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Ultrastructural alterations in scales of *Cyprinus carpio* L. induced by azole fungicide tebuconazole

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ABSTRACT

Fish scales act as 'dermal armour' that protects the skin. Fish scales can serve as essential bioindicators of aquatic pollution, as they are always in direct contact with the external aquatic environment. The present study aimed to evaluate the toxic potential of the fungicide tebuconazole on *Cyprinus carpio*. Electron microscopic analysis of carp scales was used to determine the toxicity on exposure to two different concentrations (6.47 and 8.09 µL/L) of tebuconazole for 30 days. The scale topography depicted time and dose-dependent responses on exposure to the fungicide. Severe alterations were observed in the carp scales, with significant damages in radii, focus, circuli and fractured lepidont. Fungicide exposure on scales leads to depletion of calcium and compromises the fish health by loosening of scales.

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1. Introduction

Pesticides play a crucial role in modern agriculture. Pesticides are used to improve pest management, including disease control, reduce crop loss and to increase agricultural productivity. Many kinds of insecticides, fungicides, herbicides and other chemicals have been extensively used to control pests and to ensure crop safety (Nayak and Solanki, 2021). Unfortunately, the hazards of using pesticides have already outweighed their relative advantages. In addition to improving crop yield, pesticides have a detrimental impact on the environment. Concerns have been raised on the environmental threats associated with pesticide exposure through residues in food, drinking water and surface water. It is known that overuse of pesticides have caused critical damage to biodiversity. Pesticide spills in the environment affect many non target species since their chemical activity are not species specific. Destructive pesticides pose threats to the survival of many aquatic and other animals, including birds (Kumar and Kumar, 2019).

The potential of a pesticide to cause harm to fish and other aquatic animals is primarily determined by its toxicity, duration of exposure, rate of dosage and environmental persistence. Fish are among the various aquatic species that are most susceptible to environmental water contamination (Choudhury, 2018). Therefore, contaminants like pesticides have the potential to cause substantial impact by seriously harming fish health. (Banaee, 2013). Tebuconazole is a broad-spectrum systemic fungicide, with Demethylase inhibitors (DMI) that obstruct the formation of fungal cell wall's structure. It is an active component of the globally popular fungicide family, triazoles. Triazole, having the molecular formula $C_2H_3N_3$, is a heterocyclic compound with a five-membered ring made up of two carbon atoms and three nitrogen atoms. Its actions are eradicative, curative and protective. It is quickly taken up by the plant's vegetative parts, primarily through acropetal translocation. Tebuconazole residues have been found in soil, water and foods like grapes, apples and pepper due to its widespread use, raising the risk of exposure to non-target organisms (Patyal *et al.,* 2013). Through surface runoff or spraying, it can enter rivers, lakes, oceans, and surface waters, affecting all living organisms that are present in these environments (Castro *et al.,* 2018). Previous studies also have revealed that tebuconazole can pass from exposed parents to their offspring, resulting in thyroid endocrine disruption and developmental toxicity (Li *et al.,* 2019).

Scanning electron microscopy (SEM) has been applied in numerous fields worldwide. It can be considered a useful technique for analyzing both organic and inorganic materials on the nanometer to micrometer scale. SEM is a tool that allows one to observe the invisible realms of nano and micro space. SEM can reveal details and complexity that are not visible with light microscopy (Mohammed and Abdullah, 2018). SEM is a practical tool for determining the impact of environmental stressors on fish structures that are essential to their survival and fitness.

In studies on various fish species, scales have been proven to be useful markers for the classification of species and the determination of age and growth. In addition, scale morphology is used for sexual dimorphism (Ganzon *et al.,* 2012), age determination (Dwivedi *et al.,* 2016), migration and the distinction between hatchery-raised and wild populations (Unwin and Lucas, 1993). Fish scales are dermal derivatives, that are subjected for significant morphological investigations. Fish scales get direct contact with the environment and its pollutants and suffer pathological alterations in their structure. The study of scales does not require sacrificing the fish and the scales can be effectively employed as biomarkers of pollution. Evaluating the toxicity of fish scales yields valuable insights into the state of aquatic ecosystems, the impact of pollutants on fish populations, and potential health hazards to humans.

