

# Urea in Plants: Metabolic Aspects and Ecological Implications



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**Abstract** Urea is widely used as a crop fertilizer because of its low cost. However, how this organic compound is absorbed and assimilated by plants is still poorly characterized. This chapter gives a general overview of how plants deal with urea and use it to support growth, covering the high- and low-affinity transport systems, which accounts for external urea uptake, intracellular remobilization and transport, as well as urea assimilation through the action of plant ureases. In addition, the ecological relevance of urea is discussed through some examples of organisms that live in habitats in which urea may be a preferential source of nitrogen. We believe that it is very important to study organisms that have a high potential for efficiently

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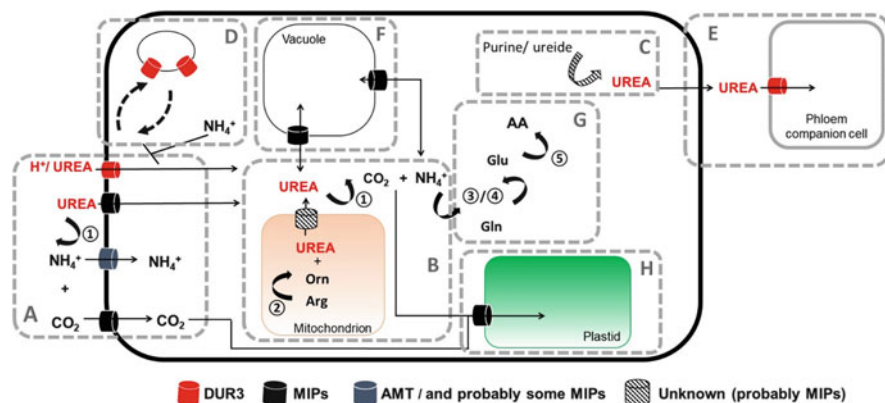
metabolizing urea in order to apply this knowledge to improve the nitrogen use efficiency (NUE) in plants, which is the topic of the last section of this chapter.

## Abbreviations

AA	Amino acids
AMT	Ammonium transporter
AQP	Aquaporins
ASN	Asparagine synthetase
DUR3	High-affinity urea transporter
GIPs	GlpF-like intrinsic proteins
GOGAT	Glutamate synthase
GS	Glutamine synthetase
HIPs	Hybrid intrinsic proteins
MIPs	Major intrinsic proteins
NIPs	Nodulin-26-like intrinsic proteins
NUE	Nitrogen use efficiency
PEPC	Phosphoenolpyruvate carboxylase
PIPs	Plasma membrane intrinsic proteins
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SIPs	Small basic intrinsic proteins
TIPs	Tonoplast intrinsic proteins
XIPs	X intrinsic proteins

## 1 Introduction

Urea is a small, neutral organic compound formed by two ammonia ( $\text{NH}_3$ ) and one carbon dioxide ( $\text{CO}_2$ ) molecules. In the environment, urea can originate as a degradation product from organic matter or from the urine of animals, such as mammals, as a key detoxification product of nitrogenous compounds (Wang et al. 2008). Additionally, the low cost of industrial manufacture and the high nitrogen content of urea (46% N of total urea molecular mass) make this organic compound a frequent fertilizer used in arable soils (Kojima et al. 2006; Pinton et al. 2016). As a result, the addition of nitrogen to the soil has increased through the application of urea fertilizers, which is one of the main strategies for improving crop production. In the coming years, urea demand is expected to increase at an average annual rate of 3.2% (<http://www.ceresana.com/en/market-studies/chemicals/urea/>). Unfortunately, more than half of the nitrogen applied in agriculture is not absorbed by plants, resulting in the polluting of water bodies and the atmosphere by ammonia and nitrogen oxides. The significant losses of urea applied as fertilizer in certain soils are due to the high urease activity of the microorganisms (Dawar et al. 2011; Krajewska 2009; Zanin et al. 2016) and/or the secretion of ureases by plants to the



**Fig. 1** Processes of urea absorption and metabolism in plant cells. **(a)** Extracellular urea can be absorbed in its intact form either through the low-affinity system (mediated by MIPs) or through proton symport (mediated by DUR3). Alternatively, extracellular urease (①) may generate  $\text{NH}_4^+$ , which can be absorbed mainly by AMT transporters and  $\text{CO}_2$  that may diffuse into the plastids through MIPs. **(b)** Intracellular urea can also be originated from arginine catabolism in the mitochondria by arginase (②) or **(c)** through ureide degradation (see text for details). **(d)** DUR3 can be recycled into the endomembrane system, a process that seems to be related to intracellular  $\text{NH}_4^+$  concentrations. **(e)** Urea may be exported from source tissues, mainly senescent leaves through phloem to other organs, possibly entering in the vascular system through DUR3. **(f)** Intracellular urea can be stored inside the vacuole through TIPs to avoid toxicity and be remobilized whenever needed. **(g)** Upon urease action, the resulting  $\text{NH}_4^+$  is assimilated by GS and GOGAT (③ and ④, respectively), forming glutamate, and through transaminases (⑤), originating the other amino acids (AA), while **(h)** the resulting  $\text{CO}_2$  may diffuse

external environment (Inselsbacher et al. 2007; Cambuí et al. 2009), which quickly causes the conversion of urea to ammonia (Fig. 1a), a form of nitrogen that is normally lost through volatilization.

In addition to external sources of urea (such as fertilizers, degradation of organic matter, and animal excreta), inside plant cells, urea is an important metabolite generated mainly by the catabolism of the amino acid arginine through the enzyme arginase (Fig. 1b) (Polacco et al. 2013; Winter et al. 2015). Arginine has the highest nitrogen-to-carbon ratio (4:6), which makes it an outstanding compound during protein mobilization for seed germination since it is an important constituent of storage proteins (Zonia et al. 1995). In source tissues, such as senescent leaves and seeds during germination, urea can accumulate; the nitrogen from this organic source is, subsequently, remobilized through urease activity to support the growth of the plant (Polacco and Holland 1993; Zonia et al. 1995; Bohner et al. 2015). In addition to arginine degradation, other endogenous sources of urea in plant cells, such as the degradation of ureides (which are intermediates in purine degradation pathways), are still controversial since it seems that this process does not necessarily lead to the generation of urea in all plants (Fig. 1c) (Werner et al. 2010; Werner and Witte 2011).

Despite the importance of urea as a metabolite in plant cells and its extensive use as a nitrogen-releasing fertilizer in arable soils, little attention has been given to the study of its absorption and assimilation compared to its inorganic counterparts,

ammonium and nitrate. Moreover, few studies have examined the ecological relevance of urea as a nitrogen and carbon source for plants.

This review highlights the main metabolic aspects of urea regarding its absorption and assimilation as well as the ecological relevance and potential use of urea transporters in plants that are naturally exposed to this organic source as a strategy to improve nitrogen efficiency in crops.

