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UV-B Resistant Yeast Inhabit the Phyllosphere of Strawberry

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Authors' contributions

This work was carried out in collaboration between all authors. Author AGBC performed the experiments and analyzes and wrote the first draft of the manuscript. Author VNK corrected the first draft of the manuscript, helped with the discussion section and with the elaboration of the manuscript. Author TDZ helped with the phylogenetic analysis and reviewed the manuscript. Author MLS helped with UV-B tests. Author RSN helped with the development of the experiments. Author RTSF helped with the maintenance of the field experiments. Author RGT helped with T-RFLP analysis and reviewed the manuscript. ISM designed the experiments. All authors read and approved the final manuscript.

Short Research Article

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ABSTRACT

The increase on the worldwide influx of solar ultraviolet radiation (UV-B) has inflicted a considerable challenge, due to its deleterious effects to live beings and pose a special threat to phyllosphere communities. However, UV-B influence on epiphytic yeasts associated with agricultural crops remains limited. Main aim of the present study was to determine the effect of ultraviolet-B radiation on the epiphytic yeast populations associated with strawberry under field conditions. Thus, strawberries (*Fragaria* x *ananassa* Duchesne cv. Oso Grande) were grown under three different treatments: a) environmental UV-B, b) enhanced UV-B and c) decreased UV-B; thereafter, their yeast epiphytic populations were analyzed by T-RFLP prior to yeast isolation, identification and *in vitro* test for the sensitivity against UV-B. Our results demonstrated that UV-B radiation did not significantly affect the strawberry epiphytic yeast populations. However, isolates directly exposed to radiation, generally revealed morphological abnormalities and a diminishing

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value in the percentage of survival, although they remained constant after 240 min of exposure. The increase in UV-B radiation was not sufficient to affect the dynamics and composition of epiphytic yeast communities from strawberry, there was a clear morphotype shift towards the selection of pigmented isolates.

Keywords: Global warming; field conditions; T-RFLP; leaf surface; phyllosphere.

1. INTRODUCTION

Currently, world climate has been negatively affected by the global warming influence. This worldwide phenomenon also affects the ozone layer in which its depletion usually leads to noxious effects [1]. The decrease in the ozone level is more evident in the polar regions, although its effects can be observed all around the world and as a direct consequence the daily influx of solar radiation has increased on many ecosystems [2]. Within these radiations, ultraviolet-B (UV-B; 290-320nm) is one of the most harmful to biologically active molecules due to its direct absorption by nucleic acids and proteins [3-5] and therefore, poses a special threat to living organisms.

Plants can negatively respond to UV-B radiation and leaf surfaces (phyllosphere) are constantly exposed to this type of radiation [6-7]. Phyllosphere harbors a wide diversity of microorganisms and its population density is characterized by constant fluctuations in nutritional and physical conditions [8-9]. Among the phyllosphere inhabiting microorganisms, yeasts are considered as important epiphytic colonizers due to their ability to adapt to many nutritional and environmental conditions [10-11]. These organisms have the capacity to adapt readily to harsh environments, due to their ability of reorganization of gene expression [12]. The repair mechanisms displayed by yeasts are different from other organisms: radiation exposure leads to cell response activation, regardless DNA damages, which trigger the physiological response that generates a faster signaling system and, consequently, a more effective repair mechanism [13].

When such organisms colonize the phyllosphere, they offer protection to the host plant through natural biological control. This occurs due to the establishment of a symbiotic relation with plants, as they interact with other microorganisms as well as phytopathogens [14]. Although the constantly increasing UV-B has led to many researches into phyllosphere microbial responses to this waveband [15], its effect on yeast epiphytic communities of agricultural crops is still scarce. For instance, UV radiation has been reported to change the diversity and/or distribution of bacteria on maize (*Zea mays*) [16] and peanut (*Arachis hypogea*) [15]; fungi on pedunculate oak (*Quercus robur*) [17] and microbial population on *Lactuca sativa* [18].

Thus, considering the possible impacts caused by climate changes, this work aimed to test the hypothesis that UV-B radiation would have an effect on epiphytic yeast communities associated to strawberry in field conditions as well as to determine the sensitivity of some yeasts exposed to this wavelength (280-320nm).

