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Isolation and identification of fungi associated with seeds of chickpea (*Cicer arietinum* L.)

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Abstract
Due to various fungal diseases, production of chickpea crop has been decreased in recent years. In the present study, chickpea seeds were collected from different districts of Rajasthan (Bharatpur, Alwar, Dholpur, Karauli and Kota). These seeds were subjected for isolation and identification of the associated fungi with this. Percent frequency of the associated fungi was also calculated. For each seed sample, dry seed examination was done. Besides normal seeds, some seeds had abnormal colour (blackish) (4-25%), seeds with white mycelial growth (0.60-11%), shriveled seeds (2-8%) and damaged seeds (5-45%). In the present study different fungi were found to be associated with chickpea seeds. Total 56 isolates were obtained which were identified morphologically as 16 species of fungi. The isolated fungi were identified as *Alternaria alternata, Fusarium oxysporum, Aspergillus flavus, Penicillum species*, *Rhizopus sp.*, *Botryodiplodia theobromae, Curvularia lunata, Chaetomium globosum, Rhizoctonia solani, Macrophomina phasaelina, Stemphylium sarciniforme, Aspergillus niger*, Sclerotium *rolfsii, Verticillium sp., Cladosporium sp.*, and *Negrospora sp.* Among all the associated fungi, 5 fungi i.e. *Alternaria alternata, Fusarium oxysporum, Aspergillus flavus, Penicillum species* and *Rhizopus sp.* were present in the maximum frequency.

Keywords: Chickpea, fungi, isolation and identification, frequency

Introduction
Chickpea (*Cicer arietinum*) is a self-pollinating diploid (2n=2x=16) pulse crop with a 738 Mbp genome (Varshney *et al.*, 2013) [3]. Chickpea primarily extended from *Cicer reticulatum* Ladizinsky approximately 11,000 years ago (Zohari and Hopf, 2000) [4], a variable wild species that originated in several regions of South-Eastern Turkey (37.3–39.3°N, 38.2–43.6°E; Kerem *et al.*, 2007) [5].
Globally, it is one of the most cultivated pulses in terms of world production with a total production of 14.2 million tonnes and an average yield of 0.96 tonnes ha^{-1} (Warude *et al.*, 2016). Currently, India represents the largest producer of chickpeas accounting for around 70% of the global production. India is followed by Australia, Pakistan, Myanmar, Ethiopia, Turkey, Iran, Mexico, Canada and Russia. Among the top exporting countries, Australia represents the biggest exporter of chickpeas accounting for more than one-third of the total global export volumes. Australia is followed by Russia, India, Mexico, Canada, the United States, Ethiopia, Argentina, Tanzania and Iran. At present, India represents the biggest importer of chickpeas accounting for around one-fifth of the total global import volumes. India is followed by Bangladesh, Egypt, the United States, Algeria, Pakistan, Spain, United Kingdom and Turkey (Lev-Yadum *et al.*, 2000).
Chickpea is a highly nutritious pulse crop with low digestible carbohydrates (40–60%), protein (15–22%), essential fats (4–8%), and a range of minerals and vitamins. The fatty acid composition of the seed adds value because fats govern the texture, shelf-life, flavor, aroma, and nutritional composition of chickpea-based food products (Madurapperumage *et al.*, 2021) [1].
In the last several years, chickpeas cultivation area and production has sharply declined because of some major constraints (Bakr *et al.* 2002) [2, 8]. Most of the diseases (more than 30) are caused by fungi (Bakr *et al.* 2007) [2, 8]. Chickpea is often attacked by fungi during both pre and post-harvest (during transport and/or in storage), significantly affecting its productivity. Seeds and infected harvest debris are the main sources of primary infections, and the level of seed damage depends on environmental conditions such as high relative humidity, dew, and temperatures above 25 °C (Jukanti *et al.*, 2012; Brase *et al.*, 2009) [6].
In this research work, an attempt was made to isolate and identify fungi associated with chickpea seeds.

