https://doi.org/10.35662/unine-thesis-3246

Molecular detection of smut and bunt pathogens

A dissertation submitted to the
University of Neuchâtel (Switzerland)
Faculty of Science, Institute of Biology
for the degree of

DOCTOR OF SCIENCE

Presented by

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Defended January 30^{th}, 2026

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Neuchâtel, le 9 mars 2026

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Summary

Increasingly strict regulations on synthetic plant protection products (PPPs) make the management of seed- and soil-borne cereal diseases more challenging. Agricultural policies, such as the European Green Deal and the "Farm to Fork" strategy, aim to reduce the use and risk of synthetic pesticides to protect human health and the environment. Although sustainable agriculture requires this ecological transition, it may increase crop vulnerability to the reemergence of pathogens that were historically controlled with prophylactic synthetic seed treatments. These pathogens include *Ustilago nuda* (loose smut of barley), *Tilletia caries* (common bunt of wheat), and *Tilletia controversa* (dwarf bunt of wheat). No low-risk or organic treatments available on the market control these diseases as effectively as synthetic PPPs. Additionally, methods to assess the presence of these pathogens are labor-intensive, and the biological relevance of the tolerance threshold used to guide management decisions is unclear. Together, these factors hinder the practical application of integrated pest management (IPM) strategies to control these bunt and smut diseases; IPM relies on pathogen assessments to make decisions regarding treatment applications and prevent pathogen transmission.

Disease management with reduced synthetic pesticide inputs requires accurate and timely assessments of pathogen presence and pressure. Current surveillance and detection methods, such as field inspections and seed health tests, often lack the sensitivity required to detect low levels of seedborne infection or tools to measure soilborne inoculum. These methods are also poorly suited for contributing to risk-based decision-making or evaluating new plant protection strategies because they are labor-intensive, time-consuming, and costly. Thus, improved detection methods are necessary to support both pathogen prevention and treatment development. With improved pathogen detection, targeted treatments can be applied more precisely when pathogen levels exceed tolerance thresholds. Tolerance thresholds are an integral part of IPM strategies to prevent pathogen transmission.

Molecular methods, such as qPCR and dPCR, have the potential to streamline the seed health certification process. In my dissertation research, I developed a qPCR protocol to detect *U. nuda* in barley seeds that can serve as a seed health test. Compared to the conventional barley seed health test based on visual embryo inspection, the qPCR method showed a stronger correlation with the observed disease incidence in the field. The qPCR-based assessments enabled a precise and accurate classification of tested the seed lots' infection as above or below the tolerance threshold, which corresponds to 0.01% infected embryos. I linked the qPCR measurements from seed lots to subsequent disease occurrence in the field to determine the biological meaning of the qPCR results. Although seed-based detection methods and seed health tests are suitable for detecting seedborne pathogens, these tests do not address the potential presence of soilborne inoculum. Pathogens transmitted through both seeds and soil, such as *T. caries* and *T. controversa*, require detection methods that consider both transmission pathways. I developed a PCR-based protocol to detect these *Tilletia* spp. on seeds and in soil. This protocol is applicable to both dPCR and qPCR assays and uses primers targeting mitochondrial

and nuclear regions to discriminate between the two bunt species. I incorporated an exogenous internal control to improve the reliability of soil DNA extraction. These PCR-based protocols detected low levels of *T. caries* in contaminated seed lots (1-2 teliospores per sample of 250 seeds) and low levels of *T. controversa* teliospores in soil (100 teliospores per gram of soil).

Effective treatments are required when pathogen levels exceed tolerance thresholds to prevent disease outbreaks. However, few organic or low-risk treatments are currently available to control *U. nuda*, *T. caries*, and *T. controversa*. Moreover, because these pathogens show symptoms only at late stages of plant growth, the evaluation of new treatments — and consequently their development — has a long time horizon. To accelerate the evaluation of treatment efficacy, I adapted the qPCR *U. nuda* protocol used on seeds for a laboratory screening procedure that quantifies *U. nuda* copies in DNA extracted from barley seedlings. The simultaneous quantification of *U. nuda* and barley DNA in seedling samples enables a normalization of pathogen levels to its host tissue and reduces the variability related to sampling. Based on field and laboratory experiments, I found that qPCR measurements obtained from seedling samples grown from treated seed for 10 or 14 days on filter paper or in potting soil serve as useful predictors of treatment efficacy in the field. This qPCR-based screening procedure can enable the selection of promising new treatments at an early plant stage, reducing the time and space required for treatment efficacy evaluation. Due to the dual transmission pathway of *T. caries* and *T. controversa*, treatments that are effective against both seed- and soil-borne inocula are needed. To improve the treatment efficacy against *T. caries*, I optimized a qPCR-based screening method by adapting the PCR protocol used to detect bunt pathogens on seeds and in soil. This screening method correctly distinguished infected from uninfected plants based on the eventual development of bunt balls, it provided a less reliable estimate of treatment efficacy than early leaf symptoms of *T. caries*. One limitation is that pathogen DNA was not normalized to host tissue DNA within each sample, which may have reduced the accuracy of the qPCR-based assessment of treatment efficacy.

The PCR-based detection methods developed for *T. caries* and *T. controversa* on seeds, in soil, and in plants provide additional tools for managing bunt pathogens transmitted through seed and soil pathways under increasingly restrictive synthetic pesticide regulations. Further optimization of these methods is required, particularly regarding sampling strategies, the biological interpretation of PCR-based measurements, and performance across soil types. However, these approaches enable more informed, risk-based decision-making within IPM strategies. The frameworks for methodological development presented in this dissertation can inform the improvement of detection methods for other plant pathogens. Ultimately, the work of my dissertation work strengthens molecular detection approaches that are grounded with data acquired from the field, thereby reducing the reliance on prophylactic synthetic pesticide applications and supporting more sustainable crop production.

Keywords: plant protection strategies, seed treatment evaluation, seed health testing, seed- and soilborne pathogenic fungi, quantitative and digital PCR, *Tilletia caries*, *Tilletia controversa*, *Ustilago nuda*, wheat, barley.

Résumé

Le renforcement de la réglementation sur les produits phytopharmaceutiques synthétiques (PPP) rend toutefois la gestion des maladies céréalières transmises par les semences et le sol plus difficile. Les politiques agricoles, comme le « Green Deal » pour l'Europe et la stratégie « Farm to Fork », visent à réduire l'utilisation et les risques liés aux pesticides synthétiques afin de protéger la santé humaine et l'environnement. Cependant, cette transition écologique nécessaire à l'agriculture durable peut accroître la vulnérabilité des cultures au retour d'agents pathogènes qui étaient historiquement contrôlés par des traitements prophylactiques synthétiques des semences. Ces agents pathogènes comprennent *Ustilago nuda* (charbon de l'orge), *Tilletia caries* (carie commun du blé) et *Tilletia controversa* (carie naine du blé). Aucun traitement à faible risque ou biologique sont disponible sur le marché se révèle aussi efficace que les PPP synthétiques pour lutter contre ces maladies. De plus, les méthodes d'évaluation de la présence de ces agents pathogènes nécessitent beaucoup de travail et la pertinence biologique du seuil de tolérance utilisé pour orienter les décisions de gestion ne est pas claire. Ensemble, ces facteurs entravent l'application pratique des stratégies de lutte intégrée (IPM) pour contrôler ces maladies du charbon et carie; l'IPM étant basée sur l'évaluation des agents pathogènes pour prendre des décisions concernant l'application des traitements et la prévention de la transmission de ces agents pathogènes.

La gestion des maladies avec une réduction des applications de pesticides synthétiques, nécessite des évaluations précises et rapides de la présence et de la pression des agents pathogènes. Les méthodes actuelles de surveillance et de détection, comme les inspections sur le champ et les tests de santé des semences, manquent souvent de la sensibilité nécessaire pour détecter de faibles niveaux d'infection transmise par les semences ou des outils pour mesurer l'inoculum transmis par le sol. Ces méthodes sont également peu adaptées pour contribuer à la prise de décision fondée sur les risques ou à l'évaluation de nouvelles stratégies de protection des végétaux, car elles sont laborieuses, chronophages et coûteuses. Il est donc nécessaire d'améliorer les méthodes de détection afin de soutenir à la fois la prévention des agents pathogènes et le développement de traitements. Une meilleure détection des agents pathogènes permet d'appliquer des traitements ciblés de manière plus précise lorsque les niveaux d'agents pathogènes dépassent les seuils de tolérance. Les seuils de tolérance font partie intégrante des stratégies de IPM visant à prévenir la transmission des agents pathogènes.

Les méthodes moléculaires, telles que la qPCR et la dPCR, ont le potentiel de simplifier le processus de certification sanitaire des semences. Dans le cadre de ma thèse, j'ai développé un protocole qPCR pour détecter *U. nuda* dans les semences d'orge, qui peut servir de test sanitaire des semences. Comparée au test sanitaire conventionnel des semences d'orge basé

sur l'inspection visuelle des embryons, la méthode qPCR a montré une corrélation plus forte avec l'incidence observée de la maladie dans les champs. Les évaluations basées sur la qPCR ont permis une classification précise et exacte de l'infection des lots de semences testés comme étant supérieurs ou inférieurs au seuil de tolérance, qui correspond à 0.01% d'embryons infectés. J'ai établi un lien entre les mesures qPCR des lots de semences et la suivant développementde la maladie dans les champs afin de déterminer la signification biologique des résultats qPCR. J'ai lié les mesures qPCR des lots de semences et le suivant développement de la maladie dans les champs afin de déterminer la signification biologique des résultats qPCR. Bien que les méthodes de détection basées sur les semences et les tests de santé des semences soient adaptées à la détection des agents pathogènes transmis par les semences, ces tests ne tiennent pas compte de la présence potentielle d'inoculum transmis par le sol. Les agents pathogènes transmis à la fois par les semences et par le sol, comme *T. caries* et *T. controversa*, nécessitent des méthodes de détection qui tiennent compte des deux voies de transmission. J'ai développé un protocole basé sur la PCR pour détecter ces espèces de *Tilletia* sur les semences et dans le sol. Ce protocole peut être utilisé avec la dPCR et la qPCR, et utilise les primers ciblant les régions mitochondriales et nucléaires pour distinguer les deux espèces de carie. J'ai intégré un contrôle interne exogène afin d'améliorer la fiabilité de l'extraction de l'ADN du sol. Ces protocoles basés sur la PCR ont permis de détecter de faibles niveaux de *T. caries* dans des lots de semences (1-2 téliospores par échantillon de 250 graines) et de faibles niveaux de téliospores de *T. controversa* dans le sol (100 téliospores par gramme de sol).

Des traitements efficaces sont nécessaires lorsque les niveaux de pathogènes dépassent les seuils de tolérance afin de prévenir les épidémies. Cependant, il existe actuellement peu de traitements biologiques ou à faible risque pour contrôler *U. nuda*, *T. caries* et *T. controversa*. De plus, comme ces agents pathogènes ne provoquent des symptômes qu'à un stade avancé de la croissance des plantes, l'évaluation de nouveaux traitements — et par conséquent leur développement — s'inscrit dans le long terme. Pour accélérer l'évaluation de l'efficacité des traitements, j'ai adapté le protocole de qPCR utilisé pour détecter *U. nuda* dans les graines à une procédure de dépistage en laboratoire qui permettant de quantifier les copies de *U. nuda* dans l'ADN extrait de semis d'orge. La quantification simultanée de l'ADN de *U. nuda* et de l'orge dans les échantillons de semis permet de normaliser les niveaux de pathogènes par rapport au tissu hôte et de réduire la variabilité liée à l'échantillonnage. Sur la base d'expériences menées en champ et en laboratoire, j'ai constaté que les mesures qPCR obtenues à partir d'échantillons de semis issus de graines traitées cultivées pendant 10 ou 14 jours sur du papier filtre ou dans du terreau constituaient des prédicteurs utiles de l'efficacité du traitement en champ. Cette procédure de dépistage basée sur la qPCR permet de sélectionner de nouveaux traitements prometteurs à un stade précoce de la plante, réduisant ainsi le temps et l'espace nécessaires à l'évaluation de l'efficacité du traitement. En raison de la double voie de transmission de *T. caries* et *T. controversa*, des traitements efficaces contre les inoculums transmis par les semences et par le sol sont nécessaires. Pour améliorer l'efficacité du traitement contre *T. caries*, j'ai optimisé une méthode de dépistage basée sur la qPCR en adaptant le protocole PCR utilisé pour détecter les agents pathogènes du carie sur les semences et dans le sol. Cette méthode de dépistage a permis de distinguer correctement les plantes infectées des plantes non infectées en se basant sur le développement éventuel de grains cariés. Toutefois, l'estimation de

l'efficacité du traitement fournie avec la qPCR était moins fiable que les symptômes précoces de *T. caries* sur les feuilles. Une des limites est que l'ADN du pathogène n'a pas été normalisé par rapport à l'ADN du tissu hôte dans chaque échantillon, ce qui a pu réduire l'exactitude de l'évaluation de l'efficacité du traitement basée sur la qPCR.

Les méthodes de détection par PCR développées pour *T. caries* et *T. controversa* sur les semences, dans le sol et dans les plantes fournissent des outils supplémentaires pour gérer les agents pathogènes responsables de la carie transmise par les semences et le sol dans un contexte de réglementation plus restrictive concernant les pesticides synthétiques. Une optimisation supplémentaire de ces méthodes est nécessaire, notamment en ce qui concerne les stratégies d'échantillonnage, l'interprétation biologique des mesures avec PCR et les performances selon les types de sol. Cependant, ces approches permettent une prise de décision plus éclairée et fondée sur les risques dans le cadre de stratégies IPM. Les méthodes de développement présentées dans cette thèse peuvent contribuer à l'amélioration de la détection d'autres agents pathogènes végétaux. En fin de compte, les travaux de ma thèse renforcent les approches de détection moléculaire fondées sur des données acquises sur le champ, réduisant ainsi la dépendance aux applications prophylactiques de pesticides synthétiques et favorisant une production agricole plus durable.

Mots-clés: stratégies de protection des végétaux, évaluation du traitement des semences, tests de santé des semences, champignons pathogènes transmis par les semences et le sol, PCR quantitative et digital, *Tilletia caries*, *Tilletia controversa*, *Ustilago nuda*, blé, orge.

Contents

Introduction

Policies on synthetic pesticides and their consequences

Agricultural policies regarding plant production products (PPPs) aim to balance food security with protecting human health and environmental sustainability [1]. In the European Union (EU), this balance is achieved through Directive 2009/128/EC [1]. Its adoption establishes a common framework to reduce the use of synthetic PPPs and the associated risks and impacts on human health and the environment, while promoting sustainable crop protection strategies [1, 2]. This directive recommends implementing Integrated Pest Management (IPM) strategies, prioritizing alternative solutions to synthetic PPPs and restricting their use only in situations where pest or pathogen populations exceed defined tolerance thresholds [1].

Recently, diverse strategies related to food systems and biodiversity, including the European Green Deal [3], EU Biodiversity Strategy for 2030 [4], and the Farm to Fork policy [5], have set quantitative synthetic PPPs reduction goals. An example of these goals is the 50% reduction in synthetic PPPs use and their associated risk by 2030. These policy targets are expected to promote increased use of low-risk and alternative PPPs and to support the conversion of at least 25% of agricultural land to organic farming [4, 5]. The EU Harmonized Risk Indicator 1 — the metric for quantities of synthetic PPPs weighted by risk that are placed on the market [6] — showed a yearly reduction and decreased 61% between 2011 and 2023 [7, 8]. However, this reduction in risk and use of synthetic PPPs contrasts with the number of emergency authorizations granted for PPPs that are not otherwise approved for use [1]. The emergency authorization requests fluctuated — greatly increased and then decreasing — between 2011 and 2023 based on the EU Harmonized Risk Indicator 2 [7]. This metric is based on the number of authorizations granted [6], but weighted by hazard. The discrepancy between the consistent reduction of risk associated with synthetic PPPs and the high fluctuation in emergency authorization requests for unapproved PPPs may be due to increased pressure from plant pathogens and the unavailability of effective, low-risk PPP alternatives. Together, the restriction in PPPs and absence of effective low-risk products can contribute to crop yield losses. The Farm-to-Fork and EU Biodiversity strategies for 2030, which combine limiting the synthetic pesticide application and risk with increasing the area of organic farms to 25%, could potentially impact crop yields in EU member states [9, 10].

In low-input agricultural systems, where the use of synthetic PPPs is already restricted, crop yield losses can range from 15% to 35% depending on the crop and country [11]. In principle, IPM guidelines should mitigate the yield losses and ensure that PPPs — synthetic or biological — are applied only when the level of a harmful organism exceeds a tolerance

threshold[1]. In practice, the transition towards a low-input agriculture while maintaining food security is limited due to the lack of low-risk PPPs or other alternatives control options to synthetic PPPs. The identification, testing, registration, and commercialization of PPPs, including low-risk or organic PPPs, requires considerable time [12, 13]. Consequently, regulatory restrictions may leave crops vulnerable to plant diseases or pests due to the lack of effective alternatives. Therefore, the prevention of pest and pathogen establishment during early stages of crop production is necessary. Improved tools are needed to assess the presence of pathogens in soil and seeds before the growing season begins in order to support timely and appropriate management decisions.

Exposure to synthetic PPPs can have adverse effects on humans and the environment [14, 15]. However, when the use of PPPs is restricted and economically viable crop production should be maintained, alternative strategies — cultural, biological, genetic, and diagnostic approaches — must take on a greater role in pest and disease management [16]. This transition from relying on synthetic PPPs to using more preventive crop protection strategies may lead to the re-emergence of pathogens that have been well controlled and additional crop losses, particularly if farmers lack sufficient support for implementing new crop management strategies [17, 18, 19]. These challenges have implications for production and security of food because global yield losses caused by pathogens and pests in the most relevant crops — wheat, maize, rice, potato, and soybean — are estimated to range from 9% to 74% [20]. The inclusion of additional preventative measures, such as early detection of plant pathogen in seeds and soil, can help mitigate these potential crop losses.

Methods to detect seedborne pathogens

A seed health test uses a representative sample to determine pathogen incidence within a seed lot. Seed health primarily refers to the presence or absence of pathogens associated with seeds, including fungi, bacteria, viruses, and animal pests (nematodes and insects) [21, 22].Because national and international seed trade can unintentionally spread seedborne pathogens over long distances, they can impact the dissemination of the pathogen and epidemiology of plant diseases [23]. Pathogens can be carried with seed as sclerotia (e.g., *Claviceps purpurea*), on the seed surface (e.g., *Microdochium nivale*), or within the seeds (e.g., *Ustilago tritici*), and under suitable environmental conditions can cause seedborne diseases [24]. Seed health tests should help prevent the introduction of pathogens into new regions and reduce seedborne inocula, which can lead to disease development in the field. Additionally, seed health tests can identify the causes of poor seedling germination or field establishment [22], which complements standard germination tests. These tests also inform decisions on whether seed lots require treatment to eradicate seedborne pathogens or to reduce the risk of disease transmission [21, 22].

In seed certification, the seed health tests may be used in combination with field inspections for seed production [19]. However, both field inspections and seed health tests have limitations in terms of speed, sensitivity, and reliability for pathogen detection. Field inspections may underestimate infection levels due to environmental conditions that can suppress or conceal symptom expression. Infected seeds are often asymptomatic and seed health tests may fail

to detect the pathogen. In addition, infected seeds can have low pathogen levels and uneven pathogen distributions within a seed lot [25]. As a result, neither seed health tests nor field inspections can ensure that a seed lot is completely free of seedborne pathogens because both methods depend on representative sampling rather than exhaustive testing [22, 26]. To achieve absolute certainty of no seedborne pathogens within a seed lot, every individual seed would require testing [21]. Because this exhaustive testing is not possible, accurate assessment of disease risk from seedborne pathogens depends on statistically valid sampling strategies and detection methods capable of reliably identifying pathogens at predefined thresholds [19, 21, 22].

In parallel to conventional seed health tests, non-destructive approaches have been explored as rapid screening tools for pathogen detection. Visual and non-destructive methods provide rapid methods to detect plant pathogens while preserving plant material viability. Techniques such as hyperspectral imaging, machine vision, near-infrared spectroscopy, and infrared thermography enable a rapid pathogen detection. However, their performance is often influenced by environmental conditions, and they generally have limited sensitivity or an insufficient ability to discriminate among pathogen species. Furthermore, these methods when applied to seeds are typically restricted to the detection of pathogens located on the seed surface [27, 28, 29, 30, 31]. X-ray imaging can overcome this limitation by detecting internal infections [32], but its effects on seed viability are unclear [33], and it requires costly equipment. Electronic nose technologies, which rely on pathogen-specific volatile organic compound signatures, are affected by environmental variability and limited sensor specificity [34].

Available seed health tests differ in term of sensitivity, reproducibility, expertise, and equipment required. The choice of the test depends on the target pathogen, the seed species, and the specific objectives (e.g., certification and pathogenicity test). The application of a seed health test and the interpretation of its results, therefore, require technical knowledge and expertise [22]. Most currently validated seed health tests rely on visual seed examinations or pathogen identification following seed incubation, washing, or grow-out procedures [22]. These tests are often labor-intensive and time-consuming, particularly when large sample sizes are required and tolerance thresholds are low (e.g. one infected seed per 1000) [19, 35]. Ideally, these tests should be sensitive, specific, rapid, robust, cost-effective, and simple to implement and interpret [25]. Relatively few validated seed health tests meet these attributes. To address the low efficiency and reliability associated with many currently used seed health tests, the International Seed Testing Association (ISTA) has started revising traditional protocols and integrate molecular techniques, such as quantitative polymerase chain reaction (qPCR) for *Ditylenchus dipsaci* and *Fusarium oxysporum* forma *specialis lycopersici* [36, 37] and Enzyme-Linked Immunosorbent Assay (ELISA) for Pea early browning virus and pea seedborne mosaic virus [38]. Although most validated seed health tests are based on visual examinations, non-validated seed health tests have also been developed using molecular detection methods, such as ELISA, PCR, and Loop-mediated isothermal amplification (LAMP). Even though these immunological and molecular detection methods are promising, many of the previously developed protocols have short-comings, including inconsistent amplification, insufficient sensitivity, or a lack of biological relevance.

Field inspections for seedborne diseases

Field inspections of plants before harvest are a preventative measure that minimizes the risk of seed contamination or infection by seedborne pathogens transmitted from the parent crop, such as *Tilletia caries* and *Ustilago nuda* [19]. These inspections, which detect regulated seedborne pests and pathogens with visible symptoms, are conducted concurrently with the evaluations for other characteristics required for certification (e.g., variety purity and weed density) [39]. For an effective field inspection, the evaluated crop should be at a growth stage that shows the symptoms of relevant diseases. Furthermore, inspectors receive training to accurately identify diseased plants [39, 40]. To ensure the field inspection is statistically representative of the entire field, systematic field-walking schemes are employed [41]. These standardized schemes, such as a random or clockwise travel patterns, ensure maximum field coverage (60%-85%) and reduce the probability that localized disease occurrence remains undetected. The number of plants to inspect depends on the crop. For example, at least 100 plants should be inspected for maize, and a total of 2000 plants or ears should be inspected for wheat and rice [41]. Despite the importance of field inspections, they are unable to detect asymptomatic or latent infections. The subsequent generation of seeds may be infected with a seedborne pathogen without showing visible symptoms in the plant during seed development [42]. Consequently, field inspections should be complemented using visual seed inspections (seed health tests) to reliably detect and quantify seedborne pathogens [19].

Visual seed inspections for seedborne pathogens

A direct visual inspection of the seed surface is not considered a reliable detection method, even though some seedborne pathogens cause visible lesions or discolorations. Infected seeds often are asymptomatic or exhibit barely visible symptoms, particularly in seeds with dark seed coats [43]. Incubation methods are commonly used to detect the seedborne pathogens located on the seed surface that can grow under artificial conditions. These methods allow for the simultaneous assessment of pathogen presence and viability. Seeds are incubated in Petri dishes with culture media or placed on moist filter paper (grow-out method) and maintained under controlled environmental conditions to promote pathogen growth [43, 44, 45, 46]. After the incubation period, colonies can be identified based on macroscopic and microscopic characteristics [44, 46]. In the grow-out methods, the developed seedlings or cotyledons are individually inspected for symptoms [43].

The visual examination of a seed wash solution is used to detect some seedborne pathogens that are located on the seed surface and do not easily grow on artificial media or cause visible symptoms in seedlings[37, 47, 48]. This method involves washing the seeds in a liquid solution that separates spores or pests (i.e. nematodes) from the seed surface [37, 47, 48]. The resulting suspension can be filtered and inspected under a microscope [37, 47], or centrifuged to obtain a pellet that can be observed under a microscope [48]. For internally seedborne pathogens, such as *Ustilago nuda*, visual detection requires dissecting the seed to access the pathogen-infected tissues. The seed processing enables microscopic examination of the infected seed internal part [49]. Visual seed examination is time-consuming and requires technical expertise to accurately identify different seedborne pathogens and perform multiple protocols. Furthermore, species-

level discrimination is often difficult with these protocols. If greater restrictions of synthetic PPP applications are enforced, the number of seed samples evaluated for health will increase, requiring more rapid, large-scale testing [50].

Immunological and molecular detection of seedborne pathogens

Immunological and molecular detection methods enable more samples to be processed within a shorter time frame than visual inspections [50]. The detection of seedborne pathogens with immunological and molecular methods — ELISA, PCR, and LAMP — compared to the visual seed examination have increased scalability, sensitivity, and analytical precision [51, 52]. Among molecular approaches, ELISA has been widely applied for the detection of seedborne pathogens [53, 54], particularly viruses [38]. ELISA relies on polyclonal or monoclonal antibodies produced in response to specific antigens of plant pathogens [55, 56]. The antigen-antibody binding is then detected through an enzyme-mediated reaction coupled to a chromogenic or fluorescent signal. A major limitation of ELISA is the antibody production, which typically involves animal immunization and spleen extraction [56]. Additionally, ELISA generally has moderate sensitivity and specificity, whereas they are high to very high in PCR-based assays [25].

PCR-based assays detect seedborne pathogens through the amplification of specific DNA sequences using short oligonucleotide primers designed to anneal the target regions of the seedborne pathogen genome. Quantitative PCR (qPCR) enables the absolute quantification of the target using calibration curves generated from known target concentrations [57]. The infection level of a seed lot can be estimated with pathogen quantification using qPCR and then compared with tolerance thresholds defined for seed certification. QPCR has been widely adopted in research for the detection and quantification of numerous seedborne pathogens in different host seeds, such as wheat [50, 58], barley [59, 60, 61, 62], spinach [63, 64] and in soybean [65].

Recently, digital PCR (dPCR) and droplet digital PCR (ddPCR) were developed as alternative PCR-based assays. Both dPCR and ddPCR rely on the partitioning of samples into thousands of individual reactions, in which single nucleic acid molecules are amplified to endpoint [66]. These PCR-based assays do not require calibration curves because Poisson statistics is used to quantify the absolute target based on the proportion of positive partitions [67]. Researchers have reported that ddPCR produces more robust results, improved tolerance to inhibitors, and lower variability among replicates than qPCR and dPCR at low target concentrations [68]. However, dPCR and ddPCR are currently more expensive than qPCR, which limits their routine application in seed health testing.

A limitation for implementing PCR-based assays in seed health test is the lack of a standardized, rigorous methodology to design experiments, particularly for newly developed dPCR and ddPCR protocols. Although the Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines have been established for qPCR [69], their implementation remains inconsistent across studies [66]. A common issue in qPCR pathogen detection methods is the amplification of excessively long target fragments, which can compromise efficiency, sensitivity, and reproducibility [70, 71, 72]. Many immunological and molecular detection protocols have been developed for the detection of seedborne pathogens, such as fungi (e.g., *Plasmopara halstedii* [56], *Verticillium dahliae* [63, 64], *Cladosporium variabile*,

Peronospora farinosa f. sp. *spinaciae*, *Stemphylium botryosum* [63], *Colletotrichum truncatum*, *Corynespora cassiicola*, and *Sclerotinia sclerotiorum* [65], *Fusarium oxysporum* f.sp. *lycopersici* [36]), nematodes (e.g., *Ditylenchus dipsaci* [37]), bacteria (e.g., *Erwinia carotovora* ssp. *carotovora* [73]) and viruses (e.g., pea seedborne mosaic virus [38]). However, few of these protocols were developed for practical implementation and application for seed health testing [36, 37, 38]. Statistically appropriate sample sizes and practicality on a large scale are often overlooked. Furthermore, immunological and molecular results may lack direct biological interpretation, which is a limitation that also affects some of the current non-molecular seed health testing protocols used [19, 74]. Some seedborne pathogens, such as *T. caries*, are difficult or impossible to culture *in vitro* or occur at very low infection levels. These biological characteristics, combined with symptom development at mature stages of plant growth [Biologische Bundesanstalt, Bundessortenamt und CHemical Industry (BBCH) 51-92], complicate linking immunological and molecular results to actual disease occurrence. Thus, the biological relevance of seed health tests can be difficult to interpret, which pose additional challenges to integrate immunological and molecular detection methods into seed certification.

Currently, among the validated seed health tests provided by the International Seed Testing Association (ISTA), only the ELISA method is used to directly detect seedborne viruses in seed samples [38], while PCR is used in conjunction with visual examination procedures to confirm the detected species [37, 36]. However, some seedborne pathogens can also persist and spread through the soil, such as *Helminthosporium solani* [75], *Tilletia horrida* [76], and *Fusarium oxysporum* [77]. Therefore, even if they are not detected in inspected seeds, the potential presence of these pathogens in the soil poses additional challenges.

Methods to detect soilborne plant pathogens

Soilborne plant pathogens can persist in soil for periods ranging from weeks to several years, depending on their biology [78]. These pathogens have specialized survival structures, such as resistant spores or melanized hyphae that enable them to survive in the soil without a host for extended periods [79]. The physicochemical and biological properties of the soil influence these pathogens. Soilborne diseases may remain asymptomatic during the early stages of infection and are highly dependent on environmental stressors or may exhibit long latent periods that delay the detection [80].

Factors that influence the reliability of pathogen detection methods

The detection and identification of soilborne pathogens encounter unique challenges due to the soil environment. In agricultural soils, these pathogens are often unevenly distributed with patchy or "mosaic" spatial patterns rather than uniform distributions [81]. Therefore, the number of samples to collect and the extent of sampling within a field are important for successful detection and reliable data [82]. These pathogen populations can also fluctuate seasonally or in response to short-term environmental events, such as rainfall or irrigation, which further complicates achieving an accurate assessment of pathogen abundance. Additionally, soils are diverse in terms of physical, chemical, and microbial characteristics within and among different soil types. Within soils, heterogeneous matrices offer ecological niches for soilborne

pathogens [83]. The uneven distribution and differential survival of these pathogens in the soil makes their detection in soil more variable and uncertain [78].

Conventional methods to detect soilborne pathogens

Conventional methods to detect soilborne fungal pathogens include baiting techniques and the use of selective culture media, which are relatively inexpensive and do not require advanced technical expertise [84, 85]. Sucrose-centrifugation methods applied to soil samples have been used to detect dormant teliospores of bunt fungi [86]. Despite their usefulness, these conventional methods are generally slow, labor-intensive, and require a high level of expertise for accurate pathogen identification [78]. These method are poorly adapted to process large numbers of samples or provide rapid detection, and they may not detect pathogens that are either non-culturable or present at low abundances.

Immunological and molecular detection methods for soilborne pathogens

Molecular methods detect soilborne plant pathogens more reliably and precisely than conventional culture-based methods [87]. However, accurately detecting and quantifying pathogens in soil remains challenging due to the complexity of soil matrices, which can interfere with target recovery, detection chemistry, and signal interpretation [78, 88, 89, 90, 91, 92, 93, 94, 95, 96]. These challenges increase when large soil volumes are processed. Although a larger sample size may theoretically contain more target DNA (i.e., soilborne pathogen DNA), it also introduces co-extracted inhibitors, such as humic substances. These inhibitors can exceed the enzymatic tolerance limit of molecular assays and reduce the amplification efficiency [97], which can lead to an underestimation of pathogen quantities [96].

Immunological approaches, such as ELISA, are used to detect pathogens in soil [98, 99], but the antigens used in these approaches can bind to soil particles — especially clay — and cause nonspecific absorbance [78, 88, 89, 90]. In contrast, PCR-based assays can be more specific and sensitive to detect pathogens in soil [78], and portable qPCR methods have been adapted for the detection of soilborne pathogens in *situ* [79]. However, co-extracted soil compounds can still inhibit qPCR amplification and reduce the signal detection [75, 91, 92, 93, 94, 95]. Compared to qPCR, ddPCR is more tolerant of inhibitors [100]. However, ddPCR can underestimate spore densities at high infestation levels and performs optimally with low target DNA concentrations [96]. LAMP assays are more reliable in the presence of soil-derived inhibitors [101, 102] and require less technical expertise and sophisticated instrumentation than PCR-based methods [103], but they can have issues of false positives [104]. Field-based LAMP assays typically provide qualitative presence-absence results [105], while quantitative LAMP (qLAMP) assays can determine pathogen amount. However, qLAMP requires similar levels of infrastructure as qPCR and often do not have the same level of accuracy at low pathogen concentrations [96].

Life cycles of bunt and smut pathogens

The effectiveness of detecting soil and seedborne pathogens, however, regardless of methodology chosen depends on their biological characteristics, such as survival structures, dispersal

mechanisms, and longevity in soil. Therefore, the development of detection and control strategies results requires an understanding of the plant pathogen life cycles and epidemiology. In cereal production, especially in wheat production, synthetic PPP reductions have been estimated to contribute to annual yield losses of up to 28.1% because of increased pathogens [106]. In wheat and barley, the absence of seed dressings facilitates the resurgence of seed- and soil-borne diseases, which had not been problematic after the widespread adoption of synthetic pesticides [107, 108]. Among these pathogens, smut and bunt diseases — *Ustilago nuda*, *Tilletia caries*, and *Tilletia controversa* — are some of the most economically relevant and historically destructive diseases that affect cereals [107, 109, 110]. Indeed, in regions with low wheat productivity where seed treatments are not routinely applied, these diseases cause most of the yield loss [20].

The increasing incidence of common bunt is a problem in low-input and organic farming systems [18], causing crop losses up to 80% [111] and compromising seed quality for subsequent sowing and consumption [19, 112, 113]. The life cycles of bunt and smut pathogens directly influence the strategies used to detect and control them in seeds and soil. These globally distributed fungi belong to the phyla Basidomycota and subphylum Ustilaginomycotina, and their infection cycles are closely linked to crop development and environmental conditions. Detecting these pathogens requires sampling strategies that target different substrates — plant tissue, seeds, and soil — and account for the development stages of the pathogens and the crops. Therefore, to develop reliable detection methods and effective control strategies, it is necessary to understand the life cycles of *Tilletia caries* (Figure 1), *Tilletia controversa*, and *Ustilago nuda* (Figure 2).

Tilletia caries

Tilletia caries (de Candolle) Tulasne & C. Tulasne [syn. *Tilletia tritici* (Bjerkander) G. Winter, *Tilletia foetida* J. G. Kühn, *Tilletia leavis* (Wallr.) Liro], the causal agent of common bunt of wheat, replaces the contents of developing seeds with teliospores, which causes direct yield and quality losses [111, 114, 115]. During harvest, teliospores contaminate healthy seed, which are then unsuitable for sowing. In addition, the *T. caries* trimethylamine odor compromises the marketability and suitability of seeds for consumption [111, 114, 115]; for this reason, the disease is also known as stinking smut of wheat. Teliospores undergo a dormancy period and serve as the primary source of inoculum in subsequent cropping seasons. The teliospores can germinate across a temperature range from 5 to 20 °C in the laboratory [116], but field studies indicate that infection is favored by temperatures around 6–7 °C during seedling germination [117]. Fungal infections in the greenhouse occur between 3 and 15 °C [118]. Because these temperature conditions frequently coincide with the germination period of both winter and spring wheat, advanced or postponed sowing time can be insufficient to avoid infection. Within three days after sowing, seed- or soilborne teliospores germinate rapidly and form a promycelium that produces primary sporidia, which undergo fusion through characteristic "H-shaped" conjugation structures [119, 120]. The fused sporidia infect wheat seedlings with a pathogenic hypha at the coleoptile stage (BBCH 7)[120, 121]. Initially, the hyphae of *T. caries* diffusely colonize most of the seedling tissues before concentrating in the stem tissues near the apical meristem [122]. As the plant grows, the fungus spreads systemically and colonizes the

leaves and culm tissues [123]. The leaf infections can produce early symptoms, such as chlorotic flecking on leaf blades [123]; however, these symptom may depend on the wheat cultivar and pathogen strain [124]. Similar symptoms in the field can be caused by abiotic stress or other diseases. Consequently, early leaf symptoms are unreliable for field diagnosis.

During seed development, *T. caries* replaces the developing seed tissues with teliospores, from the pericarp to the embryo [118]. This replacement may be complete and produce the typical dark bunt balls, or be partial and develop partially infected seed [118]. These partially bunted seeds are particularly difficult to detect visually in the field because the typical symptoms, such as shortened ears and spread spikelets, may be absent [113, 118]. This variability in wheat symptom development complicates field monitoring and increases the risk of the disease spreading unnoticed. At harvest, teliospores are dispersed from broken bunted kernels, which contaminates harvested seed and soil. Although contaminated seeds are the primary source of inoculum [113], *T. caries* can persist in soil for up to ten years under laboratory conditions [125]. In field conditions, teliospores buried below the plow layer can remain viable for at least five years under organic management systems [126]. In addition to soilborne inoculum, teliospores of *T. caries* can survive the passage through the digestive tracts of several animals, including livestock whose manure contributes to dissemination [125, 127]. Although teliospore viability decreases after ingestion by collembola [128], animal-mediated dispersal poses an additional challenge for disease containment in agricultural systems.

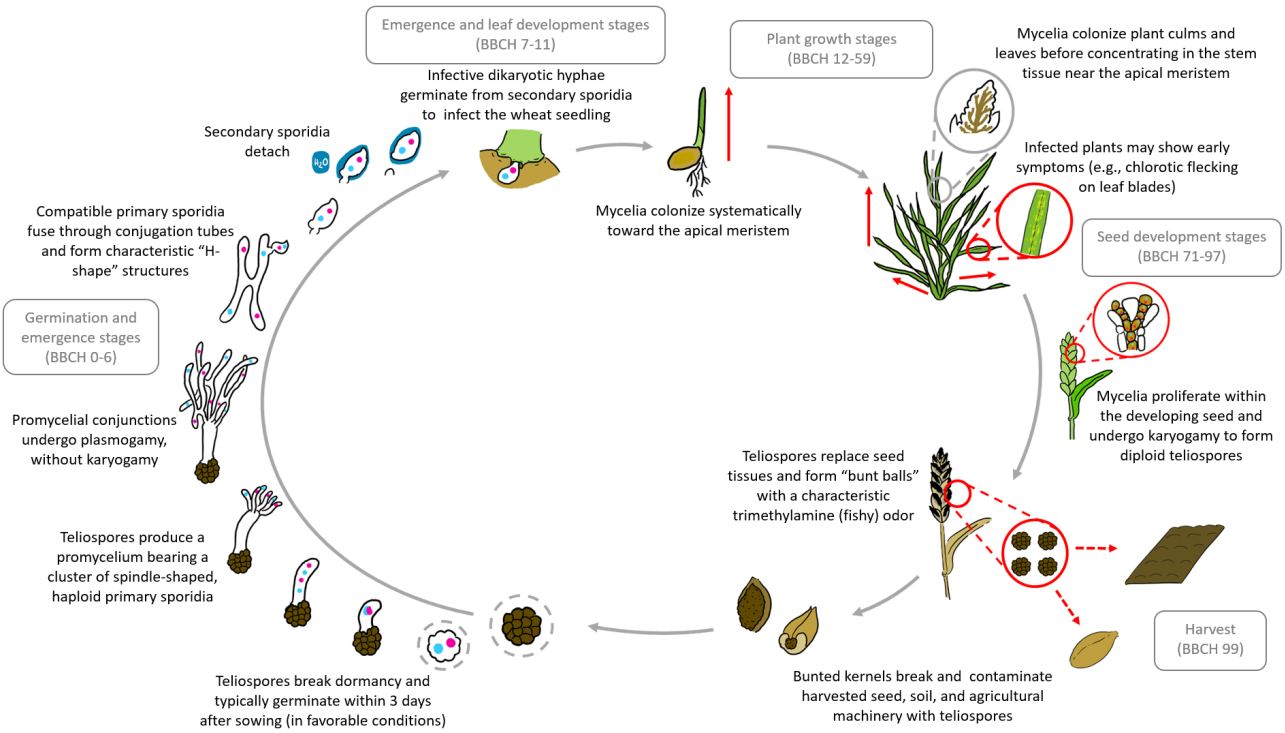

Figure 1: Scheme of *Tilletia caries* life cycle. Drawing: Cecilia Panzetti

Tilletia controversa

The causal agent of dwarf bunt in winter wheat is *Tilletia controversa* J. G. Kühn [syn. *Tilletia brevifaciens* G. Fischer and *Tilletia nanifica* (Wagner) Savulescu], this fungus causes symptoms similar to *T. caries* by replacing the contents of seeds with teliospores and causing

direct crop losses [107, 121, 129, 130]. The seed coat, which is filled of bunted teliospores, usually breaks apart during harvest. The released teliospores contaminate the harvested seed, the soil, and the agricultural machinery. Although infected seeds can act as vehicles to spread the pathogens to new areas and fields [121, 131, 132], the soilborne inoculum is considered to cause more plant infections and disease outbreaks than seedborne teliospores [131]. *T. controversa* teliospores can remain viable in soil for up to ten years [121, 127, 133]. This long-term persistence poses a significant challenge for dwarf bunt control: contaminated fields can remain infectious across multiple cropping seasons. While routine seed health tests allow for a relatively simple detection of teliospores on seeds, there are no routine methods for detecting teliospore density in soil. Due to the difficulty in detecting *T. controversa* teliospores in soils, accurate risk assessments of soilborne inoculum before sowing are unfeasible to obtain for individual fields.

T. controversa teliospore germination is slow, and environmental conditions affect its speed and infection success. The germination occurs over a period of one to three months and is favored by persistent snow cover combined with relatively mild winter temperatures, generally between 1 and 10 °C [130]. The snow cover prevents soil freezing and maintains a cool, moist environment with limited light penetration: conditions that promote teliospore germination [130]. These conditions can be reproduced in winters with cool, wet, and cloudy weather [121]. The specific environmental requirements partly explain the geographically restricted distribution of dwarf bunt. According to the European and Mediterranean Plant Protection Organization (EPPO) Global Database, *T. controversa* is widespread in Switzerland and the Czech Republic, but it is reported only sporadically or with a restricted distribution in many other countries (e.g., seventeen other countries in Europe, three countries in North Africa, eleven Asia, twelve states in the United States, and two provinces in Canada) [134]. The favorable winter weather conditions for *T. controversa* teliospore germination also impact disease occurrence and frequency between growing seasons.

During germination, the teliospore produces a promycelium that forms at its apex a cluster of spindle-shaped primary sporidia [130]. Pairs of compatible sporidia typically fuse through a conjugation tube while still attached to the promycelium and go through plasmogamy, without karyogamy. The fused sporidia detach and germinate as binucleate hypha capable to infect wheat seedlings [121, 130]. Infection usually occurs when the seedlings are between the one- and three-leaf stages (BBCH 10-13). In the compatible sporidia absence, the promycelium produces mononucleate, nonpathogenic hyphae that do not contribute to disease development. The teliospore germination does not occur synchronously, which complicates the control of dwarf bunt. Instead, *T. controversa* shows a consistent, low level of germination from December to April in the boreal zone in the Northern hemisphere [130]. This extended germination period increases the time during which the fungus can infect seedlings. Once infection is established, binucleate hyphae slowly grow toward the apical meristem within host tissues [129]. During vegetative growth, the hyphae colonize the internodes of the culm and the leaves that grow from infected internodes (BBCH 31-33) [129]. At the wheat boot stage (BBCH 41-49), *T. controversa* replaces the contents of developing seeds with teliospores enclosed within the seed coat, which produce the bunt [130]. The bunted ears are the only clear symptom of infections with this pathogen. This symptom appears during ear maturation (BBCH 71-89), and no

curative intervention is possible.

Infected plants can show earlier symptoms, but they are inconsistent. In the tiller phase (BBCH 21-29), infected plants often produce about twice as many tillers as healthy plants. Infected plants also show reduced height, a characteristic reflected in the common name "dwarf bunt" [130]. Unlike to common bunt (*T. caries*), early leaf symptoms such as mottling or flecking occur irregularly [130]. *T. controversa* bunt balls are generally larger and more spherical than *T. caries* bunts, and infected ears often produce a higher number of infected florets. The larger size of *T. controversa* bunt balls causes greater opening of the lemma and palea, which may facilitate the local dispersal of teliospores within the same field.

T. controversa, similarly to *T. caries*, can remain viable after passing through the digestive tracts of several animals — earthworms, grasshoppers, cattle, and chickens — that can disseminate teliospores via animal-derived fertilizers [127, 135]. This additional dispersal pathway increases the risk of *T. controversa* spread outside of infected fields or increase the inoculum in already infected fields. Furthermore, *T. controversa* can potentially infect multiple grass species within the Poaceae family [136]. These alternative hosts to wheat can act as inoculum reservoirs during the crop rotations, which maintain the pathogen populations in the absence of wheat and thereby reducing the effectiveness of crop rotation as a disease control strategy.

Ustilago nuda

Ustilago nuda (Jensen) Rostrup, the causal agent of loose smut of barley, is a strictly seed-borne pathogen that remains as dormant mycelium within the embryo of infected seeds until germination. Unlike bunt diseases, infected seeds and seedlings show no visible signs of infection — no teliospores on surfaces or early leaf symptoms. The loose smut symptoms are visible only at the ear emergence and flowering stages (BBCH 51-69) when *U. nuda* replaces the entire inflorescence with masses of dark teliospores, which causes a direct yield losses up to 25% [137]

During seed germination (BBCH 0-9), the mycelium grows and colonizes the seedling's apical meristem and ear primordia [60, 138]. Although the seedling apex initially appears free of fungal structures, hyphae actively invade the apical meristem and leaf primordia within the first days after germination [60]. The pathogen may infect the meristem and leaf tissue even before visible germination [139]. Because the fungal colonization occurs within the seedling and exhibits no external symptoms, infected plants cannot be identified during their growth. The fungal hyphae are passively carried upward with the stem elongation during the plant development (BBCH 9-39). The mycelium is also in stem nodes and can infect the tillers that develop from the main culm, causing multiple smutted ears per plant and increase the inoculum production for the next seed generation [60, 140].

In the ear development (BBCH 41-49), *U. nuda* mycelium grows within the developing inflorescence, even in the ear primordium. Tangled hyphae occupy large regions of ear primordia, which later are more concentrated within florets [138]. While developing, *U. nuda* destroys the inflorescence tissues and replaces them with hyphae that subsequently segment to form teliospores. At ear emergence (BBCH 50-69), all floral structures — except the rachis — are replaced with teliospores [138], which cause smutted ears. Environmental factors such as wind, insect, and rain promote the release and dispersion of teliospores [141]. Wind and rain enhance the spread of teliospores to healthy plants and can remove the smutted ears so that only the

rachises are visible. The reduction of visible smutted symptoms limits the detectability of infected ears during flowering. The infection of the next generation's seed occurs only during the flowering stage (BBCH 61-69). The teliospores reach open flowers, germinate on the ovary wall surface, and penetrate the ovary tissues with a dikaryotic infectious hyphae without cause visible tissue damage [139]. The hyphae grow intercellularly within the ovary and colonize the scutellum and embryo [139]. The infected seeds remain externally indistinguishable from healthy ones, and differentiation is only possible after embryo excision [142]. However, the detection of an infected embryo only identifies the presence of the disease and not its severity in the subsequent flowering season. Multiple teliospores can infect a single flower, resulting in an infected seed with several independent hyphae [139], which can increase disease severity.

The temperature and moisture levels in the soil and air can influence the development of *U. nuda* in seedlings and its infection efficiency during the flowering stage. The *U. nuda* mycelium grows within seedlings in a temperature range from 10 to 25 °C, and higher infection levels seem to occur at warmer temperatures during the seedling germination [143]. During this seedling stage, soil moisture can affect disease development: drier soils favor mycelial growth compared to excessively moist conditions [143]. These environmental conditions often coincide with the sowing periods of both winter and spring barley. During the flowering stage, cool and humid weather can prolong the flower opening and extend the infection period.

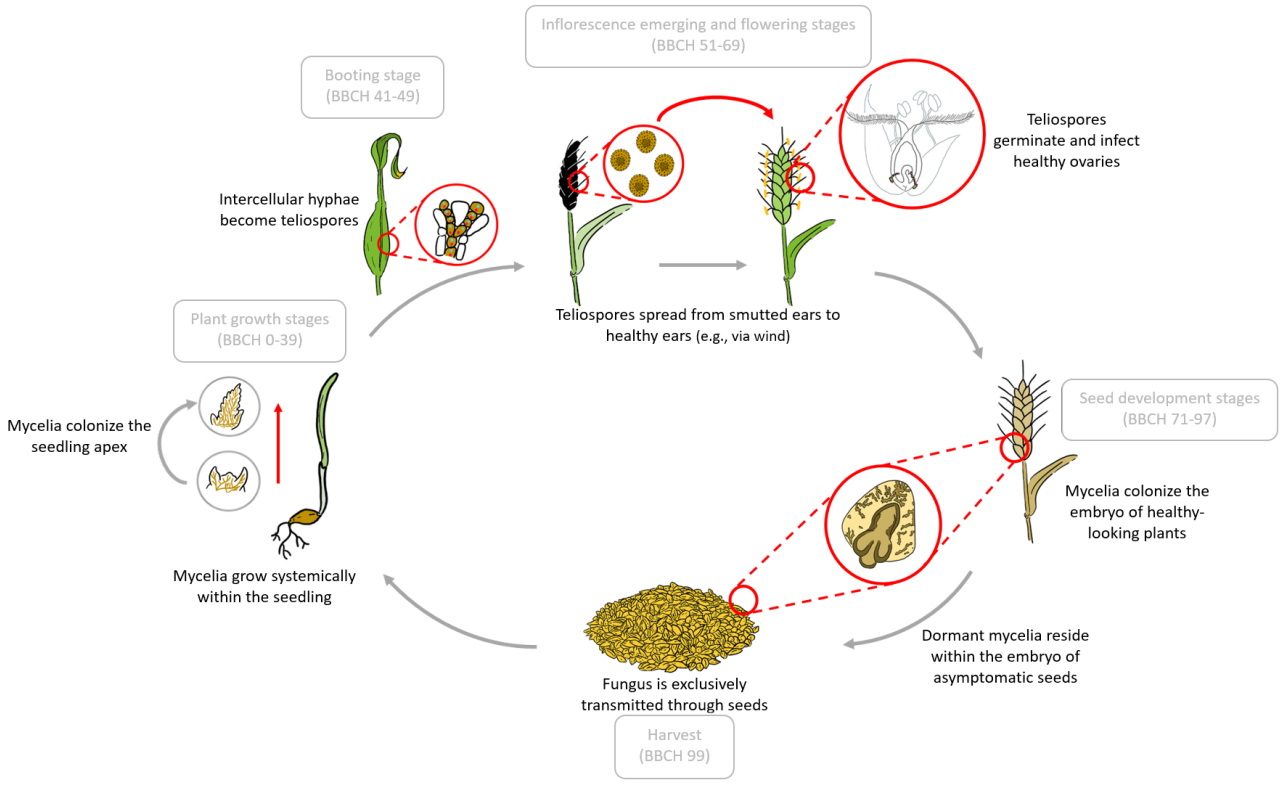

Figure 2: Scheme of *Ustilago nuda* life cycle. Drawing: Cecilia Panzetti

Current management strategies of fungi causing bunt and smut

Current management strategies for *Tilletia caries*, *Tilletia controversa*, and *Ustilago nuda* should follow integrated pest management (IPM) principles to maintain pathogen populations below economically damaging levels, while minimizing risks to human health and the environ-

ment [144, 145]. According to IPM guidelines, plant protection products (PPPs) should be applied only when pathogen levels exceed established tolerance thresholds [144, 145]. Consequently, the use of untreated seed should be prioritized whenever possible. Preventive strategies form the foundation of these bunt and smut disease management, particularly through seed health certification procedures to avoid the introduction of these pathogens [146]. Certified healthy seeds with a low presence or absence of *T. caries*, *T. controversa*, or *U. nuda*, resistant cultivars, and appropriate crop rotation, can reduce primary inoculum and limit disease establishment in the field [18, 144]. However, when these preventive measures fail and pathogen levels exceed economic thresholds, PPPs may be required to suppress pathogen development. When the application of PPPs is necessary, organic and low-input treatments should be prioritized, and synthetic plant protection products should be used only as a last option [144, 145]. Nevertheless, several synthetic pesticides that were previously effective against *T. caries*, *T. controversa*, and *U. nuda* are being progressively ban or restricted (e.g. Ipconazole [147], Pentachloronitrobenzene [148]) or are showing reduced efficacy, such as Carboxin based fungicide [137].

Resistant cultivars

Agronomic practices form the basis of IPM strategies and include crop rotation, crop management (e.g., altering the sowing date), and the use of resistant cultivars [144]. However, crop rotation provides limited control of *Tilletia caries*, *Tilletia controversa*, and *Ustilago nuda*. Because *U. nuda* is strictly seedborne, its transmission is independent of the previously grown crop. Crop rotation also is not effective for *T. caries* and *T. controversa* because their teliospores can survive in soil for three to ten years [126, 149]. Adjustments in sowing date or variety (e.g, winter or spring cultivar) may also be ineffective or pose agronomic risks. Delayed planting of winter cultivars can increase the risk of winterkill [150], and substituting winter cultivars with spring cultivars only solves the problem for *T. controversa*, but not for *T. caries* and *U. nuda* because spring cultivars can be susceptible [151, 152].

Consequently, the development of resistant cultivars is the most reliable agronomic measure for managing these pathogens. Cultivars that develop 0-10% diseased ears are generally considered resistant [153]. In wheat, resistance to both *T. caries* and *T. controversa* is mainly conferred by the same set of 17 bunt or (Bt) resistance genes [113, 149]. Due to the relative simplicity of developing *T. caries* infections compared to *T. controversa*, breeders have focused more on testing common bunt instead dwarf bunt [149]. A recent study of 46 winter wheat cultivars in three locations over two growing seasons observed that resistance to common bunt remains limited. These cultivars' resistance was tested against 128 *T. caries* populations, and only six cultivars maintained effective resistance [113]. The variability found in resistance against different *T. caries* races reflects the well-documented differences in virulence among bunt populations across regions [149].

For *U. nuda*, eight major (Un) resistance genes have been described. However, some of these resistance genes (e.g., gene Un8 [59]) can negatively affect seed germination [59, 152] or quantity and quality of the yield [154]. Recently, breeders developed three winter two-row barley cultivars that combine loose smut resistance with high yield and grain quality [154]. Despite the advances in breeding resistant wheat and barley cultivars to these bunt

and smut diseases, pathogen evolution limits the durability of host resistance. Therefore, resistant cultivars alone cannot ensure stable pathogen control. Complementary measures to the deployments of resistant cultivars remain essential within an IPM strategy, but the use of certified seeds is central to preventing pathogen dispersal and spread.

Untreated seed and certification process

Low-input or organic farms prioritize the use of untreated seeds [155]. However, common bunt, dwarf bunt, and loose smut can remain asymptomatic in seeds. To prevent infection of these smut and bunt pathogens, the producers should sow certified seed, which should ensure that yield losses are limited. Many seed certification standards are harmonized at the EU level, especially for varietal identity, weed contamination, and minimum germination capacity [156]. However, seed health requirements for seedborne pathogens are not harmonized among EU countries [19].

