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Induced resistance in potato plants against verticillium wilt invoked by chitosan and Acibenzolar-S-methyl

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Abstract

Verticillium wilt of potato is a major limiting factor in potato production caused by *Verticillium dahliae*. The objective of this study was to evaluate the efficacy two elicitors including Acibenzolar-S-methyl (ASM) and Chitosan as plant activator in controlling of potato verticillium wilt *in vitro* and greenhouse conditions. ASM and chitosan were tested *in vitro* using Potato dextrose Agar (PDA) amended with five concentrations (0, 5, 50, 100, 500 µg a.i./ml). All the concentration of Chitosan reduced radial growth of *V. dahliae in vitro* significantly ($p < 0.05$) after 120 h (9 to 57.5%), Whereas, ASM did not significantly reduce on the growth of pathogen *in vitro* and reduced radial growth of pathogen very less (5.4 to 16.9%). The effective concentration of Chitosan that reduced the radial growth of *V. dahliae* to 50% (EC_{50}) was 258.28 µg/ml. In greenhouse conditions, potato tubers c.v Agria dipped in aqueous solution of different concentration of elicitors and sown in soil of pots artificially infested with *V. dahliae*. Also, ASM and chitosan applied by foliar spray on potato seedlings at concentration 100 µg a.i./plant; 15, 25 and 40 days after sowing. Results shown all treatment significantly reduced disease severity and increased tubers fresh weights, but ASM was more effective than chitosan and reduced the potato verticillium wilt. The biggest disease reduction and increasing yields of potato belong to concentration of 500 µg a.i./ml of ASM by 67.8% and 56.7%, respectively. Results showed that ASM and chitosan may be inducing plant resistance and protected tubers of potato against Potato verticillium wilt. These chemical could provide a new approach for suppression of Potato verticillium wilt, but its practical use needs further investigation.

Keywords: Potato verticillium wilt, Acibenzolar-S-methyl, Chitosan.
Abbreviations: ASM_ Acibenzolar-S-methyl, PDA_Potato Dextrose Agar, a.i/ml_ active ingredient per ml, CRD_ completely randomized design.

Introduction

Verticillium wilt disease, caused by *Verticillium dahliae* Kleb. and *Verticillium albo-atrum* Reinke & Berthod, is the most important disease of potato in Iran and other countries (Aminaee et al., 2006; Mansoori and Smith, 2005). It is soil borne pathogen and causing problems in wide range of herbaceous and woody plant hosts such as Potato, Strawberry, cauliflower, lettuce, cotton, olive and spinach (Iakovos et al., 2009; Bilodeau et al., 2012; Olesen et al., 2014). Control of pathogen is difficult to eradicate from soil, because they produce resting spore (microsclerotia) that can survive in the soil for more than 10 years in the absence of a suitable host as resting structures (Olesen et al., 2014). Therefore, management of Verticillium wilt is challenging due not only to the endogenous growth of the pathogen, but also its ability to infect multiple hosts and survive for several years (Uppal et al., 2008). Management strategies of disease are mainly focused on the use of resistant hosts and cultural practices, but are not always available or effective (Thanassoulopoulos and Hooker, 1968). One of the potential methods of reduction of disease in plants against pathogens is the induction of plant resistance (Gozzo, 2003; Malolepsza, 2006). Plant can be induced locally and systemically to become more resistant to disease by virulant, avirulent or non-pathogenic microbes or artificially by various chemical agents such as salicylic acid (SA) (Radhakrishnan et al., 2011). Use of bioactive products, commonly referred to as plant activators that induced systemic acquired resistance (SAR) in plant to limit pathogenesis of many plant pathogen (Sticher et al., 1997). SAR can be induced by biotic and abiotic elicitors, which are the initial biochemical signal recognized by the plant cell being infected by fungi or other pathogens (Davis et al., 1993). Several products have been used as inducers of resistance in many plants species against various pathogens, including Acibenzolar-S-methyl (ASM) (Kamal et al., 2008) and chitosan (Eikemo et al., 2003). The ASM with commercial name Bion or Actigard^{TM} is the most potential SAR activator discovered to date (Kessmann et al., 1994) and elicits the same SAR pathway which includes the same pathogenesis related (PR) proteins as observed in SA (Friedrich et al., 1996). It has been commercially released in some countries as a plant health promoter of annual, which protects against a broad spectrum of pathogen in several crops in field conditions (Gorlach et al., 1996; Malolepsza, 2006). Application of ASM suppressed Phytophthora blight on pepper caused by *P. capsici* (Matheron and Porchas, 2002) and reduced other diseases caused by Oomycetes fungi (LaMondia, 2008; Leskovar and Kolenda, 2002). Chitosan, a natural substance derived from chitin is a biopolymer with antifungal properties, which has been proved against several types of fungi (Ziani et al., 2010; Munoz et al., 2009). It induces genes to produce phytoalexin as defensive mechanism (Martinez Pena Alejandro, 2002). Chitosan induces systemic resistance in plants against different fungus by accumulation of components such as Phytoalexins and pathogenesis-related (PR) proteins such as β-1,3-glucanases and chitinases has been reported in diferent plant (Stone et

