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Research Article

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Exploitation of some plant extracts for ecofriendly management of Net Blotch of Barley

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ABSTRACT

The antifungal activity of ten plants extracts was tested in controlling Pyrenophora teres the causal organism of net blotch of Barley in vitro and in vivo. Effects of the aqueous extracts varied depending on concentrations. In in vitro study the aqueous extracts of Anacyclus valentinus and Tetraclinis articulate at 1500 ppm caused highest reduction of mycelia growth of P.teres(72.27 and 87.05 % respectively), while extracts of Mentha pepirita and Foeniculum vulgare caused the lowest inhibition of the pathogen. In in vivo experiments the plants extracts were tested for their preventive and curative efficacy against net blotch. Barley plants were treated either aqueous extracts 1 day before or2 days after artificial inoculation. The highest reduction of diseases severity was achieved by the extract of Tetraclinis articulata. The same extracts were then tested as seed treatments, against seed-borne fungi. The best control against barley seed mycoflora was obtained with the extract of Inulavis cosa(72.8%). Results revealed that the selected plant extracts were active on both Pyrenophora teres in vitro, on disease severity in vivo and can be used as potential seed treatments for net blotch disease control.

Key words: barley, *Pyrenophora teres*, antifungal activity, biological control, aqueous extracts

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INTRODUCTION

Barley net blotch, caused by *Pyrenophora teres* Drechs. (anamorph *Drechslerateres* (Sacc.) Shoemaker),is one of the most serious constraints to barley production worldwide **[1, 2]**. Under warm and humid conditions, expression of disease symptoms can increase rapidly, causing substantial economic losses **[1]**. The infected seed is an important means by which *D. teres* survives, spreads, and initiates primary foci for net blotch epiphytotics. Seed infection contributes to disease symptoms on young plants and influences further disease development depending on vegetation conditions and/or varietal susceptibility **[3]**. Several control methods against *P. teres* had been recommended, such as crop rotation, the application of fungicides and the use of resistant cultivars. The use of genetic resistance is the favored method for controlling this disease, however; it is complicated by the existence of several pathotypes of the pathogen **[4, 5]**. An alternative control approach against net blotch would be the use of natural products that would inhibit or reduce the pathogen development. This approach should be based on economically and technically feasible and environmentally safe strategy. Plant extracts seem to be an alternative to currently used fungicides to control phytopathogenic fungi, as they are rich sources of bioactive chemicals, biodegradable in nature, non pollutant and have no residual or phytotoxic effects. Extracts of many higher plants have been reported to exhibit *in vitro***[6,7, 8]**, and *in vivo***[9, 10, 11]**antifungal activities. Thus, this study was

undertaken to determinate the antifungal activity of ten plants extracts against *P. teres*, the causal organism of barley net blotchin an attempt to contribute to the biological control of this pathogen.

EXPERIMENTAL SECTION

Plant materials

Seedlings at two to three leaf stage of Saida183, a highly susceptible local cultivar of barley from Algeria, were used for *in vivo* antifungal assays. The barley seeds were supplied by ITGC (Technical Institute of Field Crops of Sidi-Bel-Abbes, Algeria).

Fungal isolate and culture conditions

The fungal isolate "R8" was obtained from monoconidial culture of *P. teres*, the causal organism of barley net blotch. This isolate was certified by Phytopathology Laboratory of University of Mascara (Algeria) asbeinghighly aggressive and causing severs foliar chlorosis and necrosis on barley plant.

Collection of Medicinal Plants

Ten medicinal plants species: *Anacyclus valentinus* L., *Ammoïdes verticillata* Briq, *Eucalyptus* sp, *Foeniculum vulgare* Mill, *Inula viscosa* (L.) Aiton, *Mentha pepirita* L., *Rosmarinus officinalis* L., *Salvia officinalis* L., *Tetraclinis articulate* (Vahl) Masters and *Thymus vulgaris* L. were collected during spring and summer of 2012 from various locations of the north western Algeria (Mascara) in order to select samples showing a potent antifungal activity against *P. teres*, except *Anacyclus valentinus* which is originally from Adrar (South of Algeria).

