CHAPTER 11

DETECTION, QUANTIFICATION AND IMMUNOLOCALISATION OF *BOTRYTIS* SPECIES

Frances M. Dewey (Molly)[1] and David Yohalem[2]

[1]Department of Viticulture and Enology, University of California at Davis, Davis CA95616, USA;
[2]Horsekildevej 38. 1 tv, Valby DK-2500, Denmark

Abstract. Classical methods of detection of *Botrytis* species include plating-out of surface sterilized infected plant tissues, soils and airborne conidia on selective media and the identification, by microscopy, of the sclerotia, conidia and conidiophores, based on their characteristic shape, size and colour. Other methods are now available such as nucleic acid-based methods that can be used to track individual isolates or specific species. The determination of biomass levels in samples using these methods, however, is problematic because of the multinucleate nature of *Botrytis* conidia and thallus. Immunological methods employing genus-specific monoclonal antibodies, particularly quantitative laboratory-based plate-trapped antigen ELISAs, allow large numbers of samples to be processed easily within a few hours. These methods, combined with the modified plate spore trap, the Micro-Titre Immuno Spore Trap (MTIST), enable the quantification of conidia in microtitre wells. A rapid semi-quantitative immuno-chromatographic lateral flow device designed for use in the field or office promises to be a useful screening device for *Botrytis*. Development of species-specific monoclonal antibodies remains a challenge. The usefulness of Fourier transform infrared spectroscopy, nuclear magnetic resonance, liquid chromatography-mass spectroscopy and enzymic methods to detect and quantify specific secondary metabolites produced by *Botrytis* remains to be fully demonstrated.

1. Introduction

Detection and quantification of *Botrytis* infections in plants, seeds, air-borne conidia and sclerotia in soils has, until recently, depended on the plating out of infected material and the microscopic identification of sclerotia, conidia and conidiophores on the basis of their size, shape and colour. Although these methods yield valuable information, they are limited. Plating out is a time-consuming process in which surface sterilization is a general pre-requisite. Other methods are now becoming available that either yield more specific information, as is the case with molecular methods, or are faster and more easily replicated, as with immunological methods. These various methods will be addressed separately along with other quantitative methods.

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Sampling is a problem inherent to any detection assay. There are no sampling techniques unique to *Botrytis* spp., which has a generally rare (Poisson) spatial distribution and/or clumped (negative binomial) and sampling should be performed with the biology and epidemiology of the system being examined in mind (e.g. Marois et al., 1993). In the case of epidemiological studies, some stratification is often necessary to make meaningful inferences and different techniques are needed for different environments to address specific questions.

Assessing the extent of infection within a sample is also problematic. Care has to be taken when making comparisons that employ different methods. For example, the level of infection in a sample of grape berries assessed on a weight for weight basis will be different from that assessed on a number basis because rotted berries generally weigh less (Dewey et al., 2000). Estimates of fungal biomass of foliar infections are commonly determined by measuring lesion area (Elad et al., 1994), but these estimates are clearly different from levels based on percentage of leaf area that is sporulating (Köhl et al., 1995), the numbers of conidia derived from sporulating tissues by shaking at high speed in tap water with a detergent (Gerlagh et al., 2001) or CFU/cm^2 from macerated leaf samples plated out by limiting dilution (Lennox et al., 2003). The use of immunological methods to determine fungal biomass in individual and massed infections is promising (Sect. 3).

2. Classical plating out method

Common methods of surface sterilizing plant material prior to plating out include immersing excised tissues/seeds or fruits sequentially in sodium hypochlorite, ethanol and sterile distilled water (Meyer et al., 2000; Coertze and Holz, 2001). Where infected material is already sporulating identification can be confirmed by plating out single conidia picked up with a sterile needle. Special methods are employed for the detection of latent infections. Dipping berries in alcohol, freezing them at –20ºC for a short time and then incubating in a moist chamber at room temperature for 7-10 days until the fungus has sporulated has proved to be an effective simple method (Mundy and Beresford, 2003). Others have induced sporulation by treatment of surface sterilized tissues or fruits with paraquat to reveal latent infections in grape berries (Gindrat and Pezet, 1994; Pezet et al., 2003), strawberries (Sutton et al., 1997), sweet cherry fruit (Adaskaveg et al., 2000) and fallen rose petals (Morandi et al., 2000).

