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Alterations in isozyme patterns induced by different levels of iron, manganese and boron on white lupin

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RESUMO

Tremoceiros brancos, quando cultivados em soluções nutritivas com vários níveis de ferro, manganês ou boro, apresentaram perfis isoenzimáticos diferentes, de aspartato-aminotransferases, fosfatases ácidas e carboxilesterases.

Concentrações elevadas de ferro ou manganês induziram a síntese de novas isoenzimas com actividade de aspartato-aminotransferase. Estas apresentaram mobilidades electroforéticas menores do que as das enzimas normalmente presentes. Altas concentrações de ferro também inibiram a actividade de uma carboxilesterase. Uma fosfatase ácida foi estimulada nas folhas, por altos níveis de manganês.

A falta de ferro na solução nutritiva conduziu à inibição de uma fosfatase ácida e a de manganês à de uma carboxilesterase.

Elevados níveis de boro inibiram a actividade de todas as aspartato-aminotransferases normalmente presentes nas raízes.

SYNOPSIS

White lupin plants grown in nutrient solutions with different levels of iron, manganese or boron presented distinct zymographic stains of aspartate aminotransferase, acid phosphatase and carboxylesterase isozymes.

High levels of iron or manganese induced the synthesis of new aspartate aminotransferase isozymes which had lower electrophoretic mobility than the enzymes usually present. High iron levels also inhibited the activity of one isocarboxylesterase. An acid phosphatase isozyme was stimulated in leaves grown in the presence of high levels of manganese.

Lack of iron or manganese in the nutrient solutions inhibited one acid phosphatase and one carboxylesterase isozymes, respectively.

High boron concentrations caused the inhibition of all root aspartate aminotransferase isozymes.

1. INTRODUCTION

The technique of isozyme electrophoresis has been frequently used to discriminate among species or cultivars of several plants as grape (WALTERS *et al.*, 1989), maize (SMITH, 1988), rice (GLASZMANN, 1987), lettuce (MEJIA and MCDANIEL, 1986), raspberry (COUSINEAU and DONNELY, 1989) and lupin (CABRAL, 1990).

As far as white lupin is concerned, since it is mainly autogamous and multiplied by seed, several isozyme phenotypes would be expected. In fact, CABRAL (1990) characterized nine distinct white lupin cultivars based on aspartate aminotransferase (2.6.1.1.) acid phosphatase (3.1.3.2.) and carboxylesterase (3.1.1.1.) isozyme patterns. She calculated a low polymorphism index (MARSHALL and ALLARD, 1970), varying from 0.060 to 0.097, proving the existence of genetic stability in these cultivars.

Stability of isozyme patterns to environmental factors is also essential if these are to be used for taxonomic purposes. CABRAL (1990) showed that the expression of the isozymes studied was maintained, albeit with some quantitative differences, when white lupin plants were subjected to water deficit.

In the present study, we investigated if white lupin isozyme patterns of aspartate aminotransferase *(AAT)*, acid phosphatase *(AP)* and carboxylesterase *(CE)* were stable when plants were grown in the presence of different levels of iron, manganese or boron. The results obtained showed that iron or manganese deficit inhibited some isozyme species. Furthermore, high levels of all micro-nutrients studied (levels not frequently encountered in normal growing conditions) also affected the zymographic stains of these enzymes.

