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Research Article

Echiniscus testudo (Doyère, 1840): A promising biological control agent against Aflatoxin contamination of *Aspergillus flavus*

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Abstract

While Mashhad's drinking water is up to par with national standards, several locations have elevated levels of certain fungus, including *Aspergillus flavus*. A facultative parasite called *Aspergillus flavus* contaminates a number of significant food crops. Moreover, the pathogen that causes aspergillosis in humans and animals is opportunistic. One of the most dangerous contaminants of raw food commodities throughout pre- and post-harvest crops is *Aspergillus flavus*, which produces aflatoxins. Metabolites of aflatoxins are very carcinogenic. Even though aflatoxins are often produced during fungal colonization, precipitation may carry them into surface water. The presence of fungus in water has caused considerable alarm since it might endanger water quality and increase the risk of illness in humans. Dosti Dam is the primary water source for Mashhad. *Echiniscus testudo* specimens have been documented from the rivers that flow to this dam. Tardigrades are a class of microscopic, ubiquitous metazoa distinguished by their extreme intolerance. As an adaptation to extreme environmental conditions, cryptobiosis enables organisms to endure periods of extremely low temperatures or water scarcity, which are inhospitable to life. Due to the resistance and potential for extensive presence of this animal in the Target rivers, we investigated the possibility that it could be utilized to biologically control any form of water pollution in this study. The findings derived from the current investigation demonstrate the considerable potential of *Echiniscus testudo* as a biological control agent against *A. flavus*. Mycelial growth was observed to be 98% reduced and spore germination was inhibited by 100% under in vitro conditions.

Keywords: Fungi, Biological control, Water quality, water bears, Iran, Mashhad

Introduction

Aspergillus, classified within the Trichocomaceae family, is a genus of fungi that causes human illness by being saprotrophic and opportunistic (Davati & Ghorbani, 2023). *Aspergillus flavus* is a significant pathogenic species that poison numerous crops and is a primary contributor to the contamination of those crops with aflatoxin (Podgórska-Kryszczuk, 2023). Synthetic fungicides are the most frequently employed method of fungus control. Long-term fungicide application, on the other hand, is deemed unsuitable and may promote the development of fungicide-resistant mutants (Oufensou et al., 2023). Despite this, In the majority of nations, the proliferation of microorganisms in potable water systems is impeded through the residual addition of a disinfectant during distribution. Microbial proliferation nevertheless takes place in potable water systems (Van-der et al., 2023). The presence of *Aspergillus flavus* in the potable water of various Iranian cities has been documented and is significant when discussing personal and public health (Molavi et al., 2023).

At present, the phylum Tardigrada comprises over 1,400 species (Guidetti et al., 2023). With more than 160 species, the genus *Echiniscus* Schultze, 1840, is the most extensive and morphologically diverse within its family. Tardigrada, a collection of diminutive protostomata, are renowned for their capacity to transition into an anhydrobiotic state and withstand severe desiccation. Complete cessation of metabolism and loss of body water are the defining features of this condition (Krakowiak et al., 2023). Regardless of their life stage, water bears are recognized as the most prodigious species at anhydrobiosis. In contrast, Tardigrada employ distinct approaches to existence. The majority of published research indicates that tardigrade species exhibit a considerable capacity for survival following brief periods of anhydrobiosis (Poprawa et al., 2022). Furthermore, the optimal air humidity and temperature for dehydration may vary among these species (Roszkowska et al., 2023). Testudo individuals can resume their normal activities following 120 days in the tun stage (Roszkowska et al., 2023). While Tardigrades commonly consume microalgae, rotifers, or nematodes as food, the availability of food significantly influences their food selection (Arakawa, 2020). Additionally, the oral appendages and pharynx structure of this animal dictate the food it consumes. The species *Echiniscus testudo* possesses six lateral filaments (Fig1 A,B). Absence of cephalic appendages and a solitary placoid within the pharynx. Cephalic in shape in the absence of cuticular dorsal plates, the buccal tube consists of four peribuccal papillae and four peribuccal lamellae surrounding the oral orifice. The posterior diameter of the buccal tube is approximately 90% of the anterior diameter and is funnel-shaped. Pharyngeal bulb elongated, pear-shaped, and without placoids or septum. four cephalic papillae, positioned laterally. These structures indicate that this animal can feed on the *Aspergillus flavus* spores (Molavi et al., 2018). The animal has various physiological traits and exhibits the capacity to use diverse food sources, making it a viable contender for biological control. The resistance of these animals made this research investigate the possibility of removing the most important fungal pollutant of Mashhad's drinking water by this animal.