Semi-selective and differential media are based on the selective inhibition of competing microbes, the encouragement of the target organism's growth and/or the expression of a characteristic property of *Botrytis.* Viable conidia will germinate on many media, in the presence of free water, nitrogen and phosphate, and form colonies, but do not always sporulate. Confirmation of growth of any species of *Botrytis* requires microscopic examination. A medium based on Martin's rose bengal agar amended with several fungicides and high concentrations of antibiotics was found satisfactory for assaying organic soils (Lorbeer and Tichelaar, 1970). Kritzman and Netzler (1978) developed a medium for isolation of *Botrytis* species from soil and onion seed based on the development of dark pigments in the medium

due to degradation of tannic acid and resistance to PCNB and maneb. This medium was also used to monitor *Botrytis* populations in vineyards and green houses, and as a medium amended with fungicides to detect resistant populations in Israel (Elad et al., 1992). For the detection of conidia of *B. cinerea* caught in spore traps, Kerssies (1990) used a very similar medium to which fenarimol was added to inhibit growth from airborne conidia of *Penicillium* spp. However, this medium also shows the same browning with *B. aclada* and *B. allii* as with *B. cinerea* (D. Yohalem, unpubl.). A new *Botrytis*-selective medium and *Botrytis* spore trap medium have been developed by Edwards and Seddon (2001). Several basal media amended with a suite of fungicides have been used to monitor fungicide resistance within populations of *Botryotinia fuckeliana* (Baroffo et al., 2003).

3. Immunological methods

Development of immunological methods for the detection of *Botrytis* species has had a relatively long and chequered history with many unpublished reports. This is because antisera (polyclonal antibodies) raised to mycelial fragments or crude extracts lack the necessary specificity and commonly cross-react with related and unrelated fungi as well as with extracts from plant tissues (Dewey, 1996). However, Linfield et al. (1995) and Cousins et al. (1990) did raise antisera to *B. allii* and *B. tulipae* respectively that cross-reacted only weakly with unrelated species of fungi and extracts from host tissues. In testing antiserum from rabbits immunized with *B. cinerea*, Ricker et al. (1991) found that while antiserum from early bleeds was relatively specific, that from later bleeds cross-reacted strongly with *Aspergillus niger* and other fungi. Using purified, deglycosylated invertase from *B. cinerea* as the immunogen in chickens, Ruiz and Ruffner (2002) found that antibodies from the egg yolks did not cross-react with unrelated fungi or with extracts from uninfected grape berries. However, their antibodies did not recognize native invertase; they only recognized invertase in samples that had first been partially denatured by heat treatment.

The advent of hybridoma technology has made possible the production and selection of antibodies that are near-genus- or genus-specific to *Botrytis* (Bossi and Dewey, 1992; Meyer and Dewey, 2000). Attempts to raise species-specific monoclonal antibodies have not been successful but Salinas and Schots (1994) did produce three antibodies to *B. cinerea* each of which recognized a different array of *Botrytis* species. Despite comparative studies, it is still not clear what is the best source of antigens for raising taxonomically specific antibodies for detection purposes (Meyer and Dewey, 2000).

A number of different types of immunoassays have been used to detect, and to a lesser extent quantify, levels of *Botrytis* infections in plants. By far the most common are enzyme-linked immunosorbent assays, particularly plate trapped antigen-immunosorbent assays (PTA-ELISAs). Using antisera raised in rabbits, Ricker et al. (1991) demonstrated that PTA-ELISAs could be used to determine levels of *Botrytis* antigens in juice from infected grapes. More recently, the same protocol, with the genus-specific monoclonal antibody BC-12.CA4, was used to

