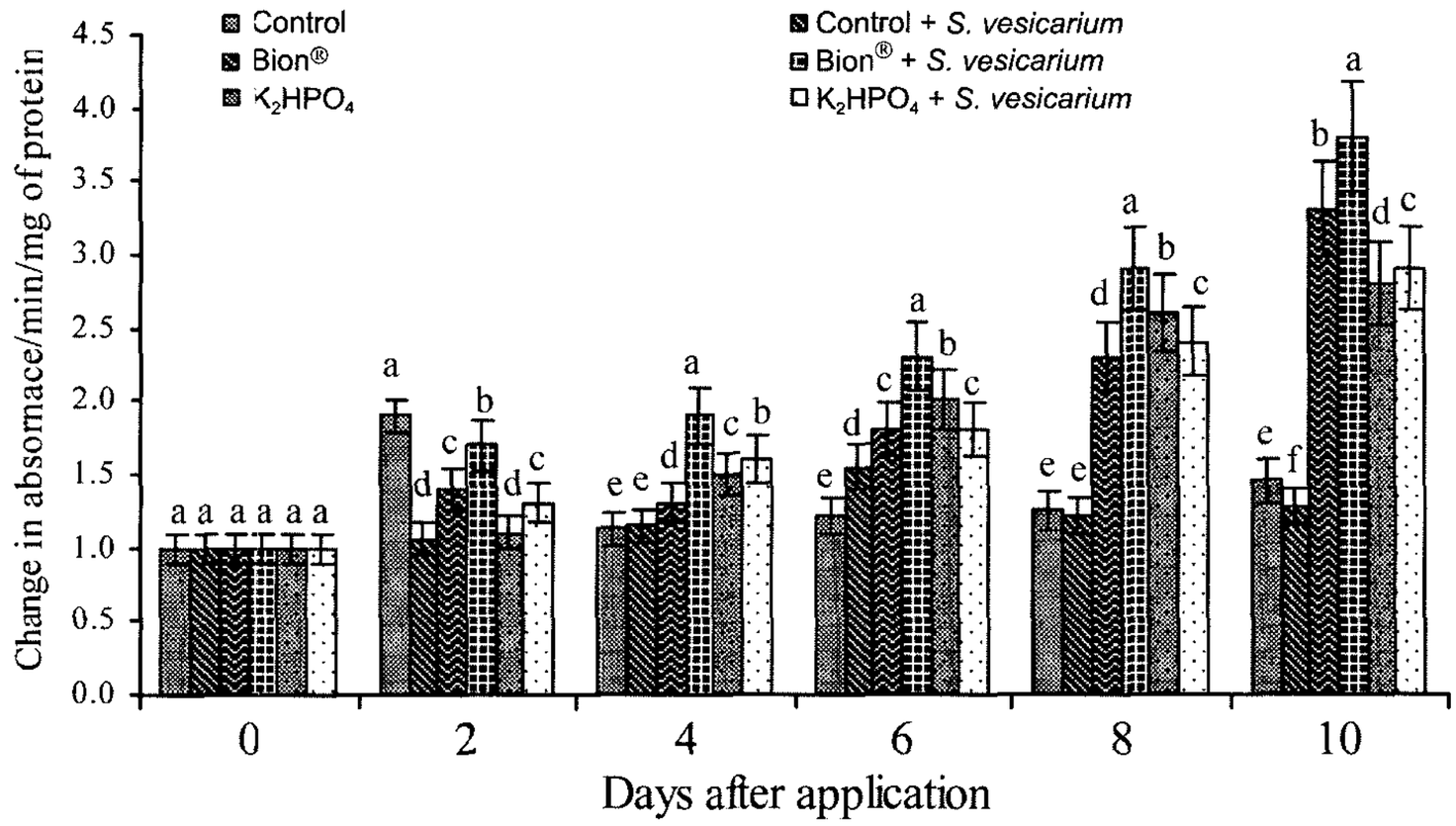

Fig. 2. Effect of Bion® and K_2HPO_4 treatment on the induction of peroxidase activity in the leaves of onion plants. Different letters indicate significant differences between treatments according to L.S.D. test (P=0.05).

