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An Enzyme-Linked Immunosorbent Assay (ELISA) for the Detection of *Tuber albidum* Ectomycorrhiza

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Abstract

$F(ab^1)_2$ indirect ELISA using an antiserum prepared in rabbit against an acetone precipitate of a *Tuber albidum* culture fluid was effective in discriminating *T. albidum* ectomycorrhiza from *T. magnatum*, *T. aestivum*, *T. brumale* and an unidentified ectomycorrhiza on *Quercus pubescens* and *Pinus pinea*.

Keywords: $F(ab^1)_2$ indirect ELISA, *Tuber albidum*, serological assay of ectomycorrhizal fungi

1. Introduction

Seedling mycorrhizal inoculation with *Tuber* spp. is extensively used in Italian truffle cultivation. Mycorrhiza formation on the root system is usually identified by morphological methods. However, as first observed by Palenzona (1969), ectomycorrhiza produced by different *Tuber* spp. have numerous morphological features in common, which can only be distinguished by a specialist. In particular, *Tuber albidum* and *Tuber magnatum* ectomycorrhiza are very similar in colour, size, shape and mantle ornamentation, and are easily confused.

Several biochemical methods have been attempted for the identification of mycorrhiza of individual *Tuber* species, such as pyrolysis (Papa and Balbi, 1988), electrophoretic analysis of gene-enzyme systems (Palenzona et al., 1990) and electrophoresis of soluble proteins (Papa and Polimeni, 1990; Dupré and Chevalier, 1991). None of these methods are suitable when a large number of seedlings are to be examined because they are too laborious. In addition, plant tissues interfere in these analyses.

On the other hand, serological techniques have been developed as diagnostic methods for fungi in plant pathology (Mohan, 1988; Amouzou-Alladaye et al., 1988; Unger and Wolf, 1988; Kitagawa et al., 1989) and several immunochemical

approaches have recently been made to characterize and detect endo- and ectomycorrhizal fungi (Wright et al., 1987; Cleyet Marel et al., 1989; Perotto et al., 1992).

The aim of this study was to determine whether an enzyme-linked immunosorbent assay (ELISA) is suitable for the identification of *Tuber* ectomycorrhiza. Despite the fact that *T. magnatum* is more important economically, we applied the ELISA technique to *T. albidum* because the latter grows much better in pure culture than *T. magnatum* and thus is suitable for antigen preparation.

2. Materials and Methods

An isolate of *T. albidum* was obtained from fruit bodies collected under *Pinus pinea*. The isolate was cultured in 250 ml flasks containing 100 ml of modified Melin-Norkrans nutrient solution (MMN) (Marx, 1969) inoculated with fungus culture agar plugs (PDA, Difco).

After four weeks under static conditions at 22°C in the dark, the culture fluid and mycelium mat were separated by filtration on a Bückner funnel and used, respectively, to obtain an extracellular precipitate and a soluble mycelial protein fraction for rabbit immunization. The contents of 15 flasks were processed.

Extracellular antigens were obtained by adding three volumes of acetone to the liquid medium and kept at −18°C overnight (Nachmias et al., 1979). The precipitate was then collected by centrifugation at 10,000 g for 15 min and the resulting pellet was resuspended in 5 ml of 0.1 M phosphate saline buffer (pH 7.0).

The soluble mycelial protein fraction was obtained according to Amouzou-Alladaye et al. (1988). The mycelium was washed in water and freeze-dried. The freeze-dried mycelium powder was ground with a Polytron blender in 50 ml of extraction buffer (Amouzou-Alladaye et al., 1988) with 40 mg/ml polyvinylpolypyrrolidone.

After centrifugation for 15 min at 10,000 g the supernatant was centrifuged at 35,000 rpm for 90 min in a Beckman, 60 Ti rotor and the supernatant was concentrated by dialysis with PEG 6,000 until the volume was reduced to about 2 ml.

The protein concentrations determined using the Lowry method (1951) were 0.5 mg/ml for the liquid medium precipitate and 2 mg/ml for the soluble mycelial protein fraction. The solutions were stored at −18°C until further use.

