BIOLOGIA PLANTARUM 40 (3): 345-350, 1997/98

Effects of ferulic acid on L-malate oxidation in isolated soybean mitochondria

M.A. SERT, M.L.L. FERRARESI, Y.R. BERNADELLI, A.M. KELMER-BRACHT, A. BRACHT and E.L. ISHII-IWAMOTO*

Department of Biochemistry, University of Maringá, Av. Colombo 5790, 87020900 Maringá, Brazil

Abstract

The effects of ferulic acid on L-malate oxidation in mitochondria isolated from soybean (*Glycine max* L.) seedlings were investigated. Oxygen uptake and the products of L-malate oxidation were measured under two conditions: pH 6.8 and 7.8. At acidic pH, the activity of the NAD^+-linked malic enzyme (L-malate:NAD^+oxidoreductase [decarboxylating] EC 1.1.1.39) was favoured, whereas at alkaline pH a predominance of the L-malate dehydrogenase activity (L-malate:NAD^+oxidoreductase EC 1.1.1.37) was apparent. Ferulic acid inhibited basal and coupled respiration during L-malate oxidation either at acidic or alkaline pH, reducing also the amounts of pyruvate or oxaloacetate produced. The results suggest that the site of ferulic acid action is situated at some step that precedes the respiratory chain. An interference with the L-malate entry into the mitochondria could be an explanation for the effects of ferulic acid, but the possibility of a direct inhibition of both enzymes involved in L-malate oxidation cannot be ruled out.

Additional key words: allelopathy, *Glycine max*, phenolic acid, respiration.

Introduction

Many natural phenolic acids have been suggested as natural growth inhibitors, not only of the plant in which they are produced, but also of other plants when released into the environment (Bonner 1950, Muller 1966, Kefeli and Kadyron 1971, Whittaker and Feeny 1971, Van Sumere *et al.* 1972, Sathiyamoorthy 1990). It seems that they alter several aspects of plant metabolism (Van Sumere *et al.* 1972, Glass 1973). Effects on ion transport, protein synthesis, hormone action and energy

---

Received 2 April 1997, accepted 25 June 1997.

Abbreviations: EDTA - ethylenediaminetetraacetic acid.

Acknowledgements: This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

* - Author for correspondence; fax (044)2614396

metabolism have been reported (Glass 1973, Harper and Balke 1981, Einhellig 1986, Moreland and Novitsky 1986). Demos *et al.* (1975) reported a correlation between inhibition of respiration or coupling processes in isolated mitochondria of mung bean and inhibition of hypocotyl growth by tannic, gentisic, *p*-coumaric and vannilic acids but not by ferulic acid. However, ferulic acid is commonly found in plants and has been frequently implicated as the agent responsible for allelopathy (Wang *et al.* 1971, Demos *et al.* 1975, Einhellig and Muth 1980, Sathiyamoorthy 1990). Demos *et al.* (1975) measured only the effect of 230 μM ferulic acid on succinate driven respiration. The effects of ferulic acid on mitochondrial respiration during the oxidation of NAD^+-linked substrates, as L-malate, for example, have not yet been examined. L-malate, a respiratory substrate produced from sugars, appears to be ubiquitous in plants and is often accumulated in large amounts. It is well established that in plant mitochondria at least two mitochondrial enzymes, the malate dehydrogenase (L-malate:NAD^+oxidoreductase EC 1.1.1.37) and the malic enzyme (L-malate:NAD^+oxidoreductase [decarboxylating] EC 1.1.1.39), contribute to L-malate oxidation. The malic enzyme provides a mean by which plants can oxidize tricarboxylic acid cycle intermediates without the supply of pyruvate from glycolysis (Palmer 1976). The presence of both enzymes in the matrix space causes oxaloacetate and pyruvate to accumulate in the medium during L-malate oxidation. The balance between both enzymes competing for L-malate oxidation is believed to be controlled by pH (McRae 1971). Since L-malate oxidation by means of both enzymes seems to perform an important role in plant metabolism (Wedding *et al.* 1976), we decided to investigate the effects of ferulic acid in mitochondria isolated from soybean seedlings. Our purpose was to obtain informations about a possible site of action of ferulic acid.