2. MATERIAL AND METHODS

PLANT MATERIAL – White lupin seeds (*Lupinus albus* L. cv Estoril) were germinated in moist cottonwool, at room temperature in the dark, for four to five days. Seedlings were transplanted to nutrient solutions which contained: macro-nutrients – $6\,mM\ Ca(NO_3)_2$, $6\,mM\ KNO_3$, $2.5\,mM\ MgSO_4$ and $1\,mM\ KH_2PO_4$; micro-nutrients – $30\,\mu M\ ZnSO_4$, $1\,\mu M\ Na_2MoO_4$, $0.1\,\mu M\ CuSO_4$, $0.1\,\mu M\ CoCl_2$ and variable amounts of iron, manganese and boron supplied as *Fe-EDTA*, $MnSO_4$ and H_3BO_3, respectively. Normal concentrations of these micro-nutrients were $75\,\mu M\ Fe$, $100\,\mu M\ Mn$ and $100\,\mu M\ B$. Besides these concentrations, 0, 750 and $7500\,\mu M\ Fe$; 0, 1000 and $10000\,\mu M\ Mn$ and 1000, 2000 and $4000\,\mu M\ B$ were also used. Since continuous growth in the presence of $7500\,\mu M\ Fe$ inhibits the establishement of a normal root system (VARENNES, 1990), in some experiments plants were grown for one week with $75\,\mu M\ Fe$, and then grown for a further week with $7500\,\mu M\ Fe$.

Plants were kept in a growth chamber at 18°C and 55% humidity, with 14/10 h dark/light periods at a light intensity of $500\,\mu mol\,foton\ m^{-2}\ s^{-1}$ supplied by day light fluorescent tubes.

Samples were collected one, two and three weeks after transplant and analysed for enzyme activity.

PROTEIN EXTRACTION – Protein extraction and all posterior handling of samples was carried out at 0°C. Leaves or roots were homogenised in *0.1 M* Tris-HCl buffer, pH 7.5, containing 0.25% sodium thioglycollate and 0.25% potassium disulfite. The homogenates were centrifuged for 20 min at 14 000 *rpm*. The supernatants were immediately used or stored frozen at −20°C until the following day.

ISOZYME ELECTROPHORESIS – Protein samples were resolved by polyacrilamide gel electrophoresis according to DAVIS and ORNSTEIN (1961) using 8% acrylamide in the resolving gel and 2.5% acrylamide in the stacking gel. Electrophoresis was performed at 200 *V* whilst the samples went through the stacking gel and then at 500 *V* until the tracking dye reached the bottom of the gels.

ENZYMIC STAINING – Enzymatic activities were detected using the staining procedures described by SCHWARTZ *et al.*(1963), SCANDALIOS (1969) and MARKERT and HUNTER (1959). Briefly, for *AAT* activity, gels were reacted with 2-oxoglutaric acid, aspartic acid and pyridoxil-5'-phosphate. After the reaction had proceeded for 15 min, the gels were developed with Fast Blue *BB* salt, at 37°C until the bands had the desired intensity.

To detect acid phosphatases, the substrate used was α-naphtyl phosphate and after 15 min its products were detected at 52°C with Fast Garnet *GBC*.

Carboxylesterases were detected using as substrates α- and β-naphtyl acetates. The products were developed with Fast Blue *RR* salt, at room temperature.

All the developing reactions were stopped by transfer of the gels to 50% ethanol.

MICRO-NUTRIENT ANALYSIS – Leaves or roots were dried at 105°C and then burned at 500°C. To determine iron and manganese concentrations, the ash was digested in 3 *M HCl* and the amount of these elements determined with an atomic absorption spectrophotometer.

To determine boron concentration, the ash was dissolved in 0.1 *M HCl* and reacted at 55°C with 5% oxalate and 0.04% curcumin in ethanol. It was then dissolved in ethanol and the solution was centrifuged. The absorvance of the supernatant was measured at 540 *nm* and the results obtained were compared with a standard curve.

3. RESULTS

IRON, MANGANESE AND BORON LEVELS – The concentration of micro-nutrients in the solutions used had a direct influence in the elements levels, in the plants. As can be seen in Table I, iron levels were higher in the roots than in the corresponding leaves, whilst the opposite was true for boron and manganese.

