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Research Article

Evaluation of Eleven Olive Cultivars Tolerance to *Verticillium dahliae* kleb

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ABSTRACT

In this study, two effective screening methods were used to compare 11 Mediterranean olive varieties for their tolerance to Verticillium dahliae Kleb. (i) inoculation method using butanolic extract of Verticillium dahliae Kleb liquid culture (ii) A natural phytotoxin as a new rapid selection tool. The purpose of these in-vitro tests was to compare their effectiveness and their capacity to differentiate levels of tolerance /susceptibility to Verticillium wilt in Olive varieties. Result indicate that the two screening method have the same sensitivity in identifying variable levels of olive tolerance to Verticillium wilt. Both methods confirmed that Bel Elanza is highly tolerant cultivar. Hence, using natural phytotoxin was less costly and requires less time and space than the standard inoculation and evaluation methods like pot immersion, bare-root dipping or stem injection using a conidial suspension of a defoliating pathotype of Verticillium dahliae isolate.

Keywords: *olive cultivars, Verticillium dahlia kleb, butanolic extract, natural phytotoxin, selection*

INTRODUCTION

The olive plant has many pests and diseases, although only a few of them cause serious economic losses on olive groves. One of the main diseases of olive is Verticillium wilt caused Verticillium dahliae Kleb (VWO), This soilborne fungus disease is a one of the most destructive disease widespread in all olive growing countries including Morocco; causing serious concern to olive oil and companies growers (López-Escudero & Mercado Blanco, 2011; Jiménez-Díaz et al., 2012; Tsror 2011). Verticillium is also one of most destructive plant diseases affecting various species worldwide such as cotton, tomatoes, potatoes (Fradin & Thomma 2006; Jiménez-Díaz et al., 2012, Kaliterna et al., 2016).

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Over the past 30 years, the prevalence of this disease has increased as a result of the expansion of irrigation in the olive groves (Perez-Rodríguez et al., 2015), the use of infected planting material (Blanco-Lopez et al., 1984; Jimenez-Díaz et al., 2012) and the establishment of orchards in fields beforehand cropped with susceptible hosts of the pathogen (Tsror, 2011).

The control of VWO is arduous due to the absence of specificity of host, the extreme variability of pathogenicity and it's microsclerotia which are also the primary inoculum of the pathogen in natural conditions and have been documented to survive more than 20 years in the soil (Pegg & Brady 2002; Jimenez-Dıaz et al., 2012; Inderbitzin & Subbarao, 2014).

Due to the complications mentioned above and the lack of an efficient method to control VWO, an integrated management strategy is needed to reduce both disease incidence in olive orchards and pathogen dispersal (Lopez-Escudero & Mercado Blanco, 2011). Within this the use of both precautionary and subdued control emerges as a promising alternative like the use of biological control agents such Paenibacillus alvei (Markakis et al., 2015), the use of fungicides represent an environmental threat and have little effect, soil solarization (García-ruiz et al., 2015; Sesli et al., 2010). In the midst of all these control measures, using resistant/tolerant plant material is considerate to be the most effective method, the least expensive, the easiest and the safest (Agrios 2005; Klosterman et al., 2009; Bubici & Cirulli 2012; Tsror, 2011). To our awareness, there is no single method satisfactory efficient when applied independently.

Numerous studies have concentrated on the establishment of an effective screening sources of resistance to Verticillium wilt in olive under controlled or field conditions (Seyed Javad Sanei & Seyed Esmael Raza, 2017; Agrios 2005; Klosterman et al., 2009; Bubici & Cirulli, 2012; López-Escudero and Mercado-Blanco2011; Tsror 2011; Jiménez-Díaz et al.2012). However, most of them, are susceptible or extremely susceptible to Verticillium wilt including cultivars broadly grown such as 'Moroccan Picholine', 'Arbequina (spain)' (Tous, 2011; Laouane et al., 2013). Finding a simple and precise screening method is important to successfully evaluate disease resistance (Infantino et al., 2006; Trapero et al., 2013(a); Calderón et al., 2015).

The screening methods should achieve major functions for instance to easily differentiate between tolerant and susceptible varieties, to generate results that are remarkably correlative with the attainment of plants in the field and to reduce the number of infected plants (Grau et al., 1991; Johnson & Jellis 1992; Debode et al., 2005; Gordon et al., 2005; Trapero et al., 2013 (a)). The evaluation period of Resistance to V. dahliae may last up to 24 months if the resistance assessment is conducted with soil inoculums other technic are also used in olive like using drilling to infect the plants trunk with spore suspensions, soil Root dipping or drenching artificial inoculations. In general, these methods are labor exhaustive (López-Escudero and Blanco-López 2007; Antoniou et al., 2008), pricey and highly time-consuming (Hiemstra, 2015).

