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Novel methods for quarantine detection of karnal bunt (tilletia indica) of wheat

Prem Lal Kashyap[1], Satvinder Kaur[1], Gulzar S. Sanghera[2], Santhokh S. Kang[1] and PPS Pannu[1]

[1] Molecular Diagnostic Laboratory, Department of Plant Pathology, Punjab Agricultural University, Ludhiana- 141004

[2]SKUAST (K) - Rice Research and Regional Station, Khudwani, Anantnag, 192102, Jammu and Kashmir, India

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ABSTRACT

Prior knowledge about the presence of a plant pathogen in an infected plant material and natural reservoir is the first requirement for a successful disease management strategy. This becomes more crucial in case of quarantine pathogen like *T. indica* in order to alleviate unnecessary restrictions that prevent the movement of wheat across the globe and tells how this pathogen hinders the wheat trade of India. More over the potential risk of its dissemination in international wheat trade and germplasm exchange, there is a need for quick, sensitive, reliable and alarming method to identify *T. indica* to facilitate implementation of specific disease control strategies and for accurately selecting areas for quarantine. The detection of Karnal bunt (KB) is based primarily on the presence of teliospores on wheat seeds. However, accurate and reliable identification of *T. indica* teliospores by spore morphology alone is not always possible. Research based on genomic advances and innovative detection methods as well as better knowledge of the *T. indica* life cycle will facilitate their early and accurate detection, thus improving the sanitary status of cultivated plants in the near future. A new, novel, highly accurate molecular tests are emerging which help in surveillance of KB. This brief review will present the overview of classical and emerging *T. indica* detection and diagnosis assays and a repertoire of molecular diagnostic tools that can serve as a foundation stone for identifying and detecting *T. indica* inoculum load on multiple, rapid-cycling, real-time, PCR platforms both *in vitro* and *in vivo* conditions. This also provide an efficient way for disease surveillance and disease forecasting

Introduction

The fungal pathogen Tilletia indica incites diseases of Karnal bunt (KB) in wheat all over northwest plains of India (Mitra 1935) imparting the quality of grain and the products. The disease has come under concern because of the quarantine restrictions imposed by the importing nations (Sansford *et al*. 2008). Because of the risk of spread, wheat trade is only accepted after declaring the wheat lots, which are having zero tolerance limits against KB (Babadoost 2000). To overcome the problems in exports, a sensitive, rapid and novel method for detection and diagnosis of KB pathogen is required.

These types of protocols enables one to make decisions regarding cultivar choice and chemical control that can be used most effectively to prevent development of a potential plant disease epidemic (Ward *et al.* 2004). Diagnosis of the causal agent is also important for studies on epidemiology, yield loss relationships and designing new strategies for disease management (Lapoz *et al*. 2008).

Disease symptoms often aid with making decisions, but a definitive diagnosis requires unambiguous pathogen identification. More over population of T. indica is more difficult to identify from other Tilletia species using morphological criteria, which can be time consuming and challenging and requires extensive knowledge in taxonomy which varies from person to person. In addition, these traditional methods of appropriate disease control may be taken to prevent plant injury especially when high value cereal crops like wheat is at stake. The situation become more complicated after itsintroduction into a new location, where it would be almost impossible to eradicate the fungus since teliospores can remain viable in the soil for five years (Agarwal *et al.* 1993). High humidity and low temperature factors are necessary for infection to occur (Kaur *et al*. 2005; Workneh *et al.* 2008), and making situation more aggravating where decision regarding disease prediction become difficult, and create hurdles in taking decision regarding the occurrence of KB, and how severe it will become in wheat crop. As a consequence fungicides are often applied inappropriately, at the wrong time, or when they are not required. Moreover, conventional diagnosis and identification protocols are relatively slow, often requiring skilled taxonomists to reliably identify the pathogens at the genus or species level.

As diagnostic technology is moving from qualitative to quantitative phase and there is no doubt that most tests will be quantitative in the future. Methods that are based on the use of monoclonal antibodies or the polymerase chain reaction (PCR) are the best example, which have the potential to replace traditional technologies.

