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Original article

# Temperature mediated influence of mycotoxigenic fungi on the life cycle attributes of *Callosobruchus maculatus* F. in stored chickpea

Tatheer Zahra [a], Hamed A. Ghramh [b,c,d], Muazzama Batool [a], Allah Ditta Abid [e], Sohail Shahzad [e], Muhammad Shahbaz [f], Abuul Hassan Faiz [f], Qamar Saeed [a,*], Sarfraz Hashim [g]

[a] Department of Entomology, Faculty of Agricultural Sciences & Technology, Bahauddin Zakariya University, 60800 Multan, Pakistan
[b] Research Center for Advanced Materials Science (RCAMS), King Khalid University, P.O. Box 9004, Abha 61413, Saudi Arabia
[c] Unit of Bee Research and Honey Production, Faculty of Science, King Khalid University, P. O. Box 9004, Abha 61413, Saudi Arabia
[d] Biology Department, Faculty of Science, King Khalid University, P. O. Box 9004, Abha 61413, Saudi Arabia
[e] Department of Plant Protection, Ministry of National Food Security and Research, Pakistan
[f] Department of Zoology, The Women University Azad Jammu and Kashmir, Pakistan
[g] Department of Agriculture Engineering, MNS University of Agriculture, Multan, Pakistan

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ABSTRACT

Environmental factors (biotic and abiotic) are major depletion reasons in granaries. Fungi and insect pests act synergistically in deterioration of grains in storages which results in nutritional damage to the stored food which becomes unpalatable for the consumer. There is a need to establish a timeline for synergistic damage caused by insect pests and mycotoxigenic fungi for better management. For this purpose, interaction of mycotoxigenic fungi (*Aspergillus flavus, Aspergillus niger*, *Penicillium digitatum* and *Alternaria alternata*) with *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae) was studied at different temperatures. Development of *C. maculatus* was observed on fungus inoculated and uninoculated *C. arietinum* seeds. In fungus inoculated grains the development (Fecundity, larval emergence, pupation rate and adult emergence) of *C. maculatus* was found more better as compared on uninoculated grain. The population of *C. maculatus* was decreased by increase in temperature but high temperatures favours more fungi developments. More egg laying was observed at 27 °C and 33 °C. At tested temperatures, larval emergence was high as compared to other observed life attributes. Infestation of *A. flavus* and *A. niger was* also increased with increase of temperatures. *Penicillium digitatum* and *A. alternata* infestation were increased in the *C. arietinum* at 27 °C and 30 °C respectively. This study will help in measuring the control practices of fungi and insect pest infestations in stored *C. arietinum* (chickpea) in Pakistan.


## 1. Introduction

Pakistan is fourth in chickpea (*Cicer arietinum* Linnaeus) production worldwide. It has high carbohydrate (62.34 %) and protein contents (23.67 %) (El-adawy, 2002) (Alajaji and El-Adawy, 2006; FAO, 2018). Pakistan faces considerable losses (15–55 %) in chickpea crops during storage (Vanzetti et al., 2017). Contamination of stored commodities is mostly due to microflora and insect infestations (Bhat, 1988; Delouche, 1980; Mills, 1986; Tuda, 1996). Mycotoxins are non-volatile secondary metabolites, produced by filamentous fungi which reduce the quality of stored food by damaging their physical appearance and chemical composition (Bräse et al., 2009). Mycotoxins mediated semiochemicals are considered as an indicator of rotten odor in grains and facilitate interaction among insects and fungus species (Bennett and Inamdar, 2015; Bennett et al., 2012).

In stored commodities, the species of genus *Aspergillus* and *Penicillium* are more proliferating due to high relative humidity and mycotoxins (Dawar et al., 2007; Kumar et al., 2009; Patil et al., 2012; Shukla et al., 2012). *Aspergillus flavus* is responsible for 64 % more aflatoxin production in stored chickpea (Ramirez et al., 2018). Chickpea contaminated with toxigenic fungi have an detrimental effects on human health and animals (Urooj et al., 2015).

* Corresponding author.
*E-mail address:* qamarsaeed@bzu.edu.pk (Q. Saeed).
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The granivorous cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae) is the considerable causative agent of severe losses in seed germination, weight and nutritional level of legumes (Généfol et al., 2018; Staneva, 1982; Valencia et al., 1986; Murugesan et al., 2021). The *C. maculatus* can destroy dry beans in tropical and arid climatic zones, especially in stores (Tuda et al., 2006). Resistance against the stress condition of store houses is found in *C. maculatus* (Dongre et al., 1996). The penetration of storage fungi in stored commodities occurs due to the mishandling after harvest, presence of dust residues, cracks in seed coat because of mechanical handling and insects (Woloshuk and Martínez, 2012).

Temperature is also a fundamental aspect related to insect physiology (Ratte, 1984) and biochemistry (Downer and Kallapur, 1981). The various ranges of temperature affect the survival of Bruchidae species and insect activities (Giga and Smith, 1987; Miyatake et al., 2008; Soares et al., 2015). Development of *C. maculatus* is highly responsive to ranges of temperature which are also responsible for fungal development during post-harvest practices (Kistler, 1995; Sautour et al., 2001; Umoetok Akpassam et al., 2017). The well-studied temperature variables for all pathogenic microbes ranges from 15 to 37 °C. The optimum temperature for growth of *A. flavus* is 37 °C, while *Penicillium* species are also developed at lower temperatures i.e., from room temperature to 0 °C (Asurmendi et al., 2015; Lahouar et al., 2016; Palou, 2014).