The requirements for seed certification and acceptable tolerance thresholds for pathogens in seed health testing or field inspections depend primarily on three factors: the seed category, the pathogen of concern, and national regulations [19]. Seed health tests or field inspections are generally conducted for pre-basic, basic, certified, and organic seed categories [19, 156]. Pre-basic seeds are produced before the basic seed stage and are usually inspected according to the same criteria as basic seeds [19, 156]. Basic seeds serve as the source for certified seed production, while certified seeds are primarily used for commercial crop production [19, 156]. Organic seeds differ from conventional certified seeds because they are produced under an organic farming system. Pre-basic, basic, and organic seeds are subject to more stringent health requirements than certified seed because they are used for propagation or cannot be treated. For example, Switzerland's tolerance threshold for *U. nuda* in basic seeds is up to two diseased ears per 100 m^2, whereas certified seeds allows up to five smutted ears within the same area [19].

The seed health assessment for *U. nuda*, *T. caries*, and *T. controversa* during seed certification relies on field inspections conducted at specific crop growth stages to score diseased ears within a defined area (e.g., 100 m^2). This certification may also incorporate direct seed testing to detect teliospores or internal mycelia [19, 39]. For *T. caries* and *T. controversa*, seed testing generally involves washing a working sample of the seed lot (e.g., 250 seeds), filtering the wash suspension, and microscopically counting the teliospores retained on the filter [47]. The results are expressed as teliospores per seed [19, 74]. The certified seed tolerance thresholds for contamination with these *Tilletia* spp. vary by country and seed category. In Europe, the certified seed tolerance thresholds range from zero teliospores per seed in Sweden to 300 teliospores per seed in Austria [19]. However, in Austria, seed lots exceeding ten telisopores per seed in the tested samples must be treated [19]. These two *Tilletia* species are not always distinguished during the certification process [19].Some countries (e.g., China) regulate *T. controversa* as a quarantine pathogen, so they have zero tolerance for its presence [134]. Seed health testing for *U. nuda* is based on the visual analysis of extracted embryos [49], for which at least 1000 embryos are individually examined under a microscope. The embryos that contain mycelium within them are counted as infected, regardless the amount of mycelial colonization. National tolerance thresholds for *U. nuda* in seeds vary among countries, seed categories and

use. For example, Denmark requires zero infected embryos for certified seed that will be used as untreated in conventional farming, whereas United Kingdom allows up to 0.5%. Austria has the highest tolerance threshold (2% of infected embryos), but seed treatment is mandatory if more than 0.1% of the embryos are infected [19]. Seed health assessments for seed certification that rely exclusively on field inspections usually do not test harvested seeds for infection from the neighboring fields [19]. In many cases, seed lots that fail the seed health test can still be marketed after seed treatment.

The development of new bunt and smut management strategies requires reliable and efficient seed health testing. Molecular methods are often cited for their inability to distinguish between viable and non-viable pathogens or virulent and avirulent pathogen strains [25, 157]. While some traditional seed health tests can simultaneously test for pathogen presence and viability through promoting the pathogen to grow, such as the test for snow mold (*Microdochium nivale* and *Microdochium majus*)[44], other currently used seed health tests only inspect for pathogen presence [22]. Therefore, similar limitations apply to seed health tests that only rely on visual detection rather than pathogen growth. For example, seed washing and filtration methods used to detect *Tilletia* spp. [47], as well as the embryo excision test for *U. nuda* [49], indicate the pathogen presence rather than its viability or virulence. Therefore, for some pathogens, molecular detection methods and visual seed inspection have comparable constraints regarding the interpretation of pathogen viability and pathogenicity. Molecular methods can also bring advantages, including an increase in potential throughput and specificity. For both molecular detection methods and visual seed inspection, the biological relevance of their results (e.g. disease development) is needed to understand the implications of the test results.

Organic and low-input plant production products

Organic plant protection products, or biopesticides, include naturally derived active compounds such as botanicals, microbial agents, semiochemicals, and plant-incorporated protectants [158]. These products may enhance plant defense by inducing systemic resistance —for example, certain *Bacillus* spp. can trigger plant responses against a range of pathogens and pests [159]. However, the systemic control of loose smut and common and dwarf bunt with these types of PPPs has not yet been showed.

The management of these smut and bunt diseases is challenging in organic and low-input cereal production systems because there are limited options for effective, non-synthetic seed treatments, especially for *Ustilago nuda*. Currently, there are no biological seed coatings on the market that reach the internal mycelium of *U. nuda* and prevent its growth during seedling development. The only effective non-synthetic treatment against *U. nuda* is warm water [160, 161]. In this treatment, the seeds are soaked for approximately two hours in preheated water at 45 °C [161]. Water penetrates the seeds and the heat reaches the embryo, while the prolonged exposure of the seed to the heat suppresses mycelial growth without damaging seed viability. The water uniformly distributes the heat within the seed lot, which reduces the risk of localized thermal damage. Despite the efficacy of a warm water treatment, it is not economically or logistically feasible to apply to seeds on a large scale. The seeds have to be dried at $\sim$ 37 °C for about 12 hours for large scale seed distribution, which requires substantial energy input and specialized infrastructure.

The control of seed-transmitted *Tilletia caries* and *Tilletia controversa* has historically been easier than preventing disease from soilborne inoculum. In fact, contact fungicides, such as copper [162], that are effective against seedborne inoculum were developed in the 1800s. One and a half centuries later synthetic products were developed that either work systematically or sufficiently persist on the seed to work against soilborne inoculum [107, 162]. Currently available physical, mechanical, and biological treatments can reduce the incidence of bunted ears from seed sources, though they do not completely eliminate infection or contamination. Commercially available physical treatments include steam [163] and electron treatments [164], while seed brushing is a mechanical approach [19]. A mustard-based product marketed as Tillecur is available for seed coating and has demonstrated high efficacy against *T. caries* [74]. In field trials, this treatment had an efficacy between 98.4% to 100% depending on the winter wheat cultivars (susceptible, moderately, and resistant), the inoculum (20, 100, and 1000 spores/seed), and field location [74]. The bio-pesticide based on *Pseudomonas chlororaphis* isolate MA342 (Cerall) is another seed coating treatment that effectively suppresses seedborne infections of *T. caries* [165, 166]. However, this bio-pesticide is ineffective against *T. caries* soilborne infections [166]. The scarcity of broadly effective, non-synthetic seed treatments capable of managing *T. caries* and *T. controversa* soilborne inoculum highlights the need for methods that support the development of new treatments and prevention strategies.

Synthetic plant production products

The first synthetic fungicide used against bunt diseases was a copper-based solution developed in 1807. However, routine seed treatment became widespread only after 1913, when inexpensive and effective organomercurial seed dressings were developed [162]. Neither copper- nor mercury-based treatments could control soilborne infections of *Tilletia caries* and *Tilletia controversa*, which were controlled only after 1956 with the discovery of polychlorobenzenes. In particular, hexachlorobenzene and pentachloronitrobenzene showed effective against both the common and dwarf bunt fungi from soil- and seed-borne inoculum [107].

Developing effective synthetic treatments to control *Ustilago nuda* infected seeds was more challenging than for *T. caries* contaminated seeds due to the difference in pathogen location (i.e. in versus on the seed). Contact fungicides are sufficient for *T. caries* because the teliospores are located on the seed surface, while *U. nuda* mycelium colonize embryo tissue. The first systemic fungicide, Carboxin, was commercialized in 1969. This fungicide controlled surface bunt fungi and, by penetrating the seed embryo, controlled loose smut infections [162]. However, over time, these pathogens developed resistance to synthetic fungicides (e.g., *U. nuda* to carboxin [137]), and several active ingredients have raised concerns about their toxicity to humans and the environment. Consequently, some compounds were banned globally (e.g., hexachlorobenzene [167]), banned in the EU [168, 169], or with restricted use in other countries (e.g., carbendazim in the US [170]). Other synthetic pesticides have also been proposed for withdrawal in countries outside of the EU, such as pentachloronitrobenzene in the US [171].

The withdrawal of these effective treatments, together with the emergence of pathogen resistance to fungicides, has complicated the maintenance of effective synthetic control options. Currently available synthetic PPPs are limited and are mostly effective against seedborne inoculum. A seven year study showed that triazole- (tebuconazole and prothioconazole) and

benzimidazole- (thiabendazole) based fungicides are highly effective against *U. nuda*, but the efficacy seems to be modulated by abiotic factors such as temperature, rainfall, and relative humidity during flowering [172]. Additionally, the same study found that seed treatment with sedaxane and fludioxonil achieved efficacy levels ranging from 84.21% to 100% against *U. nuda*. The combination of sedaxane, difenoconazole, and mefenoxam is marketed as effective against *U. nuda* [173], likely due to sedaxane's contribution, since formulations containing higher proportions of difenoconazole and mefenoxam alone are not commercialized to control loose smut [174]. Tebuconazole also effectively controls seedborne infections of *T. caries* [175], but it is not marketed as being effective against *T. controversa*. Difenoconazole [174, 176] appears to be one of the few synthetic fungicides with consistent efficacy against *T. controversa* from both seed- and soilborne inoculum sources [150], likely because other synthetic PPPs do not remain active long enough to prevent infection [177]. Nevertheless, difenoconazole-based fungicides showed efficacy against *T. caries*, though they have shown limited efficacy against *U. nuda* [174, 178]. Prothioconazole controls *U. nuda* development and both seed- and soilborne inoculum of *T. caries* [179]. Fludioxonil, when used as a seed coating, can control seed- and soilborne *T. caries* [180], and when combined with difenoconazole, it can effectively control *T. controversa* [181, 182].

However, the efficacy and applicability of currently available synthetic fungicides is subject to possible limitations in the future. The progressive loss of effective active ingredients and increasing regulatory restrictions further highlight the growing constraints on synthetic PPPs within integrated pest management strategies. Constantly evolving fungal resistance and changes in permitted synthetic fungicides increase the need to strengthen preventive approaches, improve detection methods, and develop new treatments that are low-risk and environmentally compatible to manage bunt and smut diseases without harming humans or the environment.

Rationale

The regulatory and agricultural policies aimed at reducing the use and risk of synthetic plant protection products (PPPs) did not consider whether alternative control solutions with comparable efficacy and practical applicability for plant pathogens or pests were available. This gap in available control solutions is particularly problematic for cereal production, which represents a major component of global food security [183]. Cereals contribute between 22% and 49% of dietary energy worldwide [183, 184]. Restrictions on synthetic PPPs in cereals increase the risk of re-emerging seed- and soil-borne diseases, such as common bunt (*Tilletia caries*) and dwarf bunt (*Tilletia controversa*) in wheat [18], and loose smut (*Ustilago nuda*) in barley. Therefore, new tools that support disease prevention while reducing synthetic PPP use are necessary to enable effective implementation of Integrated Pest Managment (IPM) guidelines. According to IPM guidelines for these smut and bunt disease, the priority is preventing the pathogen introduction through the testing of potential inoculum sources, particularly seed and soil. When pathogen levels exceed defined tolerance thresholds, organic or low-risk PPPs should be applied, whereas the synthetic PPPs should be used only as a last option [1].

However, the implementation of IPM guidelines for loose smut, common bunt, and dwarf bunt is currently hindered by two main bottlenecks. First, current seed health certification relies on slow, expert-dependent visual assessments of pathogens [50], and there are no validated methods to monitor soilborne inoculum of *Tilletia* spp. Second, the development of organic or low-risk PPPs is impeded by the time and resources required for growing the plants to the ear emergence or seed maturation stages in field and greenhouse trials to assess treatment efficacy.

This dissertation addresses these specific IPM bottlenecks through the development of PCR-based detection tools applicable to samples from seeds, soil, and plant tissues. The dissertation is organized into two complementary parts that follow the logic of the IPM guidelines. The first part focuses on improving the prevention of pathogen dispersal (Chapter 1 and Chapter 2). In Chapter 1, I develop a qPCR protocol for detecting *U. nuda* in seeds and link the qPCR measurements to field disease occurrence. For Chapter 2, I extend a qPCR-detection approach to *T. caries* and *T. controversa*, which includes the development of a protocol to detect soilborne inoculum and addresses the current lack of standardized soil monitoring methods. In the second part, I focus on accelerating treatment development through the application of molecular methods (Chapter 3 and Chapter 4). In these chapters, I aimed to reduce the time and resources required to evaluate alternative seed treatments by developing plant-based qPCR screening approaches to measure treatment efficacy against *U. nuda* (Chapter 3) and *T. caries* (Chapter 4) months before disease occurs.

To evaluate the effectiveness of these PCR-based tools within the IPM framework, this

dissertation addresses the following research questions and hypotheses:

1. To what extent can PCR-based methods provide sensitive, specific, and high-throughput detection of *U. nuda* in seeds and *T. caries* and *T. controversa* in seeds and soil?

   H1 Optimized PCR-based protocols will achieve higher throughput and sensitivity than the current visual method for seeds and enable reliable quantification of soilborne inoculum.

2. Can PCR measurements be used as a predictor of disease occurrence?

   H2 Although subject to influence of abiotic factors, PCR measurements of pathogen DNA in seeds can indicate disease incidence, allowing PCR results to support IPM decision-making.

3. Can plant-based qPCR screening reliably predict the efficacy of seed treatments?

   H3 The quantification of pathogen DNA in plant tissues will provide an accurate measurement of pathogen infection, thereby enabling its use to calculate a proxy for treatment efficacy observed during disease occurrence.

In summary, this dissertation aims to provide molecular tools to efficiently apply IPM strategies to loose smut, common bunt, and dwarf bunt management. Streamlining pathogen detection and treatment evaluation supports the transition from synthetic fungicides to low-risk PPPs while protecting crop health.

Chapter 1

A new molecular seed assay to predict *Ustilago nuda* field infection levels

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Published manuscript doi: https://doi.org/10.1038/s41598-025-18544-3

Abstract

Seed health tests are performed to prevent sowing untreated seeds with problematic pathogen levels. The detection of internal seedborne pathogens like *Ustilago nuda*, causing loose smut in barley (*Hordeum vulgare*), is challenging because symptoms only appear when teliospores replace barley inflorescences and smutted ears develop. Currently, *U. nuda* seed infection levels are determined from the visual inspections of extracted embryos or fields of plants used for seed production, both of which are laborious and can be unreliable. To improve *U. nuda* detection, we developed a multiplex qPCR method targeting *U. nuda* and *H. vulgare* DNA. Naturally infected seed lots were tested using this qPCR method and the visual analysis of embryos. We grew the same seed lots in the field over two seasons and used the observed smutted ears as the reference infection level. The qPCR results, *U. nuda* DNA normalized to *H. vulgare* DNA, better correlated with observed field infections than the number of infected

embryos. Our qPCR method more accurately distinguished seed lots with infections above and below the field tolerance threshold. It offers a reliable alternative to the visual analysis of extracted embryos. The integration of our qPCR method with field observations can enhance *U. nuda* management and reduce unnecessary prophylactic seed treatments, thereby bolstering integrated pest management strategies.

Keywords: *Ustilago nuda*, Loose smut of barley, qPCR, Molecular detection, Plant pathology, Seed testing

Introduction

Seed health tests detect seedborne pathogens and can serve a foundational role in integrative pest management (IPM) strategies. Plant pathogen detection on untreated seed and the subsequent avoidance of infected material can prevent the spread of seedborne diseases. Indeed, a core IPM principle is the targeted use of synthetic or organic plant protection products (PPPs) only when a harmful organism's level exceeds a tolerance threshold [1]. Seed health tests detect pathogens, carried on or within seeds [2], and help determine when these tolerance thresholds are reached and whether seed treatments are necessary [3]. However, often seed suppliers routinely treat seeds with synthetic PPPs by default [3, 4], regardless of the pathogen's level. Seed treatments are seen as an inexpensive, low effort method to protect crops [5]. As more policies aim to reduce synthetic PPP applications [1], an increased reliance on seed health tests can reduce unnecessary seed treatments. This targeted approach would ensure that PPPs are applied only when needed based on a predefined tolerance threshold to prevent the resurgence of seedborne diseases [6, 7, 8, 9].

Seedborne pathogens, unlike infections in other plant tissues, can be asymptomatic and difficult to detect in seeds. Undetected pathogens can develop within healthy-appearing plants and then manifest disease symptoms at different plant growth stages [10, 11]. Internally located fungal seed pathogens are a particular threat to agricultural production because they are difficult to detect and can spread unnoticed. For example, *Ustilago nuda* and *Ustilago tritici*, which cause loose smut in barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) respectively, can cause significant yield losses [12]. In these diseases, the symptoms become visible when the fungus replaces the plant inflorescence with its teliospores, causing smutted ears, and spreads to nearby healthy plants [10, 13, 14]. Because these pathogens are asymptomatic through plant growth until ear development, early detection and control remain challenging [15]. Due to their difficulty in early detection, these seedborne pathogens confer a high risk of disease transmission in seed production [16, 17]. Since crop losses from *U. nuda* can reach up to 25% in barley and only up to 5% in wheat due to *U. tritici* [18], loose smut of barley is typically more problematic in cereal seed production.

In barley seed production, as part of the seed certification process, field inspections are conducted to identify diseases. Loose smut infections are assessed during the flowering stage to minimize its propagation to the next generation of barley seed [19]. In Switzerland, for example, no more than 2 infected ears per 100 m^2 are allowed in plants that produce first-generation certified seed and up to 5 infected ears per 100 m^2 in plants that produce second-generation certified seeds that are commercially available [20, 21, 22], but the tolerance thresholds are

country-dependent. However, infection detected during the field inspection may not accurately correlate with infection levels in the subsequently harvested seed; environmental factors, such as wind and rain, can hinder the visual detectability of infected ears and increase teliospores dispersion [23]. Although an inspected field may meet the seed certification criterion regarding loose smut, its barley inflorescences may hide *U. nuda* latent infections due to teliospore dispersal from a neighboring field. An unexpectedly high infection rate in the harvested seed may occur despite the appearance of healthy-looking plants [16, 17, 19, 23]. Therefore, weather conditions and teliospore dispersion may obscure the actual number of diseased ears and may lead to an underestimation of the harvested seed's true infection rate [23].

A direct assessment of the harvested seeds can avert a potential mismatch between the harvested seed's *U. nuda* infection rate and the visible field infections during seed production. Although not required for seed certification in many countries [20], the *U. nuda* infection rate can be determined directly from the inspections of harvested seed rather than an inference based on observed smutted ears in the field during seed production. A validated seed testing protocol for *U. nuda* detection in embryos of barley seed is used in some countries either for seed health certification or as a complement to it [7, 20, 22, 24]. The percentage of infected embryos tolerated for seed health certification depends on the country and ranges from 0.1% to 1.0% [20, 22]. Nevertheless, the detection of *U. nuda* mycelia within embryos remains challenging due to limited mycelial visibility, constraints on sample size, and the labor-intensive nature of the process [24, 25, 26].

Molecular methods can improve the detection of seedborne pathogens because such methods increase scalability, sensitivity, and specificity compared to visual inspections [27, 28]. Detection protocols for other seedborne fungal pathogens have employed molecular methods successfully [29, 30, 31, 32] and have shown promising results for detecting *U. nuda* in plant material [10, 33, 34, 35]. Previous research on *U. nuda* detection employed enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) methods [10, 33, 34, 35]. Among the published techniques that focused on *U. nuda* detection in seed [33, 34], seeds were individually tested, which is neither economical nor feasible for a large-scale application. To facilitate an increased throughput, the bulk analysis of milled seeds can simplify seed sample preparation for PCR-based assays [34, 35]. However, initial attempts at using PCR with bulk milled seed samples encountered challenges; in particular, nonspecific fluorescence in SYBR Green-based quantitative PCR (qPCR) assays impaired at the detectability of low *U. nuda* infection levels [35]. This nonspecific fluorescence was observed with a previously published *U. nuda* primer set used on seedling [10]. Within a later publication, this primer set was modified with the addition of three base pairs, and it was applied to seed using a SYBR Green-based qPCR assays[36]. A drawback to this published protocol is that SYBR Green dye binds to all double stranded products and can result in decreased specificity. Therefore, a fluorogenic probe can enhance *U. nuda* assay specificity by providing an additional specific-binding region of the amplified product required for a florescent signal's production [35].

No established molecular method can reliably detect *U. nuda* in bulk milled seed samples with the sensitivity and specificity required for seed health testing. In this study, we developed a multiplex qPCR method using newly designed primers and fluorogenic probes that quantify *U. nuda* and *H. vulgare* DNA in bulk milled seed. The host DNA acts as an internal control for the

DNA extractions and qPCR reactions. It also normalizes the pathogen DNA in each sample. Furthermore, no prior study has directly connected *U. nuda* seed infection rates detected with molecular methods to infection rates observed in the field. This link is needed to translate the qPCR results directly to field infection levels and, thus, potential yield losses. We used field observations as a reference to evaluate our qPCR detection method's performance. Additionally, we evaluated the performance of the embryo test — the recognized seed health test for *U. nuda* [24] — on the same seed lots. Compared to the embryo test, the qPCR method more accurately distinguished seed lots with infection above or below its tolerance threshold, reflecting the classification of the observed field infection levels with respect to the field tolerance threshold. Overall, our newly developed qPCR method showed an improved ability to detect *U. nuda* and predict field infections compared to the embryo test. Due to its increased scalability compared to the embryo test, our qPCR method is also highly applicable for large-scale seed health assessments. It provides a quantitative evaluation of *U. nuda* infections, which enables informed seed management decisions based on a tolerance threshold. Our qPCR method's incorporation into the barley seed certification process would strengthen IPM strategies to limit loose smut's spread and reduce the reliance on PPP seed treatments.

Results

Sensitive and specific primers and probes

We developed a new multiplex TaqMan qPCR protocol that targets COX3 in the pathogen, *Ustilago nuda*, and GADPH in the host, *Hordeum vulgare*, enabling the normalization of pathogen to host DNA. The multiplex reactions demonstrated average amplification efficiencies of 99.7% (R^2 = 98.3%)for COX3 and 90.6% (R^2 = 99.7%) for GADPH (Supplementary Information, Table 4). Our protocol's limit of detection (LOD) for the *U. nuda* COX3 gene is 4 copies per reaction based on a dilution series of its gBlock gene fragment tested eight times. We also compared our *U. nuda* primers in a multiplex TaqMan reaction with the most recently published ITS primers in a SYBR Green singleplex reaction[36]. Both protocols successfully amplified *U. nuda* teliospores in reactions with template DNA concentrations of 0.8, 0.08, and 0.008 ng/µL (Table 1). From our tests to determine the primers' species-specificity, we observed that our COX3 primers also amplified *Ustilago hordei* mycelium, which was expected from the sequence similarities of *U. nuda* and *U. hordei* at the primer binding sites (Supplementary Information, Figure 1). We tested DNA from mycelial samples of other basidiomycetes that had been isolated from single teliospores to determine the COX3 and ITS primers' species-specificity. The published ITS protocol consistently amplified all *Ustilago* species tested with the DNA template concentration of 0.8 ng/µL per reaction (Table 1). In contrast, our newly developed protocol amplified *Ustilago maydis* in one of three replicates with a quantification cycle (Cq) of 36.42, and it did not amplify another non-target *Ustilago* spp., showing increased species-specificity. Additionally, the ITS protocol amplified an unidentified *Pseudozyma* species (Cq = 37.51 ± 0.36), while our protocol showed no amplification. The other six non-target fungal taxa exhibited either unstable amplification (i.e. amplification in only some replicates) or no amplification in both protocols (Table 1). We found that the coefficient of variation

(CV) was 89% lower with our protocol than with the ITS protocol when applied to *U. nuda* infected seed and seedling samples (Supplementary Information, Table 1), showing in more stable measurements.

Source	Fungal taxa[a]	Concentration (ng/µL)[b]	Newly developed COX3 TaqMan protocol			Published ITS SYBR green protocol [36]		
			Cq Mean[c]	Cq Std. Dev [d]	CV (%) [e]	Cq Mean	Cq Std. Dev	CV (%)
Teliospores	*Ustilago nuda* (Ustilaginomycotina, Ustilaginomycetes)	0.8	14.84	0.23	1.52	11.92	0.11	0.90
		0.008	22.05	0.05	0.21	19.18	0.25	1.29
		0.0008	26.41	0.31	0.66	29.27	0.17	0.57
Mycelia	*Ustilago hordei* (Ustilaginomycotina, Ustilaginomycetes)	0.8	17.30	0.12	0.70	13.37	0.22	1.63
		0.008	25.01	0.08	0.30	23.99	0.19	0.77
		0.0008	29.11	0.18	0.61	29.71	1.20	4.04
	Ustilago maydis (Ustilaginomycotina, Ustilaginomycetes)	0.8	$36.42_{1/3}$	-	-	33.67	1.10	3.27
		0.008	-	-	-	-	-	-
	Ustilago spp. (Ustilaginomycotina, Ustilaginomycetes)	0.8	-	-	-	30.09	0.07	0.22
		0.008	-	-	-	-	-	-
	Pseudozyma spp. (Ustilaginomycotina, Ustilaginomycetes)	0.8	-	-	-	37.51	0.36	0.95
		0.008	-	-	-	-	-	-
	Tilletiopsis spp. (Ustilaginomycotina, Exobasidiomycetes)	0.8	-	-	-	-	-	-
		0.008	-	-	-	-	-	-
	Entyloma spp. (Ustilaginomycotina, Exobasidiomycetes)	0.8	-	-	-	$38.39_{2/3}$	1.39	3.62
		0.008	-	-	-	-	-	-
	Holtermanniella spp. (Agaricomycotina, Tremellomycetes)	0.8	$36.42_{1/3}$	-	-	-	-	-
		0.008	-	-	-	-	-	-
	Tilletia caries (Ustilaginomycotina, Exobasidiomycetes)	0.8	-	-	-	-	-	-
		0.008	-	-	-	-	-	-
	Tilletia controversa (Ustilaginomycotina, Exobasidiomycetes)	0.8	-	-	-	-	-	-
		0.008	-	-	-	-	-	-

Table 1: The assessment of COX3 and ITS primer species-specificity for *Ustilago nuda*. The newly designed COX3 primers in multiplex and the published ITS primers in singleplex were tested with DNA extracted from mycelia and teliospores of the target (*U. nuda*) and non-target fungal species (all other mycelial isolates listed that come from species other than *U. nuda*). Three technical replicates were used for both protocols. The number of amplified replicates is denoted as a subscript in the quantification cycle (Cq) mean when fewer than three amplified. The higher Cq value indicates less amplification. Samples with no amplification are indicated by the symbol "-".

[a] Taxonomic classification with subphylum and class in parenthesis
[b] Final DNA concentration in each qPCR reaction
[c] Average cycle threshold of triplicates
[d] Standard deviation of the quantification cycle among triplicates
[e] Coefficient of variation $(^{\text{Cq Std. Dev.}}/_{\text{Cq Mean}})*100$

We tested non-target plant DNA with our newly developed GADPH primers to determine which plant species' DNA would be suitable to include in the non-template DNA control and the standard curves. The DNA extracted from barley (*H. vulgare*), wheat (*Triticum aestivum*), and lentil (*Lens culinaris*) seeds were tested in our multiplex reaction. The primers successfully amplified barley DNA (Cq = 24.97 ± 0.24), but they showed reduced amplification of wheat DNA (Cq = 34.53 ± 0.37), and no amplification of lentil DNA, demonstrating that lentil can be used as the non-template DNA in the barley standard curve and control. The data acquired for the main experiments in this paper showed minimal variation of *H. vulgare* detection based on the Cq values of the HEX-labeled *H. vulgare* GADPH probe (Supplementary Information, Tables 2, and 3)

A representative seed lot sample for *Ustilago nuda* qPCR detection

Our qPCR method relies on DNA samples extracted from milled seeds, so we evaluated different sample preparation procedures to ensure an accurate seed lot representation. We tested three key parameters to establish a representative seed lot sample: 1) the number of milled seeds, 2) the amount of seed flour for DNA extraction, and 3) the number of milled seed batches. The evaluation of these key parameters was conducted on three seed lots, each from a different cultivar: Esprit, KWS Orbit, and Maltesse. The qPCR results, based on the ratio $^{\textit{U. nuda}}/_{\textit{H. vulgare}}$ DNA copies, showed no significant differences between any of the parameters

tested within each cultivar (Figure 1, Supplementary Information, Table 5). However, wide variability in infection levels was observed among subsamples, especially as the average infection level of the seed lot increased (Supplementary Information Table 5).

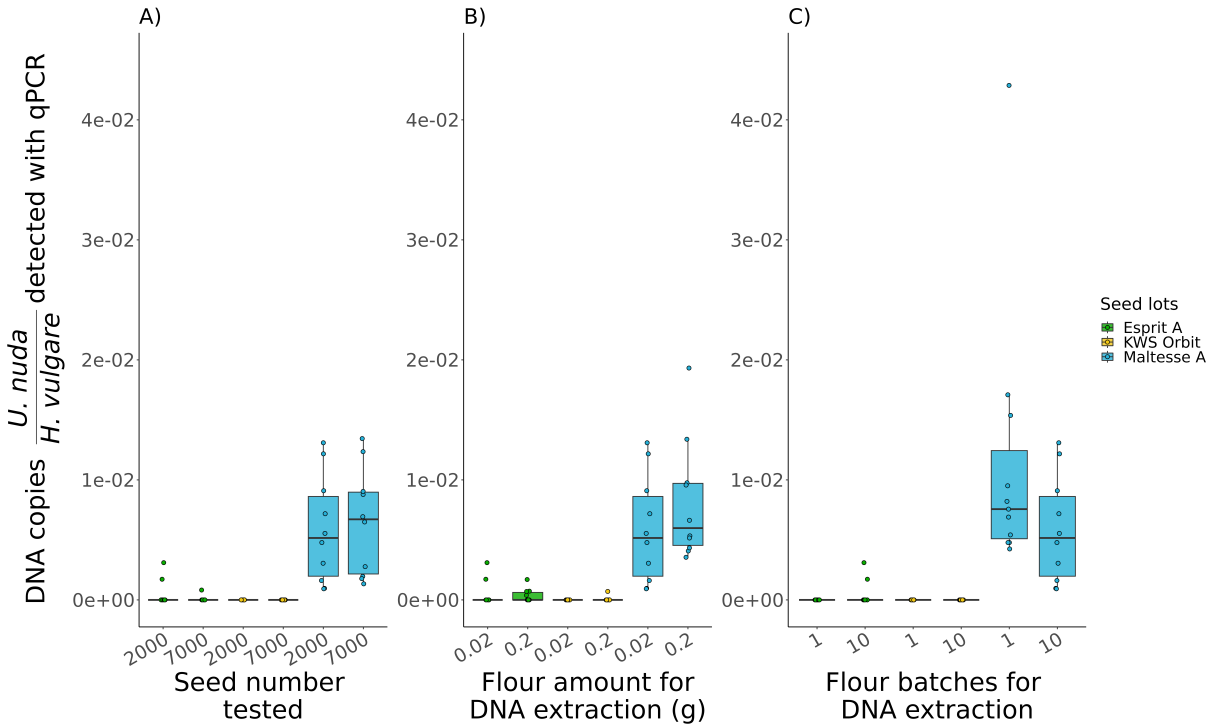

Figure 1: Key parameters evaluated to establish a representative *Hordeum vulgare* seed lot sample for *Ustilago nuda* qPCR detection. The parameters included: (A) the number of seeds tested (2000 versus 7000), (B) the amount of flour used for extraction (0.02 g versus 0.2 g), and (C) the number of flour batches used for the ten DNA sub-sample extractions (i.e. one batch extracted ten times or ten batches extracted one time each). The later analysis was used to assess inter- and intra-flour batch *U. nuda* DNA variation. The parameters in (A) and (B) were assessed with ten DNA extractions, each from a separate flour batch. *U. nuda* infection levels were quantified with the qPCR method developed in this study using the ratio *U. nuda* to *H. vulgare* DNA, which normalizes the pathogen DNA to its host DNA. Three different *H. vulgare* seed lots, each of a different cultivar, are shown in different colors. Kruskal-Wallis tests showed no significant differences in the detected *U. nuda* infection levels between each of the three key parameters within cultivars.

The relationship between laboratory detection methods and field results

The relationships between *Ustilago nuda* infection levels observed in the field and those measured by laboratory detection methods, including the embryo test and the qPCR method, were evaluated with three barley cultivars: Azrah, Semper, and SU Celly. All seed lots included had similar field germination rates ($\chi^2 = 17.00$ p $= 0.45$). The seed lots were chosen for this experiment based on our access to naturally infected seeds with an infection level greater than 1%. Samples of each cultivar were composed of four different infection levels, which were achieved by using naturally low-infected (certified seed lots) and high-infected seed lots (based on observed field infection levels of the previous generation) and mixing them. The two mixtures for each cultivar were made with either 10% or 1% of seed from the high-infected lot and 90% or 99% of seed from the low-infected lot, respectively, totaling twelve seed samples. The two naturally infected seed lots and their mixtures with different infection levels were used to study the correlation between field observations and laboratory detection methods. Additionally, this relationship was used to derive a tolerance threshold for the qPCR method.

To determine which model type best fits the relationship between the qPCR measurements and the field observations, we tested three models (linear, polynomial and exponential) and compared them (Supplementary Information, Table 6). The linear model was chosen for its predictive performance (highest adjusted R^2) and lower complexity (lowest AIC and BIC) compared to the polynomial and exponential models. The positive linear relationships showed that the laboratory detection methods are able to predict the field infection level (Figure 2) and that the correlation is about 22% higher in the qPCR method compared to the embryo test based on the adjusted R^2 values. The correlation between the qPCR measurement and the field observations was then used to develop a tolerance threshold for the qPCR method. The field tolerance threshold used in our study was 35 infected ears based on 350 seed/m^2 sowing density (equivalent to 0.1% infected embryo and the assumption that one infected embryo develops one infected ear, Supplementary Information, Figure 2). This tolerance threshold correlated with 7.50×10^{-5} *U. nuda*/*H. vulgare* DNA copies.

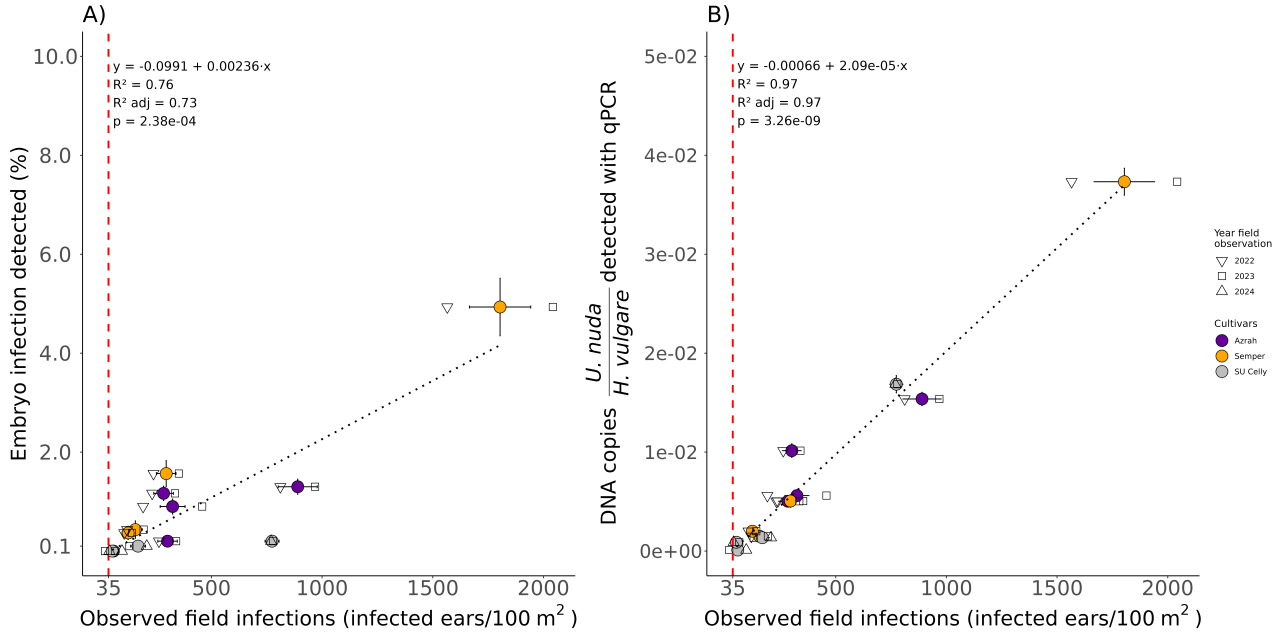

Figure 2: Relationship between the *Ustilago nuda* infection observed in the field and measured with laboratory detection methods: (A) embryo test and (B) qPCR method. Observed field infections, used as the reference infection level for each sample obtained from naturally infected seed lot or their mixtures, are plotted on the x-axis as the independent variable. The *U. nuda* measurements from the laboratory detection methods that are evaluated in this study are plotted on the y-axis as dependent variables. The linear models' lines of best fit between field observation and the laboratory detection methods are illustrated with black dotted lines. The linear model equations, R^2, adjusted R^2 (R^2 adj), and p-value are shown in the top left corner of each plot. Each sample from each cultivar was sown in two of the three field growing seasons. The different shapes indicate the two years of observed field infections for each sample, and the average infections of each sample are shown as a circle. In both plots, the red vertical dashed line indicates the field tolerance threshold value (35 ears per 100 m^2).

The comparative performance of laboratory *Ustilago nuda* detection methods to predict disease levels in the field

The qPCR method and the embryo test were evaluated for their ability to detect *Ustilago nuda* infections in six commercially available *Hordeum vulgare* seed lots from four cultivars: Esprit (two seed lots, A and B), KWS Orbit, Maltesse (two seed lots, A and B), and SU Celly (seed lot C). Three of the seed lots — Esprit A, KWS Orbit, and Maltesse A — were the

same used to establish a representative seed lot sample for the *U. nuda* qPCR detection method. Each seed lot was sown in four 20 m^2 field plots in two growing seasons. The observed field infection levels across the two growing seasons were averaged and served as the reference for the performance of the embryo test and qPCR method to detect *U. nuda* in seed (Table 2). The field infection levels ranged from 0.00% to 0.44% across all of the seed lots in both growing seasons (Supplementary Information, Figure 2).

We created confusion matrices to compare the performance of the embryo test and qPCR method. For each matrix, we classified the infection levels measured by the embryo test and qPCR method as either above or below their respective tolerance thresholds: 0.1% infected embryos and 7.50×10^{-5} $^{U.\ nuda}/_{H.\ vulgare}$ DNA copies. These classifications served as the predicted values (Supplementary Information, Table 8). In parallel, the seed lots' infection levels were classified as above or below the tolerance threshold based on their observed infected ears in the field (Table 2), and these were used as the actual (or true) values. Based on the comparisons between the actual values and predicted values, we assessed which samples they accurately categorized to have infection levels above or below their tolerance thresholds (Table 2, Supplementary Information, Table 8). Based on the infection detected by the embryo test, all tested seed lots were classified as having infection levels below the embryo test's tolerance threshold (0.1%). However, two of these seed lots had field infection levels exceeding the field's tolerance threshold, so the embryo test produced false negatives by failing to detect those infections. The seed lot infections detected using the qPCR method, on the other hand, were correctly identified as above or below its tolerance threshold and matched the actual values.

	Field			Embryo test				qPCR method				
Seed lots	**Observed infection (infected ears per 100 m²)**	**Tolerance threshold (infected ears per 100 m²)[a]**	**Infection compared to the tolerance threshold**	**Measured infection (%infected embryo per 1000 embryos)**	**Tolerance threshold (%infected embryo per 1000 embryos)**	**Infection compared to the tolerance threshold**	**Outcome**	**Measured infection (*U. nuda*/*H. vulgare* DNA copies)**	**Tolerance threshold (*U. nuda*/*H. vulgare* DNA copies)[b]**	**Infection compared to the tolerance threshold**	**Outcome**	**Projected infected ears[c]**
Kws Orbit	0 ± 0		Below	0.1		Below	TN	0.0 ± 0.0		Below	TN	≤ 31
Espirt B	2 ± 1		Below	0.0		Below	TN	0.0 ± 0.0		Below	TN	≤ 31
Espirt A	10 ± 2	35	Below	0.1	0.1	Below	TN	0.0 ± 0.0	7.5×10^{-5}	Below	TN	≤ 31
SU Celly C	11 ± 3		Below	0.0		Below	TN	0.0 ± 0.0		Below	TN	≤ 31
Maltesse B	44 ± 12		Above	0.0		Below	FN	$9.1 \times 10^{-4} \pm 9.9 \times 10^{-5}$		Above	TP	75 ± 36
Maltesse A	136 ± 14		Above	0.0		Below	FN	$1.2 \times 10^{-2} \pm 3.7 \times 10^{-3}$		Above	TP	612 ± 206

Table 2: Comparison of *Ustilago nuda* infections observed in the field to those measured using the laboratory detection methods, the embryo test and qPCR method, on six commercially available seed lots. The field infection levels of each seed lot were evaluated in two years from four replicate plots per year, and these values served as a reference for the true infection levels. The percentage of infected embryos was based on one evaluation of 1000 seeds per seed lot. The infections detected with qPCR were based on ten extractions per seed lot, each with 0.02 g of flour per extraction. The flour for the ten extractions was obtained from 2000 milled seeds of the same seed lot. The standard errors are shown for the field observations and qPCR measured infection. All measured infection levels were determined to be either above or below the method-specific tolerance threshold for each seed lot, and these binary classifications were then compared with confusion matrices (Supplementary Information, Table 8). The binary classification for the field observations served as the actual values, and for the laboratory detection method, it served as the predicted values. The predicted values were then categorized as true positive (TP), true negative (TN), false positive (FP), or false negative (FN) depending on whether they reflected the actual values. These categorizations are reported in the outcome column.

[a]Tolerance threshold (infected ears per 100 m^2) calculated from the number of infected plants derived from 0.1% infected seeds at a sowing density of 350 seeds/m^2.

[b]Threshold (*U. nuda*/*H. vulgare* DNA copies) corresponding to 35 infected ears/100m^2 and derived from the relationship *U. nuda* qPCR assay results with observed field infections (Figure 2)

[c]Projected infected ears: estimated from the measured infection by qPCR and its corresponding field infection level according to their linear relationship (Figure 2)

We then used the confusion matrices to evaluate the sensitivity, specificity, precision, false discovery rate, and accuracy of the *U. nuda* detection methods in six commercially available seed lots. These attributes served as performance metrics. Together, the performance metrics showed that the qPCR method classified the *U. nuda* infection levels better than the embryo test (Table 3). Two of the six seed lots had infection levels exceeding the field's tolerance threshold so have actual values that are above its threshold (Table 2). The qPCR method showed a sensitivity and specificity of 1.0, accurately identifying the infection of these two seed lot infections as above its tolerance threshold and the other four seed lots as below it. On the other hand, the embryo test identified the infection of these two seed lot samples as below its tolerance threshold when they should be above it. Because the embryo test yielded no predicted values that were above its tolerance threshold, we cannot calculate the embryo test's precision and false discovery rate in this study. However, the embryo test successfully identified the seed lots with actual infection values below the field tolerance threshold, resulting in a specificity of 1.0 and a sensitivity of 0.0. Overall, the qPCR method consistently detected *U. nuda* infection levels more accurately and precisely in the evaluated six commercially available seed lots than the embryo test (Table 3).

	Sensitivity	Specificity	Precision	False discovery rate	Accuracy
Embryo test	0.0	1.0	-	-	0.6
qPCR method	1.0	1.0	1.0	0.0	1.0

Table 3: Performance evaluations of the embryo test and our qPCR method in the detection of *Ustilago nuda* infections across six commercially available seed lots. The performance metrics include sensitivity, specificity, precision, false discovery rate, and accuracy. These performance metrics were based on the six seed lots' agreement between their predicted values, which classified the seed lot as above or below the method-specific tolerance threshold, and their actual values based on the classification of the infections observed in the field as above or below its tolerance threshold (Table 2). The outcome of each seed lot's classification according to the confusion matrices (Supplementary Information, Table 8) was used to evaluate the laboratory methods' performance. Metrics marked with "-" were incalculable due to the absence of positive test results in the embryo test.

Discussion

Seed health tests help prevent seedborne disease outbreaks and reduce reliance on plant protection products (PPPs) [37] because they indicate whether untreated seed lots present low risk of disease outbreaks. However, traditional methods — such as field inspections and visual seed assessments — are often time-consuming, resource-intensive, and prone to inaccuracies [25, 26, 38]. These challenges are particularly problematic for detecting cryptic seedborne infections, such as *Ustilago nuda* in barley (*Hordeum vulgare*), in which infected seeds are asymptomatic [39], making it difficult to determine whether seed lots require treatments or should not be sown. To address these limitations, we developed a multiplex qPCR method that quantifies *U. nuda* DNA relative to host DNA in bulked milled seed samples, thereby improving both detection accuracy and scalability.

We tested barley seed lots with our qPCR method and the existing validated test, the embryo test, to evaluate their ability to detect *U. nuda*. The direct comparison of a new qPCR method to an existing test can be problematic; if the validated test performs poorly, it provides an unreliable indicator of the seed lot's infection status. Because of the drawbacks associated with

directly comparing detection methods, we used the observed number of infected ears in the field as a reference to determine the true *U. nuda* infection rate in seed lots. Based on the adjusted R^2 values in our study, the embryo test explained 24% less variation in the field infection rates compared to our qPCR method. The difficulty of visualizing *U. nuda* mycelia with the embryo test may have contributed to its reduced ability to predict field infection levels. Therefore, the seed inspectors may underestimate the seed infection levels (Supplementary Information, Table 9). Although recent protocol improvements to the embryo test, including methyl blue staining, have been introduced to enhance mycelium visibility [24], these protocol adjustments still do not overcome the longer working time required to obtain results compared to a DNA-based test.

Molecular based detection methods on seed can offer higher sensitivity and specificity with faster throughput compared to visual seed inspections [27, 28]. Our multiplex qPCR amplifies both the host and the pathogen DNA to improve the relative quantification of *U. nuda* detection in seed. Previous studies have amplified host DNA as an external control in separate qPCR reaction or within a multiplex reaction as an internal control to confirm the quality of the DNA extraction and PCR amplification [31, 32]. However, their protocols have not used host DNA to normalize the pathogen DNA. Our qPCR method not only uses an internal control of host DNA, but it also provides a means to normalize the pathogen DNA. The normalization step is useful to quantify an internal embryo infection, such as *U. nuda*, that is asymptomatic and lays dormant in the seed as mycelia. Normalizing *U. nuda* mitochondrial DNA to *H. vulgare* nuclear DNA in each reaction strengthens the relationship between *U. nuda*'s qPCR measurement and the infected ears observed in the field (Supplementary Information, Figure 4). Although mitochondrial copy numbers may vary across different cell types and growth stages [40, 41], the mycelia in seed samples are dormant [10], suggesting that the mitochondrial copy number should be constant. The pathogen to host ratio additionally reduces the reliance on quantification cycle (Cq) values, also known as threshold cycle (Ct) values. The use of Cq values as a cutoff for pathogens' detection does not account for possible variability in DNA extraction efficiencies and intra- and inter-plate differences [42, 43].

Another benefit of using the host DNA within a multiplex reaction is that *U. nuda*'s mycelial biomass in the embryo can vary among seeds, and this variability is not considered during the visual embryo test. Moreover, most seedborne pathogenic fungi are quantified as spores or colonies per seed without destructive sampling methods [29, 31, 44], which is not possible for *U. nuda* due to its location in the seed embryo. Therefore, when pathogenic fungi are measured as spores or colonies per seed based on visual assessments, it is easier to quantify the infection rate of the seed and observe the subsequent field infection levels. However, due to the *U. nuda* location within the seed, this relationship between seed and field infection levels is difficult to assess using both the embryo test or our qPCR method. Neither laboratory detection method can distinguish between viable and non-viable mycelia in the seed, potentially complicating the connection between *U. nuda* detection results in seed and the observed infected ears in the field. The *U. nuda* infection level in the field correlated better with our qPCR results than the embryo test results, suggesting that the qPCR measurement provides a greater biological relevance. Further studies are needed to clarify under which seed storage conditions *U. nuda* mycelia in barley seeds lose viability.

An especially challenging aspect of *U. nuda* detection is that the tolerance level of an *U. nuda* infection in seed is low — for example, it is 1 in 1000 for many countries[20, 22] — so that destructively sampling enough seeds to estimate the field infection rate quickly becomes labor-intensive and cumbersome. Moreover, the relationship between the *U. nuda* mycelial biomass and the number of infected ears in the field remains unknown because each tested seed requires its destruction. To address the challenge to related seed and field infection levels, we established equivalent tolerance thresholds for the field, the qPCR method and the embryo test based on the assumption that one infected seed produces one infected ear (Supplementary Information, Figure 2B).

We used a tolerance threshold to assess the ability of our qPCR method and the embryo test for predicting field infections in commercially available seed lots. Although both laboratory detection methods showed high specificity, the qPCR method was more sensitive because it detected infections that the embryo test missed. These findings suggest that the embryo test may underestimate *U. nuda* infections, potentially failing to prevent its spread and future economic losses. Additionally, the qPCR results closely matched the observed infections in the field. One of the tested seed lots had an infection level close to the tolerance threshold, and it showed concordance between the observed field infections and the measured *U. nuda* infection with qPCR: both measurements were not consistently above or below their respective tolerance threshold (Supplementary Information, seed lot Maltesse B in Tables 7 and 10).

Overall, our qPCR method showed a higher precision and accuracy than the embryo test. The embryo test's lower performance can be attributed to its detection limit of 0.1% infected embryo based on analyzing 1000 embryos per sample. However, the International Seed Testing Association suggests increasing the sample size to 2000 – 4000 embryos to decrease the test's detection limit to 0.05% — 0.025% [24]. Doubling or quadrupling the sample size would add time and cost, making the test impractical for large-scale applications [25]. In contrast, we can predict down to 31 infected ears per 100 m^2 at a sowing density of 350 seeds/m^2 with our qPCR method, which corresponds to an embryo infection rate of 0.009%. We reached this improved detection sensitivity because our qPCR method uses pooled seed samples to enable the detection of even small amounts of *U. nuda* DNA. Indeed, qPCR-based detection methods for other seedborne pathogens show the advantages to pooling seed samples [28, 29, 30, 31, 32, 45]. Additionally, unlike previous *U. nuda* molecular detection methods that involve single embryo extraction or labor-intensive techniques (e.g., mortar and pestle) [10, 33, 34, 35], our approach involved seed milling to pool seed samples. This sample processing facilitates potential large-scale applications and improves detection at low infection rates [32, 34, 35, 46].

Despite the potential for high-throughput, we noticed that our qPCR method overestimated the projected number of infected ears in seed lots that were above the field tolerance threshold (Table 2). This overestimation may be reduced if more seed lots are tested for the correlation between qPCR results and field observations (Figure 2 B). Another possible explanation is that highly infected seed lots may contain infected seeds with more mycelial biomass, which could increase the number of infected ears per plant (Supplementary Information, Figures 2). To evaluate whether non-target fungi could lead to an overestimated *U. nuda* infection level, we investigated the species-specificity of our qPCR method. Our qPCR method showed improved species-specificity because it amplified non-target fungi in fewer instances than the

most recently published qPCR protocol to detect *U. nuda* [36]. We consistently amplified *Ustilago hordei* (covered smut of barley) with our qPCR method, but no other fungal species isolated from Swiss seed lots. Since *U. hordei* and *U. nuda* cause similar damage and the field tolerance threshold is based on the number of infected ears of either pathogen in the field, the *U. hordei* amplification does not compromise the practical application of our qPCR method [21]. Another common limitation in qPCR methods is the presence of inhibitors that can cause false negatives [47]. Although we did not include any additional inhibitor-removal steps during DNA extraction [32], our tests showed no inhibition in detecting *U. nuda* in seed (Supplementary Information, Table 11). To help validate the method's reliability and facilitate its adoption in routine seed health testing, ring testing across multiple laboratories and sowing seed lots under diverse conditions would further ensure the robustness of our qPCR method [48, 49, 50].

In conclusion, our qPCR method for untreated barley seed could provide a more accurate, scalable, and practical alternative to the existing validated seed health test for detecting *U. nuda* in embryos. It may serve as a robust secondary check on harvested seed certified through field inspections; rather than relying on visually infected ears in field, which do not necessarily capture the next generation's infection rate, it detects the actual infection in harvested seed. Additional testing of our qPCR method on more cultivars grown in diverse field conditions would contribute further knowledge on its application to practice. The combination of field inspections on plants grown from first-generation certified seed and our qPCR method on second-generation seed could help farmers and seed producers minimize the *U. nuda* infection in their barley fields. Additionally, rather than the use of prophylactic seed treatments, the incorporation of more accurate seed health testing prior to treatments can improve targeted PPP application. Targeted seed treatments follow the principles of integrated pest management and support disease management practices within the seed sector [1].

Methods

Primer and probe development for multiplex qPCR to detect *Ustilago nuda*

Primers and probes for the multiplex qPCR reaction were developed to specifically target the cytochrome c oxidase subunit III (COX3) gene from the plant pathogen *Ustilago nuda* (GenBank accession number: HQ013017) and the glyceraldehyde-3- phosphate dehydrogenase (GAPDH) gene from its host, *Hordeum vulgare* (GenBank accession number: MT933276). The primers were designed using the Primer3 software tool [51] as a basis for primer selection (Table 4). The potential amplification of non-*U. nuda* species was first checked using the NCBI nucleotide database and Primer-BLAST [52]. All primers and probes were synthesized by Microsynth (Balgach, Switzerland). The annealing temperatures for the primer and probe sets were first optimized in the range of 58 °C to 68 °C in singleplex reactions, followed by a test gradient of 63 °C to 66 °C for multiplex reactions. Both singleplex and multiplex reactions demonstrated comparable amplification efficiencies: 99% for COX3 and 90% for GAPDH.

Gene target	Target organism	Oligo name	Sequence (5' - 3')	Amplicon length (bp)
COX3	*Ustilago nuda*	UN_COX3_F	TCC GTT TGA GCT ACC ACT GC	101
		UN_COX3_R	CTG CAC GTC GAT TTC CTT GG	
		UN_COX3_P	FAM-CGC TGC TGC TTC TAT CTT CA-MGB-Q500	
GADPH	*Hordeum vulgare*	HvGADPH_F	CAG TTC ACG GCC ATT GGA AG	105
		HvGADPH_R	ATC AAT ACT ACC TGA CGC CAA AG	
		HvGADPH_P	HEX-AAG ACG ACA AGA CGC TGC TCT TCG-BHQ-1	

Table 4: Primers and probes used in this study. The primers and probes target the plant pathogen, *Ustilago nuda*, and its host, *Hordeum vulgare*. The COX3 probe has a minor groove binder (MGB) modification on its 5' end.

The newly developed *U. nuda* primer sets were compared to the most recently published *U. nuda* primers [36], which amplifies the ITS region of *U. nuda* in a singleplex reaction using SYBR Green dye. The published ITS protocol was adapted for the Opus qPCR system (BioRad, USA) with a reaction volume of 10 µL, and reactions were run using the GoTaq qPCR Master Mix (Promega, USA). To evaluate the specificity of the *U. nuda* primers, both protocols were tested on DNA extracted from mycelia of various basidiomycetous fungal species (Table 1). The qPCR protocols with either our newly developed COX3 or the published ITS primers were run using a range of DNA concentrations, including 0.8, 0.008, and 0.0008 ng/µL. The positive control for *U. nuda* originated from teliospores collected from the *H. vulgare* cultivar Cassia during a 2022 field trial at Reckenholz (ZH), Switzerland.

