

Leaf Urea Metabolism in Potato. Urease Activity Profile and Patterns of Recovery and Distribution of ^{15}N after Foliar Urea Application in Wild-Type and Urease-Antisense Transgenics¹

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The influence of urease activity on N distribution and losses after foliar urea application was investigated using wild-type and transgenic potato (*Solanum tuberosum* cv Désirée) plants in which urease activity was down-regulated. A good correlation between urease activity and ^{15}N urea metabolism (NH_3 accumulation) was found. The general accumulation of ammonium in leaves treated with urea indicated that urease activity is not rate limiting, at least initially, for the assimilation of urea N by the plant. It is surprising that there was no effect of urease activity on either N losses or ^{15}N distribution in the plants after foliar urea application. Experiments with wild-type plants in the field using foliar-applied ^{15}N urea demonstrated an initial rapid export of N from urea-treated leaves to the tubers within 48 h, followed by a more gradual redistribution during the subsequent days. Only 10% to 18% of urea N applied was lost (presumably because of NH_3 volatilization) in contrast to far greater losses reported in several other studies. The pattern of urease activity in the canopy was investigated during plant development. The activity per unit protein increased up to 10-fold with leaf and plant age, suggesting a correlation with increased N recycling in senescing tissues. Whereas several reports have claimed that plant urease is inducible by urea, no evidence for urease induction could be found in potato.

Urea is the most frequently used N fertilizer globally. For example, in China and India urea accounted for 53% and 83%, respectively, of total N fertilizer consumption in 1998. Together, both countries consumed 41% of all N fertilizer used worldwide in that year (Food and Agriculture Organization of the United Nations, 1998).

The reaction catalyzed by urease is essential to make urea N accessible to plants (Gerendás et al., 1999). Urease activity has been detected in many plants (Frankenberger and Tabatabai, 1982; Hogan et al., 1983; Witte and Medina-Escobar, 2001) and is reported to be inducible by urea in rice (*Oryza sativa*; Matsumoto et al., 1966), jack bean (*Canavalia ensiformis*; Matsumoto et al., 1968), and barley (*Hordeum vulgare*), where in addition the formation of different urease isoforms was observed (Chen and Ching, 1988).

Urease catalyzes the hydrolysis of urea to carbamate and NH_3 . Carbamate is unstable and yields a second molecule of NH_3 and carbonic acid. The release of NH_3 during the urease reaction leads to a pH

rise because at neutral pH most NH_3 becomes protonated [$\text{NH}_3 + \text{H}^+ \leftrightarrow \text{NH}_4^+$]. Gaseous NH_3 may escape from the system (volatilize) especially when the chemical equilibrium is driven toward NH_3 at higher pH.

Urea can also be supplied to plants through the foliage, facilitating optimal N management, which minimizes N losses to the environment (Haverkort and MacKerron, 2000) without affecting yield (Giroux, 1984; Millard and Robinson, 1990). Most plants absorb foliar applied urea rapidly (Wittwer et al., 1963; Nicoulaud and Bloom, 1996) and hydrolyze the urea in the cytosol. The NH_3 released may be transported into the chloroplast and be assimilated by the chloroplastic Gln synthetase (Lam et al., 1996). Alternatively, NH_4^+ may be assimilated directly by the cytosolic Gln synthetase, which has been reported to be limited to the phloem parenchyma cells in leaves (Edwards et al., 1990).

NH_3 can also escape into the apoplast (Nielsen and Schjoerring, 1998). The apoplastic NH_4^+ concentration is an important determinant of NH_3 volatilization from plants (Schjoerring et al., 2000). It has been assumed that volatilization of NH_3 is a major cause of the often low recoveries of fertilizer N (about 30%–70%) after foliar urea application (e.g. Vasilas et al., 1980; Gooding and Davies, 1992). However, in some studies N losses were comparatively small (5%–15%; Morris and Weaver, 1983; Klein and Weinbaum, 1984; Smith et al., 1991).

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Given these contradicting reports, one aim of this study was to establish the fate of foliar-applied urea N (including losses) in potato (*Solanum tuberosum* cv Désirée) using a ^{15}N mass balance technique. Transgenic potato plants with down-regulated urease activity were used to assess the potential influence of urease activity on N distribution and recovery from applied urea. Furthermore, the leaf urease activity profile during plant development was elucidated providing clues about the physiological role of this enzyme. The possibility of urease induction by urea was also tested.

