Original Research Article

In-Vitro Evaluation of Antagonists and Fungicides in Controlling Citrus Gummosis Caused by *Phytophthora*, *Phytopythium* and *Pythium* species in Tunisia

ABSTRACT

Aims: This study investigated the efficiency of some bioagents and fungicides against causals agents of gummosis of citrus.
Study design: Antifungal activities of bioagents and effect of fungicides.
Place and Duration of Study: Department of Biological Sciences and Plant Protection, Higher Institute of Agronomy of Chott Mariem, 4042, Sousse, Tunisia, between June 2015 and July 2015.
Methodology: Antifungal activity was carried out using dual culture and sandwich plate methods. Antibacterial activity was carried out using bacterial suspension; while fungicide effect was carried out using food poisoned technique. The mycoparasitim of fungal antagonists was also determined.
Results: The tested bioagents and fungicides showed significantly effect on mycelial growth inhibition of the pathogens compared to the control. In the dual culture assay, the highest percent of the mycelia growth inhibition (90%) was obtained by *T. viride* and *T. harzianum.* However, *P. digitatum* and *P. italicum* revealed to be the least effective with, respectively, 70% and 60% of growth inhibition. The effect of volatile substances of these bioagents showed also that *T. viride* exhibited the highest percent of inhibition (85%), followed by *T. harzianum* (75%), *P. digitatum* (60%) and *P. italicum* (50%). The mycoparasitim was also noted. *Bacillus* isolates were found significantly effective in reducing the mycelial growth of the *Oomycota* species, with a percent of mycelium inhibition verging from 95% to 54%. However, copper fungicides generated the highest percent of the mycelial growth inhibition (100%), following by the acid citric and borax fungicides which recorded a percent of the mycelial growth inhibition of 10.82% and 12.93%, respectively.
Conclusion: Thus the study indicated that the integration of bioagents can provide a better management of the gummosis of citrus.

Keywords: Antagonists; biological control; mycelial growth; Phytophthora spp.; *Phytopythium* spp.; *Pythium* spp.

1. INTRODUCTION

Citrus is one of the most important tropical fruit crops of the world [1]. Citrus fruits occupied the third place after apple and banana and accounts for the production of about 100 million ton ~~T~~ with massive area of cultivation spread over 7.2 million hectares [2]. In Tunisia, citrus production is also important. However, this culture is susceptible to a large number of diseases due to fungi and virus, causing serious losses to the yield. Gummosis becomes one of the most destructive diseases to citrus production in Tunisia.

To control this disease copper fungicides are largely used~~.~~, ~~However~~however, this metal is heavy and could be accumulated in the soil. Besides environmental concerns, the repeated applications of these fungicides could lead to a resistance by pathogens [3]. Thus, the reduction in the use of chemicals products against plant pathogens became an inevitable task due to their severe risks on humans, animals, plants and environment [4].

Therefore, alternative treatments for control of gummosis are needed. Biological control offers the chance to improve crop production within the existing resources, besides avoiding the problem of pesticide resistance [5].The development of formulation and delivery systems for biocontrol by using antagonistic microorganisms to suppress soil borne pathogens is of great importance [6]. Thus, promising strategies of managing plant pathogens include microbial antagonists such as fungi, and bacteria have been used [4].

Sivan [7] showed that *Bacillus* spp. gave a highly antagonistic effect against some pathogenic fungi. Whipps and Lumsden [8] and McLean et al. [9] demonstrated that *Trichoderma* spp. are effective biological control agents of plant diseases caused by soil-borne fungi.

This disease management, as mediated by biocontrol agents, is the consequence of the interactions between the plant, pathogens and the microbial community [10].

Moreover, Anitha and Dass [11] reported that the soil-borne plant pathogens, affecting agricultural plants could be controlled by using *Trichoderma* species and *Bacillus subtilis.*

Thus, the objectives of the present research were to study the efficiency of some native antagonistic bioagents and some alternative fungicides against *Phytophthora*, *Phytopythium* and *Pythium* species causals agents of citrus gummosis in Tunisia.

2. MATERIAL AND METHODS

2.1. Pathogens used

Two species of *Phytophthora* (*P. nicotianae*, *P. cryptogea*), three species *of Pythium* (*P. dissotocum*, *P. aphanidermatum*, *and P. ultimum)* and 2 species *of Phytopythium (P. mercuriale, P. vexans)* were used during the assays *in-vitro*. These isolates had been obtained from infected fields of citrus by gummosis in Tunisia (Table1).

Table1. Characteristics of the pathogens used for the biocontrol assays.

Species	Reference code	Date of collection	Origin	Source
Phytophthora nicotianae	P.119	27/09/2013	Slimen	Soil
Phytophthorara cryptogea	P.101	04/09/2013	Hawaria	Soil
Phytopythium vexans	P.25	22/02/2012	Bnikhaled	Trunk
Pythium dissotocum	P.61	01/06/2012	Bouargoub	Soil
Phytopythium mercuriale	P.121	29/09/2013	Takelsa	Trunk
Pythium aphanidermatum	P.38	28/05/2012	Bouargoub	Trunk
Pythium ultimum	P.30	31/05/2012	Takilsa	Soil

2.2. *In-vitro* assays of bioagents

Four isolates of antagonists fungi (*Trichoderma viride*, *T. harzianum, Penicillium italicum* and *P. digitatum)* and 16 isolates of Bacteria (*Bacillus* spp.) has been used during the assays *in-vitro*. The fungal antagonist was taken from the fungal collection of the Phytopathological laboratory, I.S.A of Chott Mariem, Sousse, Tunisia. Bacterial antagonists were kindly provided by Pr. Slim Tounsi, from the Biotechnology Center of Sfax, Tunisia. Fungal antagonist cultures were grown in Petri plates containing PDA medium (potato dextrose agar) for 5 days and the bacteria antagonist were grown in the medium NA (nutrient agar) for 2 days, before used.