detect and quantify *B. cinerea* in grape juice (Dewey et al., 2000), wines (Dewey, 2002), pear stems (Meyer et al., 2000), strawberries (L. Mehli, Institutt for Biologi, NTNU, Trondheim, Norway, pers. comm.), grape berries (K. Williamson, F.O. Obanor and M. Walters, HortResearch, Lincoln, NZ, pers. comm.), raspberries (Dewey, 2000), tomato fruit (Lurie et al., 2003) and latent *B. aclada* infections in onion leaves (Yohalem et al., 2004). The antigen detected by this antibody is produced constitutively, is highly stable, is not degraded by heat or freezing and is not metabolised during fermentation (Dewey, 2002). As standards for quantitative assays, extracts from freeze-dried mycelium of the fungus grown in liquid culture on grape juice have been used. The assay gives a linear correlation between absorbance values and mycelial extracts in the range of 10 ng/ml to 20 µg/ml (Dewey et al., 2000). This antibody has also been used to develop a 20-min tube assay for on-site quantification of *Botrytis* antigens in grape juice at wineries at harvest time (Dewey and Meyer, 2004), and to develop a semi-quantitative 4-minute immuno-chromatographic assay or lateral flow device (B-LFD) (M. Dewey and C. Danks, UC Davis, CA, USA and Central Scientific Laboratory York, UK, unpubl.). The latter, which is technically similar to LFDs developed for the detection of viruses in potatoes (Danks and Barker, 2000), is a very simple "user friendly" device that can be used in the field or office without any electrical power; the time taken for the appearance of the positive test band is related to the level of the *Botrytis*-antigen in the sample. The device is highly sensitive and has been used to detect early symptomless infections in artificially inoculated grape vine leaves (C. Aguero and M. Dewey, UC Davis, USA, unpubl.), pine seedlings (K. Capieau and E. Stenstrom, Swedish University of Agricultural Sciences (SLU), Upssala, Sweden, pers. comm.) and tomato fruits (Lurie et al., 2003). Development of a "stand-alone" scanner that measures the level of reflectance or intensity of the test band is under way (K.G. Wilson, KGW Enterprises, Indiana, USA).

Immunofluorescence techniques have been used to immunolocalize and follow infection paths of *B. cinerea* and *B. fabae* in *Vicia faba* (Cole et al., 1998a, b). In an elegant study, Kessel et al. (1999), using the same antibody (BC-KH4), determined effectively the biomass of *B. elliptica* in lily leaves co-inoculated with the biocontrol agent *Ulocladium atrum* by digital image analysis. *Botrytis* antibodies that have worked well by immunofluorescence have also proved useful in studies at the ultra-structural level. For example, Cook et al. (2000) by use of BC-KH4 which recognizes antigens present in the extracellular matrix of *Botrytis* species (Cole et al., 1998a, b) were able to show the active attachment of a potential biocontrol bacterium, *Enterobacter aerogenes*, to the extracellular matrix of geminating hyphae of *B. cinerea* and E. Zellinger and M. Dewey (Oxford Brookes University and University of Oxford, UK, unpubl.) have shown that the *Botrytis* antibody BC-12.CA4 strongly immunolabels the walls of *B. cinerea* hyphae, but not the conidia (Figure 1).

