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Development and evaluation of an enzyme-linked immunosorbent assay (ELISA) for the detection of loose smut of barley (*Ustilago nuda*)

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Abstract

Polyclonal antibodies were raised against mycelium from the logarithmic growth phase of a shake culture of *Ustilago nuda*, and a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) with biotinylated detection antibodies was developed. The detection limit of the assay was 15 ng total protein ml^{-1} for the homologous antigen and 50 ng ml^{-1} for a spore extract, respectively. Other species of *Ustilago* reacted with the antibodies. Cross-reactivity was highest with *U. tritici*. No signal was obtained with the tested isolates of *Tilletia*, *Rhizoctonia*, *Pythium* and *Fusarium*. With naturally infected barley seeds, the results of the ELISAs were always in good agreement with those obtained with the routinely used seed embryo test. However, when seeds grown from artificially inoculated florets were used, the ELISA indicated significantly higher infestation levels than the embryo test. Results of assays with halved seeds from the same lot showed that high amounts of mycelium were present in the non-embryo half. This and especially the relatively long duration of the assay suggested that the ELISA (as conducted here) may not be suitable as a routine method for analysing seed infection with *U. nuda*. With samples from barley seedlings grown from infected seeds the results of the immunoassay again corresponded very well with the infection level determined by staining of the seed embryo, irrespective of the mode of floret inoculation (natural or artificial). Potential fields of application of the ELISA include the early prediction of the efficacy of protection agents, e.g. in screenings for seed treatments, the elucidation of the biology of the fungus and characterisation of resistance mechanisms.

Abbreviations: cv. – cultivar; EC – growth stage according to Zadoks et al., 1974; IgG – Immunoglobuline G; ISTA – International Seed Testing Association; PBS – phosphate buffered saline.

Introduction

Ustilago nuda is a basidiomycete fungus that infects barley during flowering (floral-infecting smut). Teliospores are carried into the flower by wind and settle mainly at the shoulder of the ovary. The spores germinate and dicaryotic infectious hyphae invade the developing seed by penetration of the pericarp. The fungus grows through the seed coat into the scutellum and embryo at the base of the seed (Malik and Batts, 1960a). After germination of the seed, the mycelium permeates the crown node and enters the growing point of the tillers. The fungus is carried passively up with the plant growing point, which eventually develops into a smutted ear (Malik and Batts, 1960b).

Neither the infected seeds nor the developing plants show any obvious or unambiguous macroscopic symptoms until appearance of infected ears.

Detection of *U. nuda* in barley seed stocks is routinely done by an embryo test (Morton, 1961; Rennie, 1990). Embryos are extracted from seeds by soaking in NaOH, cleared in boiling lactophenol and stained with trypan blue. Infected embryos can be identified under a dissecting microscope because of selective uptake of the stain by fungal hyphae. The correlation between the results of this test and the incidence of loose smut in the field is high (Rennie and Seaton, 1975). However, the test is laborious and time consuming. To our knowledge, development of other methods for determining the infection rate of seed lots with *U. nuda* has not been attempted. Similarly, methods alternative to tracing the mycelium of *U. nuda* in green plants by histological examination are not available. Because *U. nuda* colonises the plant without obvious disease symptoms, development of such methods would be advantageous for resistance breeding, for development of control measures and for studies on the relationship between the fungus and its host plant.

Modern diagnostic tools are increasingly used for the detection of viral, bacterial and fungal pathogens (Schots et al., 1994; Dewey and Thornton, 1995; Goulter and Randles, 1997). Enzyme-linked immunosorbent assays (ELISAs) are routinely applied for the detection of various plant viruses (e.g. Goth et al., 1999; Perry et al., 2000) and have also been developed for a number of fungal pathogens (e.g. Thornton et al., 1999; Meyer et al., 2000). ELISAs allow sensitive and specific detection of taxonomic groups or species and, unlike most DNA-based techniques, quantification of pathogens in the host tissue.

The objectives of this study, which represents parts of a doctoral thesis (Eibel, 2002), were to develop an immunological method to detect *U. nuda* and to test its potential use in seed health testing and experiments involving diseased plants. For this purpose polyclonal antibodies were raised against *U. nuda* and used for the development of a double antibody sandwich ELISA. Sensitivity and specificity of the assay were characterised, and detection of *U. nuda* in seeds was evaluated in relation to the traditional embryo test. Furthermore, the potential of the ELISA to diagnose *U. nuda* in green plant tissue was examined.

Materials and methods

Cultivation of fungi on solid media

The fungal cultures originated from culture collections or were freshly prepared from teliospores taken from the respective hosts (Table 1). For preparation of fresh cultures, the spores were suspended in sterile water (+0.05% Tween 20) and incubated on water agar. After germination had occurred (usually within 24 h) the isolates were viewed under a microscope to confirm the absence of contaminants and a piece of agar containing a single germinated spore was transferred to malt extract agar (MPA; 30 g l^{-1} malt extract, 3 g l^{-1} peptone from soybean [Merck], 1.6% w/v agar). All cultures of smut fungi were maintained at 15–18 °C on MPA. *Rhizoctonia solani* and *Fusarium graminearum* were kept on the same medium at 20 °C, whereas *Pythium ultimum* was cultured at 20 °C on Czapek-Agar (Difco Czapek-Dox broth solidified with 1.6% agar).