An *in silico* analysis with the COX3 primers indicated the highest likelihood of cross-amplification with *Ustilago hordei* (Supplementary Information, Figure 1). To check potential cross-amplification, *U. hordei* mycelia (strain Mat1, provided by Gunther Doehlemann, University of Cologne) was tested in qPCR reactions with the same DNA concentrations as *U. nuda*, 0.8, 0.008, and 0.0008 ng/µL. DNA from non-target basidiomycetous mycelia was tested at concentrations of 0.8 and 0.008 ng/µL in qPCR reactions. Non-target basidiomycetes — *Ustilago maydis*, *Tilletia caries*, *Tilletia controvera*, *Tilletiopsis* spp., *Pseudozyma* spp., another *Ustilago* spp. with teliospores approximately 9 μm in diameter, *Entyloma* spp., and *Holtermanniella* spp. — were included in this analysis to assess primer specificity. *Tilletiopsis* spp., *Pseudozyma* spp., another *Ustilago* spp., *Entyloma* spp., and *Holtermanniella* spp. were isolated from Swiss seeds or smutted ears (Supplementary Information, Subsection Supplementary Methods), and their identity was deduced based on sequence similarity to other Basidomycetes using BLAST searches against the core nucleotide database and whole genome shotgut contigs (Supplementary Information, Table 12). DNA from *T. caries* and *T. controversa* was extracted from single spore mycelial isolates from bunt-infected seeds collected during a 2022 pot trial in Reckenholz (Zurich, Switzerland) and a 2023 field trial in Unterwasser (St. Gallen, Switzerland), respectively. The *U. maydis* strain FB2 was provided by G. Doehlemann. Sanger sequencing of the ITS1 region confirmed the species identity of the fungi isolated in this study (NCBI Accession numbers to be provided upon publication). DNA extractions of the mycelia and teliospores were performed using the NucleoSpin Plant II Mini kit (MACHEREY-NAGEL, Germany), and DNA concentration was quantified with Nanodrop (Thermo Fisher Scientific Inc, USA). Primers and probe targeting the *H. vulgare* GAPDH gene were tested to determine whether the plant material DNA from wheat (*Triticum aestivum*) or lentil (*Lens culinaris*) seed could be used as the control and the standard curve's non-template DNA.

Seed material and subsample preparation

Untreated naturally infected seeds from two-row and six-row barley cultivars were used in this study. For the experiment to test a representative seed lot sample, we used three commercially available seed lots from the cultivars: Esprit, Maltesse, and KWS Orbit. To assess the correlations between the observed field infection level and *Ustilago nuda* detected in seeds with the embryo test and qPCR method, we focused on three different cultivars: Azrah, Semper and SU Celly. For each of these cultivars, we used two seed lots including a more infected and less infected lot. The more infected lots were mixed with the less infected lots in the following proportions: 10:90 and 1:99. The original two seed lots plus these two mixtures resulted in four infection levels for each cultivar, which were then sown in the field and analyzed using the qPCR method and the embryo test.

Next, we conducted an experiment to identify whether the results from the qPCR method or the embryo test on barley seed provided a more accurate prediction of *U. nuda* infection levels in the field. For this experiment, we used the same three commercially available lots used to establish a representative seed lot sample and three additional commercially available seed lots from the cultivars, Maltesse, Esprit, and SU Celly. The commercially available SU Celly seed lot used in this part of the study differed from the two seed lots used in the previous experiment explained above.

In all experiments, the seeds from each 25 kg seed lot were placed in 12 cm x 60 cm x 80 cm containers so that the subsamples could be collected randomly. Sufficient subsample quantities for all field trials and laboratory detection methods (i.e. embryo test and qPCR method) were collected together at the beginning of our study to ensure that the same subsample was used throughout the study. For each seed lot and seed lot mixtures, 200 g of seed (approximately 4000 seeds) were sent to the Mycology Laboratory at the Bavarian State Research Center for Agriculture, where the embryo test was performed. For the qPCR method, the seeds — 2000 or 7000 seeds depending on the test conducted — were milled (CT 293 Cyclotec; Labtec Line, Denmark). To avoid cross-contamination a thorough cleaning of the mill with compressed air was performed between each seed sample.

Field trial set-up

The field trials were conducted at Agroscope in Zurich, Switzerland over two growing seasons for each seed lot. The trials took place during 2021-2022 and 2022-2023 for the cultivars Azrah and Semper, while SU Celly and the six commercially available seed lots were sown for the growing seasons 2022-2023 and 2023-2024. Each growing season, the field location was changed to comply with crop rotation practices. Seeds were sown in the field during the first week of October (Supplementary Information, Figure 3). Each seed lot and seed lot mixture was sown with four replicates in completely randomized blocks. Seeds were sown at a density of 350 seeds/m^2 and at a depth of 3-4 cm.

Seed lots and their mixtures, used to establish the correlation between the results of the laboratory detection methods and the observed field infection levels, were sown in 9.0 m^2 plots. Prior to the flowering stage, approximately 0.65 m from the short ends of each plot was trimmed with a motor mower. The shortened plots facilitated the assessment of infections and ensured

plot separation. Following the trimming, the average final plot size was 5.1 m^2 that was based on the measured plot length and assuming a fixed width for each plot of 1.1 m. Commercial seed lots, used to evaluate the laboratory methods' performance for *Ustilago nuda* detection, were sown in larger plots of approximately 20 m^2. Gaps were left between plots during the sowing to ensure consistency in plot size. Plot dimensions were measured at plant maturity, and the average plot size was 19.1 m^2, which was calculated by measuring each plot length and considering a fixed plot width of 1.1 m.

At the second leaf stage (Supplementary Information, Figure 3), germination rates were measured by counting seedlings along a linear meter multiple times within each plot. To minimize boundary effects, the counts were taken at two points along the second and sixth rows (of seven rows) and approximately 1 meter from the short plot edges. In the larger plots, this procedure was slightly adjusted, and counts were conducted at six points per plot instead of two to account for the size difference. During the flowering stage, ears were counted in a meter long frame that covered an area of 0.3 m^2. For the ear counts, plot edges were avoided, and ears were counted in the second and sixth rows (of seven rows). Two and six sampling points per plot were used for the 5.2 m^2 and 19.4 m^2, respectively. From this ear count, the total number of ears per plot was estimated by averaging the number of ears within the each 0.3 m^2 sampling area and scaling it to the measured plot size. In the flowering stage (Supplementary Information, Figure 3), the total number of infected ears was recorded within each entire plot. The number of infected ears was divided by the estimated total number of ears to calculate each plot's infection rate.

Embryo test implementation

The International Seed Testing Assosiation (ISTA) method "Detection of *Ustilago nuda* in *Hordeum vulgare* subsp. *vulgare* (barley) seed by dehulling and embryo extraction" [24] was used for the embryo test. This examination was conducted on 1000 embryos from the seed lots and seed lot mixtures that had been sown in the field and tested with the qPCR method. For the seed samples of the cultivars Azrah and Semper, the same extracted embryos were examined by three different evaluators (Supplementary Information, Table 9).

Sample preparation for the DNA extraction

To establish a representative flour sample from each seed lot, a series of tests was conducted on the following milled commercial seed lots: Maltesse A, Esprit A, and KWS Orbit. The optimal seed quantity for milling and the appropriate amount of flour for DNA extraction were assessed using ten subsamples each collected from ten different milled flour batches. Additionally, the uniformity of infection across milled flour batches was evaluated by extracting ten subsamples from either one or ten milled flour batches. For the seed quantity test, we analyzed two sample sizes: 2000 seeds and 7000 seeds. The 2000 seed sample corresponds to the number of seeds used in the working sample of the embryo test [24], while the 7000 seed sample corresponds to the number of seeds sown in a field trial in 20 m^2 plot and sowing density of 350 seed/m^2. To establish the flour amounts required for DNA extraction, 0.2 g and 0.02 g samples of 2000 milled seeds were used; the number of seeds was selected based on the previous

test's results that showed no significant difference between 2000 and 7000 seeds in *Ustilago nuda* detection. To assess the uniformity of infection within and between flour batches, DNA was extracted from 0.02 g of flour samples obtained by milling 2000 seeds; the amount of flour was established in the previous test that showed no significant difference in *U. nuda* detection between the two flour amounts. These flour samples were collected in two ways: ten times from a single flour batch, and once from ten separately milled flour batches. Based on this series of tests, a sampling protocol was established that consisted of milling 2000 seeds and extracting DNA from ten 0.02 g subsamples of flour from a single batch.

Seed sample DNA extraction

The milled seed samples of 0.02 g and 0.2 g were extracted using the NucleoSpin 96 Plant II Mini kit (MACHEREY-NAGEL, Germany) according to the manufacturer's protocol with the following modifications. For both flour amounts, a 3 mm tungsten bead (Retsch, Germany) was added to facilitate mechanical homogenization during the extraction process. For the extractions of 0.02 g flour, the flour was added to the NucleoSpin lysis tube strips. The initial homogenization and mechanical destruction of the sample was performed with the Tissue Lyser II (Qiagen, Germany) at a rate of 30 1/s for four consecutive 1-minute intervals, and following each interval, the orientation and position of the samples were changed. Cell lysis was performed with 600 µL of PL1 buffer provided with the kit, and the samples were mixed four times with the Tissue Lyser II (Qiagen, Germany) before adding 12 µL of RNase A (concentration 12 ng/µL). Subsequently, the samples were gently mixed and placed for 1 hour at 65 °C in a LAUDA E200 heated water bath (Lauda Dr. R. Wobser GMBH & CO. KG, Germany) and then gently mixed. Following the washing steps as described in the manufacturer's protocol, each sample was eluted two times with 100 µL of elution buffer from the kit, yielding a final volume of 200 µL.

For the extractions of 0.2 g flour, further modifications were taken in the cell lysis step to account for the increased volume of material. A 50 mL Falcon tube was used for the homogenization and mechanical disruption step, which was conducted with the Bead Ruptor 24 (Omni International, USA) at a speed of 2.1 m/s for three 1-minute intervals. After each 1-minute interval, the sample orientation and position were changed. The amounts of PL1 buffer and RNAse A were increased to 4.03 mL and 66.78 µL, respectively, to account for the increased sample dry weight. The three different sized NucleoSpin Plant II kits' maximum recommended sample dry weights were used to interpolate the reagent volumes for the 0.2 g sample. Following centrifugation, 400 µL of lysate was transferred to the next step according to the kit's instructions for extractions of 0.02 g of plant material. The remainder of the extraction was carried out as described as above for the 0.02 g flour DNA extraction.

Ustilago nuda and *Hordeum vulgare* DNA quantification

A series of gBlock^{ő} double-stranded synthetic oligonucleotides fragments (Integrated DNA Technologies, USA) was prepared with a 5-fold serial dilution to create a standard curve for absolute quantification (Supplementary Information, Table 13). Distinct 125 bp gBlock^{ő} fragments were synthesized for both the *Hordeum vulgare* and the *Ustilago nuda* targets. These

fragments included 12 bp and 10 bp flanking regions on both sides of the COX3 and GAPDH amplicons, respectively. The gBlock^{ő} fragments were initially diluted to a concentration of 0.03 ng/µL and stored at -20 °C with 0.2 mg/mL Poly(A) (Sigma-Aldrich, Germany). The diluted gBlock^{ő} fragments were then divided into aliquots suitable for a single plate to limit freeze-thaw cycles. The standard curve copy numbers, calculated from the molecular weight of the gBlock^{ő} fragments (Supplementary Information, Table 13), ranged from 62500 to 0.8 for COX3 and from 1563000 to 500 for GAPDH.

To replicate the PCR reaction conditions, template DNA from uninfected *H. vulgare* seedlings was included in the *U. nuda* standard curves, and *Lens culinaris* flour was added to the *H. vulgare* standard curve. The final amount of DNA in the standard curves was 50 ng in each reaction. Each qPCR plate included technical triplicates of the standard curves, experimental samples, and controls. The controls run on each plate included water and the non-target template DNA (i.e. uninfected *H. vulgare* seedlings and *L. culinaris* seed for the COX3 and GADPH standard curves, respectively) that had been used in the standard curves.

DNA sample concentrations were measured using a Varian Cary Eclipse Spectrophotometer (Agilent, USA). Samples were diluted to a concentration of 12 ng/µL using a Pipetmax Gilson 268 system in combination with Trilution software (Gilson, USA). The qPCR reactions were conducted in a final volume of 10 µL per well, containing 50 ng of DNA, 0.2 μM of each COX3 primer and probe, 0.1 μM of each GAPDH primer and probe, and 5 µL of GoTaq Probe qPCR Master Mix (Promega Corporation, USA). The qPCR was performed using white 384-well plates sealed with adhesive optical sealing foil (Nolato TreffLab, Sweden). The thermal cycling conditions were initiated with a denaturation step at 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 15 seconds and 65.3 °C for 30 seconds. All reactions were run on the CFX Opus 384 Real-Time PCR System (Bio-Rad, USA). The gene copy numbers for COX3 and GAPDH in each sample were determined using CFX Maestro 2.2 software (version 5.2.008.0222) by comparing the quantification cycle (Cq) values to the corresponding standard curve.

The limit of detection (LOD) for *U. nuda* was established by running eight series of standard curve dilutions. Experimental samples were repeated when the standard deviation of the Cq values across triplicates exceeded 0.5 for either gene's quantification. However, samples were not repeated if the average triplicate measurement for *U. nuda* was below the LOD for COX3, as the high standard deviation was likely due to the low levels of *U. nuda* target DNA. In cases where *U. nuda*'s COX3 measurement was below the LOD, *U. nuda* was considered to be undetectable, and a copy number of 0 was used in the statistical analysis. We chose to use 0 as the copy number for samples with undetectable levels of *U. nuda* to ensure consistency among the different detection methods (embryo test, qPCR method, and field observations). Both the field and embryo tests consider detection from 0, meaning that an undetectable result does not necessarily indicate the absence of infection, but rather an infection load below the detection threshold. The same baseline was used to provide a meaningful comparison to the reference (field) and embryo tests, which both start at 0. The infection level in each sample was calculated by taking the ratio of the mean copy number of *U. nuda* COX3 to the mean copy number of *H. vulgare* GAPDH.

Data and statistical analyses

Establishment of a representative seed lot sample for *Ustilago nuda* qPCR detection

All statistical analyses were conducted using R version 4.3.3. Three key sampling parameters were compared and analyzed with Kruskal-Wallis test to establish the sampling procedure used in the rest of the study. The tested parameters were: the seed number (2000 versus 7000), the flour amount (0.2 g versus 0.02 g), and the number of flour batches used for the ten DNA sub-sample extractions (i.e. one batch extracted ten times or ten batches extracted one time each).

Determination of a tolerance threshold

The minimum number of embryos examined for the embryo test is 1000 per sample [24]. With a sample size of 1000 embryos, infections levels under 0.1% cannot be detected [25]. Based on the lowest infection rate possibly detected with the embryo test from 1000 seeds, a minimum field tolerance threshold (number of infected ears acceptable in a given field area) was derived using the equation below:

$$\text{Field area } (m^2) \times \text{Tolerable embryo infection rate } (\%) \times \text{Field sowing density } (\text{seeds}/m^2) \times \text{Number of infected ears per infected seed}$$

Although one infected seed potentially results in zero or multiple infected ears, we conservatively assumed that one infected seed produces one infected ear to calculate the field tolerance threshold. This assumption was supported by our observations (Supplementary Information, Fig. 2). Additionally, based on our sowing rate of 350 seed/m^2 and the use of 0.1% as the tolerable embryo infection rate, the field tolerance threshold was calculated to be 35 infected ears per 100 m^2 of field area. The germination of all seeds was assumed because the embryo test does not account for a potential reduction in germination. This field tolerance threshold was used to derive the qPCR method tolerance threshold. To connect field infection levels to expected results of the qPCR method, we used the relationship between the naturally infected Azrah, Semper and SU Celly seed lots and their mixtures' field data to their qPCR results. We used these data to derive a qPCR tolerance threshold that corresponds to 35 infected ears per 100 m^2. Linear, polynomial and exponential correlations were tested to evaluate the relationship between the field observations and the qPCR method. The linear correlation was selected based on the adjusted R^2, the mean squared error (MSE), the Akaike information criterion (AIC) and the Bayesian information criterion (BIC). The conversions of the tolerance thresholds from embryo test to the field observations and from the field observations to the qPCR method allowed us to indirectly compare both laboratory detection methods to each other.

Performance evaluation of the laboratory detection methods: qPCR method and embryo test

The observed infected ears in the field were used as a reference to evaluate the performance of the laboratory detection methods, including the qPCR method and embryo test, to detect

Ustilago nuda in six commercially available seed lots. Seed germination rates were compared among seed lots with a Kruskal-Wallis test. We classified whether a seed lot was below or above each respective tolerance threshold: 0.1% for the embryo test, 7.50×10^{-5} *U. nuda*/*H. vulgare* DNA copies for the qPCR method, and 35 infected ears per 100 m^2 for the field observation.

We constructed confusion matrices to compare the actual values from the binary classifications of the field results to the predicted values from the binary classifications of each laboratory detection method (Supplementary Information, Table 8). Samples for which the laboratory detection method correctly classified as above the threshold (based on the reference field results), were assigned as true positives (TP), while samples that both methods classified as below the threshold were considered true negatives (TN). False negatives (FN) were assigned to cases with infections above the threshold in the field but incorrectly measured as below the threshold according to the respective laboratory detection method. Conversely, samples that were below the tolerance threshold in the field but were classified as above the threshold by the respective laboratory detection method were considered false positives (FP).

Each laboratory detection method's confusion matrix was used to calculate performance metrics — sensitivity, specificity, precision, the false discovery rate, and accuracy — to directly compare the qPCR method and the embryo test. Sensitivity and specificity measure the ability of the laboratory detection methods to correctly classify the samples as above or below the tolerance thresholds, respectively, while precision and false discovery rate evaluate the reliability of the laboratory detection methods' predicted value. Accuracy, on the other hand, represents the overall proportion of the correctly classified samples by the laboratory detection methods. We calculated these performance metrics with the following formulas:

$$\text{Sensitivity } (\frac{\text{TP}}{\text{TP + FN}});$$

$$\text{Specificity } (\frac{\text{TN}}{\text{TN + FP}});$$

$$\text{Precision } (\frac{\text{TP}}{\text{TP + FP}});$$

$$\text{False discovery rate } (\frac{\text{FP}}{\text{FP + TP}});$$

$$\text{Accuracy } (\frac{\text{TP + TN}}{\text{TP + TN + FP + FN}}).$$

Data Availability

All data collected from the experiments and used in the analyses are available in the Supplementary Information or the provided data tables.

Acknowledgements

We thank Nicole Bischofberger and Damian Amrein for assistance in acquiring seed material, and Francesco Bassi and Magnus Wagner for help with sample processing. We are grateful to Prof. Gunther Doehlemann (University of Cologne) for *Ustilago hordei* and *Ustilago maydis*

strains, and Marco Wüthrich for providing cultures of *Tilletia caries*, *Tilletia controversa*, *Ustilago* spp., *Tilletiopsis* spp., *Pseudozyma* spp., *Entyloma* spp., and *Holtermaniella* spp. We acknowledge Matteo Selmi for providing uninfected barley seeds from the "Mixture Ceccarelli" unknown cultivar, grown in Loritto, Italy. We thank Daniel Fuchs and his team for assistance with field trials and German Bonilla-Rosso for statistical discussions. We thank Joëlle Schläpfer for her comments on the manuscript, and we appreciate the early discussions with Annette Büttner-Mainik.

Funding

This research was supported by Fondation Sur-la-Croix, IP Suisse, the Swiss Association of Cereal Producers, Swiss granum, and swisssem.

Author contributions statement

Conceptualization: C.P. and K.E.S; Formal analysis: C.P. and K.E.S; Funding acquisition: K.E.S with support from S.V.; Investigation: C.P., E.J., I.B., P.B., A.K., and K.E.S.; Methodology: C.P., E.J., and K.E.S; Project administration K.E.S.; Resources: S.V., T.H., and F.W.; Supervision: K.E.S. and D.C.; Visualization: C.P. and K.E.S.; Writing-original draft preparation: C.P. with support from K.E.S; Writing-review & editing: C.P., K.E.S., D.C., S.V., T.H., F.W., P.B., E.J., I.B., and A.K.

Additional information

Competing interests The authors declare no competing financial and/or non-financial interests in relation to the work described.

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Supplementary Information: A new molecular seed assay to predict *Ustilago nuda* field infection levels

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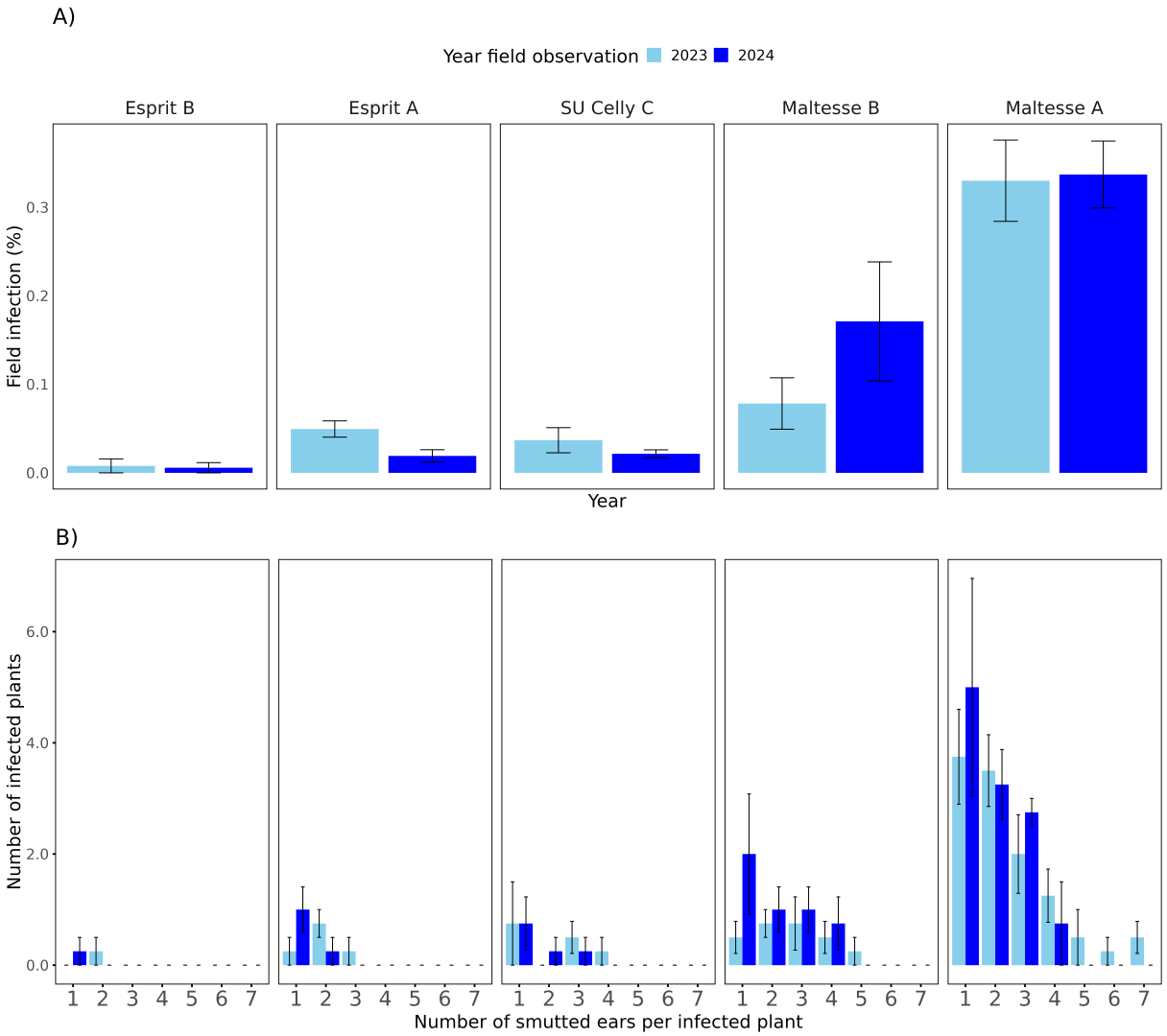
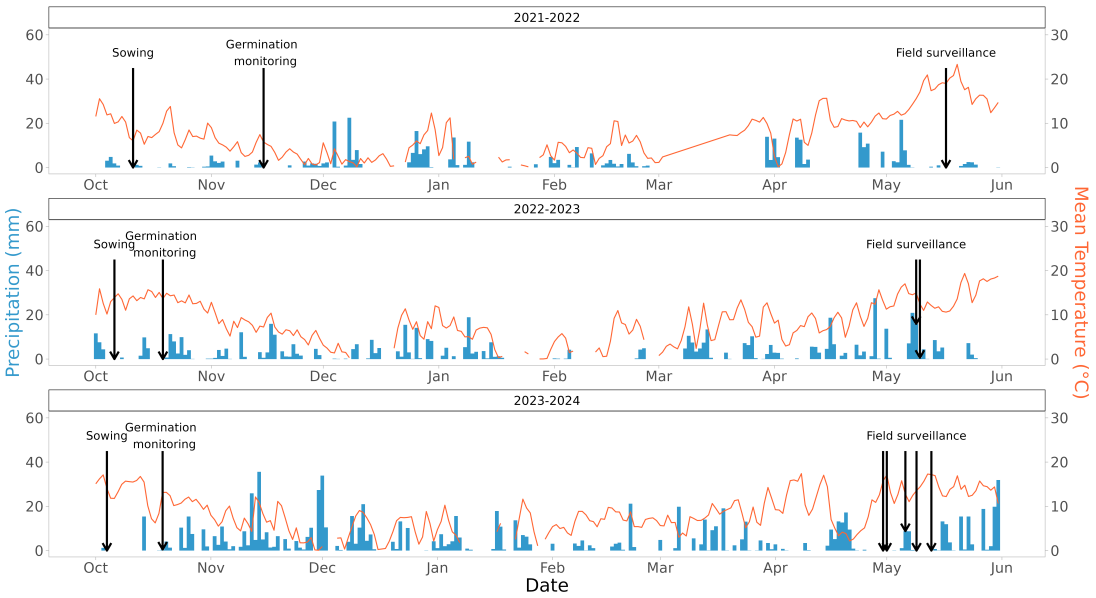
Supplementary Figures

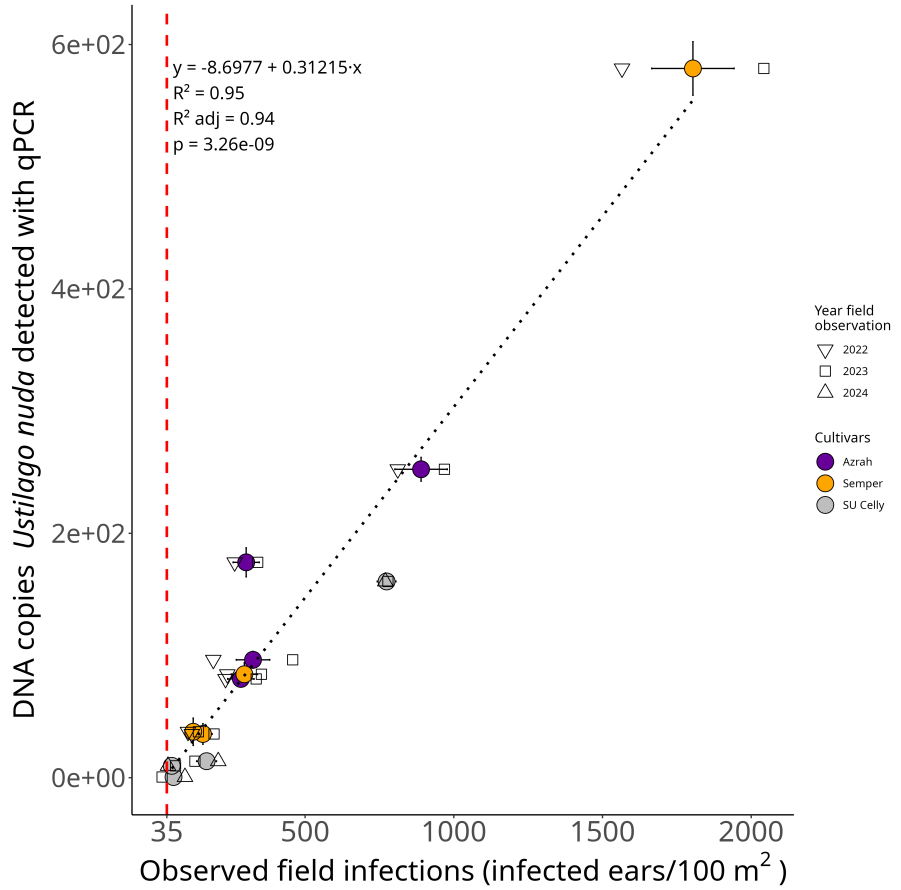
Supplementary Figure 1: Alignment of COX3 target gene sequences. The GenBank accession numbers for sequences included in the alignments are shown in sequence names, including the *Ustilago nuda* reference sequence, HQ013017.1. We included closely related *Ustilago* species and additional fungi species that may be amplified based on the Primer BLAST program. Primers (unCOX3_F and unCOX3_R) are highlighted in two green colors, while the probe is highlighted in light blue (unCOX3_P).



Supplementary Figure 2: *Ustilago nuda* field infection levels and number of smutted ears per infected plant. Five of the six commercially available seed lots showed smutted ears. The number of smutted ears per plant was evaluated across two years of field observations: 2023 (light blue) and 2024 (dark blue). (A) Field infection levels from each seed lot, and (B) the distribution of smutted ears per infected plant. In both panels, error bars represent the standard error. The sixth commercially available seed lot, KWS Orbit, had no observed field infections.



Supplementary Figure 3: Weather data collected during field trials. The weather measurements were taken at the MeteoSwiss station Zurich/Affoltern, which was closest to the fields. Daily mean temperature (°C) is shown as an orange line and total precipitation (mm) per day as blue bars. Arrows indicate the dates of seed sowing, germination monitoring, and field surveillance.



Supplementary Figure 4: Evaluation of the relationship between the *Ustilago nuda* infection observed in the field and measured using qPCR method without *Hordeum vulgare* normalization. Compared to Figure 2, which shows the relationship between the observed infection in the field and the *U. nuda* normalized to *H. vulgare* DNA copies measured, this figure shows non-normalized COX3 measurements.

Supplementary Tables

Source	Infection status	Concentration (ng/µL)[a]	Newly developed COX3 TaqMan protocol			Published ITS SYBR green protocol [b]		
			Cq Mean[c]	Cq Std. Dev [d]	CV (%) [e]	Cq Mean	Cq Std. Dev	CV (%)
***Hordeum vulgare* seedling**	*Ustilago nuda* infected	5.00	25.43	0.11	0.44	27.92	1.13	4.05
	Uninfected		-	-	-	-	-	-
***Hordeum vulgare* seed**	*Ustilago nuda* infected	5.00	29.74	0.09	0.29	27.97	0.74	2.66
	Uninfected		-	-	-	-	-	-

Supplementary Table 1: Comparison of relative variance in *Ustilago nuda* amplification with our newly designed COX3 primers in a Taqman protocol and the published ITS primers in a SYBR green protocol. Our COX3 primers were included in a multiplex reaction, and the published ITS primers were run in a singleplex reaction to detect *U. nuda*. A difference between the coefficients of variation (CV) were found between the tested protocols. A sample each of infected and uninfected *Hordeum vulgare* seeds and seedlings was used. Seed DNA was extracted from a 0.02 g flour subsample of 2000 milled seeds. The seeds were of the cultivar KWS Orbit; the seed lot was not otherwise included in this study. Seedlings were grown from a SU Celly seed lot used in this study, which had been treated with warm water to obtain an uninfected sample. The tested seedling DNA samples were extracted from a pool sample of 20 seedling segments (0.5 cm each) cut from the base of seedlings grown in soil for 5 days (uninfected seedlings) and 14 days (infected seedlings). Each sample was analyzed with triplicate reactions using the same template DNA concentration for both protocols. All uninfected samples that showed no amplification and are indicated by the symbol '-'.

[a] Final DNA concentration in each qPCR reaction

[b] SYBR Green Protocol with ITS primers published in a previous study[59]

[c] Average cycle of quantification across replicates

[d] Standard deviation of the cycle of quantification among replicates

[e] Relative variance or coefficient of variation ($^{\text{Cq Std. Dev.}}/_{\text{Cq Mean}}$)*100

Low-infected seed lot (%)	High-infected seed lot (%)	Cultivars		
		Azrah	SU Celly	Semper
		($\overline{Cq}$ $\overline{Mean}$ ± Std. Dev.[a])		
100	0	24.26 ± 0.52	25.48 ± 0.16	24.42 ± 0.42
99	1	24.36 ± 0.66	25.63 ± 0.24	24.53 ± 0.45
90	10	24.62 ± 0.17	25.53 ± 0.25	24.38 ± 0.43
0	100	24.39 ± 0.46	25.61 ± 0.28	24.50 ± 0.36

Supplementary Table 2: Evaluation of *Hordeum vulgare* GAPDH target stability in three cultivars with different *Ustilago nuda* infection levels. This evaluation is based on HEX fluorescence measurements targeting *H. vulgare* GAPDH and are reported as the average of the mean quantification cycle (Cq Mean) ± standard deviation. Average Cq Mean values were calculated for high- and low-infected seed lots from three cultivars and their mixtures. These seed lots and mixtures are part of an experiment designed to assess the relationship between *U. nuda* infection levels observed in the field and those detected using laboratory methods (Figure 2).

[a] Average of the mean quantification cycle (Cq) values across tested seed lots ± standard deviation among the seed lot subsample Cq Mean

Seed number tested	Flour amount for DNA extraction (g)	Number of flour batches tested	Cultivars					
			Esprit A	KWS Orbit	Maltesse A	Esprit B	SU Celly C	Maltesse B
			($\overline{Cq\ Mean}$ ± Std. Dev.[a])					
2000	0.02	1	24.70 ± 0.17	24.66 ± 0.52	25.74 ± 0.52	24.68 ± 0.38	25.49 ± 0.21	25.75 ± 0.38
		10	24.50 ± 0.18	24.64 ± 0.32	25.42 ± 0.21			
	0.2		24.70 ± 0.31	24.60 ± 0.34	25.63 ± 0.45			
7000	0.02		24.58 ± 0.30	24.63 ± 0.33	25.65 ± 0.39			

Supplementary Table 3: Evaluation of *Hordeum vulgare* GAPDH target stability in six commercial seed lots. This evaluation is based on HEX fluorescence measurements targeting *H. vulgare* GAPDH and the results are reported as the average of the mean quantification cycle (Cq Mean) ± standard deviation. Average Cq Mean values were calculated for six commercial *H. vulgare* seed lots. These seed lots were used for the comparison of *U. nuda* infection obeserved in the field to those measured using laboratory detection method 3. In this comparison, each seed lot was represented by 10 technical replicates from 1 milled flour batch of 2000 seeds, and 0.02 g of flour used for each DNA extraction. Three of the six seed lots (Espirit A, KWS Orbit, and Maltesse A) were additionally used in an experiment to test key parameters for establishing a representative *H. vulgare* seed lot samples for *Ustilago nuda* qPCR detection (Figure 1). For these three seed lots, the average Cq Mean values are reported for each tested parameter level, which included different seed number (2000 and 7000 seeds), flour amount for DNA extraction (0.02 and 0.2 g),the number of flour batches used for ten DNA sub-sample extraction (one batch extracted ten times or ten batches extracted one time each).

[a]Average of the mean quantification cycle (Cq) values across tested seed lots ± standard deviation among the seed lot subsample Cq Mean

qPCR plate	*Ustilago nuda* standard curve (COX3)				*Hordeum vulgare* standard curve (GADPH)			
	Efficiency	Coefficient of determination (R^2)	Slope	y-intercept	Efficiency	Coefficient of determination (R^2)	Slope	y-intercept
mpl31	0.996	0.986	-3.331	37.488	0.923	0.998	-3.521	39.114
mpl32	1.042	0.993	-3.225	37.279	0.923	0.996	-3.522	38.934
mpl33	0.985	0.981	-3.358	37.827	0.882	0.998	-3.640	40.330
mpl34	1.043	0.969	-3.222	43.496	0.923	0.993	-3.520	46.483
mpl35	0.978	0.977	-3.375	37.877	0.905	0.998	-3.579	39.837
mpl36	1.019	0.988	-3.277	37.699	0.900	0.999	-3.587	39.813
mpl37	0.961	0.974	-3.418	37.971	0.885	0.999	-3.634	40.369
mpl38	0.955	0.998	-3.434	38.417	0.905	0.994	-3.574	40.459

Supplementary Table 4: Standard curve parameters. In each qPCR plate, gBlock double-stranded synthetic oligonucleotide fragments prepared in a 5-fold serial dilution were used to generate standard curves for absolute quantification of the *Hordeum vulgare* GADPH gene region and the *Ustilago nuda* COX3 gene region. The *U. nuda* COX3 target was detected with a FAM-labeled probe, and the *H. vulgare* GADPH target with a HEX-labeled probe. For each standard curve were calculated using CFX Maestro software (Bio-Rad) the parameters: amplification efficiency, coefficient of determination (R^2), slope, and y-intercept.

Seed lot	Key parameter	Parameter level	Mean measured infection (*U. nuda* / *H. vulgare* DNA copies)	Min measured infection (*U. nuda* / *H. vulgare* DNA copies)	Max measured infection (*U. nuda* / *H. vulgare* DNA copies)	Median measured infection (*U. nuda* / *H. vulgare* DNA copies)	Std. Error	Std. Deviation	CV (%)[1]	Kruskal test	
										χ^2	p-value
Esprit A	Seed number tested[2]	2000 seed	4.8×10^{-4}	0.0	3.1×10^{-3}	0.0	3.4×10^{-4}	1.1×10^{-3}	221.3	0.53	0.47
		7000 seed	8.2×10^{-5}	0.0	8.2×10^{-4}	0.0	8.2×10^{-5}	2.6×10^{-4}	221.3		
	Flour amount for DNA extraction[3]	0.02 g flour	4.8×10^{-4}	0.0	3.1×10^{-3}	0.0	3.4×10^{-4}	1.1×10^{-3}	221.3	0.31	0.58
		0.2 g flour	3.5×10^{-4}	0.0	1.7×10^{-3}	0.0	1.8×10^{-4}	5.6×10^{-4}	159.2		
	Flour batch number for DNA extraction[4]	1 batch	0.0	0.0	0.0	0.0	0.0	0.0	-	2.11	0.15
		10 batches	4.8×10^{-4}	0.0	3.1×10^{-3}	0.0	3.4×10^{-4}	1.1×10^{-3}	221.3		
KWS Orbit	Seed number tested	2000 seed	0.0	0.0	0.0	0.0	0.0	0.0	-	-	-
		7000 seed	0.0	0.0	0.0	0.0	0.0	0.0	-		
	Flour amount for DNA extraction	0.02 g flour	0.0	0.0	0.0	0.0	0.0	0.0	-	1.00	0.32
		0.2 g flour	8.7×10^{-5}	0.0	7.1×10^{-4}	0.0	7.1×10^{-5}	2.2×10^{-4}	256.4		
	Flour batch number for DNA extraction	1 batch	0.0	0.0	0.0	0.0	0.0	0.0	-	-	-
		10 batches	0.0	0.0	0.0	0.0	0.0	0.0	-		
Maltesse A	Seed number tested	2000 seed	5.8×10^{-3}	9.3×10^{-4}	1.3×10^{-2}	5.2×10^{-3}	1.4×10^{-3}	4.5×10^{-3}	76.5	0.21	0.65
		7000 seed	6.5×10^{-3}	1.3×10^{-3}	1.3×10^{-2}	6.7×10^{-3}	1.4×10^{-3}	4.4×10^{-3}	68.5		
	Flour amount for DNA extraction	0.02 g flour	5.8×10^{-3}	9.3×10^{-4}	1.3×10^{-2}	5.2×10^{-3}	1.4×10^{-3}	4.5×10^{-3}	76.5	1.120	0.290
		0.2 g flour	8.0×10^{-3}	2.7×10^{-3}	1.9×10^{-2}	6.0×10^{-3}	1.6×10^{-3}	5.1×10^{-3}	63.8		
	Flour batch number for DNA extraction	1 batch	1.2×10^{-2}	4.3×10^{-3}	4.3×10^{-2}	7.9×10^{-3}	3.7×10^{-3}	1.2×10^{-2}	95.1	2.64	0.10
		10 batches	5.8×10^{-3}	9.3×10^{-4}	1.3×10^{-2}	5.2×10^{-3}	1.4×10^{-3}	4.5×10^{-3}	76.5		

Supplementary Table 5: Evaluation of key parameters to establish a representative *Hordeum vulgare* seed lot sample for *Ustilago nuda* qPCR detection. The evaluated key parameters included: the number of seeds tested (2000 versus 7000), the amount of flour used for the DNA extraction (0.02 g versus 0.2 g), and the number of flour batches used for the ten DNA sub-sample extractions (one batch extracted ten times versus ten batches extracted one time each). The later analysis was used to assess inter- and intra-flour batch *U. nuda* DNA variation. The key parameters were tested with three different seed lots, which each consist in a different cultivar. The mean, minimum, maximum, and median measured infections detected in all parameter level are reported as the ratio $^{U.\ nuda}/_{H.\ vulgare}$ DNA copies. For each parameter level, the standard errors, standard deviation, coefficient of variation, and the Kruskal-Wallis test statistics (χ^2 and p-values) are reported. The Kruskal test p-values were used to evaluate whether differences between parameter levels were significantly different within cultivars. Coefficients of variation and Kruskal test results that were not possible to calculate due to zero values are indicated using the symbol "-".

[1] Coefficient of variation ($^{\text{Std. Dev. infected embryo observed}}/_{\text{Infected embryo observed Mean}}$*100

[2] For the key parameter of seed number tested, 10 separate flour batches were used for the DNA extractions of 0.02 g of flour.

[3] For the key parameter of flour amount, 10 separate flour batches were used for the DNA extractions of 2000 seeds.

[4] For the key parameter of flour batch number for DNA extraction, 0.02 g of flour milled from 2000 seeds were used.

Tested model	Lab method	R^2	R^2 adj	MSE[a]	AIC[b]	BIC[c]
Linear	qPCR	0.974	0.972	2.69×10^{-6}	-113.860	-112.405
	Embryo	0.756	0.732	0.420	29.645	31.099
Polynomial	qPCR	0.974	0.969	2.69×10^{-6}	-111.867	-109.927
	Embryo	0.844	0.810	0.268	26.253	28.193
Exponential	qPCR	-1.677	-	2.80×10^{-4}	40.129	41.584
	Embryo	0.791	-	0.361	31.767	32.675

Supplementary Table 6: Model evaluations used to derive the tolerance threshold for the qPCR method. The models are based on the correlation between *Ustilago nuda* infection detected with the qPCR method and field observations. The linear model was chosen for its predictive performance (highest adjusted R^2) and lower complexity (lowest AIC and BIC) compared to the polynomial and exponential models.

[a] Mean squared error
[b] Akaike information criterion
[c] Bayesian information criterion

Seed lot	Plot repetition	Year collecting data	Infection observed (infected ears per 100 m^2)	Tolerance threshold (infected ears per 100 m^2)	Plot infection compared to the tolerance threshold	Average infection (infected ears per 100 m^2)	Average infection compared to the tolerance threshold
KWS Orbit	1	2023	0	35	Below	0	Below
	2		0		Below		
	3		0		Below		
	4	2024	0		Below		
	5		0		Below		
	6		0		Below		
	7		0		Below		
	8		0		Below		
Esprit B	1	2023	0	35	Below	2	Below
	2		0		Below		
	3		0		Below		
	4	2024	10		Below		
	5		5		Below		
	6		0		Below		
	7		0		Below		
	8		0		Below		
Esprit A	1	2023	16	35	Below	10	Below
	2		10		Below		
	3		10		Below		
	4	2024	15		Below		
	5		16		Below		
	6		5		Below		
	7		0		Below		
	8		10		Below		
SU Celly C	1	2023	0	35	Below	11	Below
	2		16		Below		
	3		15		Below		
	4	2024	21		Below		
	5		11		Below		
	6		10		Below		
	7		0		Below		
	8		16		Below		
Maltesse B	1	2023	36	35	Above	44	Above
	2		10		Below		
	3		77		Above		
	4	2024	15		Below		
	5		97		Above		
	6		53		Above		
	7		57		Above		
	8		5		Below		
Maltesse A	1	2023	83	35	Above	136	Above
	2		203		Above		
	3		143		Above		
	4	2024	164		Above		
	5		129		Above		
	6		105		Above		
	7		163		Above		
	8		99		Above		

Supplementary Table 7: Consistency of *Ustilago nuda* observed infections in individual field plots compared to the field tolerance threshold. For six commercially available seed lots, the number of infected ears per 100 m^2 were assessed over two years in four field plot each year. The Infection level observed in each plot and their averages were classified as either above or below the field tolerance threshold.

A)

Predicted values (qPCR method)	**Actual values (Field reference)**	
	Above tolerance threshold	Below tolerance threshold
Above tolerance threshold	True Positive (TP): - Maltesse A - Maltesse B	False Positive (FP): —
Below tolerance threshold	False Negative (FN): —	True negative (TN): - Esprit A - Esprit B - SU Celly C - KWS Orbit

B)

Actual values (Field reference)	
Above tolerance threshold	Below tolerance threshold
True Positive (TP): —	False Positive (FP): —
False Negative (FN): - Maltesse A - Maltesse B	True negative (TN): - Esprit A - Esprit B - SU Celly C - KWS Orbit

Predicted values (Embryo Test)
Above tolerance threshold
Below tolerance threshold

Supplementary Table 8: Confusion matrices comparing the predicted values of the qPCR method and the embryo test with the actual values of the field observations. For the confusion matrix based on the (A) qPCR method, the predicted values of the *Ustilago nuda* infection levels were classified as either above or below the qPCR tolerance threshold: 7.50×10^{-5} *U. nuda/H. vulgare* DNA copies. For the confusion matrix based on the (B) embryo test, predicted values were classified as either above or below the tolerance threshold of 0.1% infected embryos. Seed lots were classified as either above or below the field tolerance threshold of 35 infected ears per 100 m^2. based on the number of observed ear infections in the field. These classifications served as actual values in the confusion matrices. The predicted values that matched the actual values were either true positive or true negative, while the predicted values that did not match the actual values were either false positive or false negative.

Cultivar	Low-infected seed lot (%)	High-infected seed lot (%)	Evaluator	Infected embryos observed	CV(%)[a]
Azrah	100	0	1	20	29.4
			2	22	
			3	12	
	99	1	1	28	21.6
			2	24	
			3	18	
	90	10	1	6	50.0
			2	6	
			3	2	
	0	100	1	22	20.4
			2	32	
			3	24	
Semper	100	0	1	10	63.0
			2	10	
			3	2	
	99	1	1	14	70.5
			2	10	
			3	2	
	90	10	1	40	28.8
			2	32	
			3	22	
	0	100	1	120	20.4
			2	96	
			3	80	

Supplementary Table 9: Inter-evaluator result differences in visual embryo test. Discrepancy in the number of *Ustilago nuda* infected embryos detected in eight seed samples by three independent evaluators with the validated International Seed Test Association test[185]. The seed samples were composed of 1000 embryos extracted from two different cultivars (Azrah and Semper), including low- and high-infected seed lots and their mixtures. The variability in the number of infected embryos among evaluators and seed samples was assessed using the coefficient of variation.

[a]Coefficient of variation ($^{\text{Std. Dev. infected embryo observed}}/_{\text{Infected embryo observed Mean}}$)*100

Seed lot	Sub-sample repetition	Measured infection (*U. nuda*/*H. vulgare* DNA copies)	Tolerance threshold (*U. nuda*/*H. vulgare* DNA copies)	Sub-sample infection compared to the tolerance threshold	Average infection (*U. nuda*/*H. vulgare* DNA copies)	Average infection compared to the tolerance threshold
KWS Orbit	1	0.0	7.5×10^{-5}	Below	0.0	Below
	2	0.0		Below		
	3	0.0		Below		
	4	0.0		Below		
	5	0.0		Below		
	6	0.0		Below		
	7	0.0		Below		
	8	0.0		Below		
	9	0.0		Below		
	10	0.0		Below		
Esprit B	1	0.0	7.5×10^{-5}	Below	0.0	Below
	2	0.0		Below		
	3	0.0		Below		
	4	0.0		Below		
	5	0.0		Below		
	6	0.0		Below		
	7	0.0		Below		
	8	0.0		Below		
	9	0.0		Below		
	10	0.0		Below		
Esprit A	1	0.0	7.5×10^{-5}	Below	0.0	Below
	2	0.0		Below		
	3	0.0		Below		
	4	0.0		Below		
	5	0.0		Below		
	6	0.0		Below		
	7	0.0		Below		
	8	0.0		Below		
	9	0.0		Below		
	10	0.0		Below		
SU Celly C	1	0.0	7.5×10^{-5}	Below	0.0	Below
	2	0.0		Below		
	3	0.0		Below		
	4	0.0		Below		
	5	0.0		Below		
	6	0.0		Below		
	7	0.0		Below		
	8	0.0		Below		
	9	0.0		Below		
	10	0.0		Below		
Maltesse B	1	0.0	7.5×10^{-5}	Below	9.1×10^{-4}	Above
	2	1.3×10^{-3}		Above		
	3	6.7×10^{-4}		Above		
	4	1.1×10^{-3}		Above		
	5	1.2×10^{-3}		Above		
	6	9.1×10^{-4}		Above		
	7	1.6×10^{-3}		Above		
	8	0.0		Below		
	9	8.1×10^{-4}		Above		
	10	1.5×10^{-3}		Above		
Maltesse A	1	1.5×10^{-2}	7.5×10^{-5}	Above	1.2×10^{-2}	Above
	2	5.4×10^{-3}		Above		
	3	9.5×10^{-3}		Above		
	4	8.2×10^{-3}		Above		
	5	4.3×10^{-2}		Above		
	6	4.3×10^{-3}		Above		
	7	1.7×10^{-2}		Above		
	8	6.9×10^{-3}		Above		
	9	7.6×10^{-3}		Above		
	10	7.8×10^{-3}		Above		

Supplementary Table 10: Consistency of *Ustilago nuda* infections detected with qPCR in individual DNA extractions compared to the qPCR tolerance threshold. For six commercially available seed lots, the measured infections were assessed with the ratio *U. nuda*/*H. vulgare* DNA copies in ten DNA extractions. The Infection level measured in each DNA extraction and their averages were classified as either above or below the qPCR tolerance threshold.

Template DNA	Template DNA concentration (ng/µL)[5]	Test	Spiked gBlock gene fragments	Spiked gBlock gene copy numbers	*Ustilago nuda* COX3		*Hordeum vulgare* GADPH	
					Cq Mean	Cq Std. Dev.	Cq Mean	Cq Std. Dev.
H. vulgare seed	5.0	qPCR inhibition	UnCOX3_stdcurve	62500	21.15	0.06	24.79	0.46
	2.5				21.23	0.18	26.04	0.63
	0.8				21.43	0.32	28.1	0.48
	5.0	Control	-	-	0.00	0.00	25.11	0.10
H. vulgare seedling	5.0	qPCR inhibition	UnCOX3_stdcurve	62500	21.16	0.13	24.20	0.28
	2.5				21.33	0.04	25.42	0.36
	0.8				21.29	0.10	28.94	0.27
	2.5	Control	-	-	0.00	0.00	25.78	0.15
Lens culinaris seed	5.0	qPCR inhibition	UnHvGADPH_stdcurve	1563000	0.00	0.00	17.51	0.12
	2.5				0.00	0.00	17.58	0.21
	0.8				0.00	0.00	17.45	0.18
	5.0	Control	-	-	0.00	0.00	0.00	0.00

Supplementary Table 11: Evaluation of potential qPCR inhibition. DNA from *Hordeum vulgare* seeds and seedlings, and *Lens culinaris* seeds were spiked with gBlock gene fragments. Uninfected *H. vulgare* seeds were obtained from an undefined cultivar "mixture Ceccarelli" grown in Loritto, Italy. DNA was extracted from a 0.02 g flour subsample of 2000 milled seeds. *H. vulgare* seedlings were grown in soil for 5 days from a seed lot of cultivar SU Celly that had been treated with warm water. DNA was extracted from a pooled subsample of 20 seedling segments (0.5 cm each). *L. culinaris* DNA was extracted from seeds intended for consumption.

Plant	Isolation sources	Closest accession and region	Database	Percent similarity (%)	Query cover (%)	Classification of closest BLAST result
Triticum aestivum	Seed	MN901739: 352-1175	Core nucleotide database (core_nt)	95.3	100	*Entyloma* spp.
Hordeum vulgare	Smutted head	JALCCE020000007:289349-290325	Whole genome shotgut contigs (Basidiomycetes)	100	100	*Pseudozyma flocculosa*
Triticum aestivum	seed	MW248454: 151-1076	Core nucleotide database (core_nt)	100	100	*Holtermanniella festucosa*
Hordeum vulgare	Smutted ear	DQ835992: 75-563	Core nucleotide database (core_nt)	96.33	100	*Tilletiopsis minor*
Glycine max	seed	LVYE01000169:3537-4519	Whole genome shotgut contigs (Basidiomycetes)	100	100	*Ustilago trichophora*

Supplementary Table 12: Information about fungal isolates from Swiss cereal samples. The fungi were isolated from either seeds or smutted ears and cultured on PDA plates to obtain mycelia. DNA was extracted from each isolate's mycelia and used to test the *Ustilago nuda* primer specificity (Table 1).

Gene target	Target organism	Oligo name	Sequence (5′ - 3′)	Length (bp)	Molecular weight (g/mol)	Genbank accession number
COX3	*Ustilago nuda*	UnCOX3_stdcurve	TCC GTT TGA GCT ACC ACT GCT TAA TAC GCT GCT GCT TCT ATC TTC AGG TGC AAC TGT AAC ATA TTC TCA CCA TGC TCT TAT CCA AGG AAA TCG ACG TGC AG	125	77105.0	HQ013017
GADPH	*Hordeum vulgare*	HvGADPH_stdcurve	CAG TTC ACG GCC ATT GGA AGC ACA GTG ACA TCA AGC TCA AAG ACG ACA AGA CGC TGC TCT TCG GCG AGA A G CCA GTT ACT GTC TTT GGC GTC AGG TAG TAT TGA T	125	77110.9	MT933276

Supplementary Table 13: Sequences of gBlock gene fragments used as the standard curve. The standard curves were included in all qPCR plates for the absolute quantification of *Ustilago nuda* and *Hordeum vulgare*. The copy numbers of each target were calculated from the absolute quantification and used to normalize the pathogen DNA to its host's DNA. The GenBank accession numbers identify the sequences used as the basis of the gBlock gene fragments.

Supplementary Methods

The fungal isolates provided by Marco Wüthrich were isolated from Swiss cereal, either from seeds or smutted ears. These fungal isolates were grown on PDA plates to obtain mycelia from which DNA was extracted with the NucleoSpin Plant II Kit (MACHEREY-NAGEL, Germany). The extracted DNA was amplified using the primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and LR5 (5′-TCCTGAGGGAAACTTCG-3′). Each PCR reaction had a total volume of 20 μl, containing 1× GoTaq Flexi Colorless Buffer, 0.4 μM of each primer, 2.5 μM $MgCl_2$, 0.2 mM dNTPs, and 0.05 U/μl GoTaq G2 Hot Start Polymerase (Promega, USA). A total of 15 ng of DNA template was used per reaction. Sanger sequences was performed by Microsynth (Balgach, Switzerland) using the ITS1 primer. Low quality regions at the sequence end were trimmed until the base pair quality scores were greater than 40. Sequences were then searched using BLAST against both the core nucleotide database (core_nt) and the whole-genome shotgun contigs (wgs) databases restricted to Basidiomycetes. The closest matches are reported and were used to deduce the isolate's identity that were used for primer specificity testing. Sequences will be deposited in NCBI (accession numbers of sequences to be provided prior to acceptance).