2.2.1. Effect of fungal antagonist on the mycelial growth inhibition of pathogens

The dual culture method was used to evaluate the antagonistic effects of the fungi. Plug of 5-mm of diameter of pathogen and a similar size of plug of antagonist fungi, of 5-day-old-cultures, were placed at the periphery of Petri plate (–90 mm), amended with PDA medium, with a 30 mm of distance between the two plugs (Fig. 1.a). For the control, just a plug of pathogen was placed in the Petri plate. The plates were incubated at 25°C in the dark. There were four replicates for each pathogen–antagonist combination and the test was repeated twice. The ability of the fungi either to inhibit or colonize pathogen growth was taken as hyper parasitism. Linear growth of the tested pathogen was recorded when the control treatments covered the plate surface. Antagonist reaction on mycelia of the pathogens such as penetration and twisting was observed under Leica microscope. These observations were made, after seven days of inoculation of the dual cultures of each association pathogen/antagonist. A mycelium were taken from the interaction zones, of each dual culture, and fixed into slides with methylene blue. Then, slides were putted directly under light microscope.

2.2.2. Effect of volatile compounds of antagonist fungi on the radial growth of tested pathogens

The effect of volatiles compounds produced by the selected four antagonist fungi against the growth of *Oomycota* was studied using a sandwich plate technique [12]. Plugs of 5-mm diameter, of 5-days-old cultures, of pathogen and antagonist isolates were kept in the middle of Petri plates containing 20 mL of PDA medium. Petri plate containing PDA medium without antagonist served as control. The two plates were superposed together and covered with Parafilm under aseptic condition and were incubated at 25±1ºC. Four plates were prepared for each treatment beside the control and the experiment was repeated twice. The radial growth of each pathogen was measured when the control treatments covered the plate surface (Fig.1.b).

2.2.3. Effect of bacterial bioagents on the mycelial growth inhibition of pathogens

In Petri plates (90 mm), two straight perpendicular lines were drawn. Two plugs of each pathogen were putted in one line. These plugs were opposite and were drawn each at 1.5 cm from plate edge. However, the bacterial suspension (approximately 10^8cfu/ml) of each *bacillus* isolate was dropped on the PDA medium tracing these two lines. Separate control plates, containing just 2 plugs of pathogen were also maintained to verify the inhibition percent. The inoculated Petri plates were incubated at 25±1°C for 5 days, in the dark. There were four replicates for each pathogen–bacteria combination and the test was repeated twice. The antagonistic effect was determined by measuring the longest and shortest free growth zone between the antagonistic bacteria and the tested pathogen (Fig.1.c).

2.3. *In-vitro* assay of fungicides

Five fungicides were evaluated for its efficiency against *Oomycota* species (Table 2). The copper-based fungicides have been used as positive controls in this assay. The fungicides efficiency against *Phytophthora*, *Phytopythium* and *Pythium* species was evaluated using the 'Food Poisoned Technique', as described by Nene and Thapliyal [12].

Table2. Fungicides characteristics used *in-vitro* test for *Oomycota* control.

Active ingredients	Concentration	Commercial names	Manufacturers	Doses of uses
Citric acid + Mint oil + Adjuvant	16 % + 0,8 % + 48 %	Fungastop	AGRIPROTEC	200cc/hl
Borax	10g/L	Pre-vam	ALMAWSEM ALFILAHI	300cc/hl
Copper metal	50 %	Cuivox	STEC	500 g/hl
Copper metal	20 %	Bouillie Bordelaise	SEPCM	1,5 kg/ha
Copper hydroxide	35 %	Kocide 2000	AGRIPROTEC	150g/L

Approved concentration of each fungicide was used and adjusted to 250 ml. A PDA medium (250 ml) at 45°C, was mixed with this concentration of fungicide, poured into sterilized Petri plates and allowed to solidify. A disc of 5-mm-diameter of each pathogen, of 5-days-old culture grown on a PDA medium, was cut with a sterilized corn borer and placed aseptically in the center of each Petri plates containing the fungicides and incubated at 25±1°C for 6 days (Fig.1.d).The plates of Petri containing basal medium with plugs of pathogens were served as control. Four replicate of each treatment was made, and the assay was repeated twice. Colony diameter in each treatment was measured after control plates were fully covered with mycelial growth of the test pathogen and percent growth inhibition of the test pathogen over control was measured.

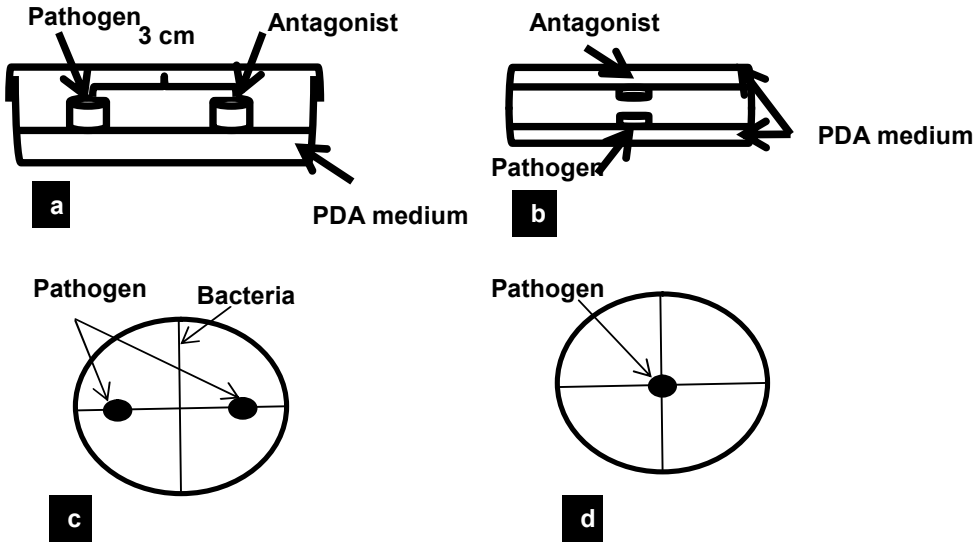


Fig. 1. Techniques used during the different assays: **(a)** Dual culture technique for antagonist fungi assay; **(b):** Sandwich plate technique for antagonist fungi assay; **(c)** Technique for antagonist bacteria assay; **(d):** Technique for fungicides assay.

