Pergamon

PII: S0031-9422(98)00188-5

Phytochemistry, Vol. 49, No. 2, pp. 347–354, 1998

Printed in Great Britain
0031-9422/98/$–see front matter

METHIONINE SULPHOXIMINE DOES NOT INHIBIT PEA AND WHEAT GLUTAMATE DEHYDROGENASE

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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(*Received* 5 *December* 1997; *received in revised form* 3 *March* 1998)

Key Word Index—*Pisum sativum*; Leguminaceae; pea; *Triticum vulgare*; Graminae; wheat; glutamate dehydrogenase; inhibition; methionine sulphoximine

Abstract—The addition of methionine sulphoximine (MSX) for 2 days at concentrations ranging from 0.6 to 2.0 mM, completely inhibited glutamine synthetase (EC 6.3.1.2) in the roots and leaves of 12 day old wheat (*Triticum vulgare*) and pea (*Pisum sativum*). However MSX had no effect on glutamate dehydrogenase (GDH; EC 1.4.1.2) activity or subunit composition in the roots of wheat or pea. In the leaves of pea, MSX stimulated a 4.5 fold increase in NADH-dependent glutamate dehydrogenase activity, that correlated with the synthesis of a second enzyme subunit and a dramatic alteration in the GDH isoenzyme pattern following native PAGE. Although MSX also caused a 2.4 fold increase in GDH activity in wheat leaves, this increase was not accompanied by a change in subunit composition or isoenzyme pattern. It is concluded that MSX does not inhibit GDH in the leaves or roots of wheat and pea seedlings, but it may cause the onset of premature senescence, which results in an increase in GDH activity in the leaves, as has been shown previously.

INTRODUCTION

It is now well established that the pathway of ammonia assimilation in higher plants is via the operation of the two enzymes glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase. Evidence to support this statement is based on enzyme activity measurements, labelling experiments, inhibitor studies and more recently the use of mutants deficient in the enzymes [1, 2]. *L*-Methionine-*S*-sulphoximine (MSX) and phosphinothricin, are potent inhibitors of the first enzyme GS, which catalyses the incorporation of ammonia into the amide position of glutamine. This inhibitory action forms the basis of the commercially important herbicide 'Basta'. Following the application of either of the two GS inhibitors there is a dramatic increase in the concentration of ammonia within the plant tissue [3]. Using MS as part of a detailed ^{15}N NMR spectroscopic study, it has been proposed recently that ammonia released during active phenylpropanoid metabolism is rapidly recycled back to regenerate phenylalanine, via GS and glutamate synthase [4, 5]. GS is normally present as an octamer with subunits of M_r values ranging from 39–45 k, which are under the control of a multigene family [6]. A major form of the enzyme is localised in the chloroplast of leaves, but separate cytosolic forms have been shown to be active in the vascular system, roots and root nodules [6–8]. Mutants of barley that lack the chloroplast isoenzyme of GS, accumulate high concentrations of ammonia in leaves in the light, due to photorespiration [9].

Glutamate dehydrogenase [GDH, EC 1.4.1.2] catalyses the reversible amination of 2-oxoglutarate to yield glutamate. The role of the enzyme in higher plants has been the subject of continuous debate. There is now a concensus of opinion that the enzyme may be important in the deamination of glutamate to yield ammonia and 2-oxoglutarate, particularly during times of stress and senescence, although a role in ammonia assimilation is still proposed [10–14]. GDH is localised in the mitochondria, and the native enzyme has been shown to be composed of six subunits with M_r values of 42–45 k [15]. At least two genes regulating

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GDH activity have been shown to encode distinct subunits, which are able to assemble randomly to form seven isoenzymes, which can be readily identified by polyacrylamide gel electrophoresis [15–17]. A mutant of maize lacking one enzyme subunit and containing less than 10% normal GDH activity was unable to metabolise ^{15}N-glutamate to ammonia [18]. A similar mutant of *Arabidopsis thaliana* displayed retarded root growth when the plants were grown on media containing high concentrations of nitrate and ammonium ions [19].