Growth conditions of U. nuda *and preparation of antigens*

For the production of sufficient quantities of antigen, various media and growth conditions were tested (data not shown in the results section). Antigens were prepared from culture filtrate or mycelium from the logarithmic growth phase of shake cultures of *U. nuda* BV (Table 1) (20 °C, 130 rpm). The filtrate was taken from cultures of *U. nuda* in MPB (malt extract broth: 30 g l^{-1} malt extract, 3 g l^{-1} peptone from soybean; Merck), whereas the mycelium was harvested from a culture in CzPepV (Czapek medium [Difco Czapek-Dox broth] supplemented with 5 g l^{-1} casein hydrolysate and, added after autoclaving as stock solution [prepared in sterile distilled water], with 2 mg l^{-1} nicotinic acid, 2 mg l^{-1} pyridoxol hydrochloride and 20 mg l^{-1} thiamine dichloride). In the latter medium the ingredients were dissolved in straw extract instead of water. The straw extract was prepared by placing chopped barley straw (length approximately 1 cm) in distilled water (40 g l^{-1}), boiling the suspension for about 5 min and separating the straw from the extract by filtration.

Antigens from mycelium were extracted as described below (see 'Preparation of extracts from

Table 1. Fungal isolates used

Isolate	Newly isolated	Culture collection	Origin
Ustilago nuda BV	X		Field (Bad Vilbel, D)
U. nuda 67019		X	BBA, PS-D[a]
U. nuda 67079		X	BBA, PS-D
U. nuda 67121		X	BBA, PS-D
U. avenae	X		Field (St. Aubin, CH)
U. hordei	X		Field (St. Aubin, CH)
U. tritici W	X		BBA, BI[b] (Greenhouse-grown wheat)
U. tritici 25	X		BBA, BI (Greenhouse-grown wheat)
Tilletia caries W		X	BBA, BI
T. controversa 67201		X	BBA, PS-D
T. controversa 67202		X	BBA, PS-D
Rhizoctonia solani		X	BBA, BI
Pythium ultimum		X	BBA, BI
Fusarium graminearum		X	BBA, BI

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different fungi and from spores of *U. nuda*'). Antigen from culture filtrate was prepared by dialysing approximately 400 ml culture filtrate against pre-cooled distilled water overnight at 4 °C (Visking dialysis tubing 20/32, Serva, Heidelberg), followed by lyophilisation of the culture filtrate. The lyophylisate was re-suspended in approximately 4 ml PBS (NaCl 8 g, KH_2PO_4 0.2 g, $Na_2HPO_4 \cdot 2\ H_2O$ 1.44 g, KCl 0.2 g, NaN_3 0.2 g, distilled water ad 1000 ml, pH 7.2–7.4) and the resulting solution dialysed overnight at 4 °C against PBS.

Preparation of IgGs

The sera were kindly supplied by Dr. W. Huth and Karin Balke, Federal Biological Research Centre for Agriculture and Forestry (BBA), Institute for Plant Virology, Microbiology and Biological Safety (Braunschweig). Rabbits were immunized by injection of antigen emulsified with Freund's adjuvant (first injection complete, all others incomplete). Three injections were given at 2-week intervals and a booster injection about four months after the first. Blood samples were taken one week after the second, third and last injection. Unless otherwise stated, all results reported here were obtained with antibodies prepared from the last sampling. Purification of antibodies was based on the method described by Clark and Adams (1977). To a 1:10 dilution of serum in distilled water an equal volume of 3.9 molar ammonium sulphate was added, and the precipitating proteins were pelleted by incubation on ice for 60 min followed by 20 min centrifugation at 12,000 g (4 °C). The pellet was re-suspended in PBS and desalted by 15 h dialysis (Visking dialysis tubing, 13 kD pore size, Serva, Heidelberg) at 4 °C against four changes of half concentrated PBS. Proteins were fractionated by ion-exchange-chromatography (DEAE-Fractogel [S], Merck, Darmstadt). The gel was equilibrated, and the protein solution eluated with half-concentrated PBS + 0.02% NaN_3. Eluate fractions with $A_{280} > 0.6$ were combined and stored at −20 °C or, after addition of NaN_3 (final concentration 0.05%) at 4 °C.

Part of the antibodies was labelled with biotin according to Bieber (1990). Purified IgG-solution (1.5 ml) was dialysed for about 24 h at 4 °C against coupling buffer (10 g l^{-1} NaCl, 10 g l^{-1} $NaHCO_3$, pH 7.5). Coupling was done at room temperature by addition of 75 µl biotinylization-reagent (1 mg Biotin-Amido-Caproyl-*N*-Hydroxysuccinimide-Ester [Sigma] per ml Dimethylformamide [Serva]) and stopped after 30 min by adding 75 µl 1 M Tris–HCl (pH 7.4). Finally the solution was dialysed for 15 h at 4 °C against a solution of 0.85% NaCl to remove free biotin molecules. Biotinylated IgGs were stabilised by addition of bovine serum albumin (BSA, final concentration 1%) and stored as described above.

