



RESEARCH ARTICLE

Detection of seed-borne pathogens in sesame and their management through seed biopriming

Anand Theerthagiri* & Senthilraja Govindasamy

Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore 641 003, India

*Email: barathiana@yahoo.com



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Abstract

Sesame is a significant oilseed crop cultivated extensively in the tropical and subtropical areas of India. Seed-borne pathogens are the most important biological constraints in seed production worldwide. Seed health testing to detect seed-borne pathogens is an important step in the management of crop diseases. In addition, it is also essential to adopt environmentally friendly strategy for preventing and managing seed-borne diseases. The main objectives of the present investigation including testing the seed health status of different sesame seed samples, standardizing the seed health testing methods and assessing the efficacy of different biological agents against seed -borne pathogens and seed quality in sesame through seed biopriming and dry seed treatment. To find the mycoflora that was positively correlated with seeds, samples of sesame seeds were collected from farmers, research stations and seed production plots. According to the findings, the most common types of fungi that are associated with sesame seeds are Macrophomina phseolina, Alternaria sesami, Aspergillus flavus, Aspergillus niger, Rhizopus sp., Curvularia sp. and Fusarium sp. Among the seed-borne fungi, the infection of M. phaseolina was observed highest in most of the sesame seed samples followed by A. sesami, A. flavus and A. niger. Various techniques used to detect seed mycoflora, the blotter technique was the most efficient method, with a maximum recovery of 45.1 %, followed by the potato dextrose agar (PDA) plate method (35.2 %), while the minimum recovery was observed in deep freezing blotter method (16.4 %). However, the detection of seed- borne pathogens of significance like A. sesami and M. phseolina was best in PDA plate method (9.82 and 14.2 %) followed by standard blotter method (7.9 and 11.8 %). Among the different bioprimed seeds individually and in combination, Bacillus subtilis (1%) alone primed seeds recorded lesser seed infection (0.66 %) and higher germination per cent (92 %) and seedling vigour (1186). When compared to dry seed treatment, sesame seeds primed with biocontrol agents generally showed lower seed infection (%) and higher seed quality parameters. The findings revealed the presence of several mycoflora in all sesame seed samples and these seed mycoflora can be detected effectively by the standard blotter paper method and the PDA plate method. Furthermore, the biopriming technique has the potential to mitigate the detrimental impact of these microbes, thereby improving seed quality characteristics.

Keywords

biopriming, detection, dry seed treatment, sesame, seed-borne pathogens

Introduction

Sesame (Sesamum indicum L.) is a significant oilseed crop cultivated extensively in tropical and subtropical climates globally. It is also known as the "Queen of Oilseeds" because of its superior storage quality and high level of resistance to oxidation and rancidity (1). The quality of the seed used can have a direct impact on crop productivity (2). Pathogen-free, healthy seeds support the intended plant populations and a satisfactory harvest. Numerous seed-borne diseases can reduce plant development and crop productivity (3-5).

Seed-borne fungi are transmitted by infected sesame seeds, causing seed deterioration, affecting germination in soil, leading to seedling death and further leaf infection occurs in mature plants (6). Sesame seeds have been reported to be associated with fungi such as Alternaria, Curvularia, Fusarium, Bipolaris, Macrophomina, Penicillium and Rhizophus sp. (7). The most harmful seedborne pathogens are Macrophomina and Alternaria because they damage the plant throughout its early growth stages and impair seed-germination as well as seedling vigour (8). Fusarium and Aspergillus also decreased seed germination by causing seed rot, later invading young seedlings, causing the sesame plant to suffer from root rot as well as seedling blight (9). In addition, farm-saved sesame seeds used by most farmers are infected with seed-borne fungal pathogens. Therefore, it is crucial to test sesame seeds in order to understand the different associated fungi and how they affect the quality of the seeds. Recently, Lakshmi Pravallika et al. (10) analysed the presence of seed-derived mycoflora associated with sesame using various standard detection methods like blotter paper method, deep freezing blotter method, 2, 4-D blotter method, water agar method, agar plate method with potato dextrose agar (PDA) and paper towel method. Among the six methods tested, the standard blotter method proved superior by recording the highest mycoflora infection (31.48 %) in all tested samples.