2. MATERIALS AND METHODS

2.1 Experimental Design

The experiment was conducted in an area of 30x40 m in Embrapa Environment (Jaguariúna, São Paulo, Brazil). In this area nine parcels were assembled and bounded by galvanized steel structures (1.20m wide, 2.30m long and 1.80m high). In each plot, seedlings of strawberry cv. Oso Grande were transplanted during the stage of leaf development, with the third leaf unfolded, under the following three treatments: 1) "decreased UV-B" (negative control) which received a polyester filter (0.125 mm thick; DuPont Co.) over the whole plot to block wavelengths (λ) less than 320 nm radiation from natural sunlight; 2) "environmental UV-B" (positive control) with UV-B and UV-A radiation from the sun; and 3) "enhanced UV-B" with extra UV-B radiation provided by eight fluorescent 40 W lamps (Model UV, Q Lab Corporation) spaced apart by 250mm and surrounded by cellulose acetate filter (type crystal, 0.10 mm thick) to block the UV-C radiation (λ<280 nm). These treatments were performed in triplicate with randomized blocks as shown in Fig. 1A.

The filters were changed every seven days to avoid the effect of photodegradation caused by UV-B radiation. The distance from the top of the plants were kept constant (30cm) provided by the sheave system that supported the structure of the lamps. The use of programmable timers installed in electrical panels with dimmable reactors lit the lamps with different light intensities from 09:00 am to 05:00 pm, i.e., the time at which the sun emits the highest incidence of radiation (12:00 pm) and considering the different intensities that occur during the day (Fig. 1B).

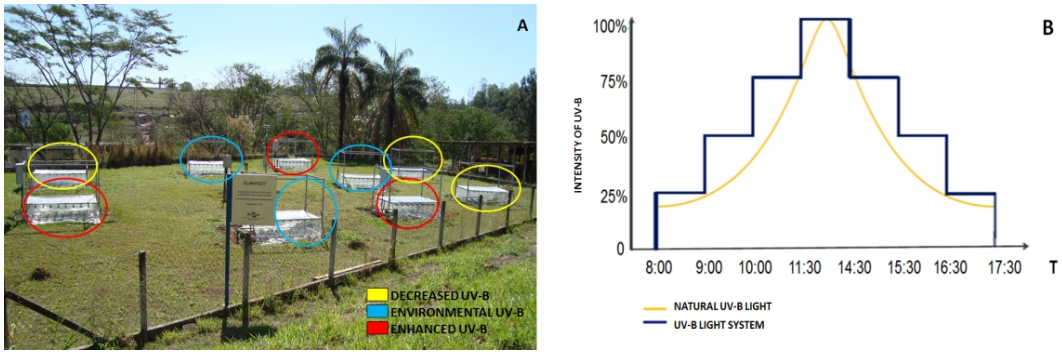

Fig. 1. A) View from the field experiments indicating the three different treatments (yellow - decreased UV-B, blue - environmental UV-B and red - enhanced UV-B); B) Comparison between the incidence of solar UV (yellow) and incidence of UV-B provided by the lamps (blue)

The incident radiation on plants was measured by using a spectroradiometer (Ocean Optics ® Model USB2000 + rad). Also, the lamps of the "increased UV-B" treatment provided, on average, 266mWm² during the months of July and August, according to the method of Quaite et al. [19].

2.2 Terminal Restriction Fragment Length Polymorphism (T- RFLP) Analysis

DNA was extracted from the surface of adult strawberry leaves after washing them in sterile 200ml NaCl solution (0.85%) with a drop of Tween 80. The samples were subjected to ultrasound for 30s and to 60 min of agitation at 150rpm and then centrifuged at 13,000 xg, resuspended in 1 ml of ultrapure Milli-Q water and subjected to successive centrifugation steps at 6,000 xg for 5 min until the achievement of a microbial pellet. The DNA extraction was performed as described by White et al. [20] and the fungal internal transcribed sequence (ITS) region was sequenced by using the following specific primers: ITS1-forward (5' TCCGTAGGTGAACCTGCGG 3', with 6-FAM – Carboxyfluorescein added to the 5' end) and ITS4-reverse (5' TCCTCCGCTTATTGATATGC 3´) [20]. Each reaction contained a mixture of 1µl of DNA, 3.0mM magnesium chloride, 0.2mM of each dNTP, 20pmol of each primer, 1X PCR buffer and 2.5 units of Taq DNA polymerase and ultrapure Milli-Q water to a volume of 50 µl. PCR was performed in a thermocycler (PTC 200, MJ Research) following the recommendation of Gardes and Bruns [21].