Materials and methods

Isolation of mitochondria: Mitochondria from soybean (*Glycine max* L. Merr. cv. Abyara) seedlings, grown for 3 d in the dark at 25 °C were prepared according to the procedure described by Day and Hanson (1977). The radicular hypocotyl axes were cut and subsequently homogenized with a cell desintegrator in ice-cold extraction medium consisting of 0.4 M mannitol, 10 mM Tris (pH 7.2), 1.0 mM EDTA, 0.5 % (m/m) fatty acid-free bovine serum albumin and 4.0 mM cysteine. The homogenate was filtered through several layers of cheesecloth. The pH of the filtrate was adjusted to 7.2 with KOH and centrifuged at 1 000 *g* for 5 min. The supernatant was centrifuged at 10 000 *g* for 10 min. The mitochondrial pellet was washed with medium containning 0.4 M mannitol, 1.0 mM EDTA and 10 mM Tris (pH 7.2) and centrifuged again for 10 min, firstly at 1 000 *g* and then at 10 000 *g*. The resulting pellet, containning intact mitochondria, was ressuspended in a small volume of washed medium. The whole procedure was performed at 0 - 4 °C.

Mitochondrial respiration: Oxygen uptake was measured at 25 °C using a Clark-type electrode positioned in a closed plexiglass chamber. The reaction medium contained

0.4 M mannitol, 10 mM KH_2PO_4, 10 mM KCl, 5.0 mM $MgCl_2$, 0.5 % (m/m) fatty acid-free bovine serum albumin, 10 mM Tris (pH 6.8 or 7.8) and known amounts of mitochondrial protein (0.2 - 1.0 mg protein per cm^3). L-malate and NAD^+ were added for final concentrations of 10 mM and 1.0 mM, respectively. Coupled respiration was initiated by adding ADP for a final concentration of 380 μM.

Protein content of the mitochondrial suspensions was determined using the method of Lowry *et al.* (1951). Bovine-serum albumin was used as a standard.

Assay of products of L-malate oxidation: Mitochondria were incubated at 25 °C in Enlermeyer flasks containning the same medium used for oxygen measurements at pH 6.8 or 7.8. 3.0 mM arsenite was included to inhibit pyruvate oxidase (Wedding *et al.* 1976). The reaction was initiated by the addition of 12 mM L-malate and stopped after 15 min by the addition of ice-cold 0.3 M $HClO_4$. After centrifugation at 4 000 *g* for 10 min, the supernatant was neutralized with K_2CO_3 . The neutralized extract was used for pyruvate and oxaloacetate determination as described by Wedding *et al.* (1976). Pyruvate was determined with L-lactate dehydrogenase by following the oxidation of NADH at 340 nm. Oxaloacetate was determined in a second aliquot of the extract after incubation with 10 mM $NiCl_2$ for 20 min at 45 °C. The pyruvate formed was then determined as described above. The amount of pyruvate found in the first aliquot was subtracted from that found after $NiCl_2$ treatment, giving an estimative of the oxaloacetate content.

Statistical analysis: Data in the graphs are presented as means of three or four experiments. The vertical bars represent standard errors of the mean. Statistical analysis was performed by means of the *Primer* program (version 1.0, McGraw-Hill 1988). Analysis of variance and Student's *t*-test were applied and the results are given in the text as probability values (*P*). $P < 0.05$ was adopted as a criterion of significance.

Chemicals: The L-lactate dehydrogenase, coenzymes (ADP, NAD^+, and NADH) and ferulic acid were purchased from *Sigma Chemical Co.* (St. Louis, USA.). The other reagent grade chemicals were from *Carlo Erba* (São Paulo, Brazil) and *Reagen* (Rio de Janeiro, Brazil).

Results and discussion

Effects of ferulic acid on oxygen uptake during L-malate oxidation: Ferulic acid affected the L-malate dependent respiration of soybean mitochondria (Fig. 1*A*,*B*). The rates of oxygen uptake significantly decreased with increasing concentrations of ferulic acid, both in the absence of ADP (basal respiration) and in the presence of ADP (coupled respiration), independently of the pH in the reaction medium. At pH 6.8 (Fig. 1*A*), the maximal inhibition (5.0 mM ferulic acid) was 52.2 % and 48.4 % for the coupled and basal respiration, respectively. At pH 7.8 (Fig. 1*B*), the corresponding values were 43.1 % and 35.2 %. Although the decreases in the rates of oxygen uptake at pH 6.8 tended to be more pronounced than those at 7.8, the differences were not statistically significant at the 5 % level.