Osuji and Madu [20], demonstrated that in maize, three distinct subunits of GDH were present that could assemble into 28 isoenzymic forms following separation by isoelectric focusing. MSX was shown to inhibit GDH activity and to alter the native form of the enzyme from a hexamer to a tetramer. In a paper published at the time that this manuscript was being completed, Osuji and Madu [21] also demonstrated that MSX severely depressed GDH activity in peanut seedlings following incubation with 1 mM MSX. In their original paper Osuji and Madu [20] made the statement "Evidence showing the MSX inhibition of GDH, now means that the role of the enzyme in NH_4^+ ion salvage reactions has been grossly underestimated; and there is the need to review and revise the physiological functions of the enzyme". Due to the importance of the latter statement, we have reexamined the effect of MSX on GDH activity in wheat and pea seedlings.

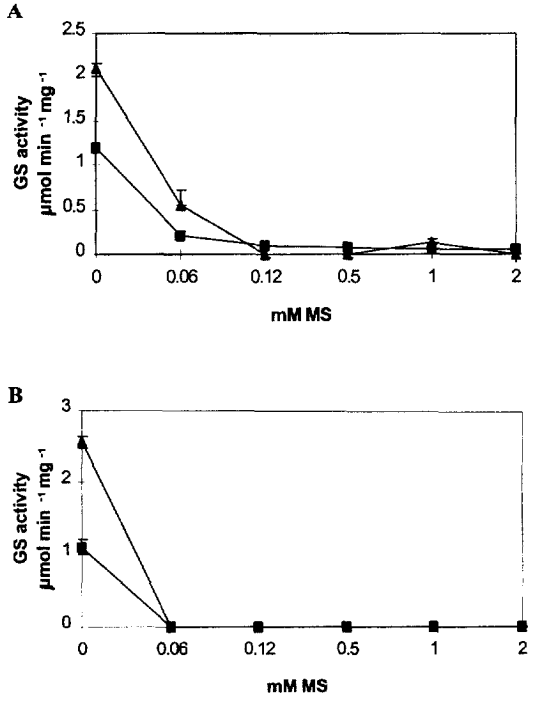

Fig. 1. The effect of increasing concentrations of methionine sulphoximine (MS) on the activity of glutamine synthetase (GS) in the leaves (■) and roots (▲) of A. wheat and B. peas.

RESULTS AND DISCUSSION

Following the addition of MSX (calculated as the active *L*-methionine-*S*-sulphoximine isomer only) to the growth medium for 2 days, the wheat leaves displayed evidence of chlorosis and the pea plants began to wilt at concentrations above 1 mM. The activity of GS in the roots and leaves of both the wheat and pea was completely inhibited even by the lowest concentration of 0.06 mM MSX (Figure 1), as would be expected from previous work [2, 3]. The data does, however, provide clear evidence that MSX is rapidly taken up by the plants and is able to reach enzymes in both the roots and leaves. In the roots of both wheat and pea, only one major GS protein band was detected following Western blotting, the concentrations of which were not altered during the MSX treatments (Figure 2A and B). It is likely that this protein band represents the cytosolic form of the enzyme and that the chloroplastic form is only present in low concentrations [7, 22]. In the leaves of wheat and pea, two GS proteins were detected (Figure 2C and D), it is probable although not definite, that the faster running lower molecular mass protein is the cytoplasmic isoenzyme and the slower running higher molecular mass protein is the chloroplast form [7, 8]. In the wheat leaves and to a greater extent in the pea leaves, there was evidence that the lower concentrations of MSX induced the synthesis of the cytoplasmic form of GS (see lanes 2,3 and 4 Fig. 2D), this is probably due to an early senescence response, which has been previously shown in rice [23] and other plants [8]. Interestingly there was no evidence of senescence induced proteolysis of the GS protein as has been demonstrated in wheat [24], probably due to the stabilisation of the GS protein by the inhibitor MSX [25].