Chapter 2

Optimization of *Tilletia caries* and *Tilletia controversa* detection in soil and seed

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Unpublished manuscript

Abstract

Seed coatings with synthetic treatments can effectively protect crops from seed- and soilborne diseases. However, due to environmental concerns, there are increased efforts to reduce synthetic pesticide application. The prospect of reduced synthetic seed treatments underscores the need for effective preventive strategies. Sensitive and reliable pathogen detection tools can support such reductions. Current detection methods for seedborne pathogens, such as *Tilletia caries* (common bunt) and *Tilletia controversa* (dwarf bunt), primarily rely on seed health tests that often overlook soilborne teliospores as an inoculum source. Bunt teliospores in soil are typically not assessed because the existing methods are labor-intensive and technically demanding. To improve the detection of *T. caries* and *T. controversa* in seed and soil — their main transmission pathways, we developed multiplex qPCR and dPCR protocols targeting both pathogens with mitochondrial and nuclear primers. We quantified *T. caries* in naturally contaminated seed lots using a visual inspection method and dPCR. We then applied the

newly developed protocols to detect *T. caries* and *T. controversa* in artificially inoculated and naturally infested soils. We integrated an exogenous internal control in the qPCR soil protocol to monitor DNA extraction and PCR reaction efficiency. Both pathogens were successfully detected in seed and soil samples, and our results suggest that dPCR may be more sensitive than qPCR in detecting low amount of teliospores in soil. Mitochondrial primers were more specific and sensitive than nuclear primer sets, enabling the detection of 1-2 teliospores per seed sample and 100 teliospores per gram of soil. Overall, the proposed PCR-based protocols can provide a reliable tool that can improve *T. caries* and *T. controversa* detection in seed and soil. This approach supports more effective pathogen prevention through the avoidance of contaminated seeds and infested soil, thereby reducing a reliance on synthetic fungicides.

Introduction

Ensuring the health of cereal seeds is fundamental to plant disease prevention because it reduces disease incidence and the reliance on synthetic fungicidal treatments [1]. Seed health testing identifies potentially problematic seedborne pathogens and limits their spread in agricultural systems [2]. The growing adoption of preventive measures, such as seed health tests, aligns with current agricultural policies that aim to reduce the use of synthetic plant protection products (PPPs), including synthetic seed coatings [3, 4, 5, 6, 7, 8, 9]. Consequently, stakeholders are increasingly interested in the use of untreated seeds or seeds coated with low-risk or organic treatments [10, 11]. Reliable and sensitive diagnostic tools are an integral component of plant protection that are especially needed to support the use of untreated seeds.

Although seed health tests can identify many seedborne pathogens [12, 13], some of these pathogens have additional transmission pathways, such as through soil or wind, that tests on seeds cannot address (e.g., *Claviceps purpurea* [14] and *Alternaria* spp. [15]). Soilborne pathogens are challenging to manage because they can survive for extended periods, either as saprophytes on plant residues or as resistant or dormant structures [16, 17]. The viability of these pathogens can vary depending on their biology and abiotic and biotic factors [16, 17]. Spore survival can range from months (e.g. *Pythium Deliense* [18]) to even years (e.g. *Sclerotinia trifoliorum* [19]). Consequently, pathogen risk assessments based solely on seed health may underestimate the infection risk to crops when soil inoculum is present.

The wheat pathogens *Tilletia caries* (common bunt) and *Tilletia controversa* (dwarf bunt) have dual primary transmission pathways: seed and soil [20, 21]. The teliospores of *T. caries* and *T. controversa* can persist in the soil for up to five and ten years, respectively [21, 22]. *T. controversa*, in particular, can contribute long-term sources of soil inoculum [23, 24]. While both species have a similar life cycle, they differ in their ecological requirements for teliospore germination and infection. *T. controversa* requires prolonged periods of cool temperatures (5 °C for 20-30 days [23]) and low light intensity (30 µmol/s/m^2) for germination and infection [25] — conditions often associated with continuous snow cover [21]. In contrast, *T. caries* teliospores germinate within 3 days [26] and infect wheat coleoptiles shortly after seedling emergence [26, 27]. After the infection, both pathogens grow systemically within the host and ultimately replace healthy seeds with masses of teliospores that produce trimethylamine and are enclosed within the pericarp [28, 29]. Bunt diseases can cause direct yield losses of up to

80% and the presence of their teliopores reduces flour quality due to the fish-like odor associated with trimethylamine production [29, 30].

Current seed health testing for *T. caries* and *T. controversa* relies on seed visual inspections and the morphological identification of teliospores [12, 31]. These methods can produce results within a short time frame ($\simeq$ 24 h), but they require trained personnel [31]. The visual examination of a microscope slide is a laborious process, which restricts the sample throughput [31]. Even if seeds are considered healthy based on visual seed inspections, these seeds can still be susceptible to soilborne pathogens. Direct visual inspections of soil samples for *Tilletia* spp. [32] are not feasible on a large scale due to the time required to process and microscopically examine the samples.

Several diagnostic approaches have been developed to improve the detection of *T. caries* and *T. controversa* and support their management. These approaches include polymerase chain reaction (PCR)-based assays [31, 33, 34, 35, 36], enzyme-linked immunosorbent assays (ELISA) [34], and loop-mediated isothermal amplification (LAMP) [37, 38]. Most molecular assays target nuclear regions to achieve species discrimination [31, 33, 36, 37]. However, the published molecular assays have several limitations, including insufficient species specificity and an inability to distinguish between *T. caries* and *T. controversa* [31, 33, 37], amplification of excessively long fragments that can compromise qPCR efficiency [35, 39, 40], lack of quantitative output [33, 41], or limited validation in relevant matrices such as seed and soil [38].

Despite the utility of molecular assays, many of them lack the sensitivity required to detect low pathogen levels, such as a single teliospore per sample. This limitation in sensitivity probably results from the use of single-copy nuclear markers. Mitochondrial DNA is a promising target for pathogen detection due to its higher copy number relative to nuclear DNA. The higher copy number may improve sensitivity in detecting very low pathogen levels in a sample [42]. In parallel, advances in PCR technologies, particularly digital PCR (dPCR), reduce the impact of inhibitors and stochastic variation, thereby improving robustness and sensitivity [43]. The relationship between mitochondrial and nuclear targets of*T. caries* and *T. controversa* is unclear. Furthermore, a better understanding is needed of qPCR and dPCR assay performance in detecting these pathogens in naturally contaminated or infested samples. The complexity of soil DNA extraction underscores the importance of including non-target internal controls to verify extraction efficiency and amplification performance [44].

To overcome the limitations of existing diagnostic approaches, we developed and evaluated PCR primer sets that can detect and differentiate *T. controversa* from *T. caries* in both seeds and soils. We compared the performance of mitochondrial and nuclear primer sets, and assessed the robustness and sensitivity of qPCR and dPCR assays using naturally contaminated seed samples, artificially inoculated soil, and naturally infested field soil. Due to the difficulty of extracting and detecting DNA from soil, we further incorporated a synthetic exogenous internal control to monitor the efficiency of DNA extraction from soil and amplification performance. Through the examination of both seed- and soilborne transmission pathways, we aim to implement our primer sets as a complementary tools for use in either seed health certification protocols and environmental pathogen-risk level evaluations.

Material and Methods

Sample origin and preparation

Fungal mycelial and teliospore samples to test for primer specificity

The fungal mycelial samples from *Tilletia bromi* (CBS 123002), *Tilletia fusca* (CBS 122992), *Tilletia goloskokovii* (CBS 122995), *Tilletia puccinelliae* (CBS 122993), and *Tilletia vankyi* (CBS 121954) were acquired from the Westerdijk Institute (Utrecht, The Netherlands). *U. maydis* strain FB1 and *U. hordei* strain Mat2 were provided by G. Doehlemann (University of Cologne, Germany). The other mycelial samples isolates from unidentified species assigned to the genera *Pseudozyma*, *Tilletiopsis*, *Entyloma*, *Holtermaniella*, and *Ustilago* based on ITS and large ribosomal subunit regions, had been collected from Swiss seed samples. Their identities were deduced based on sequence similarity to other basidomycetes using BLAST searches against the core nucleotide database and whole genome shotgut contigs (Genbank Accession numbers: PX369863-PX369867). Further information about their isolation and amplification can be found in a previous study [45].

T. caries and *T. controversa* teliospores from bunted kernels collected during a 2022 pot trial in Reckenholz (Zurich, Switzerland) and a 2023 field trial in the Canton of St. Gallen, Switzerland, respectively. *U. nuda* originated from teliospores was collected from the *Hordeum vulgare* cultivar Cassia during a 2022 field trial at Reckenholz (ZH), Switzerland. K.-J. Müller (Cereal Breeding Research Darzau) provided the teliospores of *U. avenae*, *U. tritici*, and *U. horidei*.

Tilletia caries bunt panel samples

We used diverse bunt populations of *T. caries* to test newly designed primers that amplify *T. caries* or *T. caries* and *T. controversa*. We tested bunt populations collected from forty-seven bunted wheat ears across different countries, including Austria (5 samples), Denmark (8), Germany (15),Iran (6), Italy (2), Latvia (1), Sweden (6), and Switzerland (1). Three samples had unknown origins, two of which belonged to a historical collection from Sweden. The bunted ears were stored at room temperature ($\simeq$ 25 °C). Two bunt sori were removed from each bunted ear and placed in 2 ml screw-cap Eppendorf tube containing $\simeq$ 20 mg of diatomaceous earth and $\simeq$ 400 µL 1 mm glass beads for DNA extraction.

Wheat seed sample origin and preparation

Twenty-two wheat seed lots from the Swedish Board of Agriculture and ten wheat seed lots from Frökontrollen Mellansverige AB were tested in this study. The presence of *Tilletia caries* and *Tilletia controversa* teliospores in these seed lots was assessed using the seed wash and filtration method [12] prior to visual inspection. Briefly, 250 seeds were washed in $\simeq$ 15 mL of 0.2% sodium dihydrogen phosphate solution, and the wash solution was filtered through a 5 µM Millipore filter with 64 mm^2 area. The filter was checked in ten equal sectors with a 0.81 mm^2 area of field of view, and *Tilletia* spp. teliospores were counted under a light microscope at 100× magnification. Each filter was visually evaluated by three inspectors. The mean number of teliospores per seed was calculated using the following formula:

$$\frac{\left(\frac{\text{Number of } \textit{Tilletia} \text{ spp. teliospores counted on the filter}}{\text{Number of evaluated filter sectors}} * \left(\frac{\text{Filter area}}{\text{Field of view area}}\right)\right)}{\text{Number of tested seeds}}$$

The seed wash samples for DNA extraction were prepared following a modified version of the procedure outlined in Appendix D of the Laboratory Procedures for Karnal Bunt [46]. From each seed lot, 5 g of seeds ($\simeq$ 80/100 seeds) were collected in a 50 mL centrifuge tube (Sarstedt AG & Co, Germany) and mixed with 30 mL of extraction solution (deionized water containing 0.0667% [v/v] Tween 20). The samples were shaken with the extraction solution for 10 minutes at 250 rpm on an orbital shaker (Forma Scientific, USA). The suspension of the shaken samples was filtered through a 40 µM cell strainer (Falcon, USA) positioned over a clean 50 ml centrifuge tube (Sarstedt AG & Co, Germany). To recover residual teliospores, the centrifuge tubes containing the 5 g of seeds were rinsed with 2 ml of extraction solution. These tubes were then shaken briefly to re-suspend the possible remaining teliospores, and poured through the same 40 µM cell strainer. This rinse step to recover residual teliospores was repeated three times, during which approximately 1 mL of liquid was lost. The cell strainer was then rinsed once with 2 mL of extraction solution to maximize teliospores recovery before being discarded. The 40 µM filtrate was centrifuged at 1500 rpm ($\simeq$ 310 x g) for 5 min. Most of the supernatant was rapidly decanted to avoid disturbing the loose pellet. Additional centrifugation and decanting steps were applied as needed until the entire sample settled as a pellet. The wet pellet was transferred to 2 mL screw-cap containing $\simeq$ 20 mg of diatomaceous earth and $\simeq$ 400 µL of 1 mm glass beads. To maximize teliospore recovery, 800 µL of extraction solution (deionized water containing 0.0667% [v/v] Tween 20) was added to the 50 mL tube, and the rinse was transferred to the corresponding 2 mL screw-cap tube. The 2 mL screw-cap tubes were then centrifuged at 2000 rpm for 5 min, and the residual extraction solution was removed. Seed pellet samples were stored at -20 °C until DNA extraction.

Soil sample collection and preparation

Field soil was collected at the end of March 2022, from an experimental field at Agroscope (Zurich, Switzerland) with no recorded history of *Tilletia caries* or *Tilletia controversa* infection or experiments conducted with these pathogens. The collected soil was sieved through a 0.8 × 0.8 cm mesh and then stored at 5 °C. This served as the uninfested soil to which the *Tilletia caries* and *Tilletia controversa* inoculum was added for the dilution assays. We used this artificially infested soil as a proof of concept that the primer sets with probe TcarARP2 and RPB2, and the primers Tc2.5 without probe can be applied in soil.

The *T. caries* teliospores used to artificially inoculate the soil were obtained from bunted wheat ears grown in a pot experiment, which had been conducted in climate chambers in 2019 and 2024 using the cultivar Apogee. The *T. controversa* teliospores were collected in 2023 and 2024 as sori originating from a naturally infested field in the Canton of St. Gallen, Switzerland. The uninfested field soil was inoculated to achieve a concentration of 10^5 teliospores per gram of soil. Serial ten-fold dilution series of the inoculated soil were prepared for soil samples with the following concentrations: 10^5, 10^4, 10^3, 10^2, and 10^1 teliospores per gram of soil. Before each dilution, the inoculated soil sample ($\simeq$ 10 g) was homogenized for two minutes using a TURBULA Type T2A machine (WAB-GROUP, Switzerland).

The same sieved uninfested soil ($\simeq$ 278.5 g) was then inoculated with three different amount of *T. controversa* teliospores: 0.1 g ($\simeq 10^5$ teliospores/g soil), 0.001 g ($\simeq 10^3$ teliospores/g soil), 0.0001 g ($\simeq 10^2$ teliospores/g soil). Each of these amount of teliospores was inoculated in ten individual repetitions. All the repetitions of inoculated and uninoculated soil were individually homogenized for four minutes using TURBULA Type T2A machine (WAB-GROUP, Switzerland). Approximately 6 g of each homogenized sample was then collected and stored at -20 °C.

Soil naturally infested with *T. controversa* was collected in September 2023 in the Canton of St. Gallen, Switzerland, from a field with a documented history of this pathogen infection. A section of the cultivated area of such field (48 m x 4.5 m) was divided into 24 plots (6 m x 1.5 m), which were evenly distributed in three rows. Each row included eight plots, each measuring 6 m in length. One soil core (20 cm long and 2.5 cm in diameter) was collected from the center of each plot using a one-piece gouge auger. Each core was placed in a plastic bag and stored in a cooler during transport. The soil samples were sieved using a 5 × 5 mm mesh and frozen at –20 °C prior to DNA extraction.

DNA extraction of mycelial, *T. caries* bunt panel, seed washes, and soil samples

DNA extraction of mycelia and teliospores for testing primer specificity

DNA was extracted from mycelium and teliospores using the NucleoSpin Plant II Mini Kit (MACHEREY-NAGEL, GmbH & Co, Germany) according to the manufacturer's instructions. The following modifications were made to the initial disruption and lysis procedures for each sample source.

For mycelium DNA extraction, 100-200 mg of mycelium was carefully removed from the growth substrate, avoiding carry-over of substrate material, and transferred to a 1.5 µL tube containing approximately 0.5 g of 0.10 mm glass beads. The mycelium was mechanically disrupted using a FastPrep-24™ 5G (M.P. Biomedicals, USA) at 6 m/s for 45 s, twice. Then, 400 µL of PL1 buffer was added to the powdered mycelium, which was then vortexed for 5 s, and 12 µL of RNase A (12 ng/µL) was added. The samples were gently mixed manually before the incubation for 15 min at 65 °C with a Thermomixer Comfort (Eppendorf, Germany) at 300 rpm. After centrifugation at 11000xg for 10 min, 400 µL of the supernatant was recovered in a new 2 mL tube.

To extract teliospore DNA, approximately 100 mg of teliospores were placed in a 1.5 mL tube and re-suspended in 100 µL of PL1 buffer. The teliospores were crushed using a micropestle (Eppendorf, Germany). The micropestle was rinsed with an additional 300 µL of PL1 buffer to recover all of the teliospores. The samples were vortexed for 5 s, after which 12 µL of RNase A (12 ng/µL) was added and gently mixed. The samples were incubated for 15 min at 65 °C with shaking at 500 rpm. Then, the samples were centrifuged at 11000xg for 7 min, and 200 µL of the supernatant was transferred to a new 2 mL tube.

From this step, both sample sources were processed identically. The sample supernatants were mixed with 450 µL of binding buffer, and the subsequent purification steps were performed according to the kit instructions. Each sample was eluted in 50 µL of preheated elution buffer (65 °C). The purified DNA was stored at -20 °C until analysis.

DNA extraction of *T. caries* bunt panel and seed material

Both bunt sori and seeds underwent the same DNA extraction protocol using the NucleoMag Plant kit (MACHEREY-NAGEL, GmbH & Co, Germany, Germany) and the Mealstrom 4800 extraction robot (TANBead, Taiwan). A volume of 500 µL of lysis buffer MC1 was added to the crushed bunt sori or seed pellet samples. Each sample was homogenized using Precellys® 24 Touch (Bertin Technologies, France) at 4000 rpm for 40 seconds and centrifuge for 5 seconds at 13000 rpm. After the lysis step, 8 µL RNase A (12 ng/µL) and 1-3 µL proteinase K solution was added to each sample. The samples were vortexed and then incubated at 56 °C for 90 min using a Thermolyne thermostat block (Branstead/Thermolyne, USA). The samples were inverted every 30 minutes during the incubation period. After the incubation, the samples were centrifuged for 5 min at 130000 rpm.

The DNA extraction and purification steps were conducted using a Maelstrom 4800 automated nucleic acid extractor (TANBead, Taiwan) with the program 6SC, which required a 96-deep-well plates loaded with the kit reagents and magnetic beads. The wells were filled in the indicated order and volumes with the following reagents and magnetic beads: 400 µL MC2, 500 µL MC3, 500 µL MC4, 500 µL 80% ethanol, 20 µL of magnetic beads diluted in 400 µL water, and 100 µL of elution buffer MC6. The reagents and the magnetic beads were added to eight wells, and this filling process was repeated for the second half of the plate following the same order. After the automated purification step, the plate was placed on a magnetic rack. The purified DNA was transferred to a 1.5 ml tube (Eppendorf, Germany) and stored at -20 °C until use.

DNA extraction of soil material

Before DNA extraction, both the naturally and artificially infested soils — $\simeq$ 500 g and $\simeq$ 10 g, respectively — were homogenized manually and with a vortex for 2 min and 5 s, respectively. The homogenized soil samples were then placed in NucleoSpin Bead tubes provided in the NucleoSpin 96 Soil kit (MACHEREY-NAGEL, GmbH & Co., Germany). Each tube, which already contained ceramic beads, was filled with soil sample until it reached the 1 mL mark ($\simeq$ 500 mg), in accordance with the limit provided in the manufacturer's protocol. The metal spatula used to transfer the soil was sterilized using 70% ethanol and a flame between each sample. An exogenous internal control composed of NADH dehydrogenase 2 protein (ND2) from *Delphinapterus leucas* was added to each soil sample prior to DNA extraction. An amount of 5µL with the concentration of (4497512 copies/µL) was used. The soil samples were vortexed for 5 s each to homogeneously distribute the added synthetic target.

Because the NucleoSpin 96 Soil kit includes different suggested combination of lysis buffers and enhancers, we tested various combinations to determine the most efficient extraction for *Tilletia* infested samples. The final DNA extraction protocol for the soil samples followed the manufacturer's instructions with the following adjustments. Each soil sample received 700 µL of lysis buffer SL1 and 150 µL of the SX enhancer. The samples were then homogenized twice at 5 m/s for 30 s using a FastPrep-24™ 5G (M.P. Biomedicals, USA). For the inhibitor precipitation step, 400 µL of the supernatant was transferred to the precipitation plate. When the supernatant was less than 400 µL, the entire available supernatant was used. All centrifuge

steps were conducted at 6000 x g. After following the manufacturer's instructions for the washing steps, the silica membranes were incubated at 37 °C for 20 minutes to ensure complete drying. Each sample was eluted twice with 100 µL of the kit's elution buffer, which yielded a final volume of 200 µL per sample. The extracted DNA was stored at -20 °C until use.

Primer and probe development

Oligonucleotide primers and probes that target three different genomic regions, including two nuclear and one mitochondrial targets of *Tilletia caries* and *Tilletia controversa* were developed for single- and multiplex PCR assays (Table 1) using the Primer3 software tool [47]. Primer and probe specificity was checked using the NCBI nucleotide database and Primer-Blast [48]. The TcarARP2 primers and probe were designed to target the actin-related protein 2 (ARP2) nuclear gene (GenBank accession: JALUTQ010000006.1) of *T. caries*. The RPB2 primers and probe target the RNA polymerase II subunit (RPB2) nuclear gene of both *T. controversa* (Genbank accession: NC_073546.1) and *T. caries* (GenBank accession: EU257596.1). A primer set that targets *T. controversa* and *T. caries* was designed from a mitochondrial non-coding region (GenBank accession: NC_073546.1:49086-49237) and (Genbank accession: BK074390.1:109911-110053), respectively. A Tc2.5 probe within this mitochondrial region can specifically bind to *T. controversa*. The oligonucleotide primers and probes were synthesized by Microsynth (Balgach, Switzerland).

Gene target	Target organism	Oligo name	Sequence (5' - 3')	Amplicon length (bp)
ARP2	*Tilletia caries*	TcarARP2_479F	AGC GGC TCC AAA CCA TC	128
		TcarARP2_601R	TTG ACC TCC TTC TCC AGA CG	
		TcarARP2_544P	FAM-TCT CCG GT[**LNA-G**] GAT CGA GCA TTA CC-BHQ-1	
mtDNA	*Tilletia caries* and *Tilletia controversa*	Tc2.5_F	AGG GGT GAC AAA GAT CGG AT	152
		Tc2.5_R	CGC CGA TTC TGT CCT TGT TT	
	Tilletia controversa	Tc2.5_P	HEX-ATC TCT GCT TTA CGG CTC CGC CGA T-BHQ-1	
RPB2	*T. caries* and *T. controversa*	RPB2_236F	GAC ATG CGG ACT GTC TTG G	151
		RPB2_386R	AAA GGT CCC GAT GTC GTA GG	
		RPB2_271P	FAM-TTG GGT GTC AAG GAC GGT GCC-BHQ-1	
ND2	*Delphinapterus leucas*	DleND2_663F	AAA TTC CAC CAC CAC CAC AC	125
		DleND2_787R	TGG GTA TAA ATC CTG TGA GTG G	
		DleND2_721P	Cy5-ACA ACC TTC ACC ATA CTC ACC CTC CT-BHQ-2	

Table 1: Primers and probes used in this study. The primers and probes target the plant pathogens, *Tilletia caries* and *Tilletia controversa*. The ARP2 probe has a locked nucleic acids (LNA) modification on the third G in the 5' end, annotated in the table as [LNA-G].

A synthetic gBlock fragment based on the NADH dehydrogenase subunit 2 (ND2) gene of beluga whale *Delphinapterus leucas* (GenBank accession: NC_034236.1) was used as an exogenous internal control for DNA extraction and PCR amplification. A species-specific primer-probe set (DleND2) had been developed for this fragment (Table 1). The DleND2 primer-probe set was tested with DNA extracted from uninfested soil to verify the absence of undesired amplification, and with barley DNA, which was used as a DNA matrix within its standard curve.

DNA quantification and analysis

DNA quantification of mycelial and teliospore samples

The DNA concentration of mycelia and teliospores used to test the primer specificity were quantified using a Nanodrop One Microvolume UV-Vis Spectrophotometers (Thermo Fisher Scientific Inc, USA). Samples from mycelia and teliospores of fungi were manually diluted at

the concentrations 0.8, 0.008, and 0.0008 ng/µL. These sample DNA were amplified with the qPCR CFX Opus 384 Real-Time PCR System (Bio-Rad, USA) targeting the nuclear genes, ARP2 and RBP2, and a mitochondrial region. Each qPCR plate included technical triplicates of all biological samples, positive control (gBlock synthetic gene fragments (Integrated DNA Technologies, USA) of target genes), and negative controls (nuclease-free water).

The Tc2.5 primers that should amplify mtDNA of both *T. caries* and *T. controversa* were run in singleplex reactions with DNA extracted from fungal mycelia and teliospores. The reaction was composed of a final volume of 10 µL per well, containing 4.2 µL of DNA template, 0.3 µM of each Tc2.5 primer, and 5 µL of SYBR qPCR Master Mix (Promega Corporation, USA). The RBP2 primers and probe that amplify both *T. caries* and *T. controversa* were used in singleplex reaction with the same fungal DNA extracted from mycelia and teliospores. For this reaction, a final reaction volume of 10 with 4.2 µL of DNA template, 0.3 µM of each RBP2 primer and 0.25 µM of probe, and 5 µL of GoTaq Probe qPCR Master Mix (Promega Corporation, USA). To test the specificity of primer-probe sets of Tc2.5, TcarARP2, and DleND2, multiplex qPCR reactions were set in a final volume of 10 µL per well, containing 4.2 µL of DNA template. Each reaction included 0.3 µM primers and 0.25 µM probes for both Tc2.5 and TcarARP2, 0.2 µM of each primer and probe for DleND2, and 5 µL of GoTaq Probe qPCR Master Mix (Promega Corporation, USA). Primer and probe concentrations were previously optimized for both singleplex and multiplex assays.

The qPCR program for both singleplex and multiplex reactions consisted of an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 63 °C for 1 min.

The fluorescent intensity baselines were adjusted manually for all the runs depending on the background noise at 200 RFU, 600 RFU and 250 RFU, respectively for primer-probe set Tc2.5, TcarARP2, and DleND2. The background noise was higher in the reactions using Tc2.5 without probe and the fluorescent baseline was manually adjusted to 2500 RFU.

The species specificity of primers or primer-probe were evaluated using quantification cycle (Cq) values. Fungal DNA that was amplified in all three technical replicates with a Cq standard deviation of less than 0.5 was considered a target for primers or primers–probe.

Testing DNA quantification protocols with *T. caries* in bunt panel and on seed wash samples

The concentration of DNA extracts from the *T. caries* bunt panel and seed washes were measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). The concentrations of the DNA extracts from the *T. caries* bunt panel were manually diluted to 0.5 ng/µL and the seed DNA extracts were diluted to 4.0 ng/µL. Samples were run on the dPCR QIAcuity One (Qiagen, Germany) in singleplex and in duplex reactions.

The singleplex reaction quantified *T. caries* DNA from seed and bunt panel samples with the Tc2.5 mitochondrial primer sets, without the specific probe, using a 24-well Nanoplate 26K. The final volume of each dPCR reaction for these samples was 40 µL per well, 0.5 µM of Tc2.5 primer set, and 13.3 µL of QIAcuity EvaGreen (Qiagen, Germany). The DNA extracted from seed samples and *T. caries* bunt panel were run with a reaction concentration of 1 ng/µL and 0.024 ng/µL, respectively. The amplification program included an initial denaturation of 95 °C

for 2 min, followed by 35 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s, and a final step of 40 °C for 5 min. The imaging parameters were set to an exposure time of 300 ms and a gain of 3 to avoid signal saturation, particularly in analyzing the DNA extracted from the bunt samples.

Duplex reactions were also run with the bunt panel DNA using the mitochondrial primer set Tc2.5 with the *T. controversa* specific probe and the nuclear primers TcarARP2 and RPB2 with their respective probes. In the 96-well Nanoplate 8.5K, technical duplicates were included of the biological samples, a negative control (nuclease-free water), positive control (synthetic gBlock fragment based on the ARP2 gene or the Tc2.5 mtDNA target). To determine whether two or three technical replicates were sufficient based variance in dPCR results, serial dilutions of the synthetic targets for the regions of TcarARP2 or Tc2.5 were run (Supplementary Table 5, Supplementary Table 3). The intra-plate coefficients of variation were less than 25%, indicating that duplicate measurements were appropriate and used for dPCR analyses within this study. The final volume of each dPCR reaction was 12 µL per well. Each well contained 4.2 siµl of DNA, 0.3 µM of each primer and probe (Tc2.5 and either TcarARP2 or RPB2), and 5 µL of QIAcuityDx Universal MasterMix (Qiagen, Germany). The two reactions for each sample were previously prepared in a PCR plate, mixed, and centrifuged. Two replicates per sample were then transferred into a 96-well Nanoplate 8.5K (Qiagen, Germany) with a Nanoplate Tray to protect the chip during loading. Each plate was then sealed with Nanoplate seal foils (Qiagen, Germany). The amplification program included an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 63 °C for 1 min. To improve the detection of low-contaminated samples, the imaging parameters were set to an exposure time of 500 ms and a gain of 6.

The copy numbers were quantified for each sample using QIAcuity Software Suite (version 3.1.0.0). The number of target DNA copies per µL was assessed for the DNA template volume (4.2 µL). The threshold values to distinguish positive and negative signals were set manually. To quantify the copy number using the singleplex reaction, the threshold of fluorescent intensity was manually set individually for each sample. For the duplex reaction, the threshold was set at 36.55 RFU for Tc2.5, 30.00 RFU for TcarARP2, and 44.00 RFU for RPB2. These threshold values were set by evaluating the results of nine dPCR plates.

We correlated the number of *T. caries* copies/µL amplified from the bunt panel DNA using Tc2.5 primer to dPCR measurments of the same samples obtained with the nuclear primer sets (either RPB2 or TcarARP2). To evaluate the relationship between the mitochondrial and nuclear primer sets, we tested power-law, linear, polynomial, exponential, and logarithmic models. The power-law correlation was selected based on the adjusted R^2, p-value, Akaike information criterion (AIC) and Bayesian information criterion (BIC) (Supplementary Table 4). We assessed differences between the linear and the power-law relationships for the Tc2.5 mitochondrial primer set relative to the RPB2 or the TcarARP2 nuclear primer sets based on the 95% confidence interval (Coefficient $\pm$ ($t_{(0.05,44))}$ $\times$ Standard Error).

The performance of dPCR in detecting *T. caries* in contaminated seed lot was evaluated using a visual seed inspection method [12] as the reference. These two method were used to detect *T. caries in thirteen seed lots*. The differences in *T. caries* copies among the seed lots within each primer set were evaluated with Kruskal-Wallis test, followed by Fisher's least

significant difference (LSD) test for post-hoc comparisons with Benjamini-Hochberg p-value adjustment. All statistical analyzes were performed using R version 4.5.1 in this section and others.

We classified each seed lot as contaminated or not contaminated for both the visual seed inspection and the dPCR methods. For dPCR, the samples were considered contaminated if the primer sets Tc2.5 detected at least 1.17 *T. caries* copies/µL and 1.14 *T. caries* copies/µL for TcarARP2 primers and probe. The number of copies/µL is referring to the DNA template volume in the reaction. The limit of detection (LOD) was assessed through serial dilutions of gBlock synthetic fragments of ARP2 gene and mitochondrial targets (Supplementary Table 3). A seed lot was considered contaminated using the visual seed inspection if at least one *T. caries* teliospore was observed. To ensure comparability among the dPCR assessments and pathogen detection in seed, we used 0 as the copy number for samples with undetectable levels of *T. caries*.

We constructed confusion matrices to compare the actual values from the binary classifications of the visual seed inspection results to the predicted values from the binary classifications of each primer sets (Supplementary Table 5). Samples for which the primer sets correctly classified as positively detected (based on the reference of visual seed inspection results), were assigned as true positives (TP), while samples that both methods classified as not detected were considered true negatives (TN). False negatives (FN) were assigned to cases with detected teliospores in the seed visual inspection but incorrectly measured as not detected according to the respective primer set. Conversely, samples that were not detected in the seed visual inspection but were detected by the respective primer sets were considered false positives (FP).

Each primer set's confusion matrix was used to calculate performance metrics — sensitivity, specificity, precision, the false discovery rate, and accuracy — to directly compare the Tc2.5 mitochondrial primer set, the TcarARP2 nuclear primer-probe set, and the RPB2 nuclear primers and probe. Sensitivity ($\frac{\text{TP}}{\text{TP + FN}}$) and specificity ($\frac{\text{TN}}{\text{TN + FP}}$) measure the ability of the primer sets to correctly classify the samples as contaminated or not contaminated, respectively. Precision ($\frac{\text{TP}}{\text{TP + FP}}$) and false discovery rate ($\frac{\text{FP}}{\text{FP + TP}}$) evaluate the reliability of the primer sets' predicted value. Accuracy ($\frac{\text{TP + TN}}{\text{TP + TN + FP + FN}}$), on the other hand, represents the overall proportion of the correctly classified samples by the primer sets.

DNA quantification of soil samples

The DNA extracted from soil samples was measured with a Varian Cary Eclipse Spectrophotometer (Agilent, USA). The soil DNA extracts were not diluted prior to quantification. Instead, a fixed volume of undiluted DNA was used to maximize sensitivity for samples with low pathogens loads (DNA concentrations of each DNA extraction can be found in supplementary table, 7). Samples were run in duplex reaction on the dPCR QIAcuity One (Qiagen, Germany) with the primers Tc2.5 and TcarARP2 as described above for detecting *T. caries* in seed and bunt panel samples in muliplex dPCR assay. The same DNA extracts were tested with qPCR CFX Opus 384 Real-Time PCR System (Bio-Rad, USA) in singleplex reaction using the primers Tc2.5 without specific probe and in triplex reaction using the primer-probe sets of Tc2.5, TcarARP2, and DleND2.

qPCR was used to quantify copy numbers of*Tilletia caries*, *Tilletia controversa*, and *Delphinapterus leucas* (exogenous internal control) in soil samples. Absolute quantification relied on standard curves generated from 5-fold serial dilutions of gBlock synthetic gene fragments (Integrated DNA Technologies, USA) that contained the respective target DNA sequence (Supplementary Table 1). Standard curves were included on all plates. For triplex reactions, all three standard curves were used on each plate. The standard curve copy numbers per reaction, calculated from the molecular weight of each gBlock fragments, ranged from 62500 to 0.8 for the ARP2 gene target, 114800 to 1.47 for the mitochondrial target, and from 1563000 to 500 for ND2 gene. In each dilution point of all three standard curves, 12 ng/µL of DNA extracted from *Hordeum vulgare* seedlings was added as non-template DNA to simulate the sample matrix. Each qPCR plate included technical triplicates of all dilution points of the standard curves, biological samples, and negative controls (nuclease-free water and non-target DNA used in the standard curves), while the singleplex reaction plates only included the standard curve of the single targeted amplicon. Reactions were run in 384-well plates sealed with optical adhesive foil (Nolato TreffLab, Sweden).

The amplification of *T. caries*, *T. controversa*, and *D. leucas* DNA in soil was performed using singleplex and multiplex qPCR with the same reaction conditions, program, and fluorescent intensity baselines setting previously described for testing primer specificity on DNA extracted from mycelium and teliospores. The copy numbers for each target gene were then calculated using CFX Maestro 2.2 software (version 5.2.008.0222) based on the quantification cycle (Cq) values and standard curve regression.

Samples were reanalyzed if the standard deviation of the triplicate Cq values exceeded 0.5 for any target. The limit of detection (LOD) was defined as the lowest measurable dilution point of the standard curve consistently amplified in all triplicates, which corresponded to 4.00 copies per reaction for *T. caries* and 7.35 copies per reaction for *T. controversa*. Samples with mean Cq values higher than the mean Cq value of the lowest reliably quantifiable dilution point of the standard curve were considered below the quantification range and were not reanalyzed. The quantification of *T. caries* in soil samples was based on nuclear (ARP2) and mitochondrial target amplified without specific probe, while *T. controversa* was quantified using the mitochondrial primers and species-specific probe (Tc2.5). The DNA extraction homogeneity and reaction performance were assessed using the copy number of *D. leucas* ND2.

The copy number of *T. caries* and *T. controversa* per µL in artificially inoculated soil samples was quantified using mitochondrial and nuclear primer sets to identify the most suitable gene target for detecting the pathogens. Additionally, DNA extracted from naturally and artificially infested soils was analyzed to quantify *T. controversa* using both dPCR and qPCR. The detection frequency was calculated as the percentage of soil samples in which the pathogen was detected by each PCR assay. Frequencies were then compared directly between methods.

Results

Primer and probe specificity and sensitivity based on the *T. caries* bunt panel and non-target basidiomycetes

To determine the species-specificity of the Tc2.5 without probe and RPB2 with probe were run in singleplx reaction, while the primer-probe set of Tc2.5, TcarARP2, RPB2, and DleND2 were tested in triplex reactions. The primers and probes were tested using DNA from mycelial samples of other non-target basidiomycetes (Table 2) and smut and bunt teliospores.

Based on a fist analysis, the primers Tc2.5 with SYBR Green singleplex assay and no probe amplified the intended target of *T. caries* and *T. controversa*. Among non-target basidiomycetes only *Tilletiopsis* at 0.08 ng/µL was detected in one out of three replicates with a quantification cycle (Cq) of 31.31. Most of the non-target *Tilletia* species from mycelia DNA extracts were detected across all tested concentrations (0.8 ng/µL, 0.08 ng/µL, and 0.008 ng/µL per reaction). In contrast, *Tilletia vankyi* was less readily amplified and only detected at the highest concentration of 0.8 ng/µL per reaction.

The RPB2 primer-probe set, tested in singleplex reaction, amplified the DNA from fewer *Tilletia* spp. than the TcarARP2 primer set, but it consistently amplified the DNA of *Tilletia goloskokovii* across all concentrations. Occasional late and unstable amplification (Cq of 38.6 in one out of three replicates) was observed for the DNA extracted from *Tilletia bromi* at a concentration of 0.8 ng/µL, while 0.8 ng/µL of DNA extracted from *Tilletia puccinelliae* was amplified with mean Cq of 36.8 and a standard deviation of 0.6. For reactions that included only 0.08 ng/µL of DNA extracted from *T. puccinelliae*, the Cq was 39.24 in one replicate of three replicates, but the other two replicates were not amplified (Table 2).

Tc2.5 primer set when run with the probe amplified only the DNA extracted from *T. controversa* teliospores because the probe was indeed specific for *T. controversa*. The TcarARP2 primer-probe set stably amplified *T. puccinelliae* and *T. vankyi* across concentrations from 0.8 ng/µL to 0.008 ng/µL (Table 2). The DleND2 primer-probe did not amplify any of the tested fungi (data not shown).

The amplification efficiency of Tc2.5 primer set without probe in singlplex reaction using SYBR Green was 97.2% and R^2 of 0.995 (Supplementary Table 5). The triplex reaction showed mean amplification efficiencies of 100.18% ($R^2 = 0.989$) for Tc2.5, 100.30% ($R^2 = 0.988$) for TcarARP2, and 99.63% ($R^2 = 0.998$) for DleND2. These efficiencies and coefficients of determination were calculated from three independent plates, which were comparable to the efficiencies of each primer-probe singleplex reaction.

The limit of detection (LOD) for Tc2.5 in qPCR was 7.35 copies per reaction (1.76 copies/µL) with a Cq standard deviation ranging from 0.4 to 0.8. This LOD was determined using four independent dilution series of the mitochondrial gBlock fragment; three of which were tested with TaqMan chemistry in triplex reaction and one with SYBR Green chemistry in singleplex reaction. The LOD for TcarARP2 using qPCR was 20 copies per reaction (4.80 copies/µL), based on three indipendent dilution series of the ARP2 gBlock fragment in triplex reaction using TaqMan chemistry. In dPCR, the LOD of Tc2.5 was established at 1.17 copies/µL (Supplementary Table 3), and TcarARP2 LOD as 1.14 copies/µL (Supplementary Table 3).

			qPCR results ($\widetilde{Cq\ Mean}$ ± Std. Dev.)			
			SYBR Green chemistry	TaqMan chemistry		
Source	**Tested fungus**	**Concentration per reaction (ng/µL)**	**Tc2.5 primer set**	**Tc2.5 primer set**	**TcarARP2 primer set**	**RPB2 primer set**
Mycelium	*Tilletia bromi*	0.8	13.95 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	$38.6_{1/3}$ ± 0.00
		0.08	17.38 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		0.008	20.77 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Tilletia fusca	0.8	29.4 ± 0.45	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		0.08	32.45 ± 0.60	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		0.008	0.00 ± 0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Tilletia goloskokovii	0.8	13.52 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	22.5 ± 0.20
		0.08	16.71 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	26.3 ± 0.07
		0.008	20.16 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	29.65 ± 0.25
	Tilletia puccinelliae	0.8	12.15 ± 0.28	0.00 ± 0.00	21.13 ± 0.01	36.8 ± 0.60
		0.08	15.08 ± 0.10	0.00 ± 0.00	24.45 ± 0.10	$39.24_{1/3}$ ± 0.00
		0.008	18.62 ± 0.05	0.00 ± 0.00	27.84 ± 0.14	0.00 ± 0.00
	Tilletia vankyi	0.8	34.15± 0.55	0.00 ± 0.00	21.84 ± 0.28	0.00 ± 0.00
		0.08	0.00 ± 0.00	0.00 ± 0.00	25.28 ± 0.24	0.00 ± 0.00
		0.008	0.00 ± 0.00	0.00 ± 0.00	28.68 ± 0.11	0.00 ± 0.00
	Ustilago maydis	0.08	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Ustilago spp.	0.8	0.00 ± 0.00			
	Pseudozyma spp.	0.8	0.00± 0.00			
		0.08		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Tilletiopsis spp.	0.08	$31.31_{1/3}$ ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		0.008		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Holtermaniella spp.	0.8	0.00 ± 0.00			
	Entyloma spp.	0.8	0.00 ± 0.00			
Teliospores	*Tilletia caries*	0.8	17.12 ± 0.02	0.00 ± 0.00		
		0.08	24.41 ± 0.10			
		0.008	38.26 ± 0.36			
	Tilletia controversa	0.8	19.73 ± 0.26	22.17 ± 0.12		
		0.08	27.16 ± 0.11			
		0.008	35.47 ± 0.62			
	Ustilago tritici	0.8	0.00 ± 0.00			
	Ustilago nuda	0.8	0.00 ± 0.00			
	Ustilago horidei	0.8	0.00 ± 0.00			

Table 2: Species-specificity assessment of Tc2.5, TcarARP2, and RPB2 primer-probe sets for *Tilletia caries* and *Tilletia controversa* using qPCR. Tc2.5 primers without a probe with SYBR Green chemistry and the RPB2 primer-probe set with TaqMan chemistry in were tested in singleplex reactions. The primer-probe sets of Tc2.5, TcarARP2, and DleND2 were tested in a triplex reaction using TaqMan chemistry. The DNA templates used for testing the primers and probes were extracted from teliospores of the target species (*T. caries* and *T. controversa*) and non-target fungal species (all other mycelial isolates listed that come from species other than *T. caries* or *T. controversa*). The samples were tested in three technical replicates. The number of amplification is denoted as a subscript next to the mean quantification cycle (Cq) when fewer than three replicates showed amplification. High mean Cq values indicate lower target DNA abundance.

We quantified the copy numbers of *T. caries* mitochondrial and nuclear targets in DNA extracted from bunt panel with dPCR. Then, we assessed the relationship between the quantification of the mitochondrial and nuclear gene targets. The mitochondrial target was amplified using Tc2.5 primers without probe in singleplex and the nuclear targets (ARP2 or RPB2) using their respective primer-probe sets in duplex with Tc2.5 primers with probe.

The linear relationship between *T. caries* quantified with mitochondrial primers and both nuclear primer-probe sets was significant with p-values of $1.15\text{x}10^{-12}$ for ARP2 target and $1.06\text{x}10^{-11}$ for RPB2 target (Supplementary Table 4). However, the power-law model described the relationships between mitochondrial (Tc2.5 primers) and nuclear (TcarARP2 or RPB2 primers) targets better than the other models tested (Supplementary Table 4). For TcarARP2, the exponent of power-law model had a 95% confidence interval of 0.97-1.30, which indicated an approximately linear relationship between mitochondrial and nuclear copy numbers (Figure 1). One ARP2 nuclear copy corresponded to 140 mitochondrial copies/µL, due to *T. caries* teliospores are a dikaryotic, $\simeq$ 280 mitochondrial copies/µL should correspond to one teliospore. For RPB2, the exponent had a 95% confidence interval that ranged from 1.11 to 1.61, indicating positive allometry (Supplementary Table 4). A dikaryon teliospore — two nuclei — amplified with RPB2 nuclear primer set should corresponded to $\simeq$ 917 mitochondrial copies per µL. These relationships established a direct conversion between mitochondrial and nuclear targets, which enabled accurate estimation of teliospore numbers at low concentrations.

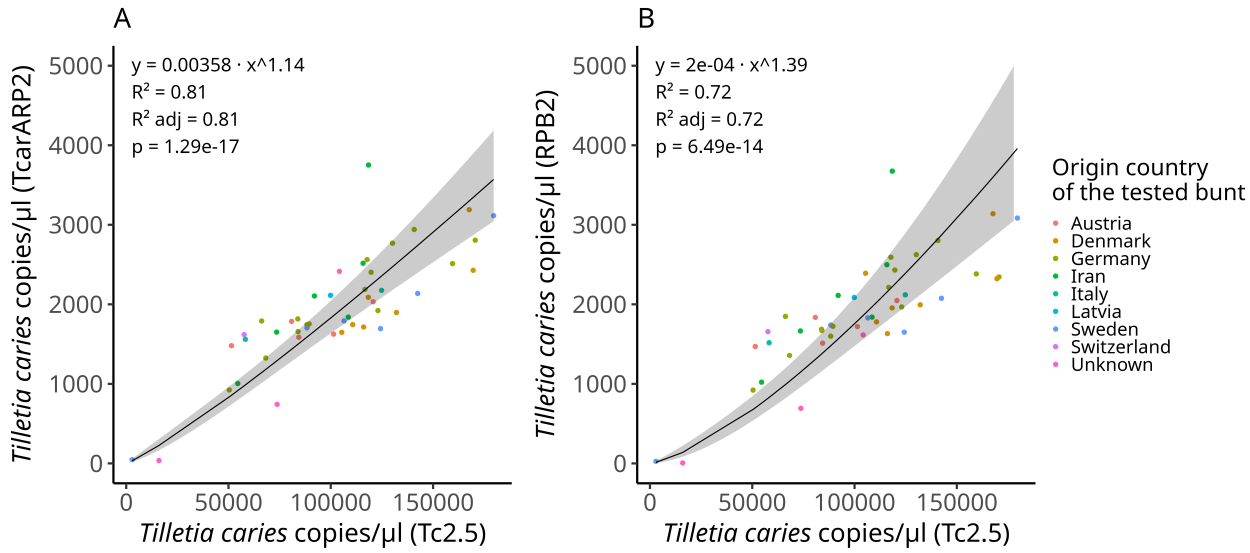

Figure 1: Relationship between the *Tilletia caries* copies/µL amplified in DNA extracted from bunt panel using the mitochondrial primer set Tc2.5 and with the nuclear primer sets: (A) TcarARP2 and (B) RPB2. For each nuclear gene, the power-law model line of best fit between nDNA and the and mtDNA target is illustrated with black lines. The power-law model equations, R^2, adjusted R^2 (R^2 adj), and p-value are shown in the top left corner of each plot. The different color indicate the origin country for each tested bunt sori.

Identification of *Tilletia caries* contamination in seed lots

We assessed the presence of *Tilletia caries* in twenty-two samples of wheat seed lots using both visual inspection of seeds and dPCR (Figure 2). At least one teliospore of *T. caries* was observed by at least one inspector in seventeen of these twenty-two samples. Across the seed lot samples, the mean number of teliospores per kernel ranged from 0 to 26.7 for evaluated seed subsample (Figure 2 A, Supplementary figure 1). We detected *T. caries* DNA in fourteen seed lot subsamples using dPCR with the mitochondrial primer set Tc2.5 without probe in a reaction using EvaGreen chemistry (Figure 2 B). Twelve of these fourteen seed lot samples that were considered contaminated based on the dPCR results were also classified as contaminated through visual inspection of the seeds (Figure 2). In contrast, both of the nuclear primer sets — TcarARP2 and RPB2 — with TaqMan chemistry detected *T. caries* in only one seed lot sample that contained the highest level of *T. caries* teliospores per kernel (Supplementary figure 2). This sample had also been reliably detected *T. caries* with the mitochondrial primer set and through seed visual inspection. The *T. caries* quantification differed significantly among primer sets ($p = 1.51 \times 10^{-7}$, $\chi^2 = 31.41$).

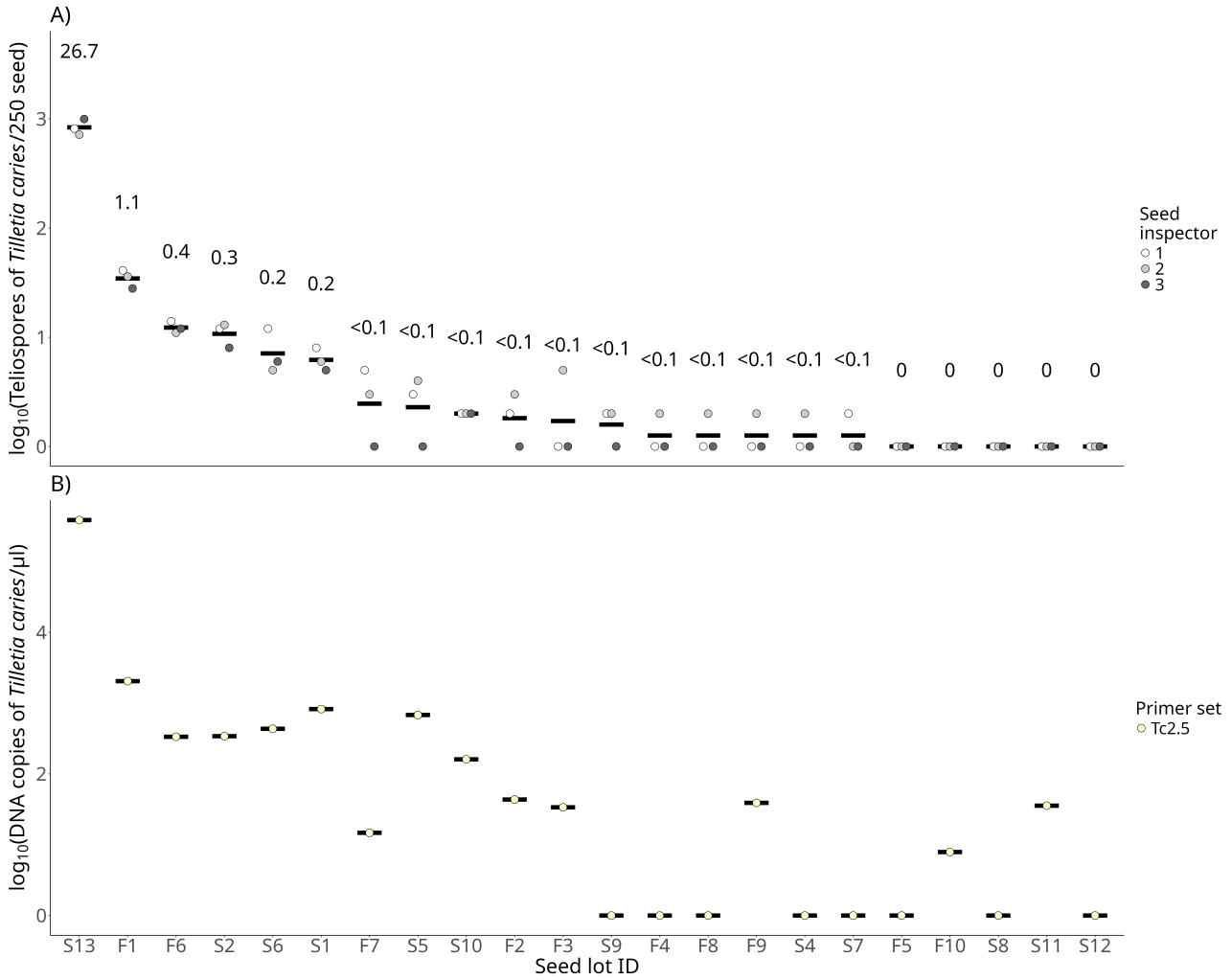

Figure 2: Examination of *Tilletia caries* teliospore contamination in twenty-two wheat seed lots using two methods: A) visual seed inspection and B) dPCR assay. Each seed lot is reported on the x-axis with an identification name. A) The results of the visual seed inspection are expressed as log_{10}(Teliospores of *T. caries*/250 seed). The mean number of *T. caries* teliospores per kernel counted from the seed inspections are plotted with the black lines, and the different colored points show the individual results from each of the three seed inspectors. B) The dPCR quantification using the primer set Tc2.5 (EvaGreen chemistry) without replication.

To evaluate *T. caries* detection agreement between seed visual inspection results and dPCR measurements using mitochondrial or nuclear primer sets, we classified the seed lots as either contaminated or uncontaminated with *T. caries* teliospores based on each detection method (dPCR with RBP2 or TcarARP2 primer-probe sets, qPCR with Tc2.5 primers and visual seed inspection). We based this classification for the dPCR methods on the limits of detection (LOD) specific to each primer set. We considered the lot contaminated when at least one teliospore was observed during visual inspection of the seeds by one or more inspectors. We created confusion matrices for each primer set using the results of the seed visual inspection to determine the true contamination status of the seed lot subsamples (Supplementary Table 5). The nuclear primer sets — TcarARP2 and RPB2 — identified only one contaminated seed lot, which was also classified as contaminated by the seed visual inspection, resulting in sixteen false negatives. Both nuclear primer sets correctly classified five uncontaminated seed lots according the the visual seed inspection. In contrast, the mitochondrial primer set (Tc2.5) correctly classified twelve contaminated and three uncontaminated seed lot samples using the

visual seed inspection as a reference. The mitochondrial primer set showed higher sensitivity (0.71) and accuracy (0.68) in classifying contaminated seed lots with *T. caries* than the nuclear primer sets did (Table 3). However, the nuclear primer sets had higher specificity (1.00) and precision (1.00) along with a lower false discovery rate (0.00) than the mitochondrial primer set, which was based only on one positive sample.

	Sensitivity	**Specificity**	**Precision**	**False discovery rate**	**Accuracy**
Mitochondrial primer set Tc2.5	0.71	0.60	0.86	0.14	0.68
Nuclear primer set TcarARP2	0.06	1.00	1.00	0.00	0.27
Nuclear primer set RPB2	0.06	1.00	1.00	0.00	0.27

Table 3: Evaluations of the mitochondrial primer set (Tc2.5) using SYBR Green chemistry and the nuclear primer sets (TcarARP2 and RPB2) using TaqMan chemistry were used to detect *Tilletia caries* contamination across twenty-two seed lots. The performance metrics include sensitivity, specificity, precision, false discovery rate, and accuracy. These performance metrics were based on the twenty-two seed lots' agreement between their visually observed contamination status based on the observation of one or more teliospore and the dPCR-measured contamination. The outcome of each seed lot's classification according to the confusion matrices (Supplementary Table 5) was used to evaluate the primer sets' performance.

Detection of *Tilletia caries* and *Tilletia controversa* in artificially inoculated soil

Dormant teliospores of *Tilletia caries* and *Tilletia controversa* can persist in soil and serve as a source of inoculum. Therefore, we evaluated the mitochondrial primer set Tc2.5 with and without probe and the nuclear primer-probe sets (TcarARP2 and RPB2) in detecting both species in artificially inoculated soil using qPCR and dPCR assays.

