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First Report of in Vitro Biological Agent to Biocontrol of Date Palm Stipe Rot Affected by *Fusarium brachygibbosum* by Using *Pergularia tomentosa* L., Aqueous Extract

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ABSTRACT

Palm (*Phoenix dactylifera* L.) is a specie cultivated in Mauritania. The present study is focused on the potential of the aqueous extract of the *Pergularia tomentosa* L., as biological control agent against *Fusarium brachygibbosum,* the main agent of the date palm stipe rot disease in Mauritania. Fungal pathogen was isolated from symptomatic date palm stipe rot disease in Mauritania. The morphological characterization and the molecular identification by sequencing ITS1, ITS2 5.8 RNAr region showed homology of 98% with *Fusarium brachygibbosum* strain UOA/HCPF 16982 s during *in vitro* tests on leaves performed to verify its phytopathogenicity. The inhibitory effect of aqueous extract of *Pergularia tomentosa* L. on the phytopathogenic isolate (*Fusarium brachygibbosum*) of date palm, on mycelial growth and spore germination was observed. In fact, in vitro on PDA, mycelial growth ranged from 39.23 to 67.7%, depending on the concentration of the aqueous extract of *P. tomentosa*. The aqueous extract showed a reduction of spore germination varying from 5.3 to 23.8%. The minimum inhibitory concentrations varied from 1 to 100 mg/ml. The various extracts give high antioxidant activities exhibited by DPPH assay. Multivariant analysis by PCA plot and the heatmap were done, to evaluate the correlation between the tested parameters. These results suggest the use of *Pergularia tomentosa* L., extract as a biological agent to control and reduce damage caused by *Fusarium brachygibbosum.*

Keywords: *Pergularia tomentosa* L., antifungal activity, antioxidant, extract, *Fusarium brachygibbosum*; stipe rot

INTRODUCTION

Phoeniculture covers around 1.1 million hectares, with a total production of 8,500,000 tones, distributed mainly between Asia 56%and Africa 43% [Rabaaoui et al., 2021]. As a result of this policy, tens of hectares of date palms have been planted and large modernization projects have been initiated. Bayoud disease, (derived from the Arabic abiadh "white"), is a real scourge in the fruit-growing areas of parts of North Africa and a threat to all countries that are free of it [Sedra and Lazrek, 2011].

Despite the date palm's great resistance to abiotic factors, palm crops are threatened by Bayoud disease, a fungal disease characterized by chlorosis of the affected leaves, giving them a whitish color [Zaid et al., 2002]. Bayoud disease

is a disastrous disease for date palms; the most pathogenic fungi belong to the *Fusarium* genus [Boussalah et al., 2013]. Several studies have identified that this disease is caused by *Fusarium oxysporum f. sp. albedinis*, *Thielaviopsis paradoxa* and *Fusarium brachygibbosum* [Dihazi et al., 2012a].

Date palm stipe rot, caused by *Fusarium brachygibbosum*, is the cryptogamic disease of date palms [EFSA Panel on Plant Health (PLH) et al., 2021].

Since its appearance during the past years, the disease has been rampant in traditional Mauritanian oases, causing considerable economic losses and genetic erosion that threatens the fragile balance of the oasis ecosystem. Efforts to combat the epidemic have focused on chemical control [Alblooshi et al., 2021].

Since 1995, a high prevalence has been observed in Adrar, in northern Mauritania. Control of this disease is mainly based on the use of chemicals such as carbendazim [Chanda et al., 2009]. Control using environmentally friendly approaches is very limited [Dihazi et al., 2012b]. Thus, biological control methods present a promising alternative [Fitrianingsih et al., 2015]. They are based on the use of plant extracts and certain antagonistic micro-organisms. The effective effect of chitinase, produced by *Bacillus licheniformis*, has been observed in Fusarium head blight caused by *Fusarium pseudograminearum* [Essghaier et al., 2021a].

Several methods have demonstrated the ability of certain plant extracts to inhibit pathogen development in vitro, in soil, and in vivo [Saeed et al., 2016].

The plant extract obtained must confer protection against phytopathogens [Mezouari et al., 2019]. It is therefore imperative to find new molecules that are effective, active, and less toxic [Abdel-Monaim et al., 2011].

Plants are the main source of pharmacotherapy in traditional medicine. The toxic, therapeutic, and antimicrobial properties of several plants have been well demonstrated [Subba and Rai, 2018]. Northern Mauritania, with its rich floral resources and ethnobotanical history, is an ideal location for screening plants with high biological activity [Yebouk et al., 2020]. *Pergularia tomentosa* L. is one such plant, rich in antifungal compounds and traditionally used in Mauritania for the treatment of various types of dermatological diseases, especially fungal.

