

Evolution under Ecological Stress: Fungal Divergent Adaptive Melanization at Evolution Canyons in Israel

Eviatar Nevo*

Institute of Evolution, University of Haifa, Mount Carmel, Haifa, Israel

*Corresponding author: Eviatar Nevo, Institute of Evolution, University of Haifa, Mount Carmel, Haifa, Israel, Tel: +97248240448; E-mail: nevo@research.haifa.ac.il

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Abstract

Environmental stress, both biotic and antibiotic, is a major driving force of evolution. I revisit our earlier study on the effects of solar radiation on melanin concentration in the soil fungus *Aspergillus niger*, at Evolution Canyon I (EC I), Mount Carmel, the first and most explored of four Evolution Canyons, investigated in Israel. The Evolution "Canyon" model is a microsite, optimal for studying biodiversity evolution, adaptation and incipient sympatric ecological speciation. At AC I, the tropical African (AS), south facing slope (SFS) receives 200-800% more solar radiation than the temperate European slope. We measured conidial melanin concentration of 80 strains of *A. niger* from the opposite slopes and from sunny versus shady micro niches in each slope. *A. niger* on AS had threefold more melanin than on the ES. As expected, AS strains of *A. niger* resisted UVA irradiation better than ES strains and the same was demonstrated by *A. niger* strains from sunny as compared with abutting shady micro niches on the ES. We concluded that melanin shelters *A. niger* adaptively from solar radiation. We conduct reciprocal transfer experiment of *A. niger* from AS to the ES and vice versa, in an attempt to highlight melanin evolutionary dynamics on the opposite slopes.

Keywords: Environmental stress; Evolution; Evolutionary dynamics

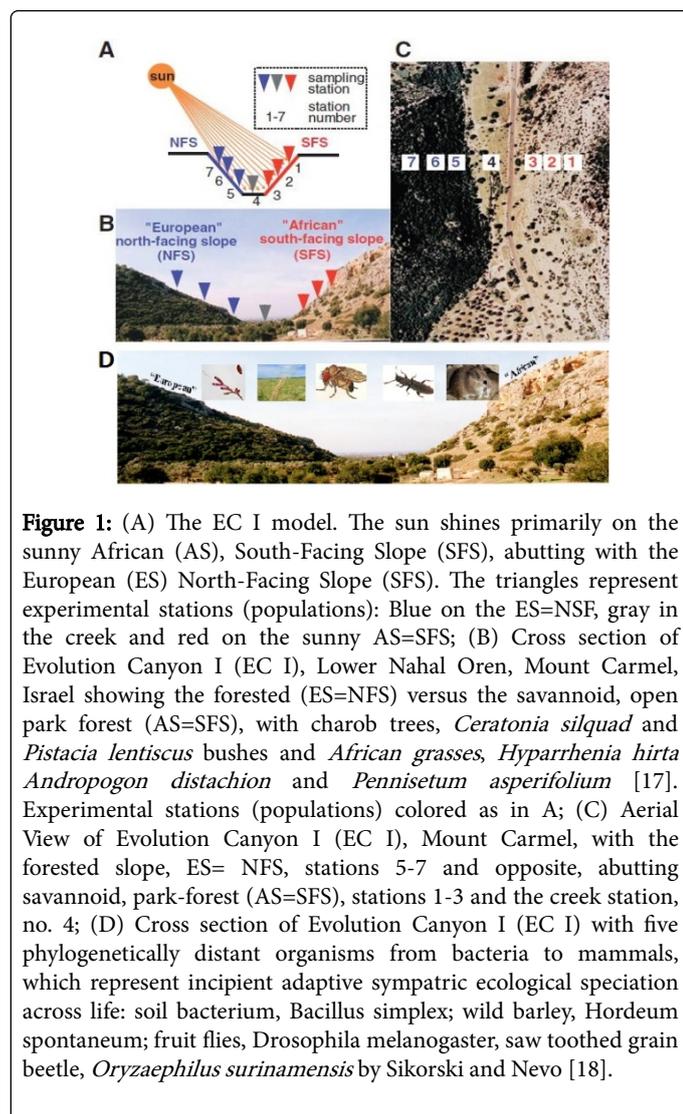
Introduction

Stressful environments are major drivers of evolution [1,2]. This is true both for abiotic and biotic stresses at macro and microscales, like the "Evolution Canyons" (EC) by Nevo [3-9]. The EC research project is a long-term project that started in 1990 [2]. The Evolution Canyon model is investigated in Israel in four EC's Carmel (EC I); Galilee (EC II), Negev (EC III) and Golan (EC IV) [3]. Evolution Canyon I, in lower Nahal Oren, Mount Carmel, is the most advanced EC studied to date (Figure 1, A-*+D).

