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Role of malonate in chickpeas

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Abstract

Analysis of the content and distribution of organic acids in chickpea plants (*Cicer arietinum* L.) showed that malonate was the most abundant acid in roots and nodules, whereas malate was the main acid in leaves and stems. The highest concentration of malonate in roots was in the apices. Malonate metabolism did not appear to be directly related to abiotic stress. We suggest that malonate has a role as a defensive chemical in roots and nodules of chickpeas. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Cicer arietinum*; Leguminosae; Chickpea; Malonate; Malate; Organic acids; Defensive chemicals

1. Introduction

Malonate has long been known to be abundant in legumes, but the function of this toxic organic acid is still unclear. In some legume tissues, malonate accounts for as much as 4% of the dry weight and up to 50% of the total acidity (Bentley, 1952; Soldatenkov and Mazurova, 1957; Stumpf and Burris, 1979; Streeter, 1987). Malonate is also present in non-leguminous plants, but in general, is less abundant than in legumes (Bentley, 1952). Knowledge of the biosynthesis of malonate in plants is limited, although it has been suggested to be formed from malonyl-CoA, which is a biosynthetic precursor of fatty acids and other metabolites, such as flavonoids (Stumpf and Burris, 1981).

Dicarboxylic acids are considered to have a role in maintaining pH balance and as counter ions for the transport of cations in the xylem. However, on the basis of the distribution and fluxes of organic acids in legume tissues, malate but not malonate was concluded to have the major involvement in these functions (Stumpf and Burris, 1979; Israel and Jackson, 1982). Organic acids are also often present in root exudates that are produced in response to nutrient deficiencies or heavy metal stress. Considerable exudation has been noted to occur from chickpea roots, but the main acid component seems to be citrate (Marschner, 1995). The high concentration of malonate in root nodules of legumes has led to the suggestion that this acid has an essential role in nitrogen-fixing symbioses (Schramm, 1992; Kang and Kim, 1994). However, radiolabelling studies of organic acid pools in intact root nodules, studies on the utilisation of malonate, and metabolite uptake studies with intact symbiosomes tend to rule out malonate as a significant carbon source for bacteroid metabolism (Streeter, 1991, 1995; Udvardi and Day, 1997). In this paper, we describe a study of the temporal and spatial distributions of organic acids in chickpea plants. Further, since plants may accumulate a range of low molecular weight metabolites in response to stress, we have also investigated the influence of different kinds of abiotic stress on organic acid content. Our results are consistent with malonate having a role as a plant defensive chemical, but not as a chemical that accumulates as a primary response to stress.

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2. Results

Malonate was the most abundant organic acid in chickpea roots and nodules, whereas malate was the major acid in leaves and stems (Fig. 1). The malonate content in nodules and roots was greatest 3 weeks after planting and declined to close to zero in nodules of 8-week-old plants (Fig. 1). The malate content of nodules was low compared with malonate, whereas in roots, malate was present at high levels only in 1-week-old seedlings (Fig. 1). Malate levels in stems and leaves fluctuated, with peaks occurring in young plants and 5–6 weeks after planting (Fig. 1). Succinate and fumarate were detected at low concentrations in all the tissue extracts but the amounts did not exceed 1 μmol g^{-1} fresh weight (Fig. 1). Trace amounts of other acids were detected in the extracts by gas chromatographic analysis, but were not identified.

Analysis of 1-cm-long segments of roots of 10-day-old seedlings indicated that the maximum malonate content occurred in the apex (Fig. 2). The malonate content was 8 μmol g^{-1} fresh weight in the apical segment, declined to 2.5 μmol g^{-1} fresh weight in the fourth subapical segment and then increased to 4.5 μmol g^{-1} fresh weight in the fifth subapical segment (Fig. 2). Malate was distributed more evenly throughout the root than malonate, with the concentration ranging between 1.5 and 3.0 μmol g^{-1} fresh weight (Fig. 2). Succinate and fumarate were present at very low concentrations and did not change significantly along the length of the root (Fig. 2).

To further investigate the role of malonate in chickpea plants, studies were performed to determine how different types of stress affected the organic acid content of nodules and roots. When plants were subjected to drought stress by withholding water for 7 days before harvesting, nodule fresh weight declined by 50% and nitrogen fixation, as measured by acetylene-reducing activity (ARA), was almost completely lost (Fig. 3). Under these conditions, both the malonate and malate content per dry weight of the nodules decreased by approximately 40–50% compared with watered control plants (Fig. 3), although the acid content expressed on a fresh weight basis did not change significantly (results not shown). Reducing the carbon allocation to the nodules by partial defoliation of the plants caused both the malate and malonate content of the nodules to increase by 30–50% (Fig. 4). In contrast, the malonate and malate content of the nodules decreased by 60–70 and 30–40%, respectively, after the application of 10 mM KNO_3 or 150 mM NaCl to chickpea plants (Fig. 5). Partial defoliation and application of KNO_3 and NaCl both resulted in ARA being reduced by approximately 50% compared to untreated control plants, although nodule fresh weight was not significantly changed.

