Potential of near infrared spectroscopy for the classification of fungal endophytes of grasses

A. García Ciudad,[a] I. Zabalgogeazcoa,[a] B.R. Vázquez de Aldana,[a] M.M. Canedo[b] and B. García Criado[a]

[a]*Instituto de Recursos Naturales y Agrobiología, CSIC, Apdo. 257, 37071 Salamanca, Spain*

[b]*Facultad de Ciencias Químicas, Universidad de Salamanca, 37008 Salamanca, Spain*

Introduction

Clavicipitaceous fungal endophytes infect a large number of grass species. Plants infected by *Neotyphodium* and certain *Epichloë* species do not show any symptom, but may contain toxic alkaloids. As a result, some endophyte-infected grasses are toxic to livestock. Endophyte toxicity represents a serious problem for animal production in the United States and New Zealand, this is due to the importance of the grasses *Festuca arundinacea* and *Lolium perenne* in grazing pastures in these countries. It is quite common for these grasses to be infected by clavicipitaceous endophytes.

In natural grasslands of Western Spain, there are several species which are hosts of fungal endophytes. In particular, more than 50% of the plants of *Festuca rubra* and *Dactylis glomerata* are infected by *Epichloë* species.[1] *Festuca rubra* is infected by *Epichloë festucae*, while *Dactylis glomerata* and other grasses are hosts of *E. typhina.*[2]

In terms of morphology and radial growth rates, pure cultures of *E. festucae* and *E. typhina* are virtually identical. In general, *Epichloë* species are very similar morphologically, and their species identification may involve techniques such as nucleotide sequencing of partial sequences of ribosomal RNA genes, a process which involves multiple steps and is expensive.

Near infrared (NIR) spectroscopy has been widely used to determine forage quality components.[3] However, few works dealing with the use of NIR to detect and quantify the presence of pathogens in forages and other agricultural products have been published. Asher and his associates,[4] applied this technology to estimate the number of spores of several species of fungi in grains of wheat and barley. Roberts *et al*.[5,6] estimated the amount of mould in alfalfa by quantifying chitin, and also quantified glucosamine by NIR, finding that the amount of glucosamine in barley was correlated to mycelial growth and spore production. Hill *et al*.[7] used NIR to try to estimate the amount of seeds of tall fescue infected by *Neotyphodium coenophialum*.

Recently, there has been a proliferation of papers related to the application of NIRS to qualitative analyses in several fields.[8–11] The main types of approach used to address the qualitative problems include discriminant procedures (both supervised and unsupervised), spectral library searching and modelling methods. It is assumed that compounds of similar chemical or physical properties are grouped closely together, that dissimilar compounds are separated in hyperspace and that the degree of separation between any two groups is inversely related to their degree of similarity.[12]

The aim of this study was to evaluate the potential of NIR spectroscopy for the classification of two species of the endophytic genus *Epichloë* (*Epichloë festucae* and *E. typhina*). If accurate, this method could then be used for the identification of morphologically similar fungal species.

Near Infrared Spectroscopy: Proceedings of the 11th International Conference.

Materials and methods

Fungal endophytes were isolated from surface-sterilised pieces of plants of *Festuca rubra* and *Dactylis glomerata*.[13] A total of 25 isolates were obtained from plants of *F. rubra* sampled at several locations, and 17 isolates were obtained from *D. glomerata* plants. Five to seven cultures of each isolate were grown in cellophane disks placed on top of potato dextrose agar plates. In total 117 cultures of *Epichloë festucae* and 71 of *E. typhina* were prepared with this method. When these cultures were about four weeks old, the disks of mycelium were lifted from the cellophane and their NIR spectra were recorded.

The spectral recordings of all 188 cultures were made by placing each disk of mycelium representing a sample between a stainless steel platform and a glass cover plate. This device was designed to fit in the sample stage of the spectrophotometer. Spectra of the range 1100–2500 nm were recorded at 4 nm intervals (351 data points) in an InfraAlyzer 500 (Bran & Luebbe, Germany), the instrument was operated by the software package IDAS. Spectral data were imported into Sesame Software, versión 3.1 (Bran & Luebbe), which was used to develop the calibrations. Before calibrations were made, the spectra of each isolate were averaged, and outliers were deleted.