All the selection work for the resistance character is carried by direct inoculation of olive plants or by dipping the roots of the olive tree in a solution containing a suspension of verticillium dahliae. This technique is laborious, expensive and time consuming. In order to establish a rapid and efficient protocol for studying the sensitivity of olive varieties to V. dahliae Kleb**;** two methods were used: the inoculation method using butanolic extract of Verticillium dahliae Kleb liquid culture and a natural phytotoxin as a new rapid selection tool to compare 11 varieties from 5 Mediterranean countries (Morocco, Algeria, Italy, Egypt and Croatia), and two screening method have been evaluated for their capacities in discriminating different

characteristics of tolerance/susceptibility in those olive cultivars.

MATERIALS AND METHODS

Plant materials

Seeds and twigs are obtained from Olive World Germplasm Bank of Marrakech, Morocco (OWGB). Those olive cultivars are: Toffahi, AbouMonkar, Cairo 7 and Bez Elanzafrom (Egypt), Pontoza (Croatia), Grosse du Hamma and Aberkanefrom (Algeria), ZDH7, Picholine Marocaine (Morocco) and Ottobratica (Italy), Picholine du Languedoc (France) were investigated according to their tolerance to V. dahliae.

Fungal Material

The defoliant isolate of Verticillium dahliae Kleb Vd18 from laboratory of genetic phytopathology and microbial control INRA Marrakech was used for olive trees inoculation. V. dahliae (V18) was provided by the laboratory of INRA-Marrakech Phytopathology. An 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 K_2HPO_4, 2 g of $NaNO_3$, 0.5 g KCl, 0.5 g of $MgSO_4$, 0.01 g of $FeSO_4$. 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 (Laouane et al., 2013).

n-butanol extract

The extract was made according to laouane et al. 2013. 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. 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 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.

n-butanol extract screening Method

The tests were made by transplanting germinated seeds from the Petri's dishes to the test tubes containing a mixture of water agar (15%) and butanolic extract at a concentration of 40 µg/ml. The test was performed on a one seedling per tube. Knowing that10 tubes for each variety were used. The tubes were placed in a chamber at a temperature of 25°C. The control was transplanted in the same conditions with only the water agar. The results were rated by the number of deaths plants. Control seedling was subjected to the same treatment using sterile water.

Inoculation of plants tolerant to butanol extract with the strain Vd18

In order to confirm and complete the previous test, the plants which showed a tolerance to the butanol extract are transplanted into the soil, and were incubated in a growth chamber at 25°C, with a12h photoperiod. After two months, the tolerant varieties to butanolic extract were inoculated with a suspension of the strain Vd18 at a concentration of 105 spores/mL. The roots of the olive plants are soaked in inoculums containing the spore suspension for about 5 minutes. The plants are then transplanted into plastic bags, and placed in a growth chamber at a temperature of 25°C with a 12h photoperiod. The same treatment for the control was maintained by replacing the spore suspension with sterile water. The test was performed on ten plants per variety.

Screening by using natural phytotoxin

Twigs cuttings were taken from 11 olive tree cultivars. The bioassays were made according to the method of Sedra (2002) the young twigs were taken from olive trees and then were put in sterile glass test tubes. The tests were

performed using the natural phytotoxin "Vdt" (Laouane et al., 2011) at 10 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 twigs with chlorosis, necrosis and leaf curl.

Disease severity

Symptoms of V. dahliae were estimated by calculating per each plant or twigs, the disease severity, percentage of dead plants. They were assessed each two days. The symptoms were evaluated on a score ranging of 0 to 5, following a scale as described in table 1 the arbitrary scale (Cirulli et al., 2008). The disease severity was determined according to the modified formula of El Said (2012):

$Disease\ severity$ = $[(Ax0) + n(Bx1) + nx(Cx2) + nx(Dx3) + nx(Ex4)]/ Totalnumbersofplants$

Where n: number of plants in categories of A-E.

The percentage of dead plants (PDP) was calculated according to the following formula:

PDP = $[(Numberofinfectedplants)/ (Totalnumberofplants)]$ *100

Table S1: **Severity of external symptoms**

	Description of symptoms / Categories	
Value	Leaf alteration index	Disease severity
0	i0: healthy leaf	A: Healthy plants
1	i1: yellowing	B: Light foliar symptoms in 1-9% of the plants
2	i2 : chlorosis	C: Severe foliar symptoms and moderate defoliation
3	i3 : necrosis	D: Defoliation (26- 50)
4	i4 : wilting	E: Total defoliation
5	i5 fallen	F: Dead plant

Tolerance categories:

The tested cultivars were arranged on the base of evaluation scales under the following categories

HT= Highly Tolerance

T= Tolerance

MS= Moderately Susceptible

S = Susceptible

HS= Highly Susceptible.

