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Isolation and Characterization of a New Phytotoxic Molecule from Culture Fluids of Verticillium Dahliae

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I. Introduction

Phytotoxins are microbial metabolites which, at low concentration are harmful to plants. Many different fungi and bacteria are known to produce a wide range of metabolites in culture, which are toxic to plants. These include certain substance with varied biochemical structures, belonging to certain groups with height molecular weight such as quinines, polyketides, sterols, terpenoids, glycopeptides and glycoproteins (Mansoori and Smith, 2005; Buchner et al., 1987; Nachmias et al., 1982, 1987). Small molecules such as fusaric acid (Chawla and Wenzel 1987a; 1987b) eutypine and sterehirsutinal (Perrin-Cherioux et al., 2004) also have been described.

The most important disease of olive-tree growing in several Mediterranean Basin countries is Verticillium wilt (Zazzarini and Tosi, 1994; Cirulli et al., 1998; Tosi and Zazzarini, 1998; Vigouroux et al., 1975; Matallah et al., 1996; Saydam and Copu 1972; Ahmad et al., 1988), which is caused by Verticillium dahliae (Kleb). The later is a pathogenic agent of large variety of plants (Koike et al., 1995).

In Morocco, the disease was first observed in the region of Meknes (Serrhini, 1992). Since then, it has spread extensively in the main olive-growing belt of Morocco (Lachger and Sedra, 1996; Sedra, 2002). The very sensitive variety, "Picholine marocain", is widely cultivated, being approximately 98% of the total olive cultivation in Morocco and 5% of international cultivated territory of olive tree.

The difficulty of the verticillium dahliae control depends on the absence of host specificity and extreme pathogenicity's variability.

Most effective and economical means of reducing the disease impact are offered by varietal resistance. But, critical evaluation of olive trees and of segregating material for resistance to *V. dahliae* under field is time-consuming and expensive. In the same context, reports of 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), and their potential use as tools for rapid screening for resistance in different hosts (Michail and Can 1966; Irland and Leath, 1987) have been of great interest.

In this context, a toxic precipitate with acetone from the culture verticillium filtrate was obtained (Nachmias et al., 1982). This precipitate contains molecules with high molecular weight such as a protein-lipopolysaccharide toxin (Nachmias et al., 1985). The toxin with low molecular weight of this fungus had never been determined. The main objectives of this study were to show the presence of low molecular weight toxin in the filtrate after precipitation of all macromolecules by methanol. The filtrate was extracted with butanol and we have tested the toxicity of this fraction on stem cuttings of olive tree cultivar and compared with the toxicity of acetone precipitate obtained according to the method used by Nachmias in 1982.

II. Materials and Methods

a) Fungal Culture

V. dahliae (V10) was provided by the laboratory of INRA-Marrakech Phytopathology. A isolate kept in sterile sand was sown on PDA and incubated for 8 days at 25 ° C. Part of colony was suspended in sterile water. The Czapek medium (5 L) was made (for 1 L): 30 g of sucrose, 1 g of K2HPO4, 2 g of NaNO3, 0.5 g KCl, 0.5 g of MgSO4, 0.01 g of FeSO4. The pH was adjusted to 7 with NaOH and HCl. The medium was distributed as aliquots of 100 ml in 250 ml Erlenmeyer flasks which were then autoclaved at 121 ° C for 20 min. Each flask was inoculated with 1 ml of conidia suspension

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(10^6 conidia/ml) and incubated at room temperature (25 ° C) on a rotary shaker maintained at 80 rpm for 15 days. After centrifugation at 4200 g for 20 min and the supernatant was used for the butanol extraction. All chemicals and solvents were pure and were purchased from Sigma-Aldrich and Fluka.

b) Butanolic Extract (BE)

The obtained supernatant was evaporated under reduced pressure at 45 ° C to 250 ml. An equal volume of methanol was added and the mixture was kept for 48 hours at 4 ° C. The precipitate was filtered off and washed with methanol / water (1:1, v / v). The white precipitate was discarded, while the fractions resulting from filtrate and rinsing were mixed and evaporated under vacuum at 45 ° C to a volume of about 200 ml (F1). A glass column (3.5 x 60 cm) was filled with a mixture of 24 g of norite and 37.5 g of Celite homogenized in distilled water.

