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Isozyme gene markers in the dioecious species *Asparagus officinalis* L.

E. Maestri [1], F.M. Restivo [1], G.P. Marziani Longo [2], A. Falavigna [3] and F. Tassi [1]

[1] Institute of Genetics, University of Parma, Viale delle Scienze, I-43100 Parma, Italy
[2] C.N.R. Study Center for the Cellular and Molecular Biology of Plants, Department of Biology, General Botanical Section, Via Celoria 26, I-20133 Milano, Italy
[3] Research Institute for Vegetable Crops, Via Paullese 28, I-20075 Montanaso Lombardo (MI), Italy

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Summary. Extracts from phylloclads of *Asparagus officinalis* were electrophoretically analyzed for isozyme polymorphism. Fourteen enzyme systems were examined using four buffer systems: seven enzymes (acid phosphatase, catalase, glutamate-oxaloacetate transaminase, isocitrate dehydrogenase, malate dehydrogenase, peroxidase, and 6-phosphogluconate dehydrogenase) exhibited clear and consistent banding patterns. Isozyme polymorphism was studied in seven pairs of male and female doubled haploids and in their male F_1s. Segregation of polymorphic loci was examined in the backcross progenies and was found to be consistent with a simple Mendelian inheritance in all cases, except for three anodical peroxidases, where two factors have been hypothesized. No linkage could be found between isozyme markers that were segregating in the same cross, but association was demonstrated between one malate dehydrogenase locus and the sex determining genes. The availability of isozyme markers may be useful in breeding and, in particular, the localization of one malate dehydrogenase locus on the sex chromosomes may be helpful in mapping the sex genes.

Key words: *Asparagus officinalis* – Isoenzymes – Marker genes – Sex-linked inheritance

Introduction

Asparagus officinalis ($2n = 2X = 20$) is by nature a dioecious species, but cultivars may vary in their degree of "maleness," ranging from pure males to males showing some stylar growth up to true hermaphroditic plants. Genetic experiments have demonstrated that females are homogametic (conventionally XX), while male plants are heterogametic (XY) for sex chromosomes with "maleness" dominant (Rick and Hanna 1943). Primary sex determining factors have been associated with the homomorphic chromosome pair *L5* (Loptien 1979) and, on the basis of the rare occurrence in some crosses of hermaphroditic and neuter plants, a bipartite function (dominant suppressor of female and dominant activator of male organs) has been suggested (Marks 1973; Westergaard 1958). Modifying genes that influence pistil development have been also described; these genes can be manifest in male plants producing hermaphrodites (Franken 1970; Peirce and Currence 1962). Association between modifying genes and sex chromosomes is at present uncertain.

In a project aimed towards understanding the genetic and physiological bases of sex expression (Bracale et al. 1990; Galli et al. 1988; Marziani Longo et al. 1990), we have undertaken the search for isozyme gene markers in *Asparagus*. This would be useful for several reasons: (i) first of all, since a genetic map of *Asparagus* is lacking, it will allow the establishment of linkage groups which can be useful in breeding; (ii) it may serve to map sex chromosomes and to find associations between markers and sex-modifying genes; (iii) since, from the commercial viewpoint, male plants are more productive and females represent a source of unwanted seedlings, sex-linked markers will allow the plant sex to be determined in a nondestructive way at the seedling stage.

Materials and methods

Plant material

The *Asparagus officinalis* genotypes used in the present work are reported in Table 1. Parents came from the Research Institute for Vegetable Crops (Section of Montanaso Lombardo) and are doubled haploid clones obtained from in vitro anther culture (Falavigna et al. 1983). Seven F_1s (cross A to E, G, and S-795)

were produced; since the male parents were homozygotes (YY), all F_1s were male plants. F_1 seeds were sown and, at flowering, one individual F_1 plant for each cross was used to pollinate the same female plant originally entered in the cross. BC_1 seeds were grown in greenhouse and, at flowering, the sex of each plant was determined. Occasionally, plants with male flowers bearing well-developed ovaries and styles were observed and, in these plants, rare berries were present: these plants were classified as hermaphrodites (Table 1).

