ISOENZYME ANALYSIS: A TOOL FOR CHARACTERIZING SUBPOPULATIONS OF *PLASMOPARA HALSTEDII*

Hedvig Komjáti, Department of Plant Protection, Szent István University, 2103 Gödöllő, Páter K. u.1. Hungary
E-mail: komjatih@fau.gau.hu

József Bakonyi, Plant Protection Institute, Hungarian Academy of Sciences, 1525 Budapest, P.O. Box 102., Hungary
E-mail: jbak@nki.hu

Ferenc Virányi, Department of Plant Protection and Group of Mycology, Hungarian Academy of Sciences, Szent István University, 2103 Gödöllő, Páter K. u.1. Hungary
E-mail: viranyi@fau.gau.hu

Abstract

The variability of *Plasmopara halstedii* populations have been intensively studied at the molecular level by different nucleic acid-based technologies, with no or very few polymorphisms. In the present work we analysed the isozyme pattern of some Hungarian subpopulations by means of CAE–electrophoresis. It was found to be a quick, simple, and inexpensive method, which had been successfully applied by others to oomycete organisms in the past and used for comprehensive population studies. Two isozymes, GPI and PGM, proved to be polymorphic within the samples tested, and both of these appeared to have two alleles present in our fungal populations. The characteristics of the allozymes, their frequency and distribution, as well as the possible use of this technique are discussed.

Introduction

Plasmopara halstedii (Farl.) Berl. and de Toni, the downy mildew pathogen of sunflower (*Helianthus annuus* L.) causes a devastating disease of this crop worldwide. Mildewed sunflower plants do not usually produce viable seed, and once they are systemically infected, they cannot recover from the disease. Over the last two decades, a dramatic change has become evident in the *P. halstedii* populations in many countries, as far as virulence phenotype and sensitivity to phenylamide fungicides are concerned. In Europe, an increasing number of pathotypes, each with distinct virulence structure, have been identified and at least six such variants have already been distinguished from Hungary (Virányi and Walcz, 2000). Furthermore, two alternative hosts of this fungus, the common cocklebur (*Xanthium strumarium* L.) and the common ragweed (*Ambrosia artemisiifolia* L.) have recently been found in Hungary both having the potential of serving as an additional source of inoculum and/or a gene pool for new pathogenic forms (Virányi, 1984; Virányi and Walcz, 2000).

Since fungal diversity of this kind has consequences in both disease epidemiology and breeding for resistance, there is a need to identify the virulence structure of local fungal

populations and to monitor the changes over time. The traditional methodology for virulence testing (Gulya et al., 1998; Tourvieille et al., 2000b) requires a standard set of sunflower differential lines and considerable bench space, and the results may sometimes be uncertain. However, little is known so far of the molecular nature of *P. halstedii* and the published data so far show very little variation between pathotypes and isolates (Roeckel-Drevet et al., 1997; Tourvieille et al., 2000a; Intelmann and Spring, 2002).

Cellulose-acetate electrophoresis (CAE) has been successfully used for estimating the amount of variation of oomycete fungi studying population structure, tracing origins of new pathogenic forms and analysing crosses (Micales et al., 1992; Goodwin et al., 1995). The success in using isozyme polymorphism among *Phytophthora* has prompted us to initiate similar experiments for studying the diversity of *P. halstedii*. To the best of our knowledge, this is the first report on allozyme genotype identification in this fungal pathogen.

Materials and Methods

The subject of investigation was single sporangial isolates (SSI) of *P. halstedii* derived from field isolates. The majority of the field isolates were collected in Hungary and one originated from Serbia-Montenegro, each belonging to one of the five pathotypes currently existing in Hungary (Virányi and Gulya, 1995). The field isolates and their virulence codes were as follows: Bi02 (100), 911 (100), 98 (700), 130 (700), 29-13 (700), 145 (710), 01/A2 (710), 101 (730), 129 (730), 68 (330), and 114 (330). An additional isolate, X02 (717), collected from *X. strumarium* in 2002 and maintained on the generally susceptible sunflower cultivar GK-70, was also included in the experiment. Genetically uniform material was obtained by producing SSIs using a modified methodology of Spring et al. (1998). SSIs were then propagated on their respective compatible sunflower line, and were subsequently re-tested for virulence by inoculating each on a set of standard differentials (Gulya et al., 1991) (Table 1).

Sufficient amount of sporangia were produced by culturing the fungal strains on cv. GK-70, the spores collected by means of a homemade vacuum-cyclone and stored at –20C prior to further use. Protein extraction was carried out by mixing 25 mg of sporangia with 25 mg of glass beads (Sigma Co.), homogenised for 2-3 min by a plastic pestle on ice in a microfuge tube using 200 µl extraction buffer (Ládai et al., 2000). Homogenates were kept on ice for approximately one hour prior to three centrifugations for 10 min each at 20000 rpm at 4C. Supernatant was collected successively without disturbing the upper layer and pellet and cleared solution was stored at –20C until use. Cellulose Acetate Gel Electrophoresis (CAE) was carried out following the protocol of Herbert and Beaton (1993) by using the CEA system of Helena Laboratories (Beaumont, TX), and Super Z-12 Applicator kit. Gels were stained for the enzymes GPI and PMG using their appropriate buffer system (Table 2). Chemicals used were purchased from Sigma-Aldrich Co. Photographs of gels were taken and stored under dry conditions. The relative mobility of bands was calculated based on the % of mobility of each line from the anodal base line. The most common band was chosen to be Rf=100. All isozyme polymorphisms were confirmed by a second protein extraction and staining for enzyme reactions to exclude misinterpretation of such polymorphic bands from enzyme degradation.

