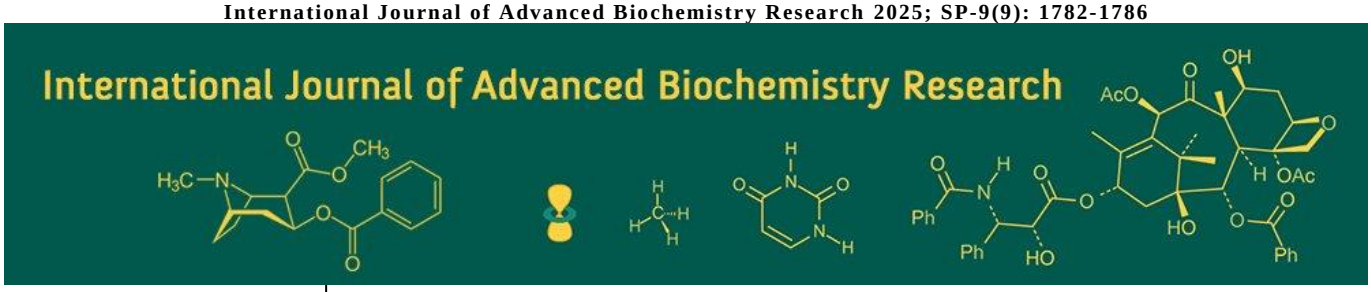


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**Swetha Priya Gali**
Ph.D. Scholar, Department of Food Packaging & Storage Technology, National Institute of Food Technology, Entrepreneurship and Management, Thanjavur (NIFTEM-T), formerly IIFPT, Ministry of Food Processing Industries, Government of India, Pudukkottai Road Thanjavur, Tamil Nadu, India

**S Bhuvana**
Associate Professor, Department of Food Safety and Quality Assurance, National Institute of Food Technology, Entrepreneurship and Management, Thanjavur (NIFTEM-T), formerly IIFPT, Ministry of Food Processing Industries, Government of India, Pudukkottai Road Thanjavur, Tamil Nadu, India

**R Meenatchi**
Associate Professor, Department of Food Packaging & Storage Technology, National Institute of Food Technology, Entrepreneurship and Management, Thanjavur (NIFTEM-T), formerly IIFPT, Ministry of Food Processing Industries, Government of India, Pudukkottai Road Thanjavur, Tamil Nadu, India

**Corresponding Author:**
**R Meenatchi**
Associate Professor, Department of Food Packaging & Storage Technology, National Institute of Food Technology, Entrepreneurship and Management, Thanjavur (NIFTEM-T), formerly IIFPT, Ministry of Food Processing Industries, Government of India, Pudukkottai Road Thanjavur, Tamil Nadu, India

# Impact of moisture content of the bedding material on the survival & growth of Superworms (*Zophobas morio*)

**Swetha Priya Gali, S Bhuvana and R Meenatchi**

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**Abstract**
Moisture is a crucial environmental factor influencing the physiology, metabolism, and survival of insect species. This study aimed to evaluate the effect of varying moisture levels in wheat bran bedding (10 ± 2%, 20 ± 2%, 30 ± 2%) with and without a wet cotton ball on the Survival rate (%), weight (g), and length (mm) of *Zophobas morio* larvae over a 28-day period. Six experimental groups were established with triplicates, and larval parameters were recorded at 7-day intervals. Results revealed that *Z. morio* reared in 20 ± 2% moisture with a wet cotton ball exhibited the highest survival (74.64 ± 12.84%) and minimal weight loss (final weight: 10.30 ± 1.44 g), while the lowest survival and weight were observed in 10 ± 2% moisture without cotton (10.64 ± 6.08%; 0.91 ± 0.35 g). Statistical analysis indicated significant effects of moisture and cotton presence on larval survival ($p < 0.05$). These findings suggest that moderate moisture content (~20 ± 2%) supplemented with a water-retaining aid optimizes *Z. morio* performance, providing insights for sustainable insect farming practices.