The calibration was developed by means of a supervised method, therefore, each spectrum in the training set of samples is identified as belonging to a particular group. In this case, the 42 average spectra were separated in two classes, each one representing a different endophyte species. These classes were divided into two sets, the training set and the prediction set. The training set containing 16 samples from Class 1 (*Epichloë festucae*) and 11 samples from Class 2 (*Epichloë typhina*) respectively. The prediction set was composed of the remaining samples, nine samples from Class 1 and six from Class 2, respectively. The qualitative calibration was made using a cluster model based on principal component analysis (PCA).

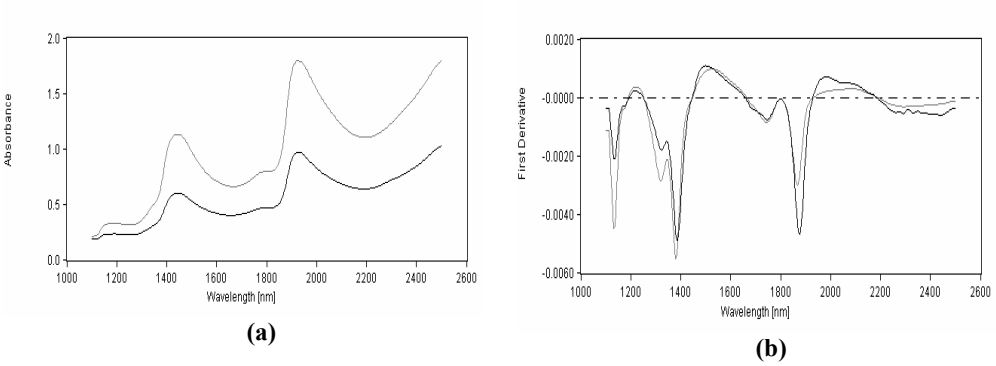
In this work two data transformations were tested to develop the calibration: absorbance and first derivative. The optimum number of components to be used in the calibration development was automatically determined by the software; and based on a correlation index, the most relevant components were selected for clustering.

Results and discussion

Figure 1 shows NIR mean spectra (absorbance and first derivative) of samples of two endophytic fungi species: 117 cultures of *Epichloë festucae* and 71 cultures of *Epichloë typhina*. Along the whole spectrum, *Epichloë typhina* endophyte species showed absorbance values higher than those of *E. festucae.* Spectra of both species had a strong band close to 1430 nm which is characteristic of water content and of substances high in crystalline sugar, and another band next to 1940 nm which is also characteristic of water.[14]

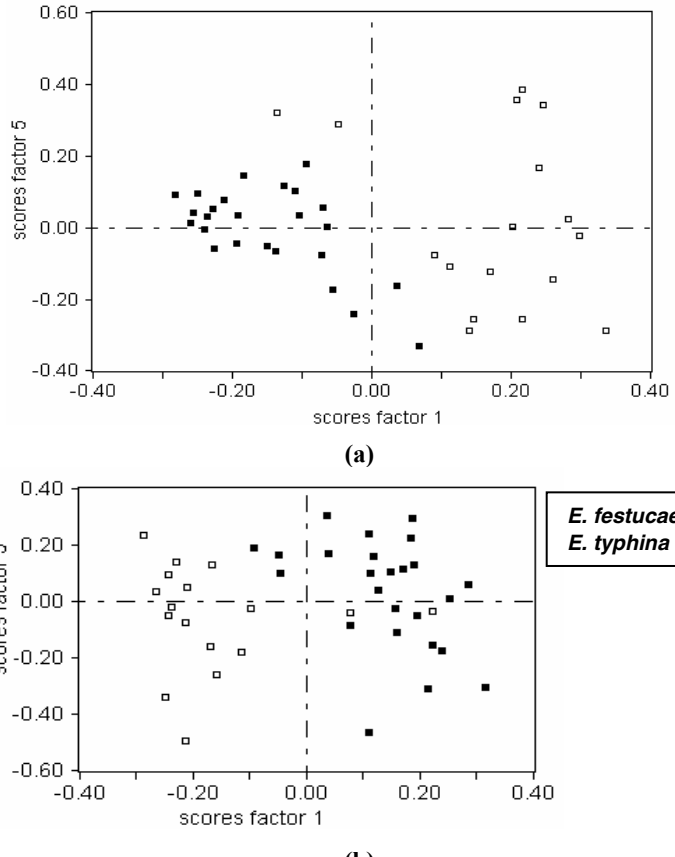
According to the results of cluster analysis over individual spectral data, both endophytic fungi species can be discriminated considering five principal components (Figure 2 and Table 1). On the basis of the correlation index, components 1 and 5 were the most relevant for the clustering and the highest influence was found for factor 1. The results obtained with the absorbance treatment (log $1/R$) were better than those of the first derivative. Using the absorbance treatment, a more clear separation, with only one cluster per class was obtained, and 93% of the test sample set were properly classified. Component 1 accounted for 98.5% of the total variance of the data. However, using the first derivative treatment (five factors), two clusters per class were obtained and 86% of the test sample set were properly classified. In this case, Component 1 accounted for 66.6% of the variance.

