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Application of molecular and immuno-diagnostic tools for detection, surveillance and quarantine regulation of Karnal bunt (*Tilletia indica*) of wheat

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Disease diagnostics and pathogen detection, a primary component of any crop management programme, is also helpful in monitoring sanitary and phyto-sanitary measures to seed quality. Molecular diagnostics based on immunological and DNA techniques can provide an efficient way for disease surveillance and disease forecasting. Karnal bunt (KB) of wheat has become a major disease of economic importance and an important issue in the international wheat trade. Due to its seed borne, soil borne and air borne nature, KB can be diagnosed in three pathogenic forms of pathogens viz. teliospores, sporidial and mycelial forms which occur in infectious or proliferative entities at different stages of the disease cycle. Suitable sampling and extraction procedures can improve the detection limits of modern molecular diagnostic methods used to test KB. For the extraction of teliospores from both infected and infested seeds, highly efficacious detergent washing techniques have been developed and extracted pathogenic entities were diagnosed by involving several species of specific primers and immuno-chemical reagents. Nucleic acid and antibody based techniques have been developed for the detection of KB pathogen in different forms. These techniques can also be successfully employed for differential diagnosis, disease surveillance of seed borne pathogens of quarantine importance and determination of teliospore load in wheat seeds. These tests can also be designed to assist in the trade of wheat, development of disease forecasting models and molecular and immuno-pathotyping of *Tilletia indica* isolates collected from different agro-climatic regions. Moreover, these formats could also be employed for molecular characterization of disease determinants and in comprehending their role in molecular basis of host-pathogen interaction. The present paper highlights the use of the principle of molecular diagnostics in exerting disease surveillance and quality assurances of seeds.

Keywords: Karnal bunt; molecular diagnostics; immunoassays; seed certification; plant quarantine; teliospore load

Introduction

Production of wheat is severely hampered by seed borne diseases like bunts and smuts, which result in reduced crop yield and poor grain quality. Amongst these diseases, Karnal bunt is the major disease of wheat (*Triticum aestivum* L., *T. durum* and triticale). It is caused by the sporadic fungus, *Tilletia indica* (Syn *Neovossia indica*), which occurs frequently in the northwestern plains and Tarai regions of Himachal Pradesh, Punjab,

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Uttar Pradesh and Uttarakhand states of India. Karnal bunt (KB), also called partial bunt, was first detected at Karnal (Haryana) by Mitra (1931), and hence the name. Since then, the disease has spread to northwestern states of India, parts of Bihar, West Bengal and Gujarat (Gill, Sharma, & Aujla, 1983; Singh, 1994). The disease has been reported to occur in other parts of the world such as Pakistan, Nepal, Iraq, Iran, Afghanistan, Mexico (Begum & Mathur, 1989; Gill et al., 1983; Joshi, Singh, Srivastava, & Wilcoxon, 1983; Matsumoto & Bell, 1989; Singh, 1994; Warham, 1986) and in 1996, it was reported to occur in several limited areas of the southwestern US (Bonde, Tooley, & Schaas, 1997; Ykema, Floyd, Palm, & Peterson, 1996) and now KB pathogen is considered as a major threat to the annual six billion dollar wheat industry of the USA. Moreover, this disease has the potential of causing great devastating effects. The movement of wheat seeds from one country to another is strictly regulated through quarantine measures to restrict the entry of KB infected wheat grains. Therefore, KB acts as a genuine non-tariff trade barrier in commercial seed trade in the international markets and thus threatens wheat trade because export of KB infected wheat grains would disseminate seed borne pathogens within and between regions, countries and continents. Since, 1996 when the USA strengthened its quarantine regulations against KB some countries like Mexico imposed domestic quarantine regulations and also legal restrictions on planting wheat in areas with high incidence of KB. Nevertheless, an effective disease management strategy necessitates stepping up the disease surveillance programme through the use of modern tools of molecular and immuno-diagnostics.

A disease of economic importance

Karnal bunt affects mainly common wheat, durum wheat, triticale and other related species (Royer, Rytter, & Matsumoto, 1986; Warham, Mujeeb-Kazi, & Rosas, 1986). The loss due to KB is difficult to estimate because the disease reduces seed quality, inflicts changes in the chemical composition of infected grains, and renders seed useless for consumption. Nevertheless, in India, loss in yield due to the disease has been calculated as $1/3 \times \text{yield} \times \text{per cent infection}$ as the disease covers one third of an area under wheat cultivation in India (Munjal, 1975). The financial losses caused by the disease are substantial, ranging from 5–20%.

Since the disease is seed borne, many countries have a zero tolerance, indirect cost is incurred in prevention of its spread and results in reduction of marketing opportunities. This is, in addition to the loss incurred due to non-certification of the infected seeds, costs on seed treatment, inability to grow high-yielding varieties susceptible to KB and the costs of quarantine, inspection and export losses.