The goals of studying the EC model are to highlight evolution in action, encompassing biodiversity evolution, adaptation and incipient ecological sympatric speciation across life from bacteria through fungi, plants and animals reviews in Nevo [3-9]. The EC model consists of two abutting slopes: The "African"(AS), south facing slope(SFS) is a tropical, savannoid, hot and dry slope, due to the higher solar radiation on it, 200-800% higher than on the "European" (ES), north facing slope (NFS) which is temperate, forested, shady, cool and humid slope (Figures 1A-1D). I will refer to the tropical slope as AS=SFS and to the temperate slope as ES=NFS to avoid confusion [10-12].

Fungi biodiversity has been studied extensively in EC I Wasser et al. [10] and across all ECs in Israel by Grishkan et al. [13-16]. The spatiotemporal distribution of soil microfungi in Evolution Canyon I, Mount Carmel, has been studied by Grishkan et al. [14]. A total of 166 micromycetes species belonging to Deuteromycotina (142 species), Ascomycotina (9) and Zygomycotina (15) were isolated. The highest species number and diversity were found on the African slope (AS=SFS).

The greatest melanic differences was identified in the intraslope differences in the comparison of sunny open niches with shady niches, meters apart, under Carob trees, *Ceratonia siliqua*, on the AS=SFS. The dominance of Fusarium species and high frequency dark colored species, including *Aspergillus niger* (Figures 2A and 2B), characterized sunny soils, also on ES=NFS. Penicillium species prevailed in the shady slope ES=NFS, but also occur in the sunny slope (AS=SFS) in shady niches. The results of this study demonstrated clearly the qualitative and quantitative effects of microclimate both inter slopes and intra slopes. However, the sun-shade differences were accentuated on the ES=NFS: The percentage of *A. niger* on the forested ES was 27% in the sunny niche but only 19% in the shady niche. By contrast, it was 37% and 35% in the sunny and shady niches, respectively, on the savannoid AS=SFS by Grishkan et al. [14]. Clearly, micromycetes are very sensitive to divergent microclimatic stresses. The higher species diversity on the African hot and dry slope (AS=SFS) is caused by its larger climatic and ecological niche diversities. More microclimatic homogeneity on the European slope (ES=NFS) resulted in a stable micromycetes complex. The major fungal divergence is due to sunny-shady microsites, especially on the ES. Most soil fungi at EC are cosmopolitans. Seasonal variation was inferior to spatial variations (Figure 1).

