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In Vitro Evaluation of Verticillium Dahliae – Derived Cinnamyl Acetate for Phytotoxic Effects on Alfalfa, Potato and Tomato Plantlets

KEYWORDS: *Verticillium dahliae*, toxin, cinnamyl acetate, alfalfa, tomato, potato

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ABSTRACT *Cinnamyl acetate obtained from the culture filtrate of strain of Verticillium dahliae, grown in liquid Czapeck medium, was synthesized and tested for its phytotoxicity on seedlings and plantlets of tomato and alfalfa and on potato leaves. The results showed that cinnamyl acetate (VdT) is a phytotoxic substance that induces wilting, dehydration, chlorosis and necrosis on alfalfa, tomato and potato via laboratory bioassays. Similar to those symptoms observed on the same hosts naturally infected with V. dahliae. This is the first report of the phytotoxicity of this compound on alfalfa, tomato and potato.*

INTRODUCTION

Verticillium dahliae is an economically important vascular wilt pathogen widespread in temperate climates. Strains of this soil borne fungus attacks over 150 species of plants, including tomato, potato and alfalfa. *Verticillium* wilt is a serious problem of tomato, potato and alfalfa production throughout the world. In Morocco, a tomato export annually generated almost 10 million Euros and creates an average of 9 million working days per year. In Morocco potato occupies 25% of the vegetable production is about 200.000 ha, including 40.000 ha of sown potato with a production of 1.700.000 tons. The market for potato is so strategic for the future of local agriculture, since it absorbs 75% of national production. Alfalfa extends over an area exceeding 135.000 ha and it is the main forage crop in irrigated area. *Verticillium* wilt is a limiting factors for each these crops in most regions of Morocco and throughout the world. Control of *Verticillium dahliae* is difficult due to the wide host range of most strains of the fungus which limits the efficacy of crop rotation for management of the disease and the extreme variability of *Verticillium dahliae* pathogenicity (Pegg, 2002). Host resistance is the most effective and economical means for controlling this disease. However, evaluation of alfalfa, tomato and potato *germplasm* for resistance to *Verticillium dahliae* by the commonly used root-soak screening procedure (Peaden et al., 1984) is laborious, time-consuming and expensive. Toxins produced by *Verticillium dahliae* have been shown to play a major role in the development of disease symptoms. Studies on the in vitro production of phytotoxins by *Verticillium spp.* (Green 1954; Mc Lead 1961; Malysheva and Zel'tser, 1968; Keen and Long, 1972; Cronshaw and Pegg, 1976; Nachmias et al., 1982), demonstrated possible involvement in pathogenesis and the potential for use in rapid screening for hosts resistance (Michail and Carr, 1966; Sheffer and Livingstone 1984; Nachmias et al., 1982, 1985, 1987; Irland and Leath, 1987, Koike, 1998; Clovis et al., 2006).

In a recent study (Laouane et al 2011), cinnamyl acetate a new phytotoxin (VdT) has been extracted from culture fluids filtrate of strains of *V. dahliae* isolated from olive tree. The structure of VdT was determined using GPC-MS. The VdT was chemically prepared; in subsequent testing in vitro on stem cutting was show that this compound play a role in the development of the Verticillium wilt disease symptoms in olive tree. The present study was undertaken to determine whether this purported phytotoxin is involved in verticillium wilt pathogenesis in alfalfa, tomato and potato through the use of in vitro plant bioassay.

MATERIAL AND METHODS

The method used to evaluated phytotoxicity in this study was described by Laouane et al (2011) using olive tree stem cutting and was developed by Sedra and Lazrek (2011) using date palm. The synthetic VdT (Laouane et al., 2011) was tested for its effect on the seeds and plantlets of the alfalfa (cultivar 'Pioneer') and tomato (cultivar 'Marmara') and leaves of potato (cultivar 'Desiree') which represent the major cultivated varieties in Morocco. The used concentration varied from 10 to 40 µg/mL.

BIOASSAYS:

Bioassay for alfalfa and tomato seedling

Seeds of alfalfa and tomato cultivars susceptible to *V. dahliae* were surface sterilized by rinsing in 70% ethanol for 30 s washed by swirling in 1% sodium hypochlorite solution for 15 min, and vigorously rinsed in two changes of sterile distilled water for 2 min each. The seeds dried were then placed individually in tubes containing 20 ml of sterilized medium consisting of 15g/l of agar, 0.5% DMSO, and varying concentrations of synthetic toxin (cinnamyl acetate) (10, 20, 30 and 40µg/ml). The control consisted of the same liquid medium with no toxin added. The tubes were capped tightly and incubated at 25 °C in the dark. Symptoms were recorded after 20 days by measuring root length. Twenty-four replicates were used per each concentration tested.