Loss in grain weight can range from 2% when infection occurs at the embryo end with less than 10% of the endosperm destroyed, to 43% where the entire endosperm is destroyed. There is a progressive decline in fertility with increasing grades of KB infection (Mitra, 1935; Munjal & Chatrath, 1976). Severely infected grains show considerable reduction in the viability of seeds, which produce weak seedlings (Rai & Singh, 1978). Depending on the level of infection, the quality of wheat grains can be affected. Wheat grains with less than 3% infection remain unaffected for the quality of appearance and palatability of bread, cookies, and chapattis, while wheat grains above 5% infection are not acceptable for human consumption (Mehdi, Joshi, & Abral, 1973). Karnal bunt contains no known mycotoxins or ergot alkaloids having known toxicological effects (Bhat, Rao, Roy, Vijayaraghavan, & Tulpule, 1983; Rai, Singh, & Rai, 1991).

Disease symptoms

Symptoms of the disease become evident only when the grain develops fully. The disease is first noticed when broken and/or partially smutted kernels are seen in threshed grain, although the first symptoms appear at the soft-dough stage in the form of blackened areas surrounding the base of the grain and extending upward to varying degrees. However, Duran and Cromarty (1977) could detect grain infection in the ear head by its swollen appearance and slightly wider opening of glumes. Unless the disease is severe, only a few florets per spike are affected, and diseased spikes are not conspicuous because infected kernels do not noticeably distort the glumes. The infected grains are partially or completely converted into bunt sori. The sori are oblong to ovoid, 1–3 mm long, brown to black and contain black powdery spore mass enclosing the pericarp which emit a fishy odour (due to the presence of trimethylamine) similar to common bunt (Mitra, 1931; Mundkar, 1940, 1943). When the grains are partially attacked, infection is confined to the groove side destroying embryonic tissues. In an advanced attack, tissues along the groove and the adjacent endosperm are replaced by spores (Mundkar, 1940) but the embryonic tissues are not entirely destroyed (Mitra, 1931). With the development of the fungus in the infected grain, the pericarp ruptures and the black spore mass is released into the air (Mitra, 1935). Partial conversion of the grains into spores is a rule and complete conversion an exception.

Infection cycle for disease development

Sources of inoculums are diseased seed and soil contaminated with spores from the previous crop. The delicate pericarp surrounding each sorus of teliospores is easily broken during harvest, dispersing the spores to contaminate the soil. There is no data available to determine the relationship between the teliospore load in wheat lots and the subsequent appearance of KB disease in the field, though environment plays a significant role in its development. The teliospores germinate in response to free moisture and produce numerous sporidia at the soil surface, which are forcibly ejected and dispersed by the wind, running water and insects.

Sporidia have a short life span even at a high relative humidity. Generally they survive for short periods when airborne. Long distance dispersal of secondary sporidia can probably occur, but only during moist periods and under cloud cover at night. Plants are most susceptible to infection when spikes emerge from the boot, though infection can occur throughout the flowering period.

Sporidia infect the ovaries, directly penetrating the glumes and ovary wall. Infection can move across all florets of a spikelet and to the spikelets above and below the initial infection site. Sporulation starts at the embryo end and moves along the suture of the grain. Teliospores develop from enlarged thickened and pigmented sporogenous end cells of the hyphae in a hymenium-like layer over all the surfaces of the pericarp tissue surrounding the cavity caused by the disintegration of the middle layers of parenchymatous cells of the pericarp. The extent of teliospore production depends on the length of favourable temperature and the amount of moisture after the initial infection. Promoted by cool, humid or wet weather, diseased kernels may be partially or completely displaced by masses of teliospores. Fresh teliospores require a dormancy period of 6 months (though a low level of germination (6.7%) of teliospores from freshly harvested kernels has been observed after 1 week of storage (McRae, 1933)). Teliospores remain viable up to 45 months in the soil (Zhang, Lange, & Mathur, 1984). Teliospores survive longer when

buried in dry soil than when buried in moist, cropped soils or when present on the soil surface.

Factors governing disease development

The spread of the disease and successful seed infection in an area is dependent upon the abundant presence of the pathogen, susceptible hosts, crop management and favourable climatic conditions (moisture, temperature, wind velocity, rainfall, and irrigation). Even if the transmission rates are low, pathogen load on, in and with seeds is an important determinant for disease initiation. For effective management of the disease it is necessary to cultivate crops from disease free seeds and to prevent the pathogens from entering and spreading through crops. This puts increased responsibility on disease surveillance programmes in the form of seed health testing and seed certification, which forms a link between variety registration and seed production and assures the quality of seed marketed to farmers by having rigid standards for varieties identity, purity and freedom from pathogens.

Impact of Karnal bunt in wheat trade – a seed borne, quarantined disease

In China, owing to the fear of importing KB infected wheat from other countries only wheat lots declared as having zero infection levels are imported. Exporting countries are thus required to meet the challenge posed by Karnal bunt. In the USA, there is concern about the possibility of export losses due to the recent introduction of Karnal bunt in Arizona, Texas and New Mexico (Ykema et al., 1996). As a result, the USA has declared *T. indica* as a quarantine pest. The European and Mediterranean Plant Protection Organization (EPPO/EOEPP) has imposed several standards for regulating diseases in the international trade. In 1995, European and Mediterranean Plant Protection Organization (EPPO) prepared a list of 46 A1 quarantine organisms including *T. indica*. Kehlenbeck, Motte, and Unger, (1997) have analysed the risk of introduction of *T. indica* to Germany and have also pointed to the risk of contamination of seeds from grain that has previously been stored in the same premises.