The addition of MSX had little effect on the activity of GDH in the roots of either wheat or pea (Figure 3A and B) and this was confirmed by native PAGE staining for enzyme activity (data not shown). Western blot analysis indicated that there was no change in the concentration of the major GDH subunit protein in either wheat or pea roots (Figure 4A and B). However, in the leaves of both wheat and pea (Figure 3C and D) there was clear evidence of elevated GDH activity, following the addition of increasing concentrations of MSX. Western blot analysis of the GDH proteins in the leaves indicated that the response of wheat and pea to the action of MSX was quite different. In wheat leaves, one major and one minor lower molecular mass GDH protein band were detected (Figure 4C), the concentration of the major band did not alter, and there was slight evidence that the minor band was reduced following MSX treatment. As the invariance of the GDH protein did not correlate with the increase in activity, wheat leaf GDH was also analysed by native PAGE (Figure 5A). Although seven isoenzyme bands were detected, the

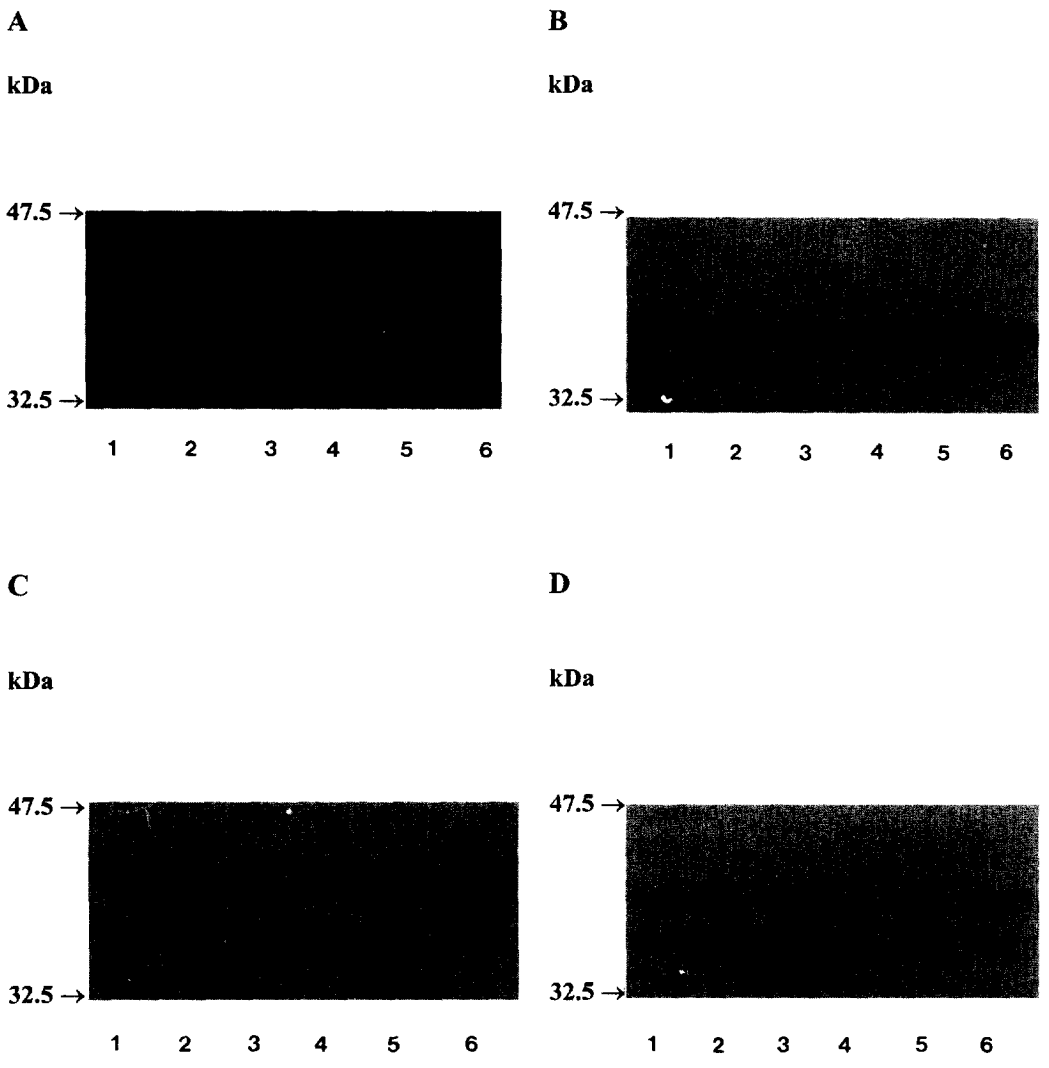

Fig. 2. Western blot analysis of the glutamine synthetase (GS) proteins of A. wheat roots, B. pea roots, C. wheat leaves and D. pea leaves following treatment with 1. zero, 2. 0.06 mM methionine sulphoximine (MS), 3. 0.12 mM MS, 4. 0.5 mM MS, 5. 1 mM MS and 6. 2 mM MS.

pattern was somewhat different from that described previously for other plant species [17, 26], in that there is a major basic band, two very faint bands and four more acidic bands. The pattern did not change following the addition of MSX, although an increase in activity was clearly visible, confirming the data in Fig. 3C.

In pea, only one GDH subunit protein was detectable in the untreated control leaves (lane 1, Fig. 4D), however following the addition of MSX, there was clear evidence of an increased synthesis of the original GDH protein and the formation of a second protein with a higher molecular mass. Native PAGE of GDH isolated from the untreated control leaves of pea, showed again the presence of seven bands, which are just visible in lane 1 of Fig. 5B. However the addition of MSX induced the appearance of a new set of six heavily staining isoenzyme bands, that were more basic than those observed in the untreated control leaves (lanes 2–6, Fig. 5B). Control gels developed in the absence of glutamate (not shown) did not exhibit any banding pattern, confirming that the isoenzymes detected were GDH. Gels developed in the presence of malate, exhibited a totally different banding pattern (not shown), confirming that the ubiquitous and highly active malate dehydrogenase was not responsible for any of the GDH isoenzymic bands observed [26].

The addition of ammonium ions to plants has frequently been shown to stimulate an increase in measurable GDH activity [8, 27] and was at one time taken as evidence for an assimilatory role for the enzyme. However the addition of carbon sources such as glucose and sucrose cause a decrease in activity and it has been argued that the role of GDH is to supply 2-oxoglutarate for oxidation in the tricarboxylic acid