al., 2003; Asgar Ali et al., 2014). Chitosan has been shown to control post harvest infection of *Botrytis cinerea* and *Rhizopus* sp. in strawberry fruit (Reddy et al., 2000) and wheat and rice seeds treated with chitosan increased yield 5-20% (Freepons, 1997). Also, chitosan could potentially provide a protective antifungal coating in postharvest production (Jiang et al., 2005). To date, ASM and chitosan have not been tested in potato/*V. dahliae* pathosystems. The objective of this study was conducted in order to evaluate the effectiveness of two elicitors, namely ASM and chitosan for the control of potato Verticillium wilt under *in vitro* and greenhouse conditions.

Results

Pathogenicity test of pathogen on potato plant

Results of Pathogenicity test showed the isolate of *Verticillium dahliae* was able to caused symptom of diseases (Fig 1). Some leaves turned brown and Yellowing, chlorosis and necrosis of lower leaves are usually the first symptoms of Verticillium wilt, which these symptoms can occur on one or both sides of the leaf or the whole potato plant. (Fig 1-a). The disease index of potato wilt in the pathogenicity test was expressed as 2.8 which represent severe wilting or death. Also a discoloration of the vascular tissue was observed when plant samples were cut longitudinally and plants were stunted under severe infestation (Fig 1-b). Evaluation of severity of vascular discoloration of potato stems shown 2.2 which represent 12.8% of the stem cross-section showing a vascular discoloration. Symptoms on tuber appeared as brown discoloration of the vascular ring (Fig 2).

Effect of ASM and chitosan on growth of V. dahliae in vitro

Result showed that concentration of chitosan from 5 to 500 µg a.i./ml reduced radial growth of pathogen, progressively. Fungal growth of pathogen was significantly affected by all chitosan doses after 120 hours. Concentration of 100 and 500 µg a.i./ml inhibited the mycelial growth by 46.2 and 57.5%, respectively compared to the control (Table 1). But, all concentration of ASM reduced mycelial growth of *V. dahliae* less 20%. Therefore, ASM had less effective than chitosan *in vitro* (Table 1). The regression line indicated the equation $y = - 0.076x + 67.74$ ($r^2 = 0.7$) (Fig 3) and $y = - 0.018x + 73.33$ ($r^2 = 0.5$) (Fig 4) for chitosan and ASM, respectively. The EC_{50} values for chitosan against *V. dahliae* according to the liner relation between inhibitory probit and logarithm of concentration was 258.28 µg/ml.

Effect of ASM and chitosan on growth V. dahliae in greenhouse conditions

Results in the greenhouse experiments indicated that, treatments with ASM and chitosan significantly ($P < 0.05$) reduced disease severity of potato Verticillium wilt as compared with infected control (Table 2). The best result of protection of potato plants against *V. dahliae* was conferred by ASM and chitosan at dose of 500 µg a.i./ml which reduced Disease severity 67.8 and 53.6%, respectively. Also, vascular discoloration was progressively reduced with increasing concentration in both ASM and chitosan from 5 to 500 µg a.i./ml (Table 2). All concentration of ASM and chitosan increased tubers fresh weights of potato compared to control pathogen (Table 2).

