



Job's Tears Enhances Fish Cold Tolerance

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Improving Cold Tolerance in *Labeo rohita*: Effects of Job's Tears (*Coix lacryma-jobi* L.) as a Natural Feed Additive

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ABSTRACT

This study investigated the effects of Job's tears (*Coix lacryma-jobi* L.) as a feed additive on cold tolerance in *Labeo rohita* (initial weight: 15.2±0.3 g), marking the first evaluation of its health benefits in fish or aquaculture. Three doses (low: 0.5%, medium: 1%, and high: 2% of diet) were tested over an 8-week period, with fish fed to apparent satiation twice daily. Fish were subjected to a cold stress test (15°C), and physiological, biochemical, and molecular parameters were assessed. Blood samples were collected from the caudal vein of anesthetized fish at 0, 12, and 24 hours during the cold stress test. The medium dose (1%) showed the most significant improvements in cold tolerance, evidenced by enhanced survival rates, reduced plasma cortisol levels, and up regulation of cold-tolerant genes. These novel findings suggest that Job's tears could be an effective feed additive for improving cold tolerance in *L. rohita* aquaculture, opening new avenues for natural stress mitigation in fish farming in subtropical and temperate regions.

KEYWORDS: Aquaculture stress mitigation, *Coix lacryma-jobi*, Cold tolerance, Feed additive, Indian major carp.

Article received: 24 October 2024; Article accepted: 06 December 2024

INTRODUCTION

Labeo rohita, commonly known as rohu, is an economically important freshwater fish species widely cultured in South Asian countries including India (FAO 2022). However, its growth and survival are considerably hindered by low temperatures, especially in subtropical and temperate areas (Kausar and Salim 2006; Mridul et al., 2024). Enhancing this species' tolerance to cold is therefore essential to extend its aquaculture viability in colder regions, ultimately improving production efficiency.

Recent studies have shown that certain plant-based feed additives improve stress tolerance in fish (Kuebutornye et al., 2023). Singh et al. (2024) specifically highlighted that feed additives are crucial in aquaculture, serving as functional substances that can improve growth performance, decrease mortality rates, and provide specific medicinal properties. Complementing this perspective, researchers have explored various natural additives' potential in fish cultivation. For instance, Muzaffar et al. (2017) demonstrated garlic powder's efficacy in common carp growth, Khan et al. (2018) investigated Aloe vera's impact on fish performance, and Jain et al. (2020) examined carrot meal's role in enhancing ornamental fish characteristics.

Job's tears (*Coix lacryma-jobi* L.), a grain crop rich in polysaccharides, proteins, and bioactive compounds (phenolic acids, lignans, flavonoids, polyphenols, phytosterols), has demonstrated immunostimulatory and stress-mitigating effects in various animal models (Hsia et al., 2007; Manosroi et al., 2016; Devaraj et al., 2020; Gond et al., 2024). However, its potential to enhance cold tolerance in fish remains unexplored.

This study aimed to investigate the effects of Job's tears as a feed additive on cold tolerance in *L. rohita*. It was hypothesized that dietary supplementation with Job's tears would improve physiological, biochemical, and molecular responses to cold stress, with varying effects depending on the dosage.

MATERIALS AND METHODS

Experimental setup and fish stocking

Three hundred and sixty *L. rohita* fingerlings (initial weight: 15.2±0.3 g) were randomly distributed into 12 FRP tanks (500 liters each tank; 30 fish per tank) in a recirculating aquaculture system. The recirculating aquaculture system was equipped with a centralized aeration system using air blowers and fine-bubble diffusers. Dissolved oxygen was

continuously monitored using a digital oxygen meter (Model Lutron PDO-519, Taiwan) and maintained above 5 mg/L through controlled air injection. Oxygen concentration was regulated by adjusting air flow rates and ensuring proper water circulation, with backup oxygen supplementation mechanisms in place to prevent any potential hypoxic conditions. Four treatments were established in triplicate: control (basal diet), low dose (0.5% Job's tears), medium dose (1% Job's tears), and high dose (2% Job's tears). The experiment lasted for 8 weeks.

