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METHODS OF FINDING AND IDENTYFICATION OF PATHOGENS IN SEEDS

ABSTRACT

Methods for detection and identification of seedborne pathogens are discussed. Traditional, immunodiagnostic and nucleic acid–based methods are briefly described including their use for fungi, bacterial and viral pathogens finding and identification in seeds.

Key words: detection, identification, methods, seedborne pathogens

INTRODUCTION

Investigations of methods of seed health testing have been started since the second half of 19th century when the first official seed testing station was organised (Germany, 1869) (Malone and Muskett, 1964). A number of seed health testing methods were developed in Russia (Dorogin 1923, Budrina 1935) and modified in Germany (Klemm 1926) and the USA (Orton 1931). According to Doyer, in the „First Interna–tional Rules for Seed Testing" published in 1928, special attention was paid to seedborne pathogens and pests on peas, cereals, beans and flax (Wold 1983). Doyer worked at the Official Seed Testing Station, Wageningen, the Nertherlands, and developed in 1930–ies basic meth–ods of seed health testing (Doyer 1938).

In the middle of the twenteeth century interest in seed pathology ex–panded from the following reasons:

1. A few research programmes dealing with elaboration of practical methods for detection of seedborne organisms were started,
2. Seedborne parasites were listed,
3. Seed testing practise was so extended and covered 'health' of seed therefore further research on rapid methods for detection of seedborne organisms was necessery (Porter 1949, Noble 1951).

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At that time common objectives of seed pathology were recognised in–ternationally and they were directed towards seed improvement, seed trade and plant protection. Later, de Tempe (1970) listed the methods for routine evaluation of seed health condition. Namely:

1. Examination of seeds – dry seed, softened or soaked, and the seed washings.
2. Examination of seeds and seedlings by the moist chamber methods – germinated on paper or textile substrata or in essentially inert media, and after emergence in non–sterile soil.
3. Examination after incubation on agar media.
4. Examination of plants or their organs in greenhouse or in the field.
5. Testing for serological or other biochemical reactions combined serological methods for viruses and bacteria and also phage–plaque method for bacteria.

Soon, Neergaard (1977) described routine methods, similar to those of de Tempe (1970), suitable for pathological seed analysis. These seed health tests were used for: quarantine, and certification purposes, eval–uation of planting value, advisability of seed treatment, storage quality, feeding value, resistance of cultivars and testing of chemically treated seeds (Neergaard 1977).

These methods include:

1. Direct observation of a seed with unaided eye, hand lens or under low power stereoscopic microscope.
2. Microscopic examination of suspensions obtained by washing of seeds under microscope.
3. Microscopic examination of seed after clearing and /or staining.
4. Examination of seeds and seedlings after incubation on blotter or within blotting paper, on agar media or any other essentially sterile media.
5. Observation of symptoms developed on seedlings grown in soil, sand or similar material.
6. Examination based on growing–on tests, carried out in greenhouse, environment–controlled chamber or in the field.
7. Serological tests.
8. Bioassays (e.g. indicator test, phage plaque test).
9. Tests under field conditions.

Most of the above mentioned methods are rather time and space con–suming, so during the past 25–30 years a marked progress was made in development of rapid and accurate methods for detection of seedborne pathogens. However, some methods described earlier (de Tempe 1970, Neergaard 1977), now called ‘traditional’, have dominated for a consid–erable time and still have been in use for various purposes. Now techno–logical advance have provided many specific methods for organism recognition, especially essential for bacteria and viruses, however, one has to remember that direct observation of seed samples and incubation

tests of seeds are important basic laboratory methods of parasite detection for general seed pathological research and seed health testing.

Over 100 years of seed health studies many new methods were developed, or older methods were modified, but all of them used for detection and identification of seedborne organisms have to fulfil six main requirements (Ball and Reeves 1991):

1. Specificity – the ability to distinguish a particular target organism from others occurring on tested seeds.
2. Sensitivity – the ability to detect organisms at low incidence in seed stocks.
3. Speed
4. Simplicity – minimalization of a number of examination stages to reduce error and enable testing by a staff not necessarily highly qualified.
5. Cost effectiveness – costs should determine acceptance ot the test.
6. Reliability – methods must be sufficiently robust to provide repeatable results within and between samples of the same stock regardless who performs the test (within statistical probability and sample variation).