Discussion

All the concentration of chitosan reduced radial growth of pathogen *in vitro* significantly after 120 hours (9 to 57.5%), whereas ASM had less effect on reduction of radial growth of pathogen (5.4 to 16.9%). ASM did not directly suppress mycelial growth of pathogen and effected on pathogen in the present of plant and reduced disease severity, because it activated plant nature defense against disease. This result is agreement with another researcher (Kone et al., 2009; Ji et al., 2011).Studies indicated that chitosan significantly inhibited the radial growth of *Colletotrichum sp* on tomato (Munoz et al., 2009), *Botrytis cinerea* and *Plasmopara viticola* on grapevine (Ben-Shalom and Fallik, 2003). Other researcher demonstrated antifungal activity of chitosan and its ability to reduce growth of pathogen *in vitro* in many fungi (Allan and Hadwiger, 1979; Romanazzi et al., 2001). Chitosan is one of the potential candidates which have been reported with antimicrobial properties and its effectiveness by numerous authors (Ali and Mahmud, 2008; Reddy et al., 2000; Rhoades and Roller, 2000). Results of effect of elicitors on disease in greenhouse indicated that, all treatments significantly reduced both the disease severity and browning of vascular tissues in lower stem section and increased fresh weights of tubers compared to untreated control (pathogen only) (Table 2). These treatments improve plant health through reducing wilt symptoms and vascular invasion. These results are in agreement with other researches (Hofgaard et al., 2005; Munoz et al., 2009; Asgar et al., 2014; Urszula Malolepsza, 2006). ASM was more effective than chitosan. Concentration 500 µg a.i./ml of ASM and chitosan was the most effective against *V. dahliae* and reduced severity of disease 67.8 and 53.6%, respectively (Table 2). ASM and analogues have suppressed Verticillium and Fusarium diseases on some crop under field conditions (Gorlach et al., 1996) and it was effective in controlling tomato Corky root and stem canker on pepper caused by *Pyrenochaeta lycopersici* and *Phytophthora capsici*, respectively (Bubici et al., 2006; Matheron and Porchas, 2002). Resende et al. (2002) shown that application 200 mg l^{-1} ASM induced resistant in cocoa against *V. dahliae*. *In vitro* studies indicated that application ASM as rhizome pre-treatment was effective in controlling rhizome rot disease of turmeric (*Curcuma longa* L.) caused by *Pythium aphanidermatum* (Radhakrishnan et al., 2011). Rhizome pre-treatment by ASM enhanced activities of proteases, peroxidase, protease inhibitors, soluble and ionically bound peroxidase already infected with *P. aphanidermatum* (Radhakrishnan et al., 2011) and may be play a key role in restricting the development of disease symptoms in plant. There is a correlation between reduction of disease severity in plants and increased activities of protease, protease inhibitors and peroxidase in plant pre-treated with ASM, because peroxidases and protease inhibitors have been demonstrated to possess antifungal activity (Chen et al., 1999; Kim et al., 2005; Wang et al., 2006). Reports have indicated that chitosan has the capacity to induce resistance to *Fusarium oxysporum* in susceptible tomato plants when applied as a root dressing, foliar spray and seed dressing by restricting pathogen growth to the outer rot tissues and eliciting a number of defense reaction, including structural barriers (Benhamou et al., 1998). Our results indicated that chitosan was effected on *V. dahliae in vitro* and greenhouse experiments and reduced severity disease and increased

Table 1. Percentage of growth inhibition of *V. dahliae* on PDA media supplemented with different concentrations of ASM and chitosan after 120 hours.

Treatment, concentration (µg a.i./ml)	Acibenzolar-S-methyl (ASM)		Chitosan	
	Mean of radial growth (mm)	Growth inhibition (%)	Mean of radial growth (mm)	growth inhibition (%)
Control pathogen	78.6[a]	-	78.6[a]	-
5	74.3[b]	5.4	71.5[b]	9.0
50	69.8[c]	11.2	63.2[c]	19.6
100	66.5[d]	15.4	42.3[d]	46.2
500	65.3[e]	16.9	33.4[e]	57.5

Mean in the column followed by different letters indicate significant differences among treatments at 0.05 according to Duncan's multiple range test. Data are means of five replicates. Treatment control pathogen includes only pathogen (without ASM and chitosan).

