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Characterization of lichen Xanthoria parietina and Isolation of the associated photobiont and mycobiont

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Abstract: Lichens are one of the most promising sources for bioactive compounds which exhibit different functions including antioxidant, antimicrobial, anti-inflammatory and anticancer effects. However, these beneficial bioactive compounds never make it to the industrial application due to the lichens' extremely slow growth rate which was the drive for in vitro cultivation of the lichen symbionts. In this study, lichen samples were collected from *Calligonum polygonoides* old shrubs and were identified as *Xanthoria parietina* following the study of morphological characteristics and performance of spot test (K, C, & KC tests) with reference to the identification keys. Additionally, the lichen internal structure and anatomy were studied in lichen thin sections which clearly illustrated the algal and the fungal layers (the cortex and medulla). Both the photobiont and the mycobiont were isolated through thallus derived culture on BBM and PDA media respectively then were preserved in axenic cultures for further analysis and characterization. The results of this study spot the light on the main associates of *Xanthoria parietina* paving the way to explore the interaction between them and the secondary metabolites that might be attained from such association.

keywords: : lichen, photobiont, mycobiont, Xanthoria parietina

1.Introduction

Mutualism between fungi and other organisms have been reported in several cases including plant endophytes and lichens [1].

Lichens are generally identified as a symbiotic relationship between a fungus (the mycobiont) and an alga or in some cases a cyanobacterium (the photobiont). In this association the mycobiont is mainly responsible for nutrient acquisition from the surrounding environments while the photobiont provides organic matter required for the fungus growth through photosynthesis [2]. Lichens inhabit a wide range of ecosystems extending from hot deserts to the Antarctica and are recognized for their ability to tolerate harsh conditions such as nutrient scarcity, extreme drought, toxic levels of metals and high UV radiation [3].

There are about 18,500 known lichen species [4] that are categorized in primarily three groups; crustose lichens which are found firmly attached to the substrate they grow on and usually lack rhizines (root like structures responsible for adherence), foliose lichens which are leafy in appearance and are loosely attached to their substrate specially at the margins and fruticose lichens in which the large portion of the thallus is found hanging resembling a tiny shrub while being attached to their substrate at a single point [5].

Lichens have been used as traditional medicines for centuries [6] while recent work showed the bioactivity of lichen metabolites and their potential use as antibiotics [7, 8], antioxidants [9], antiviral [10] and as anticancer [11, 12]. However, lichens are one of the most slow-growing organisms with a

generalized growth rate of 0.5–5 mm per year [13] and any mass harvesting of lichens for exploitation as industrial resource might lead to species extinction. In addition, many species are listed

endangered owing to climate change and increased air pollution [14], hence the need arises for in-vitro cultivation of lichens.

During the past two decades many successful trials for the isolation and axenic cultivation of both symbionts have been carried out. In 2013, McDonald and co-workers were able to culture the mycobiont in twenty-five different lichen species using five growth media (malt extract-yeast extract (MY), Bold's basal medium (BBM), yeast extract with supplements (YES), oatmeal and potato-carrot medium), these media were proven efficient in the isolation of mycobionts. Examples of cultivated mycobionts include *Astrothelium galbineum, Phaeographis elliptica, Polymeridium subcinereum, Pyrenula cruenta* and *Usnea strigosa* [15].

As for the photobionts, approximately 40 genera of eukaryotic microalgae and cyanobacteria have been reported as lichens' photobionts. *Trentepohlia, Trebouxia* and *Nostoc* are considered the most frequent genera [4]. They are generally isolated and maintained on Bold's basal medium (BBM), BBM amended with high nitrogen or on Trebouxia medium [16].

Xanthoria parietina is a broadly distributed foliose lichen, it can be found on rocks, walls, roofs and stems of shrubs and trees [17]. It is characterized by the production of parietin which is an anthraquinone responsible for its yellow-orange color and the protection of the photobiont from high radiation [18]. Parietin was also found to have antimicrobial and anti-cancer effects [10]

Due to its wide spread, *Xanthoria parietina* became a model species in lichenology; it was extensively studied, found to be amenable to in vitro cultivation [19] and was the first lichen to be subjected to total genome sequencing [20].

This work aims at studying *Xanthoria parietina* collected from the Egyptian habitat and obtaining axenic cultures of its two symbionts

2. Materials and methods

1. Collection of samples

2. Lichen samples were collected from Ash Shehabeyah, Al Burlos, Kafr El Sheikh Governorate, Egypt (latitude: 31.539117, longitude: 31.294286) intact as found grown on *Calligonum polygonoides* old shrub and stored at 4°C till future analysis.