Statistical analysis

The Data was subjected to an analysis of variance for a randomized block design, using the SPSS 20.0 (software package). Waller-Duncan examined the means values. Differences between treatments were determined using least significant difference (LSD) test at P< 0.05. The experiment was repeated tree time with similar results, thus only data from the final trial is submitted.

RESULTS

Effect of n-butanol extract

No Verticillium wilt symptoms were observed in non-inoculated control plants. The results of the tests made by transplanting germinated seeds from the Petri's dishes to the test tubes containing a mixture of water agar (15%) and n-butanolic extract at a concentration of 40µg/ml (Figure 1) showed that all varieties

were susceptible to the extract except Pontoza, Picholine de languedoc and Bez Elanza. Those three varieties were tolerant to the butanolic extract with 8 out of 10 seedlings tolerant for Bez Elanza (Figure 2) 7 out of 10 for Pontoza and 5 out of 10 for Picholine de laguedoc.

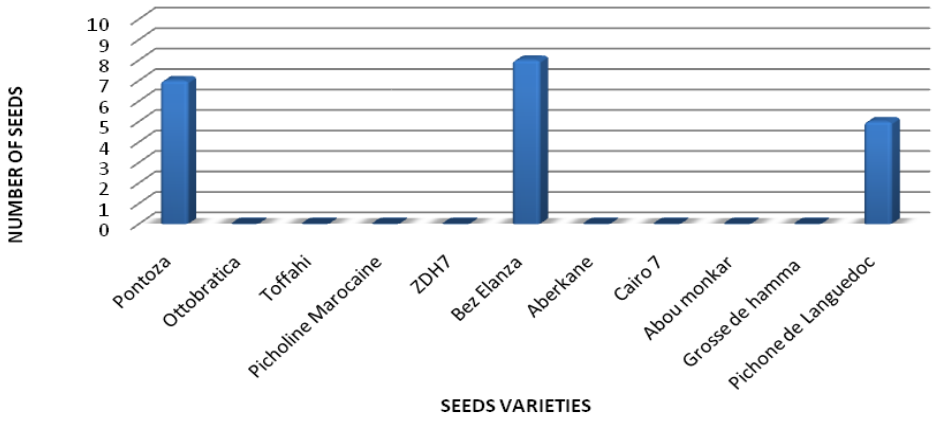

Figure 1: difference of tolerant seedlings among 9 varieties against butanolic extract, the test was performed on 10 seedlings per variety

Figure 2: Appearance of tolerant seedlings (Bez Elanza variety) in the n-butanolic extract

Inoculation of plants tolerant to n-butanol extract

The first result to note is the absence of seedling mortality during the first four months of testing. The observations are conducted 10 months after which the test was completed. For Pontoza variety, we noticed that from the fifth month there is a reduction in the size of the variety compared to controls. In the sixth month, other symptoms began to develop which were yellowing, and leaf curl beginning with the 1st falls on the base of the twigs. Then the symptoms were developed by drying and falling leaves from the bottom to the top of the twigs. In the eighth month, there was the death of the totality of Pontoza's variety; the same observations were also made for Picholine de languedoc where there was the death of the totality of the seedling. Whereas for the batch of 10 seedlings of Bez Elanza variety eight survived and two died. The tolerant seedlings were incubated in the culture room, after that, they were transplanted into large bags, and kept in ponds for watering to prevent the flow of water from the bags on lands that may contain spores of the fungus (Figure 3).

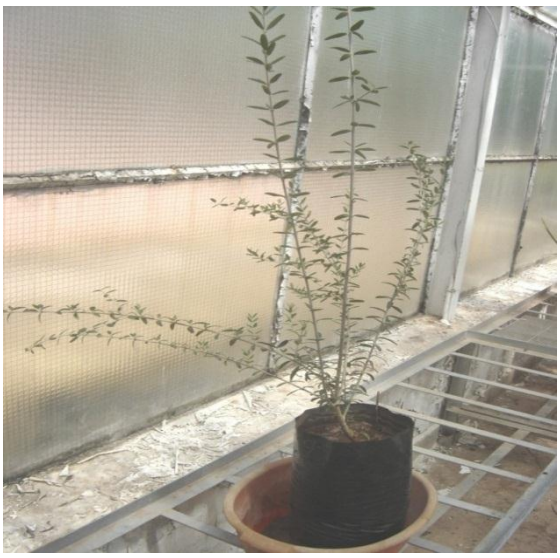

Figure 3: seedlings from the germination of Bez Elanza variety that has stood the n-butanol extract in vitro tests and resisted even after inoculation with *Verticillium dahliae*.