Many of the diseases that cause a decrease in sesame yield are seed-borne diseases. Controlling these diseases at the seed-derived stage is considered the cheapest disease control strategy (11). Various chemical agents are employed to manage seed-borne pathogens; nevertheless, the utilization of biocontrol agents for the management of seed-borne diseases is essential for future crop production. Fungicides are effective, but when used, bioactive agents are an economically viable and environmentally friendly approach. Biocontrol agents are microorganisms that safeguard young plants and seeds from several seed-borne fungus (12,13). Seed treatments are the most economical and safe approach for controlling seed-borne diseases (14). For fungicides or biological control agents used as seed dressers or seed primers.

Seed priming promotes germination, enhances plant performance and increases yield potential, even under adverse conditions (15,16). This technology has emerged as a viable strategy to mitigate the effects of modern agriculture by protecting plants against both

biotic and abiotic stresses (17). Biopriming uses a variety of beneficial microorganisms to increase plant growthpromoting activity and fight disease. Microorganisms used for biopriming include beneficial fungi and bacteria such as various types of Trichoderma, Pseudomonas and Bacillus. They improve seed germination rates, increase seedling vigour and control soil and seed-borne pathogens. Biopriming of seeds with Trichoderma harzianum has been reported to reduce the occurrence of root rot in cowpeas (18). So, with this view, the present investigation was undertaken (i) to document the significant seed-borne pathogens that affect sesame seeds collected from different locations, (ii) to develop standardized procedures for assessing the health of seeds for detecting significant seed-borne pathogens and (iii) to assess the effectiveness of commercially available biocontrol agents, both individually and in combination, as seed treatments against fungal pathogens and other seed quality traits in sesame.

Materials and Methods

Collection of sesame seeds and analysis of seed mycoflora

Sesame seed samples belonging to five varieties were collected from research stations, seed production plots and farmers' fields of Cuddalore and Villupuram districts of Tamil Nadu, India. Using the conventional blotter method, a total of thirty sesame seed samples were collected and examined for the presence of seed-borne fungi linked to sesame seeds (19). The mycoflora associated with sesame seeds were identified through different cultural and morphological characteristics.

Standardization of seed health testing methods

To standardize the detection of fungi in sesame seed samples, a number of seed health testing techniques were used, including the standard blotter technique, the 2, 4-D blotter technique, the deep-freezing blotter method, the water agar method, the PDA (Potato Dextrose Agar) plate techniques, along with the rolled paper towel technique (2).

Standard blotter method

In this technique, sterilized Petri plates were filled with three layers of 90 mm-sized blotter paper that had been soaked with distilled water and drained of surplus water. On the wet blotter paper, 25 sesame seeds were evenly distributed, with 16 seeds on the outside circle, 8 seeds inside the circle and 1 seed in the centre. The plates were exposed to 12 h NUV light-dark cycles at 25 °C. After 7 days of incubation, seeds were examined using a stereo-binocular microscope for related fungi. Fungal species were identified by morphology and colony features (19). Four replications were maintained with 100 seeds per replication.

2, 4-D Blotter method

In the 2,4-D blotter method, placed 400 sesame (VRI 2) seeds on a wet blotter soaked in 0.2 percent sodium salt of 2,4-dichlorophenoxy acetic acid at 25-seeds per Petri dish. The seeded Petri dishes were incubated in accordance

with the standard blotter method. Seeds were assessed for fungal development after a 7-day incubation period utilizing a stereo-binocular microscope (20).

Deep freezing blotter method

In this procedure, 400 sesame seeds (25 per plate) were placed on a wet blotter as in the normal method. Inoculated Petri dishes were incubated at 25±2 °C for 24 h, alternated between UV light and darkness for 12 h and then incubated at -20 °C for 24 h. The process was repeated for 5 days. Incubated seeds were checked for fungal growth after 7 days.

Water agar method

Four hundred sesame seeds were placed at a rate of 25 seeds per Petri dish in 20 mL of 1 % water agar. Four replicates were maintained; each composed of 100 sesame seeds. Petri dishes were incubated and inspected using the conventional blotter method.

PDA plate method

After disinfecting with 2 % sodium hypochlorite, the sesame seeds were rinsed on three occasions with sterilized distilled water. Once sterilized, the seeds were transferred to a PDA medium and incubated at 22–25 °C in a cycle of twelve hours of day light and darkness for five to seven days. Following the incubation period, the fungi developing from seeds on the growth medium are then examined and characterized.