Bioassay for alfalfa and tomato plantlets

Plantlets of the cultivars evaluated were obtained by germinations seeds of alfalfa and tomato in culture pots at 25°C containing sterilised soil and 50% of peat and placed in glass house (25°C). Month-old tomato and alfalfa plantlets were used in a bioassay to evaluate the toxicity of VdT. Roots of the plantlets were immersed in sterile water containing 10-40 µg/ml of toxin with 0.5% DMSO. The control was done using sterile water containing 0.5% DMSO. Symptoms were scored 10 days after treatment (0 = no symptom to 4 = severe chlorosis and necrosis with leaf collapse). Differences in symptom severity were assessed using the ANOVA (in SPSS Software). The differences were considered significant at $p < 0.05$.

Bioassay for potato leaves

The phytotoxicity of VdT was evaluated on potato using a detached-leaves bioassay on the based method of Amraoui et al., (2005). The first true leaves of 3-6 week-old potato plants were excised under water and placed in sterile glass test tubes containing 0, 10 and 20 µg/ml of the synthetic toxin (5 ml/tube) and loosely closed with autoclaved cotton plugs. Three replicates were used per concentration.

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The tubes were placed in growth chamber and maintained at 25 ± 2°C with 16 h of light intensity. The susceptibility of detached leaves to VdT assessed after a 7 day incubation, during which the appearance of symptoms similar to those of verticillium wilt were evaluated.

RESULTS

Effects of toxin on alfalfa and tomato root-length

The response of seedlings to the toxin was correlated with the toxin concentration. At 20 µg toxin/ml alfalfa root length was reduced to 50% of the control. Similar results were obtained with tomato roots at 10µg/ml (Tables 1). No seeds of either crop germinated at 40 µg/ml.

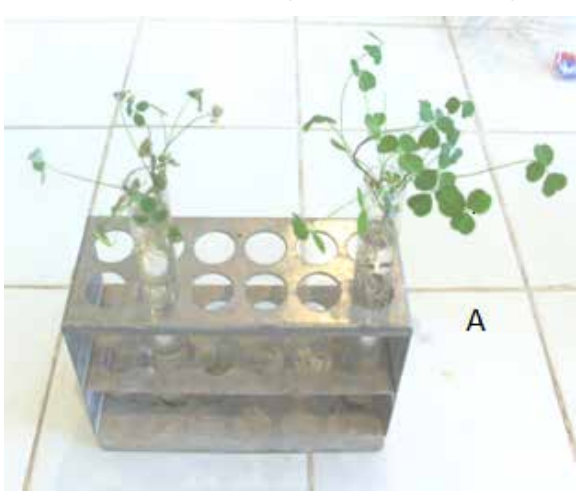
Table 1: Effects of different concentrations of *Verticillium dahliae* Toxin (VdT) on root length of Alfalfa and tomato (p<0,001).

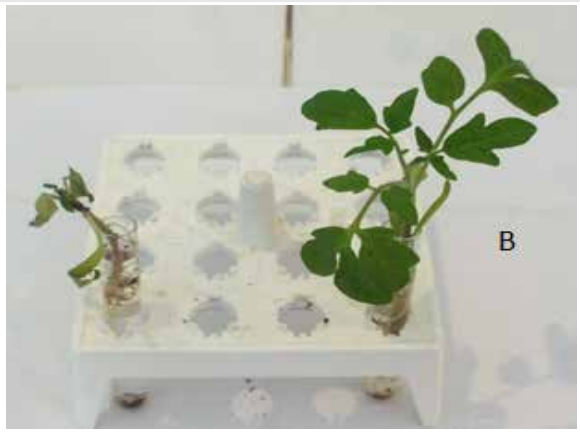
Plants	VdT Concentration	quantity	Length average (mm)	SD
Alfalfa	Control	14	41,7	8,5
	10 µg/ml	24	30,7	8,8
	20 µg/ml	24	20,0	11,1
	40 µg/ml	24	0,0	0,0
Tomato	Control	12	40,7	22.6
	10 µg/ml	24	20.2	15.0
	20 µg/ml	23	8.9	10.9
	40 µg/ml	24	0,0	0,0