2.4. Data analysis

Radial growths of the pathogens were used for calculating percent growth inhibition according to the formula: **PGI = 100 (DC-DT)/DC**

PGI = percent of inhibition of mycelia growth;

DC= growth of mycelium for the control (mm);

DT = length of mycelial growth for the treatment.

Differences between percent of growth inhibition data registered by antagonists and fungicides used were subjected to analysis of variance (ANOVA) using SPSS Statistics software for Windows 18 (IBM Corporation, Somers, NY, USA). Mean differences were separated according to Student Newman-Keuls test (P <0.05). As no significant differences had been noted, between the experiments based on preliminary analysis of variance, data from the repeated experiments had been combined.

Using STATISTICA version 12.0., PGI data of all bioagents and fungicides tested were submitted to a multivariate statistical method of cluster analysis, in order to classify in groups of closer similarity, according to their ability to reduce growth of pathogens tested. Via the same analyze, the data of pathogens were classify in groups, according to their sensibility to bioagents and fungicides used. Hierarchical cluster analysis was applied on standardized means of the evaluated variables by using the single linkage method, and is shown on a dendrogram.

3. RESULTS

3.1. *In-vitro* assays of bioagents

3.1.1. Effect of fungal antagonist on the mycelial growth inhibition of the pathogens

The dual culture assay generated significant inhibitory effects on the radial growth of the seven tested pathogens. This inhibition was clearly discerned by a limited growth or by a complete absence of pathogen mycelium.

T. viride has exhibited the highest inhibition of mycelia growth with an average of inhibition between 49% (*P. mercuriale*) and 90% (*P. nicotianae*). *T. harzianum* showed an average of inhibition between 45% (*P. mercuriale*) and 90% (*P. nicotianae*). For *P. digitatum,* the level of inhibition growth varied between 45% (*P. aphanidermatum*) and 70% (*P. cryptogea*). However, the lowest inhibition rate was recorded by *P. italicum* with an average between 60% (*P. nicotianae* and *P. cryptogea*) and 40% (*P. mercuriale*).

The data presented in Fig. 2 indicated clearly that there was significant reduction in mycelial growth of pathogens. The highest percent of inhibition of mycelial growth was observed in the case of *P. nicotianae*, with 90% of inhibition observed in treatment with *T .harzianum*. However, the lowest percent of growth inhibition was observed in the mycelium of *P. mercuriale*, with 40% of inhibition observed in treatment with *T. italicum* (Fig. 3).

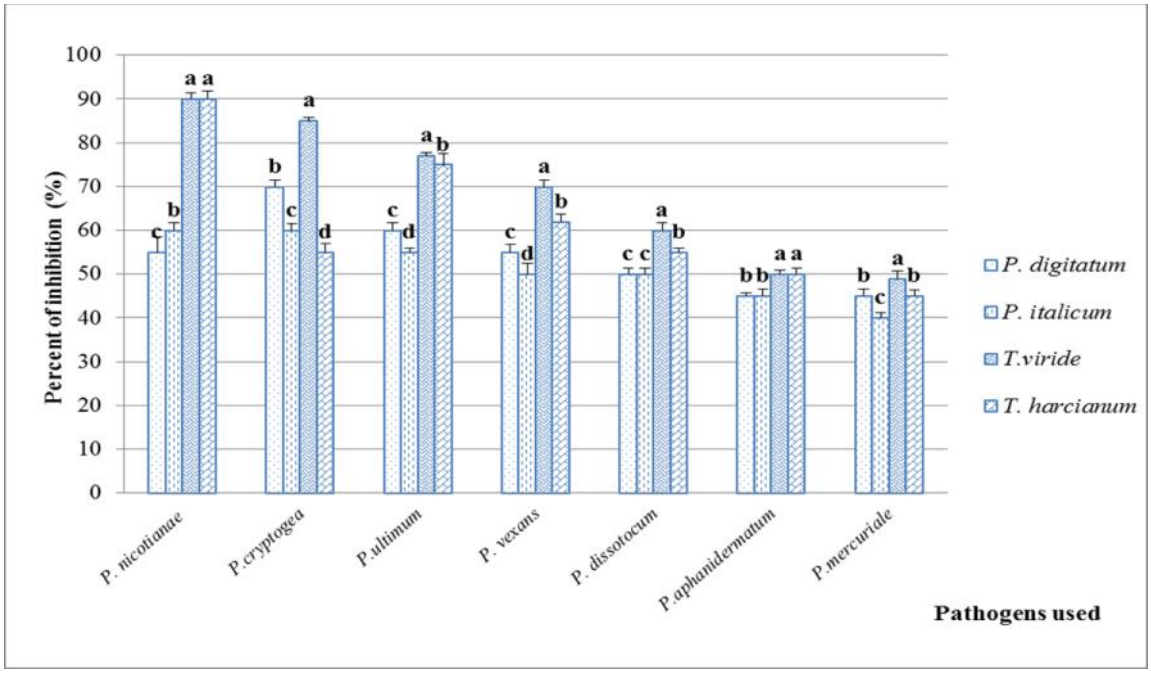


Fig. 2: Effect of dual cultures of fungi bioagents on mycelial growth of *Oomycota* species, on PDA medium, after 4 days of incubation at 25±1°C (error bars represent standard error of the mean).