Figure1. A, General view of the oral part *Echiniscus testudo***;** B, Image of pharyngeal section *Echiniscus testudo*

Material and methods

Species used in the experiment

The experiments were conducted with strains of Aspergillus that were isolated from drinking water distribution systems in Mashhad during a previous study (Molavi et al., 2023). The strains of Aspergillus were pre-grown in a mineral medium with added glucose (0.25 mg/L) and KNO3 (6 mg/L) (Molavi et al., 2023).

The total of 65 individuals of *E. testudo* were extracted from 2 moss samples collected from Dehbar region (36°25′32″N, 59°28′77″E; 45m asl/ Fig.2A) and from four moss samples collected From the Harirood border river in Sarakhas county (35°82′99″N, 61°25′34″E; 90m asl/ Fig 2.B) in the northeast of Razavi Khorasan May 2023. Moss samples were placed into plastic beakers containing 200 ml of tap water. After 24 hours, the water-saturated moss was strongly shaken with tweezers and all plant particles were removed. Water with tardigrades was then poured into a 200 ml plastic cylinder and left to settle for 30 minutes, after which the upper portion of water (ca. 200 ml) was decanted and discarded and the remaining 50 ml was poured into Petri dishes for tardigrade extraction under a stereomicroscope (Olympus SZ)(Perry et al., 2019).

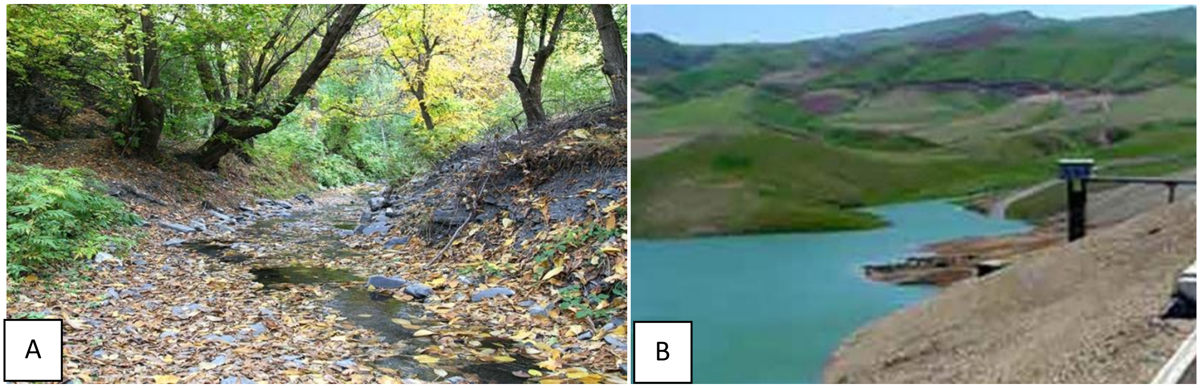

Figure 2. Image of sampling areas. A: Dehbar region, B: Harirood border river in Sarakhas

To determine the effect of this species on the desired fungus, the collected taridgrades were kept for ten days in water without organic matter (distilled water) at a temperature of 25 C° (Molavi et al., 2018). 50 tardigrades were counted under a light microscope (10x). The tardigrades were placed equally in ten Petri dishes containing 1 cc of water. Two Petri dishes without samples were used as controls. One millilitre of the suspension of 10^4 fungal spores per millilitre was added to each of the twelve Petri dishes. After three days of heating at 25°C, spores were counted in Petri dishes by Thoma Lam (Bagheri et al., 2017).

