OPINION PAPER

Internal efficiency of nutrient utilization: what is it and how to measure it during vegetative plant growth?

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Abstract

Efficient use of the resources required by plants to sustain crop production is considered an important objective in agriculture. In this context, the idea of developing crops with an enhanced ability to utilize mineral nutrients already taken up by roots has been proposed. In recent years powerful tools that allow the association of phenotypic variation with high-resolution genetic maps of crop plants have also emerged. To take advantage of these tools, accurate methods are needed to estimate the internal efficiency of nutrient utilization (ENU) at the whole-plant level, which requires using suitable conceptual and experimental approaches. Here we highlight some inconsistencies in the definitions of ENU commonly used for ENU 'phenotyping' at the vegetative stage and suggest that it would be convenient to adopt a dynamic definition. The idea that ENU should provide information about the relationship between carbon and mineral nutrient economies mainly during the period under which growth is actually affected by low internal nutrient concentration is here advocated as a guide for the selection of adequate operational ENU formulae for the vegetative stage. The desirability of using experimental approaches that allow removal of the influence of nutrient acquisition efficiency on ENU estimations is highlighted. It is proposed that the use of simulation models could help refine the conclusions obtained through these experimental procedures. Some potential limitations in breeding for high ENU are also considered.

Key words: Acquisition, efficiency, nutrient, utilization, vegetative, whole plant.

Introduction

A crucial issue in contemporary agriculture is to maintain and whenever possible improve crop yield while minimizing the costs of cultivation and side effects on the structure and dynamics of ecosystems. In this context, enabling the efficient use of essential mineral elements originally present in soils as well as those added as fertilizers is considered a research priority (White and Brown, 2010; Andrews and Lea, 2013). To fulfil this goal, two complementary strategies have been devised: improving the efficiency of nutrient acquisition by roots from the soil solution and improving the efficiency by which the acquired elements are utilized to generate vegetative biomass or edible parts of plants. Important advances have been made

in our understanding and the genetic manipulation of the efficiency of nutrient acquisition (ENA), whereas progress in the comprehension of the internal efficiency of nutrient utilization (ENU) has been comparatively slow. Interestingly, some recent advances in the study of ENU (discussed below) have guided researchers to key questions regarding both conceptual and experimental problems involved in measuring this efficiency. These pivotal matters are likely to be critical in breeding for traits associated with ENU as well as with biomass accumulation in low-nutrient environments. Here, we first discuss the concept of ENU for whole plants during the vegetative stage and highlight inconsistencies arising from

some definitions currently in use. Next, we consider some aspects of the protocols used in 'phenotyping' for improved ENU and discuss the potential of simulation models to interpret results in ENU estimation. Finally, some possible constraints involved in selection for high ENU are discussed.

Internal efficiency of nutrient utilization: what is it?

The need for use of dynamic definitions of ENU

The basic idea behind the ENU concept is to connect the economy of carbon with that of a given mineral nutrient. Several alternative definitions, with their corresponding formulae, have been proposed to attain a quantitative assessment of that link during vegetative growth, thus enabling researchers to compare different plant species or diverse populations within the same species, each of them emphasizing a particular aspect or a specific purpose [see Good *et al.* (2004) and Rose and Wissuwa (2012) for a detailed list]. Here we use the acronym ENU to refer to the general concept of internal efficiency of nutrient utilization and add a lower case suffix to identify different ENU estimates (Table 1).

The existence of a wide spectrum of ways to express efficiency could potentially contribute to a better understanding of utilization efficiency, as recently proposed for expression of leaf photosynthesis on a different basis of normalization (Poorter *et al.*, 2014). However, most formulae designed to account for ENU use a similar basis and aim to describe the same phenomenon. Still, differences among these overlapping definitions should not be an important problem unless they prove to be mutually contradictory. In this regard, however, experimental estimations of ENU based on the use of similarly targeted formulae commonly employed in large screenings during vegetative growth (i.e. ENUo and ENUu: see Table 1) did not always lead to the same conclusion. For example, Siddiqi and Glass (1981) found that at low potassium concentrations, barley cv. Fergus displayed a lower value of ENUo than cv. Conquest (37.7 and 46.7 g mmol^{-1}, respectively) while

the opposite was observed when the ENUu formula was used (14.7 and 11.7 g^2 mmol^{-1}, respectively). Similarly, Gurley *et al.* (1994) found differences at growth-limiting concentrations of phosphorus between white clover cultivars with ENUu but not with ENUo. Furthermore, on the basis of work with theoretical models, it has recently been shown that under certain circumstances some ENU indicators that hypothetically must serve equal purposes may render intrinsically different results (Moriconi and Santa-María, 2013), an observation that could be a critical impediment to breeding for high ENU.