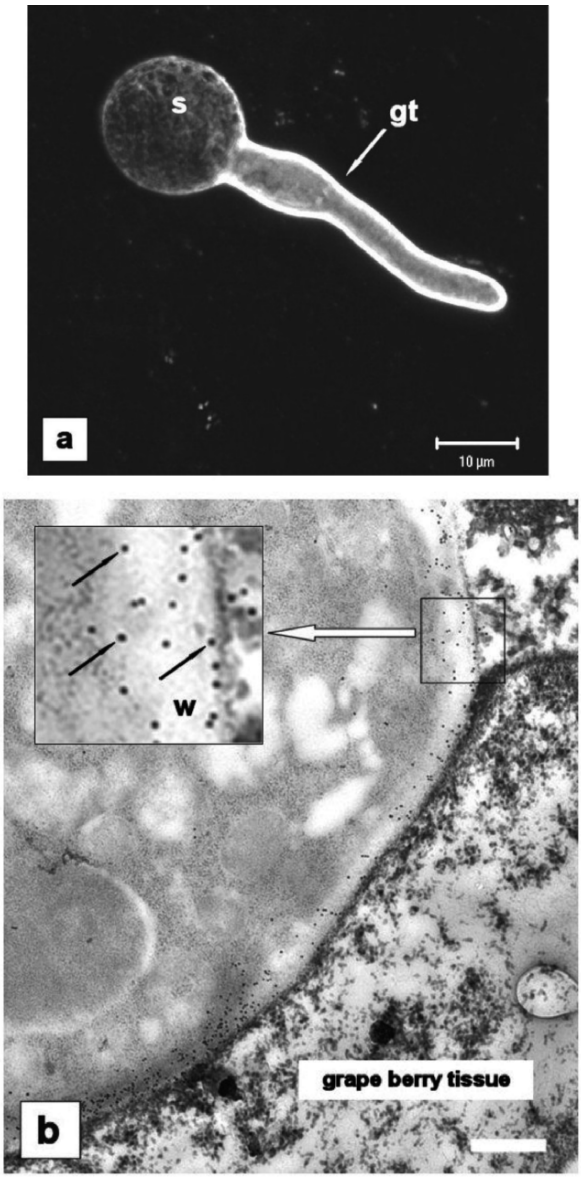

Figure 1. *Botrytis cinerea* immunolabelled in plant material. a. Confocal microscopy of *Botrytis cinerea* conidium germinated on outer surface of detached grape berry skin and immunolabelled with the monoclonal antibody BC-12.CA4 and anti-mouse FITC; note poor labelling of conidial wall but intense labelling of germ tube wall (gt); b. TEM of hypha of *B. cinerea* growing within detached grape berry tissue immunolabelled with BC-12.CA4 and anti-mouse 10 nm gold conjugate; note labelling within the wall of the hypha (w)

Immunofluorescent labelling techniques have also been used to detect, differentiate and enumerate air-borne conidia of *B. cinerea* trapped on tape in a Burkard 7-day volumetric suction spore trap (Dewey, 1996). Other methods of trapping and quantifying air-borne conidia have also been tried such as use of a modified Burkard portable air sampler for agar plates, the Micro-Titre Immuno Spore Trap (MTIST) (Kennedy et al., 2000). This device uses a suction system to directly trap air-particulates and conidia by impaction into micro-titre wells. The conidia are then allowed to germinate overnight in an appropriate buffer and are later quantified by PTA-ELISA.

4. Nucleic acid-based methods

With the development of nucleic acid-based techniques, modern epidemiology has moved closer to population genetics and population biology. Methods based on nucleic acids have been developed for the specific detection and diagnosis of sub-sets of the form genus (Nielsen et al., 2002), species of *Botrytis* (Mathur and Utkhede, 2002; Nielsen et al., 2002; Rigotti et al., 2002), sub-species (Giraud et al., 1999; Fournier et al., 2003; Martinez et al., 2003), populations (Luck and Gillings, 1995; Moyano et al., 2003) and individual isolates (Kerssies et al., 1997). Several of the methods are critical for proper identification of the causal organism (e.g. *B. cinerea* subspp. *vacuma* and *transposa* (Giraud et al., 1999) and *B. aclada* and *B. allii* (Yohalem et al., 2003), while others hasten diagnosis. In general, the broader groupings are detected with primer sets designed from random-sequence characterized fragments, that is, modifications of various sequence characterized amplified regions (SCAR) or from sequencing with arbitrary primer pairs (SWAPP). The finer groupings rely on either known genetic differences (e.g. Giraud et al., 1999) or randomly amplified polymorphic DNA (RAPD) or related fingerprint methods. Specific RNA transcripts have been detected, which can be used to reveal differential expression of *Botrytis* genes (Choquer et al., 2003).

4.1. Different types of molecular detection assays

Several methods have been used to detect and diagnose *B. cinerea.* Both Mathur and Utkhede (2002) and Rigotti et al. (2002) report direct detection of *B. cinerea* with specific primers sets. Mathur and Utkhede (2002) designed their primers based on sequence data obtained from the internally transcribed spacer (ITS) regions of ribosomal DNA (rDNA). They screened their primers against isolates of other common fungi associated with greenhouse tomato production and found little or no cross-reactivity. In contrast, Nielsen et al. (1998) did not find sufficient variation among ITS sequences within the genus to design primers for the detection of neck rot-associated *Botrytis* spp. Mathur and Utkhede (2002) used their primer set in a dot-blot assay which allows for the simultaneous detection of the pathogen in many samples, but also recommended it as part of an array in a reverse dot-blot test, which allows for the detection of many pathogens simultaneously in a single sample. Rigotti et al. (2002) designed 20-mer primers based on the sequence of a randomly amplified fragment common to all *B. cinerea* isolates screened. A single 0.7-kb band

was produced from all *B. cinerea* isolates tested while a 0.6-kb band was amplified from the DNA of *B. fabae.* They were able to detect 2 pg fungal DNA when mixed with 1 μg plant DNA.

