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Title: Isozyme Banding Patterns and Embryo Development in Interspecific Crosses of Phaseolus.

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Dr. David W. S. Mok

Interspecific hybridization between Phaseolus vulgaris and P. coccineus results in the formation of either mature F_1 seeds or abnormal embryos, depending on the direction of the cross. However, normal as well as abnormal embryos occur in the reciprocal F_2 populations. The exact cause of abnormal embryo development is unknown. To determine if there is a correlation between the genomic composition and embryo development (normal vs. abnormal), isozyme banding patterns were examined in the reciprocal F_1 and F_2 progenies of P. vulgaris cv. Great Northern (GN) and P. coccineus cv. Scarlet Runner (SR). Three enzyme systems, acid phosphatase (AP), esterase (EST), and malate dehydrogenase (MDH) were analysed in extracts of immature embryos at the cotyledonary stage. Parental species and reciprocal F_1 and F_2 embryos exhibited clear differences in electrophoretic patterns. The embryos of reciprocal crosses displayed banding patterns characteristic for their developmental type (normal or abnormal). AP and MDH zymograms of normal embryos were closer to those of the GN parent, whereas abnormal embryos had isozyme bands similar to those of SR. Differences in EST banding patterns were apparent between normal and abnormal F_2 embryos, however,

these patterns did not resemble either parent. The results indicate that embryo development can be correlated with particular isozyme patterns which may at least in part reflect the genetic combination. They also seem to substantiate the hypothesis that the skewed distributions of quantitative traits in interspecific progeny populations could be related to selective survival of seeds containing normal embryos.

Isozyme Banding Patterns and Embryo Development in Interspecific Crosses of *Phaseolus*

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In dedication to
my husband, my son and my parents

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ISOZYME BANDING PATTERNS AND EMBRYO DEVELOPMENT IN INTERSPECIFIC CROSSES OF PHASEOLUS

INTRODUCTION

Interspecific hybridization between Phaseolus species is one of the methods of incorporating desirable traits from other species for the improvement of the common bean, P. vulgaris. The most frequently attempted cross involves P. vulgaris and P. coccineus. The favorable characteristics of P. coccineus include disease resistance, hypogeal germination and cross pollination. The primary barriers to introgression are: sterility of P. vulgaris x P. coccineus, abortion of P. coccineus x P. vulgaris hybrid embryos, and the occurrence of both abnormalities in later progeny populations. Seeds containing abnormal embryos do not mature and fail to germinate in most cases. The recovery of hybrid plants has been enhanced with the aid of embryo culture (Mok et al, 1978; Shii et al, 1982) and F_1 hybrids with sufficient fertility have been identified. However, abnormal embryos continue to occur in reciprocal F_2 and later populations. It has been noted that if hybrid seeds are planted, the resulting populations tend to resemble one or the other parent in many morphological characteristics. The reversion to parental phenotype may be the results of elimination of seeds containing abnormal embryos (Smartt, 1970; Wall ant York, 1957; Mok et al, 1986). Although the control of abnormal embryo development is unknown, the genomic combination must play a role (Mok et al, 1987). As an initial step to study the genetic basis of abnormal embryo development, the present investigation was undertaken. The objective is to identify

quantitative parameters which can be correlated with the type of embryo development. The parameters chosen are isozyme banding patterns. The work presented in this thesis includes the identification of appropriate isozyme systems, the discovery of a correlation between specific banding patterns and the type of embryo development, and a discussion regarding the possible significance of the findings and future experiments.

LITERATURE REVIEW

Interspecific Hybridization of *Phaseolus*

Interspecific hybridization in *Phaseolus* is a useful method to transfer desirable characters and to increase the genetic variability of cultivated species (Mok *et al*, 1986). According to the earlier taxonomic classification, the genus *Phaseolus* comprised 470 species (Small, 1933). Recently, the Asiatic beans were seperated and assigned to other genera. The genus *Phaseolus* is now considered to include about 50 species (Miranda, 1966; Evans, 1978). Of these, *P. vulgaris* (common bean, including both dry and green beans), *P. coccineus* (runner bean), *P.lunatus* (lima bean) and *P. acutifolius* (tepary bean) are the major cultivated species. Improvement of *P. vulgaris* has been the primary goal of interspecific crosses. Some desirable traits which are potentially useful are disease or insect resistance of *P. coccineus* (Rudorf, 1955; Hubbeling, 1957; Baggett and Frazier, 1959; Baggett *et al*, 1965), *P. acutifolius* (Coyne and Schuster, 1973; CIAT, 1977; Yoshii *et al*, 1978), and *P. lunatus* (McFarlane and Rieman, 1943; Wolfenbarger and Sleesman, 1961; CIAT, 1976); hypogeal germination and cross pollination habit of *P. coccineus* (Gates, 1951; Honma and Heeckt, 1962; Ibrahim and Coyne, 1975); high temperature and drought tolerance of *P. acutifolius* (Rachie and Robert, 1974) and efficient nitrogen-fixing capability of *P. lunatus* (Henzell, 1977).

The most common and successful interspecific cross has been *P. vulgaris* x *P. coccineus* and the two parental species are considered to be closely related (Hucl and Scoles, 1985). The first interspecific

hybrid in Phaseolus was between these two species as reported by Mendel in 1865. Consistent success has been limited to crosses using P. vulgaris as the female parent from which mature seeds were routinely recovered (Lamprecht, 1941; Thomas, 1964; Smartt, 1970). In reciprocal crosses, only a few studies reported the recovery of mature seeds. In those cases, either the success was limited to particular genotypic combinations (Lamprecht, 1948; Thomas, 1964; Al-Yasiri and Coyne, 1966; Smartt, 1970) or intraspecific hybrids of P. vulgaris (Wall and York, 1960) and P. coccineus (Smartt and Haq, 1972) were used as parents. When P. coccineus was used as the female parent, pod growth was slower than in the reciprocal cross but seeds could sometimes still develop up to near normal size. However, these seeds contained abnormal embryos. The failure to obtain mature seeds has been attributed to various causes such as the lack of fertilization (retarded growth of P. vulgaris pollen tubes in P. coccineus style) and the slow development of interspecific hybrid embryo and endosperm (Thomas, 1964). However, other studies demonstrated that pre-fertilization events (pollen germination and pollen tube growth) were normal (Hawkins and Evans, 1973; Shii et al, 1982). It was concluded that the primary failure of P. coccineus (female) x P. vulgaris (male) crosses resided in the abnormal development of the hybrid embryos (Shii et al, 1982).

The hybrids of P. vulgaris x P. coccineus (derived from mature seeds) had lower fertility (17%-45%) than those of the reciprocal cross (75% or higher) (Thomas, 1964; Smartt, 1970; Ibrahim and Coyne, 1975; Haq et al, 1980; Shii et al, 1982). The reduced fertility could not be attributed to either lack of chromsome homology (Shii et al, 1982) or cytoplasmic-nuclear interaction, since chromosome pairing in F_1s was

near normal and both reciprocal F_2 populations contained plants with low fertility (Mok et al, 1986). In addition, pollen sterility and restricted embryo development appeared to be under separate genetic controls as there was no correlation between the extent of embryo development and the degree of pollen fertility among plants in later generations (Shii et al, 1982). A skewed distribution towards the maternal parent was observed in F_2 and backcross generations for cotyledon position (Wall and York, 1957) and other morphological characters (Lamprecht, 1948; Smartt, 1970). It was suggested that the reversion to the parental phenotype could be in part due to the elimination of seeds containing abnormal embryos which may represent specific genotypic combinations (Mok et al, 1987). Thus the selective recovery of certain embryos combined with different pollen fertility may contribute to the skewed distribution of quantitative characters observed in generations following P. vulgaris - P. coccineus crosses (Wall and York, 1957; Smartt, 1970).

Abnormal plants of interspecific hybrids of Phaseolus have been described in several cases. F_1 populations of P. vulgaris x P. coccineus were either uniformly abnormal or segregated into normal and "crippled" plants (Lamprecht, 1948; Rudorf, 1961). The "crippled" phenotype included dwarfism, abnormal leaf development and in extreme cases, seedling lethality. The frequencies of these plants could be explained by a two-gene system (Rudorf, 1961).

Crosses between P. vulgaris and Phaseolus species other than P. coccineus have been less successful. Hybridization between P. vulgaris and P. acutifolius was first investigated by Honma (1956). Crosses using P. vulgaris as female parent resulted in pod formation, but pod

abscission occurred before seed maturity (Honma, 1956; Al-Yasiri and Coyne, 1966; Smartt, 1970). It was shown that fertilization and division of the embryo and endosperm occurred regularly in reciprocal crosses involving these two species but embryos aborted during early stages of development (Rabakoarihanta et al, 1979). Culturing the immature embryos on artificial medium was required to recover hybrid plantlets (Honma, 1955; Mok et al, 1978). An exception to these observations was the formation of mature hybrid seeds using specific cultivars of P. vulgaris and P. acutifolius (Smartt, 1970).

The low fertility of P. vulgaris x P. acutifolius hybrids was related to incomplete chromosomal homologies (Rabakoarihanta et al, 1980). In other cases, genetic factors other than insufficient chromosome homology were thought to be the cause of sterility (Parker and Michaels, 1986).