RESULTS

Leaf Urease Profile during Plant Development

One month after plant emergence, three potato plants were selected randomly from a group of 200 field-grown plants. On each plant, all leaves were tagged and numbered from top (=1) to bottom. Urease activity was measured on leaves from several nodes for each plant at four dates during the growing season (at approximately monthly intervals). The sampling procedure used (leaf discs) allowed urease measurements on the same leaves at various sampling dates. The results of enzyme activity and total protein measurements for one representative plant is shown in Figure 1 (note that at the first sampling date not all nodes were developed and at later dates some leaves had abscised). In general, urease activity per unit protein increased with leaf and plant age, whereas total protein levels declined proportionally. Thus, relative urease levels rose because of declining protein levels and probably not because of an induction. Expressing the activity on a fresh weight basis, which stays relatively constant per leaf disc during development, clarifies this point. For example, for node 4, urease activity per gram fresh weight was 254 milli-units, 288 milli-units, 283 milli-units, and 124 milli-units at successive sampling dates. The decline

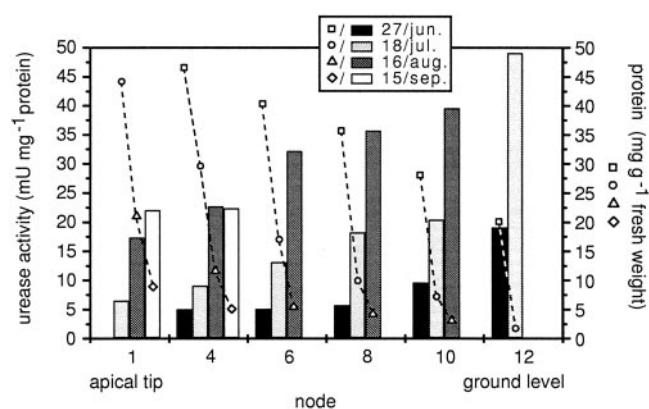


Figure 1. Leaf urease activity (histogram) and total protein (symbols) at different nodes of a field-grown potato plant during a growing season. The insert assigns the data labels to the different sampling dates.

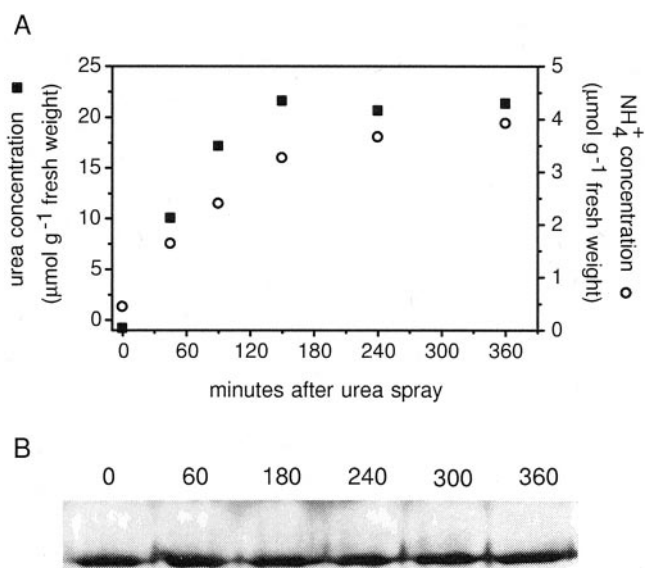


Figure 2. Urea, ammonium, and urease levels in leaves at different times after a 2% (w/v) foliar urea treatment. A, Urea (■) and ammonium (○) concentrations in the leaves. B, Native PAGE gel stained for urease extracted at different times after a 2% (w/v) urea application. Samples were loaded on a constant fresh weight basis.

between the two last dates shows that urease is eventually subject to net degradation in very senescent leaves, which can also be seen from the patterns of enzyme activity and protein concentration in Figure 1 (e.g. compare August 16 with September 15 for node 4).

Is Potato Urease Inducible by Urea?