Antisera production

One New Zealand white rabbit was given intramuscular injections in several sites with the extracellular precipitate suspension and one was injected with the soluble mycelial protein fraction. The immunization scheme included three injections of 1 mg antigen suspension at one week intervals; two months later, another series of injections was given with half the original dose. The antigen suspensions were mixed with an equal amount of Freund's complete adjuvant for the first injection,

and with incomplete adjuvant for subsequent injections. The rabbits were bled five weeks after the first injection and two weeks after the final injection.

The antisera titres were determined by indirect ELISA with $F(ab^1)_2$ immunoglobulin fragments using antigens as prepared for immunization.

ELISA tests

The indirect ELISA procedure using $F(ab^1)_2$ immunoglobulin fragments was performed as described by Barbara and Clark (1982). Immunoglobulins G (IgG) were purified from antisera by column chromatography through protein A – Sepharose (Pharmacia). $F(ab^1)_2$ fragments of IgG were then prepared by pepsin digestion (Barbara and Clark, 1982).

ELISA tests were performed in microtiter plates (Dynatech) coated with $F(ab^1)_2$ fragments of IgG diluted in carbonate buffer (Clark and Adams, 1977).

Trapped antigens were detected with whole IgG diluted in PBS-Tween 20 (Clark and Adams, 1977) followed by Protein A-alkaline phosphatase conjugate (Sigma) diluted 1:1,000 in the same buffer.

Results were recorded at 405 nm, 2 hr after the addition of substrate at room temperature. The optimal dilutions of $F(ab^1)_2$ fragments of IgG and the whole IgG, used as second antibody, were selected to minimize non-specific reactions.

The reaction was considered positive if the mean absorbance of the sample was higher than the mean absorbance of three non-mycorrhizal control samples plus three times the standard deviation of the control.

Preparation of ectomycorrhizal samples

The tests were performed using *Quercus pubescens* and *Pinus pinea* seedlings inoculated with *T. albidum* under controlled conditions and uninoculated seedlings as controls.

Young, branched root tips, about 2 mm long were excised under a dissecting microscope and ground with a pestle and mortar with extraction buffer (Clark and Adams, 1977). Varying numbers of root tips from both plant species, colonized by *T. albidum*, were ground in 1 ml of extraction buffer and tested by ELISA to determine the sensitivity of one of the antisera (see below). The possibility of detecting the ectomycorrhizal fungus *T. albidum* in batches of non-mycorrhizal root tips, was tested by grinding varying numbers of root tips with *T. albidum* mycelial colonization with varying numbers of tips from non-mycorrhizal plants in a standard volume of 2 ml extraction buffer.

The reactivity of the antiserum was also tested against mycorrhiza produced by other fungus species, viz. *T. magnatum, Tuber aestivum, Tuber brumale* and against uncharacterized field ectomycorrhiza on *Q. pubescens* and *P. pinea.* For this purpose, fungi were extracted by grinding 30 root tips of *P. pinea* or 100 tips of *Q. pubescens* in 2 ml of extraction buffer.

All the trials were performed in adjacent triplicate wells and each trial was repeated three times.

3. Results

In $F(ab^1)_2$ indirect ELISA, the antiserum against the extracellular fraction showed a higher titre with homologous antigens, and a very low non-specific binding with non-mycorrhizal root tissues, than with the antiserum to the soluble mycelial protein fraction. The mean absorbance values, from 3 experiments with 3 replicates for each ELISA plate, were respectively: 1.45 O.D. and 0.31 O.D. for homologous antigens; 0.09 O.D. and 0.08 O.D. for controls. For this reason, all the subsequent results refer to the antiserum to the extracellular fraction, collected after the booster injections. For this antiserum, the optimal conditions for $F(ab^1)_2$ indirect ELISA was found to be 10 ng/ml $F(ab^1)_2$ fragments of IgG for plate coating and 1:500 dilution of the whole IgG. Under these conditions, the detection limits for the homologous antigens and the soluble mycelial protein fraction antigens were 40–60 ng and 100–150 ng protein/ml, respectively.