**Keywords:** Superworms, *Zophobas morio*, moisture content, survival, weight, bedding, insect farming

## 1. Introduction
Superworms are the larval stage of a darkling beetle called *Zophobas morio (Z. morio)*. They are being increasingly recognized for their ecological and commercial value as a protein-rich insect that convert organic waste into a high-value biomass. They serve a dual purpose, as sustainable animal feed, and potential bioconversion agents for organic waste decomposition. It is essential to optimize rearing conditions to allow reproducible larval performance. Among the several environmental factors that impact insect growth, the moisture in bedding is an important factor (Oonincx *et al*. 2015; Katchali *et al*. 2024) [15, 10].

The moisture of the bedding material can directly affect the insects. It influences hydration, palatability, microbial activity, and the overall microenvironment of the rearing environment. With too little moisture, desiccation and reduced metabolism may occur, while excess moisture can lead to increased fungal and bacterial activity and subsequently higher levels of larval mortality (Tomberlin *et al*. 2002; Cheng *et al*. 2017) [18, 4]. Moisture can also influence the rate of breakdown of the organic substrate, which in turn mediates the availability of the nutrients and metabolites necessary to support larval development.

Scientific studies using insects such as black soldier fly (*Hermetia illucens*), housefly (*Musca domestica*), and yellow mealworm (*Tenebrio molitor*) have indicated that creating a good moisture content (wetness) in the rearing environment (a substrate) for optimal growth and survival (Diener *et al*. 2009; Cammack and Tomberlin 2017; Gligorescu *et al*. 2020) [6, 3, 7]. Although significant research has focused on insects such as black soldier fly and mealworms, *Z. morio* are relatively unexplored in the context of relationship between substrate moisture and supplementary moisture-retaining materials. The use of a cotton ball as a localized humidity stabilizer is a fairly simple mechanism for providing sufficient moisture in small rearing containers. The cotton ball passively releases water maintaining surrounding humidity to the larvae, without adding moisture to the substrate or changing its texture, also it prevents mold formation which can be a challenge especially in reducing stress in circumstances requiring moisture stability.

The present study explores the influence of three moisture levels (10 ± 2%, 20 ± 2%, and 30 ± 2%) in wheat bran, and the presence or absence of a wet cotton ball, on the survival,

weight, and length of *Z. morio*, and useful information to improve superworm farming sustainability.

## 2. Materials and Methods

### 2.1 Insects

*Z. morio* larvae were purchased for the study from a commercial insect farm, M/s. Promeal, Nasik, Maharastra. To ensure proper acclimatization and optimal health, larvae were placed in wheat bran (sole substrate) for 20 days after shipping, as suggested by (Harsanyi *et al*. 2020) [8].

### 2.2 Experimental Design

Three moisture levels (10 ± 2%, 20 ± 2%, 30 ± 2%) with (Y) and without (X) wet cotton ball resulted in six experimental treatments and depicted in the groups as shown in Table 1.

Each treatment group was set up with three replicates in plastic containers, each containing 25 actively feeding *Z. morio* larvae of similar size and age.

**Table 1:** Treatment Groups Based on Moisture Level and Hydration Method

| Group | Moisture Content | Wet Cotton Ball | Label |
|---|---|---|---|
| A | 10 ± 2% | No | A X |
| A | 10 ± 2% | Yes | A Y |
| B | 20 ± 2% | No | B X |
| B | 20 ± 2% | Yes | B Y |
| C | 30 ± 2% | No | C X |
| C | 30 ± 2% | Yes | C Y |

### 2.3 Environmental Conditions and Monitoring

The experiment was conducted in a containers (12 cm h x 16 cm d) with ambient environmental conditions (30 ± 4 °C temperature, 40–50% relative humidity and 12 hours each of light and dark photoperiod alternatively) (Beesigamukama *et al*. 2022) [2]. Cotton balls saturated using distilled water were set in 'Y' configuration containers to buffer environmental humidity without wetting the substrate. The cotton balls were replaced once every two days and the wheat bran with respective moisture percentages were replaced once every week during the experiment to continue to provide moist conditions.