TABLE I – *Micronutrient levels present in white lupin plants grown for three weeks in nutrient solutions with variable concentrations of these elements.*

	Level of *Fe* in the nutrient solution ($\mu M\ Fe$)			
	0	75	750	7500
Leaves (*mg Fe/g* dry weight)	0.12	0.29	0.73	1.6
Roots (*mg Fe/g* dry weight)	0.21	1.5	4.1	5.1
	Level of *Mn* in the nutrient solution ($\mu M\ Mn$)			
	0	100	1000	10 000
Leaves (*mg Mn/g* dry weight)	0.07	1.5	5.5	7.6
Roots (*mg Mn/g* dry weight)	0.02	0.14	0.79	5.7
	Level of *B* in the nutrient solution ($\mu M\ B$)			
	100	1000	2000	4000
Leaves (*mg B/g* dry weight)	0.05	0.3	0.6	1.1
Roots (*mg B/g* dry weight)	0.05	0.1	0.2	0.4

NORMAL ZYMOGRAMS – Leaf and root zymograms from plants grown with normal levels of all micro-nutrients presented some bands that were always observed and additional bands that sometimes were not present, presumably due to different incubation times during the staining procedures. Therefore, any variations in these bands, when different levels of micro-nutrients were tested, were discarded.

Lupin leaves and roots showed two main bands with *AAT* activity, with *Rf* values of 0.18 and 0.25. An additional band (*Rf* 0.13) was observed, specially in the root material, but frequently also in the leaf extracts. Leaves usually presented a darker *Rf* 0.25, whilst the roots showed a stronger *Rf* 0.18.

Zymographic stains of *AP* isozymes from leaves or roots were almost undistinguishable and consisted of four main bands with *Rf* values of 0.2, 0.42, 0.45 and 0.55. The band with lowest electrophoretic mobility (*Rf* 0.2) was very wide and was probably composed of two or more proteins (in fact, sometimes two bands with *Rf* values of 0.16 and 0.2 were observed). The second and third proteins (*Rf* 0.42 and 0.45) were often observed as a merged large band. Additional minor bands, like those with *Rf* values of 0.05 and 0.08, were also frequently detected.

CE zymograms from leaves or roots were easily differentiated. Both consisted of six main bands with *Rf* values of 0.09, 0.22, 0.38, 0.42, 0.55 and 0.58. However, *Rfs* 0.55 and 0.58 from roots were always wider and darker than the corresponding leaf bands. On the other hand, leaf bands with *Rf* values of 0.09 and 0.22 were more intense.

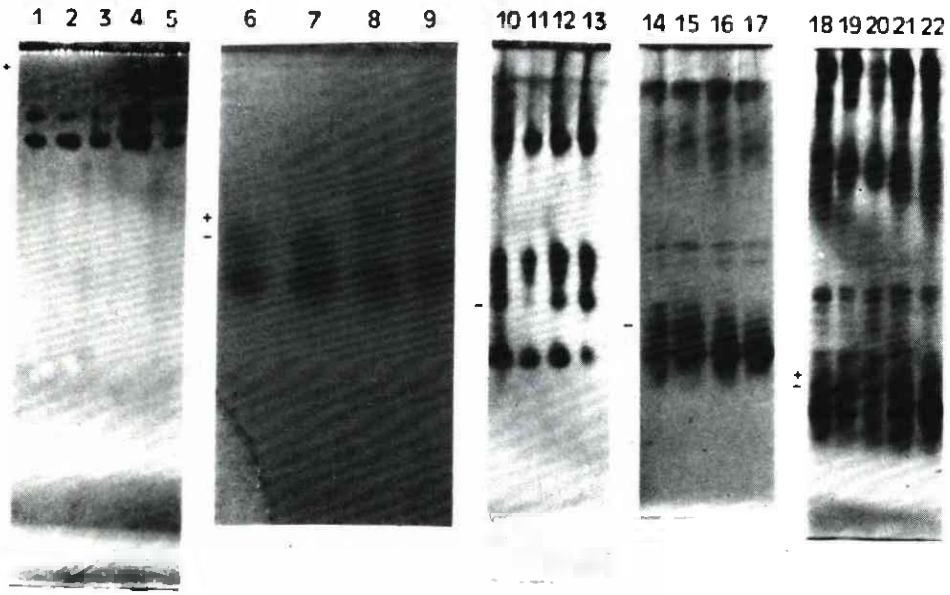
Additional minor bands, like those with *Rf* values of 0.37 and 0.50 were sometimes observed.

