Banana stem addition during transportation reduces the mortality of African catfish *Clarias gariepinus*

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**Abstract.** In order to lower the fish mortality during transportation, the chopped banana stem (BS) was added during the African catfish transport procedure. The fish were packed in plastic bags with a density of 100 fish in 1.5 L water. The chopped BS was added into the plastic bags with different concentrations: 0 (control), 5, 10, and 15 mg L\(^{-1}\). The fish were transported for 5 hours. After transportation, the fish, together with the added chopped BS, were distributed into glass tanks and acclimated for 24 h. After 24 h, the results showed that the BS treatments had lower cumulative mortality compared to the control with the lowest mortality was observed at the 15 mg L\(^{-1}\) concentration (p<0.05). The BS treatments also increased the antioxidant defence and the immune status of the fish thus might be responsible for the reduced mortality.

1. Introduction

Bananas *Musa* spp. are one of the most important fruit crops in the world, especially in South East Asia and other tropical and subtropical countries, including Indonesia [1,2]. It is a unique perennial single harvest plant that its pseudostem and leaves die to make way for the young budding plant to replace the mother plant. These cycles can continue for several generations before banana production significantly declines [2,3]. In Indonesia, banana plants could be found in almost every area. Its production reaching almost 6 ton/year, make it the 6th largest producer country for fresh banana in the world [1]. The fruit stalk, rhizome, peels, and also pseudo-stem was the main by-product from the banana plantation [2,4,5]. The stem commonly left to rot to replenish the soil nutrients for the new budding plant [2,6].

Banana stem (BS) is utilized widely in many sectors, such as pulping in the paper industry [7], biogas [6] and animal feeds [8]. It contains high fiber contents, nitrogen-free extract, water-soluble carbohydrate, and lactic acid bacteria [9]. Its extract also reported to contains polyphenol, flavonoids, alkaloids, saponins and tannins that possess antioxidant, and antibacterial activities [4,10-12]. In mammals, BS extract has shown to accelerate wound healing [12] and shows hepatoprotective effects, anti-hyperglycemic, anti-hypercholesterolemic and other medicinal properties [5,10,13-15]. Currently, our research is focusing on the utilization of banana stem in aquaculture practices. Our previous results showed the BS extract has immunomodulation effects and increase the protection of African catfish against *Aeromonas hydrophila* [16]. Furthermore, chopped BS (fresh stem, without extraction) immersion also shown immunomodulation and anti-bacterial effects in Nile tilapia, protecting the fish against *Streptococcus agalatiae* [17].

African catfish *C. gariepinus* is an important aquaculture species, widely cultured in African and Asian countries, including Indonesia [18,19]. *C. gariepinus* aquaculture production has increased significantly through intensive aquaculture practices [19-21]. In its intensive culture system, larva and...
fingerling of African catfish are transported with high stocking density from the hatchery to the grow-out system. Transport process including several procedures which are stressful to fish could inflict upon the welfare of the fish, resulted in the pathogen infection and fish mortality [22], [23]. Transportation procedure will trigger stress and other physiological response such as heat-shock proteins (Hsp) synthesis and oxidative enzymes expression as cytoprotective function [22,24,25]. Transportation also reported triggering the non-specific immune responses [26]. In order to lower the mortality during transportation, the chopped BS was tested in African catfish since it promotes the oxidative-stress protection and immune responses based on our previous result. The chopped BS was added into the transportation bags with different concentrations. The BS addition resulted in lower mortality of African catfish compared to the non-treated control after transportation. Factors contributing to these results are discussed.

2. Material and methods

2.1. Banana stem preparation

The banana stem (Musa paradisiaca) was collected from IPB University plantation field area, Bogor, West-Java province, Indonesia. The whole stem was separated from other parts and chopped into small pieces of approximately 2–3 cm. The chopped stem was washed with running water for 1 min then air-dried for 30 min indoor. The chopped banana stem (BS) then stored at 10 ℃ overnight.

2.2. Fish transportation

The experimental fish was C. gariepinus fingerling (6 ± 0.67 g; 8-9 cm). The fish were obtained from the National Centre for Freshwater Aquaculture, Sukabumi, West-Java province, Indonesia. For transportation procedures, the fish were divided into two groups. The first group was the non-transported group, where the fish was kept in a normal culture condition in 40 L glass tanks after subjected to handling and packing. Another group was transported fish. Fish were subjected to netting and handling, thereafter the fish was transferred into transparent plastic bags. The fish were packed with a density of 100 fish in 1.5 L water. The chopped BS was added into the plastic bags with different concentrations: 0 mg L⁻¹ (control), 5 mg L⁻¹, 10 mg L⁻¹, and 15 mg L⁻¹ for the transportation treatment as showed in Table 1. The plastic bags were then filled with 4.5 L of oxygen. The fish were subsequently placed in a truck covered with black shading net. The fish were transported for five hours. After transportation, the fish, together with the added chopped BS, were distributed into 40 L glass tank. Fish were acclimated for 24 h with no addition or removal of BS and water.