The EPPO (2004) has amended standards for Karnal bunt in its diagnostics protocols for regulated pests which are intended to be used by national plant protection organisations in respective countries, in their capacity as bodies responsible for the application of phyto-sanitary measures to detect and identify the regulated pests of the EPPO and/or European Union lists. The following general provisions apply to all diagnostic protocols: (1) laboratory tests may involve the use of chemicals which present certain hazards. In all cases, local safety procedures should be strictly followed; (2) the use of the names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may be suitable; and (3) laboratory procedures presented in the protocols may be adjusted to the standard of individual laboratories, provided that they are adequately validated with the inclusion of proper positive and negative controls. With reference to *T. indica* regulatory measures have been suggested for isolation, sampling as well as for diagnostics schemes including morphological comparisons and molecular confirmations.

In Russia, there is a domestic quarantine restricting the movement of wheat planting material from one zone to another. Other countries like the USA, Canada, Russia and China are also cautious regarding the spread of this disease. Since KB quarantine blocks

the free exchange of germplasm from CIMMYT, Mexico, into other countries, salvage techniques have been developed to clean the contaminated seed before shipment (Prescott, 1986; Zhang et al., 1984). With the globalisation of trade, there is a need to rationalise quarantine restrictions which hamper the spirit of the free market. The FAO and other global organisations are of the view that quarantine should be based on risk analysis using appropriate molecular testing procedures. Even *Tilletia indica* listed under the category of plant pathogens can be used as biological weapons (Beck, 2003)

Disease surveillance programme for the prevention of Karnal bunt spread

From seedling to harvest, constant vigilance against KB is required in order to grow a healthy wheat crop to its potential yield and quality. KB can reduce profits, and if detected late, may cause total crop loss. An effective disease surveillance programme is thus mandatory to combat major outbreaks of the disease. These measures can be well placed before any symptoms are seen in the fields by detection of the main disease entities of the causative KB pathogen. These entities viz. teliospores, sporidia and mycelia are found to be associated with different stages of the disease cycle.

Teliospores are 22–49 µm in diameter, somewhat spherical and black having a slightly rough surface. The teliospores are nearly twice the size of those of related species *Tilletia caries* and *Tilletia controversa*, causal organisms of common and dwarf bunt of wheat, respectively. The teliospores bear truncate projections surrounded by a delicate membranous sheath. They are composed of three distinct layers of walls, viz. perisporium (sheath), episporium and endosporium. The hyaline perisporium is more predominant in immature spores and becomes loose as they mature. Episporium has appendages projecting into winged margins at the circumference (4–7 µm x 3.6 µm) (Khanna & Payak, 1968). Teliospores are mixed with numerous yellowish or hyaline, round, angular or pear-shaped sterile cells, which are smaller than mature spores and have comparatively thinner walls. Sterile cells fail to germinate under similar conditions (Begum & Mathur, 1989; Joshi et al., 1983).

Germinating teliospores form promycelia which bear numerous haploid, sickle-shaped sporidia (frequently over 100) are produced in a whorl at their apex. The sporidia do not fuse, but germinate directly to form hyphae or uninucleate allantoid secondary sporidia which are forcibly discharged into the air. Primary sporidia measure 64–79 µm x 1.6–1.8 µm while secondary sporidia measure 12–13 µm.

Liberated sporidia must reach a wheat spike where they germinate to produce fungal mycelium. The colonisation leads to infection of the embryo.

Morphologically similar bunt fungi of economic importance

Spore morphology, germination and ultra structure have traditionally been important characters for classification and identification of bunt and smut fungi. Spore size and spore surface ornamentation provides important taxonomic information (Vanky, 1991). Ryegrass bunt *Tilletia walkeri* teliospores morphologically similar to *T. indica* were discovered in pasture and in mixed seed containing wheat and rye grass from Oregon, USA (Carris, Castlebury, & Goates, 2006). This fungus tested positive for *T. indica* using the molecular test in current use at the time although no infected wheat kernels were found. However, differences in colour, surface ornamentation, and size were noted when these teliospores were thoroughly compared with *T. indica*. However, the spores/teliospores, the causal agent

of smuts and common bunts are very similar microscopically, although some differences are apparent when the spores are exposed to certain tests like germination type assay etc. (Stockwell & Trione, 1986; Trione, 1973). Hence, the detection techniques must be efficient in order to differentiate them from similar bunt species like *T. caries*, *T. controversa*, *T. foetida* and rice bunt like *T. barclayana*.

Regulatory measures for disease surveillance and prevention

Karnal bunt spreads through seed, soil and air and hence it becomes difficult to manage. Agronomic adjustments such as crop rotation (Mitra, 1937), non-cultivation of wheat for 2 consecutive years (Padwick, 1939), adjustment in water and fertiliser balance and mulching (Singh Sharma, Hand, Srivastava, & Aggarwal, 1992) have been suggested as a comprehensive package to manage the disease. However, under the present conditions of intensive and extensive cultivation of wheat, recommendations involving long rotations or avoiding irrigation and fertilisers are not practical. An integrated package incorporating the use of fungicides against seed and air borne inoculum, adoption of appropriate cultural practices and bio suppression techniques against the soil borne inoculum and use of host resistance is the key approach (Singh, 1994). Seed health testing and seed certification using modern diagnostic tools is highly advocated as an integral component of this package (Kumar, Singh, & Garg, 1998).

