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Short term physiological implications of NBPT application on the N metabolism of *Pisum sativum* and *Spinacea oleracea*

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ABSTRACT

The application of urease inhibitors in conjunction with urea fertilizers as a means of reducing N loss due to ammonia volatilization requires an in-depth study of the physiological effects of these inhibitors on plants. The aim of this study was to determine how the urease inhibitor N-(n-butyl) thiophosphoric triamide (NBPT) affects N metabolism in pea and spinach. Plants were cultivated in pure hydroponic culture with urea as the sole N source. After 2 weeks of growth for pea, and 3 weeks for spinach, half of the plants received NBPT in their nutrient solution. Urease activity, urea and ammonium content, free amino acid composition and soluble protein were determined in leaves and roots at days 0, 1, 2, 4, 7 and 9, and the NBPT content in these tissues was determined 48 h after inhibitor application. The results suggest that the effects of NBPT on spinach and pea urease activity differ, with pea being most affected by this treatment, and that the NBPT absorbed by the plant caused a clear inhibition of the urease activity in pea leaf and roots. The high urea concentration observed in leaves was associated with the development of necrotic leaf margins, and was further evidence of NBPT inhibition in these plants. A decrease in the ammonium content in roots, where N assimilation mainly takes place, was also observed. Consequently, total amino acid contents were drastically reduced upon NBPT treatment, indicating a strong alteration of the N metabolism. Furthermore, the amino acid profile showed that amidic amino acids were major components of the reduced pool of amino acids. In contrast, NBPT was absorbed to a much lesser degree by spinach plants than pea plants (35% less) and did not produce a clear inhibition of urease activity in this species.

Introduction

Urease, the only Ni-dependent metalloenzyme in eukaryotes, catalyzes the hydrolysis of urea to ammonium and carbon dioxide, thereby allowing these organisms to use external or internally generated urea as an N source (Andrews et al., 1984; Mobley and Hausinger, 1989; Mobley et al., 1995). In plants, urea is mainly derived from arginine (Polacco and Holland, 1993), although it can also be generated by ureide catabolism (Todd and Polacco, 2004; Muñoz et al., 2006). Plants are able to utilize urea applied to foliage (Leacox and Syvertsen, 1995) or they can take it up through the roots as a whole molecule, as demonstrated by hydroponic studies (Harper, 1984).

It is well known that the rapid hydrolysis of urea-based fertilizers by bacterial ureases in the soil results in substantial N loss due to ammonia volatilization. Indeed, it has been estimated that more than 50% of the N fertilizer applied is lost this way (Terman, 1979). One approach to improving the efficiency of urea application is to combine it with urease inhibitors, which delay the hydrolysis process and thereby extend urea availability by avoiding nitrate leaching and reducing NH_3 loss. Among the various types of urease inhibitors that have been identified and tested, N-(n-butyl) thiophosphoric triamide (NBPT) has proved to be significantly effective at relatively low concentrations under laboratory conditions (Gill et al., 1999).

NBPT shows similar solubility and diffusivity characteristics to urea (Carmona et al., 1990), and its application in conjunction with urea can affect plant urease activity and cause some leaf-tip scorch, although these effects are transient and short-lived (Watson and Miller, 1996). When urease activity is low due to inadequate Ni supply or urease inhibitor application, urea may accumulate to considerable levels, particularly in urea-treated plants (Gerendás and

Abbreviation: NBPT, N-(n-butyl) thiophosphoric triamide.

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Sattelmacher, 1997). This accumulation, as well as some physiological effects and the disruption of amino acid metabolism, has been described in wheat, soybean, sunflower, ryegrass and pecan (Watson and Miller, 1996; Gerendás and Sattelmacher, 1997; Bai et al., 2006). Previous studies from our group found inter-specific differences in ammonium sensitivity that seemed to be related to differences in the organ where ammonium is assimilated, as well as to the assimilation pathway (Lasa et al., 2002). In the current study, the short-term physiological implications of NBPT application for N metabolism in pea and spinach plants were investigated.

Materials and methods

Plant growth conditions and experimental set-up

Pea (*Pisum sativum* L., "Snap-pea") and spinach (*Spinacea oleracea* L., "Gigante de invierno") seeds were sown in vermiculite:perlite (2:1) and irrigated with distilled water. Pea seeds were previously surface sterilized as described by Labhilili et al. (1995). After 10 days, seedlings were transplanted to a continuously aerated hydroponic culture with eight seedlings/8-L tank. The nutrient solution used was that described by Rigaud and Puppo (1975) (N-free solution) supplemented with urea (5 mM for pea and 1.5 mM for spinach), as previous studies have shown that these concentrations are optimal for maximum growth of each species. The hydroponic solution was changed every 7 days during the first 2 weeks for pea and 3 weeks for spinach. After that time, treated plants were supplemented with NBPT at a final concentration of 100 μM. Urea isotopically labeled with ^{15}N (5%) was applied in the last solution change just before the application of NBPT. Hydroponically cultured plants were grown under controlled conditions at 22/18 °C (day/night), 60/80% relative humidity, 16/8 h photoperiod and 600 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux. Plant material from leaves and roots was collected at days 0, 1, 2, 4, 7 and 9 after treatment initiation, frozen in liquid N_2 and stored at −80 °C. Younger pea leaves in their early stages of development were also taken separately at day 9. Dry material was obtained by drying in an oven at 80 °C for 48 h.