TECHNIQUES CURRENTLY USED FOR THE DETECTION OF SEEDBORNE PATHOGENS

I. TRADITIONAL METHODS (CONCERN MOSTLY MORPHOLOGY OR PHYSIOLOGY OF PATHOGENS AND CHARACTER OF SYMPTOMS PRODUCED ON PLANTS)

Fungal pathogens

1. Direct inspection – sample of seeds may be examined:
   - dry for the presence of sclerotia, e.g. *Sclerotinia sclerotiorum*, *Claviceps purpurea* or smut balls (*Ustilaginales*) and also oospores of *Peronospora manshurica* encrusted on soybean seeds (with or without stereomicroscope)
   - wet (immersed in water or other liquid) to make fungal fruiting bodies or symptoms more visible (under stereomicroscope) or promote liberation of spores which are next counted in concentrated extract. Useful for different species of Ascomycota.
2. Incubation methods – 2a. Testing on agar media:
   - This method gives an information on viability of inoculum in the infected seed sample.
   - The procedure is preferable when a blotter test does not provide adequate conditions for development of mycelial growth, sporulation or symptoms of the pathogen on seedlings and seeds but on nutrient agar characteristic colonies are developed.
   - Surface sterilised seeds (commonly in 0.5–1% sodium hypochlorite for 1–5 min. or other disinfectants), to free or reduce superficial mi–

croorganisms (Sharma *et al.* 1997), are usually plated into Petri dishes on sterile standard media, like PDA (Filipowicz 1976, Pyndji *et al.* 1987, Czyżewska 1991, Kućmierz and Gorajczyk 1991, Osorio and McGee 1992, Babkiewicz 1993, Wójcik 1993, Błaszkowski 1994, Tseng *et al.* 1995, Stompor–Chrzan 1996, Wiwart and Korona 1997), MEA (Ali *et al.* 1982, Bathgate *et al.* 1989, Wiewióra and Prończuk 2000), or specific media, e.g. Coon's (Marcinkowska 1998), SNA (Wakuliński and Chełkowski 1993). The seeds are left on the medium for a few days (5–8), usually at ca. 20°C, to promote growth of seedborne necrotrophs. Time, temperature and light for plate incubation differ as there are many variations of the agar test depending on species requirement (Yeh and Sinclair 1982, Nowicki and Strzelec 1986). Usually 400 seeds consist a standard sample for testing. In general acidic agars are produced to reduce bacterial contaminants (Maude 1963, Pyndji *et al.* 1987). Agar media may be semi–selective when specific chemicals (Kritzman and Netzer 1978) and/or antibiotics (Bathgate *et al.* 1989, Janas *et al.* 1993, Tseng *et al.* 1995) and/or fungicides (Byford and Gambogi 1985) are added.

Agar tests are the most effective for detection of high–incidence pathogens such as: *Ascochyta pisi* on pea, *Botrytis allii* on onion, *Septoria (Stagonospora) nodorum* on wheat, *Colletotrichum lindemuthianum* on bean and *C. linicola* as well *B.cinerea* on flax (Anon.1993b). These and the other fungi occurring in seed sample at the level greater than 1% can be detected by the standard seed health tests.