Current study was designed to interpret the relationship between fungal species (*Penicillium digitatum, Aspergillus flavus, Aspergillus niger, Alternaria alternata*) and population build-up of *C. maculatus* in stored chickpea at different temperatures (25, 27, 33 and 35 °C) at constant level of R.H. (70 %). Current findings will be helpful in developing effective IPM strategy for *C. maculatus* and fungal infestation in stored products.

## 2. Materials and methods

### 2.1. Insect culture

*Callosobruchus maculatus* was cultured on sanitized chickpea grains under constant laboratory conditions (25 ± 5 °C and 55 ± 5 % R.H.) to obtain a uniform population. Males and female beetles were separated using the standard procedures (Beck and Blumer, 2011).

### 2.2. Collection of chickpeas

For experiment, stored chickpea ('kabuli' variety) (stored for one year) was obtained from the retailor shop at four different locations (Fig. 1) (Dera Ghazi Khan, Lodhran, Muzaffargarh, and Multan) and was kept at 25 ± 5 °C and 55 ± 5 % R.H. in Ecotoxicology Laboratory, Department of Entomology, Bahauddin Zakariya University (BZU) Multan, Pakistan. All the samples were stored in autoclaved cylindrical glass jars (32 × 23 × 23 cm).

### 2.3. Isolation of mycotoxigenic fungi

To isolate fungi from *C. maculatus*, seven adult insects were made sterile with 2 % solution of sodium hypochlorite, washed twice with distilled water, dried on blotter paper, crushed and placed on PDA (Potato Dextrose Agar) plate. The PDA plates were prepared by potato starch (125 g), dextrose (10 g) and agar (7.5 g) in 500 ml distilled water.

To isolate fungi from *C. arietinum*, five grains were disinfected with 2 % sodium hypochlorite, washed with distilled water twice, dried on blotting paper and placed on PDA plates for fungal growth observation (Bosly and Kawanna, 2014; Taylor and Sinha, 2009).

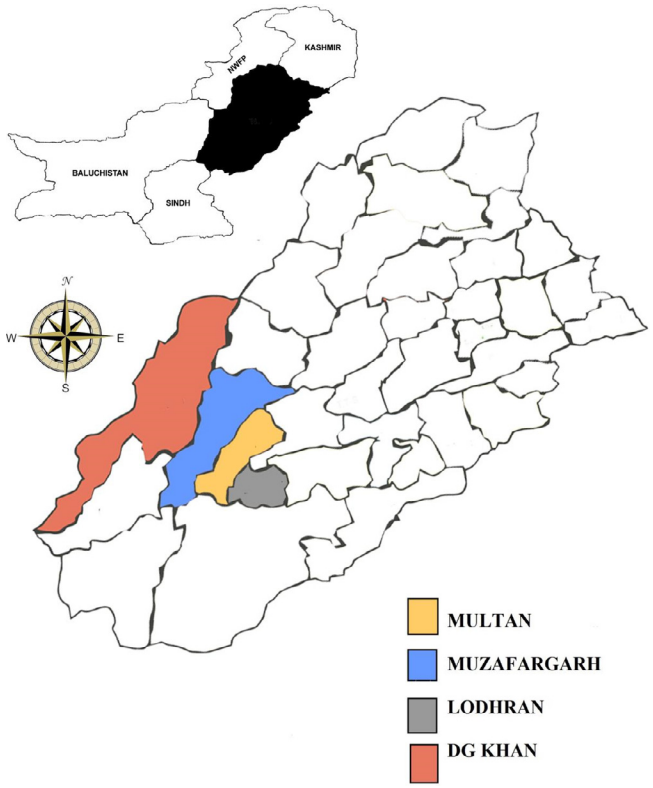


**Fig. 1.** Locations for *C. arietinum* sampling in Punjab Pakistan.

All the isolation procedures were done in a horizontal laminar flow cabinet (Airstream® LHG) (Lamboni and Hell, 2009). The isolation procedure for both insects and grains were replicated 10 times.

### 2.4. Identification of mycotoxigenic fungi

Identification of different fungi spp. was performed at Plant Pathology Department of Bahauddin Zakariya University (BZU) Multan, Pakistan. Mycological evaluation through microscopic examination was done by staining the hyphae with methylene blue on glass slides from fresh fungal cultures (Morishita and Sei, 2006).

### 2.5. Purification of mycotoxigenic fungi

Fungal cultures obtained through isolation were purified to avoid other microbes. The PDA plates with required fungal species were separated through sterile needles and incubated at 25 °C and observed daily (Ko et al., 2001).

### 2.6. Percentage of fungus

Fungal growth frequency (isolated from *C. maculatus* and *C. arietinum*) was determined by the following equation (Eq. (1)) (Ahmad and Singh, 1991):

$$\text{Frequency of Fungus}\,(\%) = \frac{\text{Total no. of seeds containing particular fungus}}{\text{Total no. of seeds used}} \times 100$$

### 2.7. Spore suspension

Fungi isolated from *C. arietinum* and *C. maculatus* were used for spore suspension. The suspensions were prepared from 7 days old

cultures of fungus. Fungal spores of mycotoxigenic fungi species were scraped with the help of glass slide by adding 20 ml autoclaved distilled water, and then solution was stirred in magnetic stirrer until conidia become separated from PDA. After agitation, impurities of suspension were removed by filtering it through filter paper. The numbers of spores were counted under a light microscope through haemocytometer.