Cyprinus carpio is the most widely cultivated aquaculture species and is comparatively hardy and tolerant to external environmental changes. The present study was aimed to use *C. carpio* as a test model to determine the tebuconazole induced alterations in its scales and to evaluate the health status of the fish and its environment.

2. Materials and Methods

The fish *Cyprinus carpio* of weight 150-200 gm and length up to 20±2 cm were collected from Fish Seed and Breeding Farm Deoli, District Bilaspur, Himachal Pradesh. The experiments were conducted after obtaining approval from the Fisheries Department of Himachal Pradesh. The fish were acclimatized to laboratory conditions in glass aquaria filled with 80 L of dechlorinated tap water, for 15 days. The water was dechlorinated using an anti-chlorine (sodium thiosulphate) solution and was then exposed to air for 24 h. All aquaria were equipped with aerators and filters. 40% of the volume of the water was changed after every 24 h. They were fed with commercial supplementary feed twice a day (10:00 and 17:00 h). Unconsumed feed and excreta were siphoned daily. The physicochemical properties of water were temperature 22 ºC, pH 7.8±0.2, D.O. 8±2 mg L^{-1}, TDS 150±8 ppm (using water analyzer kit, model no. CK 710), total alkalinity170±10 mg L^{-1} (using titration method, APHA, 2017).

After acclimatization, fish were divided into 3 groups based on the exposure to tebuconazole (25.9% EC). During the experiment, the water in each aquarium was aerated and the temperature was maintained at 22 ºC. Tebuconazole (25.9% EC), a commercial grade was purchased from the local market.

Group I served as control, kept in a normal water environment and Groups II and III were in 6.47 and 8.09 µL/L (one-fifth and one-fourth of 96 h LC_{50}) tebuconazole added water tanks, respectively, for 30 days. No water was changed during the experimental procedure. To study the tebuconazole induced changes in the common carp, 8-10 scales were gently removed from the left side of the body, from the second row above the lateral line and below the dorsal fin at each exposure period. After removal, they were mechanically cleaned with a fine brush and then rinsed in distilled water. Then scales were treated with a 2% mild potassium hydroxide solution.

For scanning electron microscopy, cleaned scales were fixed in Karnovsky fixative for 24 h and rinsed in 0.2 M buffer. Then dehydrated in ascending grades of alcohol (30-90%) and dried on Whatman filter paper. To prevent margins from curling, the scales were not put in absolute alcohol. In addition, scales were kept between the microslides for two to three days after 70% ethanol to prevent curling.

The cleaned and dried scales were then mounted on the metallic stubs using double adhesive tape, with the ventral surface adhering to the tape and the dorsal surface facing upward. Proper care was taken to avoid the trapping of air bubbles under the tape. The scales were then covered with a thick layer of gold using the SPUTTER COATER, a gold coating unit. The gold coating eliminates beam damage and charging. Also, it improves the strength of secondary electron signals from the specimen's surface.

The scale specimens were studied under vacuum using EVO 18 scanning electron microscope, at an accelerating voltage of 20 kV and low probe current at SAIF, All India Institute of Medical Sciences, New Delhi. The images obtained on the fluorescent screen of SEM were photographed. The specimens were stored in a desiccator when not being viewed to avoid the uptake of moisture and deposition of dust particles.

3. Results

Cycloid scales of *C. carpio* are large and thick. 33 – 39 scales on the lateral line; 5 – 6 scale rows above and below the lateral line; and 16 around the caudal peduncle are present. The cycloid scales vary in shape. The scale may be an oval, semioval, oblong or circular shaped. The deposition of calcium salts has resulted in the rough, opaque, and hard posterior part of the scale. It has two surfaces: ventral surface is smooth and shiny and is attached to the skin; and the exposed dorsal surface, which is rough and non-shiny because it displays the growth lines.

Electron micrograph of scales from the control group demonstrated the rostral (anterior), lateral and caudal (posterior) fields. There is a noticeable focus in the front half of the scale. Numerous circuli have been observed to radiate from focus and cover the anterior and lateral regions of the scale. These anterior circuli were widely separated from one another. It has been noticed that the radii arising from the focus disrupt the continuity of circuli. Lepidonts were tiny, unevenly shaped tooth-like processes that were present in the anterior region of scales. On the other hand, minute swellings called tuburcle are restricted to the posterior half. Thick, widely spaced, irregular circuli have been observed in the post-focus region (Figs. A, B, C).