ELISA protocol

For detection of antigens a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with biotinylated detection antibodies was used. Working volume was 100 µl per well. Ninety-six-well microtiter plates (U96 Maxisorp, Nunc, Wiesbaden) were coated with a 1:1000 dilution of IgGs (unless stated otherwise) in coating buffer (Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, NaN_3 0.2 g, distilled water ad 1000 ml, pH 9.6) (100 µl per well) and incubated at 4 °C overnight. Each well was filled with 200 µl blocking buffer (coating buffer containing 0.2% BSA [Fraction V, Serva, Heidelberg]) to prevent non-specific adsorption of protein to the well surfaces and the plates incubated for 2 h at room temperature (blocking step). After the plant or fungal extracts were applied (100 µl per well) the plates were incubated at 4 °C overnight and filled with 100 µl per well biotinylated IgGs (diluted 1:1500 in PBS-Tween [PBS containing 0.05% Tween 20] + 0.2% BSA, unless stated otherwise). Following incubation at 4 °C overnight, 100 µl streptavidin-alkaline phosphatase (StrAP) (Roche, Mannheim) solution (diluted 1:7500 in PBS-Tween + 0.2% BSA) were added to each well and the plates incubated for 30 min at 37 °C. Finally, 100 µl substrate (para-nitrophenylphosphate, pNPP; Serva, Heidelberg) solution (1 mg pNPP per 1 ml substrate buffer [1 M diethanolamine in distilled water adjusted with HCl to pH 9.8.]) were added to each well.

Each of the above-mentioned steps (addition of blocking reagent, addition of plant or fungal extract, addition of biotinylated IgGs, addition of StrAP, addition of substrate) was preceded by three washes for 3 min each with half-strength PBS-Tween.

For development of the colour reaction the plates were incubated at room temperature in the dark for 2 h (if not stated otherwise). Absorbances were measured at 405 nm (A_{405}; reference wavelength 592 nm) with a microplate reader (Spectra Mini, Tecan, Crailsheim, Germany). Each sample was at least tested twice and the mean taken for evaluation.

For the determination of total protein content of samples, the homologous antigen with known total protein concentrations (see 'Protein assay' below) was serial diluted in PBS. The dilutions were used as positive standards and run in parallel with the samples in the ELISA. From the resulting absorbances calibration curves were generated and the unknown protein content of samples extrapolated.

Data processing, generating of calibration curves from protein standards and determination of protein content of samples were done with the software 'Easfit' v 7.31 (Tecan).

Classification of samples as 'infected' or 'healthy' was determined in relation to the value and variation of the controls. Values higher than the threshold '$Mean_{(controls)} + 3 \times$ standard $deviation_{(controls)}$' were regarded as 'infected' (Clark, 1981; Sutula et al., 1986).

Preparation of extracts from different fungi and from spores of U. nuda

Fungal extracts were prepared from mycelia produced in liquid shake cultures. Fungi were grown at 20 °C (*T. caries* and *T. controversa* at 15 °C) in MPB except *P. ultimum*, which was cultured in Czapek medium. Mycelial mass was separated from the culture with a Büchner funnel equipped with a filter paper circle (No. 595, Schleicher & Schuell, Dassel, Germany). The mycelium was pulverised with quartz sand and liquid nitrogen and homogenised with a low volume of extraction buffer (PBS containing 0.05% Tween 20 and 0.2% polyvinyl-pyrrolidone 40,000) in a mortar. The homogenate was centrifuged for 10 min at 30,000 g (4 °C) followed by 30 min at 45,000 g (4 °C) and the supernatant stored at −20 °C until used. The same procedure was used for preparation of an extract from *U. nuda* spores collected from smutted ears of field-grown barley.

The fungal extracts were used as samples in the ELISA to test the specifity of the antibodies. Dilutions of the spore extract and the homologous antigen (mycelial extract of *U. nuda* isolate BV) were used in the ELISA for the determination of the sensitivity of the antibodies.

Protein assay

Total protein content of mycelial extracts (including the homologous antigen preparation) and the spore homogenate was determined in a spectrophotometer (Uvikon Spectrophotometer 922, Kontron Instruments) or in a microplate reader (Spectra Mini, Tecan) (Bradford, 1976).

Seed material and embryo test

The seed lots used are listed in Table 2. The naturally infected seeds were derived from field-grown barley. Artificial inoculation was based on the partial vacuum method (Moore, 1936) or injection of spore suspensions into single florets with a syringe (Poehlman, 1945).

Seed infection with *U. nuda* was determined under a dissecting microscope after separation of embryos from kernels in 10% NaOH, clearing of embryos and staining of the mycelium with boiling lactophenol containing trypan blue (seed embryo test, Morton, 1961).