To test our ability to detect *T. caries* and *T. controversa* in DNA extracted from soil using the developed primer sets with qPCR and dPCR, we extracted DNA from soil samples inoculated with a known number of *T. caries* or *T. controversa* teliospores per gram of soil. Using one sample of each artificial inoculation level, we detected 1000 *T. caries* teliospores/g soil using a qPCR assay with SYBR Green chemistry and the mitochondrial primer set Tc2.5 without probe. The nuclear primer-probe set TcarARP2 using TaqMan chemistry amplified *T. caries* in the soil DNA extracts starting at 10000 *T. caries* teliospores/g soil (Supplementary Figure 3). Using the dPCR assay, we detected *T. controversa* DNA extracted from soil only with the species-specific mitochondrial primer set Tc2.5 and its associated probe, while the nuclear primer-probe set RPB2 using TaqMan chemistry did not yield detectable amplification at concentrations ranging from 10 to 100000 teliospores/g soil (Supplementary Figure 4).

Due to the relevance of *T. controversa* in soil, subsequent tests focused on this *Tilletia* species. We then used Tc2.5 primers with a specific-probe to compare *T. controversa* detection with qPCR and dPCR assays with a larger sample size of inoculated soil. We tested with both PCR assays using DNA extracted from soil samples inoculated with 100, 1000, or 100000 *T. controversa* teliospores per gram of soil (ten independent replicates per concentration). We detected *T. controversa* with both qPCR and dPCR assays at 100 teliospores/g of soil (Figure 3).

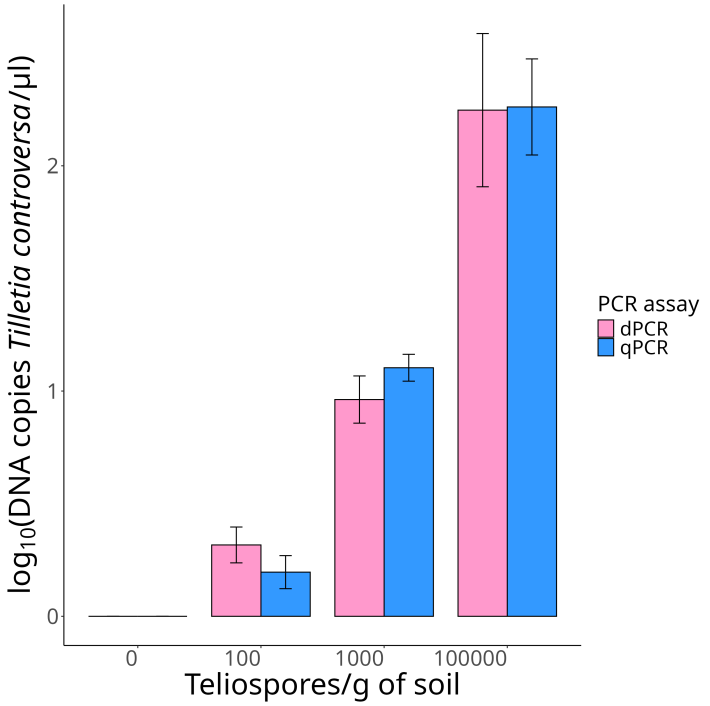

Figure 3: The quantification of *Tilletia contorversa* with dPCR and qPCR in soil samples. Each inoculum level consisted of ten individual replicate mixtures of teliospores and soil (Supplementary Figures 5) for which duplicate dPCR and triplicate qPCR reactions were performed. Bars are standard errors of the mean *T. controversa* quantity detected in the DNA extracted from the replicate mixtures.

The two PCR assays differed in their detection frequency of *T. controversa*. In DNA samples extracted from soil inoculated with 100 teliospores/g of soil, *T. controversa* was detected in 50% of the samples using dPCR, compared to 20% with qPCR (Figure 4). Though, among these positive samples the dPCR measurements showed greater variability than the qPCR measurements, as reflected by larger standard errors (Supplementary Figures 5). At higher inoculum levels, the detection frequency increased for both PCR assays. *T. controversa* was detected in 100% of DNA samples extracted from soil inoculated with 1000 teliospores/g of soil and 80% of DNA samples extracted from soil inoculated with 100000 teliospores/g of soil using dPCR. The difference in detection frequency between qPCR and dPCR decreased to 10% at both soil inoculum concentrations (1000 and 100000 teliospores/g of soil).

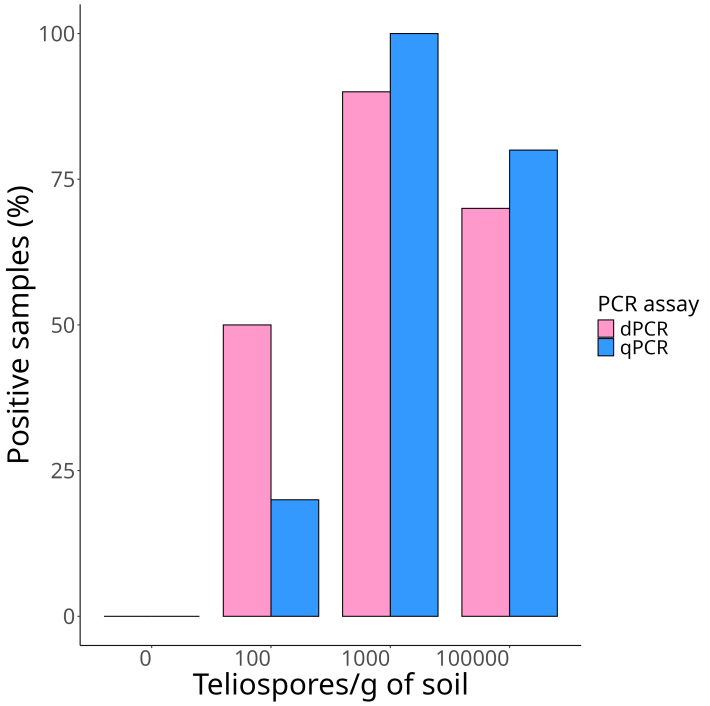

Figure 4: Percentage of samples in which was detected *Tilletia controversa* using qPCR or dPCR. The samples consisted of DNA extracted from soil that was artificially inoculated with 100, 1000, and 100000 teliospores/g of soil. Ten separate repetitions were tested for each inoculum level (Figure 5). The DNA extractions were tested in duplicate using dPCR (pink) and in triplicate using qPCR (blue).

Validation of *Tilletia controversa* detection in naturally infested soil

To evaluate the applicability of the mitochondrial primer set using the *T. controversa*-specific probe on field-collected soil, samples were collected from a naturally infested *T. controversa* field that was divided into 24 plots. One soil core was taken from the center of each plot and tested using both dPCR and qPCR with the Tc2.5 primer set and probe.

Both PCR assays showed a heterogeneous distribution of *T. controversa* across the field. The spatial patterns of *T. controversa* detection obtained with dPCR and qPCR were highly similar, showing comparable trends in soil inoculum distribution among field plots (Figure 5). dPCR detected the presence of *T. controversa* in all 24 soil samples. In contrast, qPCR detected *T. controversa* in 19 of the 24 samples. However, one of the soil sample that was deemed uninfested with qPCR was consistently amplified in two out of three technical replicates (Supplementary Table 6). *T. controversa* copies/µL differed between qPCR and dPCR with a relative differences in estimated copy numbers ranging from -100% to +35.86%, on average -19.98% (Supplementary Table 6).

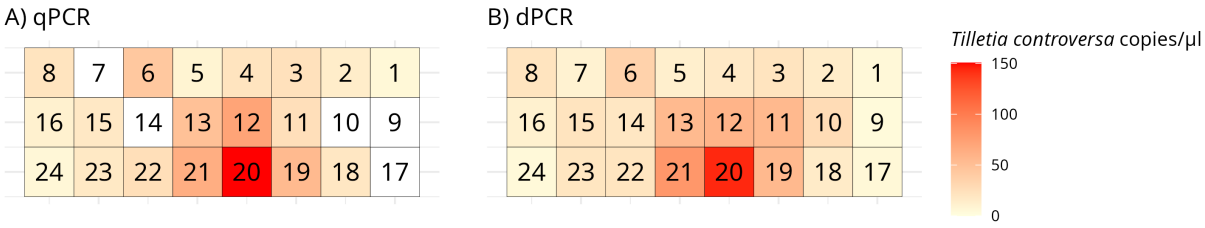

Figure 5: *Tilletia controversa* DNA quantified using dPCR and qPCR in naturally infested soil samples. A core was taken from each plot, and the extracted soils were tested with (A) qPCR and (B) qPCR (Supplementary Table 6). The field plots are shown as they are spatially arranged within the field with color representing *T. controversa* infestation level based on PCR measurements. An identifying number is show within each plot (6 m x 1.5 m). The samples were run in duplicate with the dPCR (pink) and in triplicate with the qPCR (blue).

Performance of *Delphinapterus leucas* synthetic target as an exogenous DNA control in soil samples

A synthetic *Delphinapterus leucas* DNA target was added to soil samples prior to DNA extraction to control for DNA extraction and qPCR amplification. The *D. leucas* synthetic target, which was intended to serve as an internal exogenous control, was consistently amplified in artificially inoculated soil samples, yielding an overall mean quantification cycle (Cq) value of 24.77 ± 0.44 (Supplementary Table 7, Supplementary Figure 7), which indicate uniform DNA extraction procedure. In naturally infested field soil samples, the amplification of *D. leucas* target showed greater variability with mean Cq values, ranging from 24.50 to 27.10 and an overall mean Cq value of 25.36 ± 0.70 (Supplementary Table 7). The highest mean Cq values were 26.66, 26.66, and 27.10 that were obtained analyzing the DNA extracted from field soil samples 1, 9, and 17, respectively. These increased Cq values suggest differences in the DNA extraction procedure or possible errors during the amplification step. In these field soil samples, *T. controversa* was consistently amplified with dPCR, whereas qPCR amplification was absent or was the lowest amount detected among the positive samples (Supplementary Table 7). *D. leucas* synthetic target was not included in the dPCR assay.

Discussion

Common bunt (*Tilletia caries*) and dwarf bunt (*Tilletia controversa*) remain important threats to wheat production because disease occurrence reduces both yield quality and quantity [29, 30, 49]. Teliospores can survive for long-term periods as dormant teliospores in contaminated seed or infested soil [21, 22, 49], complicating their control. Additionally, since these pathogens infect seedlings during early stage of plant development [21, 26], effective management should rely on preventive measures, such as the identification and avoidance of contaminated seed lots [49]. Historically, synthetic seed treatments provided reliable control of *T. caries* and *T. controversa* [21]. However, current agricultural policies aim to reduce the use of synthetic plant protection products (PPPs) [3, 4, 5, 6, 7, 8, 9]. The reduction of synthetic PPPs, including fungicidal seed coatings, may cause the reemergence of these *Tilletia* species [49]. The reliable detection of *T. caries* and *T. controversa* inocula in seed lots and soil is essential to support Integrated pest management (IPM) strategies, including risk-based management and disease control with PPPs only when economically necessary. Although molecular detec-

tion methods have been proposed as complementary approaches to traditional seed inspection tests [31, 45], their routine implementation remains limited by challenges related to sensitivity, possible inhibition due to the sample material [50], and uncertainties in interpreting molecular results in terms of biological risk [51]. Molecular-based assays targeting nuclear gene regions have been developed for detecting *Tilletia* spp. in seed [31, 37, 52]. However, mitochondrial regions, which have higher copy numbers, have been observed to increase detection sensitivity in the detection of other pathogens [42]. We observed the benefit of using mitochondrial DNA due to its high-copy-number, which increases the sensitivity for detecting *T. caries* and *T. controversa* either in seed or soil using qPCR and dPCR assays.

Seed transport is the primary pathway for long-distance dissemination of many seedborne pathogens [53], including Tilletia spp. In some European countries, such as Denmark and Sweden, the detection of a single teliospore in a seed lot is sufficient to reject it for the seed health certification [54]. Therefore insufficient assay sensitivity may result in false negative classifications. The variation observed among seed inspectors in teliospore counts highlights the inherent variability of visual seed inspection, which may depend on the evaluation procedures and the inspector's expertise [31]. To improve the sensitivity of seed health testing and reduce the variability in results of visual seed inspections, we evaluated both mitochondrial and nuclear primer sets with dPCR. The mitochondrial primer set detected *T. caries* in a greater number of seed lot samples that were positive in the visual seed inspection than the nuclear TcarARP2 and RPB2 primer sets. The reduced detection of *T. caries* DNA using nuclear primers was most evident in seed lots with low teliospore numbers (i.e., less than 26 teliospore per seed) identified through the visual seed inspection. Limited sensitivity at low number of teliospores restricts the reliability of nuclear primer set application for seed health certification. This limitation is particularly relevant because tolerance thresholds used for seed health certification vary widely among Europe countries, ranging from zero tolerance in Sweden and Denmark to 20 teliospores per seed in Germany [54]. PCR-based seed health testing should therefore align with national certification requirements. Rather than replacing visual seed inspection, PCR assays can serve as high-throughput screening tool to distinguish seed lots with high contamination from those with low contamination. Seed lots classified with low contamination using the PCR-based test, especially when the number of teliospores detected approaches the regulatory tolerance thresholds, should be confirmed with visual seed evaluation. PCR-based seed testing would streamline the certification process because it would reduce the number of seed lots that require a visual inspection. Ongoing validation of this PCR-based protocol includes testing additional seed lots, including those contaminated with *T. controversa*, a quarantine pathogen in China and among other countries [55].

In comparison to *T. caries* and *T. controversa* detection on seeds, pathogen detection in soil has been studied less extensively, despite the fact that soilborne teliospores contribute to disease transmission as a long-term inoculum sources, particularly for *T. controversa* [20, 21, 23, 24, 27, 56]. Although the detection of *T. controversa* DNA in soil is feasible [57], the relationship between detectable *T. controversa* DNA and the corresponding number of teliospores per gram of soil remains unclear. We analyzed soil samples artificially inoculated with a known number of teliospores per gram of soil using the same species-specific mitochondrial primer-probe set for both dPCR and qPCR assays. Direct comparison of these assays revealed that the choice of

PCR assay may influence detection at low inoculum levels. At the lowest inoculum level tested (100 teliospores/g of soil), dPCR detected *T. controversa* in 30% more samples than qPCR, likely due to the partition-based amplification, which reduces the stochastic variation near the detection limit and improves the target detection in dPCR [43]. At higher teliospore inoculum levels (1000 and 100000 teliospores/g soil), the number of samples in which *T. controversa* was detected converged between the two PCR. These results indicates that qPCR remains suitable for moderate to high *T. controversa* soil inoculum levels. QPCR and dPCR measurements should be interpreted as indicators of *T. controversa* inoculum presence rather than direct predictors of yield losses or disease occurrence, which depend on multiple factors (e.g., environment and cultivar). Further research is needed to better understand the relationship between the number of *T. controversa* teliospores in a gram of soil and subsequent disease occurrence in order to better interpret PCR-based detection results into biological meaning.

Analysis of naturally infested field soil with *T. controversa* using both qPCR and dPCR showed the applicability of the mitochondrial primers and probe to samples collected from agricultural fields. Both PCR assays revealed that *T. controversa* was heterogeneously distributed across the sampled field area. In the season following the soil sampling, disease occurrence in the field showed a heterogeneous pattern (Supplementary Figure 6), reflecting the PCR results from the soil sampling. This distribution of *T. controversa* in soil is consistent with findings for other soilborne bunt pathogens, such as *Tilletia indica*, whose teliospores distribution in soil is frequently aggregated or random rather than uniform [58]. The detection of aggregated or random distributions of *Tilletia* spp. teliospores is difficult using conventional field surveillance based on disease occurrence [58] or visual soil inspection, both of which are labor-intensive and poorly suited for routine, large-scale sampling. In contrast, PCR-based detection of *T. controversa* teliospore in soil may enable systematic sampling strategies and quantitative comparisons across multiple fields, thereby supporting field-level risk assessment. The reliability of observed variability of *T. controversa* in infested soil — based on PCR measurements — appears to be further supported by the synthetic exogenous internal control. This internal control was consistently amplified at the same quantification cycle across soil samples, despite differences in DNA concentration. Therefore, variation in detected *T. controversa* DNA copy numbers among samples most likely reflects a biological heterogeneity in telisopores distribution rather than PCR inhibition [44]. Consequently, we interpreted low or absent signals as indicating low or absent presence of *T. controversa* teliospores in soil at specific sampling points. We will further validate the use of this internal control across diverse soil types and other substrates of interest, such as the seed wash pellet, for improving the robustness and comparability of the PCR protocols. In addition, we will normalize the soil samples based on dry-weight correction to improve quantitative precision and comparability among field samples.

Despite the improved sensitivity in detecting *T. caries* and *T. controversa* with mitochondrial primer set than with nuclear primer sets, soil heterogeneity and sampling scale remain constraints for pathogen detection in soils — especially at low pathogen abundance. Even high-sensitive PCR assays cannot compensate for uneven distribution of teliospore in soil samples from fields, which has been observed with other soilborne pathogens [59], or low efficiency of DNA extraction [57]. The adaptability of developed PCR protocol to both qPCR and dPCR assays enables laboratories with different technical infrastructures to harmonize *Tilletia* spp.

detection. In addition, this adaptability allows for the most appropriate PCR-based assay to be selected for the regional certification or phytosanitary requirements (e.g., number of teliospores per seed). The PCR-based detection of *T. caries* and *T. controversa* in seeds and soils before sowing can improve bunt management strategies.

Acknowledgments

We thank Nicole Bischofberger and Damian Amrein for assistance in acquiring seed material, and Jilla Schneider, Jan Glauser and Julia Weidmann for help with sample processing. We are grateful to Prof. Gunther Doehlemann (University of Cologne) for *Ustilago hordei* and *Ustilago maydis* strains, and Marco Wüthrich for providing cultures of *Tilletia caries*, *Tilletia controversa*, *Ustilago davisii*, *Tilletiopsis* spp., *Pseudozyma* spp., *Entyloma* spp., and *Holtermaniella* spp. We acknowledge Karl-Josef Müller (Cereal Breeding Research Darzau) for providing smutted ears of *Ustilago avenae*, *Ustilago tritici*, *Ustilago horidei*, and *Ustilago nuda*. We thank COST action T0PAGRI (Towards zer0 Pesticide AGRIculture: European Network for sustainability) for having founded the Short Term Scientific Mission at Swedish University of Agricultural Sciences. This research was supported by Fondation Sur-la-Croix, IP Suisse, the Swiss Association of Cereal Producers, Swiss granum, and swisssem.

Supplementary Material

Detailed test of DNA extraction procedures for soil samples information as well as additional data analyses can be found in the Supplementary Material.

Author contributions

Conceptualization: C.P. and K.E.S; Formal analysis: C.P. with support from K.E.S.; Funding acquisition: K.E.S; Investigation: C.P., K.I.; Methodology: C.P., A.B., K.I, and K.E.S; Project administration K.E.S.; Supervision: K.E.S. and D.C.; Visualization: C.P. with input from K.E.S.; Writing-original draft preparation: C.P. with support from K.E.S; Writing-review & editing: C.P., K.E.S., D.C., A.B., K.I.

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Supplementary Material: Optimization of *Tilletia caries* and *Tilletia controversa* detection in soil and seed

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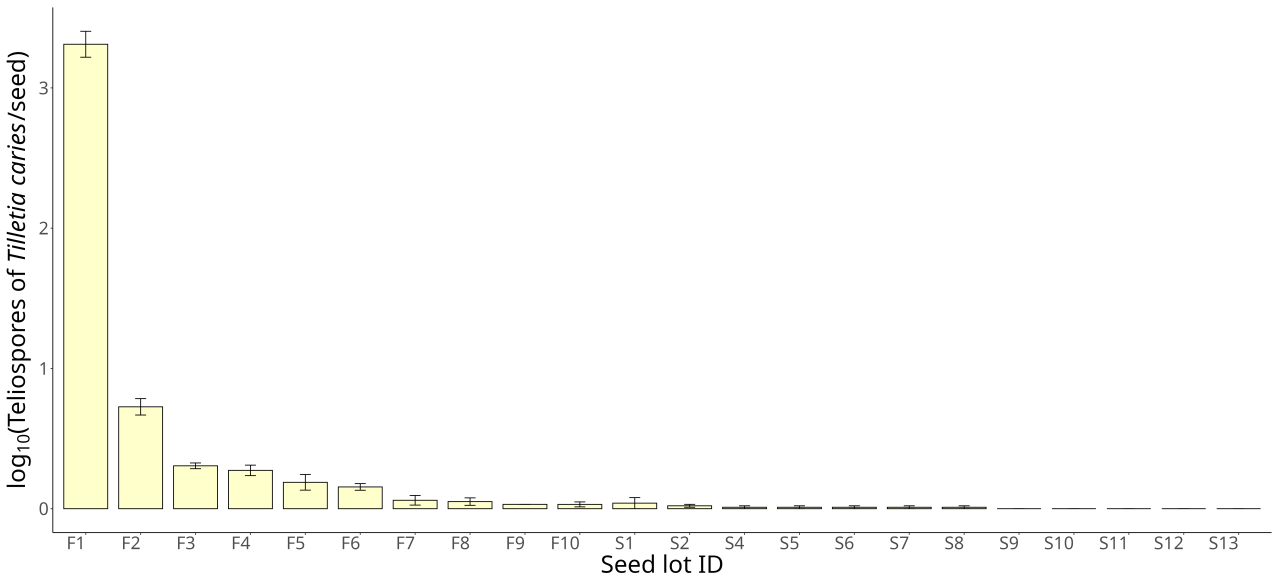
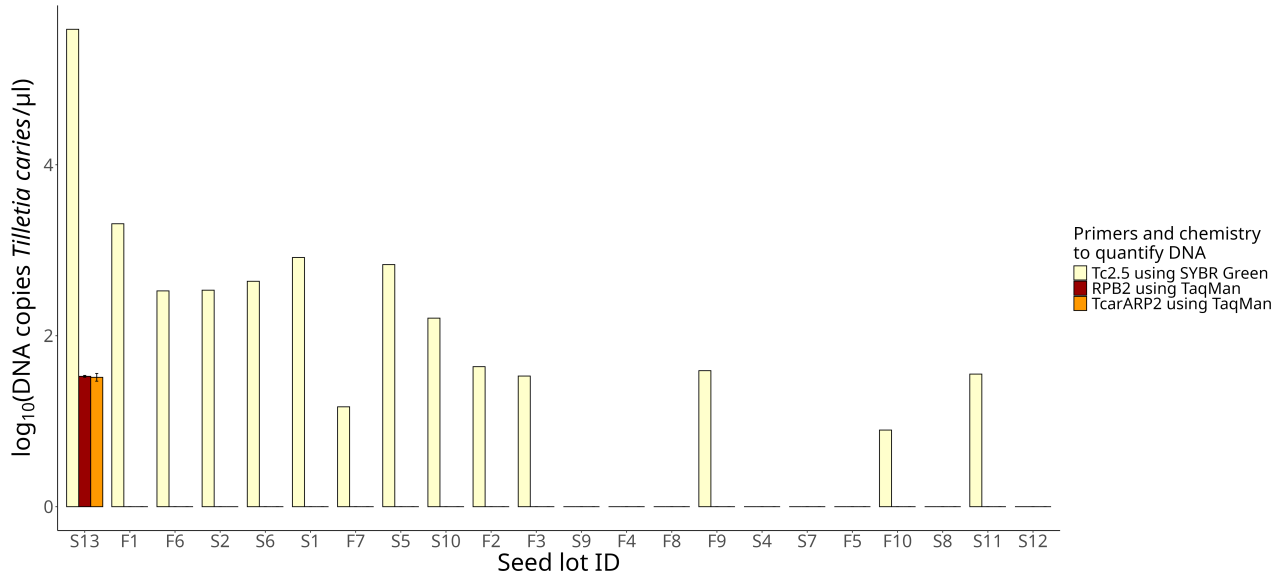
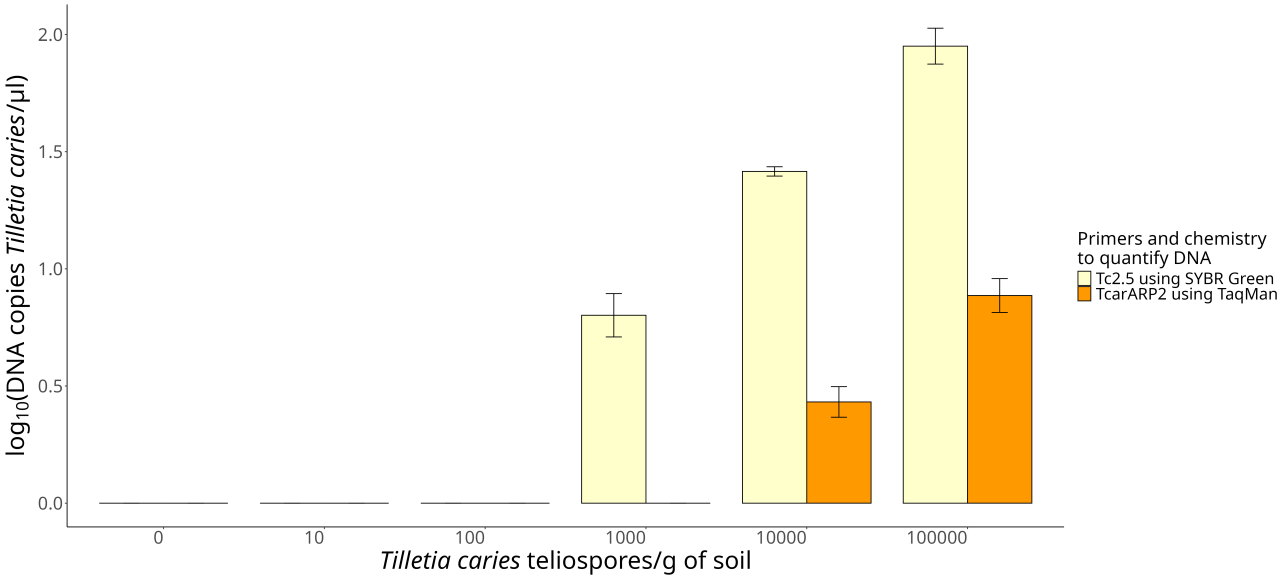
Supplementary Figures



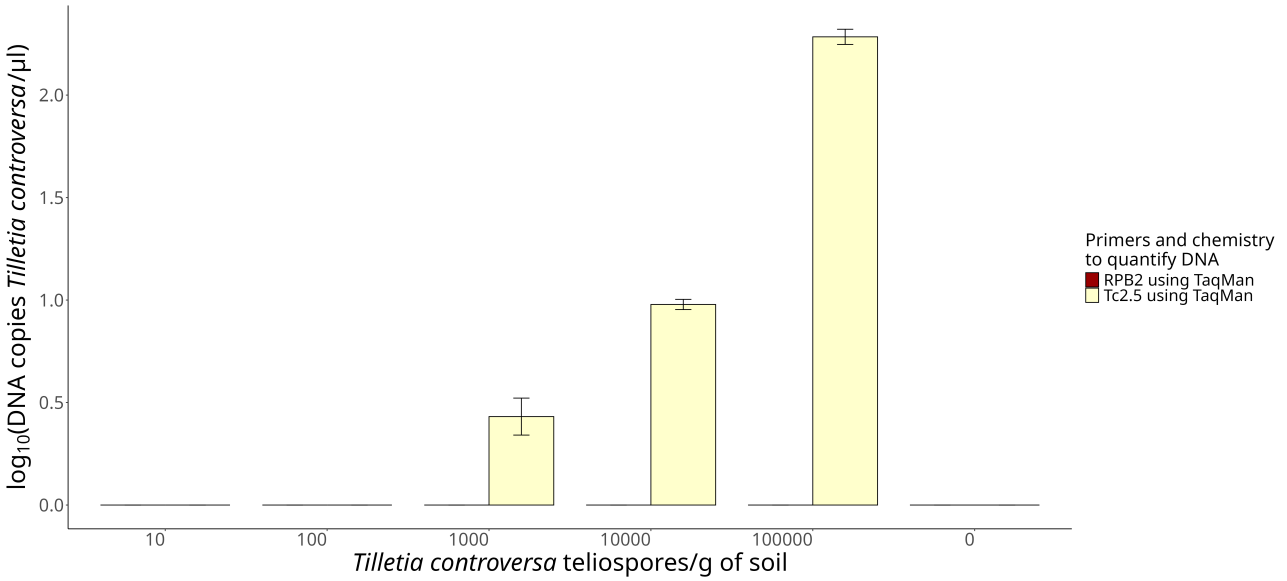
Supplementary Figure 1: Number of *Tilletia caries* teliospores per seed assessed using visual seed inspection [47] in 22 wheat seed lots. Error bars represent the variation among three independent inspectors who evaluated the same sample.



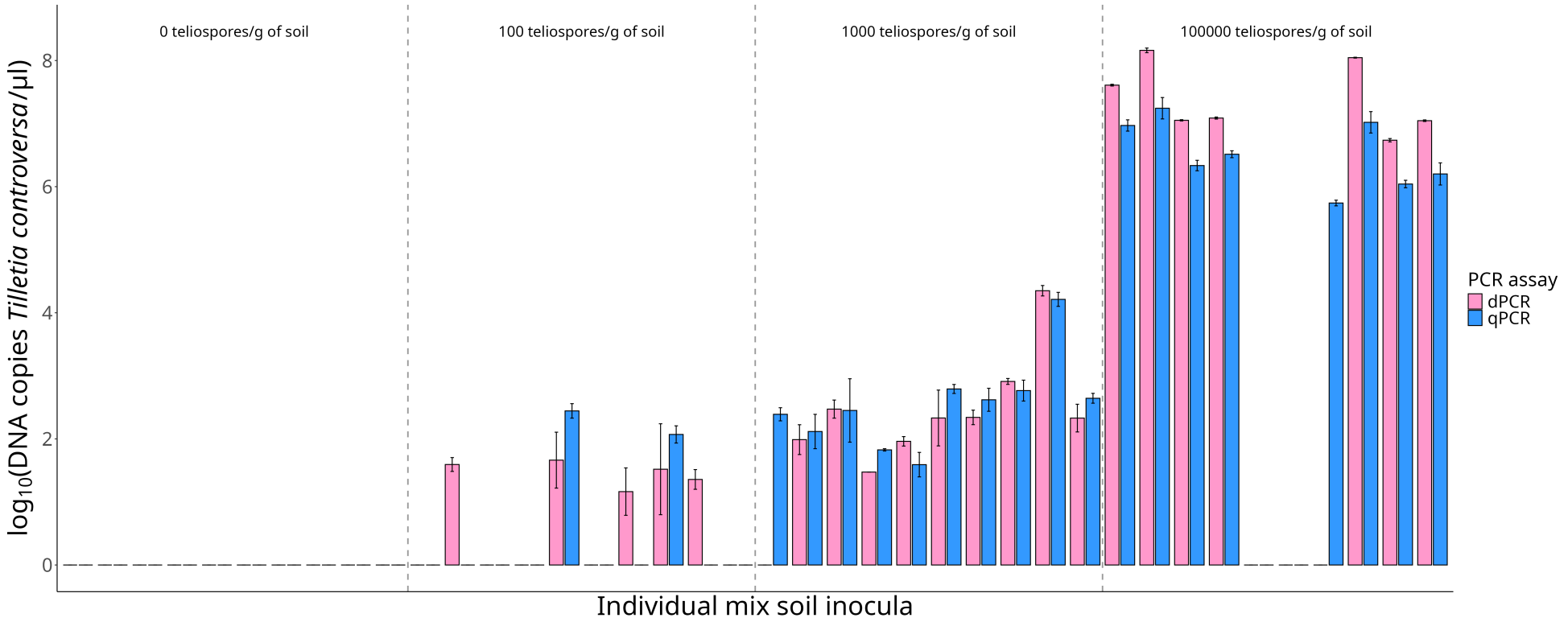
Supplementary Figure 2: *Tilletia caries* infection detected using dPCR in pellet seed DNA extracted from thirteen *Triticum aestivum* seed lots. The same extraction were tested with three primer sets, shown in different colors. The nuclear primer sets (TcarARP2 and RPB2) were run in duplicates, while for the mitochondrial primer set (Tc2.5) without replicate, as shown from the error bars. The difference in the quantification of *T. caries* were significant among primer sets base on the Kruskal-Wallis ($p = 1.51 \times 10^{-7}$, $\chi^2 = 31.41$) that was followed by a Fisher's least significant difference test with Benjamini-Hochberg p-value adjustment. There was no significant difference between the nuclear primer sets.



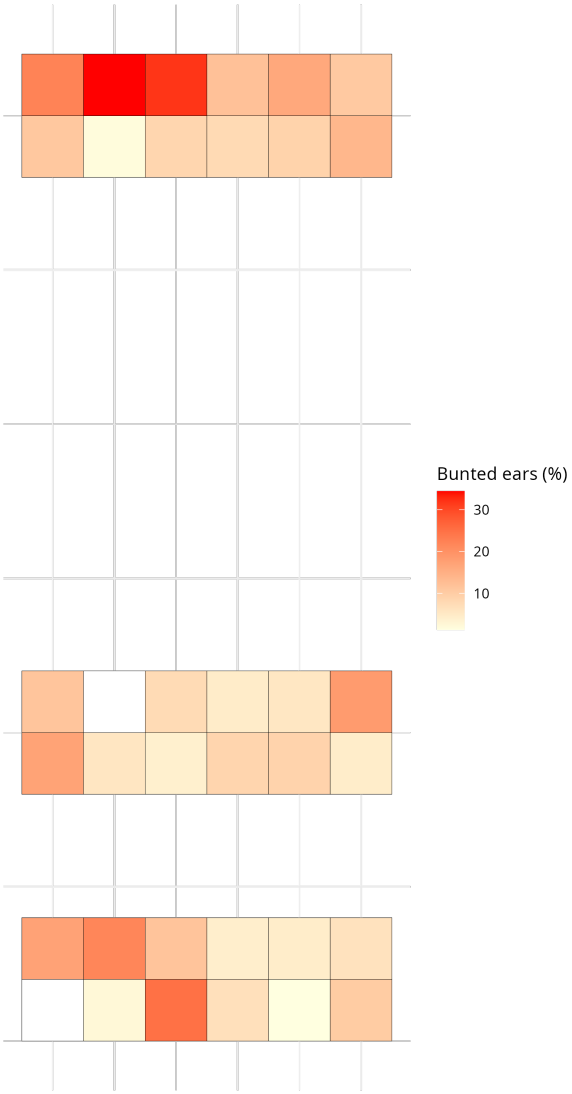
Supplementary Figure 3: Quantification of *Tilletia caries* DNA in soil using mitochondrial (Tc2.5) and nuclear (TcarARP2) primer sets. One DNA extraction was performed for each level of soil artificial inoculum. The six soil samples of the different levels of infestation were analyzed with qPCR using the Tc2.5 primer set using SYBR Green chemistry and TcarARP2 primer set using TaqMan chemistry with a probe. The mean of the triplicate qPCR reactions are shown and the error bars correspond to the standard error.



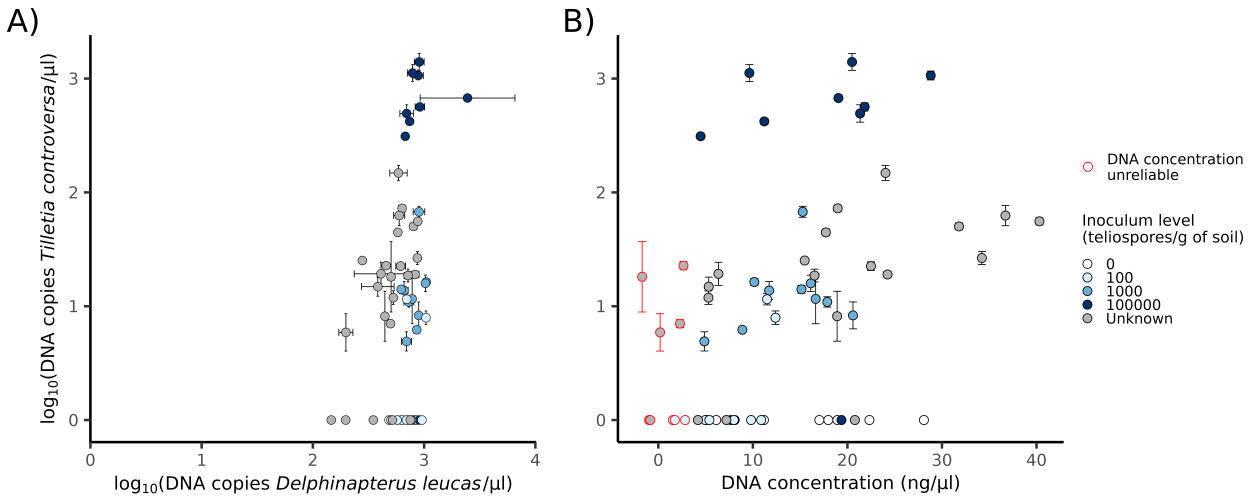
Supplementary Figure 4: Detection of *Tilletia controversa* DNA in artificially inoculated soil using mitochondrial (Tc2.5) and nuclear (RPB2) primer sets. The same DNA extractions were tested with both Tc2.5 and RPB2 primer-probe set. For each inoculum level a single soil sample was analyzed, and the error bars represent the standard error of the triplicate qPCR measurments.



Supplementary Figure 5: Detection of *Tilletia contorversa* DNA in artificially inoculated soil samples using dPCR and qPCR. Each inoculum level was tested with ten individual replicates of separately extracted DNA. The same extraction was tested in duplicate with dPCR (pink) and triplicate with qPCR (blue). The error bars shown the standard errors of PCR replicate measurements.



Supplementary Figure 6: Spatial distribution of *Tilletia contorversa* disease incidence observed in a naturally infested field (July 2024). The monitored area comprised three untreated control rows (1.5 m x 48 m) from a field trial evaluating plant production products against *T. controversa* (data not shown). Disease incidence was assessed within a 0.3 m^2 frames, positioned twelve times per row (six per side), by counting the total wheat ears and bunted ears and calculating their ratio. A subsection of this field was sampled in September 2023, prior to sowing, to quantify *T. controversa* DNA in the soil using qPCR and dPCR (Figure 5).



Supplementary Figure 7: Correlation between the number of DNA copies of *Tilletia controversa* and A) *Delphinapterus leucas* and B) DNA concentration in soil samples. The copy numbers of *T. controversa* and *D. leucas* were quantified using a qPCR triplex reaction and TaqMan chemistry. Artificially inoculated soil samples are shown across a range of *T. controversa* teliospores per gram of soil (0-100000 teliospores/g) and colored according to the inoculum level. Naturally infested soils with an unknown inoculum level are shown in gray. B) Samples with a DNA concentration below 3 ng/µL were considered unreliable. Error bars indicate the standard error among triplicates for A) both targets and B) *T. controversa* target.

Supplementary Tables

Gene target	Target organism	Oligo name	Sequence (5′ - 3′)	Length (bp)	Molecular weight (g/mol)	Genbank accession number
ARP2	*Tilletia caries*	TcarARP2_stdcurve	GAC GTC GAA CAA CCA AGA GCG GCT CCA AAC CAT CCA AGC AGC CGC AGT CGA CAC CCG CTC CGA GCT CTA CAA GCA CAT CGT CCT CTC CGG TGG ATC GAG CAT TAC CCC GGC CTG CCC TCC CGT CTG GAG AAG GAG GTC AAG CTG CCC ATT TCG ATC GGA	159	98139.2	JALUTQ010000006.1:41834-41992
Unknown	*Tilletia controversa*	Tc2.5_stdcurve	AGA TGC TAG GGG TGA CAA AGA TCG GAT TCT GT TTC TGG TCC TGA CTT TCC TCA CTT TCT TGT CGT TCG TCT TTA GCT CGT TAT ATC TCT GCT TTA CGG CTC CGC CGA TTA TAC TCT TCT ATT ATA TTT ACA TTG TAA AAA AAC AAG GAC AGA ATC GGC GGA GCG CCA ATC ATT TTC TTT TAC TAC ATT TA	190	117258	NC_073546.1:49162-49186
ND2	*Delphinapterus leucas*	DleND2_stdcurve	ATC CAA AAT TCC ACC ACC ACC ACA CTA CTA CTA TCC CAA ACA TGA AAT ACA ATA CCA ATT ATA ACA ACC TTC ACC ATA CTC ACC CTC CTA TCC ATA GGA GGA CTC CCT CCA CTC ACA GGA TTT ATA CCC AAA TGA	135	83281	NC_034236.1

Supplementary Table 1: Sequences of gBlock gene fragments used to create the standard curve. These standard curves were included in qPCR plates for the absolute quantification of *Tilletia caries*, *Tilletia controversa*, and *Delphinapterus leucas*. Copy numbers of each target were calculated from the absolute quantification. The GenBank accession numbers identify the sequences used as the basis of the gBlock gene fragments.

qPCR plate	Assay	*Tilletia controversa* standard curve (Tc2.5)				*Tilletia caries* standard curve (ARP2)				*Delphinapterus leucas* standard curve (ND2)			
		Efficiency	R^2	**Slope**	**y-intercept**	**Efficiency**	R^2	**Slope**	**y-intercept**	**Efficiency**	R^2	**Slope**	**y-intercept**
mpl18	TaqMan	0.999	0.985	-3.324	39.079	0.108	0.983	-3.152	35.239	0.103	0.995	-3.247	36.605
mpl19	TaqMan	0.101	0.989	-3.299	39.303	0.104	0.984	-3.229	35.896	0.101	0.997	-3.302	38.088
mpl20	TaqMan	0.102	0.989	-3.285	39.702	0.977	0.993	-3.378	36.928	0.980	0.999	-3.370	38.055
spl27	SYBR	0.972	0.995	-3.392	36.107	—	—	—	—	—	—	—	—

Supplementary Table 2: Information about standard curves from each qPCR plate tested in multiplex (mpl) or singleplex (spl). Standard curves were generated with gBlock double-stranded synthetic oligonucleotide fragments prepared in a 5-fold serial dilution for absolute quantification. *Tilletia controversa* mitochondrial DNA was amplified with the Tc2.5 primer set, part of the ARP2 gene was amplified for *Tilletia caries* quantification, and the ND2 gene for amplification of *Delphinapterus leucas*. The *T. contorversa* mitochondrial target was detected with a HEX-labeled probe (TaqMan chemistry) in mpl qPCR plates, while without probe the primer set Tc2.5 (SYBR Green chemistry) detects both *T. caries* and *T. controversa* in spl qPCR plate. The *T. caries* ARP2 target with a FAM-labeled probe, and *D. leucas* ND2 target was detected with a Cyn-labeled probe. For each standard curve, the parameters: amplification efficiency, coefficient of determination (R^2), slope, and y-intercept were calculated using CFX Maestro software (Bio-Rad).

		dPCR results			
		Intra-plate variance (triplicate)		Intra-plate variance (duplicate)	
Primer set	**Dilution series**	**($\overline{Copies}/\mu$l$\pm$ Std. Dev.**	**Coefficient of Variation (%)**	**($\overline{Copies}/\mu$l$\pm$ Std. Dev.**	**Coefficient of Variation (%)**
Tc2.5	S1	26909.68 ± 744.88	2.77	27876.86 ± 2116.58	7.59
				28419.00 ± 899.21	3.16
				29282.46 ± 5055.26	17.26
	S2	5278.59 ± 9.82	0.19	5386.65 ± 400.91	7.44
				5209.03 ± 180.34	3.46
	S3	1059.99 ± 75.76	7.15	916.54 ± 88.21	9.62
				1032.48 ± 16.18	1.57
TcarARP2	S1	13401.81 ± 594.60	4.44	12636.01 ± 77.30	0.61
				13716.89 ± 435.16	3.17
				13387.71 ± 6.47	0.05
	S2	2527.20 ± 136.54	5.40	2374.38 ± 107.76	4.54
				2494.37 ± 113.73	4.56
	S3	522.61 ± 63.87	12.22	496.06 ± 28.52	5.75
				522.61 ± 63.87	12.22

Supplementary Table 3: Evaluation of intra-plate variance among replicates of the standard curves using dPCR. To compare intra-plate variation, coefficients of variation for dPCR plates that contained either duplicates or triplicates of three dilution points of the standard curve (S1-S3) were examined. The standard curves were composed of gBlock fragments that are amplified with the primers Tc2.5 and TcarARP2. Duplicates of these dilution points were tested on two or three plates for each synthetic target while triplicates of these dilution points were tested on one plate.

Tested model	Nuclear primer set	R^2	R^2 adj	p	$F_{(df1,df2)}$	Residual Standard Error	AIC	BIC
Power-law	TcarARP2	0.81	0.81	$< 2.20 \times 10^{-16}$	$191.60_{(1,44)}$	0.37	42.62	48.10
	RPB2	0.73	0.73	3.68×10^{-14}	$120.1_{(1,44)}$	0.56	81.21	86.69
Linear	TcarARP2	0.69	0.68	1.15×10^{-12}	$96.54_{(1,44)}$	403.20	686.44	691.93
	RPB2	0.65	0.65	1.06×10^{-11}	$83.19_{(1,44)}$	410.10	688.00	693.49
Polynomial	TcarARP2	0.70	0.69	4.07×10^{-12}	$51.32_{(2,43)}$	396.10	685.75	691.93
	RPB2	0.69	0.67	1.54×10^{-11}	$46.94_{(2,43)}$	395.30	685.56	692.88
Exponential	TcarARP2	0.52	0.51	1.84×10^{-8}	$47.05_{(1,44)}$	0.60	86.86	92.34
	RPB2	0.44	0.43	5.43×10^{-7}	$34.33_{(1,44)}$	0.83	116.98	122.47
Logarithmic	TcarARP2	0.60	0.59	3.49×10^{-10}	$64.7_{(1,44)}$	458.50	698.26	703.75
	RPB2	0.60	0.59	3.39×10^{-10}	$64.84_{(1,44)}$	443.30	695.16	700.65

Supplementary Table 4: Model evaluations used to correlate *Tilletia caries* copy/µL of bunt population from mitochondrial primer set and nuclear primer sets. These models are based on the correlation between *Tilletia caries* copy/µL measured with dPCR using the nuclear primer sets with their probes (RPB2 or TcarARP2) and the mitochondrial primer set without probe (Tc2.5). The power-law model fits best for its predictive performance (highest adjusted R^2) and lower complexity (lowest AIC and BIC) compared to the linear, polynomial, exponential, and logarithmic models. Because of the significant linear relationship, an mtDNA target can be used as a proxy for quantification based on a nuclear target.

A) Mitochondrial primer set Tc2.5 with SYBR Green chemistry

Predicted (*Tilletia caries* copies/µL)	Actual values (Seed visual inspection): Contaminated	Actual values (Seed visual inspection): Uncontaminated
Contaminated	True Positive (TP): F1 F2 F3 F6 F7 F9 S1 S2 S5 S6 S10 S11 S13	False Positive (FP): F10 S11
Uncontaminated	False Negative (FN): F4 F8 S4 S7 S9	True Negative (TN): F5 S8 S12

B) Nuclear primer set TcarARP2 with TaqMan chemistry

	Actual values (Seed visual inspection): Contaminated	Actual values (Seed visual inspection): Uncontaminated
Contaminated	True Positive (TP): S13	False Positive (FP): —
Uncontaminated	False Negative (FN): F1 F2 F3 F4 F6 F7 F8 F9 S1 S2 S4 S5 S6 S7 S9	True Negative (TN): F10 F5 S11 S12 S8

C) Nuclear primer set RPB2 with TaqMan chemistry

Predicted (*Tilletia caries* copies/µL)	Actual values (Seed visual inspection): Contaminated	Actual values (Seed visual inspection): Uncontaminated
Contaminated	True Positive (TP): S13	False Positive (FP): —
Uncontaminated	False Negative (FN): F1 F2 F3 F4 F6 F7 F8 F9 S1 S2 S4 S5 S6 S7 S9	True Negative (TN): F10 F5 S11 S12 S8

Supplementary Table 5: Confusion matrices used to evaluate the dPCR-based classification of *Tilletia caries* contamination in wheat seed lots using the primer sets Tc2.5, TcarARP2, and RPB2 (predicted values), compared to visual seed inspection results as the reference method (actual values). Seed lots were classified as contaminated or uncontaminated based the visual observation of at least one *T. caries* teliospore from one of three seed inspectors. For dPCR, the contamination status of the seed lots depended on the primer set's limit of detection. (A) For the mitochondrial primer set Tc2.5, seed lots were classified as contaminated when the measured *T. caries* DNA copies exceeded 1.17 per µL. For the nuclear primer set (B) TcarARP2 and (C) RPB2, contamination was defined as more than 1.14 *T. caries* DNA copies/µL. Agreement between classifications of dPCR-based and visual seed inspection was considered true positives or true negatives, whereas disagreement between these two classifications was considered false positives or false negatives.

	dPCR results		qPCR results		
Sample number	*Tilletia controversa* copies/μL [a] ± Std. Dev.	Coefficient of variation (%)	*Tilletia controversa* copies/μL ± Std. Dev.	Coefficient of variation (%)	Differences between the results of qPCR and dPCR (%)
8	21.70 ± 8.14	37.54	21.90 ± 3.36	15.36	0.91
7	10.10 ± 2.70	26.68	0.00 ± 0.00	—	-100.00
6	35.61 ± 8.62	24.21	43.72 ± 5.21	11.91	22.75
5	12.64 ± 4.22	33.39	9.01 ± 6.10	67.74	-28.73
4	17.61 ± 3.18	18.04	21.69 ± 3.55	16.37	23.18
3	21.91 ± 7.52	34.32	24.29 ± 2.43	9.99	10.89
2	15.17 ± 1.44	9.50	14.45 ± 5.53	38.31	-4.75
1	5.21 ± 2.62	50.23	5.65 ± 3.39	59.93	8.50
16	11.29± 6.35	56.24	11.10 ± 2.68	24.15	-1.69
15	21.72 ± 2.67	12.28	18.08 ± 2.11	11.68	-16.74
14	19.20 ± 1.69	8.82	0.00 ± 0.00	—	-100.00
13	54.72 ± 17.95	32.80	49.49 ± 6.10	12.32	-9.56
12	59.11 ± 6.64	11.23	71.75 ± 8.23	11.47	21.39
11	55.48 ± 11.16	20.11	25.22 ± 19.54	77.47	-54.55
10	28.10 ± 9.98	35.51	$0.00_{102.39} \pm 0.00_{6.53}$	$—_{6.37}$	$-100.00_{264.36}$
9	4.08 ± 0.89	21.83	0.00 ± 0.00	—	-100.00
24	4.48 ± 0.01	0.32	6.08 ± 0.99	16.31	35.56
23	20.36 ± 4.24	20.83	17.92 ± 4.49	25.03	-11.98
22	21.08 ± 3.60	17.08	25.96 ± 5.95	22.91	23.16
21	80.96 ± 4.61	5.69	64.20 ± 21.98	34.23	-20.70
20	143.72 ± 6.13	4.27	150.67 ± 40.59	26.94	4.84
19	55.44 ± 2.01	3.63	55.00 ± 6.90	12.55	-0.81
18	16.22 ± 8.60	53.04	19.27 ± 7.48	38.78	18.80
17	5.65 ± 3.25	57.54	0.00 ± 0.00	—	-100.00

Supplementary Table 6: Percentage differences between qPCR and dPCR measured *Tilletia controversa* copies/µL in naturally infested soil samples. Each sample was analyzed in triplicate with qPCR and duplicate with dPCR. For samples with DNA amplification detected in only one replicate, the quantified copies and standard deviation are reported as a subscript.

