



Isolation and evaluation of fruit and seed mycoflora of bottle gourd (*Lagenaria siceraria* (Molina) Standl.)

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Abstract

Cucurbits include the major vegetables group cultivated widely in India during summer and rainy seasons. Out of different cucurbitaceous crops grown, bottle gourd (*Lagenaria siceraria*) occupies a highest place in human diet but prevalence of various mycoflora acts as a major limiting factor in deteriorating the crop qualitatively as well as quantitatively. In the present study, mycoflora associated with seeds and fruits of bottle gourd was isolated using different methods and characterized on the basis of colony colour, type of spore, spore size and spore shape. The identity was further confirmed through microbial identification services of CABI (Centre for Agriculture and Biosciences International). The isolated fungal flora was enumerated on the basis of per cent isolation frequency of individual mycoflora. A total of fourteen fungal species belonging to eight genera were isolated with prevalence of *Fusarium proliferatum* as external as well as internal seed borne and *Talaromyces pinophilus* as external seed borne mycoflora. Amongst three methods used for seed mycoflora isolation, moist blotter paper method was found to be the best followed by deep freezing and agar plate methods, respectively.

Keywords Bottle gourd · *Fusarium proliferatum* · Isolation · Mycoflora · Seed-borne

Introduction

India is one of the major vegetable producing countries in the world with second rank in the global vegetable production next only to China. Amongst various vegetable crops cultivated in India, cucurbits include the largest group of summer vegetables (Sharma et al. 2014). The family Cucurbitaceae is composed of around 130 genera and 800 species of edible and ornamental plants (Saboo et al. 2013). The main cucurbits cultivated in India include cucumber, snack squash, watermelon, snake melon, bottle gourd, bitter gourd, pumpkin, sponge gourd, squirting cucumber, round gourd etc. Out of different cucurbits cultivated, bottle gourd (*Lagenaria siceraria*) occupies a high place in human diet (Gajera and Joshi 2016). The genus *Lagenaria* possesses a total of six cultivated and wild species out of which the domesticated sweet flavoured genotype is used as vegetable (Attar and Ghane 2018). It is an important cucurbitaceous vegetable occupying an area of 157.08 thousand ha with the

production of 2572.01 thousand MT in India and Haryana occupied area of 25.77 thousand ha with the production of 347.81 thousand MT during 2016–2017 (www.indiastat.com).

The crop is cultivated during warm and rainy season and does not require complex management strategies. It performs well even with low amounts of nitrogenous fertilizers and acts as a natural weed smother (Chimonyo and Modi 2013). The fruits are harvested at tender stage and before 100% maturity for vegetable purpose (Heiser 1979). Various parts of this cucurbitaceous vegetable (leaves, roots, shoots, seeds, flowers and fruits) can be consumed in raw or cooked form as curries, juice, pickles, pudding, salads, soup etc. De-oiled seed of bottle gourd (*Lagenaria siceraria*) has been found to be a highly nutritious by-product of edible oil industries which contains a substantial proportion of dietary fiber, minerals, essential amino acids and quality protein (Ghosh et al. 2018).

Bottle gourd comprises of various pharmaceutically important compounds along with tetracyclic triterpene-cucurbitacin I which is known for its bioactive properties (Attar and Ghane 2018). All the plant parts, viz., leaves, fruits, stem, seeds and oil are used traditionally for the treatment of various ailments like diabetes, ulcer, jaundice,

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hypertension, piles, colitis, congestive cardiac failure, disorders of digestive and urinary tract, skin diseases etc. (Minocha 2015).

Besides these important characteristics, bottle gourd is prone to attack by various microorganisms including fungi that lower down its productivity. The pathogens contaminate economic parts such as seeds and fruits, particularly, by adhering to their surface externally or infecting them internally that lead to reduction or complete loss of germination, mortality of seedlings in nursery beds, developing seed borne-diseases and damage to seedlings at later stages etc. (Avinash and Rai 2013; Khani et al 2019). A variety of mycoflora harbouring the bottle gourd fruit and seed reduces yield and quality by discolouration, decreased seed germination etc. leading to seedling mortality. The pathogenic fungal flora assumes major threat to bottle gourd cultivation (Maheshwari et al. 2013). The infection of fruits and seeds of bottle gourd reduces market value of crop and resultantly may cause harm to consumers (Nayyar et al. 2014). The present study was therefore conducted to enumerate fruit and seed mycoflora associated with bottle gourd involving its isolation, identification and evaluation for per cent frequency of isolation in addition to other parameters during the course of study.