The present study is the first to exploit these proprieties described in *Pergularia tomentosa* L., by evaluating the antifungal activity of the aqueous extract of the whole plant against Fusarium brachygibbosum, the fungus responsible for date palm stipe rot.

MATERIALS AND METHODS

Plant material and preparation of aqueous extract

The plant *Pergularia tomentosa* L., (Fig. 1) was harvested in 2019 from the Inchiri region (X:22.153805, y: -10.783081,9). Harvested plants are rapidly transported to the Plant Physiology and Biotechnology laboratory. They are then laid out on the bench in the open air at a temperature of 25°C. Once dried, the whole plant is reduced to powder form. The aqueous extract is obtained by adding 50 grams of the plant drug to 300 ml liter of distilled water.

The mixture, heated for two different times: 1 hour and 24 hours, is filtered on a double layer of filter paper. The filtrate obtained was evaporated on a rotary evaporator. Four filtrates were used: two from the aerial part, heated for 1h and 24h, and two from the root, heated for 1h and 24h. Four concentrations were tested: (0.1 mg/mL, 1 mg/mL, 10 mg/mL, and 100 mg/mL).

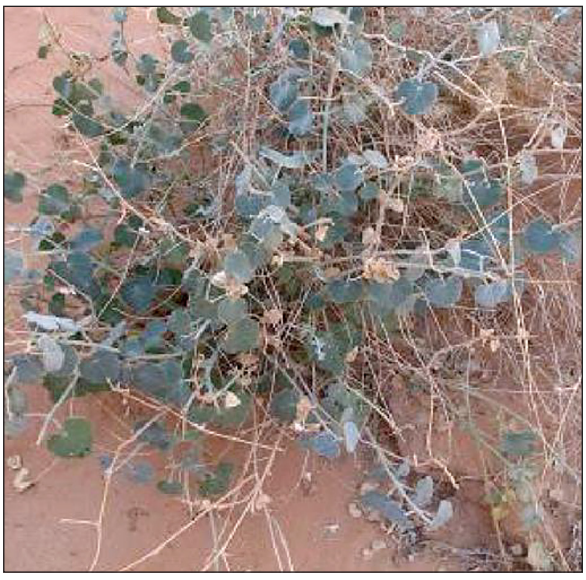

Figure 1. *Pergularia tomentosa* L. from the region of Inchiri

Isolation and identification of fungi

Fusarium brachygibbosum was isolated from infected roots and palms. 10-fold serial dilutions were made from 1 g of each sample diluted in 9 ml sterile saline solution (0.9% NaCl, Carlos Erba Reagents Srl, Barcelona, Spain). Different dilutions were made (10^{-1}–10^{-6}) and 0.1 ml of each dilution was spread on Petri dishes containing the appropriate medium. 50 µg/L streptomycin and tetracycline were added to malt agar extract (MEA, VWR-Prolabo Chemicals, Barcelona, Spain) to inhibit bacterial contamination [Robledo-Mahón et al., 2020]. Three replicates were performed. Plates were incubated for 96 hours at 28°C. Macroscopic and microscopic identification was confirmed by analysis of the internal transcribed spacer (ITS1-ITS2) regions of rRNA 5.8. The resulting sequence was analyzed by BLAST. The phylogenetic tree was constructed using the rDNA method. To verify their pathogenicity, the 7-day fungal growth disc in PDA of the isolate was inoculated onto the leaf surface of *Nicotiana tabacum*.

Evolutionary relationships of taxa

Evolutionary history, the phylogenetic tree, and evolutionary distances were studied following the methods described by Saitou [Naruya Saitou, 1987; Tamura et al., 2004]. The analysis focused on 12 nucleotide sequences, deleting all ambiguous positions for each pair of sequences (one deletion option per pair). Evolutionary analyses were performed with MEGA11 [Tamura et al., 2021].

Preparation of pathogenic fungal suspension

Fungal spores, collected from the surface of a 7–10-day-old *Fusarium brachygibbosum* fungal culture in the presence of 15 ml sterile water, are filtered to remove mycelium and agar debris. The concentration of the pathogenic spore solution is assessed by adjusting it to 10^5 spores/ml using the Malassez cell.

Well agar diffusion method

Petri dishes containing PDA medium were inoculated with 0.1 ml of the spore suspension adjusted to 10^5 spores/ml. Then, wells drilled on the agar surface were filled with 50 µl of each extract dilution (0.1 mg/ml, 1 mg/ml, 10 mg/ml and 100 mg/ml) individually per well. Three independent replicates were performed for each test [Essghaier et al., 2021b]. Distilled water was used as a negative control. Inhibitory action was indicated by the appearance of a halo zone around the well and measured in mm.