Seed formation in crosses between P. vulgaris and P. lunatus was reported by Honma and Heeckt (1959), but all later attempts failed to confirm this observation (Smartt, 1970). The discrepency may have been due to the fact that Honma and Heeckt (1959) used intraspecific hybrids as parents. In other reports, the cross between P. vulgaris (female) and P. lunatus (male) resulted in formation of pods which abscised in early stages of development, while the reciprocal cross did not set pods (Al-Yasiri and Coyne, 1966; Mok et al, 1978). P. vulgaris x P. lunatus embryos could be rescued using embryo culture. However, hybrids obtained by embryo culture were abnormal and did not advance to the flowering stage (Mok et al, 1978).

Embryo Development

Limited embryo development was found to be the primary barrier in interspecific hybridization (Mok et al, 1987). The only exception was in crossing P. vulgaris (female) with P. coccineus (male) which resulted in the formation of mature seeds. The seeds produced by these crosses were as plump as selfed P. vulgaris seeds and germinated easily. On the other hand, seeds produced in the reciprocal cross were shrivelled, contained abnormal embryos, and had a low germination rate (Thomas, 1964). The causes of embryo abortion and the reciprocal cross difference are not known.

Examination of the embryo development of interspecific hybrids of P. coccineus x P. vulgaris revealed that the abnormalities occurred at very early stages of development (Kroh, 1962; Thomas, 1964). During the immediate post-fertilization period, the interspecific embryos arising from P. vulgaris x P. coccineus grew faster and had a higher mean nuclei number than selfed embryos. On the contrary, hybrid embryos from P. coccineus x P. vulgaris attained little additional growth during the immediate post-fertilization period and the mean nuclei number was lower than in selfed embryos. Ten days after pollination, hybrid embryos of P. vulgaris x P. coccineus developed at a slower rate than selfed embryos. And at this time embryos from P. coccineus x P. vulgaris had the lowest nuclei number. It was suggested that abnormal embryos might result from the interaction of embryo and endosperm (Thomas, 1964).

Abnormal embryos of P. coccineus x P. vulgaris could be classified in at least two categories, the shrunken and the underdeveloped embryos (Shii et al, 1982; Mok et al,1986). The shrunken embryos were

characterized by wrinkled cotyledons and a compressed embryonic axis. The underdeveloped embryos were characterized by their small size at the cotyledonary stage as compared to those of the reciprocal cross at the same stage. In addition, the embryos were surrounded by a thick layer of endosperm tissue while reciprocal embryos at comparable stages completely filled the cavity of the seed. The occurrence of the particular type of abnormal embryos, shrunken or underdeveloped, depended on the genotype of the P. coccineus parent. Underdeveloped embryos were observed in all P. coccineus x P. vulgaris crosses examined, while the shrunken embryos occurred only when particular genotypes of P. coccineus were used. Three types of F_2 embryos, normal, shrunken and underdeveloped, were observed. The degree of abnormality of the shrunken embryos varied, ranging from those with extremely wrinkled cotyledons to those with only rough edges. There were no significant reciprocal cross differences in the frequency of the different classes of F_2 embryos. It was suggested that the interaction between the embryo and maternal parent was likely the cause of abnormal development (Shii et al, 1982).

Honma (1956) reported that interspecific embryos of P. vulgaris x P. acutifolius aborted within 3 to 24 days following pollination. The reciprocal cross did not form pods or seeds. When intraspecific F_1 plants of P. acutifolius were used as female parents, a few pods were set but embryos failed to attain sufficient size for in vitro culture. Smartt (1970) also reported that reciprocal hybrid embryos between P. vulgaris and P. acutifolius were formed but pods collapsed at 17 to 21 days after pollination.

Embryo development in reciprocal crosses of P. vulgaris - P.

acutifolius has been further investigated by Mok et al (1978) and Rabakoarihanta et al (1979). Interspecific embryos of P. vulgaris x P. acutifolius usually developed to the early cotyledonary stage. A distinct characteristic of these embryos was the uneven development of the two cotyledons which were apart rather than closely adjoining each other (as in selfed embryos). Hybrid embryos derived from the reciprocal cross developed until the late heart or early cotyledonary stage. The final size of the hybrid embryos ranged from 2.5 to 3.5 mm at 24 days after pollination. The rate of growth and final size of these hybrid embryos seemed to be influenced by the genotype of both parents (Mok et al, 1978). It was suggested that the failure of hybrid embryos to develop normally may be caused by the relatively low levels of cytokinins (Nesling and Morris, 1979).

Formation of mature seeds from P. vulgaris x P. lunatus crosses has been reported (Honma and Heeckt, 1959). However, other studies indicated that hybrid pods usually abscised at early stages of development (Al-Yasiri and coyne 1966; Smartt, 1970). A later investigation (Mok et al, 1978) revealed that all embryos obtained from P. vulgaris x P. lunatus crosses developed only to the pre-heart stage. The maximum length of the hybrid embryo at the time of pod abscission was 0.4 mm. The reciprocal cross (P. lunatus x P. vulgaris) resulted in embryos which ceased to divide at the four-celled stage. It was suggested that the severe delay in embryo and endosperm divisions could be the major cause of early pod abscission in P. lunatus x P. vulgaris crosses. As addition of cytokinins to the female parent in a hydroponic culture advanced the four-celled embryos to the pre-heart stage (Rabakoarihanta et al, 1979), lower levels of hormone may play a role in

embryo abortion and pod abscision.

The Use of Biochemical Markers as Quantitative Parameters

Isozymes are multiple forms of an enzyme occurring within a single species as a result of the presence of more than one structural gene (Commission on Biochemical Nomenclature, 1977). Isozymes occur in several different ways: they can be derived from different gene loci, the same gene loci having different alleles, or the presence of the same gene alleles but with modifications in the expression of genes in somatic cells (Moss, 1982). These multiple forms of enzymes are different in structure and some catalytic properties, although they catalyze the same reaction.

Most isozymes are controlled by several gene loci that are usually distributed randomly among the chromosomes. They may be more representative of the genomic constitution than qualitative characters which are controlled by a few loci. Also, isozymes are gene products that reflect gene activity more directly than the morphological markers which in many cases are influenced by environmental factors. Therefore, from a genetic point of view, isozymes are useful as quantitative parameters to measure genetic composition. There are a number of other advantages of using isozymes as biochemical markers over conventional morphological traits. Isozyme variations often occur spontaneously and these variations normally do not show deleterious effects. Variant alleles are generally codominant and it is possible to distinguish heterozygotes and homozygotes.

It has been shown that electrophoretic separation of isozymes can

provide information regarding the distribution of allelic variation in natural populations. This is because the variation in the electrophoretic banding patterns of enzymes can be directly equated to the variation in gene structure or codon sequence.

Isozymes have been used to estimate the degree of genetic polymorphism present in populations, the genetic differences within and between species, differential gene expression during development, gene complementation and heterosis within and between genera, and the evolution of genes and organisms (Scandalios, 1974).

One of the most successful uses of isozymes is as markers in linkage studies leading to the construction of genetic maps of plant chromosomes. For example, in tomato, an extensive linkage-map has been constructed with this technique. Several isozyme loci have been marked on each chromosme (Tanksley and Rick, 1980). Identification of isozyme markers linked to certain genotypes is also very useful in crop improvement as it facilitates the selection of desirable genotypes. It has been estimated that in backcross programs, by using an isozyme as a selection marker, two or more generations might be eleminated. If this technique is combined with selection for morphological traits in later generations, even greater potential might be achieved (Tanksley and Rick, 1980). Isozymes are superior to other techniques for the detection of abnormal segregation patterns (Tanksley and Rick, 1980) and have been used to demonstrate the occurrence of differential gene recovery in an interspecific backcross in Phaseolus (Wall, 1968). Isozyme banding patterns have been used successfully to identify sexual and somatic hybrids (Gates and Boulter, 1979; Tanksley and Jone, 1981; Gamborg, 1981). The hybrid zymogram may be the sum of the parents or

may include a hybrid band. Some parental bands may be missing in the hybrid zymogram (Wetter, 1977). Isozymes may also be used to estimate the degree of hybridity or recombination in progeny plants. It may be possible to use isozymes to screen F_1 plants for maximum heterozygosity or favorable heterotic combinations. Isozyme and protein banding patterns have been used to study the evolutionary relationships between species by comparing their pattern of variation, to verify the phylogenetic origin of polyploids, and also to study the selection pressure placed on plant populations by environmental conditions (Brown, 1978; Tanksley and Rick, 1980; Gottlieb, 1982).

Restriction fragment length polymorphism (RFLP) represents another method of measuring genomic composition. RFLPs are variations in the length of DNA fragments generated with a particular restriction endonuclease digestion. As molecular markers, RFLPs can be used in the same way as isozymes or morphological markers, but with additional advantages. As RFLPs represent differences at the DNA level, they provide the means to directly measure the genomic variation. Also, the measurement is not likely to be affected by environmental factors. RFLPs are also useful in studying quantitative traits and to construct linkage maps. The latter has been established for tomato, maize (Helentjaris et al, 1986) and lettuce (Landry et al, 1987). Co-segregation of particular RFLPs with important traits in a segregating population will have potential use as selection indices. It is possible that RFLPs may also facilitate the identification of particular genomic compositions associated with abnormal embryo development in interspecific population of Phaseolus.

