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Research Article

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Antifungal activity of a Moroccan plant extract against pathogenic fungi *Pyrenophora teres*, the causal agent of Net Blotch of barley

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ABSTRACT

The extract of a medicinal plant Daphne gnidium , was tested for its in vitro and in vivo antifungal activity against Pyrenophora teres Drechs. f. teres the fungi causing Net blotch of barley .The extract obtained by plant decoction is tested in vitro on the growth diameter of five Moroccan isolates of Pyrenophora teres Drechs. f. teres. Twelve concentrations were tested: 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 55 and 60 g /L . The concentration of 40g/L of D.gnidium extract induced complete inhibition of 4 pathogen isolates in vitro conditions . The isolates inhibited by the medicinal plant extract and transferred in PDA media only have not revived. Therefore the effect of D.gnidium on Pyrenophora teres f. Sp. Teres is fungicidal. The most active concentration of the extract in vitro studies was tested, afterwards, in vivo against Net blotch on barley leaves . Incidence of Net blotch was decreased to a rate of 0 on the scale of Tekauz , while control barley plants showed a rate up to 9 on the severity scale of the disease . This study demonstrated that plants extracts have a high potential to control Net blotch of barley. Therefore such natural products represent a sustainable alternative to the use of chemical fungicides.

Key words: Net blotch, *Pyrenophora teres Drechs. f. teres*, *Daphne gnidium*, aqueous extract, antifungal activity

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INTRODUCTION

Pyrenophora teres is an haploid ascomycete [1] that causes Net blotch on barley leaf blade and sheath [2] . The disease is found almost in all regions of the world where barley is grown and usually occurs in cool and moist areas. Even so, this disease is considered serious in the dry lands of North Africa and the Mediterranean region , where Net Blotch is economically important [3].

Due to Net blotch , yield loss can reach up to 56% in Morocco [4] and goes up to 44 % in Australia [5] even under fungicide treatment .

The fungal agent infects leaves and stems of plants, either by direct penetration or through wounds caused by cultivation practices. Infestation is stimulated by high humidity.

Although *P.teres* is a classical 'high risk' pathogen in the sense of resistance management [6,7] disease control is generally achieved by the use of synthetic fungicides [8].

Although the synthetic fungicides are effective, their continued or repeated application has disrupted biological control by natural enemies and led to outbreaks in diseases, widespread development of resistance to various types of fungicides [9], toxicity to non-target organisms and environmental problems. The reducing efficacy and

increasing concern about the negative environmental effects of synthetic fungicides lead to the necessity to the development of new types of selective control solutions and methods of crop protection .

Medicinal plants have been used in developing countries as alternative treatments to health problems . Many plants extracts and essential oils have been shown to exert biological activity *in vitro* and *in vivo* , which justified research on traditional medicine focused on the characterization of the antimicrobial activity of these plants [10].

Plants extracts may be an alternative to currently used disease control agents, since they constitute a rich source of bioactive chemicals. In fact, there are many studies revealing the antifungal activities of plants extracts against plant pathogenic fungi .

The Eastern Mediterranean area of Morocco has rich flora in native aromatic and medicinal plant species [11] , such as *Daphne gnidium* which belongs to the Thymeleacea family widely grown in the Mediterranean basin of Morocco since antiquity and known for its medicinal properties [12,13].

In the present study, antifungal effect of plant extract from aerial parts of *Daphne gnidium* has been investigated against fungal disease agent *P.teres* . The effect of different concentrations of aqueous extract on the mycelia growth was determined *in vitro* conditions.

The impact of the most efficient concentration of the extract , as determined *in vitro* studies, was evaluated further on Net blotch disease control in greenhouse conditions (*in vivo* tests) following its application as a foliar spray with a broader objective of identifying eco-friendly tactics to manage this disease.

EXPERIMENTAL SECTION

Plant material

D.gnidium was collected from their natural habitat during February 2011 in the forest of Maamora. The botanical identification was performed in the laboratory of botanic, mycology and environment , in the university of science of Rabat , where the plants were dried in the shade for fifteen days and stored until use .