A 2% (w/v) urea solution was applied to a group of field-grown plants as a foliar spray. Before spraying, and at intervals thereafter, several leaves (second–fourth node from the top) were removed from these plants at random for quantification of urea, ammonium, and urease activity (see “Materials and Methods”). Figure 2A shows that urea enters the leaf and is converted to ammonium. However, leaf urease activity remained constant over the 6-h period studied (at 236 ± 11 milli-units g^{-1} fresh weight) and no new isoforms of urease were detectable (Fig. 2B). This result contrasts with findings in barley (Chen and Ching, 1988). The lack of different isoforms is not surprising because urease is a single-copy gene in potato (C.-P. Witte, S.A. Tiller, M.A. Taylor, and H.V. Davies, unpublished data) and plant ureases are not known to undergo posttranslational modifications that could lead to modified electrophoretic mobility. Further tests of urease induction within 8 h after urea application gave similar results (data not shown). However, urea application led to an increase in leaf protein of $19.0\% \pm 3.3\%$ after 8 h (five replicate analyses) and in some cases, urease activity was also increased by approximately 20% if expressed on a

fresh weight basis, but not when expressed on a total protein basis. Thus, the synthesis of new protein because of the abundant availability of ammonium may lead to some parallel increases in urease synthesis. The protein concentrations were determined on gel-filtered extracts (Witte and Medina-Escobar, 2001) eliminating the possibility of interferences from low- M_r substances.

Urea Metabolism, N Redistribution, and N Losses

In 2 years (1999 and 2000), three plants of similar size were selected from a group of 200 field-grown plants. ^{15}N urea was applied at 9,000 μg per plant to a total of 10 leaves (see "Materials and Methods" for details). In 1999, plants were harvested after 36 to 48 h. In 2000, plants were presprayed with 1.8% (w/v) non-labeled urea 30 min before the application of the labeled urea, and were harvested after 8 d. The prespray was used to trace the metabolism of the ^{15}N substrate under conditions where endogenous urea and NH_3 pools were manipulated (i.e. to mimic the normal situation after applying urea through the foliage). At harvest, as much of the plant as possible was recovered and separated into several components: (a) the leaves to which the urea had been applied; (b) the remainder of those shoots (stem and leaves) to which the treated leaves were attached; (c) all other shoots; (d) roots, stolons, and underground shoots; (e) mother tuber; and (f) new tubers. The ^{15}N content of each plant component was determined by mass spectrometry (Fig. 3).

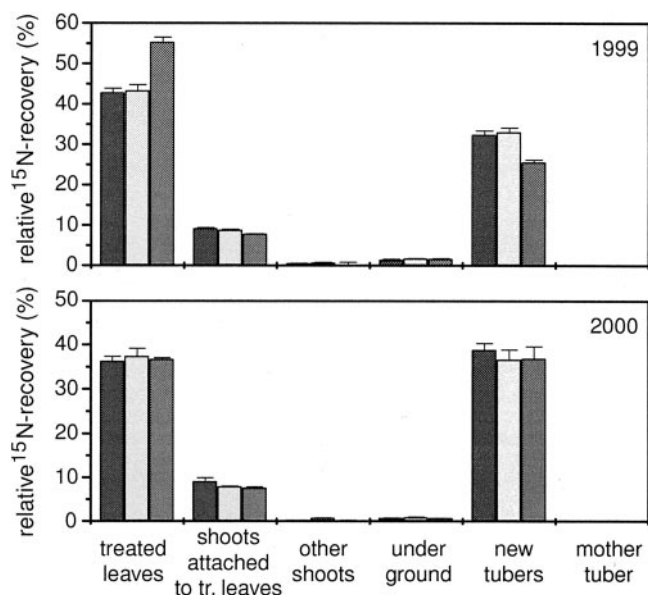


Figure 3. Urea ^{15}N recovery from field-grown plants. Plants in 1999 (upper) were harvested 36 to 48 h after urea application. In 2000 (lower), plants were harvested 8 d after. Results are shown for three replicate plants indicated by different column shading. Error bars indicate the confidence interval ($P = 95\%$) for the mass-spectrometric determination of ^{15}N .

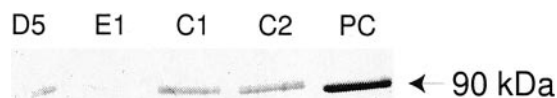


Figure 4. Western blot of protein extracts from leaves of urease antisense transgenics and controls. D5 and E1 are antisense lines. C1 and C2 are control lines, transformed with pBIN19 only. PC is a positive control containing purified jack bean urease (Boehringer).

The pattern of ^{15}N distribution between different plant parts was very similar in the 1999 and 2000 experiments. Most of the label was recovered from the treated leaves and new tubers. In 1999, the average recovery (percent of total supplied) from tubers was 30.3% (37.4% in 2000), whereas the average recovered from treated leaves was 47.1% in 1999 (36.8% in 2000). Thus, the export to tubers of N derived from leaf-applied urea was rapid within the first 48 h (1999), continuing at a slower rate subsequently (comparing 1999 with 2000, assuming that plants were physiologically similar in both years). Some label (8%–9% in both years) was recovered in the shoots to which the treated leaves were attached, indicating that some N was intercepted in shoots during the export of N from leaves to tubers. Recovery of labeled N was very low from all other plant parts.