Isozyme analysis

Extraction. Phylloclads from adult parents and F_1 plants grown in open fields and from 1 year-old BC_1 plants grown in greenhouse were collected in late spring 1989 at flowering. Phylloclads were frozen in liquid nitrogen and stored at −80 °C until analysis.

Extracts for peroxidase isoenzyme separation were prepared from frozen phylloclads homogenized with a cell dismembrator (Braun Mikro Dismenbrator II) and suspended in a buffer containing 10 m*M* Hepes (pH 7.5), 1 m*M* dithiothreitol, and 1 m*M* EDTA (200 µl per 100 mg of tissue). The extracts were clarified by centrifugation in a microcentrifuge (Hereaus Biofuge A) and stored at −20 °C. The extraction procedure for the analysis of the remaining enzymes involved grinding with carborundum powder in 0.05 *M* Tricine-NaOH (pH 7.8), 0.01 *M* $MgSO_4$, and 0.1% 2-mercaptoethanol. The frozen extracts were thawed only once for electrophoretic analysis.

Starch gel electrophoresis. Three buffer systems were employed for resolving the various isoenzymes tested: (1) histidine-citrate, pH 6.5, (Cardy et al. 1983) for the dehydrogenases and acid phosphatase; (2) lithium borate-TRIS citrate, pH 8.3 (Cardy et al. 1983), for peroxidase; (3) TRIS-glycine, pH 8.7 (Scandalios 1969), for catalase and glutamate-oxaloacetate transaminase. Gels were prepared as described by Cardy et al. (1983) with 11.5% (w/v) starch (Sigma) and 3% (w/v) sucrose. Samples were loaded on Whatman 3 wicks inserted in wells cut approximately 5 cm from the cathodal edge of the gel. Electrophoresis was performed at 4 °C on LKB 2117 Multiphor II at constant power (40–50 W for a 15 × 15 × 1 cm gel). After 1 h wicks were removed and gel continued to run for 2 h. Staining procedures for isocitrate dehydrogenase, malate dehydrogenase, 6-phosphogluconate dehydrogenase, catalase, and acid phosphatase were performed essentially according to the recipes of Cardy et al. (1983); peroxidase staining was performed according to Graham et al. (1964). Gels to be stained for glutamate-oxaloacetate transaminase activity were incubated for 30 min in a mixture composed of 0.2 *M* TRIS-HCl (pH 8.0), 2 mg/ml each of aspartic acid and 2-oxoglutaric acid, 0.2 mg/ml of pyridoxal-5-phosphate; they were then stained with 1.5 mg/ml Fast Blue BB salt in 0.2 *M* TRIS-HCl, pH 8.0. With the exception of catalase and peroxidase, all staining mixtures contained 0.4% agarose.

Results

Sex segregation in backcrosses

BC_1 plants deriving from crosses A, B, D, G, and S-795 segregated male and female plants with a ratio close to 1 : 1, as expected from the cross of a heterozygous F_1 (XY) with the homozygous (XX) female (Table 1). In cross C, a 3 : 1 ratio male : female was observed. In crosses E and G three and two hermaphrodites were present, respectively; these plants were considered male plants with tendency to hermaphroditic behaviour.

Electrophoretic patterns

Out of 14 enzymes tested, 7 (alcohol dehydrogenase, esterase, glutamate dehydrogenase, glucose-6-phosphate dehydrogenase, glutamine synthetase, phosphoglucomutase, and phosphohexoseisomerase) proved to be unsatisfactory for isozyme analysis, either because bands were faint or indistinct or did not occur consistently. For the remaining enzymes, where clear bands were reproducibly obtained, a search for polymorphism in isozyme patterns among the parental genotypes and, in the favorable cases, a study of the inheritance of the different phenotypes were undertaken.

