

Ecological Determinants of Fungal Growth and Colonization in Indian Millets

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ABSTRACT

Millets are nutritionally rich and climate-resilient crops widely consumed in India, yet they are highly vulnerable to fungal contamination during post-harvest storage. The present study aimed to isolate potentially mycotoxigenic fungi from stored millet grains collected from major millet-producing regions of India and to evaluate the influence of environmental and nutritional factors on their growth under laboratory conditions. Aged millet samples exhibiting visible fungal infestation were subjected to standard mycological isolation techniques using potato dextrose agar. The dominant fungal genera identified based on morphological characteristics were *Aspergillus*, *Fusarium*, and *Penicillium*, with *Aspergillus* species occurring most frequently across all sampling sites. Optimization studies revealed that potato dextrose agar supported maximum radial growth of fungal isolates compared to other tested media. Temperature significantly influenced fungal growth, with optimal growth observed at 27–30 °C, while growth declined at higher and lower temperatures. The isolates exhibited maximum biomass production under slightly acidic conditions, particularly at pH 5.5–6.5. Incubation period studies indicated peak fungal growth between 7 and 10 days. Among nutritional factors, glucose and sucrose were the most favorable carbon sources, while yeast extract and peptone supported higher biomass production compared to inorganic nitrogen sources. The optimized growth conditions closely resemble traditional grain storage environments in rural India, highlighting a substantial risk of fungal proliferation and potential mycotoxin contamination in stored millets. The findings emphasize the need for improved post-harvest handling, storage practices, and regular monitoring to ensure the safety and quality of millet and millet-based food products.

Keywords: Mycotoxin-producing fungi; Millets; Storage fungi; Optimization studies; post-harvest safety

1. Introduction

Millets are increasingly recognized as climate-resilient, nutrient-dense cereal crops that play a vital role in ensuring food and nutritional security, particularly in semi-arid and resource-limited regions of the world. In India, millets such as pearl millet, finger millet, sorghum, foxtail millet, and little millet are widely consumed and contribute significantly to dietary protein, minerals, dietary fiber, and bioactive compounds [1,2]. The renewed global interest in millets, supported by initiatives such as the International Year of Millets 2023, highlights their potential for sustainable agriculture and public health. However, despite their agronomic and nutritional advantages, millets are highly susceptible to fungal contamination during post-harvest handling and storage, posing serious food safety concerns. One of the major challenges associated with stored millet grains is contamination by potentially mycotoxigenic fungi. Fungal genera such as *Aspergillus*, *Fusarium*, and *Penicillium* are commonly reported in cereal grains and are known to produce toxic secondary metabolites including aflatoxins, ochratoxins, fumonisins, and zearalenone [3,4,19]. These mycotoxins are chemically stable and can persist during processing, thereby entering the food chain and posing chronic health risks to consumers. Long-term exposure to mycotoxins has been associated with hepatotoxicity, immunosuppression, carcinogenesis, and growth retardation [5,6].

India's tropical and subtropical climatic conditions, characterized by high temperature and humidity, create a favorable environment for fungal growth and mycotoxin production in stored grains. Traditional storage practices, inadequate drying, poor ventilation, and prolonged storage further exacerbate the risk of contamination [7,8]. Several studies have reported the presence of aflatoxigenic *Aspergillus flavus* and *A. parasiticus* in Indian food grains, including millets, indicating a serious public health concern [9,10,34,35,37]. Despite increasing awareness, surveillance of potentially mycotoxigenic fungi in millets remains limited compared to major cereals such as rice and wheat. Accurate evaluation of growth conditions for potentially mycotoxigenic fungi is essential for understanding their behaviour and potential risks in stored millets. Environmental and nutritional factors such as temperature, pH, moisture, incubation period, and nutrient availability significantly influence fungal growth and metabolite production. Many storage-associated fungi exhibit optimal growth under moderate temperatures, slightly acidic pH, and nutrient-rich substrates, conditions commonly found in traditional grain storage environments [11,12,36]. The type of carbon and nitrogen sources further regulates fungal biomass accumulation and colonization potential [13]. In this context, the present study focuses on optimizing cultural and environmental parameters for fungal isolates from Indian millets to better understand contamination risks and support

improved post-harvest management strategies aimed at minimizing fungal contamination and ensuring the safety and quality of millet and millet-based food products.