Materials and methods
The present study was carried out on storage seeds of chickpea. Seeds samples of Cicer arietinum L. were collected from 5 different districts of Rajasthan (Bharatpur, Alwar, Jaipur, Ajmer and Kota), India. Samples were placed in clean brown paper bags, labelled properly and preserved at room temperature for subsequent use.
Location-wise codes of chickpea seeds are mentioned in Table 1.

Table 1: Details of chickpea seeds collected from different districts of Rajasthan

S. No.	Name of district	Latitude	Longitude	Code for chickpeas seeds	Number of seeds
1.	Bharatpur	27.2152° N	77.5030° E	Ch-Bh	20
2.	Alwar	27.5530° N	76.6346° E	Ch-Al	20
3.	Dholpur	26.702518°N	77.893394°E	Ch-Dh	20
4.	Karauli	26.491680°N	77.017670°E	Ch-Kr	20
5.	Kota	25.2138° N	75.8648° E	Ch-Ko	20
Total number of seeds					100

The fungi associated with seeds samples were isolated by using tissue planting method on Potato Dextrose Agar medium (CAB 1968) and blotter method of ISTA (1996). 100 seed (20 from each site) sampled were placed on 3 layers of moist blotting paper (Whatman No. 1) in petri plates. The seeds were surface sterilized by dipping in 10% Chlorox solution for 5 minutes and then washed 3 times with sterilized water. Seeds were placed in each plate and incubated at 25ºC for 5-7 days.

Fungi isolated from seeds inocula were transferred to separate PDA plates for further studies and preservation. Identification of the isolates were determined based on morphological characteristics observed under a compound microscope following the standard literature (Barnett and Hunter 2000; Benoit and Mathur 1970; Booth 1971; Ellis 1971, 1976). Percent frequency of the occurrence of the fungal isolates was calculated by adopting the following formula (Spurr and Wetly, 1972).

% frequency = (no. of inocula from which fungi isolates were raised/ no. of inocula cultured) *100

Results and discussion

For each seed sample, dry seed examination was done. Besides normal seeds, some seeds had abnormal colour (blackish) (4-25%), seeds with white mycelial growth (0.60-11%), shriveled seeds (2-8%) and damaged seeds (5-45%).

In the present study different fungi were found to be associated with chickpea seeds. Total 56 isolates were obtained which were identified morphologically as 16 species of fungi. The isolated fungi were identified as *Alternaria alternata, Fusarium oxysporum, Aspergillus flavus, Penicillum species, Rhizopus sp., Botryodiplodia theobromae, Curvularia lunata, Chaetomium globosum, Rhizoctonia solani, Macrophomina phasaelina, Stemphylium sarciniforme, Aspergillus niger, Sclerotium rolfsii, Verticillium sp., Cladosporium sp.,* and *Negrospora sp.*

Seeds having blackish colours mainly have *Alternaria alternata*, seeds with mycelial growth were infected with *Fusarium oxysporum, Aspergillus flavus*, shrieveled seeds had *Aspergillus flavus* and *Penicillum sp*. while broken or insect affected seeds were infected with *Rhizopus sp., Penicillum sp.,* and *Fusarium oxysporum*.

The frequency of these fungi in different seeds of chickpea collected from different locations are given in Table 2. The highest total frequency of occurrence was found to be of *Fusarium oxysporum* (65%), *Alternaria alternata* (30%), *and Aspergillus flavus* (25%), *Penicillum sp.* (20%) and *Rhizopus sp.* (20%) while other fungi show very lower occurrence in the chickpea seeds.

The appearance of different fungal species Are given in Figure 1. *Alternatia alternata* appeared white on petri plates and brownish-black on microscopic view. *Fusarium oxysporum* showed cottony white growth on plate and bluish under microscope. The growth of *Aspergillus flavus* was green on plate and it was also blue coloured in microscopic view. *Penicillum sp*. appeared greenish (centre) white (periphery) and blue after staining. The growth of *Rhizopus sp*. was blackish cottony on plate and deep purple under microscope.