2b. Blotter testing:

- The blotter test detects infection of seeds and, in some tests, also infection of germinated seedlings.
- The blotter method is widly used when regular seed health testing is carrired out and the agar test is nonpracticable. The test combines advantages of *in vitro* examination with *in vivo* observations.
- In general, standard sample of 400 nonsterilised seeds are placed on (Zad 1987, Nowicki 1997) or between (Neto and West 1989) 1–3 layers of water– or buffer– soaked paper (Małuszyńska, Wiewióra 2000) or cellulose pads (Yeh and Sinclair 1982) for a couple of days depending on a fungus and plant tested (Mariotto *et al.* 1987, Mishra *et al.* 1999, Nsemwa and Wolffhecheel 1999). In some cases seeds were surface sterilized (Yeh and Sinclair 1982, Sharma *et al.* 1997). Standard blotter method was the best for expression of a number of micro–organisms and their incidence either on small or large seeds of *Lagestroemia microcarpa* and *Pterocarpus marsupium*, respectively (Sharma *et al.* 1997). For some tests seed germination is suppressed by temperature (deep freezing) (Janas *et al.* 1993, Nowicki *et al.* 1996, Nowicki 1997) or chemicals (2,4–D) which disrupt seed tissues and seedborne fungi grow easier

(Limonard 1966, Jorgensen 1977, Maguire *et al.*, 1978). Blotter freezing tests are used for example: for carrot seeds to detect *Alternaria dauci* and *A.radicina,* for brassica seeds (1000 per sample) to detect *Phoma lingam* (Anon., 1993b), for *Ricinus communis* seeds to identify several fungi (Mariotto *et al.* 1987). Freezing blotter or osmotic methods have been applied in the Nordic countries for *Drechslera* spp. detection in barley seeds (Scheel 1997). In the Central European countries blotter tests have been used for detection of *Ascochyta* spp. in peas, *Fusarium* spp. in beans, broad beans or pea seeds (Tylkowska 1997), but incubation only on agar media has been able to be used as well for these pathogens (Stompor–Chrzan 1996, Marcinkowska 1998, Marcinkowska and Borucka 2001) or both methods have been applied (Grzelak and Iłłakowicz 1973).There have been some more examples for applying both incubation methods for detection seed pathogens of various plants: soybean (Yeh and Sinclair 1982, Esentepe *et al.* 1985, Zad 1987), dill (Janas *et al.* 1993), lupin (Nowicki 1995) or triticale (Małuszyńska, Wiewióra 2000). Janas *et al.* (1993) noted that results of both tests on dill seeds were comparable.

3. Staining methods:

– These methods are used to detect seedborne pathogens which are biotrophs or may grow on artificial substrate but very slowly, and generally are not able to develop fruiting structure (e.g. *Ustilaginales*) or their development takes many (usually over 10) days (e.g.*Septoria*).

– Staining barley embryos for the presence of *Ustilago nuda* mycelium is a standard method for seed health testing (Anon., 1993). Fluorescent method is applied for identification of *Septoria (Stagonospora) nodorum* identification (Małuszyńska, Wiewióra 2000). Percentage of kernels with fluorescent mycelium is counted after incubation of wheat seed (Scheel 1997).

When detection of seedborne fungi is performed by the incubation and staining methods stereomicroscope and microscope are main necessary tools. However, for identification of species with incubation tests one has to be skilful at recognition of etiological signs, especially fruiting bodies and/or sporulation on infected seeds or when characteristic pure culture of a fungus on media is well developed. Keys and descriptions of organism morphology are necessary for identification (Arx von 1974, Descriptions of Pathogenic Fungi and Bacteria. Sets 1–154, 1964–2002, Hawksworth *et al.*, 1995). For a qualified person in fungal diagnostics these methods are easy to perform but since species differ in sporulation ability the time needed to obtain results may last a few days, eg. *Colletotrichum lindemuthianum, Alternaria* spp, or even over 2 weeks when besides spores also mycelium structures characteristics for their

identification (chlamydospores of *Phoma* spp., microsclerotia of *Verticillium* spp.) are needed.