The concentrated fraction (F1, 200ml) was then passed through this column, and was washed with 100 ml of distilled water. The filtrates were collected (about 300 ml) and subjected to extraction with n-butanol (3 x 100 ml). The butanolic extract (BE) was evaporated to dryness, and gave 200mg of crude toxin (Figure 1). The later was subjected to purification by preparative TLC and analysed by GC-MS.

c) Acetone Precipitate (AP)

According to the procedure outlined by Nachmias et al. (1982, 1985), the crude toxin (AP) was obtained by precipitation with acetone from the in vitro culture of the V. Dahliae. A sterile Czapek liquid medium (100 ml) in Roux bottles was inoculated with 1 ml of conidia suspension (10^6conidia/ml) and incubated at room temperature (25°C) on a rotatory shaker maintained at 80 rpm for 15 days. The culture medium was centrifuged at 4200g for 20 mn, and the supernatant was concentrated under vacuum at 45°C in an evaporator. The concentrated fraction (one tenth of original volume) was dealt with four volumes of cold acetone (-18°C) and permitted to stand overnight at this temperature. The AP was collected by centrifugation at 10000g for 10 min at 5°C. The supernatant was discarded and the pellet was air dried and stored at 4°C.

d) 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 described by Sedra (2002) and Amraoui (2005); 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, with the stem bases dipped in a *40 µg/ml* solution of the butanolic extract. The second assay was carried out in the same manner with the acetone precipitate (AP) at *50µg/ml as a positive control*. 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. Thereafter, appearance of symptoms of *Verticillium* wilt were observed: brown leaves, leaf necrosis, wilted stem with chlorosis, necrosis and leaf curl.

e) Preparative TLC and GC-MS Analysis of BE Fraction

A sample of the BE extract was purified on a silica gel TLC preparative plate (Kiesegel, 60 F 254) with a mixture of CH_2Cl_2/MeOH (9.5:0.5, v/v) as eluent. The silica gel band revelled with UV at 254 nm (Rf = 0.95) was scratched and the obtained powder was washed with methanol. The filtrate was analysed by gas chromatography on a Varian 3800 analyzer fitted with a fused silica capillary column BP5 (0.25 mm id, 25 m, 0.25 µm coating thickness), directly coupled to a Saturn 2000 MSD mass spectrometer. The GC/MSD was operated under the following conditions: injector temperature 220°C; transfer line 240°C; oven temperature programmed from 60 °C to 240 °C (3°C/min); carrier gas He at 1.0 ml/min; injection 0.1µl.

Compound identification was done by GC coupled to MS and a computer system that managed a library of mass spectra NIST (National Institute of Standards and Technology).

III. Results

a) Stem Cutting Assays

In susceptible reactions, cuttings displayed *Verticillium* wilt-like symptoms after uptake of the AP (acetone precipitate) and B.E (butanolic extract). The high toxicity of AP was obtained at 50 µg/ml, with 80% of mortality. The same toxicity symptoms observed with butanolic extract at 40 µg/ml with 80% of mortality (figure 2).