The amplification products were cleaved with the restriction enzymes Hinf-I and Mbo-I, according to manufacturer´s instructions. The reaction was performed in a thermocycler (Applied Biosystems) at 37ºC for 90 s and 35 cycles at 65ºC for 30s. After digestion, the products were precipitated by the addition of 2ml of EDTA (125 mM), 2 ml of sodium acetate (3 M) and 50 ml of ethanol (100%) according to the method suggested by the manual of BigDye Terminator v3.1 Cycle Sequencing Kit. Samples were stored at 22ºC until they were analyzed. Terminal Restriction Fragments (T-RFs) analysis was performed using ABI PRISM 3500 Genetic Analyzer (Applied Biosystems). Data obtained from sequencing were analyzed with GeneMapper v.4.1 (Applied Biosystems) software. For T-RFs, a limit baseline of 50 fluorescence units was used to discriminate ''true'' peaks from the background noise in this technique. We examined T-RFs that were 50bp and 800bp. Peak heights were transformed into relative data (percentage of detection). T-RFLP profiles were compared among different samples using T-RF relative abundance (0.1%). Analysis of Similarity (ANOSIM) was performed with Bray-Curtis, offering a value ranging from -1 to 1. Values close to 0 indicate no differences among groups, while values close to 1 indicate a distinction among groups [22]. Non-metric Multidimensional Scaling (NMDS) graphs indicate the relative similarity of samples through distance ordering, in which similar samples are found to be very close. Analyzes were performed using Past 2.12 software [23].

2.3 Isolation of Epiphytic Yeasts

The first sampling of leaves was performed after 24 hours of radiation exposure, during the stage of beginning of flowering, with about 10% of the flowers open and the remaining samplings were performed with a 7-day difference up to the last sampling, in a total of five samplings. Twenty adult strawberry leaves from the upper part of the plant under three different treatments were excised, placed in sterile plastic bags and transported to the laboratory for processing. The methodology was based on Olive and Kevin [24], with some modifications. Briefly, leaves were aseptically cut into small disks of 16mm. Ten discs per treatment were added to 25 ml of sterile NaCl solution (0.85%) with a drop of Tween 80 per liter of solution. Subsequently, the samples were sonicated by using an ultrasound bath for 30s, kept under stirring for 60 min at 150rpm and serially diluted. Then, 100 µl of the 10^{-3} dilution were plated on YMA culture medium [25], supplemented with chloramphenicol ($0.1\mu g.ml^{-1}$) and incubated at 25ºC for 10 days. Colonies were selected based on their morphologies and kept on YMA plates at 4ºC.

2.4 ITS Gene Sequencing and Phylogenetic Analysis

After isolation, colonies of distinct morphologies were inoculated into 50 ml of YEPD culture broth (0.5% yeast extract, 2% peptone and 2% glucose) and kept under agitation for 48 hours at 25ºC. The cells were harvested by centrifugation (12,000xg for 5min) and genomic DNA was extracted following the procedure described by White et al. [20]. PCR amplification and ITS gene sequencing were achieved following the methods described previously. The quality of sequences was checked using FinchTV 1.4.0 program (Geospiza Inc.) and the sequences of each sample were manually aligned into a consensus sequence using BioEdit 7.1.3.0 [26]. Sequences were compared with entries deposited in the GenBank database. The ITS gene sequences were aligned manually using MEGA version 5 software [27] against corresponding sequences of closely related yeast species retrieved from the GenBank database. Phylogenetic trees and bootstrap analysis were inferred following the procedures described by Zucchi et al. [28].