Materials and methods

Collection of seed and fruit samples

The unhealthy fruits and seeds of bottle gourd showing any blemishes, deformities, discoloration or having any piece of disease adhering to their surface fruits and seed samples were collected from farmer's fields, experimental fields, local market of Hisar. Isolation of fruit and seed (internal as well as external) mycoflora was carried out in Department of Plant Pathology, CCS Haryana Agricultural University,

Hisar as per the rules of International Seed testing Association (www.seedtest.org).

Isolation of external seed borne mycoflora

Three standard methods, viz., agar plate, moist blotter paper and deep freezing method were used (Fig. 1) for isolation of seed mycoflora of bottle gourd as per the description below.

Moist blotter paper method

Thick white blotter paper was cut into a number of small circular pieces according to the diameter of a Petri plate and three pieces of blotter paper were placed over one another in each Petri plate and moistened with sterile distilled water. The left-over paper from all the sides of Petri plate was removed and the plates were covered with lid, wrapped in aluminium foil and autoclaved at 15 psi pressure for 20 min. Five seeds were placed in each autoclaved Petri dish and the plates were incubated at 25 ± 2 °C. The incubated seeds were then observed for presence of various fungi after 48 h till 10 days. The Petri dishes were watered regularly and frequently at an interval of 2–3 days with sterile distilled water to maintain appropriate moisture level in order to prevent blotter papers from drying.

Agar plate method

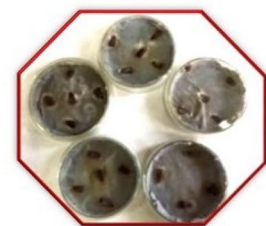
The water agar medium with 2% agar in distilled water was prepared, poured in Erlenmeyer volumetric flasks and autoclaved at 15 psi pressure for 20 min. The autoclaved medium was poured aseptically into Petri dishes sterilized, previously, in the hot air oven at 180 °C for 2 h and allowed to solidify. Five seeds were placed in each Petri dish and incubated at 25 ± 2 °C in BOD incubator. The incubated plates were visualized under a stereoscopic microscope after 48 h up to 10 days for determining the presence of seed borne mycoflora.



Moist blotter paper method



Agar plate method



Deep freezing method

Fig. 1 Methods of isolation of seed borne

Deep freezing method

The procedure followed was almost the same for this method as in moist blotter paper method with the difference that in deep freezing method, the 24 h after incubation of Petri dishes at 25 ± 2 °C was followed by deep freezing at -20 °C for one day and the plates were again incubated at 25 ± 2 °C for 5 days. Observations for presence of mycoflora were recorded after one week till ten days.

Isolation of internal seed borne mycoflora

All the above three methods were followed with the difference that seeds were surface sterilized by using 0.1% sodium hypochlorite solution for 2–3 min and washed 2–3 times with sterile distilled water serially. The surface sterilized seeds were placed in the Petri plates with five seeds in each plate. Observations and calculations for presence of various fungi were recorded in the same manner as in case of external seed borne mycoflora.

