Utilization of Respiratory Energy in Higher Plants

Requirements for 'Maintenance' and Transport Processes

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Stellingen

1 Wetenschappelijke stellingen in een proefschrift met betrekking op dat proefschrift zijn dubbelop en kunnen dan ook beter weggelaten worden.

2 Een positieve koppeling van de financiering van universiteiten aan het aantal studenten in plaats van een koppeling aan het belang van de aanwezigheid van kennis - waarbij onderwijs een positieve spin-off vormt - heeft als risico dat veel toekomstige werkelozen duur worden opgeleid om een klein aantal banen te behouden.

3 Aangezien de mensvriendelijkheid van het beleid helaas vaak een negatieve relatie vertoont met de beschikbare middelen, is humaan "resource-management" te verkiezen boven "human-resource management".

4 Beleid voor een duurzaam milieu is veelal niet duurzaam.

5 Door de relatie tussen leeftijd en de hoogte van het loon om te draaien, kunnen maatschappelijk wenselijke regelingen als de VUT en lage pensioengerechtigde leeftijden gehandhaafd worden, terwijl er financieel per saldo voor niemand iets verandert.

6 De moeilijkheid van een eenvoudig probleem is om te begrijpen waarom het een probleem was nadat je de oplossing vond.

7 De honorering binnen het AIO-stelsel voldoet aan de waarneming in de psychologie die toont dat als de beloning te gering is, mensen zich meer inzetten om toch een bepaalde vorm van rechtvaardiging te krijgen.

8 Als bij het besturen van een land de kans op ernstige brokken even groot is als bij het besturen van een auto, ligt het in de rede boven een bepaalde leeftijd ook een geestelijke gezondheidsverklaring te vereisen voor het passieve kiesrecht.

9 Het aanstellen van AIO's zonder een passend post-doc beleid is voor universiteiten een 'goedkope' manier van bezuinigen, die echter door de maatschappij duur wordt betaald.

10 Naarmate de premies voor gezondheidsverzekeringen, bijvoorbeeld door toepassing van een genetische kaart, steeds gedifferentieerder worden, vervalt de oorspronkelijke solidariteitsgedachte steeds meer tot een betalingsregeling met gescheiden termijnen, waarin een ieder betaalt wat hij 'kost'.

11 Het nakomen van verkiezingsbeloften om daarmee het vertrouwen van de burgers in de politiek te verbeteren is te realiseren door de politieke partijen financieel aansprakelijk te stellen voor eventuele contractbreuk met de kiezer.

12 Onderzoek naar onderhoud is onderhoudend.

Stellingen behorende bij het proefschrift:
Utilization of Respiratory Energy in Higher Plants
Requirements for 'Maintenance' and Transport Processes

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Abstract

Quantitative knowledge of both photosynthesis and respiration is required to understand plant growth and resulting crop yield. However, especially the nature of the energy demanding processes that are dependent on dark respiration in full-grown tissues is largely unknown. The main objective of the present study was to establish the identity and energy requirements of the most important of these (maintenance) processes, and to gain insight in methods of determining the rates and specific costs of these processes. Developing such methods is more important than obtaining data on the rates of maintenance processes for certain crops, as these rates are likely to vary as a function of e.g. the environmental conditions, developmental stage and species.

Leaf respiration rates of 15 potato cultivars (*Solanum tuberosum* L.) differed significantly (chapter 2). To examine whether growth and maintenance requirements differed, two cultivars were compared. After synchronizing their development, leaf protein content, shoot and leaf respiration, photosynthetic light response curves and the growth parameters (i.e. RGR, LAR, SLA, and LWR) were similar, thus excluding potential differences in growth and maintenance respiration. It was concluded that it is important to study the physiological cause of respiratory differences, before starting to select genotypes for low respiration rate.

Protein turnover is generally regarded as an important maintenance process. The component of dark respiration rate associated with overall protein turnover of tissues was quantified *in vivo* by the use of an inhibitor of cytosolic protein synthesis (chapter 3). The *in vivo* effect of this inhibitor was assessed by monitoring the inhibition of the induction of the ethylene-forming enzyme activity. The respiratory costs of protein turnover were maximally 17 - 35% of total respiration. The maximum degradation constants (i.e. K_d-values) derived for growing and full-grown leaves were up to 2.42×10^{-6} and 1.12×10^{-6} s^{-1}, respectively.

Nocturnal carbohydrate export is another process requiring respiratory energy. The potential contribution of the energy requirements associated with nocturnal carbohydrate export to *i)* the fraction of dark respiration affected by leaf nitrogen concentration and *ii)* the dark respiration of mature source leaves, was explored (chapter 4). The estimate of the specific energy cost involved in carbohydrate export (0.70 mol CO_2 [mol sucrose]$^{-1}$), agrees well with both literature data (0.47 to 1.26) and the theoretically calculated range for starch-storing species (0.40 to 1.20). Maximally 42 to '107'% of the effect of the leaf organic nitrogen concentration on the dark respiration of primary bean leaves, is ascribed to the energy costs associated with nocturnal export of carbohydrates. Total energy costs associated with export account on average for 29% of the dark respiration rate for various starch-storing species.

The respiratory energy requirements of maintaining ion gradients were quantified on plant roots (chapter 5). Combining the anion efflux rate (35 neq [g dry weight]$^{-1}$ s^{-1}) with literature

data on the specific costs of ion transport, suggests that energy costs associated with re-uptake of ions may account for up to 66% of the total respiratory costs involved in (an)ion influx. A value of 34% of the total respiratory costs involved in (an)ion influx was obtained if the net uptake rate was based on the relative growth rate observed for potato, and assuming phosphate and sulfate to be both 10% of nitrate in- and efflux. Comparison of relative values of the respiration of root and shoot is not useful, as in both tissues other processes add to the total.

Estimating the respiratory energy requirements of maintaining ion gradients is complicated by lack of knowledge on efflux kinetics. Therefore, efflux kinetics was studied, using a dynamic simulation model (chapter 6). Simulations showed that the overall efflux kinetics observed in the medium may differ significantly, even if actual efflux rates (and thus costs involved in maintaining ion gradients) in the simulations were equal. Similarly, the relative contribution of ions originally located in the apoplast, cytoplasm and vacuole of different cell layers to these efflux kinetics and the observed cumulative efflux originating from the symplast were different. All these differences were due to the presence or absence of an endodermis, different pathways involved in net uptake and different number of cell layers involved in efflux.

Integration of the available knowledge on maintenance, growth and uptake processes enabled to explain the respiration of potato roots. The costs calculated for protein turnover could explain total maintenance requirements (10.2 to 14.8 nmol O_2 [g DW]$^{-1}$ s^{-1}). It was deduced that overall costs for maintaining solute gradients (i.e. re-uptake balancing efflux) account for up to 33% of the overall costs of nitrate influx (i.e. 1/U is up to a factor 1.5 higher if efflux takes place). This agrees well with the results of chapter 5.

Index words - adaptation, ATP, bean, costs, carbohydrate export, cycloheximide, compartmental analysis, computer simulation, dark respiration, efflux, efflux kinetics, electrical conductivity, electrolyte leakage, energy costs, ethylene, ethylene-forming enzyme, growth, growth analysis, influx, ion gradients, ion uptake, leaf, leaf dark respiration, leaf protein turnover, leaves, maintenance, maintenance respiration, modelling, nitrogen concentration, *Phaseolus vulgaris* L., potato, protein biosynthesis, protein turnover, regulation, respiration, respiratory costs, re-synthesis, root, roots, *Solanum tuberosum* L., specific energy cost, synthesis.

aan mijn ouders
aan Ingrid

Toets alles en behoudt het goede

1 Thessalonicenzen 5 vers 21

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Contents

Account

Parts of this thesis have been included in part or entirely, in the following publications:

Chapter 2 Bouma, T. J., Spitters, C.J.T. and De Visser, R. 1992. Variation in leaf respiration rate between potato cultivars: effect of developmental stage. - In Molecular, Biochemical and Physiological Aspects of Plant Respiration (H. Lambers and L.H.W. van der Plas, eds), pp 515-522. SPB Academic Publishing, The Hague. ISBN 90-5103-079-7.

Chapter 3 Bouma, T.J., De Visser, R., Janssen, J.H.J.A., De Kock, M.J., Van Leeuwen, P.H. and Lambers, H. 1994. Respiratory energy requirements and rate of protein turnover *in vivo* determined by the use of an inhibitor of protein synthesis and a probe to assess its effect. - Physiol. Plant. 92: 585-594.

Chapter 4 Bouma, T. J., De Visser, R., Van Leeuwen, P.H., De Kock, M.J. and Lambers, H. 1994. The respiratory energy requirements involved in nocturnal carbohydrate export from starch-storing mature source leaves and their contribution to leaf dark respiration. - submitted.

Chapter 5 Bouma, T. J. and De Visser, R. 1993. Energy requirements for maintenance of ion concentrations in roots. - Physiol. Plant. 89: 133-142.

Chapter 7 Bouma, T. J., Broekhuysen, A.G.M. and Veen, B.W. 1994. Analysis of root respiration of *Solanum tuberosum* as related to growth, ion uptake and maintenance of biomass: a comparison of different methods. - submitted.

Chapter 1

General introduction

General background

In plant leaves, light energy is captured driving the photosynthetic reduction of CO_2 with H_2O to carbohydrates. This energy can become available again as ATP at a different time and/or place, by oxidizing these carbohydrates to CO_2 and H_2O in the respiratory process. The rate of respiration is determined by the metabolic activity of the tissue, and is a measure for the tissue's energy (i.e. ATP) consumption. Respiration can be observed as either the production of CO_2 or as O_2-consumption.

$$C_6H_{12}O_6 + 6\ O_2 \longrightarrow 6\ CO_2 + 6\ H_2O + (12_{alt}\text{ to }36_{cyt})\ ATP \qquad (1.1)$$

The subscripts 'alt' and 'cyt' indicate the range of ATP production resulting from the exclusive participation of the alternative and cytochrome pathway, respectively (Lambers 1985). Plant dry weight increment is largely the balance of photosynthetic gains and respiratory losses, which are up to 50% of the photosynthates produced daily (Lambers 1985). Therefore, detailed *quantitative* information of both processes is required for understanding plant growth and crop yield. The available knowledge of biochemistry, physiology, plant morphology and crop architecture enables accurate estimation of the photosynthetic rate and daily photosynthetic gains for relevant environmental conditions and to 'design' a favorable crop. Although the biochemical nature of respiration is known, present knowledge is insufficient to derive the respiration rate from the processes utilizing energy. That is, quantitative data on the nature, rate and costs of such processes are lacking. Such quantitative knowledge is needed to understand the relationship between assimilation and plant growth and for related scientific and agricultural applications like describing and predicting plant growth with process-based simulation models (e.g. SUCROS87; Spitters et al. 1989), studying competition e.g. between fast- and slow-growing species in nature (Poorter et al. 1991), or improving yield by breeding (e.g. Wilson 1975, 1982 as compared to Kraus et al. 1993). To investigate the possibilities of breeding for low respiratory costs, knowledge on the energy-requiring processes underlying respiration and the necessity of these processes is required.

Respiration as related to energy-requiring processes; a physiological model

Based on the processes that use the respiratory energy, respiration has been divided into the

functional components growth, maintenance (Pirt 1975, Thornley 1970), and - in the case of roots - (ion) uptake (Veen 1980). Growth, maintenance and uptake respiration are defined as the respiration associated with the synthesis of new biomass, the maintenance of existing biomass and the uptake of nutrients, respectively. The biochemical nature of the respiration (and all other processes involved), is identical for each of these components. The respiration rates for each of these three functional respiratory components (r) is the sum of the products of the rates of the processes involved (v_i) and their specific costs (e_i), defined as the respiration per unit activity (i.e. the overall specific costs for growth, maintenance and uptake are the respiration per gram growth, the respiration to maintain a gram existing biomass per second and the respiration per mol ions taken up, respectively).

$$r = \Sigma (v_i \times e_i) \qquad (1.2)$$

where i is the number of all processes involved (cf. Penning de Vries et al. 1974, Penning de Vries 1975). For growth and maintenance there are several experimental estimates of their overall specific costs (e.g. the data for shoots in Amthor 1984, Hansen and Jensen 1977, Mariko 1988, Szaniawski 1981, Szaniawski and Kielkiewicz 1982), whereas for ion uptake such experimental data are still limited to the monocots, maize (Veen 1980) and two *Carex* species (Van der Werf et al. 1988).

The overall specific costs for both growth and ion uptake may be theoretically derived from the individual processes involved, whereas for maintenance this is not yet completely possible (Penning de Vries 1975). The overall specific costs for growth are determined by the chemical composition of the new biomass and the various (thermodynamically most efficient) biosynthetic pathways involved to produce this biomass (Penning de Vries et al. 1974). Similarly, the overall specific costs involved in ion uptake may be calculated from the number of active root-membrane passages involved in ion uptake and the proton-ion and proton-ATP stoichiometries of these membrane passages (e.g. chapter 5). However, although it is well documented that 20 - 60% of the respiratory energy may be used for maintenance (Amthor 1984, Lambers 1985, Lambers et al. 1983[a], Penning de Vries 1975, Van der Werf et al. 1988), knowledge about the nature, specific costs and rate of the individual processes underlying maintenance is poor. That is, the identity of the energy-requiring maintenance processes, the efficiency of the biochemical pathways involved as well as their spatial and temporal activities are obscure. On the basis of biochemical information, Penning de Vries (1975) suggested that a major part (60 - 80%) of maintenance respiration may be needed for protein turnover and a smaller part for maintaining solute gradients. However, experimental evidence to support this contention is scarce.

Objectives

The general objective of this thesis is to obtain a better understanding of the rates and (specific) energy costs of the (maintenance) processes depending on respiration, especially in non-

growing tissues. This respiration might be expected to be mainly associated with maintenance. Present methods to determine maintenance respiration (for a review, see Amthor 1984, Lambers, 1985) are 'black-box' approaches that do not deal with the individual processes involved. In contrast to such 'black-box' approaches, in the present work the individual processes contributing to the maintenance component of respiration have been studied. The first objective is to quantify the relative importance of protein turnover and maintenance of ion gradients in terms of respiratory energy, as Penning de Vries (1975) suggested that these processes account for a major part of the maintenance component of dark respiration. Alternative methods have been used from those used for the existing data (e.g. the importance of protein turnover derived from double labelling experiments), to investigate the effect of different methods on the conclusions. When appropriate, experiments were combined with simulation studies. Maintenance processes do not necessarily account for the total dark respiration of non-growing tissues, especially not in leaves. Therefore, the second objective was to quantify the contribution of carbohydrate export to the dark respiration rate of non-growing leaves. The third objective was to obtain a better understanding and more data on the specific costs for ion uptake, as only two estimates were available at the start of this study. Finally, the knowledge on individual maintenance, growth and uptake processes obtained in the present study was integrated by comparison to the estimates of the specific costs of these processes obtained by regression.

For all processes studied, rates and thus overall costs might vary as a function of the environmental conditions, developmental stage and species. Therefore, insight in how to determine the rates and specific costs of the processes of interest, is more important than obtaining data on certain crops. The present quantitative values of certain species are used to explore whether the process is quantitatively important, so that its relation with the environmental conditions, developmental stage and species should be established in the future, or whether the process can be ignored.

Outline of the thesis

To establish the relative importance of different maintenance processes like protein turnover and maintaining ion gradients, it would be useful to have genotypes differing with respect to these traits. Preliminary (dark-decay) measurements showed large and reproducible varietal differences in (maintenance) respiration of potato leaves. Therefore, initially the cause of different dark respiration rates of two extreme cultivars (chapter 2) was analyzed. Subsequently, the relative importance of protein turnover, carbohydrate export and maintaining ion gradients in terms of respiratory energy was quantified separately, in the chapters 3, 4 and 5, respectively. The factors needed to quantify the energy requirements of an individual process are its rate and its specific energy costs. In the present study, experimental measurements on these factors were combined with theoretically derived values. The approaches applied are *i)* to establish the maximum contribution to respiration by blocking the process while measuring

respiration, and calculating the rate of the process using its theoretical specific costs (used for protein turnover; chapter 3), *ii)* to quantify the specific costs (theoretically and experimentally) and relative contribution to respiration by measuring both the process and (its effect on) the respiration under a range of conditions (used for carbohydrate export; chapter 4), and *iii)* to determine the maximum rate of the process and to calculate its contribution to the respiration using its theoretical specific costs (used for maintaining ion gradients; chapter 5). The required specific costs for protein turnover (chapter 3) were obtained as the sum of the costs of underlying processes and by a regression of leaf nitrogen over respiration, assuming the average protein turnover rate from the literature (De Visser et al. 1992). Theoretical values of specific costs involved in carbohydrate export (chapter 4) and maintaining ion uptake (chapter 5) were derived from the literature. Both present results and literature data suggested that a better understanding of efflux kinetics is the key to a better quantification of costs involved in maintaining ion gradients (chapter 5). Therefore, a simulation study was performed that visualizes the efflux kinetics from cell walls, cytosol and vacuoles at different locations in the root, and the factors that influence these efflux kinetics (chapter 6). To obtain a better understanding and more data of the specific costs for ion uptake, the respiration of potato roots was partitioned into functional components by multiple regression (chapter 7). This allowed the integration of knowledge on individual maintenance, growth and uptake processes by comparison to the (black box) estimates of the specific costs of these processes obtained by regression. Finally, the results and conclusions of the separate elements of this thesis are discussed and summarized (chapter 8).

Regarding the present objectives and the three approaches proposed, the respiratory energy (i.e. ATP) utilization has to be estimated from either the production of CO_2 or the consumption of O_2. In appendix 1, the advantages and disadvantages of both methods are compared in relation to nitrate reduction and the tissue studied (roots versus full-grown leaves).

Chapter 2

Variation in leaf respiration rate between potato cultivars: effect of developmental stage

with C.J.T. Spitters† and R. De Visser

Abstract - Leaf respiration rates of 15 potato cultivars (*Solanum tuberosum* L.) differed significantly both in the field and in the greenhouse, be it with a different ranking. The correlation between leaf protein content and earliness of the cultivars in the field, suggested that differences in respiration rate might be affected by the developmental stage. The relationship is not necessarily proportional, as the respiration rate and earliness did not correlate. To derive whether growth and maintenance requirements differed, the cultivars were compared under conditions that synchronized their development. Under these conditions, leaf protein content, shoot and leaf respiration, photosynthetic light response curves and the parameters of the growth analysis were similar, excluding differences in growth and maintenance respiration. It was concluded that differences in respiration rate do not necessarily originate from different energy costs of maintenance and/or growth costs. The outcome of selecting for low respiration rate cannot be predicted, unless the physiological cause is identified.

Abbreviations - Alc., cultivar Alcmaria; DW, dry weight; FW, fresh weight; LAR, leaf area ratio; LWR, leaf weight ratio; NAR, net assimilation rate; Pimp., cultivar Pimpernel; PPFD, photosynthetic photon flux density; RGR, relative growth rate; RWR, root weight ratio; SLA, specific leaf area; SWR, shoot weight ratio

Introduction

Although it is well-documented that a major part of photosynthates is used for maintenance of plant biomass (Amthor 1984, Lambers et al. 1983[a], Penning de Vries 1975), knowledge about the nature of the underlying processes is poor (chapter 1). Wilson (1982) showed that it is possible to select high-yielding ryegrass populations by screening for low respiration rate of mature leaves without knowing the underlying processes. However, more insight in the processes of maintenance respiration is required to *i)* understand plant and crop production and

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ii) develop new plant breeding criteria. Insight in the quantitative aspects of plant and crop production may form the basis of plant breeding.

For identification of the quantitatively most important maintenance processes it would be helpful to have varieties/cultivars/lines differing in (maintenance) respiration rate. Various potato cultivars are available which differ in earliness, a commonly used index for time of tuber formation/harvest (0 = late, 10 = early). For example, Alcmaria (index 8) is an early cultivar, whereas Pimpernel (index 3.5) is about 30 days later (Spitters et al. 1988). Preliminary measurements showed large varietal differences in leaf respiration rate of potato (by a factor two; F.W.T. Penning de Vries, pers. comm.). There may be several causes for these differences in respiration rate. General metabolic activity may differ due to different optima for temperature or other conditions. Alternatively, the efficiency of one or more of the many energy-consuming processes (e.g. growth, maintenance, ion transport) may be responsible.

We examined possible causes of the varietal differences in leaf respiration rate, hypothesizing that different energy requirements of the cultivars for growth and/or maintenance are involved.

Materials and methods

Plant material

Greenhouse and field experiments with 15 cultivars - For both the *greenhouse* experiment (March-May 1988) and the *field* experiment (April-June 1989), plants were grown from pre-sprouted tubers. Fifteen potato (*Solanum tuberosum* L.) cultivars were used: Alcmaria, Alpha, Bintje, Civa, Désirée, Katahdin, Kennebec, Krostar, Maritta, Multa, Pimpernel, Saturna, Spunta, Veenster and Woudster. In the *greenhouse* experiment, plants were grown on recirculating aerated Steiner nutrient solution (Steiner 1966). The growth conditions in the *greenhouse* were : 18 ± 1°C constant, and 65% RH.

Growth room experiments with 2 cultivars - Plants of the potato (*Solanum tuberosum*) cvs Alcmaria and Pimpernel, were propagated by in vitro culture, and grown on recirculating aerated Hoagland solution (macro nutrients 1/2 strength according to Hoagland and Snyder [1933] and micro nutrients 1/2 strength according to Lewis and- Powers [1941] with iron as Fe[III]-EDTA). Entangling of roots of neighbouring plants was prevented, and a uniform supply of nutrients was provided. Other conditions were: 12 hours 240 μmol m^{-2} s^{-1} PPFD (fluorescent light from Philips TLD 36W/54 and incandescent light from Pope 60W in a ratio 12:1) for growth and 6 h incandescent light (Pope 60W) for day length extension to prevent tuber induction. The temperature was maintained at 18 ± 2°C, and the air humidity at 70%. Photosynthesis was measured, and a growth analysis was carried out. Fresh and dry weight of roots, shoots and leaves and leaf area were determined.

Respiration measurements

Dark respiration rate was measured (1) manometrically with a Warburg apparatus (*greenhouse, field and growth room*-experiments) and (2) by infra-red gas analysis (IRGA; Analytical Development Co. Ltd., type 225-2B-SS) as described by Louwerse and Van Oorschot (1969) with computerized control and data processing (*growth room*-exp.). Measurements were made on leaf discs, in the dark at 20°C, on humid filter paper to prevent desiccation. O_2 uptake was monitored for one hour after equilibration. No dark-decay measurements were performed, as this kind of measurements causes artefacts (data not shown). By IRGA, respiration rates were determined of leaves of intact plants or for whole shoots under growth conditions. Warburg and IRGA measurements showed no significant differences. Dark respiration rates were expressed on a dry weight basis.

Leaf respiration rate was measured on the youngest fully-expanded leaves of two months old plants (*greenhouse* and *field*-exp.) and 19, 34 and 44 days old plants (*growth room*-exp.). The respiration rate of whole shoots was determined on 34 days old intact plants (*growth room*-exp.). The *greenhouse* and *field* experiment were performed during 14 days in May 1988 and June 1989, respectively.

As it is known that previous illumination may influence respiration (Azcón-Bieto and Osmond 1983), care was taken to sample the leaves from the different cultivars in *greenhouse* and *field* at the same time of the day throughout the experimental periods.

Protein determination

Leaf tissue was boiled for 1 hour. Protein was precipitated overnight in 2.5% (w v^{-1}) TCA. The N content of the precipitate was determined by Kjeldahl analysis. Protein content of the leaf was calculated by multiplying the N content of the precipitated protein with 6.25.

Growth analysis and Statistics

For growth analysis the following equations were used:

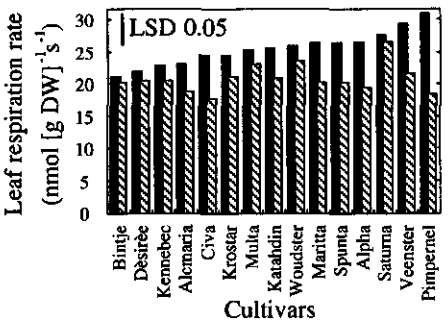
$$W_2 = W_1 \times \exp(RGR \times [t_2-t_1]) \quad (2.1)$$

$$RGR = NAR \times LAR \quad (2.2)$$

$$LAR = SLA \times LWR \quad (2.3)$$

where W_i is plant weight at time t_i, RGR is the relative growth rate (mg plant [g plant]$^{-1}$ day^{-1}), NAR is the net assimilation rate (g [m^2 leaf]$^{-1}$ day^{-1}), LAR is the leaf area ratio (m^2 leaf [kg plant]$^{-1}$), SLA is the specific leaf area (m^2 leaf [kg leaf]$^{-1}$), and LWR is the leaf weight ratio (g leaf [g plant]$^{-1}$).

Cultivar differences were tested in *greenhouse* and *field* experiments by analysis of variance (ANOVA) and regression analysis. In the *growth room* experiments, cultivar differences were tested by Student's t-test (95%-probability intervals, unless indicated otherwhise).



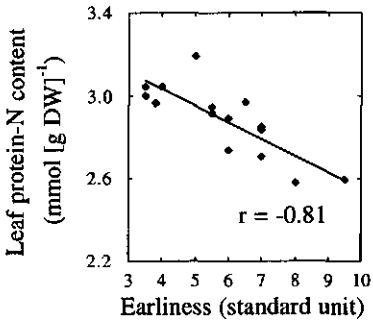
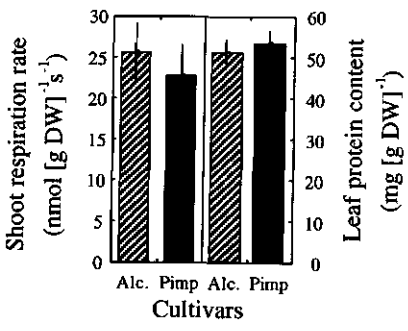


Fig. 2.1 (left) Dark respiration rates (nmol O_2 [g DW]$^{-1}$ s^{-1}) of fully expanded leaves of 15 potato cultivars grown in the (■) greenhouse in 1988 and (▨) field in 1989 with n = 6 and 8, respectively. The cultivars are ranked in order of ascending respiration rate in the greenhouse. LSD stands for least significant difference.

Fig. 2.2 (right) Relationship between leaf protein content and earliness of 15 potato cultivars grown in the field.



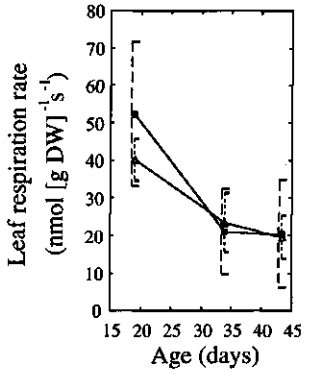


Fig. 2.3 (left) The respiration rate of shoots (nmol CO_2 [g DW]$^{-1}$ s^{-1}) and the protein content of fully expanded leaves of 34 days old, intact potato plants of cv. Alcmaria (▨) and cv. Pimpernel (■) (n = 5). Bars indicate 95%-probability intervals.

Fig. 2.4 (right) Respiration rate (nmol CO_2 [g DW]$^{-1}$ s^{-1}) of fully expanded leaves of the cvs Alcmaria (- - ▲ - -) and Pimpernel (—■—). Plants of various ages (19, 34 and 44 day old plants) grown under controlled conditions (n = 4 or 5). Bars indicate 95%-probability intervals.

Results

Greenhouse and field experiments with 15 cultivars

Figure 2.1 shows significant differences in leaf respiration rates between 15 potato cultivars in the *greenhouse* experiment ($P < 0.01$; $n = 6$). In accordance with preliminary measurements (F.W.T. Penning de Vries, pers. comm.), Alcmaria (early cv) and Pimpernel (late cv) showed different leaf respiration rates ($P < 0.01$; $n = 6$). We found a negative trend between respiration rate on a dry weight basis and earliness ($r = -0.49$; $P < 0.07$).

Also in the field experiment significant differences in leaf respiration rate between the 15 cultivars existed (Fig. 2.1; $P < 0.01$; $n = 8$). However, respiration rates differed from those of the plants grown in the *greenhouse*. No significant correlation between respiration on a dry weight basis and earliness was found ($r = -0.32$). In contrast to the preliminary results and *greenhouse* experiment, Alcmaria and Pimpernel did not show a significantly different respiration rate (Fig. 2.1; $n = 8$).

Leaf senescence is generally accompanied by a decrease in protein content. So, leaf protein is an indicator of leaf age/developmental stage (Field 1983, Field and Mooney 1983, Friederich and Huffaker 1980, Osman and Milthorpe 1971). The relation between the protein content of the leaves of plants grown in the *field* and earliness is shown in Fig. 2.2. In the *field* experiment the protein content of the leaves showed a negative correlation with earliness ($P < 0.001$); the earliest cultivars (high numbers) had the lowest protein content. Thus, in this experiment cultivars appeared to develop in accordance with the earliness-index of the Dutch National Lists of Varieties.

Significant cv-differences in leaf respiration rate are not constant but change with growth conditions (Fig. 2.1). These differences between the cultivars might be partially due to differences in developmental stage. The correlation between leaf protein content and earliness support the idea of different developmental stages in our experiments (Fig. 2.2). However, differences in growth and maintenance respiration cannot be excluded. Therefore we examined the respiration rate of plants grown under controlled conditions in a *growth room*, where developmental rate can be regulated by manipulating the growth conditions. Tuber induction can be delayed by high nitrate nutrition and long day-lengths (Bodlaender 1963, Lorenzen and Ewing 1990, Sattelmacher and Marschner 1978). The cultivars selected for the growth room experiments, Alcmaria and Pimpernel, strongly differed in leaf respiration rate in both the preliminary and the greenhouse experiments. Growth analysis was performed *i)* to trace the causes of possible differences in respiration rate and *ii)* to check whether developmental differences occurred.

Growth room experiments with two cultivars

Alcmaria and Pimpernel showed similar shoot respiration rates (95%-probability intervals; Fig. 2.3). Fully expanded leaves of young plants (19 days old) of both cultivars had a significantly higher rate of respiration than the older (34 and 44 day old) plants (99%-probability intervals; Fig. 2.4). However, leaves of the same age had similar respiration rates in both cultivars (90%-

probability intervals; Fig. 2.4).

The cultivars showed similar light responses of photosynthesis (Fig. 2.5), leaf protein content (34 days old plants; 95%-probability intervals; Fig. 2.3) and RGR (Fig. 2.6). There were also no differences in the SWR , RWR, SLA, LWR and the calculated LAR (Tab. 2.1).

Discussion and conclusions

The relative variation in the leaf respiration rate between the potato cvs (Fig. 2.1) is of a similar magnitude as found between *Lolium perenne* populations by Wilson (1982). A quantitative comparison of our respiration measurements with values in the literature is difficult to interpret, because respiration rate strongly depends on the measuring conditions (e.g. temperature), and respiration rates are expressed on various bases (g DW, g FW or m^2 leaf) even though plant morphology (e.g. SLA, percentage dry matter) is influenced by growth conditions. Recalculated respiration rates of the potato cultivars on a leaf area basis are for both the *field* experiment (0.7-1.1 μmol O_2 m^{-2} s^{-1}; average SLA 30 m^2 kg^{-1}) and *greenhouse* experiment (0.4-0.6 μmol O_2 m^{-2} s^{-1}; average SLA 50 m^2 kg^{-1}) quantitatively of a similar magnitude as the leaf respiration rates of 8 plant species mentioned by Lambers et al. (1983[b]; 0.6-1.2 μmol O_2 m^{-2} s^{-1}).

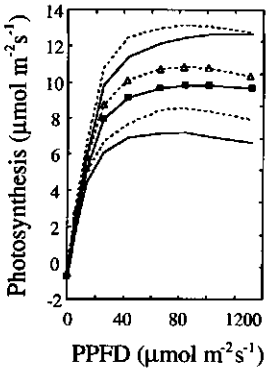


Fig. 2.5 (left) Light response curves of photosynthesis (μmol CO_2 [m^2 leaf]$^{-2}$ s^{-1}) of cvs Alcmaria (- - Δ- -) and Pimpernel (—■—). Lines without points indicate 95%-probability intervals.

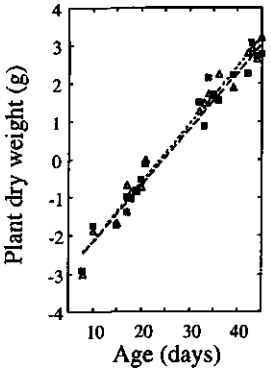


Fig. 2.6 (right) Dry matter accumulation of plants of the cultivars Alcmaria (- - Δ- -) and Pimpernel (—■—). Note that the Y-axis is a natural log (ln) scale The RGR is the slope of the line, being 0.153 for Alcmaria and 0.149 g g^{-1} day^{-1} for Pimpernel, respectively.

Table 2.1 Growth parameters of cvs Alcmaria and Pimpernel, with 95%-probability intervals; n = 11 to 18. The RGR is calculated from the data in Fig. 2.6.

Cultivar :	Alcmaria	Pimpernel
measured:	mean	mean
LAR (m^2 [kg DW plant]$^{-1}$)	26.5 ± 1.7	25.9 ± 1.4
SLA (m^2 [kg DW leaf]$^{-1}$)	47.0 ± 3.5	46.1 ± 2.7
LWR (kg DW leaf [kg DW plant]$^{-1}$)	0.57 ± 0.03	0.56 ± 0.02
SWR (g DW shoot [g DW plant]$^{-1}$)	0.82 ± 0.02	0.80 ± 0.02
RWR (g DW roots [g DW plant]$^{-1}$)	0.17 ± 0.01	0.18 ± 0.01
RGR (g DW [kg DW]$^{-1}$ day^{-1})	153	149
calculated:		
NAR (g m^{-2} day^{-1})	5.8	5.8

The results of the growth analysis show that development is identical for Alcmaria and Pimpernel when grown under long day conditions in a *growth room*. No tuber induction or flower initiation occurred during the experiment. Respiration measurements on leaves (Fig. 2.4) and shoots (Fig. 2.3) of both cultivars were also similar under these growth conditions. Table 2.1 shows that RGR and LAR (measured or calculated from the SLA and LWR) are similar for both cultivars. This results in a similar NAR (RGR = NAR x LAR). The NAR is the difference of leaf gross photosynthesis and total plant respiration (i.e. day plus night). As can be seen in Fig. 2.5, the cultivars have similar light responses of leaf photosynthesis. Therefore we conclude that at a similar light interception, due to identical LAR, SLA and LWR and irradiance, total plant respiration rates of both cultivars must be equal during the experimental period. This is in agreement with the respiration measurements on both leaves and intact shoots (Figs 2.4 and 2.3).

In case of a similar total respiration rate, differences in maintenance and growth respiration between the two cultivars can only exist if a higher growth respiration is accompanied by lower maintenance costs or vice versa for one of the cultivars. Differences in growth respiration are not likely, as the cultivars have similar leaf protein contents (Fig. 2.3), one of the most abundant, energetically important compounds (Penning de Vries et al. 1974). Furthermore, in the growth room experiment no differences in respiration rate were found for *i)* full-grown leaves (34 and 44 day old plants; Fig. 2.4) and *ii)* shoots consisting of both growing and fully developed parts (Fig. 2.3). It is not clear why the respiration rate of the fully expanded leaves of 19 day old plants is higher than the respiration rate of similar leaves in 34 and 44 day old plants (Fig. 2.4). Nevertheless, again no differences between the cultivars existed. Therefore, we conclude that differences in growth and/or maintenance respiration between the cultivars

Alcmaria and Pimpernel can be excluded, as long as they are in the same stage of development.

The varietal differences in respiration rate measured on plants grown in the *greenhouse*- and *field*, are probably related to different developmental stages of the cultivars, caused by the interaction between genotype and environmental conditions. It cannot be excluded that differences in respiration for growth and maintenance are absent for all cultivars. Also, energy costs for maintenance and/or growth may depend on the growth conditions. To answer such questions, a detailed study of growth, development, and respiration with various cultivars under various conditions is needed.

Differences in respiration rate do not necessarily originate from different energy costs of maintenance and/or growth. Therefore the outcome of selecting for low respiration rate cannot be predicted. In order to select for low maintenance costs, identification of the processes and a direct method of selection are required.

Chapter 3

Respiratory energy requirements and rate of protein turnover in vivo determined by the use of an inhibitor of protein synthesis and a probe to assess its effect

with R. De Visser, J.H.J.A. Janssen, M.J. De Kock, P.H. Van Leeuwen and H. Lambers

Abstract - Protein turnover is generally regarded as a major maintenance process, but experimental evidence to support this contention is scarce. Here we quantify the component of dark respiration rate associated with overall protein turnover of tissues *in vivo*. The effect of an inhibitor of cytosolic protein synthesis (cycloheximide, CHM) on dark respiration was tested on a cell suspension from potato (*Solanum tuberosum* L.) and quantified on leaf discs of expanding and full-grown primary leaves of bean (*Phaseolus vulgaris* L.). The *in vivo* effect of CHM on protein biosynthesis was assessed by monitoring the inhibition of the induction of the ethylene-forming enzyme (EFE) activity. The present method yields the energy costs of turnover of the total pool of proteins irrespective their individual turnover rates. Average turnover rates were derived from the respiratory costs and the specific costs for turnover.
Inhibition of respiration by CHM was readily detectable in growing-cell suspensions and discs of expanding leaves. The derived respiratory costs of protein turnover in expanding leaves were maximally 17 - 35% of total respiration. Turnover costs in full-grown primary leaves of bean amounted to 17 - 21% of total dark respiration. The maximum degradation constants (i.e. K_d-values) derived for growing and full-grown leaves were up to 2.42×10^{-6} and 1.12×10^{-6} s^{-1}, respectively.