Leaves first became chlorotic, then developed necrosis that usually began at the margins of leaflets and progressed inward. Leaflets often curled inward or twisted around the midvein as they became necrotic and dried. Sometimes, leaflets or entire petioles abscised while the stem remained green and upright, and new green leaves emerged. The earliest symptoms were observed 5 days after treatment, and severe symptoms developed after 10 days. In contrast, no symptoms were observed on controls with water. These observations provide the first evidence that the butanolic extract is a toxic fraction composed with new phytotoxic compound present in the *Verticillium. dahliae* culture medium.

b) Extraction and Identification of the Natural Toxin

The purification of BE using the preparative TLC, according the method used by Akor and Anjori (2009), gave two major bonds (Rf = 0.95 and Rf=0.96

after migration using 95/5 methylene chloride/methanol as solvent). These compounds were analysed by GC-Mass spectroscopy according to the method used by Imelouane (2009) for identification of essential oil of *Lavadula dentata* and thyme (*Thymis vilgaris*) from Eastern Morocco. The figure 3 shows the chromatographic profiles. The identification of these two compounds was based on the mass spectrum and confirmed by the data from library of mass spectra NIST (National Institute of Standards and Technology), and were assigned as: two isomers E and Z of cinnamyl acetate ($C_{10}H_{12}O_2$) with an abundance of 70% and 30% respectively (figure 4).

IV. Discussion

The BE have potential for use to induce symptoms of *verticillium dahliae*. Both the stem cutting assay could be reduce the time required for a selection compared with the currently used root-soak method.

Studies of metabolite production by *Verticillium albo-atrum* and *Verticillium dahliae* showed that they both produce high molecular weight toxic substances in liquid culture media (Nachmias *et al*., 1982, 1985, 1987; Riaan *et al*., 1994; Clovis *et al.,* 2006). Some of these toxins, are peptidic nature, were purified from culture fluids of potato isolates of *Verticillium dahliae* (Buchner *et al*., 1989), and induced interveinal chlorosis, followed by necrosis, when injected into excised leaves from disease-susceptible potatoes. In the present work, we showed that *Verticillium dahliae*, which is a plant pathogenic agent, is capable of producing a toxin that can able to induce the characteristic symptom of *verticillium dahliae* disease. Moreover, BE showed to be more toxic than either the AP fraction. The BE induced severe symptoms more rapidly. The purification of BE using the preparative TLC gave two major compounds which were analysed by GC-Mass spectroscopy and were assigned as two isomers E and Z of cinnamyl acetate ($C_{10}H_{12}O_2$) with an abundance of 70% and 30% respectively.

In our laboratory we have synthesised the major product, E-cinnamyl acetate (laouane 2011). This product was used in a screening program and we showed that 'Picholine Languedoc' has developed a susceptibility to the phytotoxin at 20 µg/mL, moreover, the same symptoms were obtained only at 10 µg/mL for 'Picholine Marocaine'.

This result suggests that the *verticillium dahliae* produces low molecular weight toxic substances in liquid culture. In addition, the stem cutting assay could provide an additional tool for screening plants for resistance to *verticillium dahliae*.

Susceptible olive tree (picholine marocaine) cutting treated with BE developed typical symptoms for *Verticillium Dahliae*-infected olive tree in the field (Lachger and Sedra, 1996, Serhini, 1992). This result supports that the BE is producing the same symptoms as those produced by fungal inoculation. Others host plants showed similar symptoms (Clovis S *et al* , 2006; Nachmias *et al.,* 1987; Scheffer, R. P., 1976; Irland, K. F. and K. T. Leath, 1987) when treated with toxins and fungal filtrate.

V. Conclusion

In the present study we described for the first time a protocol for the extraction and the determination of the structure of a new phytotoxin produced by a strain of Verticillium Dahliae which is pathogenic on olive tree. We have provided evidence that the butanolic fraction play a role in the development of the Verticillium wilt disease symptoms in susceptible olive tree more rapidily. The butanolic fraction showed the presence of two isomers: E-cinnamyl acetate (major product, VdT) and Z-cinnamyl acetate (minor product). The toxicity of E-cinnamyl acetate (major product, VdT) was confirmed on steam cutting olive tree.