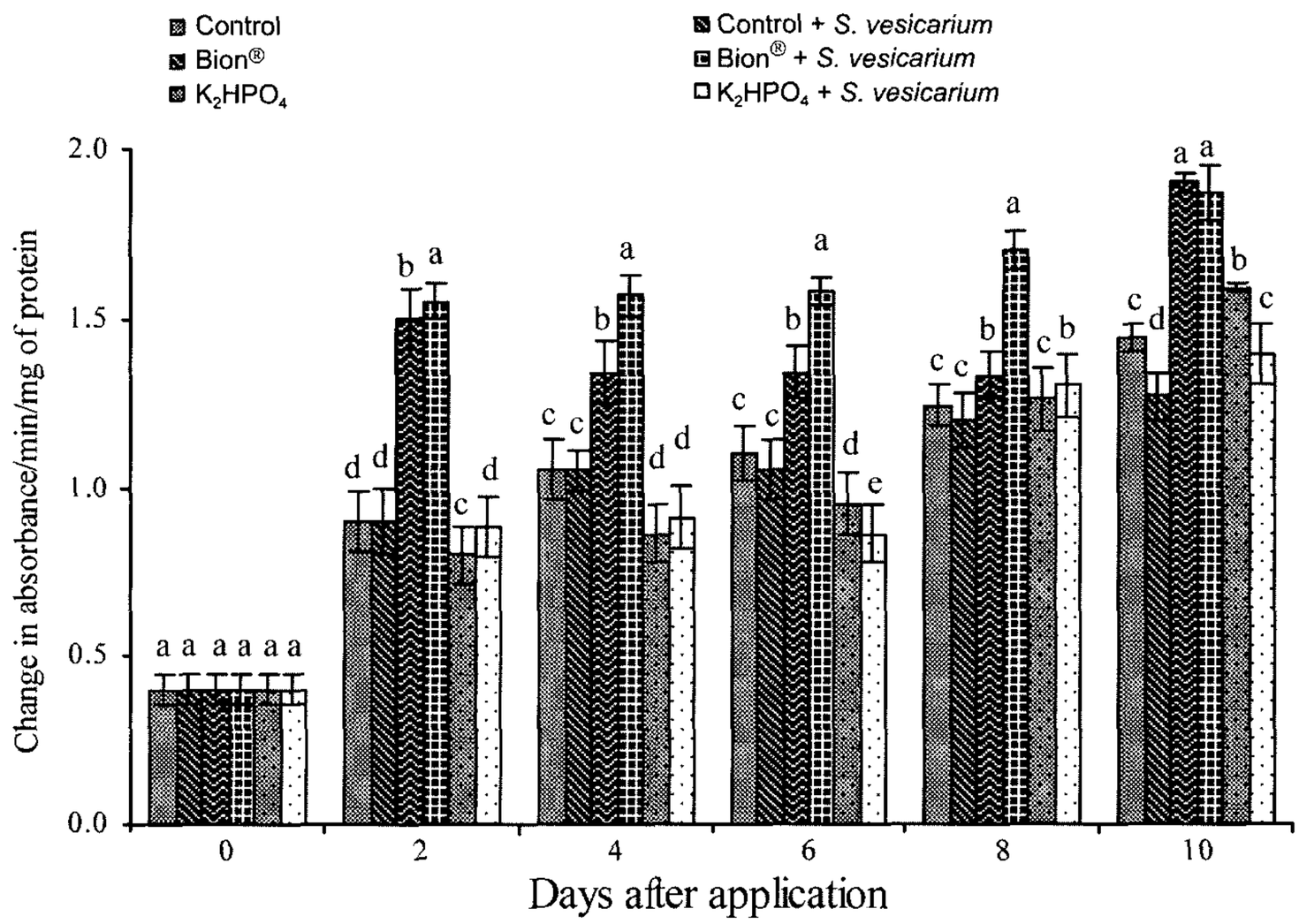

Fig. 3. Effect of Bion® and K_2HPO_4 treatment on the induction of polyphenoloxidase activity in the leaves of onion plants. Different letters indicate significant differences between treatments according to L.S.D. test (P=0.05).

the pattern of PAL activity (Fig. 4). In the inoculated plants, enzyme induction was highest on 8 and 10 days after application of K_2HPO_4. Untreated plants showed slightly higher activity of PAL. There was a highly significant increase in induction of PAL in the treated onion plant with Bion® and K_2HPO_4 compared with the untreated ones.