Abbreviations - ACC, 1-amino-cyclopropane-1-carboxylic acid; BA, N^6-benzyladenine; CHM, cycloheximide; EFE, ethylene-forming enzyme; HEPES, N-[2-hydroxylethyl] piperazine-N'-[2-ethanesulfonic acid]; MES, 2-[N-morpholino]ethanesulfonic acid; NAA, 1-naphtaleneacetic acid; RER, relative ethylene production rate; SHAM, salicylhydroxamic acid; for other abbreviations see Table 3.2.

Introduction

The relation between respiration rate and protein content and its physiological basis are not fully understood. A positive correlation between the rate of leaf respiration and leaf protein content

has been found (e.g. De Visser et al. 1992, Lambers et al. 1989, Pons et al. 1989), but not always (Byrd et al. 1992 and references therein). It has been hypothesized that the energy costs of protein turnover, defined as a cycle of degradation and 're-synthesis', may explain the positive relationship between respiration and protein content (De Visser et al. 1992, Lambers et al. 1989, Pons et al. 1989), as both protein degradation (Vierstra 1993) and (re-)synthesis (De Visser et al. 1992 and references therein) are energy dependent. The energy costs of protein turnover were estimated at 30 - 60% of leaf dark respiration, both by experimental and theoretical analyses (De Visser et al. 1992), whereas Byrd et al. (1992) concluded these costs to be of minor importance. A thorough testing of the hypothesis is hampered by the absence of a more direct method to determine the energy costs of protein turnover *in vivo*. Also, *in vivo* estimates on the average protein turnover rates are hard to obtain. Our objective is to introduce an experimental method alternative to the approach of De Visser et al. (1992), for estimating the energy requirements associated with overall *in vivo* protein turnover in plant tissues, irrespective the turnover rates of individual proteins. From this value the average protein turnover rate is derived.

Energy costs of protein turnover may be estimated from the decrease in respiration rate after complete inhibition of cytosolic protein synthesis, in combination with a test to quantify the *in vivo* action of the inhibitor. Such an approach seems adequate since *i)* at least 80% of the energy costs of protein turnover is associated with the re-synthesis of proteins (Tab. 3.1, cf. De Visser et al. 1992), *ii)* plant proteins, including most of the mitochondrial (Leaver and Gray 1982) and chloroplast proteins (Ellis 1981, Mullet 1988), are predominantly encoded by the nucleus and synthesized on cytosolic ribosomes and *iii)* the method is independent of the turnover rates of individual proteins. Here we examine whether this approach can be used *in vivo*, assuming similar protein turnover rates in the light and dark. The application of the inhibitor is optimized and validated by examining the induction of ethylene-forming enzyme (EFE) activity as a probe. The respiratory efficiency (i.e. P/O_2-ratio) is determined both in the absence and presence of the inhibitor. Estimates of turnover costs in leaves are compared with the theoretical inhibitor-effect on respiration.

Materials and methods

Plant material

Bean (*Phaseolus vulgaris* L. cv. Berna) was used because of the similar growth rate of its two primary leaves. Plants were grown from seeds on tap water in a climate room. Temperature was maintained during day and night at 20 ± 2°C, and the relative humidity at 75%. During germination, seeds were covered with humid filter paper and transparent plastic for 5 days. Seedlings received 165 μmol m^{-2} s^{-1} PAR fluorescent light (Philips TLD 36W/33) during 16 h per day. After 7 to 9 days, the seedlings were transplanted to pots filled with 0.5 l aerated Hoagland solution (macro nutrients 1/2 strength according to Hoagland and Snyder [1933] and micro nutrients 1/2 strength according to Lewis and Powers [1941] with iron as Fe(III)-

Table 3.1 The expected effects of CHM on processes of protein turnover and on respiration rate (E_{CHM}; mol ATP [mol peptide bond]$^{-1}$). The sum of the costs of all processes, or of synthesis processes only, are the specific energy costs [mol ATP (mol peptide bond)$^{-1}$] of protein turnover (E_{sp}), and protein synthesis (E_s), respectively. The listing of the processes involved is according to De Visser et al. (1992).

Process	CHM-effect	Specific energy costs (mol ATP [mol peptide bond]$^{-1}$)	
		Low	High
Protein biodegradation	-	1.0	2.0
Protein biosynthesis:			
Amino acid activation	+	2.0	2.0
Error correction (by aminoacyl-tRNA synthetases)	+	5.0	8.0
Peptide bond formation and translocation	+	2.0	2.0
Tool maintenance	+	1.0	1.0
Signalling sequences	+	0.6	1.9
Amino acid turnover	-	0	3.5
Post-translational processing			
Methylation, acetylation, etc.	+	0.1	0.1
Phosphorylation	+	0.1	0.3
Protein turnover (E_{sp})		11.8	20.8
Protein synthesis (E_s)		10.8	18.8
Expected CHM effect (E_{CHM})		10.8	15.3

EDTA). Light conditions were 16 h 600 µmol m^{-2} s^{-1} PAR SON-T light (Philips, 400W). The effect of cycloheximide on the activity of the alternative, non-phosphorylating pathway, was, due to technical constraints, determined on plants grown at a daylength of 14 h at 170 µmol m^{-2} s^{-1} PAR fluorescent light (Philips TL-D-HF).

The time of maturity of the primary leaves was determined from the time course of dry and fresh weight and leaf area. To reduce variation, leaf expansion of individual primary leaves was also monitored non-destructively, with a video camera (SONY AVC 3250 CE, Japan) and monitor (SONY PVM 90 CE, Japan) connected to an integrator (TFDL nr 720314,

Wageningen, The Netherlands).

A cell suspension was obtained from callus of leaves of potato (*Solanum tuberosum* L. cv. Alcmaria). The nutrient medium contained MS macro- and micro-elements (Murashige and Skoog 1962), MS vitamins, 88 mM sucrose, 555 µM inositol, 27 µM NAA, 444 nM BA and 5 g l^{-1} agar in case of callus culture. Weekly, 9 ml of cells of a seven days old growing suspension culture were transferred to 40 ml fresh medium in a 250 ml Erlenmeyer flask closed with a cotton plug. The flasks were kept on an orbital shaker at 20°C under continuous 10 µmol m^{-2} s^{-1} PAR fluorescent light (Philips TLD 36W/33). Growth of the cells was measured after Gilissen et al. (1983).

Assessment of the in vivo action of cycloheximide

Cycloheximide (CHM), is known to block total cytosolic protein synthesis (Lewin 1990). CHM also inhibits the induction of ethylene-forming enzyme (EFE) activity by its substrate, 1-amino-cyclopropane-1-carboxylic acid (ACC; De Visser and Spencer 1986, Philosoph-Hadas et al. 1986). This induction of EFE activity was used as a probe to establish the *in vivo* response of protein synthesis to CHM. Whether this inhibition of EFE activity is due to blocked *de novo* synthesis of either EFE itself (De Visser and Spencer 1986, Philosoph-Hadas et al. 1986) or some activator or regulatory protein, is not relevant to the present objective. Generally, ACC production limits *in vivo* ethylene biosynthesis, whereas EFE is largely constitutive (Yang 1980, Yang and Hoffman 1984). Therefore, EFE activity was measured as the ethylene production rate at saturating ACC concentrations.

We determined the lowest concentrations of ACC and CHM yielding maximal ethylene production and inhibition of protein synthesis, respectively. The optimal ACC concentration was determined from ethylene production during 8 h by both leaf discs and detached intact leaves of bean (17 to 20 days old). This ACC concentration was used to establish the dose-response relationship of CHM and protein synthesis, with 11 and 13 days old leaf discs and 10 to 12 days old detached leaves and CHM concentrations up to 80 µM. Both ACC and CHM were dissolved in demineralized water. To minimize variation, various sets of leaf discs were made up from the same set of leaves. Ethylene production rates were expressed as percentage of the control without ACC or CHM (relative ethylene production rate; RER).

Directly after some of the respiration measurements on 10, 12 and 19 days old discs (see next section), the effect of CHM on protein biosynthesis was assayed on line, using the induction of EFE activity as a probe. Ethylene production by EFE at saturating ACC concentrations was determined from 2 to 21 h. CHM was added at the start of the respiration measurements, but no CHM was present in the medium during the following ethylene production measurements. Therefore, the effects of CHM on the induction of EFE activity were solely due to CHM taken up during the respiration measurements. No such measurements were done with the cell suspension, because the only purpose of this system was to test the detectability of CHM effects on respiration.

Ethylene was collected in a vessel, placed upside down in a petri dish with two concentric compartments. The outer compartment was filled with water, to obtain a gas-tight seal under

atmospheric pressure. The center compartment contained either leaf discs floating on the solution or detached leaves incubated with the basal end of the petiole submerged. Samples were taken with a 1 ml syringe from the headspace, through a septum in the glass vessel (500 ml gas volume). Ethylene was measured by gas chromatography (Packard model 427, Canberra, Australia) with a flame ionization detector (FID). Oven temperature was 220°C. The stainless steel column (length 2 m, ∅ 1/8 inch) contained aluminium oxide (80 mesh). The carrier gas was N_2.

Respiration and inhibition of protein synthesis

The activity of the alternative, non-phosphorylating pathway was determined both in the absence and in the presence of 80 µM CHM. Titration curves for salicylhydroxamic acid (SHAM, 1 M stock in 2-methoxyethanol) were made with a Clark-type oxygen electrode (Yellow Springs Instruments, Ohio, USA), to assess possible side effects such as stimulation of peroxidases or inhibition of the cytochrome path (Møller et al. 1988). Slices of primary leaves of bean of approximately 2 mm^2 were incubated in a buffer (pH 6.6; temperature 20°C) containing 10 mM MES, 50 mM HEPES and 0.2 mM $CaCl_2$ (Azcón-Bieto et al. 1983).

The effect of CHM on the oxygen uptake of discs of growing (10 - 12 days old) and full-grown (17 - 19 days old) leaves was measured manometrically during the normal dark period using a Warburg apparatus at 22°C. Discs (∅ 9.5 mm) were floating on demineralized water with 0 to 80 µM CHM. To avoid induction of senescence, ethylene accumulation was prevented by the presence of $KMnO_4$ in the side arm of the vessel. The activity of the alternative path was determined manometrically, on 2 mm^2 slices of 12 - 16 days old leaves floating on MES/HEPES buffer (pH 6.6), either half-way or at the onset of the light period. SHAM was added from the side arm after approximately 3 hours; there was no $KMnO_4$ present in these short-term measurements. The stock solutions of SHAM and the buffer were the same as those used for the titrations. The CHM effect on the respiration rate of growing (2, 3 or 7 days after transfer to a new medium) cell-suspensions of potato was also measured manometrically. CHM (20 µM) was added to one half of the cells, 5 h after start of the measurement. The non-sterile conditions were no problem, as the respiration rates of medium without cells remained negligible (data not shown). All respiration rates on leaves were expressed on initial leaf area, to avoid effects of changes in weight of the discs during long incubations.

Calculations of costs of protein turnover and synthesis

Maintenance and synthesis of the protein pool involves various processes, most of which are inhibited by CHM (Tab. 3.1). CHM affects the energy utilization in cytosolic turnover and synthesis of all proteins, irrespective their individual rates and pathways of turnover and synthesis. The theoretically expected CHM-inhibition of the respiration rate for *i)* cytosolic protein turnover (m_{CHM}; mol O_2 m^{-2} s^{-1}), *ii)* net cytosolic protein synthesis for growth (s_{CHM}; mol O_2 m^{-2} s^{-1}) and *iii)* total protein synthesis (r_{CHM}; mol O_2 m^{-2} s^{-1}), were calculated by equation 3.1 (after De Visser et al. 1992), 3.2 and 3.3, respectively. Note that in full-grown

leaf, s_{CHM} equals zero so that r_{CHM} is equal to m_{CHM}.

$$m_{CHM} = (E_{CHM} \times K_d \times N_t) / [(P/O_2) \times N_p \times SLA] \quad (3.1)$$

$$s_{CHM} = [E_{CHM} \times (RGR_l \times N_t + RN_tC)] / [(P/O_2) \times N_p \times SLA] \quad (3.2)$$

$$r_{CHM} = m_{CHM} + s_{CHM} \quad (3.3)$$

where E_{CHM} is the sum of the energy costs of the processes blocked by CHM (mol ATP [mol peptide bond]$^{-1}$), K_d is the degradation constant of protein turnover (s^{-1}), N_t is the protein-N in the leaf (mol N-protein [g DW]$^{-1}$), P/O_2 is the ATP/O_2 ratio of oxidative phosphorylation (mol ATP [mol O_2]$^{-1}$), N_p is the average nitrogen content of leaf protein (1.26 mol N-protein [mol peptide bond]$^{-1}$; De Visser et al. 1992), SLA is the specific leaf area (m^2 [g DW]$^{-1}$), RGR_l is the relative growth rate of the primary leaf (g DW [g DW]$^{-1}$ s^{-1}) and RN_tC is the rate of change of the protein-N in the leaf (mol N-protein [g DW]$^{-1}$ s^{-1}). The resultant of (RGR x N_t) + RN_tC gives an estimate of net protein synthesis.

As CHM inhibits only part of the processes in Table 3.1, its effect on respiration is an underestimation of the overall costs of protein turnover and net protein synthesis. The actual overall respiratory costs of protein turnover (m_p; mol O_2 m^{-2} s^{-1}) and net protein synthesis (s_p; mol O_2 m^{-2} s^{-1}) were obtained from the CHM inhibition of the respiration rate (m_{CHM} and s_{CHM}) using equations 3.4 and 3.5, respectively. The overall respiratory cost of *total* synthesis (r_p; mol O_2 m^{-2} s^{-1}) is the sum of m_p and s_p (eq. 3.6).

$$m_p = m_{CHM} \times (E_{sp}/E_{CHM}) / (1 - F_{org}) \quad (3.4)$$

$$s_p = s_{CHM} \times (E_s/E_{CHM}) / (1 - F_{org}) \quad (3.5)$$

$$r_p = m_p + s_p \quad (3.6)$$

where, E_{sp} is the specific energy costs of protein turnover (mol ATP [mol peptide bond]$^{-1}$; Tab. 3.1), E_s is the specific energy costs of synthesis (mol ATP [mol peptide bond]$^{-1}$; Tab. 3.1) and F_{org} is the fraction turnover or synthesis in the organelles, i.e. mitochondria and chloroplasts. In full-grown leaves, s_{CHM} is zero so that r_p equals m_p.

We used the range of K_d values given in the literature (i.e. 0.31 x 10^{-6} to 6.02 x 10^{-6} s^{-1}; Barneix et al. 1988, Davies 1982, Dungley and Davies 1982, Eising and Gerhardt 1987) as input for some of our calculations. Tab. 3.1 (after De Visser et al. 1992) shows the processes expected to be affected by CHM and the range of possible values of E_{CHM}. CHM inhibits the peptidyl transferase on the 60S subunit of the cytosolic ribosomes which blocks protein elongation (Lewin 1990) and, thus, any polypeptide processing. F_{org} was estimated to be about 30% of overall protein synthesis (see discussion). Other parameters (i.e. RGR_l, N_t, RN_tC, P/O_2 and SLA) were derived from the present experiments. The symbols and abbreviations used are listed in Tab. 3.2.

Chemical determinations

Total nitrogen and NO_3^--nitrogen content (mg N [g DW]$^{-1}$) were determined by CHN analysis

Table 3.2 List of symbols and abbreviations used in the calculations of expected CHM effects on the respiration rate and overall respiratory costs of protein synthesis and turnover, with dimensions.

Abbreviation	Description	Unit
K_d	degradation 'constant' of protein turnover	s^{-1}
m_{CHM}	CHM-inhibited (maintenance) respiration rate for cytosolic protein turnover	mol O_2 m^{-2} s^{-1}
r_{CHM}	CHM-affected respiration rate for the total of cytosolic protein synthesis and turnover	mol O_2 m^{-2} s^{-1}
from literature		
E_{CHM}	sum of the specific energy costs of the processes blocked by CHM	mol ATP (mol peptide bond)$^{-1}$
E_s	specific energy costs of protein biosynthesis	mol ATP (mol peptide bond)$^{-1}$
E_{sp}	specific energy costs of protein turnover	mol ATP (mol peptide bond)$^{-1}$
F_{org}	fraction of protein turnover or synthesis in organelles	-
N_p	average nitrogen content of leaf protein	mol N-protein (mol peptide bond)$^{-1}$
measured		
N_t	protein-N content of the leaf	mol N-protein (g DW)$^{-1}$
P/O_2	ATP/O_2 ratio of oxidative phosphorylation	mol ATP (mol O_2)$^{-1}$
RGR_l	relative growth rate of a leaf	g DW (g DW)$^{-1}$ s^{-1}
RN_tC	relative change rate of leaf protein-N	mol N-protein (g DW)$^{-1}$ s^{-1}
SLA	specific leaf area	m^2 (g DW)$^{-1}$
calculated		
m_p	overall (maintenance) respiration rate for protein turnover, in cytosol and organelles	mol O_2 m^{-2} s^{-1}
r_p	overall respiration rate for the total of protein synthesis and turnover in cytosol and organelles	mol O_2 m^{-2} s^{-1}
s_{CHM}	CHM-inhibited respiration rate for net cytosolic protein synthesis	mol O_2 m^{-2} s^{-1}
s_p	overall respiration rate for net protein synthesis, in cytosol and organelles	mol O_2 m^{-2} s^{-1}

on a Heraeus CHN-rapid (Hanau, Germany) and a TRAACS 800 continuous flow system (Bran and Luebbe Analyzing Technologies, Elmsford, NY, USA), respectively. The organic N content, i.e. the difference between the total nitrogen content and NO_3^--nitrogen content, was taken as upper estimate of protein-N content in the leaf (N_t).

Statistics

All experiments (except those for the SHAM titrations) had a block design. For the titration curves with SHAM the standard error was calculated. In all other experiments, differences were tested by ANOVA, using the statistical package GENSTAT 5 (Payne 1987). LSD values were calculated only if the corresponding F-tests of ANOVA were significant (protected LSD method). LSD values were calculated for $P < 0.05$.

Results

Characteristics of primary leaves of bean

The primary leaves of bean grew exponentially from day 9 up to day 16 after germination, and started yellowing after day 20. Therefore, we selected leaves of 17 to 19 days old plants as full-grown primary leaves. The rate of change of the protein-N concentration in the leaf (RNtC) during growth till the early mature stage, was obtained by linear regression of N_t over the period of 10 to 14 days. Both N_t and the SLA decreased with increasing age of the primary leaves (Tab. 3.3).

Assessment of the in vivo action of cycloheximide

In order to determine the energy costs of protein turnover, the *in vivo* action of CHM was assessed using EFE as a 'reporter' protein. The induction of EFE-activity was maximal at 2 and 10 mM ACC for leaf discs and detached bean leaves, respectively (Fig. 3.1). CHM effectively

Table 3.3 Characteristics of primary bean leaves, used in calculating energy costs of protein turnover (eq. 3.1) and synthesis (eq. 3.2). RGR_l and RN_tC were calculated by linear regression of the dry weight and N_t, respectively, on plant age. Plant age was 9 to 16 and 10 to 14 days after germination, for RGR_l and RN_tC, respectively.

Characteristic	Unit	Values	
		Leaf type	
		Growing	Full-grown
Age plant	d	9 to 12	17 to 19
N_t	mmol N-protein (g DW)$^{-1}$	2.93 ± 0.11	2.02 ± 0.11
SLA	m^2 (g DW)$^{-1}$	0.046 ± 0.006	0.023 ± 0.002
RGR_l	g DW (g DW)$^{-1}$ day^{-1}	0.312 ($r^2 = 0.96$; n = 16)	
RN_tC	mmol N-protein (g DW)$^{-1}$ day^{-1}	-0.299 ($r^2 = 0.78$; n = 18)	

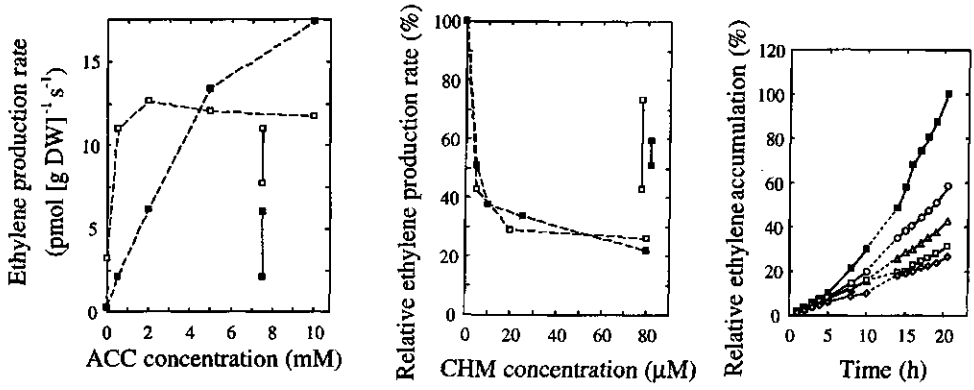


Fig. 3.1 (left) Ethylene production rate (pmol (g DW)$^{-1}$ s^{-1}) of leaf discs (open symbols) and detached leaves of bean (filled symbols), as dependent on external ACC concentration. LSD values are indicated by the vertical bars (n = 3 for both the leaf discs and detached leaves).

Fig. 3.2 (middle) Relative ethylene production rate of leaf discs (open symbols) and detached leaves (filled symbols) of bean, as dependent on the external CHM concentration. The discs and leaves were incubated in 2 and 10 mM ACC, respectively. LSD values are indicated by the vertical bars (n = 2 and 4 for leaf discs and detached leaves, respectively).

Fig. 3.3 (right) Ethylene production of primary leaves of bean in the absence (■) and presence of 5 (○), 10 (Δ), 25 (□) or 80 (◇) μM CHM. In all treatments 10 mM ACC was present and CHM was added at the start of the measurement. The 24 h time course of each treatment was obtained from different leaves for the various periods, separated by the dashed lines. Data from all leaves used in one 24 h time course (i.e. 4 treatments with two leaves per treatment) were expressed as a fraction of the control (0 μM CHM). The slopes of the lines give the relative ethylene production rates (n = 2).

inhibited this induction of EFE-activity (Fig. 3.2), which became only evident after more than 4 h following ACC addition (slopes in Fig. 3.3). At 80 μM CHM, no increase in EFE activity was found in detached leaves. In leaf discs 20 μM had a similar effect.

Respiration and inhibition of protein synthesis

In the presence of CHM, dark respiration of growing leaves was inhibited by approximately 20 to 34% of the control ($P < 0.05$; Fig. 3.4A). In full-grown leaves, CHM inhibition was at most 11% of the control ($P < 0.05$; Fig. 3.4B) and only present after 6.9 and 11 hours. SHAM blocked the alternative path (Fig. 3.5), but did not affect the respiration of 12 to 16 days old bean leaves in the presence or absence of 80 µM CHM (data not shown). Thus, the P/O_2-ratio was 6 and CHM did not cause a decrease in respiration by an effect on the efficiency of oxidative phosphorylation, assuming that the external NADH oxidation and the rotenone insensitive bypass are not operating (cf. Lambers 1985). The respiration rate of the cell suspension was inhibited by up to 37%, after addition of 20 µM CHM ($P < 0.05$; Fig. 3.6). This shows, similar to the data of discs of growing leaves, that the present method has adequate sensitivity to detect the respiratory costs of protein synthesis (involved in turnover), when the rate of synthesis is quantitatively important.

CHM supplied during the respiration measurements, was sufficient to block the induction of EFE-activity determined the first 6 h afterwards, for discs of both growing (10 and 12 days old) and full-grown (19 days old) leaves (data show a similar pattern as in Fig. 3.2). Thus, the absence of a CHM effect on respiration was not due to poor penetration during the respiration measurements.

Calculations of respiratory costs of protein turnover

For full-grown leaves, the theoretical decrease of the respiration rate after CHM treatment was calculated following equation 3.1 (input data in Tab. 3.3; in the absence of growth s_{CHM} equals zero and thus r_{CHM} equals m_{CHM}). This theoretical CHM effect varied by an order of magnitude, depending on the literature values of K_d and E_{CHM} (Tab. 3.4; derivation E_{CHM} in Tab. 3.1). Despite this variation, the CHM effect should be detectable as shown by a comparison of the theoretical values of m_{CHM} (Tab. 3.4) to both the lowest (0.06 µmol O_2 m^{-2} s^{-1}) and highest (0.09 µmol O_2 m^{-2} s^{-1}) just detectable difference between respiration rates (i.e. the LSD values in Fig. 3.4B). Furthermore, with the highest detection limit of respiration observed as input in equation 1 (s_{CHM} equals zero), K_d-values higher than 0.7×10^{-6} s^{-1} should cause a significant decrease of respiration (Tab. 3.5). The highest CHM inhibition of respiration observed yields a K_d of 1.1×10^{-6} s^{-1} (Tab. 3.5). These K_d-values are both within the range of literature values (Tab. 3.4). The present calculations show that non-significant CHM effects on the respiration rate of full-grown leaves cannot be ascribed to insensitivity of the method, but are due to relatively low turnover rates.

For growing leaves, the significance of the costs of protein turnover was examined by calculating m_{CHM} (eq. 3.3; Tab. 3.5), as the difference between the observed CHM effect (r_{CHM}; Fig. 3.4A) and the calculated s_{CHM} (eq. 3.2; Tab. 3.5). The K_d-values corresponding with this m_{CHM}, as derived from equation 3.1, are $\leq 2.42 \times 10^{-6}$ s^{-1} (Tab. 3.5). These estimates are within the range of literature values (values and references in Tab. 3.4; average $K_d = 2.6 \times 10^{-6}$ s^{-1}), also indicating that the sensitivity of the present method is adequate for detecting differences in respiration rate due to CHM inhibition of protein (re-)synthesis.

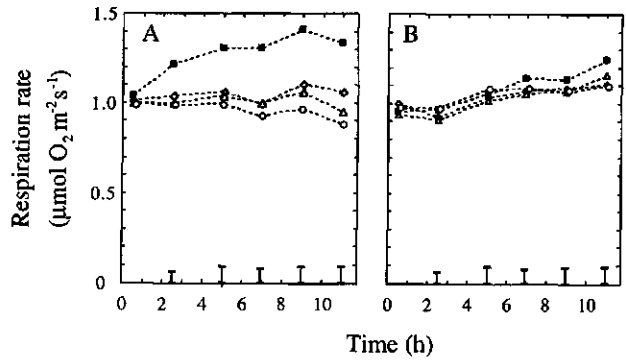


Fig. 3.4 Effect of the absence (■) and presence of 5 (○), 20 (Δ) or 80 (◇) μM CHM on the respiration rate of leaf discs of growing (A) and full-grown (B) primary bean leaves. CHM was added at the start of the measurement. LSD values, based on the combined data set of A and B (same residual variance), are given by the vertical bars at the bottom of the figure (n = 9 for both ages).

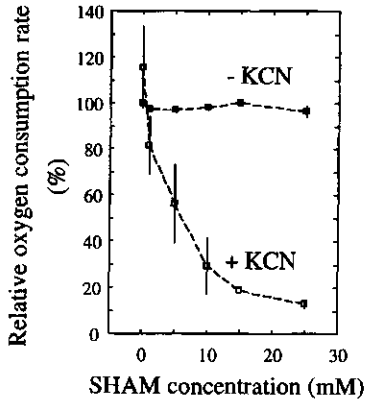


Fig. 3.5 (left) Response to SHAM of respiration of bean leaf slices. The absence and presence of KCN is represented by ■ and □, respectively. The standard error is given by a vertical bar, unless this value is smaller than the symbol size (n varies for different points from 1 to 10).

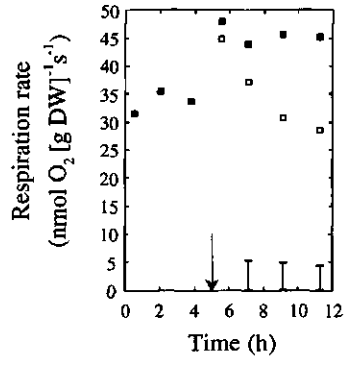


Fig. 3.6 (right) The effect of 20 μM CHM (□) on the respiration rate of cell suspensions compared to that of untreated cells (■). The time of CHM addition (5 h) is indicated by an arrow. LSD values are indicated by the vertical bars at the bottom of the chart (n = 16).

Table 3.4 Theoretical values of m_{CHM} (CHM-affected respiration for protein turnover), as dependent on protein degradation constants (K_d) and specific costs of protein synthesis as affected by CHM (E_{CHM}). Calculation is according to equation 3.1, for full-grown primary leaves of bean. The values of m_{CHM} significant in all measurements (i.e. values higher than largest LSD in Fig. 3.4B) are underlined.

K_d x 10^6 (s^{-1})	m_{CHM} (nmol O_2 m^{-2} s^{-1})		References
	E_{CHM} = 10.8	E_{CHM} = 15.3	De Visser et al. (1992)
0.306	38	54	Dungley and Davies 1982
0.389	49	69	Ibid.
1.113	139	197	Ibid.
2.265	284	402	Ibid.
2.377	298	422	Eising and Gerhardt 1987
3.241	406	575	Barneix et al. 1988
4.861	609	863	Davies 1982
5.961	748	1060	Eising and Gerhardt 1987

Table 3.5 Derivation of the degradation constants (K_d; using eq. 3.1) corresponding with the various maintenance respiration rates for protein turnover (m_{CHM}). For growing leaves, s_{CHM} was calculated according to equation 3.2. In full-grown leaves no net synthesis occurs (s_{CHM} = 0). m_{CHM} is the difference of r_{CHM} and s_{CHM} (eq. 3.3). The values of s_{CHM}, m_{CHM} and K_d are based on an E_{CHM} value of 10.8 or 15.3 mol ATP (mol peptide bond)$^{-1}$ (cf. Tab. 3.1 and De Visser et al. 1992) as indicated by the superscript a or b, respectively.

Leaf tissue	r_{CHM}	s_{CHM} (nmol O_2 m^{-2} s^{-1})	m_{CHM}	K_d x 10^6 (s^{-1})	Remark on the value of r_{CHM}
Growing leaves	260	221^a - 313^b	$-^b$ - 39^a	$-^b$ - 0.43^a	lowest observed effect (Fig. 3.4A)
	442	221^a - 313^b	129^b - 221^a	0.99^b - 2.42^a	highest observed effect (Fig. 3.4A)
Full-grown leaves	91	0	91	0.51^b - 0.73^a	highest detection limit (Fig. 3.4B)
	140	0	140	0.79^b - 1.12^a	highest observed effect (Fig. 3.4B)

Discussion and conclusions

Assessment of the *in vivo* action of cycloheximide

The optimal ACC (2 mM) and CHM (20 µM) concentrations for leaf discs, agreed with those used by Philosoph-Hadas et al. (1986; 3 mM ACC and 20 µM CHM for discs of tobacco leaves). The induction of EFE-activity by its substrate ACC became only evident after a lag-time of 4 h (cf. the slopes of the curves in Fig. 3.3). A period of 4 h is sufficient to enable induction of *de novo* protein synthesis (Lewin 1990). From the results presented in Figs 3.2 and 3.3 and from the observations following the respiration measurements (similar pattern as shown in Fig. 3.2), it is concluded that induction of EFE-activity is a useful probe for evaluating the *in vivo* action of CHM.

The effect of CHM on respiration

The respiration rate of leaf discs after approximately 5 h in the absence of CHM follows the trend we observed on primary leaves of intact bean plants: respiration (µmol O_2 m^{-2} s^{-1}) = -0.051 x age (days) + 2.11 (n = 31) for ages between 10 and 20 days. Therefore, leaf discs are considered representative of intact leaves.

CHM inhibits 80% of the energy costs involved in protein turnover (Tab. 3.1, cf. De Visser et al. 1992) of all nucleus-encoded proteins, irrespective their individual rates and pathways of turnover and synthesis. The fraction of protein synthesis in the organelles (F_{org}) was assumed to be 30%, because *i)* less than 10% of all the mitochondrial proteins are encoded by and synthesized in the mitochondria (Leaver and Gray 1982), and *ii)* ribulose bisphosphate carboxylase/oxygenase, which constitutes approximately 1/3 of total organic nitrogen in mesophyll cells (Dalling 1987), is for approximately 75% of its subunits encoded by DNA of the chloroplast (Ellis 1981, Mullet 1988) and *iii)* most of the other chloroplast proteins are encoded by the nucleus (Ellis 1981).

After correcting the *measured* CHM effect on respiration of growing leaves (r_{CHM}; Tab. 3.5) for all processes involved in protein synthesis and for the synthesis in the organelles by using equation 3.5, total costs of overall protein synthesis (including protein turnover when present) were up to 59% (i.e. r_p = 0.77 µmol O_2 m^{-2} s^{-1}) of total dark respiration in the absence of CHM (approximately 1.3 µmol O_2 m^{-2} s^{-1}; Fig. 3.4A). Similarly, overall theoretical costs of *net* protein synthesis of a growing leaf, without regarding turnover, were 24 to 42% (0.32 to 0.55 µmol O_2 m^{-2} s^{-1}; using equation 3.5 and s_{CHM} given in Tab. 3.5) of the respiration measured in the absence of CHM. Thus, using equation 3.6, m_p is found to be up to 17 to 35% of total respiration.

In full-grown leaves, the inhibition of respiration in the presence of CHM (6.9 and 11 h; Fig. 3.4B) occurred later than expected, as both ethylene production and respiration of growing leaves showed a CHM response after 4 and 2.5 hours, respectively. From the largest CHM effect observed in full-grown leaves (r_{CHM} equal to m_{CHM} as s_{CHM} is zero; 11%; Fig. 3.4B), the fraction of respiration associated with protein turnover in all cell organelles (m_p) was calculated to be up to 17 - 21% of leaf dark respiration rate (cf. eq. 3.4). These values represent an upper

limit, as *i)* the largest CHM effect was used in the calculation and *ii)* secondary effects cannot be excluded completely, despite the short-term measurements (see next paragraph on non-specific effects of CHM). From the relatively low values for the upper limit, we conclude that protein turnover does not require a major amount of respiratory energy in full-grown primary bean leaves. These low costs must be due to a relatively low turnover rate in this tissue, as it is unlikely that the specific costs of protein turnover (values in Tab. 3.1; after De Visser et al. 1992) have been overestimated.

The fraction of respiration associated with protein turnover obtained by the present approach, is in the lower part of the range of experimental values derived from relationships between respiration rate and protein-N (5 to 76%; after Tab. 2 in De Visser et al. 1992). This supports the contention that such a relationship between respiration rate and protein-N may be determined by more than the process of protein turnover alone. For example respiratory costs involved in nocturnal carbohydrate export might be higher in leaves with higher protein content (cf. De Visser et al. 1992). Also the theoretical estimates of the fraction of leaf respiration associated with protein turnover (De Visser et al. 1992) are somewhat higher than the present experimental values. For full-grown leaves, this is due to the higher turnover rate as assumed by these authors (K_d 1.39 x 10^{-6} s^{-1}), than calculated from our measured CHM inhibition of dark respiration rate (Tab. 3.5) For growing leaves, the differences might be attributed to the relative high overall dark respiration rates in our growing leaves (i.e. high value for 100%). Clarkson et al. (1992) observed 20 and 31% inhibition of the respiration of apical and basal parts of barley roots, respectively, after a 2-h incubation with CHM. A direct comparison of this relative CHM effect to the present results is hampered by the functional differences of leaves and roots. Assuming E_{CHM} to be 10.8 and 15.3, this respiratory inhibition would be explained by K_d values of 2.7 to 1.9 x 10^{-6} s^{-1}, respectively (i.e. assuming 10% dry matter, P/O_2 = 5 and N_t = 3). These values are within the range of experimental estimates in the literature (see Tab. 3.4), but relatively high compared to the K_d value of our tissue, especially for full-grown leaves (Tab. 3.5). In conclusion the present values for relative costs and rates of protein turnover are relatively low compared to literature data.

The relatively low values derived for the turnover rates (Tab. 3.5; especially for full-grown leaves) compared to literature values (e.g. Tab. 3.4, Clarkson et al. 1992), might be due to a discrepancy in the turnover rate between the light and dark period. Unfortunately, the present method does not allow the determination of the respiratory costs of protein turnover in the light. Therefore, the present values are based on the assumption that turnover rates are constant during the diurnal cycle. The differences between the results mentioned above, do not necessarily indicate that this assumption is incorrect. For example, the K_d-values that can be derived from the regression data in Tab. 2 in De Visser et al. (1992) when using an E_{sp} of 11.8 to 20.8 (i.e. 1.5 to 7.5 x 10^{-6} s^{-1}), are also based on measurements in the dark. There is clearly a need for more information concerning the diurnal pattern of protein turnover.

Evaluation of the action of cycloheximide

The induction of EFE activity proved to be a useful probe for evaluating the *in vivo* action of CHM. To justify the present experimental approach, primary effects of CHM on the energy transfer should also be excluded (Ellis and MacDonnald 1970). Primary effects of CHM on energy metabolism have been posed (Dheidah and Black 1976), be it on indirect evidence like the reaction time. On the other hand, several studies support the hypothesis that the effects of CHM on respiration are indirect. Renosto and Ferrari (1975) found that the mechanism of sulfate transport inhibition by CHM in plant tissue is a blocking of the synthesis of new proteins, not a disrupting of energy metabolism. A primary effect of CHM on energy supply was also excluded in studies with heterotrophic tobacco cells (Rennenberg et al. 1989). Clarkson et al. (1992) concluded from the absence of an effect of CHM on the uptake of Pi that the general effect of CHM on energy metabolism is minimal over a period of at least 4 to 8 h. Summarizing, CHM does not affect respiration by a primary effect in the present short-term experiments. This conclusion is supported by the absence of an effect of CHM on the respiration rate of full-grown leaves (Fig. 3.4B). Inhibition of metabolic processes by CHM due to the disappearance of the proteins involved, is unlikely in the present short-term measurements. Even so, the implication of such indirect effects would be that the upper limits of energy costs for protein turnover are overestimated, and thus does not affect the present conclusions.