3. Lichen identification

Lichen identification was carried out on the basis of morphology and performance of spot tests. The external morphology of the whole lichen was examined directly using a stereo microscope (Olympus SZ-PT, Japan). Also, three spot tests were performed, few drops of each reagent were added to the lichen thallus and color change was observed [5].

K-test: 10% KOH

C-test: Sodium hypochlorite (NaOCl)

KC-test: 10% KOH followed immediately by drops of (NaOCl)

Studying the lichen anatomy

In order to study the lichen anatomy, thin sections were prepared following the method of Leśniewska *et al*. [21].

A. lichen fragments were soaked for 24 hours in a mixture of 96% ethanol, glycerol, and distilled water in 1:1:1 ratio then they were dehydrated by putting them in a series of different ethanol concentrations (10% to 96%) followed by a mixture of xylene and absolute ethanol at ratios of 1:2, 1:1, 2:1 (fragments were left in each solution for 30 minutes), after that they were placed in pure xylene for 45 minutes then another two times (fragment were left in each for an hour).

B. fragments were saturated with paraffin by placing them on cold paraffin in an evaporating dish containing xylene and were placed in a thermostat at 65°C. After

two hours, the paraffin melted and the fragments fell to the bottom of the vessel. C. fragments were transferred to a preheated metal mold that was covered with glycerol and filled with hot paraffin, then the mold with paraffin was slowly immersed in water with ice. After about 15 minutes, the paraffin block was removed from the mold and small pieces with

the fragments were cut out from it in a pyramid shape.

D. paraffin pyramids containing fragments were fixed with a hot scalpel to the microtome base and 15 µm thick cross sections through both the thallus and apothecia (the fruiting body of the mycobiont) were made using a rotatory microtome. For microscopic examination, slides were covered with heated chicken egg white that served as a binding agent and sections were transferred onto a drop of water on each slide then slides were allowed to dry at 36°C. later, sections were stained by lactophenol and examined under microscope at 40X, 100X, 200X,400X.

4. Isolation of the two symbionts

Prior to isolation of the mycobiont, small segments of the lichen (both the thallus and the apothecia) were cut and surface sterilized by placing them in 70% ethanol (for 5 seconds), 4% sodium hypochlorite (90 seconds) then washed by sterilized dist.H_2O [22]. Next, these fragments were transferred on PDA medium (Potato Dextrose Agar: potato infusion 200 g/L, dextrose 20 g/L, and agar 20 g/L) under aseptic conditions and incubated at 28 °C for 2 weeks. After multiple purification steps, axenic culture was obtained and the mycobiont was preserved on PDA slants at 4°C.

For the photobiont isolation, small segments of the lichen thallus were washed then homogenized in 1 ml sterilized dist.H_2O. Afterwards, a loopful of this suspension was streaked on BBM and 3N BBM media and incubated at 26°C 16/8 hrs light/dark cycle [2].

Colonies appeared after approximately one month and an axenic culture was obtained after numerous subcultures.

3. Results and Discussion

The lichen was positively identified as *Xanthoria parietina* depending on growth forms and the type of fruiting bodies as described in several identification keys and based on the results of spot tests where color change is an indicator for presence of substance produced exclusively by lichens [4, 23, 24].

The thallus was found to be smooth, yellowish and lacking cilia whilst apothecia were found rounded, stalked, concave with average width of 2.4 mm and deep orange color as shown in figure 1.

Upon the addition of 10% KOH on the lichen thallus, red color was developed which indicates the presence of anthraquinone pigments; parietin in case of *Xanthoria parietina.* No color change was observed in both C-test and KC-test (figure 1).

Examination of the lichen cross section clearly describes its internal structure; an upper cortex present in direct contact with outer environment and formed by the fungal filaments containing spores and

responsible for the reproduction in the lichen, the upper cortex is followed by a layer of algal cells which were found

heteromerously distributed and integrated with the horizontally arranged fungal hyphae in medulla. Finally, there is a lower cortex layer of fungal hyphae which comes in contact with the lichen substrate completing the so-called sandwich structure of foliose lichens as shown in figure 2.

After more than two weeks of surface sterilized lichen thallus incubation on PDA at 28 °C, the mycobiont hyphae began to grow. It was noted that its growth was relatively slow in comparison with other fungal species. A three-week old mycobiont culture presented in figure 3.B shows the white fluffy appearance of fungus hyphae.