An important problem with ENUo and ENUu, as well as with similar ratios, is the fact that they do not provide a dynamic perspective (Aerts and Chapin, 2000). The use of static approaches in estimating ENU is arguable because differences in these ratios among plants could result from variation in biomass accumulation as well as from disparities in the accumulation of the *j* element (where *j* corresponds to a given element). An indication that this is not only a theoretical issue is provided by the barley experiments mentioned above showing that for cv. Conquest accumulation of biomass between successive harvests was nil (Siddiqi and Glass, 1981). The obvious interpretation of this finding was that utilization of potassium present in the plants was zero under these conditions (i.e. no biomass was produced per unit of that nutrient), an aspect not reflected by any of the static formulae used and which indicates the convenience of adopting a dynamic approach that specifically takes into account the changes in biomass accumulation. There could also be another important advantage in using dynamic definitions. As recently highlighted by Rose and Wissuwa (2012), the ENU concept should primarily be focused on the range of internal nutrient concentrations below the optimum concentration (Fig. 1A, B). Measurements of ENU made above the optimal concentration correspond essentially to a numerical exercise because changes in ENU over that value are not associated with biomass production. This restriction has important consequences, for example in the case of plants previously exposed to adequate external nutrient supplies where a prominent effect of nutrient scarcity on whole-plant growth takes some time to show up (Fig. 1C), while the actual ENU corresponds to that displayed by plants

Table 1. *List of acronyms and corresponding formulae relevant to ENU, RNR, and MRT*

Acronym	Formula	Reference	Observations
ENUo	$W\,n_j^{-1}$	Steenbjerg and Jakobsen, 1963	–
ENUu	$W^2\,n_j^{-1}$	Siddiqi and Glass, 1981	–
ENUi	$(dW/dt)\,n_j^{-1}$	Ingestad, 1979	–
ENUb	$(dW/dt)\,n_j^{-1} * \text{MRT}$	Berendse and Aerts, 1987	–
MRT	RNR^{-1}	Berendse and Aerts, 1987	–
RNR	$(dn^j/dt)\,n_j^{-1}$	Berendse and Aerts, 1987	–
ENUh	$(dW/dn^u j)$	Hirose, 1971	–
ENUa	$(t_2 - t_1)^{-1} \int (dW/dt)\,n_j^{-1}dt$	Moriconi and Santa-María, 2013	Corresponds to the mean value of ENUi over the period from t_1 to t_2 during which plant growth is limited by internal nutrient concentration
ENUe	$[\ln(W^f) - \ln(W^i)] / [(t^f - t^i)\,n_j\,W^{-1}]$	Moriconi and Santa-María, 2013	–