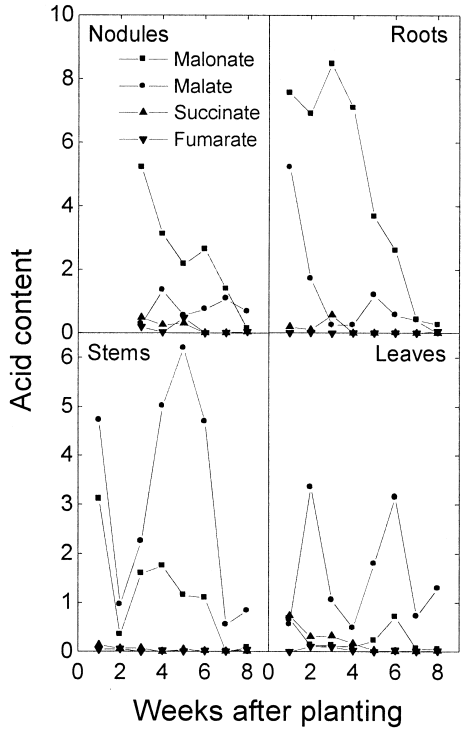

Fig. 1. Developmental changes in organic acids in nodules, roots, stems and leaves of chickpea plants. Each extract was prepared from at least five plants and acid content is in μmol g^{-1} fresh weight. The results are from one of the duplicate experiments.

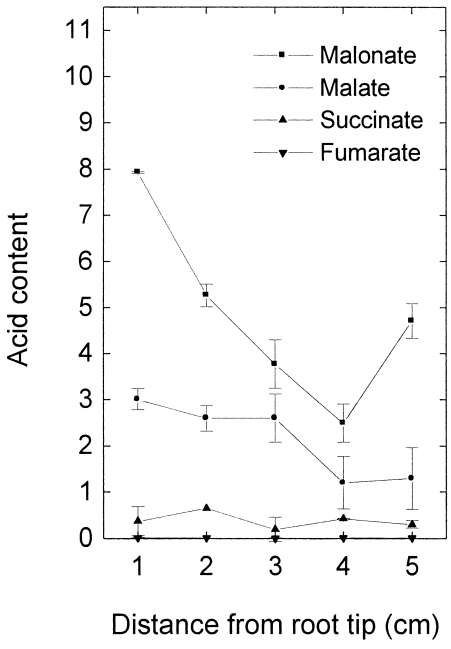

Fig. 2. Distribution of organic acids in roots of 10-day-old chickpea seedlings. Acid contents are in μmol g^{-1} fresh weight and are the means of two separate extracts, each prepared from 1-cm root segments from at least five plants. Error bars represent standard deviations.

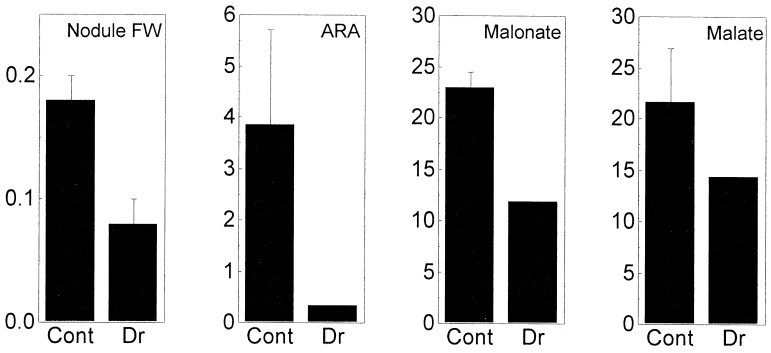

Fig. 3. Effect of drought stress on organic acid content of chickpea nodules. Water was withheld from stressed plants at day 21 after planting and the plants harvested at day 28. Nodule fresh weight (mg plant^{-1}), ARA (μmol ethylene g^{-1} fresh weight h^{-1}) and organic acid content (μmol g^{-1} fresh weight) are the means of two separate samples, each prepared from at least five control (Cont) and drought stressed (Dr) plants. Error bars represent standard deviations.