2.5 Sensitivity of Strains to UV-B Radiation

The isolates were subjected to UV-B radiation (λ 280-320 nm) to determine the minimum dose required for growth inhibition of the colonies in the culture medium. The test consisted of exposing 100µL of cell suspension (10^4cell.ml^{-1}) inoculated at the center of Petri dishes containing YMA culture medium prior to radiation exposure. The plates were distributed in a chamber of UV-B radiation which held two fluorescent lamps (EL UVB -313, Q -Lab , USA) with a cellulose acetate filter (0.1mm, Málaga, SP) that inhibited UV-C spectrum (λ 280-290nm) installed 40 cm from the base. The incidence of UV-B obtained in the chamber was measured using a spectroradiometer (Ocean Optics Model USB2000 rad +) and calculated according to the method of Quaite et al. [19]. The lamps were switched on and left stabilizing for 30s before the UV-B radiation exposure. The isolates plates (without lids) were exposed in a range of 0 (control) to 240 minutes. The treatments were performed in triplicate and after the exposure the plates were incubated for 96 hours at 25 °C in the dark to prevent photoreactivation. After incubation, the determination of colony forming unity (c.f.u.) was performed, determining the dose of UV-B radiation that resulted in the inhibition of growth, thereby obtaining the percentage of survival of each isolate. *Escherichia coli* was used as a negative control due to it's highly susceptibility to ultraviolet wavelengths [29].

2.6 Statistical Analyzes

Data from T-RFLP data were analyzed according to Rees et al. [30] whereas the isolation data were analyzed through Tukey´s test at 5% of probability.

3. RESULTS AND DISCUSSION

3.1 T-Rflp Analysis

ANOSIM indicated that there were no significant differences between the treatments based on the profiles obtained by using the restriction enzyme *Hinf*-I (r=-0.048; p=0.642) nor those obtained with *Mbo*-I (r=-0.121; p=0.963). Furthermore, there was no significant difference between the used enzymes. It can be seen from Fig. 2 an absence of distinct groups, once samples from each treatment were randomly distributed. This clearly indicated that the structure of epiphytic yeast communities was not affected by the UV treatments. The low values of stress (<0.15) obtained for the enzymes *Hinf*-I (Fig. 2A) and *Mbo*-I (Fig. 2B)

corroborate with the reliability of the results. Although other studies have demonstrated that UV-B alters microbial communities [15-16,18], in a similar field experiment, the main factor involved in the differentiation of the structure of phyllosphere bacteria from soybean was its developmental stage, regardless of UV treatments (Sáber, unpublished). Also in field experiments, Stapleton and Simmons [31] observed that the genotype was more important than UV-B in explaining patterns of bacterial rRNA of microbial diversity on *Zea mays* (maize) leaves.

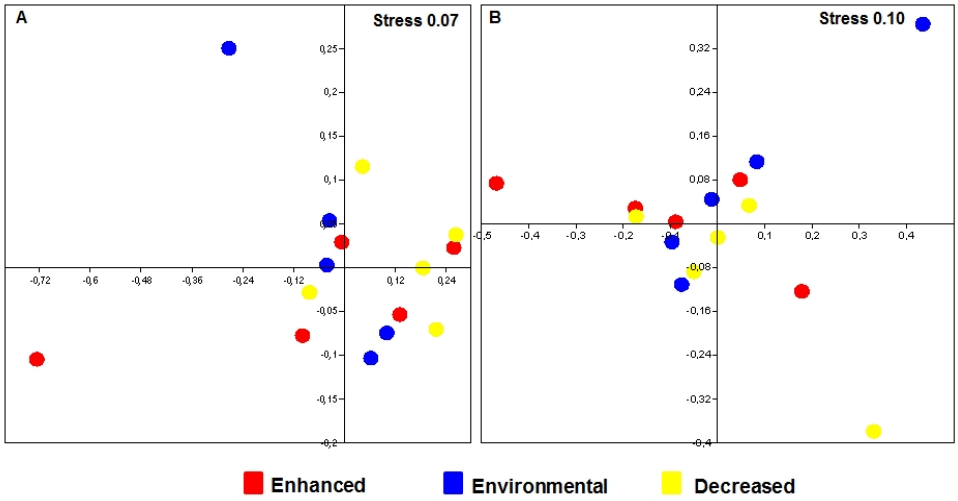

Fig. 2. NMDS analysis for T-RFLP profiles from epiphytic yeast communities. Comparison between enzymes *Hinf* -I (A) and *Mbo*-I (B)

3.2 Isolation of Epiphytic Yeasts

A total of thirteen yeast morphotypes were recovered from strawberry leaves. This result is in agreement with Guimarães [32] and Mautone [33] who also observed a relatively low density on the number of yeasts. The quantities of yeasts that colonize the phylloplane is constantly influenced by biotic and abiotic factors [34], and in general, varies according to the age of the plant, season of the year and time of the day [35-37].

