

COMPARATIVES STUDY OF BLOTTER, AGAR-PLATE, AND DEEP-FREEZING METHODS FOR THE DETECTION OF SEED-BORNE FUNGAL PATHOGENS IN WHEAT (TRITICUM AESTIVUM L.) CULTIVARS OF THE DISTRICT MANSEHRA REGION, KHYBER PAKHTUNKHWA, PAKISTAN

QUDSIA A¹, JAMSHAD^{2*}, SHIREEN F^{3*}, AHMAD S¹, SARDAR S⁴, KIRBAG S⁵

¹ Department of Microbiology, Hazara University, Mansehra, Khyber Pakhtunkhwa, Pakistan

² Sarhad University of Science and Information Technology, Peshawar, Pakistan

³ School of Allied Health Sciences, Iqra National University Peshawar, Pakistan

⁴ Women University Marden, Khyber Pakhtunkhwa, Pakistan

⁵ Department of Biology, Faculty of Science, Firat University Firat 23119 Elazig, Turkey

Correspondence Author Email Address: farahshireen65@gmail.com; jamshaidhu100@gmail.com

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Abstract Seed-borne fungal pathogens are major issues limiting wheat (*Triticum aestivum* L.) productivity, affecting germination potential, vigour of seedlings, grain quality, and also serving as a primary source of fungal inoculum for field epidemics. This study was conducted to compare the efficacy of three most popular methods, such as the blotter test, deep-freezing blotter method, and agar-plate method for detection of seed-borne fungal pathogens in Wheat (*Triticum aestivum* L.) seeds. Fifty wheat seed samples from 14 different areas in District Mansehra of Khyber Pakhtunkhwa, Pakistan, were collected and belonged to 9 commercial cultivars. A total of 9 species of fungi were isolated from 50 wheat seed samples of nine wheat varieties, including *Alternaria alternata*, *Ustilago tritici*, *Fusarium oxysporum*, *Penicillium chrysogenum*, *Curvularia lunatus*, *Aspergillus niger*, *Tilletia indica*, *Mucor mucedo*, and *Rhizopus stolonifera*. All nine species were isolated by the agar-plate method compared to the other two methods, which also had the highest overall isolation frequency (96%), blotter test (84%), and the deep-freezing method (80%), respectively; three species (*A. niger*, *P. chrysogenum*, and *R. stolonifera*) were only isolated by the agar-plate method. In all methods, the most common species was *A. alternata*, detected in 90% of the blotter test and 94% of the deep-freezing test. A supplementary wash test found *T. indica* in 90% of samples, and the deep-freezing method showed the highest recovery of this quarantine-significant pathogen in the comparative tests. In location-wise analysis, the highest fungal growth was found in Dhodial, while the cultivar-wise analysis found that the cultivar with the highest fungal load was Pirsabak-2013 and the cultivar with the highest germination percentage was Paseena-2017, while Pirsabak-2013 had the lowest. *A. alternata*, *Curvularia lunatus*, *Tilletia indica*, *Mucor mucedo* and *Fusarium oxysporum* were symptomatically transmitted from infected seed to emerging seedlings of cultivars Zincol and Paseena-2017 in a seed-to-seedling transmission assay. The results indicated that the agar-plate method is the most reliable single test to use for routine phytosanitary screening in this agro-ecological zone, and that this test, when used in combination with the blotter, freezing and wash test, can be most effective in detection of saprophytic and quarantine pathogens.

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Introduction

Wheat (*Triticum aestivum* L.) is the most widely grown cereal in the world and the main source of calories and protein in the diets of people in South and Central Asia, the Middle East and North Africa. Since the beginning of the Green Revolution, production has steadily increased, but yields have been steadily decreasing because of biotic constraints, including seed-borne fungal pathogens, which are one of the

least visible, but yet most economically important threats (Borlaug, 2007; Aboukhaddour et al., 2020). In addition to being the propagative unit of the crop, seed is also the primary means of spreading many fungal pathogens between seasons, regions, and country borders, and seed health testing is an essential component of agronomic management and phytosanitary regulation (Munkvold, 2009). Seed-