Scanning electron micrograph of scales of *C. carpio* Control group A: Normal scale of *C. carpio* showing focus (F), tubercle (T), anterior (AF), posterior (PF) and lateral field (LF). (X 50) B: Magnified focus (MF) region (X300) **C:** Circuli (C), radii (R), and inter circuli space (IS) (X100).

Scanning electron micrograph of scales of *C. carpio* treated with 6.47µL/L TBZ for 10-30 days -- D: At 10 days showing damaged circuli (DC), loss of circuli from center (LC) and damaged focus (DF). E: At 20 days, demonstrating irregular circuli (IC) and sloughing of circuli (SC). F: At 30 days, revealing widening of intercircular space (WIS), curling of circuli (SC) and broken circuli (BC) (X300).

C. carpio treated with 8.09µL/L TBZ for 10-30 days -- G: At 10 days, fish demonstrating broken circuli (BC) and broken lepidont (BL). H: At 20 days, exhibiting distorted circuli (DC) and damaged circuli (DC). I & J: At 30 days, group exhibiting broken ciculi (BC), damaged radii (DR), broken lepidont (BL) and broken ciculi (BC) (X300).

Alterations in the structure of scales were observed on exposure to fungicide. It could also be noticed that the exposure to both concentrations caused scale loosening. This may be attributed to the widespread damage to the calcareous structures known as lepidonts in the anterior portion of the scale. The treatment of fish with tebuconazole at a lower concentration of 6.47 µL/L demonstrated the focus region got damaged, becoming deformed and losing circuli from most of the central region. Radii were also found to be slightly damaged. A few circuli were found