Pathogen culture

The five isolates of *P.teres* BF1,BF2,BF3,BF4 and BF5 were collected respectively from five Moroccan regions Chaouia, Zemmour-Zaer, Gharb,Rif and Tadla, and isolated from infected barley leaves showing characteristic symptoms of the disease

The isolates were incubated on filter paper moistened with distilled water , under favorable conditions of spore-producing , at 22°C and a photoperiod of 12h . After two days of incubation, the Petri dishes were observed under a binocular lens . Once the typical spore-producing of *P.teres* was observed, a transfer of conidia on PDA(potato dextrose agar) medium was realized .

Effect of aqueous extract on mycelia growth *in vitro* conditions

The extraction of the active substances for the *in vitro* test was made by the decoction method. In brief, different amounts of leaves of the plant were added to 1000mL of melted PDA medium, to obtain final concentrations of 5,10,15,20,25,30,35,40,45,50,55 and 60g /L.

The resulting suspensions were stirred and autoclaved for 15 min at 121 °C and subsequently filtered before being dispensed into 9-cm diameter Petri plates.

Pathogen grown on PDA without plant powders was used as control.

The prepared Petri plates were inoculated aseptically with 5 mm diameter disks of five *P.teres* isolates taken from a one week old culture and incubated at 22 C° .

The antifungal activity was expressed in terms of percentage of mycelia growth inhibition and calculated according to the Leroux and Gredet [14] formula : P.I.C.D = (Øt-Øe/Øt) 100

P.I.C.D : Inhibition percentage of the diametrical growth
Øt = mean diameter of the control colonies
Øe = mean diameter of the colonies in the presence of the plant extract

In order to determine whether plant extracts have fungistatic (temporary inhibition) or fungicidal (permanent inhibition) effect on *P.teres* , fungal discs from treatment with no growth were

Re-inoculated into fresh medium and revival of their growth was observed. A fungicidal effect was where there was no growth after additional nine days of incubation at 22 °C, while a fungistatic effect was where temporary inhibition of mycelia growth occurred.

Effect of the aqueous extract on the disease development in vivo conditions

The concentration of *Daphne gnidium* extract, having the highest effect on the pathogen isolates, was selected for greenhouse trial to study the effect of the aqueous extract on the disease caused.

All experiments were arranged in a completely randomized split-plot design with five replicates of one isolate (BF3) per treatment and repeated at least twice.

The concentration of 40g/L was prepared by dissolving the requisite amounts of *D.gnidium* leaves in sterile distilled water and adding Tween 20 (0.1%, v/v) solution.

Two weeks barley plants were sprayed with this emulsions (10 mL for each plant) uniformly with a manually operated plastic sprayer .

For protective activity of the extract , barley plants were treated with *D.gnidium* extract 48 hours before pathogen inoculation.

For curative activity of the extract, barley plants were inoculated with suspension of 10^4 conidia per ml of *P.teres* BF3 isolate.

These plants were incubated for 48 h in dark at 20 ° C (± 1) under a relative humidity of 100% and then, were treated with the plant extract .

The control plants were sprayed uniformly with 10 ml of sterile distilled water and Tween 20 or pathogen suspension used as negative or positive control groups of the experiments .

Control and plant extract treated plots were assessed seven days after treatments.

The disease severity index of Net Blotch on the barley leaves was rated using the 10- point scale of Tekauz [15] for barley leaf diseases .

Statistical analyses

Statistical comparisons were performed using Excel2007 and XLSTAT 2014 software. Five Petri dishes were used for each test and each test has been repeated at least three times.

RESULTS AND DISCUSSION

***In vitro* antifungal activity of plant extract on mycelia growth**

The diametrical growth of the isolate was remarkably inhibited under the effect of the lowest concentration 5 g /L, with an inhibition percentage of 50%. At the concentration of 10g /L, isolates, BF2, BF3 and BF5 have a PICD exceeding respectively 69% , 62% and 61% .

A similarity was recorded in the level of inhibition reactions among both isolates BF3 and BF5 at the concentrations of 15g / L and 20g /L. Thus, at the concentration of 15 g /L both isolates have almost the same PICD, 68%, and at 20 g /L the PICD reached roughly 72% **(Fig.1)**.