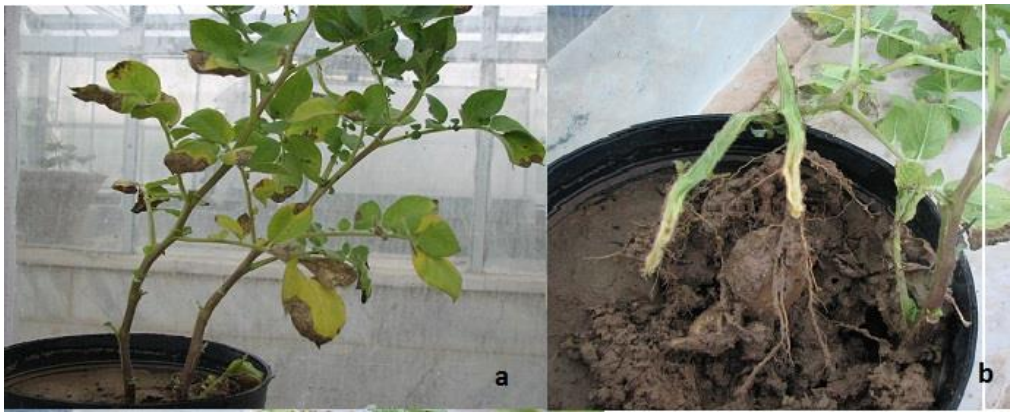

Fig 1. Symptoms of diseases on leaves (a) and vascular tissue (b) of potato

Table 2. Effect of ASM and Chitosan on *V. dahlia* under greenhouse condition after 60 days.

Treatments concentration (µg a.i./ml)	Disease severity	Disease reduction (%)	Vascular discoloration	Tubers fresh weights (g)
Control water	0[h]	-	0[h]	280.0[a]
Control pathogen	2.8[a]	-	2.5[a]	80.0[i]
ASM 5	2.3[c]	17.8	2.0[b]	115.0[g]
ASM 50	2.0[d]	28.6	1.8[c]	142.0[e]
ASM 100	1.4[f]	50	1.0[e]	168.0[c]
ASM 500	0.9[g]	67.8	0.3[g]	185.0[b]
Chitosan 5	2.5[b]	10.7	2.1[b]	102.0[h]
Chitosan 50	2.0[d]	28.6	1.6[d]	119.0[f]
Chitosan 100	1.6[e]	42.8	1.1[e]	143.0[e]
Chitosan 500	1.3[f]	53.6	0.7[f]	158.0[d]

Means in the column followed by different letters indicate significant differences among treatments at 0.05 according to Duncan multiple-ranges test (DMRT). Data are means of five replications in each experiment.

Fig 2. Appearance of symptoms on tubers of potato as brown discoloration of vascular ring.

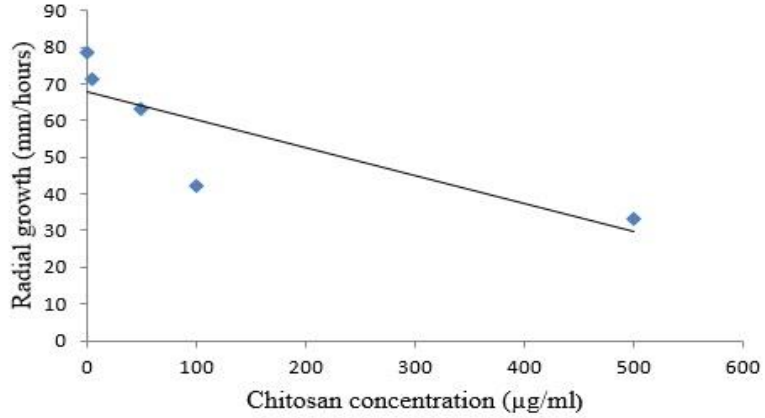

Fig 3. Linear regression of mean radial growth induced by five chitosan concentrations (0, 5, 50, 100, 500). Colony diameter of *V. dahlia* was determined 120 h after treatment. The pathogen was progressively inhibited with increasing concentration of Chitosan from zero (control) to 500 µg/ml. Fungal growth was affected by all chitosan doses ($p < 0.05$).

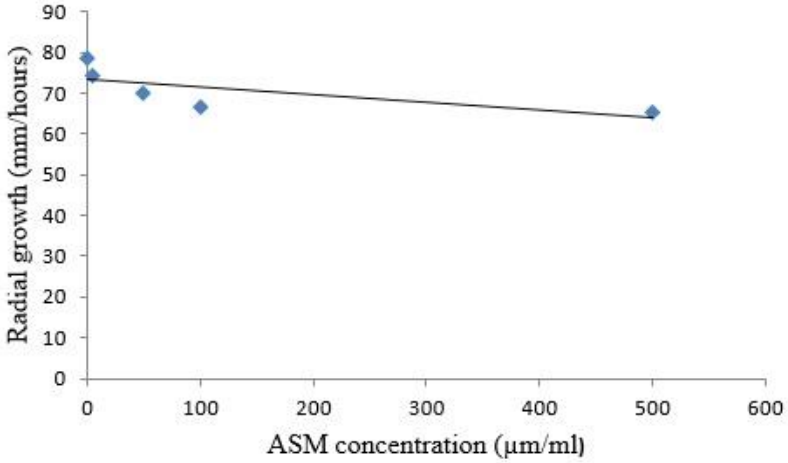

Fig 4. Linear regression of mean radial growth induced by five ASM concentrations (0, 5, 50, 100, 500). Colony diameter of *V. dahlia* was determined 120 h after treatment. The pathogen was inhibited with increasing concentration of ASM from zero (control) to 500 µg/ml. Fungal growth was less affected by all ASM doses ($p < 0.05$).