Effects of ferulic acid on pyruvate and oxaloacetate production during L-malate oxidation: In the absence of ferulic acid and at pH 6.8 the rate of pyruvate production was higher than that of oxaloacetate (Fig. 2*A*). Conversely, at pH 7.8, the rate of pyruvate production was reduced and that of oxaloacetate was raised (Fig. 2*B*). These

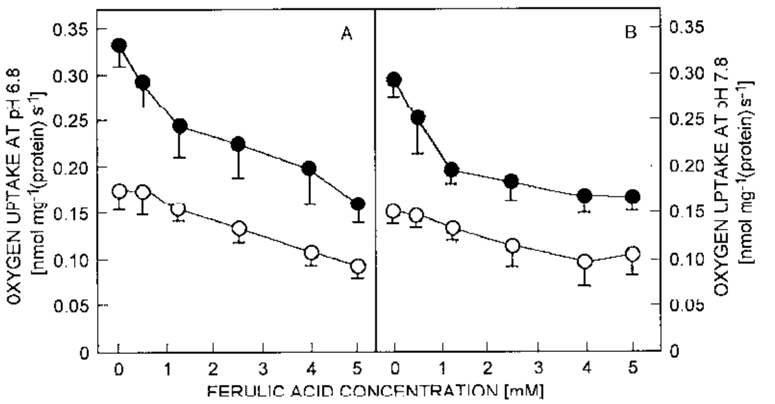

Fig. 1. Effects of ferulic acid on oxygen uptake during L-malate oxidation at pH 6.8 (*A*) and 7.8 (*B*). Mitochondria [0.2 - 1.0 mg(protein) cm^{-3}] were incubated at 25 °C in the reaction medium. *Open symbols* - the rates of oxygen consumption in the absence of ADP (basal respiration), *closed symbols* - rates in the presence of 380 μM ADP (coupled respiration).

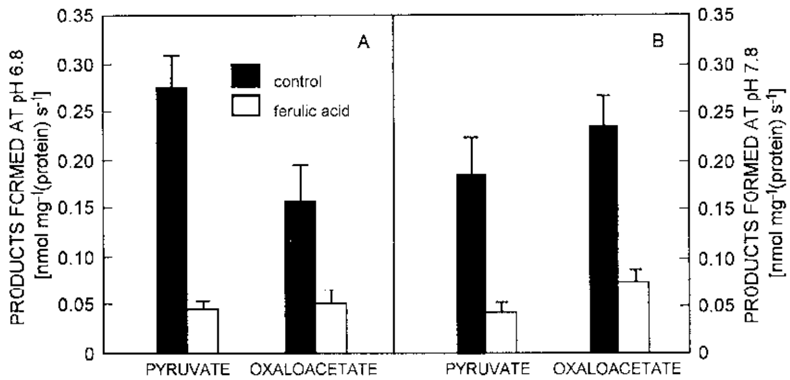

Fig. 2. Effects of ferulic acid on the production of pyruvate and oxaloacetate during L-malate oxidation at pH 6.8 (*A*) and 7.8 (*B*). Mitochondria [1.0 mg(protein) cm^{-3}] were incubated in reaction medium at 25 °C, during 15 min. The reaction was initiated by the addition of 12 mM L-malate. Pyruvate and oxaloacetate in the medium were measured enzymatically. *Solid bars* - the rates of oxaloacetate plus pyruvate production in the absence of ferulic acid, *open bars* - rates in the presence of 4.0 mM ferulic acid.

results demonstrate that in soybean mitochondria L-malate oxidation takes place through the action of both malate dehydrogenase and malic enzyme and that these two enzymes are operating at both acidic and alkaline pH. However, as expected, at acidic pH, there was a predominance of the malic enzyme activity and at alkaline pH, of the malate dehydrogenase activity. 4.0 mM ferulic acid inhibited the rates of pyruvate and oxaloacetate production, independently of the pH of the reaction medium (Figs. 2 *A*, *B*). At pH 6.8 and 7.8 the pyruvate production was reduced by 84 % and 77.9 % and the oxaloacetate production by 67.6 % and 67.2 %, respectively.