Conclusions

The ACC-induction of EFE activity is a useful probe to calibrate and verify the *in situ* action of inhibitors of protein synthesis like CHM. Protein turnover in a *growing primary leaf* of bean requires maximally 17 - 35% of total dark respiration. Turnover costs in *full-grown primary leaves* are relatively low (i.e. at most 17 - 21% of total dark respiration), but in the range of theoretical values (De Visser et al. 1992).

The present method offers perspectives to determine respiratory costs associated with protein synthesis and turnover for different species in various environments. Other inhibitors of cytosolic protein synthesis (e.g. an inhibitor of transcription like cordycepin; Galling 1982), might also be applied as long as they do not have primary effects on respiration. It should be noted that a disadvantage of transcription inhibitors is a continuing translation from existing mRNA's, which will result in a delayed effect on protein synthesis.

Chapter 4

The respiratory energy requirements involved in nocturnal carbohydrate export from starch-storing mature source leaves and their contribution to leaf dark respiration

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Abstract - The present study explores the potential contribution of the energy requirements associated with nocturnal carbohydrate export to *i)* the fraction of dark respiration correlating with leaf nitrogen concentration (upper limits) and *ii)* the dark respiration of mature source leaves (upper and lower limits). To this end, we determined the nocturnal carbohydrate-export rates from leaves with an optimal nitrogen supply, and the correlation between the nitrogen concentration and the dark respiration of leaves. The specific energy costs of carbohydrate export from starch-storing source leaves were determined both experimentally and theoretically. The present estimate of the specific energy cost involved in carbohydrate export as obtained by linear regression (0.70 mol CO_2 [mol sucrose]$^{-1}$), agrees well with both literature data obtained by different methods (0.47 to 1.26) and the theoretically calculated range for starch-storing species (0.40 to 1.20). The conversion of starch in the chloroplast to sucrose in the cytosol is a major energy-requiring process. Maximally 42 to '107'% of the slope of the relationship between respiration rate and organic nitrogen concentration of primary bean leaves, may be ascribed to the energy costs associated with nocturnal export of carbohydrates. Total energy costs associated with export were derived as the product of the specific costs of carbohydrate export and the export rates, either measured on full-grown (primary) leaves of potato and bean or derived from literature. These export costs account on average for 29% of the dark respiration rate in starch-storing species. We conclude that nocturnal carbohydrate export is a major energy-requiring process in starch-storing species.

Abbreviations - summarized in Table 4.2.

Introduction

The rate of dark respiration of leaves is often positively correlated to their nitrogen concentration (e.g. De Visser et al. 1992, Makino and Osmond 1991, Pons et al. 1989, Van der Werf et al. 1994). Lambers et al. (1989) hypothesized that this relationship is associated

with energy costs for *i)* export of carbohydrates and *ii)* protein turnover. In starch-storing species, nocturnal carbohydrate export involves the transformation of starch in the chloroplast to sucrose in the cytosol and the subsequent phloem loading of this sucrose. Phloem loading of sucrose is an active process (Gardner and Peel 1972, Geiger 1975 and references therein). Protein turnover involves the synthesis of new proteins not resulting in an increase of the protein concentration. De Visser et al. (1992) calculated that protein turnover can account for the relationship between dark respiration and nitrogen concentration of leaves. However, there are reasons to hypothesize that in addition to protein turnover, the energy cost involved in export of carbohydrate also contributes to this relationship. Firstly, the costs of protein turnover may vary, in proportion to the degradation constants, by a factor of 20 (e.g. references in. chapter 3 and De Visser et al. 1992). Secondly, photosynthesis and thus carbohydrate availability increase with the nitrogen concentration of the leaf, due to a higher photosynthetic capacity (e.g. Evans 1983, 1989, Makino and Osmond 1991, Nátr 1975). If the energy costs involved in export of carbohydrate do not affect the relationship between dark respiration and nitrogen concentration of leaves, these costs might still contribute significantly to the overall dark respiration of leaves. For example, the positive correlation between the rate of leaf dark respiration and the level of irradiation during the preceding photoperiod (Irving and Silsbury 1988, Mullen and Koller 1988) has been ascribed to the energy costs for the export of carbohydrates (Irving and Silsbury 1988).

The aim of the present study is to assess the potential contribution of the energy costs of nocturnal carbohydrate export from mature source leaves to the fraction of dark respiration correlating with leaf nitrogen concentration and to the overall dark respiration rate of leaves grown with an optimal nitrogen supply. To this end, we determined *i)* the relationships between nitrogen concentration of leaves and both leaf dark respiration and photosynthesis, *ii)* nocturnal carbohydrate-export rates from leaves with an optimal nitrogen supply, and *iii)* the specific costs of carbohydrate export.

Materials and methods

Plant material and growth conditions

Experiments were carried out with youngest full-grown leaves of potato (*Solanum tuberosum* L. cv. Alcmaria) and primary leaves of bean (*Phaseolus vulgaris* L. cv. Berna). Potato plants were propagated by *in vitro* culture (chapter 2) and bean plants were grown from seeds (chapter 3). Growth conditions and objectives of the individual experiments are summarized in Table 4.1. Plants were grown in a nutrient solution containing macro-nutrients at 1/2 strength according to Hoagland and Snyder (1933) and micro-nutrients at 1/2 strength according to Lewis and Powers (1941) with iron as Fe(III)-EDTA.

Table 4.1 The parameters derived and growth conditions in the present experiments. For potato, the youngest full-grown (YFG) leaves were used. The abbreviations used for the different light types are: d = natural daylight in the greenhouse, F & I = fluorescent light (Philiphs TLD36W/54) and incandescent light (Pope 60 W) in a ratio of 12 : 1, H = HPI light (Philiphs HPI/T 400W). The relative humidity was 70 - 75%.

parameters	experiment	species	leaf age	light			temperature
			(days)	period (h)	PPFD (μmol m^{-2} s^{-1})	type	(°C)
α	b1 and b2	bean	17 - 20	16	500	H	21
α	b3	bean	21 - 24	16	500	H	21
E_e & C_e	P1	potato	YFG	14 or 9		d & H	20
C_e	P0	potato	YFG	12 or 18	240	F & I	18
C_e	b4	bean	14 - 17	16	240	F & I	21
				16	500	H	21
C_e	b5	bean	17 - 20	16	500	H	23

The correlation of photosynthesis and dark respiration with leaf-nitrogen concentration (α)

The effect of the leaf organic nitrogen concentration on the dark respiration (α; nmol CO_2 (mmol N_{org})$^{-1}$ s^{-1}) was described by a linear model:

$$R_d = \alpha \times N_{org} + R_r \qquad (4.1)$$

where R_d is the dark respiration (nmol CO_2 m^{-2} s^{-1}), N_{org} is organic nitrogen concentration (mmol organic nitrogen m^{-2}) and R_r is a constant rest component of respiration (nmol CO_2 m^{-2} s^{-1}). The relationship between photosynthesis and nitrogen concentration was described by a similar equation. Respiration and photosynthesis rates were measured under growth conditions (Tab. 4.1), on primary leaves of 17 to 20 (experiments b1 and b2) and 21 to 24 (experiment b3) days old bean plants. A range of leaf-nitrogen concentrations was obtained by daily supply of nitrogen at different relative nitrate addition rates (RAR) to aerated N-free nutrient solution (RAR = 0.05, 0.085, 0.115 and 0.15 day^{-1}; after Ingestad 1979). In this solution, calcium and potassium were available at half the normal strength, as $CaCl_2$ and KH_2PO_4 or KCl, respectively. Nitrate was added as $Ca(NO_3)_2$ and KNO_3 in the ratio present in the standard nutrient solution.

Table 4.2 List of abbreviations. If indicated in the text that the values are expressed on a dry weight basis, the m^{-2} should be substituted by $(g\ DW)^{-1}$ in the units given below.

abbreviation	description	unit
α	slope of the relationship of respiration versus organic nitrogen concentration	nmol CO_2 $(mmol\ N_{org})^{-1}$ s^{-1}
a_e	fraction of α explained by carbohydrate export only	-
C_e	(observed) carbohydrate export rate	nmol sucrose m^{-2} s^{-1}
E_e	specific energy costs for carbohydrate export	mol CO_2 $(mol\ sucrose)^{-1}$ or, mol ATP $(mol\ sucrose)^{-1}$
N_{org}	organic nitrogen concentration	mmol m^{-2}
ΔN_{org}	range of organic nitrogen concentrations underlying α	mmol m^{-2}
P	photosynthesis rate	nmol CO_2 m^{-2} s^{-1}
RAR	relative addition rate	day^{-1}
R_d	(observed) dark respiration rate	nmol CO_2 m^{-2} s^{-1}
R_e	dark respiration rate associated with carbohydrate export	nmol CO_2 m^{-2} s^{-1}
R_r	constant rest component of respiration	nmol CO_2 m^{-2} s^{-1}

Experimental estimation of the specific energy costs for carbohydrate export (E_e)

The specific costs for carbohydrate export (E_e; mol CO_2 $[mol\ sucrose]^{-1}$) were estimated by analyzing the linear regression of dark respiration rate (R_d; nmol CO_2 m^{-2} s^{-1}) on the nocturnal carbohydrate export rate (C_e; nmol sucrose m^{-2} s^{-1}):

$$R_d = E_e \times C_e + R_r \qquad (4.2)$$

where R_r is a constant rest component of respiration (nmol CO_2 m^{-2} s^{-1}). Respiration was measured on full-grown potato leaves with different carbohydrate export rates, assuming constant costs for other processes. Carbohydrate export was varied by changing the root temperature (*i.e. changing the sink activity; cf. Verkleij and Challa [1988] and references therein*). In a preliminary experiment (P0), root temperature and leaf respiration rate were correlated, but E_e could not be estimated as the variation in carbohydrate concentration between potato leaves of a single plant was larger than the decrease due to carbohydrate export. In the next experiment (P1), nocturnal export rates were derived as the decrease in specific dry weight of samples of the youngest full-grown leaves on plants with root temperatures changed from 7.5 to 20°C and vice versa, after subtracting the carbohydrates respired. In these leaves, net

export of amino acids was assumed to be negligible. Nocturnal respiration rates of these leaves were determined simultaneously, at a constant leaf temperature. Leaf discs were sampled during the dark period from the leaf fraction outside the respiration cuvette.

Theoretical estimation of the specific energy cost for carbohydrate export (E_e)

The specific cost for carbohydrate export (E_e) is calculated from the fraction of the carbohydrates exported that originates from starch (f_{st}), the specific energy costs of the conversion of starch in the chloroplast to sucrose in the cytosol (E_{st}; mol CO_2 [mol sucrose]$^{-1}$) and the specific costs of sucrose loading into the phloem (E_{pl}; mol CO_2 [mol sucrose]$^{-1}$).

$$E_e = f_{st} \times (E_{st} + E_{pl}) + (1 - f_{st}) \times E_{pl} \quad (4.3)$$

In starch-storing species, the nocturnal carbohydrate export originates for 70 - 100% from starch (i.e. f_{st} = 0.7 to 1.0; e.g. data in Caspar et al. 1985, Fondy and Geiger 1982, Grange 1985, Kalt-Torres et al. 1987). The remaining carbohydrates may originate from e.g. sucrose in the cytosol. Carbon moves to the cytosol from the chloroplast as *i)* triose-phosphate, *ii)* hexose, or as *iii)* the disaccharide maltose (Beck 1985, Servaites et al. 1989, Stitt 1990). All pathways from starch in the chloroplast to sucrose in the cytosol require 3 ATP per sucrose, except when maltose is degraded by maltose phosphorylase (i.e. E_{st} = 2 ATP per sucrose; Beck 1985). These values are based on the assumption that hexoses and maltose cross the chloroplast envelope passively, down their concentration gradient resulting from the continuous biochemical conversion. In the present species, phloem loading is expected to occur apoplasticly (Van Bel 1992, 1993 and pers. com.). The specific costs associated with apoplastic phloem loading (E_{pl}) are 1 ATP per sucrose in plasma-membrane vesicles (Slone and Buckhout 1991). Thus, E_e ranges from 2.4 to 4 mol ATP (mol sucrose)$^{-1}$.

Measurements of carbohydrate export (C_e) and simultaneous respiration rates (R_d)

Nocturnal carbohydrate export rates (C_e; nmol sucrose m^{-2} s^{-1}) were determined on bean leaves, which accumulate significant amounts of starch (Plaut et al. 1987). Our treatments to manipulate carbohydrate export rates were insufficient to obtain a range suitable for estimating E_e.

In experiment b4, dark respiration was monitored during three successive parts (2.67 h) of the normal dark period (8 h), using different plants for each period (Tab. 4.1). At the end of each period, the leaf used for the respiration measurements and the control leaf of each plant were harvested. Export rates during the three dark periods were derived from the decrease in carbohydrate concentration between succeeding periods, after subtraction of the carbohydrates respired.

In experiment b5, the shoot was pruned just above the primary leaves, and a metal cooler (4 °C) was placed around the stem directly under the primary leaves, at the start of the light period. The leaf temperature was kept constant. The light period preceding the respiration measurements was 2 or 16 h. At the onset of the dark period, one leaf per plant was harvested,

and stem coolers were removed. Respiration was followed on the remaining leaf, which was harvested 2 h later. Dark export rates were derived from the differences in carbohydrate concentration between the leaves, after subtracting the carbohydrates respired.

Calculations on the contribution of the energy costs of carbohydrate export to α and R_d

The fraction of the effect of the leaf organic nitrogen concentration on the dark respiration (α) that may be explained by the energy costs involved in carbohydrate export (a_e) was calculated from the specific costs for export (E_e; mol CO_2 [mol sucrose]$^{-1}$) and the decrease in carbohydrate export rate (ΔC_e; nmol sucrose m^{-2} s^{-1}) at decreasing organic nitrogen concentration (ΔN_{org}; mmol organic nitrogen m^{-2}):

$$a_e = (E_e \times \Delta C_e / \Delta N_{org}) / \alpha \qquad (4.4)$$

The upper limit of a_e was calculated by estimating ΔC_e as the carbohydrate export rate at optimal nitrogen supply (C_e; nmol sucrose m^{-2} s^{-1}), i.e. assuming a zero export rate at the lowest organic nitrogen concentration.

The respiration involved in carbohydrate export (R_e; nmol CO_2 m^{-2} s^{-1}) was obtained from the specific costs for carbohydrate export (E_e; mol ATP [mol sucrose]$^{-1}$), and the carbohydrate-export rates (C_e; nmol sucrose m^{-2} s^{-1}) determined on youngest full-grown leaves of potato (experiments P0 and P1) and primary leaves of bean (experiments b4 and b5) with an optimal nitrogen supply:

$$R_e = E_e \times C_e \qquad (4.5)$$

Analysis of leaf development

In bean, expansion of individual primary leaves was monitored non-destructively, with a video camera (SONY AVC 3250 CE, Japan) and monitor (SONY PVM 90 CE, Japan) connected to an integrator (TFDL nr 720314, Wageningen, the Netherlands), to establish the full-grown stage of the leaf. For potato, the youngest full-grown leaves were selected, using older leaves as a reference.

Dark respiration and photosynthesis measurements

Dark respiration and photosynthesis were measured on leaves of intact plants by infra-red gas analysis (IRGA; Analytical Development Co., type 225-2B-SS, Hoddesdon, UK) as described by Louwerse and Van Oorschot (1969) with computerized control and continuous data processing. To enable reliable determination of nocturnal CO_2-production, flow rates were low ($\geq$ 0.12 m^3 s^{-1}) and leaf area was large (25 to 64 cm^2). During these dark respiration measurements, leaf temperature was closely regulated by the air temperature, as there was no increment due to light interception. The enclosure of the leaf in the respiration cuvette had no effect on the concentration of total reducing soluble carbohydrates and starch in the leaf (data not shown).

Chemical analyses

Plant material was dried at a temperature of 70°C, in an oven with forced ventilation. Total soluble carbohydrates were extracted by 10 minutes boiling in demineralized water, which does not affect cell walls. Reducing monosaccharides were determined in the extract by titration with sodium thiosulfate (AOAC, 1984). The total soluble carbohydrates were obtained by hydrolyzing the extract by boiling in 0.05 M H_2SO_4 (i.e. 0.25 ml 5 M H_2SO_4 in 25 ml extract), for 30 minutes. The resulting reducing monosaccharides were determined by titration (AOAC, 1984). To determine starch, the plant material was extracted twice with 40% ethanol (15 minutes, 20°C) to remove low molecular weight carbohydrates. The residue was boiled for 1 h at 125°C at 0.13 MPa. Starch was enzymatically hydrolyzed during 1 h at 60°C by amylglucosidase containing negligible sugar contamination. Subsequently, the glucose formed was determined by titration (AOAC, 1984). Starch concentration was expressed in mg CH_2O $(g\ DW)^{-1}$.

The organic nitrogen concentration (N_{org}) of leaves of various ages was determined as the difference between the total nitrogen and NO_3^--nitrogen. Total nitrogen and NO_3^--nitrogen were determined by CHN analysis on a Heraeus CHN-rapid (Hanau, Germany) and a TRAACS 800 continuous flow system (Bran and Luebbe, Germany), respectively.

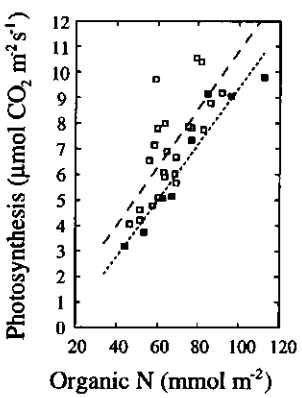
Statistics

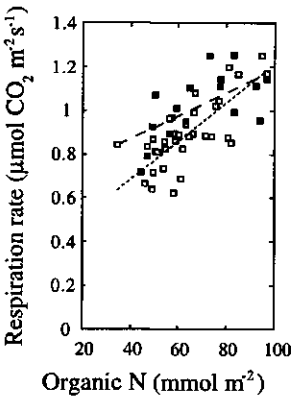
The statistical package GENSTAT 5 (Payne 1987) was used for the linear regression analysis.

Results

The correlation of photosynthesis and dark respiration (α) with leaf-nitrogen concentration

Photosynthesis correlated strongly with the organic nitrogen concentration of the primary leaves of bean (Fig. 4.1). The slope of the regression lines (0.11 μmol CO_2 (mmol organic $N)^{-1}\ s^{-1}$) was similar for both the 17 to 20 days and 21 to 24 days old leaves, whereas the intercepts were -0.64 and -1.74 μmol $CO_2\ m^{-2}\ s^{-1}$, respectively. A correlation of photosynthesis and organic nitrogen concentration has generally been found for several species, whereas a relationship between respiration and organic nitrogen concentration is less often studied (references in introduction). The present regression of respiration on organic nitrogen concentration for 17 to 20 days and 21 to 24 days old primary leaves (eq. 4.1), also yielded a strong correlation (Fig. 4.2), both on a leaf area and a leaf dry weight basis (Tab. 4.3).





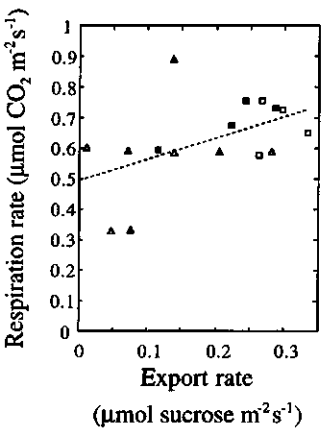


Fig. 4.1 (left) Relationship between the photosynthesis rate and the organic nitrogen concentration (N_{org}) for 17 to 20 (□) and 21 to 24 (■) days old primary leaves of bean. The lines indicate the regression equations: $P = 0.11 \times N_{org} - 0.64$ ($r = 0.72$; $df = 21$) and $P = 0.11 \times N_{org} - 1.74$ ($r = 0.96$; $df = 6$). Each symbol represents an independent measurement on an individual plant at growth conditions (Tab. 4.1). Different nitrogen concentrations were obtained by nitrogen supply at different relative addition rates.

Fig. 4.2 (middle) Relationship between the respiration rate and the organic nitrogen concentration for 17 to 20 (□) and 21 to 24 (■) days old primary leaves of bean. The lines indicate the regression equations: $R_d = 8.8 \times N_{org} + 331$ ($r = 0.73$; $df = 30$) and $R_d = 5.3 \times N_{org} + 655$ ($r = 0.62$; $df = 14$) with the slope (α) and the intercept (R_r; cf. eq. 4.1). Each symbol represents an independent determination on an individual plant at growth conditions (Tab. 4.1). Different nitrogen concentrations were obtained by nitrogen supply at different relative addition rates.

Fig. 4.3 (right) Relationship between the respiration rate and the carbohydrate export for full-grown leaves of potato. Roots were transferred from 20 to 20°C (□), 20 to 7.5°C (■), 7.5 to 20°C (Δ) and 7.5 to 7.5°C (▲). The dashed line indicates the regression equation: $R_d = 0.70 \times C_e + 0.49$ ($r = 0.498$; $df = 14$). Each symbol represents an independent determination on an individual plant.

Measurements of carbohydrate export (C_e) and simultaneous respiration rates (R_d)

For both potato and bean, the carbohydrate concentration in the leaf decreased mainly due to export, whereas the effect of respiration was at least a factor of two lower (Tab. 4.4). The strong decrease in carbohydrate concentration in experiment b4 can at least be partly ascribed to growth, as up to day 17 the primary leaves of bean continued to expand (data not shown). For this reason, in experiment b4 the export rate may have been overestimated. In all other experiments leaves did not grow.

Specific energy costs for carbohydrate export (E_e); experimental and theoretical estimates

Linear regression of the respiration rate on carbohydrate export rate (eq. 4.2) yielded a value of 0.7 $\pm$ 0.2 mol CO_2 (mol sucrose)$^{-1}$ for the specific cost of carbohydrate export (Fig. 4.3 derived from experiment P1). The theoretical estimate of these specific costs (E_e) ranges from 2.4 to 4 mol ATP per mol sucrose (calculation in Materials and Methods). Regarding the values generally observed for the ATP/O_2-ratio (6 to 4; e.g. Tab. 2 in Lambers et al. 1983[b]) and the RQ (1 to 1.2 but most often close to 1; Yemm 1965) of full-grown leaves, this equals a range of E_e from 0.4 to 1.2 mol CO_2 per mol sucrose. Thus, although the correlation in Fig. 4.3 is not strong, the value of E_e agreed well with theoretical estimates.

Table 4.3 The fraction of α (i.e. the effect of leaf organic nitrogen on the dark respiration) that can be explained by carbohydrate export rates (a_e), for a range of 50 to 100 mmol organic N m^{-2} in bean leaves. To obtain the upper limit of a_e, we used specific cost for carbohydrate export (E_e) of 1.2 mol CO_2 per mol sucrose. All regressions were significant (r from above to below was 0.73, 0.82, 0.62 and 0.53; df is 30 and 14 for 17-20 [exp. b1 plus b2] and 21-24 [exp. b3] days old leaves, respectively).

age	regression of leaf respiration versus organic nitrogen (cf. eq. 4.1)			a_e (cf. eq. 4.4)
	basis	α	R_r	upper limit
(days)		(nmol CO_2 [mmol org N]$^{-1}$ s^{-1})	(nmol CO_2 [m^2 or g DW]$^{-1}$ s^{-1})	
17 - 20	m^2	8.8	331	0.54
	g DW	11.3	3.6	0.42
21 - 24	m^2	5.3	655	0.89
	g DW	4.4	13.2	1.07

Table 4.4 The percentage dark respiration explained by the cost for carbohydrate export for potato (P0 and P1) and bean (b4 and b5). R_e was calculated according to equation 4.5, using the theoretically derived range of the specific cost for carbohydrate export of 0.4 to 1.2 mol CO_2 per mol sucrose. To express the rate of respiration and carbohydrate export measured in the preliminary potato experiment (P0) on a leaf area basis, we used a specific leaf area of 47 m^2 per (kg DW)$^{-1}$ (chapter 2). The decrease in carbohydrate concentration due to export was calculated as the difference between the total decrease and respiratory losses. The export rate calculated in experiment b4 (*) might have been overestimated, as leaves were still expanding.

experiment	treatment	dark respiration		decrease in carbohydrate concentration		
		total (R_d) (nmol CO_2 m^{-2} s^{-1})	export (R_e) (%)	total (nmol sucrose m^{-2} s^{-1})	export (%)	respiration (%)
P0		379	7 - 20	96	67	33
P1	20 -> 20	676	17 - 51	343	84	16
	20 -> 7.5	688	12 - 37	272	79	21
	7.5 -> 20	526	9 - 26	158	72	28
	7.5 -> 7.5	602	8 - 23	168	70	30
b4	HL	1156	18 - 54*	616	84*	16
	LL	719	18 - 55*	392	85*	15
b5	2 h light	1072	8 - 23	296	70	30
	16 h light	1022	7 - 22	272	69	31
average		760	12 - 35	290	76	24

Calculations of the contribution of the energy costs of carbohydrate export to α: upper limits

The highest fraction of the effect of the leaf organic nitrogen concentration on the dark respiration (α) that can be explained by respiratory energy costs associated with carbohydrate export (a_e) was calculated with equation 4.4, as described in the materials and method. The decrease in nocturnal carbohydrate export rate (ΔC_e) was taken to be equal to the export rate observed in experiment b5 (i.e. 197 nmol sucrose m^{-2} s^{-1}; after Tab. 4.4), for a decrease of the organic nitrogen concentration (ΔN_{org}) with approximately 50 mmol m^{-2} (data values in Figs 4.1 and 4.2). Table 4.3 shows the upper limits of a_e, based on the highest estimate of the specific costs of carbohydrate export (E_e = 1.2 mol CO_2 [mol sucrose]$^{-1}$). When using the

experimentally derived estimate of E_e (0.7 mol CO_2 [mol sucrose]$^{-1}$), the value for a_e ranges from 0.24 to 0.31 and 0.52 to 0.63, for 17-20 days old and 21-24 days old leaves, respectively. We conclude that carbohydrate export may account for a significant part of the effect of the leaf organic nitrogen concentration on the dark respiration (α), in the present tissue (a_e in Tab. 4.3).

Calculations on the contribution of the energy costs of carbohydrate export to R_d: upper and lower limits

Combining the theoretically calculated range of the specific costs for carbohydrate export (E_e = 0.4 - 1.2 mol CO_2 [mol sucrose]$^{-1}$) with the observed carbohydrate export rates (C_e; cf. eq. 4.5), leads to the conclusion that export costs account for approximately 7 to 51% of the observed dark respiration rates (R_e in Tab. 4.4). When using the experimentally derived estimate of E_e (0.7 mol CO_2 [mol sucrose]$^{-1}$), the average value of this fraction is 20%. Thus, carbohydrate export can account for a significant part of R_d.

Discussion and conclusions

Specific energy costs for carbohydrate export (E_e)

Estimates of the specific cost for carbohydrate export (E_e) are required to calculate the limits of the contribution of the energy costs of nocturnal carbohydrate export from mature source leaves to *i)* the effect of the leaf organic nitrogen concentration on the dark respiration (α) and *ii)* the overall nocturnal dark respiration rate (R_d). The estimate of E_e obtained by linear regression (0.7 mol CO_2 [mol sucrose]$^{-1}$; cf. eq. 4.2) was within the theoretically calculated range (0.4 - 1.2 mol CO_2 [mol sucrose]$^{-1}$). Table 4.5 compares the present estimates of E_e to the values in the literature. These literature values were obtained by (1) linear regression of respiration rate versus carbohydrate export rate, (2) dividing the export rate by the total respiration rate or (3) dividing the export rate by the respiration rate corrected for maintenance respiration as estimated by the dark-decay method. Method 2 yields an upper limit of the specific costs of export, as all respiration is ascribed to carbohydrate export. Method 3 lacks power, as it is largely unknown which processes underlie the respiration determined by the dark-decay approach. In the present experiment P1, we used method 1. The sometimes observed asynchronous time course of the respiration and export rates during the dark period (Grange 1985), might be seen as a possible objection against method 1 (Hendrix and Grange 1991). Apparently, the activities of the various energy-requiring processes in the leaf do not always have the same time pattern during the dark period. Even the starch degradation and the phloem loading do not necessary occur synchronous. However, as long as the overall nocturnal energy demand of all processes other than carbohydrate export remains constant between treatments, it is valid to use average export and respiration rates. In our opinion, this is best achieved when the environmental conditions of all source leaves studied are similar (i.e. previous illumination period and light intensity, relative air humidity, temperature and CO_2-concentration). Therefore, we varied the export (and

respiration) rates of potato leaves by manipulating root temperatures. The average export and respiration rate of Grange (1985) agree with present experimental observations and the fitted regression line.

Table 4.5 Theoretical and experimental estimates of the specific costs for carbohydrate export in starch-storing species. The literature values were expressed on an ATP-basis using a combination of the P/O_2-ratio and RQ of either 4 and 1.2 or 6 and 1, respectively; these combinations give the maximum range. The specific cost of export based on Grange (1987) was derived by regression, using his unpublished respiration data of plants, 0, 4 and 8 days after transfer (pers. com. R.I. Grange). Hendrix and Grange (1991) calculated E_e from the values after 4 h in the dark, as presented by Fondy and Geiger (1982).

specific costs for carbohydrate export (E_e)		species	references
(mol CO_2 [mol sucrose]$^{-1}$)	(mol ATP [mol sucrose]$^{-1}$)		
E_e = theoretically calculated			
0.4 - 1.2	2.4 - 4.0	species with apoplastic loading	present chapter
E_e = slope regression			
0.70	2.3 - 4.2	potato	present chapter
0.79	2.6 - 4.7	pepper	after Grange (1987)
1.20	4.0 - 7.2	cotton	Hendrix and Grange (1991)
0.96	3.2 - 5.8	barley	Hitz (1982)
2.64	8.8 - 15.8	tomato	Ho and Thornley (1978)
0.47	1.6 - 2.8	soybean cv Amsoy 71	after Mullen and Koller (1988)
3.84	12.8 - 23.1	soybean cv Wells II	after Mullen and Koller (1988)
E_e = respiration rate / export rate			
1.00	3.3 - 6.0	sugar beet	after Fondy and Geiger (1982), as derived by Hendrix and Grange (1991)
0.60 - 1.26	2.0 - 7.6	field bean	calculation method 1 Irving and Silsbury (1988)
E_e = (respiration rate - dark decay respiration) / export rate			
0.60 - 0.77	2.0 - 4.6	field bean	calculation method 2 in Irving and Silsbury (1988)

Table 4.6 The percentage dark respiration (R_d) explained by costs for carbohydrate export, for data derived from the literature on starch-storing species. The costs for carbohydrate export (R_e) were calculated using equation 4.5, with C_e derived from the literature and a range of 0.4 to 1.2 mol CO_2 per mol sucrose for E_e.

dark respiration for export (R_e)		species	data recalculated after the references given
(nmol CO_2 m^{-2} s^{-1})	(% of R_d)		
98	9 - 27	Arabidopsis	Caspar et al. (1985), Fig. 1 & Tab. III
177	23 - 69	sugar beet	Fondy and Geiger (1982), Fig. 1a
71 - 174	12 - 78	pepper	Grange (1985), Fig. 4 & Tab. 2
8 - 180	1 - 38	cotton	Hendrix and Grange (1991), Fig. 6
171 - 343	7 - 60	cotton	Hendrix and Huber (1986), Fig. 1
310 - 388	32 - 96	field bean	Irving and Silsbury (1988), Fig. 1
257	11 - 33	maize	Kalt-Torres et al. (1987), Figs. 1 & 2
83 - 500	10 - 116	soybean cv Amsoy 71	Mullen and Koller (1988), Figs. 5 & 7
93 - 208	12 - 40	soybean cv Wells II	Ibid
90	12 - 35	cucumber	Verkleij and Baan Hofman-Eijer (1988), Fig. 2
23 - 46	3 - 17	cucumber	Verkleij and Challa (1988), Fig. 1 & Tab. 1
165	12 - 55		average of literature data
45 - 201	11 - 32	potato	present experiments, Tab. 4.4
131 - 365	13 - 39	bean	present experiments, Tab. 4.4

Most experimental estimates of the specific costs of carbohydrate export agree well with the theoretical values, despite the different methods used (Tab. 4.5). The high values for tomato (Ho and Thornley 1978) and soybean cultivar Wells II (after Mullen and Koller 1988) are difficult to explain. In many cases the energy costs for carbohydrate export would exceed the actual respiration rates when multiplying these high specific costs for carbohydrate export with the observed export rates shown in Table 4.6. The reasons for these deviating results remain obscure, but may be additional energy-requiring processes, correlating with carbohydrate export. The good agreement between the theoretical range and most of the experimental data, indicate that the present theoretical range of the specific energy costs of carbohydrate export is a sound basis for calculating the contribution of the energy costs of carbohydrate export to α and

R_d.

Calculations of the contribution of the energy costs of carbohydrate export to α: upper limits

The correlation between the dark respiration rate and the nitrogen concentration of (mature) leaves was hypothesized to be due to energy costs for *i)* export of carbohydrates and *ii)* protein turnover (Lambers et al. 1989). Assuming an average degradation constant of 1.39 x 10^{-6} s^{-1}, De Visser et al. (1992) showed that protein turnover often accounts for major part of the effect of the leaf organic nitrogen concentration on the dark respiration (α). If the costs of protein turnover are lower, e.g. due to lower degradation constants, the energy costs associated with carbohydrate export may easily account for the remaining part of α (upper limits a_e in Tab. 4.3). This is especially the case in 21 - 24 days old leaves (a_e up to 1). For 17 - 20 days old leaves a_e was approximately 50% lower, due to the higher value of α: 8.8 nmol O_2 [g DW]$^{-1}$ compared to 5.3 for 21 - 24 days old leaves (Tab. 4.3). We cannot account for this high value of α, but note that it is also high compared to most observations in (full-grown) leaves of other starch-storing species (up to 7.3 nmol CO_2 or O_2 [mmol org. N]$^{-1}$ s^{-1}; De Visser et al. 1992 and references therein, Irving and Silsbury 1988, Makino and Osmond 1991, Pons et al. 1989); only the value obtained by Irving and Silsbury (1987) is as high as that for 17 - 20 days old bean leaves .

Calculations on the contribution of the energy costs of carbohydrate export to R_d: upper and lower limits

The present export rates (C_e) and respiratory costs involved in export (R_e) agree with literature data, be it that the present export rates are in the lower part of the range (Tab. 4.6). Combining the present estimates of the specific costs for carbohydrate export (E_e = 0.4 - 1.2 mol CO_2 [mol sucrose]$^{-1}$) with literature data (cf. eq. 4.5), shows that, on average, carbohydrate export explains 12 to 55% of leaf dark respiration (R_e in Tab. 4.6). Some of these high values based on the literature, might be partly due to carbohydrate use for leaf growth, which is a relatively expensive process (Poorter et al. 1991). In conclusion, carbohydrate export is an important energy-requiring process in leaves of starch-storing species, at least in the dark. Together with the costs of protein turnover (17 to 35% of the leaves dark respiration as calculated in chapter 3; the range of 5 to 76% of leaf dark respiration as calculated in Tab. 2 in De Visser et al. [1992] may include some costs for export), it may account for the major part of dark respiration in full-grown leaves.

Are there energy costs of other processes contributing to α and R_d ?

The sum of the average range of costs associated with carbohydrate (12 to 55% of R_d; Tab. 4.6) plus protein turnover (17 to 35% of R_d; chapter 3) does not exclude that other energy-dependent processes account for part of the dark respiration of leaves (R_d). For example, maintaining ion gradients was shown to be important in roots (chapter 5). There is also no conclusive evidence to exclude that processes, other than carbohydrate export and protein

turnover, may contribute to the effect of the leaf organic nitrogen concentration on the dark respiration (α). One of these processes may be nitrate reduction. Regarding a plant with a relative growth rate of 0.25 g g^{-1} day^{-1} and an organic nitrogen concentration of 60 mg N (g DW)$^{-1}$, a reduction rate of 12.4 nmol NO_3^- (g DW)$^{-1}$ s^{-1} is required to produce the new biomass. The relative importance of nitrate reduction in the shoot increases with increasing nitrate supply (Gojon et al. 1994). Assuming that leaves reduce only the nitrate for their own demand, and using a specific leaf area as observed for potato (47 m^2 [kg DW]$^{-1}$; chapter 2), the reduction rate is 264 nmol NO_3^- m^{-2} s^{-1}. Since the reduction of NO_3^- to NH_3 gives 2 mol CO_2 [mol NO_3^- reduced]$^{-1}$, the calculated nitrate reduction rate gives a CO_2 production rate of 528 nmol CO_2 m^{-2} s^{-1}. In general and in the present experiments, its contribution is expected to be much lower than calculated here, for the following reasons: *i)* nitrate reduction in the leaves occurs predominantly in the light (Beevers and Hageman 1969; Rufty et al. 1984), *ii)* up to 1 CO_2 per nitrate reduced in the shoot may be exported to the root via the malate shuttle (Ben Zioni et al. 1970, 1971, Kirkby and Knight 1977) and *iii)* nitrate reduction rate in full-grown leaves is much lower than in growing leaves (Beevers and Hageman 1969). Moreover, the relative growth rate and organic nitrogen concentration assumed in present calculations were relatively high compared to that of potato (chapter 2), so that an upper limit was obtained.