Isolation of mycoflora was carried out from 5 Petri plates placed with 5 seeds for each of the above methods. The observed fungal growth was picked up individually from every seed and transferred to separate Potato Dextrose Agar (PDA) slants [Dextrose (20 g), agar powder (20 g), peeled potatoes (200 g), distilled water (1000 mL)]. The slants were incubated at 25 ± 2 °C in a BOD incubator until the fungal colonies developed. The different types of colonies were examined visually and per cent isolation frequency for each of the isolated mycoflora was calculated using the formula given by Shakoor et al. (2011)

$$\text{Per cent frequency of isolation} = \frac{\text{No. of seeds infested}}{\text{Total no. of seeds plated}} \times 100$$

Isolation and evaluation of fruit mycoflora

The fruit tissues were washed thoroughly with sterile distilled water. The infected tissues, along with adjacent small unaffected area, were cut into small pieces (2–5 mm squares) with a sterilized scalpel and transferred to sterile Petri dishes containing 0.1% sodium hypochlorite solution by using flame-sterilized forceps. The sodium hypochlorite solution was used to surface sterilize the plant tissues by immersing the tissues in it for a period of 30–60 s. The pieces were then washed 2–3 times with distilled water serially. The sterilized pieces @ 3–5 per Petri plate were transferred aseptically to Petri dishes containing PDA medium and incubated at 25 ± 2 °C. Observations for the presence of mycoflora were recorded after 48 h till 10 days.

Purification and maintenance of fungal cultures

The fungal isolates obtained from seeds and fruits of bottle gourd were purified by hyphal tip culture and single spore isolation method. In hyphal tip culture, fragments of fungal mycelia were picked up by using hook type inoculating needle and transferred aseptically to the PDA slants under the laminar air flow cabinet. In single spore isolation method, few drops (5–6) of sterile distilled water were taken on a sterile glass slide and spores of the fungus, to be recovered as pure culture, were placed to make a spore suspension. The suspension was, then, transferred aseptically to the Petri plate containing water agar medium with a Pasteur pipette and shaken so as to spread it uniformly. The mycelial growth established from single spores was sub-cultured and maintained in pure form on PDA slants.

To prevent mite infestation, the cultures were subjected to -20 °C deep freezing for seven days. The deep frozen cultures were taken out after seven days and then sub-cultured, using the hyphal tip culture method, in PDA slants. The pure culture obtained was maintained by repeated sub-culturing at fortnightly intervals.

Identification of fruit and seed mycoflora

All the fungal isolates were identified in the laboratory by visualizing the morphological and cultural characters, viz., colony colour, type of spore, spore size, spore colour and spore shape. Identification of fungal cultures was further confirmed by microbial identification services, CABI (Centre for Agriculture and Biosciences International), Wallingford, UK through ITS rDNA sequence analysis and matching the sequences with those of global databases.

Results and discussion

Isolation, identification and characterisation of mycoflora

In present study twelve species of mycoflora belonging to eight genera were isolated from bottle gourd seeds and fruits using different methods viz., agar plate method, moist blotter paper method and deep freezing method. Identification of the fungal isolates was done through morphological analysis and it was further confirmed through microbial identification services provided by CABI (Centre for Agriculture and Biosciences International), Wallingford, UK (United Kingdom).

Amongst the isolated mycoflora, external seed borne mycoflora were *Fusarium proliferatum*, *Fusarium incarnatum-equiseti* (species complex), *Aspergillus tubingensis*, *Rhizopus oryzae*, *Penicillium polonicum*, *Aspergillus flavus*, *Talaromyces pinophilus* and *Colletotrichum lagenarium*

while internal seed borne mycoflora were *Meyerozyma guilliermondii*, *Clonostachys rosea* f. sp. *catenulata*, *Aspergillus* sp. (I), *Aspergillus* sp. (II) in addition to those isolated as external seed borne. The mycoflora isolated from fruits of bottle gourd were *Fusarium proliferatum*, *Fusarium incarnatum-equiseti* (species complex), *Aspergillus tubingensis*, *Aspergillus* sp. (I), *Aspergillus* sp. (III) alongwith one other species of *Fusarium* (Table 1).

The characterization of the isolated mycoflora based on colony colour, spore colour, spore shape and spore type is as under.

Fusarium proliferatum

This isolate was characterised by purple-white to completely purple dense mycelium, hyaline sickle shaped septate macroconidia and small, aseptate but elongated microconidia. Entirely white mycelium was also observed under artificial culture conditions (Fig. 2A). The identity of this isolate was confirmed through ITS rDNA sequence analysis using FASTA algorithm. The ITS sequence obtained from this sample produced top matches with > 99% identity to sequences from members of the genus *Fusarium*, mainly to members of clade V, including 99.6% to *Fusarium proliferatum* (CABI Number: E0000142001).