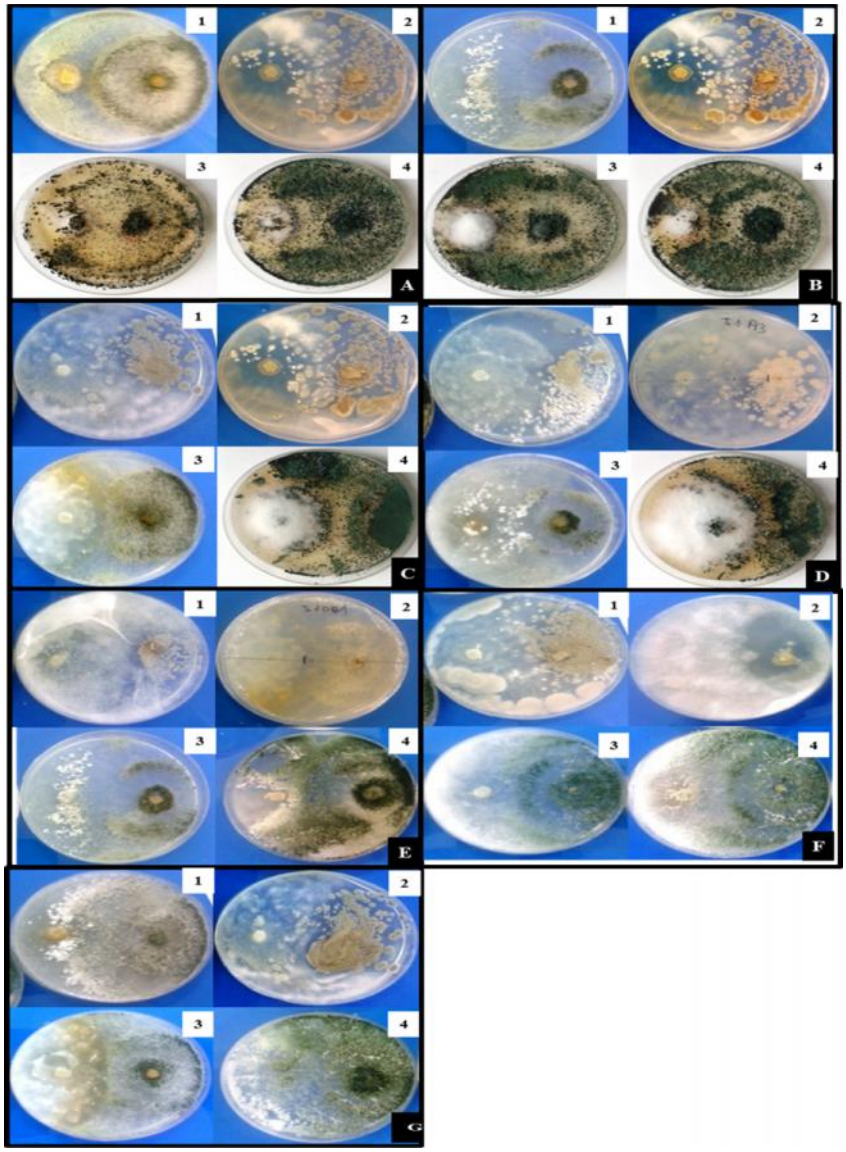


Fig.3: *In-vitro* evaluation of fungi bioagents (**1:** *T. viride*; **2:** *T. harzianum*; **3:** *P. italicum*; **4:** *P. digitatum*) on radial growth of *Oomycota* species **(A:** *P. nicotianae*; **B:** *P. cryptogea*; **C:** *P. vexans*; **D:** *P. dissotocum*; **E:** *P. mercuriale*; **F:** *P. aphanidermatum*; **G:** *P. ultimum*) in dual culture assay after 4 days of incubation, at 25±1°C.

Microscopic observations, in dual culture method, were examined after 7 days of incubation showing an interaction between the antagonistic fungi and the pathogen. This interaction caused by *Trichoderma* species was manifested by a hyphae interaction (*T. viride*) and a lysis of hyphae (*T. harzianum).* However, the interaction caused by *Penicillium* species showed the concentration of the conidia of antagonist around the mycelium of each pathogen (Fig. 4).

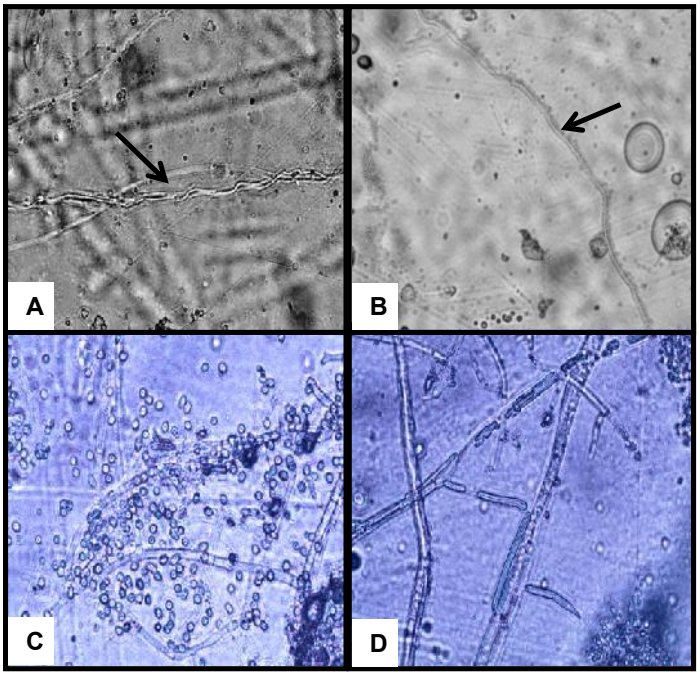


Fig. 4: Antagonistic effects of fungi on the mycelium of *Oomycota*: Hyphae interaction caused by *T. viride* **(A)**; Attachment of conidia of *P. digitatum* (B,) and P. *italicum* **(C)** to hyphae of pathogen; Lysis of hyphae of the pathogen caused by T. *harzianum* **(D)**.