Rolled paper towel method

One hundred seeds per replicate have been dispersed out on two sheets of damp germination paper, arranged in ten rows with ten seeds per row. The setup was then carefully placed on a sheet of polythene paper as well as rolled to prevent any excessive pressure on the seeds. The towels were left to incubate at room temperature for 6 days in four separate sets. Following a 6-day incubation period, fungal development on the seeds was examined using stereo-binocular microscope.

Effect of biopriming and dry seed treatment with fungal and bacterial bioagents on seed infection and seed quality parameters in sesame

Sesame seeds (var. VRI2) were obtained from Regional Research Station, Vridhachallam, Cuddalore district, Tamil Nadu and the formulation of biocontrol agents viz., Trichoderma asperellum, Bacillus subtilis and Bacillus amyloliquefaciens were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University (TNAU), Coimbatore. The treatments of the experiment were T_1 - Trichoderma asperellum (0.4%), T_2 - Bacillus subtilis (1%), T₃ - Bacillus amyloliquefaciens (1%), T₄ - T. asperellum + B. subtilis (1%), T_5 - T. asperellum + B. amyloliquefaciens (1%), T_6 - Thiram (0.4%) and T_7 Untreated control. The biocontrol agents were applied individually and in combination with seed biopriming (Experiment I) and dry seed treatment (Experiment II) as per the treatment schedule. For biopriming, sesame seeds were soaked for twelve hours in water, followed by the previously soaked seeds being separately treated/ mixed with talc-based formulation of fungal and bacterial bioagents individually

and in combination. Seeds were preserved for 24 h at 28 °C in warm and moist conditions prior to radical emergence. The fungicide thiram was used as a comparison for both experiments. Untreated seeds were considered as control. Each treatment has four replicates. Each replication had 100 seeds. The observation on seed infection (%) was recorded by using standard blotter paper method. In both experiments, observation on the percentage of germination, root length, shoot length and vigour index was also recorded by using the paper roll towel method (7). The vigour index was determined using the formula provided by Abdul Baki and Anderson (21).

Vigour Index = Germination percentage × Seedling length (cm).

Statistical Analysis

The data were analysed using analysis of variance (ANOVA) through the "IRRISTAT version 92-1 program", which was developed by the Biometrics Unit of the International Rice Research Institute, the Philippines.

Results and Discussion

Infection of seed mycoflora in different sesame seed samples

The findings showed that seven fungal species, classified in to six genera including Macrophomina phaseolina, Alternaria sesami, Aspergillus flavus, A. niger, Rhizopus sp., Fusarium sp. and Curvularia sp. were discovered to be associated with sesame seeds, suggesting that these species are seedborne. Among the seed-borne fungi, the infection of M. phaseolina was highest (0.00 to 18.90 %), followed by A. sesami (0.00 to 12.78 %), A. flavus (0.00 to 15.04 %) and A. niger (0.00 to 10.08 %), Curvularia (0.00 to 7.10 %) and Rhizopus (0.00 to 5.88 %) and whereas, the infection of Fusarium was least (0.00 to 3.98 %) in all tested sesame seed samples (Table 1; Fig. 1). Similarly, the major seed-borne pathogens viz., A. sesami, M. phaseolina, Curvularia sp., Fusarium sp., Rhizopus sp., A. flavus and A. niger was reported in sesame by applying standard blotter method (10,22). According to Haider et al. (23), the mycoflora, including Alternaria alternata, M. phaseolina, Fusarium moniliforme, Cercospora sesami, C. lunata, Aspergillus spp., Penicillium citratum and Rhizopus sp. were predominantly associated with the seeds of local sesame variety. Recently, Gawarkar et al. (24) isolated six fungal species belonging to five genera, viz., Fusarium oxysporum, A. niger, A. flavus, A. alternata, C. lunata and Cladosporium sp. from different sesame varieties. In all seed health studies, the standard blotter method was used for the detection of seed-borne fungi in sesame.