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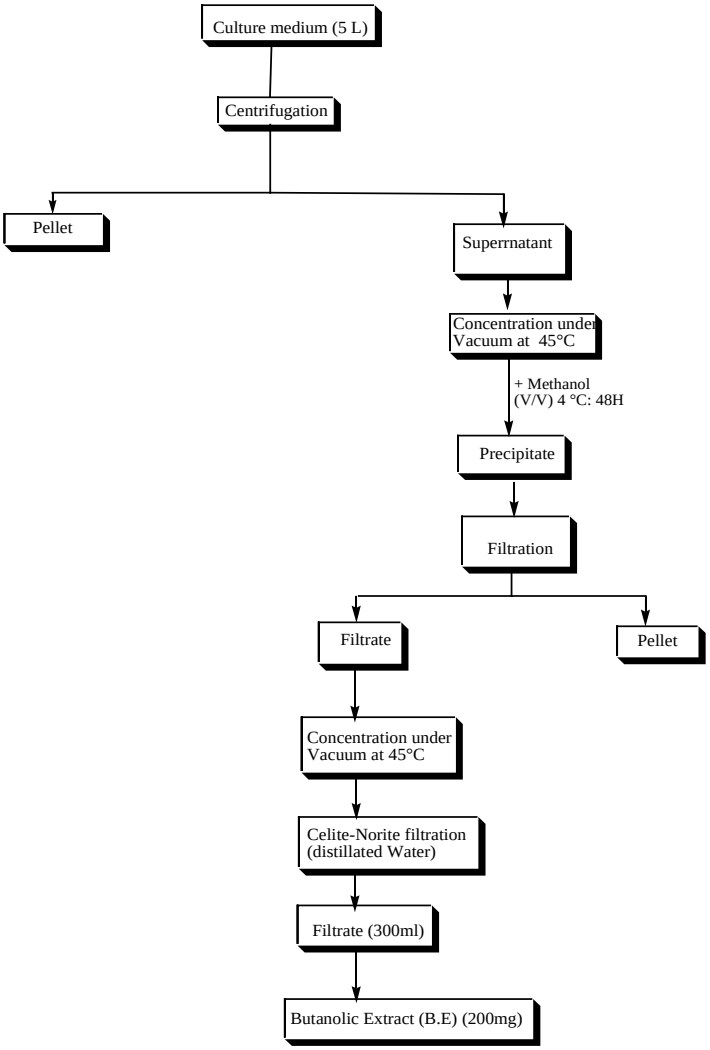

Figure1 : Protocol of phytotoxin extraction

Figure 2 : Appearance of cuttings of the susceptible olive variety (Picholine marocaine) 10 days after treatment with: BE, AP and control