[a] Average of DNA copies/μL ± standard deviation among triplicates

Inoculum type	Teliospores per g of soil	ID number	DNA concentration (ng/µL)	*Delphinapterus leucas* qPCR results			*Tilletia controversa* qPCR results	
				$\overline{Cq\ Mean}$ ± Std. Dev.[a]	$\overline{\text{copies}/\mu\text{L}}$ ± Std. Dev.	CV (%) [b]	$\overline{\text{copies}/\mu\text{L}}$ ± Std. Dev.	CV (%)
Artificial inoculum in series	100000	1	27.8	25.50 ± 0.15	633.81 ± 70.04	11.05	157.34 ± 21.51	13.67
	10000	2	18.3	25.40 ± 0.12	678.58 ± 57.11	8.42	6.28 ± 0.67	10.61
	1000	3	6.8	25.51 ± 0.12	628.06 ± 53.14	8.46	0.00 ± 0.00	—
	100	4	5.9	25.42 ± 0.05	665.79 ± 24.46	3.67	0.00 ± 0.00	—
	10	5	6.7	25.62 ± 0.02	580.34 ± 6.77	1.17	0.00 ± 0.00	—
Artificial inoculum individually	0	1	5.1	24.66 ± 0.20	789.85 ± 108.96	13.80	0.00 ± 0.00	—
		2	8.1	24.62 ± 0.15	807.19 ± 85.14	10.55	0.00 ± 0.00	—
		3	18.0	25.36 ± 0.12	483.77 ± 40.84	8.44	0.00 ± 0.00	—
		4	22.3	24.99 ± 0.10	624.70 ± 40.47	6.48	0.00 ± 0.00	—
		5	28.1	24.96 ± 0.13	639.97 ± 58.67	9.17	0.00 ± 0.00	—
		6	18.9	24.58 ± 0.18	831.33 ± 100.38	12.07	0.00 ± 0.00	—
		7	17.0	24.85 ± 0.19	694.08 ± 88.38	12.73	0.00 ± 0.00	—
		8	6.1	24.62 ± 0.14	810.71 ± 78.48	9.68	0.00 ± 0.00	—
		9	5.1	25.11 ± 0.20	580.42 ± 83.67	14.41	0.00 ± 0.00	—
		10	11.2	24.52 ± 0.04	865.71 ± 24.70	2.85	0.00 ± 0.00	—
	100000	1	28.8	24.49 ± 0.27	893.37 ± 170.49	19.08	1072.02 ± 165.35	15.42
		2	20.5	24.46 ± 0.25	911.83 ± 152.04	16.67	1437.41 ± 396.24	27.57
		3	21.8	24.44 ± 0.25	923.90 ± 160.93	17.42	566.75 ± 82.16	14.50
		4	19.0	23.01 ± 2.46	6437.81 ± 9556.31	148.44	675.70 ± 65.15	9.64
		5	19.4	24.45 ± 0.09	906.79 ± 54.14	5.97	0.00 ± 0.00	—
		6	7.7	24.75 ± 0.06	735.33 ± 32.33	4.40	0.00 ± 0.00	—
		7	4.5	24.88 ± 0.12	677.22 ± 59.19	8.74	310.87 ± 24.15	7.77
		8	9.6	24.66 ± 0.27	795.12 ± 148.41	18.67	1151.00 ± 340.65	29.60
		9	11.2	24.75 ± 0.11	741.33 ± 58.16	7.85	421.10 ± 44.67	10.61
		10	21.3	24.83 ± 0.35	709.83 ± 182.35	25.69	508.71 ± 165.58	32.55
	1000	1	17.9	24.77 ± 0.26	736.61 ± 134.26	18.23	10.03 ± 1.91	19.04
		2	20.6	24.47 ± 0.09	894.32 ± 54.44	6.09	7.88 ± 3.57	45.35
		3	16.6	24.67 ± 0.16	782.73 ± 88.28	11.28	13.20 ± 8.63	65.40
		4	8.9	24.53 ± 0.17	861.31 ± 97.35	11.30	5.21 ± 0.20	3.79
		5	4.9	24.84 ± 0.26	700.72 ± 122.21	17.44	4.09 ± 1.56	38.09
		6	10.1	24.25 ± 0.12	1046.28 ± 82.06	7.84	15.39 ± 1.95	12.67
		7	11.7	24.91 ± 0.21	664.19 ± 100.30	15.10	13.18 ± 4.27	32.43
		8	16.1	24.27 ± 0.14	1030.22 ± 99.29	9.64	15.33 ± 4.69	30.58
		9	15.3	24.46 ± 0.30	910.63 ± 177.18	19.46	67.34 ± 12.50	18.57
		10	15.1	25.00 ± 0.07	621.58 ± 28.66	4.61	13.15 ± 1.91	14.49
	100	1	10.9	24.80 ± 0.23	718.15 ± 117.32	16.34	0.00 ± 0.00	—
		2	8.0	24.75 ± 0.09	738.13 ± 47.15	6.39	0.00 ± 0.00	—
		3	$NA_{1.5}$	24.97 ± 0.06	631.65 ± 25.50	4.04	0.00 ± 0.00	—
		4	5.0	24.38 ± 0.11	956.59 ± 74.34	7.77	0.00 ± 0.00	—
		5	11.5	24.83 ± 0.12	697.76 ± 56.82	8.14	10.67 ± 2.33	21.88
		6	$NA_{1.7}$	24.69 ± 0.02	767.47 ± 11.16	1.45	0.00 ± 0.00	—
		7	$NA_{2.8}$	25.30 ± 0.01	503.04 ± 4.93	0.98	0.00 ± 0.00	—
		8	12.4	24.26 ± 0.04	1038.45 ± 25.92	2.50	7.07 ± 1.77	25.05
		9	9.8	24.84 ± 0.17	696.56 ± 78.36	11.25	0.00 ± 0.00	—
		10	5.4	25.12 ± 0.09	571.62 ± 37.55	6.57	0.00 ± 0.00	—
Natural	Unknown	8	$NA_{2.7}$	25.45 ± 0.12	455.64 ± 36.17	7.94	21.90 ± 3.36	15.36
		7	7.2	25.26 ± 0.05	518.71 ± 15.96	3.08	0.00 ± 0.00	—
		6	17.7	25.10 ± 0.07	579.38 ± 27.50	4.75	43.72 ± 5.21	11.91
		5	18.9	25.49 ± 0.11	443.80 ± 34.28	7.72	9.01 ± 6.10	67.74
		4	22.5	25.01 ± 0.25	620.62 ± 112.64	18.15	21.69 ± 3.55	16.37
		3	15.5	26.16 ± 0.07	277.78 ± 13.64	4.91	24.29 ± 2.43	9.99
		2	5.3	25.70 ± 0.85	431.25 ± 269.50	62.49	14.45 ± 5.53	38.31
		1	$NA_{0.2}$	26.66 ± 0.37	201.38 ± 52.39	26.02	5.65 ± 3.39	59.93
		16	5.3	25.23 ± 0.14	528.94 ± 49.95	9.44	11.10 ± 2.68	24.15
		15	24.2	24.58 ± 0.21	832.69 ± 115.34	13.85	18.08 ± 2.11	11.68
		14	20.8	24.73 ± 0.18	753.48 ± 95.79	12.71	0.00 ± 0.00	—
		13	31.8	24.63 ± 0.17	804.08 ± 91.68	11.40	49.49 ± 6.10	12.32
		12	19.0	24.97 ± 0.06	633.25 ± 26.97	4.26	71.75 ± 8.23	11.47
		11	$NA_{-1.7}$	25.30 ± 0.18	506.24 ± 61.01	12.05	25.22 ± 19.54	77.47
		10	4.2	25.83 ± 0.09	348.52 ± 20.55	5.90	0.00 ± 0.00	—
		9	$NA_{-0.8}$	27.10 ± 0.12	145.59 ± 12.21	8.39	0.00 ± 0.00	—
		24	$NA_{2.3}$	25.32 ± 0.15	499.68 ± 52.19	10.45	6.08 ± 0.99	16.31
		23	16.5	24.79 ± 0.20	720.86 ± 100.00	13.87	17.92 ± 4.49	25.03
		22	34.2	24.51 ± 0.08	869.62 ± 49.37	5.68	25.96 ± 5.95	22.91
		21	36.7	25.06 ± 0.29	604.16 ± 126.24	20.90	64.20 ± 21.98	34.23
		20	24.0	25.08 ± 0.45	605.60 ± 177.49	29.31	150.67 ± 40.59	26.94
		19	40.3	24.50 ± 0.14	878.90 ± 87.53	9.96	55.00 ± 6.90	12.55
		18	6.4	25.60 ± 1.39	542.72 ± 463.10	85.33	19.27 ± 7.48	38.78
		17	$NA_{-1.0}$	26.66 ± 0.10	196.70 ± 13.48	6.85	0.00 ± 0.00	—

Supplementary Table 7: Concentration of DNA extracted from soil samples and their corresponding quantification of DNA copies/µL and cycle (Cq) of *Delphinapterus leucas*, the exogenous internal control, and *Tilletia controversa*. *D. leucas* and *T. controversa* were quantified together in a multiplex qPCR reaction, and each sample was measured in triplicate. Samples with a concentration under 3.0 ng/µL are shown with a "NA" because their measurements (shown as subscript) were considered unreliable.

[a] Average of the mean quantification cycle (Cq) values across tested soil ± standard deviation among the soil subsample Cq Mean

[b] Coefficient of variation

Supplementary Material and Method

Design and validation of synthetic gBlock standards and qPCR optimization

The gBlock fragments used for the standard curves (Supplementary Table 1) were selected from the same target area as the developed primers (Table 1). The gBlock fragments that target ARP2 nuclear gene of *Tilletia caries* included 5 bp of flanking sequence on the left side and 15 bp on the right side of the target region. The gBlock fragment based on ND2 gene of *Delphinapterus leucas* contained 5 bp flanking regions on both sides. The synthetic fragment corresponding to the mitochondrial region of *Tilletia controversa* targeted by Tc2.5 primers included 5 bp of flanking sequence on the left side and 31 bp on the right side.

The mitochondrial region targeted by Tc2.5 primers could not be synthesized directly as a gBlock fragment due to its sequence features were incompatible with gBlock synthesis. To overcome this issue, additional codons were added to the terminal region, and the sequence was manually modified while maintaining the exact primer binding sites and amplicon length (i.e., the same number of base pairs flanking the target region). Although the resulting gBlock fragment had a higher melting temperature than the target sequence (80.5 °C and 73.5 °C respectively), the amplification efficiency of gBlock dilution series using Tc2.5 primers without probe (efficiency = 97.2%, R^2 = 0.995, Slope = -3.392, y-intercept = 36.107) was comparable to the efficiency obtained with the same primer sets from a dilution series of DNA extracted from *T. caries* mycelia (efficiency = 96.2%, R^2 = 0.997, Slope = -3.417, y-intercept = 13.732).

All the gBlock fragments were initially diluted to a concentration of 0.03 ng/µL and stored at -20°C with 0.2 mg/ml Poly(A) (Sigma-Aldrich, Germany). The diluted gBlock fragments were then divided into aliquots suitable for a single plate to limit freeze-thaw cycles.

Annealing temperatures for primer and probe sets were optimized in singleplex reactions using a gradient from 53 °C to 67 °C, followed by a test gradient from 59 °C to 63 °C for multiplex reactions.

Chapter 3

Optimization of a lab-screening procedure to evaluate seed treatment efficacy: detection of *Ustilago nuda* in *Hordeum vulgare* seedlings

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Submitted manuscript

Abstract

Recent changes in agricultural policies promote the reduced application of synthetic plant protection products (PPPs), including their use as prophylactic seed treatments. To prevent disease outbreaks and economic losses due to reduced synthetic seed treatments, rapid and reliable tools to evaluate newly developed, low-risk seed treatments are needed. Visual inspections of plants grown in greenhouses or fields are used to assess seed treatment efficacy. This process is labor-intensive and costly — especially for internal seedborne pathogens, such as *Ustilago nuda* in barley, which remain asymptomatic until the flowering stage when smutted ears develop. To accelerate the evaluation of seed treatments against *U. nuda*, we developed a lab-screening procedure that combines optimized barley seedling growth procedures and qPCR to quantify *U. nuda* DNA in seedlings. We grew seedlings for 5, 10 and 14 days with two

cultivation techniques: growth on filter paper in the dark and in potting soil under light–dark conditions. The seedlings were grown in a laboratory from two naturally infected seed lots, which were treated with a synthetic fungicide or warm water or not treated. The treated and untreated seeds were also grown during two seasons in the field. The infection levels in the seedlings were based on the relative amount of *U. nuda* DNA normalized to *Hordeum vulgare* DNA quantified using qPCR. These infection levels were then compared to *U. nuda* disease occurrence observed in the field. Treatment efficacies were calculated using field-observed disease incidence and laboratory-measured infection levels. The seedling cultivation methods were compared using a lab-to-field predictability factor. Treatment efficacies assessed using qPCR on seedlings grown for 10 days on filter paper and 14 days in potting soil most accurately represented field results and showed clear treatment effects, particularly in the seed lot with the higher infection rate. Overall, our lab-screening method enables an early and efficient evaluation of seed treatment efficacy, which can aid in the development of new, low-risk control strategies to help reduce synthetic PPP applications.

Keywords: loose smut, early detection, plant protection, seed coating evaluations, cereals and grains, seedborne fungi, fungicide testing, pathogen detection

Introduction

A resurgence of seedborne fungal diseases poses a threat to crop production as fewer synthetic seed treatments are used due to the agricultural policies that aim to reduce the synthetic plant protection products (PPPs) [1, 2, 3]. Developing sustainable, alternative solutions to synthetic PPP applications, such as mechanical treatments or natural product-based formulations, is necessary to control and prevent outbreaks of seedborne disease [4]. However, the assessment of new seed treatments is often slow because treatment efficacy is typically evaluated when the pathogen causes symptoms in the host. In some seedborne diseases, the symptoms only appear in the late stages of plant development [5, 6]. Furthermore, treatment efficacy trials depend on seed lots that are ideally naturally infected [7]. Seedborne pathogens that have low rates of seed contamination (e.g., *Verticillium longisporum* [8]). require a larger sample size to generate statistically meaningful differences in symptom development between treatment and control plants [9, 10, 11]. The requirement for a large sample size can make the field and greenhouse evaluations time-consuming and resource-intensive, especially when the evaluations must take place at the late stages of plant development.

Ustilago nuda (loose smut) is pathogen that causes symptoms only late in the plant's development, and PPPs can control reduce disease occurrence to below 1% [12]. For *U. nuda*, synthetic PPPs can prevent systemic infections and the development of its teliospores. In a smutted ear, teliospores replace inflorescences and can cause a yield loss of 25% or more [13]. Currently, there are no alternative treatments to synthetic PPPs available on the market for controlling *U. nuda*. Although warm water treatment of seeds shows efficacy against loose smut, it remains impractical on a large scale [14]. A wide range of treatments should be tested to develop non-synthetic PPPs that provide protection against *U. nuda* development and can be applied on a large scale. However, conducting these treatment tests in the greenhouse and field

requires space and time. Therefore, high-throughput screening tools are needed to rapidly and accurately evaluate efficacy of new and feasible treatments before proceeding with greenhouse and field testing.

A molecular screening method on DNA extracted from seedlings can reduce resources needed for seed treatment development and offers a promising approach to select the most effective control strategies for further field testing. Previous research on *U. nuda* detection in seedlings used enzyme-linked immunosorbent assay (ELISA) and quantitative PCR (qPCR) [5, 15]. A challenge of the previously used ELISA- and PCR-based methods is their low throughput because they were used to analyze individual seedlings. These individual seedlings were grown in soil to the second leaf stage, approximately 14 days after seedling emergence [5, 15]. Due to the space, labor, and energy required to grow seedlings in soil for 14 days, the protocol's scalability is limited. Furthermore, in the previously published qPCR method to detect *U. nuda* in seedlings, a nonspecific fluorescence in the negative control was observed [5], which can compromise accuracy in pathogen detection.

In this study, we established a streamlined qPCR-based lab-screening procedure that quantifies *U. nuda* DNA in barley seedlings grown under controlled conditions for 5, 10, and 14 days to estimate treatment efficacy against *U. nuda*. We aimed to adapt a multiplex qPCR protocol originally developed for seed testing that targets *U. nuda* and *Hordeum vulgare* DNA [16] to enable the *U. nuda* detection in seedling tissue. For the application of a qPCR protocol to seedling, we tested the seedling growth and sampling procedures and to implement a pooling strategy that combines multiple seedlings into a single sample. After establishing the lab-screening procedure, we evaluated whether its measurement of *U. nuda* infection represented *U. nuda* disease occurrence in the field. We compared the calculated treatment efficacy derived from field observation and estimate from laboratory results using a lab-to-field predictability factor. In addition, we measured seedling lengths to determine whether the early plant growth was affected by pathogen infection, given the reported effects of *Ustilago* species on phytohormones involved in germination and cell development in the hosts [17, 18]. The developed lab-screening procedure could provide a reliable estimate the treatment efficacy against *U. nuda* development and potentially support the prioritization of effective candidate seed treatments before resource-intensive field treatment evaluations. Through reducing time and resources needed to develop new treatments, this approach could facilitate the development of new effective fungicidal seed treatments against *U. nuda*, which addresses the growing need for alternative disease control strategies as the availability of synthetic seed treatments decreases.

Material and Methods

Seed material and seed treatments

Two naturally infected seed lots of the six-row barley (*Hordeum vulgare*) cultivars, Azrah and Semper, were used to evaluate treatment efficacy against *Ustilago nuda* under field and controlled conditions in the laboratory. Both the seed lots were part of a previous study, during which they were analyzed with embryo inspections for detecting *U. nuda* [16]. Based on visual evaluations of infected embryos [16, 19], the Azrah and Semper seed lots used had natural *U.*

nuda infections of 2.6% and 8.8%, respectively. Neither of these cultivars is known to have resistance genes against *U. nuda* [20].

The seed lots from both cultivars were treated with either warm water or a synthetic fungicide. Additionally, an untreated control was included in the experiments to measure treatment efficacy. The warm water treatment was applied as previously described [21]. In short, seeds were soaked for 2 hours at 45 °C in a stainless-steel water bath with an immersion circulator (HAAKE W46E8 Fisons, Thermo Fisher Scientific, USA) and then dried overnight ($\simeq$ 12 h) at 38 °C ± 2 °C in an oven (WTB binder type FED400, Müller&Krempel, Switzerland). For each growing season, the warm water treatment was conducted within the week prior to sowing. The synthetic fungicide Rubin®Plus (BASF Schweiz AG, Switzerland) was applied to seeds (1.5 ml/kg seed) using the Hege 11 liquid seed treater (Hans-Ulrich GmbH & Co., Germany) in September 2021. The fungicide-treated seeds were stored in the dark at 10 °C until their sowing in the field in October 2021 and October 2022 and their use in the laboratory in November 2022. Based on information from BASF [22], seed treated with Rubin®plus can be stored and used as long as the seed quality is high and has germination rate is over 90% . Both seed lots were stored for maximum of one year after the treatment and maintained a high germination rate.

Field trial set up and infection rate evaluation

Field trials to evaluate the seed treatment efficacy against *Ustilago nuda* were conducted at Agroscope in Zurich, Switzerland. Due to Swiss crop rotation practices, the field locations within Agroscope were changed between growing seasons. Untreated and treated seeds were sown the first week of October during the two growing seasons: 2021-2022 and 2022-2023. Each treatment was sown in four replicate plots in a randomized complete block design. Each 9 m^2 plot was sown with 350 seeds/m^2 at a depth of 3-4 cm.

At the second leaf stage, the germination rate was evaluated by counting the number of seedlings along a linear meter two times within each plot. These seedling counts were taken in the second and sixth (of seven) rows and at least 1 meter form the short plot edges to minimize the boundary effects. During both growing seasons and prior to the flowering stage in April (BBCH 30-41), approximately 0.65 m of the plot was trimmed from the short ends of each plot using a motor mower. The shortened plots ensured plot separation to facilitate visual observations of diseased smutted ears. Following the plot trimming, the average final plot size was 5.2 m^2. At flowering stage in May (BBCH 61-69), the ears were counted in a 0.3 m^2 frame with the same counting procedure used for the germination rate. This ear count was used to estimate the total number of ears per plot. Ears were counted multiple times in the field during ear emergence (BBCH 47-69) to overcome differences in emergence times and weather conditions. The maximum number of smutted ears was used in each plot for the assessment of disease occurrence. (Supplementary Figure 3). The disease incidence was than calculated as the percentage of infected ears in each plot. Disease incidences observed in the field were used as a reference to evaluate the laboratory-based seedling growth methods conducted under controlled conditions.

Seedling cultivation and sample preparation

Seedlings were cultivated using a total of six combinations of growth conditions (Figure 1), including two growth substrates (potting soil or filter paper) and three growth time periods (5, 10 or 14 days). Seeds grown in the potting soil substrate were sown in 25 cm x 25 cm x 4 cm plastic square potting trays without holes that were filled with a universal potting soil for perennials and potted plants (Ökohum, Switzerland, article number 4392379). In each tray, 50 seeds were sown at a depth of 2 cm. The trays were then randomly distributed in a Kälte 3000 climate chamber (CLIMECAB, Switzerland). The climate chamber was set at 20 °C with a 16-hour light cycle (light intensity: 300 µmol/s/m^2) and 80% humidity followed by an 8-hour dark cycle with 85% humidity. Throughout the experiment, each tray received 0.3 L of water every 48 hours.

Seeds grown on the filter paper substrate were sown in transparent boxes (20 cm x 13 cm x 4 cm) on three stacked sheets of filter paper (Paper Sheets Grade 100/N, Ahlstrom, Germany) that had been wetted with a 0.05% gibberellic acid solution [23]. A vacuum seeder was used to evenly distribute 50 seeds in each box. Two additional sheets of filter paper that had been wetted with the same gibberellic acid solution were placed on top of the seeds. The boxes were closed with a plastic lid and then incubated in a RUMED Type 3501 S climate chamber (BLANC-LABO SA, Switzerland) set at 18 °C, with no light, and 85% humidity. No additional water was added to the filter paper or their boxes during the seedlings' growth on the filter paper substrate.

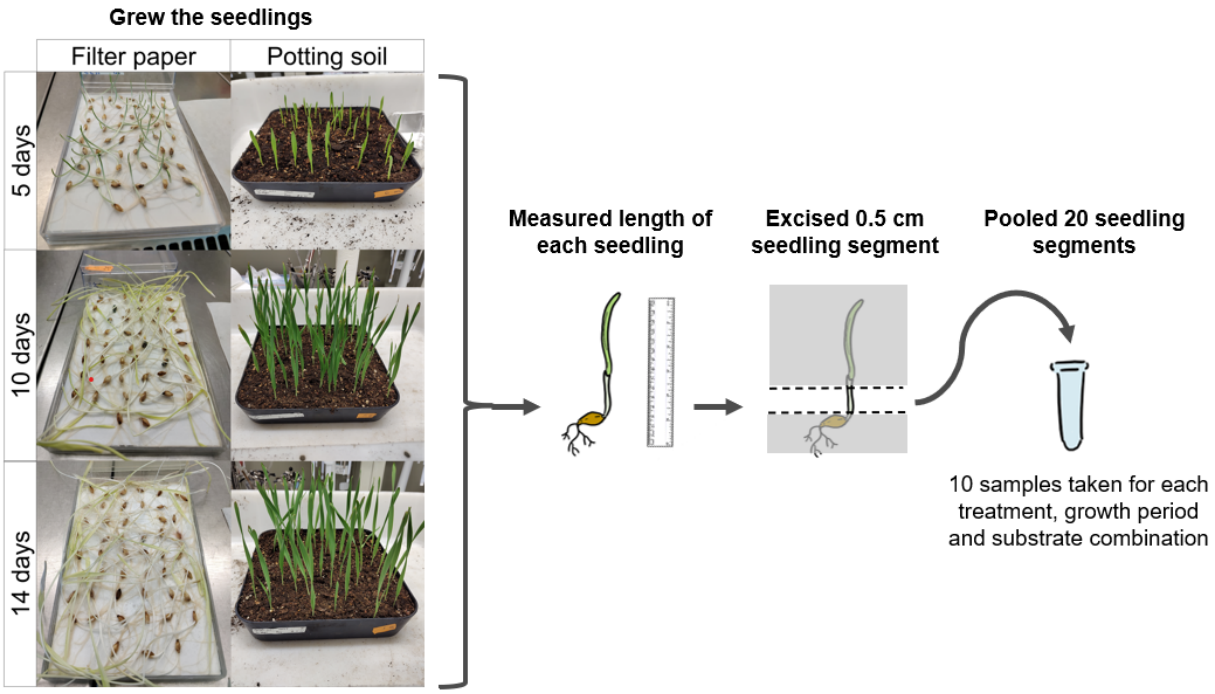

Figure 1: Diagram of seedling sample growth and processing. Seedlings were either grown on filter paper or in potting soil for 5, 10, or 14 days. The boxes used for the filter paper growth methods were 20 cm x 13 cm x 4 cm and the potting soil trays were 25 cm x 25 cm x 4 cm. The warm water treated Azrah seeds that were grown for 10 days have 9 rather than 10 samples.

At the end of each growth period, the total length of each seedling was recorded and then a 0.5 cm plant tissue segment was cut from the seedling base toward the apex . A scalpel and

forceps were used on a cutting board to cut the seedling segments (Figure 1). The scalpel and forceps were sterilized with 70%[v/v] ethanol and a Bunsen burner flame between each sample, and the cutting board was also cleaned using 70% [v/v] ethanol. The excised seedling segments were then added to the NucleoSpin lysis tube with a 3 mm tungsten bead (Retsch, Germany). A total of two hundred seedlings per treatment group and growth condition were collected and divided into ten samples, each sample consisted of twenty pooled seedling segments. The collected seedling segments were lyophilized for 24 hours using the VirTis Benchtop K Freeze Dryer (SP Industries, USA) and subsequently stored at -20 °C until DNA extraction.

DNA extraction

DNA was extracted from each sample, consisting of a pool of twenty 0.5 cm seedling segments, using NucleoSpin 96 Plant II kit (MACHEREY-NAGEL, GmbH & Co., Germany). The manufacturer's protocol for the DNA extraction was used with the modifications described below. A Tissue Lyser II (Qiagen, Germany) at a frequency of 30 s^{-1} during four consecutive 1 min intervals was used to homogenize the sample. The orientation and position of the sample batches were changed between each interval to ensure a uniform processing.

After homogenization, cell lysis was performed on each sample with 600 µL of PL1 buffer, which was provided with the kit. The samples were then mixed with a Tissue Lyser II at a frequency of 20 s^{-1} for four consecutive 30 s intervals, after which 12 µL of RNase A (concentration 12 ng/µL) was added. The samples were gently mixed by hand and incubated at 65 °C for 1 h in a LAUDA E200 heated water bath (Lauda Dr. R. Wobser GmbH & Co. KG, Germany). After the water bath incubation, the samples were gently rotated 20 times by continuously inverting the samples to facilitate complete sample coverage with the lysis buffer. The samples were then centrifuged at 6000 rpm for 20 min. Following the washing steps, each sample was eluted twice with 100 µL of the kit's elution buffer, yielding a final volume of 200 µL per sample.

Ustilago nuda and *Hordeum vulgare* DNA quantification with qPCR

Ustilago nuda development within the seedlings was quantified adapting a published qPCR approach [16]. The ratio of *U. nuda* Cytochrome c oxidase subunit 3 (COX3) copies to *H. vulgare* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) copies was used as a proxy for the *U. nuda* infection levels in the seedlings. The COX3 forward primer (5′-TCC GTT TGA GCT ACC ACT GC-3′) and the reverse primer (5′-CTG CAC GTC GAT TTC CTT GG-3′) with the probe (5′-[FAM]-CGC TGC TGC TTC TAT CTT CA-[MGB-Q500]-3′) that contained a minor groove binding residue. For the GAPDH reaction, the forward primer (5′-CAG TTC ACG GCC ATT GGA AG-3′) and reverse primer (5′-ATC AAT ACT ACC TGA CGC CAA AG-3′) were used with the probe (5′-[HEX]-AAG ACG ACA AGA CGC TGC TCT TCG-[BHQ-1]-3′). Quantification of *U. nuda* and *H. vulgare* genes was calculated from standard curves of 5-fold serial dilutions of gBlock synthetic gene fragments containing the target genes (Integrated DNA Technologies, USA), following the methods outlined in a previous publication [16]. The pathogen limit of detection (LOD), which was established as 4 copies per reaction for the *U. nuda* COX3 gene [16].

The concentration of extracted DNA was measured using a Varian Cary Eclipse Spectropho-

tometer (Agilent, USA). DNA extracts with concentrations above 12 ng/µL were then diluted to 12 ng/µL using a Pipetmax Gilson 268 system with Trilution software (Gilson, USA). Samples with DNA concentrations below 12 ng/µL (4 out of 359) were directly run without further dilution, and one sample with a DNA concentration below 5 ng/µL was excluded from the analysis. Since the ratio of *U. nuda* to *H. vulgare* DNA was used as a proxy of *U. nuda* infection level in seedlings, the lower initial template concentration should not affect the infection level measurements in these samples because both the host and pathogen DNA concentration are decreased.

Each qPCR plate contained technical triplicates of the standard curves, biological samples, and controls. The controls included water and the non-target template DNA of *H. vulgare* and *Lens culinaris* used in COX3 and GAPDH standard curve, respectively. The qPCR reactions were set in a final volume of 10 µl per well, containing 50 ng of DNA, 0.2 µM of each COX3 primer and probe, 0.1 µM of each GAPDH primer and probe, and 5 µl of GoTaq Probe qPCR Master Mix (Promega Corporation, USA). The qPCR reactions were run in 384-well plates sealed with adhesive optical sealing foil (Nolato TreffLab, Sweden). All reactions were performed on the CFX Opus 384 Real-Time PCR System (Bio-Rad, USA). The amplification program included an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 65.3 °C for 30 s. The baseline was manually adjusted for all the runs to 500 and 250 RFUs, respectively for *U. nuda* and *H. vulgare*. In each sample, the gene copy number of COX3 and GAPDH was determined using CFX Maestro 2.2 software (version 5.2.008.0222) based on the quantification cycle (Cq) value and its comparison to the standard curve.

All positive samples fell within the range of the standard curve. The experimental samples were rerun if the standard deviation of the triplicates' Cq exceeded 0.5 in either gene quantification measurement. However, samples were not rerun if their mean triplicate Cq measurements for *U. nuda* were below the Cq measurements of the lowest measurable dilution point of the standard curve (4 copies per reaction) because any *U. nuda* DNA present in the sample was considered undetectable. The Cq measurements for the lowest measurable dilution point of the standard curve employed were determined based on each qPCR plate from its lowest dilution point of the standard curve that was consistently amplified across all three technical replicates with a Cq standard deviation below 0.5 cycles.

A sample was considered uninfected with *U. nuda* when its Cq value was less than the Cq value of the standard curve's lowest concentration from the same qPCR run. We used 0 as the copy number for samples with undetectable levels of *U. nuda* to ensure comparability among the qPCR assessments and field observations. The infection level in each sample was quantified per reaction using the ratio of the mean copy number of *U. nuda* COX3 divided by the mean copy number of *H. vulgare* GAPDH. The ratio values obtained from pooled seedling samples grown from seeds treated with warm water or synthetic fungicide were compared to the ratios measured in the untreated control to calculate the infection levels and treatment efficacy.

Statistical Analyses

All statistical analyzes were performed using R version 4.5.1. We used the ratio of *Ustilago nuda* to *Hordeum vulgare* DNA copies as an indicator of infection levels in seedlings grown in the lab. We based the disease incidence in the field on the number of smutted ears in

each plot. To compare treatment efficacies in the lab to those found in the field, the efficacies of synthetic fungicide and warm water treatments were calculated based on the reduction in disease development and occurrence following treatment compared to the untreated control, using the adapted formulas [24]:

$$\text{Field-calculated treatment efficacy (\%)} = \frac{\left(\frac{\text{Infected ears}}{\text{Total ears}}\right)_{\text{Untreated}} - \left(\frac{\text{Infected ears}}{\text{Total ears}}\right)_{\text{Treated}}}{\left(\frac{\text{Infected ears}}{\text{Total ears}}\right)_{\text{Untreated}}} \times 100$$

$$\text{Lab-calculated treatment efficacy (\%)} = \frac{\left(\frac{\text{Copy number } \textit{U. nuda}}{\text{Copy number } \textit{H. vulgare}}\right)_{\text{Untreated}} - \left(\frac{\text{Copy number } \textit{U. nuda}}{\text{Copy number } \textit{H. vulgare}}\right)_{\text{Treated}}}{\left(\frac{\text{Copy number } \textit{U. nuda}}{\text{Copy number } \textit{H. vulgare}}\right)_{\text{Untreated}}} \times 100$$

The actual treatment efficacy, which we hereafter call the field-calculated treatment efficacy, was based on the field results from the two growing seasons (2021-2022 and 2022-2023). The treatment efficacy measured from the laboratory growth procedure (lab-calculated treatment efficacy) was calculated for each substrate-growth period combination and compared to the field-calculated efficacy that had been averaged across the two years. To compare the treatment efficacy from the field trial to each seedling substrate and growth period combination, we adapted a published formula to calculate a lab-to-field predictability factor [25].

$$\text{Lab-to-field predictability factor (\%)} = \frac{\frac{(\text{Field efficacy})_{\text{Treated}} + (\text{Lab efficacy})_{\text{Treated}}}{2}}{(\text{Field efficacy})_{\text{Treated}}} \times 100$$

To determine which substrate–growth period combination used in the laboratory best represented the treatment efficacy observed in the field, we employed the lab-to-field predictability factor. It measured the ability of the treatment efficacy obtained from each laboratory growth method to serve as a proxy measurement for the field treatment efficacy. Values close to 100% indicate high accuracy, while values above or below 100% indicate that the laboratory growth method over- or underestimated the treatment efficacy, respectively. We further assessed the overall reliability of predictions based on each substrate–growth period combination using the coefficient of variation, for which a lower value indicates a lab method's higher precision.

The rate of seed germination in the field was compared among treatments using Kruskal–Wallis tests. The disease incidence observed in the field was compared among treatments within each seed lot and year using Kruskal–Wallis tests, followed by Fisher's least significant difference (LSD) test for post-hoc comparisons with the Benjamini–Hochberg (BH) p-value adjustment to account for multiple testing. The differences in field infection rates between years within each treatment were assessed using two-sample t-tests. The infection levels of *U. nuda* assessed using qPCR results were compared among treatments within each seed lot, growth substrate, and growth period using Kruskal–Wallis tests followed by Fisher's LSD post hoc test with the BH adjustment of p-values. The effects of treatment, growth substrate, and growth period on seedling length were analyzed using a three-way analysis of variance (ANOVA). The

ANOVA assumptions of normality and independence were met, but the data showed slight heteroscedasticity. The seedling length measurements were further compared among treatments within growth period, substrate, and cultivar using Kruskal–Wallis tests followed by Fisher's LSD post hoc test with the BH adjustment of p-values. The correlations between seedling length and corresponding *U. nuda* infection levels assessed by qPCR were evaluated using Spearman's rank correlation coefficient.

Results

Treatment efficacy based on *Ustilago nuda* infection levels observed in the field

We evaluated whether *U. nuda* qPCR measurements in seedlings grown for 5, 10 and 14 days in potting soil or on filter paper in the lab accurately captured seed treatment efficiency observed in the field. We tested seedlings grown from seed lots of Azrah and Semper cultivars, which were treated with either warm water or synthetic fungicide or not treated. The two treatments were applied to determine our screening procedure's ability to measure treatment efficacy from seedlings.

In the field, we observed an disease incidence of 7.5% in the Semper seed lot and of 3.5% in the Azrah seed lot (Figure 2). Germination rates within the Semper seed lot ranged from 59.4 ± 8.0 to 70.8 ± 6.0 while germination rates for Azrah ranged from 62.7 ± 11.8 to 77.0 ± 16.0 (Supplementary Table 1). Although germination rates among treatments within the Azrah lot were found to differ marginally ($p = 0.06$, $\chi^2 = 5.57$) in the growing season 2021-2022 and significantly in the growing season 2022-2023, the number of ears measured within a 0.3 m^2 transect did not differ among any treatments in either cultivar. In both cultivars, the synthetic fungicide- and warm water-treated seed showed a significant reduction in *U. nuda* smutted ears at the flowering stage (Figure 2). In the Azrah seed lot, the warm water treatment was 100% effective, while the synthetic fungicide treatment was only 74.6% effective at reducing *U. nuda* smutted ears (Supplementary Table 2). The disease incidence observed between 2022 and 2023 differed significantly in the untreated Semper seed lot ($t_{(df=6)} = -2.91$, $p = 0.027$) and the synthetic fungicide-treated Semper seed lot ($t_{(df=6)} = -2.65$, $p = 0.038$), as well as in the Azrah fungicide-treated seeds ($t_{(df=6)} = -6.20$, $p < 0.001$). Although, the disease incidence in the untreated Azrah seed lot observed in 2022 and 2023 showed no significant difference ($t_{(df=6)} = -1.98$, $p = 0.094$), the number of smutted ears was generally higher in 2023 (Figure 2, Supplementary Table 3).

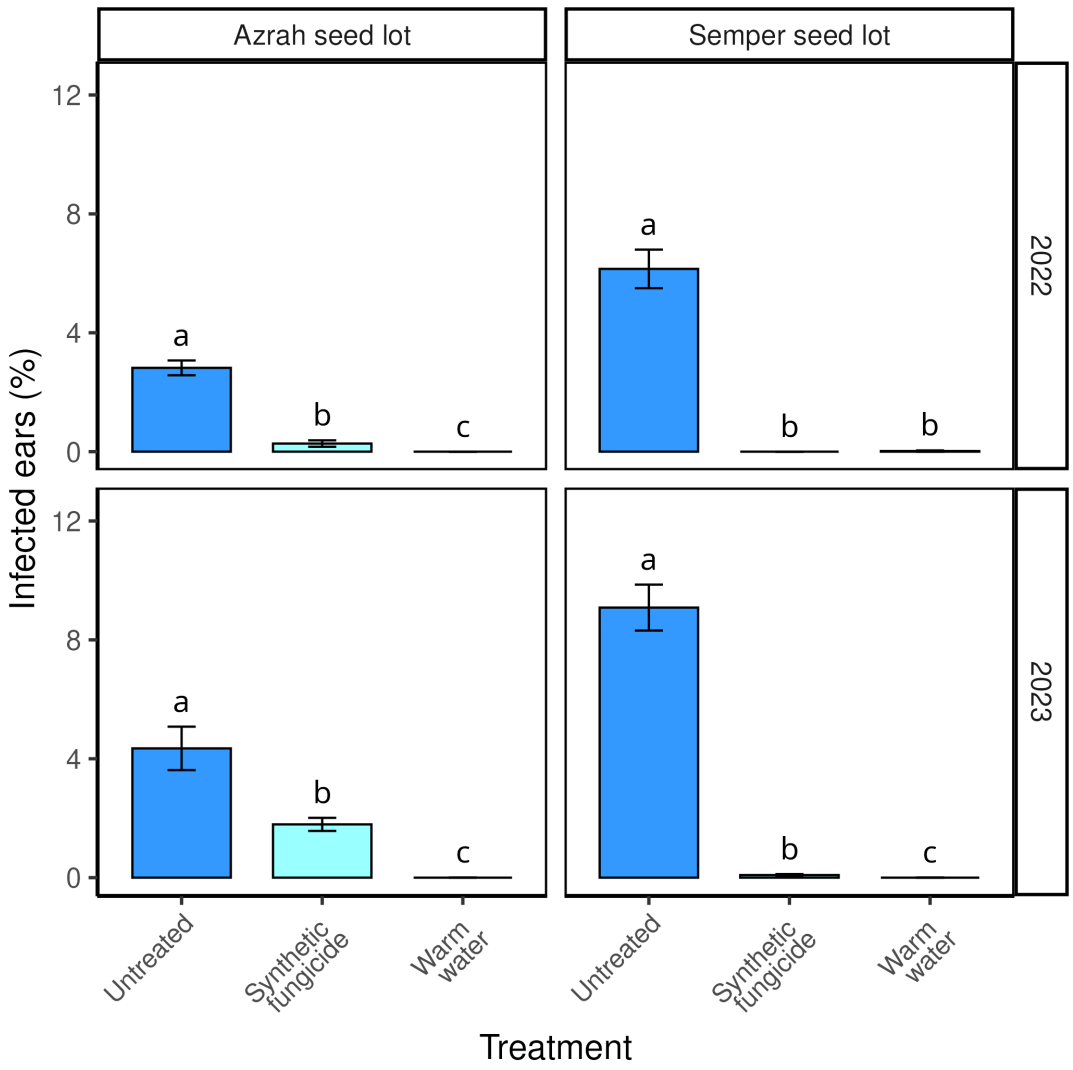

Figure 2: Field disease incidence of *Ustilago nuda* from two *Hordeum vulgare* seed lots of the cultivars Azrah and Semper assessed in 2022 (top panels) and 2023 (bottom panels) based on four replicate field plots per year. The barley plants were grown from untreated seeds or seeds that had been treated with synthetic fungicide or warm water. Within each seed lot and year, the letters indicate significant differences ($p < 0.05$) in the observed infection rate among treatments based on Kruskal–Wallis tests followed by Fisher's least significant difference tests. Bars indicate standard error of the means.

Treatment efficacy based on qPCR-measured *Ustilago nuda* infection levels in seedlings

The seed lots treated and sown in the 2022 field trial were also grown in the lab to evaluate whether the *U. nuda* infection detected with seedling-based qPCR reflected the disease incidence observed in the field. The treatment efficacies based on the qPCR measurements were obtained from comparing the infection levels in the seedlings grown from treated seeds to the seedlings grown from untreated seeds (Supplementary Table 2). *H. vulgare* DNA copies showed minimal variation among the seedling samples (Supplementary Table 4). The average qPCR reaction efficiencies based on the standard curves were 98.4% for COX3 and 89.4% for GAPDH (Supplementary Table 5).

To optimize seedling growth period and substrate, we determined whether the infection levels of the treated seedlings reflected the disease incidence in the field (Figure 2, Figure 3). The

differences in the infection levels among the treatments detected using qPCR were inconsistent between the seed lots and among the experimental growth periods (i.e. testing after 5, 10 and 14 days of seedling growth) (Figure 3, Supplementary Table 6). For Semper seedlings, there was a clear correspondence to the field results for 10 and 14 day growth periods (Figure 2, Figure 3). Both the synthetic fungicide and warm water treatments significantly reduced *U. nuda* infection compared to the untreated control in seedlings grown for 10 and 14 days on both substrates (Figure 3, Supplementary Table 6). The infection levels found in seedlings grown from the Semper seed lot did not differ between the growth substrates (Supplementary Table 7). However, infection levels of seedlings grown for 10 days from fungicide-treated Azrah seeds and for 14 days from untreated Azrah seeds significantly differed when grown on different substrates.

Although three combinations of growth period and substrate for the cultivar Azrah resulted in seedling infection levels that followed the general trends of the field disease incidence (Figure 3), this result was most visible in seedlings grown for 14 days in potting soil (Supplementary Table 6), but the differences were marginally significant ($p = 0.06$, $\chi^2 = 5.76$).

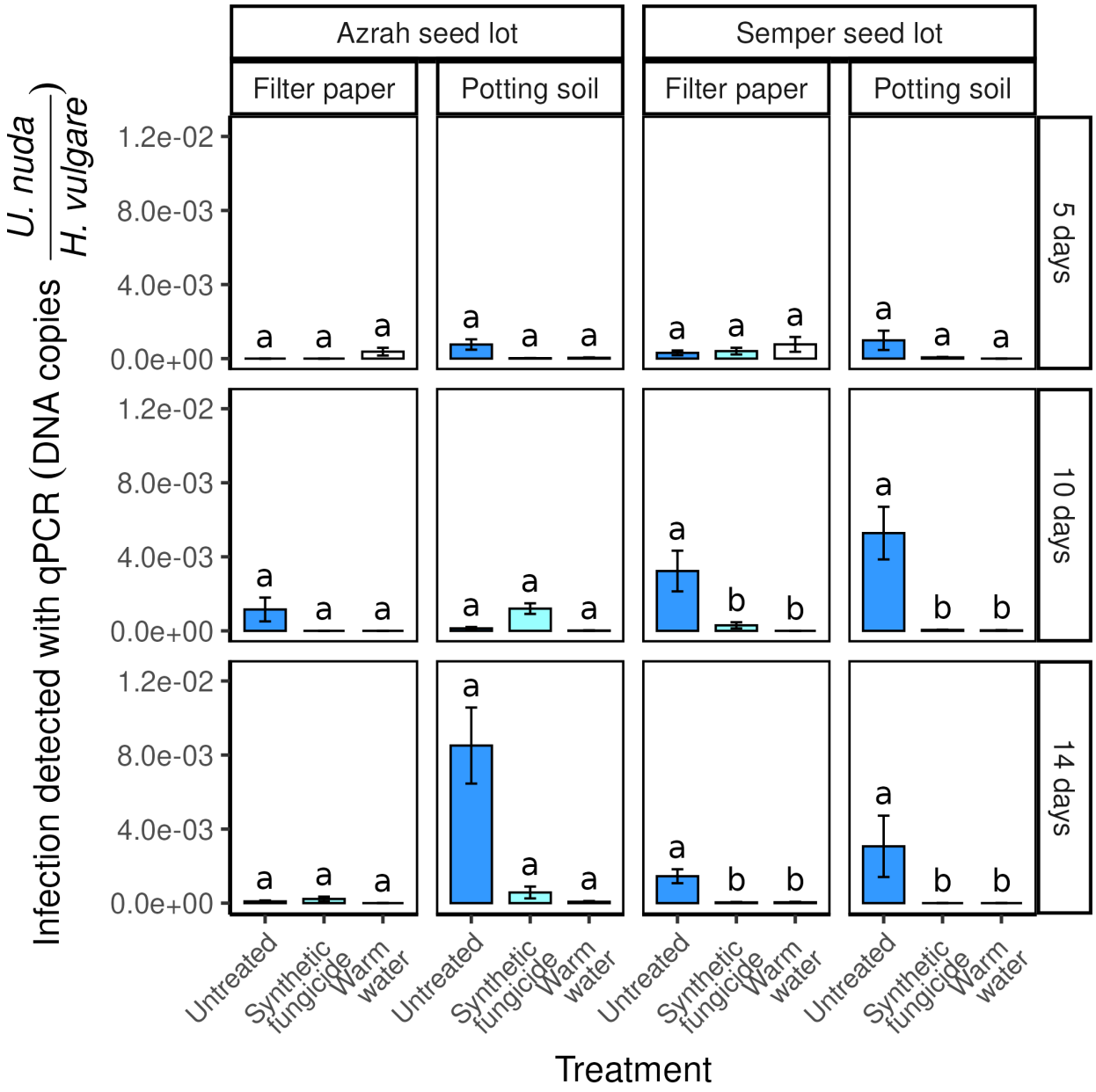

Figure 3: *Ustilago nuda* infection levels assessed using qPCR in seedlings grown from two *Hordeum vulgare* seed lots (cultivars Azrah and Semper). The seed lots were untreated or treated with a synthetic fungicide or warm water, then sown on filter paper or in potting soil (vertical panels) and grown for 5, 10, or 14 days (horizontal panels). The infection levels are expressed as the ratio of *U. nuda* to *H. vulgare* DNA copies, and bars indicate standard error of the means. The letters indicate significant differences ($p < 0.05$) of infection levels among treatments within each seed lot, growth period, and substrate. Most combinations included 10 pooled samples of 20 seedling segments each. Azrah seedlings grown from warm water treated seeds that had been grown for 10 days on filter paper included 9 samples. The significant differences were assessed with Kruskal–Wallis test followed by Fisher's least significant difference test with Benjamini–Hochberg p-value adjustment.

Comparison of treatment efficacy using lab-screening methods and field assessments

Treatment efficacy was estimated from the qPCR measurements of DNA extracted from seedlings grown under controlled conditions on either filter paper or in potting soil and sampled after 5, 10, or 14 days (Supplementary Table 2). We used a lab-to-field predictability factor as a measure to compare the treatment efficacies estimated with the lab results to the treatment efficacies assessed with the field observations (Table 1). No infection was detected

in the Azrah seedlings grown on filter paper for 5 days from untreated and synthetic fungicide-treated seed (Supplementary Figure 1), which prevented the calculation of treatment efficacy (Supplementary Table 2) and the lab-to-field predictability factor (Table 1).

Among the seedling growth conditions tested, the treatment efficacies assessed using the qPCR results from seedlings grown for 5 or 14 days in potting soil and for 10 days on filter paper most closely matched the treatment efficacies obtained in the field. The lab-calculated treatment efficacies that closely matched field-calculated treatment efficacies produced a lab-to-field predictability close to 100%. The lab-to-field predictability factors for these growth period and substrate combinations ranged from 102.4 to 103.4%. These three growth period and substrate combinations showed the greatest precision and consistency across replicates; they had lab-to-field predictability factors close to 100% and showed the lowest variation among replicates based on their coefficients of variation, ranging from 8.6 to 10.7. The lab-to-field predictability factor's performance is also dependent on field conditions, which can affect the development of *Ustilago nuda*. Therefore, the field conditions can lead to a reduced agreement between the qPCR and field results, which occurred in 2023 (Supplementary Table 8).

Growth period (day)	Substrate	Treatment	Cultivar	Lab-to-field predictability factor (%)	Standard deviation	Coefficient of Variation (%)
5	Filter paper	Synthetic Fungicide	**Cumulative**	—	—	—
			Arzah	—	—	—
			Semper	34.9	166.5	477.5
		Warm Water	Arzah	—	—	—
			Semper	-23.9	366.5	-1530.8
	Potting soil	Synthetic Fungicide	**Cumulative**	**102.4**	**9.8**	**9.5**
			Arzah	115.3	5.6	4.9
			Semper	96.9	7.1	7.3
		Warm Water	Arzah	97.2	8.8	9.1
			Semper	100.1	0.0	0.0
10	Filter paper	Synthetic Fungicide	**Cumulative**	**103.3**	**11.0**	**10.7**
			Arzah	117.1	0.0	0.0
			Semper	95.6	14.7	15.4
		Warm Water	Arzah	100.0	0.0	0.0
			Semper	100.1	0.0	0.0
	Potting soil	Synthetic Fungicide	**Cumulative**	**-47.5**	**465.6**	**-981.2**
			Arzah	-482.5	811.6	-168.2
			Semper	99.8	0.8	0.8
		Warm Water	Arzah	93.1	22.0	23.6
			Semper	99.8	0.8	0.8
14	Filter paper	Synthetic Fungicide	**Cumulative**	**63.2**	**253.6**	**401.2**
			Arzah	-44.61	511.2	-1146.1
			Semper	98.9	4.35	4.4
		Warm Water	Arzah	100	0.0	0.0
			Semper	98.5	4.9	5.0
	Potting soil	Synthetic Fungicide	**Cumulative**	**103.1**	**8.8**	**8.6**
			Arzah	112.5	14.3	12.7
			Semper	100.2	0.0	0.0
		Warm Water	Arzah	99.6	1.3	1.4
			Semper	100.1	0.0	0.0

Table 1: Comparison of treatment efficacies against *Ustilago nuda* based on qPCR detection in *Hordeum vulgare* seedlings and field observations using the lab-to-field predictability factor. Seeds from two seed lots (cultivars Azrah and Semper) that had been either untreated or treated with warm water or a synthetic fungicide were sown in the field for two growing season (2021-2022 and 2022-2023) and in the laboratory under controlled environment. In the controlled environment, the seeds were grown on filter paper or in potting soil, and the seedling samples were collected after 5, 10, or 14 days. The sample consisted of 20 pooled 0.5 cm seedling segments. Each combination of treatment, growth period, substrate, and seed lot had 10 samples, except for Azrah seeds warm water treated and grown 10 days on filter paper (9 samples). The lab-to-field predictability factor indicates the correspondence between the treatment efficacy based on qPCR and field results; a value of 100% indicates a better alignment. The standard deviation and coefficient of variation of each treatment were calculated to evaluate precision and consistency, respectively. A dash (—) indicates that the lab-to-field predictability factor could not be calculated because no infection was detected in the untreated control (Supplementary Figure 1).

Effects of growth condition, treatment, and infection level on seedling length

Because *Ustilago* species have the potential to influence their hosts' growth, before we excised the 200 seedling segments for the DNA extraction within each combination of growth period and substrate, we measured the lengths of all 7200 seedlings. The main factors, including treatment, growth period, and growth substrate, all had a significant effect on the seedling length (Table 2). The seedlings grown in soil were longer on average than those grown on filter paper. The seed treatments had a weaker effect but seemed to increase the length of the seedling compared to the untreated control (Supplementary Table 9). The interaction terms, including treatment x growth period and growth substrate x growth period, also influenced seedling length. The interaction of growth substrate x treatment had no effect on seedling length, while the interaction of all three terms was marginally significant.

	$F_{(df1,df2)}$	p
Growth substrate	$162.906_{(1,18)}$	< 0.001
Treatment	$5.453_{(2,18)}$	0.014
Growth period	$623.953_{(1,18)}$	< 0.001
Growth substrate x Treatment	$1.736_{(2,18)}$	0.205
Growth substrate x Growth period	$3.612_{(2,18)}$	0.048
Treatment x Growth period	$3.406_{(4,18)}$	0.031
Growth substrate x Treatment x Growth period	$2.896_{(4,18)}$	0.052

Table 2: The effects of growth period, substrate, and treatment on the mean seedling length of *Hordeum vulgare* grown from the Azrah and Semper seed lots. A three-way ANOVA shows the effects of the following factors: treatment (untreated, synthetic fungicide, or warm water), substrate (filter paper or potting soil), growth period (5, 10, or 14 days), and their interactions on seedling length

We further examined the treatment effect within each growth period, substrate, and cultivar (Supplementary Figure 2, Supplementary Table 9). Seedlings grown for 5 and 10 days from warm-water–treated seeds were generally longer than those from untreated or synthetic fungicide-treated seeds, although these differences were not always significant (Supplementary Table 9). The influence of treatment on seedling length varied among seed lots, substrates, and growth periods, showing no consistent trend (Supplementary Table 9).

From the Spearman's correlation between seedling length and *Ustilago nuda* infection levels detected using qPCR, which was assessed on seedlings from each seed lot, substrate, and growth period, we saw no clear overarching pattern between the pathogen level and seedling length (Supplementary Table 10). The treatment, growth period and substrate combinations that indicated a strong association between length and infection level — for example, the seedlings grown from synthetic fungicide treated Semper seeds on potting soil for 10 days — typically had 2-3 positive samples, making them less reliable (Supplementary Figure 1). Therefore, no clear relationship between seedling growth and infection rate was found within each growth period and substrate from this work.

Discussion

Efficient and reliable methods are needed to evaluate the efficacy of new, low-risk plant protection products (PPPs) against plant pathogens [26, 27]. Traditionally, PPP efficacy is

assessed through time- and resource-intensive field trials [28, 29], which are subject to environmental variability that can affect performance and pathogen development [30, 31, 32, 33, 34]. Additionally, the typical evaluation of treatment efficacy against plant pathogens, such as *Ustiliago nuda*, requires months; the disease can only be evaluated at the flowering stage, when the smutted ears are visible. To accelerate the treatment screening and reduce associated costs, we developed a laboratory screening procedure that uses qPCR to quantify *U. nuda* DNA in barley seedlings and evaluate treatment efficacy under controlled conditions.

To evaluate seed treatment efficacy, we adapted a seed-based multiplex qPCR assay [16] to detect *U. nuda* in pooled seedling tissues. Because synthetic fungicide treatments and seed certification programs effectively control *U. nuda* infections [12, 35], it is difficult to find seed lots with sufficiently high infection levels to discriminate among treatment efficacies. Typically seed lots with *U. nuda* infection levels of 4.9–24.2% based on embryo inspections are used for treatment assessments in the field [36, 37, 38]. However, when seed lots with low infection levels are used, small sample sizes can reduce the accuracy of the treatment evaluation. The pooling approach increases the number of tested seedlings per sample while using the same quantity of reagents and materials required for an individual seedling. Therefore, pooling provides an efficient, large-scale screening protocol [39].

Although previous studies have evaluated treatment efficacy against other pathogens with qPCR measurements of DNA extracted from seedlings [31, 40], we expand upon this work to focus on *U. nuda* and to determine the effect of substrate and growth period on seedling development and, ultimately, treatment assessment. In a total of six combinations of growth period and substrate, we evaluated the treatment efficacy in seed lots with infection levels as low as 2.6% and 8.8% based on the percentage of infected embryos. Assessing the treatment efficacy in seed lots with low infection rates allows for the testing of naturally infected seed lots, which aligns with the recommendations of the European and Mediterranean Plant Protection Organization (EPPO) for evaluating treatment efficacy against seedborne disease [7].

Overall, seedlings grown for 5 or 14 days in potting soil and for 10 days on filter paper showed cumulative lab-to-field predictability factors close to 100%, and Semper seedlings grown for 10 to 14 days also show significant differences in qPCR measured infections among treatments. No significant differences in *U. nuda* infections among treatments on both seed lots were found in seedlings grown for 5 days in either growth substrate, likely reflecting insufficient pathogen development within the seedlings at this stage. The high variability within the Azrah seed lot may be due to its the lower infection rate (2.6%), which makes it more susceptible to sampling stochasticity. These results suggest that a larger sample size is necessary to reliably detect *U. nuda* in seedlings using our qPCR lab-screening protocol for seed lots with low infection rates, such as the Azrah seed lot [9, 10, 11] due to the lower number of plants tested than included in a field trial. The high infection variation across Azrah seedling samples tested with qPCR reduced our ability to detect treatment differences.

The warm temperature and mild field conditions during barley germination in 2023 promoted *U. nuda* development (Supplementary Figure 3, Supplementary Table 8) [33, 34] and appeared to affect the qPCR-based treatment efficacy for the Azrah seed lot. For Azrah, the synthetic fungicide in the field was 31.5% less effective in 2023 compared to 2022. The increased temperature potentially promoted the pathogen's early growth [34], possibly allowing

it to escape the synthetic fungicide's effect. However, the Semper seed lot had been treated under the same conditions as the Azrah seed lot and only showed a 1% decrease in treatment efficacy. Therefore, favorable temperatures during germination alone cannot explain the decreased efficacy of the synthetic fungicide on the Azrah seed lot. Future work should more closely investigate the interactions among cultivar, *U. nuda* population, and infection levels to determine the applicability of this qPCR lab-screening procedure to all seed lots.

Although seedlings grown for 10 or 14 days from the Semper seed lot on filter paper or potting soil showed comparable treatment efficacy results to the field, the choice of growth substrate influences the practicality of the lab-screening method. Seedlings grown on filter paper offer a rapid and resource-efficient option for a high-throughput lab-screening of treatment efficacy. The use of seedlings grown in potting soil to assess treatment efficacy requires more labor, but it can more accurately simulate field conditions. The potting soil setup can serve as a second lab-screening step to evaluate how different environmental conditions influence the treatment efficacy, which is relevant for zonal efficacy assessments [41, 42, 43]. Lab-screening tests on seedlings grown in controlled environments reduce the variability in results due to environmental conditions in the field so that treatment differences become more apparent [13, 32, 44]. These lab-screening procedures can support the selection of the most promising treatments for field validation, which remains essential for the confirmation of the treatment performance under true field conditions and regulatory approval.

Targeting the mitochondrial COX3 gene increased the sensitivity of the qPCR reaction and improved detection of low pathogen levels. However, mitochondrial copy numbers can sometimes vary depending on the developmental stage and cell type [45, 46]. In this study we compared seedlings among the same growth stages, so that any influence of mitochondrial copy numbers should be minimized in our testing. The use of a nuclear target to detect *U. nuda* could help ensure that mitochondrial targets are stable at early stages of seedling growth and that the host or fungal growth did not affect the ratio of *U. nuda* to *H. vulgare* DNA copies. However, the use of a nuclear target would reduce the sensitivity of the qPCR reaction and its ability to detect low levels of *U. nuda*. Future comparisons between detection protocols using mitochondrial DNA and nuclear targets would help confirm their suitability for *U. nuda* detection in seedlings.