IRON-RELATED ISOZYME PATTERNS – High levels of iron in the nutrient solution induced the appearance of a new leaf band with low electrophoretic mobility and presenting *AAT* activity. As can be seen in Fig. 1, plants grown with 0, 75 or 750 $\mu M\ Fe$ in the nutrient solution only revealed the three usual proteins (the upper band was very weak and can not be observed in the photographed gel). On the other hand, plants grown with 7500 $\mu M\ Fe$ and specially those grown for a week with 75 $\mu M\ Fe$ and another week with 7500 $\mu M\ Fe$ presented an additional band with a *Rf* value of 0.08. On one occasion, in one week old plants, instead of this pattern, the protein with a *Rf* value of 0.18 seemed to have migrated less, remaining in a position corresponding to a *Rf* value of 0.15 (Fig. 1, lanes 6-9).

FIGURE 1 – *Isozyme patterns of white lupin plants grown in nutrient solutions with different levels of iron.*

1– 9 leaf aspartate aminotransferase isozymes;
10–13 leaf acid phosphatase isozymes;
14–17 root carboxylesterase isozymes; and
18–22 leaf carboxylesterase isozymes.

1, 6, 10, 14 and 18 – plants grown with 75 μM *Fe*
2, 7, 11, 15 and 19 – plants grown with no iron
3, 8, 12, 16 and 20 – plants grown with 750 μM *Fe*
4, 9, 13, 17 and 21 – plants grown with 7500 μM *Fe*
5 and 22 – plants grown for a week with 75 μM *Fe* and another week with 7500 μM *Fe*.



The root *AAT* isozyme pattern was not affected by the different levels of iron tested (data not shown).

Leaf acid phosphatases were not affected by high levels of iron in the nutrient solution. On the other hand, in plants one week or more old, lack of iron in the solution lead to a decrease in the activity of a protein with a *Rf* value of 0.45 (Fig. 1, lanes 10-13).

Root acid phosphatases were not affected by the different levels of iron tested (data not shown).

A root isocarboxylesterase (*Rf* 0.55) was affected by high levels of iron. Its activity sharply decreased in roots grown for one or more weeks with 750 or 7500 $\mu M\ Fe$ (Fig. 1, lanes 14-17). In leaves, its activity was less affected, though sometimes a decrease was also observed (data not shown). Alternatively, in plants grown for a week with 75 $\mu M\ Fe$ and another week with 7500 $\mu M\ Fe$, this protein seemed to migrate less, remaining in a position corresponding to a *Rf* value of 0.53 (Fig. 1, lane 22).

MANGANESE-RELATED ISOZYME PATTERNS – Zymographic assays from one week old plants, grown in the presence of different levels of manganese lead to identical results. This was not the case with older plants. Leaves or roots from two or three week old plants, grown with 10000 $\mu M\ Mn$, presented an additional *AAT* species with a *Rf* value of 0.1 (Fig. 2, lanes 1-8). Plants with the same age but grown with no manganese in the nutrient solution showed a marked decrease in the activity of one isocarboxylesterase (*Rf* 0.42), (Fig. 2, lanes 17-24).

Three week old plants, but not younger plants, grown with 10000 $\mu M\ Mn$, showed an increased activity in a leaf *AP* isozyme with a *Rf* value of 0.45 (Fig. 2, lanes 9-12).

Root acid phosphatases were not affected by the levels of manganese used (Fig. 2, lanes 13-16).

BORON-RELATED ISOZYME PATTERNS – The activities of *CE* and *AP* isozymes were not affected by the levels of boron tested (data not shown). The same was true for leaf *AAT* species (Fig. 3, lanes 1-4). On the other hand, root *AAT* isozymes, in one or more week old plants grown with 2000 or 4000 $\mu M\ B$, were strongly affected. In the latter, this enzyme activity was not detected at all, whilst in the former weak bands were still observable (Fig. 3, lanes 5-8).