Preparation of seed and plant extracts for the ELISA

Complete seeds were placed singly in the cavities of 96-well microtiter plates, and 300 µl extraction buffer were added to each cavity. After incubation of plates at 4 °C for 24 h the kernels in the cavities were cut up into approximately 3 mm diameter pieces using small pointed scissors. After addition of another 300 µl extraction buffer to each cavity, the plates were shaken for about 5 min using the shake mode of a microplate reader. The resulting suspension was pipetted into reaction tubes, centrifuged for 20 min at 20,000 g (4 °C) and the supernatant used for the ELISA. The ELISAs with extracts from complete seeds were done with seed lots I (105 samples), II (103 samples) and III (30 samples) (Table 2). The number of control samples (extracts prepared from seeds of healthy seed lots from the same cultivars and tested in parallel) was in each case 6 for seed lots I and II and 8 for seed lot III.

In another experiment 31 seeds from seed lot III (control: 7 seeds of a healthy seed lot from the same cultivar) were first cut across before they were placed separately in the cavities of the microplate. The resulting two halves ('embryo-half' and 'non-embryo-half') were extracted in parallel as described above for complete seeds.

Seeds of barley cv. Comtessa were sown in a mixture (1:3) of sand: commercial potting substrate (FRUHSTORFER® ERDE Typ T; Industrie-Erdenwerk Archut, Lauterbach, Germany) in plastic pots. The pots were placed in a growth chamber at 20 °C and 16 h lighting from fluorescent lamps. At growth stage EC 12 (Zadoks et al., 1974) the seedlings (83 grown from infected seed lot IV, 29 grown from a healthy control batch) were removed from the pots and rinsed with tap water. Seed coat, endosperm, scutellum and roots were carefully removed with a scalpel, and the part of the plant above the base of the first leaf was cut off. The remaining portion was weighed and after addition of ice-cold extraction buffer (1:5 w/v) homogenised with a mortar and a pestle. The homogenate was centrifuged for 20 min at 20,000 g (4 °C) and the supernatant stored at −20 °C until use.

For testing of the optimum dilution of samples, plant extracts were diluted between 1:5 and 1:500 in extraction buffer and used in the ELISA. Extracts from infected and healthy barley seedlings were used as well to compare the quality of the different antibody fractions.

Results

Sensitivity and specificity of antisera

Quality of the antisera from rabbits injected with antigens from mycelial extract (in the following termed 'My') and from rabbits injected with antigens from culture filtrate ('Cf') was compared using the ELISA with extracts of seedlings (EC 12) (Figure 1). Antibodies collected from the second

Table 2. Seed lots used

Number	Type	Mode of inoculation or infection	Embryo infection [%][a]
I	Winter barley cv. Tapir	natural	2.6 (n = 227)[b]
II	Winter barley cv. Tapir	natural	14.4 (n = 449)
III	Spring barley cv. Comtessa	vacuum	54 (n = 39)
IV	Spring barley cv. Comtessa	injection	37 (n = 92)

[a] As determined by the embryo test (Morton, 1961).
[b] Number of seeds used for determination of infection.

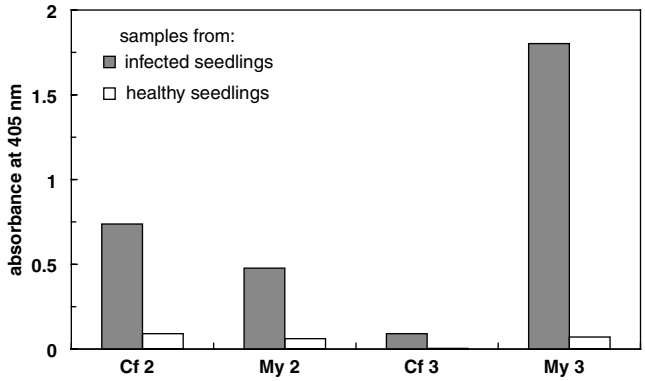

Figure 1. Reactivity of antibody preparations in ELISAs with extracts from healthy or *U. nuda*-infected barley seedlings; Cf 2 (3) = preparations from culture filtrate, second (third) bleeding; My 2 (3) = preparations from mycelium, second (third) bleeding. Dilution of coating and detection antibodies 1:1000 each.

bleeding and directed against antigens in culture filtrates (Cf2) were slightly more reactive than the respective antibodies directed against antigens in mycelial extracts (My2). However, in sera collected after the booster injection (third bleeding) sensitivity of antibodies raised against culture filtrate (Cf3) had dropped, whereas the reactivity of antibodies directed against antigens in mycelium (My3) had sharply increased. With extracts from healthy seedlings absorbances of ELISAs with all four antibodies remained below $A_{405} = 0.1$. Based on these results, all following assays were run with antibody preparation My3.

In dilutions of the extracts of infected seedlings the antigen was detected up to a dilution of 1:100 (Figure 2). At the dilution 1:500 the resulting absorbance was not different from the absorbance in the assay with the extract from healthy plants. Dilution of extracts of healthy plants had no significant effect on the resulting absorbance (data not shown). No interfering background reactions were detected even with the lowest sample dilution tested. Therefore, the optimum dilution of plant extracts for fast and clear detection of the pathogen was 1:5.