Isocitrate dehydrogenase. [IDH; E.C.1.1.1.42; Threo-D_s-isocitrate: $NADP^+$ oxidoreductase (decarboxylating)]. Variations in the band number (from two to eight bands) were observed among parental genotypes. In most cases, however, F_1 and BC_1 patterns were not amenable to a simple Mendelian analysis. Cross C instead proved to be satisfactory; in this cross the female parent showed a three-banded phenotype, whereas in the male only the slowest and the fastest migrating bands were present (see

Table 1. Genotypes used for isoenzyme analysis

Cross	Parentals		F_1	BC_1	Sex of BC_1 plants			χ^2 1:1	χ^2 1:3
	♀	♂			♀	♂	⚥[a]		
A	1396-33	1666-10	H387-1	1396-33 × H387-1	43	44		0 NS	
B	1847-8	1666-10	H423-1	1847-8 × H423-1	46	45		0 NS	
C	1767-10	2038-10	H404-1	1767-10 × H404-1	6	17		4.35 *	0 NS
D	1396-33	1979-1	H390-1	1396-33 × H390-1	40	34		0.34 NS	
E	1796-1	1559-6	H415-1	1796-1 × H415-1	39	29	3	0.51 NS	
G	109-21	127-1	H236-1	109-21 × H236-1	34	32	2	0.01 NS	
S-795	46-1	127-1	H99-24-1	46-1 × H99-24-1	23	21		0.02 NS	

[a] ⚥, plants with male flowers which present well-developed ovaries and styles and occasionally bearing berries
NS – not significant
* $P<0.05$

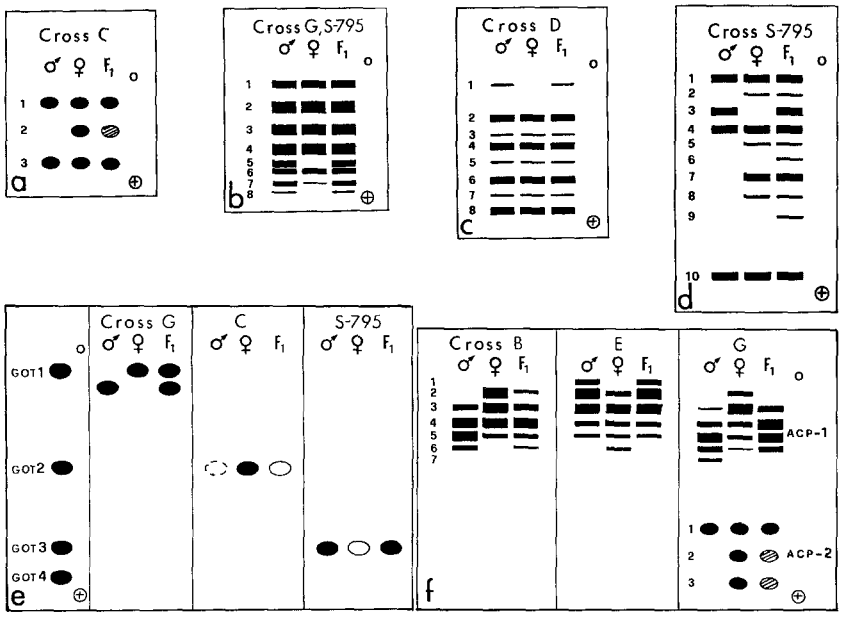

Fig. 1 a–f. Diagrammatic representation of banding patterns for: **a** isocitrate dehydrogenase; **b** malate dehydrogenase; **c** 6-phosphogluconate dehydrogenase; **d** catalase; **e** glutamate-oxaloacetate transaminase; **f** acid phosphatase. *Top row* indicates the cross(es) in which the polymorphism was evidenced; phenotypes of the male (♂) and female (♀) doubled haploids and of their F_1s are reported. Bands are numbered from the origin (o) towards the anode (⊕)

Table 2. Single-locus segregation of the different isoenzymes

Isozyme	Cross	Progeny phenotype[a]		Total	χ^2	P
		Mat_T	F_{1T}			
IDH-2	C	8	8	16	0.06	NS
MDH	G	14	25	39	2.56	NS
	S-795	25	19	44	0.57	NS
	Pool	39	44	83	0.19	NS
6PGD	D	11	9	20	0.05	NS
CAT	S-795	19	25	44	0.57	NS
CP1	C	9	5	14	0.64	NS
	D	7	10	17	0.24	NS
	E	4	8	12	0.75	NS
	Pool	20	23	43	0.09	NS
GOT-1	G	20	17	37	0.11	NS
GOT-2	C	8	8	16	0.06	NS
GOT-3	S-795	21	23	44	0.02	NS
$ACP\text{-}1_B$	B	13	13	26	0.04	NS
$ACP\text{-}1_E$	E	11	9	20	0.05	NS
$ACP\text{-}1_G$	G	17	22	39	0.41	NS
ACP-2	G	21	17	38	0.24	NS