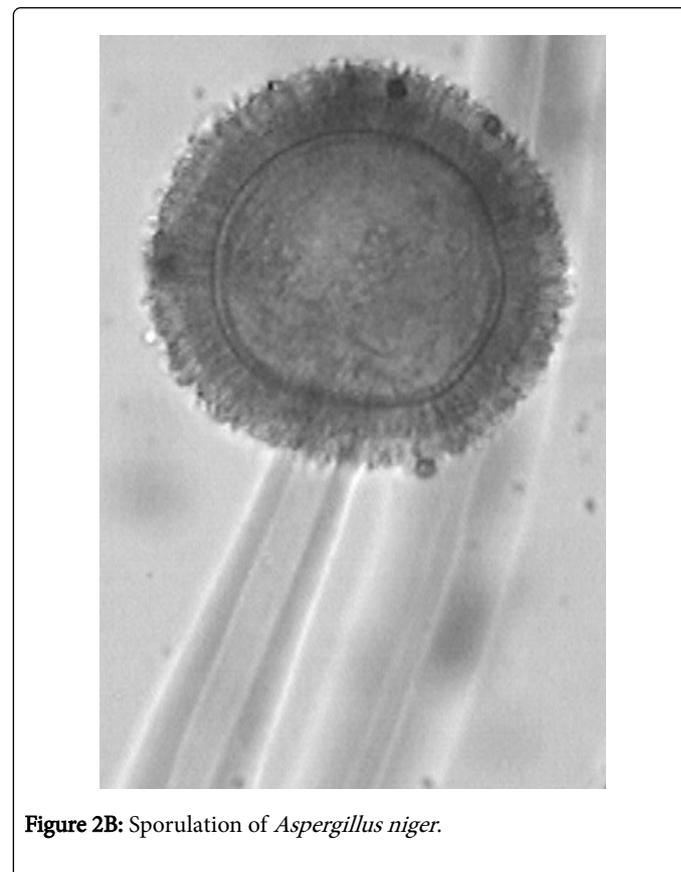
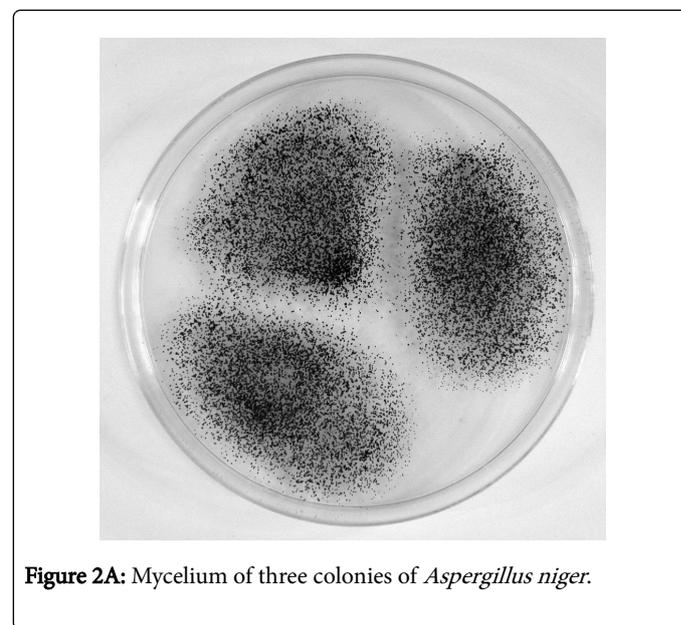


Aspergillus niger Melanization Strategies at Evolution Canyon

Aspergillus niger (Figures 2A and 2B) shows (A) colonies and (B) sporulation, of *A. niger*, the black mould. The genus *Aspergillus* consists of a few hundred mould species found in various climates. *Aspergillus* species are generally considered asexual, although sexual species are known [19]. The section Nigri includes black or near black colored conidia and 26 species, with *A. niger* being the most common species isolated. Aspergilli are ubiquitous in nature. They are geographically widely distributed, living in diverse habitats and thrive on numerous substances. *A. niger* is prevalent as a saprophyte, growing on dead leaves, often associated with organic material and is ubiquitous in soil, as in EC.

Remarkably, however, it also grows in the hypersaline Dead Sea (~34-35% salinity) by Selezka et al. [20]. Differential adaptive growth rates of *A. niger* strains in the Dead Sea and strains growing on the opposite slopes of Evolution Canyon by Perl et al. [21] were unfolded by Singaravelan et al. [22]. *A. niger* is one of the most important microorganisms used in biotechnology Schuster et al. [23]. The

primary uses of *A. niger* are for the industrial production of enzymes and organic acids by fermentation, particularly citric acid and gluconic acid. The 33.9 megabase genome of *A. niger* was sequenced by Pel [24], demonstrating a *Aspergillus* high level of synteny with other sequenced aspergilli.