Plate growth inhibition assay

In a 10 cm diameter Petri dish, we mixed 10 ml of PDA melted at 45°C with a 0.1 ml volume of the extract tested. After solidification at room temperature, the dishes were inoculated with a 6 mm diameter disc of the pathogen in the center of the dishes. Incubation took place at 28°C for 5 days. Plates inoculated only with the pathogen were used as a control [Zulkifli et al., 2014]. Linear growth of the fungus was monitored, and the rate of inhibition of fungal growth was calculated according to the following formula:

$$PI = (C - CT)/C \times 100\%$$

where: PI – percentage of inhibition (%),
CT – growth of the fungus in the presence of the extract (mm),
C – control corresponds to the growth of the fungus without the addition of the extract (mm).

Rapilly's (1968) method, modified by Howell, was used to estimate mycelial growth [Chihat et al., 2021]. It involves measuring the daily linear mycelial growth of colonies up to day 7, according to the following formula:

$$L = (D - d)/2$$

where: L – mycelial growth (mm), D – diameter of the colony (mm), d – diameter of the explant (mm).

Antibiofilm activity

The anti-biofilm effect of the aqueous extract was assessed using flat-bottomed microliter plates as described by [Woo et al., 2017]. 200 µl of 10^6 cells/ml PBS were added to the wells and incubated at 37°C for 24h. Subsequently, non-adherent cells were removed by washing with PBS and 20 µl of each extract dilution was added and incubated at 37°C for a further 24h. The negative control consisted of 200 µl of PBS. To the biofilm biomass, 20 µl of Crystal violet (0.1% (w/v) were added. After

15 min, unbound dye was removed by three washes with sterile water. The dissolution step was performed by 200 µl ethanol for 15 min and observed directly at 570 nm optical density with BioTeK [Gulati et al., 2018].

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The determination of MIC values of each aqueous plant extracts was calculated according to the liquid dilution method [Omidbeygi et al., 2007]. The plant extract was diluted from 100 to 1 mg/ml in distilled water. Eppendorf tubes containing 1ml of culture medium was used and inoculated with 100 µl of the spore suspension of *F. brachygibbosum* (10^5 spores/ml). The negative control was inoculated with the fungal pathogen suspension without the addition of the extract. The test tubes, thus prepared, were incubated for 24 hours at 28 °C for the fungal growth thus, we determined the MIC defined as the lowest concentration showing growth inhibition compared to the negative control. After revealing the MIC values, the determination of the MFC (minimum fungicide concentration) was calculated, which corresponds to the minimum concentration, after subculture on solid medium (PDA), inducing the absence of visible fungal growth.

Free radical scavenging activity (DPPH) assay

The DPPH (2, 2-diphenyl-1-picryl-hydrazyl) free radical scavenging activity of each aqueous extract was performed by the DPPH reduction of (1,1-diphenyl—picrylhydrazyl), using the spectrometric method described by (Dridi et al., 2021). The absorbance was measured at 517 nm and scavenging activity was calculated as the percentage of inhibition (PI) at the tested concentrations at mg/ml. The IC50 was calculated as the concentration able to reduce 50% of tested radical [Sellam et al., 2015].

Statistical analysis

Values are presented as mean ± Standard Error Means. Comparison of means (n=3) was carried out using Student Newman-Keuls SNK tests at the 5% threshold. The same letter indicates that the difference is not significant.

To determine the adequate plant extract a multivariante analysis was performed using all tested extracts and measured parameters. For this, the freely available Heatmapper web server (http://www.heatmapper.ca) was used to generate a heat map to interactively visualize the antioxidant and antifungal activities of the tested plant extracts [Babicki et al., 2016]. In addition, the principal component (PCA) was conducted using the same data to explore the variation of measured parameters using plant extracts; this analysis was performed by using XLSTAT software (www.xlstat.com).

RESULTS

Fungal isolation and identification

The fungal isolate was identified by sequencing the rDNA-ITS marker. The blast analysis shows homology of 98% with *Fusarium brachygibbosum* strain UOA/HCPF 16982 s (Figure 2). Moreover, the *in vitro* test on leaves was done to verify its phytopathogenicity (Figure 3).

Antifungal potential by agar well diffusion method

The aqueous extracts of the plant *Pergularia* showed inhibitory effect on *Fusarium brachygibbosum* growth at 10 mg/ml. The maximum inhibition fungal growth diameter of 13.5 mm was presented by the extract obtained from the aerial parts after one hour (PA1H) (Figure 4a). As for the extracts of the underground parts, they showed activity at 1 mg/ml and 10 mg/ml after 24 h of extraction (PR24H).

