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Variability of *Peronospora tabacina* A. - An isoenzyme study

by

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SUMMARY

The variability of *P. tabacina* A. populations was studied by using the isoenzymes of peroxidase (PO), malate dehydrogenase (MDH) and superoxide dismutase (SOD) as molecular markers. *P. tabacina* conidia/conidiophores from *N. tabacum* crops cultivated in distant areas of Europe (France and Bulgaria) were investigated during a long period of time (1978–1992). It was found that no variations of *P. tabacina* as a function of space and time occurred. The data point to the genetical stability of the presently spread strain of the pathogen and the lack of processes of new strain formation. However, a significant variability of *P. tabacina* as a function of host plant was established. Suspension of conidia/conidiophores of *P. tabacina* originating from *N. tabacum* (P 48 cv.) was used to inoculate the wild species *N. repanda*. First conidia/conidiophores of *P. tabacina* produced on the new host plant (designated as Pt/Nr) as well as those from the "classical" host, *N. tabacum* (designated as Pt/Nt) were analyzed. Thus, two types of *P. tabacina* (according to their origin) were compared. It was shown that one MDH and most PO isoenzymes present in Pt/Nt were not observed in Pt/Nr; these isoenzymes repressed following the transfer were common for Pt/Nt and its *N. tabacum* host. Moreover, in conidia/conidiophores of Pt/Nr new isoenzymes appeared, namely one major PO, one MDH and a block of three SOD isoenzyme components. These isoenzymes were common for Pt/Nr and the new host, *N. repanda*. It is noteworthy that all fungus specific isoenzymes present in Pt/Nt (one PO, five MDH and seven SOD components) were conserved in Pt/Nr. The results reveal molecular mechanisms underlying relationships between host plants and obligatory fungal pathogens, such as *P. tabacina*. They could be used in blue mold epidemiology research.

ZUSAMMENFASSUNG

Die Variabilität der Populationen von *P. tabacina* A. wurde mit Hilfe der Isoenzyme der Peroxidase, (PO), der Malatdehydrogenase (MDH) und der Superoxiddismutase (SOD) als biologische Marker untersucht. Die Konidien und Konidiophoren von *N. tabacum*, die in voneinander entfernt liegenden Gebieten Europas (Frankreich und Bulgarien) angebaut werden, wurden über einen Zeitraum von mehr als 10 Jahren

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(1978–1992) untersucht. Veränderungen von *P. tabacina* in Abhängigkeit von Ort und Zeit wurden nicht beobachtet; dies zeugt von der genetischen Stabilität der existierenden Linie des Pathogens und zeigt, daß keine neuen Formen ausgebildet werden. Im Gegensatz hierzu konnte eine große Variabilität von *P. tabacina* in Abhängigkeit von der Wirtspflanze gezeigt werden. Suspensionskulturen von Konidien und Konidiophoren von *P. tabacina*, die von der suszeptiblen Sorte Paraguay P 48 (*N. tabacum*) abstammen, wurden benutzt, um die Wildform von *N. repanda* zu impfen. Die ersten Konidien und Konidiophoren, die auf der neuen Wirtspflanze *N. repanda* (Pt/Nr) gebildet wurden, sowie jene, die vom klassischen Wirtsorganismus, *N. tabacum* (Pt/Nt), abstammen, wurden analysiert. So wurden zwei nach ihrem Ursprung unterschiedliche Typen von *P. tabacina* miteinander verglichen und es konnte gezeigt werden, daß ein Isoenzym der MDH und die Mehrheit der Isoenzyme der PO, die im Wirtsorganismus Pt/Nt vorkommen, in der neuen Wirtspflanze Pt/Nr nicht vorgefunden wurden. Diese Isoenzyme, die nach der Übertragung reprimiert waren, sind sowohl in Pt/Nt als auch in der Wirtspflanze *N. tabacum* vorhanden. Auf der anderen Seite sind in den Konidien und Konidiophoren, die sich in *N. repanda* (Pt/Nr) gebildet haben, neue Isoenzyme entstanden: eine vorherrschende PO-Variante, eine MDH und eine Gruppe bestehend aus drei SOD-Isoenzymen. Diese Isoenzyme kommen in Pt/Nr und der neuen Wirtspflanze (*N. repanda*) vor. Es ist wichtig zu betonen, daß alle für das Pilzpathogen zu beobachtenden spezifischen Isoenzyme (ein Isoenzym der PO, fünf Isoenzyme der MDH und sieben der SOD), die in Pt/Nt vorhanden sind, auch in Pt/Nr vorkommen. Die Ergebnisse zeigen die molekularen Mechanismen auf, die die Grundlage für die Interaktion zwischen Wirtspflanzen und obligaten Pilzpathogenen, wie *P. tabacina*, bilden. Sie können zur Untersuchung der Blauschimmel-Epidemiologie genutzt werden.