### 2.4 Moisture Conditioning of Wheat Bran

Wheat bran is used as feed substrate and also as a bedding material for *Z. morio* larvae. The moisture conditioning was required to optimize the bedding material across experimental groups and remove any potential confounding variable relating to material dry or overly wet condition. Wheat bran is conditioned to 3 different moisture levels 10 ± 2%, 20 ± 2%, 30 ± 2% (Table 1).

Required volumes of distilled water to condition the moisture content of the wheat bran were calculated using the following *formula 1*

$$Q = \frac{Wi(Mf-Mi)}{(100-Mf)}$$ --------- formula 1

Where:

- Q = Mass of distilled water to be added (kg)
- $W_i$ = Initial weight of dry wheat bran (kg)
- $M_i$ = Initial moisture content of wheat bran (%, dry basis)
- $M_f$ = Desired final moisture content (%, dry basis)

Uniform Moisture content of wheat bran was maintained by adding required quantity of distilled water as per the method given by (Mahapatra *et al*. 2013) [13].

After conditioning, the moisture content was then assured by oven-drying 5 g of sample at 105 ± 1 °C for 24 hours using the gravimetric method (AOAC 2005) [1].

All batches of *Z. morio* were stored in air-tight containers kept at room temperature and used within 48 hours to avoid moisture loss or microbial growth.

### 2.5 Parameters Analysed

Survival rate (%), larval weight (g) and length (mm) were recorded on 0, 7, 14, 21, and 28 days. Weight was recorded using a precision digital balance, and larval length was measured using a vernier calipers. Survival rate% was calculated as the number of living larvae at different time intervals. Data are reported as mean ± standard deviation. Statistical comparisons were made using a two-way repeated measures ANOVA followed by Tukey's HSD post-hoc test. Significance was set at $p < 0.05$.

## 3. Results and Discussion

The C Y (30 ± 2% moisture with wet cotton ball) group, by the 3rd day, showed visible mold growth on the surface of the substrate, as well as around the cotton ball. The mold was evident as white to greenish functional patches. These observations indicated moisture build-up, or excessive microbial growth. So, the cotton balls were replaced in the containers once in every 2 days. The C X (30 ± 2% moisture without wet cotton ball) group, did not show any kind of mold growth throughout the study. The substrate did appear dense and compacted, indicating that aeration may have been reduced and it is also possible that it affected larval movement while it was active, and also oxygen supply to the resort.

### 3.1 Larval Survival Trends

Larval survival decreased over the 28-day period for all treatment groups (Table 2); however, the extent of these decreases varied across groups. Group A X had the largest decrease in larval numbers, with just 10.64 ± 6.08% surviving on day 28 indicating that the low moisture treatment has a negative impact on larval survival when there is no humidity buffering. These results are consistent with the findings of (Lalander *et al*. 2019) [12] who showed that worms raised on dry biowaste substrates without sufficient moisture to keep hydration levels, suffered high mortality due to dehydration stress and low feed intake.

Group B Y had the best performance with 74.64 ± 12.84% remaining on day 28; followed by A Y and C Y with respective survivor means of 64.00 ± 10.56% and 53.32 ± 9.24%. The presence of the wet cotton ball has showed the lowest larval mortality even at low and high moisture levels (A Y and C Y) which was likely related to the stabilization of the humidity and not allowing the cotton ball to desiccate.