2. Materials and Methods

2.1. Sample Collection

Millet grain samples used in the present investigation were collected from local markets across four major millet-producing regions of India, namely Haryana, Rajasthan, Madhya Pradesh, and Uttar Pradesh [14]. Approximately 500 g of millet grains were collected in triplicate from each location. Both unprocessed and retail-packed millet varieties were included in the study. In addition, millet grains exhibiting visible signs of fungal contamination, such as black, green, or powdery mould growth on the grain surface, were selectively collected and placed in separate sterile zipped poly bags to avoid cross-contamination. All samples were properly labelled, transported to the laboratory under ambient conditions, and stored at room temperature until further analysis.

2.2. Sample Preparation

Before fungal isolation, the millet grains were examined visually, and damaged or visibly contaminated grains were separated from apparently healthy grains. The samples were then cleaned to remove dust and other surface debris that could interfere with fungal recovery. All subsequent handling was performed aseptically to reduce the possibility of external microbial contamination and to facilitate reliable recovery of the resident grain mycoflora [15,16].

2.3. Isolation and Purification

Potentially mycotoxigenic fungi were isolated from the millet grains using standard mycological procedures with minor modifications [17,21]. Visibly contaminated grains were immersed in 1% sodium hypochlorite for 30 seconds in sterile polyethylene bags, followed by three rinses with sterile distilled water. The grains were air-dried under laminar airflow and transferred aseptically to potato dextrose agar (PDA; HiMedia, India). Plates were incubated at $27 \pm 2^\circ\text{C}$ for 7 days [18] and monitored periodically for emerging fungal colonies. Distinct colonies were purified by repeated hyphal-tip transfer (3-4 times) onto fresh PDA. Representative isolates were selected for subsequent characterization on the basis of their morphology and relative occurrence. Because mycotoxin production was not experimentally evaluated, the isolates are described as potentially mycotoxigenic based on their taxonomic identity and published evidence of toxin production by the corresponding fungal taxa. Species level confirmation would require molecular identification.

2.4. Optimization of fungal isolates

Optimization experiments were conducted with the selected fungal isolates to identify the cultural and environmental conditions that supported maximum fungal growth. Pure cultures obtained after repeated sub-culturing were used for all optimization assays. The study evaluated culture medium, temperature, pH, incubation duration, and carbon and nitrogen sources as growth-related factors.

2.4.1. Effect of Culture Media

Four routinely used fungal culture media potato dextrose agar (PDA), Rose Bengal chloramphenicol agar (RBC), malt extract agar (MEA), and Sabouraud dextrose agar (SDA) were compared

for their ability to support fungal growth [20]. A 5-mm mycelial plug taken from a 7-day old culture was placed at the centre of each plate. The plates were incubated at $27 \pm 2^\circ\text{C}$ for 7 days, and radial colony expansion was measured at defined intervals [16].

2.4.2. Effect of Temperature

PDA plates inoculated with the selected fungal isolates were incubated at 20, 25, 30, 35, and 40°C . Radial colony diameter was measured after 7 days, and the temperature associated with the greatest colony expansion was designated as the optimum for growth [13,22].

2.4.3. Effect of pH

The effect of pH was examined using potato dextrose broth adjusted to pH 4.0 - 9.0 with sterile 1 N HCl or 1 N NaOH before autoclaving. After inoculation, the cultures were maintained statically for 7 days at the optimized temperature. The fungal biomass was recovered by filtration, oven-dried, and weighed as the measure of growth [23,24].

2.4.4. Effect of Incubation Period

To determine the influence of culture duration, inoculated potato dextrose broth cultures were maintained for 3, 5, 7, 10, and 14 days. Biomass was determined at each sampling point, and the period yielding the greatest biomass was considered the optimum incubation duration [25].

2.4.5. Effect of Carbon and Nitrogen Sources

Nutritional effects were assessed by adding glucose, sucrose, lactose, or starch as carbon sources and peptone, yeast extract, ammonium nitrate, or sodium nitrate as nitrogen sources to the basal medium. Cultures were maintained under the optimized physicochemical conditions, and the resulting dry biomass was used to compare nutrient utilization [26,27].

