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Responses of cucumber plant to NH_4^+ and NO_3^- nutrition: The relative addition rate technique vs. cultivation at constant nitrogen concentration

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ABSTRACT

Different N sources (NO_3^-, NH_4^+, or NH_4NO_3) at different relative addition rates (RAR) were supplied to cucumber (*Cucumis sativus* L.), a species sensitive to NH_4^+ toxicity. For comparison, cucumber plants were also grown at constant concentrations of 1 and 5 mM NH_4^+ or 5 mM NO_3^-. The fresh weight of NH_4^+-fed plants at RAR 0.15 and RAR 0.25 day^{-1} was similar to that of NO_3^--fed plants, while at RAR 0.35 or RAR 0.45 day^{-1} growth reduction occurred. When available as a constant concentration, NH_4^+ decreased plant growth at 5 mM. It is concluded that at low rates of N supply the relative addition rate technique can be used for growing cucumber plants with NH_4^+ as sole N source without deleterious effects.

1. Introduction

The form in which N is taken up by plants affects numerous physiological processes including N-assimilation, cation–anion balance, respiration (Matsumoto and Tamura, 1981; Escobar et al., 2006), water relations (Ragab, 1980), photosynthesis (Siddiqi et al., 2002), and secondary metabolism (Wang and Below, 1996). Nitrate is generally the preferred source for crop growth (Miller and Cramer, 2004), while ammonium can be deleterious to the growth of many plant species if absorbed as the sole N source (Lamb et al., 1993; Xu et al., 2001; Kotsiras et al., 2005).

Ammonium can be a desirable source of N under certain conditions in order to reduce N leaching and accumulation of NO_3^- in edible plant parts (Gangolli et al., 1994). Injection of NH_3 into soil has been used as a tool to provide crops with a supply of NH_4^+ instead of NO_3^- (known as the CULTAN cropping system: controlled uptake long-term ammonium nutrition). In this method, soil microbial-activity and, consequently, nitrification is inhibited in the injection zone due to the high pH. The plant roots form a dense root web around the ammonium hotspots in the soil and can take up the NH_4^+ before it is nitrified. Schumacher and Sommer (2001) reported that NH_4^+ application according to the CULTAN method increased potato yield 15–30% and the amount of N required to obtain optimum yields could be reduced by 30% compared to N fertilization by top dressing.

The response to ammonium nutrition varies between plant species and with environmental conditions. For example, species such as rice (*Oriza sativa* L.) and conifers prefer ammonium as the N-source (Britto and Kronzucker, 2002; Britto and Kronzucker, 2004), whereas cucumber and tomato are tolerant only to low ammonium concentrations. Plant age, temperature, light intensity, pH and the nutrient concentrations in the growth medium affect plant tolerance to NH_4^+ (Kotsiras et al., 2005). In normal conditions, cucumber growth decreased at 5 mM NH_4^+ compared to NO_3^- and 10 mM NH_4^+ was extremely toxic (Roosta and Schjoerring, 2007). Plants seem to be more tolerant to ammonium if the assimilation takes place mainly in the roots (Lasa et al., 2001). Ammonium detoxification in the roots is related to the availability of sufficient carbohydrate reserves which provide the necessary energy and carbohydrate skeletons for its assimilation (Claussen and Lenz, 1995; Finnemann and Schjoerring, 1999). Buffering of external pH, optimization of the light regime, application of ethylene biosynthesis inhibitors, co-provision of K^+ and nitrate can alleviate the NH_4^+ toxicity in the plants (Britto and Kronzucker, 2002).

The objective of the present study was to investigate if ammonium (NH_4^+) stress in cucumber can be alleviated by matching the N supply rate to plant N requirements. For thus purpose, the relative addition rate technique developed by Ingestad (1982) was used to supply N to the plants. In this technique, N is supplied in an exponentially increasing quantity per unit time, providing plants with a relative growth rate

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approximately equal to the relative addition rate (RAR) of N as long as this is below that required to maintain maximum relative growth rate. Employing a constant RAR during the cultivation period corresponds to supplying N in a constant ratio relative to the amount of N already present in the plants and provides a means to grow plants with steady internal N status.