W, biomass; n_j, content of the *j* nutrient in the plant; *t*, time. The superscripts *l* and *u* denote nutrient loss and uptake, respectively. Noticeably, alternative acronyms for a given formula have been used in the literature for specific major elements like nitrogen, phosphorus and potassium.
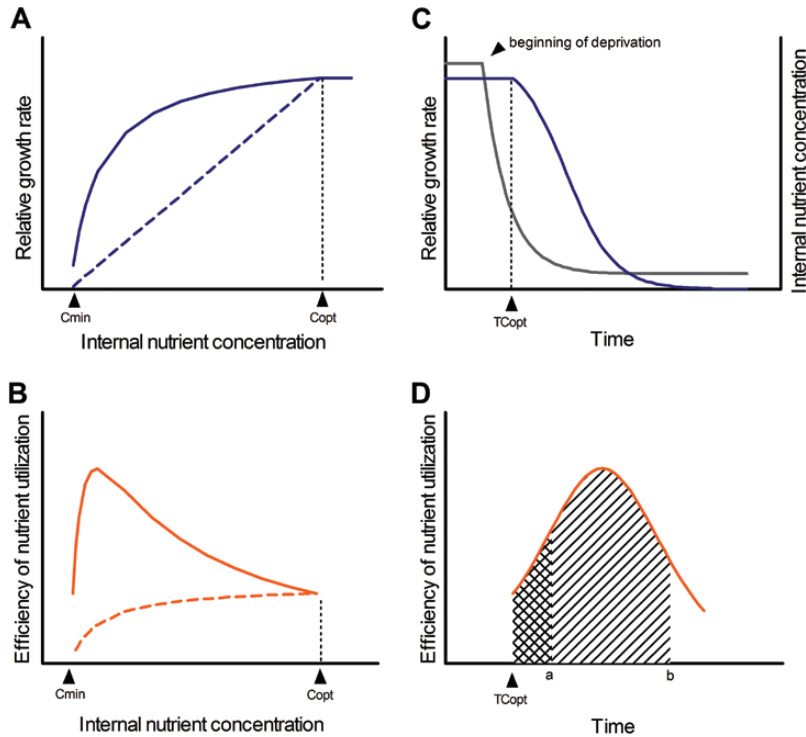
Fig. 1. Schematic representation of the dependence of plant growth and ENU on the internal nutrient concentration. (A) Plant growth, here ascribed as the relative growth rate, within the range defined by the minimal (*Cmin*) and optimal (*Copt*) concentrations, could display either a linear (dashed line) or a curvilinear (solid line) relationship with the internal concentration. (B) Hypothetical dependence of ENU on the internal nutrient concentration for the linear (dashed line) and curvilinear (solid line) responses shown in panel (A). (C) In experiments where nutrient shortage is imposed on plants formerly receiving an adequate supply of the nutrient, plant growth (blue line) usually becomes affected only when the internal nutrient concentration (grey line) drops below the optimal one which occurs at *t* = *TCopt*. (D) The time course of ENU during a deprivation experiment is shown for plants obeying the curvilinear relationship described above. The dashed area corresponds to the ENU displayed by plants once growth became limited by the nutrient under study. Integration of this area over the period of deprivation yields the mean actual ENU (ENUa). Screenings of ENUa made for short periods of growth restriction, i.e. between *TCopt* and the '*a*' timepoint, could be useful for improving ENU in nutrient-rich environments. Screenings made during longer periods of growth restriction, i.e. between *TCopt* and the '*b*' timepoint, could be useful for improving ENU in nutrient-poor environments. The slope of the linear relationship plotted in panel (A) corresponds to the intrinsic rate of conversion (P_j), while the maximum relative growth rate of the curvilinear one corresponds to A_j. Panels (A), (B), and (C) are adapted from Moriconi and Santa-María (2013). A theoretical framework to study potassium utilization efficiency in response to withdrawal of potassium. Journal of Experimental Botany **64,** 4289–4299, by permission of the Society for Experimental Biology).

from the moment that internal sub-optimal concentrations are reached (shaded area in Fig. 1D). Calculations of ENU made using static formulae necessarily result from the integration of growth at supra-optimal and sub-optimal levels, thus excluding the possibility of conceptually deriving the actual value of ENU from them. The extent to which this problem affects the above-mentioned estimations of ENU can be reduced, in some cases, by extending the time of exposure to growth-limiting levels of the nutrient under study. Unlike static definitions, dynamic formulae potentially have the capacity to be integrated over the precise period during which nutrient scarcity compromises plant growth. From this point of view, the use of dynamic definitions, in addition to helping overcome the uncertainties derived from static approaches, confers mathematical flexibility to the concept of ENU.

The ins and outs of dynamic definitions

Three main alternative dynamic definitions of ENU have been advanced, named here ENUi, ENUb, and ENUh, respectively (Table 1). According to the first one, ENUi, internal utilization efficiency should specifically consider the amount

of biomass that can be generated (*dW*) in a given time period (*dt*) by the amount of nutrient present in plant tissues (n_j). This assertion can be expressed by the following formula:

$$\text{ENUi} = (dW \, / \, dt)\,n_j^{-1}, \text{ being nj the amount of element} \quad (1)$$