The count of c.f.u.cm^{-2} did not display any significant difference when analyzed by Tukey´s test ($p>0.05$), indicating that the amount of yeasts present in the phylloplane was not affected by the different field treatments (data not shown). When leaf surfaces are exposed to high incidences of ultraviolet radiation, sensitive epiphytic microorganisms start to occupy sites that might protect them from radiation. These sites can be stomatal cavities, ridges, depressions between leaf cells and/or the base of trichomes [14].

Another feature that may favor the establishment of these populations under high incidence of UV-B is the production of carotenoids. In yeasts, the carotenoids are considered secondary metabolites and give them orange or red color [38-39]. They play an important role against oxidative damage caused by light [40]. According to Moliné et al. [41], white colonies are more vulnerable to UV-B than pigmented ones, showing that carotenoids play an essential role in photoprotection. A total of ten out of the thirteen morphotypes were pigmented and this feature may reflect the niche selection for colored species.

3.3 Phylogenetic Analysis

The thirteen morphotypes were placed within two phyla: *Ascomycota* (*n*=1) and *Basidiomycota* (*n*=12), in which there was a clear predominance of the latter phylum. A survey of epiphytic yeasts on leaves of Mediterranean plant species from Portugal yielded about 850 isolates, mostly of them with basidiomycetous affinity. Amongst the basidiomycetes, thirty-five strains showed the production of orange colonies [42]. Indeed, yeasts belonging to the phylum Basidiomycota have features that usually favor their colonization in the phylloplane, such as mucoid capsule, carotenoid pigments and wider potential for assimilation of carbon sources [11,43]. The isolates were also distributed into seven ITS gene clusters and were most closely related to six genera (Fig. 3).