Preparation of aqueous extracts

Fresh aerial parts of the plant material were dried in the laboratory at room temperature then grounded into powder form. Fifty grams of this dried powder were decocted in 1Lof distilled water during 15 min at 100°C.After filtration through Whatman filter paper No.1,the resulting extracts were evaporated at 45^{o}C and transferred into sterile bottles and kept in refrigerator until used.

Evaluation of the Antifungal Activity of the plant extracts

In vitro antifungal assays

Screening of the plant extracts for their antifungal activity against *P. teres* was conducted using the radial growth method as described in Banso et *al*. **[6]**. Each extract tested was used at different concentrations: 100, 500, 1000 and 1500 ppm. The extracts were added each to 20 ml of PDA medium before solidification into Petri dish. Mycelial discs of 5 mm diameter were taken from the periphery of 7 days old *P. teres* cultures, and were aseptically placed in the centre of each Petri dish. Control treatment was without the extracts. The plates were incubated in alternating periods of 12 h darkness and 12 h light at 22 ^{o}C for 7 days. In this study each treatment was carried out in triplicate. The efficacy of treatments was evaluated from all the plates by measuring size of fungal colony. The percent mycelial growth inhibition with respect to the control was computed from the following formula:

$$\text{Growth inhibition (\%)} = \frac{\text{Colony diameter of (Control – Treatment)}}{\text{Colony diameter of control}} \times 100$$

In vivo antifungal assays

The aqueous extracts of five plants (*Anacyclus valentinus, Inula viscosa, Salvia officinalis, Rosmarinus officinalis* and *Tetraclinis articulata*), at a concentration of 1500 ppm were tested *in vivo* for antifungal activity against *P. teres.* The selection of the five plants extracts was based on their effectiveness against mycelial growth of the pathogen.

Plant extracts prepared as described above were mixed with sterile distilled water containing 0.01% Tween 20 to obtain the desired final concentration of 1500 ppm. In control treatments, sterilized distilled water and Tween 20 were used instead of the plant extracts.

Barley plants were grown in the plastic pots (15cm diameter) in a greenhouse at 20 ± 2^{o}C for2 weeks. For the development of net blotch, plant seedlings at the 3rd leaf stage were inoculated with *P. teres* by spraying a spore suspension of the fungus adjusted to a concentration of 2×10^{4} conidia/ml **[12,13]**. The inoculated plants were incubated in the dark for 48 h at 20°C and at 100% relative humidity. They were then transferred to a growth chamber maintained at 20± 2^{o}C and 70-80 % RH with 12 hr daylight per day. Disease severity was determined as the percentage of infected leaf area 9 days after inoculation. In

this experiment the plants extracts were tested for their preventive and curative effects against net blotch according to Gyung *et al*. **[10]**.

Protective and curative effects of the active plant extracts

To further investigate the protective activity of the plant extracts showing potent efficacy against the pathogenic fungi, aqueous suspensions of the selected plant extracts were applied protectively (1 day prior to inoculation).

For evaluating the curative activity, the plant extracts tested were applied onto the foliage of plant seedlings at a concentration of 1500 ppm, 2 days after inoculation.

Pots were arranged as a randomized complete block with three replicates per treatment. Each pot was assayed for infection extent by visual estimation of the percentage area of leaves covered by chlorotic and necrotic lesions. Three estimates for each treatment were converted into percentage of fungal control as compared to the control plants.

Effect of plant extracts on seed - borne fungi

In this experiment, the activity of the selected plant extracts at 1500 ppm on seed health of barley was examined. The extracts were used for dressing barley seeds. The seeds were dressed by wetting and shaking for 10 min in a dressing device then remained for 20 hours at ambient temperature**[14]**. The barley seeds treated with sterile and distilled water were the controls.