**Table 2:** Effect of substrate moisture content and hydration on larval survival rates (%) over 28 days

| Sample | Day 0 | Day 7 | Day 14 | Day 21 | Day 28 |
|---|---|---|---|---|---|
| A X | 100.00 | 81.32 ± 2.28$^{c}$ | 53.32 ± 15.12$^{b}$ | 24.00 ± 10.56$^{b}$ | 10.64 ± 6.08$^{c}$ |
| A Y | 100.00 | 96.00 ± 6.92$^{ab}$ | 84.00 ± 4.00 $^{a}$ | 73.32 ± 4.60$^{a}$ | 64.00 ± 10.56$^{a}$ |
| B X | 100.00 | 97.32 ± 2.28$^{ab}$ | 85.32 ± 14.04$^{a}$ | 64.00 ± 21.16$^{a}$ | 29.32 ± 8.32$^{bc}$ |
| B Y | 100.00 | 98.64 ± 2.28$^{a}$ | 88.00 ± 4.00$^{a}$ | 84.00 ± 4.00$^{a}$ | 74.64 ± 12.84$^{a}$ |
| C X | 100.00 | 93.32 ± 6.08$^{abc}$ | 78.64 ± 6.08$^{ab}$ | 54.64 ± 14.04$^{ab}$ | 20.00 ± 4.00$^{c}$ |
| C Y | 100.00 | 85.32 ± 6.08$^{bc}$ | 74.64 ± 4.60$^{ab}$ | 66.64 ± 4.60$^{a}$ | 53.32 ± 9.24$^{ab}$ |

Likewise, (van Broekhoven *et al*. 2015) [19] found that *Tenebrio molitor* larvae exposed to low humidity conditions (<40% RH) declined rapidly its survival unless moisture was introduced to the substrate or given as a separate resource. The decline in survival for A X reinforces that moisture withdrawn from below a critical level impacts several physiological aspects of larvae - possibly by desiccation, metabolic suppression, or low ability to utilize dry substrates due to dry environment.

Presence of mold indicated that too much moisture (CX and CY) of the substrate, likely created conditions that are less than ideal for the substrate, such as excessive microbial activity that leads to spoilage or unpalatability of the substrate, reduced substrate aeration, larval suffocation, or reduced feeding ability.

(Johnsen *et al*. 2021) [9] observed similar results to *Tenebrio molitor*, too much moisture (water) in wheat bran resulted in poor larval performance, due to substrate solidity and microbial overgrowth. (Laksanawimol *et al*. 2024) [11]. Also, excessive moisture content can put larva under stress and subject them to more exposure to pathogen infection.

This strongly implies that a balance of sludge at an estimated 20 ± 2% moisture is nearer to effectively optimal conditions for substrate composition, especially with respect to water provisions offered as an external resource. As moisture is required, these findings also confirms that above optimal total water may induce hypoxic conditions, to pathogen risk, reducing larval survival rate, despite water being abundant. The stable survival experienced in B Y suggested a moderate balance of both internal and external moisture generated the 'most favourable' rearing environment for maintaining the larvae over the long term.

### 3.2 Weight Retention Analysis

The initial larval weight across all groups were between 12.10 ± 0.50 gm and 12.65 ± 0.30 g is presented in Table 3. From the outset, weight decreased in all treatments, with the greatest loss of weight occurring in Group A X; larvae from Group A X reached a final weight of 0.91 ± 0.35 g at day 28. The least loss of weight occurred in Group B Y, which declined to 10.30 ± 1.44 g showed the greatest hydration and feeding efficiency. The role of wet cotton as a buffer towards suboptimal microenvironments positively impacted weight retention among all hydration groups, especially in the case of 10 ± 2% and 30 ± 2% moisture groups.