3. Discussion

The most abundant organic acids in chickpea tissues were malonate and malate, with lesser amounts of succinate and fumarate also present. Malonate and malate had different temporal and spatial distribution patterns in chickpea plants. The concentration of malonate was the highest in nodules and roots, whereas the malate content of nodules was low compared to leaves and stems. A similar distribution was found in previous studies with *Phaseolus vulgaris* and soybeans (Antoniw and Sprent, 1978; Stumpf and Burris, 1981; Werner et al., 1982; Kouchi and Nakaji, 1985; Kouchi et al., 1985; Kouchi and Yoneyama 1986). Malonate remained at elevated levels in nodules during the most active nitrogen-fxing period, whereas the concentration of malate in nodules increased as the nitrogen-fixing activity declines in 6–8-week-old plants (this study, Copeland et al., 1995). The relatively low concentration of malate in actively fixing nodules is consistent with this compound being turned over rapidly as major souce of carbon for the bacteroids (Day and Copeland, 1991; Streeter, 1995; Udvardi and Day, 1997).

Malonate is a strong competitive inhibitor of succinate dehydrogenase, which is a key enzyme of the tricarboxylic acid cycle and respiratory electron transport pathway. The K_i of succinate dehydrogenase for malonate is approximately 40 μM (Tipton, 1996). Assuming that the volume of 1 g of tissue is approximately 1 ml, and that malonate is distributed throughout the cell (as might occur when a cell is disrupted), the concentration of malonate in chickpea roots and nodules could range between 2 and 8 mM. In view of the lim-

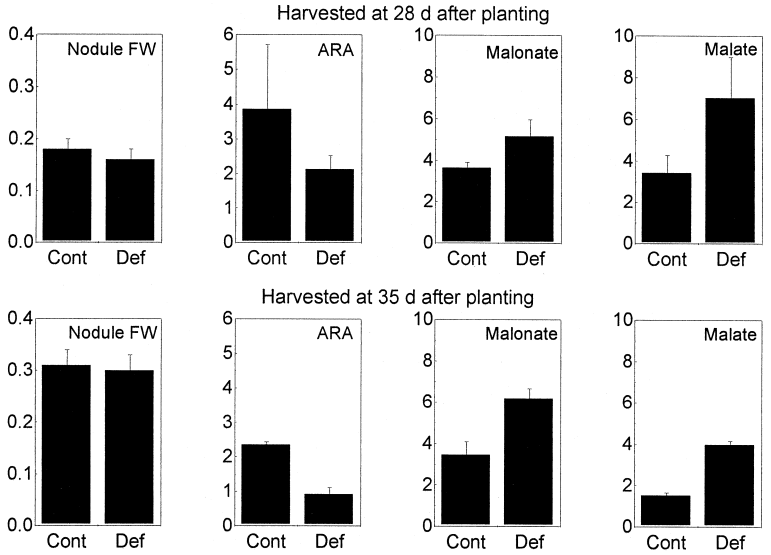

Fig. 4. Effect of partial defoliation of chickpea plants on organic acid content of nodules. Partially defoliated plants had 50% of their leaves removed at day 21 after planting and were harvested at days 28 and 35 after planting. Nodule fresh weight (mg plant^{-1}), ARA (μmol ethylene g^{-1} fresh weight h^{-1}) and organic acid content (μmol g^{-1} fresh weight) are the means from two separate samples, each prepared from at least five control (Cont) and defoliated (Def) plants. Error bars represent standard deviations.

ited availability of free oxygen in the nitrogen-fixing region of nodules, it seems curious that a respiratory poison, such as malonate, is present in such a large extent in this tissue. Presumably, malonate is compartmentalised in the abundant vacuoles in infected and uninfected cells of chickpea nodules (Lee and Copeland, 1994), but could be released when the tissue is disrupted. The toxicity of malonate, and its distribution mainly in the underground parts of the plant, suggests that this acid has a defensive role against soil pathogens and herbivory. The high concentration of malonate in root tips is consistent with such a role. The cell wall in the apex is likely to be less cross-linked and thinner than in other sections of roots, and hence, more vulnerable to attack by pathogens. A similar distribution was found for hydroxamic acids, which are suggested to have a protective role in young cereal roots (Wilkes et al., 1999). Malonyl-glycosides have been suggested to be storage forms of isoflavones involved in defensive responses against pathogens in several species of legumes, including chickpeas (Kessman et al., 1990; Mackenbrock and Barz, 1991; Park et al., 1995). However, the malonated isoflavones occur at 5- to 10-fold lower concentrations g^{-1} fresh weight than the malonate concentrations found in the present study.