Conclusions

Dark respiration of full-grown leaves correlates with the nitrogen concentration of these leaves. In starch-storing species, nocturnal carbohydrate export may account for a significant part (up to 100%) of the slope of this correlation, and on average for 29% of total dark respiration. Thus, in starch-storing species, energy costs associated with nocturnal carbohydrate export is a quantitatively important process

Chapter 5

Energy requirements for maintenance of ion concentrations in roots

with R. De Visser

Abstract - The importance of maintaining ion gradients across plant membranes in terms of respiratory energy requirements in plant tissues, was quantified. Roots of intact plants of potato (*Solanum tuberosum* L. cv Alcmaria and cv Pimpernel) were incubated in a closed circulation system. Electrical conductivity of the solution surrounding these roots was continuously monitored to determine total ion efflux into demineralized water. Anion efflux rate from the symplast was 35 neq (g dry weight)$^{-1}$ s^{-1}. In combination with literature data on the specific costs of ion transport, this efflux rate yields the respiration rate associated with re-uptake balancing efflux (i.e. maintenance of cellular ion concentrations). The results suggest that energy costs associated with re-uptake of ions may account for up to 66% of the total respiratory costs involved in ion influx.

Abbreviations - Dp, Donnan phase; EC, electrical conductivity; eq, mol equivalent charge; RGR, relative growth rate; WFS, water free space

Introduction

Up to 50% of the photosynthates produced daily are respired (Lambers 1985). Depending on plant growth rate and age, 20 - 60% of the respiratory energy may be used for maintenance (Amthor 1984, Van der Werf et al. 1988). The energy demands of the individual maintenance processes *in vivo* are not well known and estimates are scarce (Amthor 1984). Knowledge about these processes will improve our understanding of plant growth and crop yield (chapter 1). A major part of the maintenance energy costs is supposed to be associated with *i)* the maintenance of ion gradients across membranes and *ii)* protein turnover (Penning de Vries 1975). Recently, more data became available on protein turnover (De Visser et al. 1992, chapter 3) in relation to nocturnal carbohydrate export (chapter 4). However, little work has been reported on the energy costs of maintenance of ion gradients across membranes (i.e. maintenance of cellular concentrations), and it is the purpose of this chapter to approach this aspect on roots of intact plants.

The plant maintains a constant membrane potential and a constant composition of the cytosol (Lauer et al. 1989, Tomos and Wyn Jones 1988). The energy requirements for maintenance of ion concentrations are calculated as the product of the specific costs of ion uptake as derived from literature data and the fraction of active transport that compensates for efflux. This fraction of transport is estimated as being quantitatively equivalent to the rate of total ion efflux from intact roots in demineralized water, as measured by monitoring the electrical conductivity of the medium. However, efflux not involving energy-dependent re-uptake, like efflux from the free space and efflux of some cations (e.g. cellular potassium) from the symplast, must be excluded in these calculations. Therefore, the efflux kinetics from the Donnan phase and the nature of the cations in the efflux medium and root extracts have been experimentally determined.

Materials and methods

Plant material

Potato plants (*Solanum tuberosum* L. cv Alcmaria and cv Pimpernel) were propagated by *in vitro* culture, and grown in a circulating aerated Hoagland solution (macro nutrients at 1/2 strength according to Hoagland and Snyder [1933] and micro nutrients at 1/2 strength according to Lewis and Powers [1941], with 90 μM iron as Fe(III)-EDTA). Entangling of the roots of neighbouring plants was prevented. A uniform supply of nutrients was provided for each individual plant by rapid circulation from a central storage vessel. The light conditions were: 12 h 240 μmol m^{-2} s^{-1} PPFD provided by fluorescent tubes (Philips TLD 36W/54) and incandescent lamps (Pope 60W) in a ratio 12:1. Incandescent light (Pope 60W) was supplied for another 6 h after the 12-h light period, to prevent tuber induction. Temperature was 18 ± 2°C, and relative air humidity 70%. In all experiments vegetative plants were used at various ages ranging from 18 to 48 days.

Electrical conductivity measurements

Before transfer to the experimental set-up, roots of intact plants were gently rinsed in two separate volumes (500 ml) of demineralized water for less than a minute in order to remove adhering nutrient solution. The roots were then incubated in a circulation system filled with demineralized water (Fig. 5.1) to monitor net charge efflux by electrical conductivity (EC). The EC was measured using a WTW LF530 conductivity meter and WTW LTA 01 electrode, and a WTW TFK 530 sensor for automatic temperature compensation (Wissenschaftlich-Technische Werkstatten GmbH, Weilheim, Germany) and expressed in μS cm^{-1}. The reference temperature for automatic compensation (20°C) is arbitrary, as long as the equivalent conductance used for the conversion of EC to a molar bases is valid for the same temperature (calculation in next paragraph). Efflux was calculated from the slope of the change of EC against time at various moments after start of the incubation. At the end of some of the experiments (n = 5), the medium was sampled to obtain an estimate of the relative contribution

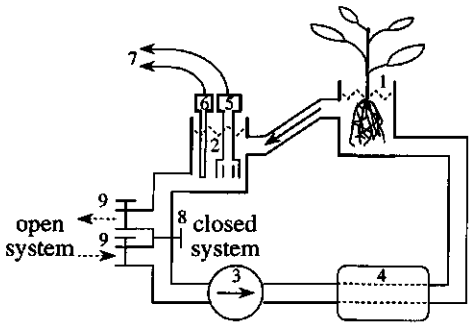


Fig. 5.1 Incubation system for monitoring electrolyte efflux from intact roots. 1, plant cuvette; 2, electrode cuvette; 3, pump; 4, temperature control; 5, EC electrode; 6, thermocouple; 7, EC meter; 8 and 9, valves.

of the different cations to the efflux.

Our objective was to prevent re-uptake during efflux by maintaining the external concentrations low compared to the k_m of the uptake system. The concentration in the circulation system (Fig. 5.1) is determined by the efflux rate integrated over the experimental time ($\int \Phi^{io} dt$, on a dry weight basis) multiplied by root dry weight and divided by the volume of the system (V). This volume (300 ml) was a compromise between *i)* the ideal infinite volume (a zero source for uptake) and *ii)* a small volume/root-weight ratio to enable detection of changes in EC. Efficient mixing is required to minimize development of boundary layers close to the roots. Since the EC electrode is sensitive to electromagnetic fields, the medium was mixed by circulation with a pump (Iwaki magnet pump model md-6, max capacity 8 to 9 l min^{-1}; Iwaki CO., LTD., Tokyo, Japan). As indicated by the rapid changes in EC after injection of salt solutions, the mixing rate was adequate for the time resolution required (equilibration time less than 1 min). All other conditions were similar to the growth conditions.

The circulation system could be changed from a 300 ml closed system (valve 8 open, valves 9 closed) to an open system (valve 8 closed, valves 9 open), in which the medium was continuously replaced (Fig. 5.1). Preliminary measurements showed no significant differences in efflux between the two systems, indicating that the closed system behaves like a zero source of ions for re-uptake over the experimental period. Further experiments were performed with the closed system.

Calculation of ion efflux from electrical conductivity measurements

The efflux measured as the change of EC of the medium (ΔEC, mS m^{-1} s^{-1}) was transformed to a charge equivalent basis. Ion efflux of roots (Φ_{eq}^{io} eq [g DW]$^{-1}$ s^{-1}; eq is one mol charge equivalents) is calculated according to:

$$\Phi_{eq}^{io} = (\Delta EC \times V) / (\Lambda_a \times W) \quad (5.1)$$

where V is the volume of the measuring system (m^3), Λ_a is the average conductance of equivalent charge (mS eq^{-1} m^2) and W is the dry weight of the root (g). Although the relationship between conductance (Λ) and concentration is complex (Chang 1981), it may be regarded as proportional for strong electrolytes at the low concentrations used here. This was verified by calibration with 0 to 1 mM KNO_3 and KCl ($r^2 > 0.99$). The molar conductances as calculated from single ions (after Anonymous 1978), agreed well with our calibrations. Because of the small differences between ion species in Λ (after Anonymous 1978), the overall conductivity of a mixture of ions as expressed on electronic charge basis is hardly affected by the chemical nature of the ions (except for protons and hydroxyl anions). Therefore, the average conductance of equivalent charge (Λ_a = 6.42 mS eq^{-1} m^2) was calculated as the average of the average equivalent conductances of K^+ and 1/2 Ca^{2+} (6.05) and the average of NO_3^-, Cl^- and 1/2 SO_4^{2-} (6.79; after Anonymous 1978) at the same temperature as used for automatic correction of the output values by the WTW LF530 (20°C).

Kinetics of loss from the Donnan phase

To enable correction for total efflux from the free space (i.e. loss from the Donnan phase [Dp] and water free space [WFS]), data on the kinetics of the slowest component of this efflux (i.e. the efflux from the Dp) were used. Cell wall calcium, a major component of the Dp, is reversibly exchangeable against magnesium (Sentenac and Grignon 1981), which is mainly located in the symplast (Hanson 1984). Therefore, we measured the reversible exchange of calcium for magnesium and used these data to calculate the half-life of the Dp ($t_{1/2}$) according to:

$$t_{1/2} = (-t \ln 2) / (\ln V_{actual} - \ln V_{t0}) \quad (5.2)$$

where t is the time from the start of the exchange, V_{actual} is the rate of loss of ions from the free space at time t and V_{t0} is the initial rate of loss, both proportional to the ion concentration in the free space. The reversible exchange was determined in vessels with a relatively small volume (100 ml), to obtain levels of ions detectable by atomic absorption spectrometry within 10 min. The medium was stirred by aeration, and could be rapidly changed without disturbing the roots, via a tap in the bottom of the vessel.

In the first experiment (Dp-1), two plants were used. With the first plant the exchange/efflux rates of calcium and magnesium were determined during incubations for one hour each of the roots into successively (1) $MgCl_2$, (2) $CaCl_2$, (3) $MgCl_2$, (4) $CaCl_2$ and (5) demineralized water. Salt concentrations were 50 mM. The second plant was used to measure the efflux of calcium and magnesium into (6) demineralized water for one hour.

In the second Dp experiment (Dp-2), the efflux/exchange rates of calcium and magnesium were measured on four plants (two of each cultivar). The media were changed in the following order: incubations for one hour each in successively (1) demineralized water, (2) $MgCl_2$, (3) $CaCl_2$, followed by incubations of 30 min each in (4) $MgCl_2$, (5) $CaCl_2$ and (6) demineralized

water. Salt concentrations were 23 mM.

During all incubations in both experiments Dp-1 and -2, we replaced the medium every 10 min, and kept it for chemical analyses. To remove adhering medium, roots were briefly washed twice in the type of medium to be used for the next treatment.

Calculation of respiratory costs of re-uptake

Efflux (Φ_{eq}^{io}, eq [g DW]$^{-1}$ s^{-1}) is expressed on a molar basis (Φ_j^{io}; mol ion$_j$ [g DW]$^{-1}$ s^{-1}) by dividing half of Φ_{eq}^{io} by the absolute value of the ion valency of the average cat- or anion j (z_j). Due to electroneutrality the ratio anion/cation equivalents is 1, and thus half of Φ_{eq}^{io} is for cations and half for anions, respectively.

$$\Phi_j^{io} = 0.5 \times \Phi_{eq}^{io} / z_j \qquad (5.3)$$

From this rate (after correction for the free space) and various literature data, the respiratory energy cost for re-uptake balancing efflux for either cat- or anions was calculated according to:

$$m_{c\text{-}j} = \Phi_j^{io} \times M_j \times [H/I_j] \times [H/P]^{-1} \qquad (5.4)$$

where $m_{c\text{-}j}$ is the energy cost for maintaining concentrations for ion$_j$ (mol ATP [g DW]$^{-1}$ s^{-1}), Φ_j^{io} is the efflux of ion$_j$ (mol ion$_j$ [g DW]$^{-1}$ s^{-1}), M_j is the number of active membrane passages of ion$_j$, $[H/I_j]$ is the stoichiometry between the protons and ion$_j$ (mol H$^+$ [mol ion$_j$]$^{-1}$) and [H/P] is the stoichiometry of the proton-ATPase (mol H$^+$ [mol ATP]$^{-1}$). By dividing $m_{c\text{-}j}$ by the P/O_2-ratio of the oxidative phosphorylation (mol ATP [mol O_2]$^{-1}$), the respiratory costs on an oxygen basis are obtained (mol O_2 [g DW]$^{-1}$). Equation 5.4 describes the calculation method for secondary active anion transport. In case of re-uptake by primary active transport, the product $[H/I_j] \times [H/P]^{-1}$ should be replaced by the stoichiometry of the ion$_j$-ATPase ($[P/I_j]$, mol ATP [mol ion$_j$]$^{-1}$).

In our calculations we assumed one membrane passage to be involved in re-uptake balancing efflux. Transport (efflux and influx) of potassium does not require metabolic energy (Hedrich and Schroeder 1989) because the K$^+$ concentration is generally near the Nernst equilibrium. The proton:ATP stoichiometry of the proton-ATPase [H/P] is 1, as derived from kinetic models (Sanders 1990, Teyerman 1992). Since the major anion absorbed is nitrate (Marschner 1989), we used the absolute value of the anion-valency ($z_j = 1$) and the proton/ion ratio ($[H/I_j] = 2$) of nitrate in our calculations. Under the present growth conditions, the potato plants had a relative growth rate (RGR) of 0.15 g (g DW)$^{-1}$ day^{-1} (chapter 2). We assumed an average alternative pathway activity of 30% (P/O_2-ratio of 4.8; after Tab. 3 in Poorter et al. [1991] for plants with a RGR less than 0.2 g [g DW]$^{-1}$ day^{-1}).

Chemical analyses of root extracts, media from exchange experiments, and media from efflux measurements

In order to relate leaking electrolytes to the cellular ionic contents, two roots of control plants were extracted by boiling for 5 min in 200 ml demineralized water. Before extraction, the roots were washed in demineralized water to remove adhering medium. Both extracts and samples of

the efflux media of the EC measurements were analyzed for K^+, $Fe^{2+/3+}$, Mg^{2+}, Ca^{2+} and Na^+, whereas the samples of Dp-1 and -2 were analyzed for Mg^{2+} and Ca^{2+}. All cation analyses were done by atomic absorption spectrometry (VARIAN SpectrAA-10, Varian Techtron Pty. Ltd., Springvale, Australia). All data were corrected for the mineral content of demineralized water samples stored under identical conditions as the efflux media and extracts.

Statistics

The 95%-probability intervals for efflux (EC measurements) and exchange (experiment Dp-2) rates were calculated using Student's t-values.

Results

Electrolyte efflux, and nature and half life of the Donnan phase

Since there were no significant differences between the two potato cultivars, the time course data of the anion *plus* cation efflux (neq $[g\ DW]^{-1}\ s^{-1}$) of both cultivars were combined (Fig. 5.2). Damaging the roots induced high ion efflux into demineralized water (data not shown). Gently removing the plants from this medium and immediate return to the original position also increased efflux (Fig. 5.3). However, comparison with efflux kinetics of untreated roots (Fig. 5.2) revealed that this increase was transient (duration less than 10 min). The high initial efflux is partly due to previous treatments (rinsing and transfer) of the roots (cf. Fig. 5.3).

A comparison of the relative contribution of the cations released into the efflux medium with those in extracts of the roots showed that, although potassium was quantitatively the most important cation in the root (ca 77%), calcium was the most abundant one in the efflux medium (ca 67%). This calcium is unlikely to originate from the cytosol, because the cytosolic calcium concentration is tightly regulated at a very low level (ca 10^{-6} M; Lühring and Tazawa 1985, Williamson and Ashley 1982). Cell walls however, contain a large amount of calcium (Demarty et al. 1984), suggesting that the calcium in the efflux medium originated predominantly from the free space. Efflux of ions from the free space is not compensated for by active transport and was, therefore, excluded in calculations of respiratory costs of re-uptake balancing efflux.

Reversible exchange for magnesium showed that the Donnan phase contained calcium (Figs 5.4 and 5.5), with a half-life of ca 9 min (cf. eq. 5.2) as it decreased by at least 90% within half an hour (Figs 5.4A and 5.5). The exchange could not be explained by adhering solution, because relatively little calcium was found in demineralized water after a $CaCl_2$ treatment (Fig. 5.4A). Thus the adhering solution was effectively removed during rinsing between treatments. Negative values for the efflux in demineralized water (Figs 5.4A, B) are due to the variation in the samples combined with the correction for the ion content of control samples of demineralized water.

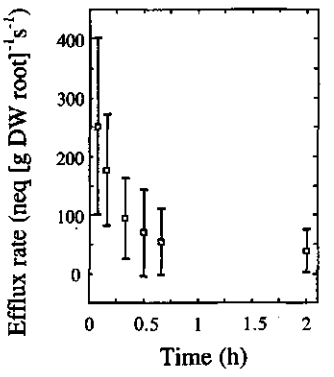


Fig. 5.2 (left) Efflux of charge equivalents (neq [g DW]$^{-1}$ s^{-1}) from potato roots incubated in demineralized water. Bars indicate 95%-probability intervals.

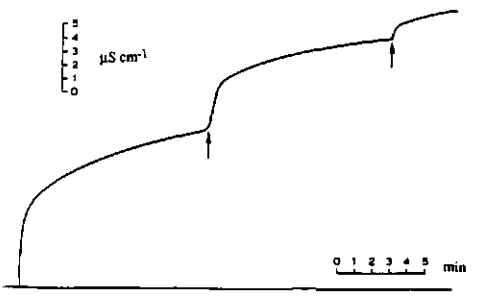
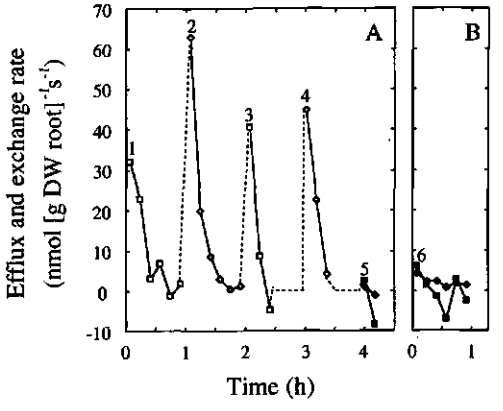


Fig. 5.3 (right) Stimulation of root efflux rate in response to removing the plants from the incubation system and then immediately returning them to the original position. The moments of the start of the treatment are indicated by arrows.



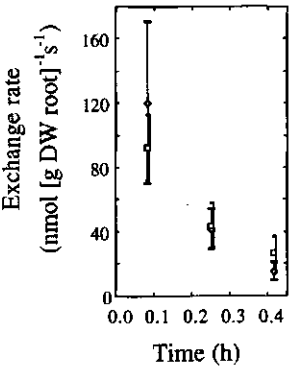


Fig. 5.4 (left) The rate of exchange of (a) calcium in $MgCl_2$ (□; 1 and 3) or (b) magnesium in $CaCl_2$ (◇; 2 and 4) and the efflux of (c) calcium (■) and (d) magnesium (◆) in demineralized water (5 and 6). The results of the different plants are shown in the figures A and B, respectively. Full details are given in Materials and methods.

Fig. 5.5 (right) The rate of efflux/exchange of magnesium in 0.023 M $CaCl_2$ (◇) and of calcium in 0.023 M $MgCl_2$. Bars indicate 95%-probability intervals.

Table 5.1 Calculation of *i)* the ATP costs, *ii)* the respiratory costs and *iii)* the fraction of literature values for total or maintenance respiration (references in last column), which are explained by re-uptake balancing efflux in roots (maintaining gradients). In the calculations we used an anion-valency (z_j), proton/ion ratio [H/I_j] and the proton:ATP-ratio [H/P] of 1, 2 and 1, respectively.

Parameter	Time (min)			ADP/O_2 -ratio	References
	10	30	120		
leakage rates anions *plus* cations					
nS m^2 (g DW)$^{-1}$ s^{-1}	1.13	0.44	0.25		
neq (g DW)$^{-1}$ s^{-1}	176	69	39		
neq (g FW)$^{-1}$ s^{-1}	11.8	4.6	2.6		
costs for re-uptake (absolute values)					
nmol ATP (g DW)$^{-1}$ s^{-1}	176	69	39		
nmol O_2 (g DW)$^{-1}$ s^{-1}	29.3	11.5	6.5	6	
nmol O_2 (g DW)$^{-1}$ s^{-1}	36.7	14.4	8.1	4.8	
fraction of root respiration					
% of 355 nmol ATP (g DW)$^{-1}$ s^{-1}	50	19.4	10.9	4.8	(unpublished data)
% of 536 nmol ATP (g DW)$^{-1}$ s^{-1}	33	12.9	7.2	5.2	Lambers et al. (1983[b])
% of 240 nmol ATP (g DW)$^{-1}$ s^{-1}	73	29	16.2	4.8	Poorter et al. (1991)
fraction of root maintenance costs					
% of 59 nmol ATP (g DW)$^{-1}$ s^{-1}	298	117	66	4.8	(chapter 7)
% of 24 nmol ATP (g DW)$^{-1}$ s^{-1}	733	288	162	4.8	Poorter et al. (1991)
% of 18 nmol ATP (g DW)$^{-1}$ s^{-1}	978	383	216	4.6	Veen (1980)

Calculation of respiratory costs of re-uptake

Respiratory costs for re-uptake of anions lost by efflux (m_c) were estimated according to equations 5.3 and 5.4 (Tab. 5.1). Efflux from the free space was excluded in calculations of respiratory costs by using the electrolyte efflux as measured after 30 min. At this moment the remaining efflux originating from the free space is found to compensate approximately for the decrease in efflux from the symplast (details in discussion). Efflux data after 10 min and 2 h are presented to show the effect of a higher and lower efflux rate on respiratory costs (Tab. 5.1). In the calculations we assumed that *i)* all calcium originates from the WFS and Dp, *ii)* the symplasmatic efflux consists of potassium accompanied by anions, resulting in overall

electroneutrality and *iii)* the anions consist predominantly of nitrate. The calculated respiratory costs for re-uptake of anions lost by efflux (m_c) after 10 min, 0.5 h and 2 h were 176, 69 and 39 nmol ATP (g DW)$^{-1}$ s^{-1} or 37, 14 and 8 nmol O_2 (g DW)$^{-1}$ s^{-1}, respectively (Tab. 5.1).

Discussion and conclusions

Evaluation of the electrical conductivity technique

Isotopic labelling is commonly used for examining in- and efflux kinetics. A limitation of this laborious technique is the small number of ion species that can be handled in one experiment. Therefore, no overall measure of ion efflux is obtained. Electrolyte leakage, measured by electrical conductivity (EC), is a commonly used index of membrane integrity and thus potentially suitable for monitoring total ion efflux from intact plant roots. It has been used for the evaluation of the extent of *i)* senescence of plant tissues (Borochov and Faragher 1983), *ii)* chilling injury (Hetherington et al. 1988), *iii)* seed germination capacity (Senaratna et al. 1988) and *iv)* injury caused by herbicide surfactants (O'Donovan et al. 1983). Here we discus the validity of measuring the efflux rate as change of EC in demineralized water, and consider the experimental problems in comparison with the labelling technique.

Calcium stabilizes membranes and shields negative charges (Ferguson 1984, Hanson 1984). No calcium was added to the efflux medium, because it lowers the sensitivity for measuring EC due to an increased background signal. In labelling experiments this technical limitation is absent. This lack of external calcium was not considered to be a problem as plants were grown with ample nutrients, the experiments were short, and requirements are only at millimolar activities (Hanson 1984).

The present method provides information on the kinetics of *net* ion efflux rates. Therefore, ion efflux may have been underestimated for two reasons. Firstly, total efflux is underestimated if re-uptake from the medium occurs. In labelling studies this problem is regarded as being small, because re-uptake of the labelled ions is statistically less likely due to the excess of unlabeled ions (Jackson et al. 1976). In EC measurements this stochastic process is absent. Re-uptake will depend on *i)* the external concentration (and thus measuring period) and *ii)* the K_m and V_{max} of the uptake system. These uptake parameters vary with nutrient supply over days (Lee and Drew 1986, Siddiqi et al. 1990). However, large changes are not likely to occur within half an hour. For example, Clarkson (1986) mentions that cells that have never been exposed to nitrate, upon exposure increase their nitrate uptake capacity from the constitutive level (1 μmol h^{-1} (g FW)$^{-1}$) so that, after a lag period of 0.5 to 1.5 hrs, they reach a 2 to 5 times higher steady state level after 4 to 6 h (Fig. 1 in Clarkson 1986). *De novo* protein synthesis is involved in these adaptations (Fig. 2A in Clarkson 1986). Although the external anion concentration rises to about 100 μM after 2 h (cf. Fig. 5.2 assuming an average root weight of 0.2 g), this concentration is only 1% of that of the growth medium (9.0 mM anions). Therefore, influx will be much lowered under these conditions. This notion is supported by our preliminary comparison, where the open and the closed system (Fig. 5.1) did not show

different efflux rates. The second possible cause of underestimating the efflux rate, is a fast re-uptake within the tissue (from the cell wall) relative to the exchange between tissue and well-stirred medium. This possibility is more likely in a complex tissue than in a unicellular system. However, this problem relates to both labelling studies (described in a model by Morgan et al. 1973) and EC measurements alike, and cannot be resolved with present-day techniques.

In summary, EC measurements have the advantage that an overall measure for all ions is obtained. Incubation for more than about an hour should be avoided, as efflux may be affected by re-uptake from the medium. However, during short-term experiments this problem is expected to be small. Re-uptake from within the tissue cannot be assessed in any of the experimental approaches presently available. Therefore, we regard the present data as an adequate estimate for calculating the energy costs for maintenance of ion concentrations in roots.

Interpretation of efflux kinetics

Efflux from the free space needs to be excluded from total root efflux when calculating energy costs of re-uptake balancing efflux. From the calcium exchange rate (Dp-1 and -2), we calculated the half-life of the slowest component of the free space (Donnan phase) to be 9 min. This half-life agrees well with literature values (Tab. 5.2) obtained by compartmental analysis (Baker and Hall 1988) in labelling experiments. Because these data have been derived for multi-cellular systems (intact roots or root segments), results should be considered with some care (chapter 6). The internal localization of the cellular compartments within the tissue might hamper a straightforward interpretation; numerous conditions must be met for a proper analysis (Baker and Hall 1988, Zierler 1981). Also, the half-life values vary strongly, e.g. depending on whether ^{15}N or ^{13}N label is used (Macklon et al. 1990). However, we regard our estimated half life reliable, as it is obtained by an alternative method, agreeing well with most of these literature values (Tab. 5.2).

The overall initial efflux (ca 250 neq $[g\ DW]^{-1}\ s^{-1}$) originates from Dp, WFS, cytosol and vacuole. As the half-life of the Donnan phase is 9 min, after 30 min at most 25 neq $(g\ DW)^{-1}\ s^{-1}$ (cf. eq. 5.2, assuming the initial efflux to be completely from the Donnan free space) originates from the free space and thus at least 44 neq $(g\ DW)^{-1}\ s^{-1}$ (i.e. 69 less 25; Fig. 5.2) from the symplast. Using the ratio between the half-lives of cytosol and of Donnan phase as calculated in Tab. 5.2, the half-life of efflux from the cytosol is expected to be between 126 (i.e. 14 x 9) and 28 (i.e. 3.1 x 9) min. Regarding these extremes, after 30 min the initial cytosolic efflux rate would be underestimated by ca 8 to 49 neq $(g\ DW)^{-1}\ s^{-1}$ (i.e. 18 to 110% of the cellular efflux after 30 min). Because this expected underestimation overlaps the expected overestimation due to efflux from the free space (i.e. at most 25 neq $[g\ DW]^{-1}\ s^{-1}$), we regard the efflux after ca 30 min as an adequate estimate of the efflux rate from the symplast, and thus suitable as input for calculating the energy costs of maintaining concentrations. Efflux rates after 10 min and 2 h were used to illustrate the relative effects of increase or decrease of the efflux on the costs for re-uptake of ions lost by efflux (Tab. 5.1).

Table 5.2 Half-life of ions in different plant compartments as derived from the literature and measured for calcium in the present experiments. The ratio Cyt/Dp indicates the ratio between the half-life of the cytosolic ions and the Donnan phase (Pi and $^{15}NH_4^+$), or the free space when no data on the Donnan phase were available ($^{13}NO_3^-$).

Ions	Half life ($t_{1/2}$; min.)						Ratio	References
	WFS	Dp	FS	Cell	Cyt	Vacuole	Cyt/Dp	
Pi	2.69			25.9				Lefebvre and Clarkson (1984)
	2.2	11.5			111	26.3 x 60	9.7	Ron et al. (1988)
$^{15}NH_4^+$	1.89	11.5			35.5	15.5 x 60	3.1	Macklon et al. (1990)
$^{15}NO_3^-$	5.8				126	140 x 60		Ibid.
$^{13}NO_3^-$			0.5		7		14.0	Siddiqi et al. (1991)
Ca^{2+}		9						present chapter

The efflux calculated after 30 min is relatively high (Tab. 5.3), especially when considering that part of the literature data from the labelling studies were not corrected for initial efflux rates from the free space. Possible reasons for this high value are: *i)* all anions are included in efflux, *ii)* nutrient supply was high during growth, *iii)* some organic ions may have been present, or *iv)* perturbation effects lasted for more than 30 min, but do not occur in labelling studies. Treatments can influence membrane potential and thus ion transport (Rincon and Hanson 1986), ion uptake (Bloom and Sukrapanna 1990) and root pressure (Miller 1987). The labelling studies in the literature show a large variation in ion efflux from roots (Tab. 5.3), probably partly due to different experimental conditions and interpretation of efflux kinetics.

However, the obtained efflux expressed as fraction of influx agrees well with values determined by means of isotopes. Efflux amounted to 50-85% ($^{36}ClO_3^-$; cited in Clarkson 1985), 63% (^{15}N and ^{13}N; Tab. 5 in Clarkson et al. 1989) or up to 88% (^{15}N; Tab. 1 in Pearson et al. 1981). In the present experiments, root net N uptake was estimated from the product of plant RGR (0.15 g g^{-1} day^{-1}; Tab. 2.1), N content (4.1 mmol N [g DW plant]$^{-1}$; chapter 7) and plant to root ratio (7 g DW plant [g DW root]$^{-1}$) at 4.3 mmol N g^{-1} day^{-1} or 50 nmol N g^{-1} s^{-1}. When assuming anion efflux to consist predominantly of nitrate and thus assuming other anions to be negligible, efflux rate equals 100 x 34.5 / (50+34.5) = 41% of nitrate influx (Influx = |Efflux| + Net uptake). Comprising other anions yields a slightly lower value (e.g. 34% assuming phosphate and sulfate both to be 10% of nitrate in- and efflux).

In summary, the efflux rate observed after 30 min is a good estimate of the initial efflux from the symplast. Although the absolute value of this efflux rate is high (Tab. 5.3), the relative efflux rate as fraction of the influx rate is lower than the highest literature values.

Table 5.3 Leakage rates for anions as derived from the literature for various species and experimental conditions. The literature data were obtained for roots by labelling techniques. Some authors indicate the origin of the efflux. This is shown by the superscripts ^{c}, $^{c+v}$ and ^{in} for cytosol, cytosol with vacuole and inner space, respectively. For some data, indicated by *, it is not clear whether weights are for fresh or dry matter.

Anion and species	Anion leakage rate from roots (nmol [g DW]$^{-1}$ s^{-1})	Anion leakage rate from roots (nmol [g FW]$^{-1}$ s^{-1})	References
Nitrate			
Pisum sativum	0.47± 0.03		Oscarson et al. (1987)
Pennisetum americanum	29.7 ± 5.64		Pearson et al. (1981)
Zea mays	12.4 ± 0.81		Ibid
Hordeum vulgare		1.03 ±0.14	Lee and Clarkson (1986)
Hordeum vulgare		3.44	Clarkson et al. (1989)
Allium cepa		0.22 −1.11^{c+v}	Macklon et al. (1990)
Hordeum vulgare		0.0083 −0.41^{c}	Siddiqi et al. (1991)
Hordeum vulgare		0.07 −0.24	Wieneke and Nebeling (1990)
Curcurbita pepo		0.41 −0.53	Ibid
Phosphate			
Carex rostrata	0.30− 0.45		Atwell et al. (1980)
Carex acutiformis	0.20− 0.32		Ibid
Tritium aest.	0.52− 11.46^{in}		Cogliatti and Santa Maria (1990)
Zea mays		0.0033 −0.0097^{in}	Elliott et al. (1984)
Hordeum vulgare		0.06^{c+v}	Lefebvre and Clarkson (1984)
Allium cepa		0.13^{c+v} (*)	Ron et al. (1988)
Hordeum vulgare		0.07 −0.16	Schjørring and Jensén (1984)
Brassica napus		0.15 −0.37	Ibid
Sulphate			
Daucus carota L.		0 −3.10$^{c(*)}$	Cram (1983)

Parameter	Anion leakage rate from roots (neq [g DW]$^{-1}$ s^{-1})	Anion leakage rate from roots (neq [g FW]$^{-1}$ s^{-1})	References
Total			
minimum - maximum	0.64− 47	0.0116 −7.7	references above
Measured by EC			
after 10 minutes	88	5.9	present chapter
after 30 minutes	34.5	2.32	present chapter
after 2 h	19.4	1.31	present chapter

Comparison of respiratory costs of re-uptake to respiration rates

Table 5.1 compares the calculated respiration rates for re-uptake with the average overall and maintenance respiration of roots. For the data of Poorter et al. (1991) we estimated an average value for the species with an RGR of less than 0.2 g (g DW)$^{-1}$ day^{-1}, as the efflux was measured on potato plants with a RGR of only 0.15 g (g DW)$^{-1}$ day^{-1}. The respiration rates of potato roots were transformed to an ATP basis by assuming a P/O_2-ratio of 4.8 (after Tab. 3 in Poorter et al. [1991] for plants with a RGR less than 0.2 g [g DW]$^{-1}$ day^{-1}). The overall root respiration of potato was measured on young roots with a higher RGR (i.e. 0.21 g [g DW]$^{-1}$ day^{-1}) than the plants used in the present experiments, whereas for maintenance respiration the average value obtained in chapter 7 was used. The data of Veen (1980) were transformed using a dry matter content of 7% (B.W. Veen, personal communication) and a P/O_2-ratio of 4.6 mol ATP (mol O_2)$^{-1}$ (B.W. Veen, personal communication).

The respiration rate calculated for re-uptake after half an hour can easily explain the total maintenance respiration rate of all plant species (Tab. 5.1). Before concluding that maintenance of ion gradients is the most important maintenance process, it is necessary to consider the estimates of maintenance respiration. All these values are obtained by the multiple regression method, as introduced by Veen (1980). In this statistical method, respiration rate is attributed to growth, ion uptake and maintenance as far as it correlates with the rate of dry weight increase, ion uptake rate, or root biomass, respectively (e.g. chapter 7). Depending on the presumed mechanism of efflux (e.g. slip of carrier or membrane permeability), re-uptake balancing efflux is expected to correlate with either ion uptake or root biomass. Thus, in the regression approach, costs for re-uptake of ions lost by efflux might be incorporated in the costs for uptake or maintenance. Our present calculations suggest that either a (major) part of these costs is included in the uptake costs instead of in the maintenance coefficient, or maintenance of ion gradients is indeed the quantitatively most important maintenance process. The latter inference is highly unlikely as Van der Werf et al. (1992) calculated that protein turnover accounts for 24-48% of the maintenance costs of roots, indicating that the maintenance of gradients is not the only maintenance process in roots. Moreover, the specific energy costs of protein turnover as used by Van der Werf et al. (1992) may have been underestimated by a factor 2 to 4 (cf. De Visser et al. 1992). Therefore, we hypothesize that energy costs for re-uptake balancing efflux are predominantly included in the regression coefficients of uptake. This contention is supported both by the results of chapter 7, and the findings of Van der Werf et al. (1988) and Clarkson (1985; based on the data of Veen, 1980), who both calculated uptake costs to be twice as high as theoretically expected.

The energy costs of re-uptake balancing efflux may be assessed relative to the costs of net uptake and influx. Clarkson (1985) presented a mechanistic account of the energy costs of root ion uptake. At a net anion uptake rate of 16.7 nmol (g DW root)$^{-1}$ s^{-1} (60 μmol g^{-1} h^{-1}; Clarkson 1985) and a single active membrane passage per ion, costs of net anion uptake are 35 nmol ATP (g DW root)$^{-1}$ s^{-1}. The calculated influx costs equal the sum of the costs of net uptake and for re-uptake balancing efflux (Tab. 5.1; assuming efflux of nitrate only), i.e. 104 nmol ATP (g DW root)$^{-1}$ s^{-1}. In this case, efflux costs are ca 66% of influx costs. Considering

the efflux of all anions, this value becomes negligibly lower (e.g. efflux costs become 66 nmol ATP [g DW root]$^{-1}$ s^{-1} and thus 65% of the influx costs, when assuming phosphate and sulfate to be each 10% of the efflux). In our plants, nitrate uptake rate was 50 nmol N (g DW root)$^{-1}$ s^{-1}, giving an ATP hydrolysis rate of 100 nmol ATP (g DW root)$^{-1}$ s^{-1}. Here, influx costs for nitrate equal 169 nmol ATP g^{-1} s^{-1}. Efflux costs are 34-41% of influx costs, and 19% (Tab. 5.1) of total root respiration of young potato plants.