Fusarium incarnatum-equiseti (species complex)

This fungus had white to peach-white coloured dense mycelium with spindle shaped macroconidia tapering at both the ends and small but elongated microconidia. Both macroconidia and microconidia were hyaline in colour (Fig. 2B). This fungal isolate was identified based on ITS rDNA region sequencing and analysing the same using the FASTA algorithm. Top matches of > 98% were obtained

to sequences assigned to members of *Fusarium* clade VII, including 98.8% to *Fusarium incarnatum-equiseti* species complex. The other species of this clade include *Fusarium graminearum*, *Fusarium culmorum* etc. (CABI Number: E0000142002).

Aspergillus tubingensis

This fungal isolate was quite similar to *Aspergillus niger* in morphology and culture characteristics but identified as *Aspergillus tubingensis* comprising of round dark pigmented spores alongwith interspersed pinkish-brown sclerotia. The inconspicuous mycelial growth was observed on Potato Dextrose Agar medium (Fig. 2C). The identity of this isolate was confirmed by CABI through ITS region and beta-tubulin gene sequence analysis using FASTA and BLAST algorithm. The sequence analysis produced > 99% match to members of *Aspergillus* section *Nigri* including 99.1% identity to sequence of *Aspergillus tubingensis*. The results of partial calmodulin sequencing showed > 97% identity to *Aspergillus tubingensis*. In addition to this, CABI Validated Sequence Database for fungi showed a top match of 99.6% identity to *Aspergillus tubingensis* while other members of *Aspergillus* section *Nigri* gave 97% or less matching sequences (CABI Number: E0000178001).

Meyerozyma guilliermondii

This isolate possessed greenish growth in clusters on Potato Dextrose Agar medium. It was identified as an yeast species that forms round green to yellowish coloured spores in several chains or groups (Fig. 2D). The sample was identified by ITS rDNA sequence analysis and the result was confirmed using CBS online yeast identification tool. Numerous top matches of > 99% were made to members of the genus

Table 1 Mycoflora isolated from bottle gourd seeds and fruit

S. no	Externally seed borne	Internally seed borne	Mycoflora associated with fruit
1	<i>Fusarium proliferatum</i>	<i>Fusarium proliferatum</i>	<i>Fusarium proliferatum</i>
2	<i>Fusarium incarnatum-equiseti</i> (species complex)	<i>Fusarium incarnatum-equiseti</i> (species complex)	<i>Fusarium incarnatum-equiseti</i> (species complex)
3	<i>Aspergillus tubingensis</i>	<i>Aspergillus tubingensis</i>	<i>Aspergillus tubingensis</i>
4	<i>Rhizopus oryzae</i>	<i>Rhizopus oryzae</i>	<i>Aspergillus</i> sp. (I)
5	<i>Penicillium polonicum</i>	<i>Penicillium polonicum</i>	<i>Fusarium</i> sp.
6	<i>Aspergillus flavus</i>	<i>Aspergillus flavus</i>	<i>Aspergillus</i> sp. (III)
7	<i>Talaromyces pinophilus</i>	<i>Talaromyces pinophilus</i>	<i>Colletotrichum lagenarium</i>
8	<i>Colletotrichum lagenarium</i>	<i>Colletotrichum lagenarium</i>	
9		<i>Meyerozyma guilliermondii</i>	
10		<i>Clonostachys rosea</i> f. sp. <i>Catenulata</i>	
11		<i>Aspergillus</i> sp. (I)	
12		<i>Aspergillus</i> sp. (II)	