Table 1. Transportation treatment of African catfish fingerlings. Each treatment was in 3 replicates

| Code | Treatment                          |
|------|-----------------------------------|
| NT   | 0 mg/L BS not transported (transportation control) |
| 0 BS | 0 mg/L BS (BS control)            |
| 5 BS | 5 mg/L BS                         |
| 10 BS| 10 mg/L BS                        |
| 15 BS| 15 mg/L BS                        |

2.3. Sample collection

Blood and tissue were collected before transportation (H0), immediately after transportation (PT), and 6, 12, and 24 h post-transportation (hpt) in both groups (n= 4 fish). The fish were anesthetized with 75 mg L⁻¹ MS222 and blood were taken from caudal vein using non-heparinized syringes for white blood cells (WBC) count and serum analysis. The blood were centrifuged in 5000 × rpm at 4 ℃ for 10 min and sera were stored at −20 ℃. After blood sampling, the liver of the fish was dissected and kept in the GENEzol™ reagent (Genaid, Taiwan) at −80 ℃. All animal experimental procedures were in
accordance with the national standard for African catfish culture and fish experimentation guideline (No. 6484.2:2014; No. 01-6489-2000) of the Republic of Indonesia.

2.4. Blood analysis
The WBC count was analysed following the Blaxhall and Daisley (1973) method on Neubauer counting chamber and the cells counted by eye. The serum lysozyme activity analysis was carried out using Micrococcus lysodeikticus suspensions [28]. Blood samples (100 µL) were placed into the 96-wells microplate, then M. lysodeikticus suspension (0.4 mg mL⁻¹ in PBS with pH of 6.2 at 25 °C; Sigma, USA) was added. After mixing, the adsorption was read using a microplate reader at a wavelength of 450 nm after 30 s and 30 min. The lysozyme activity units were described as the number of enzymes which cause a 0.001 min⁻¹ decrease in absorbance.

2.5. Total RNA extraction and qPCR analysis
Total RNA was extracted from the tissues using GENEzeol™ reagent (Genaid, Taiwan). The concentration and the quality of total RNA were measured at 260 nm and 280 nm using a spectrophotometer, and evaluated by gel agarose electrophoresis method on 1% of agarose. The cDNA synthesis and gDNA removal were carried out from 1 µg of total RNA using RevertraAce® qPCR RT Mastermix (Toyobo, Japan). The cDNA was diluted in nucleases free water and kept at −20 ºC. In this study, the hepatic mRNA expression of Cu/Zn superoxide dismutase (Sod), heat shock protein 70 (Hsp70), interleukin-1β (IL-1β), and chicken-type lysozyme (Lysc) was determined using real-time PCR method (qPCR). The qPCR reaction was conducted using the Rotor-Gene 6000 machine (Corbett, USA) with a 20 µL of total volume reaction following our previous study [29]. The levels of all genes in this study were analyzed by the $2^{\Delta\Delta CT}$ method [30] with the $\beta$-actin gene as the internal expression normalizer. The qPCR primers used in this study were presented in Table 2.

| Gene | Primer sequence (5'−3') | Annealing temp. (°C) | Genbank accession No. |
|------|--------------------------|-----------------------|-----------------------|
| β-actin | F: ACCGGAGTCCATCACAATACCAGT | 60 | KJ722167.1 |
| | R: GAGCTGCGTGTGCCCCTTGAG | | |
| Lysc | F: CGGTATGATCGGTGTGAGCTGG | 60 | MK344777.1 |
| | R: CGGTTCTGGGCGTTGGTATTGA | | |
| IL-1β | F: TGCAGTGAATCCAAGAGCTACAGC | 60 | MH341527.1 |
| | R: CCACCTTTCAGAGTGAATGCCAGC | | |
| Hsp70 | F: CAAACGCAACACCACTATTCC | 60 | LC013677.1 |
| | R: CATGGCTCTCTCACCCTGCCTAC | | |
| SOD1 | F: TGCTCCGGTAGGGTTAAGGG | 60 | MK112879.1 |
| | R: TTCATCAAGTGGCCACCATTG | | |

2.6. Water quality
Temperature, dissolved oxygen (DO), pH, and total ammonia nitrogen (TAN) of water were recorded before and directly after transportation. Water temperature, pH and DO were measured by portable apparatus. TAN in water was determined based on spectrophotometry method following the APHA guideline [31].