The stoichiometry of the proton-ATPase used in present calculations ([H/P] = 1 mol H$^+$ [mol ATP]$^{-1}$), was derived from kinetic models (Sanders 1990, Teyerman 1992). However, a value of 2 mol H$^+$ [mol ATP]$^{-1}$ could also be valid, as *i)* the proton motive force that can be generated with such stoichiometry would still allow active uptake of anions (calculations in appendix 5) and *ii)* such stoichiometry was found for the tonoplast (Bennett and Spanswick 1984, Guern et al. 1989) and sometimes obtained by kinetic models for the plasmamembrane (Sanders 1990). Assuming this more efficient stoichiometry ([H/P] = 2 mol H$^+$ [mol ATP]$^{-1}$), all values on the energy costs in Tab. 5.1, absolute and relative, would be halved. However, this does not change present conclusion that the energy costs of re-uptake balancing efflux are clearly a significant part of influx costs (34 - 66%) and respiration in roots. It might be speculated that variation in ion uptake costs in the literature, may well be explained by variation in re-uptake balancing efflux (chapter 6).

Chapter 6

A theoretical analysis of the influence of root characteristics on the efflux kinetics and respiratory requirements for maintaining ion gradients

with J.S. Geelhoed, B.O.M. Dirks and R. Rabbinge

Abstract - This study explores the effect of anatomical and physiological characteristics of complex multicellular tissues, like plant roots, on *i)* the quantification of the respiratory costs involved in maintaining ion gradients and *ii)* the comparison of such costs between roots with a different diameter. Dynamic simulation was used to visualize the contribution of the apoplast, cytoplasm and vacuole of different concentric cell layers of the root tissue, to the apparent efflux kinetics. The present approach demonstrates that different pathways of net uptake and the presence of an endodermis, cause significant differences in the apparent efflux kinetics, even if the actual total efflux is equal. Thus, for an accurate estimation of energy requirements involved in maintaining ion gradients in roots, these physiological and structural characteristics should be taken into account. Besides, the apparent efflux appeared not to be proportional to the number of cell layers involved in efflux. This may complicate the estimation of the number of cell layers involved in efflux. To allow comparison of respiratory costs between roots with different diameters, we derived a relation that visualizes the effect of the root diameter on the costs per unit dry weight of membrane-related processes like maintaining ion gradients (i.e. if not all cells have a similar efflux rate) and net uptake (i.e. if influx and efflux area differs). For a straightforward comparison of the costs of maintaining ion gradients and of net uptake between roots, the surfaces involved in influx and efflux have to be quantified.

Introduction

Respiratory costs for maintaining ion gradients, defined as re-uptake balancing efflux, were calculated to be 34 - 66% of the influx costs and 19% of total respiration (chapter 5). Determination of the exact energy requirements for maintenance of ion gradients is limited by difficulties in interpretation of the observed efflux kinetics from roots in terms of the actual total efflux from all root cells (chapter 5). That is, the overall efflux over the total of leaking membranes is not necessarily equal to the efflux initially observed in the medium. To enable quantification of such discrepancy, knowledge on how to interpretate efflux kinetics is

required. Besides the difficulties in establishing the energy requirements for maintenance of ion gradients, the comparison and interpretation of such estimates on a root dry weight basis is hampered by lack of information on the relation between the root dry weight and the membrane surface determining efflux. To enable a better quantification and comparison of the respiratory costs involved in maintaining ion gradients in plant roots, this study aims at identifying *i)* which factors affect the efflux kinetics, and *ii)* the relation between the actual total efflux on an area basis and energy costs on a dry weight basis. The first objective is studied by dynamic simulation, whereas the latter is visualized analytically.

Efflux kinetics are obtained either by monitoring the re-appearance of labelled ions after a period of pre-loading (i.e. wash-out method; e.g. Kasimir Klemedtsson and Pettersson 1990, Lee and Clarkson 1986, Macklon and Sim 1990, 1992), or by calculating the difference between influx of a freshly supplied isotope and overall net ion uptake (i.e. differential method; e.g. Pettersson and Kasimir Klemedtsson 1990, Wieneke and Nebeling 1990). Wash-out kinetics are generally analyzed by applying the compartmental analysis, characterizing the various ion pools in terms of the ion concentration, the half life or exchange constant and the transport rates over the membranes (in most of the references mentioned above). Thus, interpretation of efflux kinetics by the compartmental analysis should yield the actual efflux necessary for calculating the respiratory costs involved in maintaining ion gradients. Validation of such results is not possible, as in the differential method the nature of the efflux is generally identified using the exchange constants observed in wash-out experiments (e.g. Deane-Drummond 1986, 1987, Siddiqi et al. 1991).

Compartmental analysis has major restrictions. Firstly, numerous conditions must be met and several assumptions made, to enable a proper analysis (Cram and Laties 1974, MacRobbie 1964, Pitman 1963, Zierler 1981). Secondly, complicating factors, such as ion export (e.g. Jeschke and Jambor 1981, Lefebvre and Clarkson 1984, Presland and McNaughton 1984, for Na^+, $H_2PO_4^-$, NO_3^-, respectively), metabolism or complexation of ions (e.g. Lee and Clarkson 1986, Macklon et al. 1990, Macklon and Sim 1992, Rauser 1987, Thoiron et al. 1981, for NO_3^-, NH_4^+, $H_2PO_4^-$, Cd^{2+}, SO_4^{2-}, respectively), require modification of the compartmental analysis. This difficulty is partly due to the fact that this method was originally used for simple tissues (Pierce and Higinbotham 1970) or single cell systems (Diamond and Solomon 1959, MacRobbie 1964, MacRobbie and Dainty 1958), whereas efflux kinetics are nowadays widely studied on complex tissues like roots (most of the references mentioned above). Thirdly, sometimes unexpected efflux phases occur, like a Donnan free space for phosphate in the form of $CaH_2PO_4^+$ (Ron et al. 1988). Fourthly, the statistical resolution of an experimentally established efflux curve does not necessarily give reliable estimates for multiphasic systems (Cheeseman 1986, Rygiewicz et al. 1984, Zierler 1981). The striking variation in half-life values of the identified pools, depending on whether ^{15}N or ^{13}N label is used (Macklon et al. 1990), also raises questions about the interpretation according to the compartmental analysis.

Separate quantification of efflux from all major ion pools in the root (i.e. the cell walls, cytoplasm and vacuoles in all cell layers), would give insight in the factors determining the observed net efflux kinetics and the accuracy of compartmental analysis to analyze efflux

kinetics. Experimentally, this is not possible due to the lack of suitable isotopes and methods to load individual compartments. Analytical solutions are restricted to relatively simple systems, as the unmanageable matrix appearing in the mathematical analysis for complex multicellular systems requires major simplifications. Therefore, an alternative approach was explored. A dynamic computer simulation model was developed to separate the contribution of all compartments in the root to the efflux kinetics observed in the medium during a wash-out experiment. The at present available simulation models on in- and efflux (e.g. Morgan et al. 1973) do not separate the contribution of the compartments in all cell layers.

To improve our present understanding on how to quantify the respiratory costs involved in maintaining ion gradients of complex multicellular tissues like plant roots, the (relative) effects of some tissue characteristics on its efflux kinetics were simulated. Most important tissue characteristics studied are the presence of an endodermis, the number of leaking cell layers, and different transport rates and pathways. Secondly, the relation between the actual total efflux on an area basis and energy costs on a dry weight basis, was theoretically derived as a function of the root diameter, for roots with a different number of leaking cell layers. Such a relation of the root diameter was also derived for the net uptake on an area basis and the energy demands on a dry weight basis.

Materials and methods

Principles of the simulation model

In the present dynamic simulation model 'COMTRANS', a slice of one cell thickness from a cylindrical root is considered, excluding axial ion transport as this does not affect the efflux kinetics. The root slice consists of a number of concentric cell layers (Fig. 6.1). The number of cells per concentric cell layer follows from dividing the volume of each layer by the volume of one cylindrical cell, including the apoplast. The external medium, and the cell wall, cytoplasm and vacuole per concentric layer are distinguished as different compartments as indicated in Fig. 6.1. Cellular compartments within the same concentric cell layer are not regarded separately, as they share an equal distance to the external medium and, thus, contribute to the same extent to the efflux kinetics. Within the compartments that are distinguished, ions are assumed to be distributed homogeneously.

The model simulates the time course of *i)* the fraction of ions that become labelled in each cellular root compartment during loading from a 100% labelled medium, *ii)* the ion efflux of each compartment into the external medium, from a root with all ions initially labelled with an index indicating its original cellular root compartment, and *iii)* the reallocation of the ions remaining in the root during this efflux period. The fraction of ions that become labelled in each cellular root compartment during loading has to be simulated, as this fraction will differ between compartments. Therefore, the efflux kinetics per compartment after a limited loading

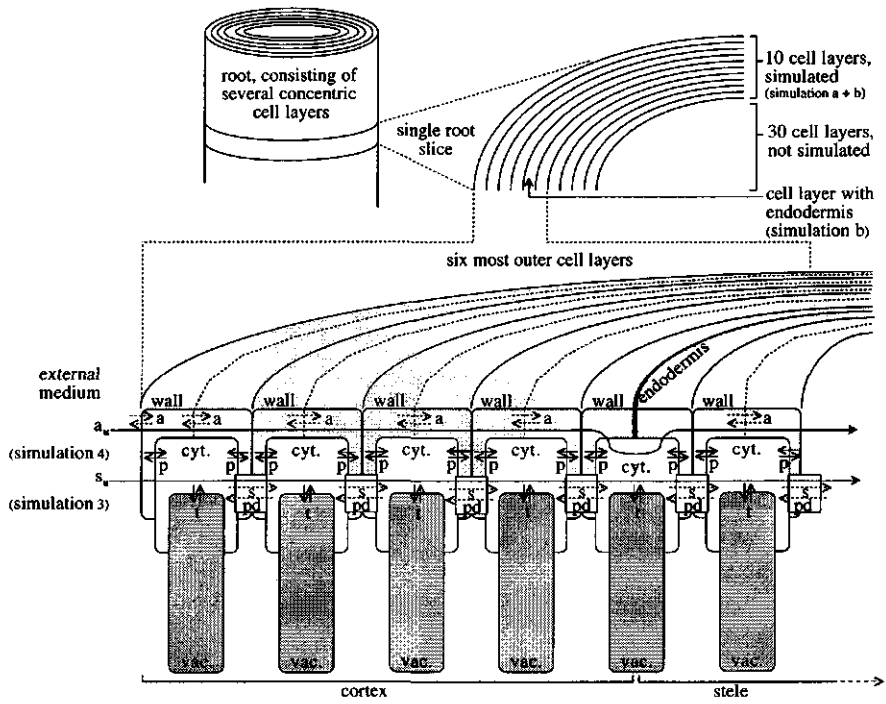


Fig. 6.1 Schematic representation of the simulated root slice. For the sake of simplicity, the model considers the cell walls between two adjacent cell layers as a functional compartment (borders indicated by the dashed lines). The locations of the apoplastic (a) and symplastic (s) diffusion transport (dashed arrows), the influx and efflux (drawn arrows) over the plasmamembrane (p) and tonoplast (t), and the apoplastic (a_u; simulation 4) and symplastic (s_u; simulation 3) net uptake are indicated. In simulation 1, net uptake occurs per cell as the influx over both plasmamembrane (p) and tonoplast (t) exceeds the efflux over these membranes. The abbreviations used for cytosol, vacuole and plasmodesmata are cyt., vac, and pd, respectively.

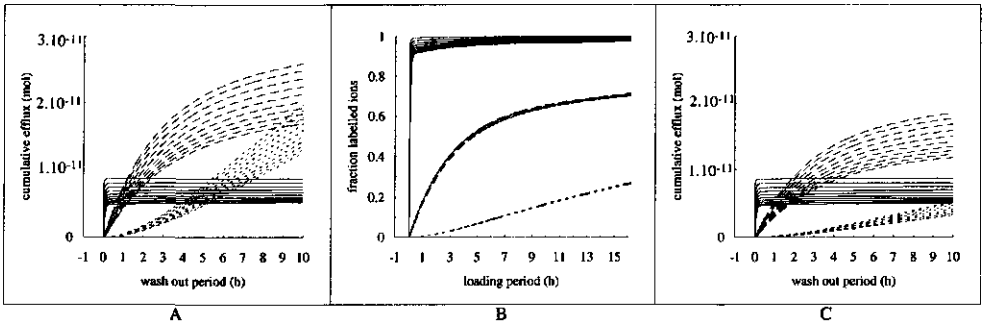


Fig. 6.2 The simulated cumulative efflux per compartment from a 100% labeled root slice (A; left) was multiplied by the label distribution after 16 h loading (B; middle), in order to obtain the cumulative efflux per compartment from a root slice after 16 h labelling (C; right). The compartments separated are the cell walls (drawn lines), cytoplasm (large dashes) and vacuoles (small dashes) from the 10 outer cell layers (uppermost lines belong to most outward cell layers).

period like applied in a wash-out experiment, are derived by multiplying the simulated efflux kinetics per compartment *(ii)* by the simulated fraction of labelled ions in each compartment at the end of the loading period *(i)* (example shown in Fig. 6.2). Adding these kinetics originating from all individual compartments yields the efflux kinetics that would have been observed in wash-out experiments (i.e. the apparent efflux).

In the model the changes in the distribution of labelled ions between the compartments that are distinguished, result from the following types of transports (Fig. 6.1): *a)* apoplastic exchange between cell walls and between the outer cell wall and medium, *b)* symplastic exchange via plasmodesmata, *c)* efflux and influx over plasmamembrane and tonoplast, and *d)* ion transport through apoplast (mass flow) and cytoplasm for net uptake. The next section on "inputs of the simulation model", describes how each type of transport is simulated. In general, for all types of transport a *maximum* rate is defined as the transport rate to an empty compartment. At each moment, the actual *(gross)* transport rate per label from a given compartment by a given type of transport, follows from multiplying the constant value of the maximum rate with the fraction of label within that compartment at that moment. The *net* label transport rate between adjacent compartments is the result of the opposite gross-transport rates. For example, diffusion between two compartments with a concentration difference of $\Delta x = (\alpha - \beta)$, is derived as the difference of two equations of Fick with $\Delta x = (\alpha - 0)$ and $\Delta x = (\beta - 0)$.

Table 6.1 Input values for the simulation program used (details explained in Materials and Methods). To obtain the inputs of the simulations 1a to 4c, the characteristics summarized by simulation 1 to 4 have to be combined with those summarized by simulation a to c, like in a matrix. The number of cell layers having efflux and the location of the cell layer representing the endodermis are indicated as "cell layer(s) - leaking" and "cell layer(s) - endodermis", respectively. In all cases the root diameter is based on 40 concentric cell layers. The asterisk (*) indicates the influx and efflux rates as given by Macklon et al. (1990), resulting in net uptake per cell.

simulation	transport rates (nmol [g FW]$^{-1}$ s^{-1})						cell layer(s)	
	plasmamembrane (p; Fig. 6.1)		tonoplast (t; Fig. 6.1)		net uptake for growth of other parts of the plant		leaking	endodermis
	influx	efflux	influx	efflux	symplastic (s_u; Fig. 6.1)	apoplastic (a_u; Fig. 6.1)		
1	0.29*	0.08*	0.37*	0.16*	-	-		
2	0.08	0.08	0.16	0.16	-	-		
3	0.08	0.08	0.16	0.16	0.42	-		
4	0.08	0.08	0.16	0.16	-	0.42		
a							10	absent
b							10	5
c							5	absent

Inputs of the simulation model

To restrict the number of combinations of input parameters for the simulation model, we focussed on the NO_3^--data from Macklon et al. (1990) as a typical example of a detailed wash-out study using the compartmental analysis to interpret efflux kinetics. As discussed further on in this section, part of the input parameters was changed because the physiological interpretation of the data derived from wash-out experiments is not clear (i.e. range of maximum influx, efflux and net uptake rates; Tab. 6.1). In addition to the data from Macklon et al. (1990), we included those morphological data that are required to describe a root, but are generally ignored in the compartmental analysis of wash-out kinetics (i.e. presence or absence of an endodermis, and the number of leaking cell layers). The remaining input parameters (i.e. maximum rates of apoplastic and symplastic exchange, the ion concentrations and cell and root dimensions) were kept constant to limit the size of the output. Although the present simulations are based on NO_3^--data (Macklon et al. 1990), the principles are also valid for other ions.

Apoplastic and symplastic exchange rates: The maximum apoplastic exchange rate between cell walls and between the outer cell walls and medium, was calculated according to the diffusion equation of Fick using a diffusion coefficient for ions in the apoplast (100 $\mu m^2 s^{-1}$; Mengel and Kirkby 1987). The diffusion surface for transport between cell walls was considered as the total number of cells per concentric layer, multiplied by the cell wall surface of the central longitudinal section of a cylindrical cell. For transport between the outer cell wall and external medium, the outer surface of the root slice was used as diffusion area. Behl and Jeschke (1982) regarded the cytoplasm as one readily mixed continuum for radial transport of $^{42}K^+$ and $^{86}Rb^+$. Because quantitative information on exchange rates through plasmodesmata is lacking, the symplastic exchange within the roots was also calculated after Fick. The diffusion surface was considered as the product of the surface of the plasmamembrane and the fraction of plasmodesmata surface (8.4×10^{-5}; in the range derived from Clarkson et al. [1971]). The diffusion coefficient of apoplastic exchange was used.

Range of influx, efflux and net uptake rates: The influx and efflux rates over the plasmamembrane and tonoplast were derived from data on NO_3^- by Macklon et al. (1990; simulation 1 in Tab. 6.1). The efflux rates were lower than the influx rates. This net uptake from the cell walls was compensated by simulating net transport through the cell walls, so that concentrations in all cell walls was constant. The difference between influx and efflux rates implies that all cells accumulated ions. This seems rather unrealistic for mature tissues. Therefore, three additional combinations (simulation 2, 3 and 4 in Tab. 6.1) were derived from the in- and efflux rates presented by Macklon et al. (1990). Independent of the different inputs in simulations 1 to 4, the costs involved in maintaining ion gradients (i.e. re-uptake balancing efflux) are identical for these simulations, as the overall efflux in these four simulations is identical (Tab. 6.1). The actual costs of re-uptake balancing efflux may be calculated according to equation 6.1 in the next section.

Simulation 2) Influx over plasmamembrane and tonoplast was assumed to equal the rate derived for efflux over these membranes (Macklon et al. 1990). Hence, there is no net ion uptake, but only bidirectional exchange of ions.

Simulations 3 and 4) In contrast to simulation 1, ions are not accumulated per cell, but transported through either the symplast (simulation 3) or apoplast (simulation 4) of the concentric cell layers towards the xylem (not necessarily into the shoot). To exclude net uptake *per cell*, in the model the influx rates over plasmamembrane and tonoplast were taken to equal the efflux rates over these membranes (Macklon et al. 1990). The transport towards the xylem to meet the requirements of growing tissues, was calculated as the difference between the influx and efflux rates derived by Macklon et al. (1990). The ions transported through the symplast were taken up by the outer concentric layer, directly adjacent to the medium, whereas the ions transported through the apoplast entered the symplast in the most inner concentric layer simulated. The net uptake was simulated separately, in addition to all other rates.

In the present simulations, it does not make a difference whether the measured values of the efflux rates are used or if these values are calculated according to the electrochemical diffusion (eq. 6.2; next section). The reason for this is that the permeability coefficient (P_c) required in

such a calculation was derived from the measured rates using the electrochemical diffusion equation (Macklon et al. 1990).

Presence or absence of an endodermis: The effect of the presence of an endodermis (Fig. 6.1) on the efflux kinetics was studied for the four situations described above (simulation 1b to 4b; Tab. 6.1). Macklon (1975) observed that the stele occupied 7.8% of the total volume (i.e. approximately 2 cell layers), but did not include this information in the compartmental analysis. Here the endodermis was assumed to be represented by the fifth concentric cell layer, to obtain a clear illustration of its effect. All apoplastic exchange rates in the cell walls of the concentric layer representing the endodermis were set at zero. In simulations 1b and 4b (Tab. 6.1), ions transported through the apoplast for net uptake were considered to pass the concentric layer representing the endodermis via the symplast. After passing the endodermis the transport proceeded through the apoplast.

Range of number of leaking cell layers: To study the effect of the number of leaking concentric layers (i.e. layers displaying efflux) on efflux kinetics, this number was varied from 10 (simulation 1a to 4b) to 5 (simulation 1c to 4c; Tab. 6.1) for a root slice of equal dimensions. The amount of 'label' present in the root at the start of the efflux period, is calculated as the sum of the amount of label present in the leaking cell layers only.

Ion concentrations and cell and root dimensions: The cell volume, cell height, cell diameter (i.e. assuming a cylindrical cell, including apoplast volume), and specific volume were $1.146\ 10^{-4}$ mm^3, 0.293 mm, 22.3 μm, and 1.1 mm^3 $(mg\ FW)^{-1}$, respectively, whereas the relative volumes (%) of free space (cell wall + air spaces), cytoplasm and vacuole were 12.4, 8.3 and 79.3 (after Macklon 1975). The diameter of the onion roots used was between 1.5 and 2 mm (Pers. com. A.E.S. Macklon), indicating that the root consisted on average of approximately 40 concentric cell layers. In all simulations, the reallocation of ions was monitored on at most the ten outer cell layers and the surrounding medium. For more cell layers the results are qualitatively the same. The ion concentration in the medium, cytoplasm and vacuole are 2.0, 50.4 and 31.3 mM, respectively (Macklon et al. 1990), whereas the cell wall concentration was assumed to be identical to the medium concentration. The loading and efflux period were 16 h and 9 h, respectively (Macklon et al. 1990).

Technical aspects of the simulation model

The model is state determined. The time constant is optimized to prevent sudden large changes in the concentrations in all compartments. The model is programmed in FORTRAN 77, using the TT-utilities of Rappoldt and Van Kraalingen (1990). All simulations were performed on an α-VAX, to enable fast simulation. The model offers good access to users, as there is a separate input file for most important input parameters, also containing switches for selecting different options (e.g. there is a possibility to set the various maximum rates to be zero, calculated according to electrochemical diffusion using equation 6.2 [i.e. for efflux over tonoplast and vacuole] or diffusion according to Fick [i.e. apoplastic and cytoplasmic exchange], or given a fixed value). Listings including explanatory comments, and copies of the programs may be obtained from the authors.

Energy requirements of maintenance of ion gradients and net uptake in relation to root diameter

The energy requirements for the maintenance of ion gradients ($m_{c\text{-}j}$; mol ATP g^{-1} s^{-1}) can be calculated, assuming that the fraction of total active transport that compensates for efflux is equivalent to the rate of total ion efflux (chapter 5):

$$m_{c\text{-}j} = \Phi_j^{io} \times M_j \times [H/I_j] \times [H/P]^{-1} \quad (6.1)$$

where Φ_j^{io} is the efflux rate of ion j (mol g^{-1} s^{-1}), M_j is the number of membranes to be passed actively by ion j, $[H/I_j]$ is the ratio between transported protons and ion j (mol H^+ [mol ion j]$^{-1}$) and [H/P] is the stoichiometry of the proton-ATPase (mol H^+ [mol ATP]$^{-1}$). Although efflux is generally expressed on a weight basis (Φ_j^{io}; mol g^{-1} s^{-1}), from a functional perspective it is more likely that efflux is proportional to the available membrane surface; cf. the electrochemical diffusion equation (Baker and Hall 1988):

$$\phi_j = -P_c \times \frac{z_j \times F \times MP}{R \times T} \times \frac{(c_j^o - c_j^i \times \exp[z_j \times F \times MP / (R \times T)])}{(1 - \exp[z_j \times F \times MP / (R \times T)])} \times 10^3 \quad (6.2)$$

where ϕ_j is the electrochemical diffusion flux of ion j (mol m^{-2} s^{-1}), P_c is the permeability coefficient (m s^{-1}), z_j is the valency of the ion j (i.e. including the + or - sign), F is the Faraday constant (96.485 J mol^{-1} mV^{-1}), MP is the membrane potential (mV), R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T is the absolute temperature (K; 293 at room temperature), c_j^o is the concentration of ion j outside (i.e. apoplast or vacuole; mol l^{-1}), c_j^i is the concentration of ion j inside (i.e. cytoplasm; mol l^{-1}). Thus:

$$\Phi_j^{io} = \phi_j^{io} \times A_j^{io} / W \quad (6.3)$$

where A_j^{io} is the membrane surface involved in efflux of ion j over that membrane, which is not necessarily equal to the membrane surface of all concentric cell layers (m^2), and W is the weight of the root slice as was also used in the simulation model (g). Combining equations 6.1 and 6.3 shows that the relative effect of root diameter on the energy requirements for maintaining ion gradients (f_m) can be derived as a multiplication factor by dividing the relative value of the new area involved in efflux (f_A) by the relative value of the new root weight (f_w), after changing the root diameter.

The effect of root diameter on the experimental estimates of the specific cost of net ion uptake can be studied, assuming that the amount of influx and efflux are both proportional to the surfaces involved. These influx and efflux surfaces are not necessarily equal. Experimental estimates of the specific cost of ion uptake of ion j ($1/U_j$; mol ATP [mol ion j net uptake]$^{-1}$) are composed according to equation 6.4, in which the denominator and numerator are estimated as the net uptake rate of ion j (mol ion j g^{-1} s^{-1}) and the respiration rate correlating to this uptake rate (mol ATP g^{-1} s^{-1}), respectively:

$$1/U_j = (\phi_j^{oi} \times A_j^{oi} \times M_j \times [H/I_j] \times [H/P]^{-1} / W) / ([\phi_j^{oi} \times A_j^{oi} - \phi_j^{io} \times A_j^{io}] / W) \quad (6.4)$$

where ϕ_j^{oi} and ϕ_j^{io} are the in- and efflux rates of ion j (mol m^{-2} s^{-1}), and A_j^{oi} and A_j^{io} are the surfaces involved in the in- and efflux of ion j (m^2). The actual specific cost of ion uptake is obtained if efflux is zero.

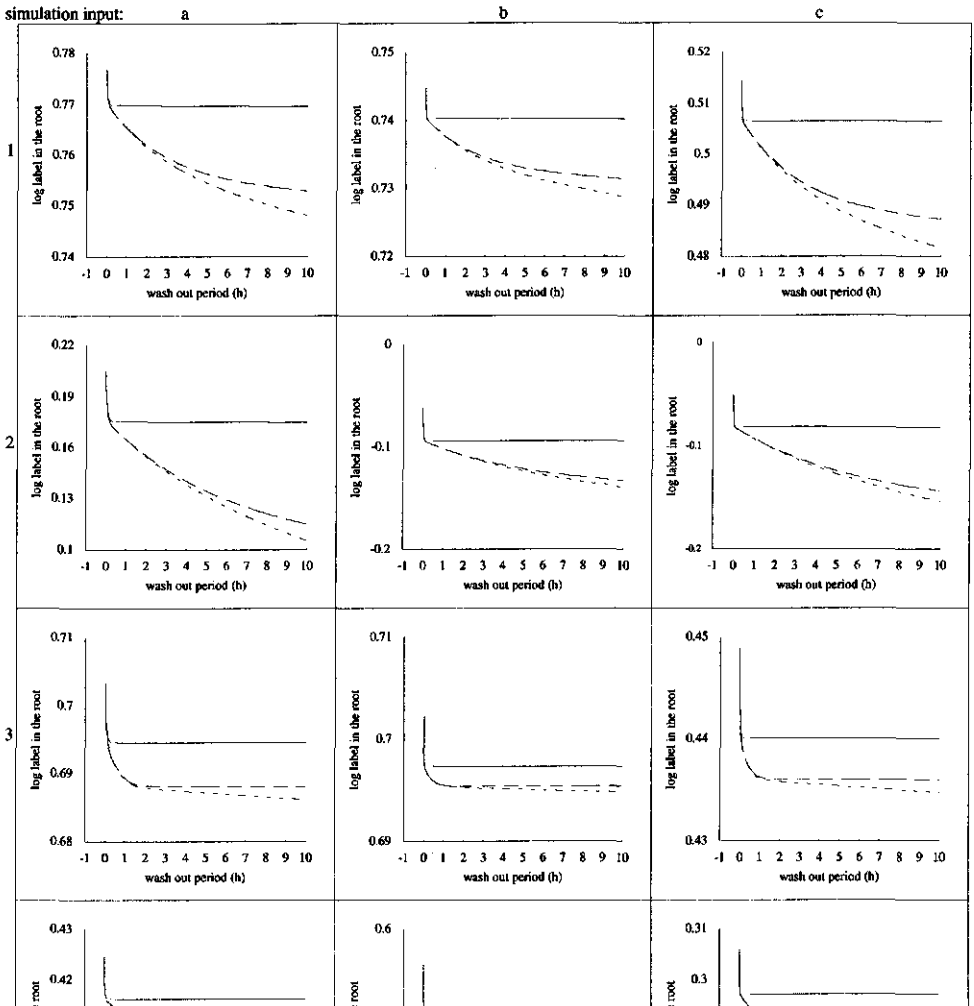
Results

Simulation of (apparent) efflux kinetics and consequences for maintenance requirements

The apparent efflux (Fig. 6.3) is the sum of all efflux kinetics per compartment, derived as illustrated in Fig. 6.2 (figures for all simulations in appendix 6). The presence of an endodermis (simulation b) and changing the number of cell layers involved in efflux from 10 to 5 (simulation c), reduced the absolute amount of apparent efflux (see appendix 6). In Fig. 6.3 this may not be obvious for the simulations 3b and 4b. The reason is that in Fig. 6.3 the apparent efflux is presented as the amount of label remaining in the tissue, as generally done in compartmental analysis. However, the labeling of the tissue is also affected by the presence of an endodermis and the number of cell layers involved in efflux (see appendix 6). The decrease in apparent efflux was not proportional to the reduction of the number of cell layers involved in efflux.

The apparent efflux kinetics differed significantly between simulations (Fig. 6.3), even when the actual total efflux was identical (i.e. simulations 1a to 4b or 1c to 4c). That is, the efflux rate over plasmamembrane and tonoplast and the number of cell layers having efflux were similar, but the rate of efflux-ions reaching the medium differed. Similar differences were present when regarding the simulated efflux kinetics from a 100% labelled root (data not shown). The differences in apparent efflux are due to differences in root anatomy and different transport rates and pathways for net uptake, between the various simulations (Tab. 6.1). Analysis of the apparent efflux without regarding these factors, yields different values for the efflux rates over plasmamembrane and tonoplast (data not shown). This suggests different costs for re-uptake balancing efflux (i.e. maintaining ion gradients), although these costs are identical for the simulations with the same actual total efflux (i.e. simulations 1a to 4b or 1c to 4c).

Fig. 6.3 (p. 83) Representation of efflux kinetics as generally used for performing a compartmental analysis, by expressing the amount of label remaining in the tissue on a ^{10}log scale versus time. With experiments, only the overall curve from vacuoles + cytoplasm + cell walls (i.e. the apparent efflux; small dashes) is obtained. The curve for cytoplasm + cell walls (large dashes) and cell walls only (drawn line) are normally not known. From above to below the individual figures represent the simulation runs 1 to 4, and from left to right the situations a, b and c (codes explained in Tab. 6.1).


simulation input: a
b
c
1
2
3
4
log label in the root
wash out period (h)

Energy requirements of maintenance of ion gradients and net uptake in relation to root diameter

Electrochemical diffusion of ion j (ϕ_j^{io}; mol m^{-2} s^{-1}) can become negligible if the internal concentration is sufficiently reduced; the outer (i.e. cell wall) concentration is less important unless membrane potentials are very low (cf. eq. 6.2). Thus, efflux from certain cell layers could be approximately zero, due to metabolism of ion j. This justifies simulation c (Tab. 6.1) and the present calculations for a smaller number of leaking cell layers.

There will be no effect of the root diameter on the efflux rate per unit weight (Φ_j^{io}) if all cell layers contribute to efflux (i.e. similar $\phi_j^{io} >> 0$), as the relative value of both the new root weight (f_W) and area involved in efflux (f_A) have an identical relationship with the root diameter. In situations where the number of cell layers involved in efflux was decreased, the relative effect of a smaller root diameter on Φ_j^{io} and thus the maintenance costs ($m_{c\text{-}j}$) were found to increase up to a factor of 4 (Fig. 6.4). Similarly, the experimental estimates of the specific costs of ion uptake (mol ATP [mol ion taken up]$^{-1}$) will also be affected by the root diameter in the case that in- and efflux area are not identical. However, the magnitude of the effect cannot be given as a simple fraction, independent of the in- and efflux rate. Assuming an in- and efflux rate p and q to be affected by a factor a and b, respectively, the specific costs of ion uptake will change with a factor (ap - bq) / (ap - aq).

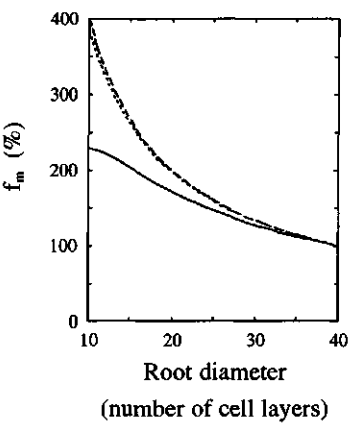


Fig. 6.4 The relative effect of different root diameters on the efflux rate per unit weight and thus the maintenance costs (f_m; %), in case of efflux over the *i)* surface directly adjacent to the medium (long dashes), *ii)* plasmamembrane of the most outer cell layer only (small dashes), and *iii)* plasmamembrane of the 10 most outer cell layers (drawn line). In all cases a root with a diameter of 40 cell layers was used as reference for deriving the relative changes.

Discussion and conclusions

Apparent efflux kinetics and consequences for maintenance requirements.

The simulations 1a to 4b and 1c to 4c.shows that differences in apparent efflux kinetics (Fig. 6.3) do not necessarily indicate a different actual total efflux and, thus, related respiratory costs for maintaining ion gradients. These costs can be calculated according to equation 6.1, by combining the efflux rates over the plasmamembrane with a value of 1, 2 and 1 for M_j, $[H/I_j]$ and [H/P], respectively, and combining the efflux rates over the tonoplast with a value of 1, 1 and 2 for M_j, $[H/I_j]$ and [H/P], respectively. For example, assuming that all membranes are involved in efflux, the ATP costs of re-uptake balancing efflux ($m_{c,j}$) are 0.24 nmol ATP [g FW]$^{-1}$ s^{-1}. These costs, based on the nitrate efflux rates as observed by Macklon et al. (1990), are relatively low compared to those derived in chapter 5. Reasons for this discrepancy are discussed in chapter 5. The observed differences in apparent efflux kinetics, despite an equal total actual efflux, might be attributed to the effect of an endodermis, symplasticly versus apoplasticly proceeding transport for net uptake or different transport rates within the root. Thus, these factors should be included in the analysis of experimentally observed efflux curves, to assess the maintenance costs. The analysis method generally used is the compartmental analysis.

Before starting compartmental analysis, the labelling of the tissue should be near steady state (Cram 1968, 1983, MacRobbie 1964). That is, in particular any rapid adjustment of the cytoplasmic phase has to be completed (Cram 1983), whereafter the specific activity of the cytoplasm should rise only very slowly, like that of the vacuole (MacRobbie 1964). Simulations 1 and 2 (a to c) meet this criterion, illustrated by the approximately similar slopes of the fractions of labelled ions in cytoplasm and vacuole (see appendix 6). However, regarding the data presented in Fig. 6.3, compartmental analysis is not allowed, as efflux from the cytoplasm still contributed to the apparent efflux after 10 h. Obviously, the 10 h period as used by Macklon et al. (1990), is too short for the present simulations. To establish a loading period, a time step for pursuing the efflux kinetics and an efflux period suitable for compartmental analysis, information on the time constants of the system is required in advance. For example, efflux from the surface solution and the free space could only be distinguished after reducing the time step (Siddiqi et al. 1991), and characterization of the last compartment was only possible if the efflux period was long enough (Kasimir Klemedtsson and Pettersson 1990). The differences in half-life values of the identified pools, depending on whether ^{15}N or ^{13}N label is used (Macklon et al. 1990), might well be due to variation in factors like loading period, efflux period and time step of observation, as in all cases the efflux curve obtained is suitable for curve peeling, irrespective of the different time scale.

Compartmental analysis has been modified to account for ion export and ion metabolism (references in introduction), which contributed to deviating kinetics e.g. as obtained in simulations 3 and 4. However, we are not aware of methods that correct for the effect of an endodermis (simulations 1b to 4b versus 1a to 4a) or different transport pathways involved in net uptake (simulation 3 versus 4). Anatomical effects are rarely included in compartmental

analysis. Exceptions are the study of Behl and Jeschke (1982; longitudinal heterogeneity for cytoplasm pools) and Siddiqi et al. (1991; reduction and efflux of nitrate occurs in epidermis and cortex, respectively). The present study shows that it is important to account for differences in root anatomy and transport pathways, as it strongly affects the apparent efflux kinetics and thus estimates of respiratory costs for maintaining ion gradients. For example, the respiratory costs of maintaining ion gradients will be significantly underestimated, if no corrections are made for efflux remaining inside the stele.

Reducing the number of leaking cell layers (i.e. a lower actual total efflux), reduced the observed efflux. The effect was not proportional to the decrease of the amount of tissue involved. This complicates the estimation of the number of leaking cell layers, which we consider as an important factor for understanding variation in the respiratory requirements involved in maintaining ion gradients.

Validity of the present simulations.