The onion neck rot-associated species of *Botrytis* can be detected, as a group, by PCR amplification of a 413-bp sequence (Nielsen et al., 2002). Digestion of the amplification product with the restriction enzyme *Apo*1 clearly distinguishes *B. aclada, B. cinerea* and *B. squamosa* (Nielsen et al., 2002). A further amplification with rDNA ITS primers and digestion with *Sph*1 serves to separate *B. byssoidea* from the hybrid species, *B. allii* (Yohalem et al., 2003). Hence, the method can be used for detection of a suite of pathogens associated with the syndrome, or for diagnosis of the specific causal agent. Nielsen et al. (2002) report a detection limit of 1 pg DNA from pure cultures.

Population structure within *B. cinerea sensu lato* has been studied with a variety of genetic markers. A method was reported for differentiating benomyl resistant from susceptible isolates using PCR (Chapter 12; Luck and Gillings, 1995). A single base substitution was discovered that correlated with the mutation that conferred resistance, which was found to be detectable by cleavage into two fragments of a restriction digest of a 381-bp amplification product, while leaving the susceptible strains' product undigested. They also designed primers that were specific to the substitution site. The method proved useful for both pure cultures and for direct assay from infected plant tissues. Kerssies et al. (1997) used RAPD markers to distinguish isolates, but found no correlation between their markers and pathogenicity, time, nor sampling source. They reported a species-specific 45-kb band generated by primer D6 (Operon Technologies Inc., Alameda, CA, USA) from which, presumably, a SWAPP set could be designed. RAPD and amplified-fragment length polymorphism (AFLP) techniques have been directly compared and found equally satisfactory for revealing the genetic structure of populations of *B. cinerea* (Moyano et al., 2003). The *vacuma* and *transposa* groups of the *B. cinerea* complex can be separated by the absence or presence of two transposable elements called *Flipper* and *Boty*, respectively (Giraud et al., 1999; Chapters 3, 4 and 12). Multiple isolates can be screened using dot-blot hybridisation. The *Boty* transposon has subsequently been found in several *vacuma* strains (Martinez et al., 2003). However, these isolates can be distinguished using a PCR-RFLP method developed by Fournier et al. (2003). Muñoz et al. (2002) developed a duplex PCR scheme to test for the presence or absence of the transposons and, combined with four known genetic markers and RAPD-RFLP, report host differentiation between *transposa* and *vacuma* populations in Chile. Baraldi et al. (2002) have used RFLP with amplification products of four genes combined with two other genetic markers to examine diversity within and among populations of *B. cinerea* found on kiwifruits. Their data indicate recombination among populations and an association between cold-temperature adapted isolates and carbendazim resistance.

Northern blot hybridizations have been used to detect gene expression related to growth or infection by *Botrytis* and are proving to be a useful tool in elucidating infection processes. Benito et al. (1998) tracked transcripts of *B. cinerea* actin and β-tubulin genes during infection of tomato leaves, while Mengiste et al. (2003) used

the β-tubulin transcript in an *Arabidopsis* model system to make inferences about the effects of a host *Botrytis* susceptibility factor also required for stress response. Choquer et al. (2003) used real time-PCR (RT-PCR) to compare expression levels of several transcripts *in vitro* and *in planta.*