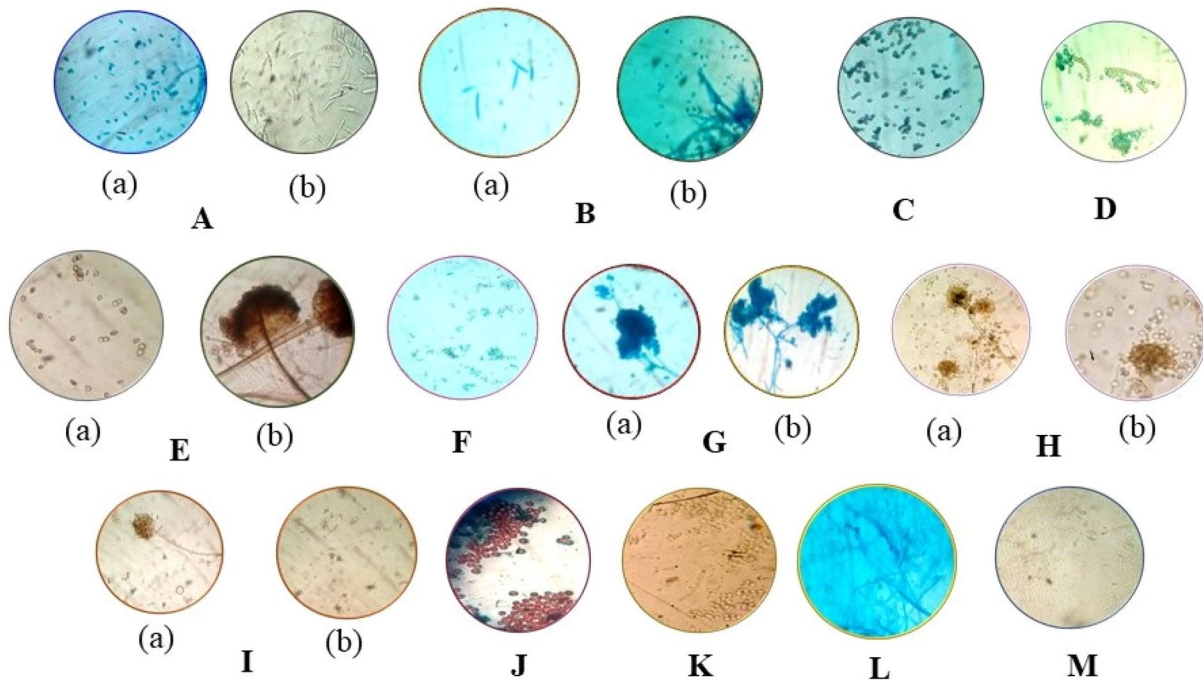


Fig. 2 **A** *Fusarium proliferatum* (a) microconidia; (b) macroconidia, **B** *Fusarium incarnation-equiseti* complex (a) macroconidia, (b) microconidia, **C** *Aspergillus tubingensis* spores, **D** *Meyerozyma guilliermondii* spores, **E** *Rhizopus oryzae* (a) sporangiospores, (b) sporangia borne on sporangiophores, **F** *Penicillium polonicum* spores, **G** *Clonostachys rosea* f. sp. *catenulate*, (a) and (b) spores borne on spo-

rophores, **H** *Aspergillus flavus* (a) conidiophores, (b) conidia borne on conidiophores, **I** *Aspergillus* sp. (I) (a) conidiophores, (b) conidia borne on conidiophores, **J** *Aspergillus* sp. (II) spores, **K** *Talaromyces pinophilus* spores, **L** *Fusarium* sp. conidia, **M** *Aspergillus* sp. (III) spores

Meyerozyma which comprises of two species only: *Meyerozyma guilliermondii* and *Meyerozyma caribbica* but the best matches were made to former one, i.e., *Meyerozyma guilliermondii* (CABI Number: E0000178002).

Rhizopus oryzae

The isolate was observed to possess white coloured, highly branching and fast growing mycelium interspersed with apparently brown to black coloured large sporangia at initial stage that soon turned brown. Each sporangium consisted of large number of hyaline to light brown globose (roughly spherical) sporangiospores and presence of rhizoids was also observed (Fig. 2E). ITS rDNA sequence analysis produced top matches at >99% identity to sequences of *Rhizopus oryzae* and *Rhizopus americanus*. Majority of the top matches were made with sequences of *Rhizopus oryzae* at >99.5% identity while upto 99.1% with the sequences of *Rhizopus americanus* (CABI Number: E0000178003).