3.1.2. Effect of volatile compounds of antagonist fungi on the radial growth of tested pathogens
Results showed the ability of the four fungi antagonist to inhibit the growth mycelium of the studied pathogens. *T. viride* exhibited, after 5 days of incubation, an inhibition rate between 40.25% (*P. mercuriale*) and 85% (*P. nicotianae*). *T. harzianum* was in the second position, with an inhibition of growth between 37% (*P. mercuriale*) and 75% (*P. nicotianae*). For *P. digitatum,* the values varied from 40% (*P. aphanidermatum)* and 60% (*P. nicotianae)*. *P. italicum* reduced the mycelial growth of *P. mercurial* and *P. nicotianae* by 40.25% and 50%, respectively (Fig. 5).

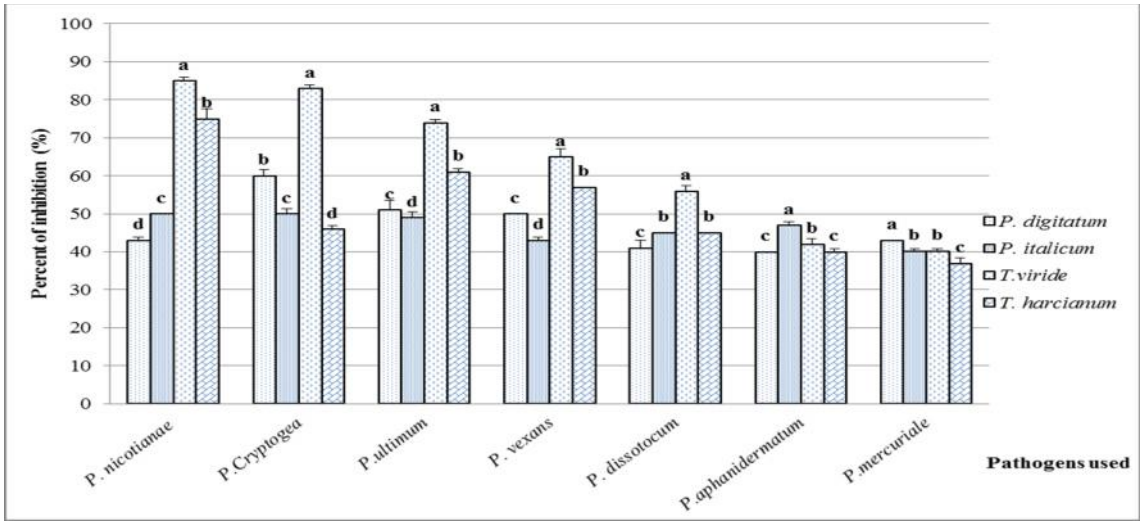


Fig. 5: Effect of Sandwich plate assembly technique of fungi bioagents on mycelial growth of *Oomycota* species on PDA medium after 5 days of incubation at 25±1°C (error bars represent standard error of the mean).

3.1.3. Effect of bacterial bioagents on the mycelial growth inhibition of pathogens

Results of the effect of *Bacillus* spp. against *Oomycota* species, tested in this investigation showed the capacity of these bacteria in reducing the radial growth (Fig. 6). No statistical difference has been observed between the isolates of *Bacillus* spp. Data present in Fig. 7 revealed that *Bacillus* spp. caused the highest mycelial growth reduction of *P. nicotianae* and *P. cryptogea* (95%), then *P. ultimum* (85%). *Bacillus* species revealed to be able to reduce the growth mycelial for *P. aphanidermatum* and *P. mercuriale* with a percent of growth inhibition of 60%. While, the least effect of *Bacillus* spp. was indicated in case of *P. vexans* (54%) and *P. dissotocum* (59%).

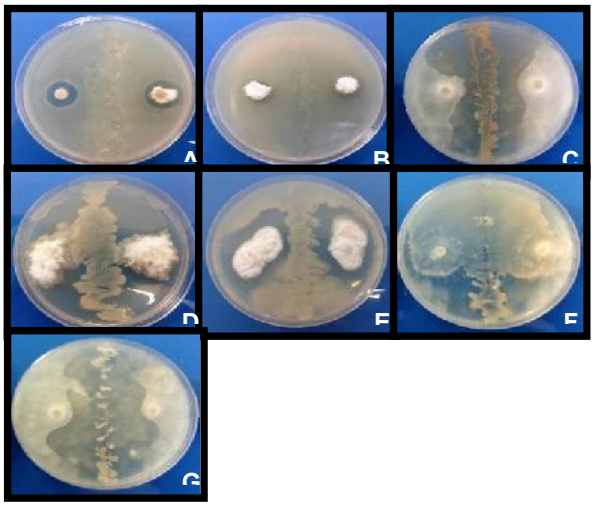


Fig.6: *In-vitro* evaluation of *Bacillus* spp. on radial growth of *Oomycota* species (**A :** *P. nicotianae* ; **B :** *P. cryptogea* ; **C :** *P. ultimum* ; **D :** *P. vexans* ; **E :** *P. mercuriale;* **F :** *P. dissotocum* ; **G :** *P. aphanidermatum*).