In the current study, a total of seven fungal species were identified, with *M. phaseolina* and *A. sesami* being the most commonly associated with the majority of the tested sesame seed samples. *Aspergillus flavus*, *A. niger*, *Curvularia* sp., *Rhizopus* sp. and *Fusarium* sp. were found in only some of the seed samples. The findings clearly showed that *M. phaseolina* and *A. sesami* were more prevalent in the major sesame-cultivating regions of Tamil Nadu. Conversely, *Curvularia* sp. and *Fusarium* sp. were the most commonly

Table 1. Mycoflora associated with different sesame seed samples

C: No	Variety	Location -	Seed infection (%)									
Sr. No.			M. Phaseolina	A. sesami	A. flavus	A. niger	Rhizopus sp.	Fusarium sp.	Curvularia sp.			
1.	VRI1		16.44	6.22	8.22	0.00	0.88	0.00	0.00			
2.	VRI2		10.22	12.78	0.00	4.12	0.00	1.33	1.07			
3.	VRI3		11.04	4.80	0.00	0.00	0.00	0.00	2.22			
4.	TMV4		14.80	10.50	3.45	2.74	0.00	2.07	0.00			
5.	TMV7		17.66	6.50	0.00	5.08	3.12	0.00	0.00			
6.	VRI2		8.21	0.00	5.65	1.30	1.44	0.00	1.84			
7.	VRI3		10.40	3.33	0.00	0.00	2.00	0.00	0.98			
8.	VRI2		18.90	8.74	8.66	0.00	0.00	1.22	2.10			
9.	VRI2	Cuddalore	17.44	7.95	0.00	3.92	0.00	3.12	2.00			
10.	TMV4	Cuddatore	15.88	12.22	0.00	5.95	1.75	0.00	1.24			
11.	TMV4		17.62	9.50	8.60	0.00	0.00	0.87	0.00			
12.	TMV4		5.84	11.20	0.00	0.00	4.33	0.50	7.10			
13.	TMV7		14.75	0.00	0.00	5.33	0.00	1.07	0.00			
14.	VRI2		13.65	0.00	0.00	0.00	0.00	0.72	0.00			
15.	VRI2		16.54	8.45	3.94	0.00	0.00	0.00	0.00			
16.	TMV7		0.00	10.60	0.00	0.00	0.00	0.00	0.00			
17.	VRI3		3.60	11.20	0.00	3.97	2.22	2.05	1.87			
18.	TMV4		12.87	10.11	4.90	0.00	5.88	2.00	0.00			
19.	VRI2		7.86	12.57	15.04	1.98	2.60	3.98	3.42			
20.	VRI2		10.44	6.66	0.00	10.08	0.00	0.00	0.00			
21.	TMV4		5.68	9.80	3.33	1.58	0.00	0.00	0.00			
22.	VRI2		3.24	5.48	0.00	6.10	2.42	0.00	5.88			
23.	VRI3	Villupuram	8.37	2.45	0.00	0.00	0.00	1.04	0.00			
24.	VRI3		11.10	3.65	3.22	0.00	1.08	2.97	0.00			
25.	TMV7		4.22	1.22	0.00	5.77	0.00	0.00	0.00			
26.	TMV7		0.00	2.87	0.00	0.00	0.00	0.00	4.15			
27.	VRI2		3.33	0.00	0.00	0.00	0.00	1.77	0.90			
28.	VRI2		5.80	2.84	7.65	2.65	2.00	0.00	1.22			
29.	VRI2		7.00	6.40	0.00	0.00	0.00	0.00	0.00			
30.	TMV7		5.50	4.87	3.20	0.00	3.07	0.00	0.00			

Values are mean of four replications



Standard blotter method



Aspergillus flavus Fusarium sp. Curvularia sp.

Fig. 1. Detection of mycoflora related to sesame seeds.

observed pathogenic fungi and species of *Aspergillus* and *Rhizopus* were the commonly found saprophytic fungi associated with seed mycoflora. The current findings are in agreement with the research of Lakshmi Pravallika et al. (10), who found that the mean incidence of *A. sesami* was highest followed by *A. flavus* and *A. niger*, while the mean incidence of *Helminthosporium* sp. was least (4.82 %) in different sesame seed samples collected from different locations of Andhra Pradesh and Telangana. The results clearly indicated that the seed-borne nature of *M. phaseolina* and *A. sesami*, which are the major fungi causing seed infection in sesame.

Standardization of detection methods for seed-borne pathogens of significance

Of the various methods evaluated for the detection of important seed-borne pathogens in sesame, the PDA plate method proved effective for the detection of *Macrophomina* (14.2 %) and *Alternaria* (9.8 %) and was found significantly superior over other detection methods (Table 2; Fig. 2). This method was followed by standard blotter method which recorded 7.9 and 11.8 % seed infection of *Alternaria* and *Macrophomina*, respectively. The present results confirm with the findings of Indra (25), who found that the PDA plate method was statistically superior for the detection of *M. phaseolina* in all the varieties of sesame. In contrast, Radha and Chattannavar (8) reported that standard blotter was the best method for the detection of *Alternaria* sp. in sesame.