Seed health testing

Karnal bunt spread restricted in a number of ways by the use of resistant or less susceptible varieties coupled with seed treatment remains the most likely measure of control. Development of disease resistant varieties or newer seed treatments to avoid selection pressure is very costly and time-consuming. Due to the unavailability of KB-resistant wheat cultivations and effective seed treatment methods, seed health testing could well be an integral component of disease management as it would ensure that only KB free seed is moved from an infected to a disease free area. Therefore, the following issues are imperative:

1. Determination of true incidence, causes and financial/personal impact of seed borne diseases.
2. Development of more effective methods of salvation and seed testing (routine monitoring/epidemiological tools).
3. Development of better means of risk assessment including pathogen load and pathogenicity/toxicity for crops and differential risks among other crops.
4. Development of better methods for controlling the risks associated with diseased seeds.

In order to limit the transmission of the pathogen to disease free areas, transportation of infected/infested seed from the disease prone areas has been restricted. To meet the requirement, the central seed certification board has formulated strict seed certification standards. To limit the dispersal and build-up of KB infection, tolerance limits (certification standards), based on visual inspection, are applied to foundation and certified seeds i.e. a maximum of 0.05% for foundation seeds and 0.25% for certified seeds. A seed-washing test to quantify the seed borne teliospores for the purpose of seed certification has also been suggested (Agarwal & Verma, 1983; Rai & Singh, 1985).

However, the seed certification standards can be improved by considering total pathogen load on seed lot rather than on percent infection, which does not account for infection grade. If the teliospore contamination is over 25 spores per grain, the seed lot is rated as contaminated (Agarwal, O. Singh, & A. Singh, 1973; Agarwal & Verma, 1983).

Seed scientists have developed techniques for analysing large number of samples for seed certification and plant quarantine. The International Seed Testing Association (ISTA) has an important role in defining and promoting standard procedures, and establishing tolerance for seed tests. The advent of newer molecular diagnostics based on nucleic acid and immunological reaction permit precision detection of KB (Kumar et al., 1998). Pantnagar University, together with the Indian Council of Agricultural Research, New Delhi and the Department of Biotechnology, New Delhi took an initiative in 1997 and in 2006 to share the know-how so as to formulate standard detection and seed certification methods. The immunological formats developed in our laboratory are quite promising not only for the detection of KB infection in seeds but also for detection of teliospore infestation. The significance of seed health testing is to ensure safe movement of germplasms, for the purpose of research and trade. It also offers a means of quality control of seedling stocks for crop production.

Requirements of molecular diagnostics

In a regulatory climate in which the presence of a single teliospore could lead to regulatory action, the need for a reliable method for detecting and identifying *T. indica* teliospores in grain cannot be overstated. Such a method must meet three requirements: (a) a detection method sensitive enough to detect one teliospore in 50g sample; (b) an extraction method that is quick and cost effective in the evaluation of a large number of samples; and (c) compatibility with available biochemical and molecular methods for identification of *T. indica* (Bonde, Pesterson, Dowler, & May, 1989; Castlebury & Carris, 1999; Ferreira, Tooley, Hatziloukas, Castro, & Schaad, 1996; Frederick et al. 1998; Luster, Bonde, Peterson, Hack, & Russo, 1995; Matsumoto & Bell, 1989; McDonald, Wong, Kristjansson, & White, 1999; Smith, Peterson, Beck, Schaad, & Bonde, 1996).

Sample preparation

Sample preparation remains crucial to the development and application of analytical methods in plant diagnostics. Therefore, discussion of some of these methods is quite relevant since many of them are distinguished by the relative lack of sample preparation before the analysis while others require sample preparation for biological amplification. The biological amplification consists of an extraction stage and culture of pathogen on an enriched medium.

Extraction of seed borne teliospores

Several methods of teliospore isolation are a modification of general seed wash assays (Agarwal, Singh, & Mathur, 1993; Begum & Mathur, 1989; Matsumoto & Bell, 1989) being currently used by most international seed health laboratories, that have been improved by the incorporation of suitable detergents commonly used for extracting fungi from soil (Adams, Burmester, Hue, & Long, 1980; Crowe & Hall, 1980; Gerdemann & Nicolson, 1963; Punja, 1985). For the extraction of teliospores from both infected and infested seeds,

Peterson and Bonde (2000) devised a size-selective sieving protocol, where 50 g grain samples are shaken with 100 ml of water and few drops of Tween 20 for 5 min. Supernatant is collected and sieved first through 53 μm and then 20 μm mesh. The teliospores collected on the 20 μm screen mesh, are taken in 100 ml of water and centrifuged at 200 g for 3 min. The pellet is resuspended on 1 ml of spores mounting medium (Booth, 1977), centrifuged, resuspended on 100 μl of mounting medium and visualised at 100 x magnification under binocular microscope. This method yields approximately 82% teliospores from the test sample. Superior methods, compared to the previous described by Matsumoto and Bell (1989) (i.e. centrifuge seed wash procedure) and Peterson and Bonde (2000) (i.e. size selective sieving protocol), give 95% recovery of teliospores from infected seeds. In one such method (Gupta, Kumar, Joshi, Agarwal, & Garg, 2002) seeds are washed with specific concentration of different reagents like 0.005% SDS, 0.001% Tween-20 and 0.05% Triton-X-100 and an yield of 90–95% teliospores is obtained. This method is simple, sensitive and takes less than an hour. Use of such a sampling and extraction procedure can improve the detection limits of KB by the application of molecular and immunological techniques (for example PCR or ELISA). However, application of these modern techniques must be more cost effective, time efficient and accurate than currently used protocols.