**Table 3:** Changes in larval weight (g)

| Sample | Day 0 | Day 7 | Day 14 | Day 21 | Day 28 |
|---|---|---|---|---|---|
| A X | 12.43±0.23$^{a}$ | 8.54±0.29$^{b}$ | 5.44±1.04 $^{b}$ | 2.30±0.68 $^{d}$ | 0.91±0.35 $^{c}$ |
| A Y | 12.65±0.30 $^{a}$ | 11.64±0.48 $^{a}$ | 10.68±0.28 $^{a}$ | 10.44±0.38 $^{ab}$ | 9.30±0.85 $^{ab}$ |
| B X | 12.38±0.29 $^{a}$ | 10.90±0.60 $^{a}$ | 9.62±1.53 $^{a}$ | 7.50±2.33 $^{bc}$ | 3.35±0.95 $^{c}$ |
| B Y | 12.10±0.50 $^{a}$ | 11.78±0.41 $^{a}$ | 11.20±0.24 $^{a}$ | 11.67±0.70 $^{a}$ | 10.30±1.44 $^{a}$ |
| C X | 12.18±0.10 $^{a}$ | 11.00±0.43 $^{a}$ | 9.45±0.39 $^{a}$ | 6.49±1.90 $^{c}$ | 2.45±0.55 $^{c}$ |
| C Y | 12.60±0.41 $^{a}$ | 10.96±0.84 $^{a}$ | 9.70±0.75 $^{a}$ | 9.07±0.54 $^{abc}$ | 7.55±1.08 $^{b}$ |

This supports findings from (Lalander *et al*. 2019) [12], who indicated that *Z. morio* larvae reared with dry organic matter without moisture supplementation exhibited limited growth and increased mortality, while moisture-enhanced substrates provided better feed acceptability and digestibility. In a similar study, (van Broekhoven *et al*. 2015) [19] observed that *Tenebrio molitor* larvae had reduced growth and nutrient conversion under low humidity conditions, but this could be reversed by increasing moisture levels or adding humidified environments.

This evidence suggests that moderate substrate moisture (20 ± 2%) is appropriate for larval development, especially in conjunction with additional moisture. The impression is that too much moisture in the substrate may lead to anaerobic or undesirable microbial activities that diminish feed quality or may be stressful by some mild degree. The findings of (Johnsen *et al*. 2021) [9] showed that *Tenebrio molitor* diminished growth rate beyond maxima relative to optimal moisture levels likely as a function of a soggy substrate, possible microbial imbalance, or impair/reduce aeration.

### 3.3 Changes in Larval Length

The length of the larvae, in comparison, experienced only minor changes over the 28-day observation period (Table 4). The two groups supplemented with wet cotton balls generally maintained or increased their larval lengths, while the two groups without cotton experienced a gradual decrease in length. Group B Y (20 ± 2% moisture plus cotton) had the greatest final length of 44.0 ± 1.7 mm, followed closely by group A Y and group C Y, which maintained similar lengths throughout the trial. The groups without cotton, especially group C X and group A X, had reduced lengths over time. These reductions likely indicate that dehydration stress occurred, along with reduced nutrient availability in the diet or even cuticular shrinkage due to time spent in less than optimal moisture for larvae. The lengths found through the cotton supplementation groups suggests enough hydration support, even just a small amount, can play a role in larval body maintenance throughout the rearing process.

**Table 4:** Variation in length (mm) of *Z. morio* larvae in response to moisture and hydration conditions

| Sample | Day 0 | Day 7 | Day 14 | Day 21 | Day 28 |
|---|---|---|---|---|---|
| A X | 41.3 ± 1.1 [ab] | 40.6 ± 1.1 [a] | 40.0 ± 0.0 [b] | 38.0 ± 0.0 [b] | 36.6 ± 1.5 [b] |
| A Y | 41.3 ± 1.1 [ab] | 40.0 ± 2.0 [a] | 43.6 ± 1.5 [a] | 43.7 ± 1.5 [a] | 43.6 ± 1.5 [a] |
| B X | 39.3 ± 1.1 [b] | 39.3 ± 1.1 [a] | 39.0 ± 1.0 [b] | 39.0 ± 1.0 [b] | 37.6 ± 2.5 [a] |
| B Y | 40.0 ± 2.0 [a] | 41.3 ± 2.3 [a] | 42.0 ± 2.0 [ab] | 43.6 ± 1.5 [a] | 44.0 ± 1.7 [b] |
| C X | 44.0 ± 1.7 [a] | 40.6 ± 1.1 [a] | 39.3 ± 1.1 [b] | 38.7 ± 1.1 [b] | 37.0 ± 1.7 [b] |
| C Y | 41.3 ± 1.1 [ab] | 40.6 ± 1.1 a | 39.3 ± 1.1 [b] | 40.3 ± 2.1 [ab] | 40.6 ± 2.3 [ab] |