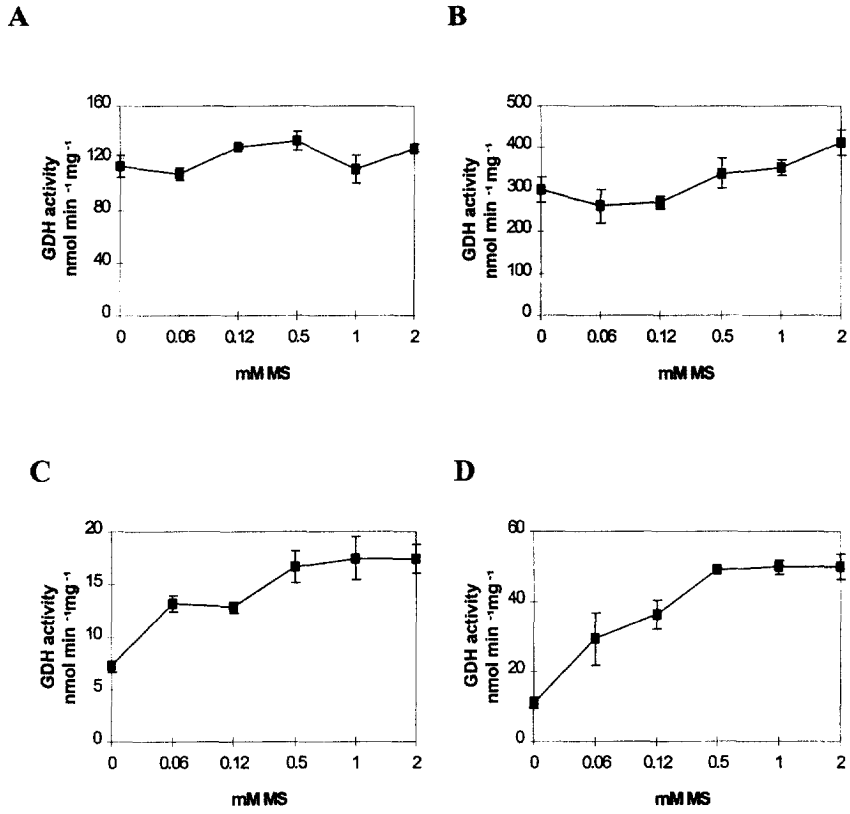

Fig. 3. The effect of increasing concentrations of methionine sulphoximine (MS) on the activity of NADH-dependent glutamate dehydrogenase (GDH) in A. wheat roots, B. pea roots, C. wheat leaves and D. pea leaves.

cycle, via the deamination reaction, when the supply of oxidisable carbohydrate is low [2, 10]. A physiological role for this increase in activity during the senescence of leaves [28], when the amino acids of proteins are mobilised as amides and the rate of photosynthetic CO_2 assimilation is low, has been proposed [2, 8]. Frequently the addition of ammonium ions or the onset of senescence has been shown to cause a dramatic change in the GDH isoenzyme pattern detected following native PAGE [17, 29, 30].

In pea leaves, the increase in GDH activity induced by the presence of MSX (Figure 3D) correlated with an increase in the GDH proteins detected by Western blot analysis. It is likely that the two protein bands visible in Fig. 4D correspond to the α and β subunits of GDH detected in both *Vitis vinifera* [15] and *Arabidopsis thaliana* [17], that have M_r values of 43 and 42.5 k respectively. The two subunit proteins have been shown to be regulated by independent genes, designated *GDH*1 and *GDH*2 [17, 19]. The increased synthesis of the α-subunit as observed in Fig. 4D, following treatment with MS has also been demonstrated after the application of ammonium ions to *V. vinifera* [30], and ammonium nitrate or glutamine to *A. thaliana* [17]. Increased rates of synthesis of *GDH*1 and *GDH*2 mRNA have also been detected in *A. thaliana*, following the application of ammonium ions or dark treatment [17, 19]. However, the induction of *GDH*1 but not *GDH*2 was repressed by the presence of sucrose in the dark [17]. It would appear therefore that the application of MS to peas induces the synthesis of both GDH subunits probably via an increase in gene transcription, caused by the inhibition of photosynthetic CO_2 assimilation [2, 3] and depletion of soluble carbohydrate, in a response similar to that observed during natural senescence. The alteration in the isoenzymic banding pattern detected following native PAGE (Figure 5B), is further confirmation of the synthesis of a new GDH isoenzyme protein. Interestingly, very similar changes in GDH activity and isoenzyme pattern have been reported in much earlier studies on peas, following treatment with high concentrations of SO_2 [31] or floating pea leaf discs on water for 24 h in the dark [32].

The changes in GDH in wheat leaves were less dramatic than in pea. Although an increase in enzyme activity was observed (Figure 3C), there was no obvious increase in either of the two GDH proteins, which presumably again correlate with the α and β subunits discussed previously [17, 19]. However, the fact that wheat is hexaploid, means that a simple interpretation of the number of GDH subunits, is difficult. The absence of any induced subunit synthesis correlates with the lack of change of the isoenzyme pattern fol-