Detection of seedborne bacteria and viruses

1. Growing–on tests for bacteria and viruses:
– These tests give an indication of total potential transmission (from external as well internal sources of inoculum) of seed sample under the experimental conditions of the test.
– In general, seed samples are sown in greenhouse or in the isolated field from related plant species to prevent transfer of inoculum. After appearance of symptoms on the emerging seedlings, isolation and identification can be performed.
Many seedborne bacteria (Schaad 1989a), for example *Pseudomonas syringae* (*savastanoi*) pv. *phaseolicola* (Grogan and Kimble 1967) were detected in this way. *Chenopodium quinoa* inoculation test was used for detection lettuce mosaic virus (LMV) in commercial seed stocks (Marrou and Messiaen 1967). The tests are easy to perform, but require time and space.
2. Laboratory tests for bacteria detection

Standard methods for detection of bacteria in seeds involve:
a. Extraction from seeds
b. Isolation in culture
c. Identification by different methods

ad. a. Procedures of extraction aims at optimization of the recovery of a target bacterium. The release of bacteria from a seed sample is mainly done by saprophytic organisms present on seeds and inhibitory compounds in seeds (Roth 1989). Bacteria are extracted from seed flour or seeds in a liquid medium. Generally, a sterile buffered saline is commonly in use, but time and temperature of soaking depend on bacterial species.

ad. b. Extracted bacteria are transfer in small volumes of the extraction media onto general or semi–selective media (Schaad 1989).

ad. c. Identification may be done by:
– standard tests – involving classic, morphological, physiological and nutritional methods (Stead 1992).
– agar plating methods – some seedborne bacteria can be identified when isolated onto general plating media, like King's medium B, on which *Pseudomonas syringae* (*savastanoi*) pv. *phaseolicola* produced a characteristic fluorescent pigment (Taylor 1970) or nutrient–starch cycloheximide agar (NSCA) on which *Xanthomonas campestris* pv. *campestris* formed typical starch–hydrolysing colonies (Schaad and Donaldson 1980, Mguni *et al.* 1999). The agar plating methods are very effective for isolation of pathogenic bacteria present in large numbers in extracts in which incidence of saprophytic bacteria is low. In case of reverse situation semi–selective media enriched with chemicals to reduce the growth of

saprophytes are recommended. Semi–selective media can be also used to detect particular bacteria from seeds directly plated on agar (Schaad 1989a). Since incidence of seedborne bacteria is frequently very low this method often can not be exclusively used. Mguni *et al.* (1999) noted that identification of *Xanthomonas campestris* pv. *campestris* colonies isolated on 3 semi–selective media (FS, NSCA and NSCAA) must be confirmed by other tests including final confirmation by the host pathogenicity test.

– host pathogenicity tests – inoculation of a different series of cultivars of the appropriate host by pure cultures of bacteria are required for pathovar and race identification (Taylor *et al.* 1989, Mguni *et al.* 1999).

– bacteriophage tests – since the relationship between a phage and its bacterial host may be specific, bacteriophages are employed for detection and identified of bacteria (Taylor 1970). Although several trials have been initiated the use of phage tests are limited by lack of true species specificity and resistance of bacteria to phages (Sheppard *et al.* 1989).

Laboratory tests for bacteria detection and their identification do not require much space and time for their performance but they require highly qualified person skilful in bacteriological methods.

II. IMMUNODIAGNOSTIC METHODS FOR BACTERIA, VIRUSES AND FUNGI DETECTION.

Former serological tests

Serology as a method was first employed for bacteria detection, as early as in 1918. It is based on the immunological principle that foreign molecules (immunizing agent or antigens) injected into bloodstream of a mammal stimulate its immune system to produce specific antibodies which recognize and bind to the antigens (Schaad 1979, Fox 1993). The antibodies recognize many chemical sites on target antigens and are known as polyclonal antibodies. Introduction of monoclonal antibodies has markedly improved specificity of serological tests as these antibodies recognize only one chemical site on target antigens. Monoclonal antibodies are prepared by cell culture techniques and produced by hybridoma specific for a single site (Stead 1992) and thus they can act selectively at the generic, species, pathovar or strain levels.