[a] Mat_T, maternal type pattern; F_{1T}, F_1 type pattern
NS – not significant

Fig. 1 a). In BC_1 plants, a segregation for the staining intensity of the intermediate band, IDH-2 (strong versus weak corresponding, respectively, to the maternal and F_1 phenotypes), was observed with a 1:1 ratio (Table 2). The simplest explanation of the results is that IDH-2 is encoded by a gene with a producing, positive allele present in the female and a null allele in the male parent.

Malate dehydrogenase. (MDH; E.C.1.1.1.37; L-malate: NAD^+ oxidoreductase). Isoenzymes of MDH resolved in two zones of activity; only the anodal zone gave distinct bands in a ladder that varied frequently in intensity among the different genotypes. In two crosses, G and S-795, the differences between parental lines concerned also the number of bands (Fig. 1 b): the common male parent 127-1 showed a pattern characterized by eight bands, whereas the female parents showed patterns lacking bands 5 and 8. Both F_1 hybrids, showing an eight-banded pattern, yielded BC_1 progenies in which two phenotypic classes were present: the maternal and the F_1 patterns. Segregation ratios for the two classes did not differ significantly from 1 : 1 (Table 2). Since MDH isoenzymes are frequently dimers (Gottlieb 1982), the results can be explained by assuming that the products of different loci concur in producing the observed phenotypes; the presence of a null allele at one of these loci would bring about the disappearance of different bands (the homodimer and some heterodimers).

6-phosphogluconate dehydrogenase. [6PGD; E.C.1.1.1.44; 6-phospho-D-gluconate: $NADP^+$ 2-oxidoreductase (decarboxylating)]. 6PGD resolved in a series of eight bands, four major and four minor, and polymorphism in band intensity occurred most frequently among genotypes. In cross D, however, the female parent lacked band 1, which was detectable in the patterns of the male parent and of the F_1 hybrid (Fig. 1 c). In the BC_1 individuals, segregation of presence versus absence of this band fits with a 1:1 ratio (Table 2), hence with a monogenic inheritance.

Catalase. (CAT; E.C.1.11.1.6; Hydrogen peroxide: hydrogen peroxide oxidoreductase). Two different phenotypes of CAT isozyme patterns were observed among the

parental lines of crosses C, G, and S-795. The first pattern consisted of four intense, well-separated anodal bands (named 1, 3, 4, and 10 in Fig. 1 d) while, in the second pattern, band 3 was missing and a faster-migrating one (number 7) appeared. In addition, three minor bands, interspersed among the major ones, were also present in this second phenotype. Inheritance of the CAT isozyme pattern was studied in cross S-795 since, in this case, the two parents showed contrasting phenotypes. F_1 progeny yielded a codominant pattern as far as bands 3 and 7 were concerned and additional bands not present in the parents became visible. In BC_1 plants, a 1 : 1 segregation for the F_1 and the maternal phenotype was observed (Table 2). A possible and simple explanation is that bands 3 and 7 are encoded by a single catalase gene, with two codominant alleles differing in the electrophoretic mobility of their products. Since catalase is reported to be a tetramer (Scandalios 1969), however, alternative hypotheses, based on two loci producing homo- and heterotetramers, are also conceivable.