borne infection can be systemic (with mycelium infesting the embryo and vascular tissue and being carried internally within seed) or non-systemic (superficial) (mycelium infecting only seed coat without deep tissue invasion). Systemic pathogens like *Ustilago tritici* (loose smut) infect at anthesis and lie dormant in the embryo until germination, when the fungus grows synchronously with the host, leading to the replacement of the developing grain by a bunch of teliospores (Malik and Batts, 1960; Wegulo, 2009). Non-systemic contaminants, on the other hand, do not enter the system and remain on the pericarp or glumes, and germinate under suitable moisture and temperature after sowing, resulting in seedling blight, damping-off, or root rot (Agrios, 2005). Several economically important seed-borne diseases of wheat have been reported from all over the world. Loose smut, caused by *U. tritici*, causes complete conversion of the entire inflorescence into a bunch of dark smut spores, resulting in the loss of yield without any external symptom on the harvested grain (Nielsen, 1987; Abraham, 2019). Bunt fungi (Common bunt: *Tilletia caries* and *Karnal bunt: Tilletia indica*) invade the kernel, displacing part or all of the kernel content and producing highly fetid sori containing spores that result in yield loss and also grade and quarantine restrictions that can lead to closure of entire export markets (Kumar et al., 2021; Kashyap et al., 2023). Black point is caused primarily by *Alternaria alternata* and *Bipolaris sorokiniana*, and is characterized by a dark discoloration at the embryo end of the kernel, which may result in a downgrade in milling and baking quality even though the germination rate may be only slightly reduced (Fernandez and Conner, 2011; Li et al., 2021). The diseases alone have been estimated to contribute significantly to the global losses of 20–25% of wheat production each year due to fungal diseases (Aboukhaddour et al., 2020).

Although smallholder farmers in Pakistan often carry and share farm-saved seed without adequate certification or treatment, there is little systematic data on the composition and prevalence of seed-borne mycoflora, especially in the wheat-growing mountainous areas of the country (Bahoo, 2020; Khan et al., 2023). District Mansehra is located in the sub-humid temperate region of the province where a wide variety of wheat cultivars are grown under both rainfed and irrigated conditions; however, the seed-borne fungal complex of locally cultivated cultivars has never been characterized in a comparative method-based approach. The selection of a seed health assay in this situation is critical, because testing by either of the three methods (blotter, agar, freezing) has different sensitivity to specific taxa, is sensitive to the presence of fast-growing saprophytes that might overgrow slower pathogens, and may apply to the detection of either external or internal borne pathogens (Diekmann and Asaad, 1989; Mathur and Kongsdal, 2003). The present study, therefore, was

designed to (i) isolate and morphologically identify the fungal species present in the seed of nine wheat cultivars grown from various areas of Mansehra region; (ii) compare the diagnostic efficiency of the traditional blotter test, deep-freezing method and agar-plate method for the recovery of the fungal flora; (iii) assess a wash test for early detection of teliospores of *T. indica* as a quarantine-relevant supplementary assay; and (iv) evaluate the effect of the fungus on seed germination and their ability to transmit from seed to seedling. The outcomes will inform the choice of appropriate and field-relevant seed-health protocols at a lower cost in wheat-growing regions having similar agro-climatic and smallholder seed-system features.

Materials and methods

Study Area and Sample Collection

A total of 50 wheat samples (~200 g) were collected from nine commercial wheat cultivars from the farmers' fields in 14 areas of District Mansehra, Khyber Pakhtunkhwa – Baffa, Bajna, Balakot, Battal, Bherkund, Dadar, Dhodial, Gandhian, Gulibagh, Jaborri, Lassan Nawab, Oghi, Peeran, Shinkari and Tanda. For processing, the samples were kept in labelled paper bags at 5–7°C at the Department of Microbiology, Hazara University, Mansehra.

Detection Methods for Seed-Borne Fungi

Wash Test

For each sample, 50 g of seed was shaken for 10 minutes with 50 mL of sterile distilled water containing a few drops of Tween-20, and the suspension was then centrifuged (3,000 rpm for 15 min), and the pellet was viewed under the microscope for the presence of fungal propagules according to Mathur and Kongsdal (2003).

Standard Blotter Test

The 25 seeds/ sample were sown on three layers of moistened Whatman filter paper in 90 mm Petri dishes for a period of seven days at 25°C. Infected seeds were counted and examined with a stereomicroscope for their characteristic fungal morphology.