FIGURE 2 – *Isozyme patterns of white lupin plants, grown in nutrient solutions with different levels of manganese.*

1– 4 root aspartate aminotransferase isozymes;

5– 8 leaf aspartate aminotransferase isozymes;

9–12 leaf acid phosphatase isozymes;

13–16 root acid phosphatase isozymes;

17–20 leaf carboxylesterase isozymes; and

21–24 root carboxylesterase isozymes.

1, 5, 9, 13, 17 and 21 – plants grown with 100 μM Mn

2, 6, 10, 14, 18 and 22 – plants grown with no manganese

3, 7, 11, 15, 19 and 23 – plants grown with 1000 μM Mn

4, 8, 12, 16, 20 and 24 – plants grown with 10000 μM Mn.

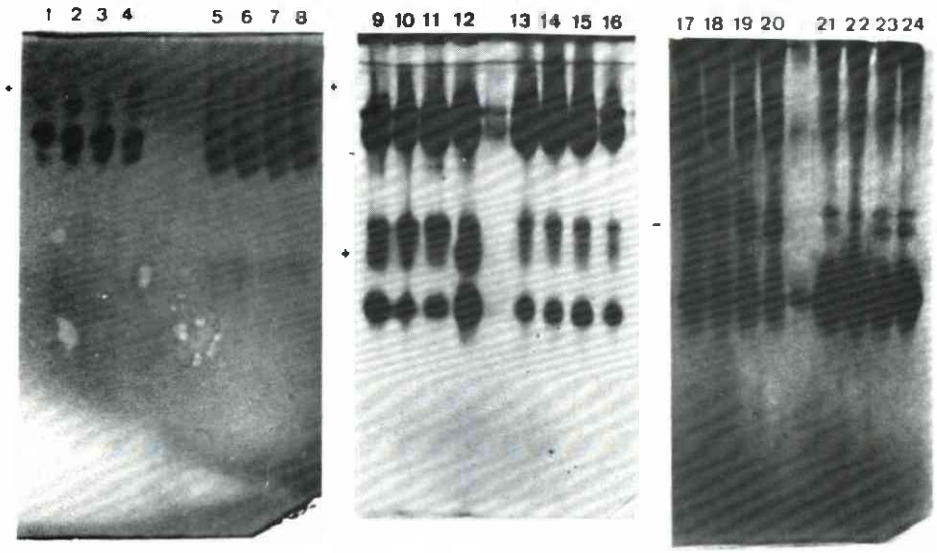
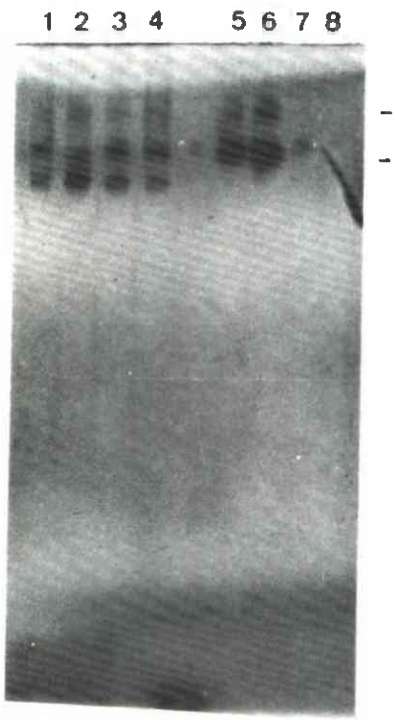


FIGURE 3 – *Aspartate aminotransferase isozymes from white lupin plants grown with different levels of boron.*

1–4 leaf isozymes and
5–8 root isozymes.
1 and 5 – plants grown with 100 μM B
2 and 6 – plants grown with 1000 μM B
3 and 7 – plants grown with 2000 μM B
4 and 8 – plants grown with 4000 μM B.