The fungus *T. albidum* could be reliably detected in extracts of two *P. pinea* root tips and in extracts of at least 25 *Q. pubescens* root tips ground in 1 ml of extraction buffer. A good correlation was found between the number of tips and the absorbance value (Table 1), although a small variability of the absorbance values was observed, due to the different ages and sizes of the ectomycorrhiza tested; young, branched ectomycorrhiza with a well-developed fungal mantle were the most reactive.

The limit for a positive reaction in batches of mycorrhizal root tips mixed with non-mycorrhizal root tips was 1:5 for both *P. pinea* and *Q. pubescens* (Table 2).

Non-mycorrhizal *P. pinea* and *Q. pubescens* root tips gave low absorbance values, below 0.1.

The results for cross-reactivity of *T. albidum* antiserum with other ectomycorrhizal species are reported in Table 3.

A very slight heterologous reaction was noticed only with *T. magnatum*, while there was no reactivity with the other species tested. The fungi in *P. pinea* and

Table 1. ELISA absorbance for different numbers of *T. albidum* mycorrhizal root tips.

Pinus pinea		*Quercus pubescens*	
Number of mycorrhizal tips	Absorbance value	Number of mycorrhizal tips	Absorbance value
Control (*)	0.09 (0.02)	Control (*)	0.08 (0.02)
2	0.29 (0.01)	25	0.41 (0.06)
4	0.29 (0.02)	50	1.01 (0.08)
8	0.41 (0.05)	100	1.72 (0.11)
16	0.61 (0.04)		
32	1.13 (0.04)		

Note: (*) 32 non-mycorrhizal *P. pinea* root tips and 100 non-mycorrhizal *Q. pubescens* root tips were used. The absorbance values were recorded at 405 nm. The data are the means of triplicate wells with three repetitions. Standard deviation in parenthesis.

Table 2. ELISA absorbance for *T. albidum* mycorrhizal root tips mixed with non-mycorrhizal root tips

Pinus pinea		*Quercus pubescens*	
Non-mycorrhizal root tips / Mycorrhizal	Absorbance value	Non-mycorrhizal root tips / Mycorrhizal	Absorbance value
30/0 (Control)	0.09 (0.03)	100/0 (Control)	0.08 (0.02)
25/5	0.29 (0.08)	80/16	0.23 (0.04)
20/10	0.57 (0.04)	66/33	0.43 (0.05)
10/20	0.75 (0.08)	33/66	1.90 (0.02)

Note: The absorbance values were recorded at 405 nm. The data are the means of triplicate wells with three repetitions. Standard deviation in parenthesis.

Table 3. Absorbance values at 405 nm $F(ab^1)_2$ ELISA with root tip extracts of *Q. pubescens* and *P. pinea* with different fungus species

	ELISA value of extracts for each fungus					
Plant host	no fungus	*T. albidum*	*T. magnatum*	*T. aestivum*	*T. brumale*	unidentified fungus
*Q. pubescens**	0.09 (0.02)	1.20 (0.33)	0.17 (0.02)	0.12 (0.02)	–**	–**
*P. pinea**	0.09 (0.02)	1.31 (0.08)	–**	0.09 (0.03)	0.07 (0.01)	0.09 (0.03)

* The root tips tested were 100 for *Q. pubescens* and 30 for *P. pinea*. Results are expressed as mean absorbance values for triplicate wells with three repetitions. Standard deviation in parenthesis.
–** Not tested

Q. pubescens trees reacted similarly, apart from a slightly reduced absorbance value constantly obtained for *T. albidum* in *Quercus*.

4. Discussion

Our results show that the antibodies obtained with the *T. albidum* extracellular fraction are able to differentiate this species from other ectomycorrhiza in both *P. pinea* and *Q. pubescens*. Moreover, they are able to detect the presence of the fungus in mixed batch samples of mycorrhizal and non-mycorrhizal root tips. This makes it possible to detect the presence of the fungus in seedling trees with ELISA even when there is irregular ectomycorrhiza development. In addition, considering the good correlation seen between the absorbance value and the number of infected tips, this technique could permit estimates to be made of the degree of ectomycorrhizal formation in roots.

ELISA thus appears to be a promising method for large-scale, routine detection of *Tuber* ectomycorrhiza.