Detection of seed borne pathogen (*P. teres*) was carried out according to the procedures published by the International Seed Testing Association **[15]**.Two hundred seeds of susceptible barley cultivar were tested using the deep freezing method. In this method, replicates of ten seeds were plated in 9 cm diameter Petri dishes containing three layers of blotters (filter paper) soaked with sterilized tap water. The plates were incubated at 22±2 °C for 24 h, then transferred to -20 °C for 24 h. This was followed by 7 days incubation at 22±2 °C for 12 h under alternating cycles of light and darkness. For each treatment three replicates were maintained. After incubation, all fungi were purified and identified. The level of seeds' contamination was determined by the percentage presence of the fungi. The percentages of inhibition were obtained based on the comparison with the control.

RESULTS

Screening of plant extracts for *in vitro* antifungal activity

The percent inhibition is one of the elements necessary for the evaluation of the effectiveness of an extract. The results obtained *in vitro* showed that the different extracts have varying influence on *P. teres* according to their concentrations. Generally, mycelial growth decreased with increase in each of the extract concentration; with the higher aqueous extracts concentration being more effective.

Table (1) shows that all treatments had positive effect on reducing the linear mycelial growth of *P. teres*. Five (50%) out of 10 plant extracts displayed disease control activity of more than 50% against the pathogen at 1000 ppm concentration.

Results revealed that extract of *Tetraclinis articulate* at 1500 ppm 7 days post-inoculation, when the control fungi completely covered the plates, reduced about 87% from mycelial growth of the fungi. Whereas, the extracts of *Anacyclus valentinus* and *Inula viscosa* at the same concentration and time reduced 72.27and 70%, respectively, of *P. teres* growth.

At 1500 ppm concentration, aqueous extracts of *Salvia officinalis* and *Rosmarinus officinalis* were also effective, with an inhibition of 68.62 and 62.04%against the pathogen. The extracts of *Thymus vulgaris* and *Mentha pepirita* showed a moderate activity; they reduced the mycelial growth of *P. teres* of more than 50% at the higher concentration, at the other concentrations their antifungal activity was less important.

However, at all tested concentration *Eucalyptus* sp. extract reduced the mycelial growth of *P. teres* of less than 50%.

At 100 ppm concentration, the extracts of *Ammoïdes verticillata* and *Foeniculium vulgare* were not effective against the pathogen, but they had low inhibitory activity at the other concentrations. Control results showed absence of fungal growth inhibition without addition of plants extracts.

Generally, none of the aqueous extracts tested had total inhibition on the growth of *P. teres*, suggesting that the control was fungistatic against the pathogen.

Table 1. Inhibition (%) of radial growth of *Pyrenophora teres* on PDA medium with ten plants extracts added at different concentrations.

Plants extracts	Mycelial growth inhibition (%) ± SD			
	100 ppm	500 ppm	1000 ppm	1500 ppm
Ammoïdes verticillata	00 ± 00 [a]	07.15 ± 0.32	13.12 ± 1.15	26.45 ± 0.25
Anacyclus valentinus	40.32 ± 1.40	57.31 ± 0.20	64.15 ± 0.49	72.27 ± 0.36
Eucalyptus sp.	09.60 ± 0.36	12.30 ± 0.55	20.42 ± 1.25	42.03 ± 1.10
Foeniculum vulgare	00.00 ± 00	00.00 ± 00	08.12 ± 0.15	16.05 ± 1.45
Inula viscosa	35.50 ± 0.62	51.12 ± 0.15	63.16 ± 0.65	70.00 ± 0.49
Mentha pepirita	15.40 ± 0.42	22.45 ± 1.15	37.89 ± 0.50	50.04 ± 1.07
Rosmarinus officinalis	22.00 ± 0.35	39.41 ± 0.22	50.13 ± 1.03	62.04 ± 0.75
Salvia officinalis	24. 50 ± 1.26	42. 25 ± 0.35	54.03 ± 0.10	68.62 ± 1.75
Tetraclini sarticulata	51.17 ± 0.82	64.10 ± 0.50	72.42 ± 0.65	87.05± 1.20
Thymus vulgaris	18.05 ± 0.82	29.15 ± 0.12	44.33 ± 1.24	54.42 ± 0.75

[a]: *Means of three replicates± standard deviation.*

Efficacy of plants extracts on fungal disease severity

The effects of the selected extracts that had the highest antifungal effects of the 10 extracts tested on mycelial growth of *P. teres* are presented in Table 2. Data shows disease severity of net blotch on barley plants as affected by the medicinal plant extracts tested. When the five active plant extracts were evaluated for their 1-day protective activity against net blotch diseases, results showed that all plants extracts, significantly reduced disease severity compared to infected control under *in vivo* condition. The greatest reduction of diseases severity was achieved by *Tetraclinis articulate* extract (79.21%), followed by *Inula viscosa*(72.55%), and the lowest reduction was obtained when barley plant was treated with *Rosmarinus officinalis* extract.