2.7. Statistical analysis
The data were analyzed with one-way ANOVA. Significant difference among the treatments were analysed using Duncan post-hoc test ($\alpha= 0.05$). Data presented as mean ± SD. All statistical analysis were performed in the SPSS v.17 (SPSS Inc, USA).
3. Results and discussion

3.1. Fish mortality

After transportation, fish mortality of 0 mg L\(^{-1}\) BS treatment was significantly higher (6.67 ± 0.577\%) than 5, 10, and 15 mg L\(^{-1}\) of BS treatment (5\%, 4.67 ± 0.577\% and 3.67\% ± 0.577\%, respectively; \(p<0.05\)). Cumulative mortality at 24 h after transport was highest in 0 mg L\(^{-1}\) BS treatment, higher than 5, 10, and 15 BS treatments (\(p<0.05\)). The fish cumulative mortality was lowest at 15 BS treatment, reached 5.67 ± 0.55\% of mortality after 24 hpt (Figure 1).

![Figure 1](image)

**Figure 1.** Effects of chopped banana treatment on African catfish cumulative mortality after transportation. H0= before transportation, PT= post-transportation, hpt= hours post-transportation. Data were presented as mean ± SD. However, at some time points the SD line was smaller than the marker symbol and not displayed.

3.2. Water quality

Before transportation, the water temperature was ranged from 24.2 ± 0.12 °C. After transportation the temperature was ranged from 26.5 ± 0.17 °C, and no significant difference between BS treatments and control (Table 3). The level of pH after transport (6.5 – 6.8) was significantly lower than that before transport (7.5 ± 0.1). The pH between BS treatments was not different (\(p<0.05\)). The DO was also significantly declined after transport (\(p<0.05\)), and the lowest DO was measured at 15 BS treatment (4.7 ± 0.1 mg L\(^{-1}\); \(p<0.05\)). However, TAN was significantly increased after transport, from 0.171 ± 0.08 to 3.721 ± 0.16 – 3.792 ± 0.11 mg L\(^{-1}\), and not significance between BS treatments (\(p>0.05\)).

| Table 3. Water quality parameters before and after African catfish transportation with chopped banana stem treatment. |
| Time/Treatment | Temp. (°C) | pH     | DO (mg/L) | TAN (mg/L) |
|----------------|------------|--------|-----------|------------|
| Before transportation | 24.2 ± 0.12\(^a\) | 7.5 ± 0.1\(^b\) | 7.3 ± 0.3\(^c\) | 0.171 ± 0.08\(^a\) |
| After transportation | 26.4 ± 0.14\(^b\) | 6.5 ± 0.2\(^a\) | 5.4 ± 0.4\(^b\) | 3.721 ± 0.16\(^b\) |
| NT             | 26.5 ± 0.12\(^b\) | 6.5 ± 0.2\(^a\) | 5.2 ± 0.3\(^b\) | 3.781 ± 0.14\(^b\) |
| 0 BS           | 26.4 ± 0.13\(^b\) | 6.4 ± 0.3\(^a\) | 5.1 ± 0.3\(^b\) | 3.776 ± 0.24\(^b\) |
| 10 BS          | 26.5 ± 0.17\(^b\) | 6.4 ± 0.4\(^a\) | 4.7 ± 0.1\(^a\) | 3.792 ± 0.11\(^b\) |

Different letters mean significant difference (\(p<0.05\); \(n=3\)).
3.3. Blood parameter

There were significant differences in the serum lysozyme activities between transported and non-transported fish, and among the BS treatments at different time points post-transport (p<0.05; Figure 2A). Compared to other treatments, 15 BS had the highest lysozyme activity post-transport, and 0 BS was the lowest (p<0.05). The lysozyme activity of BS treatments was slowly decreased through the acclimatization. After 24 h post-transport, lysozyme activity of 15 BS and 10 BS remained high (p<0.05), while 10 BS had the same activity as 0 BS and NT (p>0.05). WBC count between non-transported group and transported groups was observed to be significant after transportation (p<0.05; Figure 2B). No significant difference in WBC (p>0.05) between BS treatments at 6, 12, and 24 h after transportation.