4. DISCUSSION

The present results show that different levels of some elements in the nutrient solution induce isozyme changes in white lupin. The only cultivar tested was cv Estoril; therefore, though it is likely that similar changes will occur in other cultivars, the discussion can only be focused on this particular one.

Several lupin cultivars have been recently characterized for "normal" zymograms of the isozymes tested in the present work (CABRAL, 1990). However, some of the results we obtained with non-stressed plants are different from the zymograms previously reported for cv Estoril. Since CABRAL (1990) states that the cultivar she used was obtained in a cultivated field, it is possible that it was no longer the pure cultivar. We obtained our seed from Estação Agronómica Nacional, where this cultivar was developed, so we are confident that it truly corresponds to its designation.

No genetic analysis of white lupin has been carried out. Therefore, zymographic bands can correspond to true isozymes or be the result of post-translational modifications of these proteins. Thus, the results we obtained with *AAT* can be interpreted in two ways. Either cv Estoril has a silent *locus* that becames activated only after stress, as growth in the presence of high levels of iron or manganese, or the new bands represent iron- or manganese- induced alterations in post-translational modifications. The former hypothesis would lead to a new isozyme with a characteristic *Rf* value, which seems not to be the case. However, a rigorous determination of *Rf* values was not carried out, so it remains possible that the bands observed with high levels of iron or manganese correspond to the same species. The latter hypothesis is strengthened by the fact that a shift towards a lower electrophoretic mobility of one *AAT* species (*Rf* 0.18) was observed in one week old iron-stressed leaves. Shifts in electrophoretic mobility were reported in flax isoperoxidases as a consequence of growth in the presence of high levels of nitrogen and phosphorus or in high levels of nitrogen, phosphorus and potassium. These differences were due to heterogeneity in the carbohydrate moieties of the isozymes (GAUDREAULT and TYSON, 1986) and could be eliminated by digestion with α-mannosidase (GAUDREAULT and TYSON, 1988). In phosphate-starved tomato, elec-

trophoretic shifts of *AP* species were explained by association of the two isozymes to form a high molecular weight aggregate (GOLDSTEIN *et al.*, 1988).

It should be pointed out that, contrary to those results, in the present case the three normal species are still detected; thus, the new band is not formed at the expense of an usual one.

It is curious to note that CABRAL (1990) reported the presence of minor bands with low electrophoretic mobility in several lupin cultivars, including the one she referred as cv Estoril. In that case, a band with a *Rf* value of 0.05 was reported, which is in the mobility range of the bands detected by us in iron- or manganese-stressed plants. The plants CABRAL (1990) analysed were very probably subjected to, at least, an intense chloride stress, as she used tap water in her experiments.

High levels of boron had a negative effect in all root *AAT* isozymes. It should be noted that roots presented a lower level of boron compared with the corresponding leaves, where no inhibition was observed.

An enzyme degradation during the extraction procedure, due to high levels of boron in the sample, is therefore ruled out.

An *AP* isozyme (*Rf* 0.45) was sensitive to the levels of iron or manganese. It was inhibited in the absence of iron and stimulated by high levels of manganese.

Two isocarboxylesterases proved to be sensitive to the levels of micro-nutrients in the solution. One species (*Rf* 0.42) was absent in leaves from plants grown without manganese and another (*Rf* 0.55) was absent in roots from plants grown with high levels of iron.

Enzyme activation or inhibition in response to several types of stress has been described for some isozymes. For instance, an isoperoxidase from maize was inhibited under water stress (THAKUR *et al.*, 1981) and another isozyme from bean was inhibited under iron-deficient conditions (SIJMONS *et al.*, 1985). On the other hand, several isoperoxidases from tobacco were stimulated by wounding and Tobacco Mosaic Virus infection (LAGRIMINI and ROTHSTEIN, 1987). Salt stress can lead to an increase in the activity of high molecular weight *AP* isozymes from spinach (PAN, 1987).