This expression provides information about the instantaneous production of biomass per unit of nutrient and encompasses the concept of 'productivity' earlier proposed by Ingestad and co-workers (Ingestad, 1979; Ingestad *et al.,* 1988). Notably, formula (1) can be rewritten as:

$$\text{ENUi} = (dW \, / \, dt)\,n_j^{-1} = [(dW \, / \, dt)W^{-1}]c_j^{-1}$$

Taking into account that $(dW/dt)\,W^{-1} = (dn_j/dt)\,n_j^{-1} - (dc_j/dt)\,c_j^{-1}$, which has units of time^{-1}, the expression above can be reshaped as:

$$\text{ENUi} = [(dW \, / \, dt)W^{-1}]c_j^{-1} = [(dn_j \, / \, dt)n_j^{-1} \\ - (dc_j \, / \, dt)c_j^{-1}]c_j^{-1} \quad (2)$$

This shows that changes in ENUi can be driven by relative changes in nutrient content [given by $(dn_j/dt)\ n_j^{-1}$] as well as by relative changes in nutrient concentration [given by $(dc_j/dt)\ c_j^{-1}$]. While the first term provides information on the balance between gains and losses of j, the second one reveals changes of ENUi that operate through dilution or concentration effects. These two terms could eventually operate in the same or opposite directions, and both must be taken into consideration to dissect the sources of variation of ENUi.

It has previously been suggested that an appropriate definition of ENU should take into consideration the instantaneous capacity of each unit of nutrient to generate biomass as well as the period during which each unit of nutrient is retained in plants and can contribute to set plant growth (Berendse and Aerts, 1987). This period will be particularly important when significant losses of the nutrient under study take place, a phenomenon that could have several causes including loss and leaching from aerial and belowground parts of the plant, which are thought to play a more prominent role in perennials (Chapin, 1980; Vitousek, 1982). Following these observations, Berendse and Aerts (1987) proposed a redefinition of ENU as the product between two terms: one of them corresponds to ENUi, while the other corresponds to the mean residence time (MRT) of the j element in plants, which estimates the mean time during which a given element remains in plant tissues. This product has been termed, in the present paper, ENUb (Table 1). According to the original proposal, MRT can be calculated as the inverse of the relative nutrient requirement [$(dn_j^l/dt)\ n_j^{-1}$], RNR, which represents the amount of nutrient lost (dn_j^l) during a given period per unit of nutrient present in the plant (note that the superscript '*l*' denotes '*lost*' while the superscript '*u*', used later, denotes '*uptake*'; Table 1). In spite of the clear concept incorporated in the re-definition by Berendse and Aerts, the product between ENUi and MRT could confuse the influence of the losses of the element under study, since, according to formula (2), RNR is actually contained in ENUi as a part of the $(dn_j/dt)\ n_j^{-1}$ term because dn_j results from the balance between gains and losses of j. The precise consequences of the MRT term being both in the numerator and in the denominator of the product should be taken into consideration in data interpretation. In addition, it should be observed that the product between MRT and ENUi when dn_j^l is or cannot be distinguished from zero leads to division by zero or by an uncertain small quantity, thus imposing an important restriction on the use of ENUb as a general definition of ENU.

In an effort to make MRT consistent for both steady and non-steady state conditions, it has recently been argued that the RNR should take into consideration the amount of nutrient taken up over the period under study (dn_j^u) instead of the amount lost (Hirose, 2011). The calculation of ENU as the product between ENUi and MRT, estimated now on the dn_j^u basis, is consistent with a proposal made by Hirose (1971), who defined that efficiency (here named as ENUh) as the quotient between the variation of biomass and the amount of j taken up in the same time period, i.e. ENUh = dW/dn_j^u (Table 1). While this definition has the virtue of explicitly incorporating changes in both biomass and nutrient content, thus integrating them into a single formula, it is also faced with the restriction, relevant in some experimental approaches, that it cannot be defined when dn_j^u is zero.

Among the dynamic definitions so far advanced, ENUi seems to offer an adequate account of utilization efficiency, which could usually be, but not necessarily always, sufficient in studies with annual crop plants particularly during the early vegetative stage. The use of ENUi in combination with the definitions provided by ENUb and ENUh, especially when loss and/or uptake are respectively important contributors to nutrient economy, may offer complementary perspectives for refining phenotyping for ENU as well as for understanding the relationships between the economy of carbon and that of the nutrient under study at both the whole-plant and the organ levels. An adequate use of the proposed indices requires setting suitable experimental conditions, and these will be considered in the next section.