Penicillium polonicum

This fungal culture was characterised by grayish-green to bluish-green growth on PDA (Potat Dextrose Agar)

medium. The spores were globose to round possessing sea green colour (Fig. 2F) and the isolate was identified based on partial beta-tubulin gene sequence analysis using FASTA as well as BLAST algorithm. Beta-tubulin sequence obtained from this analysis showed top matches of >97% to *Penicillium* section *Fasciculata*. The result was confirmed using the CABI Validated Sequence Database for Fungi producing >98% identity to *Penicillium* section *Fasciculata* with a top match of 99.2% to a sequence from *Penicillium polonicum* (CABI Number: E0000142003).

Clonostachys rosea* f. sp. *Catenulata

The isolate was identified by whitish to sap green colour of culture and round to oblong hyaline spores (Fig. 2G). The sequence analysis of ITS rDNA region carried out by CABI produced top matches at >99.8% identity to members of genus *Clonostachys* and the teleomorphic state, *Bionectria*. The members of genus *Clonostachys* include *Clonostachys rosea*, *Clonostachys pseudochroleuca*, *Bionectria ochroleuca* etc. The best matches were composed of sequences of *Clonostachys rosea* f. sp. *Catenulata* (CABI Number: E0000178005).

Aspergillus flavus

The spores mass observed on Potato Dextrose Agar medium was observed to possess olive green colour with inconspicuous mycelial growth and abundant production of conidia as well as conidiophores. The globose shaped conidia were hyaline to yellow or golden in colour while conidiophores were hyaline to greenish brown in colour (Fig. 2H). The morphological and microscopic analysis by CABI based on taxonomic keys further corroborated the identity of the isolate (CABI Number: E0000178004).

Aspergillus sp. (I)

The culture of this isolate was deep green in colour on Potato Dextrose Agar medium and spores were round in shape, hyaline to greenish in colour which were commonly visualized in short chains or triplets (Fig. 2I). CABI identified the fungus based on morphological as well as microscopic analysis. The isolate was identified as *Aspergillus fumigatus*. (CABI Number: E0000142004).

Aspergillus sp. (II)

This culture was apparently green to buff (pale-yellow to brown) in colour and spores were spherical to globose having pink to red colour shade and prominently walled (Fig. 2J). The identity of isolate on the basis of morphological analysis was further confirmed by CABI. (CABI Number: E0000142005).

Talaromyces pinophilus

The spores of this isolate were globose shaped with yellow to yellowish green to completely green growth and mature cultures possessed white to yellowish growth with red fruiting body (Fig. 2K). ITS rDNA region sequence analysis showed top matches at > 98% identity to the members of genus *Talaromyces* including *Talaromyces pinophilus*. The identity has been confirmed using the NCBI database which also produced top matches of > 98% to members of this genus (CABI Number: E0000142006).

Fusarium sp.

The spores were hyaline with dense mycelial growth which was white to sandy brown (Fig. 2L). The isolate was identified using morphological analysis of the specimen on a

slide prepared from the original sample and further confirmed by CABI (CABI Number: E0000142007).

Aspergillus sp. (III)

This isolate had golden to sandy brown growth of culture on Potato Dextrose agar medium and conidia were round shaped, extremely small and golden in colour which were observed in long chains. The sporulation was found to be abundant as observed during microscopic analysis (Fig. 2M). CABI confirmed its identification based on morphological traits (CABI Number: E0000142009).

Colletotrichum lagenarium

The culture colour was white to saffron with hyaline, elongated spores which were rounded at ends (Fig. 3). This isolate was self-identified based on morphological and microscopical analysis carried out in the laboratory.

The identification method based on traditional cultural, morphological and molecular characteristics corroborates with the findings of Zhang et al (1998) where fungal isolates were identified based on type of fruiting bodies, conidial characteristics in addition to internal transcribed spacer (ITS) region DNA sequencing. Alsohaili and Bani-Hasan (2018) also identified the fungal species based on macroscopic and microscopic examinations depending on the colony color, shape, hyphae, conidia, conidiophores and arrangement of spores and molecular identification.