Deep-Freezing Method

20 seeds were sown on moistened filter paper and kept at 25°C for 24 h, then wounded at –20°C for 6–8 h to kill the embryo tissues while allowing the growth of fungal tissues and later returned to 25°C for 5–7 days for microscopic examination (Fakhrunnisa and Ghaffar, 2006). The percentage of seed infection and regional infection were calculated as:

The mean rate of seed infection (in percentage) is calculated as follows:

$$\text{Seed Infection (\%)} = \frac{\text{Total number of seeds tested}}{\text{Number of seeds infected with fungi}} \times 100$$

Mean regional infection percentage (per sample (Wheat))

$$\text{Mean Regional Infection (\%)} = \frac{\text{Total number of samples collected}}{\text{Number of samples in which seed-borne fungi were identified}} \times 100$$

Agar-Plate Method

The seeds per sample were treated with sodium hypochlorite (25 seeds per sample; 5 minutes in 1% sodium hypochlorite, followed by two 5-minute rinses in sterile water, dried on sterile filter paper, and plated (10 seeds per replicate, 3 replicates) on potato dextrose agar (PDA). The plates were set at 25°C in alternating 12 h periods of near-ultraviolet light and darkness, and the infected seeds were counted, and emerging colonies were identified under a microscope after 6 to 10 days.

Fungal Identification

Species-level identification was carried out by examining the morphology of the colonies and conidia under a compound and stereo microscope and comparing them with the pictorial keys of [Watanabe \(2010\)](#).

Seed Germination Test

The seeds were germinated in Petri dishes containing moistened filter paper with 20 seeds per dish at 25 ± 2°C for 14 days, and germination percentage was determined according to [Luo et al. \(2018\)](#).

The percentage of seed germination is calculated as follows:

Number of seeds germinated after 14 days divided by the number of seeds multiplied by 100.

Seed-to-Seedling Transmission Study

The seeds were removed from the two most heavily infected samples and were used to inoculate test-tube

Table 1. Comparative performance of the blotter, deep-freezing, and agar-plate methods for detection of seed-borne fungi in wheat samples from District Mansehra

Detection Method	Species Recovered (n)	Overall Isolation Frequency	Most Prevalent Species (max. %)	Species Unique to Method
Blotter test	6	84%	<i>A. alternata</i> (90%)	—
Deep-freezing method	6	80%	<i>A. alternata</i> (94%)	—
Agar-plate method	9	96%	<i>A. alternata</i> / <i>T. indica</i> (variable)	<i>A. niger</i> , <i>P. chrysogenum</i> , <i>R. stolonifera</i>

Morphological Characterization of the Isolated Fungi

Diagnostic morphological characteristics of the recovered representative colonies were found to be suitable for the assigned taxa, including fluffy olive-grey to black colonies with muriform conidia with a beak typical of *Alternaria alternata*, dark, powdery sori replacing floral tissue characteristic of *Ustilago tritici*, septate, sickle-shaped macroconidia characteristic of *Fusarium oxysporum*, curved, multi-celled conidia characteristic of *Curvularia lunatus* and dark ornamented teliospores characteristic of *Tilletia indica*.

Location-wise and Cultivar-wise Infection Patterns

The location-wise analysis showed significant spatial variation in fungal load. Infection varied from 8% (Pirsabak-2015, Dhodial) to 76% (Paseena-2017, Dhodial) in the blotter test, while 72% (Shahkar-2013,

slants (6 mL 2% water agar), which were then autoclaved (121°C, 15 lb, 10 min). Slants were kept at room temperature (20 ± 2°C), and the germinating seedlings were studied under the microscope for symptoms that can be attributed to the pathogens associated with them.

Statistical Analysis

Data were presented as mean and percentage and analyzed with Statistics version 8.1; descriptive statistics were presented in tabular and graphical format.

Results

Fungal Species Recovered and Method Sensitivity

In all three detection methods, nine fungal taxa were isolated from 50 wheat seed samples: *Alternaria alternata*, *Ustilago tritici*, *Fusarium oxysporum*, *Penicillium chrysogenum*, *Curvularia lunatus*, *Aspergillus niger*, *Tilletia indica*, *Mucor mucedo*, and *Rhizopus stolonifera*. The agar-plate method recovered all nine species and gave the highest overall isolation frequency across cultivars (96%), followed by the standard blotter test (84%) and the deep-freezing method (80%). There were three species (*A. niger*, *P. chrysogenum*, and *R. stolonifera*) that were recovered only on the agar plate method and not in either the blotter or deep-freezing method. The comparative performance of the three methods is summarized in Table 1.