3. Results and discussion

3.1. Isolation and Occurrence of Mycotoxin-Producing Fungi from Millet Samples:

Millet grain samples procured from Haryana, Rajasthan, Madhya Pradesh, and Uttar Pradesh exhibited heterogeneous levels of fungal contamination, largely influenced by storage duration, packaging condition, and local environmental factors. Visibly infected grains displayed characteristic black, green, and whitish mould growth, indicative of storage-associated fungi. Multiple fungal isolates were successfully recovered from contaminated samples, with *Aspergillus*, *Fusarium*, and *Penicillium* emerging as the dominant genera. Notably, *Aspergillus* spp. accounted for the majority of isolates across all sampling sites, underscoring their ecological advantage under warm, low-moisture storage environments commonly encountered in the investigated regions. The occurrence pattern observed in the present work is consistent with reports that *Aspergillus* is frequently recovered from stored cereal and millet grains. Studies of pearl millet and other cereals have documented the persistence of *Aspergillus* under storage conditions, particularly where moisture management and post-harvest handling are inadequate [4,19,28,29]. The predominance of *Aspergillus* in the present samples may therefore reflect its ability to survive and compete effectively in the storage environment. However, the present study did not directly quantify mycotoxin production, so the observed fungal occurrence should be interpreted as an indicator of potential mycotoxin risk rather than direct evidence of toxin formation.

3.2. Morphological Characterization of Fungal Isolates

The isolates showed distinguishable macroscopic and microscopic characteristics on PDA and were provisionally assigned to the genera *Aspergillus*, *Fusarium*, and *Penicillium*. *Aspergillus* colonies developed rapidly and produced dark brown to black sporulating growth with densely arranged conidiophores and radiating conidial heads. *Fusarium* isolates produced cottony white to pinkish mycelia and slender, sickle shaped macroconidia, whereas *Penicillium* isolates formed bluish-green, velvety colonies with characteristic brush-like conidiophores. These features were used for preliminary genus-level identification. Similar morphological features have been described for fungal isolates recovered from stored cereal grains and millet associated samples [30–32]. Species level confirmation, however, would require molecular identification.

3.3. Optimization of Culture Media

Culture medium affected colony development among the fungal isolates. PDA produced the greatest radial growth, followed by MEA, whereas lower growth was observed on RBC and SDA. The comparatively strong performance of PDA is consistent with its nutrient composition and its widespread use for cultivation of filamentous fungi. Similar observations have been reported for *Aspergillus* and other storage-associated fungi [25,29,33]. Because toxin production was not measured in the present study, the observed medium effect should be interpreted specifically as an effect on fungal growth rather than as evidence of enhanced secondary-metabolite production.

3.4. Effect of Temperature on Fungal Growth

Temperature strongly affected fungal proliferation. The highest radial growth was recorded at 27–30°C, while substantially less growth occurred at 20°C and at temperatures of 35°C or above. The observed temperature response is consistent with the known influence of temperature on the growth ecology of storage-associated fungi [22,29,31]. These findings indicate that moderately warm conditions can support rapid fungal development in stored grain environments.

3.5. Effect of pH on Fungal Growth

The greatest biomass accumulation occurred under mildly acidic conditions, with the highest values within the pH 5.5–6.5 range. Growth decreased at more acidic and alkaline conditions. The preference of many filamentous storage fungi for mildly acidic environments has also been documented in studies examining the combined effects of pH, temperature, and water activity [23,24,27].

3.6. Effect of Incubation Period

Biomass increased with incubation time and reached its maximum between 7 and 10 days, after which the measured growth response declined. The later reduction may be associated with depletion of available nutrients and changes in the culture environment during prolonged incubation. The present experiment assessed biomass accumulation rather than toxin production; therefore, the incubation period identified here represents the period of maximum observed fungal growth [22,25].

3.7. Effect of Carbon and Nitrogen Sources

The tested carbon and nitrogen sources produced different biomass responses.

Glucose and sucrose supported greater biomass formation than lactose and starch, while yeast extract and peptone produced higher biomass than the inorganic nitrogen sources. These observations indicate that nutrient composition can influence fungal biomass development in culture and may contribute to colonization of nutrient-containing grain substrates [25,27,29].