### 2.8. Inoculation of C. arietinum grains

Spore suspension was diluted to obtain $5 \times 10^3$ spores/ml to inoculate *C. arietinum*. *Cicer arietinum* inoculated with fungal cultures by adding 3 ml of spore suspension per 100 g grains in glass jar (12.7 × 8.1 × 8.1 cm) (Nesci and Montemarani, 2011).

### 2.9. Influence of temperature on growth of C. maculatus and mycotoxigenic fungi

The normal prevailing temperatures (27, 30, 33 and 35 °C) were tested for the development of *C. maculatus* and mycotoxigenic fungi, at 70 % R.H. All the selected temperatures were maintained in a growth chamber (Versatile environmental test chamber, MLR-352H, Japan).

### 2.10. Extent of fungi on life cycle of C. maculatus

Five pairs of surface sterilized (2 % sodium hypochlorite) adults of *C. maculatus* were introduced into glass jars (12.7 × 8.1 × 8.1 c m), each containing 100 g of inoculated *C. arietinum*, and were removed after 24 h (Tsai et al., 2007). All the experimental units were maintained in a growth chamber (Versatile environmental test chamber, MLR-352H, Japan) with four constant temperatures 27 °C, 30 °C, 33 °C and 35 °C and 70 % R.H. *Cicer arietinum* were checked for numbers of *C. maculatus* eggs, larvae, pupae and adults by dissecting grains along with growth of fungal species (Howe and Currie, 1964). Each temperature treatment was replicated 4 times.

### 2.11. Statistical analysis

Incidence (%) of fungal species in *C. maculatus* adults and *C. arietinum* were analysed via frequency equation mentioned in section 2.6. While Chi square test and two-way ANOVA of the fungal isolation frequency were performed by subjecting data to a computational based software SPSS (SPSS Version 7.0). As the data was normal so data was not subjected to normality test. Developmental activity of *C. maculatus* and fungal species at different temperatures were calculated through software (SAS Institute, 2000).

## 3. Results

### 3.1. Isolation of mycotoxigenic fungi

#### 3.1.1. From C. arietinum grains

*Cicer arietinum* was evaluated for fungal colonies. *A. flavus, F. oxysporum, P. digitatum* and *A. alternata* were prominent in seed. Samples of *C. arietinum* from all localities were high in *A. flavus, A. niger, A. alternata, P. digitatum* and *F. oxysporum*. Results revealed that samples of were significantly ($F$ = 32.009; df = 4 (20); $P < 0.001$) highly infested with *A. flavus* (52.3 %) followed by *A. niger* (27.3 %), *F. oxysporum* (21.33) and *P. digitatum* (22.0 %). While *A. alternata* (8.0 %) exhibited the lowest frequency (Table 1).

#### 3.1.2. From C. maculatus

Fungal growth observation from the body of *C. maculatus* revealed that *A. flavus* was most frequent (71.43 %) while *A. alternata* was rarely isolated from adults (4.64) (Table 2). Diversity of fungi on the bodies of *C. maculatus* was irrespective of the gender of insect ($X^2$ = 6.339[a]; df = 4; $P$ greater than 0.175).

### 3.2. Interaction among mycotoxigenic fungi and C. maculatus in C. arietinum grains at different temperatures

#### 3.2.1. Developmental period of C. maculatus

Life cycle attributes of (egg, larvae, pupae, and adult stages) *C. maculatus* were tested on inoculated *C. arietinum* grains at four different temperatures (27, 30, 33, 35 °C). Population was developed at all tested temperatures with significant responses ($F$ = 81.85; df = 3(60); $P < 0.001$). At highest tested temperature the life period of *C. maculatus* was shortened. Similarly, intensification in temperature also increased the development of *A. flavus* and *A. niger* in grains. The life period of *C. maculatus* was 33 days (highest recorded days) at 30 °C. Impact of fungal growth was found non-significant ($F$ = 1.51; df = 12 (60); $P$ = 0.146) with the life cycle attributes of *C. maculatus* and all tested temperatures (Fig. 2).

#### 3.2.2. Fecundity of C. maculatus

Oviposition rates of *C. maculatus* were significantly affected ($F$ = 564.37; df = 3(60); $P < 0.001$) at all tested temperatures. The females of *C. maculatus* preferred inoculated *C. arietinum* more than sterilized *C. arietinum* grains for oviposition at all temperatures treatments. At 27 °C and 33 °C, oviposition was 403.75 on *C. arietinum* grains inoculated with *A. flavus* and *A. niger*. Reduction in the oviposition occurs at 35 °C in inoculated and control *C. arietinum*. *P. digitatum* inoculated grains exhibited a few oviposition (33.25). Oviposition rate was highly significant ($F$ = 83.05; df = 12 (60); $P < 0.001$) with relation to fungal inoculation ($F$ = 192.23; df = 4 (60); $P < 0.001$) and temperature (Fig. 3).