How to measure ENU: problems arising from experimental protocols

Because a primary objective of research on ENU, particularly with crop species, has been to improve the agronomic use efficiency of nutrients under field conditions, measurements of both ENA and ENU have been commonly made with plants grown in soil. The underlying assumption of this procedure is that such measurements avoid potential errors arising from the use of artificial growth conditions, which will probably misrepresent the panoply of interactions that affect roots growing in the soil. While classic studies provide strong support to this view for investigations aimed at improving nutrient acquisition, this assumption may not necessarily hold in studying ENU. A seminal question to be answered in this context is to what extent measurements of nutrient acquisition and nutrient utilization efficiency are interdependent. In this regard, some data indicate that for phosphorus (Su *et al.*, 2009; Rose *et al.*, 2011), nitrogen, (Chardon *et al.*, 2010) and potassium (White *et al.*, 2010) a negative correlation between ENUo (or ENUu) and the amount of nutrient taken up by the plants can be found, suggesting the existence of a link between estimations of ENU and ENA.

Rose *et al.* (2011) argued that a possible way of revealing true differences in ENU is by ensuring that differences in accumulation are nullified or at least minimized. Since this cannot be done unambiguously in a soil system, they proposed to make the screening for phenotypic differences in ENU by growing individual plants in a nutrient solution containing a low amount of the nutrient under study. The rationale of this approach is that, under these conditions, individual plants will exhaust the solution to a similar extent and, consequently, absorb a similar amount of the element under study, avoiding the masking effect derived from competition among roots of different genotypes when they are placed together. The attempt to equalize nutrient capture among genotypes can also be done through the complete withdrawal of the element under study, since in this case capture would become zero (Moriconi and Santa-María, 2013). Each of these procedures offers benefits and disadvantages. A potential problem

with the first one is that until now no theoretical support has been built up to estimate the possible bias arising from the use of each ENU indicator or to interpret the effect exerted by previous differences in nutrient content on ENU estimations made for plants grown under that condition. In this regard, it has been argued that differences in seed nutrient content can be minimized by growing plants at a high nutrient supply before supplying a low level of the nutrient (Rose *et al.*, 2011), which would not necessarily avoid further differences among genotypes arising during this high-nutrient supply period. Although a theoretical framework has recently been developed for the second approach, particularly for potassium, it must be noted that in practice obtaining a culture solution with a very low amount of a certain element, in order to consider it virtually absent, may not always be an easy task. Another problem coming from this second approach is that the acclimation response of plants following a sudden interruption of nutrient supply may be different from the one that takes place when nutrient scarcity is gradually imposed as actually occurs in nutrient-deficient soils. Certainly, the use of these procedures does not preclude the possibility that genotypes under study intrinsically differ in nutrient leakage and/or in the affinity of their transport systems leading to differences in the retention and/or reabsorption of the nutrient under study within the plant (Aziz *et al.*, 2014). Comparisons of genotypes by using any of the protocols requires careful examination of the basic assumptions underlying their use, i.e. whether nutrient capture/leakage is very similar (not significantly different) for all genotypes under study. Whenever possible the use of these protocols will help to remove, or minimize, ENA as a potentially compromising effect when evaluating ENU.

Some notes on experimental procedures

Unless high-throughput phenotyping facilities are available, there are some limitations in the use of hydroponic culture in phenotyping for high ENU, and in most cases only small or medium-sized crop plant collections (<150 lines) can be properly handled through the procedures previously outlined, even during early vegetative growth. Therefore the success of these procedures to associate the ENU phenotype observed to molecular markers will largely depend on the careful choice of a well characterized (genotyped) germplasm collection of appropriate size.

Once the germplasm collection has been chosen, the use of hydroponics for ENU phenotyping could easily be combined with adoption of the dynamic approach proposed above. It requires culturing individual plants for a period long enough to ensure that growth for all plants becomes limited by nutrient supply, and obtaining at least two estimations of whole-plant biomass, one at the moment when low nutrient supply is imposed and another one when the effect of low nutrient supply on growth becomes evident. Alternatively the first harvest can be done once plants have grown for a time under the low-nutrient level condition, thus reducing the effect of early-acclimation responses to the sudden change of the nutrient level on ENU estimations. These harvests should be performed over the same fixed interval for all members of the collection. In order to estimate the experimental values necessary to compute dynamic formulae, the gains and losses of the nutrient must also be estimated by measuring changes in plant nutrient content between harvests, including senescent material [for further details to be considered in measuring MRT see Eckstein *et al.* (1999) and Hirose (2011)]. These measurements additionally allow a determination as to whether the assumptions made for a given screening protocol are actually met. Noticeably, screening of plants through a dynamic approach would require additional tissue nutrient analyses, which can operate as an important limitation in 'phenotyping' large populations.