4.2. Dealing with problems related to molecular detection

Problems associated with PCR-inhibitory components of the fungal milieu (particularly soil and seed associated phenolics and tannins) can be addressed through more or less elaborate extraction and purification protocols. Nielsen et al. (2002) report reduced foaming from plant tissues extracted with a potassium ethyl xanthogenate (PEX) buffer as opposed to a cetyl trimethylammonium bromide (CTAB) buffer, which was adequate for working with pure culture material. The extraction efficiency decreases with increasing sample size in a non-linear fashion, i.e. 100-µg samples produce proportionally lower yields of DNA than do 50-µg samples, increasing the probability of obtaining a false negative result. These problems can be addressed partly by enhancement protocols. Magnetic capture hybridization (MCH) uses single-stranded DNA bonded to magnetic beads as a template for capturing and concentrating target DNA. DNA is lyzed from the beads and the lyzate subjected to PCR. The sensitivity of this method is reported to enhance the detection threshold for *B. aclada* using the primers of Nielsen et al. (2002) by a factor of ten (Walcott, 2003). The primary disadvantage has to do with expense and additional handling of the samples. In any case, the relation between a positive result and the amount of fungus in tissue is unclear. Because *Botrytis* spp. have multinucleate conidia and hyphae the relationship between amplification of a specific band and the amount of fungal biomass is difficult to assess. ITS sequences are found in variable numbers, often several hundred copies are found in a genome. This makes ITS-based detection very sensitive to the presence of fungal DNA, with reports of 50-100 fg detection (Moricca et al., 1998), c. 100 times more sensitive than the detection limit for a single copy amplicon (Nielsen et al., 2002).

In addition to primer design, various strategies have been developed to reduce sample handling, to automate the extraction, amplification and detection steps and to increase sample through-put (Lévesque, 2001). Sample handling is a major source of contamination, while extraction is the major bottleneck and source of system-specific procedural variation. The localization of the putative pathogen is critical to sampling from tissues (Lévesque, 2001; Taylor et al., 2001).

Reverse dot-blot hybridisation allows the simultaneous detection of many genomes from a single sample. Several probes are immobilized on a membrane and sample extract is presented to each probe. Detection of hybridisation reveals the identity of the pathogen. Mathur and Utkhede (2002) have suggested their *B. cinerea*-specific probe could be used in this manner. Multiplex PCR, in which distinct primer sets that produce amplicons with either unique electrophoretic characteristics or ligated to different fluorescent moieties, similarly allows detection of multiple pathogens simultaneously. Real-time PCR (RT-PCR), in which amplification products release fluorophores that are detected as they approach a threshold concentration, obviating the necessity for electrophoresis, offers the

advantages of reduced sample handling and risk of contamination, a semi-quantitative detection of product and a faster turn-around time. Multiplexing is also possible, as well, with RT-PCR. Combined with automated melting point analysis of the PCR products, RT-PCR provides a check that specific amplification has occurred (Taylor et al., 2001). However, both apparatus and reagents are considerably more expensive than standard thermo-cyclers. RT-PCR following reverse transcription of mRNA was used by Choquer et al. (2003) to detect the levels of chitin synthase transcripts in *B. cinerea*-infected tissues. They calibrated their amplification products against levels of plant-associated RNAs and were able to make inferences about several pathogenicity-related systems. Electronic and mass spectrophotometric detection of hybridization products improve the sensitivity of detection, remove the need for time-consuming electrophoresis and are used for detection in bio-chips in microarray procedures. *Botrytis* is among the candidate genera being included in several on-going bio-chip projects. All DNA-based methods for detection of *Botrytis* infections in plant materials are susceptible to sampling errors, in part because of the small amount of tissue from which extractions and inferences can be made. Further, sampling is, of necessity, destructive.

5. Other detection methods

Several techniques based on the detection of characteristic metabolites (e.g. Fourier transform infra-red (FT-IR) spectroscopy (Dubernet et al., 2000; U. Fischer, SLFA, Neustadt, Germany, pers. comm.) nuclear magnetic resonance spectroscopy, and liquid chromatography-mass spectroscopy (LC-MS) equipped with electro-spray injection, have been developed that do not require destructive sampling and are capable of evaluating both large numbers and sizes of samples. These methods, however, require specific calibrations for *Botrytis* products, which have not been investigated so far. Botrydial is an infection-associated sesquiterpene produced during grey mould infection. LC-MS has been used to detect its presence in various host tissues (Deighton et al., 2001). Similarly the presence of β-1,3-glucanase in grape berries and grape vine leaves has been taken as an indicator of *Botrytis* infections (Renault et al., 2000), as have laccase (Grassin and Dubourdie, 1989), gluconic acid and glycerol in grape juice (Perez et al., 1991), but great caution should be exercised before using these methods for detection of a pathogen species because other organisms may produce the same or similar compounds in tissues. Characteristic lipid profiles have been derived from pure cultures of *B. cinerea* (Cooper et al., 2000), but these have not been used for detection of the pathogen in plant tissues.