MATERIALS AND METHODS

Plant Materials

P. vulgaris L. cv. Great Northern (GN) and P. coccineus Lam. cv. Scarlet Runner (SR) were used as parents. Plants were grown in the greenhouse (at 24°C/18°C day/night). Flowers were emasculated and pollinated one day before opening. F_1 plants of GN x SR were obtained by planting mature F_1 seeds in a growth medium, Jiffy Mix Plus. F_1 hybrids of SR x GN were recovered with the aid of embryo culture. F_2 embryos of reciprocal crosses were obtained by hand-pollinating of the F_1s.

Embryo Culture

F_1 pods of SR x GN were collected before senescence (35-45 days after pollination). Pods were surface-sterilized by immersion in 70% ethanol for 2 min and in 20% Clorox (1% sodium hypochlorite) for 5 min and rinsed in sterile water. Immature embryos were dissected under aseptic condition and the cotyledons were removed. The embryonic axes were cultured on embryo culture medium.

The culture medium contained inorganic nutrients as described by Murashige and Skoog (1962) with the addition of sucrose (30 g/l), myo-inositol (100 mg/l), glutamine (100 mg/l), thiamine (1 mg/l), nicotinic acid (5 mg/l) and pyridoxine (0.5 mg/l) (Table 1). The medium was adjusted to pH 5.7. Difco Bacto Agar was added at a concentration of 8 g/l. The medium was dispensed into bottles (25 ml/bottle) and

Table 1. Composition of Medium Used for Embryo Culture

Compound	Concentration mg/l	Concentration mM
Inorganic Compounds:		
NH_4NO_3	1650.0	20.61
KNO_3	1900.0	18.80
KH_2PO_4	170.0	1.25
$CaCl_2.2H_2O$	440.0	3.00
$MgSO_4.7H_2O$	370.0	1.50
Na_2EDTA	37.2	0.10
$FeSO_4.7H_2O$	27.8	0.10
H_3BO_3	6.20	0.100
KI	0.83	0.005
$Na_2MoO_4.2H_2O$	0.25	0.001
$CoCl_2.6H_2O$	0.025	0.0001
$MnSO_4.H_2O$	16.90	0.100
$ZnSO_4.4H_2O$	8.60	0.030
$CuSO_4.5H_2O$	0.025	0.0001
Organic Compounds:		
Sucrose	30000	87.30
Myo-inositol	100	0.555
Thiamine	1	0.003
Nicotinic acid	5	0.040
Pyridoxine	0.5	0.0025
Glutamine	100	0.684

autoclaved at 120°C for 15 min.

The immature embryos were cultured at 25°C with a photoperiod of 16 hr/8 hr (day/night) and light intensity of 10,000 lux for about 30 days and then transferred to Jiffy Mix Plus. The plantlets were covered with plastic wrap and placed in the culture room (25°C) for the first 7 days and then were transferred to the greenhouse.

Enzyme Extraction

Immature embryos of parents, F_1 and F_2 were used for electrophoretic analyses at the middle cotyledonary stage. The F_1 and F_2 embryos were classified as normal or abnormal (shrunken or underdeveloped). The analyses were limited to the normal and shrunken embryos in the cross of GN x SR (P. vulgaris x P. coccineus). All three classes of embryos were analyzed in the reciprocal cross.

Individual embryos were homogenized with a tissuemizer. Enzymes were extracted in 0.1 M phosphate buffer (pH 7.5, 1.3 ml/g) followed by centrifugation at 27,000 g for 20 min. The supernatant was collected and mixed with one-half volume of Bromphenol Blue (0.1%). Samples were stored in liquid nitrogen until use. Sample preparation was carried out at 4°C.

Electrophoresis

Discontinuous Polyacrylamide Gel Electrophoresis (PAGE) was performed using the Bio-Rad PROTEAN II slab cell system. The dimension of the vertical slab gels were 160 mm x 160 mm x 1.5 mm separated into

15 wells.

The stacking gel contained 2.5% (w/v) acrylamide, 0.617% (w/v) bisacrylamide, 0.0005 M Tris-H_3PO_4 (pH 6.9), 0.003 M ammonium persulfate and 6.25% (v/v) glycerol. The separating gel contained 8% (w/v) acrylamide, 0.0375 M Tris-HCl (pH 8.9) and 0.003 M ammonium persulfate. Running buffer consisted of 0.005 M Tris-HCl and 0.038 M glycine at pH 8.5. The sample size was 15 ul/well. Electrophoresis was run at a voltage of 150V until the tracking dye passed the stacking gel and then raised to 200V. Electrophoresis was conducted in the cold room ($4^{o}C$).

Enzyme Staining

Twelve enzyme systems were screened and three were selected for this study as their banding patterns were polymorphic between the two parents. The enzymes chosen were: Acid phosphatase (AP) (E.C.3.1.1.2.), Esterase (EST) (E.C.3.1.1.2) and Malate dehydrogenase (MDH) (E.C.1.1.1.37).

Acid Phosphatase:

Gels were stained in 100 ml acetate buffer (0.2 M, pH 4.0) containing 10 mg α-naphthyl acid phosphate, 10 mg Fast Garnet GBC and 1 mM $MgCl_2$. Gels were stained overnight in the dark at room temperature with slow shaking to allow the enzymatic reaction to occur.

Esterase:

Gels were stained in 150 ml phosphate buffer (0.1 M, pH 6.5) containing 150 mg α-naphthyl acetate, 75 mg β-naphthyl acetate dissolved in 3.0 ml of acetone, 30 mg Fast Blue RR salt and 30 mg Fast Garnet GBC. Incubation conditions were the same as those for AP.

Malate dehydrogenase:

The staining solution consisted of 20 ml malate buffer (0.05 M, pH 6.0), 20 ml Tris buffer (0.5 M, pH 8.5), 60 ml DD water, 20 mg NAD, 20 mg Nitro Blue Tetrazolium and 2 mg phenazine methosulfate. Gels were stained for about 4 hrs at 40°C.

RESULTS

P. vulgaris L cv. GN x P. coccineus L cv. SR

Forty F_1 and 275 F_2 embryos of GN x SR at the cotyledonary stage were dissected and classified as normal or abnormal. Twenty F_1 and 100 F_2 embryos (68 normal and 32 shrunken) were selected based on uniform size and subjected to electrophoretic analyses.

Representative zymograms of the three enzyme systems of parental, GN x SR, and normal and abnormal (shrunken) F_2 embryos are presented in Figures 1-3. In order to facilitate discussion of AP patterns (Figure 1) and to compare zymograms between parents and progeny, bands were grouped into four regions, A to D, with increasing mobility towards the anode. The darkly stained region A occurred in all types of embryos and will not be discussed further. Isozyme patterns of the two parents were clearly distinguishable by the darkly stained regions B and D in GN and the absence of band D but presence of band C in SR. All GN x SR F_1s examined had identical patterns, with a prominent band at region B, a light band at region D and no bands at region C. Normal F_2 embryos had a darkly stained region B and an intermediately stained band at region D. Zymograms of abnormal F_2 embryos were characterized by the absence of or a very lightly stained region B plus a light band at region C. Thus the clear qualitative differences were sufficient to distinguish parental as well as the two classes of F_2 embryos. F_1 embryos had band patterns resembling those of normal F_2 embryos. In addition, zymograms of normal F_2 embryos resembled GN, whereas those of abnormal embryos exhibited patterns characteristic for SR.

Figure 1. Zymograms of acid phosphatase (AP) of P. vulgaris cv. Great Northern (GN), P. coccineus cv. Scarlet Runner (SR), F_1 (GN x SR) and F_2 embryos.