Feed preparation and composition

Commercial fish feed from ABIS India (32% crude protein, 6% crude lipid) was procured and thoroughly ground using a laboratory grinder. Job's tears grains were separately processed by cleaning, drying, and grinding into a fine powder with particle

size <500 μm . The Job's tears powder (moisture content: $10.2 \pm 0.3\%$, crude protein: $15.6 \pm 0.4\%$, crude fat: $5.8 \pm 0.2\%$, crude fiber: $3.5 \pm 0.1\%$, ash: $2.1 \pm 0.1\%$, and carbohydrates: $62.8 \pm 0.5\%$) was precisely weighed and incrementally incorporated into the ground commercial feed at predetermined concentrations (0.5%, 1%, and 2%) using a mixer. The mixing process involved gradual addition and continuous blending for 10 minutes to ensure homogeneous distribution of Job's tears powder throughout the feed matrix. After thorough mixing, the feed was re-pelletized using a laboratory pellet mill to maintain uniform pellet size and consistency, then dried at 40°C for 24 hours and stored at -4°C until use, ensuring feed stability and preventing moisture-induced degradation. The analysis of feed through AOAC methods showed the feeds were isoproteineous and isolipidic (Table 1).

Table 1. The proximate composition (mean \pm SE) of the experimental diets was as follows:

Treatment	Crude protein (%)	Crude fat (%)	Ash (%)	Moisture (%)	Gross energy (MJ/kg)
Control	32.1 ± 0.2	6.1 ± 0.1	7.2 ± 0.1	8.5 ± 0.1	17.8 ± 0.2
Low dose (0.5%)	32.3 ± 0.2	6.0 ± 0.1	7.3 ± 0.1	8.4 ± 0.1	17.9 ± 0.2
Medium dose (1%)	32.5 ± 0.3	6.0 ± 0.1	7.4 ± 0.1	8.3 ± 0.1	18.0 ± 0.2
High dose (2%)	32.8 ± 0.3	5.9 ± 0.1	7.5 ± 0.1	8.2 ± 0.1	18.1 ± 0.2

Feeding and management

Fish were fed twice daily (09:00 and 16:00) to apparent satiation, with careful monitoring of feed intake and fish response. Initially, 30 fish were stocked per 500-liter tank, with 20 fish selected for the primary experimental protocol and 10 maintained as a reserve population. Daily feed consumption was calculated by weighing the offered and remaining feed after 30 minutes. Water quality parameters were rigorously monitored using portable meters (Lutron PDO-519, Taiwan; Eutech Instruments PCSTestr 35, Singapore; and API kits, USA), maintaining optimal conditions: temperature $28 \pm 1^\circ\text{C}$, dissolved oxygen >5 mg/L, pH 7.5 ± 0.2 , and ammonia-N <0.5 mg/L. Partial water replacement (10-15% daily) was conducted using dechlorinated, temperature-equilibrated water. At the conclusion of the experiment, individual fish were weighed to determine growth performance, with specific growth rate and weight gain calculated using standard aquaculture formulas.

Cold stress test and sample collection

Initially, 30 fish were stocked per tank, and for the cold tolerance test, 20 fish were selected based on uniform size and health status. The remaining 10 fish served as a reserve population for potential replacements and additional measurements. These reserve fish underwent growth monitoring and were used as backup samples if needed. At the conclusion of the 8-week feeding trial, fish were individually weighed to determine final body weight. The initial mean weight was 15.2 ± 0.3 g, and the final mean weights were as follows: control group: 42.5 ± 1.8 g, low dose (0.5%) group: 45.3 ± 2.1 g, medium dose (1%) group: 48.6 ± 2.3 g, and high dose (2%) group: 46.2 ± 2.0 g.