RESUME

La variabilité des populations de *P. tabacina* A. a été étudiée en utilisant les isoenzymes de la peroxydase (PO), la malate déshydrogénase (MDH) et la super oxyde dismutase (SOD) comme marqueurs moléculaires. Des conidies et conidiophores issus de plantes de *N. tabacum* cultivées dans des régions européennes éloignées (France et Bulgarie) ont été étudiés sur une période de plus de 10 années (1978–1992). Les variations de *P. tabacina* en fonction de l'espace et du temps n'étaient pas connues; ces résultats témoignent d'une stabilité génétique de la souche existante du pathogène, et de l'absence de processus de formation de nouvelles races. Par contre, une variabilité importante de *P. tabacina* en fonction de la plante hôte est démontrée. Des suspensions de conidies et conidiophores de *P. tabacina* provenant de la variété sensible Paraguay P 48 (*N. tabacum*) ont été utilisées pour inoculer l'espèce sauvage *N. repanda*. Les premières conidies et conidiophores produits sur la nouvelle plante hôte, *N. repanda* (Pt/Nr), ainsi que ceux provenant de l'hôte "classique", *N. tabacum* (Pt/Nt), ont été analysés. Ainsi, deux types de *P. tabacina* (d'après leur origine) ont été comparés et il est démontré qu'une isoenzyme de la MDH et la majorité des isoenzymes de la PO, présentes en Pt/Nt, n'étaient pas observées en Pt/Nr. Ces isoenzymes, réprimées après le transfert, sont communes pour Pt/Nt et la plante hôte (*N. tabacum*). D'autre part, dans les conidies et conidiophores issus de *N. repanda* (Pt/Nr) de nouvelles isoenzymes sont exprimées: une PO majeure, une MDH et un ensemble de trois SOD. Ces isoenzymes sont communes pour Pt/Nr et la nouvelle plante hôte (*N. repanda)*. Il est important de noter que toutes les isoenzymes spécifiques pour le champignon observées en Pt/Nt (une isoenzyme de la PO, cinq isoenzymes de la MDH et sept de la SOD) sont conservées en Pt/Nr. Les résultats révèlent des mécanismes moléculaires constituant les bases des relations entre les plantes hôtes et les champignons pathogènes obligatoires tels que *P. tabacina*. Ils peuvent être utilisés pour l'étude de l'épidémiologie du mildiou du tabac.

INTRODUCTION

Variability of pathogens is a field of intensive research because of its crucial importance for epidemiological and environmental problems. To estimate *P. tabacina* variability, CORESTA collective experiments have been carried out for more than 33 years (5). They were based on assessment of the pathogenicity of *P. tabacina* and pointed that no aggressive pathotypes have arisen that could bypass the blue mold resistance genes in tobacco. The present work was intended to yield additional information on this problem by using isoenzymes as molecular markers for variability; the isoenzyme approach has long been recognized as a tool for assessment of biological diversity (6, 12, 18). The aim of our investigation was to test whether natural populations of *P. tabacina* vary as a function of space, time and host plant. To this end *P. tabacina* originating from distant geographical zones (France and Bulgaria) was studied covering a long period of time (1978–1992). To ascertain the host plant dependent variability, *P. tabacina* parasitizing on different tobacco hosts, namely the cultivated species *N. tabacum* L. and the wild species *N. repanda* W., was examined.

In a next step of the work, the isoenzyme patterns of the pathogen originating from *N. tabacum* and from *N. repanda* were compared with the patterns of the corresponding host plants. The molecular forms of the following enzymes were resolved: peroxidase (PO; E.C.1.11.1.7), NAD-malate dehydrogenase (MDH; E.C.1.1.1.37), NAD-glutamate dehydrogenase (GDH; E.C.1.4.1.2), glutamate oxalacetate transaminase (GOT; E.C.2.6.1.1) and superoxide dismutase (SOD; E.C.1.15.1.1).

MATERIALS

Conidia/conidiophores of natural populations of *P. tabacina* were analyzed. They were collected from *P. tabacina*- infected sporulating leaves of *N. tabacum* plants (VSP$_{26}$, Virgin D, Virgin Gold, P 48, Bergerac C cvs.) grown and inoculated under field conditions in two localities: France (Seita , Institut du Tabac, Bergerac) and Bulgaria (Institute of Genetics, Sofia). Experiments were carried out in the period 1978–1992. A dry brushing technique for collecting conidia/conidiophores was applied.

Another set of experiments was carried out in the Tobacco Institute, Bergerac (1993–1994). The following procedure was applied. Plants of the cultivated species *N. tabacum* (P 48 cv.) and the wild species *N. repanda,* blue mold susceptible (10), were grown under greenhouse standard conditions. At the age of 2 months, plants were transferred in growth chambers (temperature 24° C, relative humidity 60–80 %, illumination 3000 lx, day/ night photoperiod 12/12), and inoculated by spraying the leaves with a conidial suspension (5.10^4 conidia/ml) of *P. tabacina,* maintained on inoculated *N. tabacum* plants (P 48 cv.) in growth chamber conditions. 11–12 days after inoculation abundant sporulation of *P. tabacina* occurred. The first conidia/conidiophores produced on both hosts were collected from sporulating leaves (using a dry brushing technique) and analyzed. Leaves of healthy, not inoculated plants of *N. tabacum* (P 48 cv.) and *N. repanda* were also used for analysis.

METHODS

Enzyme extracts from both *P. tabacina* conidia/conidiophores and tobacco leaves were done with 0.05 M sodium phosphate buffer pH 7.8, containing 1 mM EDTA-Na$_2$ (for SOD), and with 0.05 M tris-glycine buffer pH 8.3 containing 17 % saccharose (for PO, MDH, GDH and GOT). The isoenzymes were separated by disc electrophoresis in 7.5 % polyacrylamide gel pH 8.3, or by gradient (4 % – 30 %) slab polyacrylamide gel electrophoresis pH 8.3 (4). An equal amount of protein (200 mg) per each sample was applied.

Staining of the isoenzymes of PO was carried out after SEEVERS *et al.* (16), of MDH and GDH after RUBIN *et al.* (15), of GOT after GOTTLIEB (7), and of SOD after BEAUCHAMP and FRIDOVICH (2). Protein content was determined after LOWRY *et al.* (9). The similarity between the pathogen and the corresponding host plant was estimated by pair affinity index (1).