At 40 g/L the extract has led to a complete inhibition of diametrical growth of the four isolates BF1 ,BF 2, BF3 and BF5 **(Fig.1)**.

We noted that the fungal growth inhibition increases with the concentration of the extract among all isolates except for BF4 which showed insensitivity to the concentration variation. It was found that *D.gnidium* extracts at the concentrations of 30 g /L, 35 g /L and 40 g/L induced the same PICD of this isolate. BF4 required a concentration of 60 g /L to be completely inhibited (Fig.1) .

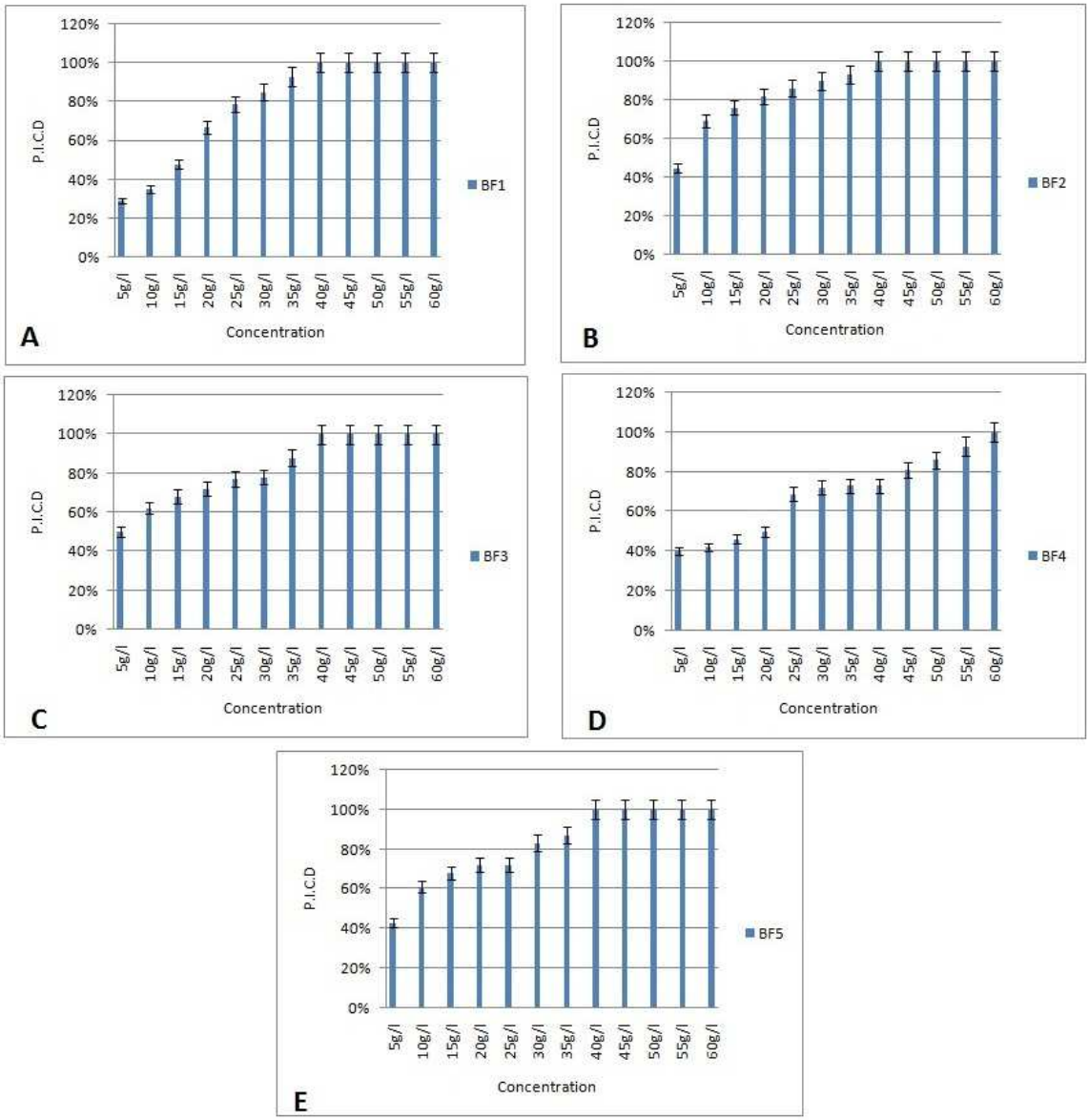

Fig.1 : Effects of different concentrations of *D.gnidium* extract on the mycelia growth of the five *P.teres* Moroccan isolates

The findings showed that *D.gnidium* extract reduced *in vitro* mycelia growth of *P.teres* with different degrees depending on the concentrations of the extract used. The pathogen response to plant extract varied from 29% to 100% inhibition level of mycelia growth.