***Aspergillus niger* in Evolution Canyon I, Mount Carmel**

Melanin concentration

The adaptive melanin response of *A. niger* to UV radiation stress at Evolution Canyon, Mt. Carmel, has been analyzed by Singaravelan et al. [22]. The divergent AS=SFS and ES=NFS, opposite and abutting slopes of Evolution Canyon, differ sharply micro-climatically by Pavlicek et al. [25] reflecting a continental scale interslope divergence by Nevo [3]. The AS=SFS, tropical, savannoid, sunny and dry biome, receives 200%-800% higher solar radiation than the ES=NFS temperate, forested, shady and humid abutting contrasting biome. This model presents sharp interslope divergence in solar radiation.

How does *A. niger* react, in terms of its melanin content on the opposite slopes, to this differential in solar radiation? By Singaravelan et al. [22] Measured Conidial melanin concentration was measured in 80 strains from EC I, from the opposite slopes, the upper soil layers (1-5 cm deep) using a spectrophotometer. The results indicated that the mean conidial melanin concentrations of AS strains were threefold higher than in ES strains. Remarkably, the AS strains' resistance to UVA irradiation was better than that of ES strains (Figures 3A and 3B). The conidia melanin of *A. niger* strains from sunny and shady microniches on the predominantly sunny AS and the predominantly shady ES clearly showed that shady conditions on the AS have no influence on melanin selection. By contrast, the sunny strains from ES displayed higher melanin concentrations (Figure 3B).

UV Resistance

We also investigated whether the adaptation of higher melanin concentration in the AS strains indeed convey increased fitness (Figures 4 and 5). The relative culturability after UVA irradiation of the AS strains (79.9%+1.36) was significantly higher than that of the ES strains (57.75%+3.71, non-parametric Mann-Whitney sum Test (T=474, n1=18, n2=19, p<0.001). The positive relationship between relative conidial culturability (after exposure to UV) and melanin concentration was indicated also by linear regression for both sunny and shady microniches by Singaravelan et al. [22] (Figures 5A and 5B).

Discussion

Overview of the experimental results of *A. niger* at evolution canyon

The Singaravelan et al. [22] study is pioneering in demonstrating experimentally a specific adaptation in soil fungi, indicating that melanin concentration in different strains at microscales (AS versus ES and sunny versus shady on the ES) respond adaptively to differential solar UVR selection pressures using the melanin as a shelter against DNA disruption by UV radiation. Highly melanized AS strains indeed resist UVA irradiation better than ES strains. Importantly, different interslope and interniche variation in melanin concentrations were due to differential exposure of UV radiation rather than due to soil temperature in both cases of interslope and interniches (Figures 3A and 3B) [19].

Thus, UVR and melanin have causal relationships. The higher is UV the higher is melanin concentrations. Melanin increases the fitness of strains under high UV radiation. Shady conditions on AS micro niches

did not affect melanin concentration. The levels of melanin in AS and ES micro niches (Figure 3B) and the experiments illustrated in Figures 4 and 5, clearly demonstrate that differences in microniche melanin concentrations resulted from different exposures of UV radiation, rather than due to soil temperature.

Melanization Adaptation in Fungi to Solar Radiation

Melanin is a pigment of high molecular weight involving various types of phenolic or indolic monomers, usually associated with a protein and often with carbohydrates [26,27]. Melanin is located in the cell wall. Melanin pigments abound across life evolution indicating their ancient origin. Melanized numerous fungal spores were found in Early Cretaceous period, when the earth lost its protective screen against cosmic radiation [28]. The radio protective role of melanin pigments is extensively discussed by Grishkan et al. [29-31]. Fungal spores abound in the atmosphere, reaching 104 spores per m³, including *A. niger* in Africa by Prospero et al. [32]. Dark fungal spores increase in the atmosphere following increases in UV-B radiation by Grishkan et al. [29-31].