Agglutination, precipitation and immunodiffusion tests belong to the earlier serological methods based on polyclonal antibodies still in use for certain bacteria (Ball and Reeves 1992). In the first test the antigen/antibody reaction results in agglutination or clumping of particular antigen (Lyons and Tylor 1990). In immunodiffusion test, usually double–diffusion, antiserum diffuses from a central well through the agar to precipitate, or no, against individual antigens diffusing from surrounding wells. When similar precipitation lines are formed antigens are consid–

ered identical (Guthrie *et al.* 1965). In the precipitation test an antigen is precipitated out of solution by specific antibody. The described techniques have been used for detection of *Xanthomonas campestris* pv. *phaseoli* and *Pseudomonas syringae* (*savastanoi*) pv. *phaseolus* and also *P. syringae* pv. *pisi* in bean and pea seeds, respectively (van Vuurde and van den Bovenkamp 1981, Trujillo and Seattler 1979).

Enzyme–linked immunosorbent assay

Enzyme–linked immunosorbent assay called ELISA was first adapted for plant viruses identification by Clark and Adams (1977). Development and principles of this method were described by Clark (1981). Both polyclonal and monoclonal antibodies could be applied for ELISA but monoclonal antibodies improved reproducibility of ELISA in detection of seed pathogens (Fox 1993).There are many different forms of ELISA (Ball and Reeves 1992) but three of them are main procedures: direct (double antibody sandwich DAS–ELISA), indirect and competitive (Lange 1986). The direct test is the most often used in the seed health assays (Lange 1986, Kazinczi and Horvath 1998). In this method a seed (or seedling) extract (i.e. an antigen) is selectively trapped and immobilized by solid–phase–specific antibody in microtitre wells. The enzyme–labelled antibody reacts with the immobilized antigen and, after removing of unreacted enzyme–labelled antibody, the bound enzyme is assayed by adding a suitable substrate (Clark 1981). Qualitative assays may be performed visually but visualization may be improved by addition of the substrates which give coloured hydrolizates. Quantitative tests are made with colorimetric or spectrophotometric equipment (Clark 1981). Fox (1993) gave descriptions and diagrams of the processes involved in direct and indirect ELISA tests. Seed testing with ELISA techniques is based on the use of polystyrene microtitre plates, each containing up to 96 wells, which are very suitable for indexing numerous samples and sub–samples of seeds.

When ELISA techniques are applied for bacteria detection mainly monoclonal antibodies are used. Candlish *et al.* (1988) developed highly specific monoclonal antibodies to *Pseudomonas syringae* pv. *pisi* for both indirect and competitive ELISA assays to distinguish between strains of this bacterium. Moreover, a commercial kit for detection of the bacteria seedborne infection was prepared and marketed (Ball and Reeves 1991). Recently Rajeshwari *et al.*(1998) developed a sensitive, specific and rapid ELISA technique for detection of *Ralstonia solanacearum* isolates from tomato seeds.

Immunofluorescence microscopy

Immunofluorescence microscopy is a highly recommended immunodiagnostic method for bacteria detection (Van Vuurde 1997). Indirect (Malin *et al.* 1983) and direct (Franken and Van Vuurde 1990) immunofluorescence cell staining involves microscope detection under

ultraviolet light of an antigen after staining with homologous antibody conjugated with a fluorescent dye (e.g. fluorescein isothiocyanate). Fluorescence is proportional to the concentration of bacteria in the seed preparation. For example, *Xanthomonas campestris* pv. *phaseoli* was identified in been seeds by indirect method but *Pseudomonas syringae* pv. *phaseolicola* by direct immunofluorescence. The same method was also used for *Clavibacter michiganensis* subsp. *michiganensis*.

Franken and Van Vuurde (1990) stated that out of the most commonly used serological methods for seedborne bacteria detection and identification (agglutination, double diffusion, ELISA and immunofluorescence microscopy) ELISA results could be easily automated, standardized and could suit for screening large numbers of seeds (Sheppard *et al.* 1986) but a second important assay is immunofluorescence. Both these methods are useful for rapid testing (Schaad *et al.* 1997) but especially ELISA lacks sensitivity (Van Vuurde 1997). However, these methods are less sensitive than agar plating assays and do not result in viable cultures for conferming identification by pathogenicity tests (Schaad *et al.* 1997). Meanwhile Van Vurde (1997) described immunofluorescence colony–staining (IFC) method for routine indexing and quantitative determination of field thresholds of pathogenic seedborne bacteria. The IFC assay is more sensitive and more specific than traditional isolation and the earlier discussed serological tests.