In the present simulations the external medium was 100% labelled during loading, whereas in wash-out experiments this percentage is much lower. This difference does not affect the conclusions of this study, as the fraction of labelled ions in the medium is proportional to the fraction of ions in the root that becomes labelled and is, thus, also proportional to the amount of efflux of labelled ions. The present simulations do not include any discrimination for isotopes. This also does not affect the conclusions of this study, as its effect is proportional to the fraction of ions within the root that becomes labelled and, thus, efflux of labelled ions.

Comparison of the present results to the original data from which the efflux rates were derived (Macklon et al. 1990), is not possible as not all input parameters required could be derived from that paper. The model and its input represent the present state of the art, and were used to visualize the effects of extremes (e.g. apoplastic versus symplastic transport for net uptake). Although the actual values of the present model may not be fully accurate due to difficulties in obtaining all input parameters, the relative differences between the different treatments are accurate, and indicate that data on root anatomy (location endodermis) and pathways of net uptake (apoplastic, symplastic or a combination) have to be included when analyzing efflux kinetics. Development and application of the model elucidated that knowledge on the number of cell layers involved in efflux is lacking and gave insight into the complexity of the system. Besides, it emphasized that also in wash-out experiments, input (i.e. loading period, efflux period and time step used in pursuing the efflux kinetics) determines the results.

Energy requirements of maintenance of ion gradients and net uptake in relation to root diameter

Our calculations show that root diameter affects the respiratory costs of maintaining ion gradients, in the case that not all cell layers contribute to efflux. The obvious reason is that these respiratory costs are expressed per unit dry weight rather than per unit surface involved in efflux. To understand differences in maintenance requirements, the way of expressing respiratory costs should be consistent with the physiological basis of the energy-requiring

processes (e.g. transport processes and its costs on an area basis). Unfortunately, it is not possible to use an area basis as long as it cannot be quantified precisely which part of the total plasmamembrane (and tonoplast) surface is involved in efflux. To resolve this, data on the ion concentrations in the different compartments, membrane permeabilities and membrane potentials for cells located in different cell layers are required (cf. eq. 6.2), but are still rare (e.g. partly in Malone et al. 1991 and Zhen et al. 1991). There is also an effect of the root diameter on the experimental estimates of the costs of net ion uptake, if the surface involved in efflux differs from that involved in influx. In general, comparison of energy requirements of membrane-related processes on a dry weight basis, derived from roots with different diameters, may easily lead to erroneous conclusions and should therefore be done using values expressed in the appropriate units.

Chapter 7

Analysis of root respiration of *Solanum tuberosum* as related to growth, ion uptake and maintenance of biomass: a comparison of different methods

with A.G.M. Broekhuysen and B.W. Veen

Abstract - The aims of this study were *i)* to determine specific respiratory costs for growth (1/Y; mmol O_2 [g DW]$^{-1}$), ion uptake (1/U; mol O_2 [mol NO_3^-]$^{-1}$) and maintenance (m; nmol O_2 [g DW]$^{-1}$ s^{-1}) of roots of potato and *ii)* to evaluate the accuracy of these estimates. Therefore, we compared different experimental estimates, theoretical calculations based on the energy costs of processes and data available from the literature. Finally, *iii)* we estimated the relative importance of protein turnover to deduce whether the costs of maintaining solute gradients in root tissue is included in the specific costs of maintenance (m) or ion uptake (1/U). Our experimental system allowed monitoring root respiration and nitrate uptake, of a single plant for several days or of a group of plants for a single day per plant. Root growth was followed by non-destructive measurements. The estimates of the specific costs of growth ion uptake and maintenance obtained from different experiments, were similar (i.e. 1/Y = 9.8 to 9.9 mmol O_2 [g DW]$^{-1}$, 1/U = 0.39 to 0.67 mol O_2 [mol NO_3^-]$^{-1}$ and m = 10.2 to 14.8 nmol O_2 [g DW]$^{-1}$ s^{-1}). The value for 1/U was affected by perturbation. The present experimental values of 1/Y agreed well with the range in the literature (6.2 to 10.9 mmol O_2 [g DW]$^{-1}$) and the theoretically calculated value (9.5 mmol O_2 [g DW]$^{-1}$). The costs calculated for protein turnover (3.7 to 16 nmol O_2 [g DW]$^{-1}$ s^{-1}) can explain total maintenance requirements, and are relatively high compared to most literature data on maintenance (4.0 to 7.0 nmol O_2 [g DW]$^{-1}$ s^{-1}). From the perturbation effects it was deduced that overall costs for re-uptake balancing efflux (i.e. maintaining solute gradients) correlates to 1/U, and accounts for up to 33% of the overall costs of nitrate influx (i.e. 1/U is up to a factor 1.5 higher if efflux takes place). The absence of these costs in the present estimates of 1/U explains the good agreement with the calculated value (0.43 mol O_2 [mol NO_3^-]$^{-1}$), and why the present estimate is lower than the values generally obtained (0.83 to 1.16 mol O_2 [mol NO_3^-]$^{-1}$).

Abbreviations - summarized in Table 7.1.

Introduction

Plant dry weight increment is largely the balance between photosynthetic gains and respiratory losses. Quantitative information on both the specific respiratory costs and the rates of various energy-requiring processes are necessary to account for the respiratory losses. The root is a suitable organ for studying this, as it is purely heterotrophic. Respiration has been divided into two functional components: growth and maintenance (Pirt 1975, Thornley 1970). Growth and maintenance respiration are defined as the respiration associated with the synthesis of new biomass and the maintenance of existing biomass, respectively. The specific costs for growth depend on the chemical composition of the new biomass (Penning de Vries et al. 1974). Maintenance respiration may be needed for up to 80% for protein turnover (De Visser et al. 1992), but much lower fractions were found for primary leaves of bean (chapter 3). Other measurements indicate that the maintenance of ion gradients is also a quantitatively important process (chapter 5).

Many estimates of the specific costs for growth (1/Y) and maintenance (m) of either roots or shoots have been obtained by two-component regression (Blacquière 1987, Hansen and Jensen 1977, Lambers 1979, Szaniawski 1981, Szaniawski and Kielkiewicz 1982). With this method specific costs for growth are determined by correlating the respiration rate to the growth rate. The respiration not correlating to growth is ascribed to maintenance. However, ion uptake should be distinguished as a third energy-requiring component (Veen 1980), especially for roots, because of the relatively large energy requirements of ion uptake of this organ. Therefore, for root respiration, estimates obtained by two-component regression may be less useful and significantly different from those obtained by three-component regression (Lambers and Van der Werf 1988). In three-component regression, root respiration is separated into functional components by correlating it to growth, ion uptake and existing biomass (Veen 1980). The specific respiratory costs involved in ion uptake (1/U) depend on the number of active root-membrane passages per ion and the proton-ion and proton-ATP stoichiometries. Experimental data on specific costs for growth, uptake and maintenance are limited to *Zea mays* (Veen 1980) and two *Carex* species (Van der Werf et al. 1988). Maintenance and growth respiration have also been estimated by other methods (Amthor 1984, De Visser and Lambers 1983, Lambers 1985), but information on respiratory costs for ion uptake is scarce. Poorter et al. (1991) suggested that specific costs for ion uptake are lower in fast-growing species than in slow-growing ones. So far, there are insufficient data to corroborate this contention.

Estimating specific costs for growth, maintenance and ion uptake by three-component regression has some limitations. Because this statistical method is correlative, it is not always exactly clear to which regression component the various processes contribute. For example, costs involved in re-uptake balancing efflux (i.e. maintaining ion gradients) might be included in the costs for uptake or in those for maintenance, depending on whether efflux rate correlates with uptake rate or with root biomass. Therefore, it is useful to examine how alternative estimates of costs of individual (maintenance) processes relate to the obtained specific costs for growth, ion uptake and maintenance. Such alternative estimates should be derived either from

direct measurements or from calculations based on specific processes.

Our main objective was to obtain parameters on specific respiratory costs for growth, uptake and maintenance by three-component regression on potato roots (*Solanum tuberosum* L. cv. Alcmaria). To test the robustness of this approach, two different experiments were compared. Secondly, we intended to validate our results by comparing the regression coefficients with estimates obtained by alternative methods (both experimental and theoretical) and literature data. Thirdly, we calculated the contribution of protein turnover to m, to evaluate whether maintaining solute gradients contributes to m or to 1/U.

Materials and methods

Requirements and assumptions of three-component regression

To estimate specific costs of growth, ion uptake and maintenance by three-component regression analysis, variable ratios between the regression components must be obtained. The success of varying these ratios determines the quality of the estimates of specific costs (indicated by their variation and the overall variance accounted for), and form the major difficulty of the present approach. If there is a constant shoot/root-ratio and constant mineral concentration of both roots and shoot, there tends to exist a fixed ratio between root growth and ion uptake. Since ion uptake by the roots is for the growth of both roots and shoot, this relation can be disturbed by changing the shoot-growth/root-growth ratio. In the present experiments, we used this approach by pruning of the shoots and/or roots. As a consequence, the regression approach is only valid if in all cases the biochemical processes underlying 1/Y, 1/U and m are identical, so that their values are independent of *i)* time and *ii)* treatments to vary the ratio between these components of respiration. Differences in growth conditions are thus expected not to affect the result.

Plant material

For both experiments, potato plants (*Solanum tuberosum* L. cv. Alcmaria) were propagated by in vitro culture and grown in aerated nutrient solution (macro nutrients at 1/2 strength according to Hoagland and Snyder [1933] and micro nutrients at 1/2 strength according to Lewis and Powers [1941], with 90 μM iron as Fe(III)-EDTA).

Experiment 1 - Growth conditions in the growth room were as described in chapter 2.

Experiment 2 - The first batch of plants used for the measuring sessions 1, 2 and 3, were grown in a growth room. Temperature was 18 ± 1 °C, and relative humidity 70%. The light conditions were: 12 h 350 μmol m^{-2} s^{-1} PPFD provided by fluorescent light (Philips TLD 50W/84HF) and incandescent light (Pope 60 W). Low-intensity incandescent light was supplied for another 6 h after the 12 h light period, to prevent tuber induction. An uniform supply of nutrients to the plants was maintained by rapid circulation from a central storage vessel. Entangling of the roots of neighboring plants was prevented. Plants were regularly rotated within the growth room to obtain an uniform growth. To prevent mutual shading, the

plants used during session 3 were moved to a greenhouse, from 15 to 43 days after transplanting (DAT) the plants from the in vitro culture to the nutrient solution. A second batch of plants used for the measuring session 4, was grown in a greenhouse up to 44 DAT. The temperature in the greenhouse was 18 ± 1 °C, and the relative humidity 70%. In addition to the natural light, there was 16 h 0.1 mmol m^{-2} s^{-1} PPFD provided by HPLR 400 W lamps (Philips). In the greenhouse, each individual plant was placed in a container of 2.5 l.

Description of the experiments

The replications required for three-component regression were obtained either by long-term frequent observations on a single plant (experiment 1) or by short-term measurements on various plants (experiment 2). The first approach allowed additional treatments to vary the constant ratio between the regression components during the experiment, but did not yield information on plant variation, whereas in the second approach plant variation was included, but the variation of the ratio between the regression components could not be evaluated during the experiment.

Experiment 1 - To break the constant ratio between the regression components, the root system of a single plant was reduced to approx. 50% at the start of the experiment (day 0) and its shoot was pruned between day 17 and 18. With pruning, the young growing, sugar-importing and expanding parts (approx. 50% of the fresh weight) were removed, leaving sufficient sugar-exporting leaves for the relatively few remaining sinks. Up to day 25, root respiration, root volume and plant weight were determined daily, except for the weekends (day 1 was on a Saturday). Nitrate, potassium and water uptake and the excretion of OH^--ions were monitored continuously. On day 25, respiration was measured in tap water (nitrate and phosphate concentrations both less than 10 μM) with 0.5 mM $CaSO_4$. On day 26 the plant was harvested. Chemical analyses were done on untreated parallel plants grown on 1/2 Hoagland solution (pH 6) under the same environmental conditions (chapter 2).

Experiment 2 - Three groups of 5 plants were randomly selected from stock at 29, 36, 43 and 46 DAT for the sessions 1 to 4, respectively. The first group was not treated, whereas from the second and third group the root and the shoot, respectively, was pruned by approx. 50%. The root system was reduced by removing the lower half. With pruning of the shoot, only the young growing, sugar-importing and expanding parts were removed. For sessions 3 and 4, the treatments were carried out at the day the plants were transferred from the greenhouse to the growth room and 2 days after transfer, respectively. After the treatments, the root and total plant weight were determined daily on all 5 plants per treatment by a non-destructive method. Root respiration, nitrate, potassium and water uptake and the excretion of OH^--ions were measured on successive plants, one plant per day. Afterwards, the plants were harvested. These measurements started 4 days after applying the treatments. During each measuring session, one or more plants per treatment were used. All observations of the four sessions were combined for the regression analysis.

Respiration and ion uptake measurements

Nitrate, potassium and water uptake, OH^--excretion, respiration and growth of the roots were monitored, using the system described by Veen (1977). Light intensity, light period and temperature during the measuring period were identical to those in the growth rooms used in experiment 1 and 2. The concentration of nitrate (0.75 mM and 1.4 mM in exp. 1 and 2, respectively) and potassium (0.3 mM in both exp. 1 and 2) and pH (4.5) were maintained by automatic titration, enabling calculation of net uptake/excretion rates. The relatively low pH was required to measure CO_2 production (data not shown), but did not affect ion uptake compared to that at pH 6 (i.e. pH during the previous growth period). Root respiration was measured with a Clark type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) in a closed system without aeration, during which the oxygen concentration did not drop below 50% of the air-saturated level. The root volume (exp. 1) or the root weight (exp. 2) was determined subsequently. Ion transport could not be derived during and directly after these determinations. The period following these determinations was required to equilibrate the titration systems (exp. 1), whereas in experiment 2 the plants were harvested.

Determination of the daily root and shoot growth

Destructive measurements in both experiment 1 and 2 included the determination of the shoot fresh weight and that of the root after mild centrifugation for 1 min. in a standard household centrifuge (2800 rpm, r = 122.5 mm) of adhering water. Subsequently, root volume was determined by a water-displacement method (Huxley 1971). Dry weights were obtained after 24 h at 70°C. Methods to monitor the growth non-destructively, differed between both experiments.

Experiment 1 - During the experiment, increment of root volume and plant weight were measured non-destructively, without removing the plant from the cuvette. The daily change in root volume was determined after Veen (1977). Instead of calculating the nutrient solution adhering to the roots by analyzing the increase in potassium concentration after spraying the roots, we measured the increase in conductivity after rinsing the root chamber with 1500 ml tap water. Root volume during the experiment was calculated from the final root volume and the daily increments. The specific root weight (g fresh weight ml^{-1}) was approximately 1, so that the root volume approximates its fresh weight. The shoot weight was calculated daily from the difference of total plant weight and root weight. Growth rate was estimated as the slope of the curve fitted through the measured weights. At the end of the experiment (day 26), the plant was harvested.

Experiment 2 - After applying the treatments, increment of root and plant weight were measured non-destructively, for all plants. The daily change in shoot weight was determined by weighing the plant with the roots floating in the nutrient solution. The root weight was calculated daily from the difference of total plant weight and shoot weight, after subtraction of the nutrient solution retained between the roots. The amount of adhering nutrient solution was obtained from the increase in conductivity after rinsing the root in 1000 ml tap water. If a plant was used to measure the respiration and ion transport rates, afterwards, this plants was

harvested destructively. Root growth rate of the different plants was estimated as the slope of the curve fitted through the measured weights (14 of the 19 times logistic, the remaining linear). In case a plant was measured (and thus harvested) shortly after the treatments were applied, parameters from the growth curves of the other plants of the same treatment in the same session were included to obtain an accurate fit of the growth rate.

Chemical analyses

For theoretical calculations of the respiratory costs for growth and protein turnover, ash, ash alkalinity, carbon, total- and NO_3^--nitrogen were determined on 17 - 45 days old plants, grown as in experiment 1. The carbon and total nitrogen concentration were determined by CHN analysis on a Heraeus CHN-rapid (Hanau, Germany). Nitrate was determined by a TRAACS 800 continuous flow system (Bran and Luebbe Analyzing Technologies, Elmsford, NY, USA). Ash concentration was determined from the change in dry weight after heating the plant material at 550 °C for at least 1 h. Addition of excess 0.1 M HCl and titration with 0.1 M NaOH to pH 5 gave the ash alkalinity (A_a; meq CO_3^{2-} [g DW]$^{-1}$).

Multiple regression model

To separate components of respiration, a linear multiple regression model was used, described by the overall equation:

$$R = 1/Y \times RGR + 1/U \times NU_NR + m \qquad (7.1)$$

where R is the root respiration rate (nmol O_2 g^{-1} s^{-1}), 1/Y is the respiration requirement for the synthesis of root material (mmol O_2 g^{-1}), RGR is the relative growth rate of the root (μg_{new} g^{-1} s^{-1}), 1/U is the respiration requirement associated with the net uptake of nitrate by the root (mol O_2 [mol NO_3^-]$^{-1}$), NU_NR is the net rate of nitrate uptake by the root, used as a measure for total ion uptake (nmol NO_3^- g^{-1} s^{-1}), and m is the respiration requirement for maintenance of root biomass (nmol O_2 g^{-1} s^{-1}). In both experiments, root size and growth rate at any moment during the experiment were derived from the fitted root growth curves.

Experiment 1 - Because ion uptake rate, respiration rate and root volume could not be measured simultaneously, the uptake rate over the 10 h directly preceding the respiration measurement was used as input for the regression analysis. This period was long enough to allow a reliable determination of the uptake rate. Day 18 and day 25 were ignored in the regression analysis, because on these days the shoot was pruned and root respiration was measured in a medium without mineral nutrients, respectively. Saturdays and Sundays were also excluded, as on these days respiration was not measured. Regression on a dry weight basis was not possible, as only one single plant was used. Where appropriate, the coefficients were expressed on a dry weight basis, using a fraction dry matter of 0.069 $\pm$ 0.009 (95% probability interval; n = 48).

Experiment 2 - The uptake rate over the 3 h directly preceding the respiration measurement was used as input for the regression analysis. In comparison with experiment 1, this period could be shorter due to a smaller nitrate amount and time-interval per titration step. The regression was done on both a fresh and dry weight basis.

Table 7.1 List of abbreviations and symbols with their descriptions, average values, units and references for the values. The references are: a, Table 7 in Penning de Vries et al. 1974; b, De Visser et al. 1992; c, Sanders 1990; d, Teyerman 1992; e, Poorter et al. 1991.

Symbol	Meaning	Value	Unit	Ref.
A_a	Amount of carbonate in the ash in dry matter		meq CO_3^{2-} (g DW)$^{-1}$ or mg CO_3^{2-} (g DW)$^{-1}$	
A_{dm}	Ash concentration in dry matter		mg (g DW)$^{-1}$	
C_{dm}	Carbon concentration of root dry matter		mg C (g DW)$^{-1}$	
C_{om}	Carbon concentration of organic dry matter		mg C (g $DW_{organic\ material}$)$^{-1}$	
$C_{NO3/NH4}$	Ratio between oxygen requirement factors for plant growth on nitrate and ammonia	1.12		a
$CPFI_{dm}$	Carbon dioxide production due to the biosynthesis of 1 g dry matter		mg CO_2 (g $DW_{substrate}$)$^{-1}$	
DAT	days after transplanting (in vitro -> Hoagland)			
E_{sp}	Specific energy costs of protein turnover		mol ATP (mol peptide bond)$^{-1}$	b
$[H/I_j]$	Proton/ion-stoichiometry required for transport		mol H^+ (mol ion j)$^{-1}$	
[H/P]	Proton/ATP-stoichiometry of proton-ATPase	1	mol H^+ (mol ATP)$^{-1}$	c,d
K_d	Degradation constant of protein turnover		s^{-1}	
m	Respiration requirement for maintenance of root biomass		nmol O_2 g^{-1} s^{-1}	
M_j	Number of membranes crossed actively by ion j			
m_p	Maintenance costs for protein turnover		(mmol or) nmol O_2 (g DW)$^{-1}$ s^{-1}	
$NO_{3\text{-}dm}$	Nitrate concentration of root dry matter		mg NO_3 (g DW)$^{-1}$	
N_p	Average N concentration of leaf protein	1.26	mol N-protein (mol peptide bond)$^{-1}$	
N_t	Protein concentration of root dry matter		mmol N-protein (g DW)$^{-1}$	
NU_NR	Net rate of nitrate uptake by the root		nmol nitrate g^{-1} s^{-1}	
$[P/O_2]$	ATP/O_2-ratio of oxidative phosphorylation	4.7	mol ATP (mol O_2)$^{-1}$	e
PV	Production value	0.645	g product (g substrate)$^{-1}$	a
R	Root respiration rate		nmol O_2 g^{-1} s^{-1}	
RGR	Relative growth rate of the root		µg g^{-1} s^{-1}	
RQ	Respiratory quotient		mol CO_2 (mol O_2)$^{-1}$	
RQ_s	Respiratory quotient for synthesis	1.97	mol CO_2 (mol O_2)$^{-1}$	a
1/U	Experimental estimation of the specific costs associated with uptake of nitrate		mol O_2 (mol nitrate)$^{-1}$	
$1/U_{theor}$	Theoretical estimation of 1/U		mol O_2 (mol nitrate)$^{-1}$	
1/Y	Experimental estimation of the respiration requirement for the synthesis of root material		mmol O_2 g^{-1}	
$1/Y_{theor}$	Theoretical estimation of 1/Y		mmol O_2 (g DW)$^{-1}$	
VAF	variance accounted for			

Alternative estimation of energy costs for growth

The CO_2 production associated with the conversion of 1 g of substrate into plant dry matter and required energy ($CPFI_{dm}$; mg CO_2 [g substrate]$^{-1}$) was calculated from the carbon concentration of the organic material (C_{om}; mg C [g $DW_{organic\ material}$]$^{-1}$) according to equation 8 in Vertregt and Penning de Vries (1987; i.e. $CPFI_{dm} = 4.24\ C_{om} - 1744$). The carbon concentration of the organic material was calculated from the carbon concentration in the dry matter (C_{dm}; mg C [g DW]$^{-1}$) and the mineral concentration in the root material:

$$C_{om} = (C_{dm} \times 1000) / (1000 - (A_{dm} - A_a + NO_{3\text{-}dm})) \quad (7.2)$$

where A_{dm} is the ash concentration (mg ash [g DW]$^{-1}$) and $NO_{3\text{-}dm}$ is the nitrate concentration of the root (mg NO_3^- [g DW]$^{-1}$). During ashing both organic anions and nitrate are converted into carbonate (Vertregt and Penning de Vries 1987), and thus included in A_a. Therefore, the mineral concentration was obtained by subtracting A_a and adding $NO_{3\text{-}dm}$ to A_{dm}. The value for A_a was transformed from equivalent to a dry weight basis (mg CO_3^{2-} [g DW]$^{-1}$) by multiplication with the equivalent weight of carbonate (30 mg [meq]$^{-1}$).

Neither the costs for transport nor those for nitrate reduction are accounted for in equation 8 of Vertregt and Penning de Vries (1987). As the plant was nitrate fed in our experiments, we used a correction factor ($C_{NO3/NH4}$) for the extra costs involved in growth on nitrate compared to those on ammonium. Furthermore, we transformed $CPFI_{dm}$ to the oxygen requirement for biosynthesis of 1 g of dry matter ($1/Y_{theor}$; mmol O_2 [g DW]$^{-1}$):

$$1/Y_{theor} = (CPFI_{dm} \times C_{NO3/NH4}) / (44 \times PV \times RQ_s) \quad (7.3)$$

where $C_{NO3/NH4}$ is the ratio between the oxygen requirement factor (ORF) of plant biomass when grown on nitrate or ammonia, 44 is the molecular weight of CO_2 (g mol^{-1}), PV is the production value of a nitrate fed plant (g DW produced [g DW substrate]$^{-1}$) and RQ_s is respiratory coefficient for synthesis with ammonia as nitrate source (mol CO_2 [mol O_2]$^{-1}$). The values of $C_{NO3/NH4}$ (1.12), PV (0.645) and RQ_s (1.24) were derived from Table 7 in Penning de Vries et al. (1974).

Alternative estimates of energy costs for ion uptake

To arrive at an alternative estimate for the specific cost for uptake, in experiment 1 the root respiration rate of a non-growing root in the presence (day 19, 20, 21) and absence (day 25) of nutrients was compared.

A theoretical estimation of the specific costs for nitrate uptake ($1/U_{theor}$; mol O_2 [mol NO_3^-]$^{-1}$) was obtained using the following equation:

$$1/U_{theor} = ([H/I_j] \times M_j) / ([H/P] \times [P/O_2]) \quad (7.4)$$

where $[H/I_j]$ is the proton/ion-stoichiometry required for a membrane passage of nitrate (2 mol H^+ [mol NO_3^-]$^{-1}$; on the basis of electrophysiological evidence, Ullrich and Novacky [1981] concluded that $[H/I_j]$ is greater than one), M_j is the number of membranes to be crossed actively (1), [H/P] is the amount of protons pumped over a membrane by the hydrolysis of one

ATP to ADP (1 mol H^+ [mol ATP]$^{-1}$ as derived from kinetic models; Sanders 1990, Teyerman 1992) and [P/O_2] is the efficiency of the oxidative phosphorylation (mol ATP [mol O_2]$^{-1}$). We used a [P/O_2] ratio of 4.7 mol ATP (mol O_2)$^{-1}$ (average value for 17 species derived from Poorter et al. 1991). In the absence of pertinent data, re-uptake to balance efflux and transport over the tonoplast for compartmentation in the vacuole were ignored in these calculations. Ignoring the transport over the tonoplast is unlikely to have a major effect on calculated costs, as most of the ions are transported to the shoot instead of to root vacuoles (i.e. the normal root/shoot-ratio is 0.21 and the nitrogen concentrations of shoot and root are approximately similar; chapter 2).

Alternative estimates of energy costs for maintenance and protein turnover

From experiment 1, maintenance costs were estimated directly by measuring the respiration rate in the absence of growth and ion uptake on day 25.

The maintenance costs involved in protein turnover (m_p; mmol O_2 [g DW]$^{-1}$ s^{-1}) was calculated after combining equations 1b and 2b in De Visser et al. (1992) from the specific energy costs (E_{sp}; mol ATP [mol peptide bond]$^{-1}$), the degradation constant of protein turnover (K_d; s^{-1}), the N-protein concentration of the root (N_t; mmol N-protein [g DW]$^{-1}$) and the average N concentration of root protein (N_p) which is considered to be approximately equal to that of leaf proteins (1.26 mol N-protein [mol peptide bond]$^{-1}$):

$$m_p = (E_{sp} \times K_d \times N_t) / ([P/O_2] \times N_p) \qquad (7.5)$$

N_t was calculated as the difference between the total nitrogen concentration (mg g^{-1}) and the NO_3^--nitrogen concentration (mg g^{-1}) divided by the atomic weight of nitrogen. The values used for the K_d of roots were 1.6×10^{-6} to 7.3×10^{-7} s^{-1} (Van der Werf et al. 1992) and for E_{sp} were 10 - 20 mol ATP (mol peptide bond)$^{-1}$ (De Visser et al. 1992).

Statistics

In experiment 1, the increase in root biomass (g FW_{root}) up to day 18, was fitted in GENSTAT (Payne 1987) by the curve: $FW_{root} = a + b \times R^{day}$, with as constraints $R < 1$. In experiment 2, the increase in root biomass of the various plants was fitted in GENSTAT as a logistic curve, except for 5 of the 19 plants which were fitted as a straight line through the last 5 available measurements. The multiple regression model (equation 1) was fitted using GENSTAT, giving the values of the specific costs for growth, ion uptake and maintenance with the standard errors (n = 11 for exp. 1; n = 19 for exp. 2). The significance of all regression terms was tested by Fischer's F-test, adjusted for other terms. For all fits, the percentages variance accounted for (VAF) were given as a measure for the reliability of the fit. For the results of the chemical determinations, the 95%-probability intervals were calculated using the appropriate Student's t-values.

Results

Growth, ion uptake and respiration

Data on the root biomass, root growth rate, nitrate uptake rate and respiration rate were collected, to arrive at an estimation of specific costs for growth, ion uptake and maintenance. In experiment 1, the root weight increased constantly, up to day 18 (VAF fitted curve = 98.7). After pruning the shoot, root growth stopped, whereas shoot growth decreased slightly. This contrasted to experiment 2, where root pruning for the majority of the plants resulted in a strongly increased root growth after a lag period of approximately 4 days; shoot growth was unchanged or decreased slightly. Shoot pruning caused a stronger decrease of the shoot growth, whereas root growth was unchanged or somewhat lower.

In experiment 1, the rate of nitrate uptake was relatively low (i.e. on the Sundays and Mondays), when the respiration rate and root volume had not been measured on the previous day (data not shown). However, the overall root growth and the respiration rate (no data for Saturdays and Sundays) were not decreased. Therefore, Mondays were excluded from regression analysis (for implications, see discussion). In experiment 2, no such deviating days were observed

Chemical analysis

Irrespective of the plant age, the ash concentration, carbon concentration, total- and NO_3^--nitrogen concentration of roots were 181 $\pm$ 9 mg g^{-1}, 385 $\pm$ 5 mg g^{-1}, 57.3 $\pm$ 2.4 mg g^{-1} and 15.3 $\pm$ 0.9 mg g^{-1}, respectively. For the ash alkalinity we obtained an average value of 1.6 $\pm$ 0.1 meq (g DW)$^{-1}$ (equal to 49.0 $\pm$ 3.0 mg CO_3^{2-} [g DW]$^{-1}$) for roots of various ages. The $\pm$ values represent the 95%-probability intervals.

Experimental respiratory energy costs for growth, maintenance and ion uptake

In both experiment 1 and 2, all regression terms of the model (eq. 7.1), were significant ($P < 0.05$). Despite different methods and different plant responses to pruning treatments, both experiments yielded similar values for 1/Y, 1/U and m (Tab. 7.2, using Student's t-test). Regarding the relative variation, the value of 1/U appeared the most difficult parameter to establish (see discussion). In experiment 1, the ratio between growth rate, ion uptake rate and maintenance varied, due to the cessation of root growth after pruning the shoot. This resulted in reliable estimates of the specific costs as the regression analysis had a high explained variation (VAF = 98.4, n = 11; Tab. 7.2) and a randomly distributed residual variance (Fig. 7.1A). By subtracting the respiration rate of day 25 from the respiration rate on day 19, 20 and 21 (in the presence of nutrients) and dividing it by the nitrate uptake rate, 1/U was 0.65 $\pm$ 0.049 (experiment 1, Tab. 7.2). Specific cost of m (9.9 nmol O_2 [g DW]$^{-1}$ s^{-1}; Tab. 7.2) was also estimated by dividing the respiration rate in the absence of ion uptake and growth on day 25 (experiment 1) by the root weight. These alternative estimates of m and 1/U agree well with the values of m found in both regression analysis and the value of 1/U obtained from the first experiment, respectively (Tab. 7.2). However, the two estimations of 1/U from experiment 1

are not completely independent. In contrast to measurements used to determine m (day 25), the measurements used for calculating 1/U (day 19, 20 and 21) were also used in the regression analysis of experiment 1. In experiment 2, the explained variation was a little lower, despite the increased number of observations (VAF = 87.8 or 86.9, n = 19; Tab. 7.2). This is possibly due to plant variation, and a less sensitive method to quantify root growth (Fig. 7.1B).

Theoretical energy costs for growth, ion uptake and maintenance

Specific costs for root growth, were calculated to be 8.44 or 9.46 mmol O_2 (g DW)$^{-1}$, for plants given ammonium and nitrate, respectively (Tab. 7.3). This agrees well with the specific costs determined by the regression analysis (Tab. 7.2). The average experimental value of 1/U (Tab. 7.2) also agrees well with the theoretically calculated costs (Tab. 7.3). Expressing uptake costs per total anions absorbed (i.e. nitrate, phosphate and sulfate) instead of only nitrate, will give an approximately 10% lower value (i.e. assuming an overall phosphate plus sulfate concentration of approx. 10% of the nitrate concentration; after Marschner 1989, Tab. 1.3). Depending on the combination of the degradation constant and specific energy costs of protein turnover used in the calculations, the maintenance costs involved in protein turnover (m_p; nmol O_2 [g DW]$^{-1}$ s^{-1}; Tab. 7.3) account for 25 - 159% of total maintenance respiration (Tab. 7.2).

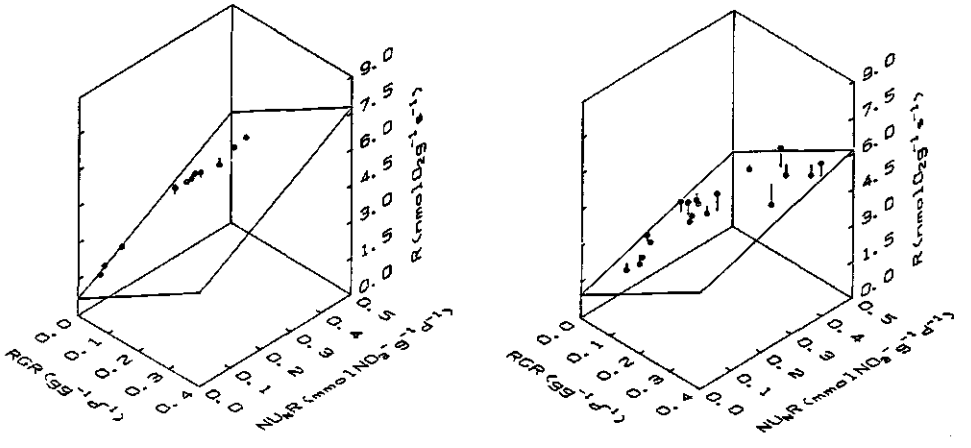


Fig. 7.1 The planes in the figures A (left) and B (right) represent the respiration rates as determined by the costs for growth, ion uptake and maintenance cf. the regression equations obtained from experiment 1 and 2, respectively. Deviations between the regression planes and the observed respiration rates (o) are indicated by the vertical lines. The RGR and NU_NR are presented on a daily basis, to enable easy comparison with literature data.

Table 7.2 The experimentally determined values ± the standard errors of 1/Y (mmol O_2 g^{-1}), 1/U (mol O_2 [mol NO_3^-]$^{-1}$) and m (nmol O_2 g^{-1} s^{-1}). All values were calculated by multiple regression analysis using equation 7.1 (n = 11 for exp. 1; n = 19 for exp. 2); the VAF is the percentage variance accounted for by the regression equation. Some values were also derived from the root respiration in the presence (1/U; n = 3) or absence (m; n = 1) of nutrients during a period when the root growth rate was zero (experiment 1). The values in parentheses represent the results on a fresh weight basis, whereas other values are on a dry weight basis.

Method	1/Y	1/U	m	VAF
Multiple regression experiment 1	9.9 ± 3.6 (0.69 ± 0.25)	0.67 ± 0.09	10.2 ± 2.4 (0.70 ± 0.17)	98.4
Multiple regression experiment 2	9.8 ± 1.7 (0.66 ± 0.12)	0.39 ± 0.10 (0.37 ± 0.11)	14.8 ± 4.6 (0.95 ± 0.31)	87.8 86.9
Zero growth, zero uptake experiment 1	-	-	9.9 (0.69)	-
Zero growth (exp. 1)	-	0.65 ± 0.049	-	-

Table 7.4 The values available from three-component regression analysis on an oxygen basis. The data of Van der Werf et al. (1988) were transformed to an oxygen basis, using their average P/O_2-ratio of 5.4. All values for 1/U were calculated on a nitrate basis. The values in brackets represent the results on a fresh weight basis, whereas other values are on a dry weight basis. For *Zea mays* we used the fraction dry matter of potato, whereas for the *Carex* species we used the fractions given in Van der Werf et al. (1988).

Method	1/Y	1/U	m	References
Zea mays	10.9 (0.77)	1.16	4.0 (0.28)	Veen (1980)
Carex acutiformis	6.2 (0.69)	0.83	4.3 (0.47)	Van der Werf et al. (1988)
Carex diandra	6.4 (0.63)	1.16	7.0 (0.69)	Van der Werf et al. (1988)

Table 7.3 Theoretical estimates of 1/Y (mmol O_2 [g DW]$^{-1}$), $1/U_{theor}$ (mol O_2 [mol NO_3^-]$^{-1}$) and m_p (nmol O_2 [g DW]$^{-1}$ s^{-1}) calculated following the equations as described in the material and methods. The $\pm$ values indicate the variation due to chemical analysis.

Method	$1/Y_{theor}$	$1/U_{theor}$	m_p
Equations 7.2 and 7.3			
without ammonium correction	8.4 $\pm$ 0.6		
with ammonium correction	9.5 $\pm$ 0.7		
Equation 7.4			
		0.43	
Equation 7.5			
$K_d = 7.3\ 10^{-7}$; $E_{sp} = 10$			3.7 $\pm$ 0.3
$K_d = 7.3\ 10^{-7}$; $E_{sp} = 20$			7.4 $\pm$ 0.6
$K_d = 1.6\ 10^{-6}$; $E_{sp} = 10$			8.1 $\pm$ 0.6
$K_d = 1.6\ 10^{-6}$; $E_{sp} = 20$			16.2 $\pm$ 1.3

Table 7.5. Other estimates of the specific costs for growth (1/Y; mmol O_2 [g DW]$^{-1}$), nitrate uptake (1/U; mol O_2 [mol NO_3^-]$^{-1}$) and maintenance (m; nmol O_2 [g DW]$^{-1}$ s^{-1}) on an oxygen and dry weight basis. The CO_2 values of 1/Y and 1/U presented in Bloom et al. (1992), were transformed to an oxygen basis using their average RQ values of nitrogen deprivated (1.12) and nitrate fed (1.17) plants, respectively (after Fig. 4). For their range of CO_2 values of m, we used their RQ values of the different genotypes, as observed under nitrogen deprivation indicated (Fig. 4 in Bloom et al. 1992).