Learning from models

Breeding for high ENU should focus on improving plant growth over the range of internal nutrient concentrations in which growth actually depends on the element studied. A focus on this range requires knowing the actual value of ENU over the period from the moment that the internal concentration of the nutrient is below the optimum concentration (Fig. 1D, C). For the case of ENUi, the mean utilization efficiency displayed by whole plants over that period has been named the actual ENU (ENUa: Table 1). The precise value of this efficiency cannot easily be measured, at least with the techniques available today, but can be approached by using mathematical models that take into account the relationship between growth and c_j for specific growth conditions. Modelling growth responses to internal nutrient concentration under specifically simulated growth conditions has already been pursued (Hirose, 1988; Hirose *et al.*, 1988). Empirically based models could serve as tools for exploring the sources of variation of ENUa. Noticeably, while ENU indicators are experimentally based on two fixed harvests, as outlined in the previous section, ENUa extends over a period that could differ among the members of the collection under analysis. Simulation models also help to assess the extent to which those ENU indicators are reliable estimators of ENUa. The latter issue is even more important in large screenings where experimental bottlenecks could force the use of non-dynamic ENU indicators. These questions have been examined in virtual plants suddenly exposed to the complete withdrawal of a given element, since for this hypothetical growth condition basic mathematical models that relate growth with the internal nutrient concentration can easily be built (Moriconi and Santa-María, 2013). Although such models are far from being representative of real plants in real soils, they help to take into account some considerations in ENUa screenings. Firstly, several parameters contained in the models contribute in different ways and to different extents to determine the time course of ENUa. In this regard, the effect exerted by the initial concentration (including that of the seed) on ENUa depends on the precise model and on the value of other parameters; which means that it cannot easily be predicted. Secondly, there are no perfect indicators of ENUa, but clearly some of them tend to better reflect the actual variation of ENUa in most scenarios while others are unlikely to

generally reflect the actual efficiency. Thirdly, in some circumstances, some indicators (among them the widely used ENUo) could be negatively associated with ENUa; this may happen particularly when the source of variation is the initial concentration. These uncertainties can be partially reduced through the careful and simultaneous use of several indicators (specifically ENUe, ENUi, ENUo, and ENUu; see Table 1). However, when only non-dynamic ENU indicators are used, precise knowledge on the degree to which they approach ENUa seems to be unavoidable and requires the use of simulation models. Overall, an important conclusion from this study (Moriconi and Santa-María, 2013) is that the factors affecting ENUa and the degree to which different indicators can approach ENUa critically depend on the structure of the model to which plant responses approach. Therefore, the extent to which a model can readily help in the screening of crops for high ENUa will largely depend on the extent to which the model actually mimics the dynamic relationship between growth and the internal nutrient concentration under given experimental conditions.

Another important issue derived from the work with different models is that ENUa cannot always remain the same within the sub-optimal range of internal concentrations (Fig. 1B; Hirose, 1988; Hirose *et al.*, 1988). This observation can be translated into different approaches according to the particular purpose of a breeding programme. If the programme is aimed at maximizing biomass production at internal concentrations close to the optimal one, ENUa estimations should be made at the very beginning of the period after growth becomes restricted (Fig. 1D). This procedure will be well suited to improve ENU in crops supplemented with fertilizers. For plants growing in nutrient-poor, non-fertilized soils, the focus should be over a more extended period of nutrient scarcity during which plants will display a wider set of strategies to cope with internal nutrient deficiency (Fig. 1D).