However, in the present study, the overall per cent recovery of seed-borne pathogens was maximum in the standard blotter method (45.1 %) followed by PDA plate method (35.2 %) and 2,4 - D blotter method (31.3 %). The deep-freezing method proved to be the least effective technique for detecting pathogens on seeds, achieving a total recovery rate of 16.4 %. The effectiveness of this

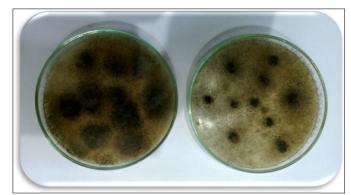


Standard blotter method

method was shown to be superior by multiple researchers working with sesame (8), chillies (26) and safflower (27). Nagaraja et al. (28) reported that standard blotter method was found to be superior over potato dextrose agar method, water agar method and 2,4- D method for detection of seed mycoflora associated with castor. Pushpavathi et al. (29) also found that standard blotter method was best for the detection of seed mycoflora associated with safflower seed followed by agar plate method. Recently, Indira et al. (30) reported that PDA agar plate method was the best method for isolation and detection of fungal pathogens associated with okra seeds followed by standard blotter method. This study found that despite the blotter paper method being superior to the PDA agar plate method, the lack of surface sterilization in the seeds resulted in the growth of both surface-contaminated saprophytes and seed-borne fungal pathogens. In the PDA agar plate method, surface sterilized seeds were used, which resulted in a significant reduction of the saprophytic mycoflora population associated with sesame seeds.

Effect of biopriming and dry seed treatment with fungal and bacterial bioagents on seed infection and seed quality parameters in sesame

Seed primed with biocontrol agents *viz.*, *T. asperellum*, *B. subtilis* and *B. amyloliquefaciens* individually and in combination recorded lesser seed infection (%) and higher germination per cent, root and shoot length and seedling vigour compared to dry seed treatment irrespective of the treatment. Among the treatments, *B. subtilis* (1 %) recorded a minimum seed infection (0.66 and 1.73 %) and a maximum germination per cent (92 and 90 %) and seedling vigour (1186 and 1116) both in bioprimed seeds (Experiment I) and dry seed treatment (Experiment II), while in untreated control recorded a maximum seed infection of 19.90 and



Potato dextrose agar plate method

Fig. 2. Standardization of seed health testing techniques for the identification of fungi associated with sesame seeds.

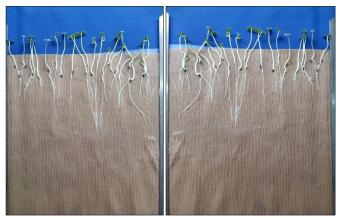
Table 2. Standardization of detection methods for seed-borne pathogens in sesame

S. No.	Method -	% seed infection									
5. NO.	method –	Alt.	Mac.	A.n	A.f	Rhi.	Fus.	Cur.	Total		
1.	Standard blotter	7.9	11.8	6.2	9.0	6.1	2.3	1.8	45.1		
2.	2, 4 - D blotter	5.3	7.2	4.2	6.7	4.7	1.7	1.5	31.3		
3.	Deep freezing	3.6	4.9	2.0	1.4	2.0	1.2	1.3	16.4		
4.	Water agar	4.8	7.0	2.2	1.1	2.7	1.9	0.9	20.6		
5.	Potato dextrose agar plate	9.8	14.2	3.4	3.2	2.0	2.2	0.4	35.2		
6.	Rolled paper towel method	4.6	6.7	2.0	4.6	1.7	1.1	1.3	22.0		
	CD(0.05%)	1.22	0.95	0.9	1.4	0.85	0.92	0.76	-		
	S. Ed	0.60	0.46	0.42	0.68	0.40	0.45	0.34	-		

Values are mean of four replications

18.78 % and a minimum seed germination of 82 and 83 %and vigour index of 877 and 904 in experiment I and II, respectively. The treatment was on par with fungicide thiram (0.4 %), which recorded 0.66 and 1.20 per cent seed infection, 92 and 90 % seed germination and 1113 and 1053 vigour index in experiment I and II, respectively (Table 3; Fig. 3). The combination of *T. asperellum* and *B. subtilis* (1 %) recorded lesser seed infection and had only moderate effect on seed germination and vigour index in both experiments. Seed priming involves a pre-sowing treatment where seeds are placed in an osmotic solution, enabling them to absorb water. During this phase, if the bioagents are subjected to the germinating seeds, it facilitates the bioagents to establish well with the seedlings in improving the health of plants. Furthermore, the enzymes and growth hormones are also triggered in seeds during this process. The current study's findings may be attributed to the fact that bioprimed seeds exhibit lower seed infection and higher seed germination rates and vigour compared to dry seed treatment methods.