Peroxidase. (E.C.1.11.1.7; Donor: hydrogen peroxide oxidoreductase). Figure 2 shows the isoperoxidase patterns of parents and F_1 of four out of seven crosses studied. In the cathodal part of the gel a major band was present, varying in electrophoretic mobility (fast or slow) among parental genotypes. In all the F_1s where the parents were polymorphic a codominant pattern was observed. In BC_1 individuals of crosses C, D, and E, a 1 : 1 segregation for the codominant and the fast or slow phenotypes, depending on the particular cross analyzed, was observed, suggesting that this cathodal peroxidase is encoded by a single gene (CP1) with two codominant alleles (fast and slow) (Table 2). [The CP1 band present in starch gels can be resolved in four bands by isoelectric focusing (IEF) separation on polyacrylamide gels (not reported), with the "fast" isoenzyme on starch gel producing four IEF bands with a pI higher than the four bands deriving from the "slow" isozyme. Similar data were also reported by Roux and Roux (1981). Since the starch "fast" and "slow" isoforms segregate in a simple Mendelian ratio, however it is likely that the IEF bands correspond to post-translational modifications of the product of a single gene.]

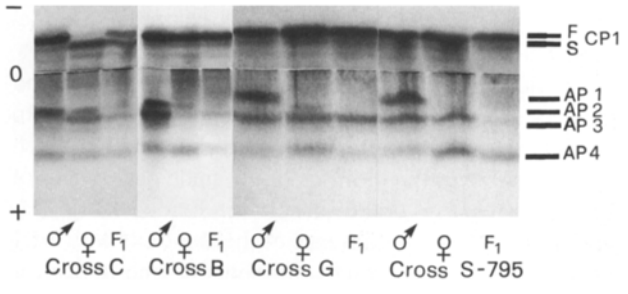

Fig. 2. Zymograms for peroxidase from the crosses reported in the *bottom row*. The schematic representation on the *right* shows the four anodal peroxidases (numbered in the direction of migration) and the two forms of the cathodal peroxidase (CP1), fast (F) and slow (S)

In the anodal part of the gel, four major peroxidase bands were present (named AP1 to AP4, see diagram in Fig. 2). Polymorphism (presence versus absence) among the parental genotypes was observed for three of these peroxidases (AP1, 2, and 3). In those crosses where the parents differed in one or another of the AP, an abnormal F_1 pattern was frequently observed. For example, AP1, present in the male of crosses G and S-795 and absent in the respective females, was absent in the F_1s, indicating a nondominant inheritance of the presence of the band versus its absence. AP2 still showed an abnormal F_1 pattern: in crosses C and B, AP2 was present in both parents, but absent in the F_1. AP3 presented a dominant inheritance in crosses B, C, and E but deviated in crosses D and S-795; in this last cross the band, present in both parents, was lacking in the F_1.

The results obtained when the BC_1 individuals were assayed for AP phenotypes can be summarized as follows: (i) AP1 was always absent in the BC_1 plants; apparently, the recurrent female genotype inhibits AP1 expression. (ii) AP2 segregated presence/absence with a ratio close to 1 : 1 in BC_1 plants of crosses B and C, even if the respective F_1 phenotypes deviated from those expected from the parental phenotypes (assuming that the presence of the band is dominant over the absence). A possible explanation of these results is that AP2 expression is under the control of two complementary, dominant inhibitory genes (I1, I2) and AP2 is suppressed only in genotypes bearing at least one dose of the two factors. Accordingly, the males of crosses B and C can be homozygous dominants for one of the inhibitory genes and recessives for the other one (I1I1, i2i2), while the females have the opposite genotype (i1i1, I2I2). In the heterozygous F_1s (I1i1, I2i2) AP2 expression is suppressed, while in BC_1 progenies a 1 : 1 ratio for presence versus absence of AP2 is expected. (iii) For AP3, BC_1 phenotypes segregated presence/absence of the band in all crosses where polymorphism was present in the parents with a ratio close to 1 : 1. In crosses B and E, the simplest hypothesis reconciling the parental, F_1, and BC_1 phenotypes is to assume that the female parents bear a null allele of the AP3 gene and the male a positive, producing allele. Accordingly, the heterozygous F_1 produces AP3 and the backcross with the null recurrent females gives plants segregating 1 : 1 presence of AP3 versus absence. This explanation however does not hold for crosses D and S-795 where the F_1 phenotypes are deviating; in these two cases, a model similar to that proposed for AP2 of crosses B and C can explain the data. AP3 of cross C remains instead a puzzle: an explanation based on a null allele seems not valid since, the recurrent female being a

producing genotype, all backcrosses should have the band; alternatively, in the two inhibitory genes hypothesis, the F_1 should be negative. Clearly a more detailed study is necessary to confirm the hypotheses and solve the unexplained data.