In curative application, the plants exhibit antifungal properties ranged from 27.45 to 61.96%. When barley leaves were sprayed with *T. articulate* and *I. viscosa* extracts, disease severity of barley net blotch (*P. teres*) was reduced from 85% (control) to 32.33% and 42.33% respectively, which corresponded to 61.96% and 50.20% reduction of infected leaf area.

However, the percent inhibition of disease severity (table 2)was generally inferior to 50% for plants treated with *A. verticillata, F. vulgare* and *R. officinalis* extracts.

Typical symptoms of barley net blotch were observed on untreated control. The leaf lesions appeared as small circular and elliptical lesions that eventually developed into dark-brown blotches containing longitudinal and transverse striations forming a net-like pattern. Susceptible reactions also included the presence of chlorotic or water-soaked areas around the dark-brown, net-like necrotic lesions. Severe infections lead to the complete death of leaves with a dry appearance.

Table 2:Effectiveness of protective and curative treatments with different plant extracts against *Pyrenophora teres*.

Plant extracts	Protective treatment		Curative treatment	
	Severity(%)	Inhibition (%)	Severity (%)	Inhibition (%)
Anacyclus valentinus	28.66CD	66.28 B	48.33 BC	43.14 BC
Inula viscosa	23.33 D	72.55 AB	42.33 C	50.20 B
Rosmarinus officinalis	50.00 B	41.17 D	61.66 B	27.45 D
Salvia officinalis	40.66 BC	52.16 C	50 BC	41.17 C
Tetraclini sarticulata	17.66 D	79.21 A	32.33 CD	61.96 A
Control	85 A	00 E	85 A	00 E

Means on the same column followed by the same letter are not significantly different, using t-test at 5% level.

Antifungal activity of plant extracts against seed borne fungi

Mycological analysis carried out revealed the presence of *P. teres* in the material tested (table 3).Generally, 13 fungal species belonging to 10 genera were isolated and identified from the seed samples using the deep freezing blotter test method. These isolated fungal taxaare ranked as follows according to the total mean percentage: *Alternaria* spp. (17.33%), *Pyrenophora teres*(16%), *Cladosporium herbarum* (11.66%), *Fusarium* spp. (11.66%), *Aspergillus* sp. (10%), *Bipolariss orokiniana*(9.33%), *Penicillium* sp. (7.66%), *Stemphylium* sp. (5%), *Mucor* sp. (4.66%) and *Trichothecium roseum* (3.33%). The genus *Fusarium* included three species: *F. graminearum*, *F. culmorum*, and *F. moniliforme*. The genus *Alternaria* included two species: *Alternaria alternata*, and *Alternaria tenuis* . The other genera isolated included only one species each.

The plant extracts selected showed different levels of inhibitory effects on seed borne fungi. Among them aqueous extract of *I. viscosa* showed maximum inhibition (72.77%), followed by extracts of *T. articulata*, *A. valentinus*, *S. officinalis* and *R. officinalis*. They reduced the seeds infection by 67.59, 65.17, 59.65 and 43.78% respectively (Fig.2).

This study showed that the extract of *I. viscosa* had a wide inhibitory spectrum of activity against seed-borne fungi. It was effective against *Alternaria* spp., *Pyrenophora teres*, *Penicillium* sp., *Aspergillus* sp., *Mucor* sp and *Trichothecium roseum*; However, it had moderate antifungal activity against *Cladosporium herbarum* and *Fusarium* spp.