2. Materials and methods

2.1. Plant material and culture conditions

The experiments were performed with cucumber (*Cucumis sativus* L., cv. Styx) plants. Seeds were germinated on moist filter paper in plastic boxes for 3 days at 20 °C. The seedlings were then transferred to black buckets containing 4 L of aerated nutrient solution. Four plants were growing together. The nutrient solution contained 0.2 mM KH_2PO_4, 0.2 mM K_2SO_4, 0.3 mM $MgSO_4{\cdot}7H_2O$, 0.1 mM NaCl. Micro-nutrients were 50 μM Fe(III)-EDTA-Na, 7 μM $MnSO_4{\cdot}H_2O$, 0.7 μM $ZnCl_2$, 0.8 μM $CuSO_4{\cdot}5H_2O$, 2 μM H_3BO_3, 0.8 μM $Na_2MoO_4{\cdot}2H_2O$. The activity was controlled at ca. pH 6–7 throughout the growth period by using $CaCO_3$ as a buffer. Nitrogen was added manually once per day or continuously supplied by peristaltic pumps as $Ca(NO_3)_2{\cdot}4H_2O$ or $(NH_4)_2SO_4$ at RAR 0.15, 0.25, 0.35, and 0.45 day^{-1}, or NH_4NO_3 at RAR 0.15 day^{-1}, using following equation:

$$N_t = N_0 \times e^{\mathrm{RAR}\times t} \quad (1)$$

where N_t and N_0 are nitrogen contents of the plants at times t and zero, respectively, and RAR is the relative rate of nitrogen addition. N_0 was estimated as 0.5 mg N $plant^{-1}$. RAR was held constant by daily additions of N calculated from $N_t - N_0$, starting after the transfer to N-free solution. The NH_4^+ concentration in the medium of plants growing at RAR ≤ 0.25 never exceeded 1 mM while at higher RAR 0.35 and 0.45 NH_4^+ accumulated in concentrations exceeding 10 and 100 mM, respectively. For comparison with the RAR approach, cucumber plants were also grown at constant concentrations of 1 or 5 mM NH_4^+ or 5 mM NO_3^-.

Solutions were changed completely every week in the first 2 weeks and subsequently every 4th day. The plants were grown in a greenhouse with 16 h light (25 °C) and 8 h darkness (21 °C). In order to keep the day light intensity above 400 μmol m^{-2} s^{-1} supplementary light was given by HQI lamps (Power Star 400 W, Osram, Munich, Germany). Plants were harvested at day 14 and at day 20 after transplanting. Plant organs (roots, leaves, and stem) were frozen quickly in liquid nitrogen and stored at −80 °C.

2.2. Plant growth

Growth was monitored as the fresh weight increment of plants from transplanting until first harvest and between the first and second harvests. The relative growth rate (RGR) was calculated on an exponential basis, using the equation:

$$\mathrm{RGR} = \frac{\ln W_2 - \ln W_1}{t}$$

where W_1 and W_2 represent the fresh weight at the beginning and end of the time interval t, respectively.

2.3. Ammonium, amino acids, total N, total C, and pigments

Organ tissue was extracted at a ratio 1:10 (w/v) with 10 mM formic acid on ice, centrifuged at 4 °C for 10 min at 21,000 × *g* and filtered (Polysulphone centrifugation filters, Micro VetraSpin; Whatman Ltd, Maidstone, UK) at 4 °C for 3 min at 4000 × *g*. Ammonium was analyzed flourometrically after ortho-phthalaldehyde (OPA)-derivatization with a columnless high-performance liquid chromatography (HPLC) system (Waters Corp., Milford, MA, USA equipped with a Nova-pak C18 analytical column 3.9 mm × 150 mm; particle size 4 μm). The analytical principle was based on detection of fluorescence upon reaction between the fluorochrome OPA and ammonium as described by Husted et al. (2000). Free amino acids were separated with a HPLC system (Waters corp., Milford, USA equipped with a Nova-pak C18 analytical column 3.9 mm × 150 mm; particle size 4 μm) after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate (Van Wandelen and Cohen, 1997).