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REFERENCES

CABRAL, F.M.M. (1990) — *Polimorfismo isoenzimático em Lupinus. Identificação e caracterização de populações (L. albus e L. luteus L.)*, Instituto Superior de Agronomia, Lisboa, 141 pp.

COUSINEAU, J.C. and DONNELLY, D.J. (1989) — Identification of raspberry cultivars *in vivo* and *in vitro* using isozyme analysis, *Hort. Sci.*, 24: 490–492.

DAVIS, B.J. and ORNSTEIN, L. (1961) — *Disc electrophoresis*, Distillation Products Industries, Rochester, USA.

GAUDREAULT, P.R. and TYSON, H. (1986) — Evidence for heterogeneity in the carbohydrate moieties of peroxidase isozymes from two environmentally induced flax genotrophs, *Can. J. Bot.*, 64: 2682–2687.

GAUDREAULT, P.R. and TYSON, H. (1988) — Elimination of differences in the mobility of flax isoperoxidases on PAGE by digestion with α-mannosidase, *Plant Physiol.*, 86: 288–292.

GLASZMANN, J.C. (1987) — Isozymes and classification of Asian rice varieties, *Theor. Appl. Genet.*, 74: 21–30.

GOLDSTEIN, A.H., BAERTLEIN, D.A. and MCDANIEL, R.G. (1988) — Phosphate starvation inducible metabolism in *L. esculentum*. II. Characterization of the phosphate starvation inducible-excreted acid phosphatase, *Plant Physiol.*, 87: 716–720.

LAGRIMINI, L.M. and ROTHSTEIN, S (1987) — Tissue specificity of tobacco peroxidase isozymes and their induction by wounding and tobacco mosaic virus infection, *Plant Physiol.*, 84: 438–442.

MARKERT, C. and HUNTER, R. (1959) — The distribution of esterases in the mouse tissues, *J. Histochem. Cytochem.*, 7: 42–49.

MARSHALL, D.R. and ALLARD, R.W. (1970) — Isozyme polymorphisms in natural populations of *Avena fatua* and *A. barbata*, *Heredity*, 25: 373–382.

MEJIA, L. and MCDANIELS, R.G. (1986) — Electrophoretic characterization of lettuce cultivars, *Hort. Sci.*, 21: 278–280.

PAN, S. (1987) — Characterisation of multiple acid phosphatases in salt-stressed spinach leaves, *Aust. J. Plant Physiol.*, 14: 117–124.

SCANDALIOS, J.C. (1969) — Genetic control of multiple molecular forms of enzymes in plants: a review, *Biochem. Genetics*, 3: 37–79.

SCHWARTZ, M.K., NISSELBAUM, J.S. and BODANSKY, O. (1963) — Procedure for staining zones of activity of glutamic oxoloacetic transaminase following electrophoresis with starch gel, *Amer. J. Clin. Pathol.*, 40: 103–106.

SIJMONS, P.C., KOLATTUKUDY, P.E. and BIENFAIT, H.F. (1985) — Iron deficiency decreases suberization in bean roots through a decrease in suberin-specific peroxidase activity. *Plant Physiol.*, 78: 115–120.

SMITH, J.S.C. (1988) — Identification of pedigrees of hybrid maize (*Zea mays* L.) cultivars by isozyme electrophoresis and reversed-phase high-performance liquid chromatography, *Euphytica*, 39: 199–205.

THAKUR, P.S., SINGH, G. and RAI, V.K. (1981) — Peroxidase isozymes in relation to developing water deficits in two *Zea mays* L. cultivars, *New Phytol.*, 89: 25–32.

VARENNES, A. de (1990) — *Effects of iron stress on lupin polypeptide patterns*, Garcia de Orta, Sér. Est. Agron. (em publicação).

WALTERS, T.W., POSLUSZNY, U. and KEVAN, P.G. (1989) — Isozyme analysis of the grape (*Vitis*). I. A practical solution, *Can. J. Bot.*, 67: 2894–2899.