It has been shown that *Alternaria alternata* cause seed borne disease in chickpea and cause Alternaria blight. The optimum temperature range was 13.7ºC to 30ºC and optimum relative humidity for its growth was 84% (morning) (Mandhare *et al*., 2008). Significant annual losses of chickpea yields are caused by epidemics of fusarium wilt, which account for ten to fifteen percent of the entire yield and can sometimes climb to one hundred percent under conditions that are favourable for the disease (Navas Cortés *et al*., 2000) [12]. *Aspergillus flavus* and *Penicillum sp.* are responsible for 64 % more aflatoxin production in stored chickpea (Ramirez *et al*., 2018) [14]. Chickpea contaminated with toxigenic fungi have an detrimental effects on human health and animals (Urooj *et al*., 2015) [13]. In a study, it was shown that "Species of *Aspergillus, Penicillium* and *Rhizopus* become predominating fungi with increase of the storage period. The fungal association with seeds of chickpea also affects germination, seedling mortality and seedling height" (Shamsi and Khatun, 2016) [15].

Table 2: Details of the isolated fungi from chickpea seeds collected from different districts of Rajasthan.

Name of Fungi	Number of inoculums to which fungi are associated					Frequency (%)				
	Ch-Bh	Ch-Al	Ch-Dh	Ch-Kr	Ch-Ko	Ch-Bh	Ch-Al	Ch-Dh	Ch-Kr	Ch-Ko
Alternaria alternata	2	1	0	1	2	10	5	0	5	10
Fusarium oxysporum	3	2	3	1	4	15	10	15	5	20
Aspergillus flavus	1	0	2	1	1	5	0	10	5	5
Penicillum species	1	2	1	0	0	5	0	5	10	0
Rhizopus sp	1	0	1	0	2	5	0	5	0	10
Botryodiplodia theobromae	0	1	1	0	1	0	5	5	0	5
Curvularia lunata	0	0	1	1	0	0	0	5	5	0
Chaetomium globosum	1	1	0	0	0	5	5	0	0	0
Rhizoctonia solani	1	1	0	1	0	5	5	0	5	0
Macrophomina phasaelina	1	0	0	0	1	5	0	0	0	5
Stemphylium sarciniforme	0	0	0	1	0	0	0	0	5	0
Aspergillus niger	1	0	1	0	1	5	0	5	0	5
Sclerotium rolfsii	0	1	0	0	1	0	5	0	0	5
Verticillium sp.	1	1	1	0	0	5	5	5	0	0
Cladosporium sp	0	1	1	0	1	0	5	5	0	5
Negrospora sp	1	1	0	0	1	5	5	0	0	5