Serial dilutions of the homologous antigen and an extract from spores in ELISAs with antibody preparation My3 resulted in an asymptotic relationship between the absorbance values and the concentration of the antigen extract. Higher ab-

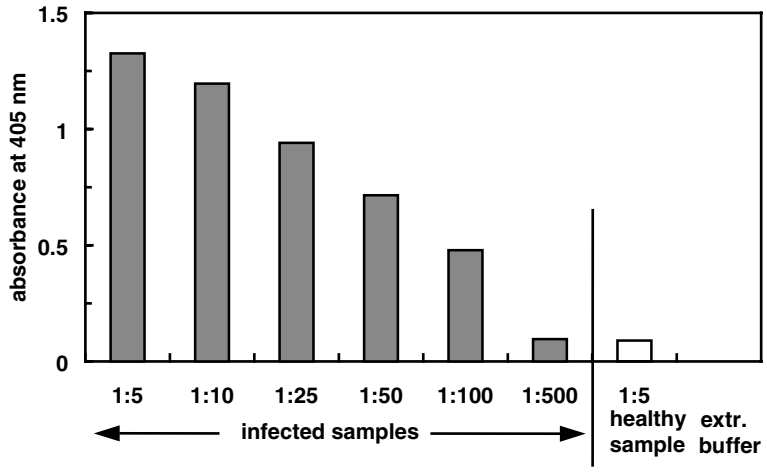

Figure 2. Reactivity of antibody preparation My3 with dilutions of extracts from healthy or *U. nuda*-infected barley seedlings, determined by ELISA.

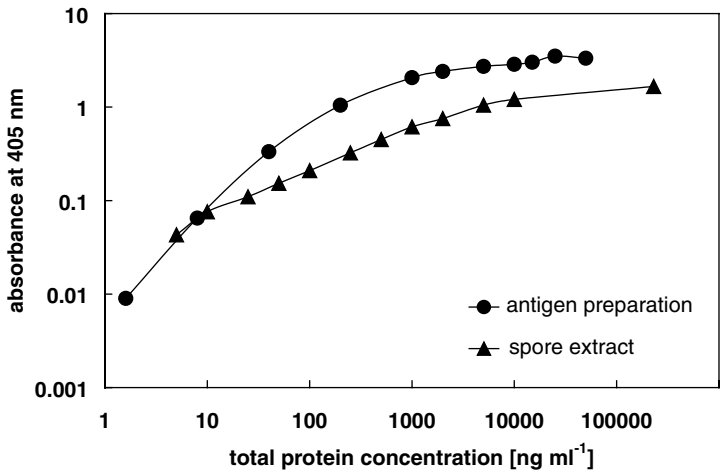

Figure 3. Reactivity of antibody preparation My3 in ELISAs with dilutions of the homologous antigen preparation and a spore extract of *U. nuda* teliospores (expressed as total protein concentration) (incubation: 3 h).

sorbances were consistently obtained in assays with the homologous antigen (Figure 3). After 3 h of incubation, the homologous antigen could be detected at a concentration of about 15–20 ng protein ml^{-1} in case of the homologous antigen, and 50 ng protein ml^{-1} in case of the spore extract ($A_{405} \leq 0.15$ unspecific; data not shown).

Specificity of antibody preparation My3 was evaluated in an ELISA with homogenates prepared from mycelia of different smut fungi and one isolate each of *R. solani*, *P. ultimum* and *F. graminearum* (Figure 4). Highest absorbances were measured in the assays with mycelial homogenates of the four *U. nuda* isolates (including isolate BV that was used for antigen preparation). Absorbances were also high in the assay with the two isolates of *U. tritici*. Some cross-reactivity also occurred in assays with extracts from *U. maydis* and *U. hordei*, whereas no signal was obtained with extracts from *U. avenae*, *T. caries*, *T. controversa*, *R. solani*, *P. ultimum* and *F. graminearum*.

Detection of U. nuda ad planta

In order to test wether *U. nuda* mycelium could be detected in seed kernels, ELISAs were performed

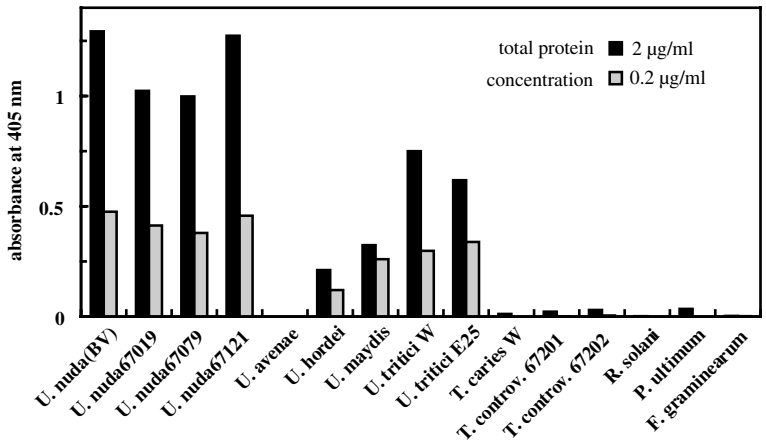

Figure 4. Reactivity of antibody preparation My3 in ELISAs with mycelial extracts of different species of *Ustilago*, *Tilletia* and other fungi. Dilution of coating and detection antibodies 1:750 each. Isolate BV was used for antigen preparation.