Overwhelming dominance of melanized fungi also occur on plant leaf surfaces, adaptively defending against UV solar radiation by Grishkan et al. [29-31]. Likewise, in rocks covered by fungi and cyanobacteria. *Cromyces* fungal strains with heavily melanized walls resistant to UV- B were suggested as good candidates for space flight simulating Mars conditions, including space vacuum, solar UV and cosmic radiation by Grishkan et al. [33]. Dominance of dark colored microfungi abound in desert soils [27] and her references, including the Negev Israeli Desert, where melanized microfungi comprised 55% of fungal species composition and showed high abundance of melanized fungal species (58-77%). Fungal spores combine adaptive multicellular spore morphology with melanin pigmentation in desert biota. Such spores can survive under UV radiation, extreme temperatures and drought. This is demonstrated in EC I by *A. niger* strains after 4 h exposure to UV irradiation plotted against conidial melanin concentration on AS (Figure 4, comparing AS and ES); and in sunny versus shady microniches on AS=SFS (Figure 5A) and on ES=NFS (Figure 5B) at Evolution Canyon I (EC I) a period. Vertical microfungi distribution in the Negev desert communities illustrates the association between solar radiation and structure of microfungi communities [28].

Uppermost fungal communities, (from 0-0.02 cm), are overwhelmingly dominated by melanin containing fungi, with many celled conidia. By contrast, a layer only 0.2 cm below is inhabited primarily by light colored species. The same phenomenon of vertical divergence in melanin-containing fungi prevailed also in the uppermost soil layer of the sunny exposed soil in AS=NFS, of two Evolution Canyons I and II Mt Carmel (EC I) and in western Upper Galilee (EC II). Both ECs demonstrate in local Mediterranean microsites, regional melanin phenomena as in the in the Negev desert, at EC I in Mount Carmel Grishkan [14] and at EC II, in Galilee by Grishkan et al [34]. However, quantitatively, the uppermost prevalence of melanic fungi in the two Mediterranean microsites of EC I and EC II, was two-fold lower than in the desert and was subjected to seasonal variations: melanics did not appear in the autumn and winter. Moreover, northern Canyons' (EC I, II) spores were mainly small and one-celled, such as in *A. niger*, as against the multi-celled spores in the extreme Negev desert.

The result indicated that mean conidial melanin concentrations of *A. niger* from the high solar radiated AS-SFS was threefold higher than in ES=NFS strains, separated on average, by only 250 m (Figures 3A and 3B).