## 2 General Features of Urea Absorption and Assimilation in Plants

Urea is a widespread molecule in nature with different physiological roles. In mammals, urea is a waste product from protein catabolism, but at the same time, its physiological role as osmolyte is essential for water reabsorption in the kidneys (Bagnasco 2005). Moreover, for enteric pathogenic bacteria, urea can serve as a buffer to survive the acidic conditions of the stomach (Weeks et al. 2000). Also, urea is a nitrogen source for bacteria, algae, fungi, and plants (McCarthy 1972; Yamanaka 1999; Endres and Mercier 2001; Valladares et al. 2002). Despite the many physiological roles of urea in different organisms, its importance as a nutrient in plants has been little investigated, in part due to the misconception about whether plants were capable of absorbing this organic nitrogen source directly. Since most arable soils possess relatively high urea-hydrolyzing activity from microorganisms, it has been suggested that the nitrogen derived from urea enters the root cells mainly in the form of ammonium (Watson et al. 1994; Marschner 1995). Therefore, it had long been thought that plants were able to uptake only inorganic sources, such as ammonium and nitrate; as a consequence, most of the studies of nitrogen plant nutrition focused precisely on these forms (Paungfoo-Lonhienne et al. 2012). Nowadays, more attention has been given to the importance of organic nitrogen sources, such as amino acids, peptides, and urea, in plant nutrition (Lipson and Näsholm 2001; Persson and Näsholm 2001; Jones et al. 2005; Mérigout et al. 2008; Ganeteg et al. 2017), and physiological evidence has demonstrated that urea is absorbed by plants in its intact form (Syrett and Bekheet 1977; Wilson and Walker 1988; Krogmeier et al. 1989; Liu et al. 2003a, b; Mérigout et al. 2008). Furthermore, urea can be translocated from the roots to other parts of the plant, as evidenced by significant amounts of this compound in the shoots or xylem sap of plants treated with urea (Hine and Sprent 1988; Gerendás et al. 1998; Mérigout et al. 2008). Moreover, urea can be transported to sink organs after foliar fertilization (Witte et al. 2002), indicating that intact urea is highly mobile throughout the vascular system of the plant.

It has been around 53 years since it was first suggested that protein transporters facilitated the transmembrane passage of urea in biological systems (Hunter et al. 1965; Wieth et al. 1974), as opposed to the low diffusion rates observed in artificial lipid bilayers (Galluci et al. 1971). Later on, it was shown that facilitated and active transporters in both eukaryotes and prokaryotes played a role in urea transport (ElBerry et al. 1993; Sands et al. 1997; Weeks et al. 2000; Liu et al. 2003a, b). In

plants, one of the first pieces of evidence that corroborated the involvement of urea transporters came through the analysis of  $^{14}\text{C}$ -labeled urea absorption by the alga *Chara australis* R. Brown, in which electrophysiological measurements and concentration-dependent uptake analysis demonstrated the existence of two urea transport systems (Wilson and Walker 1988; Wilson et al. 1988). The first one is a high-affinity system characterized by the transportation of urea against its concentration gradient, which makes it the main route of urea entry at extracellular concentrations  $<0.25$  mM. As a result, this active transport is sensitive to energy metabolism inhibitors since it uses the electrochemical gradient across membranes to energize urea transport (Liu et al. 2003a). On the other hand, low-affinity systems are the predominant mode of urea entry at extracellular concentrations above 0.5–1 mM and do not require a proton-dependent mechanism to transport urea (ElBerry et al. 1993; Liu et al. 2003b). In higher plants, it was only possible to identify and functionally characterize low- and high-affinity urea transporters at the molecular level in the late 1990s and early 2000s (Gerbeau et al. 1999; Eckert et al. 1999; Liu et al. 2003a, b).

Urea transporters are present in almost all organisms ranging from bacteria and animals to fungi and plants but are encoded by different gene classes. For example, the urea transporter (UT) family present in animals, the  $\text{H}^+$ -activated urea channel (UreI), and the urea transporter gene (Yut) present in bacteria, such as *Helicobacter pylori* Marshall & Warren and *Yersinia pseudotuberculosis* Pfeiffer, respectively, encode for low-affinity urea channels that appear to be absent in plant genomes. In higher plants, orthologs of the urea active transporter DUR3 of *Saccharomyces cerevisiae* Meyen ex E.C. Hansen and members of the aquaporin family have been identified as high- and low-affinity urea transporters, respectively (Gerbeau et al. 1999; Eckert et al. 1999; Gaspar et al. 2003; Liu et al. 2003a, b; Soto et al. 2008; Gu et al. 2012; Azad et al. 2012). These findings have brought new insights into the physiological and molecular basis of urea absorption in plants.

## 2.1 *DUR3: High-Affinity Absorption Transporter of Urea*

High-affinity urea transporters have been physiologically described in bacteria, fungi, algae, and plants (Cooper and Sumrada 1975; Syrett and Bekheet 1977; Pateman et al. 1982; Valladares et al. 2002; Liu et al. 2003a; Beckers et al. 2004). In plants, they have been identified as orthologs of ScDUR3 of *S. cerevisiae*, a member of the sodium-solute symporter (SSS) family responsible for transporting several solutes, including urea. However, to date, this kind of transporters in plants has been functionally characterized at the molecular level only in *Arabidopsis*, maize, and rice (Liu et al. 2003a, 2015; Kojima et al. 2007; Wang et al. 2012; Zanin et al. 2014).

In yeast, ElBerry et al. (1993) showed that the uptake of  $^{14}\text{C}$ -labeled urea was reestablished in a urea uptake-defective yeast mutant (*dur3*) through its complementation with the functional *ScDUR3* gene. A decade later, Liu et al. (2003a) showed that AtDUR3 from *Arabidopsis* complemented the growth of a yeast *dur3* deletion

mutant in urea concentrations below 5 mM as a sole nitrogen source. Additionally, in order to characterize the urea transport mechanism, AtDUR3 was heterologously expressed in *Xenopus laevis* Daudin oocytes (a biological system that expresses no endogenous urea transporters), and it was demonstrated that AtDUR3 mediated the active transport of urea in a proton- and concentration-dependent way. By using similar approaches, OsDUR3 and ZmDUR3 were described as high-affinity transporters responsible for absorbing urea at low concentrations in rice and maize plants, respectively (Wang et al. 2012; Zanin et al. 2014; Liu et al. 2015). In addition, the DUR3 protein family in plants appears to consist of only one member since in both rice and maize genomes *OsDUR3* and *ZmDUR3* are the only genes that show significant homology to *AtDUR3* (Wang et al. 2012; Zanin et al. 2014).

Regarding the physiological role of DUR3, both OsDUR3 and ZmDUR3 were independently capable of complementing a T-DNA insertion line of *Arabidopsis* that could not grow at concentrations below 1 mM of urea as a sole nitrogen source, confirming the key role of DUR3 in active urea acquisition (Zanin et al. 2014; Liu et al. 2015). *Arabidopsis DUR3* encodes a putative hydrophobic protein with 14 transmembrane-spanning domains (while in maize, DUR3 is predicted to have 15 transmembrane domains), and its expression is linked to the N status since it was observed that under N deficiency DUR3 is upregulated in plant root cells (Liu et al. 2003a, 2015; Kojima et al. 2007; Zanin et al. 2014; Bohner et al. 2015). Moreover, it appears that the upregulation of *DUR3* expression under N starvation is more likely due to the derepression from catabolic products of urea, such as ammonium (Fig. 1d) (Kojima et al. 2006, 2007). In addition, after supplying urea at low concentrations to N-deficient plants of rice and *Arabidopsis*, an upregulation of *DUR3* expression levels was observed in roots in a short time span, indicating that DUR3 expression is stimulated by its own substrate and reflecting its involvement in urea acquisition from the external medium (Kojima et al. 2007; Mérigout et al. 2008; Wang et al. 2012). However, in maize plants a downregulation of DUR3 expression together with an increase in urea absorption by the roots was observed (Zanin et al. 2014, 2015a; Liu et al. 2015). These results suggest that after urea is supplied, DUR3 might be regulated by other mechanisms rather than exclusively by transcriptional control. In agreement with this, some researchers have suggested that DUR3 proteins are under control of a trafficking/recycling pathway since this transporter appears to be localized in the plasma membrane (Kojima et al. 2007; Liu et al. 2015) as well as in the endomembranes (Zanin et al. 2014), indicating that the cellular localization can regulate its activity as a urea transporter (Fig. 1d).