Indirect effects of *Botrytis* infection are observed in several hosts. Increases in ethylene and ethanol with concomitant decreases in acetaldehyde have been detected in stored tomatoes and correlate with grey mould infection (Polevaya et al., 2002). The protein profiles of must from grape cultivars change as a consequence of infection with *Botrytis* and can be detected by SDS-polyacrylamide gel electrophoresis (Marchal et al., 1998).

6. Conclusions

6.1. Comparative utility of the different methods

Classical methods are useful where time and space are not limited and in laboratories where a good microscope and a certain level of expertise are available. However, rapid methods are needed where decisions have to be made about applications of fungicides and the sale of fruits and flowers on the home market versus export. Where genus-specificity is sufficient, immunological methods, such as the Botrytis lateral flow device (B-LFD) which is semi-quantitative and takes minutes rather than hours, promise to be useful. The lack of antibodies that are species-specific means that immunological methods cannot be used for the detection of one target *Botrytis* species in the presence of others, as occurs in onion crops. Nucleic acid-based methods employing species- and isolate-specific probes are the only methods currently available that can be used to track an individual species or isolate of *Botrytis* in unlabelled wild populations. Much valuable information can be gained from such methods, but they are demanding in space, time, expertise and cost. The genus-specific antibody BC-12.CA4 has proved useful for determining the biomass of *B. cinerea* and *B. aclada*.

For quantitative assays it is important that the optimum level of dilution be determined for each type of tissue or juice and that a set of standards is run with each test. Similarly, semi-quantitative nucleic acid based-methods such as RT-PCR, which have proved useful with other fungi, promise to be useful for quantification of *Botrytis.* However, correlation between quantity of amplification product and biomass is unclear for several reasons primarily associated with DNA extraction efficiency and nuclear number, which is variable within a thallus.

6.2. Problems and recommendations

Detection and quantification of latent or quiescent infections remains the most challenging and important problem because, in most cases, classical methods are too slow. The B-LFD has proved to be highly sensitive and preliminary studies have shown that, if the devices are allowed to continue developing over a 20-min period, they can detect the presence of very low levels of *Botrytis* antigens in grape juice and tissues before any visible symptoms of infection are apparent.

Determination of biomass in fungal infections is important in many fields of research, particularly in determining levels of resistance in transgenic crops and the efficacy of biocontrol agents and new fungicides. For these tests, which often involve large numbers of samples, plate immunoassays are recommended (PTA-ELISAs). Plate immunoassays could also be used for detection of seed borne infections in crops with small seeds such as onions (cf. Detection of fungi in individual rice grains by immunoassay by Dewey et al., 1992). Localization and quantification in tissues using GUS transformants is also promising for research purposes where studies with the one isolate are sufficient as are epidemiological studies using Nit and selenium mutants (Barnes and Shaw, 2003; Weeds et al., 1998, respectively, and Chapter 3).

Assessments of the levels of air-borne *Botrytis* conidia in greenhouses and polyethylene-covered tunnels, as well as in the field by direct capture into micro-titre wells, raises the possibility of using nucleic acid-based methods to quantify specific isolates for epidemiological studies. Identification of *Botrytis* sclerotia in soils, which currently depends on plating out methods involving very long incubation periods, is laborious. It is possible that immunological methods employing antibodies that recognize washings from sclerotia soaked overnight in buffer in micro-titre wells could be used to study the life cycle of the pathogen in the field. Whatever method is used however, care has to be taken at every step from sampling to interpretation of results.

Deployment of the above methods devised during the past decade to detect, diagnose and quantify species of *Botrytis* should help greatly in furthering our understanding of the biology and epidemiology of *Botrytis* infections. The usefulness of metabolomic methods, such as FT-IR, still remains to be demonstrated.

7. References

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