RESULTS

A ▸ P. tabacina from different localities (France, Bulgaria) and different years (1978, 1992)

The isoenzyme patterns of PO in *P. tabacina* originating from France and from Bulgaria are almost identical in the whole period studied. The only difference between them in 1978 is the absence of the minor component No 11 in the French origin of *P. tabacina.* Experiments carried out in 1992 point to the same tendency: the two origins differ uniquely in the expression of the minor component No 3 absent in *P. tabacina* from France (Figure 1).

When comparing the PO isoenzyme patterns of both origins in different years, no significant changes are observed. In 1992 the pattern of the French origin lacks the minor components No 2 and No 3 which it contained in 1978. In 1992 the Bulgarian origin of *P. tabacina* lacks the minor components No 2 and No 11 which were present in this origin in 1978 (Figure 1).

The isoenzyme patterns of MDH and SOD also reveal minor differences between French and Bulgarian origins of *P. tabacina* in the period studied (data not shown). In the patterns of GDH and GOT (consisting of one and two components, respectively) no variations are observed (Figure 1).

B ▸ P. tabacina from different tobacco hosts (Figure 2)

The PO isoenzyme pattern of *P.tabacina* from *N. tabacum* (Pt/Nt) contains seven components (Nos 6, 8, 11–15), while *P. tabacina* from *N. repanda* (Pt/Nr) has only two components (Nos 6, 10), the latter being the main band.

Eight components are present in the MDH pattern of Pt/Nt (Nos 3–6, 8, 9, 11, 13) and Pt/Nr (Nos 2, 4–6, 8, 9, 11, 13).

Nine bands are observed in the SOD pattern of Pt/Nt (Nos 1–3, 5, 6, 9–11, 13), and twelve bands (Nos 1–3, 5, 6, 9–11, 13, 16–18) in the pattern of Pt/Nr. The minor bands Nos 16–18 build a fast moving characteristic block.