Detection and identification of pathogens that are seed, soil and air -borne as the case with *T.* indica, presents a number of difficulties such as sampling and reaction inhibition that are beginning to be overcome. New approaches to antibody production such as phage display, the combination of immunoassays with PCR and the development of species-specific PCR primers will allow versatile and wide-scale use of these new techniques for the detection of *T.* indica inoculum.This article provides some examples of the recent emerging trends in the detection and diagnosis of KB inoculum in seed, soil and *in* planta and mainly focuses on the advances made in this field towards the development of speedy and portable protocol for pathogen detection and diagnosis.

Tele:

E-mail addresses: g_singh72@rediffmail.com

Life cycle of KB: A clue for detection and diagnosis

The life cycle of any pathogen acts as a blueprint for understanding how the pathogen functions and how it adapts to changes in diverse environments. The continued developments in the basic understanding of the biology of *T.* indica indicated that the disease cycle of KB starts when infected seed is harvested. The mechanical action of harvesting causes infected grains to rupture, liberating the resting spores (teliospores) of the fungus from the infected grains. The teliospores fall on the ground, together with infected or contaminated seed shed during harvest. Teliospores can survive in the soil for at least five years or in/on stored seed for longer periods (Agarwal *et al.* 1993; Bonde *et al.* 2004). Teliospores in contaminated or infected feed (grain, or bran from milled grain) can survive ingestion by animals, so the faeces of stock fed on such feed is also a source of inoculum for infecting susceptible crops (Smilanick *et al.* 1986). When there is a cool and moist condition, teliospores on or near the soil surface germinate to produce numerous spores called primary sporidia /or basidiospores (Nagarajan *et al.* 1997). These primary sporidia give rise to secondary sporidia by budding, which continuously multiply on leaf surfaces (Dhaliwal 1989) and infect between the boot leaf and soft dough stage, approximately a fortnight, depending on the cultivar and the weather conditions (Singh and Krishna 1982) and later on penetrate the wheat floret through stomata (Goates 1988). As the pathogen infects the immature wheat seed, hyphae infect the pericarp and form a compact layer of mycelium that gives rise to teliospores (Cashion and Luttrell 1988). Growth of the mycelium inside the pericarp eventually ruptures the connection between the pericarp and surrounding vascular bundles, and as a result, the seed atrophies to varying degrees and leading to partial bunt (Rush *et al.* 2005). When these teliospores are liberated at harvest to the soil surface, or dispersed on or in the grain, the cycle begins again.

The emerging overview regarding the basic understanding of this pathogen, life cycle has created a rapidly rising demand for new novel detection and diagnosis technologies, which are based on the detection of T. indica at three different phase viz. mycelial, teliospore and sporidial phase. As teliospores survive in seed and soil and playing a starting role in KB occurrence and later on repeated cycles of sporidia production in the ears provide more inoculum, it becomes essential to detect and quantify T. indica teliospores and sporidial population. This is only possible with the development of a quick and sensitive protocol for pre-harvest quantification of T. indica population both in soil and in planta. Moreover, such protocols will also make us aware about the extent to which the pathogen could survive, infect and perpetuate within the host system. In vision of these ideas, we have compiled the latest trend of the ongoing research mainly focusing on the emerging developments in the field of detection and diagnosis of KB in seed, soil and in planta.

Detection and Diagnosis of KB infected soil samples

Prevention is essential to avoid the dissemination of the pathogens through different vehicles, such as contaminated propagative plant material, vectors, irrigation water, soil, etc. (De Boer et al., 2007). As the soil is the birth place for every plant, so it becomes much desirable to examine the soil for prevalence of any potential pathogen even before the crop is sown because after its introduction, it become almost impossible to eradicate the fungus since spores can remain viable in the soil for a considerable time (Agarwal et al. 1993). If the pathogen is T. indica and present in areas where favourable climatic conditions exist, then obviously cause significant damage and economic losses in wheat crop. So accurate identification and early detection of pathogens in the soil become a crucial step to control the pathogen population which result in economical damage.