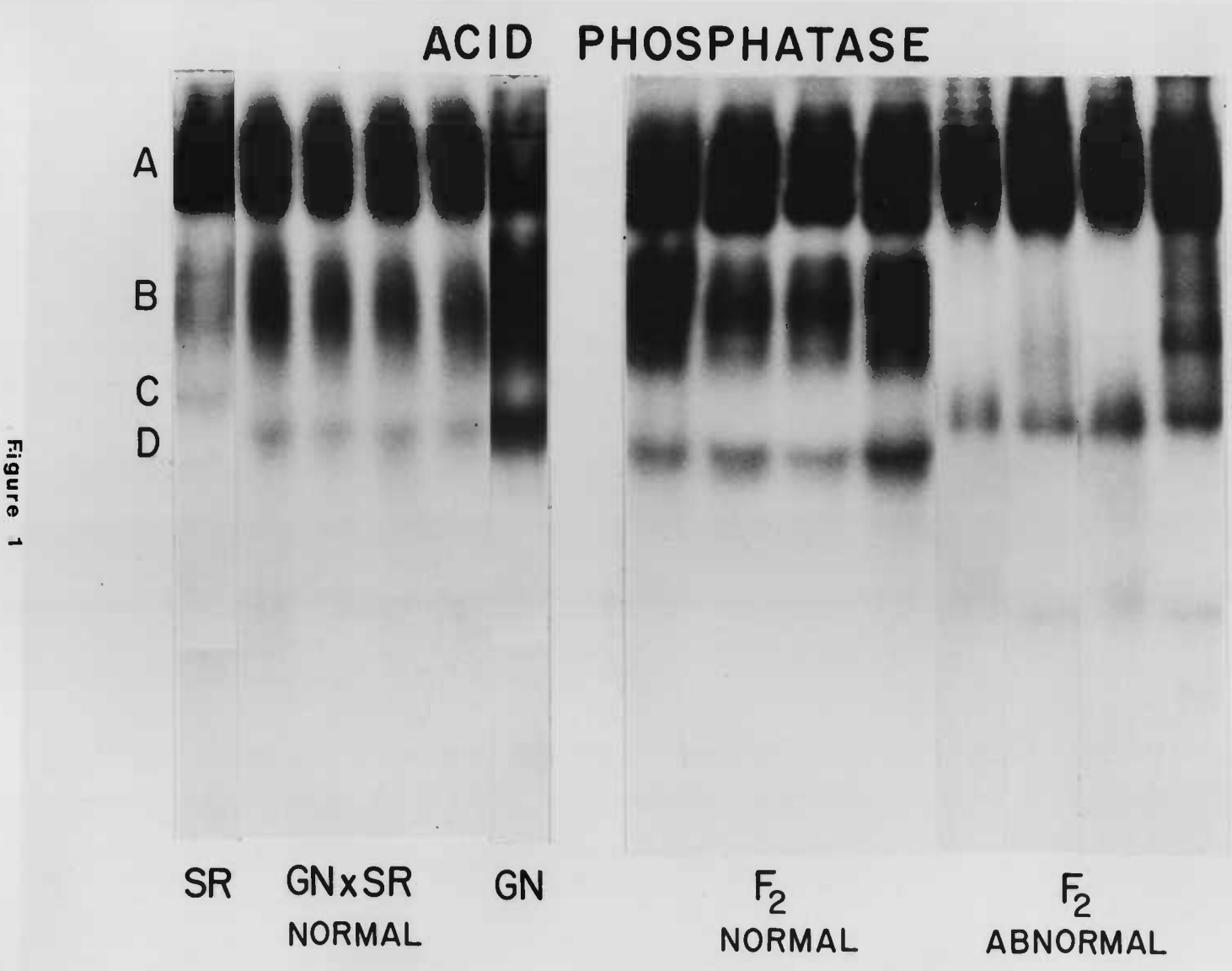
ACID PHOSPHATASE
A
B
C
D
SR
GN x SR
NORMAL
GN
F2
NORMAL
F2
ABNORMAL

Figure 1

Figure 2. Zymograms of malate dehydrogenase (MDH) of P. vulgaris cv. Great Northern (GN), P. coccineus cv. Scarlet Runner (SR), F_1 (GN x SR) and F_2 embryos.

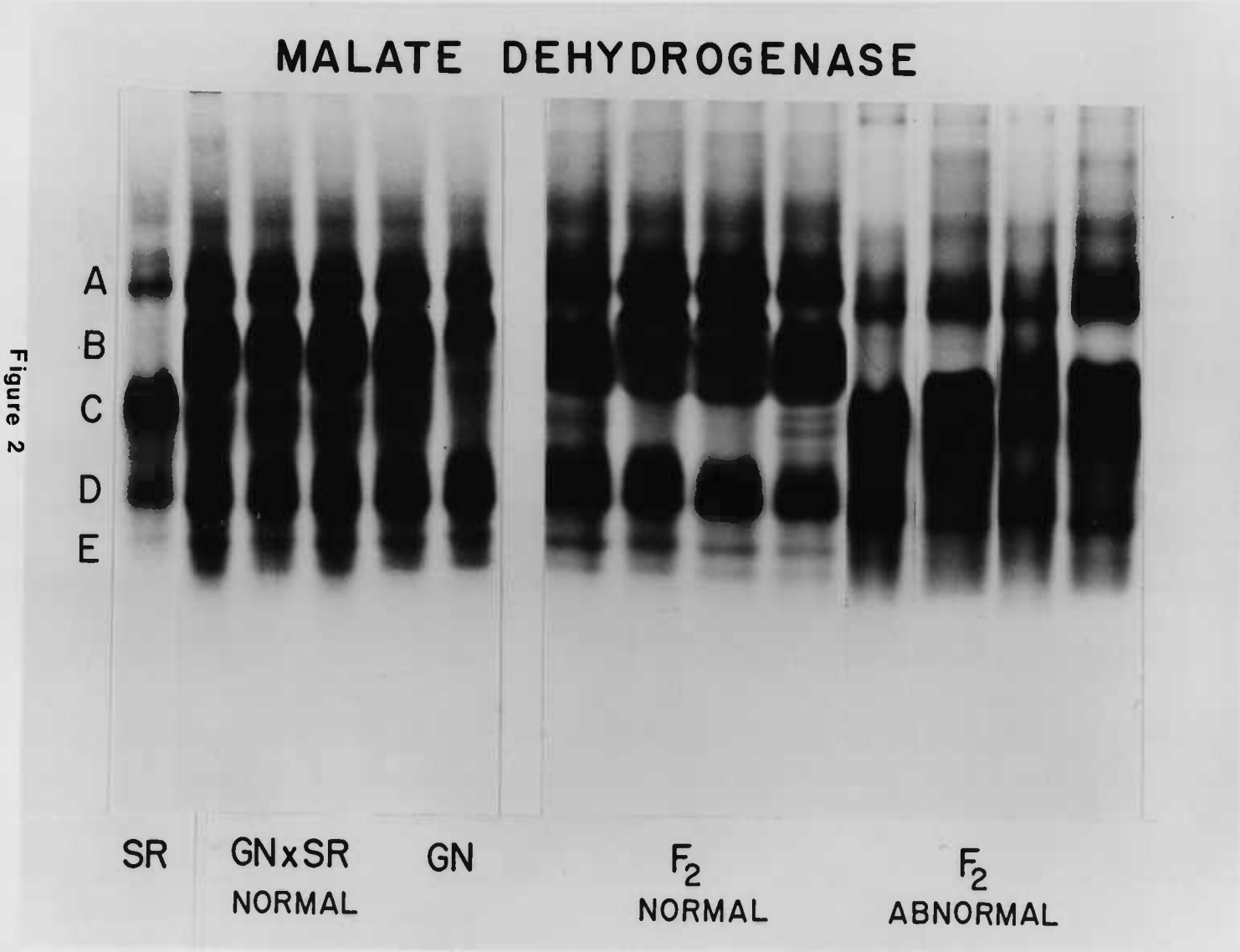
MALATE DEHYDROGENASE
A
B
C
D
E
SR
GN x SR
NORMAL
GN
F2
NORMAL
F2
ABNORMAL

Figure 2

Figure 3. Zymograms of esterase (EST) of P. vulgaris cv. Great Northern (GN), P. coccineus cv. Scarlet Runner (SR), F_1 (GN x SR) and F_2 embryos.