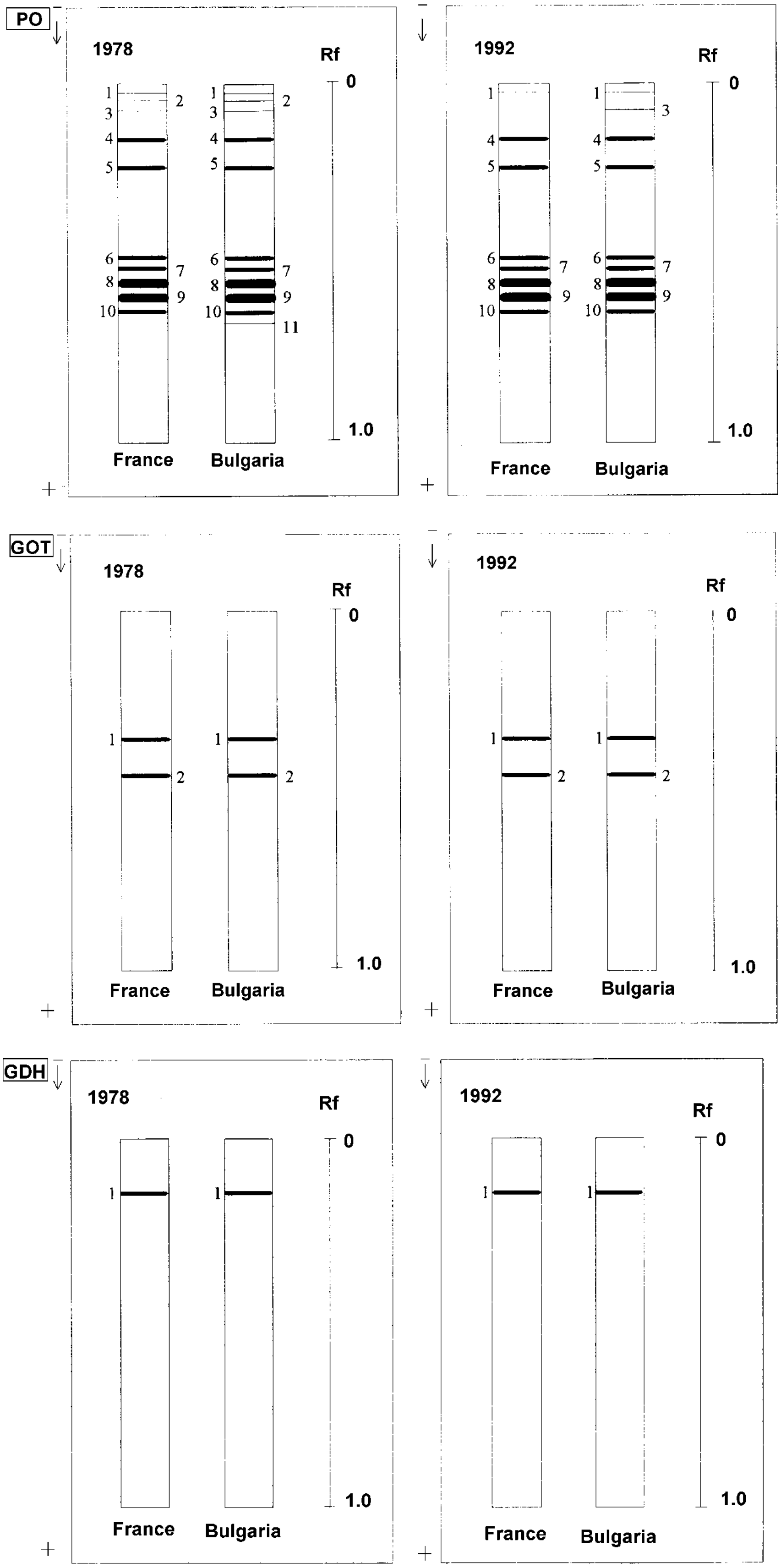

PO
1978
Rf
0
1
2
3
4
5
6
7
8
9
10
11
1.0
France
Bulgaria
1992
GOT
GDH
+

Figure 1.
Isoenzyme patterns of the peroxidase (PO), glutamate oxalacetate transaminase (GOT) and glutamate dehydrogenase (GDH) in conidia/conidiophores of *P. tabacina* originating from France and from Bulgaria. Experiments were carried out in 1978 and in 1992. Disc electrophoresis in 7.5 % polyacrylamide gel, pH 8.3.

Hence, Pt/Nt and Pt/Nr differ in both the number and the kind of components in the pattern of PO, MDH and SOD. The data point to the important variability of *P. tabacina* as a function of host plant. This finding prompted us to compare the isoenzyme complements of Pt/Nt and Pt/Nr with their host plants.

C ▸ *Comparison of P. tabacina originating from N. tabacum and from N. repanda with the corresponding tobacco hosts*

Peroxidase (Figure 3)

The pattern of PO in leaves of the cultivated tobacco species *N. tabacum* (P 48 cv.) is composed of eight bands (Nos 4, 5, 8, 11–15). *P. tabacina* parasitizing on *N. tabacum* (Pt/Nt) possesses a similar pattern sharing six common components with its host plant (No 8 and Nos 11–15). Thus, a high pair affinity index (80 %) of Pt /Nt with its *N. tabacum* host is demonstrated.

In the pattern of leaves of the wild tobacco species *N. repanda* seven iso-PO are present (Nos 1– 3, 5, 7, 9, 10), the latter being the major one. The main component No 10 in the pattern of Pt/Nr is common with its host plant, *N. repanda*.

The iso-PO No 6 is observed in both Pt/Nt and Pt/Nr but not in tobacco plants, i.e. is fungus specific.

Some components are characteristic for either *N. tabacum* (No 4) or *N. repanda* (Nos 1–3, 7, 9).

Malate dehydrogenase (Figure 4)

In the MDH pattern of *N. tabacum* leaves eleven isoenzymes are observed (Nos 1, 3, 5, 9–12 and 14–17). In the pattern of *P. tabacina* from *N. tabacum* (Pt/Nt) one MDH component (No 3) is common with the host, *N. tabacum*.

Leaves of *N. repanda* contain eight MDH isoenzymes (Nos 1, 2, 5, 7, 9, 12, 14, 17). In the MDH complement of *P. tabacina* from *N. repanda* (Pt/Nr) component No 2 is common with the corresponding host, *N. repanda*. The same four components found in Pt/Nt (Nos 4, 6, 8, 13) are also observed in Pt/Nr but not in the plants; hence, they can be considered as fungus specific.

MDH isoenzymes Nos 5 and 9 are present in both hosts and fungi, and component No 11 is present in *N. tabacum*, Pt/Nt and Pt/Nr.

Bands Nos 10, 15, 16 are characteristic for *N. tabacum*, No 7 for *N. repanda*, and Nos 1, 12, 14, 17 for both tobacco genotypes.

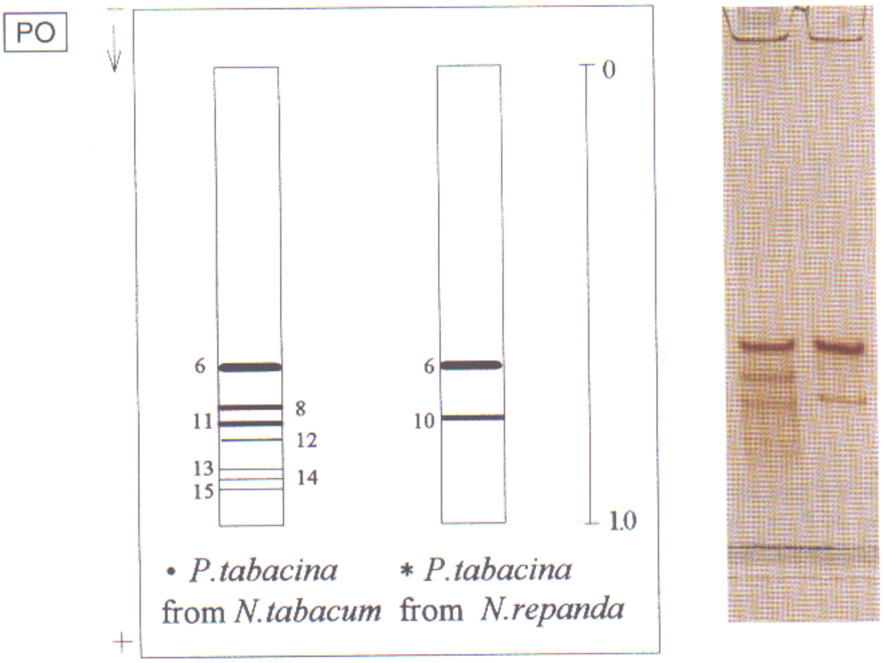


Figure 2.
Isoenzyme patterns of the peroxidase (PO), malate dehydrogenase (MDH) and superoxide dismutase (SOD) in conidia/conidiophores of *P. tabacina* originating from different tobacco hosts. *P. tabacina* from *N.tabacum* (•); *P. tabacina* from *N. repanda* (*). Slab electrophoresis in gradient (4 % – 30 %) polyacrylamide gel, pH 8.3.