Malate was the most abundant acid in stems and leaves of chickpea plants. Chickpea plants secrete large amounts of acid, primarily malate, through trichomes on leaves (Koundal and Sinha, 1983; Lazzaro and Thomson, 1995). The leaf exudate contains only a small amount of malonate (Rembold and Weigner, 1990).

Drought stress, partial defoliation and the application of salt and nitrate all severely inhibited nodule function, as seen from the effect of these stresses on ARA. However, stress-induced changes in malonate concentrations were similar to those for malate, suggesting that the responses observed were consequential rather than specific effects. Our results did not indicate that malonate accumulation or mobilisation was a primary response to abiotic stress. In conclusion, we suggest that malonate has a role as a defensive chemical in chickpea plants, to protect roots and nodules against soil pathogens and herbivory.

4. Experimental

4.1. Plant material

Chickpea (*Cicer arietinum* L. cv. Amethyst) seeds were purchased from a local market. Seedlings were grown from seeds that were soaked in 0.5% (w/v) sodium hypochlorite for 1 min, washed in running tap water for 15 min and germinated in the dark at 20–22°C in Petri dishes on filter paper moistened with deionised water. For the growth of nodulated plants, chickpea seeds were inoculated with a peat culture of *Mesorhizobium ciceri* CC1192 (BioCare, Gosford, NSW) prior to germination (Kim and Copeland, 1996). After 7–10 days, seedlings were transplanted into 15-cm pots containing a 3:1 mixture of sand and vermiculite (eight seedlings per pot). Plants were grown in a glasshouse with natural daylight supplemented to give a 15-h photoperiod and average day and night temperatures of 29 and 25°C, respectively. Each pot was supplied weekly with 200 ml of nitrogen-free nutrient solution (Evans et al., 1972) and tap water as required. At harvest, plants were rinsed thoroughly in running tap water, followed by deionised water, and blotted dry with absorbent paper.

4.2. Analysis of organic acids

Fresh tissues (0.5–1.5 g) were homogenised with a mortar and pestle in 10 ml of 95% (v/v) ethanol at 4°C. The homogenate was centrifuged at 15,000 *g* for

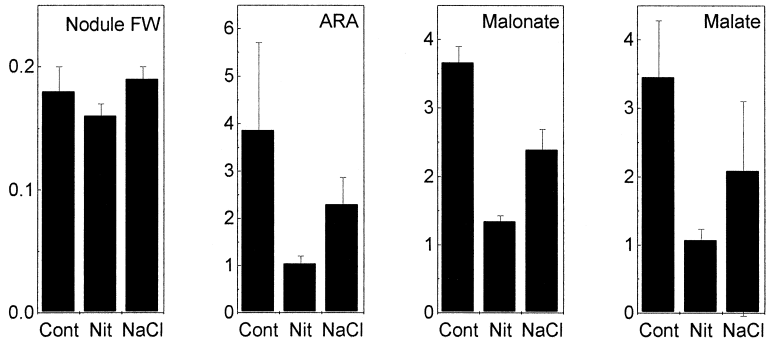

Fig. 5. Effect of nitrate and salt stress on organic acid content of chickpea nodules. Plants were supplied with nutrient solution or with nutrient solution containing 10 mM KNO_3 or 150 mM NaCl at day 21 after planting and harvested at day 28. Nodule fresh weight (mg $plant^{-1}$), ARA (μmol ethylene g^{-1} fresh weight h^{-1}) and organic acid content (μmol g^{-1} fresh weight) are the means from two separate samples, each prepared from at least five control (Cont), KNO_3-treated (Nit) and NaCl-treated plants. Error bars represent standard deviations.

10 min and the pellet was extracted again with 5 ml of 80% (v/v) ethanol. The combined ethanol extracts were dried under a nitrogen stream at 30°C. The residue was dissolved in 1.0 ml of deionised water and partitioned with 0.5 ml of chloroform to remove lipids. The acidic fraction from the aqueous phase was obtained by ion-exchange chromatography as described by Stumpf and Burris (1979). Organic acids were analysed by gas chromatography after derivatisation with *N*,*O*-bis(trimethylsiyl)acetamide (Sigma, MO, USA) according to the supplier's instruction. A 2.6-mm internal diameter and 2.7-m-long glass column packed with 5% SE-30 (Alltech Associates, NSW, Australia) was used and detection was by flame ionisation. The recovery of acids was 93–96%, as determined with homogenates to which known amounts of malonate, malate, succinate and fumarate were added.

4.3. Nitrogenase activity

Nitrogenase activity of nodules detached from root systems was estimated by acetylene reduction assay as described by Hardy et al. (1968).

Acknowledgements

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