Table 1. A list of single sporangial isolates (SSIs) used in isozyme analyses together with data on their origin and virulence phenotype.

Isolate designation	Virulence code	Year of collection	Place of origin
Bi02/A4	100	2000	Hungary
911/C12	100	1995	Serbia
911/C11	700	1995	Serbia
29-13/F3	700	1998	Hungary
145/B7	700	1994	Hungary
98/A22	700	1991	Hungary
130/B20	700	1993	Hungary
130/B15	700	1993	Hungary
29-13/F23	710	1998	Hungary
29-13/G23	710	1998	Hungary
130/B1	710	1993	Hungary
145/C9	710	1994	Hungary
145/C15	710	1994	Hungary
01/2A	710	2001	Hungary
129	730	1993	Hungary
101/C1	730	1991	Hungary
114/A10	330	1992	Hungary
61/D7	330	1989	Hungary
X02	717	2002	Hungary

Table 2. Isozymes and their characteristics used in the study.

Enzyme	Abbreviation	EC no.	Buffer	Activity	Rf values
Glucose-6-Phosphate Isomerase	GPI	5.3.1.9	TG	+	100, 113
Phosphoglucomutase	PGM	5.4.2.2	TG	+	90,100

Explanations: + = positive reaction.

Results and Discussion

The isozyme staining reactions for the enzymes GPI and PGM proved to be polymorphic among the fungal isolates. GPI has probably two alleles, 100 and 113. GPI is coded in one locus but the quaternary, active form of the enzyme is a dimer, e.g., 2 subunits should be linked together to gain enzyme activity. The sample is homozygous when two identical subunits come together to form an active enzyme. We have found strains with homodimer 100/100, and heterodimer 100/113 allels,. Only two isolates were heterozygous for GPI, Bi02/B6 and 130/B15; the other SSIs derived from either the field isolate Bio2 or 130 were all as homozygous for the GPI as all the other isolates. This might refer to the previous results we received with mixed field isolates where during single sporing we had obtained strains that differed in virulence structure from the starting material. Field isolates of *P. halstedii* used in our tests either were not genetically pure, or else mixing of sporangia happened during

propagation. Among the samples tested here we did not find any isolate homozygous for the GPI allele 113, genotype 113/113.

PGM was also polymorphic among the *P. halstedii* isolates tested. The two alleles representative for the PGM were 90 and 100 (Figure 1). PGM is also coded in one locus but the quaternary structure of the enzyme is a monomer. In this case the possible combinations for the two alleles are: 90/90, 90/100 and 100/100. The homozygous genotype produced one single band on the gel, whereas a heterozygous sample would give us two bands with PGM. We did not find any heterozygous isolate in this study. The isozyme pattern appeared to refer to location rather than to pathotype, since those SSIs either derived from the South of Hungary (Bi02/B4, Bi02/B6) or from Serbia (911/C11, 911/C12) carried the rare allele 90/90 in a homozygous manner, whereas all the other isolates had the 100/100 pattern. Interestingly, the isolate X02 collected from *X. strumarium* did not differ in isozyme pattern from those isolated in sunflower, although it is presumed that X02 might belong to a different species, *Plasmopara angustiterminalis* (Novotel'nova, 1966). Although this isolate was also collected in the South of Hungary, it did not carry the PGM 90 allele.

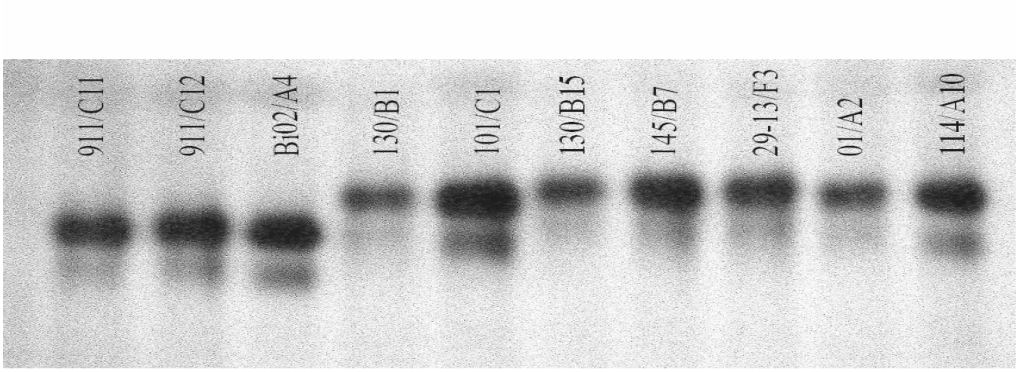

Figure 1. Isozyme pattern of PGM

For isozyme analysis of the sunflower pathogen, *P. halstedii*, CAE was found to be an appropriate technique since it required small amounts of fungal sporangia. The ability of CAE to exploit isozyme patterns from sporangia has been shown previously for *Phytophthora infestans* by Goodwin et al. (1995). In the case of *P. infestans* the polymorphic allozymes were GPI and PEPA. We tested 16 different isoenzyme systems including PEPA (data not shown). In our case the PEPA isoenzyme reaction was difficult to stain well, and produced a weak staining reaction. So, we can conclude that there might be differences between the type of polymorphic isoenzymes in these two oomycete fungi. The number of allozymes (isozymes which are allelic variants) was lower in *P. halstedii* than in the *P. infestans,* where four different allozymes were detected for the GPI isoenzyme (Goodwin et al, 1995).

In summary, the use of isoenzyme analysis seems to be an alternative tool in characterising local populations of *P. halstedii* and monitoring changes at various population levels. In the future we plan to continue our studies involving more isolates deriving from different hosts and regions in order to get better distinction within or among fungal species causing downy mildew disease in sunflower.

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