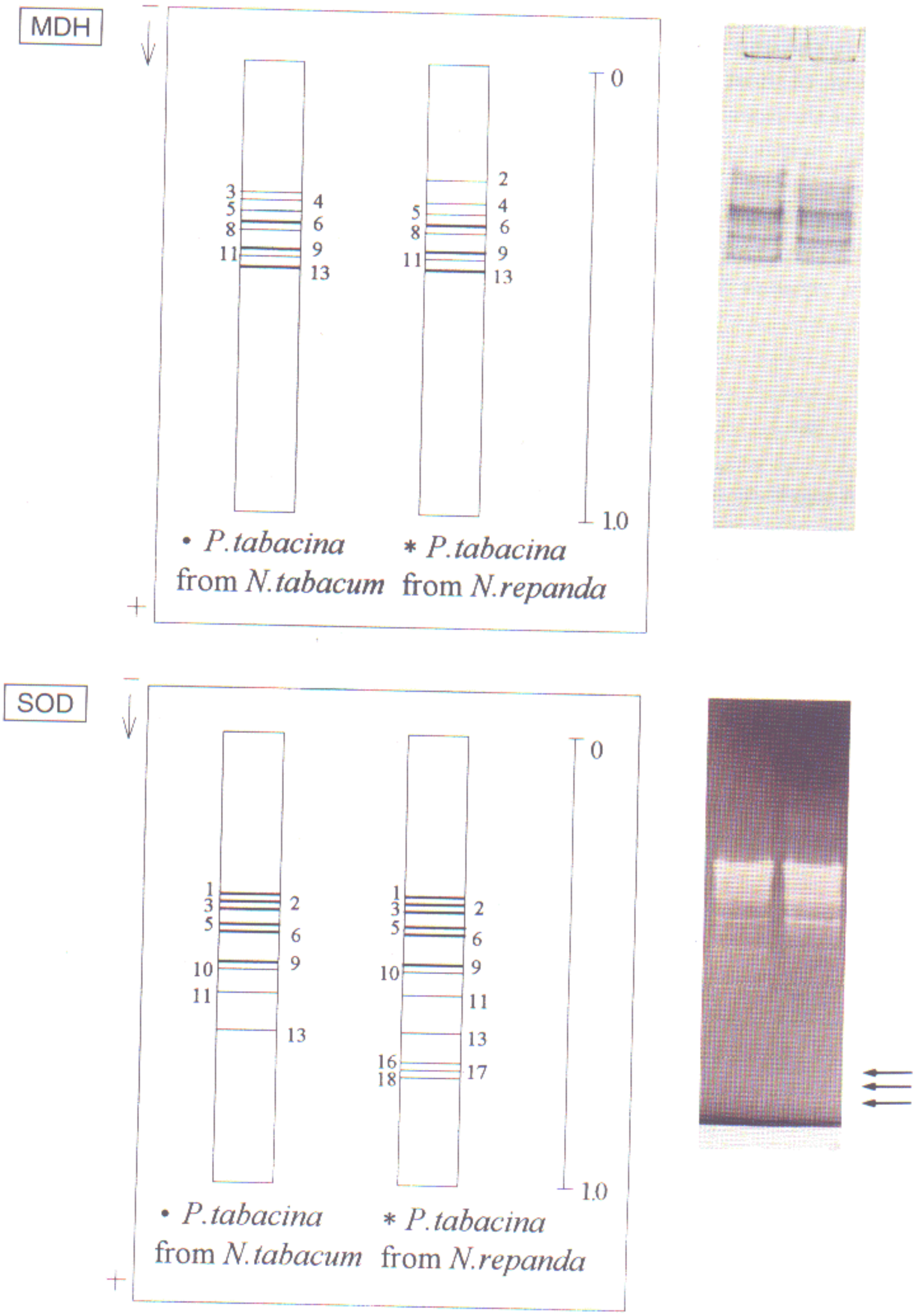

MDH
−
+
0
1.0
3 5 8 11
4 6 9 13
2 4 6 9 13
5 8 11
• P.tabacina from N.tabacum
* P.tabacina from N.repanda
SOD
−
+
0
1.0
1 3 5 10 11
2 6 9 13
1 3 5 10 16 18
2 6 9 11 13 17
• P.tabacina from N.tabacum
* P.tabacina from N.repanda

Figure 2 (contd).
Isoenzyme patterns of the peroxidase (PO), malate dehydrogenase (MDH) and superoxide dismutase (SOD) in conidia/conidiophores of *P. tabacina* originating from different tobacco hosts. *P. tabacina* from *N.tabacum* (•); *P. tabacina* from *N. repanda* (*). Slab electrophoresis in gradient (4 % – 30 %) polyacrylamide gel, pH 8.3.

Superoxide dismutase (Figure 5)

The complement of SOD in *N. tabacum* leaves contains eight components (Nos 7, 8, 10–15). *P. tabacina* from *N. tabacum* (Pt/Nt) does not share common components with its host *N. tabacum*.

In leaves of *N. repanda* six components (Nos 4, 10, 11, 16–18) are established. The last three of them (Nos 16–18) are organized in a triplet block. It is noteworthy that in *P. tabacina* from this host (Pt/Nr) the triplet is also expressed.