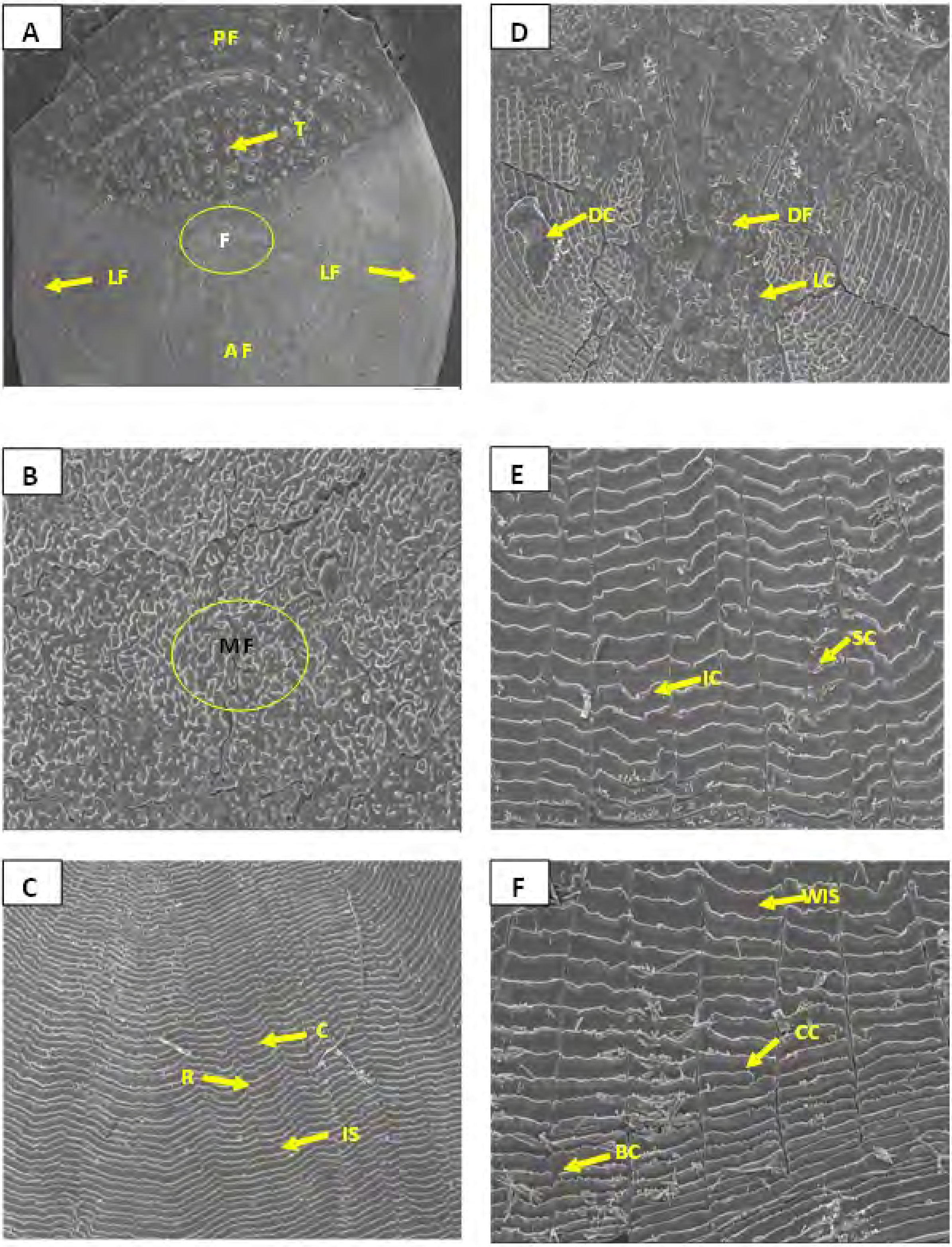

Fig. A-F.

fractured in the anterior region at the 10-day stage (Fig. D). Significant fractures, breaks, damages and irregular arrangement of the circuli could be observed. The 20-day stage showed sloughing of the circuli due to extensive damage, increased intercirculi space and widely spaced circuli (Fig. E). Curled circuli with breaks and cracks were observed after an increase in the exposure period to 30 days (Fig. F).

Scales of fish on exposure to 8.09 µL/L sublethal concentration of tebuconazole for 10 days depicted lepidonts being broken at many places, leading to irregular arrangement of circuli (Fig. G). After 20 days of exposure, increased abnormalities in the structure of circuli of the scales of fish were observed. The distorted and damaged circuli caused loosening of scales from the body (Fig. H). On prolonged exposure of 30 days, lepidonts and ciculi got