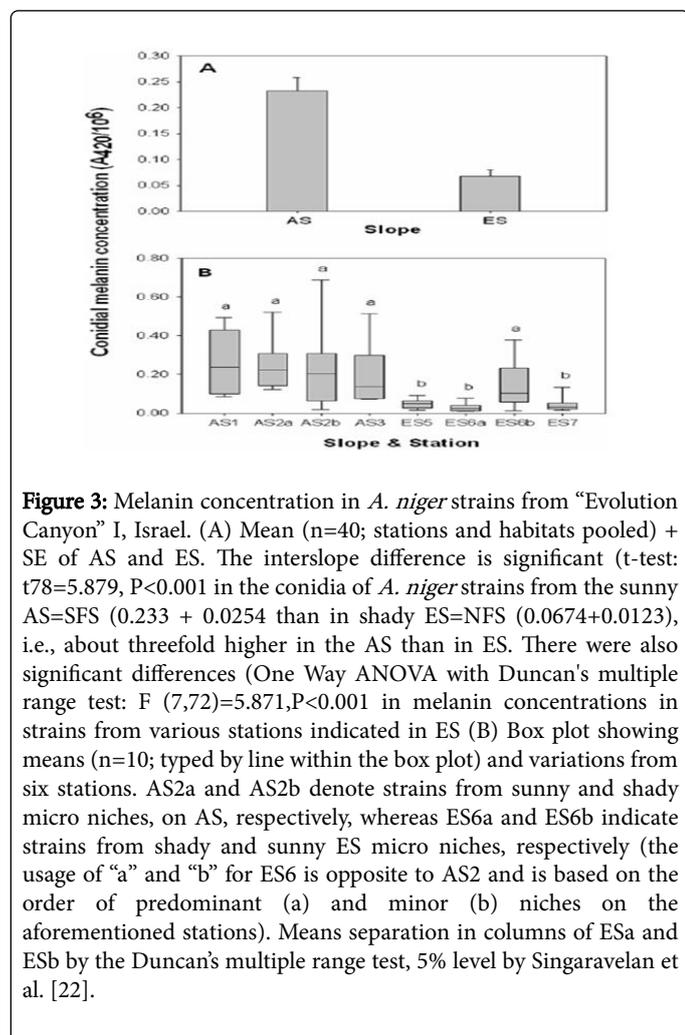


Figure 3: Melanin concentration in *A. niger* strains from “Evolution Canyon” I, Israel. (A) Mean (n=40; stations and habitats pooled) + SE of AS and ES. The interslope difference is significant (t-test: $t_{78}=5.879$, $P<0.001$ in the conidia of *A. niger* strains from the sunny AS=SFS (0.233 + 0.0254 than in shady ES=NFS (0.0674+0.0123), i.e., about threefold higher in the AS than in ES. There were also significant differences (One Way ANOVA with Duncan's multiple range test: $F(7,72)=5.871$, $P<0.001$ in melanin concentrations in strains from various stations indicated in ES (B) Box plot showing means (n=10; typed by line within the box plot) and variations from six stations. AS2a and AS2b denote strains from sunny and shady micro niches, on AS, respectively, whereas ES6a and ES6b indicate strains from shady and sunny ES micro niches, respectively (the usage of “a” and “b” for ES6 is opposite to AS2 and is based on the order of predominant (a) and minor (b) niches on the aforementioned stations). Means separation in columns of ESa and ESb by the Duncan's multiple range test, 5% level by Singaravelan et al. [22].

the ES and AS had relatively the same growth rate at 40,000 rads exposure. At 400,000 rads exposure, growth rates remained much the same for both the ES and AS exposed isolates as they were at 40,000 rads exposure. Above 106 rads, growth ceased but recovery occurred at divergent times for individual isolates of the same species from the opposing slopes of Evolution Canyon I by Voltz et al. [12] (Figure 4).

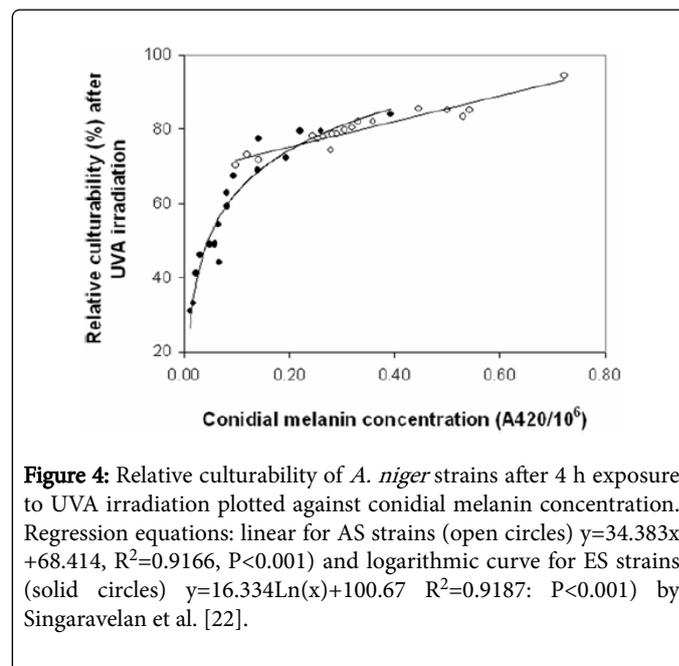


Figure 4: Relative culturability of *A. niger* strains after 4 h exposure to UVA irradiation plotted against conidial melanin concentration. Regression equations: linear for AS strains (open circles) $y=34.383x + 68.414$, $R^2=0.9166$, $P<0.001$ and logarithmic curve for ES strains (solid circles) $y=16.334\ln(x)+100.67$ $R^2=0.9187$; $P<0.001$ by Singaravelan et al. [22].

The fundamental molecular radioprotective capacity of melanin derives from the amorphous semiconductivity of melanin (dihydroxyphenylalanine-DOPA-melanin). It forms free radicals and is itself a stable-free radical [35]. DOPA melanin major adaptability is the ability to convert diverse energies into vibrational and rotational activity in the melanin molecular structure, dispersing it as heat, thereby protecting the cell from radiation damage [27]. The melanin molecular protection function derives from its capacity to absorb as sponge cytotoxic free radicals. This explanation has been experimentally demonstrated in melanized and albino mutant fungi [36,37]. Melanin can beside its oxidation-reduction potentials also function in energy capture and utilization. Remarkably, melanin can also protect fungi from temperature extremes.