with single grains from two barley seed lots naturally infected with *U. nuda*. Based on the definition of the threshold value (see Materials and methods), the results of the ELISAs gave 2.9% infection in case of seed lot I and 17.5% in case of seed lot II (Figure 5). These values correspond well with the results obtained by the embryo staining method (2.6% and 14.4%, respectively; Table 2). The absorbances of the presumably infected kernels were, in almost all cases, higher in seed lot II than in seed lot I, indicating that seed lot II not only had a higher percentage of *U. nuda* infection, but that the infected kernels in this seed lot also carried more mycelium (antigen) than in seed lot I. In the three presumably infected kernels of seed lot I, the calculated average protein content was 136 ng ml^{-1}, whereas in the 16 kernels suspected to be infected of seed lot II it was 1250 ng ml^{-1}.

When seeds originating from inoculation by the partial vacuum method (seed lot III) were employed in the assay (Figure 6), the correlation between the results of the embryo staining method and the ELISA was poor. Whereas, with the embryo staining method only 54% infected embryos were observed, the result of the ELISA, based on the absorbance threshold of 0.23, suggested that all kernels were infected (Figure 6). An explanation for this discrepancy could be the fact that the embryo staining method disregards any mycelium present in other parts of the kernel, whereas the ELISA detected antigen in whole kernels.

In order to test this assumption, kernels of the same seed lot (III) were cut across into halves that were assayed separately. Absorbances measured with the 'embryo-half' were on average higher than absorbances measured with the corresponding 'non-embryo half'. However, in the infected seed lot the absolute absorbance values of the non-embryo halves were much higher than the absorbances obtained with the healthy seed lot (Figure 7). These results indicate that in kernels from inoculation with the vacuum method, the presence of mycelium of *U. nuda* was not restricted to the embryo, confirming the above assumption.

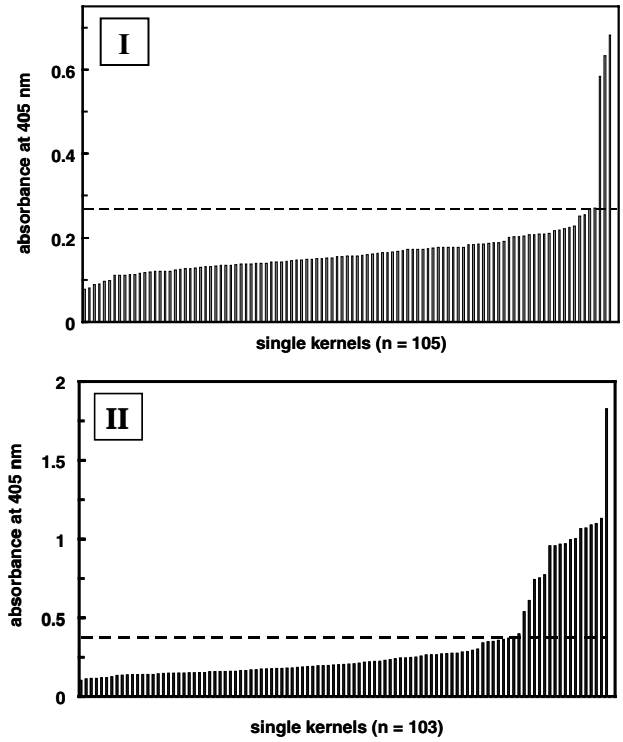

Figure 5. Detection of *Ustilago nuda* by ELISA in barley seeds from two naturally infected seed lots: (I) Infection rate according to embryo staining method 2.6%; (II) Infection rate according to embryo staining method 14.4%. The broken lines indicate the threshold between healthy and infected kernels, calculated from the respective healthy control (not shown).

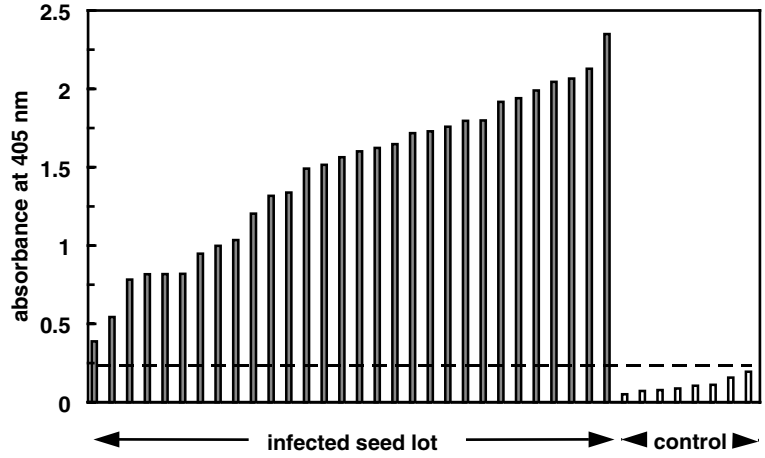

Figure 6. Detection of *Ustilago nuda* by ELISA in barley seeds (cv. Comtessa) from ears vacuum-inoculated with *U. nuda*. Infection rate according to embryo staining method: 54%. The broken line indicates the threshold between healthy and infected kernels, calculated from the healthy control.