Battal) and 60% (Kohat-2017, Lissan Nawab) had high infection. The range was even broader in the deep freezing method, ranging from 4% (Khaista-2017, Oghi; Pirsabak-2015, Dhodial) to 80% (Pirsabak-2015, Dhodial), with the maximum percentage of cases again in the Dhodial locality. When compared between the two methods, the diversity and load of fungi present in seed-borne samples from Dhodial was consistently high, suggesting the influence of local micro-climatic/storage conditions for fungal growth. Pirsabak-2013 had the highest overall fungal burden and is the least suitable cultivar of the tested cultivars for seed production in this region free from disease, whereas Paseena-2017 had comparatively high fungal infection at some stations and the best germination rate of all cultivars tested.

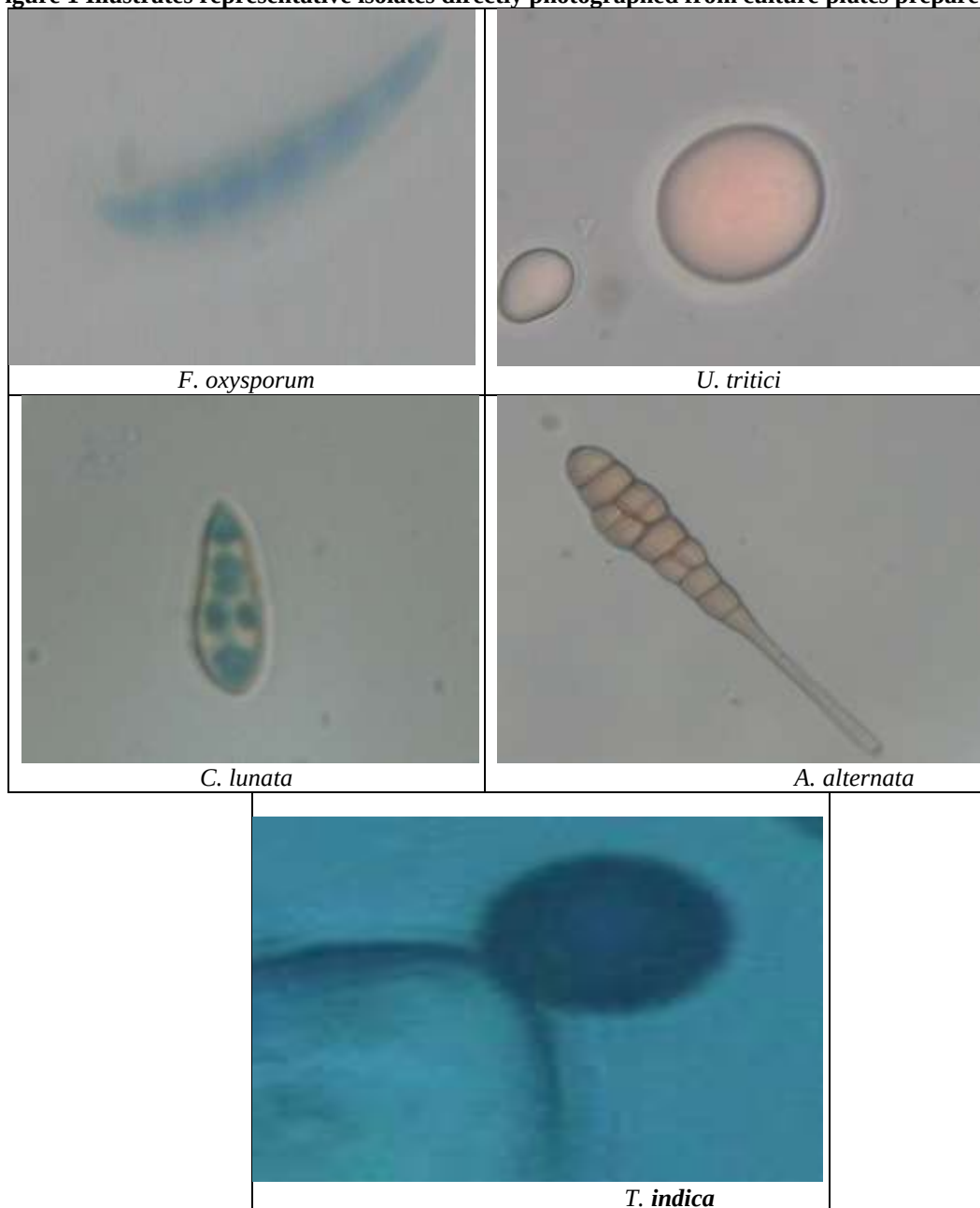
Figure 1 Illustrates representative isolates directly photographed from culture plates prepared during this study