#### 3.2.3. Incubation period

Incubation period of *C. maculatus* were not significantly ($F$ = 0.40; df = 4 (60); $P$ = 0.80) influenced by interaction of fungi and temperature ($F$ = 0.66; df = 12 (60); $P$ = 0.78). Highest incubation period was of 8.25 days at 27 °C and same number of days were observed in presence of all tested fungal species on *C. arietinum*. Temperature ranges exhibited highly significant ($F$ = 35.93; df = 3 (60); $P < 0.001$) effect on the incubation period of *C. maculatus*. Increase in temperature was also decreased the incubation period of *C. maculatus* as observed in *P. digitatum* infested *C. arietinum* grains was shows shortest incubation period (3.5 days) at 35 °C (Fig. 4).

#### 3.2.4. Larval emergence of C. maculatus

Larvae of *C. maculatus* were significantly influenced due to temperatures ($F$ = 98.15; df = 3 (60); $P < 0.001$) and mycotoxigenic fungi ($F$ = 104.49; df = 4 (60); $P < 0.001$) infestation in *C. arietinum*. Fungal prevalence in grains at different temperatures decreased the larvae emergence. Moreover, opposite results for larvae emergence were observed in instances of non-inoculated (323 at 27 °C) and *A. niger* inoculated (275.5 at 33 °C) *C. arietinum*. The successive highest rate of larvae emergence was observed in *C. arietinum* infested with *A. niger*. Numbers of larvae emergence was also significantly affected because of interaction among fungi and temperatures ($F$ = 19.38; df = 12 (60); $P < 0.001$). The results also revealed the lowest number of larvae (33.25) emergence in *P. digitatum* infested *C. arietinum* at 35 °C (Fig. 5).

**Table 1**
Mycotoxigenic fungi isolated from *C. arietinum* samples purchased from different locations of Punjab Pakistan.

| Species | Multan (n = 75) | Muzaffargarh (n = 75) | Lodhran (n = 75) | DG khan (n = 75) | Total Isolates (n = 300) | IsolationFrequency (%) |
|---|---|---|---|---|---|---|
| *A. flavus* | 72 | 28 | 27 | 30 | 157 | 52.3 ± 14.5a |
| *A. niger* | 33 | 15 | 21 | 13 | 82 | 27.3 ± 6.0b |
| *A.alternata* | 10 | 3 | 7 | 4 | 24 | 8.0 ± 2.1b |
| *F.* | | | | | | *oxysporum* |
| 30 | 8 | 7 | 19 | 64 | | 21.3 ± 7.2b |
| *P. digitatum* | 30 | 11 | 11 | 14 | 66 | 22.0 ± 6.1b |

Means within a column followed by the same letter are not significantly different from each other (SPSS software at *P = 0.05*).

**Table 2**
Isolation of fungi from both male and female adults of *C. maculatus.*

| Species | No. of isolates | | | IsolationFrequency (%) |
|---|---|---|---|---|
| | Female (n = 140) | Male (n = 140) | Total isolates (n = 280) | |
| *A. flavus* | 98 | 102 | 200 | 71.43 |
| *A. niger* | 20 | 24 | 44 | 15.71 |
| *A. alternata* | 3 | 10 | 13 | 4.64 |
| *F.* | | | | *oxysporum* |
| 24 | 37 | 61 | | |
| | | | 21.79 | |
| *P. digitatum* | 32 | 26 | 58 | 20.7 |

(Chi square test at *P = 0.05*).

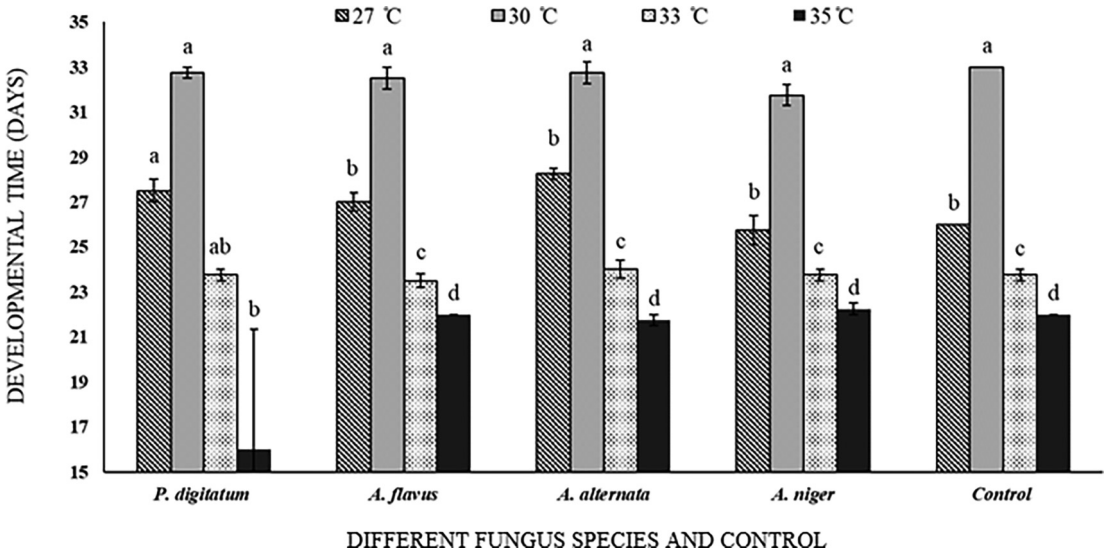


**Fig. 2.** *Callosobruchus maculatus* development rate on fungal infected and non-infected *C. arietinum* at different temperatures. Bars on each column represents standard error (±SE). Duncan test at *P* = 0.05. (Different fungus species and control are present on X-axis, developmental time at different temperatures is on Y-axis).