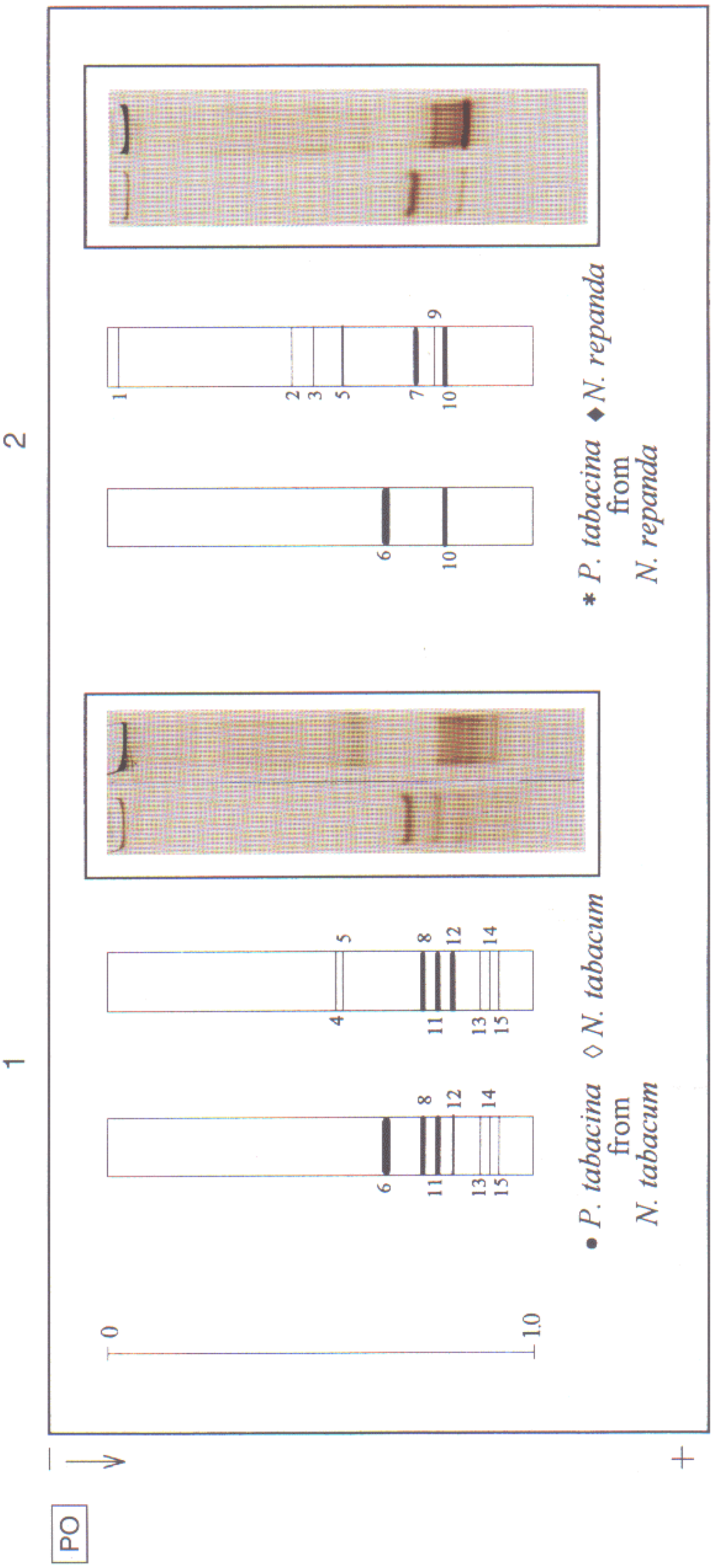
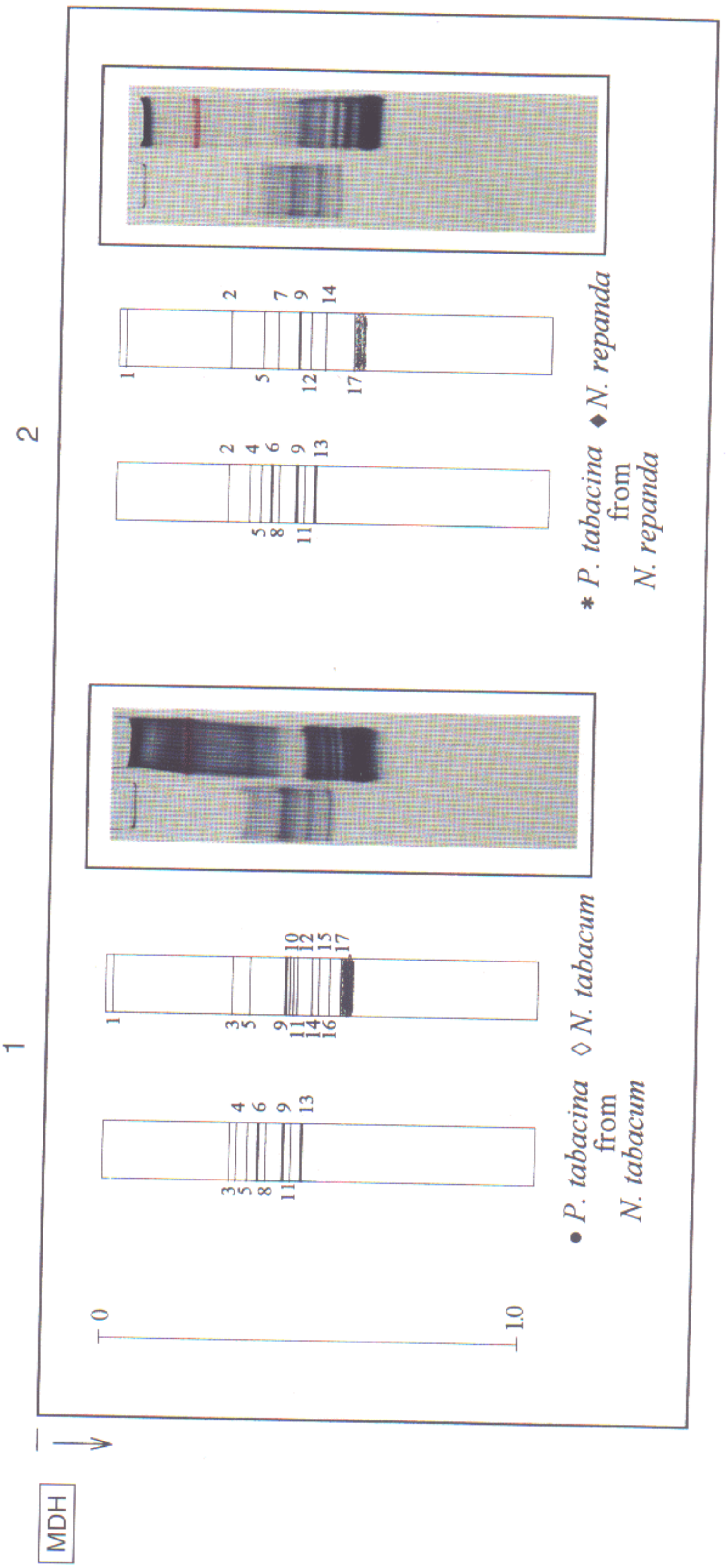