As shown in Fig.1, the percent inhibition of aqueous extracts was more than 50% against seed-borne *P. teres*, except for the extract of *R. officinalis*. Good results were obtained with *T. articulate* extract; seed treatment resulted in lower infection of barley seed with *P. teres* and *B. sorokiniana*, the causal agent of common root rot and foliar spot blotch diseases in barley.

Fusarium species showed high susceptibility to aqueous extract of *A. valentinus* and *S. officinalis.* However, moderate inhibitory effect was observed with the other medicinal plants' extracts tested. The susceptibility is also observed in *Aspergillus* sp. and *Penicillium* sp. with aqueous extracts of *A. valentinus*, *I. viscosa* and *T. articulata*.

The bioassay of treated seed showed also that extracts of different plant species completely inhibited the presence of *Mucor* sp. and *Trichothecium roseum* on treated barley seeds, however, these fungi were less frequently isolated (4.66 et 3.33% respectively).

Among the 13 taxa identified, the most resistant was found in *Stemphylium* sp. for aqueous extracts of *S. officinalis.* There was no positive effect of this extract against seed borne *Stemphylium*, the same frequency of the fungi occurred in both infected and treated barley seeds. The other plant extracts showed weak or moderate antifungal activity against this fungus.

Table 3.Effect of aqueous medicinal plant extracts on seed infection (%)

Fungus species	*A. valentinus*	*I. viscosa*	*R. officinalis*	*S. officinalis*	*T. articulata*	Control
***Alternaria* sp.**	4.33± 0.40*	3± 0.70	9± 0.70	5.66± 1.08	4.33± 1.08	**17.33± 1.77**
***Aspergillus* sp.**	3.33± 1.08	2.33± 0.81	5± 0.70	5.33± 1.08	4± 0.70	**10± 1.22**
Bipolariss orokiniana	4± 0.70	3.33± 1.08	5± 0.70	5± 0.70	2.66± 0.81	**9.33± 1.08**
***Cladosporium* sp.**	7.33± 1.08	5.33± 0.81	8.33± 0.40	6± 0.70	7.66± 1.08	**11.66± 1.47**
***Fusarium* sp.**	3± 0.50	4.66± 0.40	9± 0.50	3± 0.50	4± 0.50	**11.66± 1.77**
***Mucor* sp.**	0± 0.00	0± 0.00	0± 0.00	0± 0.00	0± 0.00	**4.66± 1.07**
Penicillium sp.	2.66± 0.40	2± 0.00	5.33± 1.00	4± 0.50	3.33± 1.00	**7.66± 1.50**
Pyrenophora teres	5± 1.41	3± 0.50	8.33± 0.81	7± 1.22	2.33± 0.50	**16± 0.70**
Stemphylium sp.	4± 0.50	2.66± 0.40	4.33± 0.50	3± 0.70	3± 0.50	**5± 1.22**
Trichothecium roseum	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	**3.33±1.08**
Total	33.65	26.31	54.32	38.99	31.31	**96.63**

** Means of three replicates ±SD*

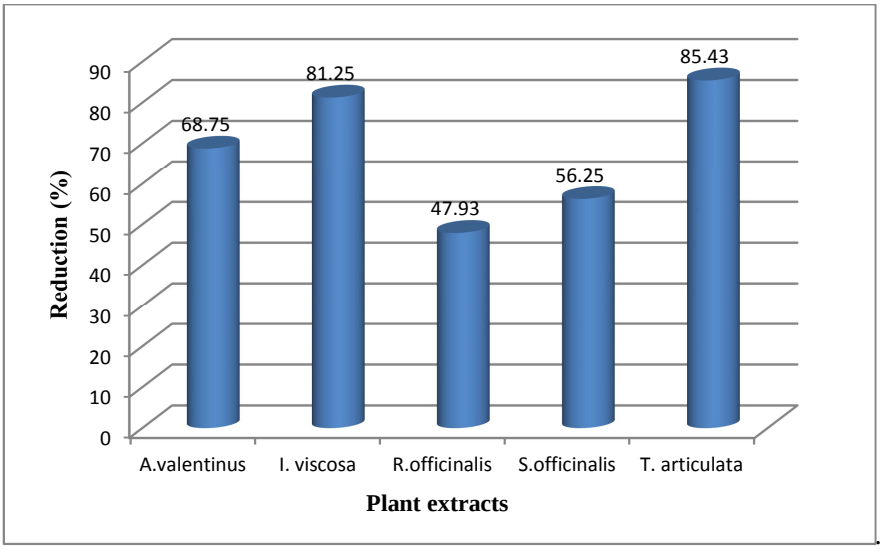

Fig.1: Reduction (%) in seed-borne *Pyrenophora teres* recorded in barley seeds treated with five plant extracts