3.8. Implications for Mycotoxin Contamination in Indian Millets

The frequent recovery of *Aspergillus* and the growth responses observed under moderately warm, mildly acidic, and nutrient-available conditions indicate that storage conditions can influence fungal colonization of millet grains. The experimental optimums identified in this study overlap with conditions that may occur during ambient grain storage, particularly when drying and moisture control are inadequate. Previous work on Indian food systems and stored grains has also demonstrated the importance of environmental and post-harvest factors in mycotoxin risk [31,29,22]. Improved drying, moisture management, ventilation, and routine monitoring can therefore help reduce the opportunity for fungal proliferation and associated contamination risks.

4. Statistical Analysis

Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test at a significance level of $p < 0.05$. Data are presented as mean $\pm$ SD of three independent experiments. Analyses were conducted using SPSS software, and graphs were generated using GraphPad Prism.

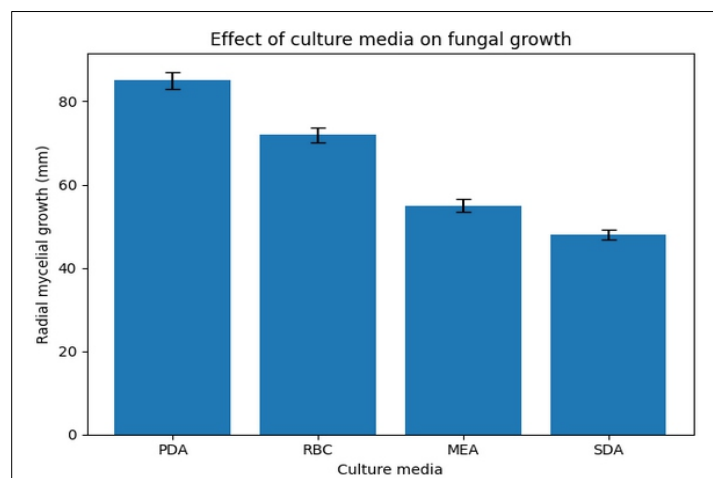


Figure 1. Effect of different culture media on radial mycelial growth of potentially mycotoxigenic fungal isolates from millets. Data represent mean values of triplicate experiments

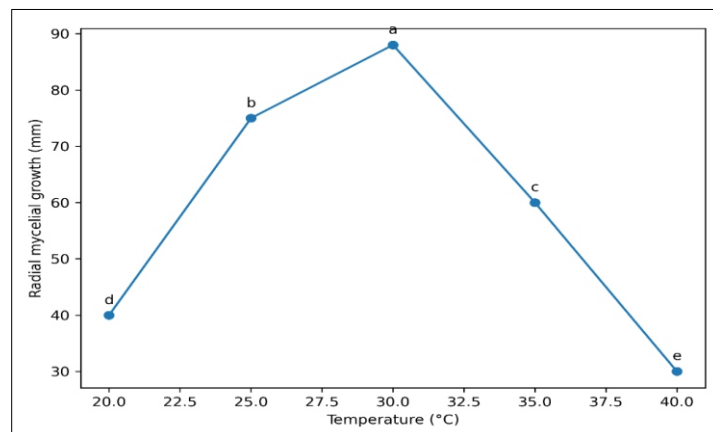


Figure 2. Effect of temperature on radial mycelial growth of fungal isolates incubated under different thermal conditions

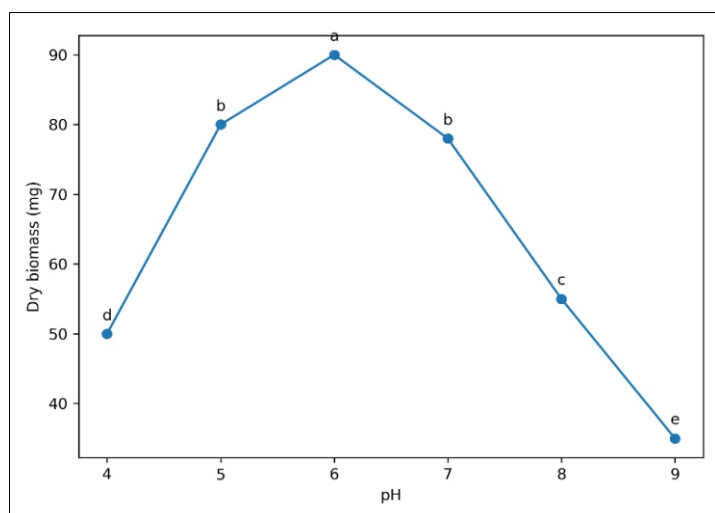


Figure 3. Influence of pH on dry biomass production of selected potentially mycotoxigenic fungi