PO
−
+
1
2
0
1.0
• P. tabacina from N. tabacum
◊ N. tabacum
* P. tabacina from N. repanda
◆ N. repanda
6 8 11 12 13 14 15
4 5 8 11 12 13 14 15
6 10
1 2 3 5 7 9 10

Figure 3.
Isoenzyme patterns of the peroxidase (PO) in two tobacco - *P. tabacina* couples:
1 - *P. tabacina* from *N. tabacum* (conidia/conidiophores) (•) and *N. tabacum* (healthy leaves) (◊).
2 - *P. tabacina* from *N. repanda* (conidia/conidiophores) (*) and N. repanda (healthy leaves) (♦).
Slab electrophoresis in gradient (4 % – 30 %) polyacrylamide gel, pH 8.3.



MDH
0
1.0
1
2
3 5 8 11
4 6 9 13
1
3 5
9 11 14 16
10 12 15 17
5 8 11
2 4 6 9 13
1
5
12
17
2
7 9
14
● P. tabacina from N. tabacum
◇ N. tabacum
* P. tabacina from N. repanda
◆ N. repanda

Figure 4.
Isoenzyme patterns of the malate dehydrogenase (MDH) in two tobacco - *P. tabacina* couples:
Explanations: cf. Figure 3.

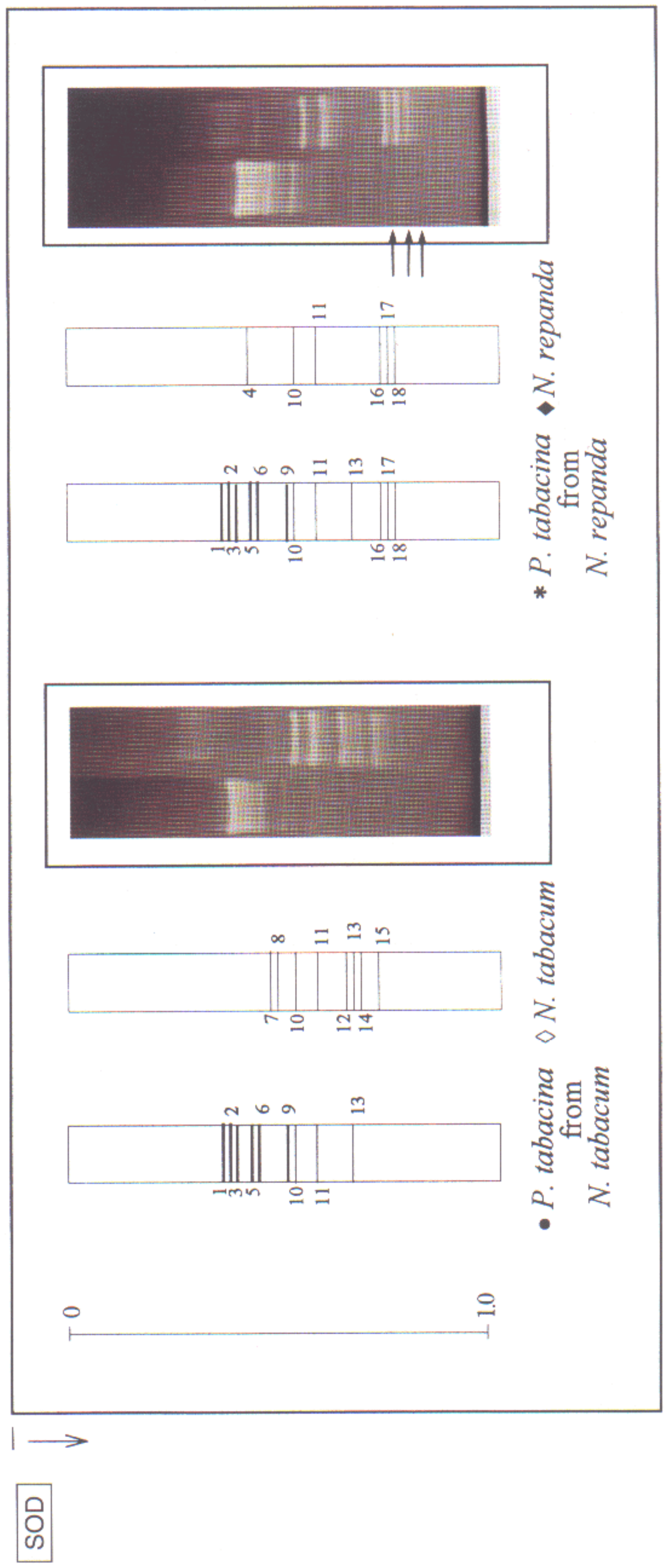

SOD
1
2
0
1.0
1 2 3 5 6 9 10 11 13
7 8 10 11 12 13 14 15
1 2 3 5 6 9 10 11 13 16 17 18
4 10 11 16 17 18
• P. tabacina from N. tabacum
◇ N. tabacum
* P. tabacina from N. repanda
◆ N. repanda

Figure 5.
Isoenzyme patterns of the superoxide dismutase (SOD) in two tobacco - *P. tabacina* couples:
Explanations: cf. Figure 3.

The same six isoenzymes found in Pt/Nt (Nos 1–3, 5, 6, 9) are observed in Pt/Nr as well (and not in tobacco) which allows to consider them fungus specific.

Components Nos 10, 11 are detected in both hosts and fungi, and component No 13 in *N. tabacum*, Pt/Nt and Pt/Nr.

Bands Nos 7, 8, 12, 14, 15 are characteristic for *N. tabacum*, and No 4 for *N. repanda*.

DISCUSSION

It may be considered that *P. tabacina* is genetically stable in a large area of Europe for a long period of time. This assumption is substantiated by the high similarity of the isoenzyme patterns of natural populations of the pathogen originating from France and from Bulgaria, and the not significant changes in these patterns between 1978 and 1992 (Figure 1). The data suggest the lack of differentiation processes and formation of new strains of *P. tabacina* in the period studied. They are in accordance with results of other authors (3, 11, 13, 19). An international research group led by C. E. Main (5) investigated the variability of *P. tabacina* pathogenicity, covering the Euro-Mediterranean and North American-Caribbean zones. The possible existence of biotypes or geographical isolates of *P. tabacina* was reported, but no data for their evolution in a uniform new strain (in terms of pathogenicity) were obtained (5).

Another finding flows from our study, namely the variability of *P. tabacina* as a function of the tobacco host. Thus, the isoenzyme patterns of conidia/conidiophores of *P. tabacina* produced on *N. tabacum* were distinct from those of *P. tabacina* produced on *N. repanda* (Figure 2). It is noteworthy that the changes in the isoenzyme patterns were expressed immediately after the transfer of *P. tabacina* from *N. tabacum* on *N. repanda*, i.e. in the first conidia/conidiophores produced on the new host.