Phenol content. Treatment with Bion® and K_2HPO_4 induced significant quantities of phenol in the leaves of onion plants at all time intervals whether they were inoculated or not. Untreated plants showed no change in phenol level (Fig. 5). In the inoculated plants, phenol induction was highest at 6 days after application of K_2HPO_4. There was a highly significant increase in induction of phenol content in the Bion®- and K_2HPO_4-treated onion plants compared with the untreated ones.

Discussion

This study assessed the effects of two compounds, Bion®

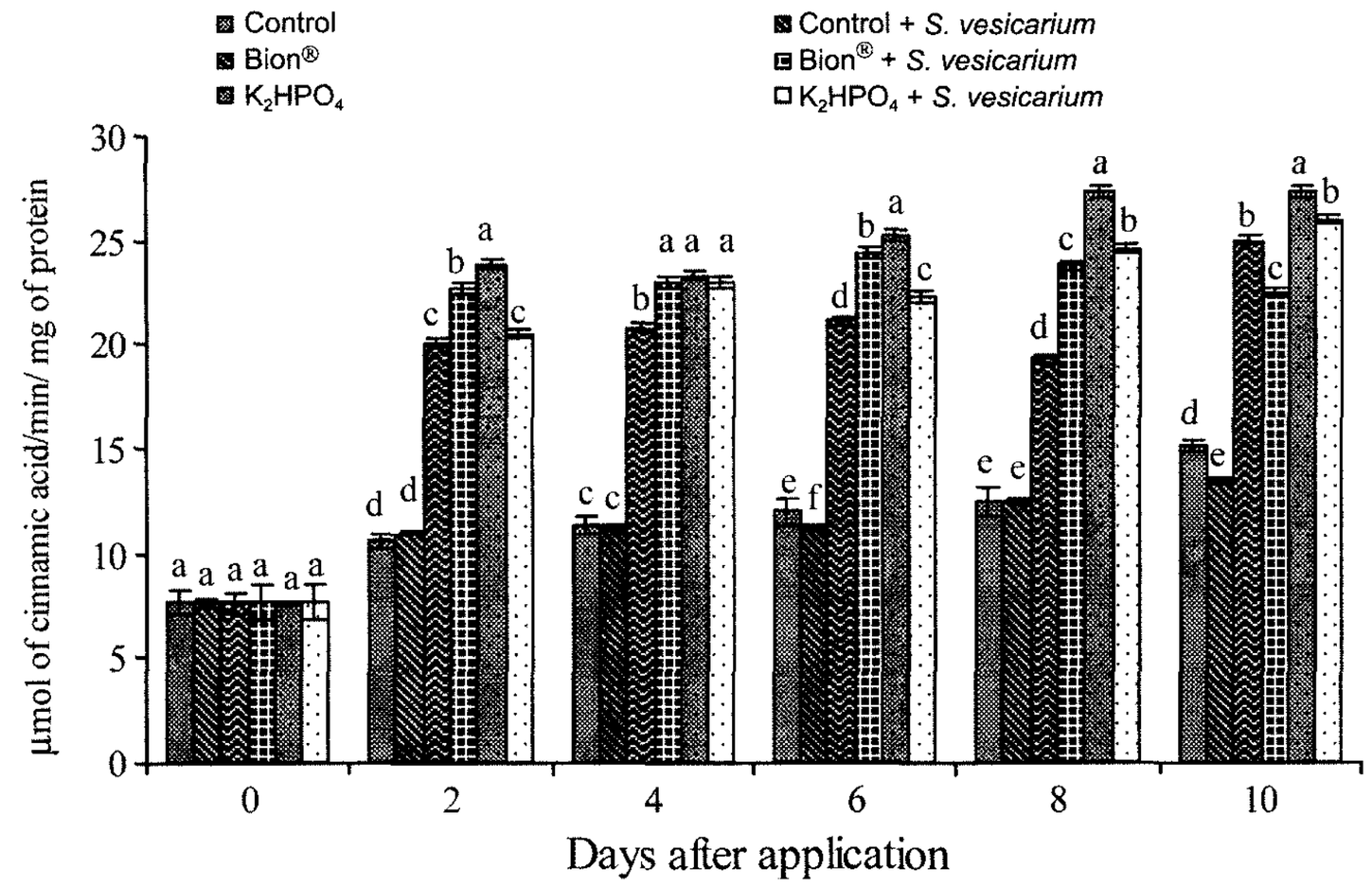

Fig. 4. Effect of Bion® and K_2HPO_4 treatment on the induction of phenylalanine ammonia lyase activity in the leaves of onion plants. Different letters indicate significant differences between treatments according to L.S.D. test (P=0.05).