Then, the contents of the Petri dishes were transferred to the flask containing Potato Dextrose Broth (PDB) culture medium. The pH of this medium was reduced to 3.3 to inactivate the tardigrades. The flasks were placed on a shaker incubator (LABTECH LIST 3016R) at a temperature of 30 C° for 7 days to allow the spores to grow. Then, the mycelium masses were separated from the specific mushroom culture medium with the help of filter paper and dried in an oven at 60 degrees Celsius. Then the dry weight of the mycelium was calculated. All the above steps were repeated twice.

Results

The samples of *Echiniscus testudo* collected from the environment were examined under a light microscope with a magnification of 40, and the food masses were observed in the digestive tube of all samples, which were mostly green food (Figure 3A). Following a period of 10 days characterized by famine, an examination of the digestive tracts revealed a total absence of contents, indicating that the animal was experiencing a condition of starvation(Figure 3B). After the samples were placed in the vicinity of the suspension of fungal spores, the digestive tract was again filled with the food masses (Fig. 3C).

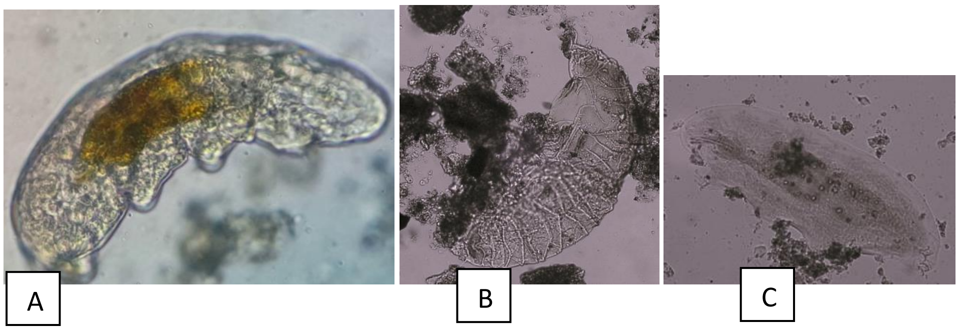

Figure 3. A, *Echiniscus testudo* samples collected from the environment. B, *Echiniscus testudo* samples after ten days of starvation. C, *Echiniscus testudo* samples after two days in the vicinity of fungal spore suspension.

The results of spore counting by Lam Toma showed that the presence of *Echiniscus testudo* can remove up to ninety-eight percent of *Aspergillus flavus* spores in the environment and prevent the growth of this fungus, and in almost one hundred percent of cases, the mycelium of this fungus did not grow with the presence of this species(Fig 4).

Figure 4. A: culture medium containing the isosuspension of fungal spores without *Echiniscus testudo*. B: culture medium containing the isosuspension of fungal spores with *Echiniscus testudo*