Indeed, in *in vitro* conditions, the results showed that the extract was found active on the mycelia growth of all isolates. The extract inhibited 100% of the isolates BF1, BF2, BF3 and BF5 at a 40g/L concentration, while BF4 isolate was completely inhibited at 60 g /L concentration.

The increasing of the inhibitory effect versus the concentration of the extract is consistent with the work of Kra *et al*. [16], which showed that the aqueous extract of the leaves of *Chromolaena odorata*, progressively inhibited mycelia growth of a *Fusarium oxysporum* isolate, at 20 and 30g /L concentrations, to completely block the growth of the fungus at a concentration of 40g /L . The aqueous extract of *C.odorata* was also found effective *in vivo* conditions in which it showed a protective antifungal activity on banana leaves .

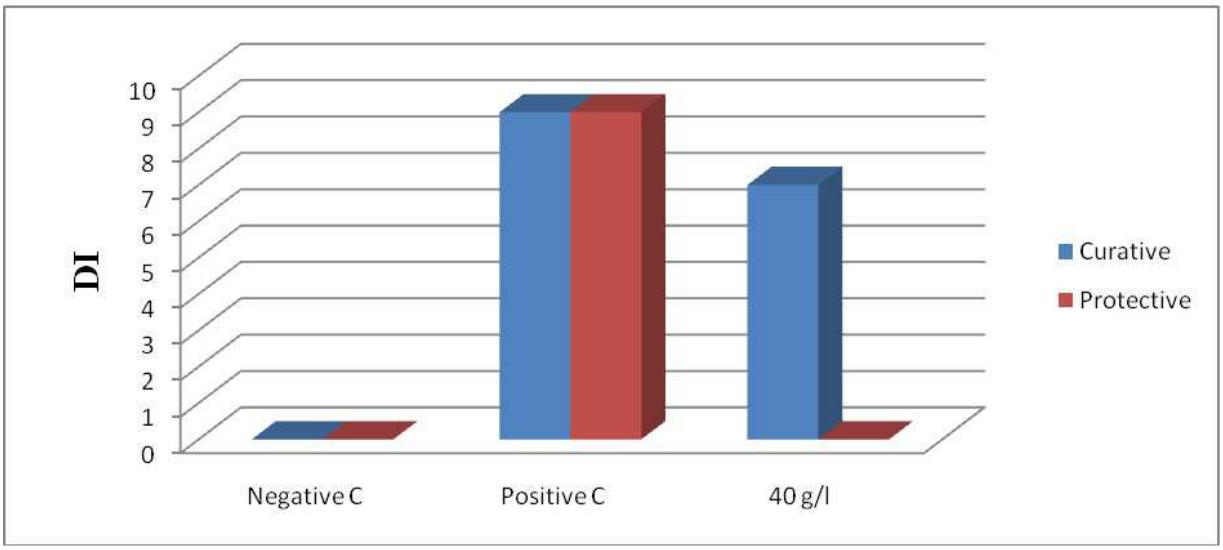

Fig.2 : Effect of aqueous plant extract on disease incidence (DI) on barley plants

Another study demonstrated that the inhibitory activity of the aqueous extract of a medicinal plant, *Citrullus colocynthis* on the pathogen fungi *Aspergillus Niger*, fluctuates with changes in the concentration of the extract [17].

