INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
10–001/ZIP/2011/13–3–444–446
http://www.fspublishers.org

Full Length Article

Synthesis and Toxicity Evaluation of Cinnamyl Acetate: A New Phytotoxin Produced by a Strain of *Verticillium dahliae* Pathogenic on Olive Tree

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ABSTRACT

A new phytotoxin (VdT), produced by a strain of *Verticillium dahliae* pathogenic on olive tree, has been purified from culture fluids. The VdT was associated with the production of disease symptoms in susceptible hosts. The structure of VdT was determined using GPC-MS. On the other hand, synthetic toxin (VdTs) was chemically prepared and used in a screening program. Our results confirmed that this new phytotoxin can be used to differentiate between *Verticillium-tolerant* and susceptible varieties of olive tree. © 2011 Friends Science Publishers

Key Words: *Verticillium dahliae*; New phototoxin; Cinnamyl acetate; Olive tree

INTRODUCTION

The *Verticillium* wilt, caused by *Verticillium dahliae* (Kleb), a pathogenic agent of large variety of plants (Koike, 1995), is one of the most important diseases of olive-trees growing in several Mediterranean Basin countries (Saydam & Copcu, 1972; Vigouroux, 1975; Ahmad, 1988; Matallah *et al.*, 1996; Cirulli *et al.*, 1998; Tosi & Zazzerini, 1998). In Morocco the disease was first noticed in the region of Meknes (Serrhini, 1992). Since then, it has spread extensively in Morocco and it is now widespread in the main olive-growing belt (Lachger & Sedra, 1996; Sedra, 2002). The very sensitive variety, Picholine, is widely cultivated, being approximately 98% of the total olive cultivation. Control of the *V. dahliae* is difficult, because of the absence of specificity of host and extreme variability of pathogenicity. Varietal resistance offers the most effective and economical means of reducing the disease impact. However critical evaluation of olive trees and of segregating material for resistance to *V. dahliae* under field is time-consuming and expensive. In this context, reports of the *in vitro* production of phytotoxins by *Verticillium* spp. (Green, 1954; McLead, 1961; Malysheva & Zel'tser, 1968; Keen & Long, 1972; Cronshaw & Pegg, 1976; Nachmias *et al*., 1982; Buchner, 1989; Riaan, 1996), their possible involvement in pathogenesis and their potential use as tools for rapid screening for resistance in different hosts (Michail & Can, 1966; Nachmias *et al*., 1982, 1985; Irland & Leath, 1987) have been of great interest. A protein-lipopolysaccharide toxin has been characterized (Nachmias, 1987; Clovis, 2006) but the low molecular weight of this fungus toxin had never been determined.

During our precedent work, the butanol extract (BE) fraction obtained from the *Verticillium dahliae* liquid culture by extraction with butanol was able to induce necrosis of cutting stem of olive tree at 40 µg/mL. The CPG profile of the fraction obtained by purification of BE with preparative TLC showed the presence of two peaks. The identification of these peaks by GC coupled to MS and a computer system managed library of mass spectra NIST (National Institute of Standards & Technology) finding the presence of cinnamyl acetate (VdTs). This method was used by Imelouane (2009) for identification of essential oil of *Lavadula dentata* and thyme (*Thymis vilgaris*) from Eastern Moroccan.

In the present study, the VdTs was chemically prepared and used in a screening program in order to provide the evidence that this compound may play a role in the development of the *Verticillium* wilt disease symptoms in susceptible olive tree and can be used to differentiate between V*erticillium*-tolerant and susceptible varieties of olive tree.

MATERIALS AND METHODS

Chemical synthesis of E-cinnamyl acetate: This molecule (VdTs) was synthesized and its structure was confirmed by NMR.

To cite this paper: Laouane, H., H.B. Lazrek and M.H. Sedra, 2011. Synthesis and toxicity evaluation of cinnamyl acetate: a new phytotoxin produced by a strain of *Verticillium dahliae* pathogenic on olive tree. *Int. J. Agric. Biol*., 13: 444–446

All chemicals and solvents were pure and were purchased from Sigma-Aldrich and Fluka. We synthesized E-cinnamyl acetate as follows (Scheme I):

Scheme I: A 1.34 g (10 mmol) of *E*-cinnamyl alcohol ($C_9H_{10}O$) (commercially avaible) and 2 mL of acetic anhydride (Ac_2O) (25 mmol) were mixed in 30 mL of pyridine with 10 mg of dimethyl aminopyridine (DMAP). The mixture was stirred overnight at room temperature, the solvent was removed under reduced pressure. The residual was purified by silica gel column chromatography using dichloromethane as eluent to give the desired compound.

Data of (E) –cinnamyl acetate.

Yield: 98% eluant dichloromethane Rf=0.95 (CH_2Cl_2/MeOH 95/0.5); H-NMR: 7.23 (m, 5 H phenyl), 6.60 (d, 1H, H_3, J CH=CH: 15.9), 6.24 (d 1H, H_2, $J_{2,3}$: 15.9, $J_{2,1}$: 6.6), 4.68 (d 2H, H_1, J_1: 5.4), 2.02 (S, 3H, CH_3). ^{13}C-NMR: 170 (C_1), 123.38-128.15 (C-Phenyl), 136.38 and 134.40 (C=C), 65.17 (C-O), 21.09 (CH_3), mass spectrum M=176.