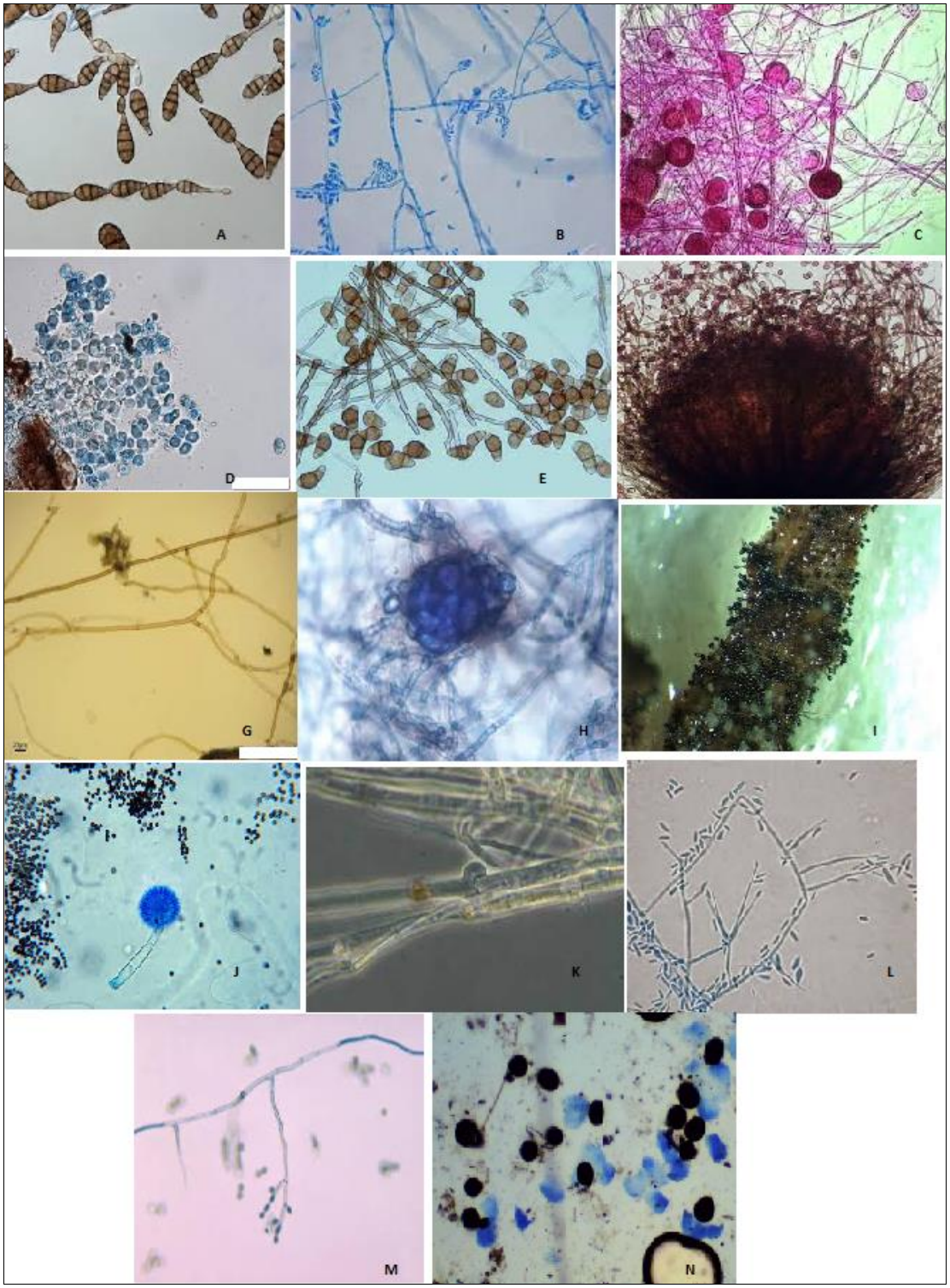

Fig 1: Microscopic view of different fungi associated with seeds of Cickpea- A: *Alternaria alternata*; B: *Fusarium oxysporum*; C: *Curvularia lunata*; D: *Chaetomium globosum*; E: *Rhizopus sp.*; F: *Botryodiplodia theobromae*; G: *Curvularia lunata*; H: *Rhizoctonia solani*; I: *Macrophomina phasaelina*; J: *Aspergillus niger*; K: *Sclerotium rolfsii; L: Verticillium sp.; M: Cladosporium sp.; N: Negrospora sp.*

Conclusion

In the present study, 16 genera of fungi were observed on the seed samples of chickpea collected from 5 different districts of Rajasthan. The samples from Bharatpur, Alwar, Dholpur, Karauli and Kota showed heavy inoculums and greater incidence of fungi. In 100 samples studied, the fungi which commonly affected seed germination were *Alternaria alternata*, *Fusarium oxysporum*, *Aspergillus flavus*, *Penicillum species* and *Rhizopus stolonifera.* To increase the yield of chickpea and to maintain quality of these seeds, control of these fungal borne diseases is must.

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