Fungi-static / Fungicidal test

The completely inhibited cuttings of isolates BF1, BF2, BF3, BF4 and BF5 respectively at the lethal concentrations 40 g /L and 60 g /L, were transferred to PDA medium only. The test shows that none of the five inhibited isolates has revived. Therefore the aqueous extract of *D.gnidium* has a fungicidal effect against *Pyrenophora teres* **(Table 1)**.

Table 1 : Results of the re-inoculation of the 5 inhibited *P.teres* isolates into a new PDA media

***P. teres* isolates**	**PICD under the effect of *Daphne gnidium***	**Result of the transfer of inhibited *P. teres* isolates**	**Effect**
BF1	100%	-	fungicidal
BF2	100%	-	fungicidal
BF3	100%	-	fungicidal
BF4	100%	-	fungicidal
BF5	100%	-	fungicidal

- : absence of mycelia growth

Plant extract activity on disease development *in vivo* conditions

The data in **Fig.1** showed that all tested concentrations of plant extract significantly reduced the incidence of Net blotch caused by *P .teres* under the laboratory conditions .

Although *in vitro* test of plant extract is an important first step in selecting concentrations with antifungal potential against Net blotch pathogen, *in vivo* tests are needed to check whether the positive results of the in vitro tests can be achieved . In fact the extract concentration of 40g/L enabled to suppress the infection of *P .teres*.

Table 2 : Rates of Net blotch on treated barley plants according to Tekauz scale

Concentration	**DI Curative Effect**					**DI Protective Effect**				
Negative Control	0	0	0	0	0	0	0	0	0	0
Positive Control	6	9	9	6	9	6	9	9	6	9
40 g/L	9	5	6	8	9	0	0	0	0	0

DI : Rate of the disease index according to Tekauz (1985).
Negative control : barley plants were sprayed sterile distilled water+ Tween 20 (0.1%, v/v)only.
Positive control : barley plants were only inoculated with P.teres + Tween 20 (0.1%, v/v) .

When we compare the curative and protective activities of *D.gnidium* extract on the infection caused, we can conclude that the protective activity has the greatest effect on the disease severity of the pathogen (**Fig.2**).

In protective treatment, the extract 40 g/L concentration displayed a significant decrease in disease severity reaching 100% , whereas the disease rate was 0 on barley plants, in comparison to negative control (**Table 2**).

Curative activity of *D.gnidium* extract had a slightly significant effect in comparison to the same concentration used in the protective activity .No sign of phytotoxicity was found on the tested plants for the concentration used in the experiment.

Results obtained in this study indicated that aqueous extract of *D.gnidium* significantly canceled the incidence and severity of Net blotch on barley .

Previous reports mentioned that secondary metabolites of plant extracts (e.g., alkaloids , phenolic , flavonoids and terpenoids compounds) may be responsible of this antifungal effects on the pathogen growth [18].

In fact , the study of Cottiglia *et al.*[19], proved that the antimicrobial activity of *D.gnidium* is due to coumarins and flavonoids . Indeed, the methanolic extract of *D.gnidium* exhibited an antibacterial activity against *Bacillus lentus* and *Escherichia coli* .

Our results are consistent with the work of Dohou *et al.* [20] on the effect of *Thymelaea lythroides*, a specie from the same family as *D.gnidium*. The extract of *T.lythroides* leaves has significantly reduced the mycelia growth and sporulation of three plant pathogenic fungus of rice. The same author has shown that among the main bioactive compounds having an antifungal activity of *T.lythroides* there are polyphenols [21,22].

CONCLUSION

This work is original as much as that we did not find any report or paper testing the impact of *D.gnidium* on *P.teres* the causative agent of net blotch of barley. The complete inhibition of *P.teres* growth both in *in vitro* and *in vivo* conditions has shown that this plant is very suitable for ethno botanical studies and confirmed that this medicinal plants could be an important component of plant disease management . Moreover, new research should focus on the phytochemical analysis to identify the active principles responsible for the antifungal effect of this plant.

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