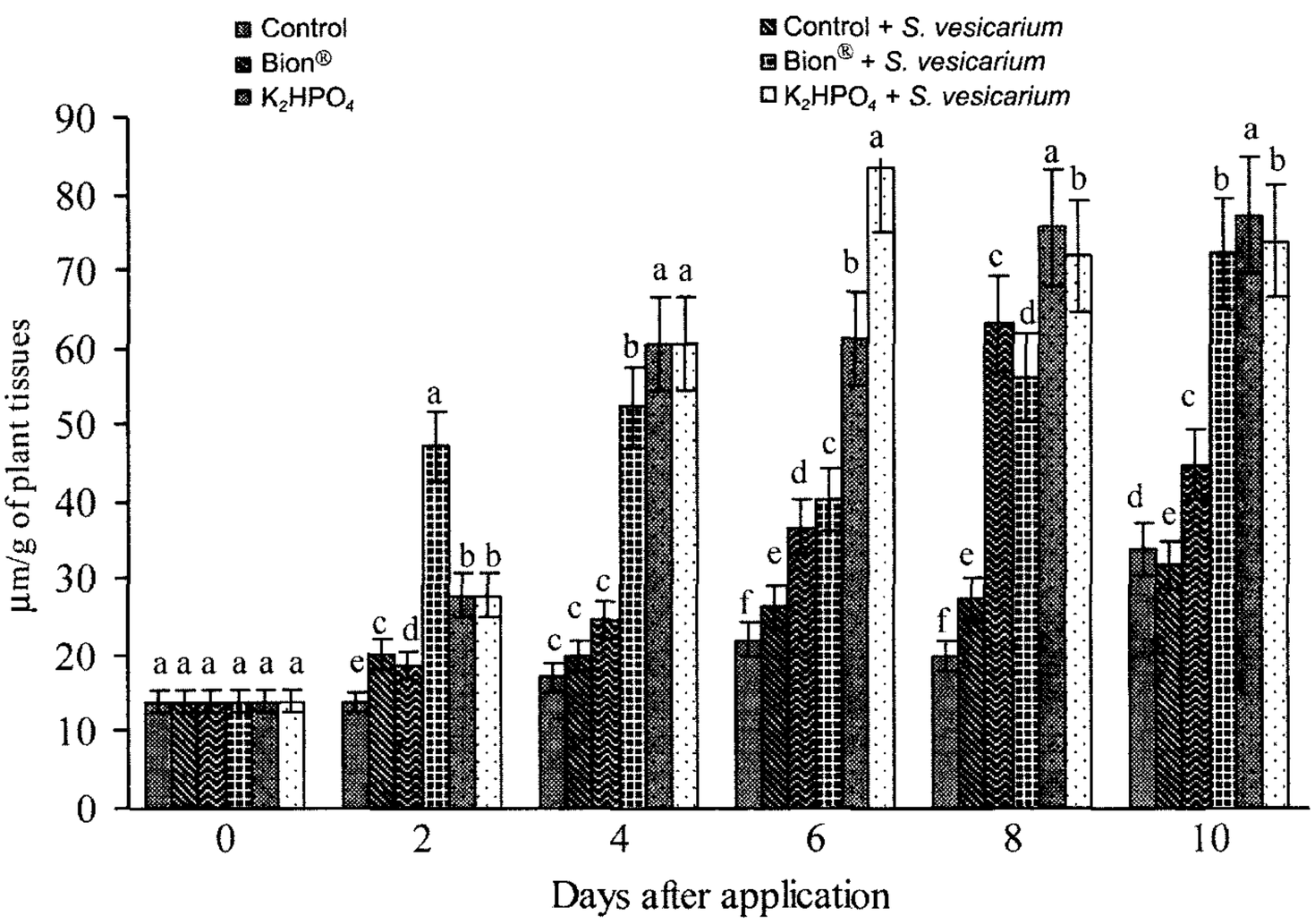

Fig. 5. Effect of Bion® and K_2HPO_4 treatment on the induction of phenol content in the leaves of onion plants. Different letters indicate significant differences between treatments according to L.S.D. test (P=0.05).

and K_2HPO_4, on *Stemphylium vesicarium* disease development in onion plants. Many reports have demonstrated the efficiency of Bion® in controlling plant diseases caused by pathogenic fungi and bacteria (Baysal et al., 2005; Ishii et al., 1999). However, the ability of Bion® and K_2HPO_4 to trigger SAR on onion against *S. vesicarium* is unknown; our data provide the first evidence that Bion® and K_2HPO_4 trigger SAR and protect onion plants against *S. vesicarium* infection.

Biochemical markers of SAR have not been previously reported in onion plants. We have chosen certain enzymes (polyphenoloxidase, phenylalanine ammonia-lyase and peroxidase activities) and total phenol content because their role in SAR has been previously demonstrated in a number of plant species (He et al., 2002).

Commercial benzothiadiazole products containing 50%

Bion® have been intensively studied for years as potential chemical resistance inducers and SAR priming (Abo-Elyousr 2006, Cavalcanti et al., 2006; Resende et al., 2002). In the current study, application of Bion® and K_2HPO_4 reduced the severity of Stemphylium leaf blight symptoms in onion plants up to 10 days after plants were inoculated. These results are consistent with the findings of Achuo et al. (2004), who showed that rustication of *Botrytis cinerea* infection on tomato was achieved after Bion® treatment, and those of the findings of Elmer (2006), who observed the suppression of Fusarium wilt of cyclamen in greenhouse.

Foliar treatment with Bion® and K_2HPO_4 induced significant increases of PO activity at all sampling times, the highest level at ten days after application. Enhanced peroxidase activity has been associated with induced systemic resistance in plants against fungal, bacterial and viral pathogens (Baysal et al., 2005; Dalisay and