weight of tubers significantly (Table 1, 2). These results are in harmony with other researches (Munoz et al., 2009; Asgar et al., 2014; Allan and Hadwiger, 1979; Romnazzi et al., 2001). Chitosan has shown antimicrobial properties (Ali and Mahmud, 2008) and induces systemic resistance against many fungus such as *Pythium* spp, *Phytophthora* spp. and *Fusarium oxysporum* by accumulation of hydrogen peroxide and phenolic compound in treated tissues of plants (Faoro et al., 2008). In addition, it was also proven that elicitors increased significantly tubers fresh weights than untreated control (Table 2), so that this result agreement with other researches (Freepons, 1997).

Materials and Methods

Isolation and identification of pathogen

V. dahliae was isolated from potato plants that showing symptoms of Verticillium wilt from Ghorveh area in Kurdistan province in Iran. Isolations of fungi were made from roots, foot and stems of potato plants. Section 3 to 5 cm long of plant tissue exhibiting vascular discoloration after surface-disinfesting in sodium hypochlorite (3%) for 2 min, were rinsed three times in sterile distilled water, dried on sterile filter paper and plated on Potato Dextrose Agar medium (PDA) with streptomycin sulphate (300 mg L^{-1}). Fungal cultures were incubated for two weeks at 24-26^{o}C. The isolate of pathogen were cleaned up by subculturing by single spores and the edge of actively growing colonies (Naraghi et al., 2010). Identification of fungi was done based on morphological and microscopic observation of the forms of colonies, conidia, conidiophore and sclerotia of *V. dahlia*e (Isaac, 1967).

Plant material

Potato (*Solanum tuberosum* L.) cultivars Agria sensitive to Verticillium wilt was used in this research. Healthy potato tubers (40-50 g) having 2-3 eyes (buds) were selected and stored at 2-4^{o}C until use. Potato tubers at first were surface-disinfected in 3% sodium hypochlorite for 3 min and immersing in sterile double-distilled water, and then Seed

tubers of potato soaked in gibberellic acid (GA3) at 1,500 ppm for 24 h to break dormancy. Finally, they were sown in pots (30 diameters and 20 high) consist of pasteurized soil-sand-peat-perlite mix (4:4:4:1, v/v/v/v). Pots maintained in a greenhouse at 22 to 28^{o}C, 60-70% relative humidity, and 16 h light, 8 h darkness. Plants were watered twice a week with sterile tap water and once a week with the fertilizer solution (Uppal et al., 2008).

Pathogenicity test

Isolate Pathogenicity of *V. dahlia* was grown on potato c.v Agria as a susceptible cultivar. Conidia of *V. dahlia* were collected from two week old cultures grown on potato dextrose broth at temperature of 24^{o}C by shaking at 150 rpm for 14 days. Then Cultures were filtered through cheesecloth and conidia concentrated from the filtrate by centrifugation at 5000 × g for 20 min and then diluted with sterile water to give a concentration of approximately 10^6 spores ml^{-1}. Tuber of potato at first were superficially disinfested with a solution of 3% sodium hypochlorite, for 3 min and rinsed abundantly with sterile distilled water. Then, tubers were inoculated with 50 ml spore suspension with a concentration of 10^6 spore/ml, for 30 min at planting (Goth and Haynes, 2000). The concentration of spores was determined using a hemacytometer. Control treatment was treated with sterile distilled water. Disease severity was calculated eight weeks after inoculation (Spink and Rowe, 1989).

Elicitors

ASM (trade name Bion WG 50; Novarits Ltd., Basel, Switzerland) and chitosan (Sigma, USA, deacetylation degree: ~85%) were tested as putative activators of resistance to Verticillium in potato. Chitosan and ASM were dissolved in distilled water and both elicitors were applied with 0.01% Triton x-100 as a dispersant. In addition to a non-treated control, water and 0.01% Triton x-100 in combination were used as control *in vitro* and greenhouse.