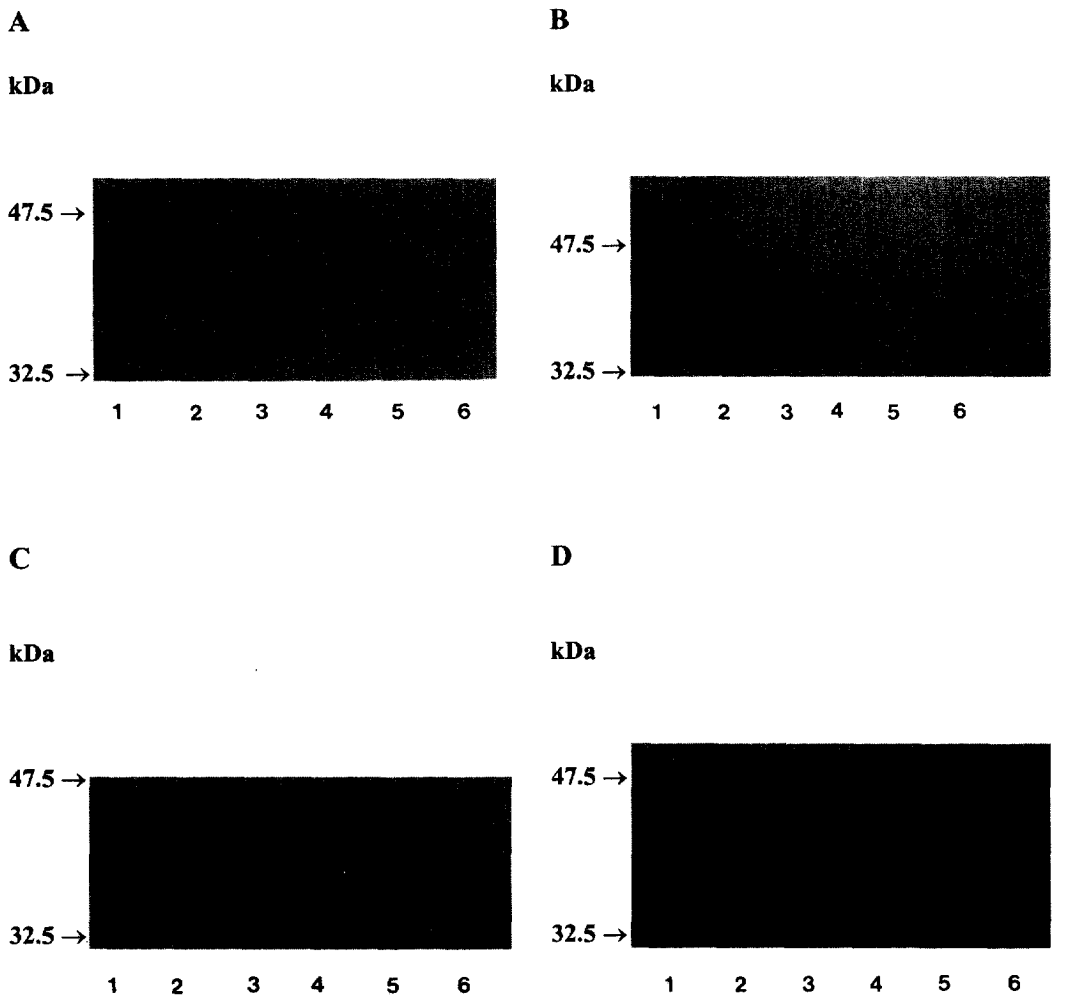

Fig. 4. Western blot analysis of the glutamate dehydrogenase (GDH) proteins of A. wheat roots, B. pea roots, C. wheat leaves and D. pea leaves, following treatment with MS, as stated in Fig. 2.

lowing native PAGE (Figure 5A). It would appear therefore that in wheat, the increase in enzyme activity is due to the activation of preexisting enzyme protein rather than *de novo* synthesis, although no such mechanism has yet been identified in plants.

In conclusion, we have shown that MSX does not inhibit GDH activity in either wheat and pea, rather there is evidence for an increase in activity which may be due to an induced senescence response. The findings are in agreement with previously published data that have indicated that MSX [33, 34] and phosphinothricin [35, 36] (an inhibitor of GS with a similar structure to MSX [2, 3]), has little effect on GDH *in vivo*. These results are clearly different from the data previously published by Osuji and Madu [20, 21], who demonstrated a strong inhibitory effect of MSX on the GDH isolated from maize and peanut. It should be noted however, that in the experiments carried out by Osuji and Madu [20, 21], the MSX was added directly to the germinating imbibing seeds, whilst in the experiments reported here, the MSX was added to the root culture medium of previously germinated seedling. It is possible that the GDH present in the early stages of germination in both maize and soybean seeds, (when the enzyme will be involved in the metabolism of amino acids released from storage proteins), responds in a different manner to MSX. However the MSX induced synthesis of GDH enzyme protein in pea leaves described in this paper, does agree with the recent data of Osuji and Madu [21], in that in peanut seedlings, there is an induction of two GDH subunits with M_r values of 45 and 46 k, following the application of MSX.

