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ORIGINAL ARTICLE

Three peronosporaceae species of the genus *Phytopythium* menace *Nuphar lutea* (Sibth and Sm.) colonies occurring in South-mediterranean freshwater wetlands (a rare species in Algeria and Africa)

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Oubeira Lake, Northeastern Algeria, is about to undergo critical changes which threaten local aquaplants populations, at the image of *Nuphar lutea*. Hereby, we took the initiative to identify aquatic microorganisms associated to *Nuphar lutea* rhizosphere, in order to identify the causes of root-rot disease, observed when sampling plant materials during the early blooming season of the current year. *In silico* cultures isolation, microscopic characterization and Molecular phylogeny based on rDNA ITS and cox_2 sequences of rhizospheric Oomycetes, have been highly significant to distinguish three species from the genus *Phytopythium*, suspected to be aggressive phytopathogens against *Nuphar lutea* population in the Oubeira. This waterborne Protist is morphologically *Fungi-like*, rather *Algae-like* in its genotype apect, sharing with the sister genus *Phytophthora*, the role of decomposing biomachines on submerged organic materials within stagnant freshwater ecosystems. Empirical results suspect this aquatic microorganism to develop a pathogenic devastating behavior against the studied helophytes' fine roots and become so on, the main causal agent of yellow lily extinction from the South Mediterranean area. We remind that *Nuphar lutea* is an excellent bio-geo-indicator, only recorded in Oubeira, as a rare species in Africa.

Keywords: Oubeira lake, Freshwater, Mediterranean autochtonous populations, *Nuphar lutea* L., Phytopyhthium, Plant pathogen, Molecular identification.

Introduction

South Mediterranean climate covers a limited number of wetlands and forests, typifying subtropical to tropical ecosystems; they accomplish an agro-ecological key-role facing aridity that continuously extends from the continental Sahara. In Algeria, the National Park of El Kala comprises wetlands which host endemic rare animal and plant species.

In our current study, we are focusing on water lilies, as submerged herbaceous plants to be a true witness of great climate changes in the Mediterranean area. Natives to the northern hemisphere, Water Lilies tend to perish from this region, as the only and unique site of their occurrence all over the African continent (Robinson, 2017), (Ferrez et al., 2014), (Birks, 2013). They are dominantly represented by white water lilies, systematically designated by *Nymphaea alba*, which recently disappeared from many points within

the National Park of El Kala; observational records where noticed, but no studies are yet realized to bring out the real causes of their decay.

In a second range, yellow *Nuphar lutea* has been at the origin of classifying El Kala among Ramsar sites, since 1980s to date, for being its only and unique propitious ecosystem in Africa, as shown on Fig. 1.
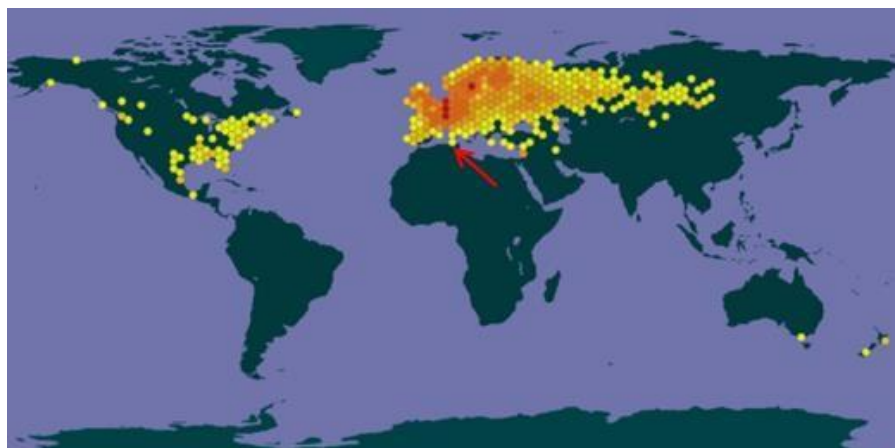

Fig. 1. *Nuphar lutea* distribution in the world, unique presence in Algeria (Hassler 2022).