Plate growth inhibition assay

The results of this test are shown in Figure 5. The obtained data indicates that the aerial part (PA1H) was inactive, while the root part (R1H) had a medium antifungal activity by applying 10 mg/ml. On the other hand, the strongest anti-*Fusarium* activities were presented by the extracts PA24H and R24H at concentrations of 10 mg/ml and 1 mg/ml, respectively. These activities were directed towards the reduction of radial growth of *Fusarium brachygibbosum*.

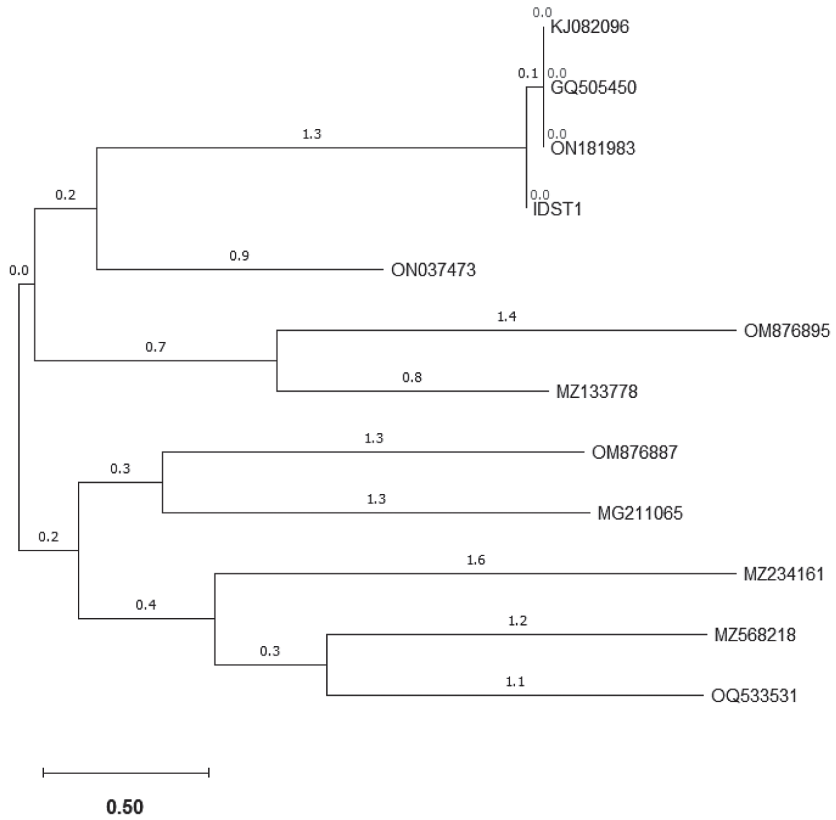

Figure 2. Neighbor joining tree showing phylogenetic relation of strain ST1 with others *Fusarium* strains, with accession number: MZ133778, MG211065, ON037473, MZ234161, OQ533531, HG327933, MZ568218, OM876895, OM876895, OM876895, OM876887, GQ505450, KJ082096, ON181983. Evolutionary analyses were conducted in MEGA11

Determination of the minimum inhibitory concentration MIC and MFC

Minimum Inhibitory Concentration (MIC) is the lowest dilution of the extract, resulting in an observable inhibition of the fungal growth and is represented in Table 1.

The rate of inhibition of mycelial growth of *Fusarium brachygibbosum* by the aqueous extracts of *Pergularia* by applying the agar well diffusion method was reported as about of 0.01 mg/ml for the aerial part (PA24H) extract. In general, PA24H was the most potent anti-*Fusarium* agent, followed by R24H (Table 1). MFC values ranging from 100 to 10 mg/ml for all tested aqueous extracts.

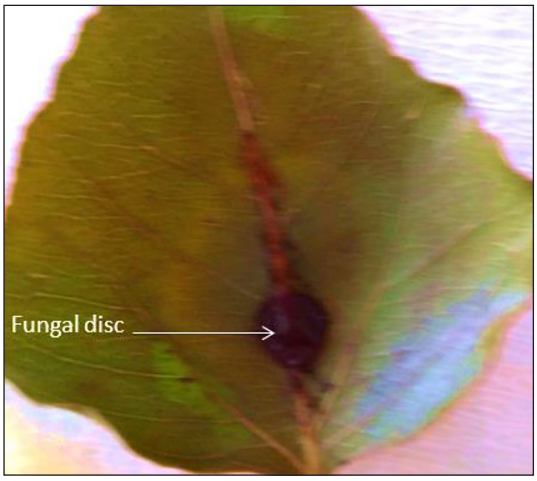

Figure 3. Phytopathogenicity test *in vitro*, on healthy leaf of the fungal isolate belonging to *Fusarium brachygibbosum* strain ST1. A fungal disc obtained from fungal growth on PDA after 7 days at 37 °C

Antibiofilm activity

The effect of *Pergularia tomentosa* extracts on biofilm development reported in figure 6 shows that both extracts, after 24 h, were more active against biofilm adherence of the tested *F. brachygibbosum*. The extract PA24H was the most active with 23.8%, followed by R24H extract with an inhibition percentage of about 21.85%. PA1H and give less antibiofilm activity don't exceed 6.0%. (Figure 6).