Glutamate-oxaloacetate transaminase. (GOT; E.C.2.6.1.1; L-aspartate: 2-oxoglutarate aminotransferase). Separation of GOT isoenzymes yielded four clearly distinct zones of activity, numbered from 1 to 4 in the direction of migration (Fig. 1 e, first panel); a fifth more cathodal zone was too weak for a satisfactory analysis. Polymorphism among the parental lines was observed for the first three zones (GOT-1, -2, and -3). For example, in cross G, male and female parents possessed a fast and a slow migrating form of GOT-1, respectively, while in crosses C and S-795, the parents differed in the intensity of GOT-2 and -3, respectively. In the three crosses, F_1 progenies always gave codominant patterns and 1 : 1 segregations for maternal and F_1 phenotypes were observed in BC_1 plants (Table 2). The results were provisionally interpreted as due to the presence of three different polymorphic loci for GOT-1, -2, and -3 isozymes in the *Asparagus* genome, even if the non-contemporary segregation in the same cross of the three forms does not allow definitive conclusions.

Acid phosphatase. [ACP; E.C.3.1.3.2; Orthophosphoryl-monoester phosphohydrolase (acid optimum)]. Two zones of staining, ACP-1 and ACP-2, were detectable on the gels. ACP-1 patterns were composed of at least three and at most seven bands among the genotypes studied. In cross B (Fig. 1 f) the male parent showed a pattern centered on bands 4–5, whereas the female one showed a greater intensity for bands 2-3-4. The F_1 hybrid had band 4 as the most prominent one. In the BC_1 progeny, a segregation for an F_1 type pattern or a maternal type pattern was observed with a 1 : 1 ratio (Table 2). Other crosses in which ACP-1 was segregating were E and G; the patterns are represented in Fig. 1 f and segregation data are listed in Table 2. Data in Table 2 cannot be cumulated since several genes are expected to be involved in determining ACP-1 patterns.

The second zone of activity, ACP-2, more anodal than ACP-1, resolved in "spots" rather than in sharp bands. The only cross in which it was found to be polymorphic was cross G (Fig. 1 f). The male parent possessed only band 1, whereas the female parent had three bands of equal intensity; their F_1 hybrid had the three bands, with the first one more intense. The segregation of this marker in BC_1 individuals fits with a monogenic inheritance (Table 2); this locus is not linked with the ACP-1 locus which was segregating in the same cross.

Table 3. Chi-square tests for linkage of isozyme markers and sexual phenotype

Isozyme	Cross	Progeny phenotypes[a]				Total	χ^2	P
		♂/P	♂/M	♀/P	♀/M			
IDH-2	C	5	5	3	3	16	0.27	NS
MDH	G	11	6	2	18	37	9.79	**
	S-795	16	5	3	20	44	15.36	***
	Pool	27	11	5	38	81	27.37	***
6PGD	D	6	4	3	7	20	0.81	NS
CAT	S-795	13	8	12	11	44	0.12	NS
CP1	C	4	5	1	4	14	0.11	NS
	D	4	4	6	3	17	0.04	NS
	E	4	2	4	2	12	0.38	NS
	Pool	12	11	11	9	43	0.02	NS
GOT-1	G	8	8	8	12	36	0.07	NS
GOT-2	C	5	5	3	3	16	0.27	NS
GOT-3	S795	13	7	10	12	42	0.92	NS
$ACP\text{-}1_B$	B	7	4	6	9	26	0.63	NS
$ACP\text{-}1_E$	E	4	6	5	5	20	0	NS
$ACP\text{-}1_G$	G	8	9	13	7	37	0.58	NS
ACP-2	G	6	11	10	10	37	0.32	NS

[a] ♂/P, ♀/M, parental classes; ♂/M, ♀/P, recombinant classes
NS – not significant
** $P<0.01$
*** $P<0.001$