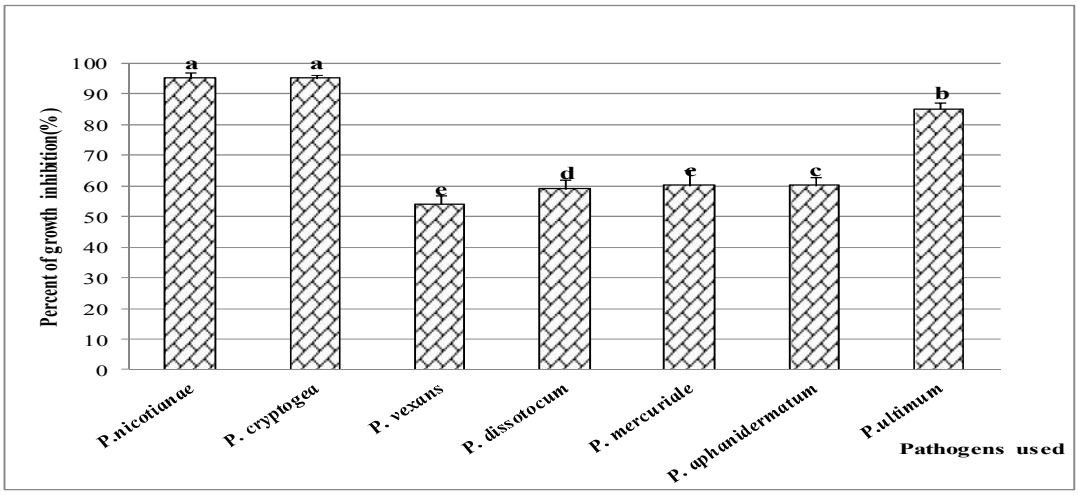


Fig. 7: Effect of *Bacillus* spp. on mycelial growth of *Oomycota* species on PDA medium, after 5 days of incubation at 25±1°C (error bars represent standard error of the mean).

3.2. *In-vitro* assay of fungicides

Obtained results (Table 3 and 4), showed a significant difference between the fungicides used at the three time of evaluation. All cooper-based fungicides showed 100% inhibition for the seven pathogenic Oomycetes evaluated.

Though there was no significant difference found among treatments and between the seven isolates of *Oomycota* used.

For the borax and citric acid, an inhibition of mycelium growth of the tested pathogens, has been observed, in the second (96h) and third time (144h) of evaluation. Thus, copper fungicides were found

most effective followed by borax. Fungicide Fungastop (citric acid) was found comparatively least effective against all pathogens used (Fig. 8).

Table.3: *In-vitro* effect of different fungicides on the inhibition of radial growth of *Oomycota* species.

	Dates of evaluation	Fungastop	Pre-vam
P. nicotianae	**48h**	0±0[c]	0±0[c]
	96h	3±0.81[b]	5±0.81[b]
	144h	5±0.81[a]	12±0[a]
P. cryptogea	**48h**	9±1.82[a]	13.5 ±0.57[a]
	96h	3±0.81[b]	9±0.81[b]
	144h	0±0[c]	0±0[c]
P. vexans	**48h**	0±0[b]	0±0[c]
	96h	4±0.5[a]	3±0.41[b]
	144h	4±0.73[a]	5±0.33[a]
P. ultimum	**48h**	0±0[c]	0±0[c]
	96h	2±0[b]	3.5±0.48[b]
	144h	3±0[a]	4±0.2[a]
P. dissotocum	**48h**	0±0[b]	0±0[b]
	96h	2±0[a]	5±0[a]
	144h	2±0[a]	5±0[a]
P. aphanidermatum	**48h**	0±0[c]	0±0[c]
	96h	3±0[b]	2±0[b]
	144h	4±0.2[a]	5±0[a]
P. mercuriale	**48h**	0±0[b]	0±0[c]
	96h	3±0[a]	3±0[b]
	144h	3±0[a]	4±0[a]

Values represent the mean of 8 replicates ±Standard deviation. For each species, values within columns followed by the same letter are not significantly different (S-N-K at $P < 0.05$).

Table.4: ANOVA table for *in-vitro* assay of fungicides against *Oomycota* species.

Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F-value**	**P-Value**
Corrected Model	3378.673[a]	41	82.407	1045.700	,000
Intercept	3170.329	1	3170.329	40229.905	,000
Species	548.505	6	91.417	1160.043	,000
Dates	368.964	2	184.482	2340.988	,000
Fungicides	160.329	1	160.329	2034.491	,000
Error	23.169	294	.079		
Total	6572.170	336			