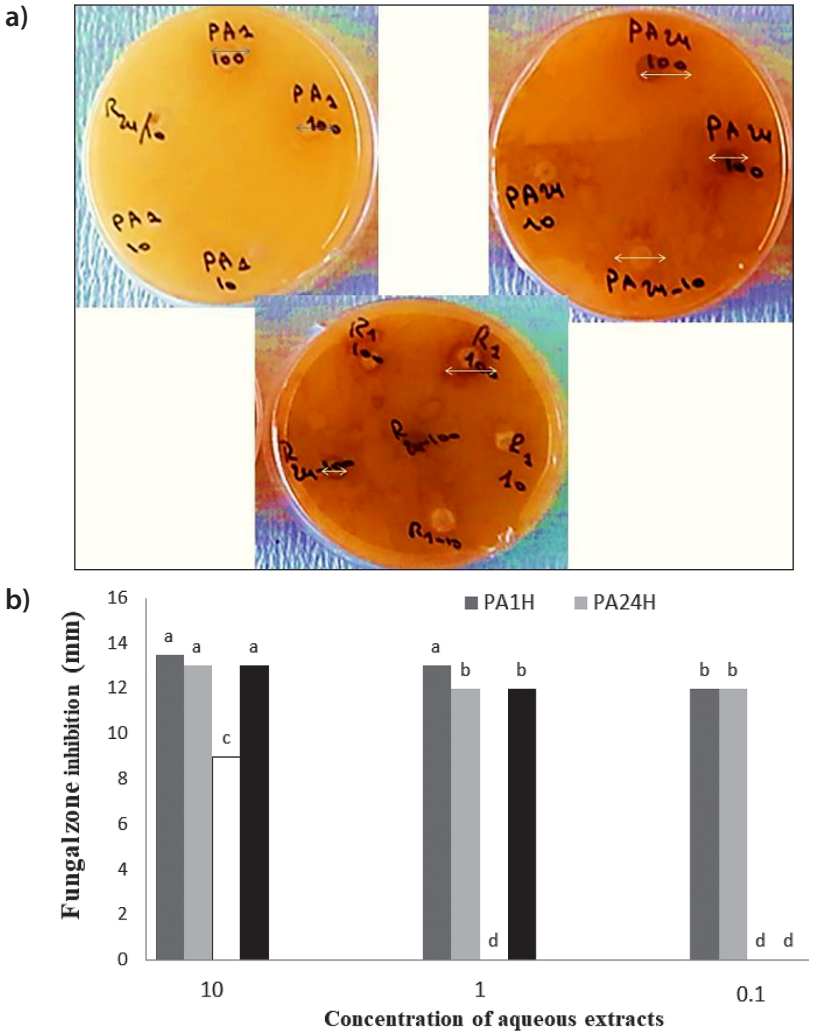

Figure 4. (a) Results of agar well diffusion methods showing the antifungal activities of the aqueous extracts of *Pergularia tomentosa* L.*,* against the mycelial growth of *Fusarium brachygibbosum.* The tested extracts, arrow indicate the observed zone inhibition around the wells containing PA1, PA24H, R1H and R24H extracts, separately; (b) Influence of different concentrations of aqueous extracts on the growth of mycelium of *Fusarium brachygibbosum*. PA1H: areal part extracted after one hour, PA24H: areal part extracted after 24 hours: Values with same letters, indicate that the difference is not statistically significant at p<0.05%.