The photobiont growth was much slower than the mycobiont and other free-living microalgae. BBM medium containing triple the amount of nitrogen present in the original BBM medium (3N BBM) was found to enhance the alga growth more than standard BBM. Future subcultures during the purification procedure was carried out on 3N BBM and after 9 months, an axenic culture was obtained (figure 3.A

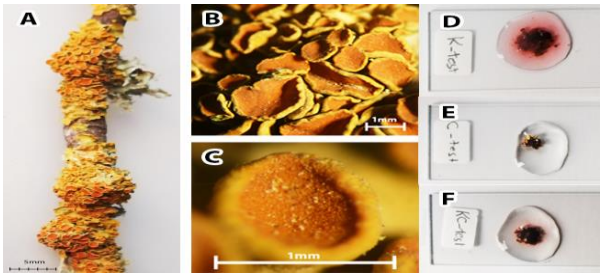

Fig 1. (A) *Xanthoria parietina* growing on *C. polygonoides* branch **(B&C)** External

morphology of *Xanthoria parietina* under stereo microscope showing the leaf like structure of the thallus and its marginal lobes **(D)** positive result for k- test (Development of red color) **(E&F)** negative C-test and KC-test respectively (no color change).

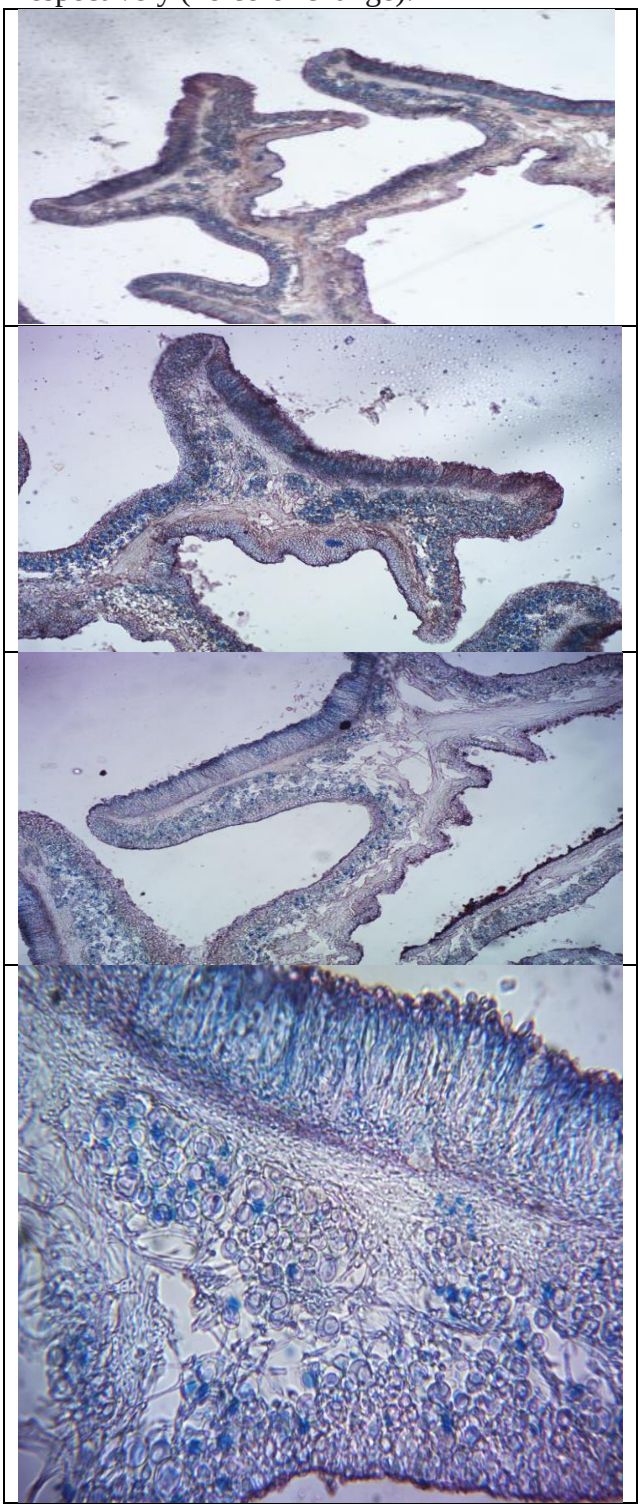

Fig 2. Cross section of *Xanthoria parietina* under microscope **(A)** at 40X**, (B)** at 100X**, (C)** at 200X**, (D)** at 400X where 1. represents the upper cortex, 2. Algal cells, 3. Medulla, and 4 is the lower cortex