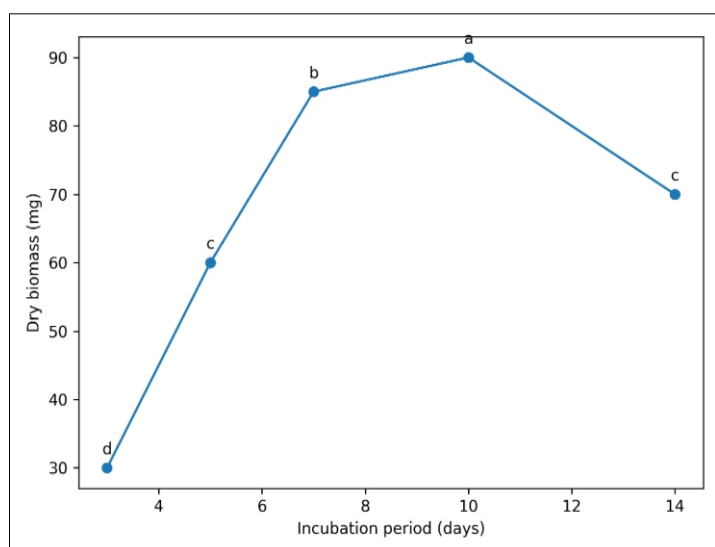


Figure 4. Effect of incubation period on fungal biomass accumulation under optimized growth conditions

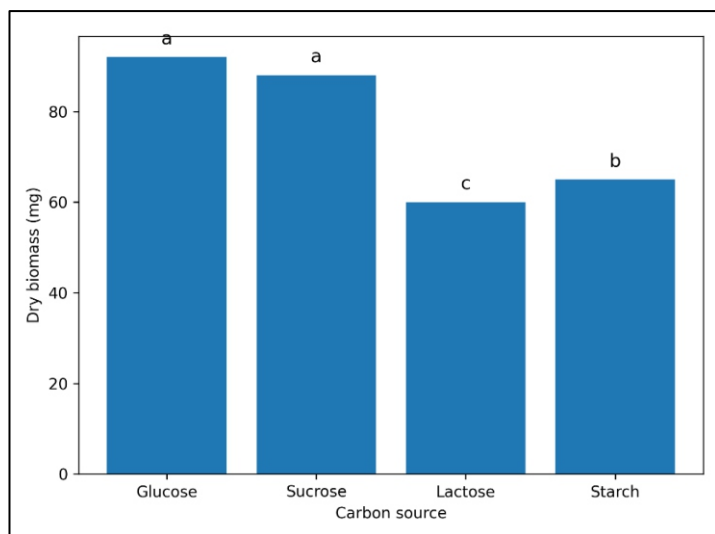


Figure 5. Effect of different carbon sources on fungal biomass production

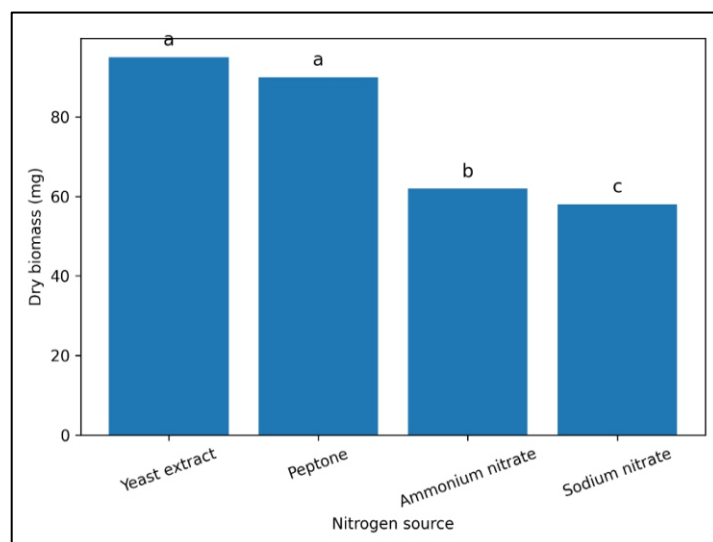


Figure 6. Effect of organic and inorganic nitrogen sources on fungal growth

Table 1: Effect of different culture media on radial mycelial growth of fungal isolates