Although it has been observed that in rice and *Arabidopsis* DUR3 increases its expression in roots after a short time of urea resupply, it is difficult to know the internal metabolic status of the plant and, therefore, to understand which elements are involved with the regulation of DUR3 activity. Moreover, DUR3 transcript levels seem to be organ/tissue specific (differing in roots, shoots, and leaves) and dependent on the ontogenetic stage of the plant (Liu et al. 2015; Bohner et al. 2015). During leaf senescence, N is remobilized to younger parts of the plant to allow growth. Along this process, urea leaks to the apoplast, while DUR3 appears to be responsible for retrieving or taking up urea from the apoplast of senescent leaves,

contributing to the translocation of N (Fig. 1e). In older/senescent leaves, both the increase in *DUR3* transcript levels in phloem cells and urea concentrations in the apoplast when *DUR3* function is lost show that *DUR3* plays an important role in endogenous urea remobilization and vascular loading during leaf senescence (Bohner et al. 2015; Liu et al. 2015).

Thus far, there is little doubt that *DUR3* represents the major active urea transporter in plants. However, the present view was restricted to the findings related only to three model plants: *Arabidopsis*, rice, and maize. Despite the obvious importance of *DUR3* as a strategy to use available diluted urea sources from soils and translocate endogenous urea during senescence, little is known about its physiological relevance in other plant systems, as well as the key elements responsible for its regulation since the studies carried out in other plants have only predicted putative *DUR3* orthologs by bioinformatics.

## 2.2 *Aquaporins as Low-Affinity Urea Transporters*

In addition to active urea transport mediated by *DUR3*, facilitated diffusion of urea can be mediated by aquaporins, whose structure and transport mechanisms are completely different. Aquaporins are channels of the major intrinsic protein family (MIPs) that exhibit specificity for small, low-weight molecules, such as urea. Since water and several neutral solutes are capable of crossing the lipid bilayer of membranes, it was initially thought that no transporters were needed to facilitate their diffusion. Later on, it was shown that aquaporins were responsible for increasing the passive flow rate of water and some small solutes through the membranes in biological systems (Maurel et al. 1993; Chrispeels and Maurel 1994; Biela et al. 1999). MIPs are present in archaea, bacteria, protozoa, yeasts, plants, and animals. In plants, based on sequence homology and subcellular localization, the aquaporins were classified into seven subfamilies (PIPs, TIPs, NIPs, SIPs, XIPs, HIPs, and GIPs) (Wang et al. 2016), with the PIPs and TIPs being the most abundant subgroups and generally localized in the plasma and vacuolar membrane, respectively (for further information about structure and classification of plant aquaporins, see Luang and Hrmova 2017).

Some of the first aquaporins identified in plants were described as facilitators of the transport of small molecules in the peribacteroid membrane of nitrogen-fixing symbiotic nodules in soybean (Sandal and Marcker 1988) and as water stress-induced proteins (Guerrero et al. 1990), and only later were they functionally characterized as water-permeable channels. In light of this finding, aquaporins have been associated with various physiological processes in plants, such as cell growth, stomatal movement, germination, and abiotic stress responses, among others (Chaumont and Tyerman 2014; Li et al. 2014; Reddy et al. 2015). However, interactions between aquaporins and plant nutrition remain poorly understood. PIPs, NIPs (present in the plasma membrane), and TIPs have been shown to play an important role in the uptake and mobilization of nitrogen in the plant (Gerbeau

et al. 1999; Liu et al. 2003b; Loqué et al. 2005; Hwang et al. 2010, Bárzana et al. 2014). Accordingly, the expression of some aquaporin genes varies according to the availability of nitrogen in the environment. For example, AtTIP2;1 of *Arabidopsis* can be induced either by the presence of ammonium or the lack of nitrogen in the environment (Loqué et al. 2005), and it has been shown to be permeable to urea (Liu et al. 2003b). In tomato, analyses carried out in a root cDNA library showed the induction of several aquaporin genes by nitrate (Wang et al. 2001), with the *ZmPIP1;5b* gene of maize also induced by this nitrogen source (Gaspar et al. 2003). To date, not a single aquaporin has been functionally characterized as a nitrate transporter; however, its induction in response to this nitrogen source may be related to the increase of hydraulic conductivity in roots (Gorska et al. 2008; Li et al. 2016).

On the other hand, the permeability of MIPs to urea and ammonia has already been demonstrated. As a result of its neutrality and low molecular weight, urea can easily be transported by aquaporins. Through stopped-flow spectrofluorimetry of purified vacuolar membrane vesicles and heterologous expression in *Xenopus* oocytes, it was possible to functionally characterize the first urea-permeable aquaporins in plants: NtTIPa (TIP) (Gerbeau et al. 1999) and NtAQP1 (PIP) of tobacco (Eckert et al. 1999). Since then, some NIPs (in *Arabidopsis*, maize, tobacco, zucchini, and cucumber), PIPs (in maize), and TIPs (in *Arabidopsis* and maize) have been shown to be permeable to urea, suggesting that the potential urea transport mediated by aquaporins may occur through the plasma membrane as well as across the vacuolar membrane in plants (Fig. 1a, f) (Gerbeau et al. 1999; Eckert et al. 1999; Gaspar et al. 2003; Kleb et al. 2003; Liu et al. 2003b; Soto et al. 2008; Gu et al. 2012; Yang et al. 2015; Zhang et al. 2016). The TIP aquaporin subfamily constitutes approximately 40% of the total vacuolar membrane protein (Maeshima 2001) and may be involved with the remobilization of N into and out of the vacuole in order to avoid toxicity and maintain nitrogen balance in the cytosol (Fig. 1f) (Liu et al. 2003b; Jahn et al. 2004; Loqué et al. 2005).

It is worth pointing out that arginine hydrolysis, which takes place at the mitochondria, is an important source of endogenous urea in plant cells. However, N release from urea occurs mainly in the cytosol via urease, indicating the essential requirement of a mitochondrial transporter for urea (Polacco and Holland 1993; Goldraij and Polacco 2000). Although several TIPs have already been characterized in tonoplast as urea carriers, others, such as AtTIP5;1, have been found in the mitochondria, likely facilitating the passage of urea into the cytosol (Fig. 1b) (Soto et al. 2008, 2010), which makes TIPs important elements in urea metabolic pathways. Interestingly, AtTIP1;3, which also facilitates urea diffusion, has been found in the plasma membrane of seed embryos (Gattolin et al. 2011) and in endomembranes of pollen tubes of *Arabidopsis* (Soto et al. 2010), while AtTIP2;1 has been found in tonoplasts and apparently in chloroplast membranes, as well (Liu et al. 2003b; Loqué et al. 2005; Ferro et al. 2003, 2010). Taken together, these findings highlight both the existence of different trafficking mechanisms for MIPs according to organ type and ontogenetic stages (since it is possible to find the same aquaporin subfamily in different types of membranes) and the relevance of MIPs in physiological processes, such as nitrogen mobilization.