The lab-screening method is feasible due to the systemic growth of *U. nuda* within its plant host [38]. It would also be possible to apply for other cereal seed-borne diseases that infect their hosts systemically, such as *Tilletia caries*, *Tilletia controversa*, *Pyrenophora graminea*, and *Ustilago tritici* [38]. Due to its systemic nature and known influence on cell regulatory processes [17], we investigated whether an infected seedling's infection level influenced its length. However, based on the few treatment combinations with enough infected seedlings, we found no clear correlation between seedling *U. nuda* infection level and length. A closer investigation of the pathogen's progress from the base to the apex in infected seedlings grown in potting soil or filter paper for 10 and 14 days among cultivars could yield more information about the biology of *U. nuda*, interactions with its host, and its host's mechanisms of resistance. Although we choose to analyze the base of the seedling due to previous work [5], future analyses may gain additional insights from including seedling segments closer to the apex.

In conclusion, our qPCR-based lab-screening method can estimate treatment efficacy in

seedlings, and it offers a resource-efficient tool to optimize the choice of treatments for field trial evaluations. The possibility to pool seedlings, shorten the growth period from 14 to 10 days, and use filter paper as a substrate can improve resource efficiency and experimental throughput. In addition, these adaptations reduce space requirements compared to non-pooled seedlings grown for 14 days in potting soil. Depending on the research priorities, high-throughput screening or simulation of field environmental conditions, the growth period and substrate for cultivating seedlings can be adjusted. Our lab-screening method can reduce the costs and time required to evaluate treatment efficacy, which supports the development of alternatives to synthetic PPPs or active ingredients with lower risks to ecosystem and human health.

Acknowledgments

We thank Susanne Vogelgsang for the valuable support and constructive feedback. We also thank Irene Bänziger, Andreas Kägi, and Linda Schlatter for their help with the climate chambers and field trials. We acknowledge Francesco Bassi, Alessio Martini, and Sara Boschi for help with field data acquisition. We acknowledge Thomas Hebeisen, Nicole Bischofberger, and Damian Amrein for assistance in acquiring seed material, and Amanda Schneller, Jilla Schneider, and Marco Wüthrich for help with seedling sample processing. We thank Daniel Fuchs and his team for assistance with field trials. We thank Joëlle Schläpfer for her comments on the manuscript. This research was supported by Fondation Sur-la-Croix, IP Suisse, the Swiss Association of Cereal Producers, Swiss granum, and swisssem.

Supplementary Material

Detailed weather and agronomic information for the two years of the field experiment as well as additional data analyses can be found in the Supplementary Material.

Author contributions

Conceptualization: C.P. and K.E.S; Investigation and Formal analysis: C.P.; Funding acquisition: K.E.S; Methodology: C.P., E.J., and K.E.S; Project administration K.E.S.; Supervision: K.E.S. and D.C.; Visualization: C.P. and K.E.S.; Writing-original draft preparation: C.P. with support from K.E.S; Writing-review & editing: C.P., K.E.S., D.C., E.J.

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Supplementary Material: Optimization of a lab-screening procedure to evaluate seed treatment efficacy: detection of *Ustilago nuda* in *Hordeum vulgare* seedlings

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Supplementary Tables

Seed lot	Growing season	Treatment	Average seedling count per linear meter ± standard deviation	Kruskall-Wallis test	
				p	χ^2
Semper	2021-2022	Untreated	59.4 ± 8.0	0.16	3.64
		Synthetic fungicide	64.7 ± 7.6		
		Warm water	60.6 ± 6.3		
	2022-2023	Untreated	67.8 ± 9.1	0.12	4.26
		Synthetic fungicide	70.8 ± 6.0		
		Warm water	64.5 ± 8.9		
Azrah	2021-2022	Untreated	71.3 ± 15.2	0.06	5.57
		Synthetic fungicide	77.0 ± 16.0		
		Warm water	63.5 ± 9.1		
	2022-2023	Untreated	62.7 ± 11.8	<0.05	6.20
		Synthetic fungicide	66.0 ± 7.5		
		Warm water	72.1 ± 9.2		

Supplementary Table 1: Differences in seedling germination rates in the field among treatments (untreated, synthetic fungicide, and warm water) across two years (2022 and 2023) between the barley seed lots Azrah and Semper using Kruskall-Wallis tests. Values are based on four replicate plots per treatment and differences were considered significant at $p < 0.05$.

Treatment	Cultivar	Treatment efficacy in the field trial (%)			Growth period (day)	Treatment efficacy in the lab trial (%)	
		Field observation 2022	Field observation 2023	Field observation average		Growth substrate filter paper	Growth substrate potting soil
Synthetic fungicide	Azrah	90.3 ± 7.7	58.8 ± 10.12	74.6± 18.8	5	—	97.4 ± 8.3
					10	100.0 ± 0.0	-794.0 ± 1210.2
					14	-141.1 ± 762.3	93.3 ± 21.4
	Semper	100.0 ± 0.0	99.0 ± 0.7	99.5± 0.7	5	-30.1 ± -331.3	93.4 ± 14.14
					10	90.8 ± 29.2	99.0 ± 1.6
					14	97.3 ± 8.7	100.0 ± 0.0
Warm water	Azrah	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	5	—	94.4 ± 17.4
					10	100.0 ± 0.0	86.1 ± 43.9
					14	100.0 ± 0.0	99.2 ± 2.7
	Semper	99.7 ± 0.7	100.0 ± 0.0	99.8 ± 0.5	5	-147.6 ± 731.8	100.0 ± 0.0
					10	100.0 ± 0.0	99.5± 1.7
					14	96.9 ± 9.8	100.0 ± 0.0

Supplementary Table 2: Treatment efficacy based on the reduction of *Ustilago nuda* infection levels observed in the field and detected in seedlings. The *U. nuda* infection levels in seedlings from the laboratory trial were measured with qPCR based on the ratio *U. nuda*/*H. vulgare* DNA copies. The evaluated treatments included a synthetic fungicide and warm water, which were tested on two barley seed lots of the cultivars Azrah and Semper. The seedlings were grown on two substrates (potting soil or filter paper) and collected after three growth periods (5, 10, or 14 days). A treatment efficacy of 100% indicates a complete suppression of *U. nuda* infection compared to the untreated control, while negative values indicate increased infection. A dash (—) shows that the treatment efficacy could not be calculated because neither the treated nor the untreated samples showed infection (Supplementary Figure 1)

Seeds lot	Treatment	T-test	
		p	$t_{(df=6)}$
Semper	Untreated	0.027	-2.91
	Synthetic fungicide	0.038	-2.65
	Warm water	0.36	1
Azrah	Untreated	0.094	-1.98
	Synthetic fungicide	0.00081	-6.20
	Warm water	—	—

Supplementary Table 3: Differences in *Ustilago nuda* infection level within treatments between two years of field observation. *Ustilago nuda* infection levels in the field were assessed across two years (2022 and 2023) in two *Hordeum vulgare* seed lots (Azrah and Semper) using two-sample t-tests (Figure 1). The efficacy of the synthetic fungicide and warm water treatments was evaluated during the same time period. Differences between years of field observation were considered significant at $p < 0.05$. The p-values and t-values reported as "—" are for seed lots in which no infection was observed.

Growth period (day)	Treatment	Growth substrate	Cultivars	
			Azrah	Semper
			($\overline{Cq}$ $\overline{Mean}$ ± Std. Dev.[a])	
5	Untreated	Filter paper	25.52 ± 0.64	25.97 ± 0.87
		Potting soil	25.71 ± 0.62	25.94 ± 0.66
	Synthetic fungicide	Filter paper	25.75 ± 0.85	25.59 ± 0.63
		Potting soil	25.57 ± 0.35	25.71 ± 0.29
	Warm water	Filter paper	25.79 ± 1.04	25.34 ± 0.43
		Potting soil	25.76 ± 0.66	25.99 ± 0.89
10	Untreated	Filter paper	25.67 ± 1.01	25.69 ± 0.36
		Potting soil	25.48 ± 0.61	25.67 ± 0.41
	Synthetic fungicide	Filter paper	25.65 ± 0.51	25.71 ± 0.41
		Potting soil	25.67 ± 0.62	25.67 ± 0.42
	Warm water	Filter paper	25.62 ± 0.44	25.71 ± 0.68
		Potting soil	26.02 ± 0.71	25.82 ± 0.93
14	Untreated	Filter paper	25.85 ± 1.15	25.61 ± 0.35
		Potting soil	25.74 ± 0.77	25.44 ± 0.32
	Synthetic fungicide	Filter paper	25.67 ± 0.48	25.67 ± 0.47
		Potting soil	25.51 ± 0.24	25.70 ± 0.51
	Warm water	Filter paper	26.06 ± 0.45	25.45 ± 0.31
		Potting soil	25.63 ± 0.66	25.65 ± 0.36

Supplementary Table 4: The mean quantification cycle (Cq Mean) for GAPDH measured in *Hordeum vulgare* seedlings from two seed lots of the cultivars of Azrah and Semper grown in two different substrates (filter paper and potting soil) for 5, 10 and 14 days. The mean Cq value ± standard deviation are shown for each treatment and reveal stable measurements of extracted seedling DNA.

[a] Average of the mean quantification cycle (Cq) values across tested seed lots ± standard deviation among the seedling subsample Cq Mean

qPCR plate	*Ustilago nuda* standard curve (COX3)				*Hordeum vulgare* standard curve (GAPDH)			
	Efficiency	**Coefficient of determination (R^2)**	**Slope**	**y-intercept**	**Efficiency**	**Coefficient of determination (R^2)**	**Slope**	**y-intercept**
mpl39	0.992	0.998	-3.342	37.338	0.892	0.996	-3.611	40.831
mpl40	0.966	0.997	-3.405	37.707	0.895	0.998	-3.603	40.598
mpl41	0.972	0.995	-3.394	37.987	0.884	0.998	-3.634	40.618
mpl42	0.983	0.990	-3.362	38.321	0.893	0.997	-3.608	41.005
mpl43	1.009	0.995	-3.300	37.730	0.895	0.995	-3.601	41.309

Supplementary Table 5: Information about standard curves from each qPCR plate. Standard curves were generated with gBlock double-stranded synthetic oligonucleotide fragments prepared in a 5-fold serial dilution for absolute quantification of the *Hordeum vulgare* GAPDH gene region and the *Ustilago nuda* COX3 gene region. The *U. nuda* COX3 target was detected with a FAM-labeled probe, and the *H. vulgare* GAPDH target with a HEX-labeled probe. For each standard curve were calculated using CFX Maestro software (Bio-Rad) the parameters: amplification efficiency, coefficient of determination (Rm^2), slope, and y-intercept.

Growth substrate	Seed lot	Growth period (days)	Treatment	Average qPCR detected infection ± sd (*Ustilago nuda*/*Hordeum vulgare*)	Kruskall-Wallis test p	Kruskall-Wallis test χ^2	Post-hoc comparisons Fisher's least significant difference
Filter paper	Azrah	5	Untreated	0.00 ± 0.00	0.37	2	a
			Synthetic fungicide	0.00 ± 0.00			a
			Warm water	$3.76 \times 10^{-4} \pm 1.19 \times 10^{-3}$			a
		10	Untreated	$1.16 \times 10^{-3} \pm 3.65 \times 10^{-3}$	0.37	2	a
			Synthetic fungicide	0.00 ± 0.00			a
			Warm water	0.00 ± 0.00			a
		14	Untreated	$9.32 \times 10^{-5} \pm 2.95 \times 10^{-4}$	0.63	0.94	a
			Synthetic fungicide	$2.25 \times 10^{-4} \pm 7.10 \times 10^{-4}$			a
			Warm water	0.00 ± 0.00			a
	Semper	5	Untreated	$3.10 \times 10^{-4} \pm 7.26 \times 10^{-4}$	0.92	0.16	a
			Synthetic fungicide	$4.04 \times 10^{-4} \pm 1.03 \times 10^{-3}$			a
			Warm water	$7.68 \times 10^{-4} \pm 2.27 \times 10^{-3}$			a
		10	Untreated	$3.23 \times 10^{-3} \pm 6.24 \times 10^{-3}$	0.02	7.70	a
			Synthetic fungicide	$2.99 \times 10^{-4} \pm 9.45 \times 10^{-4}$			b
			Warm water	0.00 ± 0.00			b
		14	Untreated	$1.45 \times 10^{-3} \pm 2.14 \times 10^{-3}$	0.01	9.04	a
			Synthetic fungicide	$3.98 \times 10^{-5} \pm 1.26 \times 10^{-4}$			b
			Warm water	$4.51 \times 10^{-5} \pm 1.43 \times 10^{-4}$			b
Potting soil	Azrah	5	Untreated	$7.61 \times 10^{-4} \pm 1.59 \times 10^{-3}$	0.32	2.25	a
			Synthetic fungicide	$2.01 \times 10^{-5} \pm 6.35 \times 10^{-5}$			a
			Warm water	$4.24 \times 10^{-5} \pm 1.34 \times 10^{-4}$			a
		10	Untreated	$1.34 \times 10^{-4} \pm 4.25 \times 10^{-4}$	0.13	4.01	a
			Synthetic fungicide	$1.20 \times 10^{-3} \pm 1.63 \times 10^{-3}$			a
			Warm water	$1.87 \times 10^{-5} \pm 5.91 \times 10^{-5}$			a
		14	Untreated	$8.51 \times 10^{-3} \pm 1.17 \times 10^{-2}$	0.06	5.76	a
			Synthetic fungicide	$5.75 \times 10^{-4} \pm 1.82 \times 10^{-3}$			a
			Warm water	$7.24 \times 10^{-5} \pm 2.29 \times 10^{-4}$			a
	Semper	5	Untreated	$9.86 \times 10^{-4} \pm 2.96 \times 10^{-3}$	0.36	2.03	a
			Synthetic fungicide	$6.53 \times 10^{-5} \pm 1.39 \times 10^{-4}$			a
			Warm water	0.0 ± 0.0			a
		10	Untreated	$5.28 \times 10^{-3} \pm 8.07 \times 10^{-3}$	0.004	10.97	a
			Synthetic fungicide	$5.05 \times 10^{-5} \pm 8.48 \times 10^{-5}$			b
			Warm water	$2.79 \times 10^{-5} \pm 8.83 \times 10^{-5}$			b
		14	Untreated	$3.07 \times 10^{-3} \pm 9.41 \times 10^{-3}$	0.047	6.12	a
			Synthetic fungicide	0.00 ± 0.00			b
			Warm water	0.00 ± 0.00			b

Supplementary Table 6: Detailed results of *Ustilago nuda* infection levels among different treatments quantified in seedlings using qPCR (Figure 3). The infection rates are expressed as the ratio of *U. nuda* to *H. vulgare* DNA copies ± standard deviation (sd). The p-values and χ^2 values were assessed among treatments within substrate, seed lots, and growth period using the Kruskal–Wallis test. Post-hoc differences with Fisher's least significant difference test are shown with letters.

Growth period (days)	Treatment	Seed lot	Wilcox rank-sum test
			p
5	Untreated	Azrah	0.078
		Semper	0.73
	Synthetic fungicide	Azrah	0.37
		Semper	0.52
	Warm water	Azrah	1.00
		Semper	0.19
10	Untreated	Azrah	1.00
		Semper	0.31
	Synthetic fungicide	Azrah	0.035
		Semper	0.39
	Warm water	Azrah	0.34
		Semper	0.4
14	Untreated	Azrah	0.05
		Semper	0.17
	Synthetic fungicide	Azrah	0.87
		Semper	0.34
	Warm water	Azrah	0.4
		Semper	0.37

Supplementary Table 7: Differences of detected *Ustilago nuda* infection levels between substrate within treatments and growth period based on the results of qPCR seedling-based pre-screening method (Figure 3). These differences were based on a pairwise comparison using continuity-corrected Wilcoxon rank-sum test. P-values were adjusted using Benjamini-Hochberg correction and were considered significant at $p < 0.05$.

Growth period (day)	Treatment	Seed lot	Lab-to-field predictability factor 2022 (%)		Lab-to-field predictability factor 2023 (%)	
			Growth substrate filter paper	Growth substrate potting soil	Growth substrate filter paper	Growth substrate potting soil
5	Synthetic fungicide	**Cumulative**	—	**99.5 ± 6.6**	—	**106.8 ± 16.6**
		Azrah	—	103.9 ± 4.6	—	132.8 ± 7.1
		Semper	34.9 ± 165.7	96.7 ± 7.1	34.8 ± 167.3	97.1 ± 7.1
	Warm water	Azrah	—	97.2 ± 8.8	—	97.2 ± 8.8
		Semper	-24.1 ± 367.2	100.2 ± 0.0	-23.8 ± 365.9	100.0 ± 0.0
10	Synthetic fungicide	**Cumulative**	**100.2 ± 8.0**	**-24.3 ± 386.4**	**107.9 ± 17.8**	**-83.1 ± 587.4**
		Azrah	105.4 ± 0.0	-389.5 ± 669.9	135.1 ± 0.0	-625.3 ± 1029.3
		Semper	95.4 ± 14.6	99.5 ± 0.8	95.8 ± 14.8	100.0 ± 0.8
	Warm water	Azrah	100.0 ± 0.0	93.1 ± 22.0	100.0 ± 0.0	93.1 ± 22.0
		Semper	100.2 ± 0.0	99.9 ± 0.8	100.0 ± 0.00	99.7 ± 0.8
14	Synthetic fungicide	**Cumulative**	**67.3 ± 210.3**	**100.4 ± 5.8**	**56.9 ± 320.2**	**107.3 ± 15.5**
		Azrah	-28.1 ± 422.0	101.6 ± 11.8	-70.0 ± 648.4	129.3 ± 18.2
		Semper	98.6 ± 4.3	100.0 ± 0.0	99.1 ± 4.4	100.5 ± 0.0
	Warm water	Azrah	100.0 ± 0.0	99.6 ± 1.3	100.0 ± 0.0	99.6 ± 1.3
		Semper	98.6 ± 4.9	100.2 ± 0.0	98.4 ± 4.9	100.0 ± 0.0

Supplementary Table 8: The lab-to-field predictability factor from each growing season (2021-2022 and 2022-2023) based on the treatment efficacies against *Ustilago nuda* quantified in *Hordeum vulgare* seedlings with qPCR compared to the infection rates observed in the field during each growing season. The treatment efficacies were assessed for two seed lots (cultivars Azrah and Semper) either untreated or treated with warm water or a synthetic fungicide (Supplementary Table 2). The seedlings for the qPCR-based pre-screening method were grown on filter paper or in potting soil, and sampled after 5, 10, or 14 days for DNA extraction. The lab-to-field predictability factor indicates the alignment between the treatment efficacy based on qPCR and field results; a value of 100% indicates a better alignment. This factor is shown as cumulative value to evaluate the growth period and substrate, as individual value per each combination of growth period, substrate, treatment, and seed lot. Each value is noted with ± standard deviation. A dash (—) shows that the lab-to-field predictability factor could not be calculated because no infection was detected in the untreated control (Supplementary Figure: 1).

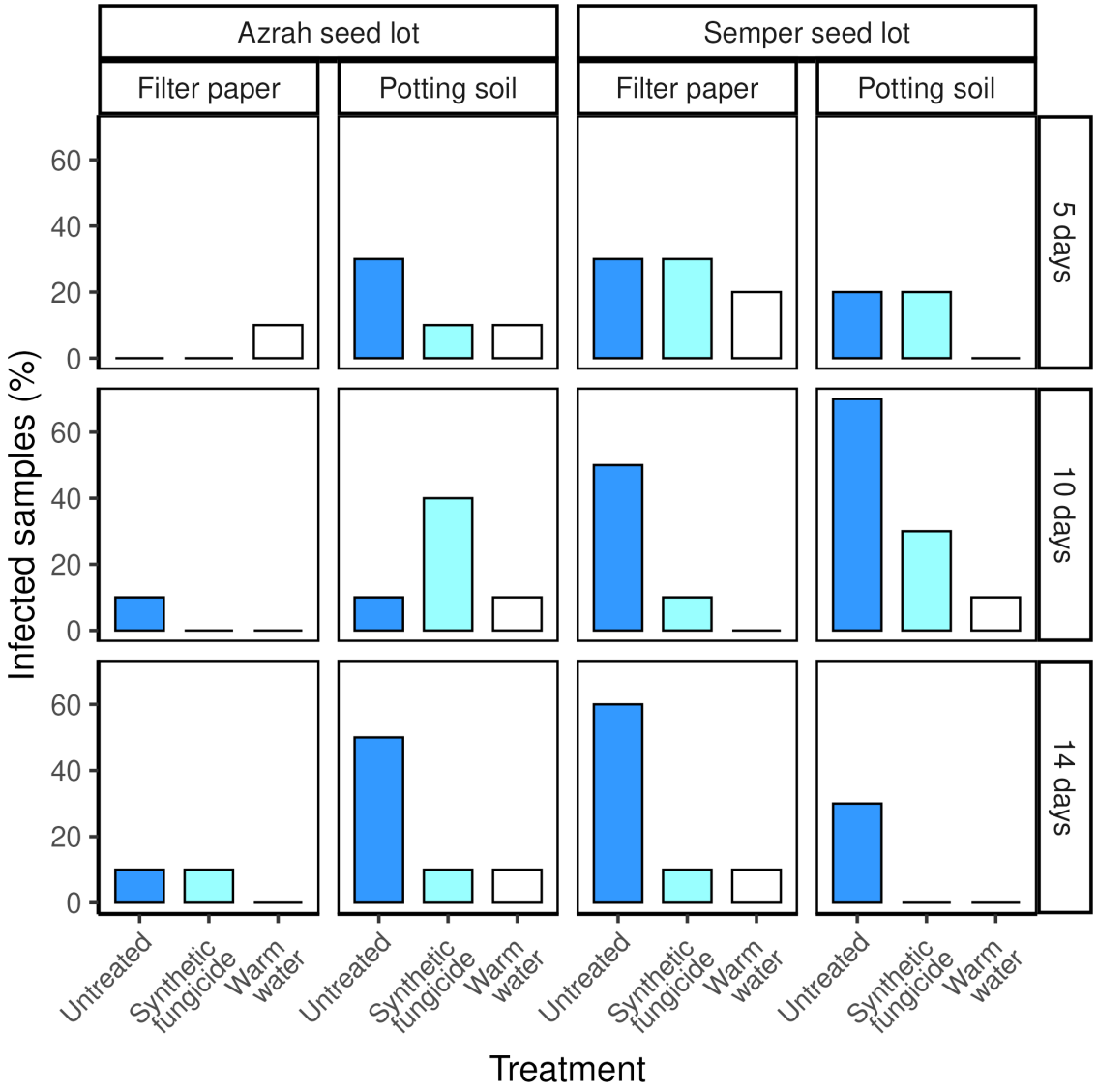
Growth period (days)	Growth substrate	Seed lot	Treatment	Average length ± standard deviation (cm)	Kruskall-Wallis test p	χ^2	Post-hoc comparisons Fisher's least significant difference
5	Filter paper	Azrah	Untreated	3.47 ± 0.54	8.48×10^{-3}	9.54	ab
			Synthetic fungicide	3.15 ± 0.47			b
			Warm water	4.14 ± 0.67			a
		Semper	Untreated	2.92 ± 0.68	2.02×10^{-1}	7.80	ab
			Synthetic fungicide	2.63 ± 0.69			b
			Warm water	3.61 ± 0.82			a
	Potting soil	Azrah	Untreated	5.37 ± 1.03	4.51×10^{-2}	6.20	b
			Synthetic fungicide	5.55 ± 0.89			b
			Warm water	6.74 ± 1.42			a
		Semper	Untreated	5.99 ± 0.89	9.68×10^{-2}	4.67	a
			Synthetic fungicide	5.79 ± 0.59			a
			Warm water	6.55 ± 0.74			a
10	Filter paper	Azrah	Untreated	11.57 ± 0.78	3.30×10^{-4}	16.03	b
			Synthetic fungicide	12.87 ± 0.68			a
			Warm water	13.16 ± 0.38			a
		Semper	Untreated	11.39 ± 1.03	7.35×10^{-2}	5.22	a
			Synthetic fungicide	9.92 ± 3.64			a
			Warm water	12.46 ± 0.80			a
	Potting soil	Azrah	Untreated	15.20 ± 1.90	3.93×10^{-2}	6.47	b
			Synthetic fungicide	16.74 ± 1.06			ab
			Warm water	17.03 ± 0.60			a
		Semper	Untreated	16.46 ± 0.92	5.66×10^{-1}	1.14	a
			Synthetic fungicide	16.68 ± 0.56			a
			Warm water	16.68 ± 0.76			a
14	Filter paper	Azrah	Untreated	13.15 ± 0.87	4.05×10^{-5}	20.23	c
			Synthetic fungicide	17.02 ± 0.52			a
			Warm water	13.76 ± 0.64			b
		Semper	Untreated	10.74 ± 3.75	6.26×10^{-5}	19.36	b
			Synthetic fungicide	16.13 ± 0.55			a
			Warm water	16.16 ± 0.71			a
	Potting soil	Azrah	Untreated	17.51 ± 0.90	3.52×10^{-3}	11.30	b
			Synthetic fungicide	19.08 ± 1.04			a
			Warm water	17.88 ± 0.49			b
		Semper	Untreated	19.26 ± 1.42	4.11×10^{-2}	6.38	a
			Synthetic fungicide	18.76 ± 0.68			ab
			Warm water	17.54 ± 1.86			b

Supplementary Table 9: Seedling length measurements from each combination of growth period, substrate, seed lot, and treatment (Supplementary Figure 2). The total length of each seedling was measured before collecting the 0.5 cm seedling segments form the base of the seedling for qPCR analysis. A total of 200 seedlings were measured for each treatment combination. The seedling length is expressed in centimeters as the average of 200 seedling length ± the standard deviation. The significances were obtained using Kruskal-test with Benjamini-Hochberg p-value adjustment followed by Fisher's least significant difference test for post-hoc comparisons within each treatment

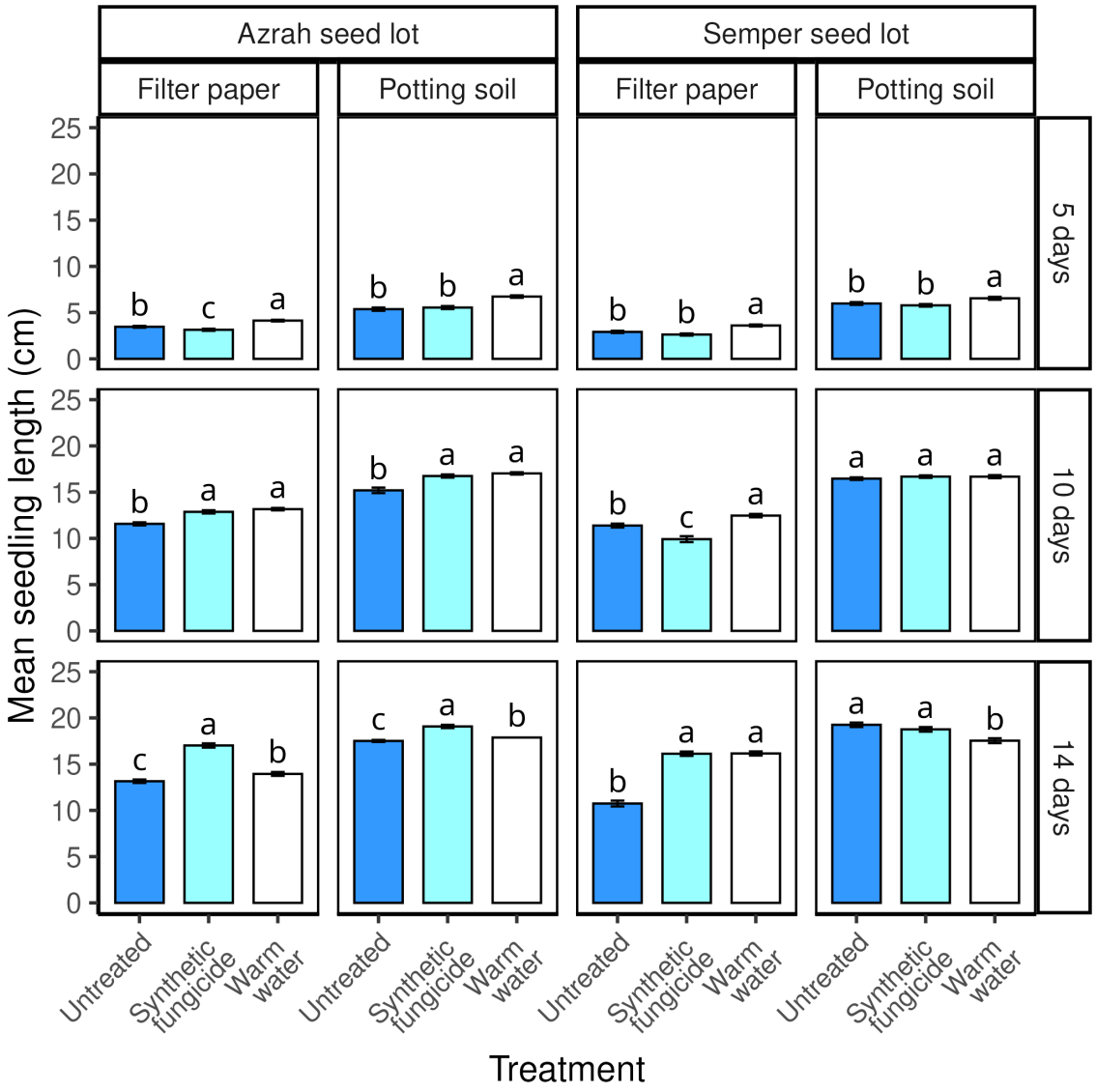
Growth period (days)	Treatment	Substrate	Seed lot	Average seedling length ± standard deviation (cm)	Average qPCR detected infection ± standard deviation (*U. nuda*/*H. vulgare* DNA copies)	Spearman's correlation between average seedling length and qPCR detected infection	
						Spearman ρ	**Strength of the correlation**
5	Untreated	Filter paper	Azrah	3.47 ± 0.54	0.00 0.00	—	—
			Semper	2.92 ± 0.68	$3.10 \times 10^{-4} \pm 7.26 \times 10^{-4}$	—	—
		Potting soil	Azrah	5.37 ± 1.03	$7.61 \times 10^{-4} \pm 1.59 \times 10^{-3}$	—	—
			Semper	5.99 ± 0.89	$9.86 \times 10^{-4} \pm 2.96 \times 10^{-3}$	—	—
	Synthetic fungicide	Filter paper	Azrah	3.15 ± 0.47	0.00 ± 0.00	—	—
			Semper	2.63 ± 0.69	$4.04 \times 10^{-4} \pm 1.03 \times 10^{-3}$	—	—
		Potting soil	Azrah	5.55 ± 0.89	$2.01 \times 10^{-5} \pm 6.35 \times 10^{-5}$	—	—
			Semper	5.79 ± 0.59	$6.53 \times 10^{-5} \pm 1.39 \times 10^{-4}$	—	—
	Warm water	Filter paper	Azrah	4.14 ± 0.67	$3.76 \times 10^{-4} \pm 1.19 \times 10^{-3}$	—	—
			Semper	3.61 ± 0.82	$7.68 \times 10^{-4} \pm 2.27 \times 10^{-3}$	—	—
		Potting soil	Azrah	6.74 ± 1.42	$4.24 \times 10^{-5} \pm 1.34 \times 10^{-4}$	—	—
			Semper	6.55 ± 0.74	0.00 ± 0.00	—	—
10	Untreated	Filter paper	Azrah	11.57 ± 0.78	$1.16 \times 10^{-3} \pm 3.65 \times 10^{-3}$	—	—
			Semper	11.39 ± 1.03	$3.23 \times 10^{-3} \pm 6.24 \times 10^{-3}$	0.39	Weak
		Potting soil	Azrah	15.20 ± 1.90	$1.34 \times 10^{-4} \pm 4.25 \times 10^{-4}$	—	—
			Semper	16.46 ± 0.92	$5.28 \times 10^{-3} \pm 8.07 \times 10^{-3}$	-0.44	Moderate
	Synthetic fungicide	Filter paper	Azrah	12.87 ± 0.68	0.00 ± 0.00	—	—
			Semper	9.92 ± 3.64	$2.99 \times 10^{-4} \pm 9.45 \times 10^{-4}$	—	—
		Potting soil	Azrah	16.74 ± 1.06	$1.20 \times 10^{-3} \pm 1.63 \times 10^{-3}$	0.03	Very weak
			Semper	16.68 ± 0.56	$5.05 \times 10^{-5} \pm 8.48 \times 10^{-5}$	—	—
	Warm water	Filter paper	Azrah	13.16 ± 0.38	0.00 ± 0.00	—	—
			Semper	12.46 ± 0.80	0.00 ± 0.00	—	—
		Potting soil	Azrah	17.03 ± 0.60	$1.87 \times 10^{-5} \pm 5.91 \times 10^{-5}$	—	—
			Semper	16.68 ± 0.56	$2.79 \times 10^{-5} \pm 8.83 \times 10^{-5}$	—	—
14	Untreated	Filter paper	Azrah	13.15 ± 0.87	$9.32 \times 10^{-5} \pm 2.95 \times 10^{-4}$	—	—
			Semper	10.74 ± 3.75	$1.45 \times 10^{-3} \pm 2.14 \times 10^{-3}$	-0.13	Very weak
		Potting soil	Azrah	17.51 ± 0.90	$8.51 \times 10^{-3} \pm 1.17 \times 10^{-2}$	-0.06	Very weak
			Semper	19.26 ± 1.42	$3.07 \times 10^{-3} \pm 9.41 \times 10^{-3}$	—	—
	Synthetic fungicide	Filter paper	Azrah	17.02 ± 0.52	$2.25 \times 10^{-4} \pm 7.10 \times 10^{-4}$	—	—
			Semper	16.13 ± 0.55	$3.98 \times 10^{-5} \pm 1.36 \times 10^{-4}$	—	—
		Potting soil	Azrah	19.08 ± 1.04	$5.75 \times 10^{-4} \pm 1.82 \times 10^{-3}$	—	—
			Semper	18.76 ± 0.68	0.00 ± 0.00	—	—
	Warm water	Filter paper	Azrah	13.76 ± 0.64	0.00 ± 0.00	—	—
			Semper	16.16 ± 0.71	$4.51 \times 10^{-5} \pm 1.43 \times 10^{-4}$	—	—
		Potting soil	Azrah	17.88 ± 0.49	$7.24 \times 10^{-5} \pm 2.29 \times 10^{-4}$	—	—
			Semper	17.54 ± 1.86	0.00 ± 0.00	—	—

Supplementary Table 10: Correlation between seedling length and *Ustilago nuda* infection levels detected using qPCR on seedling for treatment combinations with four or more infected samples (Supplementary Figure 1). The seedling lengths (± standard deviation) and their respective infections (± standard deviation) were established between seed lots and substrates within treatments and growth period. The correlation was evaluated using the Spearman ρ value. Based on ρ value were established four groups of correlation strength: strong ($\pm 0.60 \leq \rho \leq \pm 0.79$), moderate ($\pm 0.40 \leq \rho \leq \pm 0.59$), weak ($\pm 0.20 \leq \rho \leq \pm 0.39$), and very weak ($0.00 \leq \rho \leq \pm 0.19$). The Spearman's ρ and strength of the correlation are reported as "—" for the treatment combinations with fewer than four infected samples.

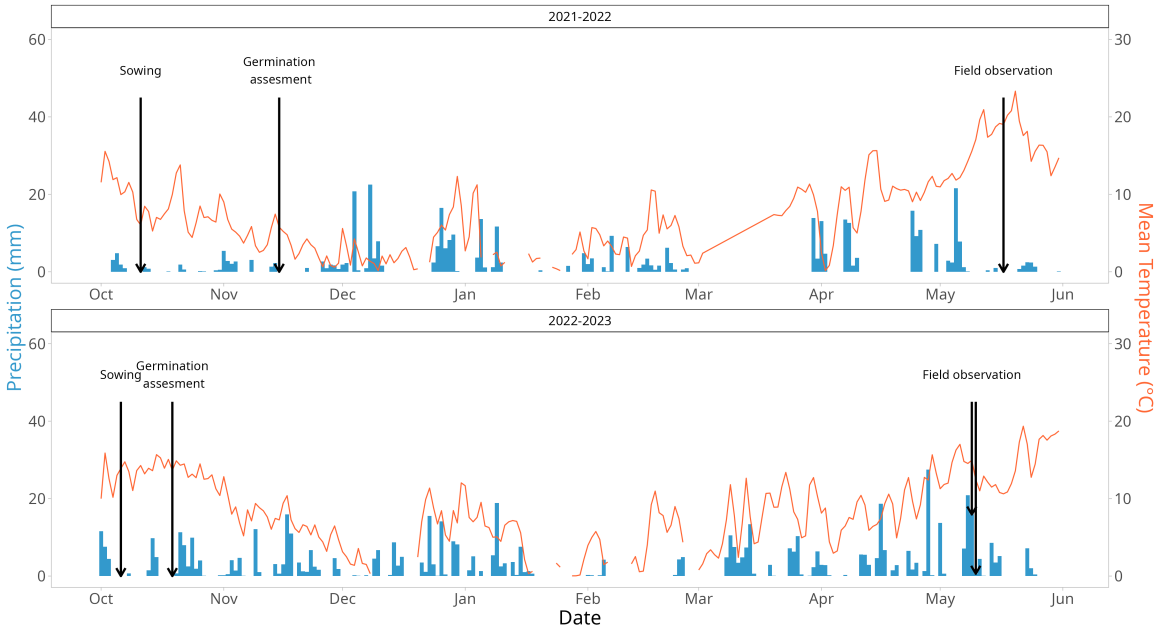
Supplementary Figures



Supplementary Figure 1: The percentage of infected samples based on qPCR *Ustilago nuda* detection in DNA extracted from seedlings across growth periods, substrates, seed lots, and treatments. The seedlings were grown in potting soil or on filter paper from two seed lots (Azrah and Semper) that were either untreated or treated with synthetic fungicide or warm water. The infection was assessed using qPCR on DNA extracted from seedlings sampled after 5, 10, and 14 days. All treatment combinations include ten extractions, except for the Azrah seed treated with warm water and grown on filter paper for 10 days, which included nine extractions.



Supplementary Figure 2: The mean seedling length (cm) of *Hordeum vulgare* seeds of cultivars Azrah and Semper grown from either untreated or treated seeds with synthetic fungicide or warm water. The seedlings were grown on two substrates (filter paper or potting soil) and sampled after 5, 10, or 14 days. The mean length was established based on 200 seedlings per treatment, and the error bars show the standard error. The different letters above each bar indicate significant differences in seedling lengths among treatments within the same growth period, seed lot, and substrate. These significant differences were assessed using a Kruskal-Wallis test, followed by a Fisher's least significant difference test for group assignment with a Benjamini-Hochberg p-value adjustment.



Supplementary Figure 3: Weather data collected during the growing seasons from the field. The weather measurements were taken at the MeteoSwiss station Zurich/Affoltern, which was the closest station to the fields. Daily mean temperature (°C) is shown as an orange line and total precipitation (mm) per day as blue bars. Arrows indicate the dates of seed sowing, germination monitoring, and field monitoring for infected plants.

Chapter 4

Optimization of *Tilletia caries* detection in plants with a qPCR-based screening method to evaluate new treatments

Unpublished manuscript

Abstract

Tilletia caries is an re-emerging seed- and soilborne pathogen in low-input wheat production. Effective management of this pathogen is necessary to prevent crop loss. However, the development of low-risk or organic treatments requires a long time-horizon because treatment evaluations are conducted only after bunted ears appear. Rapid, reliable screening methods would accelerate the evaluation of new treatments. We applied a molecular screening approach using qPCR to detect *T. caries* DNA in wheat leaves at the boot stage. In the same plant, we compared the measured *T. caries* DNA with early leaf symptoms and eventual bunted ears. The developed bunted ear was used as an indicator of the true infection level. These measurements of *T. caries* infections were used to evaluate and compare treatment efficacy for different plant-derived soil treatments. The qPCR method was more accurate and precise at discriminating between infected and uninfected plants than the use of early leaf symptoms. However, for estimating treatment efficacy, the leaf symptom-based screening method aligned more closely with the efficacy obtained using bunted ear severity. In contrast, the qPCR-based screening method underestimated the treatment efficacy. Although qPCR is a robust tool for *T. caries* detection, it requires further optimization to better estimate the treatment efficacy. Improving the qPCR-based screening method will facilitate the high-throughput screening of new treatments that can support low-input wheat production.

Introduction

Tilletia caries [(DC.) Tulasne & C. Tulasne] causes common bunt in wheat and has reemerged disease in organic and low-input wheat production [1]. The resurgence of *T. caries* is primarily associated with the limitation of synthetic seed treatments, which has provided effective control of this pathogen since the 1950s [2]. This fungus is primarily transmitted through contaminated seeds that carry teliospores on their surface. *T. caries* infects the host within the first week of seedling germination [3, 4]. During plant growth, some wheat cultivars when infected exhibit chlorotic flecks on their leaves [5, 6], although these symptoms are inconsistent due to different factors such as the environmental conditions and cultivar resistance. In a three-year greenhouse experiment, the expression of early leaf symptoms varied within the same wheat cultivars infected with the same *T. caries* population, suggesting that environmental conditions play an important role in symptom appearance [7]. Chlorotic flecks were more frequently seen at higher temperatures (15 °C than at 4 °C) and in susceptible rather than resistant cultivars [8]. Moreover, cultivar-specific responses can limit the diagnostic value of early leaf symptoms as these symptoms may occur with or without subsequent bunt balls development. Alternatively, bunt balls may develop in the absence of early leaf symptoms [9]. These chlorotic symptoms can be confused with nutrient deficiencies or abiotic stress in the field, and are only useful to assess disease in controlled environments with cultivars known to produce early leaf symptoms. The disease produces characteristic bunted ear only at ear maturity, during which the fungus completely replaces the developing seed with teliospores [10]. At this stage, subsequent treatments cannot control the disease, and the produced teliospores can spread during the seed harvest through seeds, soil, agricultural machinery and animal manure [11, 12, 13]. Teliospores in the soil can survive in the field for five to ten years [12, 14].

Effective *T. caries* control, therefore, requires treatments that can protect the plants from both seed- and soil-borne inoculum. Currently, the low-risk or organic seed control strategies available primarily protect against seedborne inoculum and include contact fungicide and physical or mechanical action, such as steam or brushing [15]. These control strategies have either no, limited or unknown efficacy against soilborne inoculum [16, 17]. For example, the biopesticide Cerall® (*Pseudomonas chlororaphis* isolate MA342) effectively controls seedborne *T. caries* but does not prevent soilborne infection [16]. The development of treatments effective against both infection pathways is time-consuming, costly, and requires space to grow plants until the disease occurs during the seed maturation, typically after 7 months in winter wheat (according to the Biologische Bundesanstalt, Bundessortenamt und CHemical Industry [BBCH] scale, 90-99). These time and space constraints highlight the need for rapid and reliable screening methods [6, 8].

Previous studies have proposed a leaf symptom-based screening method to evaluate seed treatment efficacy and cultivar resistance under controlled conditions [5, 6]. This method evaluates treatment efficacy based on the presence of chlorotic flecking on leaf sheaths and blades [5, 6] at the second- to third-leaf stages [9] (i.e., growth stage 14 BBCH [8]). However, the presence and severity of leaf symptoms can vary among wheat cultivars, *T. caries* strains, and environmental conditions, which limits the robustness of leaf symptom-based screening [8]. The immunological and molecular detection methods, such as enzyme-linked immunosorbent assay

(ELISA) and polymerase chain reaction (PCR), were applied to seedlings for accelerating the screening of resistant cultivars [8, 18]. PCR protocols were developed to detect *T. caries* in plant tissues only [19], and quantitative PCR, digital PCR, and loop-mediated isothermal amplification (LAMP) protocols were developed to detect *T. caries* from mycelial or soil samples, without specifically focusing on plant material [20, 21, 22, 23, 24, 25]. Despite these protocols, quantitative molecular methods have not yet been established as high-throughput, early screening tools to evaluate treatment efficacy in infected plants.

We tested the application of a qPCR-based screening approach to quantify *T. caries* DNA in the leaves of wheat plants grown under controlled conditions as an early indicator of treatment efficacy. We adapted a qPCR protocol, which was originally developed to detect *T. caries* in seeds and soil (Chapter 2), and implemented a sampling and pooling strategy of young plants to increase throughput and scalability. To evaluate qPCR- and leaf symptom-based screening methods, we applied botanical treatments to soils — clove, oak bark, and buckthorn — and measured their efficacy with the early-screening detection methods. Then, we compared the calculated treatment efficacy from the early screening methods and from the bunt disease occurrence. The use of reliable early-screening methods on plants can aid in the development of new seed and soil treatments or resistant cultivars and, thereby, contribute to improved integrative control strategies.

Material and Methods

Experimental set-up

Seeds were artificially inoculated with *Tilletia caries* teliospores obtained from bunted wheat ears (cultivar Apogee) grown in a climate chamber experiment in 2024. The teliospores were stored at 5 °C until their use in August 2025. Wheat plants of the susceptible and fast-grown Apogee cultivar were grown from seeds artificially inoculated with *Tilletia caries* teliospores. Negative control plants were grown from uninfected seeds and were not exposed to *T. caries* or treated soil. For the artificial inoculation, 0.72 g of *T. caries* teliospores were applied to 360 seeds and manually homogenized. The seeds were sown in potting soil for perennials and potted plant (Ökohum, Switzerland, article number 4392379).

The potting soil was pre-moistened with water one day before sowing. The soil was then left overnight at room temperature to allow the water to permeate into the soil. On the day of sowing, the pre-moistened soil was prepared according to different treatments. In addition to the negative control, a positive and six soil treatments were included. The soil treatments were amended with three plant-derived powders at two different concentrations. The positive control consisted of inoculated seeds sown in untreated, pre-moistened soil. The amended soil treatments included clove (*Syzygium aromaticum*) powder at 0.1% and 0.5% [w/w] (origin: Madagascar, Coop, Switzerland), oak (*Quercus robur*) bark powder at 1% and 5% [w/w] (material Berg Apotheke, Switzerland), and alder buckthorn (*Frangula alnus*) bark powder at 1% and 5% [w/w] (Faulbaumrinde PhEur, Dixa AG, St. Gallen, Switzerland, country of origin Poland). For each treatment, 500 g of potting soil was mixed with plant-derived powder. Each soil mixture was thoroughly homogenized manually in a glass media bottle, moistened with 300

mL of water, and mixed again to ensure a uniform distribution of the treatment amendment.

Five 11 cm diameter pots were prepared for each tested treatment and each control. Each pot was filled to approximately three-fifths of its volume with the corresponding pre-moistened soil mixture and gently compacted. Ten seeds were sown in each pot at 3-4 cm depth: eight seeds were sown equidistant along the perimeter, and two seeds were placed equidistant from each other in the center. Then, the seeds were covered with 120 g of either treated soil for the respective treatments or untreated soil for the positive and negative controls. Water was added to the pots as needed to prevent soil desiccation throughout the experiment.

After sowing, the pots were placed in a climate chamber (Climecab 1400, Kälte 3000 AG, Switzerland) and maintained for 8 days at 15 °C without light. Then, the pots were transferred to a a second climate chamber (Climecab 1400, Kälte 3000 AG, Switzerland) with a 16-hour light cycle at 20 °C (70% humidity, 29.0 kL light intensity), followed by an 8-hour dark cycle with 15 °C and 80% humidity. Seedling germination was assessed eight days after sowing (Supplementary Table 1).

Visual plant symptom and disease evaluation

Early *T. caries* leaf symptoms were evaluated on all plants at the early boot stage (41-43 BBCH), which occurred approximately one month after sowing. The flag leaf of each plant was evaluated for the early leaf symptoms: linear chlorotic spots that are typically located along the central portion of the leaf blade and oriented parallel to the midrib [26]. The severity of the leaf symptoms was scored using a scale from 0 to 5 so that 0 indicated an absence of visible symptoms and 5 indicated the highest symptom severity (Figure 1). The score given to each flag leaf in each pot was then averaged to yield a pot-level infection score.

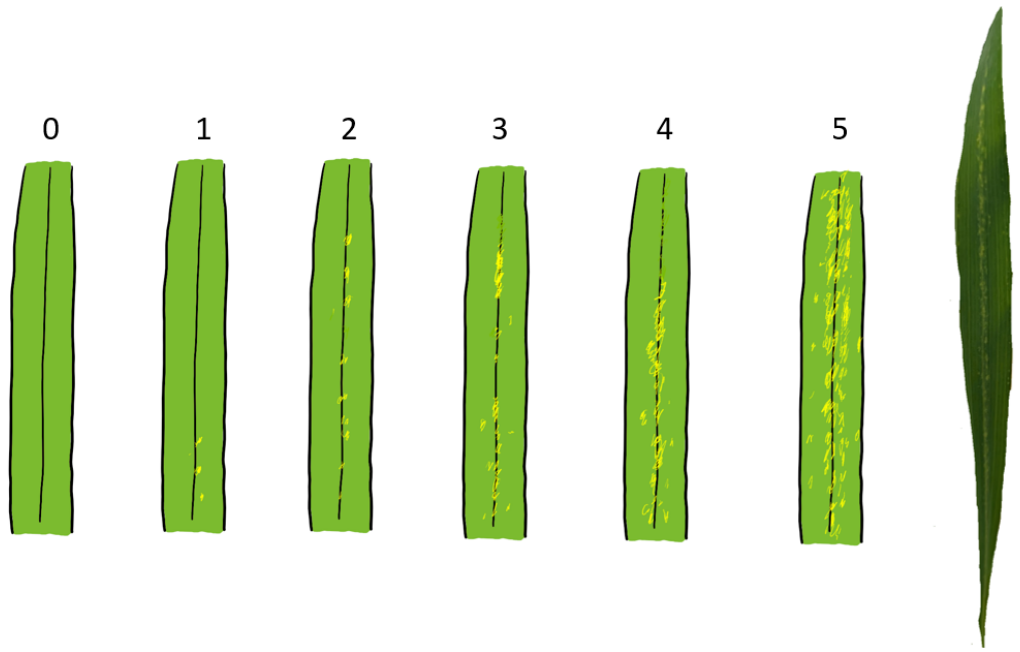


Figure 1: Schematic illustration of early leaf symptom scoring. The scale used to score the severity of early leaf symptoms caused by *Tilletia caries*. Scores range from 0 (no visible symptoms) to 5 (highest symptom severity). An example of an Apogee wheat leaf with middle-severe early symptoms (score 4) is shown on the right of the illustration. Drawing: Cecilia Panzetti.

The bunted ear infection was assessed at plant maturity. The bunted seeds were identified as seeds replaced by dark brown *T. caries* teliospores enclosed within the pericarp (otherwise known as a bunt sorus) with the characteristic fishy odor of trimethylamine. The plants developed one ear each, which were individually evaluated. The number of seeds turned into bunted sori and the number of total seeds were counted on each ear, and the disease severity was expressed as a percentage of seeds infected per ear. For each pot, the severity of the bunt infection was expressed as the average percentage across all plants.

An additional and more detailed assessment was conducted at the individual plant level for both the positive and negative control treatments to investigate the infection variation within each pot. Each plant was labeled with a unique identification number. At the boot stage, the total number of leaves per plant was counted, and early leaf symptoms were evaluated on the flag leaf as well as on each leaf of the plant individually. In mature plants, the bunted ear severity was used to assess the infection levels at the individual plant level. By testing multiple leaves on the control plants, we can better understand the variation within a plant.

Sampling and DNA extraction of plant material

The leaf samples used for the DNA extraction were collected during the leaf symptom evaluation at the early boot stage (41-43 BBCH). For each pot, a semicircular section of the flag leaf ($\simeq$ 1 cm diameter) was sampled from the main culm, which ultimately produced the ear. The leaf section was excised from the widest part of the leaf blade to minimize damage and preserve the leaf vitality. The excised section was transferred with sterile forceps into a NucleoSpin lysis tube (MACHEREY-NAGEL, GmbH & Co., Germany). To prevent cross-contamination between each sampled plant, the scalpel and forceps were sterilized with ethanol 70% [v/v] and a Bunsen burner flame and the cutting board was cleaned using ethanol 70% [v/v]. For each pot, all sampled flag leaf sections were pooled directly into the same NucleoSpin lysis tube. Additional samples were collected from the positive and negative controls to investigate individual plant-level variation in infection. In addition to the pooled sample of plants from the same pot, a composite sample of seven leaves was assembled from each plant in the controls. These leaves from each individual plant were collected in the same manner as described above and then pooled into a NucleoSpin lysis tube. From this sampling regime, both pot and plant level samples were collected from the positive and negative controls.

After sampling, a 3 mm tungsten bead (Retsch, Germany) was added to each tube for subsequent tissue disruption. The pooled flag leaf samples from individual pots and pooled seven leaf samples from individual plants were lyophilized for 24 hours using a VirTis Benchtop K Freeze Dryer (SP Industries, USA) and subsequently stored at -20 °C until DNA extraction with the NucleoSpin 96 Plant II kit (MACHEREY-NAGEL, GmbH & Co., Germany), with the following modifications to the manufacturer's protocol.

The sample homogenization and mechanical disruption were performed using a Tissue Lyser II (Qiagen, Germany) at a frequency of 30 s^{-1} for four consecutive 1 min intervals. The sample racks were repositioned between each intervals to ensure a uniform homogenization of the pooled semicircular leaf samples. To each homogenized sample was added 600 µL of the cell lysis PL1 buffer, which was provided with the kit. The samples were then mixed again with the Tissue Lyser II at a frequency of 20 s^{-1} for four consecutive 30 s intervals. The sample racks were

reoriented between each interval. Then 12 µL of RNase A (concentration 12 ng/µL) was added to each sample. The samples were gently mixed and incubated at 65 °C for one hour in a LAUDA E200 heated water bath (Lauda Dr. R. Wobser GmbH & Co. KG, Germany). After the incubation, each sample was gently rotated multiple times to ensure complete lysis before centrifugation.

The subsequent purification steps followed the kit instructions. Each sample was eluted twice with 100µL of the elution buffer provided in the kit, yielding a final volume of 200 µL per sample. The purified DNA was stored at -20 °C until analysis. The concentrations of the extracted DNA from leaves were quantified using a Varian Cary Eclipse Spectrophotometer (Agilent, USA). Leaf DNA extracts with a concentrations above 12 ng/µL were diluted to a final concentration of 12 ng/µL using a Pipetmax Gilson 268 system and Trilution software (Gilson, USA). The majority of DNA template concentrations were standardized to 12 ng/µL prior to amplification. A proportion of the DNA extracts from the leaves collected from single-plant (12 out of 84) had concentrations between 6.9 ng/µL and 12 ng/µL and were run directly without further adjustment.

DNA quantification with qPCR

The quantification of *Tilletia caries* was based on a mitochondrial non-coding region (GenBank accession number: NC_073546.1) that is specific to the plant pathogens *Tilletia caries* and *Tilletia controversa*. The primer set Tc2.5 was developed in our previous study Chapter 2 and includes the Tc2.5 forward primer (5′-AGG GGT GAC AAA GAT CGG AT-3′) and the Tc2.5 reverse primer (5′-CGC CGA TTC TGT CCT TGT TT-3′). All primers were synthesized by Microsynth (Balgach, Switzerland). Mitochondrial copy quantification relied on standard curves of 5-fold serial dilutions of gBlock synthetic gene fragment containing the target region (Integrated DNA Technologies, USA) that was used in our previous study Chapter 2 (Supplementary Table 2). The gBlock fragment included 5 bp and 31 bp flanking regions respectively on the left and right side of the mitochondrial region used for the primer set Tc2.5.