Method	1/Y	1/U	m	References
Ammonia-fed	4.9	-	-	after Tab. 7 in Penning de Vries et al. (1974)
Nitrate-fed	6.3	-	-	Ibid.
24 herbaceous species	5.5 - 8.0	-	-	Poorter et al. (1991)
Barley genotypes	7.0	-	-	cited in Bloom et al. (1992)
	-	0.15	-	Bloom et al. (1992)
	-	0.49 - 1.08	-	O_2 values in Tab. II in Bloom et al. (1992)
	-	-	23.4 - 24.7	Bloom et al. (1992)

Discussion and conclusions

Energy costs for growth

To allow comparison of the present specific costs with the data of Veen (1980) and Van der Werf et al. (1988), all values were expressed on an O_2-basis (Tab. 7.4). The costs for growth as measured in the regression analysis (Tab. 7.2) and theoretically calculated (Tab. 7.3) are within the range of values obtained by Veen (1980) and Van der Werf et al. (1988) (Tab. 7.4), but slightly high compared to most theoretical values in literature (Tab. 7.5). In general, relatively high values for experimental estimates of 1/Y, might be due to the use of inefficient pathways, which are not accounted for in calculations based on Penning de Vries et al. (1974) and derived methods (Vertregt and Penning de Vries 1987).

Energy costs for ion uptake; interpretation of calculations and measurements

The theoretically expected uptake costs, assuming one active membrane passage for nitrate uptake (Tab. 7.3), agree well with the ones derived from the present experiments (Tab. 7.2). The theoretical estimate includes two assumptions, that are still under debate. The value of [H/P] (1 mol H^+ [mol ATP]$^{-1}$) was derived from kinetic models (Sanders 1990, Teyerman 1992). However, regarding the energy concentration of ATP, a value of 2 is also possible as it still allows active uptake of nitrate, sulfate and phosphate (appendix chapter 5). A value of 2 mol H^+ [mol ATP]$^{-1}$ was also found for the tonoplast (Bennett and Spanswick 1984, Guern et al. 1989) and sometimes obtained by kinetic models for the plasmamembrane (Sanders 1990). Although the transport of anions to the xylem is theoretically expected to be passive, there are indications that this process requires energy (Marschner 1989), so that M_j might be 2, instead of 1. Changing both assumptions used in the present calculations does not affect the result, as doubling the values of [H/P] and M_j have opposites effects (cf. equation 7.4). The present results exclude the combination where [H/P] is 1 and M_j is 2, as the resulting value of $1/U_{theor}$ (0.85 mol O_2 [mol nitrate]$^{-1}$) would greatly exceed the present experimental estimates (Tab. 7.2).

Comparing and estimating costs of uptake is complicated when the ratio of efflux and influx varies. Experiment 1 indicates that perturbation affected this ratio. Net ion uptake rate was approximately 33% reduced directly after the weekends, during which root volume and respiration were not measured. However, respiration rate and respiratory requirements for growth and maintenance were virtually constant, suggesting that the requirements for nitrate influx were also constant and, thus, that efflux was higher. If, on the other days, efflux and net uptake would have been the same as on Mondays, 1/U would be a factor 1.5 higher. The reduced efflux causes experimental values to agree well with theoretical estimates. In experiment 2, root growth of all plants was determined daily. Perturbation effects, if present, were not detectable, as handlings required to determine growth were applied systematically on all days. A larger perturbation effect might be expected, as the handlings required to carry out experiment 2 were rough compared to those in experiment 1 (details in materials and methods). Perturbation is a general problem in estimating 1/U, especially because it is not always

detectable (cf. experiment 2).

Energy costs for ion uptake; comparison with literature data

The values of 1/U obtained by Veen (1980) and Van der Werf et al. (1988), were relatively high (Tab. 7.4), compared to the data found for potato (Tab. 7.2) and calculated (Tab. 7.3). These relatively high literature values for 1/U suggest substantial costs to compensate efflux (i.e. maintaining gradients) for *Zea mays* and *Carex*.

Bloom et al. (1992) obtained uptake costs of 0.15 mol O_2 per mol nitrate (Tab. 7.5). This value is equal to only one ATP per NO_3^- at a maximum P/O_2-ratio of 6 (i.e. 0.167 mol O_2 per mol nitrate), and much lower than the other estimates of specific costs of ion uptake. The low value is probably due to the high value for the maintenance respiration derived by Bloom et al. (1992), as discussed in the next paragraph. It cannot be explained by the absence of costs for nitrate reduction, because these were also not included in the other estimates, as those were derived on an oxygen basis. When regarding the primary results in Tab. II of Bloom et al. (1992), respiratory costs per unit of nitrate uptake on an oxygen basis are 0.49 - 1.08 mol O_2 (mol nitrate)$^{-1}$ (Tab. 7.5). These values are higher than theoretically estimated (0.43 mol O_2 [mol NO_3^-]$^{-1}$; Tab. 7.3). The difference might well be ascribed to costs involved in processes like re-uptake balancing efflux.

Energy costs for maintenance and protein turnover

The experimental estimate of m on day 25 agrees well with the regression data of experiment 2 and especially experiment 1 (Tab. 7.2). Compared to other regression data (Tab. 7.4), m is relatively high. However, it is much lower than the value of Bloom et al. (1992). They estimated maintenance respiration as the respiration measured under nitrogen deprivation (0.5 mM $CaSO_4$ present) of roots growing very slowly. Costs involved in continued root growth and/or sulfate uptake might have caused an overestimation of the maintenance costs, possibly explaining the more than twice higher value (23.4 - 24.7 nmol O_2 [g DW]$^{-1}$ s^{-1}) compared to ours (Tab. 7.2) and others (Tab. 7.4).

The costs for maintenance as measured in regression analyses show large variation between species (Tabs 7.2 and 7.4). The supposed major components of maintenance are protein turnover (m_p) and maintaining concentration gradients (i.e. re-uptake balancing efflux). Costs of the latter process might be included in 1/U or m, depending on whether its rate is proportional to net uptake rate or to root size (i.e. membrane surface). The present relatively high value of m (Tab. 7.2) compared to other regressions (Tab. 7.4), suggests that re-uptake balancing efflux is included. However, the effect of perturbation indicates that re-uptake counteracting leakage correlated with net uptake. Re-uptake costs are thus included in 1/U, and can account for up to 33% of the costs of total ion influx in an undisturbed potato plant (i.e. 1/U is up to a factor 1.5 higher if efflux takes place). Moreover, total maintenance costs of all species (Tabs 7.2 and 7.4) can be explained theoretically by costs for protein turnover (m_p; Tab. 7.3), be it with different combinations of the turnover rate (K_d) and specific costs (E_{sp}). About these parameters little quantitative information is available (K_d for roots of *Dactylis*

glomerata in Van der Werf et al. 1992; E_{sp} in De Visser et al. 1992). For leaves turnover rates may differ significantly (K_d $0.3x10^{-6}$ to $6.0x10^{-6}$ s^{-1}; Barneix et al. 1988, Davies 1982, Dungley and Davies 1982, Eising and Gerhardt 1987). Variation in E_{sp} is not likely, considering the conservative nature of biochemical reactions. In conclusion, m can be explained by costs for protein turnover, and variation between species (Tabs 7.2 and 7.4) might be due to different turnover rates. Respiratory costs for maintaining ion gradients are included in the estimates of 1/U.

Conclusions

Specific costs for growth, maintenance and ion uptake have been determined by different methods. Both experiments yielded similar values of 1/Y, 1/U and m. The estimates of 1/U were affected by perturbation. The estimate for 1/U in experiment 1 involved net uptake with reduced efflux, whereas in experiment 2 this is not exactly known. From the measurements on non-perturbed periods in experiment 1, the costs involved in maintaining gradients were estimated to be up to 33% of the costs of gross transport (i.e. 1/U is up to a factor 1.5 higher if efflux takes place). The absence of these costs in present estimates of 1/U explains the good agreement with theoretical calculations and the higher values obtained in other experiments. Calculations showed that m can be ascribed completely to costs for protein turnover.

Chapter 8

General and summarizing discussion

Aim of the research

Light energy captured in the process of photosynthesis leading to the synthesis of carbohydrates, may become available again at a different time and place in the plant, by degrading these carbohydrates in respiration. The rate of respiration is a measure for the energy (i.e. ATP) utilization of a tissue, and can be observed as either the production of CO_2 or as O_2-consumption. Quantitative understanding of both photosynthesis and respiration is required to understand plant growth and resulting crop yield.

In contrast to the respiration associated with biosynthesis or ion uptake by roots, the nature of the energy-demanding processes determining the dark respiration rate in full-grown tissues is largely unknown. This unexplained respiration, not correlating with growth or ion uptake, is generally referred to as 'maintenance' respiration (e.g. Pirt 1975, Thornley 1970). Only a few studies have tried to explain maintenance respiration on the basis of energy-utilizing processes (Johnson 1990, Penning de Vries 1975). Experiments aimed at the quantification of the respiratory costs of individual (maintenance) processes are even scarcer (De Visser et al. 1992, Van der Werf et al. 1992). Various approaches to quantify overall costs of maintenance (reviewed by Amthor 1984) are nowadays still in use (e.g. Byrd et al. 1992, Irving and Silsbury 1987, Thomas et al. 1993), even though the basic assumptions of some methods may be invalided (e.g. dark-decay method; Gary 1989). These approaches to quantify overall costs of maintenance are 'black-box' methods, as they give no insight into the nature of the individual processes involved. As such methods cannot explain variation in maintenance requirements (e.g. due to different species, tissue characteristics, developmental stages and environmental conditions), a process-based approach towards maintenance respiration is necessary. Respiration for growth, ion uptake and maintenance should all be regarded as the summation of the product of the specific costs (e_i) and the rate (v_i) of all process involved (cf. eq. 1.3 and Penning de Vries 1975, Penning de Vries et al. 1974).

The main objective of the present study was to establish the energy requirements of those (maintenance) processes that were expected to be quantitatively important in full-grown tissues. The processes studied are protein turnover, carbohydrate export and maintaining ion gradients. As the rates, and thus overall costs, of these processes might vary as a function of the environmental conditions, developmental stage and species, insight in ways to determine the rates and specific costs of these processes is more important than obtaining data on certain crops. The experimental approaches used are *i)* measuring the energy consumption directly and

deriving the rate using the specific costs or by *ii)* calculating the energy consumption as the product of experimentally derived rates and theoretical values of the specific energy costs.

Progress / remaining questions

The energy consumed by a (maintenance) process can be either *i)* measured directly from the effect of inhibitors of the process on the overall respiration rate or *ii)* determined as the product of the rate of the process and its specific costs. Developing such process-based methods yields progress, as maintenance respiration is no longer regarded as a function of the existing biomass like in various 'black-box' approaches. An important question inherent to a process-based approach of (maintenance) respiration, is whether all relevant processes are identified. Chapter 3 and 4 show that process-based methods are successful for studying the costs involved in protein turnover, and carbohydrate export, respectively. Together, these processes may explain a major part of the leaf's dark respiration (discussion chapter 4). The upper limit of the costs required for maintaining ion gradients is also estimated (chapter 5). The simulations in chapter 6 show that root anatomy has to be included in interpretation of efflux kinetics to obtain more accurate estimates than those in chapter 5. Integration of the present results in chapter 7, shows that the root respiration may be ascribed to growth, ion uptake and the maintenance processes studied in this thesis.

Respiratory energy costs of protein turnover

To explain dark respiration of full-grown leaves in terms of energy-demanding processes, firstly the costs of protein turnover have been quantified, as this process has been considered as the maintenance process that requires most energy (Penning de Vries 1975). The overall respiratory costs of protein turnover were measured directly, using an inhibitor and a probe to asses its *in vivo* action. Chapter 3 shows that this process accounted for maximally 17 - 35% and up to 17 - 21% of total dark respiration in expanding leaves and full-grown primary leaves of bean, respectively. This indicates relatively low protein-turnover rates; the average K_d-values ($K_d = \ln 2 / t_{1/2}$) for all leaf proteins were up to 2.4 and 1.1 x 10^{-6} s^{-1} for growing and full-grown leaves, respectively, compared to 1.4 x 10^{-6} s^{-1} for the total pool of soluble proteins in full-grown primary bean leaves (Barratt and Woolhouse 1981).

The rate of both protein synthesis and degradation depend on leaf age (Mae et al. 1983) and environmental conditions (e.g. stress effects described by Dungley and Davies 1982). Therefore, present approach to quantify *in vivo* the component of dark respiration rate associated with overall protein turnover of tissues is more important, than the value of the fraction of respiration associated with protein turnover as observed on primary bean leaves. This is especially true as alternative approaches to determine protein turnover rates are often hard to apply (e.g. ^2H_2O labelling causes stress, which might result in an altered protein turnover; Cooke et al. 1979) and to interpret (Davies 1982, Muramatsu et al. 1987, Poole 1971, Zak et al. 1977). The present approach is a useful and relatively easy alternative to

quantify respiratory costs of protein turnover, despite the limitations described in chapter 3. This contention is supported by the observation that the estimates of the costs of protein turnover in chapter 3, are within the range of the costs calculated by combining the turnover rates - estimated by other methods - with the specific costs of protein turnover (De Visser et al. 1992).

Respiratory costs of carbohydrate export

To explain the remaining part of the dark respiration of full-grown leaves in terms of energy-demanding processes, the respiratory requirements involved in carbohydrate export were derived as the product of the specific costs of export and the export rate (chapter 4). The theoretical range of the specific costs of sucrose export from starch-storing species (2.4 to 4.0 mol ATP per mol sucrose) appeared to agree well with experimental values both from the present experiments and the literature. Depending on species and conditions, sucrose export costs was shown to account on average for 29% of total dark respiration for various starch-storing species. Thus, carbohydrate export is an important process underlying dark respiration rates of full-grown leaves.

From a conceptual point of view, carbohydrate export should not be included in maintenance (cf. the dark decay method; Irving and Silsbury 1988). However, the relation between the term 'maintenance' and carbohydrate export is sometimes confusing, as its costs have been hypothesized to explain the increased 'maintenance' respiration (i.e. respiration not correlating with leaf expansion) at elevated CO_2 (Thomas et al. 1993). Carbohydrate export might also be included in the 'maintenance' component, when maintenance costs are derived on a whole plant or shoot basis. Therefore, further research to explain leaf respiration in terms of energy-requiring processes like carbohydrate export, protein turnover and growth, will benefit most from a process-based approach. When using 'black-box' approaches, a multiple regression approach equivalent to that described for roots in chapter 7, might be useful. In such a multiple regression approach costs involved in carbohydrate export can be determined separately. In functional terms this is more logical, similar to the separation of ion uptake by the roots from root maintenance. The multiple regression may even be combined with measurements involving inhibitors of protein synthesis. However, interpretation of such regression is complicated by the question whether the rates of energy-consuming processes (e.g. growth) are similar during the light and dark period. Interpretation of values of specific costs is only possible when this question is resolved by a quantitative analysis.

Respiratory costs of maintaining ion gradients: experiments and simulation

Besides protein turnover and carbohydrate export, the maintenance of (ion) gradients has been proposed to be an important energy-requiring process (Penning de Vries 1975). Maintenance of (ion) gradients is regarded as re-uptake balancing efflux. Its energy requirement may be assessed as the product of specific uptake costs and efflux rates, assuming that all efflux is observed in the medium and that no efflux-ions are retained in cell walls. Rates and energy costs of efflux were quantified in roots. The cell walls of roots have a relatively rapid ion

exchange rate with a liquid medium as compared to the cell walls of leaves. This facilitates measurements on roots. Under the assumption that anion efflux consists predominantly of nitrate, costs associated with re-uptake balancing efflux was found to contribute up to 66% of the influx costs (i.e. combining the measured efflux rates with literature data on net ion uptake rates; chapter 5). A value of 41% was obtained if the net uptake rate of nitrate was based on the relative growth rate observed for potato. Comprising other anions this value became slightly lower (e.g. 41% —> 34% assuming phosphate and sulfate to be both 10% of nitrate in- and efflux).

The present approach differs from that of Johnson (1990), who calculated the maintenance coefficient to balance efflux (m_e) in roots as the difference between the overall maintenance coefficient determined by two-component regression on root (m_r) and shoot (m_{sh}). Johnson's method is based on the assumption that re-uptake balancing efflux is a typically root related process, whereas the other part of the maintenance coefficient (m) is determined by the process of "maintaining plant structure (proteins) and ionic gradients" and is similar for root and shoot ($m = m_{sh}$ & $m_e = m_r - m$). To my knowledge, there is no experimental basis for separating the maintenance of ionic gradients from re-uptake balancing efflux and to assume that the remaining maintenance coefficient (m) is similar for shoot and root. Moreover, the validity of determining m_{sh} by regression may be questioned, as the rates of growth and maintenance during the light period might differ from that in the dark. That - for technical reasons - efflux is measured most easily on roots, does not necessarily mean that the basal process and thus maintenance costs differ between shoot and root. The energy costs are independent of whether the leached ions remain in the cell walls (e.g. under the cuticle of the leaf) or diffuse from the cell wall into the medium surrounding the tissue.

A dynamic simulation model for studying efflux kinetics was developed (chapter 6), as estimating the costs involved in maintaining ion gradients appeared extremely sensitive to the interpretation of the efflux kinetics (chapter 5). Simulations showed that *i)* the overall efflux kinetics observed in the medium, *ii)* the relative contribution of ions originally located in the apoplast, cytoplasm and vacuole of different cell layers to these efflux kinetics, and *iii)* the total amount of efflux out of the symplast that reached the medium surrounding the root, may differ significantly, even if actual efflux rates (and thus costs involved in maintaining ion gradients) are equal. These differences were due to the presence or absence of an endodermis, different pathways involved in net uptake and different numbers of cell layers involved in efflux.

The present simulations indicate that part of the variation in efflux kinetics (references and values in chapters 5 and 6) may be explained by different root anatomy and related physiological characteristics (e.g. apoplastic versus symplastic ion transport). Therefore, in future studies it may be of interest to compare efflux kinetics of roots differing in anatomy (e.g. different location of the endodermis). The present model is useful to formulate explicit hypotheses for such experiments. Besides, the model is useful for validating the results obtained by compartmental analysis (i.e. if anatomical parameters are determined) and may be useful in exploring the relative importance of apoplastic versus symplastic ion transport.

Integration of knowledge on individual processes

In chapter 7, the available knowledge on maintenance, growth and uptake processes was compared to (black-box) estimates of the specific costs of these processes. Two independent experiments yielded similar estimates for growth, ion uptake and maintenance. The estimates of the specific costs for ion uptake were affected by perturbation. Comparing the experimental estimates of specific costs to theoretically calculated specific costs enabled to explain costs for growth, maintenance and ion uptake. The costs calculated for protein turnover could explain total maintenance requirements (10.2 to 14.8 nmol O_2 [g DW]$^{-1}$ s^{-1}). Costs for ion uptake were interpreted in terms of in- and efflux costs. From the perturbation effects it was deduced that overall costs for maintaining solute gradients (i.e. re-uptake balancing efflux) accounts for up to 33% of the overall costs of nitrate influx (i.e. 1/U is up to a factor of 1.5 higher if efflux takes place). This agrees well with the maximum value derived for young growing potato plants, assuming phosphate and sulfate to be 10% of the nitrate in- and efflux (34%; chapter 5). In conclusion, integration of data on different individual processes provides an explanation of the specific costs determined by multiple regression and thus also an explanation of overall root respiration. Further research may be aimed at methods to prevent perturbation effects (e.g. see Bloom and Sukrapanna 1990), and at the mechanism of perturbation on ion uptake.

Practical applications of the present research and results

Respiration measurements as a tool for plant breeding

There is little knowledge on the physiological mechanisms underlying the negative correlation between yield and respiration (Kraus et al. 1990). For example, the differences in yield between populations of *Lolium perenne* cv. S23 selected for high and low respiration rates (Wilson 1975) were concluded not to be determined by these respiration rates (Kraus et al. 1993). The present observations on the potato cultivars Alcmaria and Pimpernel lead to analogous conclusions. The respiration and photosynthesis rates of both cultivars were similar, if grown under identical conditions that prevent tuber induction (chapter 2). It was argued that the respiratory differences observed under field and greenhouse conditions, probably reflect differences in developmental rate rather than originating from different costs for maintenance and/or growth. This may interfere with a straightforward interpretation of comparative respiration measurements on field crops. Thus, selection for low respiration does not necessary result in a favorable crop with a high growth vigor and crop yield, when it is not known which process causes the respiratory difference. Besides, a rapid respiration may reflect characteristics like the presence of secondary metabolites and a high adaptation capacity which can be useful for establishing sustainable production under dynamic climate conditions. This type of characteristics may be lost, when selecting for low respiration only. In conclusion, studying the causes of low respiration rates is useful, before using it as a selection criterion.

The importance of respiration in modelling and predicting of crop yield

The introduction of the systems analysis approach was a major step forward in agricultural and biological science. It uses dynamic simulation as a tool to *i)* quantify the relative importance and thus sensitivity for the various processes involved, and *ii)* point at gaps in the present knowledge. In building a simulation model the system becomes well defined, making all uncertainties explicit. Nowadays, simulation models can be used for both the purpose of fundamental research and making predictions. The nature of these models is often different with respect to the detail of the processes included. In general, research models have a larger number of state constants, and a smaller time coefficient and spatial scale. Here, detailed process-based crop growth simulation models are discussed, using SUCROS87 as an example.

In SUCROS87 (Spitters et al. 1989), growth is derived from the daily assimilation after subtracting respiratory costs for maintenance. Subsequently, the remaining assimilates are divided over the plant organs following fixed ratios (i.e. depending on developmental stage), and growth is calculated using given conversion factors. These growth conversions are defined as the amount of sucrose required to produce 1 gram of plant tissue, and depend on the chemical composition of the organs and the required transport of metabolites and ions. It is assumed that chemical conversions follow the most efficient biochemical pathway (Penning de Vries et al. 1974). Although maintenance respiration is an important factor determining the amount of available carbohydrates for growth, the present calculation methods are simple: *i)* maintenance is proportional to the weight of the tissues with a factor depending on the tissues activity, *ii)* maintenance is proportional to the protein content of the tissues, or *iii)* maintenance is proportional to the protein and ash content of the tissues. Thus, in the present process-based simulation models, the respiratory loss for maintenance is missing a sound physiological basis, despite the fact that it is a significant part of the total losses of carbon.

Improving a calibrated and validated simulation model by a single aspect (e.g. maintenance), requires a re-calibration and re-validation of the model to maintain accurate simulations. In this respect, it might *seem* arbitrary how well defined maintenance respiration is simulated, especially if the model is only used within the range of conditions it was calibrated for. For example, most models give accurate simulation results, despite the fact that the respiration was assumed to yield 38 ATP per glucose, as true for animals, instead of a maximum value of 36 ATP per glucose, as valid for plants. Apparently, the effect of such a difference is negligible compared to the margins of the various other input parameters of the model. Changing the input from 38 to 36 ATP per glucose, would require a re-calibration and re-validation of the model, not necessarily resulting in more accurate simulations. However, to improve insight and to decrease the number of parameters that need calibration, it *is* necessary to have a detailed process-based simulation for all processes, including maintenance respiration. Process-based simulation is essential to arrive at a complete understanding of complex systems like plant growth. Such understanding can never be obtained by descriptive models like regression models. Non-matching simulation results from process-based models, often indicate on which processes future research should be focussed.

To be able to improve the (maintenance) respiratory component of the present simulation

models, more data on more tissues (and perhaps more species) under more conditions are required than available from the present research. For example, costs for protein turnover are expected to vary between types of leaves, age and activity of the tissue, environmental conditions, etc., due to different turnover rates (but not due to different specific costs). Thus, the estimates of the respiratory costs involved in the processes studied in this thesis, should not be used for simulation. The use of the present research is *i)* advocating a general process-based approach towards (maintenance) respiration in terms of specific costs and the rate of processes (cf. eq. 1.3 and Penning de Vries 1975), *ii)* indicating values of the specific costs of the most important energy-requiring processes in mature tissues and *iii)* offering some relatively simple experimental approaches to estimate the (maximal) rate of important energy-requiring (maintenance) processes, be it with some notes in the discussion. These approaches might be of use to derive functions between the rate of the processes involved and the environmental conditions and developmental stage of the plant.

Evaluation of and speculations on the concept of maintenance respiration

Besides advocating a process-based approach to study maintenance, it is also useful to evaluate the relatively old concept of maintenance. The term maintenance respiration was introduced for microorganisms, to separate the respiration that correlated with growth, from the respiration that could not be correlated with the rate of a certain process (Pirt 1975, Thornley 1970). As illustrated in a previous part of this summarizing discussion, maintenance is a confusing term with respect to carbohydrate export. It is also confusing that maintenance may well be related with the activity of the tissue (e.g. protein turnover rates may depend on tissue activity). The static term 'maintenance' might give an erroneous impression of the dynamic character of the processes involved: "The term 'maintenance' includes the processes which maintain cellular structure and gradients of ions and metabolites, and also the processes of physiological adaptation that maintain cells as active units in a changing environment. Formation of new enzymes at the expense of others and salt accumulation in some stress conditions are examples of such adaptations. Hence, maintenance is not as conservative a process as its name suggests" (Penning de Vries 1975). In the light of the present knowledge of plant physiology and biochemistry, respiration involved in regulation (that is including adaptation, development and also the repair / replacement of damaged and worn-out products) may be hypothesized to be a more accurate description of 'maintenance' respiration. For example, the reversible (in-) activation of proteins by phosphorylation is an energy-dependent mechanism important in regulating cellular activities (Ranjeva and Boudet 1987), which will be included in 'maintenance' if using correlative methods. The present contention is also illustrated, using maintenance of ion gradients and protein turnover as examples.

The loss of ions by efflux enables the plant to regulate internal concentrations, despite the presence of only an unidirectional transport system. Efflux rates are generally found to change most under non-steady state conditions, when plants adapt to new conditions. The 'price' of

such a mechanism to regulate internal ion concentrations, is re-uptake of efflux if concentrations have to be maintained under steady state conditions.

The rate of 'protein turnover' depends on the age of the tissue. In young leaves the rate of protein synthesis exceeds that of protein degradation, whereas in older leaves the opposite is true (Mae et al. 1983). The protein synthesis balancing degradation may be defined as protein turnover. Such protein turnover accompanies the development of the plant and the adaptation to external conditions, whereas it is not clear to which extent replacement of 'worn-out' (mall-functioning) proteins contributes to turnover. Thus, protein turnover might be regarded as a regulating process enabling adaptive and developmental changes, efficiently using the nitrogen available in amino acids. Futile cycles of protein turnover seem unlikely, as protein degradation is a well-organized process only affecting specific groups of proteins (Vierstra 1993).

The integration of future developments in molecular biology and biochemistry at the whole plant level, will lead to a better understanding and - possibly - to a revision of the concept of maintenance respiration, taking us further into the fascinating world of plant growth.

Appendix Chapter 1

How to measure the respiratory energy consumption; CO_2 versus O_2 and the effect of nitrate reduction

Regarding the present objectives and the three approaches proposed in the outline of this thesis, the respiratory energy (i.e. ATP) utilization has to be estimated. This may be done from either the production of CO_2 or the O_2 consumption. However, nitrate reduction may complicate such estimation of the energy utilization from respiration measurements. Here the advantages and disadvantages of CO_2 and O_2 measurements to determine the energy (i.e. ATP) utilization in relation to nitrate reduction are compared. Firstly, the balances of the relevant chemical reactions are made. Secondly, these balances are used to calculate the under- or overestimation of the energy (i.e. ATP) production due to nitrate reduction, when measuring O_2 or CO_2, respectively. The effect of making a wrong estimation of the relative activities of the cytochrome and alternative respiratory pathway is not discussed, as it does not help to decide on measuring respiration as O_2 consumption or CO_2 production.

The first step in the degradation of carbohydrates is glycolysis in the cytosol, followed by the TCA-cycle in the mitochondria (eq. 1.3). The products with the superscripts c1-c2 and m3-m5 are formed in the cytosol and mitochondria, respectively.

$$C_6H_{12}O_6 + 6\ H_2O + 4\ (ADP + Pi) + 10\ NAD + 2\ FAD \rightarrow 6\ CO_2 + 2\ ATP^{c1} + 2\ NADH_2^{c2} + 2\ ATP^{m3} + 8\ NADH_2^{m4} + 2\ FADH_2^{m5} \quad (1.3)$$

The $NADH_2$ can be used for ATP production in mitochondria or for nitrate reduction, whereas the $FADH_2$ is only oxidized in the mitochondria. Respiration may occur following either the cytochrome (eq. 1.4) or alternative (eq. 1.5) pathway, with a P/O_2-ratio of 6 and 2, respectively. The overall P/O_2-ratio of glucose degradation using the cytochrome (i.e. eqs 1.3 plus 1.4) and alternative (i.e. eqs 1.3 plus 1.5) pathway, results also in a P/O_2-ratio of 6 and 2, respectively.

$$6\ O_2 + 2\ ATP^{c1} + 2\ NADH_2^{c2} + 2\ ATP^{m3} + 8\ NADH_2^{m4} + 2\ FADH_2^{m5} + 32\ (ADP + Pi) \rightarrow 2\ ATP^{c1} + 4\ ATP^{c2} + 2\ ATP^{m3} + 24\ ATP^{m4} + 4\ ATP^{m5} + 12\ H_20 + 2\ NAD^{c2} + 8\ NAD^{m4} + 2\ FAD^{m5} \quad (1.4)$$

$$6\ O_2 + 2\ ATP^{c1} + 2\ NADH_2^{c2} + 2\ ATP^{m3} + 8\ NADH_2^{m4} + 2\ FADH_2^{m5} + 8\ (ADP + Pi) \rightarrow 2\ ATP^{c1} + 0\ ATP^{c2} + 2\ ATP^{m3} + 8\ ATP^{m4} + 0\ ATP^{m5} + 12\ H_20 + 2\ NAD^{c2} + 8\ NAD^{m4} + 2\ FAD^{m5} \quad (1.5)$$

In case of nitrate reduction, the $FADH_2$ is respired by the cytochrome (eq. 1.6) or alternative

(eq. 1.7) pathway, whereas some $NADH_2$ may be used for the reduction of nitrate. The equations 1.6 and 1.7 represent the chemical reactions if all $NADH_2$ would be used for nitrate reduction.

$$2.5\ NO_3^- + O_2 + 2\ ATP^{c1} + 2\ NADH_2^{c2} + 2\ ATP^{m3} + 8\ NADH_2^{m4} + 2\ FADH_2^{m5} + 4\ (ADP + Pi) \longrightarrow 2.5\ NH_3 + 2.5\ OH^- + 5\ H_2O + 2\ ATP^{c1} + 2\ NAD^{c2} + 2\ ATP^{m3} + 8\ NAD^{m4} + 4\ ATP^{m5} + 2\ H_2O + 2\ FAD^{m5} \quad (1.6)$$

$$2.5\ NO_3^- + O_2 + 2\ ATP^{c1} + 2\ NADH_2^{c2} + 2\ ATP^{m3} + 8\ NADH_2^{m4} + 2\ FADH_2^{m5} + 0\ (ADP + Pi) \longrightarrow 2.5\ NH_3 + 2.5\ OH^- + 5\ H_2O + 2\ ATP^{c1} + 2\ NAD^{c2} + 2\ ATP^{m3} + 8\ NAD^{m4} + 0\ ATP^{m5} + 2\ H_2O + 2\ FAD^{m5} \quad (1.7)$$

The balances of the relevant chemical reactions are summarized in Table 1.1 under "*values in the presence of NO_3^- reduction*", separated for the extreme situations as determined by the origin of the reducing power oxidized in nitrate reduction and the pathway involved in respiration (Tab. 1.1). That is, the situations are distinguished where all $NADH_2$ (produced in cytosol plus mitochondria), all $NADH_2$ produced in the cytosol, or all $NADH_2$ produced in the mitochondria is used for nitrate reduction, whereas the $FADH_2$ and the remaining $NADH_2$ are respired by either the cytochrome or alternative pathway. Combined with the input "*ratios in the absence of NO_3^- reduction*", the maximal relative under- and overestimation of the ATP production is calculated for O_2 or CO_2 measurements, respectively. These relative values represent the maximal effects, as they are based on the assumption that all $NADH_2$ from a given origin is used in nitrate reduction.

In the present research, the respiration measurements have mainly been done on full-grown leaves (chapters 2, 3 and 4) and roots (chapter 7). In herbaceous species with sufficient nitrate supply, nitrate reduction in the shoot forms the main source of 'organic nitrogen' (Beevers and Hageman 1969, Gojon et al. 1994). The relative importance of nitrate reduction in the shoot is expected to increase with increasing nitrate supply (Gojon et al. 1994). However, in general its effect on dark respiration of full-grown leaves is small for the following reasons: *i)* nitrate reduction in the leaves occurs predominantly in the light (Beevers and Hageman 1969; Rufty et al. 1984), *ii)* the rate of nitrate reduction in full-grown leaves is much lower than that in growing leaves (Beevers and Hageman 1969) and - for CO_2 - *iii)* up to 1 mol CO_2 per mol of nitrate reduced in the shoot may be exported to the root via the 'malate shuttle' (Ben Zioni et al. 1970, 1971, Kirkby and Knight 1977). Therefore, further discussion on the consequences of the under- and overestimation of the ATP production due to nitrate reduction is focused on roots.

The effect of nitrate reduction on the estimation of ATP consumption from O_2 consumption and CO_2 production, depends on the rates of total root respiration and nitrate reduction. The effect is maximal if all available $NADH_2$ is used for nitrate reduction (Tab. 1.1). The actual underestimation will generally be much lower than the maximum relative values in Table 1.1, because: *i)* nitrate reduction is expected to use predominantly the $NADH_2$ produced in the cytosol (otherwise unrealistically high RQ values are calculated), and *ii)* the major part of

Table 1.1 Estimation of the ATP consumption from CO_2 or O_2 measurements as affected by nitrate reduction. The overall balances of the chemical reactions involved were separated for the origin of the reducing power ($NADH_2$) involved in nitrate reduction and for the electron transport pathway involved in respiration. $NADH_2$ produced in the cytosol and mitochondria is abbreviated as 'cyto' and 'mito', respectively. The alternative, non-phosphorylating respiratory pathway and the cytochrome pathway is indicated as 'alt' and 'cyt', respectively. The relative under- and overestimations are calculated using the measured respiration (C or O) combined with the ATP production in the presence of NO_3^- reduction (A) and the P/O_2-ratio (P) and RQ (R) in the absence of NO_3^- reduction. These relative errors represent the maximum values.

description	calculation	'values'					
characteristics of the metabolism							
origin of $NADH_2$ used		all		cyto		mito	
for NO_3^- reduction		(i.e. cyto + mito)					
pathway used for respiration		cyt.	alt.	cyt.	alt.	cyt.	alt.
values in the presence of NO_3^- reduction (mol [mol glucose]$^{-1}$)							
CO_2 production	C	6	6	6	6	6	6
NO_3^- reduction	N	2.5	2.5	0.5	0.5	2	2
O_2 consumption	O	1	1	5	5	2	2
ATP production	A	8	4	32	12	12	4
RQ	C/O	6	6	1.2	1.2	3	3
ratios in the absence of NO_3^- reduction							
P/O_2-ratio	P	6	2	6	2	6	2
RQ	R	1	1	1	1	1	1
maximum relative error in the estimation of the ATP production (%)							
from O_2 measurements	100x(A-OxP) / A	-25	-50	-6	-17	0	0
from CO_2 measurements	100x[(CxP/R)-A] / A	350	200	13	0	200	200

respiration generally occurs via the cytochrome pathway (data for 17 species in Poorter et al. 1991), which for O_2 results in a lower underestimation than when the alternative pathway predominates. This might lead to the conclusion that the effect of nitrate reduction on the estimation of the ATP consumption as estimated from both the O_2 consumption and the CO_2 production, is approximately negligible. However, for CO_2 production such conclusion is not allowed.

The effect of nitrate reduction on the estimation of the ATP consumption from the CO_2 production is opposite to, and, especially in roots, generally larger than the effect if the ATP consumption is derived from the O_2 consumption. The reason for this opposite effect (i.e. an

overestimation of the ATP production) is that the decreased overall ATP production (compare eqs 1.4 and 1.5 versus eqs 1.6 and 1.7, respectively) is not accompanied by any decrease in CO_2 production in glycolysis and TCA-cycle (eq. 1.3). The effect of nitrate reduction on the root CO_2 production is generally larger than the effect on its O_2 consumption, as *i)* the effect of using some of the $NADH_2$ produced in the mitochondria for nitrate reduction is relatively large and *ii)* per mol nitrate reduced in the shoot, 1 mol of CO_2 originating from the shoot may be excreted by the root. The importance of the latter process may increase with the rate of nitrate reduction in the shoot, which is especially predominant in herbaceous species (Gojon et al. 1994). The excretion of CO_2 occurs in the form of HCO_3^-, to maintain electroneutrality in nitrate-fed plants by balancing the part of the demand for anions, exceeding that of cations:

$$\Phi_{HCO}^{io} = ((\Phi_a^{oi} \times z_a) + (\Phi_c^{oi} \times z_c)) / z_{HCO} \qquad (1.8)$$

with Φ_{HCO}^{io} is the excretion rate of HCO_3^- (mol g^{-1} s^{-1}), Φ_a^{oi} and Φ_c^{oi} are the influx of anions and cations, respectively (mol g^{-1} s^{-1}), and z is the valency of the ions (i.e. including the + or - sign). The HCO_3^- excreted can be produced in either root or shoot from OH^- and CO_2. The HCO_3^- produced in the shoot, is transported to the root by the malate shuttle as proposed by Ben Zioni et al. (1970, 1971) following the work of Dijkshoorn (1958) (Kirkby and Knight 1977). The CO_2 and OH^- are produced during the degradation of carbohydrates (eq. 1.3) and nitrate reduction (eqs 1.6 and 1.7), respectively. Not all negative charge of NO_3^- is excreted after reduction. The negative charge remaining in the plant, is transformed to organic anions which may be stored in the vacuole.