Despite the high number of genes identified as encoders of MIP family members (between 30 and 71 depending on the species) (Chaumont et al. 2001; Park et al. 2012), channel selectivity and exact membrane location are difficult to predict based only on its protein sequence, which makes experimental confirmation necessary. Nevertheless, the NPA and ar/R selection filters (located in the aquaporin pore) have been widely discussed in the literature as key points in their selectivity (Froger et al. 1998; Fu et al. 2000; Sui et al. 2001; Savage et al. 2010; Hove and Bhawe 2011) but not the only control points. Channel diameter, gating control by pH, binding of divalent cations (probably  $\text{Ca}^{2+}$ ), phosphorylation, and the length and movement of the cytoplasmic MIP loops are important points in the selectivity and activity of aquaporins (Daniels et al. 1999; Kukulski et al. 2005; Törnroth-Horsefield et al. 2006; Frick et al. 2013). Likewise, homo- or heterotetramerization are key regulator mechanisms of aquaporin activity since, in part, they control the targeting of MIPs to specific plant membranes. Related to this, it has been observed that heterotetramerization of two different PIP subclasses (PIP1s and PIP2s) had a synergistic effect on water transport compared to the PIP homotetramerization (Fetter et al. 2004; Zelazny et al. 2007; Besserer et al. 2012). In a similar way, the establishment of aquaporin heterotetramers might regulate the trafficking of urea aquaporin transporters to the plasma membranes and, subsequently, control the entry of urea into the cells.

In spite of the large amount of data about the molecular components regulating plant aquaporins, it remains unclear how the urea transport through MIPs is regulated and what its physiological significance in plants is.

### 2.3 Urease as a Key Enzyme in Urea Assimilation

Two different types of enzymes are capable of degrading urea: urea amidolyase and urease. Urease is a metalloenzyme that uses nickel (Ni) ions at its active site, which is key for its activity (Dixon et al. 1975). This enzyme is found in plants, fungi, and bacteria (Mobley and Hausinger 1989; Witte and Medina-Escobar 2001) and catalyzes the hydrolysis of urea to generate ammonia and carbamate, which at physiological pH is spontaneously hydrolyzed to form  $\text{CO}_2$  and a second ammonium molecule (Mobley et al. 1995).

It is estimated that spontaneous degradation of urea has a half-life of 520 years to generate ammonia and carbamate, while the urease-catalyzed half-life of urea is 20 ms (Callahan et al. 2005). Thus, urease is one of the fastest known hydrolases. In 1926, *Canavalia ensiformis* (L.) D.C. (jack bean) urease was the first enzyme to be crystallized (Sumner 1926); however, it was not possible to determine its structure until 83 years later (Balasubramanian and Ponnuraj 2010). To date, this is the only urease whose structure has been determined in plants. Plant ureases consist of a dimer of homotrimers in which each active site has two nickel ions, totaling six active sites per active protein (Dixon et al. 1975; Balasubramanian and Ponnuraj 2010; Zambelli et al. 2011).

With the exception of *Utricularia gibba* L., a carnivorous plant with no identified ureases in its genome (Ibarra-Laclette et al. 2013), in several sequenced plant genomes, urease appears to be encoded by a single gene (Witte et al. 2005a; Ligabue-Braun et al. 2013). However, in soybean three urease isoforms have been described: the embryo-specific (Eu1), expressed highly in embryos (Polacco and Holland 1993); the ubiquitous (Eu4), expressed in all organs of the plant (Polacco et al. 1985); and the SBU-III (Eu5), expressed in the first moments of root development (Polacco et al. 2013; Wiebke-Strohm et al. 2016). It should be noted that, despite the high activity of the embryo-specific urease in soybean, this activity does not seem to be related to its ureolytic function (Stebbins et al. 1991). Instead, the involvement of this enzyme has been suggested in defense mechanisms. Nowadays, it is known that in addition to its ureolytic function, soybean and jack bean ureases have insecticidal and fungicidal properties (Follmer et al. 2004; Becker-Ritt et al. 2007; Carlini and Ligabue-Braun 2016). Despite the great similarity of plant ureases to those of bacteria, only those of plants have insecticidal properties, and, even more importantly, this characteristic is independent of its ureolytic capacity (Follmer et al. 2004; Menegassi et al. 2017; Kappaun et al. 2018).

Although the first urease was discovered in plants, its ureolytic activation process is still poorly characterized. To date, bacterial ureases (such as those from *Helicobacter pylori* Marshal & Warren) have received more attention since they are related to human and animal diseases and are better characterized than plant ureases (Farrugia et al. 2013; Burne and Chen 2000). However, due to the great similarity of the protein sequences of plant and bacterial ureases, it is believed that the urease activation and catalytic mechanisms are highly conserved (Carlini and Polacco 2008; Follmer 2008).

In *Arabidopsis*, urease activation is mediated by the accessory proteins UreD, UreF, and UreG, which form an activation complex that interacts with the inactive urease (apo-urease) (Witte et al. 2005b; Myrach et al. 2017). It is believed that UreD binds to the apo-urease and serves as a scaffold for interaction with UreF, which in turn interacts with UreG (Witte 2011), which is responsible for delivering nickel ions to the metallocenter to create functional active sites (Witte and Medina-Escobar 2001; Witte et al. 2005b; Cao et al. 2010; Myrach et al. 2017). UreG-deficient soybean mutant plants showed no ureolytic activity in either its ubiquitous isoform (present in all tissues) or its embryo-specific isoform, demonstrating that the action of UreG is vital for the activation of ureases (Freyermuth et al. 2000). Once active, urease is highly stable and insensitive to the loss of nickel ions at its active site (Park and Hausinger 1993; Balasubramanian and Ponnuraj 2010). However, some specific residues in its structure (such as Cys592) are involved in many reactions that cause its inhibition (Pearson et al. 1997; Kot and Zaborska 2006; Balasubramanian and Ponnuraj 2010).

Moreover, although the formation of the apo-urease is not compromised by the lack of nickel ions, the activity of the holo-urease (active urease) decreases as the nickel concentration reduces (Winkler et al. 1983); thus, the activation of the urease is dependent on the availability of nickel (Dixon et al. 1975; Polacco 1977; Myrach et al. 2017). The discovery of Ni as an essential component in plant urease activity has brought new interest in Ni nutrition and its role in urea metabolism in plants.

Consistent with this, it has been reported that when plants are grown under Ni-depleted conditions and supplied with urea, large amounts of urea accumulate in the leaves causing leaf tip necrosis due to urea toxicity (Shimada and Ando 1980; Eskew et al. 1983; Walker et al. 1985), failures in the production of viable grains (Brown et al. 1987), and reduced grain yield (Freitas et al. 2018). Interestingly, under natural conditions and due to a relatively constitutive activity and expression of urease in almost all plant cells (Polacco and Holland 1993; Witte 2011), large amounts of urea in the cytoplasm should be unexpected. However, under conditions in which urease is inactive due to the action of an inhibitor, nickel deficiency, or mutations in the accessory protein-encoding genes (such as *UreG*), significant urea content was observed (Krogmeier et al. 1989; Stebbins et al. 1991; Polacco and Holland 1993; Gerendás and Sattelmacher 1997; Gerendás et al. 1998; Zanin et al. 2016), indicating that transient compartmentalization of urea into the vacuole through TIPs (as mentioned above – Sect. 2.2) could be an important process to detoxify urea excesses in the cytoplasm.

Since urease is localized mainly in the cytoplasm, vacuolar storage of urea keeps this nitrogen source out of its hydrolysis and assimilation pathway (Witte et al. 2002; Cao et al. 2010). Therefore, urease activity is essential to make N available for plant metabolism during urea absorption as well as being a key component in developmental processes, such as germination (when a large flux of nitrogen from arginine pools is remobilized through arginase and urease activity), pollen tube growth, and senescence (when N remobilization is required) (Zonia et al. 1995; Witte et al. 2002; Soto et al. 2010; Polacco et al. 2013; Rechenmacher et al. 2017).