Phytotoxin bioassays: Stem cuttings were taken from the 'Picholine marocaine' olive tree cultivar, which is susceptible to *V. dahliae* and represents about 98% of the cultivated varieties in Morocco. The bioassays were made according to the method of Sedra *et al.* (2002) the young stem were taken from olive tree susceptible to *verticillium dahliae* (Picholine marocaine) and then one stem was put in sterile glass test tubes. After many trials, the tests were performed using the synthetic toxin at 10 and 20 µg/mL in autoclaved water containing 0.5% of DMSO. The toxic solution was kept sterile by loosely closing each tube with autoclaved cotton plugs. The control was made by assays in sterile water containing 0.5% of DMSO. The tubes were kept at 25-27°C in a growth chamber and were exposed to a 12 h photoperiodicity. The tests were triplicated. The cuttings were rated after a 10 days period, during which it has been observed appearance of symptoms of *Verticillium* wilt: brown leaves, leaf necrosis, wilted stem with chlorosis, necrosis and leaf curl.

Determination of toxicity threshold: Two cultivars of olive tree, 'Picholine du Languedoc' (relatively resistant to *V. dahliae*) and 'Picholine marocaine' (susceptible to *V. dahliae*) were used in this study. Stem cuttings of each cultivar were used in a bioassay to test the toxicity of the synthetic toxin. The stem cuttings were immersed in water containing 10 and 20 µg/mL of the toxin (0.06 µmol–0.12 µmol). After a 10 days period, the symptoms were scored (0 = no symptoms, 1: chlorosis & necrosis of 25% of stem, 2: chlorosis & necrosis of 50% of stem, 3: chlorosis & necrosis of more than 75% of stem). The differences in severity of aggression were assessed by the Tuckey test, using the post-hoc ANOVA (in SPSS Software). The differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

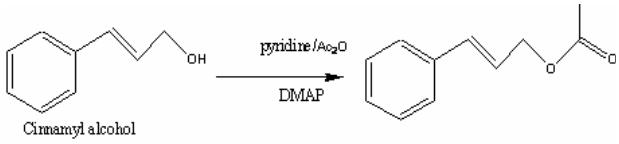
Stem cutting assays: In susceptible reactions, cuttings displayed *Verticillium* wilt-like symptoms after uptake of the VdTs (synthetic toxin). The high toxicity of VdTs (95% of mortality) at 20 µg/mL (0.12 µmol) (Fig 1).

Table I: Phytotoxicity of VDTs to olive stem cutting after 10 days

Olive Cultivar	control	10µg/ml	20µg/ml	Test F
Picholine Marocaine	0	2.20	2.8	92.5**
'Picholine Languedoc	0	1.13	2.13	20.23***

1: chlorosis and necrosis of 25% of stem, 2: chlorosis and necrosis of 50% of stem
3: chlorosis and necrosis of more than 75% of stem
(***: $p<0.001$)

Fig. 1: Appearance of cuttings from the susceptible olive variety (*Picholine marocaine*) 10 days after treatment with: VdTs: toxin; and C: control



Scheme I

Leaves first became chlorotic, then developed necrosis, which usually began at the margins of leaflets and progressed inward. Leaflets often curled inward or twisted around the midvein as they became necrotic and dry. The earliest symptoms were observed 5 days after treatment and severe symptoms were developed after 10 days. In contrast, no symptoms were observed on controls.

Determination of toxicity threshold: The concentration values from 10 to 20 µg/mL, of the synthetic toxin resulting in inter-veinal brown leaves and necrosis, followed by death of stem. These symptoms are those observed on olive trees naturally infected with virulent strains of *V. dahliae*. The sensitivity of the stem cuttings to VdTs are correlated with the susceptibility of olive tree to *V. dahliae*. In addition, the resistant 'Picholine Languedoc' cultivars exhibited a sensitivity response within 10 days at up to 20 µg/mL, whereas the same result was noticed in the susceptible 'Picholine Marocaine' cultivars at only 10 µg/mL. On the other hand, according to Scheffer (1976) concerning the

definition of host-specific toxins, the activities of the synthetic toxin on susceptible and tolerant host cultivars demonstrated that the *V. dahliae* toxin has a host-specific activity (Table I). Thus the concentration 10 µg/mL makes the difference between susceptible and resistant plantlet of olive tree.

CONCLUSION

We have prepared the cinnamyl acetate and confirmed the structure with ^{1}H and ^{13}C NMR spectroscopy. The VdTs was used in a screening program and we showed that 'Picholine Languedoc' has developed a susceptibility to the phytotoxin at 20 µg/mL but the same symptoms were obtained only at 10 µg/mL for 'Picholine Marcaine'. In addition, using VdTs instead of fungus will be considered as an additional tool for rapid and economical screening plants for resistance against *V. dahliae*.

Acknowledgment: We would like to thank Dr. O. Safarjalani, Department of Toxicology, University of Birmingham, USA for his interest in this research work.

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(Received 01 January 2010; Accepted 18 January 2010)