The species is located in a very limited area that extends to around 500 m^2, colonizing the waterway of Oubeira Lake basin and one of its permanent feeding Wadies, called El Kebir, through the so called Daï-Graâ Canal, located at 36°.81842N 8°.44179 E, GPS coordinates (Boumezbeur, 2001), (Betscoun-Muselli, 1989), (Hassler, 2022). Growing in consistent belts, these plants are excellent nests for an important community of fish, amphibians and invertebrates, and perfect habitats for planktonic populations. Some botanists describe *Nuphar lutea* as one of the eight species of the genus Nymphea, with many synonyms (Betscoun-Muselli, 1989), as listed below:

Basionyme: *Nymphaea lutea* L. *Nenuphar luteum* (L.) Link *Nymphona lutea* (L.) Bubani *Nymphozanthus luteus* (L.) Fernald.

This Macrofossil is used in ecology as a bio-geo-indicator from the Quaternary age.

It is important to evoke that the species has already disappeared from the Bay of Cannes at the extreme Southeastern Franco-Italian borders, a very similar area, geographically opposite to our site of study Northern the Mediterranean basin (Julve, Ph., 2021). We mention that in Algeria, those flowers still occur in wild contexts and have never been used in artificial ponds, neither in ornamentation nor for biotechnological targets, contrarily to European lilies, which are used for their astringent properties attributed to the tannins present in the rhizome, and the alkaloids of the flowers and rhizomes from both white and yellow waterlilies which have sedative and antispasmodic activity (Betscoun-Muselli 1989).

The problematic loss of such a patrimonial genetic resource can drastically affect the ecosystem. This current study will be the first record of aquatic phytopathogen fungi, which seem to be the causal agents of yellow lily rhizospheric disease.

Materials and Methods

Plant materials sampling

Nuphar lutea plants are preferably growing and colonizing the surface of stagnant shallow freshwater, so that flower, stalks and submerged leaves float at the surface, while the root part emerges on a growing structure of rhizomes strongly incrusted into the sediment floor (Sibth and Sm in Betscoun-Muselli 1989). The rhizosphere is built on branching, spongy, tuberous rhizomes 20-150 mm in diam., toughly attached to the sedimentary mud, so that sampling units were hardly picked at lower depth from five different water input points, along the Daï-Graâ Canal. They were taken from sediments and fine roots materials rotten by aquatic microflora biodegradation, aspictured in Fig. 2.

Biological materials were kept in clean zipper-bags and immediately transferred to the laboratory in no more than one hour time lapse, at around 7°C could.

Fig. 2. TheRotten fine roots of *Nuphar lutea* rhizome, sampled in Oubeira Lake.

Microbial culture and isolation

Microbiological labor consisted in separating fine rotten roots from the rhizome, so that disinfected pieces of about 20 mm length are plated on modified water agar medium, using 9 cm diameter Petri dishes. Water agar was prepared to be selective for Oomycetes by adding to its composition, the following antibiotics: Ampicillin, Nystatin, Pimaricyn, Rifampicyn and Pentachloronitrobenzene (Eckert and Tsao, 1962) (Samson et al., 2004). Resulting mixed colonies with fungiform hyaline soft mycelium, typical to Oomycetes, were isolated after three days to one week incubation time, at room temperature (Erwin and Ribeiro, 1996). 0.5 mm^2 Fragments of the juvenile mycelium were isolated on the same modified water agar, as described above. The isolates were cultured at room temperature and incubation took eight to ten days to be ready for microscopic visualization.

Microscopy

Microscopic morphology was obtained by using a photonic microscope BX50 from *Olympus* Company, and a *Malassez-cell* to establish lengths scale. Hyaline aseptate hyphae with obovoid to pyriform or curved shaped Sporangia, typical to the Peronosporaceae Oomycetes family, allowedus to consider the following molecular studyof the selected isolates.

DNA extraction, amplification and purification

Targeted Oomycetes DNA loci were extracted using GenEluteTM plant Genomic DNA Miniprep Kit (Sigma-Aldrich) according to Manufacturer's specifications. Internal Transcribed Spacer (ITS region) was amplified using the universal primers ITS-LR0 (GCT TAA GTT CAG CGG GT) (Moncalvo et al., 1995), while cytochrome c oxidase subunit II (cox2) was amplified using the primer cox2-F (GGC AAA TGG GTT TTC AAG ATC C) (Hudspeth et al., 2009); we added 1 µg DNA to the reactional medium furnished by Thrmoscientific company; the latter comprises the Master Mix 10 × Dream Taq Green buffer (containing 20 mM MgCl$_2$), 5µL dNTPs (0.2 mM for each nucleotide), primers (0.4 µM of each primer), 1.25 U Taq polymerase; the protocol was realized following the manufacturers' notice.