When urea is supplied, the *UreG*-encoding gene appears to be upregulated (Mérigout et al. 2008). However, due to the constitutive expression and high activity of urease, it has been suggested that this enzyme is not a limiting step in N-derived urea assimilation or remobilization (Cao et al. 2010; Wang et al. 2012, 2013) and an upregulation of either its activity or gene expression is not required. Indeed, in several studies in which potato and *Arabidopsis* plants were supplied with urea, no increases in either activity or expression of urease were observed (Witte et al. 2002; Mérigout et al. 2008; Cao et al. 2010), suggesting that urease is not inducible by its substrate. Nevertheless, in other studies significant increases in urease activity by urea have been reported (Matsumoto et al. 1966, 1968; Chen and Ching 1988; Lakkineni et al. 1995; Takahashi and Mercier 2011; Zanin et al. 2016; Matiz et al. 2017).

Taken together, these findings make it difficult to define whether or not urease is inducible by urea and raise other questions about the involvement of other variables, such as endogenous nickel levels in the plant, time of urease activation, organ type, ontogenetic stage of the plant, availability of carbohydrates for N assimilation, and/or even the N preference of the plant. In *Arabidopsis*, it was observed that with urea as the sole nitrogen source, clear N starvation symptoms develop, even with an active urease (Mérigout et al. 2008), reflecting the low preference that this species has for urea. In addition, these observations make us wonder what other processes are limiting urea utilization in plants.

## 2.4 *N* and Carbon Assimilation After Urea Hydrolysis by Urease

Although urea assimilation seems to occur primarily via GS/GOGAT, Walker (1952) noted that the alga *Chlorella pyrenoidosa* H. Chick grown in urea had a high arginine content and it was not possible to detect urease activity, hypothesizing that the incorporation pathway followed a reverse Krebs urea cycle. This idea was also proposed by Thomas and Krauss (1955), who found that urea stimulated the production of arginine in the green alga *Scenedesmus obliquus* (Turpin) Kützing. Hattori (1958) also reported having found arginine as an intermediate in the assimilation of urea in *Chlorella ellipsoidea* Gerneck and, as in the previous study by Walker (1952), was unable to detect urease activity. Baker and Thompson (1962) failed to find ureolytic activity in *Chlorella vulgaris* Beyerinck, concluding from their amino acid profile results that urea incorporation did not involve urease-catalyzed hydrolysis. Although all these studies suggested a path of direct urea assimilation without previous hydrolysis, to date it has not been possible to characterize it biochemically in living organisms. Thus, it would be interesting to test whether a direct assimilation of urea might occur in plants that are naturally exposed to this organic nitrogen source. In addition, it would be very interesting to assess how urea is metabolized in the carnivorous plant *U. gibba* L., which, as previously mentioned, shows no urease-like sequence in its genome.

Several studies conducted in crop plants in which urease had low activity (e.g., by using a urease inhibitor) have shown an increase in urea accumulation and a reduction in ammonium, amino acid, and/or N contents (Witte et al. 2002; Artola et al. 2011; Cruchaga et al. 2011; Zanin et al. 2016), indicating impairments in N-urea assimilation. In addition, by using  $^{15}\text{N}$ -labeled urea and  $^{15}\text{NH}_4^+$ , it was observed that these two N sources shared similar assimilation pathways in *Arabidopsis* plants (Mérigout et al. 2008). Taken together, these results indicate that the main assimilatory pathway of  $\text{NH}_4^+$  derived from urea follows the Gln synthetase (GS)-Glu synthase (GOGAT) cycle with previous hydrolysis by urease.

GS may be located in the cytosol (GS1 isoform) or plastids (GS2 isoform) (Mifflin and Lea 1980; Mifflin and Habash 2002) and incorporate an ammonium molecule into glutamate (Glu), giving rise to a glutamine (Gln) molecule. Subsequently, GOGAT transfers the amide group of Gln to 2-oxoglutarate, thereby generating two molecules of Glu (Bowsher et al. 2007), which allows a part of the Glu pool to be the substrate of GS, while the other part may be used to generate other amino acids through the action of aminotransferase enzymes (Fig. 1g) (Forde and Lea 2007).

In plants treated with urea as the sole nitrogen source, it was observed that the expression of the encoding cytosolic GS, GOGAT, and asparagine synthetase (ASN) genes was upregulated (Mérigout et al. 2008; Zanin et al. 2015a, b), reinforcing the idea that urea-derived N is assimilated through urease and GS/GOGAT pathways.

In addition, ammonium can be temporally stored in the vacuole and, whenever required, can be remobilized through TIPs (Fig. 1f) (Liu et al. 2003b; Kirscht et al.

2016). Related with this, Kirscht and collaborators (2016), based on simulations and the crystallographic structure of AtTIP2;1 (a known ammonia transporter aquaporin), suggested that  $\text{NH}_4^+$  is ultimately transported through aquaporins (mainly TIPs) in the form of  $\text{NH}_3$  due to its deprotonation in a side pore present in one of the aquaporin loops.

Interestingly, less attention has been given to the assimilation of  $\text{CO}_2$  derived from urea. One of the few studies that analyzed the incorporation of urea-derived C into organic molecules was performed by Webster et al. (1955), in which after 6 hours of  $^{14}\text{C}$ -labeled urea incubation, the incorporation of  $^{14}\text{C}$  into free amino acids, such as aspartate, glutamine, and glutamate, and protein-bound amino acids in pea leaves was observed. Lakkineni et al. (1995) found significant radioactivity in wheat leaves treated with  $^{14}\text{C}$ -labeled urea after 24 h. Taken together, these findings indicate that after urea hydrolysis,  $\text{CO}_2$  is assimilated. In order to form  $^{14}\text{C}$ -labeled amino acids from  $^{14}\text{C}$ -urea as claimed by Webster et al. (1955), the formation of carbon skeletons is required through the incorporation of  $\text{CO}_2$  by phosphoenolpyruvate carboxylase (PEPC) or by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in chloroplasts. Little is known about  $\text{CO}_2$  conductance into the chloroplast; however, it has been demonstrated that several PIPs mediate its conductance in leaves (Hanba et al. 2004; Uehlein et al. 2008, 2012; Mori et al. 2014; Heinen et al. 2014). Interestingly, the tobacco aquaporin NtAQP1, which is permeable to urea (as mentioned above), also regulates  $\text{CO}_2$  diffusion through membranes (Uehlein et al. 2003; Otto et al. 2010). By altering expression of *NtAQP1* or its ortholog in *Arabidopsis AtPIP1;2*, it was observed that both play a significant role in photosynthesis (Uehlein et al. 2003; Flexas et al. 2006; Heckwolf et al. 2011). The transport of  $\text{CO}_2$  by aquaporins is apparently mediated by the formation of only homotetramers instead of heterotetramers, which build an additional central pore in the middle of the four units. In plants, this central pore is lined with hydrophobic residues, which do not allow either solutes or water to permeate, only  $\text{CO}_2$  (Wang et al. 2007; Uehlein et al. 2008; Otto et al. 2010). These studies indicate that MIPs may mediate the incorporation of  $\text{CO}_2$ , and some may play an important role in carbon assimilation from urea. Thus, due to the different trafficking mechanisms of MIPs that allow its localization into diverse plant membranes (plasmatic, mitochondrial, plastidial, and vacuolar), not only might urea be transported into the cell and/or stored in the vacuoles, but its catabolic products, ammonium and  $\text{CO}_2$ , might also be remobilized from/to the plant organelles via aquaporins (Fig. 1f–h).

In addition, it has been suggested that urea may play an important role as a carbon source for bromeliads (plants with a high capacity to absorb urea), especially under conditions that limit atmospheric  $\text{CO}_2$  uptake, such as during drought conditions (Matiz et al. 2017). In the same study, through a cytochemical localization technique, it was possible to detect a high production of urea-generated  $\text{CO}_2$  near chloroplasts under drought conditions, and by using  $^{13}\text{C}$ -labeled urea, it was shown that part of the  $^{13}\text{CO}_2$  originating from urea hydrolysis was fixated into malate molecules (Matiz et al. 2017).