Effect of natural phytotoxinVdT on olive twigs

No Verticillium wilt symptoms were observed in twigs, which were put in sterile glass test tubes containing water mixed with 0.5% of DMSO. The disease severity of the obtained results indicated that the infected twigs showed typical symptoms of disease infection (Table 2). Sesli et al. 2010 reported that defoliation was the most common symptoms observed, it occurred in all the susceptible cultivars.

Table 2: Percentage of olive twigs in the different categories of external symptoms

	Severity of external symptoms					
Cultivars	0	1	2	3	4	5
Ottobratica	41.11±0.13	11.11±0.04	33.33±0.3	5.56±0.3	5.56±0.3	3.33±0.3
Toffahi	40.00±0.10	33.33±0.18	3.33±0.3	13.33±0.3	6.67±0.3	3.33±0.3
PicholineMarocaine	3.67±0.03	26.67±0.33	5.56±0.02	3.33±0.25	13.33±0.13	87.78±0.13
ZDH7	6.67±0.03	18.28±0.1	3.33±0.01	24.87±0.13	20.23±0.15	26.67±0.3
Bel elanza	87.78±0.13	06.67±0.04	5.56±0.02	0.00±0.0	0.00±0.0	0.00±0.0
aberkane	36.67±0.14	33.33±0.13	3.33±0.01	10.00±0.3	6.56±0.02	10.00±0.09
Cairo 7	60.00±0.24	26.67±0.33	3.33±0.01	0.00±0.0	6.67±0.02	3.33±0.02
AbouMonkar	36.36±0.15	6.67±0.02	34.55±0.12	10.00±0.13	9.09±0.3	3.33±0.3
Gross de hamma	09.23 ±0.1	19.23±0.12	3.35±0.03	25.38±0.13	23.08±0.21	19.23±0.12
Picholine de Languedoc	73.33±0.24	16.67±0.33	3.33±0.01	0.00±0.0	3.67±0.02	6.33±0.02
Pontoza	65.00±0.17	21,80±0.19	3.33±0.01	0.00±0.01	3.33±0.01	6.56±0.03

External symptom ± standard error.

In our study the percentage of leaves defoliation was more apparent in the susceptible cultivars like Grosse du Hammaand ZDH7 and Picholine Marocaine compared with the twigs of other cultivars. While Pontoza and Bez Elenza showed the lowest percentage. We noticed also a sudden collapse of one or few leaves which characterized by die back of twigs and some leaves become defoliated but retain some brownish color; the same aspects were found by using branches of olive cultivars (Bellahcene et al., 2000).

The Disease incidence

Results in Figure 4 showed that the percentage of total plants with disease symptoms (disease incidence) was higher in all the studied cultivars. The percentage of disease incidence ranged from 90% in ZDH 7 while Bel Elanza recorded the lowest percentage of disease incidence (6.37%).

According to the obtained results, the studied cultivars could be classified into the following tolerance: categories:

- Highly tolerant cultivars: Bel Elanza
- Tolerant cultivars: Pontoza and Cairo 7. Picholine de languedoc
- Moderately susceptible:Teffahi and Ottobratica
- Susceptible:Aberkaneand AbouMonkarcvs
- Extremely Susceptible: Grosse du Hamma and ZDH7, Picholine Marocaine