NBPT determination

NBPT was analyzed by HPLC–ESI-MS. The instrument consisted of an Agilent series 1100 chromatograph system and an ion trap SL model spectrometer. Extraction was carried out from frozen tissues in distilled water and the supernatant obtained after centrifugation was used.

Separation was performed on an HPLC column (2.1 × 30 mm; 3.5 μm, Zorbax SB-C18) at 25 °C. The mobile phase was 40:60 distilled water + 0.1% formic acid:methanol + 0.1% formic acid (flow rate: 0.1 mL/min).

All analyses were performed using the ESI interface with the following settings: positive ionization mode; 40 psi of nebulizer pressure, nitrogen flow of 8 L/min and 350 °C. MS/MS spectra of ions were obtained by collision-induced dissociation in the ion trap with helium. Quantification was based on the 151 and 74 mass ions generated from the 168 ion precursor $[M+H]^+$.

Determination of urease activity

Urease was extracted from frozen plant material in 50 mM phosphate buffer (pH 7.5) containing 50 mM NaCl and 1 mM EDTA. In-Gel detection of urease activity was performed following the methodology described by Witte and Medina-Escobar (2001) using jack bean urease (Sigma EC 3.5.1.5) as standard.

Determination of urea content

The urea concentration was determined using the method described by Witte et al. (2002). To avoid interference from other molecules, such as ammonium and some amino acids, the extracts were previously passed through ion-exchange columns (sample extraction products; Water Oasis; MCX and MAX), with 900 μL of the reagent described by Kyllingsbæk (1975) being added to 300 μL of extract.

Quantification of ammonium and protein content

Ammonium was extracted from frozen tissue by treatment with water at 80 °C for 5 min followed by centrifugation. Determination was made by isocratic ion chromatography using a DX500 system (Dionex) with IonPack CG12A and CS12A columns and 20 mM methanesulfonic acid as eluent. The protein concentration in the extracts was quantified using a Bradford-type (1976) dye-binding microassay using a commercial Bio-Rad kit (Watford, UK) and bovine serum albumin as standard.

Determination of amino acid profile

Amino acids were separated and analyzed by capillary electrophoresis using a Beckman-Coulter PA-800 system with laser-induced fluorescence detection (argon ion: 488 nm; Takizawa and Nakamura, 1998; Arlt et al., 2001). Extraction was carried out in an aqueous solution containing 1 M HCl and the supernatant obtained after centrifugation used for analysis. Samples were derivatized with fluorescein isothiocyanate and the separation was performed in a 50 μm i.d. × 43/53.2 cm fused-silica capillary at a voltage of 30 kV and a temperature of 20 °C. The migration buffer was 80 mM borax (pH 9.2) containing 45 mM α-cyclodextrin. Sample injection was accomplished by a pressurized method (5 s).

Isotopic analysis and C–N determination

$\delta^{15}N$, % N and % C were determined for shoot and root samples (approx. 1 mg dry wt) by isotope ratio mass spectrometry under continuous flow conditions. Samples were weighed, sealed in tin capsules (5 × 8 mm, Lüdi AG) and loaded into the autosampler of an NC elemental analyzer NC 2500 (CE instruments, Milan, Italy). The capsule was dropped into the combustion tube (containing Cr_2O_3 and Co_3O_4Ag) at 1020 °C with a pulse of oxygen. The resulting oxidation products (CO_2, N_xO_y and H_2O) were swept into the reduction tube (Cu wire at 650 °C), where oxides of N were reduced to N_2 and excess oxygen was removed. A magnesium perchlorate trap removed the water. N_2 and CO_2 were separated on a GC column (Fused Silica, 0.32 mm × 0.45 mm × 27.5 m, Chrompak) at 32 °C and subsequently introduced into the mass spectrometer (TermoQuest Finnigan model Delta plus, Bremen, Germany) via a Finnigan Mat Conflo II. δ (‰) Values were calculated as follows:

$$\delta = \frac{Rsample - Rstd}{Rstd} \times 1000$$

where R is the $^{15}N/^{14}N$ ratio.

The results were mathematically transformed and presented in terms of $\%^{15}N$.

Statistical analysis

All data collected were analyzed statistically. Means were tested by applying the Student's *t*-test ($p \leq 0.05$; SPSS software, version 15), and significant differences between treatments (urea-fed plants vs. urea + NBPT-fed plants) are indicated by asterisks.