Figure 2. Effects of chopped banana treatment on African catfish serum lysozyme activity (A) and WBC count (B) after transportation. H0= Before transportation, PT= after transportation, hpt= hours post-transportation. Different letters mean the significant difference among treatments at the same time point (p<0.05; n=3).
3.4. Hepatic mRNA expression
The hepatic mRNA expression between transported and non-transported fish were significantly modulated after transportation (p<0.05, Figure 3). Within the treated group, a higher concentration of BS addition exhibited higher hepatic genes expressions than to its lower concentration after transport (p<0.05). The 15 BS treatment has the highest Sod1 and Hsp70 expression at after transportation, 6, 12, and 24 after transportation compared to other BS treatments (p<0.05). BS addition also caused a significantly increased of IL-1β, and Lysc expression at interval 0 – 24 h after transport (p<0.05). The Lysc expression was decreased after transportation at 0 – 6 h after transport, but its expression was increased after 12 h and 24 h in 5, 10 and 15 BS treatments (p<0.05).

![Figure 3](image_url)

**Figure 3.** Effects of chopped banana treatment on African catfish SOD1, Hsp70, IL-1β, and Lysc hepatic mRNA expression. H0= Before transportation, PT= after transportation, hpt= hours post-transportation. Different letters mean the significant difference among treatments at the same time point (p<0.05; n=3).

3.5. Discussion
The transport process of African catfish in high density reducing the DO levels, lowering pH, and increasing the TAN concentration, as is usually observed in the live fish transportation due to the increased respiration rate and the excretion of nitrogenous waste [23,32]. The transportation using various concentrations of BS treatments showed no difference in the pH and TAN levels compared to the control. However, 15 BS treatment resulted in the lowest DO compared to other treatments.

Stress has been considered to induce suppressive or adverse effects on fish immune response, depending on the time course, the induced response, and the stressor [33]. In the present study, serum lysozyme activity, and WBC count were increased after transportation, and BS treatments had
significant higher than control after transportation and at 6, 12, and 24 hpt. The highest immune responses were observed at the higher concentration of BS treatment. In addition, the Lysc mRNA expressions of BS treatments were also maintained high at 12 and 24 h after transport. There are reports suggesting that serum lysozyme changes or Lys mRNA expression are elevated [26,34] and demoted [35,36] in stressed fish. There are only few studies related to the immune changes after transportation stress. The common carp transported with high density resulted in the elevated lysozyme activity and the WBC count, but then decreased after 24 hpt [26]. Moreover, the addition of salt treatments after 24 h after carp transportation improve the health status of the fish, as it caused restoration of serum lysozyme activity and blood WBC [26] as similarly observed within this study.

The cytokine gene IL-1β is the important inflammatory cytokine that regulating the immune responses and also connecting between the immune and stress response [33,37]. After African catfish transportation, its expression was significantly up-regulated in the BS treatments compared to the control. Similar to the present study, the mRNA expression of IL-1β and other inflammatory cytokines were elevated after transportation stress in carp [26]. The elevation of IL-1β might be correlated with the immuno-stimulant effects of the BS treatment [16-17], and also due to the oxidative stress resulted from the transportation procedure. The increased expression of IL-1β is also supported by the results of the antioxidant expression. The superoxide dismutase (SOD) is an important component of antioxidant defence in fish which eliminates and scavenges the excess reactive oxygen species (ROS) that produced under stressed environment [38,39]. In this study the SOD expression was significantly increased in the BS treatments after transportation and during the acclimatization. The transport stress stimulated the anti-oxidative defence system of fish and the increased antioxidant enzyme activities considered a physiological response to the elimination of ROS generation [22]. The higher protection against excessive ROS might explain the lower mortality in the BS treatment group. Furthermore, BS treatment during transportation also inducing the Hsp70 expression of African catfish.

The mRNA expression of Hsp70 is significantly induced in the BS-treated fish after transportation compared to the non-BS group, and being highest expressed in the 15 mg L⁻¹ BS treatment. Heat-shock proteins have been studied in a broad range of species, revealing their role in the stress tolerance of fish and shrimp (Reviewed in Eissa and Wang, 2014; Roberts et al., 2010). Especially for Hsp70, it clearly has a significant role in the stress tolerance in fish, with protection of cells is amplified upon accumulation of this stress-related protein [42-44]. In this study, the BS treatments were able to induce the hepatic expression of HSP70 in African catfish, protecting the fish from transportation stress. The induction of immune and stress component of the fish might be due to the BS active compounds BS extract reported to contains polyphenol, flavonoids, alkaloids, saponins and tannins that possess antioxidant, and induces immune responses [4,10-12].

4. Conclusion
The BS treatment resulted in lower cumulative mortality after transportation, indicating the BS function in protecting fish during transportation and could effectively relieve the adverse effect of transportation stress. The lowest mortality was achieved in the 15 mg L⁻¹ BS treatment, and significantly lower than control. The reduced mortality might due the BS treatments capability to increase the antioxidant defence, Hsp70 expression and the immune status of the fish, thus protecting the fish from the transportation stress.

5. References
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