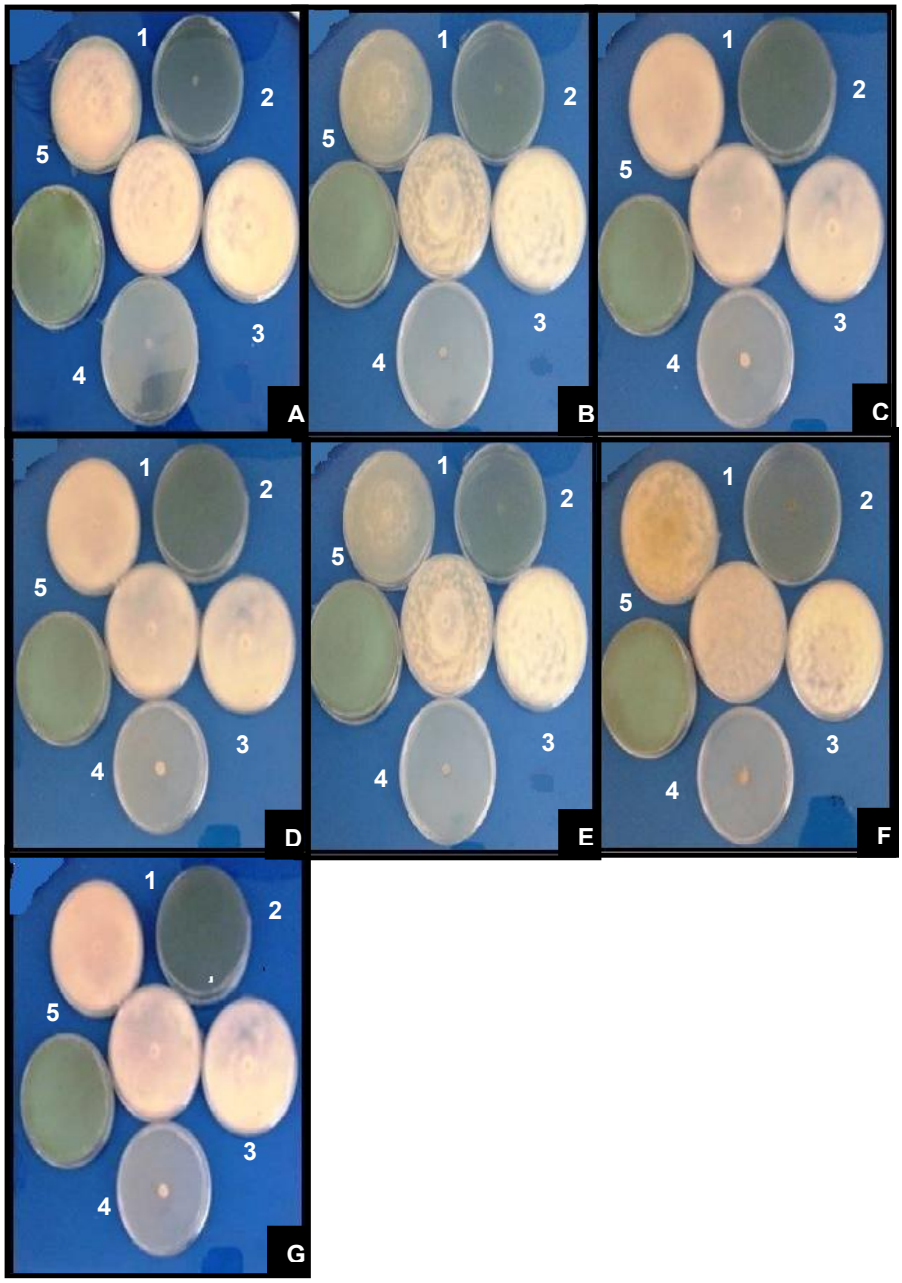
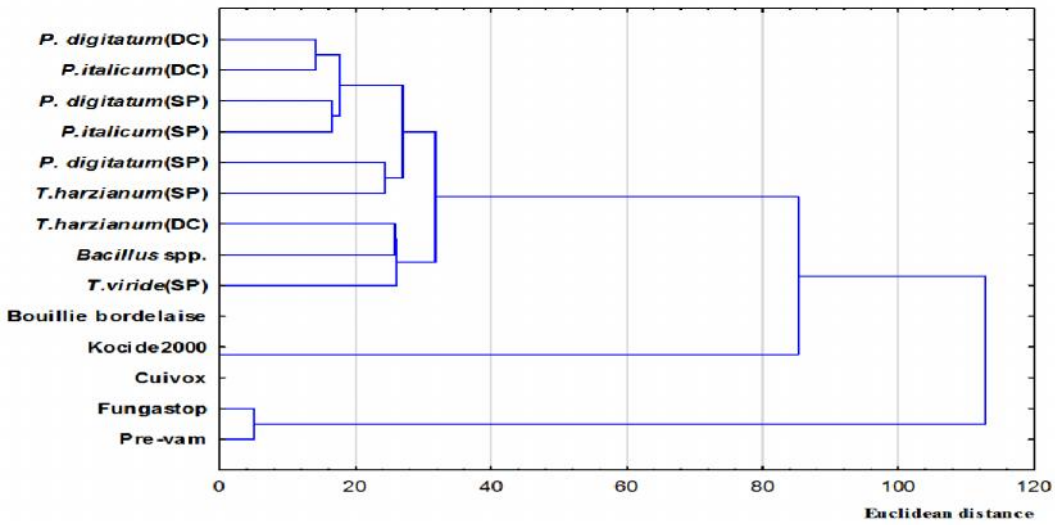


Fig.8: *In-vitro* evaluation of five fungicides (**1:** Pre-vam; **2:** Bouillie bordelaise; **3:** Fungastop; **4:** Cuivox; **5:** Kocide2000), on radial growth of *Oomycota* species (**A:** *P. nicotianae*; **B:** *P. cryptogea*; **C:** *P. ultimum*; **D:** *P. dissotocum*; **E:** *P. vexans*; **F:** *P. mercuriale*; **G:** *P. dissotocum*), after144h of incubation onto PDA medium, at 25±1°C.

Analysis of the dendrogram of similarity, using the single linkage method, generated by the bioagents and the fungicides, in the based on inhibition rate, classified the pathogens into four groups (Fig. 9). One group, includes the fungicides how have the highest percent of growth inhibition of the mycelium growth of pathogens (Kocide2000, Cuivox and Bouillie bordelaise). However, the two other fungicides (borax and acid citric) were grouped in another group. For the bioagents, the dendrogram exhibited a similarity between *T. harzianum* (DC) and *T. viride* (SP) and *Bacillus spp.* Compared to the other bioagents, these bioagents showed the highest percent of reduction of the mycelium growth. The next group was composed by *P. digitatum* (DC). Compared to the other bioagents tested, these bioagents showed a least percent of inhibition of mycelium growth of pathogens used.



DC: dual culture assay; **SP**: Sandwich plate.

Fig. 9: Dendrogram of bioagents and fungicides obtained from average values of percent of growth inhibition of mycelium, by pathogens.