*3.2.5. Pupation of C. maculatus*

The mycotoxigenic fungi ($F$ = 153.97; df = 4 (60); $P < 0.001$), temperatures ($F$ = 158.96; df = 3 (60); $P < 0.001$) and their interaction ($F$ = 38.90; df = 12 (60); $P < 0.001$) exhibited highly significant effects towards pupation rate of *C. maculatus.* However, *C. arietinum* infested with *P. digitatum* and *A. alternata* showed the lowest pupation rate was 28.5 and 38, respectively, at 35 °C. High numbers of pupae emerged at 27 °C in inoculated and un-inoculated *C. arietinum* as compared to other temperatures (Fig. 6).

*3.2.6. Adult emergence*

Adult emergence of *C. maculatus* were inversely correlated with fluctuating temperatures ($F$ = 202.02; df = 3 (60); $P < 0.001$), mycotoxigenic fungi ($F$ = 97.95; df = 4 (60); $P < 0.001$) ($F$ = 18.30; df = 4 (60); $P < 0.001$) and their interactions ($F$ = 17.37; df = 12 (60); $P < 0.001$). Results demonstrated that, at 27 °C maximum adults were emerged in inoculated and non-inoculated *C. arietinum.* However, the lowest adult emergence rate at 35 °C in *P. digitatum* inoculated *C. arietinum* was 23 (Fig. 7).

## 4. Discussion

Results of the current study revealed high frequencies of *A. flavus* and *A. niger* isolates from *C. maculatus* adults. Similar results reported that infestation of mycotoxigenic fungi in *Triticum aestivum* observed because of *T. castaneum* and *Sitophilus granarius*

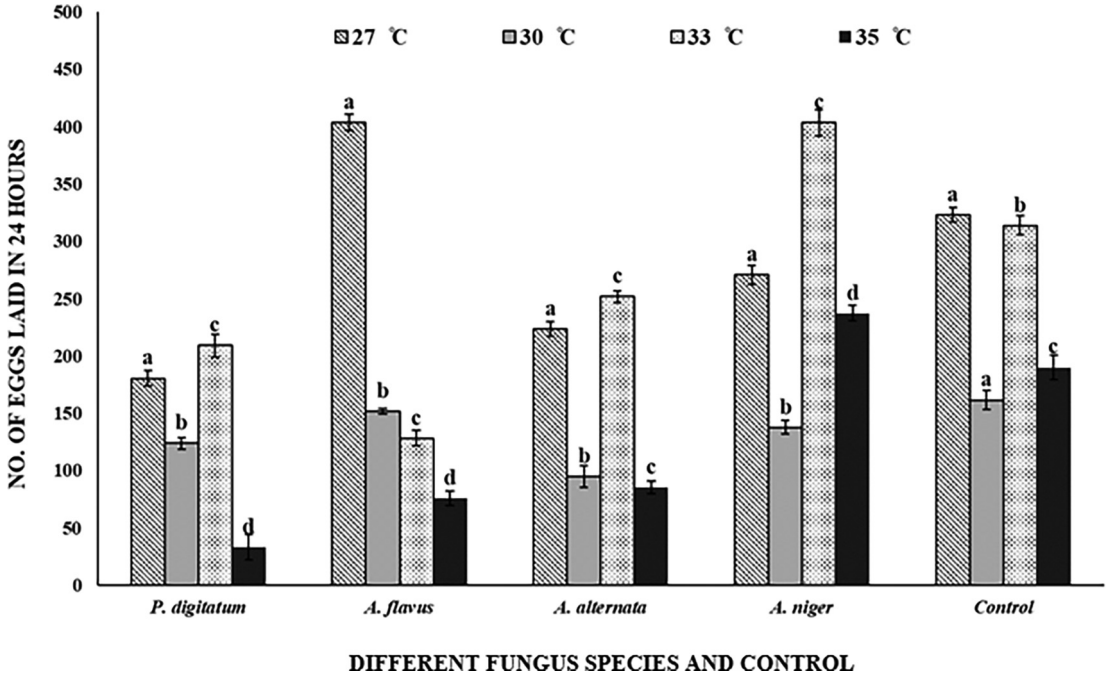


**Fig. 3.** *Callosobruchus maculatus* egg laying on fungal infected and non-infected *C. arietinum* at different temperatures. Bars on each column represents standard error (±SE). Duncan test at $P = 0.05$. (Different fungus species and control are present on X-axis, developmental time at different temperatures is on Y-axis).