This supports the findings of (Lalander *et al*. 2019) [12] who demonstrated that *Z. morio* larvae did not efficiently grow and develop under dry conditions when moisture was not offered in some other way. Age, humidity and moisture are all factors, and (van Broekhoven *et al*. 2015) [19] found low humidity environments restricted the molting and linear growth of *Tenebrio molitor*, and water is necessary to soften the cuticle and assist the stretching process that accompanies molts.

This suggests moisture alone is not enough to ensure larval elongation when placed over the long term, as bran may lose moisture over short while periods of time. The cotton ball likely provided a constant humidity environment enhancing turgor in all focal individuals, and thus improved success in molting.

This implies that although high substrate moisture may seem like an ideal conditions, too much moisture in the substrate without aeration can lead to undesired conditions (e.g., excessive microbial growth), which may ultimately decrease feeding efficiency and development time.

This was shown in the study by (Johnsen *et al*. 2021) [9] who found that too wet feed decreased *Tenebrio molitor* feeding behaviour, likely because soggy substrates may be unpalatable or there was a risk of anaerobic conditions.

Our results are in consistent with (Nyakeri *et al*. 2017) [14], who pointed out that there is a narrow line between optimal and detrimental moisture for larval rearing. A dry substrate could decrease palatability of material thus limiting larval uptake, while too much moisture could lead to hypoxic situation in the substrate that limits aerobic respiration. The desirable outcomes of wetting the substrate to achieve a moisture content of 20 ± 2% using a wet cotton ball is also likely due to the concept of controlled micro-environmental buffering as mentioned by (Cortes Ortiz *et al*. 2016) [5] which is fundamental to high density insect rearing setups.

The approach of utilizing a cotton ball as a passive humidity stabilizer is an underutilized yet potentially useful method. Very similar principles are described in fruit fly (Pascacio-Villafán *et al*. 2020) [16] and mealworm (Rovai *et al*. 2021) [17] cultures using water gels and hydrating fruits (i.e., carrots). A cotton ball has the significant advantages of not decomposing, desired placement, and being a reliable, non-nutritive source of humidity.

In addition, the link between hydration and larval metabolic efficiency requires further consideration. Previous work (Gligorescu *et al*. 2020; Zhang *et al*. 2021) [7, 20] describes how diets that allow for moisture availability directly correlate with enzymatic activity and gut microbial diversity, both of which govern digestion and nutrient utilization. It is very possible that fills of microbial symbiosis occurred in groups B Y and C Y, which allowed for weight retention and survivorship.

## 5. Conclusion

The current study found that moisture content of rearing substrates and mitigating local humidity using moist cotton had a clear impact on health and survival of *Z. morio*. The moisture level of 10 ± 2% was insufficient for larval maintenance leading to desiccation stress and inevitable mortality, particularly lacking cotton. The maximum moisture level tested (30 ± 2%) appeared neutral, if not positive, there could be some microbial imbalance. There seems to be a suitable window around 20 ± 2%, where hydration and microbial conditions have a positive effect.

It also contributes to the existing limited body of research on *Z. morio*, but still adds to the information on the care of *Z. morio* side. Commercial studies on *Z. morio* have largely looked at feed substrates, with the exception of a few studies examining abiotic parameters such as temperature and humidity or bedding moisture. Incorporating moisture content and passive humidification as standards in insect farming guidelines may eliminate inconsistencies and reduce larval mortality and inconsistencies, especially in a small-scale production system or in an environment with fluctuations.

Future research could consider the impact of microorganisms associated with various moisture regimes, as well as the feasibility for the development of large-scale insect production systems with cotton ball humidification for insect rearing systems.

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