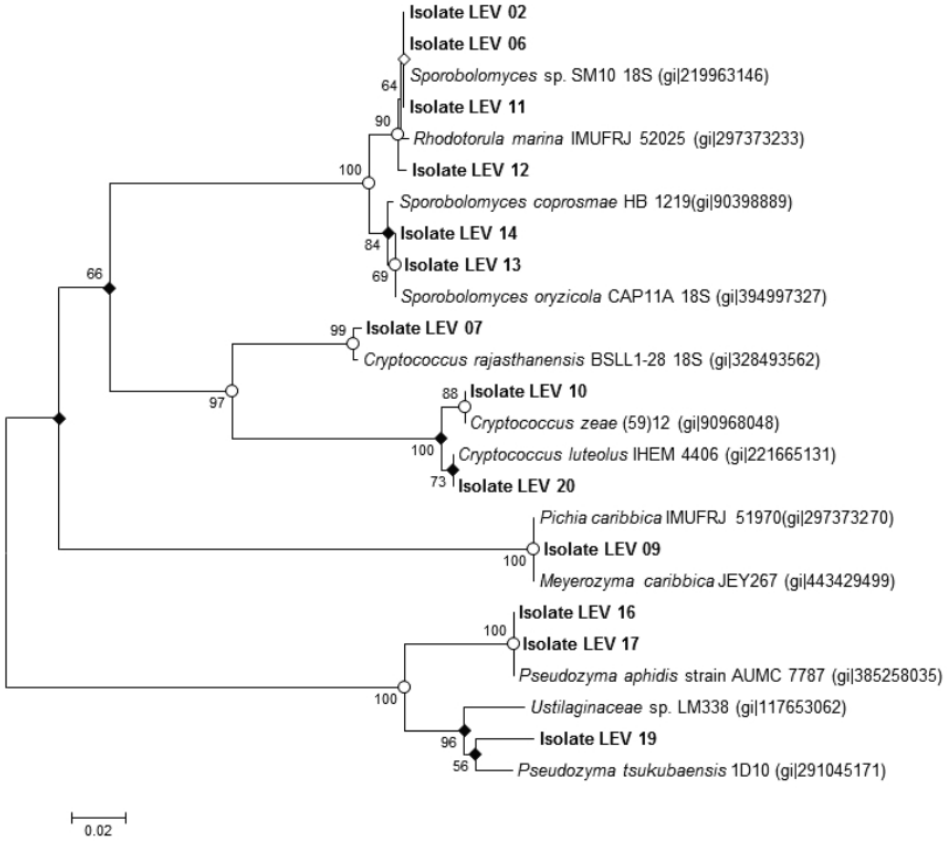

Fig. 3. Neighbour-joining tree based on partial ITS gene sequences showing relationships between yeast isolates from strawberry leaves and their phylogenetically close species. White circles indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making algorithms; the white and black diamonds stand for a branch which was recovered using the maximum-likelihood or maximum-parsimony tree-making algorithm, respectively. Numbers at the nodes are percentage bootstrap values based on a neighbor-joining analysis of 1,000 resampled datasets; only values above 50% are given. Bar 0.02 substitutions per nucleotide position

Isolates LEV 02, LEV 06, LEV 11, LEV 12, LEV 13 and LEV 14 were grouped into the same group and were closely related to *Sporobolomyces*, *Rhodotorula* and *Erythrobasidium*. Several authors have reported the frequent isolation of *Sporobolomyces* and *Rhodotorula* from the phylloplane of different plants [44-47]. The phylloplane presents polysaccharides as the main nutrients [48]. These genera show great affinity for the colonization of sites with high concentrations of this nutrient, favoring the establishment of these populations in leaf surfaces [49]. A striking feature observed in these genera is the orange or pink color due to carotenoids. It is suggested that pigment production by yeasts may favor the colonization on leaf surfaces exposed to UV-B radiation acting as a photoprotector [50].

Isolate LEV 09 was clustered together with ascomycetes genera (*Meyerozyma* and *Pichia*). Although the ascomycetes may represent important colonizing phylloplane species [51-52], they are usually found in lower amounts when compared to other genera [53]. On the other hand, members of ascomycetes are often found in larger amounts in other plant surfaces, such as fruits and flowers [52].

The genera *Cryptococcus* was closely related to isolates LEV 07, LEV 10 and LEV 20. This basidiomycota genus has been found in significant amounts on leaf surfaces [45-47]. Furthermore, this genus synthesizes light brown pigments, which provide greater protection against UV-B radiation [54-55]. Finally, isolates LEV 16, LEV 17 and LEV 19 were grouped with members of the *Pseudozyma* genus. Indeed, isolate LEV 19 formed a new phyletic line associated with *P. tskubaensis*; a position that was supported by neighbor-joining and maximum-parsimony tree- making algorithm and by a bootstrap value of 56%. There are few reports of the presence of these genera in the phyllosphere of plants [45-46, 53]. There are no reports on the behavior of the genera *Pichia*, *Meyerozyma* and *Pseudozyma* after exposure to high levels of UV-B radiation. Therefore, mechanisms of adaptation that are triggered under a stressful condition remain unknown.

3.4 Sensitivity of Strains to UV-B Radiation

Susceptibility test was performed with all strains, but only the results from isolates that displayed more resistance to UV-B than *E. coli* are shown (Fig. 4.). Differently from *E. coli,* isolates exposed to 2,636 $J.m^{-2}$ of UV-B radiation for 240 minutes, presented viable cells, with a survival percentage of 20 to 60%. Isolate LEV 09 presented the highest resistance, with survival of 79% after 240 min of exposure. On the other hand, LEV 16 showed a significant decrease in the percentage of viable cells after 30 minutes of exposure but it remained stable up to the final dose (2,636 $J.m^{-2}$; 240 minutes) with a survival rate of 16%, and therefore it was considered the most sensitive isolate among the selected resistant isolates.