Adaptive Radio and Temperature Protective Mechanisms of Fungal Melanins

Resistance of many fungi, especially melanized, to gamma radiation was found to be higher than the resistance of bacteria. Eleven soil microfungi species isolated from both the ES=NFS and the AS=SFS of Evolution Canyon I (EC I) at lower Nahal Oren, Mount Carmel, Israel, were examined for Cobalt irradiation for growth rates before and after exposure to 60 Cobalt irradiation by Voltz et al. [12]. Species of *Alternaria*, *Aspergillus*, *Humicola*, *Oidiodendron* and *Staphylotrichum* from AS=SFS grew faster than the ES=NFS isolates of the same species. By contrast, *Fusarium*, *Sordaria* and *Stachybotrys* grew at greater rates from the ES than from the AS.

The 11 isolates from each slope were then subjected to 60Co irradiation. At 40,000 rads exposure, *Alternaria*, *Fusarium* and *Stachybotrys* grew more rapidly when isolated from the ES, whereas *Humicola* and *Staphylotrichum* grew at a faster rate when isolated from the AS. *Aspergillus*, *Mucor*, *Sordaria* and *Ulocladium* from both

This, explains the threefold higher melanins in *A. niger* on the sunny AS in contrast to the shady ES at EC I and the differences in sunny and shady niches, primarily on the ES, higher melanin in sunny niches, as compared with shady ones, only a few meters apart (Figure 3b). Likewise, these neighbor sunny and shady micro niches on the ES showed significant divergence in culturability after 4 h exposure to UVA irradiation (Figure 5B). These patterns may also derive, complementarily, with heat and the drought protection of melanin, because of its water-bounding potential [38]. This is true regionally in the Negev desert where melanics abound [30,31]. The AS is by far drier than the ES, as are the sunny versus the shady niches on AS at EC I. Likewise, melanin can protect against salinity stress, explaining the adaptive melanized, black *A. niger* in the hypersaline (~35% salinity) Dead Sea (DS) and the following trend in growth rate: DS>AS>ES [20]. Remarkably, the frequency of *A. niger* is higher at microscales in upper than in lower soil layers in Evolution Canyon by Grishkan and Nevo [39] and in hot chalk than the cooler abutting basalt in the upper

Galilee [40]. Finally, there is also evidence that melanin can protect from chemical pollution and biotic pollution. Thus, the multi-potential of melanin in protecting organisms in nature may explain its universal abundance and prevalence in the tropical regions of the planet.