Analysis of linkage relationships

Among the isoenzyme markers showing a simple Mendelian inheritance we have identified in five out of seven crosses, 14 linkage tests were possible. In all cases, the data fit with independent segregation of the two markers. Since linkage analysis was not possible for every combination of the 12 markers, only preliminary information on the number of linkage groups may be provided. From this point of view, cross G was the most informative since four isozymes (MDH, GOT-1, ACP-1, and ACP-2) segregated contemporaneously. All the corresponding loci showed independent assortment, indicating a possible location on four different chromosomes. Markers segregating in the remaining crosses (C, S-795, D, and E) also identify different linkage groups for loci encoding IDH-2/CP1/GOT-2, MDH/CAT/GOT-3, 6PGD/CP1, and CP1/ACP-1, respectively. However, since it was not possible to analyze all the combinations, they can correspond to all or some of the groups identified in cross G.

As far as the linkage of isozyme markers with sex-determining factors is concerned, the null hypothesis could only be rejected for MDH (Table 3). As shown in Fig. 3, most BC_1 females showed a six-banded pattern (maternal phenotype, Fig. 1 b), whereas the majority of BC_1 males showed the eight-banded pattern (F_1 phenotype). The recombination frequency between the MDH locus segregating in crosses G and S-795 and the sex-determining genes is 19.8% when data of both crosses are pooled. All other markers segregated independently with respect to sex genes.

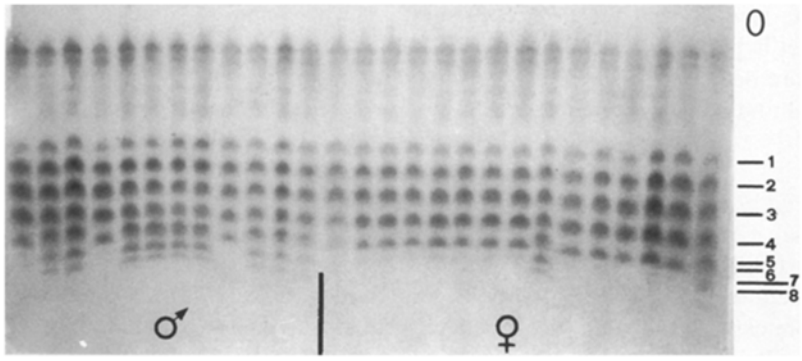

Fig. 3. MDH zymograms of the segregating progeny from the backcross of the F_1 plant H99-24-1 with the female plant 46-1 (cross S-795). The patterns on the *left* correspond to male BC_1 plants, those on the *right* to female BC_1 plants. For the schematic representation of the patterns, see Fig. 1 b

Discussion

To our knowledge, no genetic data based on biochemical markers are as yet available in *Asparagus officinalis*. Thus, our data are interesting because they demonstrate the feasibility of such an analysis in this species, due to the extensive polymorphism encountered for several isozymes among our homozygous doubled haploid clones. The availability of this material is particularly important for our study because, in a dioecious species like *Asparagus*, individuals are expected to be highly heterozygous, and this introduces an element of uncertainty in the determination of the parental genotypes. As a matter of fact, in the great majority of the isoenzyme systems analyzed in the present work, segregation data fit with simple models based on the assumption of positive/null or fast/slow migrating alleles at the various loci. The only exception encountered concerns the inheritance of the three anodal peroxidases (AP1, 2, and 3) where a two-factor genetic control has been hypothesized; the model is amenable to direct test in the following generation.

In this first study, linkage analysis between couples of isozyme markers identifies a minimum estimate of four linkage groups (those defined by markers segregating in cross G) to a maximum of ten, assuming that the loci identified in crosses C, S-795, D, and E segregate independently from each other and from those occurring in cross G. Each group at present is defined only by the association of a single locus encoding a specific isozyme, except one where major sex factors and one MDH locus are present. This last finding is noteworthy since it locates the MDH locus on chromosome 5 together with major sex genes (Loptien 1979); furthermore, the occurrence of recombination between sex genes and MDH locus implies that the two sex homologues in *Asparagus* can recombine in agreement with their homomorphic appearance. Finally, the availability of biochemical markers identifying the "X" and "Y" chromosomes will allow us to analyze in more depth the chromosomal and genetic basis of hermaphroditism in *Asparagus*.

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