EXPERIMENTAL

Plant material

Wheat (*Triticum aestivum L.* cv Chablis) and pea (*Pisum sativum L.* cv Early Onward) seeds were germinated in vermiculite and watered daily with tap water with no added nutrients. Growth conditions

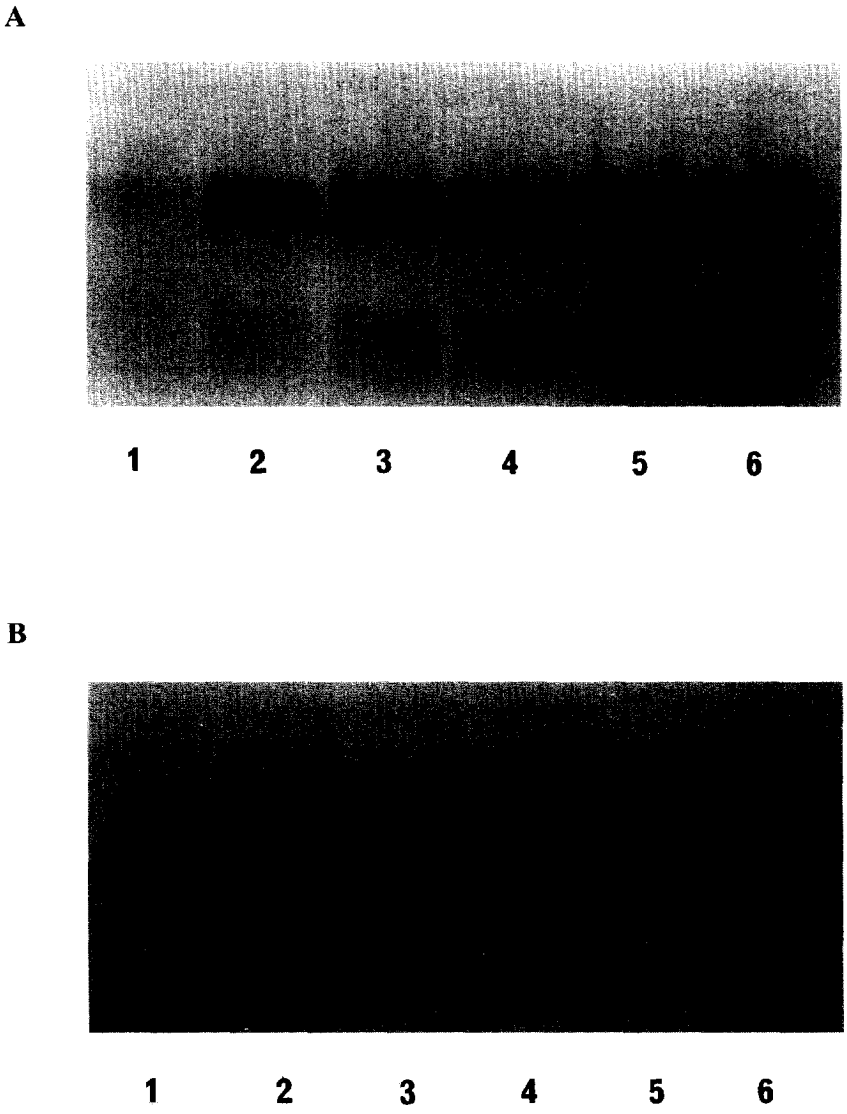

Fig. 5. Activity staining for glutamate dehydrogenase (GDH) following native PAGE of extracts of A. wheat leaves and B. pea leaves, following treatment with MS, as stated in Fig. 2.

were 16 h/8 h dark period at 23 to 25°, 60% humidity and average light intensity of 400 μmol m^{-2} sec^{-1} during the light period. After 10 days the seedlings were transferred for 2 days into 100 ml glass bottles containing 60 ml of half concentration Hoagland solution, eight plants were used for each treatment. The solution contained 3 mM nitrate as a N source and five increasing concentrations of MSX, 0.06, 0.12, 0.5, 1 and 2 mM respectively, calculated on the basis of the active L-methionine-*S*-sulphoximine isomer, only.