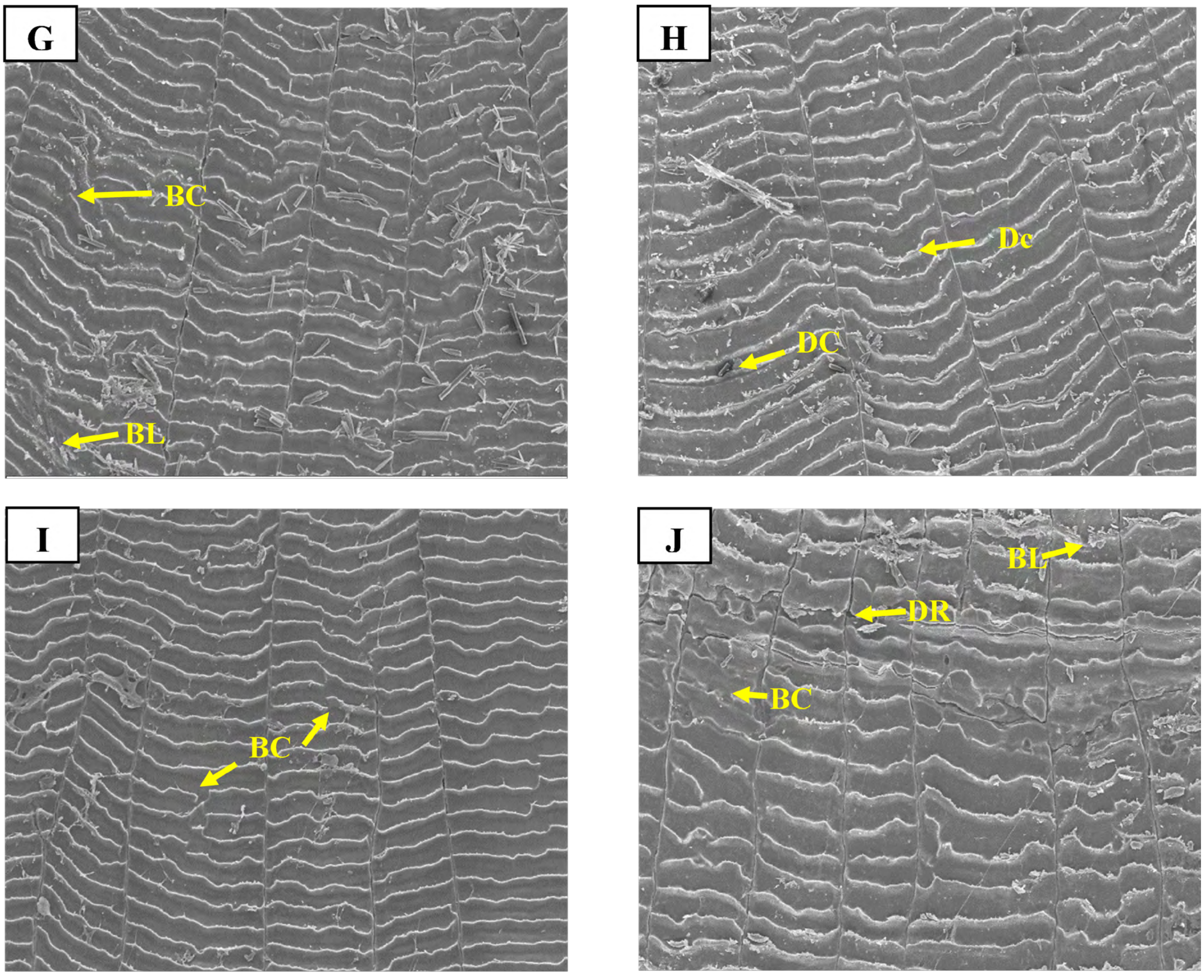

Fig. G-J.

broken due to the depletion of calcium materials. Damage to ciculi was high after increased concentration and exposure period (Figs. I & J).

4. Discussion

Fish scales are reliable bioindicators for evaluating water quality of an aquatic body (Brraich and Jangu, 2012; Jindal and Kaur, 2015). Consequently, scales are employed as vital instruments for measuring aquatic pollution. The results of the present study revealing perceptible damages to the structure of *C. carpio* scale, following exposure to tebuconazole undoubtedly demonstrate that environmental pollution can affect even the hard parts of a fish. Focus is the first part of fish scales to form, and the normal scales are composed of circuli that emerge from the focus to the anterior region which is divided by radii. The present results indicate that exposure to tebuconazole caused extensive damage and breaking down of calcareous material in the scales leading to disintegration of lepidonts, sloughing of tubercles, fracturing circuli, cracked radii, loosening of scales, etc. It could also be discernible that the damages to the scales increased with time and concentration. Various alterations, including fractured circuli and lepidonts have been reported in *Cyprinus carpio* exposed to varying levels of cadmium (Rishi and Jain, 1998) and in *Anabas testudineus* exposed to cypermethrin (Babu *et al.,* 2014). Batoye *et al.* (2021) reported scale loosening and shedding consequent to severe damages in the scales like distorted circuli, displaced or uprooted lepidonts and tubercles in *Ctenopharyngdon Idella*, exposed to fenvalerate. It was reported that broken or damaged fish scales due to lepidont disruption and subsequent loosening of scales were common in many fish species inhabiting parts of river Ganga that were noted for high human activity (Khanna *et al.,* 2007). Severe damage was reported in scales of freshwater fish collected from contaminated ponds (Rutkayova *et al.,* 2016) and from sites contaminated with the high level of aluminium and iron (Hidayati *et al.,* 2013). *Catla catla*, *Labeo rohita*, and *Cirrhinus mrigala* collected from various polluted sites of River Chenab also revealed ultrastructural deformities in the shape and scale structures like circuli, focus, radii, and annuli (Sultana *et al.,* 2017).

5. Conclusion

Fish scales perform functions like body protection and mobility assistance to fin fishes, but can also be used as effective biomarker to aquatic pollution levels that can be deduced from the structural damages suffered by the scales due to exposure to pollutants. The results of the present study indicated ultrastructural alterations in the scales of *Cyprinus carpio* consequent to exposure to fungicide tebuconazole, with structural damages increasing with both time and concentration of the fungicide. Based on the toxicity assay it may be concluded that the presence of xenobiotic compounds in the aquatic system can impact the health of fish and other organisms and bioaccumulation and biomagnification of these compounds may significantly affect the food chain and human well-being.

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