To arrive at root CO_2 production rate not related to NO_3^- reduction (c_r; mol CO_2 g^{-1} s^{-1}), the measured overall CO_2 production (c_p; mol CO_2 g^{-1} s^{-1}) has to be corrected for *i)* the CO_2 evolved during nitrate reduction in the root and *ii)* the CO_2 transported by the malate shuttle from shoot to root (i.e. the HCO_3^- excretion by the root, due to the OH^- and CO_2 production associated with the nitrate reduction in the shoot):

$$c_r = c_p - (c_{HCO\text{-}r} + c_{oa}) - c_{HCO\text{-}s} \qquad (1.9)$$

where $c_{HCO\text{-}r}$ is the amount of CO_2 produced in nitrate reduction in the root related to the excretion of HCO_3^- (mol CO_2 g^{-1} s^{-1}), c_{oa} is the CO_2 evolved during nitrate reduction in the root, that leads to the accumulation of organic anions instead of HCO_3^--excretion (mol CO_2 g^{-1} s^{-1}) and $c_{HCO\text{-}s}$ is the amount of CO_2 transported by the malate shuttle from shoot to root (mol CO_2 g^{-1} s^{-1}). In a medium with a pH that causes a complete dissociation of HCO_3^- ($pH \leq 4.5$), the rate at which HCO_3^- is excreted is equal to the titration rate to maintain the pH (mol H^+ g^{-1} s^{-1}). In this manner, $c_{HCO\text{-}r}$ and $c_{HCO\text{-}s}$ cannot be separated:

$$c_{HCO} = c_{HCO\text{-}r} + c_{HCO\text{-}s} \qquad (1.10)$$

where c_{HCO} is the total amount of CO_2 excretion in the root that is due to all nitrate reduction related to the excretion of HCO_3^-. As the titration rate to maintain the pH is equivalent to the overall nitrate-reduction rate correlating with the excretion of HCO_3^- (N_{HCO}; mol NO_3^- reduced g^{-1} s^{-1}), c_{HCO} can be derived (pers. com. B.W. Veen and A.G.M. Broekhuysen):

$$c_{HCO} = (f_r \times q \times N_{HCO}) + (f_s \times [1-q] \times N_{HCO}) \quad (1.11)$$

where f is the amount of CO_2 excreted per NO_3^- reduced leading to the excretion of HCO_3^- (mol CO_2 [mol $NO_{3\,red}^-$]$^{-1}$), and q is the fraction from such nitrate reduction, located in the root. The values of f for root (f_r) and shoot (f_s) are 2 and 1 mol CO_2 [mol $NO_{3\,red}^-$]$^{-1}$, respectively. Equation 1.12 describes the CO_2 production associated with nitrate reduction that leads to the accumulation of organic anions in the root, instead of HCO_3^--excretion (c_{oa}; mol CO_2 g^{-1} s^{-1}):

$$c_{oa} = f_r \times RGR_r \times (A_a - NO_{3\text{-}dm}) \quad (1.12)$$

with RGR_r is the relative growth rate of the root (g g^{-1} s^{-1}), A_a is the ash alkalinity of the root dry matter (meq CO_3^{2-} [g DW]$^{-1}$) and $NO_{3\text{-}dm}$ is the nitrate concentration of the root dry matter (mol [g DW]$^{-1}$). The difference between A_a and $NO_{3\text{-}dm}$ represents the concentration of reduced nitrate in the root, that has not been associated with HCO_3^--excretion (mol NO_3^- reduced g^{-1}). Assuming an RQ for all processes other than nitrate reduction equal to 1 so that c_r is equal to the O_2 consumption (r_o mol O_2 g^{-1} s^{-1}), and substituting the equations 1.11 and 1.12 in equation 1.9, enables quantification of the fraction q.

In conclusion, it seems justified to ignore nitrate reduction as process explaining root respiration when measuring O_2 consumption, whereas this is generally not so when measuring CO_2 production. This difference is due to *i)* the relatively large effect of nitrate reduction on the CO_2 production, if some of the $NADH_2$ produced in the mitochondria is used for nitrate reduction and *ii)* the excretion, by the root, of CO_2 originating from the shoot. The importance of this latter process may increase with an increasing nitrate reduction in the shoot. To understand the measured CO_2 production of a root (c_p) completely, chemical determination of the *i)* ash alkalinity and *ii)* nitrate concentration has to be combined with simultaneous measurement of the *iii)* HCO_3^--excretion rate (i.e. the titration rate to maintain the pH), *iv)* O_2 consumption (r_o; mol O_2 g^{-1} s^{-1}), *v)* CO_2 production and *vi)* root growth. The assumption that the RQ for all processes other than nitrate reduction equals 1 has to be tested and where necessary corrected. Generally, estimating the energy consumption from the CO_2 production rate will be even more complicated than discussed above, as the RQ for respiration associated with biosynthesis will be higher than 1 (e.g. the RQ for synthesis with ammonia as nitrate source is 1.24 mol CO_2 [mol O_2]$^{-1}$; Tab. 7 in Penning de Vries et al. [1974]). Chemical analysis of the (newly synthesized) biomass enables calculation of the RQ for growth respiration. For full-grown leaves complicated corrections of the CO_2 production for nitrate reduction are not necessary, as nitrate reduction occurs predominantly in the light and is located in growing leaves. Moreover, the overestimation of the ATP production will be lower due to the HCO_3^--excretion by the roots.

Appendix Chapter 5

Calculations on the required stoichiometry of the ion-carrier

The passive driving force (PDF; mV) and proton motive force (PMF; mV) give the electrochemical force for (an-)ions and protons, respectively. At diffusion equilibrium (i.e. a passive steady state), their values are zero. In steady state situations with PDF and PMF not equal to zero, active transport and diffusion occur in opposite directions.

$$PDF = z_j \times (MP - NP_j) \quad (5.5)$$

$$PMF = MP - NP_H \quad (5.6)$$

where z_j is the valency of anion j (i.e. including the + or - sign), MP is the membrane potential (mV), and NP_j and NP_H are the Nernst potential of anion j and protons, respectively (mV). The algebraic value of PDF and PMF indicates whether the diffusion flux is directed inwards (i.e. negative value) or outwards (i.e. positive value) the cytosol. That is, if $NP_j = [R \times T / z_j \times F] \times \ln [c_j^o / c_j^{cyt}]$, where c_j^o and c_j^{cyt} are the concentration of ion j outside and inside the cytosol, respectively.
The product of the Faraday constant and the absolute value of the PDF or PMF represents the Gibbs free energy of ions or protons across a membrane. Assuming that the maximal available free energy from ATP is used to transport a proton, the maximal proton concentration in the cell wall (c_H^o; mol l^{-1}) and the maximum PMF (PMF_{max}; mV) can be derived from the efficiency of proton transport over the membrane:

$$c_H^o = \exp ([H/P] \times F \times MP + [H/P] \times R \times T \times \ln(c_H^{cyt}) + E_{ATP}) / ([H/P] \times R \times T) \quad (5.7)$$

$$PMF_{max} = - E_{ATP} / ([H/P] \times F) \quad (5.8)$$

where [H/P] is the amount of protons pumped over a membrane by the hydrolysis of one ATP to ADP (mol H^+ [mol ATP]$^{-1}$), F is the Faraday constant (96.485 J mol^{-1} mV^{-1}), R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T is the temperature (293 K), c_H^{cyt} is the proton concentration in the cytosol (mol l^{-1}), and E_{ATP} is the energy charge of ATP (30.103 J (mol ATP)$^{-1}$). The "-" indicates that the proton motive force is directed towards the cytosol.
The minimal proton ion stoichiometry required for a membrane passage of anion j ($[H/I]_{min}$; mol H^+ [mol anion]$^{-1}$) is equal to the ratio between the PDF and the PMF_{max}:

$$[H/I]_{min} = -PDF / PMF_{max} \quad (5.9)$$

By this equation it is shown that the minimal required proton ion stoichiometry required for a

membrane passage of anion j ($[H/I]_{min}$) is less (Fig. 5.6) than the experimentally observed stoichiometry of nitrate, sulfate and phosphate (i.e. 2 [Ullrich and Novacky 1981], 3 [Lüttge and Clarkson 1985], 2 [Lüttge and Clarkson 1985], respectively). $[H/I]_{min}$ was calculated assuming [H/P] to be 2 mol H^+ (mol ATP)$^{-1}$, and a reasonable range for the concentrations gradient (c_j^o / c_j^{cyt}) and membrane potential (MP). Thus, theoretical calculations support the contention that in a lot of situations, a value of 2 mol H^+ [mol ATP]$^{-1}$ for [H/P] would be sufficient to enable (an)ion uptake from the medium; a stoichiometry of 1 is not required.

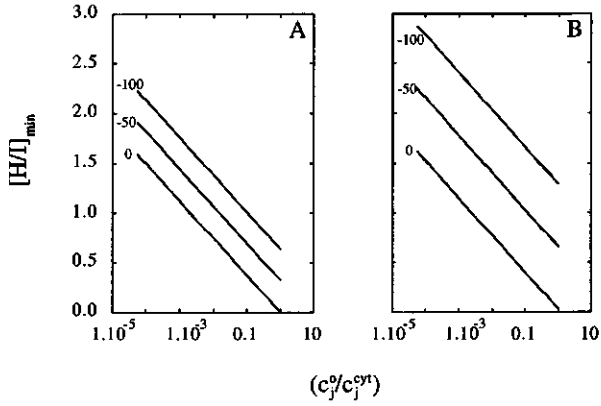


Fig. 5.6 The minimal proton ion stoichiometry required for a membrane passage of anion j ($[H/I]_{min}$; mol H^+ [mol anion]$^{-1}$), with a valency of -1 (Fig. A) or -2 (Fig. B), respectively. The value of (c_j^o/c_j^{cyt}) represents the concentration gradient of ion j from outside towards the cytosol. In both the figures A and B, the lines from above to below are derived for a membrane potential of -100, -50 and 0 mV. It was assumed that by the hydrolysis of one ATP to ADP, 2 protons were pumped over the plasmamembrane (i.e. [H/P] = 2 mol H^+ [mol ATP]$^{-1}$).

Appendix Chapter 6

The kinetics of loading and efflux per compartment

Simulation of label distribution during loading

Efflux rates over tonoplast and plasmamembrane and diffusion rates through cytoplasm and apoplast showed a ratio of 1 : 0.5 : 11 : 240, using present inputs. The ion flow through the cytoplasm (simulation 3) or apoplast (simulation 4) for net uptake towards the xylem was 52 times larger than the efflux rate over the tonoplast, whereas net uptake per vacuole and per cytoplasm (simulation 1) was 2.3 and 1.7 times larger, respectively. These transport rates and pathways combined with the root morphology (Tab. 6.1) underlie the differences between the fractions of ions that become labelled during the simulation of 16 h loading (Fig. 6.5). Differences in root morphology and pathways of ion transport resulted in a different label partitioning and concentration, even when overall transport was similar (e.g. simulation 3 versus 4). Generally, the fractions of labelled ions increased with time. In all simulations, the cell walls became approximately saturated with label, whereas the labelled fraction in the vacuole remained low. The fractions of labelled ions in the cytoplasm differed between the simulations 1, 2 and 3 (lowest in 2 and almost saturated in 3), due to the different rates of net transport through the cytoplasm (zero in simulation 2 and highest in simulation 3). Simulation 4 (a to c) showed a large difference in the fraction of labelled ions in the cytoplasm and vacuole between the different cell layers. This was caused by differences in the path length for the symplastic ion diffusion from the cell layer where the apoplastic ion flux for net uptake entered the cytoplasm.

Reducing the number of cell layers involved in efflux from 10 to 5 for a root having a total of 40 cell layers (Fig. 6.5, column c), generally increased the fraction of labelled ions. These increases are due to the absence of label transport to the cell layers 6 to 10. The relative importance of the apoplastic ion flow into the root determines the size of the increments of the labelled fractions (i.e. smallest for simulation 3 due to large symplastic ion flow, largest in simulation 4).

The presence of an endodermis caused a strong reduction of the labelling of the cell walls inside the stele (Fig. 6.5, column b). The reason for this reduction is that the label supply in the stele depended either solely on the efflux from the cytoplasm (simulations 2b and 3b), or also on the return to the apoplast of the apoplastic transport for net uptake after passing the endodermis (simulations 1b and 4b). The fraction of labelled ions in the cell walls of the cortex increased slightly, as a result of the blocked apoplastic diffusion to the cell walls inside the stele. The effect on the labelling of cytoplasm and vacuole both inside and outside the stele, varied, depending on the relative importance of the apoplastic ion flow entering the symplast at

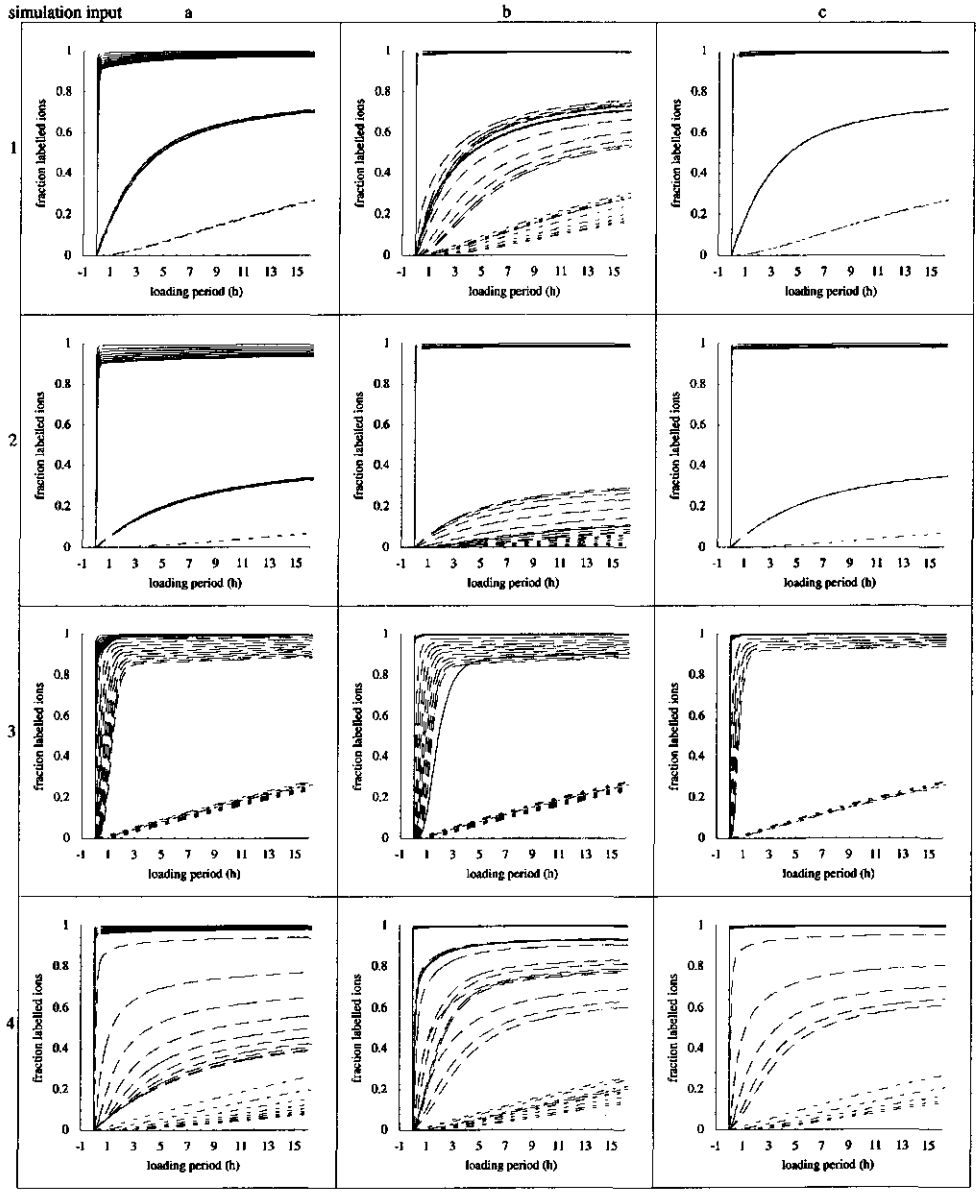

simulation input
a
b
c
1
2
3
4
fraction labelled ions
loading period (h)

Fig. 6.5 (p. 122) The effect of differences in anatomy, ion transport rates and ion transport pathways on time course of the fraction of labelled ions per root compartment during 16 h loading, is visualized by the simulations 1 to 4 (from above to below) and a to c (from left to right; codes explained in Tab. 6.1). The compartments separated are indicated similarly as in Figure 6.2. In general the uppermost lines belong to most outward cell layers, except for the simulations 1b, 4a, 4b, and 4c, where the order of lines from both the cytoplasm and vacuole were 5 to 1 + 6 to 10, 10 to 1, 5-10-6-9-4-7-8-3-2-1, and 5 to 1, respectively (lowest value is most outwards).

the endodermis, and the already occurring symplastic transport (i.e. smallest and largest effect for simulation 3 and 4, respectively).

Simulation of efflux kinetics per compartment

The contribution from the various compartments to the efflux kinetics (Fig. 6.6), differed significantly, even when the actual total efflux (i.e. simulations 1a to 4b or 1c to 4c) was equal. These differences are explained by differences in transport rates and pathways (Tab. 6.1), and the size of the ion pools and the fraction of labelled ions per compartment (Fig. 6.5). Comparing simulations 1 to 4, the cumulative efflux from cytoplasm and vacuole in simulation 3 (a to c) were relatively low (Fig. 6.6). This is due to the large symplastic transport for net uptake, moving the label to more inner pools of the root that are not subject to efflux. The higher cumulative efflux in simulation 1 (a to c) than in simulation 2 (a to c) (Fig. 6.6), was mainly due to a higher fraction of labelling in simulation 1 (Fig. 6.5). In simulation 4a, b and c, the cumulative efflux varied strongly (Fig. 6.6) as a consequence of a different fraction of labelled ions (Fig. 6.5). Thus, in simulation 4 (a to c) efflux kinetics were mainly determined by the number of the cell layer where the apoplastic ion transport for net uptake entered the cytoplasm.

Although, except for simulation 3, the cumulative efflux per compartment increased for a root with a reduced number of cell layers involved in efflux (Fig. 6.6, column c), the apparent efflux from all compartments decreased (Fig. 6.3, column c). In simulation 3c, the increased fraction of labelled ions (Fig. 6.5, column c) did not increase the cumulative efflux from cytoplasm and vacuole (Fig. 6.6, column c), as the relatively strong symplastic transport for net uptake transported this label rapidly to more inner pools of the root not subject to efflux.

In the presence of an endodermis, the cumulative efflux per compartment increased slightly for the cell walls in the cortex, but decreased for all other compartments in simulations 1, 2 and 3 (Fig. 6.6, column b). This decrease is mainly due to the relative sink activity of the cell layers inside the stele for label from the compartments in the cortex, combined with the absence of efflux from the stele via apoplastic diffusion. The increased efflux from cell walls in the cortex was due to a slightly higher label content (Fig. 6.5, column b) and the absence of apoplastic

Appendix Chapter 6

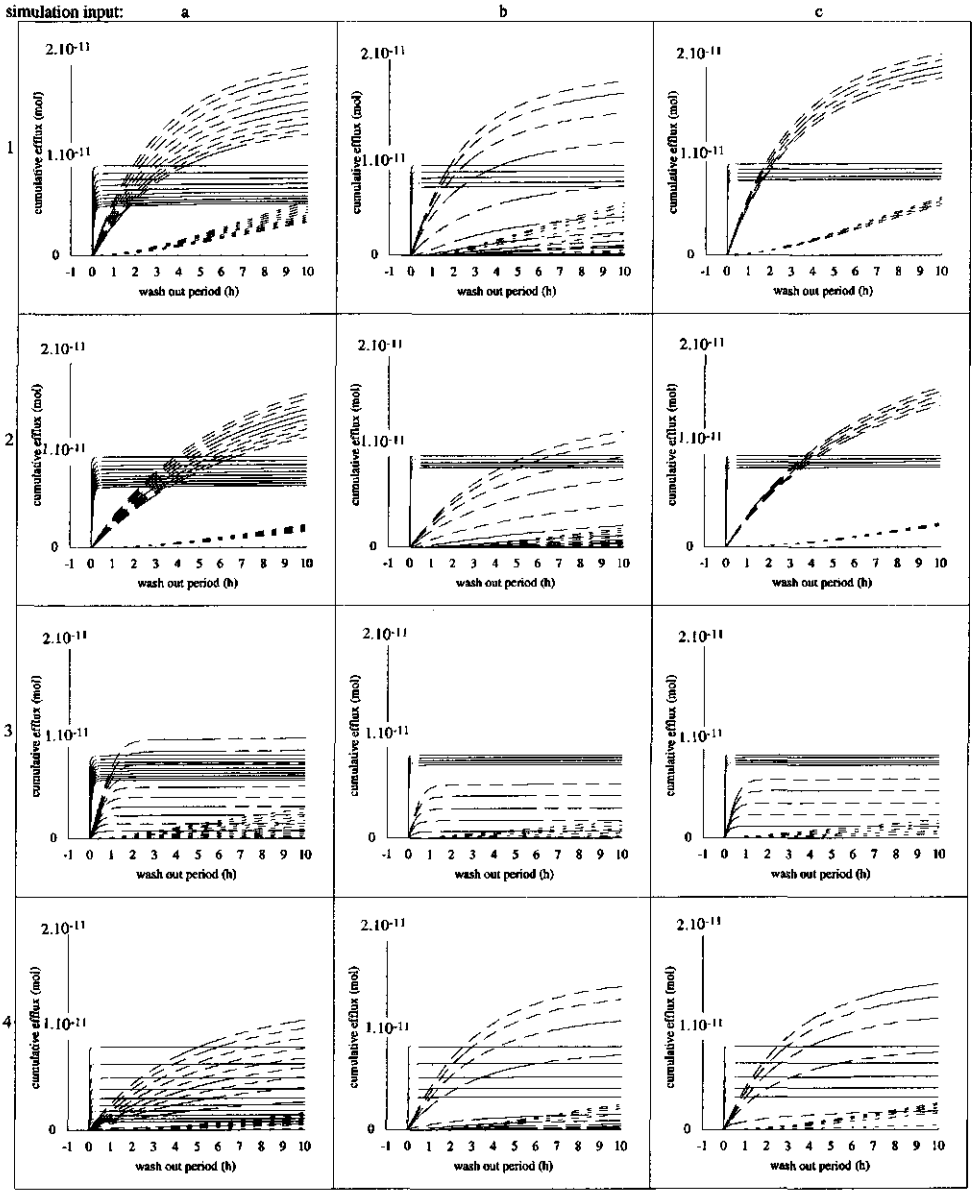


Fig. 6.6 (p. 124) The effect of differences in anatomy, ion transport rates and ion transport pathways on the origin of efflux, visualized by the simulated time course of the cumulative efflux per compartment (mol) from a root slice (0.667 mg FW) labelled for 16 h. The simulations 1 to 4 and a to c (Tab. 6.1) are shown from above to below and from left to right, respectively. The compartments separated are indicated similarly as in Figure 6.2, and the uppermost lines belong to most outward cell layers.

diffusion into the stele. The apparent efflux decreased (Fig. 6.3, column b), as the decrease in the cumulative efflux of most compartments exceeded that of the increase from cell walls in the cortex. The increased cumulative efflux in simulation 4b (Fig. 6.6, column b) followed from the almost doubled fraction of labelled ions in the cytoplasm and vacuole for the 5 cell layers outside the endodermis, and the slightly higher fraction of labelled ions in the cell walls (Fig. 6.5; note order of lines in the legend). Although cytoplasm and vacuoles were sometimes strongly labelled, efflux from compartments inside the stele hardly reached the medium as a consequence of the relative large centripetal flow, through the symplast of the endodermis.

Samenvatting

De benutting van ademhalingsenergie in hogere planten; energiekosten van onderhouds- en transportprocessen

Inleiding

Overdag gebruiken planten de energie van het zonlicht om suiker (CH_2O) en zuurstof (O_2) te maken van water (H_2O) uit de bodem en kooldioxide (CO_2) uit de lucht. Dit noemt men *fotosynthese*. Fotosynthese gebeurt voornamelijk in de bladeren, omdat die veel licht kunnen opvangen. De bij de fotosynthese gevormde suikers vormen de bouwstoffen voor de plant. Alle andere stoffen worden van deze suikers gemaakt, waarbij tevens de door de plant opgenomen meststoffen worden ingebouwd. Voor het omvormen van suikers naar andere plantstoffen en voor het opnemen van meststoffen heeft de plant energie nodig. Om die benodigde energie te verkrijgen, kan in de plant een deel van de bij de fotosynthese gevormde suikers worden afgebroken. Deze energieproduktie door suikerafbraak wordt *ademhaling* genoemd, omdat voor het afbreken van suikers zuurstof (O_2) nodig is en er kooldioxide (CO_2) bij vrij komt. Dit proces is vergelijkbaar met de afbraak van voedsel door mens en dier, die daarvoor ook zuurstof (O_2) inademen en kooldioxide (CO_2) uitademen. Het voordeel voor de plant om de lichtenergie in suikers om te zetten in plaats van die lichtenergie direct te gebruiken voor het omvormen van suikers naar andere plantstoffen en het opnemen van meststoffen, is dat suikers kunnen worden opgeslagen en naar elders kunnen worden getransporteerd. Door de vorming van suikers is er dus altijd (ook in het donker) energie beschikbaar in alle delen van de plant (ook in die delen die geen licht ontvangen, zoals de wortels). Samenvattend kunnen we de groei van een plant vergelijken met de plasticproduktie in een fabriek: olie is zowel de grondstof voor de produktie van de plastic, als de energiebron om de machines te laten werken en de overige grondstoffen aan te voeren. Het mooie en specifieke van planten is dat deze hun eigen 'olie' in de vorm van suiker produceren.

De plasticproduktie van een plasticfabriek zal afhangen van hoeveel olie dagelijks beschikbaar is, en hoe efficiënt alle benodigde machines met de brandstof omgaan. Op een vergelijkbare manier is de dagelijkse groei van een plant grotendeels de balans tussen de suikers vastgelegd in de fotosynthese en het verbruik in de ademhaling. In de ademhaling kan wel 50% van de dagelijks in de fotosynthese geproduceerde suikers worden afgebroken. Om de groei en de uiteindelijke opbrengst van planten - inclusief landbouwkundige gewassen - te begrijpen, is dus kwantitatieve informatie over zowel fotosynthese als ademhaling nodig. De dagelijkse suikerproduktie door fotosynthese kan tegenwoordig redelijk goed beschreven worden uit de weergegevens (o.a. hoeveelheid licht en temperatuur) en de waterbeschikbaarheid in de bodem

(er van uitgaand dat er voldoende meststoffen in de bodem aanwezig zijn). Ook is redelijk bekend hoeveel suiker moet worden verademd, om de voor groei benodigde plantstoffen uit suikers te kunnen maken. Over de kosten van het opnemen van meststoffen *(ionen)* door de wortels is minder informatie beschikbaar. Verder is gebleken dat de suikerafbraak benodigd voor groei en ionenopname niet de gehele ademhaling kan verklaren. Het resterende deel wordt vaak onderhoudsademhaling genoemd. Het is nog niet precies bekend welke processen in welke mate de bij de onderhoudsademhaling geproduceerde energie gebruiken. De bestaande ideeën veronderstellen dat eiwitturnover en het handhaven van ionen-gradiënten belangrijke processen zijn. Met *eiwitturnover* wordt dat deel van de eiwitsynthese (en afbraak) bedoeld, dat niet leidt tot een toename van het aantal eiwitten (d.w.z. vervanging). Dit kan worden voorgesteld als het vervangen van een versleten of te ouderwetse machine in een fabriek. Het *handhaven van ionen-gradiënten* wordt gezien als het vasthouden van de meststoffen in de plantencellen en celcompartimenten, hoewel er geen ondoorlaatbare afbakening is. Dit kan worden voorgesteld als het op spanning houden van een lekke band; het continu bijpompen van lucht vergt energie. Het doel van dit onderzoek is enerzijds meer inzicht te verkrijgen in welke processen ten grondslag liggen aan de (onderhouds-)ademhaling in niet groeiende plantendelen en anderzijds het belang van de individuele processen te kwantificeren. Het ontwikkelen van geschikte methoden om dit te onderzoeken is hierbij belangrijk.

Resultaten

Twee aardappel-cultivars, waarvoor aanwijzingen bestonden dat deze verschilden in de onderhoudsademhaling, werden vergeleken (hoofdstuk 2). De verschillen bleken echter samen te hangen met verschillen in ontwikkelingssnelheid. Er werd geconstateerd dat het niet mogelijk is om conclusies uit een verschil in ademhalingssnelheid te trekken, zonder dat de achterliggende oorzaak bekend is. Dus, bij het veredelen op planten met lage ademhalingskosten, kan op een daarmee samenhangende eigenschap worden geselekteerd, zonder dat dit vooraf duidelijk is. Dit kan geïllustreerd worden met een analoog voorbeeld uit het verleden, waar selektie voor minder temperatuur gevoelige mais-cultivars, daglengte neutrale planten opleverde. Daarom zijn in de volgende hoofdstukken de processen onderzocht, waarvan vermoed wordt dat deze de bij de ademhaling geproduceerde energie benutten.

Er zijn eerst experimenten uitgevoerd om vast te stellen in welke mate de ademhaling van volgroeide bladeren verklaard kan worden uit de energiebehoefte voor eiwitturnover (hoofdstuk 3) en de export van suiker naar overige plantdelen (hoofdstuk 4). Hiertoe werd het effekt van een remstof van de eiwitsynthese op de ademhaling gemeten, gecombineerd met een toets op de *in vivo* werking van de gebruikte remstof. Deze methode bleek relatief eenvoudig en daarom een goed alternatief voor bestaande methoden. Afhankelijk van de leeftijd van het blad, kan eiwitturnover 17 tot 35% van de donkerademhaling verklaren. Dit is gemiddeld iets lager dan de percentages die in een onlangs verschenen artikel zijn gevonden (5 tot 76%; Tab. 2 in De Visser et al. 1992). Het verschil kan gezien de uiteenlopende experimentele methoden, deels het

gevolg van suiker-export zijn. De theoretisch berekende waarde voor de specifieke energiekosten voor suiker-export (d.w.z. de kosten per geëxporteerde suiker) bleek overeen te komen met de experimenteel bepaalde waarde. Het combineren van de gevonden specifieke energiekosten met de exportsnelheden van diverse soorten, toonde aan dat suiker-export gemiddeld 29% van de donkerademhaling kan verklaren. Dus eiwitturnover en suiker-export kunnen gezamenlijk een aanzienlijk deel van de donkerademhaling van een volgroeid blad verklaren.

Welke processen kunnen ten grondslag liggen aan het resterende deel van de donkerademhaling? Naast eiwitturnover is het handhaven van ionen-gradiënten als mogelijk onderhoudsproces geopperd. De kosten voor het handhaven van ionen-gradiënten werden in eerste instantie gekwantificeerd door leksnelheden te meten, en de benodigde kosten voor heropname te berekenen (hoofdstuk 5). Dit is gedaan voor wortels, omdat ionen-uitwisseling van volgroeide bladeren niet gemeten kan worden zonder het weefsel aan te snijden (snijwonden kunnen tot overschatting van de lek leiden). Om een eerste schatting te verkrijgen van het belang van heropname van uitgelekte ionen, bleek een eenvoudige methode als het meten van de lek als verandering in geleidbaarheid van gedemineraliseerd water te volstaan. In jonge groeiende aardappelwortels (relatieve groeisnelheid 0.15 g g^{-1} d^{-1}) was $\pm$ 20% van de totale ademhaling nodig voor heropname om de lek te compenseren. Dit percentage is relatief laag door het belangrijke aandeel van groei in de ademhaling van jonge weefsels; in volgroeide weefsels zal het percentage dan ook beduidend hoger zijn. Uitgedrukt als percentage van de ademhaling voor de totale (an)ionen opname, bleken de kosten van het handhaven van ionen-gradiënten 34 tot 66% te bedragen.

Het bepalen van de exakte kosten voor het handhaven van ionen-gradiënten, bleek sterk bepaald te worden door de interpretatie van de efflux-kinetiek. Met *efflux-kinetiek* wordt het tijdsverloop van de lek(snelheid) aangeduid. Aangezien fouten in de interpretatie in de efflux-kinetiek direct leiden tot foutieve conclusies over de kosten voor het handhaven van ionen-gradiënten, is het belangrijk de efflux-kinetiek goed te kunnen interpreteren. Om meer inzicht te verkrijgen hoe de efflux-kinetiek geinterpreteerd moet worden, is door middel van computersimulatie onderzocht welke factoren de efflux-kinetiek beinvloeden (hoofdstuk 6). Hiertoe is een model ontwikkeld, waarmee alle ionen in de diverse onderdelen van de wortel (d.w.z. de celwanden, cytosols en vacuoles in de verschillende concentrische cellagen) verschillend worden gemerkt, zodat de efflux-kinetiek naar herkomst kan worden onderscheiden. De geobserveerde efflux-kinetiek bleek sterk te kunnen verschillen voor wortels met een verschillende anatomie, ondanks het feit dat de abolute lek gelijk was. De simulaties toonden dat voor een correcte interpretatie van de efflux-kinetiek in termen van kosten voor het handhaven van ionen-gradiënten, de wortelanatomie in beschouwing genomen moet worden, hetgeen tot op heden veelal niet gebeurd is.

Tot slot werd voor een wortel onderzocht in hoeverre de huidige kennis over energie verbruikende processen volstaat om de totale ademhaling te verklaren. Hiertoe werd de worteladem haling van twee datasets eerst d.m.v. multiple regressie geanalyseerd in termen van groei, onderhoud en ionen-opname (hoofdstuk 7). De overeenkomst tussen de waarden van

beide experimenten toonde aan dat de regressiemethode robuust is. De regressieresultaten werden vervolgens vergeleken met alternatieve experimentele schattingen en theoretische berekeningen gebaseerd op individuele processen (groei, ionenopname, handhaven ionen-gradiënten en eiwitturnover). Het bleek mogelijk de totale ademhaling van de wortel door deze berekeningen te verklaren. De energiekosten van netto ionen-opname werden geïnterpreteerd in termen van lek *(efflux)* en bruto opname *(influx)*. Het bleek dat efflux gecorreleerd is met (netto) ionen-opname i.p.v. onderhoud. Dit verklaart de hoge opnamekosten, zoals eerder gevonden in soortgelijke experimenten. Eiwitturnover verklaart het grootste deel van de op deze manier geschatte onderhoudscomponent in de ademhaling.

Conclusies

Concluderend blijken zowel eiwitturnover als het handhaven van ionen-gradiënten belangrijke onderhoudsprocessen van de plant te zijn. De ontwikkelde methoden om dit te bepalen bleken eenvoudig en effektief. Voor een exakte schatting van de kosten voor het handhaven van ionen-gradiënten, moet het effekt van de wortelanatomie op de gradiënten-kinetiek in beschouwing worden genomen. Het ontwikkelde simulatiemodel is een nuttig instrument om meer inzicht in dergelijke anatomische effecten te verkrijgen en vervolgonderzoek te sturen. Export van suikers verklaart een aanzienlijk deel van de donkerademhaling van volgroeide bladeren. De ademhaling van een wortel kan verklaard worden door integratie van de kennis op procesnivo. Samenvattend lijkt het er op dat de beschreven processen het belangrijkste deel van de niet aan groei en ionenopname gerelateerde ademhaling kunnen verklaren.

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Curriculum vitae

Tjeerd Bouma werd geboren op 14 mei 1964 te Utrecht. In 1984 begon hij aan de studie Biologie aan de Rijks Universiteit Utrecht. Deze werd in 1988 afgerond met als specialisatie moleculaire genetica en plantenfysiologie. Aansluitend werd hij als Assistent In Opleiding aangesteld bij de vakgroep Theoretische Produktie-ecologie (TPE-LUW), van de Landbouwuniversiteit Wageningen (1-12-1988 tot 1-5-1993) op een promotieonderwerp getiteld "Utilization of Respiratory Energy in Higher Plants - Requirements of 'Maintenance' and Transport Processes". Gedurende deze periode werd het in dit proefschrift beschreven onderzoek verricht bij het DLO-Instituut voor Agrobiologisch en Bodemvruchtbaarheidsonderzoek (AB-DLO, voorheen CABO-DLO), in samenwerking met de vakgroep Botanische Oecologie en Evolutiebiologie van de Universiteit Utrecht. Tot 1998 is hij in tijdelijke dienst bij de Pennsylvania State University te State College, in de Verenigde Staten van Amerika.