The results of the present work demonstrate that ferulic acid inhibits L-malate oxidation in soybean mitochondria. This action, however, occurred at fairly high concentrations. It is not very probable, thus, that the direct effect on L-malate oxidation contributes to the mechanism of action of ferulic acid as allelochemical agent, since there are reports of interferences with hormone actions (Ray *et al.* 1980), nutrient uptake and water potentials (Patterson 1981) in intact plants treated with micromolar levels of ferulic acid. On the other hand, in regard to its potential as natural growth inhibitor an unclear question is the concentration of ferulic acid in the intracellular compartments or organelles of the plant cells in which it is produced. Ferulic acid was detected in soybean seed leachates (Sathiyamoorthy 1990) and it is, thus, highly probable that it acts intracelularlly during germination. In this case, inhibition of L-malate oxidation could be contributing to affect seedling growth.

References

Bonner, J.: The role of toxic substances in the interaction of higher plants. - Bot. Rev. **16**: 51-65, 1950.

Day, D.A., Hanson, J.B.: On methods for the isolation of mitochondria from etiolated corn shoots. - Plant Sci. Lett. **11**: 99-1044, 1977.

Demos, E.K., Woolwine, M., Wilson, R.H., McMillan, C.: The effects of ten phenolic compounds on hypocotyl growth and mitochondrial metabolism of mung bean. - Amer. J. Bot. **62**: 97-102, 1975.

Einhellig, F.A.: Mechanism and mode of action of allelochemicals. - In: Putnam, A.R., Tang, C.S. (ed.): The Science of Allelopathy. Pp. 171-188. John Wiley & Sons, New York 1986.

Einhellig, F.A., Muth, M.S.: Ferulic acid interference with water metabolism in grain sorghum. - Proc. S. Dak. Acad. Sci. **5**: 276, 1980.

Glass, A.D.M.: Influence of phenolic acids on ion uptake. I. Inhibition of phosphate uptake. - Plant Physiol. **51**: 1037-1041, 1973.

Harper, J.R., Balke, N.E.: Characterization of the inhibition of K^+ absorption in oat roots by salicylic acid. - Plant Physiol. **68**: 1340-1353, 1981.

Kefeli, V.I., Kadyrov, C.S.: Natural growth inhibitors, their chemical and physiological properties. - Annu. Rev. Plant Physiol. **22**: 185-196, 1971.

Lowry, O.H., Rosebrough, N.J., Farr, A.C., Randal R.J.: Protein measurements with the Folin phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

MacRae, A.R.: Effect of pH on the oxidation of malate by isolated cauliflower bud mitochondria. - Phytochemistry **10**: 1453-1458, 1971.

Moreland, D.E., Novitsky, W.O.: Effects of phenolic acids, and flavonoides on isolated chloroplasts and mitochondria. - In: Waller, G.R. (ed.): Symposium on Allelochemicals: Role in Agriculture, Forestry, and Ecology. Pp. 247-261. American Chemical Society, Washington 1986.

Muller, C.H.: The role of chemical inhibition (allelopathy) in vegetal composition.- Bull. Torrey bot. Club **93**: 332-351, 1966.

Palmer, J.M.: The organization of electron transport in plant mitochondria.- Annu. Rev. Plant Physiol. **27**: 133-157, 1976.

Patterson, D.T.: Effects of allelopathic chemicals on growth and physiological responses of soybeans (*Glycine max*). - Weed Sci. **29**: 53-59, 1981.

Ray, S.D., Guruprasad, K.N., Laloraya, M.M.: Antagonistic action of phenolic compounds on abscisic acid-induced inhibition of hypocotyl growth. - J. exp. Bot. **31**: 1651, 1980.

Sathiyamoorthy, P.: Identification of vanillic and *p*-coumaric acid as endogenous inhibitors of soybean seeds and their inhibitory effect on germination. - Plant Physiol. **136**: 120-121, 1990.

Van Sumere, C.F., Cottenie, J., De Greef, J., Kint, J.: Biochemical studies in relation to the possible germination regulatory role of naturally occuring coumarin and phenolics. - Rec. Adv. Phytochem. **4**: 165-221, 1972.

Wang, T.S.C., Yeh, S.Y., Cheng, S.Y., Yang, T.K.: Behavior of soil phenolic acids. - In: U.S. Nat. Comm. IBP (ed.): Biochemical Interaction Among Plants. Pp. 113-120. National Academy of Science, Washington 1971.

Wedding, R.T., Black, M.K., Pap, D.: Malate dehydrogenase and NAD malic enzyme in the oxidation of malate by sweet potato mitochondria. - Plant Physiol. **58**: 740-743, 1976.

Whittaker, R.H., Feeny, P.P.: Allelochemicals: Chemical interactions between species. - Science **171**: 757-767, 1971.