The higher reactivity obtained with *T. albidum* mycorrhiza on *P. pinea* compared to the same mycorrhiza on *Q. pubescens*, may be due to the presence of a thicker fungal sheath composed of more layers on the *Pinus* as reported by Palenzona et al. (1972).

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REFERENCES

Amouzou-Alladaye, E., Dunez, J., and Clerjeau, M. 1988. Immunoenzymatic detection of *Phytophthora fragariae* in infected strawberry plants. *Phytopathol.* **78:** 1022–1026.

Barbara, D.J. and Clark, M.F. 1982. A simple indirect ELISA using $F(ab^1)_2$ fragments of immunoglobulin. *J. Gen. Virol.* **58:** 315–322.

Clark, M.F. and Adams, A.N. 1977. Characteristics of the microplate method of enzyme linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* **34:** 475–483.

Cleyet-Marel, J.C., Bousquet, N., and Mousain, D. 1989. The immunochemical approach for the characterization of ectomycorrhizal fungi. *Agric., Ecosystems Environ.* **28:** 79–83.

Dupré, Ch. and Chevalier, G. 1991. Analyse electrophorétique des protéines fongiques de différents *Tuber* en association ou non avec *Corylus avellana*. *Cryptogamie, Mycol.* **12(4):** 243–250.

Kitagawa, T., Sakamoto, Y., Furumi, K., and Ogura, H. 1989. Novel enzyme immunoassays for specific detection of *Fusarium oxysporum* f. sp. *cucumerinum* and for general detection of various *Fusarium* species. *Phytopathol.* **79:** 162–165.

Lowry, O.H. 1951. Protein measurements with folin reagent. *J. Biol. Chem.* **102:** 265–275.

Marx, D.H. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathol.* **59:** 153–163.

Mohan, S.B. 1988. Evaluation of antisera raised against *Phytophthora fragariae* for detecting the red core disease of strawberries by enzyme-linked immunosorbent assay (ELISA). *Plant Pathol.* **37:** 206–216.

Nachmias, A., Bar-Joseph, M., Solel, Z., and Barash, I. 1979. Diagnosis of mal secco disease in lemon by enzyme-linked immunosorbent assay. *Phytopathol.* **69:** 559–561.

Palenzona, M. 1969. Sintesi micorrizica tra *Tuber aestivum* Vitt., *Tuber brumale* Vitt., *Tuber melanosporum* Vitt. e semenzali di *Corylus avellana* L. *Allionia* **15:** 121–131.

Palenzona, M., Biocca, E., Nascetti, G., Ferrara, A.M., Mattiucci, S., D'Amelio, S., and Balbo, T. 1990. Studi preliminari sulla tipizzazione genetica (sistemi gene-enzima) di specie del genere *Tuber*. In: *Proceedings of the 2nd International Congress on Truffle*, Spoleto, 24–29 November, 1988, pp. 53–58.

Palenzona, M., Chevalier, G., and Fontana, A. 1972. Sintesi micorrizica tra i miceli in coltura di *Tuber brumale*, *T. melanosporum*, *T. rufum* e semenzali di conifere e latifoglie. *Allionia* **18:** 41–52.

Papa, G. and Balbi, P. 1988. Micorrize: riconoscimento mediante pirolisi. Abstract from: *2nd International Congress on Truffle*, Spoleto, 24–29 November, 1988.

Papa, G. and Polimeni, C. 1990. Caratterizzazione delle proteine nelle *Tuberaceae*. I. Studio preliminare di una banda elettroforetica di *Tuber magnatum* Pico. *Mic. Ital.* **2:** 37–43.

Perotto, S., Malavasi, F., and Butcher, G.W. 1992. Use of monoclonal antibodies to study mycorrhiza: present applications and perspectives. *Methods in Microbiology* **24:** 221–248.

Unger, J.G. and Wolf, G. 1988. Detection of *Pseudocercosporella herpotrichoides* (Fron) Deighton in wheat by indirect ELISA. *J. Phytopathol.* **122:** 281–286.

Wright, S.F., Morton, J.B., and Sworobuk, J.E. 1987. Identification of a vesicular-arbuscular mycorrhizal fungus by using monoclonal antibodies in an enzyme-linked immunosorbent assay. *Appl. Environ. Microbiol.* **53:** 2222–2225.