The average overall ^{15}N recovery in the whole plant was 88.0% in 1999 (range: 86.1%–90.4%) and 83.5% in 2000, ranging from 82.0% to 85.0%. Thus, N losses (presumably by volatilization) did not exceed 18% in any case and were less in most cases.

Effect of Urease Activity on Urea N Distribution and Losses

Urease activity was down-regulated using an antisense construct, based on a partial cDNA of potato urease and driven by the constitutive 35S promoter. In 15 independent transgenic lines tested, two lines exhibiting significant down-regulation were identified. In one line (D5), activity was consistently down regulated by 40% to 50% compared with the wild type, whereas the other (E1) exhibited only 20% to 30% of wild-type activity. Both lines contained two inserts of the transgene as confirmed by Southern analysis (not shown). Urease mRNA was generally not detectable by northern-blot analysis in potato (not shown), but urease could be detected (close to the detection limit) in western blots. The western-blot results (Fig. 4) confirmed the down-regulation of urease in the transgenic lines D5 and E1. Down-regulated lines were phenotypically identical to wild-type plants.

Four plants of the most strongly affected line (E1; labeled A1–A4 for antisense 1–4) were compared with controls transformed with the empty vector only (labeled C1–C3 for control 1–3) and a non-transformed wild type plant (C4) in terms of urease