Some ENU constraints

Although improving ENU at the whole-plant or aboveground levels is an important goal for plant breeders, it could eventually be in conflict with the search for other equally desirable traits, an aspect that must be taken into consideration for a comprehensive 'phenotyping' procedure. A first potential conflict to be considered for the vegetative stage could emerge between the efficiencies of acquisition and utilization as already illustrated with examples of a negative correlation between ENU and ENA. However, information obtained in screenings conducted in soils, even with the complications that the use of this system could impose for accurate ENU comparisons, suggests that in spite of being frequent this negative correlation is not always present (Su *et al.*, 2009). Thus, even in soil-based screenings, it could be possible to select for both traits, which is particularly important since the ideal phenotype for a crop plant would be a combination of high ENA and high ENU. In this regard, it should be noted that work with simulation models indicated that genotypes with high root ENU could better explore the soil leading to increased ENA (Wissuwa, 2003), thus suggesting that a physiological

trade-off between both efficiencies does not exist. In practical terms, when the occurrence of a potential bias due to ENA cannot be discarded, phenotyping for ENU would require an examination of the coincidence of genetic maps obtained for both efficiencies. Under these conditions, selection should be done with loci for high ENU that do not coincide with loci for low ENA and display enhanced biomass accumulation.

A second constraint for ENU, which has been mostly disregarded, emerged from the models outlined above, which indicate the existence of a potential conflict between high ENUa and high relative biomass accumulation. Relative 'performance' of biomass accumulation is usually expressed as the quotient W_p/W_n, i.e. between the biomass accumulated by plants grown in nutrient-poor media (W_p) relative to that measured in a non-growth-limiting nutrient medium (W_n). This quotient is an indicator of the ability of a given genotype to avoid a significant growth reduction at limiting supplies of the *j* element, and consequently an indicator of tolerance to nutrient deficiency. It differs conceptually from ENU, which just describes the capacity to generate biomass per unit of nutrient in the plant. This distinction is particularly important because a negative association between ENUa and that quotient could be expected for some (Fig. 2A), but not for all (Fig. 2B), sources of variation of ENUa (Moriconi and Santa-María, 2013). In support of this statement, it has been shown, in a soil-based screening for some Triticeae, that ENU for phosphorus can sometimes, but not necessarily always, be negatively associated with tolerance to low phosphorus supply (Osborne and Rengel, 2002). A wide screening of rice genotypes suggests that ENU and tolerance to low potassium supply are usually associated (Yang *et al.*, 2003). Thus, the relevance of this conflict in each case should be analysed. Particularly, a trade-off between both traits must be expected when plants largely differ in the 'productivity' (*Pj*) or in the maximum relative growth rate (*Aj*) as shown in Fig. 2A. Differences in tolerance, as expressed by the W_p/W_n quotient, and based on disparities in *Pj* (or *Aj*), do not necessarily result in differences in absolute biomass accumulation.

The final objective of breeding for high ENU is the selection of crop plants that maximizes yield while minimizing the requirement of nutrients. The extent to which specific traits conferring high ENU at the vegetative stage may confer enhanced yield must be carefully examined. Noticeably, the dynamic approach outlined above can eventually be extended to the reproductive stage by applying formula (1) to the process of seed biomass accumulation. In this context, it should be considered that additional constraints to those aforementioned are specific to the reproductive phase (see Barraclough *et al.*, 2010; Rose *et al.*, 2013; Manschadi *et al.*, 2014).

Towards the underlying mechanisms

Previous paragraphs have focused on the definition and measurement of ENU. While necessary to make a proper assessment, the issues discussed do not provide, per se, any insight on the mechanisms determining ENU differences among genotypes. Although analysis of these mechanisms is beyond
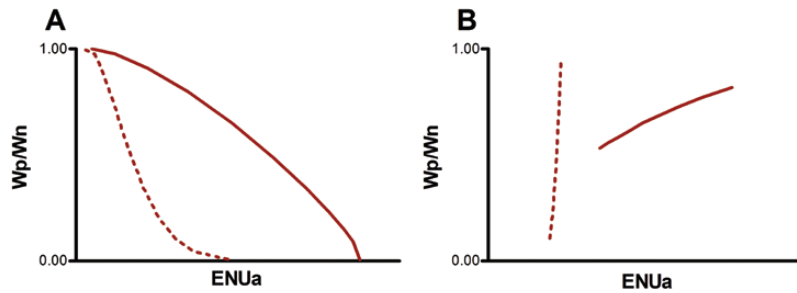
Fig. 2. Hypothetical relationship between the actual ENU (ENUa) and the quotient between the biomass accumulated by plants grown in nutrient-poor media (W_p) relative to that measured in a non-growth-limiting nutrient medium (W_n) for linear (dashed lines) or curvilinear (solid lines) models shown in the upper panel (A) of Fig. 1. In (A) the sources of variation for ENUa are P_j (the intrinsic rate of conversion for linear models) or A_j (the maximum relative growth rate for curvilinear models). In (B), the source of variation for ENUa is the pair *Copt/Cmin*. Noticeably, the effect exerted by variation of *Copt/Cmin* in linear models does not apply when *Cmin* = 0. This figure is available in colour at *JXB* online.