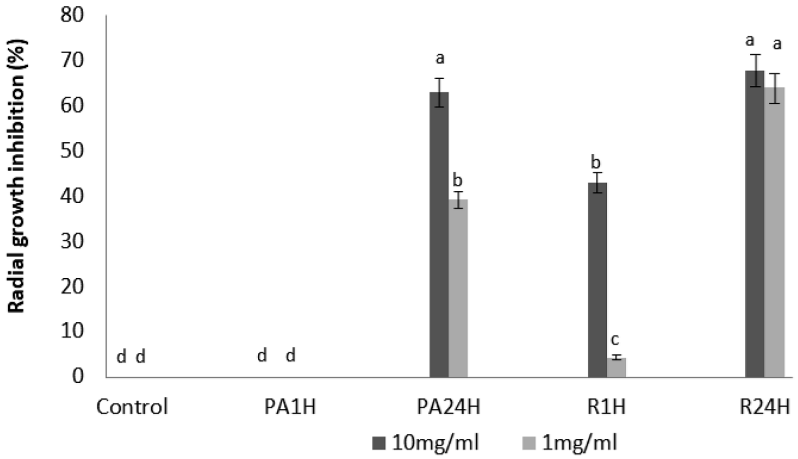

Figure 5. Radial growth inhibition of *Fusarium brachygibbosum* by *Pergularia tomentosa* L aqueous extract. PA1H: areal part extracted after one hour, PA24H: areal part extracted after 24 hours. R1H: Root part, and R24H: root part extracted after 24 hours: Values with same letter indicate that the difference is not statistically significant at p<0.05%. Error bars indicate standard errors

Table 1. MIC and MFC of the aqueous extracts expressed in mg/ml

Extracts	MIC	MFC
PA1H	0.01	10
PA24H	0.01	10
R1H	10	100
R24H	1	10

Table 2. Antioxidant activity of the aqueous extracts of *P. tomentosa*

Extracts	DPPH (IC50)
PA1H	14.306
PA24H	11
R1H	13.05
R24H	11

Table 3. Percentage of variation and parameters correlations of the two first axes of principal component analysis

Parameters	Axis 1	Axis 2
Percentage of variation	72.14	26.22
Parameters correlations		
Agar well diffusion method	**0.765**	0.181
Radial growth inhibition	**0.965**	0.027
MIC	**0.953**	0.045
MFC	**0.960**	0.026
Antibiofilm activity	**0.503**	0.494
Antioxydant activity	0.199	**0.801**

Antioxidant activity of the *Pergularia tomentosa* L. extracts

The maximum antioxidant activity was observed by PA24 H and R24H with IC50 of 11 mg/ml compared to 13.05 and 14.306 for R1H and PA1H, respectively (Table 2).

Correlation between parameters and determination of the suitable *Pergularia* extract as antifungal agent

The heat map genrated based on measured parameters (Table 3), of the tested *Pergularia* extracts showed high MIC and radial growth inhibition values of root part extracted during 1h (RP1H). The antibiofilm activity was obtained using aerial part extracted during 24h (AP24H) (Figure 7).

The PCA showed that the two first components axis 1 and axis 1 explained 98.36% of total variance. The axis 1 explained 72.14% of variance and was correlated with radial growth inhibition, MIC and MFC values.

The axis 2 explained 26.22% of total variation and was correlated with the antioxidant activity. The PCA plot of measured paramaters and plant extracts confirmed the heatmap analysis (Figure 8). It showed that the root part extracts obtained through 1 h (RP1H) was distinguishable with high radial growth inhibition, MIC and MFC values which were correlated with the axis 1 explaining 72.14% of total variation. The extracts obtained from areal and root parts after 24h (AP24H and RP24H) of extraction were distinguishable by the antibiofilm activity.

DISCUSSION

The antifungal activity of the *Pergularia tomentosa* L aqueous extract by photochemical screening was evaluated in this study based on the well disk diffusion method as reported [Riahi et al., 2013]. The obtained results show that the

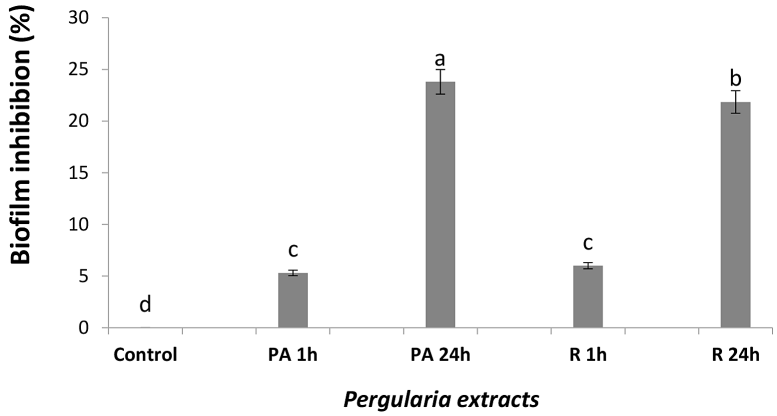

Figure 6. Antibiofilm activity of *Pergularia tomentosa* L., extracts against biofilm formation of *Fusarium brachygibbosum* strain ST. Values with same letter indicate that the difference is not statistically significant at p<0.05%