On the basis of these results, NIR spectroscopy appeared to be a promising tool for the identification of morphologically similar fungal species.



(a) (b)

Figure 1. NIR mean spectra of *Epichloë* species: *E. festucae* (black) and *E. typhina* (grey). (a), absorbance; (b), first derivative.



(a)

(b)

Figure 2. PCA score plot using data as (a) absorbance and (b) as first derivative.

Table 1. Summary of classification results using factorial discriminant analysis.

Class	Absorbance			First derivative		
	Training set		Test set	Training set		Test set
	Samples	Cluster	Total samples/ Correct identification	Samples	Cluster	Total samples/ Correct identification
Epichloë festucae	16	1	9/9	16	2	9/8
Epichloë typhina	11	1	6/5	11	2	6/5

Acknowledgments

This work was supported by Junta de Castilla y León Project CSI 6/01. The authors thank Y. Arnaiz Pita, E.M. de la Calzada Cuesta and L. Grande Pérez, for technical assistance.

References

1. I. Zabalgogeazcoa, B.R. Vázquez de Aldana, A. García Ciudad and B. García Criado, *Grass Forage Sci.* in press (2003).
2. C.L. Schardl, *Fungal Genet. Biol.* **33**, 69 (2001).
3. G.C. Marten, J.S. Shenk and F.E. Barton II, *Near infrared reflectance spectroscopy (NIRS): Analysis of forage quality*. Agric. Handbook No. 643 (revised). USDA, Washington, DC, USA (1989).
4. M.J.C. Asher, I.A. Cowe, C.E. Thomas and D.C. Cuthbertson, *Plant Pathol.* **31,** 363 (1982).
5. C.A. Roberts, K.J. Moore, D.W. Graffis, H.W. Kirby and R.P. Walgenbach, *J. Dairy Sci*. **70,** 2560 (1987).
6. C.A. Roberts, R.R. Marquardt, A.A. Frohlich, R.L. McGraw, R.G. Rotter and J.C. Henning, *Cereal Chem.* **68(3),** 272 (1991).
7. N.S. Hill, J.C. Petersen, R.A. Shelby, L.W. Dalrymple and F.E. Barton II, *Crop Sci.* **27,** 1291 (1987).
8. F.E. Dowell, J.E. Throne, D. Wang and J.E. Baker, *J. Ecol. Entomol.* **92(1),** 165 (1999).
9. S.J. Lister, M.S. Dhanoa, J.L. Stewart and M. Gill, *Anim. Feed Sci. Technol.* **86,** 221 (2000).
10. G. Downey and S.J. Flynn, in *Near Infrared Spectroscopy: Proceedings of the 10th International Conference,* Ed by A.M.C. Davies and R.K. Cho. NIR Publications, Chichester, UK, p. 239 (2002).
11. W.R. Windham, B. Park, K.C. Lawrence, R.J. Buhr and D.P. Smith, in *Near Infrared Spectroscopy: Proceedings of the 10th international Conference,* Ed by A.M.C. Davies and R.K. Cho. NIR Publications, Chichester, UK, p. 453 (2002).
12. G. Downey, *Analyst* **119,** 2367 (1994).
13. C.W. Bacon and J.F. White, in *Biotechnology of endophytic fungi of grasses*, Ed by C.W. Bacon and J.F. White. CRC Press, Boca Raton, Florida, USA, pp. 47–56 (1994).
14. I. Murray and P.C. Williams, in *Near-Infrared technology in the Agricultural and Food Industries*, Ed by P. Williams and K. Norris. American Association of Cereal Chemists, Inc., St Paul, Minnesota, USA, pp. 17–34 (1987).