KB detection techniques based on cellular and molecular differences

For differentiating KB from other pathogens many techniques can be employed. The present methods of grain testing for the presence of KB spores include microscopic analysis, pathogenicity tests, and isozyme analysis. However, the procedures have drawbacks as they rely on individual examiners and thus are highly subjective. These techniques include conventional techniques, biophysical, isozyme and DNA-based marker and immunological techniques.

Biophysical techniques

Spectrophotometric scans of the extracts of pathogens in the ultraviolet range can be a good diagnostic tool. Similarly, photoacoustic spectroscopy (PAS) is one technique to detect light-induced heat production following the absorption of pulsed radiation by the sample. The PA effect is basically an energy conversion process in which electromagnetic radiations are converted into heat energy, which in turn generates an acoustic signal (Rosencwaig & Gersho, 1976). The photoacoustic spectra for the detection and differential diagnosis study of dry spores six pathogens, viz. *Tilletia indica*, *T. barclayana*, *Ustilago tritici*, *Ustilaginoidea virens*, *Helminthosporium sativum*, and *Alternaria trititina* was generated in the wavelength range of 200–800 nm and at a modulation frequency of 18Hz using an indigenous photoacoustic spectrometer (Gupta, Kumar, Singh, & Garg, 2001). The number and the intensities of peaks in the PA spectra obtained from pathogens were compared. The results revealed that, for some diseases, a few extra peaks were observed while for others common peaks with varying intensities were observed. Apart from these, some additional peaks, characteristic of particular pathogens, were also observed. In PA spectroscopy, the characteristic peak patterns and signal strengths constitute a basis for differential diagnosis of the disease. The results clearly demonstrated the presence of various biomolecules in varying proportions in the dry spores of the six pathogens of KB teliospores. With the help of image analysis, principal components analysis (PCA) was also performed to diagnose the teliospore of *Tilletia indica*. An image

analysis system was developed for use on bleached spores. The bleached spores reveal additional morphological characters (spore profile and spore wall layers) that may be used in an image analysis system to discriminate species. The image processing software automatically locates spores on a given image and calculates perimeter, surface area, number of spines and spine size, maximum and minimum ray radius, aspect ratio and roundness (Chesmore, Bernard, Inman, & Bowyer, 2004).

Isozyme based techniques

Isozymes are enzymes which have the same substrate specificity but different enzyme kinetics and molecular weights. Isozyme analysis has been carried out to study the genetic variation for taxonomy (Micales, Bonde, & Peterson, 1986). Bonde, Pesterson, Dowler, and May (1985) used starch gel electrophoresis profile of isozyme for the differentiation between *T. indica* and *T. barclayana*. However, the use of such analysis is cumbersome and needs expertise.

Molecular diagnostics techniques

The recent and rapid developments in molecular biology have provided new opportunities in diagnostics. Modern diagnostic methods are based on high affinity biomolecular interaction between ligand and binder. These include the nucleic acid hybridisation (DNA-based) and antibody based techniques involving complementary interaction of DNA-DNA, DNA-RNA, RNA-RNA and antigen-antibody (Ag-Ab) interactions that have been applied to KB diagnostics and pathogen detection at the field level.

DNA-based techniques

DNA-based techniques are highly sensitive for the detection of small quantities of seed borne pathogens. DNA-based techniques can detect 2–3 cells per ml of original seed washing. The polymerase chain reaction (PCR) in conjunction with six short arbitrary primers of random sequences was used to perform RAPD profiling of seven seed borne fungal pathogens of wheat and rice, which exhibited distinct polymorphic DNA. A total of 117 RAPD loci were observed on polyacrylamide gel which categorised the fungal pathogens in to three different groups and established their genetic relatedness with 3.0–27.0% similarity. The six arbitrary primers could distinguish *T. indica*, a quarantine fungal pathogen from a non-quarantine fungal pathogen *T. barclayana*. A total of 89 RAPDs loci between *T. indica* and *T. barclayana* were observed, of which 18 were monomorphic and 71 loci were unique (Mishra, Singh, Goel, & Kumar, 2002).

Efforts are underway to develop an accurate molecular method to identify teliospores of *T. indica* using BIO-PCR and primer pairs Ti 17 M1/M2 (825bp) and Ti 57 M1/M2 (118bp) (Bonde et al., 1997). As few as five teliospores could be detected from per 50 g of wheat seed. PCR-based technique is important in this case because KB teliospores are morphologically similar to *T. barclayana* (Bonde et al., 1997).