The method currently used by most researchers to extract and quantify T. indica teliospores in soil (Babadoost and Mathre 1998) is very tedious and prone to error because of the microscopic analysis of each sample and the loss of teliospores during the extraction process (Bonde et al. 2004). Preliminary studies done by Stein and coworkers (2004) indicated that the Real time PCR protocol is a very much sensitive and time saving technique to quantify teliospores from T. indica in very small soil sample of 25gm and can quantify upto 100 teliospores in artificially inoculated soil samples. There is also a possibility that these types of studies will be helpful to monitor and quantify large number of teliospores (>1000) into soil samples in order to study teliospore survival (Babadoost et al. 2004).

Detection and Diagnosis of seed borne inoculum of KB

T. indica is a seed borne pathogen, therefore cultivating wheat crop from pathogen free seed is an effective means to prevent KB dissemination. The teliospores contaminating the surface of healthy seeds are not readily detected and can be carried to disease free areas and establish a primary source of inoculum (Chahal and Mathur 1992). Development of effective diagnostic protocols to identify Tilletia indica is a critical step in managing the KB threats to wheat crop. The progress and advances made in the development of protocols for detection and diagnosis of seed borne inoculum of T. indica are described in following sub-headings.

Washing technique

The washing technique is useful for rapid detection of surface-borne teliospores of T. indica (Begum and Mathur 1989). The spores are washed from seeds in water containing one or two drops of detergent. The suspension is then centrifuged, the supernatant discarded and the pellet resuspended in 5ml of Shear's solution (Mathur and Cunfer 1993). This suspension is then examined under a compound microscope for the presence of fungal teliospores (Begum and Mathur 1989). For a quantitative estimation, the spore load per seed is estimated with the help of haemocytometer.

NaOH Seed Soak Technique

Detection of KB infected seeds having a low level of infection or seeds covered with dust can be difficult using washing test. With such samples, bunted seeds can be detected using the NaOH soak technique (Agarwal and Verma 1983). The seeds are soaked in 2% NaOH solution for 20h at 20^{o}C. After this, the solution is decanted and the seeds thoroughly washed in tap water. Discoloration of infected seeds becomes clearly evident. The infected portion of the seeds appears jet black and shiny, in contrast to the pale yellow healthy seed. When black seeds are ruptured on a drop of water streams, teliospores are released. This method can be used even when seed samples have been treated with colored fungicides (the NaOH treatment removes the dye, clears the seed surface and increases the contrast between diseased and healthy seeds) (Agarwal and Mathur 1992).

Filter and Centrifuge Extraction technique

In this method, a sample of seeds is washed in a water containing 0.001% Tween 20 (Castro et al 1994). The wash water is filtered through a 60 µm pore size screen and then through 12µm pore size screen membrane. The first wash separates out debris and the second teliospores. The spores are then washed from the membrane and pelleted by centrifugation. The spore pellet is collected and suspended in water for examination under the light microscope for identification of the

spores. As few as five spores per 100 seeds can be detected using this method (Castro et al 1994). These assays took 60 minutes per sample.

Dyed latex beads Techniques

This technique is used to check the presence of certain antibodies with in a host system. The infected disease sample is analyzed in the lab by mixing it with latex beads coated with a specific antibody or antigen. If the suspected substance is present, the latex beads will clump together and indicate the presence of the pathogen. This protocol was developed by Khesari and collegaues (2005) for the detection of solubilized teliosporic antigens over intact teliospores of Karnal bunt. They prepare extempore by mixing blue color dyed latex beads (1% suspension) with equal volume of diluted anti-teliospore serum. This test was considered to be better for the teliosporic antigens solubilized using sonication and detergent extraction. For determining the sensitivity of test, antigen concentration kinetics analysis was performed by adding 15 µl of antibodies sensitized latex beads to 15 µl of different concentrations of solubilized antigens on glass slide. The detection limit of this test was 7.5 µg solubilized teliosporic antigens equivalent to 750 teliospores and suitable for single seed analysis.