Cycling protocol was accomplished by using the following program: step (1) initial denaturing for 3 min at 95°C; step (2) denaturing for 30 sec at 95°C; step (3) annealing for 30 sec at 55°C; step (4) extension for 1 min at 72 °C; step (5) final extension for 5 min at 72°C. The steps 2-4 were repeated 35 times (White et al., 1990). The PCR products were quantified by Electrophoresis on Agarose gel, then finally cleaned-up using Thermo-Fisher-Scientific kit, by adding to 5 µl of it, a mix of 0.5 µl Exonuclease 1 and 1 µl Fast-up alkaline phosphate according to manufaturer'sspecifications (Ginetti, et al., 2014), then sent for sequencing.

Bioinformatic sequence analyses

Amplicons sequences were checked in Chromas Lite v. 211, then assembled, analyzed and edited in Geneious v.8.1. in a previous step to Geneious analysis, we had to select manually the reference sequences from Genbank database. Multiple alignment was performed using Mafft 7 online aligner, by choosing Q-INS-i strategy setting and 1PAM=2k for scoring matrix. Then, we chose

Maximum Parsimony method with default parameters, to obtain the best cladogram from the produced trees (Baldwin, 1992). Bootstrap statistics and branch lengths were computedwith Mega 6 (Tamura, et al., 2013).

Results and Discussion

Our results represent the first attempt to identify a Eukaryotic lower aquatic microorganism colonizing the rhizospheric part of *Nuphar lutea*; it is suspected to be the direct phytopathogen that develops a kind of disease to the smooth tissues of the rhizome, including fine roots.

Molecular identification

Based on molecular results, *De Novo* assembly allowed us to select 12 specimens of rDNA ITS data of around 700 bp lengths. Over 100 BLAST (NCBI) analyses score permitted to select 12 most similar reference sequences belonging to the genus *Phytopythium* (Table 1).

Table 1. Best identity scores with Genebank references acquired by BLAST (ncbi) analysisaccording to rDNA ITS sequences.

Isolate	Closest reference within Genbank database	Identity %	Reference source
LK6_LR0	*Phytopythium vexans* MT611229.1	99.49%	Linaldeddu, B.T. 2020
LK9_LR0	*Phytopythium vexans* KT148890.1	99.49%	Migliorini, D. 2015
LK10_LR0	*Phytopythium helicoides* KT223574.1	95.43%	Browne, G.T. 2016
LK11_LR0	*Phytopythium vexans* KT148890.1	96.33%	Migliorini, D. 2015
LK12_LR0	*Phytopythium vexans* OK257570.1	84.02%	Panek, M. 2021
LK18_LR0	*Pythium vexans* MT611229.1	99.50%	Linaldeddu, B. 2020
LK25_LR0	*Phytopythium vexans* MF984113.1	99.75%	Chen, J.J. 2017
LK27_LR0	*Phytopythium palingenes* MN872742.1	91.96%	jung 2020
LK34_LR0	*Phytopythium vexans* MT611229.1	97.06%	Linaldeddu, B.T. 2020
LK35_LR0	*Phytopythium helicoides* KT223574.1	98.97%	Robideau 2011
LK36_LR0	*Phytopythium vexans* MT611229.1	99.49%	Linaldeddu, B. 2020
LK46_LR0	*Phytopythium vexans* MT611229.1	99.37%	Linaldeddu, B. 2020

A very high similarity reaching 99.70% with reference sequences from Genebank, allows to highlight three species of the genus *Phytopythium*, *Php. Vexans, Php. helicoides and Php. palingenes*. The latter is a newly described species that was isolated from Viet Nam forests by Jung, et al., 2020. Thus, similarity with our isolated species does not exceed 91.96%; better result will be given by Cox2 *locus* comparison. Sequences of around 530 bp lengths with over 95% similarity to 12 references belonging to the genus *Phytopythium,* are represented on Table 2.