Analysis of the dendrogram of similarity, using the single linkage method, generated by the pathogens used, in the based on percent of growth mycelium inhibition, of bioagents and fungicides used, showed the presence of four groups (Fig. 10). One group formed by *P. nicotianae, P. ultimum* and *P. cryptogea.* These pathogens exhibited the highest percent of growth reduction of mycelium. *P. vexans* was in a second group. *P. dissotocum* was in another one. However, the pathogens with the lowest percent of growth reduction of mycelium (*P. aphanidermatum* and *P. mercuriale*) were grouped in another group.

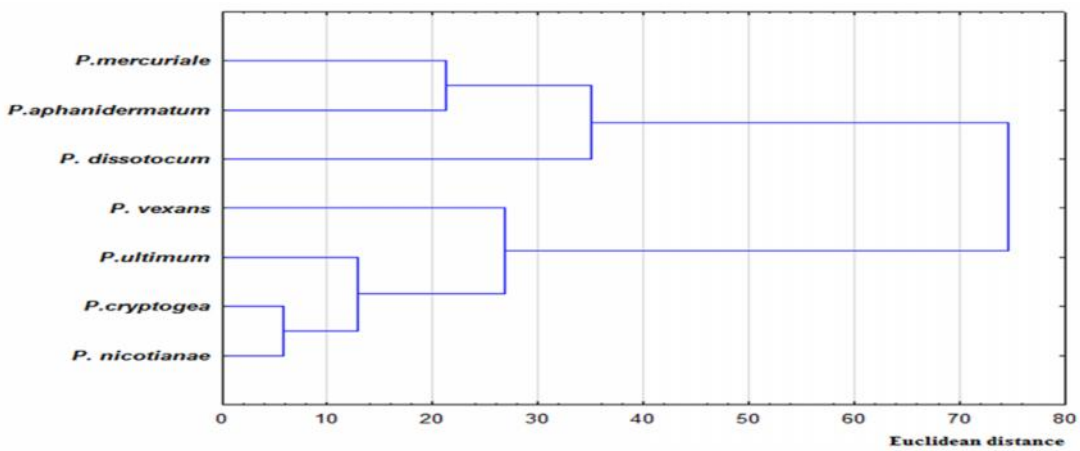


Fig.10: Dendrogram of similarity between pathogens obtained from average values of percent of growth inhibition of mycelium by bioagents and fungicides tested.

4. DISCUSSION

In the present study, the evaluated antagonists and fungicides demonstrated an inhibitor effect against gummosis pathogens under *in-vitro* conditions. Among the antagonist test, *T. viride* and *T. harzianum* proved to be the most effective against pathogens. In fact, *Trichoderma* is a well-known, valuable biological control agent against a broad spectrum of plant pathogens [14]. Puri et al. [15] reported also that mycelium of *P. parasitica var. piperina* was inhibited by *T. viride* and *T. harzianum*. Kattaleewan et al. [16] recorded 65% of inhibition of mycelial growth of *P. parasitica* by *Trichoderma* spp. Tran [17] noted the inhibitory effect of *T. harzianum* and *T. viride* to *Phytophthora* spp. Singh and Islam [18] reveled a reduction of radial growth of *P. nicotianae* of 61% by *T. harzianum* in blank sank disease of

tobacco. Kanokmedhakul et al. [19] noted inhibitory effect of Trichotoxin 9 against *P. parasitica* of sweet orange. Also, Naseby et al. [20] demonstrated that inoculation with *Trichoderma* strains reduced significantly the *Pythium* populations. Mishra et al. [21] determined the efficacy of culture filtrates of the *Trichoderma* strains and among the strains tested, *T. viride* was found most effective against *P. aphanidermatum*. Such similar observations have been made by Sharif et al. [22] about *Penicillium* spp., as a reducer of seed and seedling disease caused by *Pythium* spp.

Our results showed, an inhibition in the mycelium growth of pathogens used, in the assay of sandwich plates. This inhibition could be caused by the volatiles organic compounds emitting by *Trichoderma* [23] and *Penicillium* species [24].

For microscopic observations, it was noticed that mycelia of these fungi antagonist grew toward mycelia of the pathogens. It was also reported that in some parts fungi mycelia twisted around mycelia of the pathogen, suggesting mycoparasitim under the experimental conditions. It was demonstrated also the presence of fungi conidia around the mycelium of pathogens. Some authors have reported that this attraction may be a chemical tropism due to materials or lignin present in pathogen's cell wall or it may be due to production of aspersoria, penetrating organs or traps [25]. However, their role in the natural environment needs to be clarified.

On the other hand, data revealed that the antagonistic *Bacillus* spp. have inhibited the mycelium growth of pathogens. Similar results have been reported by many investigators [26]. *Bacillus* spp. are capable of producing an array of lytic enzymes, which is the main mechanism of bacterial biocontrol agents [27]. The inhibition in growth of pathogens, by *Bacillus* spp., could be attributed to antibiosis, hyperparasitism [28] or production of chitinase and *ß*-1,3 glucanase enzymes which degrade the cell wall leading to lysis of mycelium of the pathogen [29]. The existence of interaction between the fungicide and the pathogen propagules, especially in the field when rainfall causes redistribution of both, has been noted [30]. For best management of citrus gummosis, future studies should investigate the possibility of existence of interaction between the bioagents and the copper fungicides used in these assays.

5. CONCLUSION

In conclusion, this study has shown that both fungicides and bioagents exhibited effects against tested pathogens. The bioagents *T. harzianum* and *T. viride* and *Bacillus* spp. *was* the most effective compared to *P. digitatum, P. italicum.* However, the cupper fungicides was the most effective compared the acid citric and borax fungicides. Thus, bioagents can play an enormously important role in gummosis control. The suitable integration of these bioagents may provide a better management of the disease.

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