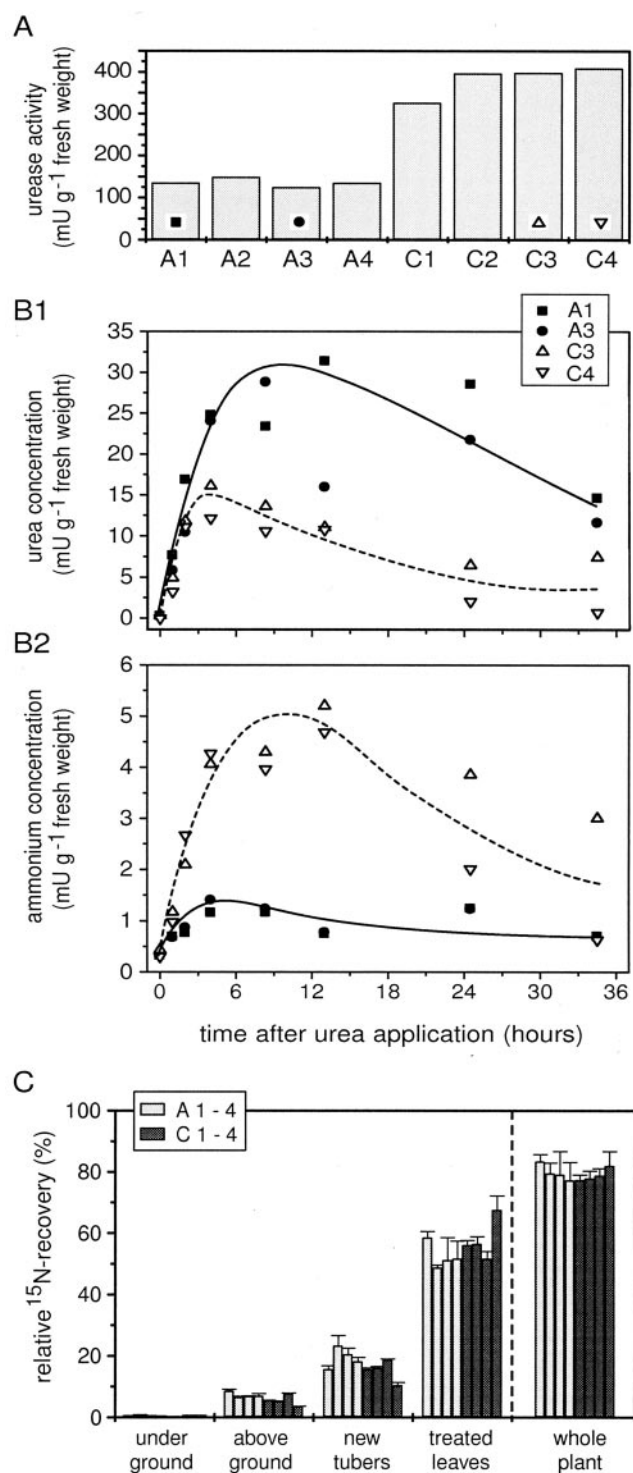


Figure 5. Urea/ammonium accumulation and N distribution/losses after urea application in control plants and transgenic plants with urease activity down-regulated. A, Urease activities in leaves of transgenic plants (A1–A4), vector-only control plants (C1–C3), and wild type (C4). B1, Urea concentration in leaves after urea application. B2, Ammonium concentration in leaves after urea application. C, ¹⁵N distribution and recovery 8 d after application of non-labeled urea followed by ¹⁵N urea. Percentages refer to total amount of ¹⁵N applied. The error bars indicate the confidence interval ($P = 95\%$) for the mass-spectrometric determination of ¹⁵N.

activity, urea and ammonium accumulation, ¹⁵N distribution, and recovery after urea application (Fig. 5).

Urease activity in leaves of the antisense plants, measured 1 d before urea application, was approximately 30% of the activity in controls (Fig. 5A). When urea (0.9%) was applied to these plants (using plants A1, A3, C3, and C4; Fig. 5B1), it accumulated to higher concentrations in the antisense plants probably because it was not as rapidly hydrolyzed by urease. However, urea degradation rates after the initial increase in urea concentrations are similar for both transgenic and non-transgenic plants. This could indicate that urea is later spatially separated from urease and that the release rate of urea from the place of storage and not the urease activity could be rate limiting for urea degradation. Ammonium concentrations in control plants were 4 to 5 times higher than in antisense plants between 4 and 12 h after urea application (Fig. 5B2). Thus, ammonium assimilation is at least initially slow compared with the rate of urea hydrolysis, leading to accumulation of ammonium that subsequently is only gradually removed.

Two days after the end of this time course experiment all eight plants were used in a ¹⁵N distribution/recovery experiment. Plants were pretreated with non-labeled urea (0.9%), left to dry, and 9,000 μg ¹⁵N urea was applied to 10 leaves per plant. After 8 d, the plants were harvested and separated into four components: (a) all underground parts including the mother tuber; (b) all green parts, except the treated leaves; (c) new tubers; and (d) treated leaves. ¹⁵N contents were then determined (Fig. 5C). The overall recoveries of ¹⁵N lay between 77.2% and 83.3% (average 79.3%), similar to the average recovery of 83.5% in the 8-d field experiment (2000). There was no significant difference in recoveries between urease antisense and control plants. Again, most of the label was recovered in the treated leaves (48.7%–67.4%; average 55.2%), a small percentage in other green plant parts (3.5%–8.5%; average 6.3%), and some label in new tubers (10.4%–23.2%, average 17.2%). Very little label was recovered in roots, stolons, mother tuber, and underground shoots. The extent to which ¹⁵N was transported from treated leaf to tubers varied slightly between different plants but was not correlated with urease activity.

DISCUSSION

Urease Activity and Plant Ontogeny

Urease appears to be a ubiquitous enzyme in plants. In potato, it is present in all organs tested (Witte et al., 2001). In planta, its substrate, urea, is generated during Arg breakdown (Gerendás et al., 1999) and in some plants also during purine/ureide breakdown (Vadez and Sinclair, 2000; Muñoz et al., 2001). Urea accumulation is increased in source tissues (Gerendás et al., 1999, and refs. therein), which remobilize N to sustain growth in developing and

growing metabolic sinks (Feller and Fischer, 1994). Urease activities per unit protein are far higher in old compared with young leaves (Fig. 1). Particularly high activity differences were also found between mother tubers (97.7 ± 11.8 milli-units mg^{-1} protein) and developing tubers (3.3 ± 0.8 milli-units mg^{-1} protein; Witte et al., 2001). These data demonstrate that potato urease levels are more stable than those of other proteins, supporting the idea of an important role for this enzyme in N recycling in tissues acting as an N source (Feller and Fischer, 1994).

Urease Induction

In potato leaves, urease activity is not induced by urea (Fig. 2). Similarly, studies by Gerendás and Sattelmacher (1999) showed that when root-applied urea and ammonium nitrate were used as N sources to support growth of oilseed rape (*Brassica napus*), plants exposed to either N source contained similar urease activities in leaves and roots. This was despite higher quantities of urea in organs of urea-fertilized plants. Polacco and Holland (1993) list several reports of plant urease induction in their review but propose that almost all cases may be explained by "induction of urease in commensal bacteria in the plant tissue or by proliferation of high-urease bacterial subpopulations." Therefore, it would be worth revisiting the evidence for inducible plant ureases and, in particular, the source of different inducible urease isoforms reported for barley leaves (Chen and Ching, 1988).