Viruses in seeds are mainly detected by different techniques of ELISA (Lange 1986). Polyclonal antibodies are effective for viruses testing when extreme specificity is not needed and strain detection is not required. Polyclonals are commonly applied first as trapping antibodies and then monoclonal antibodies in direct ELISA tests are used (Fox 1993). Modifications of the original ELISA described by Clark and Adams (1977) are necessary for effectiveness of detection of seedborne virus. Frison *et al.* (1990) stated that ELISA is a preferred method for indexing 25 out of the 35 seed–transmitted viruses of legumes listed in the technical guidelines determining safe movement of legume germplasm internationally. Such immunoassays as the biotinavidin ELISA and the enzyme–linked fluorescent assay (ELFA) were equally effective and superior to standard ELISA in detection of LMV (Lettuce mosaic virus) in lettuce seeds. Dot–ELISA (dot blot, dot immunobinding or spot immunodetection) is a newer modification of the ELISA technique (Lange 1986, Stead 1992). This is an indirect method which was found to be slightly more sensitive for pea seedborne mosaic virus than other ELISA techniques (Lange 1986).

Immunosorbent electron microscopy (ISEM) is a rapid, reliable method for testing seeds for virus infection especially when several viruses are present but not suitable when numerous samples require testing for the same virus. This method was used for detection of barley stripe mosaic virus, tobacco ringspot virus and soybean mosaic virus in seeds (Brlansky and Derrick 1979).

Immunoassays utilising antisera produced against purified patho–gens or their extracts have been very useful for virus detection (Torrance 1992) while for bacteria and especially fungi have limited value, since they contain many non–specific antibodies which may cause cross–reactions with related and unrelated species concealing the effects of specific antibodies (Dewey 1992, Miller *et al.* 1992). Therefore detection of fungi by serological methods may be achieved only when monoclonal antibody techniques are employed (Irwin 1987). Monoclonal tests are known to detect seedborne fungi of spruce (Mitchell 1988) and rice (Dewey 1992) and also *Pyrenophora graminea* (Burns *et al.* 1994) in barley.

Since 1985 in several countries the growing–on tests for bacteria and viruses have been replaced by the enzyme–linked immunosorbent assay (Dinant and Lot 1992). ELISA and its new modifications become more commonly used not only for virus detection in seeds but also for certain bacteria, as a rapid, reliable and specific method. Among serological methods the immunofluorescence colony–staining is recomended for bacteria because it is more sensitive and specific than ELISA.

III. NUCLEIC ACID–BASED METHODS

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) contain ge–netic information for synthesis of all cell compounds of living organisms. Thus identity of any organism is therefore based on nucleic acid se–quence (Oliver 1993). All viable propagules, like virus particles, bacte–rial cells or fungal spores and mycelium, have the entire nucleic complement for that pathogen.

Probes

It is possible to isolate from plant pathogen a specific sequence of DNA which only couples (i.e.hybridizes) with identical sequence of DNA of the target organism. This is called a probe. Oliver (1993) stated that for pathogen identification selection of probes with the necessary degree of specificity is very important. The most commonly used is a dot blot method because it is quick, simple and a number of samples could to be processed at the same time.

Nucleic acid probes have been widly used in plant pathology as identi–fication is very correct and fast but only with pure colonies of pathogens. Several attempts were undertaken to use this method for bacteria (Schaad *et al.* 1989), fungi (Reeves 1995) and viruses (Lange 1986) but probes have not been applied in routine seed health testing because when seed extracts are probed directly contaminating DNA may cause problems in accurate detection of organisms. In this case it is better to extract organisms from seeds and often purified before probes are able to be applied (Maude 1996).