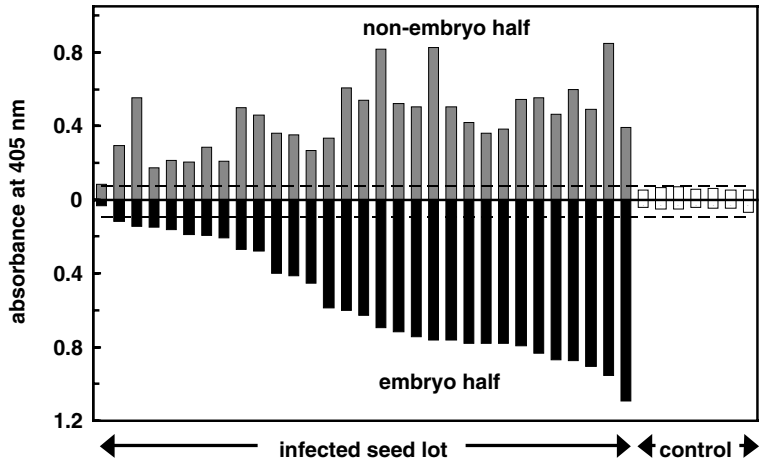

Figure 7. Detection of *Ustilago nuda* by ELISA in halved barley seeds. The seed lot was the same as in Figure 6. The broken lines indicate the threshold between healthy and infected samples, calculated from the respective healthy control.

Whereas extracts from seeds often showed some degree of cloudiness resulting from components of the grain (especially cv. Tapir), samples from seedlings were always clear. Therefore absorbances of healthy samples were in the range of $A_{405} = 0.05–0.1$ in seedlings but up to 0.36 in case of seeds. These differences are also reflected in the different threshold values for seedlings and seeds.

For the detection of *U. nuda* in plants, an ELISA was performed with barley seedlings (EC 12) grown from infected seed lot IV. Based on the absorbance threshold of about 0.1, 32 of the 83 tested seedlings were identified as infected (=38.6%) (Figure 8). Exclusion of the two samples that were only marginal above the threshold results in an infecftion rate of 36.1%. Both findings correspond very well with the value determined by the embryo staining method (37.0%).

Discussion

The antibodies obtained after injection of mycelial extracts (My2 and My3), grown in CzPepV, and from culture filtrates (Cf2) of MPB clearly detected *U. nuda* in infected seedlings. The highest

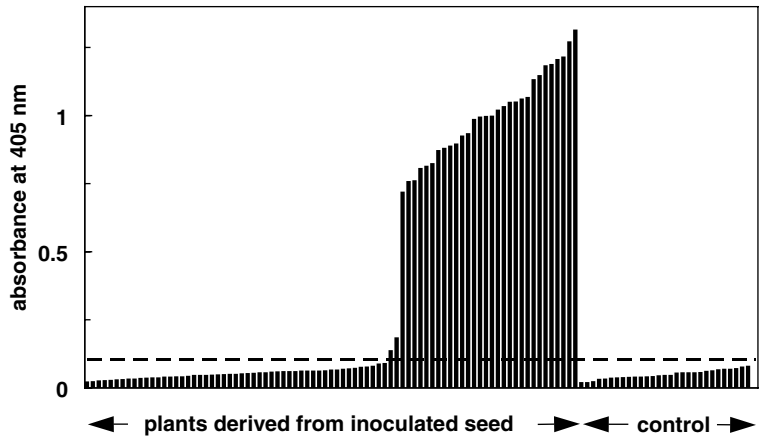

Figure 8. Detection of *U. nuda* by ELISA in extracts from barley seedlings (cv. Comtessa, EC 12). According to the embryo staining method, the infection rate with *Ustilago nuda* of the infected seed lot was 37%. The broken line indicates the threshold between healthy and infected samples, calculated from the plant samples that were derived from a healthy seed lot (control, right).

reactivity was observed with the antibody preparation directed against mycelium of *U. nuda* and taken at the last bleeding (My3) (after the booster injection). This is in agreement with studies of Brill et al. (1994) and Černuško and Wolf (1997). With fair success the authors of these reports also tested culture filtrates as sources of antigens. In our study reactivity of Cf2 was promising as well, and usually antibodies derived after the booster injection are even more reactive. But reactivity of the respective antibodies Cf3 had sharply dropped. However, as antibodies My3 could be used in further experiments for the detection of *U. nuda*, the reasons for the low reactivity of antibodies Cf3 were not investigated.

An important feature of diagnostic assays is their sensitivity. Against antigenic proteins and polysaccharides the limit of detection in ELISAs can be as low as 1 ng ml^{-1} (Clark and Adams, 1977). Usual values for plant pathogenic fungi vary between 2 and 50 ng ml^{-1} protein fraction (Mohan, 1988; Waldow, 1997). Similar limits were observed with our ELISA for detection of *U. nuda*. With 50 ng ml^{-1} the limit of detection in a spore extract was higher than in case of the homologous antigen (15 ng ml^{-1}).