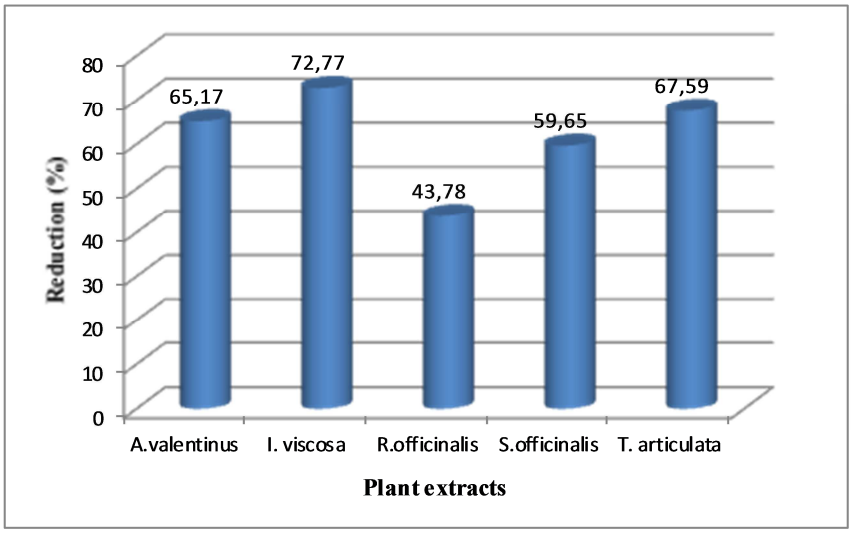

Fig. 2: Reduction(%) in barley seeds mycoflora recorded in barley seeds treated with five plant extracts

Seed infection (%) was evaluated as: (number of seeds (samples) with occurrence of fungi/total number of evaluated seeds) × 100.

DISCUSSION

In the present investigation, the aqueous extracts from ten medicinal plants were screened *in vitro* and *in vivo* for antifungal activity against an important foliar and seed borne phytopathogenic fungus. These plants were selected based on traditional medicine knowledge and on random selection from the local flora.

Data of mycelial growth inhibition recorded 7 days after inoculation at22ºCshowed that the plant extracts exhibited antifungal properties that justify their traditional use as medicinal plants. This inhibition activity may be due to the presence of active principles in the plant materials. Plants generally produce enormous amounts of secondary metabolites which constitute an important source of microbicides, pesticides and many pharmaceutical drugs **[16,17]**.

Results revealed, that the increase in the antifungal activity of the extracts was enhanced by increase in their concentration. This finding agrees with the report of Bansoet *al.***[6]**who reported that higher concentrations of antimicrobial substance were accompanied by higher growth inhibition. Highest significant effect was observed at a concentration of 1500 ppm for the ten plants extracts while the least was recorded at a concentration of 100 ppm.

The extracts differed significantly in their potential to inhibit the growth of *P. teres*, These difference can be explained by differences in the nature of the extracts, because they come from different plant species and families. The differences in the chemical composition of the extracts could also constitute an explanation.

Five of the most effective extracts were selected and evaluated *in vivo* on barley seedlings by spray inoculating 15 day-old seedlings. The preventive and curative efficacy of these extracts was assessed using a percentage disease severity 9 days after inoculation.

Data showed that all treatments significantly reduced the net blotch severity compared to the untreated control, with the greatest reduction occurring when the treatment was applied 1 day pre-inoculation. While symptoms on the leaves of control plants appeared as coherent necrotic areas, extract-treated leaves only developed smaller chlorotic spots.