At the FDWSRU, at Ft. Detrick, several real-time PCR assays have been developed using TaqMan probes for Karnal bunt of wheat. Initially developed using the AVI77100 sequence detection system, these assays have been adapted for use with both the smart cyclers and the RAPID for quick identification at remote locations or at field sites (Frederick & Snyder, 2001; Schaad et al., 2003). With the help of two-step PCR protocol

using FRET probes for the direct detection and identification of *Tilletia indica* is possible even from a very few number of spores. It involves the amplification of the ITS1 DNA segment in the highly repeated rDNA unit from any *Tilletia* species, followed by FRET analysis to detect and unequivocally distinguish *T. indica* and the closely related *Tilletia* species (Mui-Keng & Gordon, 2006).

Immunological techniques

Immunological techniques are most efficient and hence are successfully employed for the detection and differential diagnosis of the diseases.

Immunoassay techniques to detect plant pathogens have been largely adapted from methods widely used in medical diagnosis (Sikora & Smedley, 1984) and are now routinely utilised in a routine for disease indexing in plants (Ball & Reeves, 1992; Cassels, 1992; Miller, Rittenberg, Peterson, & Grathus, 1992). Immunoassays have been revolutionised by the introduction of highly specific monoclonal antibodies and enzyme linked immunosorbent assays (ELISAs) as routine methods to detect only the molecules (antigens) or antibody binding sites (epitopes) are unique to the infecting organism, or its metabolite. These diagnostic assays are rapid and can be completed in several hours instead of days or even weeks. Holland, Denny, and Bonde (1990) produced polyclonal antisera in rabbits against antigens of *T. indica* and *T. barclayana* and differences were observed between the two in their reactivity patterns.

The advent of hybridoma technology has allowed the evolution of highly specific fungal immunoassays and an increasing number of these are being used for diagnostic purposes (Dewey & Thornton, 1995). A number of workers used Mab-based immunodiagnostic assay for increased specificity (Barker, Brewer, Cook, Crossley, & Freeman, 1994; Burns, Vernon, & George, 1994; Dewey, MacDonald, & Philips, 1989; Dewey, Munday, & Brasier 1989).

It is an electron microscopic method for detecting specific antibodies. In this method specific antigens were independently covalently bonded to plastic microspheres of 0.5 microns or 0.9 microns in diameter. The antigen-linked spheres were then exposed to normal serum or serum containing specific antibody, followed by treatment with gold-labelled secondary antibodies. The binding of the gold-labelled secondary antibodies to the specific primary antibodies on the spheres acted as an indication of the presence of the specific primary antibodies. The spheres were then examined and photographed by transmission electron microscopy. The number of gold particles bound to the spheres was counted manually using the photographs. The gold labelling was found to be specific and sensitive, enabling detection of antibodies present in highly diluted antisera (Hari, Baunoch, & Das, 1990).

Immuno-detection strategies for disease surveillance of Karnal bunt

Several assay formats exist but the most common are the enzyme immunoassay based systems. These formats have many advantages as they are highly sensitive, easily replicated, automated and quantified. The only disadvantage is that they require lab facilities. A number of 'user friendly', membrane bound, dot blot and dipstick assays, which do not require laboratory facilities have been developed and are being used increasingly as 'on site' screening tests. Some immunofluorescence assays, which were developed for immunodetection, have not been adopted widely due to involvement of microscopy and a UV light

source. In our lab we have developed antibody methods for KB diagnostics such as microtitre ELISA, immunofluorescence staining test (IFST), seed immunoblot binding assay (SIBA), and dyed latex bead agglutination test and immunodipstick assay. These immunoassay systems could be allowed for reliable quantitative assessments in a high throughput manner in regulatory laboratory facilities if developed in an appropriate rapid format in the form of kits such as immune-dipstick and immune-lateral flow assays. They would be well suited for detecting KB infestations in the field. The immunodiagnostic assays for field use are inexpensive, rapid and do not require highly trained personnel.

ELISA

Most of the ELISA tests developed for fungi are simple indirect assays in which the microtitre wells are directly coated with the test sample or fungal antigen. Fungal antigens, particularly glycoproteins, bind strongly to microtitre plate wells. The binding of antigen and specific antibody is made visible through the use of a 'tag' enzyme, which acts on its substrate and generates a coloured product. Most of the commercial enzyme immunoassays involve double antibody sandwich tests in which one of the antibodies is generally a specific MAb as the other is polyclonal antisera (Nemeth, Shame, & Stier, 1990). Sensitivity in these assays can be improved, as with antigen-coated wells, by using reporter macromolecules such as biotinylated secondary antibody or biotinylated protein, a conjugate (Thornton, Dewey, & Gilligan 1993). Immunoassays incorporating magnetised beads have been used for some time in the field of medicine and plant virology (Bantari, Calpper, Hu, Daws, & Khurana, 1991) but their use in the diagnosis of fungal plant pathogens is yet to be fully exploited.

Micro-titre ELISA is being used for early detection of KB pathogen in the host when the infection levels are very low, in-planta proliferation of pathogen, determination of fungal biomass in infected tissues, characterisation of genetic races, based on their immuno-reactivity pattern (Varshney, 1999). In addition this technique has been proven to be useful for monitoring the antigenic properties in the process of population shift during the sexual development of *T. indica* together with the determination of genetic diversity among pathogens and subsequently for immuno-pathotyping (Rai, Kumar, Garg, Singh, & Lakhchaura, 1998).