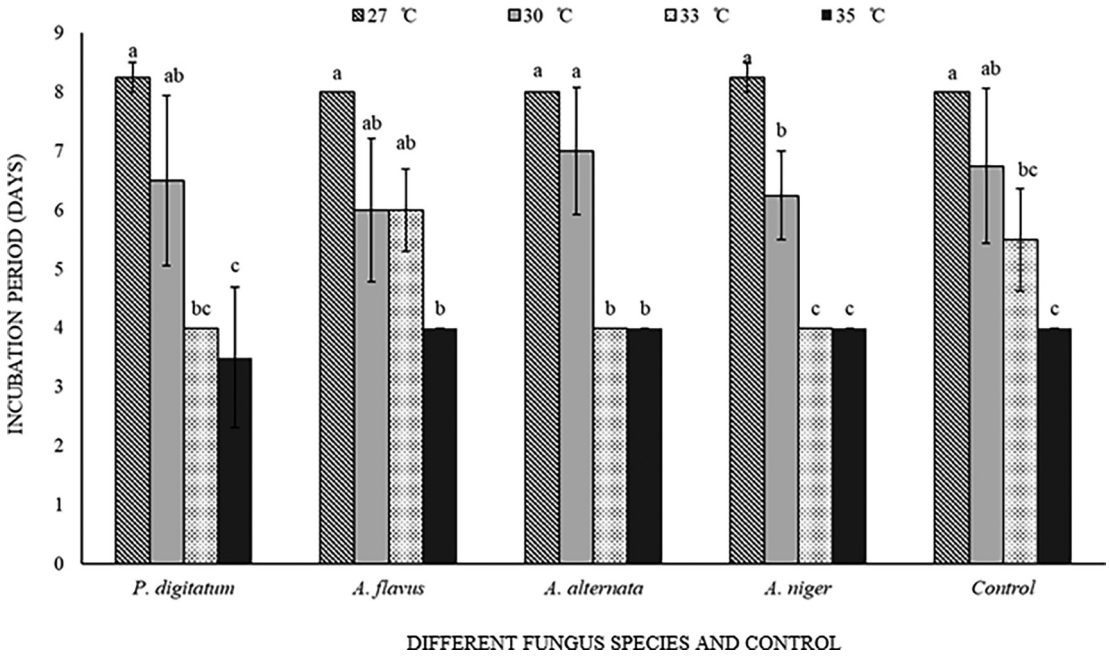


**Fig. 4.** *Callosobruchus maculatus* incubation period on fungal infected and non-infected *C. arietinum* at different temperatures. Bars on each column represents standard error (±SE). Duncan test at $P = 0.05$. (Different fungus species and control are present on X-axis, developmental time at different temperatures is on Y-axis).

activities (Agrawal et al., 1957; Bosly and Kawanna, 2014). The presence of mycotoxigenic fungi in an insect body illustrates that insects were able to transfer fungal flora in grains. Red flour beetle had been associated with dissemination of mycotoxigenic fungi to their hosts (Bosly and Kawanna, 2014) and also observed in stored rice grains (Yun et al., 2018). Larvae, pupae and adults of *C. maculatus* were significantly affected by the infestation of mycotoxigenic fungi.

Insect pests have intrinsic ability to develop and reproduce to change in temperature and time progressively (Burges, 2008). Temperature is inversely interacting with growth rate of insects. Results explain that *C. maculatus* completed its life cycle on all tested fungus and temperature parameters. The progressive period of *C. maculatus* was shorter on all infested and non-infested *C. arietinum* at 35 °C and 70 % R.H. Shortest life period of *Cadra cautella* were demonstrated at 30 °C and 70 % R.H. (Burges and Haskins, 1965). *Ahasverus advena* also complete their life cycle on different concentrations of Aflatoxin $B_1$ infested grains and the shortest life cycle was observed at 30 °C (Jacob, 1996; Zhao et al., 2018). Emergence of larvae was high at 27 °C on sterilized *C. arietinum* as well