Protein extraction

Leaves and roots of the treated plants were frozen with liquid nitrogen and ground in 3 volumes of extraction buffer containing 100 mM Tris/HCl, pH 7.8, 0.5 mM EDTA, 1 mM DTT, 10% (v/v) ethylene glycol and 0.2% (w/v) PVPP. The extracts were centrifuged at 13 000 rpm at 4° for 30 min. The supernatant was desalted on a Sephadex G 25 column, equilibrated in the same buffer and used for all enzyme assays, SDS PAGE and native gels.

Enzyme and protein assays.

All enzyme assays were carried out at room temperature using a Perkin Elmer Lamda 14 spectrophotometer. GDH was assayed as described by Fricke and Pahlich [37]. The amination reaction mixture containing 100 mM Tris/acetate, pH 8, 0.16 mM NADH, 10 mM 2-oxoglutarate, 1 mM $CaCl_2$ and 200 mM ammonium acetate in a final volume of 1 ml.

GS activity was determined by using the hydroxyl-

amine-synthetase assay as described by Lea *et al.* [38]. The reaction mixture contained 10 mM Tris/HCl, pH 7.8, 5 mM hydroxylamine, 50 mM L-glutamate, 50 mM $MgCl_2$ and 20 mM ATP in a final volume of 0.5 ml. The reaction was initiated by the addition of enzyme extract, incubated for 30 min at 30° and terminated by the addition of 0.7 ml ferric chloride reagent. After centrifugation, the absorbance at 540 nm was determined. The protein concentration of the enzyme extracts was determined using the Bio–Rad assay, with BSA as a standard, as originally described by Bradford [39]. All enzyme extractions were carried out on four different samples of leaves or roots, from each treatment and were performed in duplicate, mean values with standard deviation are presented.

SDS polyacrylamide gel electrophoresis

SDS PAGE for Western blotting was performed on a 7.5% resolving gel using a Bio–Rad Protean II system. Samples were mixed with 2 × sample buffer containing 2.3% (w/v) SDS and 10% (v/v) β-mercaptoethanol and boiled at 100° for 5 min. An equal amount of protein, roots 10 μg, leaves 20 μg was loaded per lane, the gels were run at 10° until the bromophenol blue front reached the bottom.

Western blot analysis

After SDS-PAGE, the gel was soaked in transfer buffer containing 39 mM glycine, 48 mM Tris, 20% (v/v) methanol and 0.0375% (w/v) SDS. The proteins were blotted onto a nitrocellulose membrane at 200 mA for 2 hr. After blotting the nitrocellulose was soaked in TBS with 3% (w/v) 'Marvel', washed and incubated with primary and secondary antibody and developed using the alkaline phosphatase method [40]. The primary GDH antibody was raised against the *Vitis vinifera* leaf enzyme [41] and the GS antibody against the *Phaseolus vulgaris* root nodule enzyme [42].

Native Polyacrylamide Gel Electrophoresis

Native PAGE was performed on a 4% stacking gel and 5% resolving gel using the Bio Rad mini Protean II system as described by Loulakakis *et al.* [15]. The pH of the gel buffers were adjusted to pH 7.2, for the resolving gel and pH 7.3 for the stacking gel as described by Hartmann *et al.* [43]. The gels were run at 4° for 3 hr. Staining for NAD-GDH activity using the tetrazolium system was carried out by the method described by Loulakakis *et al.* [15]. The staining solution contained 100 mM Tris/HCl, pH 9.3, 55 mM L-glutamate, 0.5 mM $CaCl_2$, 1 mM NAD, 0.13 mM PMS and 0.5 mM MTT. After incubation in the dark at room temperature, dark blue bands indicating GDH activity appeared.

Acknowledgements—We would like to thank Dr B.G. Forde and Dr J.V. Cullimore for generously providing the GS antibody and Dr K.A. Loulakakis similarly for the GDH antibody. We are grateful to our colleagues Dr Louisa Dever and Claire Skentelbery, for their continued help and support whilst this work was being carried out.

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