Kuc 1995; Slusarenko 1996) and involved in several plants defense mechanisms, such as lignin biosynthesis, oxidative cross-linking of plant cell walls and also generation of oxygen species (Bestwick et al., 1998). PPO catalyses the last step in the biosynthesis of lignin and other oxidative phenols (Baysal et al., 2005).

In the present study, pretreating plants with Bion® and K_2HPO_4 induced PAL synthesis and the activity was highest when plants were then inoculated with *S. vesicarium.* PAL is considered to be the principal enzyme of the phenylpropanoid pathway, which is the prime intermediary in the biosynthesis of phenolics and flavonoids (Dixon and Lamb, 1990). It catalyses the conversion of L-phenylalanine to trans-cinammic acid in the first step of the phenylpropanoid pathway and regulates the production of precursors for lignin biosynthesis along with other phenolic protectants in plant cells (Nicholson and Hammerschmidt, 1992).

In the present study, plants treated with Bion® and K_2HPO_4 resulted in increased accumulation of phenolic substances in response to infection by the pathogen. Accumulation of phenolic compounds at the infection site has been correlated with the restriction of pathogen development since such compounds are toxic to pathogens (Benhamou et al., 2000). Also, the resistance may be increased by a pH change in plant cell cytoplasm, due to the increase in phenolic acid content, resulting in inhibition of pathogen development (Ojalvo et al., 1987). In addition, phenolic compounds may impede pathogen infection by increasing the mechanical strength of the host cell wall (Benhamou et al., 2000).

In conclusion, the results of this study provide evidence that application of simple non-toxic chemical solutions as di-potassium phosphate and Bion® can control Stemphylium leaf blight of onion Their low toxicity to animals, comparative environmental safety and nutrient value make them ideal foliar fertilizers, which can be used for application in the field for disease control.

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