It is possible to observe different patterns of sensitivity for yeasts within the same genus (LEV 02, 12 and 13). This clearly indicated that the mechanisms involved in the tolerance response to UV-B radiation are distinct for different species of the same genus. Morphological changes of colonies such as decrease of colony size (LEV 09 and LEV 16) and loss of pigmentation (LEV 17) or both (LEV 02, 12 and 13) were observed after exposure. According to Gunasekera et al. [14], these abnormalities are a clear response to UV-B radiation exposure that kill some fraction of the population. In relation to the pigmentation loss, UV-B radiation might have affected the carotenoid biosynthetic pathway. Microorganisms have developed several mechanisms to counteract the toxic effects of UV radiation, such as DNA repair mechanisms, accumulation of carotenoids, enzymes and antioxidants [56]. Yeasts produce a wide variety of carotenoid pigments that provide them

with different colors [57]. As previously discussed, yeasts with carotenoids tend to show higher tolerance to UV radiation [41]. The accumulation of a specific type of carotenoid (torularhodin) in *Rhodotorula mucilaginosa* increased its resistance to UV-B radiation [58]. Furthermore, the accumulation of mycosporine-like aminoacids, synthesized during intense UV radiation exposure, which can increase survival of yeast cells on these harsh conditions [50, 59]. These compounds are known for their UV protection as well as their antioxidant activity [60] and their sunscreen activity is being fully investigated [61]. Furthermore, yeasts have a specific group of genes involved in environmental stress response (ESR) [62]. These genes suffer changes in their expression during the cells exposure to stressful environmental conditions [63] and are usually related to the protection of vital functions within the cell by decreasing the synthesis of proteins, assisting in energy saving [64]. This mechanism favors the resistance of yeast cells exposed to a normally lethal dose of UV-B radiation and also help their survival in different environmental conditions. However, the final answer generated towards a new stressful condition is variable among species.

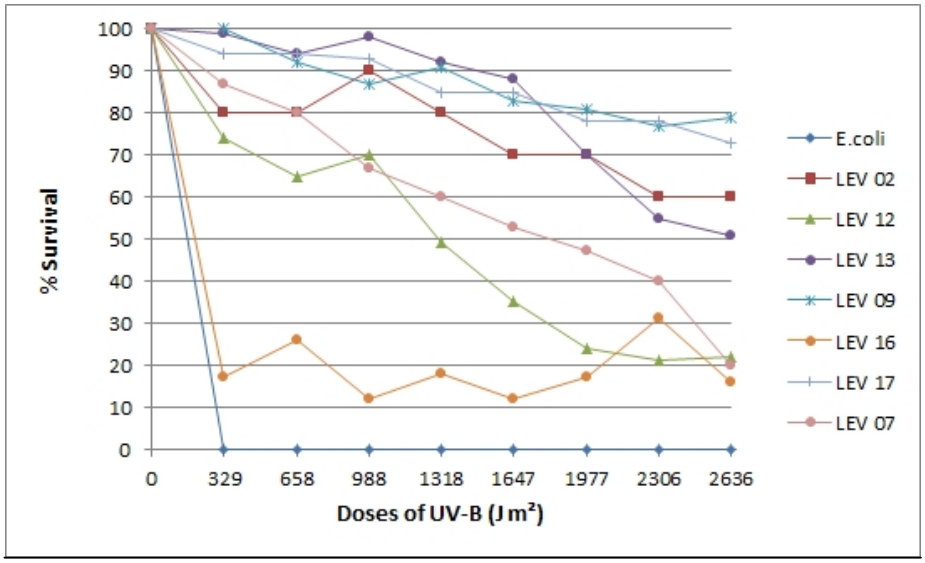

Fig. 4. Percentage of survival of seven strains after exposure to nine different UV-B doses. *E. coli* was used as a negative control

4. CONCLUSION

In summary, this study has unveiled evidence that the yeast community found in the phyllosphere of strawberry is somewhat resistant to UV-B. Since the leaf surface of this is exposed to direct sunlight the selection of organisms capable to withstand this pressure on a daily basis is expected. Although we do not know which mechanism is responsible for this, we have also shown that the organisms isolated from this environment have the capacity to resist to high dosages of this radiation. Thus, it is likely that this resistance provides the epiphytic community from strawberry the ability to survive even under increased exposure of UV-B radiation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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