Conclusion

An essential human right is access to clean drinking water. Drinking water has a direct impact on human health, sickness, and death. Each year, 485,000 people die from diarrhea due to polluted drinking water globally. Providing clean, safe drinking water is a major concern and a formidable task (Zhang et al., 2023: Wang et al., 2021). There have already been reports of fungus contamination in the research region (Molavi et al., 2023). Recurrent infections and serious illnesses that need suitable care are brought on by fungal pathogens. There aren't many antifungal resources available, and during the last several decades, pathogenic fungi have become more resistant to antifungals (Delgado et al., 2024). One of the most dangerous contaminants of raw food commodities throughout pre- and post-harvest crops is *Aspergillus flavus*, which produces aflatoxins (Dhanamjayulu et al., 2023). The findings demonstrated that *Aspergillus* species, particularly in cases of severe or critical disease, may result in co-infections in COVID-19 patients(Lai and Yu, 2021). *Aspergillus flavus* and other fungus that generate aflatoxin (AF) are the source of AFB1, a powerful carcinogen and mutagen that poses a risk to both human and animal health (Saber et al., 2022). Animals and humans may get liver cancer from aflatoxins, which are carcinogenic substances (Khan et al., 2021). Various methods, such as UV222/chlorine or fungicides can help in controlling these fungi (Wang et al., 2023). But, the possible damages and the costs of providing them should be considered. The bio-control methods are the most environmentally sustainable solution to the control of toxigenic fungi in the field (Nji et al., 2023). So far, the most promising way to manage AFs' contamination in cereal crops has been to use non-aflatoxigenic strains of *A. flavus* (AF-) as biocontrol agents (Khan et al., 2021). The outcomes of the previous investigation show that two yeast strains, Aureobasidium pullulans PP4 and A. pullulans ZD1, have a great deal of promise for biologically controlling *A. flavus* (Podgórska-Kryszczuk, 2023). Additionally, the findings show the remarkable potential of two wild yeast strains, *Saitozyma podzolicus* D10 and *Aureobasidium pullulans* PP3, in biologically controlling *A. ochraceus* and *A. parasiticus* and reducing their production of OTA and AFs. Essential oils (EOs) and natural substances produced from plants have recently shown significant promise in the fight against aflatoxin contamination and *A. flavus* deterioration (Tian et al., 2022). This study emphasizes the important potential of plant-derived natural products and peppermint essential oils to protect food and feed against aflatoxin contamination and *A. flavus* infestation, in addition to lowering the risk of aspergillosis infection (Abd-El-Hack et al., 2023). Meiofauna such as tardigrades are widely distributed and well-known for their remarkable ability to withstand extreme temperatures, pressures, and even ionizing radiation (Tibbs-Cortes et al., 2022). In several terrestrial habitats and settings, tardigrades are widespread. Prior research has shown that several factors considerably impact diversity and community composition, even though little is known about their ecological preferences (Guidetti et al., 2023). Due of their diversity, tardigrades can now live in a wide range of settings, even some which are normally inhospitable to life (Smith et al., 2023). Intense resistance to a variety of environmental stresses, such as intense heat or cold, a scarcity of water, or excessive radiation, is shown by tardigrades

(Kasianchuk et al., 2023). The data obtained suggest that the survival rate of anhydrobiosis is either extremely high or high during the tun stage, up to 120 days. However, it is important to note that there are noticeable variations in the anhydrobiosis capacities across various species/populations as the tun stage length increases (Roszkowska et al., 2023). Smith et al. (2023) shown that the anterior segments of panarthropods exhibit significant anatomical and developmental diversity, both within individuals and across different species. The findings obtained in this study provide support for the idea that carnivorous animals exhibit higher survivorship in anhydrobiosis compared to herbivorous species (Roszkowska et al., 2023). Further analysis is required to examine the inter-species relationship of tardigrades with other organisms. In laboratory cultivation, tardigrads can consume algae, bacteria, and rotifers (Stec et al., 2018). There seems to be a potential correlation between food choice and the morphology of the buccal apparatus, which in turn may be associated with the distribution of food availability (Schill et al., 2011). Empirical evidence indicates that some animals may consume a varied diet (Sugiura et al., 2022). Several kinds of algae, nematodes, and rotifers have been suggested as potential food sources for *various* Tardigrada species. However, the most often used were *Chlorococcum* sp. or *Chlorella* vulgaris for algae, *Caenorhabditis elegans* for nematodes, and *Lecane inermis* for rotifers (Wilanowska et al., 2024). Certain tardigrade species have been suggested as alternative food sources, including bacteria, diatoms, cyanobacteria, other tardigrades, and fungi. Furthermore, it has been reported by many authors that different tardigrade taxa consume the same kind of food, such as the same prey species or algae. This observation indicates that tardigrades are not solely monophagous, but rather exhibit omnivorous behavior. Nevertheless, it is important to note that the sort of food consumed may have a significant impact on both longevity and reproductive success (Roszkowska et al., 2021). The physiology of the digestive system and the resistance of these microscopic animals made us use these animals as biological controllers of fungi contaminating drinking water. According to the findings of this study, these animals can utilize fungal particles as food sources and thereby prevent the spread of contamination. Since these animals are more abundant in the muddy parts of the water floor, we cannot worry about the nutritional preference of algae over fungal spores, because the density of algae is low in their main biological areas.

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