Effects of toxin on alfalfa and tomato plantlets

At 10 and 40 µg/ml, interveinal chlorosis flowed by general desiccation and necrosis was observed on both alfalfa and tomato. Leaves first became chlorotic and then developed a pinkish necrosis that began at the margins of the leaflet and progressed inward. Leaflets curled inward or twisted around the midvein ensued of necrosis after leaflets or entire petioles abscised. The plantlets remained green and upright, and new green leaves emerged. The earliest symptoms were observed 4 days after treatment with VdT, and severe symptoms developed after 10 days both crops. These symptoms are all characteristic of those on alfalfa and tomato plants naturally infected with severe strains of *V. dahliae*, (Fig 1).

Fig 1: Appearance of plants control (right) and susceptible (left) 10 days after treatment with toxin of Verticillium dahlia (VdT) A: Alfalfa (30 µg/ml), B: Tomato (20 µg/ml).





Effects of toxin on potato leaves

Potato leaves were affected by exposure to VdT at concentration 10 to 40 µg/ml shows the high toxicity of the synthetic toxin. The early symptoms induced chlorosis and necrosis. The rate of development and severity of symptoms in detached leaves appeared to be a function of the toxin concentration. Severe chlorosis developed in 90% of leaves at 20 µg toxin/ml while only 40 % of the leaves treated with 10 µg/ml showed the symptoms. No symptoms were observed on the control leaves (Fig 2).

Fig 2: Appearance of leaves of potato 7 days after treatment with toxin of Verticillium dahliae (VdT) (4: 20 µg/ml, 3 and 2: 10 µg/ml, C: control).

DISCUSSION

An effective screening procedure must be amenable for testing a large number of plants, simple, relatively rapid, and significantly differential (Durbin, 1981). Both of the assays evaluated satisfied these criteria and could be used in breeding programs to screen large numbers of plants rapidly or to check symptomless plants for possible escapes in a conventional screening program. The root growth and plantlet and detached leaf assays used in this study could reduce the required selection time compared the currently used root-soak method (Peaden, 1984). Susceptible alfalfa, tomato and potato treated with VdT developed typical and characteristic symptoms that are similar to those observed on plants of these crops naturally infected with severe strains of *V. dahliae* in the field (Isaac, 1957). This study is an initial step in determining whether cinnamyl acetate has potential for use in verticillium wilt resistance screening. The results reported here is a similar the earlier finding of Green (1954) that *V. albo-trum* produces an extracellular metabolite in liquid culture which induces foliar symptoms on detached leaflets. The biological activities of toxins are assessed in a number of ways, depending on the apparent effect induced in susceptible plants. Mussel (1972) selected necrosis and chlorosis as a more valid signs of toxin activities than wilting in *Verticillium* infected plants.

In this study, the sensitivity of seedlings and detached leaves to VdT is correlated with toxin concentration and the root length crop species. For example, tomato appeared to be more sensitive to alfalfa. A 50% reduction on root length was observed at 20 µg/ml in alfalfa and at 10 µg/ml in tomato. Similarly, the toxin concentration resulted from the verticil-

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lium wilt symptoms in tomato and alfalfa plantlets was 20 and 30 µg/ml, respectively. In a recent study, Arpana (2014) used this technique in laboratory for to show the phytotoxic effect of *Lantana Camara* leaf extract on germination and growth of *Pistum Sativum*. Laouane et al., (2011) reported that VdT induced necrosis and chlorosis on stem cuttings of olive tree. This study is the first report of cinnamyl acetate (VTd) phytotoxicity on alfalfa, tomato and potato linking in vitro exposure to this *V. Dahliae* derived compound to symptoms of wilting, dehydration, chlorosis and necrosis on alfalfa, tomato and potato.

Conclusion

This study provides for the first time evidence that cinnamyl acetate may play a role in the development of the Verticillium wilt disease symptoms in alfalfa, tomato and potato. The seedling, plantlet and detached leaf assays could reduce the time required for verticillium wilt resistance selection cycles compared to the currently used root-soak method. In investigation, the assays are under evaluation of breeding programs to screen large numbers of plants rapidly for resistance to V. dahliae.

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