Tissue imprinting

An interesting variation of dot blot immunobinding technique is the direct blotting of the plant tissue into nitrocellulose membrane i.e. tissue print immunoblot (Lin, Y. Hsu, & H. Hsu, 1990; Makkonk, Hus, & Kumari, 1993). The principle and mechanism of dot blot or dipstick assay is same as that of ELISA and is being used successfully for detection and immunolocalisation of the pathogen within the tissues. Gwinn, Collins-Shepard, and Reddick (1991) reported that the sensitivity of the tissue immunoprint blot method for the detection of *Acremonium coenophialum* in tall Fescue was same as in protein sandwich ELISA.

To check out the teliospore load on the wheat seeds at the time of vigour testing, a technique called seed immunoblot binding assay (SIBA) was developed and optimised (Kumar et al., 1998). The principle behind this technique is that when the infected seeds are kept on the nitrocellulose membrane, the teliosporic antigens are adsorbed and diffused according to the teliosporic load on the nitrocellulose sheet. These sheets are immuno-

processed using anti-teliosporic antibodies (primary) and goat anti-rabbit immunoglobulin IgG conjugated with alkaline phosphatase (secondary) for which a substrate NBT/BCIP is used. The coloured imprint develops and appears to have direct correlation with the grade or severity of KB infection on bunted grains. Further studies are in progress to determine the specificity of SIBA by cross-reactivity with other seed pathogens (Kumar et al., 1998).

Immunofluorescence assays

In immunofluorescence, pathogen specific antibodies are conjugated with fluorescent dye molecules, commonly fluorescence isothiocyanate or rhodamine isothiocyanate. Antigens present in samples bound to microscopic slides are made visible by means of a fluorescence microscope. This type of fluorescence assay is called direct immunofluorescence assay. Specific antigen antibody reaction may be visualised by addition of labelled antibody directed against the primary antibody in a specific reaction. This is called indirect immunofluorescence assay. The indirect method has an advantage of utilising a single labelled antibody to detect many different antigen-antibody reactions occurring within a given species. Indirect assay is more specific than direct assay.

Antisera against spores, mycelium and a toxin of *R. secalis* were produced in a synthetic medium. Using the indirect fluorescence antibody techniques (IF), the percentage of infected barley seeds by pathogenic fungi was evaluated (Mill, 1990). Infection of *Botrytis cinerea* on cut flowers was detected using a monoclonal antibody based immunofluorescence test (Salinas & Schats, 1994). An immunofluorescent microscopical method has been developed for specifically detecting and counting air borne *T. sacchari* spores, using indirect labelling with fluorescein isothiocyanate (Dawson, Smythe, Schoot, Sutherland, & Egan, 1995). Resting spores of *Plasmodiophora brassicae* in soil were serologically detected using indirect immunofluorescence (Wakeham & White, 1996). Gupta et al. (2001) and also developed a fluorescent staining test for the detection of Karnal bunt teliospores.

The test aims at detecting the intact teliospores found in sori of the infected wheat seeds or as fungal contaminants on the infested seeds or in the soil. The procedure is based on the principle of indirect ELISA where polyclonal antibodies are raised against the surface teliosporic antigens, and binding is monitored by a goat-rabbit antibody conjugated to a fluorescein label. The teliospores can be seen as bright green, patchy and ring-shaped fluorescence under the microscope.

The technique is suitable for the localisation of teliospore associated molecular pattern (TAMP) of KB teliospore and for studying the antigenic configuration of the surface of teliospores and could also be employed for immunodetection of salvated teliospores present as surface contaminants in wheat lots (Gupta et al., 2001). These kinds of studies help in the understanding of the molecular basis of dormancy and teliospore germination and also provide evidence of evolutionary relationship amongst bunt and smut pathogens as well as existence of ecologically, physiologically and/or morphologically different populations within species or genus. The correct and unambiguous differentiation of bunt and smut pathogen was dependent upon the existence of qualitative and quantitative antigenic differences as in spores of fungal pathogen (Gupta et al., 2000). All these methods are suitable for mass diagnostics of bunt pathogens at lab levels as they need special instrumentation. However, easier ways to detect the KB at field level are through an immunodipstick assay and a dyed latex bead agglutination test if appropriate rapid formats are developed and commercialised.

Dot blot and immunodipsticks

The mechanism of dot blot or immunodipstick assay is essentially the same as that in ELISA. In some tests nitrocellulose membrane or nitrocellulose coated plastic tags or cards are used and in others polyvinylidene difluoride is used. Cahill and Hardham (1994) have found that positively charged nylon membranes are useful for the detection of *Phytophthora cinnamomi* because their charged surfaces attract zoospores. The 'reporter tag' in these assays may be enzyme or gold conjugates (Hsu, 1984; Rysanek, Stockey, Haeberle, & Putz, 1992). A number of workers have used such rapid formats, which are more sensitive and rapid for on site detection of pathogens (Cahill & Hardham, 1994; Dewey, MacDonald, et al., 1989; Moricello et al., 1998; Schwick et al., 1998). Immunodipstick test for KB diagnostics include the use of nitrocellulose padded plastic tags. When teliospores are kept on nitrocellulose pads, the torer-off teliosporic antigens are adsorbed due to the charged nature of the membrane. Immuno-processing of dipsticks using primary (anti-teliospore antibodies) and secondary antibodies (goat anti-rabbit immunoglobulin IgG conjugated with ALPase) lead to colour development and predicts the presence of the pathogen. The advantage associated with the use of this technique is its sensitivity as it can detect a very small number of teliospores i.e. only 5 ng of teliosporic antigen (Kesari & Kumar, 2002).