The effect of ASM and chitosan concentration on the mycelial growth of Verticillium

Effect both activators (ASM and chitosan) were assessed on the mycelial growth of Verticillium *in vitro* using PDA amended with five concentration of these substances (0, 5, 50, 100 and 500 µg active ingredient per ml in PDA). Stock solutions were 10 and 5 mg/ml for chitosan and ASM, respectively. Both elicitors were applied with 0.1% Triton X-100 as a dispersant. Media were prepared in 100 ml flasks containing 95 ml of media, and stock solutions of either ASM or chitosan were added to obtain the desired concentrations. Distilled water was added to each flask to bring the total volume to 100 ml. Chitosan was added to the media before being autoclaved, whereas ASM was added aseptically after the media had been autoclaved. Five replicates were used for each ASM and chitosan concentration. Petri dishes containing PDA amended with 0.1% Triton X-100 was used as control. An agar disc (5 mm in diameter) with mycelium of *V. dahliae* was placed in the center of each petri dish. The inoculated plates were randomized and incubated at 24-26^{o}C in incubator in the dark. After 120 hours, percentage inhibition of radial growth (PIRG) was determined by measuring colony diameters. Mycelial growth was calculated by measuring colony diameters. The regression line between colony diameter of *V. dahliae* and chitosan and ASM concentration was calculated. Then, chitosan sensitivity was measured by calculating 50% effective (EC_{50}) values.

Effect of elicitors on Potato Verticillium wilt in greenhouse conditions

The potato tubers c.v Agria were selected based on uniformity of size and absence of visible symptoms. Tubers were surface sterilized in 3% sodium hypochlorite, for 3 min, followed by two rinses in sterile water and then air dried. With a sterile scalpel made a wounds in the epidermis of potato tubers, then were treated with 15 µl of the ASM and chitosan concentrations (5, 50, 100 µg a.i./ml) or sterile distilled water (positive control). Then, tubers dried under a laminar flow hood, sown in pots consist of pathogen (thirty milliliter of suspension of *V. dahliae*/ 10^6 spors/ml added to sterile soil of pots before planting) and pots transplanted to greenhouse. Also, ASM and chitosan applied by foliar spray on potato seedlings at concentration 100 µg a.i./plant; 15, 25 and 40 days after sowing. For this work, a volume of 100 ml of ASM and chitosan solution were applied onto the foliage of plants using a hand held garden sprayer. The control plants were sprayed with distilled water.

Disease assessment

Disease severity was recorded two months after planting. All plants were rated for wilt symptoms on the scales of 0-3, where 0, no visible symptoms; 1, some chlorosis in older leaves; 2, general chlorosis coupled with some necrosis and wilting; 3, severe wilting or death (Spink and Rowe, 1989).
Also, severity of vascular discoloration of potato stems evaluated by the following scale based on the stem cross-section showing a vascular discoloration: 0, no vascular discoloration; 1, trace to less than 9% of the stem cross-section showing a vascular discoloration; 2, 10-24%; 3, 25-49%; 4, 50-74%; 5, 75-100% of the stem cross-section displaying vascular discoloration (Uppal et al., 2008). At timing of harvest of potato, in order to assess the effect of each biocontrol treatment on Verticillium wilt severity and on production, tubers in each pot were gathered from each plant, cleaned from soil particles and weighted.

Data analysis

Experiments *in vitro* and greenhouse conditions were designed as a completely randomized design (CRD) with five replications. All data before analysis changed to normalcy by the formula $Arc\sin\sqrt{\%}$ (Little and Hills 1978). All analyses were performed using the SPSS (Statistical Product and Service Solutions) versions 12.0. Means comparison were carried out by Duncan's Multiple Range Test *(*DMRT) at $P \leq 0.05$. Probit analysis was used to measure EC_{50} with POLO-PC software (2002).

Conclusions

In conclusion, this study shown the potential of using ASM and chitosan to enhance resistance of squash plants against potato Verticillium wilt caused by *V. dahliae* and they have great potential to be used as elicitors and could be a viable strategy for controlling potato verticillium wilt in the field as practical application. But additional research would be beneficial to further justify practical application of these compounds in squash production. For example, using different methods, the number, frequency and timing of

application of ASM and chitosan could be optimized and standardized for achieving maximum efficacy. Therefore, application these materials to diseases management and their practical use needs further investigation.

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