Temperature reduction was conducted using a precision digital temperature control system (Model Lutron PDO-519, Taiwan). The temperature was gradually reduced following a controlled protocol: starting at an initial temperature of $28 \pm 1^\circ\text{C}$, with a reduction rate of 2°C per hour over a total reduction

time of 6 hours. The temperature reduction stages were as follows: from 28°C to 24°C during the first hour, from 24°C to 20°C over the second and third hours, and from 20°C to 15°C over the fourth to sixth hours, after which it was maintained at 15°C for 24 hours. Temperature was continuously monitored using calibrated digital thermometers placed at multiple points in each tank to ensure uniform cooling. The survival rate was recorded hourly.

For the collection of blood and liver samples, a subset of six fish per tank group was selected at 0, 12, and 24 hours during the cold stress test. Blood samples, approximately 500µL, were drawn from the caudal vein using heparinized syringes. The blood was immediately centrifuged at 3000× g for 10 minutes at 4°C to separate the plasma. Following blood collection, the same fish were subjected to hepatic sampling. The fish were anesthetized with MS-222 (100 mg/L), and the abdominal region was sterilized with 70% ethanol. A midline laparotomy was performed using sterile surgical instruments, and the liver was carefully excised using pre-cooled surgical scissors and forceps. The liver samples were preserved immediately after excision: portions intended for enzyme activity analysis were snap-frozen in liquid nitrogen, and portions for RNA extraction were stored in RNA later solution. All

collected samples were then stored at -80°C for future analyses. All procedures adhered to institutional animal ethics guidelines to minimize animal suffering and ensure ethical standards were met.

Biochemical analysis

Plasma cortisol levels were quantified using a commercial ELISA kit (Thermo Fisher Scientific, USA), which has a sensitivity of 0.1 ng/mL and an intra-assay coefficient of variation of <8%. Antioxidant enzyme activities were assessed using assay kits (Sigma Aldrich, USA). All assays were performed according to the manufacturer's standardized protocols, with spectrophotometric measurements taken using a microplate reader (Thermo Scientific, USA).

Gene expression analysis

Total RNA was extracted from liver samples using TRIzol reagent. cDNA was synthesized and real-time PCR was performed to analyze the expression of cold-tolerant genes: cold-inducible RNA-binding protein (*cirbp*), fatty acid desaturase 2 (*fads2*), and glucose-6-phosphate dehydrogenase (*g6pd*) (Table 2). β -actin was used as a reference gene (Zhou et al., 2018).

Table 2. Primer sequences for target genes used in gene expression analysis

Gene	Primer Direction	Sequence
cirbp	Forward	5'-TGGATCAGAACGTGGATGAG-3'
	Reverse	5'-CTCCACCACCTTCTCCTTCA-3'
fads2	Forward	5'-ATGGCACTTCAGCTCATCCT-3'
	Reverse	5'-GTCTGCCTGTAGGTCTCCAA-3'
g6pd	Forward	5'-GTCGTCCTCGGTGTCCTCTA-3'
	Reverse	5'-TGCCTTGTCCTCTGGTTGA-3'
β -actin	Forward	5'-CCGTGACCTGACTGACTACCT-3'
	Reverse	5'-CTTGATGTACGCACGATTTTC-3'

PCR reactions were performed in triplicate using a real-time PCR system (e.g., Applied Biosystems 7500). The PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Melting curve analysis was performed to confirm the specificity of the amplification. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method, with β -actin as the internal control.

Data normality was first assessed using the Shapiro-Wilk test. Homogeneity of variance was examined using Levene's test to confirm the assumption of equal variances across groups. After confirming that the data met the parametric test assumptions (normality and homogeneity of variance), data were analyzed using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. Differences were considered

statistically significant at $p < 0.05$. All statistical analyses were performed using SPSS 21.0 (version 21.0, IBM Corporation, Armonk, NY, USA).