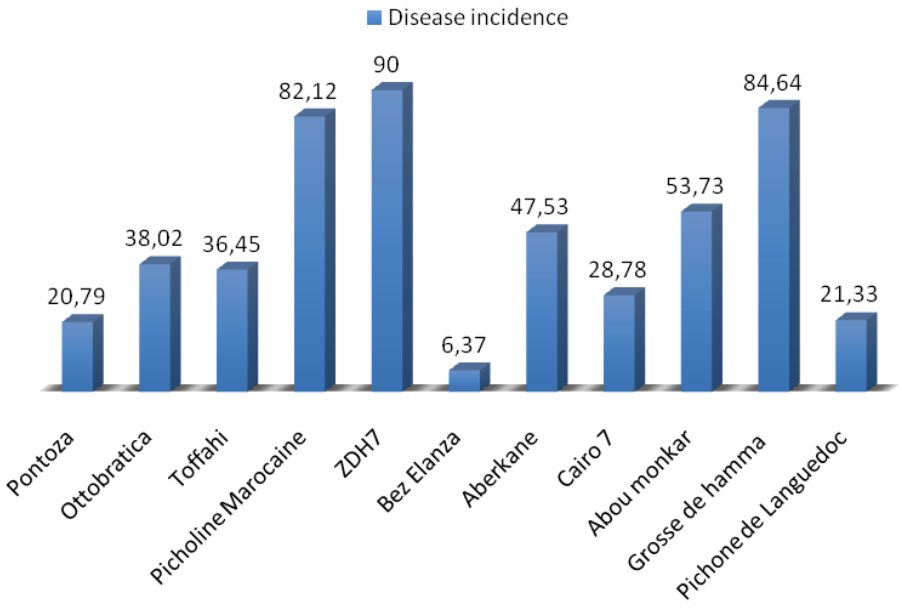

Figure 4: Percentage of disease incidence % of the studied olive cvs.

DISCUSSION

Our result is particularly relevant and in concordance with other studies since most olive cultivars is susceptible to V. dahlia (Antoniou et al., 2001 and 2008; Cirulli et al., 2008; López-Escudero et al., 2004; LópezEscudero & Mercado-Blanco, 2011). Although a number of relatively resistant/tolerant genotypes have been identified in inoculation assays (Mercado-Blanco et al., 2004; López-Escudero et al., 2004; Martos-Moreno et al., 2006; García-Ruiz et al., 2014) and also in field experiments (López-Escudero and Mercado-Blanco2011; Trapero et al., 2013(b)). Nevertheless, most of the economically and agronomical relevant olive cultivars are susceptible or extremely susceptible to highly virulent strains of V. dahliae (López Escudero and Mercado-Blanco, 2011). For instance, the susceptibility of different cultivars among them Ottobratica was investigated (Baidez et al., 2007; Markakis et al., 2010), inoculating the plantlets of 18 months with different isolates

of Verticillium dahliae Kleb, they found that Ottobratica was highly susceptible. Moreover (El Said et al., 2012), worked on the susceptibility of twelve olive cultivars by dipping the roots of one-year-old olive transplants for 30 min in the conidial suspension of the pathogen. The result showed that Teffahi was moderately susceptible while Cairo 7 was tolerant cultivars. In our study using natural phytotoxin we showed that, Ottobratica and Telffahi were moderately susceptible, while Cairo 7 was Tolerant. This may be attributed to the difference in the virulence of the pathogen (Vd18).

In several studies, crude extracts that contain Verticillium toxin complexes have been used to elicit plant defence responses or to study physiological responses of the plants (Fradin & Thomma 2006; Meyer et al. 1994). However, the exact nature of the components of the complexes is often unclear (D'Orazio et al., 2019).

The preliminary test was established at first in vitro by using seed and then carried out in vivo by incubating the tolerant seedling with n-butanol extract. The resistant/ Tolerant variety Bel Elanza resisted even after inoculation with Verticillium dahliae Kleb. These results are in concordance with our previous study (Laouane et al.2013) using the n-butanolic extract as a screening tool. Hence, the classification of studied cultivars using n-butanolic extract was divided into 3 scales: susceptible, tolerant and highly tolerant. Moreover, those results were confirmed during 10 months after direct inoculation of resistant plants with the Vd18 strain of verticillium dahliae. While when using the natural phytotoxin, we were able to conduct the selection of studied cultivars by using twigs which allowed us to conduct the test in a short time (10 days) and space, also this methodology is safer considering there is no possibility of soil or plant contamination. The natural phytotoxin allowed us to categorize the cultivars in the 5 scales. Hence, this study enables us to confirm the toxicity and the selectivity of the natural phytotoxin, and answering to the tree major functions of a good screening (Grau et al., 1991; Johnson & Jellis,1992; Debode et al., 2005; Gordon et al., 2005, Trapero et al., 2013(b)).

CONCLUSION

The main objective of this study was to develop effective methods to screen olive for tolerance to V. dahliae with the aim of differentiating tolerant from susceptible varieties, and reducing the age of the screened plants, the space and time necessary for their evaluation. Both techniques confirmed that Bel Elanza is highly tolerant cultivar. Hence, using natural phytotoxin was less costly and requires less time and space than the standard inoculation and evaluation methods conducted with seed.

This project is ongoing in the aim to show that the natural phytotoxin can be used as a pre-screening method for selection of resistance in olive cultivar.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by authors.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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