Per cent frequency of mycoflora isolation

Amongst the seed borne isolates, *Talaromyces pinophilus* was recovered, as external seed borne mycoflora, with maximum isolation frequency of 80% using agar plate method while *Fusarium proliferatum* was isolated, as external seed borne mycoflora, with maximum isolation frequency of 60%



Fig. 3 *Colletotrichum lagenarium* culture on PDA

and 80%, respectively, using moist blotter paper method and deep freezing method.

Fusarium proliferatum and *Aspergillus flavus* were isolated as internal seed borne mycoflora using agar plate method with the highest isolation frequency of 32% each while *Fusarium proliferatum* had the highest isolation frequency of 40% using deep freezing method. *Colletotrichum lagenarium* was found to have maximum frequency of isolation, i.e. 36% as internal seed borne mycoflora using moist blotter paper method.

Fusarium proliferatum and *Talaromyces pinophilus* were the most frequently recovered isolates and *Aspergillus* sp. (II) had the lowest isolation frequency of 4% using moist blotter paper method followed by *Meyerozyma guilliermondii* with 12% isolation frequency using agar plate method (Table 2).

The prevalence of a number of species belonging to *Fusarium*, *Aspergillus*, *Rhizopus* and *Penicillium* genera have been found to possess close association with bottle gourd seeds (Chandiram and Maheshwari 1992; Sultana and Ghaffar 2007) whereas the association, particularly, of *Aspergillus flavus*, *Fusarium* spp., *Aspergillus niger*, *Rhizopus oryzae* has been reported with pumpkin and bottle gourd seeds (Hegazy and Kinawy 2011). However, the fungal isolates recovered from bottle gourd during the present study, viz., *Fusarium proliferatum*, *Aspergillus tubingensis*, *Meyerozyma guilliermondii*, *Clonostachys rosea* f. sp. *catenulata*, *Penicillium polonicum*, *Talaromyces pinophilus* have not been reported yet as per the literature reviewed.

The mycoflora isolated from fruits of bottle gourd included three genera and seven species, viz., *Fusarium proliferatum*, *Fusarium incarnatum-equiseti* (species complex), *Aspergillus tubingensis*, *Colletotrichum lagenarium*

alongwith two other species of *Aspergillus* and one other species of *Fusarium*. The isolation of *Fusarium* sp. from rotten fruits of bottle gourd corroborated with results of Saha et al. (2016) where *Colletotrichum* sp. and *Fusarium pallidorozeum* have been reported to be associated with fruits of bottle gourd. The association of *Colletotrichum* sp. with anthracnose infected fruits of bottle gourd has been reported in literature (Gupta et al. 2009).

The prevalence of *Fusarium* spp. in the cucurbit seeds has been reported by Avinash and Rai (2013) as compared to other isolates which corroborates with the results of present study. The fungi *Fusarium solani* and *Aspergillus niger* were found to be the most prevalent among the cucurbitaceous crops (Abushaala et al. 2016). Fourteen fungal species belonging to twelve genera consisting of *Alternaria alternata*, *Botrytis cinerea*, *Aspergillus flavus*, *Aspergillus niger*, *Cercospora* spp., *Chaetomium* spp., *Colletotrichum* spp., *Curvularia lunata*, *Fusarium moniliforme*, *Fusarium oxysporum*, *Macrophomina phaseolina*, *Penicillium* spp., *Phoma exigua*, and *Rhizopus stolonifer* were isolated from seeds of cucurbitaceous vegetables using standard blotter paper method and identified based on colony characters (Tumpa et al. 2018).