The reduction of disease severity and increased symptom suggest that natural plant extract may have an important role in biologically based management strategies.

It can be concluded that the protective effect of the extracts against barley net blotch had resulted mainly from the inhibition of conidial germination, suppression of the mycelial growth of the pathogen accompanied with a slight

activation of the host defense mechanisms. Several prophylactic treatments of plants with different substances were reported to induce resistance against bacterial, viral and fungal diseases **[9]**.

Among the plant extracts tested, *T. articulate* and *A. valentinus* extracts showed a strong antifungal activity in comparison with other plants' extracts. The antifungal potential of these extracts had been demonstrated by Simoussa et *al*. **[18]** in relation to the reduction of date palm wilt (Bayoud) caused by *Fusarium oxysporum* f. sp. *albedinis.* Also Boungab et *al.***[19]** reported that *A. valentinus* extract showed a toxic activity against two phytopathogenic fungi: *P. teres* and *Bipolariss orokiniana* by reducing considerably the mycelial growth and by inhibiting the spore production.

Our results demonstrated that *I. viscosa* and *S. officinal is* extracts were also effective as compared with the control treatment. Several studies had shown that *I. viscosa* is an important source of bioactive compounds against different fungal species of medical or agronomic importance. Cafarchia et *al*. **[20]** reported that flower and leave's extracts of *I. viscosa* obtained with different solvents showed an antifungal activity against *Candida* species and dermatophytes. The aqueous extract of *I. viscosa* leaves was also effective against *Trichophyton mentagrophytes*, at 15 µg/ml, where the inhibition recorded was more than 90% **[21]**.

The leave's extract of *I. viscosa* have been found to be rich in sesquiterpene lactones named tayunine. This compound showed inhibitory activity against *Microsporumcanis* and *Tricho phytonrubrum***[22]**. In addition, another sesquiterpene lactone, the tomentosine, was isolated by Cafarchia et *al*. **[16]**from fresh *I. viscosa*flowers which exhibited an antifungal activity against *M. canis, M. gypseum* and *T. mentagrophytes.*

On the other hand, Wang et *al*. **[23]** showed that *I. viscosa* organic extracts were effective in controlling late blight (*Phytophthora infestans*)in potato and tomato, downy mildew in cucumber (*Pseudoperono sporacubensis*), powdery mildew in wheat (*Blumeria graminis* f. sp. *tritici*) , and rust in sunflower (*Puccinia heliathi*).

The antimicrobial activity of *S. officinalis* had been demonstrated by Yanar et *al*. **[7]**who reported that among 26plant extracts tested, *S. officinalis* extracts exhibited strong inhibitory effects on *Phytophthora infestans,* the causal agent of potato late blight, since it completely suppressed the mycelial growth of the fungus at 4% concentration.

In the case of *Rosmarinus officinalis*, The antifungal activity of ethanol extracts of this plant was tested against strains of *Aspergillus flavus* and *A. ochraceus*, The results showed, that the extracts used at low concentrations could have significant potential for the biological control of these fungi in foodstuffs **[24]**.

Net blotch pathogen was found to be seed-borne and seed transmitted. Therefore, the deep-freezing method was used to detect and isolate the associated seed-borne pathogens. Totally, 13 fungal genera including both saprophytic as well as pathogenic were encountered in the present study.

Seed-treatment trials with the plant extracts selected showed, at high concentrations, a significant reduction in seed-borne inocula. Among the five selected plant species, aqueous extract of *I .viscosa* proved to be the most effective in inhibiting the barley seeds mycoflora. This observation suggests that the aqueous extracts of different plants may be used as biofungicides against seed - borne fungi. However, their efficiency depended on the type of plant used, and the résistance or the susceptibility offered by fungal species.

CONCLUSION

The results obtained from the present investigation showed that the aqueous plant extracts tested exhibit antifungal effect on the test organism. Extracts of the plant used in this study could be exploited as an alternative treatment for future plant disease management and might contribute to the development of environmentally safer alternatives to protect plants from pathogenic fungi. However, further phytochemical researches are needed to identify the active principles responsible for the antifungal effects of each plant and to make this a practical option to be used by farmers.

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