Php. Vexans, Php. helicoides and *Php. dogmae*are the three species of this genus which identify our isolates. We have to justify that based on cox2 results, *Php. dogmae presents* 97.04% similarity is to our isolate, while ITS sequences comparison to *Php. palingenes*(Jung, et al., 2020) revealed less similarity tothe same isolate. Therefore, we argue our isolate to be rather *php. dogmae* (Bennett, et al., 2017). Both species *Php. palingenes* and *php. dogmae*, were first found in Asian tropics and seem favorably colonizing Mangrove-like wetlands (Jung, et al., 2020), (Bennett, et al., 2017) (Nam, B., and Choi, Y.J., 2019). It is herein identified in the Mediterranean basin as a first record of *Php. dogmae* in Oubeira Lake, showing their closeness to weather conditions which promote its occurrence in El Kala National park Northeastern Algeria, supposed to be Mediterranean by excellence.

This genus is one of The Peronosporaceae oomycetes, ecologically represented to be phytopathogens in agrarian ecosystems; many of them are known to attack trees roots in forestal ones (Ginetti, et al., 2012). The inventory of native species predominantly occurring in Oubeira inland freshwaters, has revealed a large distribution of the genus *Phytopythium* species almost identified, as necrotrophic microbs, decomposing dead plantmaterials and also occurring in sediments and wet soils (Kachour, et al., 2016).

Table 2. Best identity scores with Genebank references acquired by BLAST (ncbi) analysis according to cox2 sequences.

Isolate	Closest reference Within Genbank database	Identity%	Reference source
LK6_cox2	*Phytopythium vexans* MH492367.1	92.44%	Park, M.J., Back, C. 2019
LK9_cox2	*Phytopythium vexans* GU133560.1	96.57%	Spies, C.F. 2016
LK10_cox2	*Phytopythium helicoides* KT595689.1	97.33%	Chen, X.R. 2015
LK11_cox2	*Phytopythium vexans* GU133560.1	97.30%	Spies, C.F. 2016
LK12_cox2	*Phytopythium vexans* GU133560.1	97.65%	Spies, C.F. 2016
LK18_cox2	*Pythium vexans* GU133560.1	98.08%	Spies, C.F. 2016
LK25_cox2	*Phytopythium vexans* GU133518.1	95.85%	Spies, C.F. 2011
LK27_cox2	Phytopythium *dogmae* MF359561.1	97.04%	Bennett, R.M. 2017
LK34_cox2	*Phytopythium vexans* GU133560.1	97.30%	Spies, C.F. 2011
LK35_cox2	*Phytopythium helicoides* KT595689.1	96.03%	Chen, X.R. 2015
LK36_cox2	*Phytopythium vexans* GU133560.1	96.51%	Spies, C.F. 2016
LK46_cox2	*Phytopythium vexans* GU133560.1	100%	Spies, C.F. 2016

Phylogenetic reconstruction

In light of the software analyses of our resuls, maximum parsimony phylogenetic reconstruction present a cladogram organized into 3 clusters of DNA sequences for both ITS and Cox2 *loci*, respectively represented on Fig. 3 and Fig. 4.
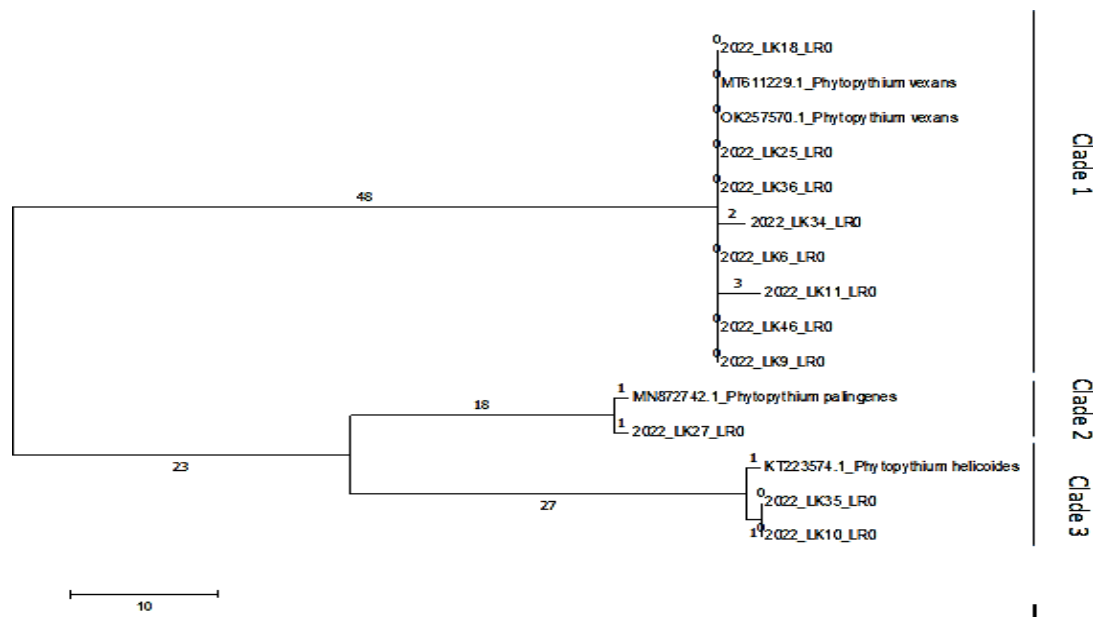