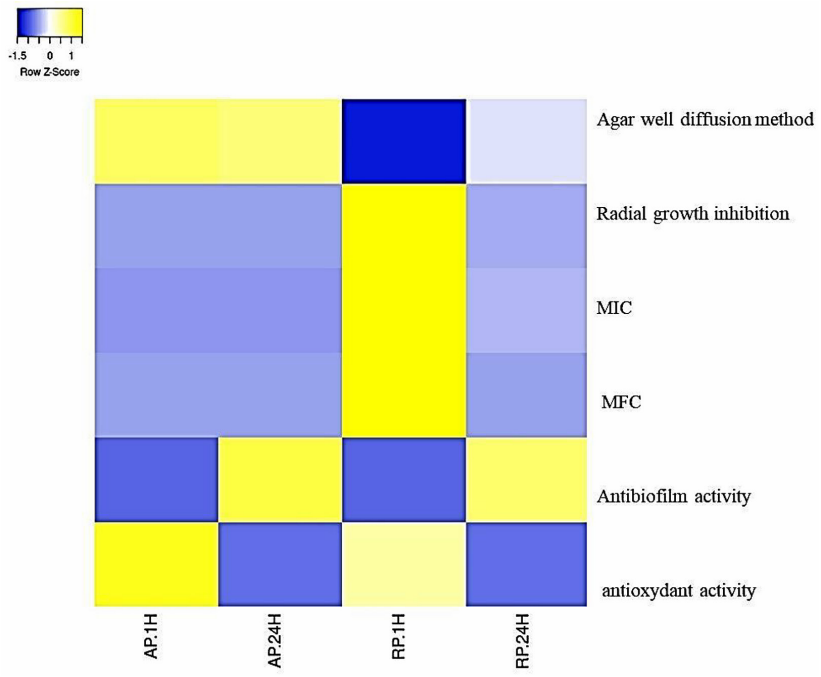

Figure 7. Heatmap plot of measured parameters according to, the tested extracts

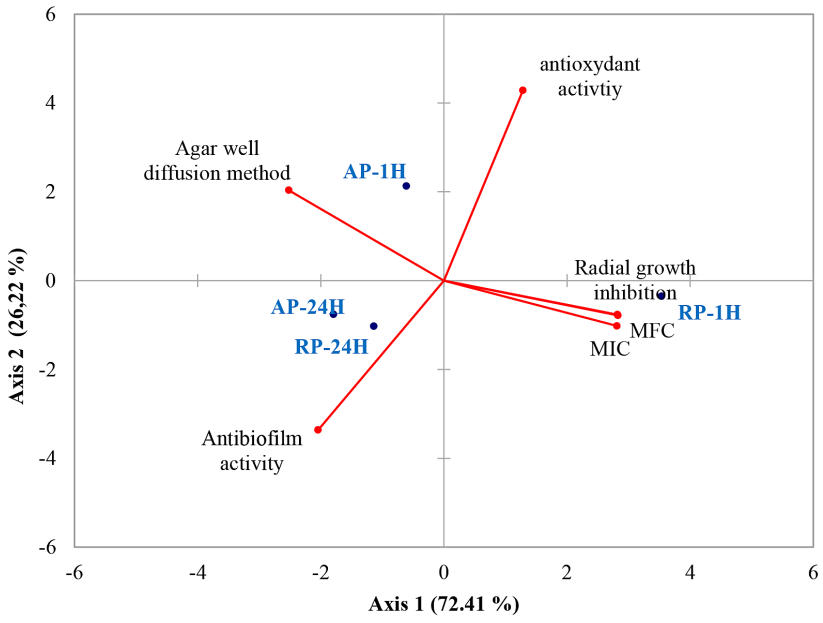

Figure 8. Principal component plot of *Pergularia* extracts and parameters

aqueous extract of *P. tomentosa* was found to be efficient against the fungal strain used in this work at various doses (10 mg/ml, 1 mg/ml, and 0.1 mg/ml), with a diameter of inhibition zone r from 12 to 13.5 mm. This finding is in agreement with the results found by Alghanem and El-Amier [2018], by using the methanolic extract obtained from the same plant. According to Rayyan et al. [2018], no inhibitory activity was obtained at 12.5 mg/ml against *Escherichia coli* strain, but a positive effect was observed at 25 mg/ml.