The accumulation of ammonium in leaf tissue after applying urea (Fig. 2A) indicates that the urease reaction is, at least initially, not rate limiting for the assimilation of exogenous urea N. Therefore, urease induction would seem "biologically unnecessary" because the leaf already contains excess amounts of this enzyme able to process far greater amounts of urea than the leaf would ever contain naturally.

Distribution of Urea-Derived N

Urea-derived N initially was distributed quickly from the treated leaves to other plant parts, mainly the tubers (Fig. 3, 1999). Between 2 and 8 d after the urea application, the urea N redistribution slowed down (comparison of 1999 with 2000 in Fig. 3, assuming that plants were physiologically similar in both years). Similar results have been obtained for hydroponically grown tomato (*Lycopersicon esculentum*) plants (Tan et al., 1999). After a rapid initial export of ^{15}N to other plant parts within 24 h after leaf application, further redistribution of label to metabolic sinks only occurred slowly. A gradual redistribution of urea-derived N from source to sink has also been observed, for example, in olive (*Olea europaea*; Klein and Weinbaum, 1984), maize (*Zea mays*; Below et al., 1985), and soybean (*Glycine max*;

Vasilas et al., 1980). The initial high mobility of the label within the plant could be because of movement of the urea molecule itself. For example, it has been shown in tomato that foliar-applied urea can be transported into the fruit (Shelp and Shattuck, 1986). Nicoulaud and Bloom (1996) observed an increase in shoot urea concentration after urea was supplied to leaves, again suggesting that urea may have entered the transport system. However, other N-rich metabolites such as Gln are the most common form of transported N (Pate, 1980), and are therefore likely to be involved in the rapid redistribution phase. If urea is transported to a significant extent, the experiments with urease antisense plants suggest that its concentration in the leaf is not rate limiting for the export (see below).

The protein content of potato leaves increased by around 20% within 8 h after urea application. Once the urea N has been incorporated into cellular components, e.g. nucleic acids and protein, it probably becomes relatively immobile. This would contribute to the explanation of slower rates of ^{15}N redistribution at later times. The immobilization of urea N in the treated leaves has also been observed in other studies. For example, 30% to 40% of the applied label remained in young cotton leaves after 1 week (Bondada et al., 1997), 60% was recovered from mature olive leaves after 9 d (Klein and Weinbaum, 1984), and about 30% of intercepted urea N was recoverable in tomato leaves 4 d after treatment (Tan et al., 1999).

The export of label from the leaves to the roots is usually very limited (Vasilas et al., 1980 [soybean]; Tan et al., 1999 [tomato]), and this is certainly true for potato (Fig. 3). This was not the case for young olive trees (Klein and Weinbaum, 1984), probably because their expanding root system represented a major sink. Thus, the extent to which N derived from urea metabolism is distributed into different organs appears to be regulated by their sink strength.

Recoveries of urea N in both experimental years were similar despite the fact that in 2000 plants remained in the field for 6 d longer than in 1999. This indicates that any major N losses are most likely to occur in the first 36 to 48 h after urea application. Maximum losses of N occur when considerable excess amounts of urea and ammonium are present in the leaves (Figs. 2A and 5B). We have observed that urea and ammonium levels in leaves of field-grown plants return to normal within 36 to 48 h after applying a 2% (w/v) urea solution (not shown). Nielsen and Schjoerring (1998) demonstrated that symplastic and apoplastic ammonium in leaves of oilseed rape are rapidly exchanged. Thus, while ^{15}N label is present abundantly as ammonium, it is particularly vulnerable to volatilization because it can easily reach the apoplast from where volatilization occurs. Once the label is incorporated into other biomolecules, losses are less likely.

In this study, the maximum possible (volatilization) losses from potato did not exceed 18% of the applied N. Low-N recoveries from urea sprays sometimes reported in the literature are probably because of additional factors. Urea might be lost because of runoff from the leaf or because it is not intercepted by the leaves in the first place. In the latter case, it might have either reached the ground directly or might have been taken away by wind drift.

Urea N Distribution and Recovery in Low-Urease Transgenics

The reduced urease activity in the transgenic plants (Fig. 5A) led to a greater persistence of urea in the leaves (Fig. 5B1) and a reduced accumulation of ammonium (Fig. 5B2) compared with controls. However, the distribution of ^{15}N label and losses of N were not significantly different between transgenics and controls (Fig. 5C). Because the kinetics of urea degradation did not influence the patterns of urea N distribution in our experiments, neither the amount of urea nor the amount of NH_3 present in the leaves could have been rate limiting for the export of urea-derived N from the leaves.