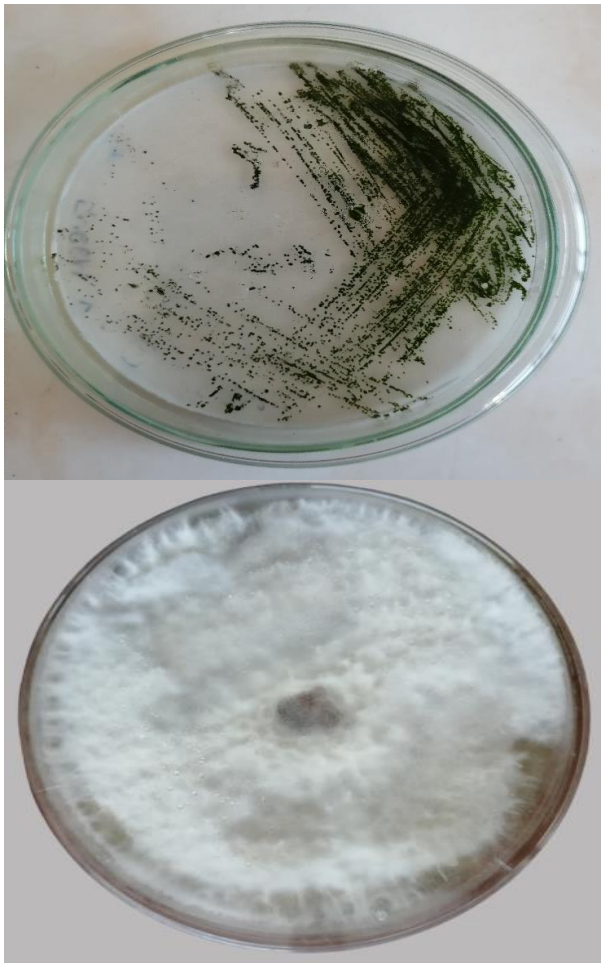

Fig 3. (A) One-month old photobiont culture on 3N BBM medium, **(B)** Three-weeks old mycobiont culture on PDA mediu

Discussion

lichens and probability of their Lichens are promising sources for diverse bioactive compounds but their very slow growth rate poses a difficult obstacle in the way of their commercial exploitation. In vitro cultivation of both symbionts individually or their co-culture under known laboratory conditions provides a worthy production of secondary metabolites application at large scale.

Lichen samples showed typical morphology of *Xanthoria parietina* thallus and apothecia, their average width was found to be 1-2 mm which is consistent with McCune estimation of lobes width between 1 and 4 mm [25]

Spot tests are simple, inexpensive yet very important in lichen identification; in K-test, the thallus turns yellow then red in presence of aromatic aldehydes or red to purple with anthraquinone pigments. In C-test, the thallus turns red with dihydroxy phenols and green with dihydroxy dibenzofurans. In KC-test, the

thallus turns yellow with usnic acid, blue with dihydroxy dibenzofurans and red with depsides and depsidones [5]. The red color developed in K-test is shows the presence of an anthraquinone pigment, parietin, and color density is directly proportional its amount in the lichen.

Despite having diverse external morphology, lichen do share a similar internal structure. A dense upper cortex functions a shield against other organism and decreases light intensity that may harm the photobiont, a loose medulla of al hyphae holding the cells of the photobiont and an additional lower cortex of hyphae in foliose lichens e.g. *Xanthoria parietina* is present and comes in contact with the substrate the lichen grows on [26].

Unlike what have been stated by Muggia and his colleagues that isolation of mycobiont requires addition of certain supplements such as metals, vitamins and amino acids [27]

our results reveal the ability of a general economic medium for fungi (PDA) to support the growth of the mycobiont as well. This corresponds to the results of Zhang & Wei. 2011 and Felczykowska *et.al.* 2017 [14, 28]

Also, results show that no light provision is necessary for the isolation of the mycobiont which supports the results of Yoshimura *et.al*. (2002) and Cristian. (2013) regarding the successful cultivation of the mycobiont without light [19, 29].

Due to its slow growth rate, the photobiont was usually outgrown by other contaminants, this made the purification process very long and extremely difficult (9 months). Further characterization of the mycobiont and the photobiont in particular is required as it was found that the algal species can differ within the same lichen species according to their geographical distribution. *Xanthoria parietina* was found to be usually associated with *Trebouxia arboricola* and *Trebouxia decolorans* as photobionts [30, 31]

Conclusion

To our knowledge, this is the first report for work on lichens isolated from the Egyptian habitat. The symbionts integration within *Xanthoria parietina*'s thallus was studied and both symbionts were obtained in pure cultures. Further investigation might be carried out to explore possible applications of the secondary metabolites obtained from this lichen.

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