Culture medium	Radial mycelial growth (mm)
PDA	85.0 ± 2.1 ^a
MEA	72.0 ± 1.8 ^b
RBC	55.0 ± 1.5 ^c
SDA	48.0 ± 1.2 ^d

Values are mean ± SD (n = 3). Different superscript letters indicate significant differences (p < 0.05).

Table 2: Effect of environmental parameters on growth of potentially mycotoxigenic fungal isolates

Parameter	Level	Growth response
Temperature (°C)	20	40.0 ± 1.6 mm ^d
	25	75.0 ± 2.0 mm ^b
	30	88.0 ± 2.4 mm ^a
	35	60.0 ± 1.7 mm ^c
	40	30.0 ± 1.3 mm ^e
pH	4.0	50.0 ± 1.4 mg ^d
	5.0	80.0 ± 2.2 mg ^b
	6.0	90.0 ± 2.5 mg ^a
	7.0	78.0 ± 1.9 mg ^b
	8.0	55.0 ± 1.6 mg ^c
Incubation period (days)	9.0	35.0 ± 1.2 mg ^e
	3	30.0 ± 1.1 mg ^d
	5	60.0 ± 1.8 mg ^c
	7	85.0 ± 2.2 mg ^b
	10	90.0 ± 2.5 mg ^a
	14	70.0 ± 1.9 mg ^c

Values are mean ± SD (n = 3). Different superscript letters within each parameter indicate significant differences (one-way ANOVA followed by Tukey's test, p < 0.05).

Table 3: Effect of different carbon and nitrogen sources on fungal biomass production

Nutrient type	Source	Dry biomass (mg)
Carbon source	Glucose	92.0 ± 2.3 ^a
	Sucrose	88.0 ± 2.0 ^a
	Lactose	60.0 ± 1.7 ^c
	Starch	65.0 ± 1.8 ^b
Nitrogen source	Yeast extract	95.0 ± 2.6 ^a
	Peptone	90.0 ± 2.3 ^a
	Ammonium nitrate	62.0 ± 1.8 ^b
	Sodium nitrate	58.0 ± 1.5 ^c

Values are mean ± SD (n = 3). Different superscript letters indicate statistically significant differences (one-way ANOVA followed by Tukey's test, p < 0.05).

5. Conclusion

The present study showed that potentially mycotoxigenic fungi, mainly *Aspergillus*, *Fusarium*, and *Penicillium*, were commonly associated with stored millet grains collected from different millet-producing regions of North and Central India. The optimization experiments showed that fungal growth was higher at moderate temperatures, particularly around 30 °C, under slightly acidic conditions, and in nutrient-rich media with longer incubation periods. These conditions may occur during improper or uncontrolled storage and can therefore favor fungal growth in millet grains. Since mycotoxin production was not measured in the present study, the findings indicate a potential risk rather than direct evidence of mycotoxin contamination. The study provides useful information on the environmental conditions that support fungal growth in stored millet grains. Proper drying before storage, control of moisture, adequate ventilation, and hygienic storage practices are therefore important for reducing fungal growth and maintaining grain quality. Further studies should include molecular identification of the fungal isolates and direct analysis of mycotoxins to determine the actual toxin-producing potential of the isolates. Future work may also focus on the effect of changing environmental conditions on fungal growth during storage and on the development of simple and low-cost methods for fungal control, improved packaging, and regular monitoring of stored millet grains.

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7. Conflict of interest

The authors declare that they have no conflict of interest related to this work.

8. Authors' Contribution

The first author completed the laboratory experiments and data collection presented in this study. The corresponding author compiled the data and prepared the manuscript in accordance with the study design and research objectives.

9. Funding

The authors received regular research funding from Shobhit University, Gangoh, Saharanpur, Uttar Pradesh, India.

10. Data Availability

All data generated or analysed during the present study are included in this manuscript and its supplementary files. No additional datasets were deposited in or are available from any external database or public repository.

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