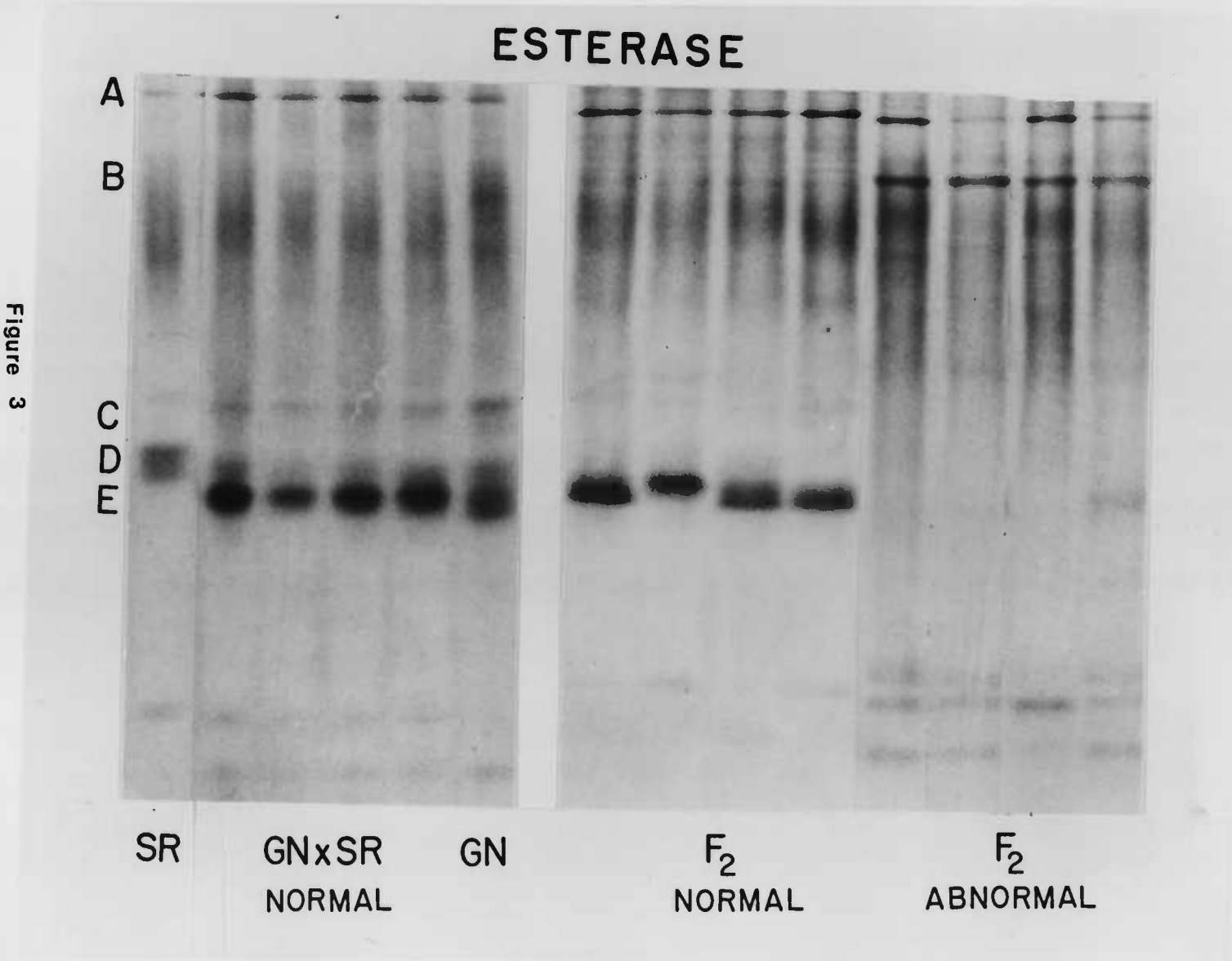

Figure 3

The MDH isozymes (Figure 2) could be described by five regions, A to E. GN exhibited broad and darkly stained bands A, B and D and a clear narrow band at region E. SR, on the other hand, had a narrow, well defined band at region A, broad and darkly stained regions C and D, but no detectable band at region B and a very light band in region E. The GN x SR embryos displayed isozyme patterns close to those of the GN parent but had a darker stained region C. Normal F_2 embryos were also more like GN, with stained regions A, B and D and a narrow band at region E. However, some variations could be observed in region C, i.e. it either had no bands or one to three well defined bands. Abnormal F_2 embryos had darkly stained regions C and D and no bands at region B, while region E was very lightly stained.

EST bands (Figure 3) were grouped into five regions, A to E. GN was characterized by a narrow band at region C, and two adjacent bands at regions D and E, with the band at region E much darker stained. SR had a darkly stained region D but no band at region E. Staining at region B in both genotypes was rather diffuse and undefined. GN x SR F_1s resembled GN, with bands at regions C, D and E. Normal F_2 embryos had a well defined band in regions D-E which showed variations in mobility between individual embryos. Abnormal embryos had a narrow and well defined band at region B but no clear dark bands in regions D and E. Although the two types of F_2 embryos could be easily distinguished, they did not resemble either of the parental patterns.

P. coccineus L cv. SR x P. vulgaris L cv. GN

In order to confirm the observed correlation between isozyme

patterns and types of embryo development, F_1 and F_2 embryos of P. coccineus x P. vulgaris were also examined. Smaller F_2 populations than those of GN x SR were analysed. Twenty F_1 and thirty F_2 embryos were first classified into normal or abnormal and then used for isozyme analyses. Zymograms of abnormal SR x GN F_1 embryos are presented in Figures 4-6. For all three enzymes, the patterns of F_1 were unlike those of its reciprocal normal F_1, but closely resemble those of the abnormal F_2 embryos.

Zymograms of AP, MDH and EST of abnormal and normal F_2 embryos are presented in Figures 7-9. For all three enzymes, F_2 embryos derived from SR x GN also displayed banding patterns characteristic of their developmental type, similar to those of reciprocal F_2s. For AP and MDH, normal embryos displayed banding patterns similar to those of GN and abnormal embryos had patterns resembling SR. Also, distinct EST banding patterns were observed between normal and abnormal F_2 embryos, however, the patterns did not resemble those of either parent. No zymogramic differences were observed between the two types of abnormal embryos, shrunken and underdeveloped.

Figure 4. Zymograms of acid phosphatase (AP) of P. vulgaris cv. Great Northern (GN), P. coccineus cv. Scarlet Runner (SR) and F_1 (SR x GN) embryos.

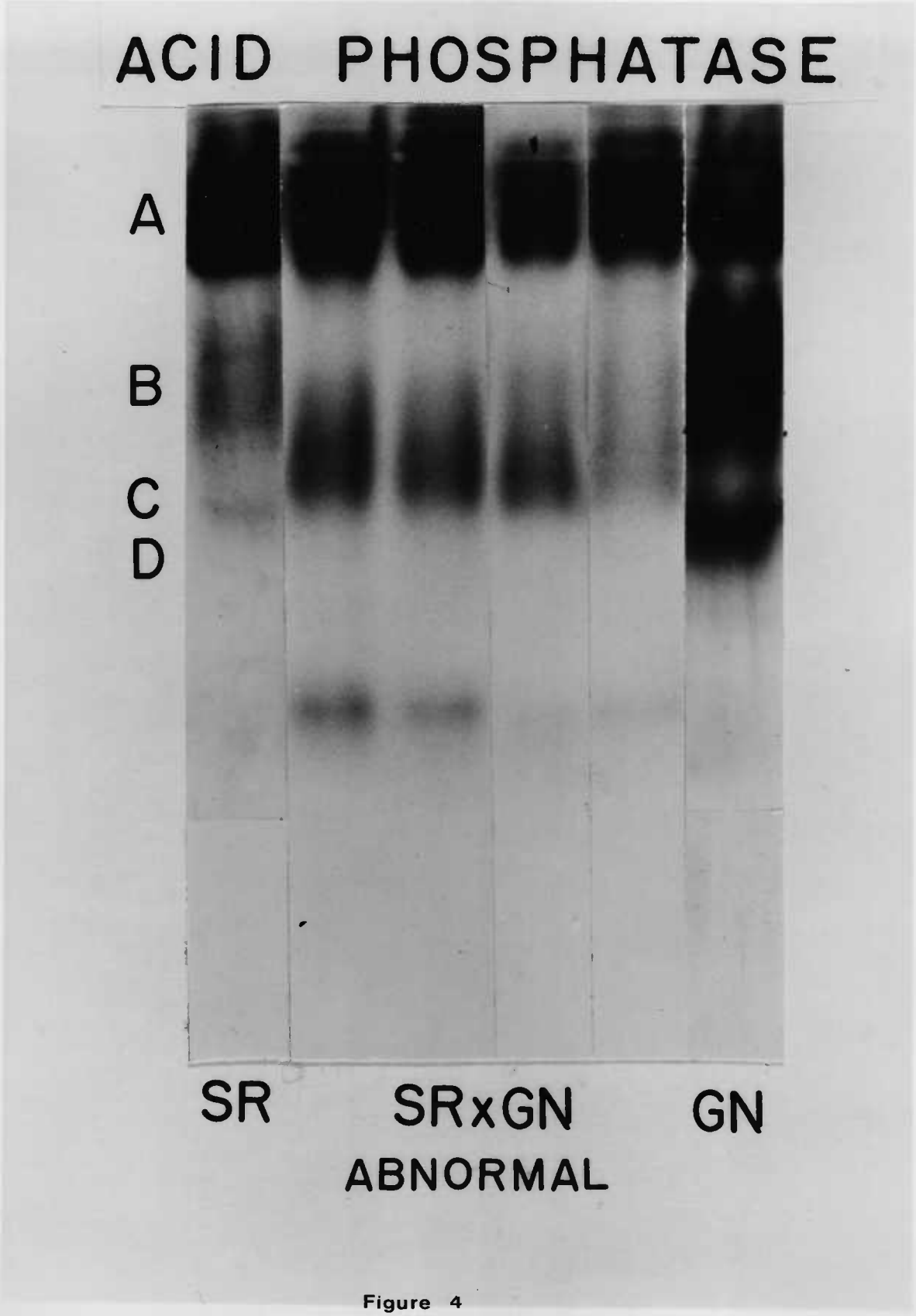

Figure 4

Figure 5. Zymograms of malate dehydrogenase (MDH) of P. vulgaris cv. Great Northern (GN), P. coccineus cv. Scarlet Runner (SR) and F_1 (SR x GN) embryos.