In the present study, suppression of seed-borne pathogen infection and improvement in seed germination and plant growth promotion in sesame may also result from B. subtilis competing with the pathogen for space and nutrients, as well as via antibiosis, lysis and the production of growth hormones. The findings of this study align with previous research, specifically the results of Begum et al. (31) and Sharma et al. (32) in soybean, which showed that seed biopriming with Pseudomonas provide better protection against damping off disease caused by Colletotrichum truncatum and enhance growth and yield parameters in soybean. The priming of seeds with PGPR increase the germination rate, root growth, yield, leaf area, chlorophyll content, magnesium, nitrogen and protein content, tolerance to drought and salt stress, shoot and root weights and delayed leaf senescence in both chickpea and rajma (33). Seed bio-priming with Trichoderma and Pseudomonas also resulted in increased production of plant secondary metabolites like phenolics in chickpeas, which aids in plant resistance to infection by Fusarium oxysporum f. sp. ciceris causal agent of chickpea wilt disease (34).



Bacillus subtilis (Bioprimed)

Bacillus subtilis (Dry seed treatment)





Untreated control

Fig. 3. Effect of biopriming and dry seed treatment using bioagents on seed germination and seedling vigour in sesame.

Conclusion

The results of this investigation indicate that the PDA plate method is a suitable technique for detecting pathogens of major concern, such as *M. phaseolina* and *A. sesami* in sesame seeds. Additionally, the standard blotter method can be utilized for examining the overall fungal diversity associated with sesame seeds. The current study concludes that the standard blotter method can be used to study the overall mycoflora associated with sesame seeds, while the PDA plate technique may be preferred for seeds health evaluation of seed-borne pathogens of significance like *M. phaseolina* and *A. sesami* in sesame. Moreover, seeds primed with *B. subtilis* had been found to be efficient in controlling seed infections, promoting seed germination

Table 3. Effect of biopriming and dry seed treatment with bioagents on seed infection and seed quality in sesame

		Ехр	Experiment II (Dry seed treatment)								
S. No	Treatments	Seed infection (%)	Germi- nation (%)	Root length (cm)	Shoot length (cm)	Vigour	Seed infection (%)	Germi- nation (%)	Root length (cm)	Shoot length (cm)	Vigour
1.	Trichoderma asperellum (0.4%)	3.00	88	7.6	4.6	1073	4.44	87	7.3	4.5	1026
2.	Bacillus subtilis (1%)	0.66	92	7.7	5.2	1186	1.73	90	7.4	5.0	1116
3.	Bacillus amyloliquefaciens (1%)	3.75	90	7.4	5.0	1116	5.22	87	7.2	4.7	1035
4.	T. asperellum + B. subtilis (1%)	0.66	87	6.8	4.6	991	1.55	86	6.4	4.5	937
5.	T. asperellum + B. amyloliquefaciens (1%)	2.78	86	6.9	4.5	980	4.05	85	6.7	4.3	935
6.	Thiram (0.4%)	0.66	92	7.0	5.1	1113	1.20	90	6.7	5.0	1053
7.	Untreated control	19.90	82	6.4	4.3	877	18.78	83	6.5	4.4	904
	CD (0.5%)	0.96	1.33	0.42	0.40	-	1.02	1.22	0.37	0.34	-
	S.Ed	0.45	0.63	0.20	0.19	-	0.50	0.60	0.18	0.16	-

and improving overall seed quality. Future research can explore the use of biopriming technology to manage various seed-borne diseases in different crops, ensuring sustainable disease control.

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Authors' contributions

AT carried out all the experiments including collection of sesame seed samples from different locations, different seed health testing methods, effect of bioagents on seed-borne fungi and other seed quality parameters in sesame. SG helped in collecting the sesame samples and data analysis.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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