the scope of this paper, a possible way to connect them with ENU definitions should be briefly mentioned. As already shown, for the vegetative stage, formula (1) can be rewritten alternatively as:

$$\text{ENUi} = (dW/dt)n_j^{-1} = [(dW/dt)W^{-1}]c_j^{-1}$$

Multiplying and dividing the last expression by the leaf area (*A*) yields:

$$\text{ENUi} = [((dW/dt)A^{-1})(AW^{-1})]c_j^{-1} \qquad (3)$$

The term $(dW/dt)\,A^{-1}$ corresponds to the net assimilation rate (NAR), while $A\,W^{-1}$ corresponds to the leaf area ratio (LAR). Dissection of differences in utilization efficiency in terms of differences in NAR and LAR (as well as of LAR components) has already been pursued (Hirose, 1984; Hirose *et al.*, 1988; van der Werf *et al.*, 1993; De Groot *et al.*, 2001). Therefore, the adoption of a dynamic approach could provide a necessary link between phenotyping and exploration of the mechanisms underlying ENU in terms of the components of the relative growth rate.

It seems worthwhile mentioning that as knowledge of the mechanisms underlying plant responses to nutrient scarcity increases, the ways to express ENU could eventually change. For the particular case of phosphorus it has recently been observed that restriction of shoot growth is partially uncoupled from total phosphorus content in this plant fraction (Rouached *et al.*, 2011). Data obtained in plants overexpressing the AtNHX1 exchanger supports a similar statement for potassium (Leidi *et al.*, 2010). These results suggest that for some nutrients a variable fraction can be in pools that only marginally contribute to set growth. In this regard, it has been proposed that for some levels of analysis the amount of nutrient considered in ENU formulae may be substituted by the amount specifically allocated to metabolically active pools (Veneklaas *et al.*, 2012).

Conclusions

Definitions of ENU are not free of inconsistencies or restrictions. The use of alternative dynamic definitions could offer

a comprehensive basis for further understanding plant responses to nutrient scarcity as well as improving ENU phenotyping. The idea that the efficiency to be estimated should correspond to that displayed by plants only when growth is actually affected by the internal concentration could serve as a guide for the selection of adequate operational formulae. New protocols have recently been advanced to minimize the masking influence of nutrient acquisition on ENU estimation, while ambiguities in screening for ENUa can be reduced through the critical use of several ENU indicators. Thus, the panorama opened up to us seems to bring innovative screenings of ENU in crop plants.

If non-biased comparisons of ENU among genotypes must preferentially be assessed under well controlled conditions, an important question is to what extent differences in ENU determined with that artificial method can be extrapolated to field conditions. The answer will operatively depend on a second question: how to infer ENU from the conditions encountered by plants in their environment without the masking effect of nutrient acquisition? Certainly, we cannot offer an adequate response yet. However as stated above, even without an unequivocal assessment of ENU it could be possible to distinguish preliminarily, in screenings performed under field conditions, some traits that influence nutrient utilization from some of those that influence nutrient acquisition.

We are witnessing the development of a notable array of tools that permits the association of wide phenotypic variation to high-resolution genetic maps of crop plants, reinforced by the development of high-throughput molecular profiling technology. These tools are increasingly used by plant breeders for the identification and selection of traits of agronomic value. To take advantage of those opportunities, equally powerful methods are needed for the screening (i.e. phenotyping) of ENU. These methods can be further facilitated by the use of non-invasive technologies (Fiorani and Schurr, 2013), once appropriate protocols for plant growth are stated and the primary phenotypic parameters (ENU indicators) to be measured are accurately validated. Identification of the conceptual and experimental problems in ENU studies, and the approaches suggested above in measuring ENU, could serve to assist with some of the challenges for conducting successful large screenings of ENU as well as to set a framework for unequivocal data interpretation.

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