Fig. 3. Maximum Parsimony analysis of taxa: Evolutionary history was inferred using the Maximum Parsimony method. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm. Branch lengths were calculated using the average pathway method and are in the units of the number of changes over the whole sequence. There were a total of 559 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura, et al., 2013).
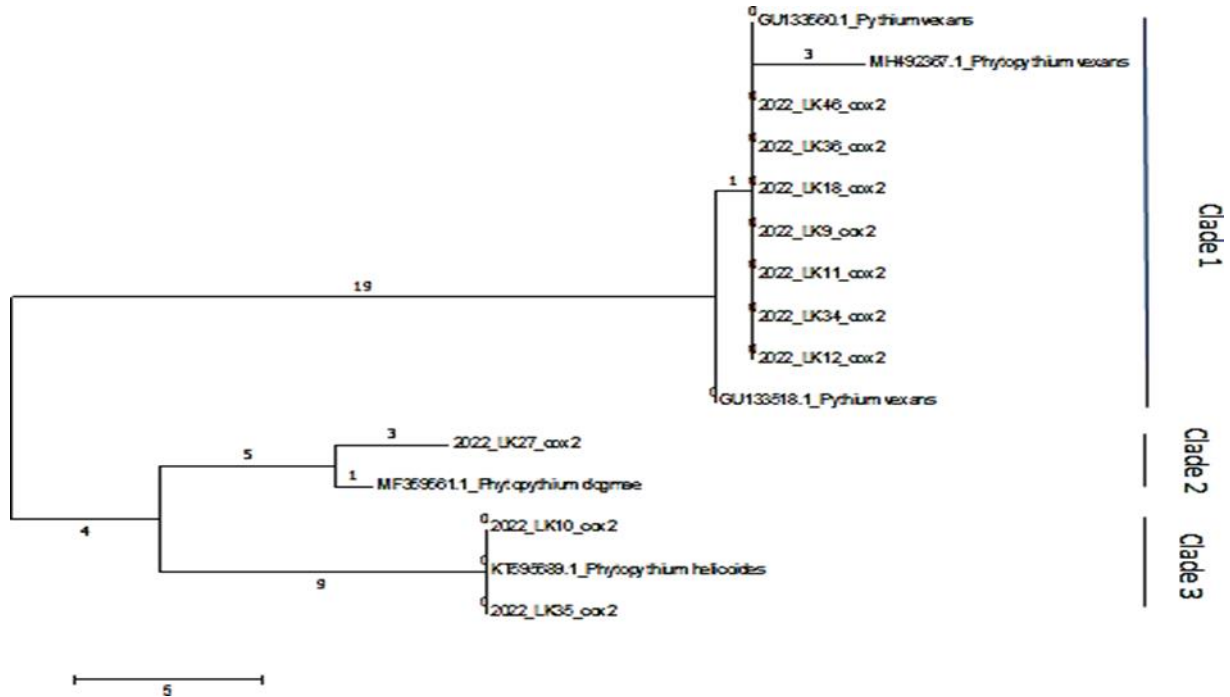
Fig. 4. Maximum Parsimony analysis of taxa, Evolutionary history was inferred using the Maximum Parsimony method. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm. Branch lengths were calculated using the average pathway method. There were a total of 468 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura, et al., 2013).

Phylogenetically speaking, *Phytopythium* has been actualised to count more than 20 sprotrophic or facultative biotrophic genera (Robideau, et al., 2011; Tkaczyk, 2020). Phylogenetic bifurcation into *Php. vexans* on one hand, and *Php. helicoides* on the other hand, is given by both ITS and cox2 sequences analyses. The new species *Php. dogmae* is established to share genetic evolution scenario with the sister species *Php. helicoides*; microscopic study will be confirmative to support the hypothesis.