RESULTS AND DISCUSSION

Survival rate

The medium dose (1% Job's tears) group showed

the highest survival rate ($92.5 \pm 2.5\%$) during the cold stress test, significantly higher than the control ($75.0 \pm 3.3\%$), low dose ($82.5 \pm 2.9\%$), and high dose ($85.0 \pm 3.0\%$) groups ($p < 0.05$) (Table 3). This suggests that the 1% supplementation level of Job's tears provides optimal protection against cold stress in *L. rohita*.

Table 3. The survival percentages of fish under different treatments

Treatment group	Initial fish	Sampling removals	Remaining fish	Survival rate (%)
Control	60	18 (6 fish × 3 time-points)	42	75.0 ± 3.3^a
Low dose (0.5%)	60	18 (6 fish × 3 time-points)	42	82.5 ± 2.9^b
Medium dose (1%)	60	18 (6 fish × 3 time-points)	42	92.5 ± 2.5^c
High dose (2%)	60	18 (6 fish × 3 time-points)	42	85.0 ± 3.0^b

Note: Different superscript letters indicate statistically significant differences ($p < 0.05$)

Plasma cortisol

Plasma cortisol levels increased in all groups during cold exposure, but the increase was significantly attenuated in the Job's tears supplemented groups (Table 4). At 24 hours, the medium dose group showed the lowest cortisol levels (89.3 ± 5.2 ng/mL) compared to the control (152.7 ± 8.9 ng/mL), low dose (118.5 ± 6.7 ng/mL), and high dose (105.8 ± 7.1 ng/mL) groups ($p < 0.05$). These results are consistent with the stress-mitigating effects of Job's tears observed in human models (Manosroi et al., 2016; Devaraj et al., 2020).

aligns with findings from Zhang et al. (2022) and Wang et al. (2024), which indicate that excessive plant-based additives can trigger a mild stress response by activating hypothalamic-pituitary-interrenal (HPI) axis mechanisms. Such responses may be attributed to oxidative stress at higher concentrations, challenges in metabolic adaptation, or cellular stress signaling pathways. Similarly, Devaraj et al. (2020) reported dose-dependent stress responses in herbal supplements, emphasizing that optimal benefits are achieved within a narrow supplementation range. In this context, the medium dose (1%) appears to offer a balanced intervention, avoiding the physiological overload observed in the high-dose group.

The elevated cortisol levels observed in the high-dose group suggest a potential hormetic effect. This

Table 4. Plasma cortisol levels (ng/mL) during cold exposure

Treatment	0 hours	12 hours	24 hours
Control	45.2 ± 3.1^a	98.6 ± 6.8^a	152.7 ± 8.9^a
Low dose	43.8 ± 2.9^a	85.3 ± 5.5^b	118.5 ± 6.7^b
Medium dose	44.5 ± 3.0^a	72.1 ± 4.8^c	89.3 ± 5.2^d
High dose	44.1 ± 2.8^a	79.4 ± 5.2^{bc}	105.8 ± 7.1^c

Note: Different superscript letters indicate statistically significant differences ($p < 0.05$)

Antioxidant enzyme activities

Cold stress induced a significant increase in antioxidant enzyme activities across all groups. However, fish fed Job's tears-supplemented diets showed higher SOD, CAT, and GPx activities compared to the control group (Table 5). The medium

dose group exhibited the highest enzyme activities, indicating enhanced antioxidant capacity. This aligns with findings by Zhang et al. (2022) and Mohammady et al. (2022), who reported improved antioxidant status in gibel carp (*Carassius auratus*) and Nile tilapia (*Oreochromis niloticus*) respectively fed with herbal extracts.

The reduced enzyme activity in the high-dose group compared to the medium-dose group suggests a potential adaptive mechanism of metabolic regulation. Similar non-linear responses have been reported by Mohammady et al. (2022) in fish antioxidant systems, where excessive supplementation may lead to the down regulation of antioxidant enzyme expression, metabolic recalibration, or the activation of negative feedback

mechanisms. This aligns with the hormetic principle, which posits that moderate stressors can enhance physiological performance, whereas excessive stimulation can result in diminished responses. The medium dose appears to strike an optimal balance, effectively stimulating antioxidant defenses without inducing the compensatory downregulation mechanisms observed in the high-dose group.