Polymerase chain reaction

The polymerase chain reaction (PCR) (Mullis and Faloona 1987) is a method which enables amplification and multiplication, up to a milionfold, of the target sequence of DNA (Saiki *et al.* 1988, Oliver 1993). Thus a previously undetectable amounts are transformed into detectable quantities. During amplification two primers are involved. They flank the DNA segment to be amplified and exposed to repeated cycles of heat denaturation of the DNA. The choice of primers is important (Fox 1993). The method leads to such a large increase in concentration that it makes more effective detection of DNA sequence by a special probe or gel electrophoresis (Old and Primrose 1985). Reactions are observed in small tubes placed in a block in a programmable thermal cycling machine. Usually about 30 cycles are used and each lasts about 2–5 min. (Ball and Reeves 1991, Fox 1993).

Several attemps have been made to develop species specific polymerase chain reaction (PCR) primers for plant pathogens (Hensen and French 1993). For identification of seed pathogens the method potentially could be used with seed washings (Vivian, 1992) without a need of extraction and isolation of the organism (Ball and Reeves 1991). However, large amount of extraneous DNA in seed samples may result in considerable non–specific hybridization with primer DNA (Vivian 1992).

The PCR techniques have been applied for detection of a few bacteria species e.g. *Pseudomonas syringae* pv. *phaseolicola* and *P.syringae* pv. *pisi* in bean and pea seeds, respectively (Prosen *et al.* 1991, 1993, Rasmussen and Wulff 1991, Reeves *et al.* 1994), *Pantoea* (*Erwinia*) *stewartii* in maize seeds (Blakemore and Reeves 1993). Schaad *et al.* (1995) developed a highly sensitive PCR technique named BIO–PCR to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seeds. Schaad *et al.* (1997) underlined advantages of BIO–PCR over classical PCR since it eliminates false positives due to dead cells and false negatives due to the presence of PCR inhibitors in seed extract and above all it is 100–fold more sensitive.

In the case of fungi, the PCR techniques have been mainly used for characterization and identification of these organisms (Reeves 1995) but so far there were only single examples of detection and identification of fungi in seeds, e.g. *Phomopsis* species in soybean seeds (Jaccoud–Filho and Reeves 1993). One of a more often applied PCR method for fungi identification involves the use of random amplified polymorphic DNA, called RAPD (Williams *et al.* 1990), but it cannot be used directly to detect seedborne organisms because of non–specific hybridization of the primer with extraneous DNA from seed extracts (Reeves 1995). As RAPD was not suitable for direct detection it was employed by Stevens *et al.* (1997) with the internal transcribed spacer (ITS) region of the nuclear ribosomal unit for development of a multiplex PCR seed health test which was able to detect and differentiate *Pyrenophora*

spp. pathogenic to barley. Jaccoud–Filho *et al.* (1997) applied RAPDs for development of the specific PCR–based test to detect *Phomopsis phaseoli* f.sp. *meridionalis* in soybean seeds. Smith *et al.* (1996) applied the BIO–PCR method for identification of *Tilletia indica*.

The majority of plant viruses have RNA genomes and as such are unsuitable for PCR (Fox 1993). Silicacapture reverse transcriptase polymerase chain reaction (SC–RT–PCR) (Boom *et al.*1990) and the immunocapture reverse transcriptase polymerase chain reaction (IC RT–PCR) (Levy and Hadidi 1991, Nolasco *et al.* 1993, Candresse *et al.* 1995) were developed to detect plant viruses. The IC–RT–PCR was applied by Van der Vlugt *et al.* (1997) for LMV detection in seed samples. In this method virus particles were trapped on the wall of an antiserum–coated tube (immunocapture, a step comparable to that of ELISA) and inhibitory plant extracts were removed by washing. RNA was released from the virus particles and used as a template for cDNA–synthesis using reverse transcriptase. The obtained cDNA is next amplified by PCR with the virus–specific primers. Analysis of the PCR product is usually performed by electrophoresis on agarose gel. The IC RT–PCR was also used to detect cherry leaf roll nepovirus (CLRV) isolates and strains from the seeds of *Betula pendula* by direct sequencing of their PCR products (Buttner *et al.* 1997).