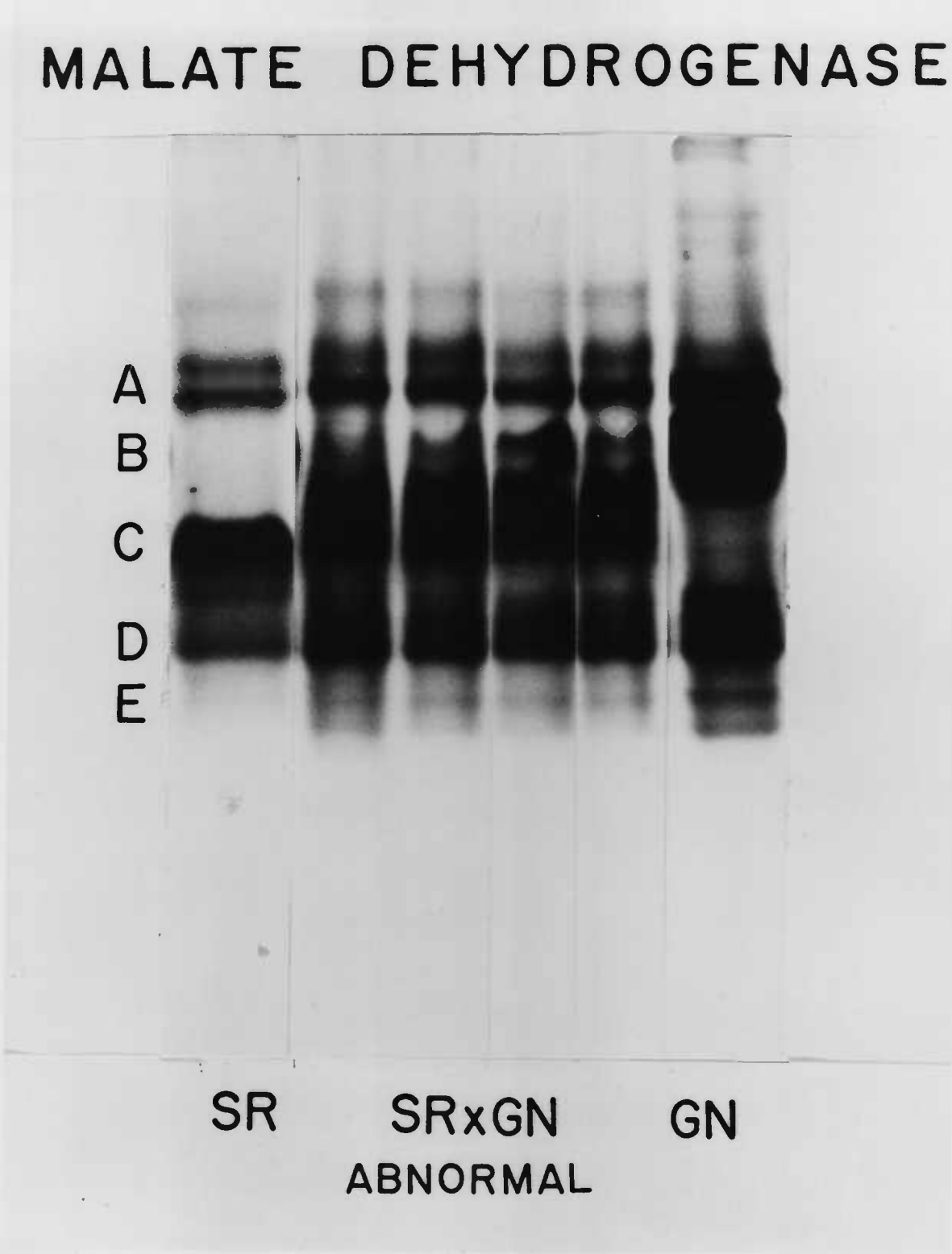

Figure 5

Figure 6. Zymograms of esterase (EST) of P. vulgaris cv. Great Northern (GN), P. coccineus cv. Scarlet Runner (SR) and F_1 (SR x GN) embryos.

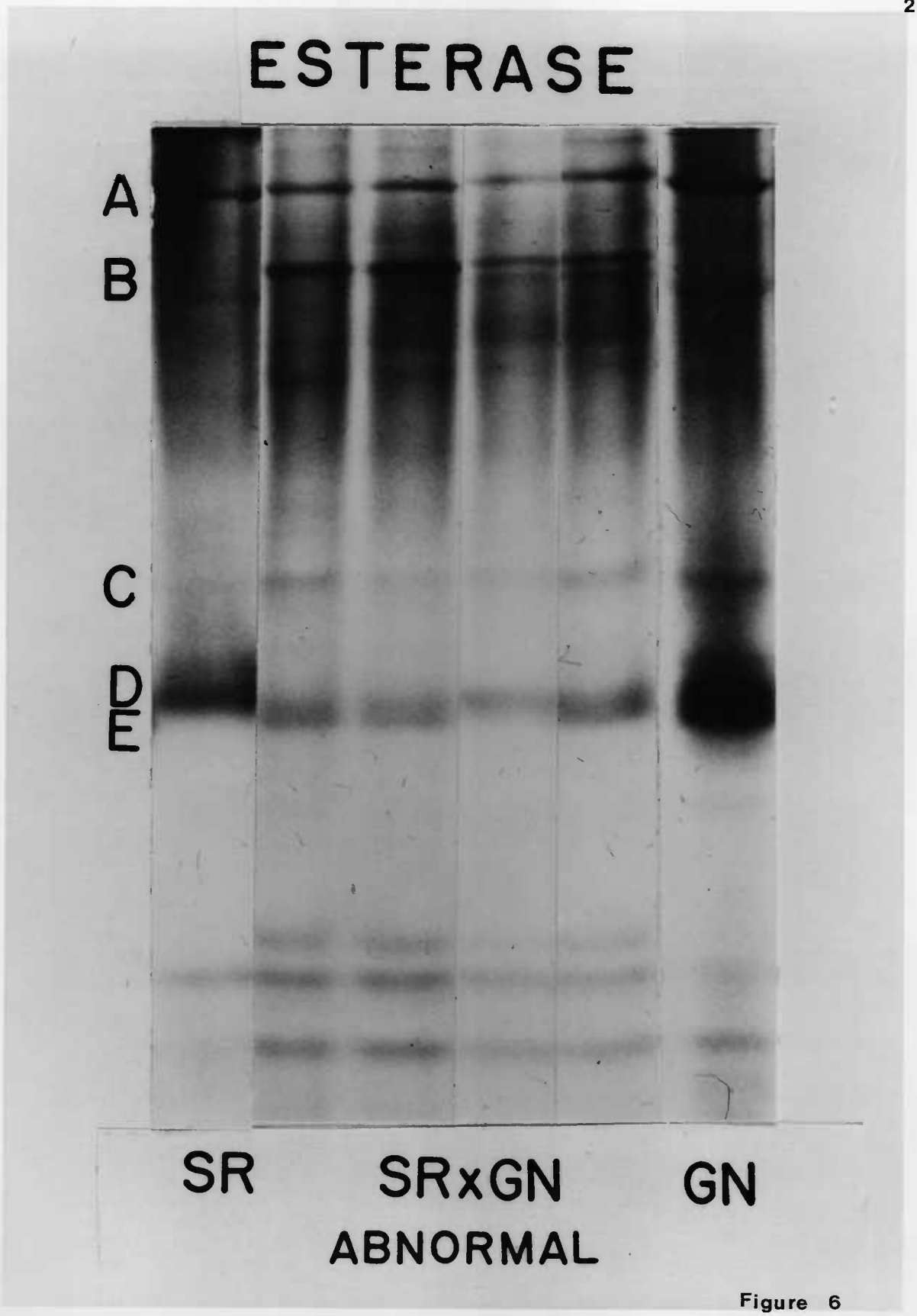

Figure 6

Figure 7. Zymograms of acid phosphatase (AP) of P. vulgaris cv. Great Northern (GN), P. coccineus cv. Scarlet Runner (SR) and F_2 (SR x GN) embryos (abnormal embryos, lanes 3-6, normal embryos, lanes 7-10).

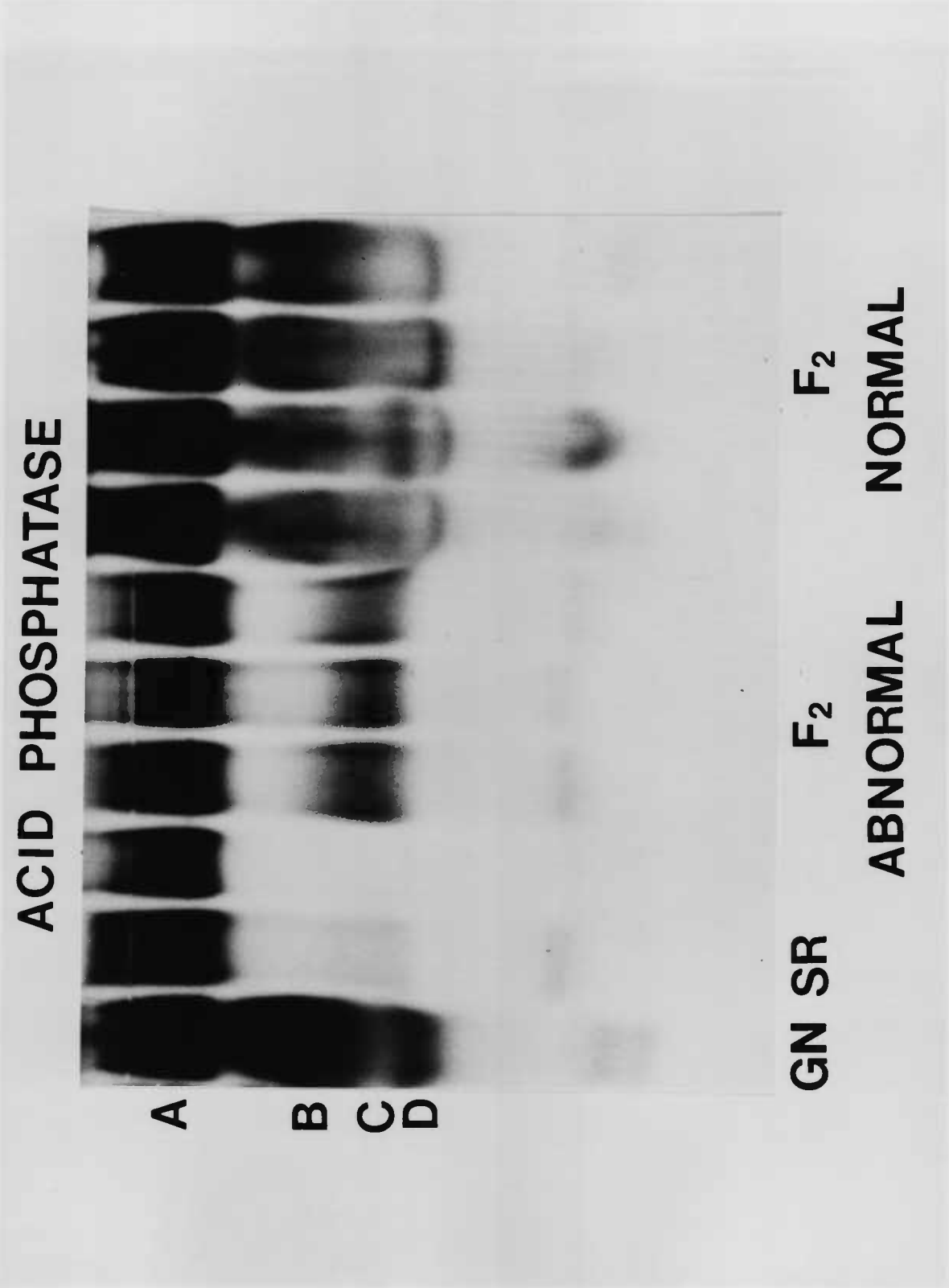

Figure 7

Figure 8. Zymograms of malate dehydrogenase (MDH) of P. vulgaris cv. Great Northern (GN), P. coccineus cv. Scarlet Runner (SR) and F_2 (SR x GN) embryos (abnormal embryos, lanes 3-6, normal embryos, lanes 7-10).