An equally important characteristic in diagnostics is the specificity of the method. As could be expected, highest absorbances were recorded for the antigen (isolate BV) and the extracts from three other isolates of *U. nuda*, whereas no signal was detected with the three non-smut fungi tested. Among the other smut fungi, serological relatedness was lowest with *U. avenae* and highest with the two isolates of *U. tritici*. Whether or not *U. nuda* and *U. tritici* are different species or simply two forms of the same species has been disputed (Nielsen, 1972). Results of more recent studies, employing biochemical (e.g. Kim et al., 1984) and molecular techniques (Menzies et al., 2003) support the separation into distinct species. Although *U. nuda* and *U. tritici* infect different host species, they offer a number of similarities in morphology and biology, in particular infection type and spore morphology. This led to the single-species concept for the loose smuts of barley and wheat (Ainsworth and Sampson, 1950; Fischer and Shaw, 1953; Lindeberg and Nannfeldt, 1959). Irrespective of their final taxonomic classification these two closely related fungi obviously share various antigenic properties, which explains their similar reaction in the ELISA.

The ELISA reacted with all isolates of *Ustilago* except *U. avenae*. With different isolates of *U. nuda*, similar, high absorbances were obtained, indicating that the raised antibodies were not singly restricted to isolate BV that was used for antigen preparation. Some cross-reactivity of the ELISA was recorded with other species of *Ustilago*. However, because of the host-specificity of the smut fungi, in tests involving plant material this is not of practical relevance.

The usability of the ELISA to detect *U. nuda* in plant material was tested on seedlings and seeds.

Since seed lots may react differently, seeds carrying different degrees of infection, derived from both natural and artificial inoculation, were employed. With naturally infected seeds, the results obtained by ELISA were in good agreement with those of the embryo test. In the presumably infected kernels of the seed lot with low infection (seed lot I), and with another seed lot carrying a moderate percentage of infection (7.7%, data not shown), the average content of the antigen was lower than in the seed lot with a higher degree of infection (seed lot II). This may indicate that under conditions favourable for infection (e.g. air humidity, stage of flower) not only a higher percentage of florets gets infected but also that colonization of the developing seeds by *U. nuda* is favoured, resulting in higher amounts of mycelium per kernel.

When artificially inoculated seeds were assayed, the correlation between the result of the embryo staining method and the ELISA was poor. This could be taken as indication that the mode of inoculation (natural vs. artificial) has an effect on the distribution of the fungus within the seed. The teliospores of *U. nuda* germinate on the ovary wall, hyphae penetrate the pericarp and grow in longitudinal direction through the seed coat into the scutellum and embryo. Consequently, mycelium may not only be present in the embryo but also in distant parts of the seed (Malik and Batts, 1960a). Indeed, results obtained in an ELISA with halved seeds from artificial inoculation showed that high amounts of mycelium were present also in the non-embryo half, which may explain the poor correlation between the ELISA and embryo test. Accordingly, the close correlation observed in case of seeds carrying natural infections may indicate that under the conditions of natural inoculation the fungus is concentrated in the embryo, with low amounts in other parts of the seed. Overall, the results confirm that mycelium of *U. nuda* is present in parts of the seed other than the embryo. Depending on the amount, this may lead to wrong conclusions in assays like ELISA or PCR that, different from the embryo test, predict ear attack based on examination of the complete seed.

The suitability of the assay to detect *U. nuda* in plants was tested on barley seedlings (EC 12) grown from an infected seed lot. In order to avoid inclusion of plant material potentially carrying antigen but irrelevant for development of smutted ears, the seed coat, endosperm and scutellum were removed before preparation of the extracts. The material examined thus included the hypocotyl, the growing point and the surrounding leaf sheaths. The result of the ELISA performed with these samples corresponded very well with the value determined by the embryo staining method.

To summarize, it can be concluded that the ELISA was able to detect *U. nuda* in seeds and in seedlings. However, the results of some tests with seeds differed from those obtained by the traditional embryo staining method and required interpretation. Furthermore, the process of extracting single kernels as well as the ELISA protocol do not appear to have advantages in terms of time and labour requirements over the traditional method. It therefore appears unlikely that the ELISA as performed in this study can replace the embryo test in routine seed testing. Variations in sample extraction (e.g. just soaking kernels for some hours) and in the applied immunoassay, e.g. plate-trapped antigen ELISA, tissue print or dot-blot immunoassay, have not yet been tested for the *U. nuda* ELISA. These methods could possibly shorten the detection procedure and therefore overcome the temporal disadvantage.

On the contrary, inspection of green plant material for the presence or absence of *U. nuda* by ELISA is much faster than the traditional histological examination, allowing the processing of large sample numbers in relatively short time. In particular, ELISAs allow the quantification of the target antigen. Therefore, potential fields of application of the *U. nuda* ELISA include early prediction of the efficacy of plant protection products, e.g. seed treatments, elucidation of the biology of the fungus, e.g. its spread and distribution in the plant, and characterisation of resistance mechanisms that are active in plants after seed germination.

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