Even if the relative contribution of C urea to the C total pool of plants remains uncertain, urea as well as other organic N sources may have a positive effect on C/N

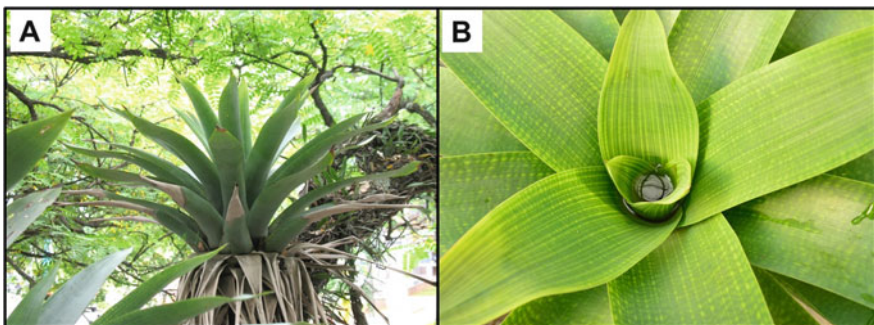
plant ratios. Thus, it is worthy considering urea not only as a N source but also as a supplier of carbon to plant cells, as well as a form of N and C for nutrient retranslocation out of senescing leaves.

### 3 Ecological Relevance of Urea

All plants studied so far, mostly crop or model plants, appear to have the capacity to obtain and metabolize urea. However, the physiological relevance of urea in plant nutrition and developmental processes is still unclear. Therefore, in order to understand the different aspects of urea metabolism and its physiological importance in plants, it would be helpful to examine other systems in which urea might play a major role, for example, plants that are naturally exposed to urea in their natural habitats.

Information regarding organic N use in non-crop or model plants comes from plants that inhabit inorganic nitrogen-limited environments, such as the canopy of tropical forests, or places where there are low rates of microorganisms acting on N cycling, such as in temperate forests (e.g., tundra plants) and coastal lands (e.g., restinga).

It is reasonable to assume that these plants are adapted to preferentially absorb the most available N form (Scott and Rothstein 2011). As an example, it has been shown that *Nepenthes hemsleyana* Macfarl, a pitcher-forming carnivorous, coprophagous-like plant, takes advantage of its mutualistic relationship with the bat *Kerivoula hardwickii* Horsfield in order to use the urea from its feces (Yilamujiang et al. 2017). By adding  $^{15}\text{N}$ -labeled urea into the pitchers of *Nepenthes* plants, it was suggested that exogenous urea is absorbed in its intact form and metabolized inside the tissues via urease since no urease activity was detected in the pitcher fluid (Yilamujiang et al. 2017). Because these plants must keep the pitcher fluid acidic, the absence of urease in this fluid prevents any urea hydrolysis, which could potentially alkalinize the pitcher solution (Yilamujiang et al. 2017). In contrast, for epiphytic tank-forming bromeliads (Fig. 2), which also establish mutualistic relationships with organisms, it



**Fig. 2** Epiphytic bromeliad. (a) *Vriesea gigantea* Gaudichaud on a host plant. (b) Detail of the tank of *Vriesea gigantea* Gaudichaud

has been suggested that ureases could be secreted into the tanks to metabolize the urea deposited into this structure (Cambuí et al. 2009).

All these findings show the importance of mutualistic relationships (also see Sects. 3.1 and 3.2) in order to acquire organic nitrogen sources in environments where inorganic N is scarce and highlight the ecological relevance of urea for plants.

### 3.1 Urea Utilization by Epiphytic Bromeliads

Epiphytic bromeliads are plants that live in the forest canopy and have no direct contact with the contents of the soil (Benzing 1990). In order to cope with this, they have developed a range of adaptations to acquire and conserve water and nutrients (Laube and Zotz 2003; Givnish et al. 2014; Leroy et al. 2016). One of these adaptations is the tank, a structure typical of some bromeliads, formed by the overlapping of the leaf bases that accumulates water and nutrients (Fig. 2) (Benzing 1990, 2000).

The establishment of the tank is associated with major changes in the bromeliad morphology and physiology, which function to maximize the capacity of the leaf to perform photosynthesis as well as to take up tank contents through specialized absorptive trichomes (Takahashi et al. 2007; Freschi et al. 2010; Takahashi and Mercier 2011; Matiz et al. 2013; Mito and Mercier 2013; Rodrigues et al. 2016; Pikart et al. 2018; Kleingesinds et al. 2018). Part of the available nutrients comes from precipitation and debris that fall into the tank, but the tank contents may also provide resources or shelter for a wide variety of organisms, forming small ecosystems within the bromeliads (Stewart et al. 1995; Ngai and Srivastava 2006; Cape et al. 2011; Leroy et al. 2016).

If we focus on the association with visiting animals, most of the available nitrogen comes in the form of organic compounds, such as urea. In fact, associations with animals, such as amphibians and spiders, can contribute a high proportion of nitrogen present in the plant (Romero et al. 2006, 2008, 2010). For example, it was estimated that individuals of *Scinax hayii* Barbour, which excrete mainly urea and visit the bromeliads to breed, contribute around 30% of the total plant nitrogen (Romero et al. 2010).

Based on this information, it is not difficult to imagine that organic nitrogen compounds, such as urea excreted by various animals, are a vital source of nitrogen for epiphytic bromeliads and are sometimes more readily available than inorganic sources (Benzing 2000; Romero et al. 2010; Gonçalves et al. 2016). Consistent with this, the overall growth of some bromeliad species is greater when nitrogen is supplied in the form of urea rather than nitrate or ammonium (Mercier et al. 1997; Endres and Mercier 2001). Accordingly, the epiphytic bromeliad *Vriesea gigantea* Gaudichaud showed a high capacity to absorb urea in its intact form through its leaves. Moreover, it has been suggested that this bromeliad may be able to secrete urease in the tank, increasing its rapid utilization (Inselsbacher et al. 2007). Later, the location of urease in *V. gigantea* Gaudichaud was determined to be in the cell walls

and cell membranes in addition to the cytosol, indicating that urease might be secreted into the tank (Cambuí et al. 2009), a very interesting feature considering that most ureases are located exclusively in the cytosol.

When urea was supplied into the tank of *V. gigantea* Gaudichaud, the apical region of the leaf showed the highest GS activity, indicating that urea-derived N assimilation occurs preferably in this leaf portion, while urea hydrolysis seems to occur mainly in the basal portion of the leaf, as evidenced by the high urease activity (Takahashi and Mercier 2011). Interestingly, even if urease activity is constitutive in all leaf tissues of *V. gigantea* Gaudichaud, its activity is inducible and considerably greater in the basal part of the leaves. Moreover, despite the elevated urease activity, urea accumulates in this leaf portion probably in the vacuoles (Takahashi and Mercier 2011; Matiz et al. 2017). However, what at first might be considered as a limitation of N-urea assimilation seems to be a very common strategy in epiphytic bromeliads, which demonstrates “luxury consumption.” This process consists of the rapid absorption of nutrients when available and their stockpiling for later use in times of nutritional deficit (Lin and Yeh 2008; Winkler and Zotz 2010; Gonçalves et al. 2016). Strategies such as these may increase the chances of survival in the epiphytic habitat.