Showing an inhibition zone of 18 mm, the inhibition diameter depends on divers factors, for example: the facility of diffusion of components in the extracts, into the surface of the agar medium; the effect of antimicrobial agent was strictly related to the disseminated substances; the characteristic of the microorganisms (growth and metabolism) [Esmaeili et al., 2022]. Besides, depending on the date of harvest, it will have very important variations in the chemical composition and activity [Athamena et al., 2010]. From

these results it is concluded that the classes of polyphenols can improve the poisoning effect of the plant extracts towards the pathogens [Maitlo et al., 2013], terpenes and steroids and phenolic compounds detected are two classes of secondary metabolites that constitute chemical control agents against pathogens such as bacteria and fungi [Benabbes et al., 2015]. In the current work, the aqueous extract of the plant species *Pergularia. tomentosa* possess high antioxidant activities and the value of IC50 ranging from 11 to 14.30 mg/ml, which translates into medium antiradical potential compared to those of the standard antioxidants tested. The value of this IC50 is related to the synthesis of the induced secondary metabolites [Salah, 2015]. Among the most active in the scavenging of the free radicals in the case of the extraction with the solvents owing moderate or hydrophilic properties, we noted both phenolic acids and flavonoids [Bahriz and Bouras, 2020]. In general, medicinal plants used in traditional pharmacopeia have antioxidant properties [Xu et al., 2012]. The research of Alghanem et al. [2017], suggest that the methanolic extract of *P. tomentosa* harvested in Egypt has an IC50 of about 2.86 mg/ml; this value is relatively low compared to our result. It can be suggested that the observed antioxidant power is due to the difference in the chemical constitution of this plant, which itself varies according to the conditions of the biotope as well as the geographical region. in fact the wealth of various biomolecules in the vegetal depends on several factors, among them the factor of geographical region, which, associated with climatic conditions such as temperature and altitude, influences the chemical composition of active constituents and antioxidant capacity of plants [Khadri et al., 2010]. The antioxidant potential showed by our ethanolic extract depends on species and organs of the same species and would be directly related to their different composition in polyphenolic compounds, as shown by the results of [Rayyan et al., 2018]. For example, when we used the extraction from stem, leaves, roots of the specie *P. tomentosa* by the ethanolic solvent, we obtained a value of IC50 about of 0.63 mg/ml, which is very much lower than that of our sample. This data is due to the chemical solvent used, as compared to distilled water used in our work, in fact in the current study, we develop a natural process of biological control by using safe extraction solvent; water distilled to avoid any chemical residues by using other chemical solvent such as methanol, hexane. Our results by DPPH method also indicate that our extract has powerful antioxidant properties compared to those found by Amit et al. [2014] on the same plant, in the order of 9.810.06 mg/ml. This result may be accounted not only just to the influence of the concentration of polyphenols on the antioxidant potential of the plant extracts [Sahouli et al., 2020].

It is well known that biofilm is more resistant to antimicrobial agents than microbial organisms. Biofilm related infections threaten human life, food safety and are considered the main cause of reduced economic productivity in industrial food [Selaimia et al., 2021].

P. tomentosa L., is known as an antimicrobial agent against a large number of bacterial and fungal species. However, few adequate studies report the potential effect of the *P. tomentosa* extracts on the microbial biofilm formation. This study, was the first that examined the action of the aqueous extracts of *P. tomentosa* L., on growth and biofilm of *Fusarium brachygibbosum*.

Both the root and aerial part extracts of 24 h showed an inhibitory effect varying between 23.8% and 21.8%, while the aqueous extract during 1 h of the aerial and root part did not exceed 6.0%.

According to the literature, several studies have shown the anti-biofilm potential of the polyphenols extracted from the medicinal plants [Riihinen et al., 2014]. Wang et al. [2019] described that the caffeic acid and chlorogenic acid among the phenolic acids, have significant inhibitory activity on biofilms. The antimicrobial and anti-biofilm activity of *P. tomentosa* L. extracts may be due to phenolic compounds or their synergy. Our data indicate that the extracts of *P. tomentosa* L., showed good efficacy in the inhibition of *F. branchygibbosum.*

The multivariant analysis by PCA and heatmap indicateS the presence of significant correlation between all tested parameters. The high correlation was obtained with radial growth inhibition, MIC and MFC values based on PCA analysis. In addition to the performance of both extracts (AP24H and RP24H) as antibiofilm agents.

CONCLUSIONS

The current study show that the used extract shows extraordinary antioxidant activity, with an IC50 ranging from 11 and 14.60 mg/ml. This finding may be largely correlated with the

composition of the extract and its richness in phenolic compounds, in particular flavonoids.

Antimicrobial activity was determined on a fungal strain (*F. brachygobbosum)* based on the standard diffusion method. The studied aqueous extract has antimicrobial activity against the tested *F. brachygobbosum*. These finding confirm that *Pergularia tomentosa* is a medicinal plant of not negligible importance in medicine but also could be used to prevent phytopathogen fungal diseases as biological control of *Fusarium* plant diseases. It would therefore be judicious in the future to identify the phytochemical substances in order to enhance its biological application.

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