The gBlock fragment was initially diluted to a concentration of 570632 copies/µL and stored at -20 °C with 0.2 mg/ml Poly(A) (Sigma-Aldrich, Germany). The diluted gBlock fragment was then divided into aliquots suitable for a single plate to limit freeze-thaw cycles. The standard curve copy numbers per reaction, calculated from the molecular weight of the gBlock fragment, ranged from 114800 to 7 for Tc2.5. In each dilution of the standard curve was added 12 ng/µL of DNA extracted from *Hordeum vulgare* seedlings as non-tamplate DNA.

Each qPCR plate included technical triplicates of all standard curve points, biological samples, and negative controls (nuclease-free water and non-target DNA). The qPCR reaction was run in 384-well plates sealed with adhesive optical sealing foil (Nolato TreffLab, Sweden). The qPCR reactions were set in a final volume of 10 µL per well, containing 50 ng of DNA, with either 0.3 µM of each Tc2.5 primers, and 5 µL of SYBR qPCR Master Mix (Promega Corporation, USA). The amplification program included an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 63 °C for 1 min. All reactions were performed on the CFX Opus 384 Real-Time PCR System (Bio-Rad, USA). In each sample, the absolute quantification of the mitochondrial target was determined using CFX Maestro 2.2 software (version 5.2.008.0222), which incorporates an efficiency correction based on the standard curve.

The samples were rerun if the Cq standard deviation of the triplicate reactions exceeded 0.5 for the fungal target. However, a sample was not rerun if the Cq mean of the triplicate reactions were below the Cq of the lowest measurable dilution point of the standard curve (7.35 copies per reaction) on the same plate. In this case, the *T. caries* DNA within sample was considered not detectable and counted as uninfected. The undetectable samples were included in the dataset as having 0 *T. caries* copies so that the qPCR measurements could be directly compared to the pathogen detection in plants. Because plants without noticeable visual infection were counted as uninfected. The RFU baseline was adjusted manually for all the runs to 2500.

Statistical Analyses

The plants were considered infected if at least 1.76 *T. caries* copies/µL were detected in the extracted DNA. In the evaluation of early leaf symptoms, the plants were considered infected when at least one leaf was symptomatic. Plants were counted as diseased when at least one bunt ball was observed at seed maturation. We constructed confusion matrices to compare the visual assessment of the bunted ears — used as the reference — to the binary classifications of either the qPCR or the early leaf symptoms assessment (Supplementary Table 3). We used the results obtained from these matrices — true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN) — to calculate the sensitivity, specificity, precision, and false discovery rate, and accuracy according to the following formulas:

$$\text{Sensitivity } (\frac{\text{TP}}{\text{TP + FN}});$$

$$\text{Specificity } (\frac{\text{TN}}{\text{TN + FP}});$$

$$\text{Precision } (\frac{\text{TP}}{\text{TP + FP}});$$

$$\text{False discovery rate } (\frac{\text{FP}}{\text{FP + TP}});$$

$$\text{Accuracy } (\frac{\text{TP + TN}}{\text{TP + TN + FP + FN}}).$$

We evaluated the use of early leaf symptoms and qPCR of plant tissue in assessing the soil treatment effectiveness. Estimates of treatment efficacy against *T. caries* based on early leaf symptoms and qPCR results were compared to the calculations of treatment efficacy derived from bunt ball development. Bunt ball incidence shows the actual disease development in the mature plant, which we used as the true infection level. Treatment efficacy based on leaf symptoms was calculated using the mean severity score of leaf symptoms per pot, while treatment efficacy based on qPCR was calculated using the mean number of *T. caries* DNA copies/µL detected in triplicate reactions of DNA extracted from pooled flag leaf samples per pot. For each treatment, the efficacy estimates from leaf symptoms or qPCR results was compared directly to treatment efficacy derived from bunt sori incidence. We quantified the ability of these early detection methods to assess treatment efficacy on bunt sori development by adapting the predictability factor described in Chapter 1. This factor expresses the degree of agreement between the estimates of treatment efficacy obtained from leaf symptoms or qPCR

results and treatment efficacy obtained from bunt sori assessments. All statistical analyzes were performed using R version 4.5.1.

Results

Treatment efficacy based on *Tilletia caries* infection levels and disease occurrence

We assessed *Tilletia caries* infections in plants grown from soil either treated with botanical amendments or untreated with two early screening procedures. Then, we compared these infection levels to disease occurrence based on bunt ball incidence at plant maturity. The early screening procedures included leaf symptom scoring and *T. caries* DNA quantification from pooled flag leaf samples within pots using qPCR. The same plants were used for all three assessments. Although this experiment did not focus on treatment performance, we used the treatments to evaluate the early plant screening procedures. The efficacy of the experimental treatments based on the amount of *T. caries* infections scored based on the early-leaf symptoms and measured with qPCR were compared to the final disease outcome.

Bunt ball incidence was significantly greater ($p < 0.05$) in the positive control than in all treatments (Figure 2A). Only the 5% alder buckthorn treatment resulted in a significantly lower percentage (5.4%) of bunted kernels on average compared to all other treatments; it did not significantly differ from the negative control. The *T. caries* infections assessed using the leaf symptom-based screening method generally reflected the results observed for bunt ball incidence (Figure 2B). In contrast, the qPCR quantification of *T. caries*/µL DNA detected from the pooled flag leaves showed that the positive control did not significantly differ from most treatments (Figure 2C)„ unlike the differences observed among treatments based on bunt ball incidence and early leaf symptom score. However, the qPCR-based measurement clearly showed that 5% buckthorn treatment reduced the infection compared to the positive control.

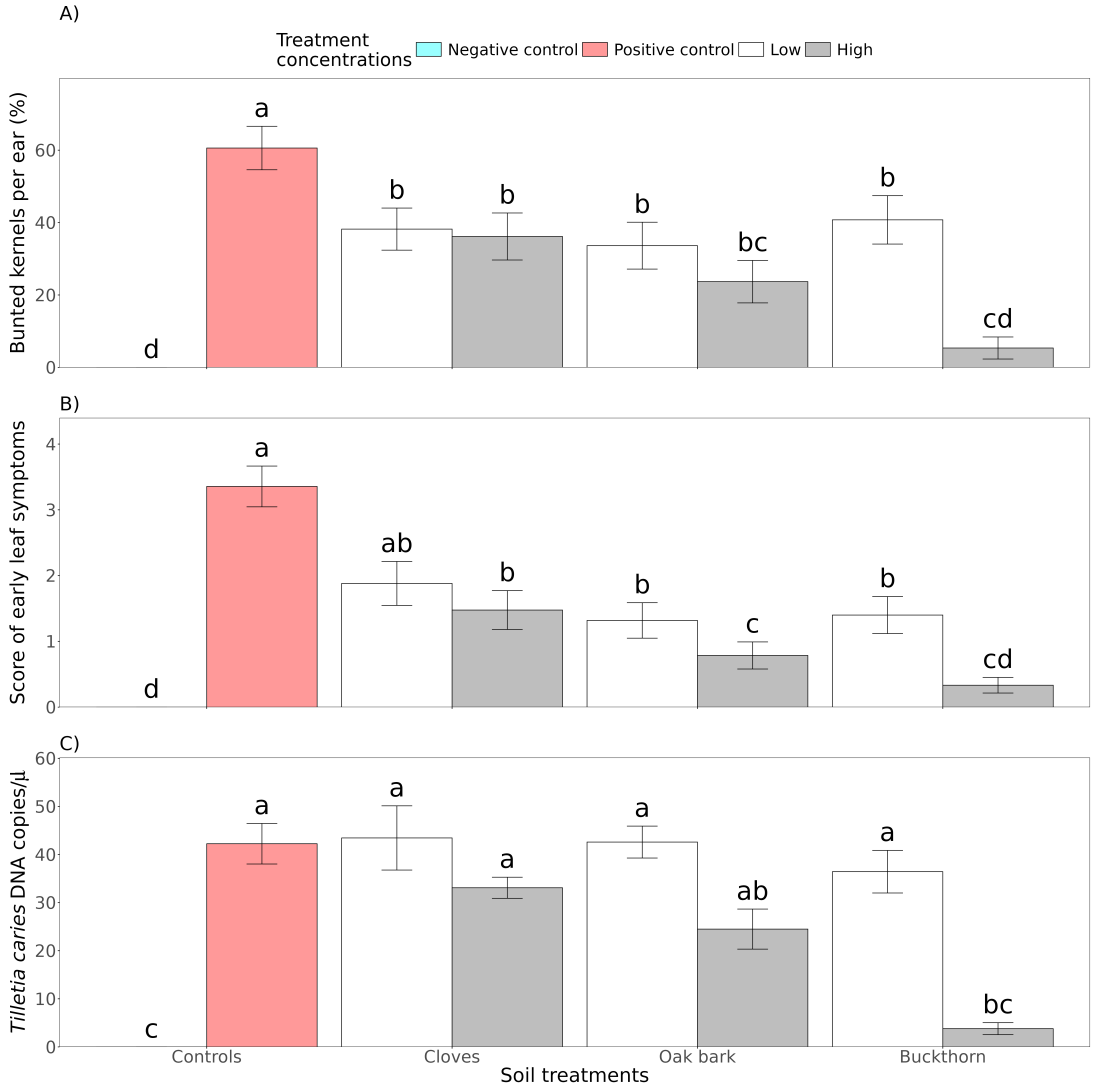


Figure 2: *Tilletia caries* infection levels are shown based on (A) the percentage of bunt ball per ear, (B) the average early leaf symptom score per pot (0 no symptoms to 5 highest level of symptoms), and (C) quantification of *T. caries* with qPCR. Plants were grown in either untreated soil for the positive (infected seeds) and negative controls (uninfected seed) or soil mixed with botanical products at two different concentrations for the treatments. The soil treatments consisted of cloves (0.1% or 0.5%), oak bark (1% or 5%), and buckthorn (1% or 5%). Error bars indicate the standard error of the mean based on five replicates. The letters indicate significant differences ($p < 0.05$) in the observed infection rate among treatments based on Kruskal-Wallis tests followed by Fisher's least significant difference tests.

Discrimination between infected and uninfected plants using qPCR and early leaf symptoms

We assessed *Tilletia caries* development in young plants in each pot using two approaches: qPCR detection of pathogen DNA in immature plant leaves and the scoring of early leaf symptoms. The evaluation of bunt ball development in mature plants was used to assess the disease level of the plants within each pot. We classified each pot as either infected or uninfected based on the detection of *T. caries* DNA or the presence of disease symptoms in plants. Based on the presence of the bunt ball in pots, only two treatments showed fewer pots with diseased plants: the 5% buckthorn treatment and the 5% oak bark treatment, which suppressed bunt ball development in 60% and 20% of the pots, respectively.

To assess whether the early leaf symptom score and the number of *T. caries* copies/µL

detected using qPCR corresponded to an increased percentage of bunted kernels per ear, we compared them directly (Figure 3). Early leaf symptom evaluation did not completely discriminate between infected and uninfected plants because it incorrectly classified two uninfected samples as infected. However, these two pots had relatively low symptom scores (Figure 3A). In contrast, the qPCR results that identified samples to be pathogen-free were indeed uninfected and showed no sori development. The qPCR-based screening accurately reflected the treatment suppression effect observed with bunt sori evaluation, while the leaf symptom-based method identified complete suppression for the 5% buckthorn treatment in only 20% of replicates. We performed a secondary evaluation of the controls on an individual plant rather than pot level. The qPCR results in those samples also consistently measured *T. caries* DNA in plants that were truly positive based on observed bunt sori (Supplementary Table 3, Supplementary Table 4).

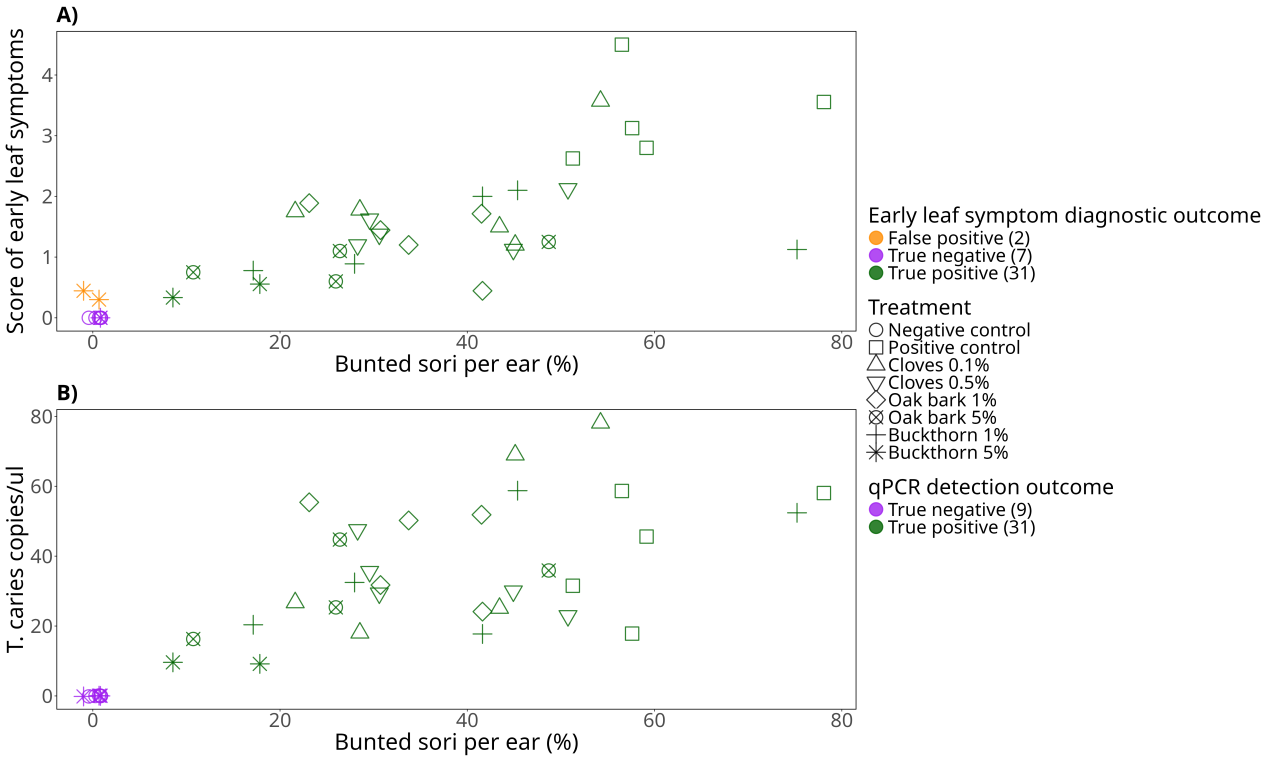


Figure 3: The correlation between the percentage of bunted seed and diagnostic outcomes based on (A) early leaf symptom and (B) qPCR methods. Early leaf symptoms were averaged for each pot. The qPCR measurements are reported as *Tilletia caries* DNA copies/µl. The percentage of bunted seeds per ear was used to classify plants as infected or uninfected. This classification was used as a reference to determine the diagnostic outcomes (true positives, true negatives, false positives, and false negatives). Different colors indicate diagnostic outcomes, and the samples belonging to each outcome are noted in parentheses. The different shapes indicate treatments.

The specificity of the early leaf symptom scoring evaluation decreased compared to the qPCR method due to discrepancies between early leaf symptom scores and bunted sori assessments in uninfected replicates. Nevertheless, the early leaf symptom evaluation showed high sensitivity (1.00), accuracy (0.95), and precision (0.94). However, the qPCR method had slightly higher accuracy (1.00) and precision (1.00) (Table 1).

	Sensitivity	Specificity	Precision	False discovery rate	Accuracy
Early leaf symptoms	1.00	0.78	0.94	0.06	0.95
qPCR method	1.00	1.00	1.00	0.00	1.00

Table 1: Evaluation of early leaf symptoms and the qPCR method to discriminate between plants infected with *Tilletia caries* and uninfected plants. Eight treatments were included in the trial, and five pots were used for each treatment. The performance metrics include sensitivity, specificity, precision, false discovery rate, and accuracy. These performance metrics were based on the agreement between the each pot's disease establishment (one or more bunted seed per pot) and the plants' infection status based on their early leaf symptoms or *T. caries* qPCR-measurements. The outcome of each pot's classification according to the confusion matrices (Figure 3) was used to evaluate the early-screening method based on observed early leaf symptoms and the qPCR results

Treatment efficacy using qPCR, early leaf symptoms, and bunted ears

We calculated treatment efficacy using the *T. caries* disease and infection levels, which were quantified based on the percentage of bunted kernels and the early detection methods: early leaf symptom assessment and the qPCR method. We evaluated the early detection methods' ability to predict treatment efficacy as determined according to the disease outcome.

Estimates of treatment efficacy based on *T. caries* DNA quantification with qPCR ranged from -2.8% to 91.0% (Table 2). In contrast, the early leaf symptom assessment showed a smaller range of variation between treatment efficacy from 41.6% to 90.3%. Both early detection methods identified the 0.1% clove treatment as least effective and the 5% buckthorn treatment as the most effective. The disease occurrence based on bunted kernels indicated that the 1% buckthorn treatment was least effective, but showed that 5% buckthorn was indeed the most effective treatment.

We further examined the degree of agreement between the early detection methods and the disease occurrence using the predictability factor. Predictability factor values close to 100% indicated close agreement between the efficacy estimates of the early detection methods and the treatment efficacy assessed with the disease reference. Larger deviations from 100% reflected under- or overestimation compared to the bunted seed evaluation. Overall, early leaf symptom assessment tended to overestimate treatment efficacy, while qPCR-based estimates tended to underestimate treatment efficacy (Table 2).

	Treatment efficacy ± Std. Dev. (%)			Predictability factor ± Std. Dev. (%)	
Treatment	Bunted kernel evaluation	qPCR method	Early leaf symptoms	qPCR method	Early leaf symptoms
Cloves 0.1%	36.5 ± 21.2	-2.8 ± 66.1	41.6 ± 27.7	-15.6 ± 180.1	105.4 ± 58.8
Cloves 0.5%	39.6 ± 17.4	21.7 ± 21.7	55.6 ± 12.0	96.4 ± 58.2	131.9 ± 35.8
Oak bark 1%	44.0 ± 13.6	-0.8 ± 32.9	60.1 ± 16.8	53.1 ± 44.6	126.8 ± 41.1
Oak bark 5%	63.2 ± 30.5	42.0 ± 41.3	77.9 ± 14.6	80.9 ± 21.2	128.0 ± 48.3
Buckthorn 1%	31.7 ± 35.5	13.8 ± 43.9	58.9 ± 18.7	75.6 ± 62.8	73.2 ± 93.6
Buckthorn 5%	91.2 ± 13.5	91.0 ± 12.3	90.3 ± 6.2	100.2 ± 4.1	100.2 ± 6.5

Table 2: Treatment efficacy against *Tilletia caries* was based on the reduction of bunted kernels and early leaf assessments of infection levels. *T. caries* infection levels were measured from DNA in leaves with qPCR and chlorotic symptoms. The treatments, which included 0.1% and 0.5% cloves, 1% and 5% oak bark, and 1% and 5% buckthorn, were applied to soil, in which seeds from the wheat cultivar Apogee were sown. A treatment efficacy of 100% indicates a complete suppression of *T. caries* infection compared to the untreated control, while negative values indicate increased infection. The predictability factor is based on the ability of the early detection methods (qPCR and early leaf symptoms) to predict treatment efficacy on bunted seed development. A predictability factor of 100% most closely matched the treatment efficacy obtained in the bunted seed evaluation.

Discussion

Integrated pest management (IPM) guidelines aim to reduce the use and risk of synthetic plant protection products (PPPs) [27], which include the sowing of certified seeds with low pathogen contamination or treating contaminated seeds with low-risk or organic alternatives to synthetic PPPs [27, 28]. However, these treatments are not currently widely available on the market. Their development is time-consuming and resource-intensive, and the infection development is often influenced from the environment [6, 29]. Treatment development for some diseases requires that the plant reach maturity when the pathogen shows clear symptoms [6], such as with *Tilletia caries*.

Two decades ago, researchers recognized the need to accelerate the evaluation of treatment efficacy against *T. caries* and proposed a leaf symptom-based screening approach [6]. Because the leaf symptom-based screening targets early pathogen development rather than final disease symptoms, it can be conducted in pots with limited space and in controlled conditions. Previous work investigated whether early leaf symptom presence corresponded to the development of bunt balls in the same plant, and only the false negatives but no false positives were recorded [6]. In contrast, our results showed no false negatives, but six false positive samples were found across all samples analyzed (Supplementary Table 3). These discrepancies underscore the variability of early leaf symptom expression, which may lead to false outcomes and a miscalculation of disease severity, which has been previously observed [26]. Here, we explore whether a molecular detection method could be used for an early screening of treatments, which could ultimately be applied to test for resistant cultivars.

We tested the application of a qPCR protocol, which we developed for detecting *T. caries* in seed and soil (Chapter 3), to plant tissue. Our qPCR-based screening method showed higher accuracy and precision than leaf symptom evaluation at discriminating between infected and uninfected plants. The detection method's success was based on whether the same plants developed bunt ball. The screening method base on qPCR-based was less accurate than early leaf symptom approach in estimating treatment efficacy consistent with the treatment efficacy based on bunt sori incidence. This reduction in the accuracy of estimating treatment efficacy using qPCR results is most likely because the treatment efficacy was calculated based only on pathogen DNA quantity and were not normalized to host DNA. Although the samples were diluted to the same concentrations, the absence of a normalization calculation means that *T. caries* could be affected by other factors. For example, some of the treatments may have reduced the number of microorganisms or influence the plant's growth. These effects would influence how much *T. caries* is measured when the same amount of DNA is added to a reaction. For example, if a plant has many endo- and epiphytes, it may dilute the pathogen's DNA quantification in the same amount of DNA. This is hard to account for because the treatments' effects on the plant's growth and microbiome are unknown. To enable a reliable quantitative comparisons of treatment efficacy, we will quantify the wheat DNA and normalize *T. caries* DNA to host DNA as done with *U. nuda* in barely (Chapter 3).

The reduced ability of the qPCR-based screening method to estimate treatment efficacy may also be related to the timing of leaf sampling. The leaves were collected at the early booting phase, by which *T. caries* mycelium has already accumulated in the developing ear

[30]. During the early stages of plant growth, *T. caries* mycelium occurs not only in the main culm but also in the leaves and secondary tillers [6]. Therefore, to identify the optimal time point for estimating treatment efficacy, samples should be collected at different stages of plant and fungus growth. Additionally, this non-destructive sampling strategy enables repeated leaf sampling from the same plant, which could further help to understand the relationship between the disease progression and seed bunt development.

The results obtained in this study suggest that the qPCR method is potentially capable of identifying clearly effective treatments — 5 % buckthorn appears to be quite effective from this first investigation based on the two screening methods and reduced bunt ball. The effectiveness of buckthorn as an organic treatment is consistent with previous studies demonstrating its activity against *Phytophthora infestans* under field and laboratory conditions [31].

The dual seed- and soil-borne transmission of *T. caries* is an additional challenge to developing and evaluating new treatments. Although the control of *T. caries* seedborne inoculum is generally more feasible [16, 32], the effective suppression of soilborne inocula remains difficult, especially under field conditions due to the environmental influences on pathogen development [10, 33, 34]. The early screening methods, including our qPCR-based screening, are ideal for the preliminary selection of promising treatments using controlled conditions (i.e., environment and pathogen inoculum). The early screening methods — leaf- and qPCR-based screening — enable the evaluation of treatment performance under conditions that promote high infection and allow the testing of a large number of treatments [6]. During the first week after seed germination (0-10 BBCH), *T. caries* grows within the plant in the absence of light [3, 4]. Therefore, further optimization to reduce space and time could potentially involve using filter paper and stacked boxes (Chapter 3). Additionally, treatment efficacy could be tested on plants grown in soil under controlled conditions in studies focusing on the efficacy of the treatments against *T. caries* soil inoculum or environmental effects on treatment success. Through using this qPCR method on plants, promising treatments or cultivars could be selected for inclusion in field trials, which would reduce the space required.

Further research is needed to refine rapid screening tools for *T. caries*. The qPCR-based screening method tested in this study shows promise, but it requires optimization. In particular through normalization of pathogen DNA to host DNA and improved sampling strategies. This qPCR method can be applied to wheat cultivars that show no early leaf symptoms. It enables the study of *T. caries* development in the plant and treatment effects across different plant growth stages. Overall, the qPCR-based screening method can offer a complementary approach to symptom-based evaluation. This screening method can accelerate and facilitate the development of low-risk and organic treatments for common bunt control.

Data Availability

All data collected from the experiments and used in the analyses are available in the Supplementary Material.

References Chapter 4

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Supplementary Material: Optimization of *Tilletia caries* detection in plants with a qPCR-based screening method to evaluate new treatments

Supplementary Tables

Treatment	Repetition	Germinated seedlings per pot (%)
Negative control	1	80
	2	60
	3	90
	4	80
	5	80
Positive control	1	90
	2	80
	3	100
	4	100
	5	90
Cloves 0.1%	1	100
	2	80
	3	70
	4	90
	5	90
Cloves 0.5%	1	80
	2	100
	3	80
	4	60
	5	80
Oak bark 1%	1	90
	2	80
	3	100
	4	90
	5	70
Oak bark 5%	1	100
	2	80
	3	70
	4	80
	5	90
Frangula 1%	1	90
	2	40
	3	70
	4	90
	5	90
Frangula 5%	1	100
	2	80
	3	80
	4	80
	5	80

Supplementary Table 1: Percentage of germinated seedlings per pot. Soils had been mixed with botanicals in the treatments. Soils in the positive and negative control pots were not treated. The positive control and the botanical treatments were sown with wheat seeds contaminated with *T. caries*

Gene target	Target organism	Oligo name	Sequence (5′ - 3′)	Length (bp)	Molecular weight (g/mol)	Genbank accession number
Unknown	*Tilletia controversa*	Tc2.5_stdcurve	AGA TGC TAG GGG TGA CAA AGA TCG GAT TCT GT TTC TGG TCC TGA CTT TCC TCA CTT TCT TGT CGT TCG TCT TTA GCT CGT TAT ATC TCT GCT TTA CGG CTC CGC CGA TTA TAC TCT TCT ATT ATA TTT ACA TTG TAA AAA AAC AAG GAC AGA ATC GGC GGA GCG CCA ATC ATT TTC TTT TAC TAC ATT TA	190	117258	NC_073546.1:49162-49186

Supplementary Table 2: Sequences of gBlock gene fragment used as the standard curve. The standard curve was included in all qPCR plates for the absolute quantification of *Tilletia caries*. The GenBank accession number identifies the sequence used for the gBlock gene fragment.

A) qPCR method

Predicted (qPCR)	Actual values (Bunted ear evaluation): Infected	Actual values (Bunted ear evaluation): Uninfected
Infected	True Positive (TP): (33) Pot 2.1: 41, 42, 44, 45, 46, 47, 48 Pot 2.2: 50, 54, 55, 56, 57 Pot 2.3: 58, 59, 60, 61, 64, 66, 67 Pot 2.4: 69, 70, 71, 74, 75, 77 Pot 2.5: 78, 79, 80, 81, 82, 84, 85, 86	False Positive (FP): (0)
Uninfected	False Negative (FN): (0)	True Negative (TN): (12) Pot 2.1: 43, 49 Pot 2.2: 52, 53 Pot 2.3: 62, 63, 65 Pot 2.4: 68, 72, 73, 76 Pot 2.5: 83

B) Early leaf symptoms

Actual values (Bunted ear evaluation): Infected	Actual values (Bunted ear evaluation): Uninfected	Predicted (Early leaf symptoms)
True Positive (TP): (33) Pot 2.1: 41, 42, 44, 45, 46, 47, 48 Pot 2.2: 50, 54, 55, 56, 57 Pot 2.3: 58, 59, 60, 61, 64, 66, 67 Pot 2.4: 69, 70, 71, 74, 75, 77 Pot 2.5: 78, 79, 80, 81, 82, 84, 85, 86	False Positive (FP): (5) Pot 2.1: 49 Pot 2.2: 53 Pot 2.4: 68, 72, 73	Infected
False Negative (FN): (0)	True Negative (TN): (7) Pot 2.1: 43 Pot 2.2: 52 Pot 2.3: 62, 63, 65 Pot 2.4: 76 Pot 2.5:	Uninfected

Supplementary Table 3: Confusion matrices comparing the predicted values of the qPCR method and the evaluation of the early leaf symptoms with the actual values of the visual assessment of the bunted ear based on single plants from the positive control (n = 45). For the confusion matrix based on (A) the qPCR method, the predicted values of the *Tilletia caries* infection levels were classified as either infected or uninfected when at least 1.14 *T. caries* copies/µl are detected. For the confusion matrix based on (B) the early leaf symptoms, predicted values were classified as either infected or uninfected when at least a leaf showed symptoms, namely chlorosis dots. Plants were classified as either infected or uninfected when at least a seed per plant was bunted. These classifications served as actual values in the confusion matrices. The predicted values that matched the actual values were either true positive or true negative, while the predicted values that did not match the actual values were either false positive or false negative.

	Sensitivity	**Specificity**	**Precision**	**False discovery rate**	**Accuracy**
qPCR method	1.00	1.00	1.00	0.00	1.00
Early leaf symptoms	1.00	0.58	0.87	0.13	0.89

Supplementary Table 4: Performance evaluations of the qPCR and early leaf symptom methods in detecting *Tilletia caries* infections across 45 plants grown from artificially inoculated seeds from the positive control. Of these plants, 41 were diseased and 4 not diseased based on the appearance of bunted kernels. The performance metrics include sensitivity, specificity, precision, false discovery rate, and accuracy. These performance metrics were based on the agreement between the plant's predicted values, which classified the plant as infected or uninfected the method-specific limit of quantification, and their actual values based on observance of disease. The outcome of each plant's classification according to the confusion matrices (Supplementary Table 3) was used to evaluate the methods' performance.

General discussion

The impacts of synthetic plant production products (PPPs) on water, biodiversity and health occur at a global scale [186], and some agricultural policies, especially in the EU (e.g., the Farm to Fork strategy), promote the reduction of synthetic PPPs use to limit their associated risks [1, 3, 4, 5, 2, 187]. However, these restrictions do not always consider the market availability of equally effective alternative solutions to synthetic PPPs and their implementation in existing management systems [188]. Additionally, the emergence of pathogen resistance to widely used synthetic PPPs can also reduce the active ingredients range of effective, low-risk synthetic PPPs options, further challenging conventional disease management strategies [17]. This misalignment between regulatory goals and practical disease management, along with increased pathogen resistance to synthetic pesticides, may contribute to the increased crop losses [9, 10], fluctuating emergency authorizations for PPPs [7], and renewed concern for plant pathogens that were historically difficult to control prior to the widespread adoption of synthetic and systemic PPPs [20, 112].

The fungi, *Ustilago nuda* in barley and *Tilletia caries* and *Tilletia controversa* in wheat, could re-emerge as a threat to cereal crops, especially in low-input and organic production systems where synthetic seed treatments are restricted or unavailable [19, 20, 112, 113]. Therefore, relying on broad, routine, prophylactic seed treatments alone is not suitable for long-term disease management. Integrated pest management (IPM) guidelines promote informed decision-making to minimize possible economic impacts on the agricultural sector while only applying treatments when necessary [17]. Therefore decisions regarding treatment applications are based on pathogen risk assessments. This dissertation contributes to improving the management of smut and bunt diseases through the integration of PCR-based protocols to measure pathogen pressure (Chapter 1 and Chapter 2) and evaluate treatments (Chapter 3 and Chapter 4). The developed protocols enable the sensitive and quantitative detection of *U. nuda* in seed and plant tissues and *T. caries* and *T. controvera* in seed, soil, and plant tissues. However, the practical value of these PCR measurements to support concrete management decisions depends on our ability to translate these pathogen measurements of DNA copies into biologically relevant information.

Contribution of quantitative PCR-based detection protocols for decision support in Integrated Pest Management

Current decision-support methods used in integrated pest management (IPM) strategies, such as field surveillance and visual seed inspection [19, 39], are often labor-intensive, time-

consuming and dependent on inspector expertise [50, 189]. Visual methods require taxonomic knowledge and expertise in distinguish pathogens based on morphological traits or disease symptoms, which often overlap among closely related species [189]. Molecular detection methods can offer greater specificity and sensitivity than the current decision-support methods. Such methods can discriminate between pathogens that are difficult or impossible to distinguish morphologically. For example, qPCR protocols can be used to differentiate formae speciales within *Fusarium oxysporum* species (e. g., *F. oxysporum* f. sp. *lycopersici* [36]), while visual seed inspection cannot reliably distinguish them. Moreover, the transition toward low-input and organic agricultural systems requires pathogen detection methods that provide quantitative information beyond simply confirming pathogen presence or species differentiation. Quantitative molecular detection methods (e.g., qPCR and dPCR) can be used to detect seed- and soil-borne fungal pathogens, such as *Ustilago nuda*, *Tilletia caries*, and *Tilletia controversa*, across different substrates — seeds, soil, and plant tissues. These quantitative measurements can facilitate the estimation of pathogen pressure, support management decisions based on tolerance thresholds, and enable targeted interventions when pathogen levels exceed these thresholds [1].

Reliable pathogen quantification is fundamental to establish and apply tolerance thresholds within IPM strategies [190]. Throughout my dissertation, I improved the quantification reliability of *U. nuda*, *T. caries*, and *T. controversa* with standardized calibration strategies based on synthetic DNA fragments for standard curves, internal controls, and sensitive PCR targets. The methods developed in this dissertation rely on absolute quantification rather than a direct comparison of quantification cycle (Cq) values, which can differ between runs, PCR assays, and laboratories [191]. The integration of endogenous and exogenous internal controls further improved the robustness of the PCR-based method is use of endogenous and exogenous internal control, which was also observed for the plant pathogen *Rathayibacter toxicus* [192]. These internal controls can be used to normalize the amount of the detected pathogen against host DNA and account for differences among DNA extractions or PCR reactions. These technical adaptations were used to develop reliable PCR-based tests that may provide more accurate decision support than the current methods.

IPM strategies to control *U. nuda* rely on the number of smutted ears in the field or infected embryos observed with the visual seed inspection. I developed a qPCR protocol targeting mitochondrial DNA (mtDNA) in seeds, and its results showed a stronger correlation with disease occurrence in the field than the number of infected embryos detected using the embryo test (Chapter 1). Additionally, the qPCR protocol had a 40% higher accuracy than the embryo test in the tested seed lots. The tested seed lots were commercially certified, meaning that they should have either no *U. nuda* or a low level of infection, but indeed two lots had a higher disease occurrence than expected in certified seed lots (more than 35 infected ears per m^2). These findings suggest that 1) the developed qPCR protocol more accurately reflects infection levels in seeds and improves classification relative to tolerance thresholds and 2) it is a test that would be useful to screen commercially certified seeds. The current visual seed inspection method for *U. nuda* is labor-intensive and has low accuracy in validating certified seed, which is consistent with what is already known about the traditional detection method [189].

Because of the absence of other detection methods for *T. caries* and *T. controversa* on seed, I used a current visual seed inspection method to compare the PCR-based detection of *T.*

caries and *T. controversa* with mitochondrial primer set and nuclear primer sets (Chapter 2). Mitochondrial primers classified a greater number of seed lots as contaminated than nuclear primers, which also better matched the reference classification. The same primer sets enabled the detection of *T. caries* and *T. controversa* in soil, in which the sensitivity of the mitochondrial primers was confirmed. Despite the higher sensitivity in detecting pathogens in seeds and soil, using mtDNA targets may complicate interpretation of the results because the number of mitochondrial copies per teliospores is unknown and may vary with spore size. Despite the uncertainty in connecting mtDNA quantities to teliospore numbers, an mtDNA target is useful for seed health tests that require increased sensitivity, such as in countries with a tolerance threshold of 0 teliospores per kernel. Additionally, mtDNA is more vulnerable to mutation than nuclear DNA [193], which may affect long-term assay robustness and necessitate periodic validation of the mitochondrial primer set across emerging pathogen populations. Nonetheless, these new molecular tools provide a number of advantages over the currently used tools.

Limitations of current seed health certification systems and tolerance threshold

The control of plant pathogens in organic and low-input agriculture is based on preventive management strategies [16]. The use of untreated certified seed can reduce the application of synthetic fungicides and prevent the introduction of seedborne inoculum [155]. The seed certification process relies on field inspections or/and seed health test [19].

In my dissertation, I compared PCR-based methods with the current seed health test in use. I observed variability in infection and contamination levels assessed by different inspectors during direct seed evaluations, even when analyzing the same embryo extractions or seed washing solutions (Chapter 1 and Chapter 2), highlighting the subjectivity of these methods. Furthermore, some seed health tests rely on binary outcomes that only report presence or absence of pathogens on the seeds [19, 44, 46, 49]. Such classifications ignore the pathogen load and its consequences for disease development. For example, the relationship between the amount of *U. nuda* mycelium within an embryo and the subsequent number of developed smutted ears remains unclear. In our field trial, seed lots with higher *U. nuda* infection levels produced more plants with multiple infected ears than seed lots with lower infection levels. The seed lots with lower infection levels mostly only developed smutted ears on the main culm. This variation in the disease development suggests that higher mycelial abundance within the embryo may promote colonization of both the main culm and tillers [60, 140], whereas lower mycelial loads may restrict the infection to the main culm. Seed health tests that use binary certification cannot capture this disease variation and its possible yield impact.

Many tolerance thresholds used in seed certification systems have unclear biological justification because few studies connect pathogen presence and infection severity in the seed to the disease pressure in the field. Furthermore, these thresholds usually do not account for variation in disease development across cultivars and environments [74]. Additionally, tolerance thresholds for seed certification vary considerably among EU countries in terms of measurement units and assessment methodologies [19]. The absence of harmonized, biologically justified tolerance thresholds limits the commercialization of untreated seed lots across EU countries and complicates EU disease management strategies [19]. Many non-standardized and non-validated seed health tests used to certify seed lots add complexity to the establishment of a biologically mean-

ingful tolerance threshold. The determination of a biologically meaningful tolerance threshold is further complicated for pathogens that can be transmitted through multiple sources, such as the seed and soilborne pathogen *T. caries* and *T. controversa*. Consequently, seed tests alone provide an incomplete picture of infection risk.

The molecular detection measurements of the pathogens represents a theoretical infection level from the fungus measured in either the seed or soil rather than the actual disease outcome in the field. The pathogen's vitality usually cannot be known using molecular detection methods with the exception of propidium monoazide (PMA)-qPCR [194, 195]. This method relies on the dye's ability to penetrate compromised membranes of dead cells and bind to DNA, thereby inhibiting its amplification [196]. PMA-qPCR may be ineffective for assessing the vitality of *Tilletia* spp. and *U. nuda* because the thick walls of teliospores and the mycelia within the embryo could prevent consistent dye penetration and the subsequent light-induced dye activation. Additionally, pathogen development and symptom manifestation at the field level depends on site-specific environmental conditions, including temperature, soil moisture, and weather conditions [107, 143, 157, 197]. In my work, *U. nuda* disease levels between growing seasons showed how field conditions can influence pathogen development (Chapter 1 and Chapter 3). Therefore, a meaningful biological interpretation of molecular measurements requires validation across multiple environments and growing seasons to account for environmental variability in pathogen development. These limitations of molecular results for biological meaning are particularly relevant for soilborne pathogens because they can be detected in the soil, but they may not infect plants, depending on field conditions [107, 198].

Limitation for soilborne monitoring application

Molecular detection methods can detect plant pathogens in DNA extracted from small amounts of soil ($\leq$ 1 g) [199]. However, this small sample size requires a precise sampling strategy because such a small quantity is usually a subsample of a larger composite soil sample. I found that targeting mitochondrial DNA was an essential step to obtain measurable values of pathogen DNA. Our sensitivity increased drastically using soil artificially inoculated when I used a mitochondrial target compared to a nuclear target (Chapter 2). However, the use of these mitochondrial primers in soil samples from known infected field showed that a representative sampling of the pathogen's spatial distribution in the field can strongly affect the results, and the most sensitive molecular assay may not compensate for an inadequate sampling design.

A practical application of a molecular detection protocol to monitor soilborne disease has to consider the design of soil sampling procedures: the number of samples to collect, the sampling time and pattern [199]. Soilborne pathogens often have heterogeneous and aggregated distributions across the field, in particular for dormant spores [96], such as *T. caries* and *T. controversa*, which complicates representative sampling. Additionally, the knowledge of the recent crop history is necessary because crop species and management practices can influence both pathogen population density and the inhibit molecular detection [96]. The selection of the sampling period may impact quantity and distribution of the pathogen may depending on the target soilborne pathogen's biology and epidemiology [199]. In row crops (e.g., wheat and barley) and orchards, sampling procedures can use either random or systematic designs. However, the high variability I observed in small-plots using a systemic design shows that

representative estimates of *T. controversa* infection at the field scale require more than a few soil samples. Instead, multiple soil samples should be collected in a well-thought out design that has been adapted to the field. This heterogeneous pattern in field distribution was observed for other soilborne pathogens [96].

Soil sampling strategies for detecting soilborne pathogens should consider the spatial heterogeneity of the inoculum and the feasibility of sampling, including sample collection and processing. Soil sampling can be conducted in conjunction with sampling to assess nutrient levels in the soil. However, if routine soil nutrient sampling is not conducted annually, the effectiveness of soil-based pathogen detection would be limited for pathogens with multiple host species or transmission pathways. Therefore, the decision to test field soil should be based on positive results obtained from other tests conducted on other transmission pathways, such as seed health tests. Testing soil from fields where infected seed lots were produced could support the earlier detection of residual inoculum and enable the timely implementation of preventive, less intensive IPM strategies.

Rapid screening of treatments to support low-risk plant protection product development and accelerate breeding

Treatment efficacy against these *Ustilago nuda*, *Tilletia caries*, and *Tilletia controversa* can only be assessed late in the crop growing season due to the biology of these pathogens. My work showed that constraints in time, space, and resources can be overcome with infection assessments in early states of plant growth (Chapter 3 and Chapter 4).

The use of molecular methods to screen treatment efficacy in the early-stage plants is a practical solution for limited space. Because molecular detection methods enables infection assessment in the first days of seedling development, they allow increased sowing density and more efficient use of climate chamber space without compromising plant growth or infection development. Higher sowing densities in *U. nuda* can also favor the disease development [200], while evaluating a greater number of plants within constrained spaces. Furthermore, *U. nuda* infections in early plant stages are unaffected by light, and seedlings can grow in darkness. Growing seedlings without light allows for additional space optimization because the growth containers can then be stacked, and the qPCR-measured infection levels were the same regardless of whether the seedlings were grown in potting soil or on filter paper (Chapter 3). A similar approach could be used to evaluate contact seed treatments for *T. caries*, whose infection can develop during the initial days following seedling germination in the absence of light.

A molecular detection method used for screening should be viewed as a "pre-screening" tool for selecting promising treatments or resistant cultivars, rather than as a replacement for fully validating treatment efficacy. However, applying these laboratory screening methods to assess cultivar resistance may yield misleading results. Resistance to bunt and smut diseases could potentially be expressed at different plant developmental stages, so analyses performed during the early stages of growth may produce inconsistent or incomplete evaluations of resistance. Furthermore, since *U. nuda* develops systemically mostly in the seedling-apex, it is difficult to sample the plant material to monitor infection dynamics while maintaining plant viability. In contrast, the method developed for *T. caries* in Chapter 4 could provide valuable insight into

the progression of infection and the underlying resistance mechanisms.

Although the molecular detection method rapidly identify promising control strategies against pathogens and eliminate ineffective treatments in early plant stages, it cannot capture the full influence of environmental factors on disease expression or treatment performance. Nevertheless, molecular screening narrows the number of candidates advanced to field trials.

Future perspective

My PhD dissertation provides a framework for integrating molecular detection methods into the management of seed- and soilborne smut and bunt diseases. However, future work, including further validation across different laboratories or testing biological meaning in different environments, can address the additional steps towards the practical implementation of the work. Specifically, the *Ustilago nuda* seed detection protocol (described in Chapter 1) should be validated across diagnostic laboratories. Inter-laboratory ring tests would evaluate reproducibility and transferability, both of which are essential prerequisites for routine application [201, 202]. In parallel, the introduction of this molecular protocol on a limited scale, alongside current field-based seed certification methods would help identify potential limitations, such as variability in qPCR measurements among cultivars or *U. nuda* strains. Beyond seed health certification, this seed-based method can verify the success of artificial seed inoculations prior to resistance screening and to assess infection levels in naturally infected seed lots before treatment efficacy trials. In this context, collaborations with breeding companies and plant protection product manufacturers would confirm the usefulness of this test. Such collaboration would also be useful to validate the laboratory-based screening methods for both *U. nuda* and *T. caries* (developed in Chapter 3 and Chapter 4, respectively) for treatment efficacy. Furthermore, within these contexts, more testing with these methods could provide insight into the mechanisms and timing of resistance expression across different cultivars.

The transition from controlled-laboratory settings to field conditions represents an important step to fully evaluate the potential of this molecular detection method, especially for evaluating treatment efficacy or cultivar resistance against *T. controversa*, which is not possible in controlled environments. For both *T. caries* and *T. controversa*, targeted soil sampling strategies to investigate the possible presence of soilborne inoculum should be assessed prior to conducting treatment efficacy assessments in the field; it could contribute to the infection development and interfere with the interpretation of the results. The detection and assessment of soilborne inoculum is especially relevant to evaluate organic or low-risk treatments that may control both seed- and soil-borne infection sources.

Future work on *T. caries* and *T. controversa* soil and seed inoculum should focus on improving the analytical performance and applicability of the detection method developed in Chapter 2. The reliability of the exogenous control and pathogen DNA recovery should be evaluated across different soil types because soil structure can influence DNA extraction efficiency and detection outcomes. Both exogenous and pathogen DNA may bind to soil particles, such as clay, which makes soil composition an important factor to consider. The test should focus on extracting *T. caries* and *T. controversa* from the soil types commonly used for wheat production by mixing defined proportions of loam, clay, and sand that reflect field-relevant soil

structures. Each soil type or reproduced soil structure should be tested in multiple replicates inoculated with the same amounts of teliospores and spiked exogenous control.

Testing a larger number of seed lots that have a broader range of infection levels would define detection thresholds and improve confidence in quantitative results. The current seed health test relies on filtering a seed washing solution and conducting inspections of filters under the microscopic to detect *T. caries* and *T. controversa* teliospores [47]. These seed health tests should be compared more directly with a PCR-based seed testing method, including qPCR and dPCR. *T. caries* and *T. controversa* DNA extracted from filter samples can be measured using a PCR-based seed method and then results from the seed health testing using filtering can be compared directly with and PCR-measured results. This direct comparison would help link results between traditional microscopic observations and molecular quantification.

On a broader scale, sharing the molecular detection protocols developed in this dissertation for *U. nuda*, *T. caries*, and *T. controversa* with the international smut and bunt research community would facilitate method validation across different field environments, cultivars, and bunt and smut populations. This coordinated research would promote method harmonization, data exchange, and strengthen collaborative research networks. In conclusion, the development of shared guidelines for molecular detection methods, in collaboration with researchers, diagnostic laboratories, and regulatory bodies, will ensure that detection results are biologically meaningful and linked to clearly defined tolerance thresholds. The coordinated validation and standardization of molecular screening tools will facilitate their integration into IPM strategies, promoting informed decision-making and reducing reliance on synthetic plant protection products in more sustainable agricultural systems.

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Curriculum Vitae

Education

01.02.2022 - ongoing **PhD in Biology**
Agroscope Reckenholz, Zurich, Switzerland (Research Group Molecular Ecology) and University of Neuchâtel, Neuchâtel, Switzerland (Laboratory of Evolutionary genomics of pathogens).

09.2018 - 03.2021 **Master's Degree in Sciences of Plant Production and Protection**
(110 Cum Laude/110) University of Milan. Milan, Italy.

09.2015 - 07.2018 **Bachelor's degree in Valorization and protection of the environment and mountain territory**
(110 Cum Laude/110) University of Milan. Edolo, Italy.

Work experience

01.09.2021 - 31.01.2022 **Scientific project assistant**
Agroscope Reckenholz (Ecological Plant Protection in Arable Crops, Research Division Plant Protection). Zurich, Switzerland.
Assisted in conducting field and laboratory trials for the detection and control of seed-borne diseases, such as *Ustilago nuda*, *Microdochium nivale*, *Microdochium majus*, and *Tilletia caries*.

01.05.2021 – 31.08.2021 **Research internship**
Agroscope Reckenholz (Ecological Plant Protection in Arable Crops, Research Division Plant Protection). Zurich, Switzerland.
Conducted seed health testing and preliminary analyses on the detection of *Ustilago nuda*, *Tilletia caries*, *Microdochium nivale*, *Microdochium majus*, and *Fusarium* spp.

01.07.2020 - 28.02.2021 **Research internship**
Agroscope Reckenholz (Ecological Plant Protection in Arable Crops, Research Division Plant Protection). Zurich, Switzerland.
Tested alternative treatments to control seed-borne diseases (*Ustilago nuda*, *Microdochium nivale*, *Tilletia caries*) using germination tests, pot and field trials.

Language skills

Italian	Native speaker
English	C1
Spanish	B2/C1
French	B2
German	A2

Publications

Panzetti C., Jenny E., Croll D., and Sullam K.E. Optimization of a lab-screening procedure to evaluate seed treatment efficacy: detection of *Ustilago nuda* in *Hordeum vulgare* seedlings. Submitted.

Panzetti C., Jenny E., Bänziger I., Kägi A., Vogelgsang S., Hebeisen T., Büttner P., Croll D., Widmer F. and Sullam K.E. A new molecular seed assay to predict *Ustilago nuda* field infection levels. Scientific Reports 15, 34755 (2025). https://doi.org/10.1038/s41598-025-18544-3

Conferences and seminars

10.12.2025 **COST TopAgri webinars**
Online. Contributed talk: "Rapid detection of bunt — development of a soil and seed health test for wheat".
Presentation of the project selected for the Short-term scientific missions (STSM) of the COST action T0PAGRI (Towards zer0 Pesticide AGRIculture: European Network for sustainability).

15.09.2025 - 17.09.2025 **XXX Congress of the Italian Phytopathological Society (SIPaV)**
Catania, Italy. Contributed talk: "A new molecular seed detection method to quantify *Ustilago nuda* and reduce prophylactic seed treatments". Awarded "Young Researchers in Training".

27.06.2025 **Swiss Mycology Symposium**
Birmensdorf, Switzerland. Contributed talk: "A new molecular detection method applied to barley seeds for quantifying *Ustilago nuda* and predicting their field infection levels".

03.06.2025 - 05.06.2025 **14th Conference of the European Foundation for Plant Pathology**
Uppsala, Sweden. Poster presentation: "Rethinking plant disease detection: comparison of soil- and seedborne *Tilletia* transmission pathways".

01.06.2025 - 02.06.2025 **XXIII International Bunt and Smut Workshop**
Uppsala, Sweden. Contributed talk: “A new molecular assay on barley seed to quantify *Ustilago nuda* and predict field infection levels”.

07.07.2024 - 10.07.2024 **4th International Conference on Wild Plant Pathosystems**
Kiel, Germany. Participant. Received a student fee waiver.

20.05.2024 **Special seminar**
Edolo, Italy. Invited talk “The role of plant pathology in the challenges of mountain agriculture [in Italian]”.

21.03.2024 - 22.03.2024 **Combined annual conference of the Swiss Society for Phytomedicine, the Swiss Agronomy Society and the Swiss Soil Science Society**
Zollikofen, Switzerland. Presented poster: "Rethinking plant disease prevention: lessons from a *Tilletia caries* soil infection study”.
Awarded 2nd best poster.

13.10.2022 **3rd Agroscope PhD/Post-doc symposium 2022**
Changins, Switzerland. Presented poster: "The HealthyStart project: the detection of cereal seed- and soil-borne diseases”. Awarded Best poster.

21.09.2022 - 23.09.2022 **XXVII Congress of the Italian Phytopathological Society (SIPaV)**
Palermo, Italy. Presented poster: "Steps to improve molecular detection of barley loose smut (*Ustilago nuda*) in seedlings”.

15.09.2022 - 16.09.2022 **Annual PhD Students’ Meeting 2022**
Neuchâtel, Switzerland. Presented poster: "HealthyStart: Detection of cereal seed- and soil-borne diseases” .Awarded 3rd best poster.

10.06.2022 **Zürich Mycology Symposium**
Neuchâtel, Switzerland. Presented poster: "Optimization of *Ustilago nuda* detection in barley seedlings”.

Research experience

06.06.2025 - 25.06.2025 **Short Term Scientific Mission (STSM)**
Swedish University of Agricultural Sciences (SLU), Uppsala and Alnarp, Sweden (Supervisors: Anna Berlin and Therese Bengtsson)
Selected for the project: "Rapid detection of bunt — development of a soil and seed health test for wheat".
Part of the Cost TOPAGRI project "Towards zer0 Pesticide AGRIculture: European Network for Sustainability".

Competences

Molecular techniques
DNA-based technology (e.g., DNA extraction methods, qPCR, dPCR, PCR), gel electrophoresis and imaging, primer development and optimization.

Phytopathogen visual detection
Ustilago nuda, *Tilletia* species, *Microdochium majus*, *Microdochium nivale*, *Fusarium* species, and *Claviceps purpurea*.

Additional laboratory methods
Culturing of fungi and bacteria, isolation of fungi, cereal germination tests (According to the International Seed Testing Association procedure).

Experimental skills
Field and climate chamber trial planning and organization.

Computer Software/tools
Proficiency in R statistical software, LaTeX typesetting system, CFX Maestro 2.2 Software for the collection and analysis of real-time PCR data;
Familiarity with AutoCAD computer-aided design software;
Base knowledge of GIS, HTML, and C++.

Specialized courses

11.02.2025 - 22.04.2025 **Developing & Validating Seed Health Assays**
American Phytopathological Society (APS). Online.

19.09.2023 - 05.12.2023 **Seed Pathology Fundamentals: Regional to Global Implications** American Phytopathological Society (APS). Online.

Additional information

03/2022 - ongoing **Member of the Italian Society of Plant Pathology (SiPaV)**

2023 - ongoing **Collector of plant pathogens samples**
As a hobby, I contribute to the ETH Fungarium collection, especially with *Claviceps* spp. samples.

01.05.2021 - 27.09.2022 **Scientific Illustrator for the Italian cultural association "Microbiologia Italia"**
As a volunteer, I collaborated with the cultural association "Microbiologia Italia" and contributed illustrations, until it ended due to its dissolution.

25.07.2021 - 25.07.2021 **Passed the Italian State Exam for Agronomist and Forester - Section A**
Not currently registered in the Italian professional registry.

A PhD teaches you to grow and to learn how to make time when it seems impossible.

This time is carved out of lost hours of sleep, vacations spent in front of a computer screen, and, most importantly, time taken away from loved ones. I thank Simone Barreca, my partner, for "agreeing" to share me with my PhD and for supporting me, even during those moments when I was unbearable. I apologize to my family and friends for missing many important moments, big and small, and for not always being physically present — although, I was always there for you at the first message or call, whenever you needed me.

However, I loved my doctoral project, and I still do. It is a love driven by curiosity, discovery, and research. A passion passed on to me by my supervisor Karen Sullam, who guided me on this journey with great patience, despite the many unexpected events along our way. I am also grateful to Daniel Croll was always able to clearly point out what was missing and to encourage me positively with just a few words. Both of my supervisors believed in me more than I did.

During my PhD, I attended several conferences — sometimes funding them by myself — because networking, staying up to date, and keeping an open mind are fundamental to research. During these events and throughout my PhD I crossed paths with many researchers, future researchers, professionals in the field, and "outsiders". I formed strong friendships with some of them and almost brotherly or family-like bonds with others. I would like to thank them all.

I would be lying if I said I'm happy to finish my PhD: I will miss it.

My PhD contract ends on January 31st, 2026 — my 30th birthday — and marks the beginning of a more mature adulthood.