Efficacy of different methods used for seed mycoflora isolation

In the present study, three methods, viz., agar plate method, moist blotter paper method and deep freezing method were used for isolation of seed mycoflora. Amongst three methods used for seed mycoflora isolation, moist blotter paper method was found to be the best for the isolation of both external as well as internal seed borne mycoflora in terms

Table 2 Per cent frequency of isolation of seed borne mycoflora using different methods

Sr. no	Fungi	Per cent frequency of isolation of mycoflora					
		External seed borne			Internal seed borne		
		AP	MB	DF	AP	MB	DF
1	<i>Fusarium proliferatum</i>	40	60	80	32	16	40
2	<i>Fusarium incarnatum-equiseti</i> (species complex)	20	32	32	24	20	8
3	<i>Aspergillus tubingensis</i>	12	28	8	4	–	4
4	<i>Meyerozyma guilliermondii</i>	–	–	–	12	–	–
5	<i>Rhizopus oryzae</i>	12	–	–	20	–	–
6	<i>Penicillium polonicum</i>	–	24	–	28	12	20
7	<i>Clonostachys rosea</i> f. sp. <i>Catenulata</i>	–	–	–	16	12	8
8	<i>Aspergillus flavus</i>	–	12	20	32	8	12
9	<i>Aspergillus</i> sp. (I)	–	–	–	–	8	12
10	<i>Aspergillus</i> sp. (II)	–	–	–	–	4	–
11	<i>Talaromyces pinophilus</i>	80	24	24	–	20	32
12	<i>Colletotrichum lagenarium</i>	76	52	42	20	36	28

AP agar plate method; MB moist blotter paper method; DF deep freezing method

of per cent isolation frequency as well as number of fungal species isolated. Deep freezing method showed significant results for the isolation of those fungal which are slow growing and deep seated into the seeds. Agar plate method was found to be the least effective out of the three methods used. Moist blotter paper method was found to be the most effective followed by deep freezing method as the total number of fungal species isolated using moist blotter paper method as internal and external seed borne mycoflora were nine and seven, respectively, while using deep freeze method, number of isolates were nine and six, respectively. The number of species isolated using agar plate method were the same as in case of deep freeze method, i.e., nine and six, respectively, but the isolation frequency of mycoflora was less in agar plate method as compared to moist blotter paper and deep freezing method.

Different methods have been reported with different efficacy by various researchers for the isolation of seed borne mycoflora. A total of 45 fungal species associated with bottle gourd seeds isolated using blotter paper method while a total of 44 and 31 fungal species isolated using deep-freezing method and agar plate method, respectively, corroborated that standard blotter paper method was more effective in isolation of seed borne mycoflora of bottle gourd (Sultana and Ghaffar 2009).

Isolation of seed borne fungi associated with bitter gourd (*Momordica charantia*) by Shakoor et al. (2011) revealed that standard blotter paper method was more efficient than agar plate method. Blotter paper method was found to be more suitable technique for isolation and detection of seed borne fungi associated with cucurbits and cowpea (Tumpa et al. 2018; Zanjare et al. 2020). However, agar plate method has been reported to be better than deep freezing and blotter paper methods for the isolation of mycoflora from seeds of *Cucurbita pepo*, *Oryza sativa*, *Pisum sativum*, *Triticum aestivum* and *Zea mays* (Rahim et al. 2013; Ghosh et al. 2018; Khani et al. 2019). Abushaala et al. (2016) isolated seed borne fungi associated with cucurbit seeds in two different regions of Libya using agar plate and deep freezing methods whereby agar plate method was reported to be better as the number of isolates recovered from agar plate isolation technique were more than those recovered using deep freezing method.

Conclusion

The prevalence of wide variety of mycoflora is a major limiting factor in deteriorating bottle gourd (*Lagenaria siceraria*) crop qualitatively as well as quantitatively. In the present study, mycoflora associated with seeds and fruits of bottle gourd was isolated, evaluated. A total of eight genera and fourteen fungal species were isolated with prevalence

of *Fusarium proliferatum* as external as well as internal seed borne mycoflora and *Talaromyces pinophilus* as external seed borne mycoflora. Out of the three methods used for isolation of seed borne mycoflora, moist blotter paper method was found to have the highest efficacy. Amongst all the bottle gourd isolates, *Fusarium proliferatum* and *Talaromyces pinophilus* had the maximum recovery and highest isolation frequency.

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Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

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