Colonies aspect and microscopicmorphology

Oomycetes are fungal-like worldwide spread, with the particularity to grow on extremely poor media, such as water agar. Typical isolates with whitish hyaline mycelia growing on modified water agar, as well as morpho-microscopic details, are represented in Fig. 5, Fig. 6 and Fig. 7, respectively corresponding to *Php vexans*, *Php. helicoides* and *Php. dogmae*.
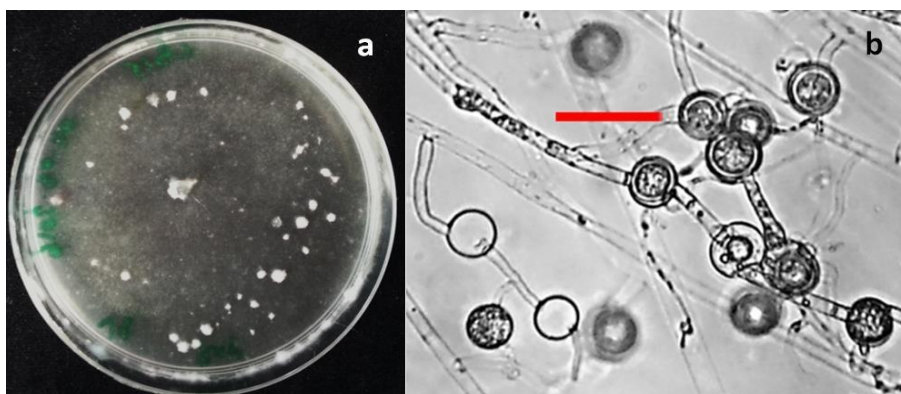

Fig. 5. *Phytopythium vexans*: (a) Hyaline filamentous colony growing on modified Water Agar, (b) Subterminal and internal germinating oogonia, with spheric subovoid or ovoid characteristic shape, thin-walled oogonium measuring 10-45 μm diameter (average 26.6 μm) by antheridium; emptied Globose sporangium with a germ tube. Scale bar: 50 μm.
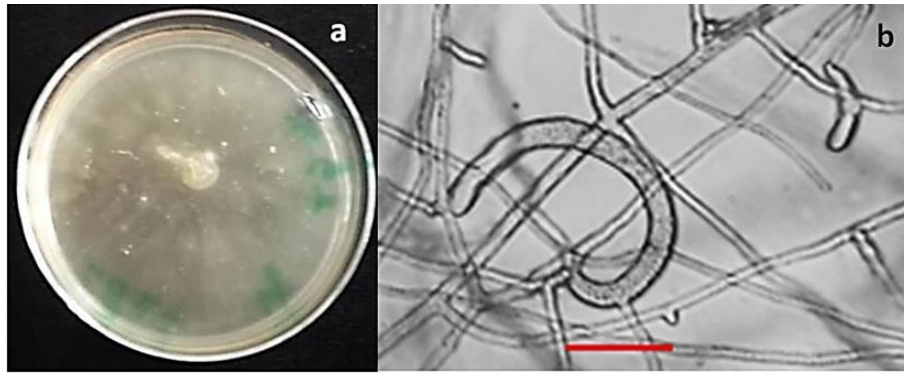
Fig. 6. *Phytopythium helicoides* hyaline filamentous colony growing on modified Water Agar (a), curved sporangia a) terminal piriform, more or less stretched to arched sporangia, thin-walled, measuring 30-60 µm length (average 50 µm). Scale bar: 50 µm.
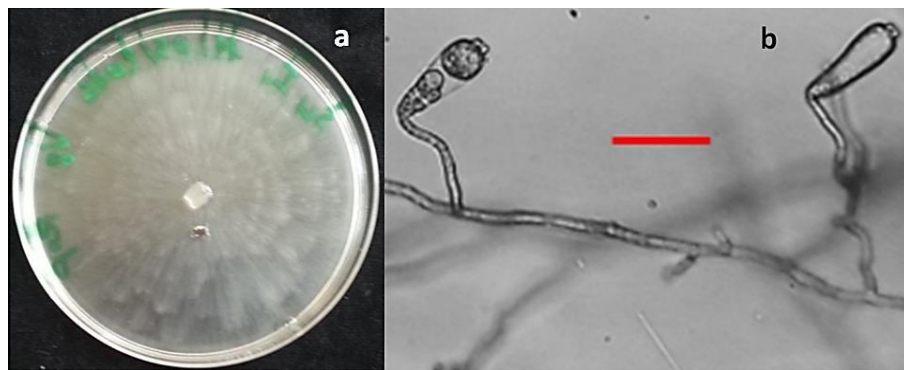