Agglutination assay

There are two types of assays, those that employ immunoglobulin bound to latex particles and those in which the antibodies are bound to proteins on the surface of formalin treated cells of *Staphylococcus aureus*. There is also a simple form known as slide agglutination test. It involves the reaction between antibody and insoluble particulate antigen. In these agglutination reactions, blood cells, viruses or bacteria are employed as the particles. These are naturally coated with antigen that can be cross-linked to make a clump or agglutinin. Recent studies suggest that for detection of solubilised antigen, antibody immobilised latex particles bentonite, or cells of *Staphylococcus aureus* can also be employed. The sensitivity of the agglutination test has been found to correspond with the Howard Mould Colony count for detection of plant fungi in seeds, animal feeds and spices (Notermans & Kamphuis, 1990). Lyons and Taylor (1990) used *Staphylococcus aureus* slide agglutination test for identification of bacteria from plants.

The dyed latex bead agglutination test developed in our laboratory is considered better for detection of solubilised teliosporic antigens over intact teliospores of KB. The teliosporic antigens are solubilised using sonication and detergent extraction and are allowed to adsorb on the surface of blue colour dyed latex beads, which are then immunoprocessed using polyclonal anti-teliospore serum. Positive agglutination reaction reflects the presence of KB infection. The method has the advantage as it is more economical, rapid and user friendly with an absence of pseudo-agglutination which is the most important factor for on-site diagnostics single step test (Kesari, Mishra, Garg, & Kumar, 2005). The test has the sensitivity to detect solubilised teliosporic antigens equivalent to 750 intact teliospores and it is suitable for immunodetection of different grades of infected seeds having teliospores mass ranging from 1000 to more than 10,000. Hence, this seed health testing procedure could also be used for single seed analysis.

Conclusion

The need to be competitive in the global market for exported food grain has brought in greater awareness for improvement of quality in terms of nutritional and pathogen-free grains. Traditional seed testing for the detection and identification of seed borne diseases/pathogens are dependent on culture practices, embryo tests, visual and microscopic examination of seeds. Enrichment and selection steps are often time consuming and the biochemical identification of a pathogen may add several days to the procedure. Such methods are inappropriate for quality assurances, seed certification and plant quarantine regulations. Thus, seed health testing using rapid and novel diagnostic methods should be employed by a national/multinational seed industry or organisation. The techniques must be developed in the form of simple, fast and reproducible molecular and immunoassay formats which can be utilised in quality control laboratories of seed industries, plant clinics and even at the hands of growers.

At present in the diagnostic sector, immunoassay products command an annual worldwide market value in billions of dollars. As compared to clinical and therapeutic fields, the applications of immunoassays in agriculture, food, veterinary and environmental sciences has developed slowly. Nevertheless, the rapid, economic, sensitive and the easy to use approach of these diagnostic tests are already making an impact in these fields. In several countries, immunoassay products for home use are available in pharmacies and even in supermarkets (Deshpande, 1996), and in a similar fashion, the single step dipstick immunoassay tests will be useful for monitoring the Karnal bunt pathogen at the field level as part of disease surveillance and quarantine regulations imposed by importing countries.

Hence, the strategy to be followed for the disease surveillance programme for KB is the identification of the disease-causing entities present in any of the forms. In the case of infected seeds or bunted seeds, the teliospores are present inside the seeds as a black powdery mass, whereas in the infested seeds the teliospores are adhered to the seed surface. The likelihood of KB developing in wheat in fields in successive years of disease incidence could only be monitored if a sensitive diagnostic format to determine the presence of teliospores are viable up to 5 years in the soil, but their disease potential for causing disease under field conditions is not known.

The diagnostic formats can help in ensuring growers who can grow and harvest wheat with minimal risk of KB. For example (i) seed tests negative for teliospores using very sensitive assay; (ii) tests which can evaluate the soil negative for teliospores. Natural movement of KB teliospore/sporidia is reported to occur via wind, insects and birds. A survey of animals capable of vectoring KB teliospores should be conducted; moreover, the relative likelihood of these vectors in relation to the spreading of disease should be determined to check if the disease spread occurs with the passing of KB teliospores into livestock/birds faeces. Further, for determining inoculum density, it is essential to use appropriate extraction and diagnostic technologies. Attempts are also being made to develop replica immunoblot binding assay (RIBA) which can provide a tool for detecting and quantifying airborne sporidia of *T. indica* after trapping on polyvinylidene difluoride membranes by spore trapping device. Monitoring of these disease-causing entities using an appropriate methodology will assist in the disease surveillance programme and plant quarantine regulations. There is an urgent need to commercialise the available immuno-diagnostic technologies for this purpose.

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