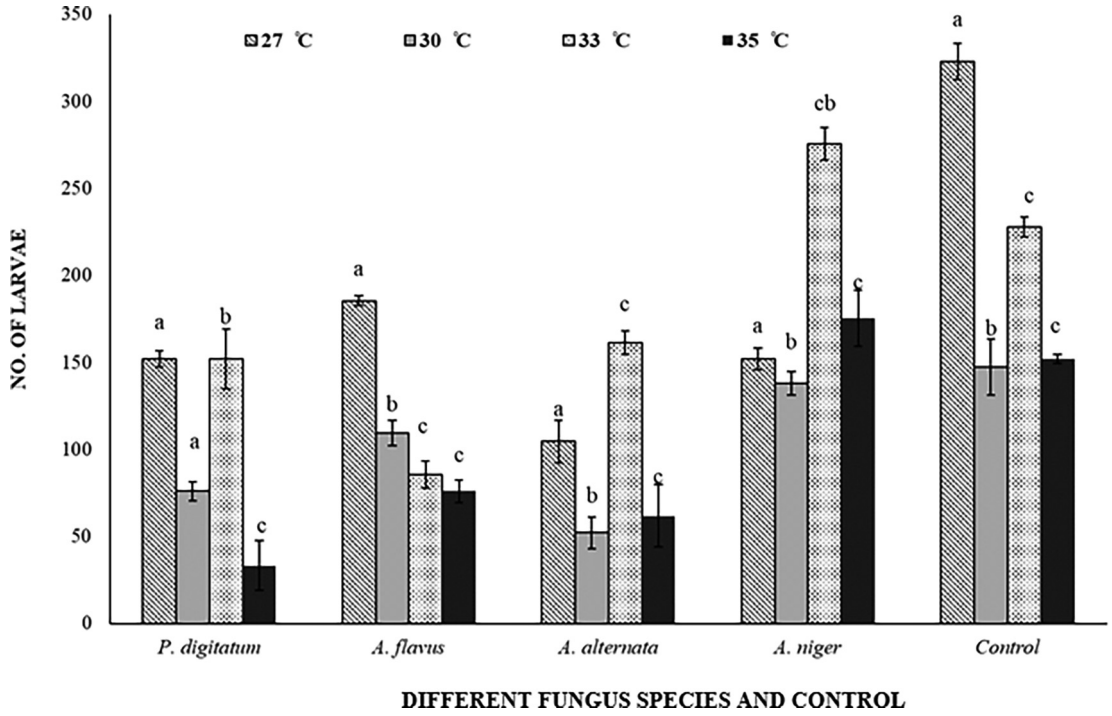


**Fig. 5.** Response of *Callosobruchus maculatus* (larval population) on fungal infected and non-infected *C. arietinum* at different temperatures. Bars on each column represents standard error (±SE). Duncan test at $P = 0.05$. (Different fungus species and control are present on X-axis, developmental time at different temperatures is on Y-axis).

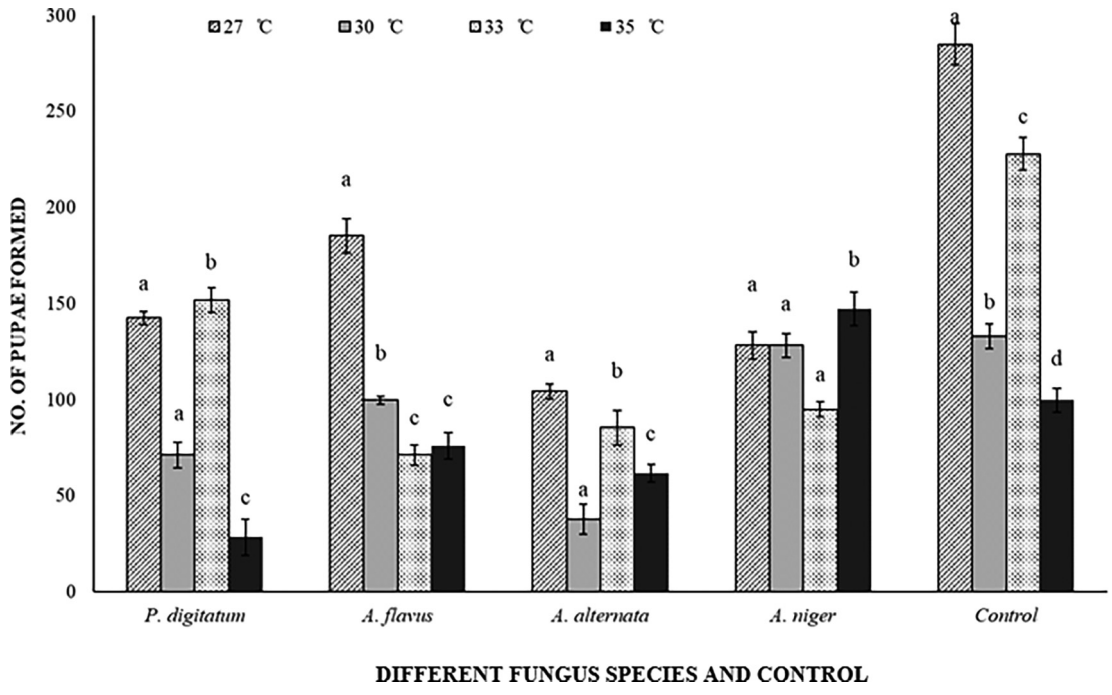


**Fig. 6.** Response of *Callosobruchus maculatus* (pupal population) on fungal infected and non-infected *C. arietinum* at different temperatures. Bars on each column represents standard error (±SE). Duncan test at $P = 0.05$. (Different fungus species and control are present on X-axis, developmental time at different temperatures is on Y-axis).

as on *C. arietinum* infested with *A. niger* at 33 °C. Similar results were observed in development of *Trogoderma granarium* on broken wheat grains at 35 °C while maximum fecundity and larvae emergence was evaluated at 30 °C (Riaz et al., 2014). The infestation of mycotoxigenic fungi was able to influence the development period of *C. maculatus* in stored grains under selected temperature ranges.

Longest development period of *C. maculatus* was analysed 33 days at 30 °C and more than 25 days at 27 °C on inoculated and non-inoculated *C. arietinum*. *C. maculatus* showed an incubation period of more than 6 days at 30 °C on all tested parameters. This is higher than other pests including *Chilo partellus* was showed shortest incubation period (4 days) at 30 °C but a similar development period of more than 30 days was observed at 30 °C at 80 % R.H. (Tamiru et al., 2012). Maximum progress rate of *A. flavus* was noticed at 35 °C (Mannaa and Kim, 2018). *A. alternata* shows maximum growth rate at 25 °C on *Glycine max* (Oviedo