Dipstick Immunoassay

When teliospores suspected of being T. indica are found in wheat samples, often they will not germinate (Bonde et al 1999). This is most common with young, dormant teliospores of T. indica found in newly harvested grain and can make confirmation by molecular methods difficult, or impossible, when only small numbers are present (Bonde et al. 1997). Dipstick tests are very inexpensive, rapid, and portable, and do not require technical skill to perform

PCR based detection technique

In a conventional PCR, only the final concentration of the amplicons may be monitored using a DNA binding fluorescent dye, but for quantification of a pathogen, we have to stop PCR reaction at various points to generate standard curves, which results in a laborious, low throughout process. So, real-time monitoring with specific instruments and fluorescent probes amplification, detection and quantification in a single step is the best arsenal to detect plant pathogens. Due to the advancement of fluorogenic chemistry, a second generation PCR known as real time PCR has become an emerging technique for the detection and quantification of plant pathogens (Mumford et al. 2006). This tool provides the highest levels of sensitivity on the diagnostic scene, opening up new detection possibilities and is becoming the new gold standard for the molecular detection of plant pathogens. Using Real Time PCR, it is possible not only to detect the presence or absence of the target pathogen, but also possible to quantify the amount present in the sample allowing the quantitative assessment of the number of the pathogen in the sample ( ). A quenched FRET probe is a rapid, highly sensitive, species-specific fluorescent molecular tool. A two-step PCR protocol using FRET probes was developed by Tan and Murray (2006) for the direct detection and identification of T. indica from less than 10 spores.

Detection and Diagnosis of in planta T. indica inoculum

Seed or soil-borne teliospores and their subsequent germination are believed to play only a starting role in Karnal bunt epidemics (Dhaliwal, 1989) but repeated cycles of sporidial production in the ears provide sufficient inoculum of T. indica (Bains and Dhaliwal 1989) to cause KB epidemics. As the pathogen infects the immature wheat seed, hyphae infect the pericarp (Cashion and Luttrell 1988) and growth of the mycelium inside the pericarp eventually ruptures the connection between the pericarp and surrounding vascular bundles, and leads to the seed atrophication to varying degrees. So, there is a need for the development of T. indica specific primers, which can help in monitoring the presence of pathogen mycelium and detect teliospores inside the host system. The wave of current research in the Molecular Diagnostic Laboratory, Department of Plant Pathology, Punjab Agricultural University is moving towards the development of novel molecular protocol for detecting and quantifying secondary sporidia prevailing in nature on the leaves surfaces of wheat. The potential of these methods for detecting sproridia has been recognized, but little progress in their use for this purpose has been made to date. In this study, we will try to establish a technique with real-time PCR conjugated with the Tilletia indica -specific primer and fluorescent SYBR® green I dye to quantify the pathogen population associated with wheat crop.

KB Detection and Diagnosis: Moving out of the lab to the field

In spite of the great advances in sensitivity and specificity of the available techniques and protocols, there is an evident lack of information on the hidden life of pathogen in the soil. To resolve this hidden mystery, researcher from Allied Signal Federal Manufacturing and Technologies in collaboration with the Kansas Department of Agriculture had succeeded in the development of an automatic KB detection system (Linder et al. 1998).

This system utilizes pattern recognition, feature extraction, and neural networks to prototype an automated detection system for identifying KB teliospores, and later own the spore like images reviewed by trained technicians. This protocol has some advantages over previous methods as this system help in the reduction of the overall cycle-time; making intelligent decision making by utilizing technicians in comparison of manual searching of KB infected teliospores; helpful in framing a quantifiable and repeatable regulatory standard and guarantees 100% coverage of the cover slip; and ultimately result into the significant enhancement of detection accuracy.

Conclusion and future prospects

The goal in the crop health research field is to have “point of care” hand-held devices for detection and diagnosis of plant pathogens. If robust assays with these characteristics become available at an affordable price in plant pathology, we believe that molecular devices for pathogen detection can also become a routine tool for almost everyone involved in field research with a plant pathology component or in making disease management decisions. Adding innovative molecular tools for differentiating viable from non-viable organisms should be given emphasis in developing diagnostic assays for T. indica.

Continued progress in automated nucleic acid detection technologies will make real-time testing a reality under any environmental and permit more rapid and accurate monitoring of Tilletia indica inoculum load under field conditions. Portable, DNA-based devices are being designed that will identify naturally occurring or deliberately released disease organisms in under an hour instead of taking days or weeks, as recent methods require.

Their adoption would allow extension agents or consultants to make on-site, real-time recommendations on how best to curtail a crop disease outbreak before it spreads. The devices would also be useful in checking imported material for hidden plant disease organisms that could imperil domestic crops.

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