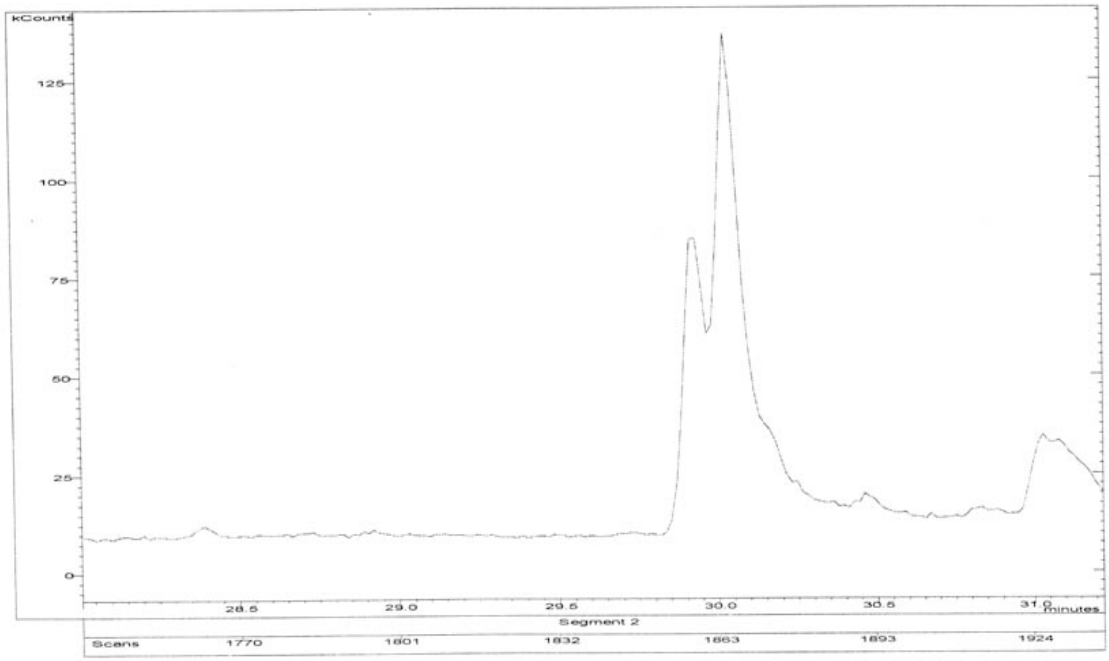

Figure 3 : chromatogram of two isomers compound extracted from *Verticillium dahliae* culture

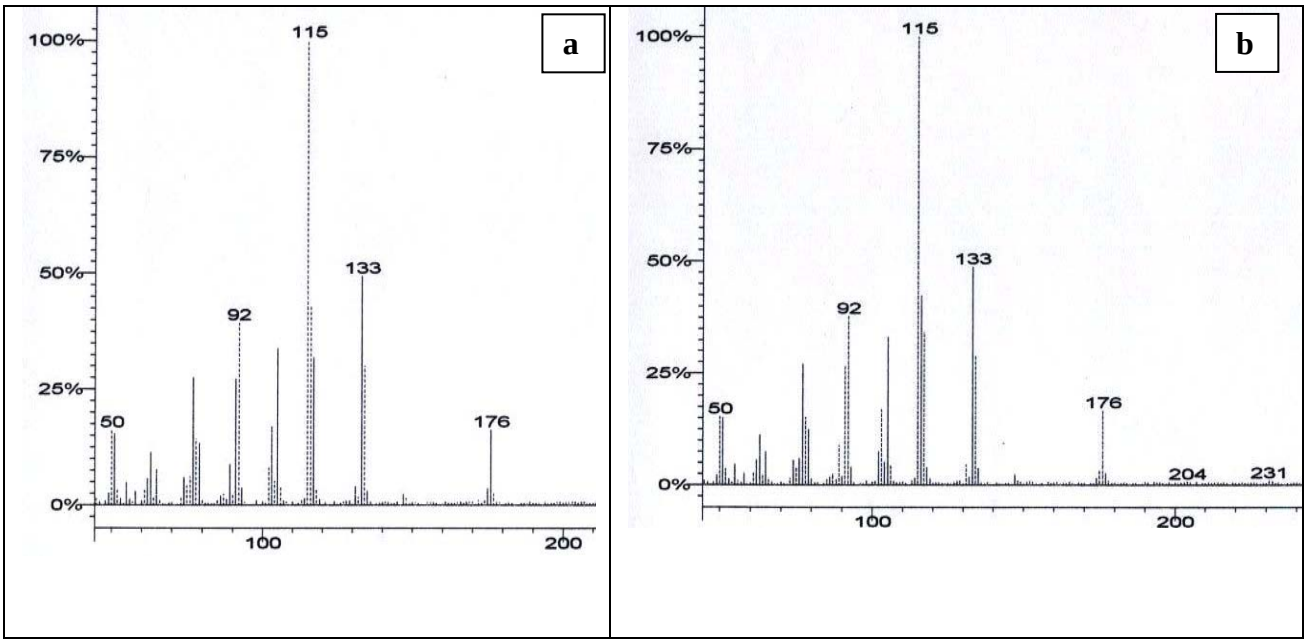

Figure 4 : **a)** Mass Spectrum of the first isomer, **b)** Mass Spectrum of the second isomer