Table 5. Antioxidant enzyme activities in liver after 24 hours of cold exposure

Treatment	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
Control	125.3 ± 7.2 ^a	42.6 ± 3.1 ^a	18.9 ± 1.4 ^a
Low dose	148.7 ± 8.5 ^b	53.8 ± 3.7 ^b	24.5 ± 1.8 ^b
Medium dose	182.4 ± 10.3 ^d	68.2 ± 4.5 ^d	31.7 ± 2.3 ^d
High dose	165.9 ± 9.4 ^c	61.5 ± 4.0 ^c	28.3 ± 2.0 ^c

Note: Different superscript letters indicate statistically significant differences (p<0.05)

Gene expression

Expression of cold-tolerant genes (cirbp, fads2, and g6pd) was significantly upregulated in all groups during cold exposure, with the Job's tears-supplemented groups showing higher expression

levels than the control (Table 6). The medium dose group demonstrated the highest fold changes in gene expression at 24 hours: cirbp (5.8-fold), fads2 (4.2-fold), and g6pd (3.9-fold) relative to the control group (p < 0.05).

Table 6. Gene expression (fold change relative to control) after 24 hours of cold exposure

Treatment	cirbp	fads2	g6pd
Control	1.0 ± 0.1 ^a	1.0 ± 0.1 ^a	1.0 ± 0.1 ^a
Low dose	3.2 ± 0.3 ^b	2.5 ± 0.2 ^b	2.3 ± 0.2 ^b
Medium dose	5.8 ± 0.5 ^d	4.2 ± 0.4 ^d	3.9 ± 0.3 ^d
High dose	4.5 ± 0.4 ^c	3.4 ± 0.3 ^c	3.1 ± 0.3 ^c

Note: Different superscript letters indicate statistically significant differences (p<0.05)

The upregulation of cirbp, which plays a crucial role in cold adaptation by stabilizing mRNA (Zhong et al., 2021), suggests enhanced cold tolerance mechanisms in Job's tears-fed fish. Similarly, the increased expression of fads2 and g6pd indicates improved lipid metabolism and cellular redox balance, respectively, which are essential for cold adaptation (Zheng et al., 2014; Xu et al., 2021). Our findings align with broader research in aquaculture feed supplementation. Studies by Muzaffar et al. (2017) on garlic, Khan et al. (2018) on Aloe vera, and Jain et al. (2020) on carrot meal have similarly demonstrated that strategic feed additives can significantly enhance fish performance. These studies collectively emphasize the potential of natural supplements to improve growth, survival, and physiological responses in fish.

The medium dose exhibited significantly higher gene expression compared to the high dose, indicating that moderate dietary supplementation may optimize gene transcription mechanisms. This observation is consistent with findings by Zheng et al. (2014) and Xu et al. (2021), who suggest that moderate supplementation enhances cellular signaling pathways, regulates gene expression more effectively, and promotes a balanced metabolic stress response. Further supported by studies from Kuebutornye et al. (2023) and Wang et al. (2024), the 1% supplementation level appears to create an ideal physiological environment for the expression of genes associated with cold adaptation.

CONCLUSION

This study demonstrates that dietary supplementation with Job's tears, particularly at a

1% inclusion level, significantly enhances cold tolerance in *L. rohita*. The improved survival rate, reduced stress response, enhanced antioxidant capacity, and upregulation of cold-tolerant genes suggest that Job's tears is a promising feed additive for mitigating cold stress in aquaculture. The findings reveal the potential of Job's tears to address the critical issue of cold stress in *L. rohita* cultivation, which could have far-reaching implications for aquaculture in subtropical and temperate regions.

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