Urea absorption in epiphytic bromeliads appears to be mostly mediated by aquaporins since exposure to HgCl<sub>2</sub>, an aquaporin inhibitor, reduced the absorption of urea by 78% (Inselsbacher et al. 2007). Recently, it was possible to confirm the involvement of aquaporins in nitrogen-acquisition strategies in these plants (H. Mercier, personal communication). Taken together, the increased, inducible activities of urease (with its potential secretion into the tanks) and aquaporin participation in urea absorption and/or accumulation (in the vacuoles) might be important elements that lead epiphytic bromeliads to prefer this organic nitrogen source.

Since one of the most studied species in terms of urea nutrition has been *Arabidopsis* and this species appears not to use urea in an efficient way (Kojima et al. 2007; Mérigout et al. 2008), it would be interesting to compare the affinities for urea (Km) of urease and urea transporters of plants that are naturally exposed to this nitrogen source with their orthologs in crop and model plants. These data could bring new insights into the key elements that make some plants prefer and metabolize urea efficiently.

### **3.2 *Mycorrhization as Facilitator of N-Urea Absorption in Plants***

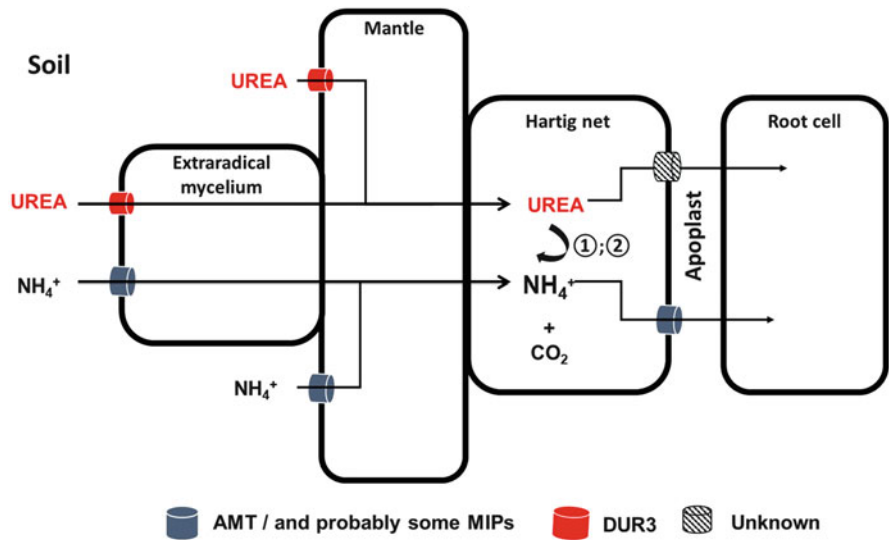
Another possibility to increase the efficiency of urea nutrition in plants could rely on the association with organisms that are capable of absorbing nitrogen from organic compounds, such as fungi. Mycorrhiza refers to a fungus associated with plant roots, which provide carbon compounds to the fungus in exchange for mineral nutrients. It



is estimated that around 80% of angiosperms form some kind of mycorrhizal association, the more common being the arbuscular mycorrhizas and the ectomycorrhizas (ECMs) (Brundrett et al. 1996; Moore et al. 2011).

Since ECMs apparently are capable of absorbing organic nitrogen sources to a much larger extent than arbuscular mycorrhizas (Bago et al. 2001; Smith and Read 2008), they are the focus of this section. In fact, ECMs are dominant in boreal and temperate forests, in which a high proportion of the available nitrogen is in organic form (Wu 2011). Despite this, these associations are not restricted to these biomes and can also be found in the tropics, as highlighted by recent studies (Smith et al. 2011, 2013, 2017; Alvarez-Manjarrez et al. 2017; Corrales et al. 2018). The ECMs form a complex structure with three defined regions: the extraradical mycelium, the mantle, and the Hartig net (Fig. 3) (Smith and Read 2008). The Hartig net develops between the root cortex cells and represents the main site of nutrient exchange between plant and fungus, while the mantle is formed by the wrapping of the hyphae around the roots, which is connected to the extraradical mycelium that extends toward the soil.

The preferred nitrogen form varies among the different ECM symbionts. There are reports of nitrogen use from organic sources, such as amino acids, proteins, and urea (Yamanaka 1999; Guidot et al. 2005; Smith and Read 2008; Shah et al. 2013). In some cases, it has been shown that ECM fungi without the host can grow fairly



**Fig. 3** Proposed model for nitrogen transfer from soil urea to roots through ectomycorrhizal fungi (adapted from Hacquard et al. 2013). Urea is mainly absorbed by the extraradical mycelium and the mantle by DUR3 transporters, while ammonium can be absorbed by AMT or MIPs. After urea is absorbed, it is transferred to the Hartig net and hydrolyzed by urease or urea amidolyase. The resulting ammonium is then excreted in the plant apoplast (possibly by AMTs or MIPs) and absorbed by the root cell through the mechanisms described in Fig. 1. ① = urease ② = urea amidolyase

well on urea as the sole source of nitrogen (Yamanaka 1999; Guidot et al. 2005). There are also a few studies that have investigated whether and how ECMs may absorb this compound and translocate N to the plant, but the data seem somewhat limited, and many questions remain to be answered.

Hacquard et al. (2013) compared microarray data from three different stages of *Tuber melanosporum* Vittad development: free-living mycelium, fruiting body, and ectomycorrhizas. A DUR3-like urea transporter was significantly more expressed when this fungus was forming ECMs. Moreover, microarray data comparing laser-dissected mantle and Hartig net compartments in the same species showed that DUR3-like gene expression was higher in the mantle cells, while urease and urea amidolyase were upregulated in the Hartig net (Hacquard et al. 2013). This suggests that the fungus is capable of absorbing urea from the soil, breaking it down (through urease or urea amidolyase action) and transferring the derived nitrogen to the plant in the form of ammonium (Fig. 3). This is somewhat similar to what happens in arbuscular mycorrhizas, in which the fungus transfers  $\text{NH}_4^+$  to the plant, likely originating from internal urea breakdown (Govindarajulu et al. 2005; Pérez-Tienda et al. 2011; Tian et al. 2010).

Morel et al. (2005) quantified nitrogen-containing organic compounds in the ECM *Paxillus involutus* (Batsch ex Fr.) Fr.-*Betula pendula* Roth association, comparing the mycelium directly associated with the roots (mantle + Hartig net) to the extraradical mycelium about 10 cm from the root tips. Strikingly, urea was present in high concentrations in both the extraradical mycelium and the root mycelium. In the same experiment, a microarray analysis of approximately 1200 genes was performed, revealing that the urea transporter *PiDur3* was approximately four times more expressed in the extraradical mycelium than in the ECMs. Again, this points toward a significant participation of urea in the nutrition of ectomycorrhizal plants. Later, *PiDur3* was functionally characterized by complementation growth assays in a urea-defective uptake yeast strain, showing that this transporter is, in fact, capable of transporting urea (Morel et al. 2008). Moreover, these authors showed that *PiDur3* expression was negatively regulated by glutamine and, possibly, intracellular urea and ammonium (Fig. 3).

It is likely that aquaporins also control metabolite transport as well as other processes between host plants and fungi, but there is very little information on the subject. Dietz et al. (2011) showed that three aquaporins are capable of transporting  $\text{NH}_4^+/\text{NH}_3$  through yeast complementation assay and that one of these (Lacbi1:317173) was highly expressed in the ECM compartment when compared to the extraradical mycelium and the isolated fungus. This could indicate that this MIP may play a role in  $\text{NH}_4^+$  transfer to the host plant. On the other hand, Lacbi1:387054 and Lacbi1:391485, which also appear to transport  $\text{NH}_4^+/\text{NH}_3$ , were more expressed in the extraradical mycelium (Dietz et al. 2011).