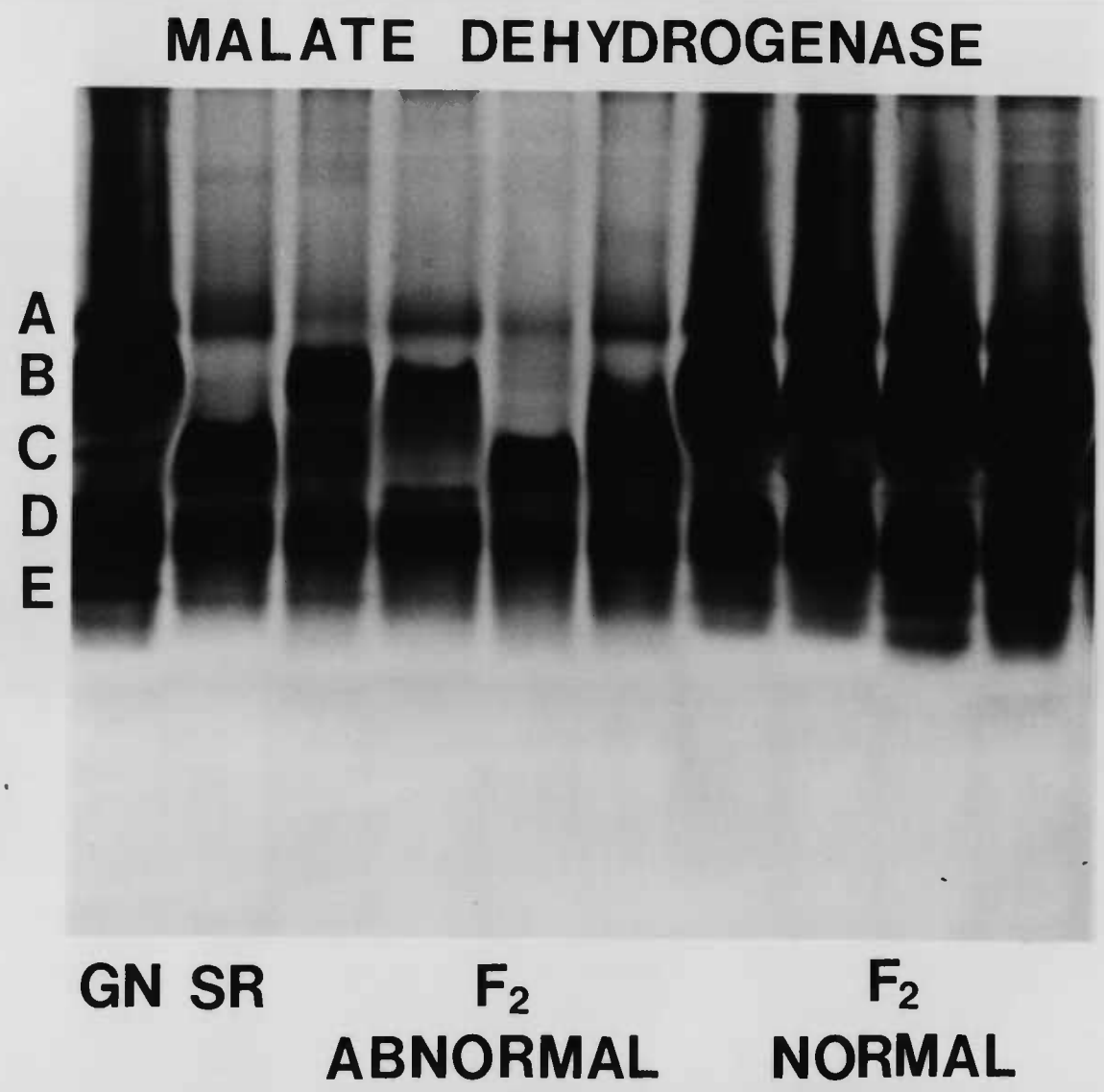

Figure 8

Figure 9. Zymograms of esterase (EST) of P. vulgaris cv. Great Northern (GN), P. coccineus cv. Scarlet Runner (SR) and F_2 (SR x GN) embryos (abnormal embryos, lanes 3-6, normal embryos, lanes 7-10).

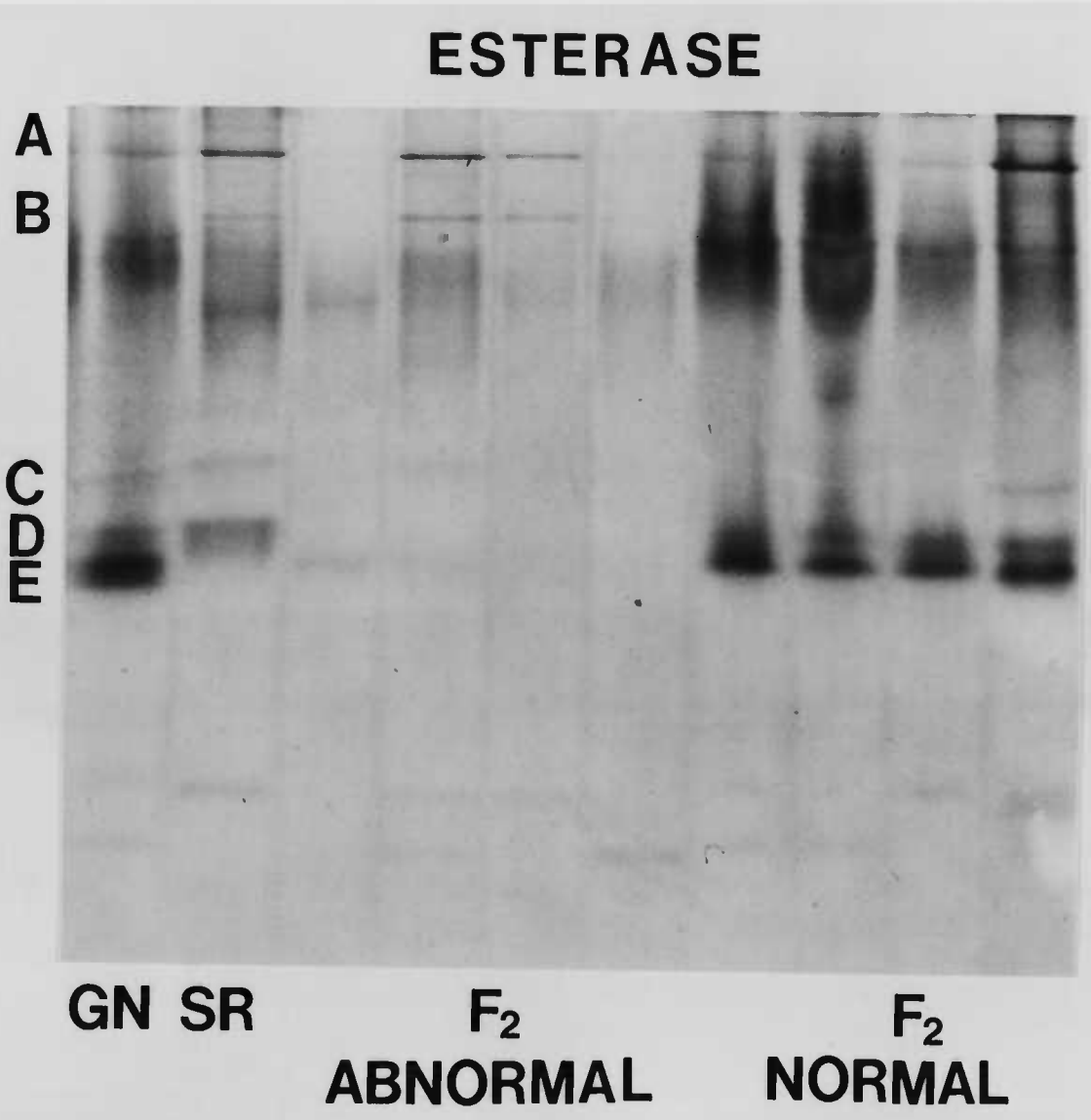

Figure 9

DISCUSSION

The primary objective of this study was to identify quantitative, biochemical parameters associated with different classes of interspecific embryos of P. vulgaris and P. coccineus. The three enzymes selected were polymorphic and were suitable for the identification of parental, reciprocal F_1 and F_2 embryos. As the analyses required very small amounts of protein, extracts of a single embryo could be used to examine all three enzyme systems. When the abnormal and normal classes of embryos were compared with regard to the isozyme bands of the three enzymes, it became apparent that embryos of each classes could be identified by their distinct isozyme banding patterns. Thus the type of embryo development seems to be associated with the presence and/or expression of particular sets of genes.