Summarizing, in some cases different diagnostic methods for pathogen detection and identification in seeds should be employed to be sure of proper results, as exemplified by *Pantoea stewartii*, the cause of Stewart's disease of corn (Block *et al.* 1998). Seed transmission of this bacteria was evaluated by assays in greenhouse and in the field, by agar plating and ELISA of individual–kernels. Mguni *et al.* (1999) performed identification of *Xanthomonas campestris* pv. *campestris* strains on semi selective media, viscosity test, Biolog GN MicroPlate system, and also by pathogenicity tests. Identification of many fungi is performed both on blotter paper as well as agar media.

Diagnostic methods should be internationally standardized as they have to provide repeatable results (with statistical probability) within samples analyzed in different laboratories (Hewett 1987). When new methods are introduced it is usually too early to evaluate their usefulness (Langerak 1997). According to this author techniques based on immunology (ELISA and immunofluorescence microscopy) looked promising for introduction in 1980–ties as routine seed health testing methods but the progress in international standardization was rather slow due to poor availability of specific high quality antisera and differences in interpretation of the results and in consequence they became not as common as could be expected.

The choice of a method to be used for detection and identification of seed–borne pathogens depends on expected parasite. Majority of fungal pathogens still are commonly identified by incubation methods especially when incidence of a fungus is not very low. Bacteria detection and identi–

fication using laboratory tests may also be performed if the samples do not contain too low concentration of pathogenic and not too high concentration of saprotrohpic once. One has to remember that these traditional methods have to be performed by qualified staff and although they consume much time and space but till now they are specific enough and reliable to be employed as basic tests for detection and identification, especially of fungi. Serological methods are mainly useful for viruses detection and used also for bacteria. The nucleic acid–based methods have been tried for identification all three ethiological groups of pathogens since they are highly sensitive, very specific, fast and reliable. Several variants of PCR techniques present new approaches for seedborne bacteria, fungi and viruses but it should be known that with these methods it is still difficult to quantify infection levels and no direct interpretation can be given about viability and/or pathogenicity of detected pathogen.

Also the expences of performed tests should be considered. Traditional methods for seed pathogen detection, still well working for some fungal and bacterial pathogens, are much more cheaper as they do not require very sophisticated and expensive equipment for identification of many fungi and some bacteria. On the other hand accurate identification of suspected organisms is often difficult and time consuming which is also expensive. Furthermore, these methods normally work well when seed samples contain proportionally more target pathogens than saprotrophs. As the time to complete a diagnostic test is usually very important, development of rapid and specific methods for detection and identifing seedborne organisms is needed to increase frequency of tests and is benefitial, especially for commercial testing of seeds. Nucleic acid–based methods are relativelly expensive but they are rapid and require shorter period to achive a pathogen identification. In a long term nucleic acid tests could be cheaper, but quantification is a main disadvantage of these tests of seed health. Another problem is connected with seed material which may inhibit amplification of DNA because PCR is only reliable when pure DNA is amplified (Reeves 1995).

On the other hand when new and more advanced methods are introduced they should be at least as good as the techniques which are to be replaced (Sheppard 1993), e.g. IC–PCR and DAS–ELISA versus Pea Seedborne Mosaic Virus (PSbMV) detection. Latter test proved to be more sensitive (Phan *et al.* 1997). The immunofluorescence colony–staining method is a combination of isolation by pour plating with serology and this test offers sensitivity and quantitative detection of culturable target bacteria (Van Vuurde 1997).

Serological and nucleic acid–based methods are characterized by high simplicity, short time of performance, specificity and relability, but not all their variants are highly sensitive. Nethertheless, recent development in seed pathology technology allow for more ecofriendly seed treatments and more reliable seed health testing. Because the use of sophisticated DNA amplification techniques makes possible detection of

seedborne pathogens that might be undetected by conventional methods a research of the nucleic acid–based methods will be fundamental in guaranteeing seed health quality standards and achieving phytosanitary requirements throughout the world in the new millennium (Nameth 1998).

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