Assuming that increases in symplastic ammonium concentrations (in controls) influence apoplastic levels, greater volatilization losses might be expected in controls compared with low-urease transgenics. For example, increases in leaf ammonium content through reduced Gln synthetase activity in transgenic barley resulted in increased NH_3 emission (Mattsson et al., 1997). It is unclear why N losses after urea applications to potato leaves do not correlate with leaf ammonium concentrations. It may be that the effect of symplastic ammonium levels on apoplastic concentrations is small in potato or that the bulk of ammonium is compartmentalized in the vacuole as reported for maize roots (Lee and Ratcliffe, 1991). Alternatively, when losses are high, factors other than the (apoplastic) ammonium concentration may become rate limiting.

Results presented here show that the reduction of leaf urease activity does not influence N distribution and losses, at least within the activity range tested and with the application regimes explored. In preliminary experiments with leaf tissue from transgenic plants with urease activity overexpressed (more than 2-fold), there were no indications of increased N losses compared with controls when urea is supplied. Thus, losses appear to be similar whatever leaf urease activity prevails. Further experimentation is required to determine the impact of up- or down-regulating urease activity on N use efficiencies and crop growth and development in potatoes supplied with urea N through the foliage using commercial practices.

MATERIALS AND METHODS

Plant Material

Transgenic potato (*Solanum tuberosum* cv Désirée) plants (urease antisense plants and vector-only controls) were grown in compost (5-L pots) supplemented with 50 μmol nickel chloride 1 week after planting. Growing temperatures were 21°C (day) and 18°C (night), with a 14-h day-length. During the day, supplementary lighting ensured a minimum intensity of 250 W m^{-2} if natural daylight was insufficient (growing period was from August to October). For experiments on field-grown plants (non-transgenic), tubers were planted locally at 4 plants m^{-2} in soil fertilized before ridging with 147 kg ha^{-1} N.

Production of Transgenic Plants

An 843-bp fragment of potato urease (C.-P. Witte, S.A. Tiller, M.A. Taylor, and H.V. Davies, unpublished data) was amplified by reverse transcriptase-PCR from total leaf RNA using primers ATATTGTTTCCCTTTGTAAGGC-AGG and TTCTACAGATCACCTCTCCAATTCC (Super-script II from Life Technologies/Gibco-BRL [Cleveland] for reverse transcriptase step, 35 PCR cycles, 62°C annealing temperature) and cloned into pGemT Easy (Promega, Madison, WI). The fragment was excised from pGemT Easy with *EcoRI* and cloned into the *EcoRI* site of pJIT60 in antisense orientation respective to a promoter with two 35S enhancers, present in this plasmid. The expression cassette of pJIT60 with the cloned urease fragment was excised with *KpnI* and *XhoI* and introduced into pBIN19 (digested with *KpnI* and *Sall*). *Agrobacterium tumefaciens* (strain LBA4404) was transformed with the resulting binary vector construct and a control vector (pBIN19). Plant transformation and tissue culture were performed as described in Kumar et al., 1996. Putative transgenic lines were tested by Southern-blot analysis and the degree of urease down-regulation was evaluated by leaf activity measurements (Witte and Medina-Escobar, 2001) and confirmed by western-blot analysis.

Western-Blot Analysis

Proteins were extracted from leaves according to Witte and Medina-Escobar (2001). Extracts were heated for 5 min to 60°C, which precipitates several proteins producing background staining but does not affect urease activity. Protein (30 μg) from the supernatants was separated by SDS-PAGE and blotted. Commercial polyclonal anti-jack bean (*Canavalia ensiformis*) seed urease antibodies (Europa Bioproducts Ltd., Cambridge, UK) were used for urease detection.

Urea Application to Leaves

Leaves of field-grown plants were sprayed on their adaxial surface with a hand-held sprayer (Hozelock, model 4075) until they were completely wetted. Spray treatments of field plants were routinely performed with a 1.8%

or 2.0% (w/v) urea solution (300 or 330 mM urea). Greenhouse-grown plants were more susceptible to leaf damage caused by high urea concentrations; thus, a 0.9% (w/v) solution was used. Sprays contained 0.05% (w/v) Agral (Zeneca, London) as a wetting agent.