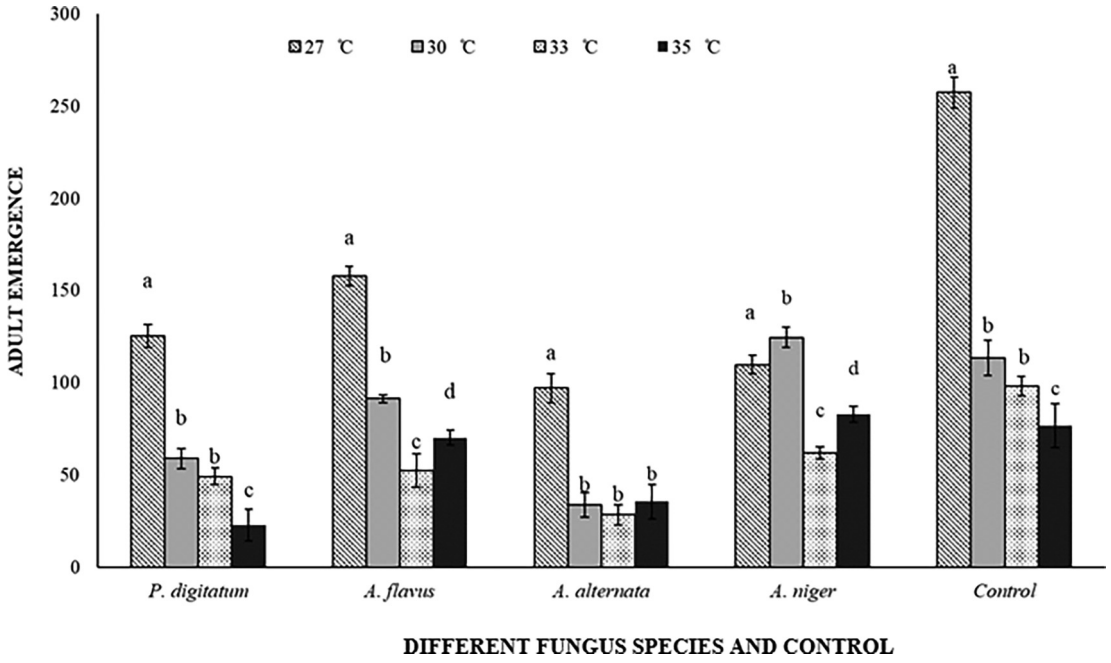


**Fig. 7.** Response of *Callosobruchus maculatus* (adult emergence) on fungal infected and non-infected *C. arietinum* at different temperatures. Bars on each column represents standard error (±SE). Duncan test at $P = 0.05$. (Different fungus species and control are present on X-axis, developmental time at different temperatures is on Y-axis).

et al., 2011). Meanwhile germination of *Penicillium* spp. was observed at 30 °C at 75 % humidity (Pasanen et al., 1991). *Fusarium* spp. was not showed any germination at temperature up to 25 °C on *G. max* medium (Garcia et al., 2012) that's why we do not select this species as the medium for *C. maculatus* growth on these temperature ranges.

Temperature influenced the fecundity of *C. maculatus* more than humidity. Temperature and suitable host preferences are the most considerable factors related to the development and oviposition of *C. maculatus* (Giga and Smith, 1987; Mam and Mohamed, 2015). The results presented maximum oviposition and larvae rate of *C. maculatus* on *A. flavus* inoculated *C. arietinum* at 27 °C as well as on *A. niger* at 33 °C. High numbers of larvae, pupae and adults were observed on sterilized *C. arietinum*. *C. maculatus* preferred *A. flavus* and *A. niger* infested *C. arietinum* more than control for oviposition. This is in contrast to corns infested with *A. halophilicus* were more suitable for the oviposition of *P. interpunctella* while a high development rate was observed at autoclaved corn (Abdel-Rahman and Hodson, 1969). *T. stercorea* showed minimum oviposition and maximum numbers of larvae on *A. flavus* at 30 °C. Average oviposition rate of *C. maculatus* on the *P. digitatum* (33–209) was highest as compared to *T. stercorea* was lowest on *P. purpurogenum* (42). Minimum larvae emergence was observed in *T. stercorea* on *P. purpurogenum* (Jacob, 1988; Tsai et al., 2007) as the same results were also observed for *C. maculatus* on *P. digitatum C. arietinum*.

*C. maculatus* preferred to oviposit at 30 °C and 35 °C while maximum oviposition was observed at 30 °C on sterilized *C. arietinum* (Chandrantha et al., 1987; Lale and Vidal, 2003). Researchers also found that *A. advena* and *Cryptolestes ferrugineus* were not able to oviposit on the *A. flavus* and *A. niger* isolates as observed in case of *C. maculatus* (David, 1974; Loschiavo and Sinha, 1966).

## 5. Conclusion

Management of stored product pests is necessary to prevent postharvest losses (Batool et al., 2021) and development of mycotoxigenic fungi. More than 70 % of *C. arietinum* deteriorates because of mycotoxigenic fungi and *C. maculatus* in houses, markets and stores. All the identified fungi species are well known to produce mycotoxins and reduction in the nutritional value of *C. arietinum*. A reduced amount of fungal growth on non-infested autoclaved chickpea grains (control) was observed at all selected temperatures even in presence of weevil. Preventive measures for both pests should be applied at commercial levels on the tested temperatures ranges in stores, houses and markets.

1. *C. maculatus* was able to reproduce in both inoculated and non-inoculated grains on all selected temperatures.
2. Market stored chickpea grains were found with more than 50 % frequency of mycotoxigenic fungi while this percentage increased to 70 % when insects come in contact with the grains in storages
3. *C. maculatus* carr. fungus in body and the relationship between both developed early at 35 °C causing high quality damage of *C. arietinum*.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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