Figure 1. Representative fungal species isolated from wheat from agar-plate and blotter-test cultures. Colony growth on PDA plates showed characteristic radial spread and pigmentation patterns that allowed rapid presumptive identification prior to microscopic confirmation (Figure 2).



Figure 2. Mixed fungal colonies developing on potato dextrose agar (PDA) plates inoculated with surface-disinfected wheat seeds, illustrating the diagnostic colony morphology used for presumptive species identification (agar-plate method, 6–10 days at 25°C).

Wash Test for *Tilletia indica* and Comparative Recovery

Teliospores of *T. indica* were detected in 90% of the 50 seed samples, suggesting a high level of contamination with this quarantine-significant pathogen from the cultivars and localities sampled; 10% of seed samples were negative. The deep freezer was compared with the three incubation-based assays for the recovery of *T. indica* specifically, and the deep-freezer method gave a higher comparative recovery of teliospores (66%) than the blotter and agar-plate methods, which is consistent with the capacity of the freeze–thaw cycle to break open teliospore-bearing tissue to release spores for microscopic identification.

Seed Germination

Three cultivars had more than 90% germination, while five others had less than 10%. The highest germination percentage was observed in Paseena-2017, while the lowest one was obtained for Pirsabak-2013 having highest fungal load throughout the experiment, indicating that seed vigour was negatively associated with seed-borne fungal load.

Seed-to-Seedling Transmission

Alternaria alternata, *Curvularia lunatus*, *Tilletia indica*, *Mucor mucedo* and *Fusarium oxysporum* were transmitted from seed to seedling from infected seeds in the test-tube seedling assay, which resulted in the appearance of seedling symptoms of discoloration, stunted radicle growth and necrotic lesions on the coleoptile in both cultivars (Zincol and Paseena-2017) (Figure 3). This validates the finding of a seed infection, as determined by the comparative assays, as a real phytosanitary risk when the seedlings are placed in the field.



Figure 3. Seed-to-seedling transmission of seed-borne fungal pathogens in test-tube seedling assays, showing symptomatic seedlings derived from infected wheat seed of cultivars Zincol and Paseena-2017

Discussion

Selection of the detection method had a significant impact on the estimated composition and prevalence of the mycoflora that occurs naturally on seed, a result consistent with the overall literature on seed pathology ([Diekmann and Asaad, 1989](#); [Mathur and Kongsdal, 2003](#)). In this study, the agar-plate method recovered all nine species and the highest overall infection frequency (96%), consistent with recent comparative studies that found the agar-plate method superior to standard and modified blotter methods in terms of total mycoflora recovery, such as in Maharashtra, India where the agar-plate method found 93.25% recovery, compared with 87% for standard blotter and 84% for modified blotter ([Discovery Editorial, 2025](#)). The exclusive isolation of saprophytic and opportunistic genera such as *Aspergillus niger*, *Penicillium chrysogenum*, and *Rhizopus stolonifera* on PDA in the present study is due to their nutrient dependence for growth and sporulation, which can be suppressed on water-only blotter media ([Munkvold, 2009](#)).