F_1 embryos of GN x SR which were normal showed isozyme patterns similar to those of normal F_2 embryos, whereas F_1 embryos of SR x GN which were abnormal exhibited isozyme patterns resembling those of abnormal F_2 embryos. The isozyme patterns of the reciprocal F_1's indicate that gene expression in the heterozygotes may vary depending on the type of embryo development. Therefore, it is difficult to identify the F_1 types in the F_2s, based on isozyme patterns alone. Normal F_2 embryos displayed AP and MDH bands similar to those of GN, whereas abnormal embryos were closer to SR. The fact that typical GN patterns occurred only in normal F_2 embryos and typical SR patterns only in abnormal embryos indicates that the normal embryos may contain or express higher proportions of the P. vulgaris genome and the abnormal embryos may contain or express higher proportions of the P. coccineus

genome. Previous observations that plants obtained from viable F_2 seeds (with normal mature embryos) were morphologically more similar to P. vulgaris seem to lend support to this hypothesis. EST patterns also differed between the normal and abnormal embryos, but the association with the parental types was not as clear. Of particular interest is the occurrence of EST band B in abnormal F_1 and F_2 embryos since this band was not present in the parental types. This may indicate that abnormal development is associated with the expression of certain sets of genes.

Results obtained from the cross of SR x GN indicate that the relationship between isozyme banding patterns and embryo development exists regardless of the direction of the cross. The absence of differences in the reciprocal F_2 populations suggests that cytoplasmic-nuclear interactions did not influence the type of embryo development. In addition, the observation that normal F_2 embryos of SR x GN showed isozyme patterns similar to GN but not SR suggests that abnormal embryo development is not caused by the incompatibility between the cytoplasm of P. vulgaris and the nucleus of P. coccineus as noted in earlier studies (Wall and York, 1957; Smartt, 1970). If that were the case, the normal F_2 embryos of SR x GN should have isozyme patterns closer to those of SR.

We are interested in identifying the genetic and physiological mechanisms leading to abnormal embryo development. Since there are reciprocal cross differences in the F_1 generation, interactions between the genotype of the embryo and either the cytoplasm or maternal plant seem likely. The latter interaction could better explain the abscence of reciprocal cross differences between the F_2 populations. However, possible gametic and zygotic selection may further complicate this

situation. Although it is difficult to distinguish between the possible causes of abnormal development at present, the preliminary results described here indicate that isozyme analysis may provide important information in elucidating the factors associated with abnormal development of the interspecific embryos.

In future studies, the significance of these characteristics in relation to genomic distributions can be explored by studying the genetic control of embryo development and/or genomic constitution of hybrids.

CONCLUSION AND FUTURE DIRECTION

1. Polymorphism for AP, EST and MDH isozymes between P. vulgaris, P. coccineus and their progeny suggests that selected isozymes are useful as biochemical markers to distinguish the parents and classes of interspecific hybrids.

2. The observation that normal embryos show isozyme banding patterns more like P. vulgaris and abnormal embryos show isozyme banding patterns more like P. coccineus substantiates the hypothesis that selective recovery of seeds containing normal embryos contributed to the reversion to parental types observed in subsequent populations of interspecific hybrids.

3. As distinct isozyme patterns are associated with abnormal embryos regardless of the generation (F_1 or F_2) or the direction of the cross, abnormal embryo development may be associated with either the presence of specific genes or aberrant gene expression regulating embryo development.

4. In order to further investigate the regulation of embryo development, saturated RFLP mapping is being used to estimate genomic composition and hopefully to identify particular fragments associated with a specific type of embryo development.

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APPENDIX

Appendix I: Procedures for Isozyme Analyses

ENZYME EXTRACTION

Extraction Buffer (0.1 M Na_2HPO_4 pH 7.5):

Na_2HPO_4	1.42g
DD H_2O	100 ml

Bromphenol Blue Solution (0.5%):

Bromphenol Blue	50 mg
DD H_2O	5 ml
Glycerol	5 ml

Extraction Procedure:

1. Buffer was added (1:1.3, W:V).
2. The embryo was homogenized with a tissuemizer for 90 sec.
3. Samples were centrifuged at 27,000 g at 4°C for 20 min.
4. Supernatant was collected and 0.5 ml of Bromphenol Blue was added to 1 ml of sample.

GEL PREPARATION

1. Stock Solutions.

Stock A: (0.3 M Tris-HCl pH 8.9)

Tris (Trisma base)	3.6342 g
DD H_2O	100 ml

Adjust to pH 8.9 with 1.0 N HCl

Stock B: (0.004 M Tris-H_3PO_4 pH 6.9)

Tris	0.0533 g
DD H_2O	100 ml

Adjust to pH 6.9 with 1.0 M H_3PO_4

Stock D: (4.2 M acrylamide + 0.065 M bisacrylamide)

Acrylamide	29.8536 g
Bisacrylamide	1.0021 g
DD H_2O	100 ml

Stock E: (1.4 M acrylamide + 0.16 M bisacrylamide)

Acrylamide	9.9512 g
Bisacrylamide	2.4667 g
DD H_2O	100 ml

Stock F: (0.006 M ammonium persulfate)

Ammonium persulfate	0.1369 g

DD H_2O	100 ml

Stocks D and E were degassed. All stock solutions were stored at 4°C. They remained stable for about two months.

2. Gel Preparation

Running gel: stock solutions were added in the following order:

Stock D	8.52 ml
Stock A	4.0 ml
DD H_2O	3.5 ml
TEMED	0.016 ml
Stock F	16.0 ml

(The Bio-Rad PROTEAN II slab cell system was used. This amount was sufficient for one gel, 160 mm x 160 mm x 1.5 mm).

1. All the ingredients were mixed except TEMED and stock F.
2. TEMED and stock F were added immediately before pouring the gel They were mixed well by shaking gently.
3. The solution was dispersed into the gel mold and overlaid with DD H_2O immediatly after casting. The gel was left to polymerize for at least 30 min.

Stacking gel: stock solutions were added in the following order:

Stock E	6.0 ml
Stock B	3.0 ml
50% (v/v) glycerol	3.0 ml
TEMED	0.024 ml
Stock F	12.0 ml

(This amount was sufficient for two gels).

1. Water was removed from the running gel and the comb was insert
2. Solution for the stacking gel was added to the running gel with a pasture pipet.
3. The gel was allowed to polymerized for 30 min.
4. The comb was removed.

ELECTROPHORESIS

Running Buffer (50x concentrated):

Tris	30.0 g
Glycine	144.0 g
H_2O to	1000 ml

Use 20 ml to make 1 liter of running buffer (pH 8.5).

The same amount of samples was loaded in each well. Electrophoresis was carried out at 150V constant voltage till the tracking dye passed through the stacking gel and then run at 200V till the dye reached 1 cm from the bottom. Gels were removed from the glass plates and stained to detect individual enzymes.

ENZYME STAINING

1. Buffers Needed For Staining:

Acetate buffer (0.2 M pH 4.0):

Sodium acetate	2.95 g
Acetic acid 0.2 M	820 ml
H_2O to	1000 ml

Phosphate buffer (0.1 M pH 6.5):

Na_2HPO_4	3.04 g
KH_2PO_4	7.77 g
H_2O to	1000 ml

Malate buffer (0.05 M pH 6.0):

L-malic acid	6.705 g
H_2O to	1000 ml
Adjust to pH 6.0 with	1.0 N NaOH.

Tris-HCl buffer (0.5 M pH 8.5):

Tris	60.5 g
H_2O to	1000 ml
Adjust to pH 8.5 with	1.0 N HCl.

2. Stanining Solutions:

Acid phosphatase:

10 mg Fast Garnet GBC
10 mg α-naphthyl acid phosphate
1 ml $MgCl_2$ (0.1 M)
100 ml acetate buffer (0.2 M pH 4.0)

The gels were incubated overnight in the dark at room temperature with gentle shaking.

Esterase:

150 mg α -naphthyl acetate
75 mg β -naphthyl acetate
Dissolve these in 3.0 ml acetone.
150 ml phosphate buffer (0.1 M pH 6.5)

30 mg Fast Blue RR salt
30 mg Fast Garnet GBC

Staining procedure was the same as that for acid phosphatase.

Malate dehydrogenase:

20 ml malate buffer (0.05 M pH 6.0)
20 ml Tris buffer (0.5 M pH 6.0)
60 ml DD H_2O
20 mg NAD
20 mg NBT (Nitro Blue Tetrozolium)
2 mg PMS (Phenazine Methosulfate)

Gels were incubated in the dark at 40°C for about 4 hr or until bands were clearly developed.

Stained gels were washed with distilled water and photographed. Gels were dried with a gel drier or wrapped with plastic wrap and stored at 4°C.