In an attempt to explain this phenomenon, we compared the isoenzyme patterns of Pt/Nt and Pt/Nr with those of the corresponding tobacco hosts. This approach was inspired by the idea of protein homology in compatible plant-pathogen couples (17). Our data are in harmony with this idea. Thus, a striking similarity (80 % pair affinity index) of Pt/Nt with *N. tabacum* host was observed in the iso-PO patterns (Figure 3). Congruent results were obtained by other authors (8, 14); they demonstrated common antigens between host plants and obligate pathogens. Moreover, after the transfer of the fungus from *N. tabacum* on *N. repanda*, important changes occurred: repression of isoenzymes common with the former host, *N. tabacum* (Figures 3 and 4), and expression of isoenzymes common with the new host, *N. repanda* (the major iso-PO No 10, the SOD triplet Nos 16, 17, 18, and the MDH component No 2) (Figures 3–5).

These data are logical taking into account that *P. tabacina* is an obligate pathogen, i.e. its development is dependent on a specific host plant. The similar traits in the isoenzyme complements of the pathogen *P. tabacina* and the cultivated host, *N. tabacum,* may be explained in terms of commonality between genetic and metabolic machinery of both long coexisting partners.

The shifts in the isoenzyme patterns of *P. tabacina* as a function of tobacco host could be interpreted by assuming the coevolution of the fungus and *Nicotiana* plants. It may be admitted that genes of different species of *Nicotiana* genus are also present (in a silent state) in *P. tabacina* genome, and are induced by stimuli (signals) from the corresponding hosts. Hence, a switch of signals upon the transfer of *P. tabacina* on *N. repanda* could trigger changes of gene expression in the fungus; in these terms the repression of isoenzymes common with the former host, *N. tabacum*, and the expression of new isoforms, common with the new tobacco host, *N. repanda*, could be explained. Different but not-conflicting explanation (according C.E. Main, personal communication) could be that *P. tabacina* population consists of two pathotypes, Pt/Nt and Pt/Nr, and each of them becomes expressed as dependent on the availability of the corresponding host – *N. tabacum* or *N. repanda*.

An intriguing finding is the conservative character of the fungus specific isoenzymes, persisting independently of the change of host plants; this pointed to a certain autonomy of obligate pathogens, such as *P. tabacina*.

The data obtained reveal a part of the molecular mechanisms underlying obligatory parasitism, and could be used in tobacco blue mold epidemiology research. They could also serve as a basis for future studies in the field.

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