Urea and NH_4^+ Extraction and Quantification

Several leaves were removed from the treated plants, washed briefly in excess water, superficially dried, and ground in liquid N to a fine powder. The gradual rise of urea concentrations (Figs. 2A and 5B1) shows that the washing procedure was efficient in removing most surface attached urea (otherwise highest values would have been obtained directly after urea application). Approximately 0.1 g of frozen leaf material was placed in a 2-mL microfuge tube and 1.0 mL of water was added. The tube was immediately frozen and stored at -70°C until further processing. For extraction, tube cap holders were put in place and the tubes transferred to a boiling water bath for 3 min followed by placement on ice. Freezing and boiling inactivates urease. Each sample was vortexed for 30 s, centrifuged (15,000g, 1.5 min), and the supernatant removed into a fresh tube. The pellet was re-extracted twice by adding 0.5 mL of water, vortexing, centrifuging, and pooling the supernatant with the previously obtained supernatant.

For urea measurements, 20 μL of aqueous extract was mixed with 180 μL of distilled water in a microfuge tube. Then, 600 μL of color reagent (Kyllingsbæk, 1975) was added and the tube incubated for 30 min at 80°C . The samples were cooled in a water bath at room temperature and the A_{527} determined with a Cecil CE 7200 spectrophotometer (Cecil Instruments Ltd., Cambridge, UK). Standards were prepared by mixing 200 μL of 18.8, 37.5, 75, and 150 μM urea solutions with 600 μL of color reagent and assaying as described above.

Phenol hypochloride reagent was used for the determination of ammonium (Witte and Medina-Escobar, 2001). To eliminate interferences, ammonium was bound to a strong cation exchange column, eluted, and measured. SCX Bond Elut columns (50 mg, Varian, Palo Alto, CA) were wetted with 1.0 mL of methanol. Two microliters of 4 M NaCl was passed through to elute ammonium contaminating the columns, followed by 4 \times 1.0-mL volumes of water. Extract (0.3–0.5 mL) made up to 1.0 mL with distilled water was applied, the column washed with 1.0 mL of water, and the bound ammonium eluted with 0.5 mL 4 M KCl into microfuge tubes. Distilled water (0.5 mL) was added, followed by 100- μL prepared phenol and a 200- μL hypochloride reagent. Samples were placed for a minimum of 15 min in a water bath at 50°C and the absorbance determined at 636 nm.

Determination of Urease Activity and Total Protein

Urease in-gel detection and activity measurements were performed as described by Witte and Medina-Escobar (2001), using a correction factor of 1.2 for the calculation of activities per unit fresh weight. Seven leaf discs ($d = 8$ mm) were taken from different leaflets of each leaf per sample.

Total protein was determined using a commercial Bradford assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard.

^{15}N Distribution and Recovery Experiments

The five main shoots of a plant at the flowering stage were selected and the two leaves at the second and third node under the apical shoot tip were tagged. Very senescent leaves and flowers were removed to ensure that no plant material was lost during the experiment. A 300 mM urea solution was prepared with 98+ % (w/v) ^{15}N -enriched urea (Cambridge Isotope Laboratories, Andover, MA) containing 0.05% (w/v) Agral (Zeneca) as a wetting agent. The urea content of the applied solution was confirmed chemically as described above. In the late afternoon, 100 μL of this solution was applied with a micropipette to the adaxial surface of each of the 10 tagged leaves on a selected plant. Special care was taken to spread the solution on each leaf with the pipette tip to avoid any runoff. Assuming a ^{15}N enrichment of 100% and using a molecular mass of 62 g mol^{-1} for ^{15}N urea in calculations, this treatment applied 9,000 μg ^{15}N per plant. This mass balance approach is probably more exact than attempting to measure the intercepted ^{15}N amount directly after application because losses of the liquid adhering to the leaf surface would be difficult to avoid at harvest and during processing for mass spectrometry.

In the field, treatments were carried out in both years during a period of warm and dry weather at the end of July near Dundee, UK.

At harvest, complete plants were recovered and separated into different parts as indicated in individual experiments. Each component part was freeze dried, weighed, and ground to a fine powder in a hammer mill. A subsample was milled further in a laboratory ball mill to obtain very fine debris. One to 5 μg of samples (in three–five replicates) was used for ^{15}N determinations using a Europa Roboprep coupled to a Europa Tracermass mass spectrometer. ^{15}N contents were calculated using the formula: ^{15}N content = dry weight \times (atom% N/100) \times (atom% ^{15}N excess/100).

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