Fig. 7. *Phytopythium dogmae* cultivated on WA agar: (a) hyaline filamentous colony growing on modified Water Agar; (b) piriform to baseball-shaped sporangia measuring 10-45 µm length (average 26.6 µm), thin-walled, papillate apex ornamented by a tight saw-toothed plug, emptied baseball sporangium is shown at the right upper corner. Scale bar: 50 µm.

Frequency

Among the 12 *Phytopythium* isolates, we could identify 9 specimens which dominantly much to *Pph. vexans*, and only 2 isolates corresponding to *Pph. helicoides*; one species was identified as *Pph. dogmae*, genotypically and phenotypically very similar to the *pph.helicoides* (Fig. 8). Phytopathological testswere not realized during the current study.
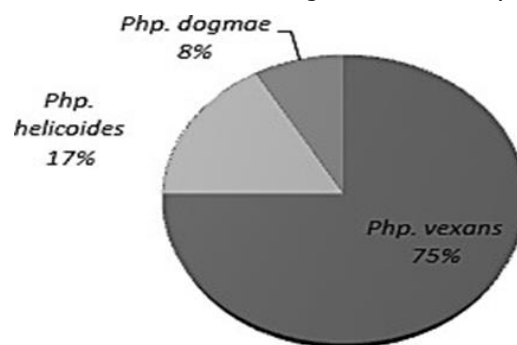

Fig. 8. Quantitative predominance of *Php. Vexans* frequency.

Resistance to the plant immunity

Nuphar luteum biochemical features are diverse, and many chemical compounds showing promise for antifungal, and antibacterial actions, including "alkaloids, terpenoids, saponins, flavonoids, tannins sind phenolic compounds, proteins, amino acids, carbohydrates, starch and vitamin C, were listed in many previous reports (Robinson, 2017). Biotechnologically speaking, yellow lilies are widely used in phyto-opharmacology and toxicology as reported in Betscoun-Muselli Isabelle, 1989:

- C15 quinolizidine and piperidinesesquiterpene alkaloids
- Thiaspirane alkaloids in C30
- A glucoside: Nymphaline

These molecules are reported to gatherantagonistic properties against pathogens, antibacterial antifungal and antiparasitic virtues. Alcaloïdes are efficient antibacterial antifungal molecules positively tested on phytopathogen bacteria, as *Corynebacterium michiganense* and many fungi like *Histoplasma*, *Blastomyces*, and *Tricophyton*. Eventhough its biochemical virtuous properties, to elaborate aroma produced by the flowers which is similar to stale alcohol, we could clearly diagnose root rot symptoms on rhizomes and hypothetically suspect oomycetes for being able to escape the biopesticide properties of the elaborated molecules.

Conclusion

The Algerian climate tends to semi-arid, even arid stages, which increasingly causes the scarcity of natural water resources. Along the Tellian-Atlas in the North, numerous inland waterplans still persist and play a vital role against drought. Inside the National Park of El Kala (PNEK) situated at the Tunisian borders, Oubeira Lake, is the largest waterplan, and mainly contributes in annual dynamics maintain of many living populations, such as migrant birds crossing the Mediterranean forth and back. Currently, the lake undertakes a kind of hemorrhagic loss of rare endemic aqua-plants like in our case of study, *Nuphar lutea.* Three microbial species belonging to the genus *Phytopythium* are identified to infect fine roots and lead to the plant death; for the first time, *Pythium dogme* is recorded among the phytopathogens, as a sister pecies with tight similarity to *Phytopythium helicoides*, with a different behavior and phenetic features.

As the disease is not supposed to be controlled by human interference, the possibility to naturally introduce plant growth promoting rhizobia that may react as an antagonistic agent to the phytopathogen fungi, seems yet, to be one a suggestion to prevent water lily extinction from Oubeira Lake.

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