Total N and C in organ tissue that was dried at 80 °C for 20 h was estimated by mass spectrometry with an elemental analyzer (20–20; Europa Scientific, Crewe, UK) according to the Dumas method (Schjoerring et al., 1993).

Chlorophyll *a*, chlorophyll *b* and caretonoids were extracted from leaf tissue with methanol and estimated according to Lichtenthaler and Wellburn (1983).

2.4. Activity of nitrate reductase and glutamine synthetase

Frozen organ tissue was pulverized in a mortar under liquid nitrogen. Nitrate reductase (NR, EC 1.6.6.1) was extracted with 100 mM HEPES (pH 7.5), 1 mM EDTA, 7 mM cystein, 3% polyvinyl polypyrrolidone (PVPP), 10 μM leupeptin and 1 mM phenyl methyl sulfonyl fluoride (PMSF). Extractions were filtered through miracloth (Calbiochem) into a falcon tube on ice and then reacted with 50 mM HEPES (pH 7.5), 100 μM NADH, 5 mM KNO_3 and 6 mM $MgCl_2$ at 30 °C for 10 min to obtain the actual NR activity. Reagents of 0.5% sulfanilamide and 0.01% N-(1-naphthyl)-ethylenediamine dihydrochloride in 1.5 M HCl were used for color development, and the amount of NO_2^- produced was determined spectrophotometrically at 540 nm and then nitrate reductase activity was calculated (unit μmol NO_2^- g^{-1} FW h^{-1}) (Mack and Schjoerring, 2002).

Glutamine synthetase (GS, EC 6.3.1.2) was extracted with triethanolamine (TEA) 100 mM, EDTA 1 mM, $MgSO_4{\cdot}7H_2O$ 10 mM, glutamate 5 mM, glycerol 10% v/v, triton X100 0.1% and dithiothreitol (DTT) 6 mM. After centrifugation (21,000 × *g*, 15 min, 4 C°) enzyme activity was assayed for 30 min at 30 °C in TEA 100 mM, glutamate 70 mM, hydroxylamine, HCl 6 mM, $MgSO_4{\cdot}7H_2O$ 20 mM, EDTA 4 mM, ATP 10 mM and terminated by addition of acidic Fe(III)Cl_3. The γ-glutamylhydroxamate produced was quantified spectrophotometrically (Finnemann and Schjoerring, 2000). Thus, both enzyme assays were for in vitro activities.

2.5. Mineral elements

Mineral elements were measured by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) after digestion of plants with HNO_3 in a Mod Block (Husted et al., 2004).

3. Results

3.1. Growth

The fresh weight of plants grown with NH_4^+ as the sole N source at RAR 0.15 and 0.25 day^{-1} was similar to that of NO_3^--fed plants, while plants grown at the very high NH_4^+ supply rate of 0.35 and 0.45 day^{-1} attained lower fresh weights compared to the NO_3^--grown plants (Fig. 1). When the two N forms were applied in a constant concentration of 5 mM, fresh weights were higher than those obtained in any of the RAR treatments with NH_4^+ still causing a considerable weight reduction compared to nitrate (Fig. 1).

The relative growth rate expressed on a fresh weight basis was in all N treatments somewhat lower than that expected theoretically according to the RAR of N. Plant growth responded to increasing N supply up to RAR 0.35 although maximum relative growth rate was about 0.25 day^{-1}. In the last part of the