Multiple recent surveys indicate *Alternaria alternata* as the most common fungus associated with wheat seeds all over the globe in a wide variety of production regions, including south Punjab, Pakistan, where it was the predominant species (up to 94% by the deep-freezing method; [Khan et al., 2023](#)) and winter wheat production systems in Eastern Europe, where *Alternaria* was the dominant endophytic fungus associated with black point symptoms (52–79% of all screened wheat seed genotypes; [Zhukova et al., 2026](#)). An overall trend seen in the present germination data was that infection level and germination percentage were not always reflected in direct correlation, and that this was also the case for some cultivars, which is in line with the effect of black point in downgrading the milling quality despite its comparatively low impact on germination ([Fernandez and Conner, 2011](#); [Endes et al., 2025](#)). The phytosanitary importance of the presence of *Tilletia indica* in 90 % of the samples by the wash test and the better recovery rate (66 %) of *T. indica* by the deep-freezing method over wash and blotter and agar-plate assays is of particular importance because Karnal bunt is a regulated quarantine disease which can limit grain trade internationally even at very low incidence ([Kumar et al., 2021](#)). Conventional morphological assays, like those used here, are a useful method to identify infection, and in future quarantine surveillance programmes, might be complemented with contemporary molecular diagnostics like a GAPDH-based quantitative real-time PCR assay, which has recently been validated for the pre-symptomatic detection of *T. indica* in the field with a limit of detection of 4 pg of pathogen DNA ([Kashyap et al., 2023](#)).

This strong locality effect seen in Dhodial, where fungal loads were consistently higher on the blotter and deep-freezing assays than other cultivars, means that the post-harvest storage humidity, seed-handling practices, or microclimatic conditions during grain filling have a greater influence on fungal load in seed than cultivar genetics alone, and this locality should be targeted for specific seed-treatment interventions. In the same way, the fact that Pirsabak-2013 was the highest infected and lowest-germinating cultivar compared to relatively high germination of Paseena-2017 offers an evidence base for cultivar-specific seed-certification recommendations in this district. The ability to infect a seed with a fungus and create a seedling infection, as demonstrated for the five fungi, is not simply an in vitro artefact but does reflect the potential for seed infection that is recorded by laboratory assays to cause a real risk of establishment from seed in the early season of a disease epidemic, as has been shown for wheat pathogens (Khanzada et al., 2002; Li et al., 2021). These results give justification for the use of a tiered wheat seed-health testing approach for smallholder wheat systems in KPK: the agar-plate method for a general survey of the diversity and level of mycoflora present; the deep-freezing method as an additional supplement when there is a specific interest in detecting Karnal bunt or other freeze-sensitive pathogens; and the wash test as a rapid, low-cost field-level triage test before sending samples to the lab for more expensive and time-consuming confirmation. If resources allow, molecular assays for regulated pathogens, like *T. indica*, could also be integrated into the diagnostic pipeline developed in this study.

Conclusion

A total of nine seed-borne fungal species were identified among wheat cultivars cultivated in the entire District Mansehra, Khyber Pakhtunkhwa province of Pakistan, with *Tilletia indica*, *Mucor mucedo*, and *Rhizopus stolonifera* being the most prevalent. The agar-plate method yielded the highest infection frequency (96%), species diversity, and the recovery of species not recovered by the other two comparative assays, *A. niger*, *P. chrysogenum*, and *R. stolonifera*. The most ubiquitous species overall was *A. alternata*, with 94% of the deep-freezing samples. In addition, Dhodial was found to be the most fungal-diseased locality for disease-free wheat production, while Pirsabak-2013 was the most infected wheat cultivar in comparison with Paseena-2017, which had the highest germination percentage with acceptable fungal infection. The wash test resulted in a high percentage of positive results for *T. indica* (90%), and the deep-freeze method had the most comparable recovery of this pathogen (66%). The seed-to-seedling transmission of *A. alternata*, *C. lunatus*, *T. indica*, *M. mucedo*, and *F. oxysporum* was confirmed in cultivars Zincol and Paseena-2017, which is a direct phytosanitary risk. These findings justify the use of the agar-plate method along with deep-freezing

and wash tests of quarantine pathogens as an integrated seed-health testing system for wheat growing areas of Khyber Pakhtunkhwa and other small farmer production sectors.

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Data Availability statement

All relevant data are within the manuscript file.

Author's Contribution Statement

AQ, and Jamshaid collected data and wrote manuscript equally. AQ, and Jamshaid make final editing. Jamshaid supervised the whole research work. All authors have read the final manuscript and approve its submission.

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Ethical Statement

Not applicable

Conflict of interest

The investigation was undertaken without any financial conflicts of interest or any other commercial relationships that could be seen as such by any of the authors.



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