It is very likely that the role of aquaporins in ECM formation is not fully understood. If we take the example of other symbioses, such as the interaction between plants and nitrogen-fixing bacteria, the NIP class of aquaporins plays an important role not only in metabolite transport but in nodule development as well (Uehlein et al. 2007; Afzal et al. 2016). Therefore, we can assume that a similar role may exist for mycorrhizal associations.

Despite the lack of information, there is evidence that at least some ectomycorrhizal associations can use urea from the environment, but how the nitrogen is transferred to the plants is still unclear. Thus far, it appears that urea is absorbed in the extraradical mycelium and mantle and then transported to the Hartig net, where it is broken down into ammonium and then transferred to the plant (Fig. 3). It is important to remember that there are many fungal species capable of forming ECMs and that, accordingly, the mechanisms of urea absorption and N transfer to the plant may vary. Nevertheless, discovering how these associations function could lead to major breakthroughs in how to make plants more effectively absorb and use urea by focusing not only on the physiology of the plants but also on the ECMs.

#### **4 Biotechnological Aspects and Challenges to Improve Nitrogen Use Efficiency (NUE) in Plants**

One of the main goals in agricultural production is the increase of NUE of plants. A logical way to achieve this is either by increasing nutrient availability or by engineering crops with a high capacity to utilize N sources. Since urea is the main N fertilizer used nowadays in arable soils, it is worth investigating how to improve its availability and utilization by crop plants.

Regarding urea availability to cultivated plants, the use of large amounts of urea fertilizers has been the main strategy to sustain high productivity in arable soils. However, as mentioned above, due to the rates of urea hydrolysis by microbial ureases in soils, the use of large amounts of urea-based fertilizers causes nitrogen losses by volatilization, which results in water and atmospheric pollution (Zaman et al. 2008, 2009). Therefore, in order to avoid or at least reduce this process, urea is being supplied to crops in conjunction with urease inhibitors, which increases the persistence of urea (Zaman et al. 2008; Sanz-Cobena et al. 2011; Abalos et al. 2012; Soares et al. 2012; Saggari et al. 2013; Ahmed et al. 2018). As a consequence of a longer permanency of urea in the soils, an improvement in its absorption by plants is expected. However, it has been demonstrated that urease inhibitors, such as N-(n-butyl) thiophosphoric triamide (NBPT), have a negative effect on DUR3 transporters and, consequently, on urea uptake (Zanin et al. 2015b). Additionally, it appears that NBPT can also be absorbed and translocated to other tissues of the plant, affecting the endogenous urease activity of the plant (Cruchaga et al. 2011; Zanin et al. 2015b, 2016). Indeed, under NBPT treatments, urea-derived N assimilation is impaired in plants, as evidenced by a reduction in ammonium and amino acid pools, as well as the activity and gene expression of glutamine synthetase (Artola et al. 2011; Cruchaga et al. 2011; Zanin et al. 2015a, 2016).

On the other hand, in Ni-poor soil, impairments of urea utilization might be caused by a hidden Ni deficiency, which impedes the correct function of plant ureases. Fertilization with Ni has been shown to have a positive effect on plant

growth and development, N assimilation, and grain yield (Roach and Barclay 1946; Gerendás and Sattelmacher 1997; Freitas et al. 2018). Moreover, since Ni is an essential element for urease activation, it has to be translocated to the different tissues of the plant in order to allow urea metabolization, a process that may take several days after its application (Myrach et al. 2017). Therefore, it would be interesting to carry out prior applications of Ni in urea fertilization programs, which may benefit the N metabolism of plants and, consequently, production, particularly in soils with low Ni content.

Significant quantities of mineral (ammonium and nitrate) and dissolved organic N (amino acids, peptides, and urea) are present in fertilized soils; consequently, plants are exposed to this combination of N sources (Garnica et al. 2009; Brackin et al. 2015). Therefore, we have to keep in mind that in order to improve our understanding of urea nutrition in plants, it is necessary to know how interactions with other N sources (inorganic and organic N pools) function in the root uptake since this appears to modify urea uptake and assimilation (Bradley et al. 1989; Mériçout et al. 2008; Garnica et al. 2009; Zanin et al. 2015a; Pinton et al. 2016). For example, nitrate supply in conjunction with urea enhances cytosolic and plastidial N-assimilatory pathways, leading to a positive N status and plant growth in maize plants (Zanin et al. 2015a). However, ammonium and ammonium nitrate caused a reduction in urea absorption in wheat seedlings (Bradley et al. 1989). In addition, in *Arabidopsis AtDUR3* gene expression was induced by urea but only in the absence of other N forms (ammonium nitrate) (Mériçout et al. 2008). These results indicate that the response to a combination of N sources depends on the plant species and may be influenced to some extent by initial N status. Therefore, it would be interesting to consider strategies focusing on synchronizing soil N supply with N status of the plant, as well as focusing nutrition plant research on interactions among N forms in crops to achieve a more sustainable use of fertilizers.

On the other hand, urea uptake and metabolism studies that identify key elements as targets for NUE have not received adequate attention. With respect to urea acquisition by plants, as mentioned above, high- and low-affinity transport systems for urea have been identified only recently. Thus, it makes them an obvious target for improving urea absorption. By analyzing the phenotypic changes of wild-type plants of *Arabidopsis* overexpressing rice or maize DUR3 transporters (OsDUR3 or ZmDUR3, respectively) and exposed to micromolar concentrations of urea, it was possible to show that *Arabidopsis*, a plant with low capacity to use urea as a sole N source, was capable of growing better than the untransformed wild-type plants (Wang et al. 2012; Liu et al. 2015).

Considering that in most agricultural soils the urease activity is high, urea would be present in micromolar concentrations; thus, engineering crops overexpressing DUR3 transporters might be an interest strategy to improve NUE. In contrast, since aquaporins manage millimolar concentrations of urea, they may be interesting targets for improving NUE in foliar fertilization programs. Consistent with this, overexpression of AtTIP4;1, a urea-transporting aquaporin of *Arabidopsis* which is detectable in roots but not in leaf tissues, was capable of increasing the absorption of urea in leaf tissues (Kim et al. 2008). These findings show a clear correlation

between the overexpression of urea transporters and the increase of urea uptake, further reinforcing the potential of engineering crops by modifying the expression of urea transporters to positively affect its acquisition.

In addition, overexpression of NtAQP1, an aquaporin that mediates the transport of CO<sub>2</sub>, has led to an increase in net photosynthesis and leaf growth (Uehlein et al. 2003). Interestingly, this same aquaporin has been proven to mediate urea transport when expressed in oocytes (Eckert et al. 1999), indicating that overexpression of aquaporins might improve urea absorption as well as membrane permeability for CO<sub>2</sub>.

Moreover, as mentioned above, an additional interesting way to increase N-urea acquisition by plants might be achieved through mycorrhizal interactions; however, this research field has been little explored.

Besides effective urea absorption by plants, an efficient assimilatory pathway is also required. As mentioned above, glutamine synthetase-, glutamate synthase-, and asparagine synthetase-encoding genes are upregulated under urea supply (Mérigout et al. 2008; Zanin et al. 2015a, b, 2016); these, too, might be targets to improve NUE under urea supply.

Due to the scant knowledge about the regulatory elements of urea metabolism in plants, further investigation into the elements involved in urea metabolism in plants that naturally receive this organic source of nitrogen, such as *V. gigantea* Gaudichaud, could potentially allow a better understanding of the importance of urea for plant nitrogen nutrition as well as identify key targets that can be used to modify urea usage in plants of economic interest.

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