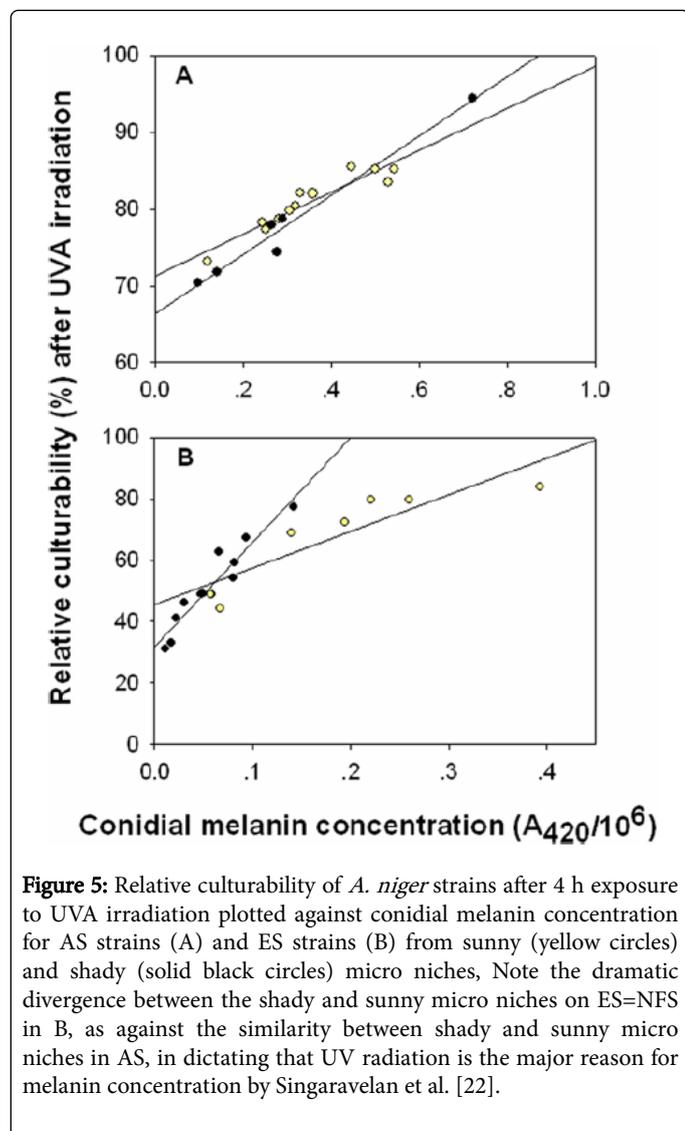


Figure 5: Relative culturability of *A. niger* strains after 4 h exposure to UVA irradiation plotted against conidial melanin concentration for AS strains (A) and ES strains (B) from sunny (yellow circles) and shady (solid black circles) micro niches, Note the dramatic divergence between the shady and sunny micro niches on ES=NFS in B, as against the similarity between shady and sunny micro niches in AS, in dictating that UV radiation is the major reason for melanin concentration by Singaravelan et al. [22].

Conclusion and Prospects

Stress and evolution are tightly intertwined. The Evolution Canyon (EC) model is an optimal microsite for studying evolution in action across life from viruses and bacteria to mammals. The EC project highlights biodiversity evolution, adaptation and incipient sympatric ecological speciation. The higher solar radiation on the African (AS), south-facing (SFS) slope, results in a tropical, sunny, hot, dry and savannoid biome. The opposite slope, on average 250 m apart, is temperate, shady, cool, humid and forested. The EC project started in 1990 and includes four ECs now, in the Carmel, Galilee, Golan and Negev Mountains (EC I-IV). The 250 publications to date appear in Nevo Evolution publications at <http://evolution.haifa.ac.il>. I reviewed our earlier study, "Adaptive Melanin Response of the Soil Fungus *Aspergillus niger* to UV Radiation Stress at "Evolution Canyon", Mount Carmel, Israel by Singaravelan et al. [22]. The results indicated

that mean conidial melanin concentration of AS strains were threefold higher than that of ES strains. *A. niger* populations "on the AS" resisted UVA irradiation better than ES populations.

Comparison of conidial melanin concentration in strains of *A. niger* populations of sunny and shady micro niches on the predominantly sunny AS and predominantly shady ES indicated that shady conditions on the AS have no influence on the melanin concentration. By contrast, the sunny strains from sunny sub-populations on ES had significantly higher melanin concentrations. This indicates that UVA radiation, rather than temperature, is the driving force of melanin evolution. We concluded that melanin in *A. niger* is an adaptive trait against UVR, both interslope and dramatically so, in sunny and shady micro niches on the ES-NFS.

Melanin is highly adaptive across life, including fungi, against environmentally extreme climatic stresses, including harmful UVR. Melanin absorbs diverse electromagnetic radiations, transforms them, thereby shielding organisms from DNA and other damages. This led to the idea that melanin probably has functions analogous to other energy harvesting pigments, such as chlorophyll [41].

What next? We started to conduct interslope transplant experiments at EC I and transformed ten AS strains to ES and vice versa. Will the concentration of conidial melanin change in accordance of the new environment, sunny to shady and shady to sunny as we expect, i.e., high AS melanin concentration will decline on ES and that of ES will increase on AS? We also plan to re-sequence the genome of AS and ES populations of *Aspergillus niger* at EC I to highlight their complex divergent genomic and epigenomic adaptations and regulation.

Melanization abounds in nature as a protective shield against diverse environmental stresses. It abounds in fungi and protects them adaptively against environmental stresses, both abiotic (climatic, edaphic, chemical) and biotic (pathogens).

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