Le point de compensation stomatique de l'ammoniac: comprendre et modéliser la relation au métabolisme azoté de la plante

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Résumé

L'ammoniac atmosphérique (NH_3) est principalement émis par l'agriculture. C'est le principal composé alcalin de l'atmosphère et il joue un rôle essentiel dans la chimie de l'atmosphère. Les dépôts humides et secs d'NH_x (NH_3 + NH_4^+) ont des effets négatifs directs (toxicité à fortes doses) ou indirects (acidification, eutrophisation, baisse de la résistance aux stress) sur les écosystèmes naturels ou cultivés.

La plante peut être un puits ou une source d'NH_3 pour l'atmosphère. Le sens et l'intensité des échanges dépendant du gradient de concentration entre la cavité sous stomatique et l'air. La concentration en ammoniac dans la cavité sous stomatique appelée aussi point de compensation stomatique pour l'ammoniac est déterminée par la température de la feuille, la concentration en ammoniaque ($[NH_4^+]_{ap}$) et le pH du liquide apoplastique. Ces deux derniers paramètres dépendent du métabolisme azoté de la feuille et des diverses entrées et sorties de composés à travers le xylème et le phloème. Jusqu'à présent, seuls des modèles empiriques ont été conçus pour modéliser le point de compensation stomatique pour l'ammoniac.

Pour analyser le determinisme du point de compensation pour l'ammoniac, nous avons conçu une expérimentation pour mesurer sa variabilité pour de jeunes plantes de colza en fonction de la nutrition azotée et des conditions jour et nuit. Deux méthodes de mesures ont été utilisées : l'extraction d'apoplaste et la chambre à flux. Les deux méthodes montrent une corrélation positive entre la concentration en ammonium dans la solution nutritive et le point de compensation. Par contre la concentration en nitrate a peu d'influence sur le point de compensation de même qu'une alimentation mixte ammonium et nitrate réduit le point de compensation par rapport a une alimentation a même concentration en ammonium. Le point de compensation mesuré de jour n'est pas significativement différent du point de compensation mesuré de nuit même si il a tendance à être plus fort.

Le modèle construit est un modèle a compartiment ; il est couplé à un modèle de continuum sol plante atmosphère donnant des flux d'eau et en particulier la conductance stomatique. Il intègre un modèle de photosynthèse et les principales sources et puits d'ammoniac dans une cellule. Le modèle montre une forte dépendance du point de compensation stomatique du flux d'eau, de la température et de la concentration en ammonium dans le xylème.

Mots Clés: NH_3, NH_4^+, pH, apoplaste, xylème, fertilisation azotée, échanges stomatiques.

Abstract

Agriculture is the major source of atmospheric ammonia (NH_3). NH_3 is the most abundant alkali component of the atmosphere and it plays an essential role in air chemistry. Dry and wet depositions of NHx (NH_3 + NH_4^+) have direct negative effects (toxicity at high levels) and indirect effects (acidification, eutrophication, etc.) on natural and semi natural ecosystems.

Plants can either be a source or a sink of NH_3 for the atmosphere. The direction and intensity of the exchange depends on the ammonia gradient between the sub-stomatal cavity and the atmosphere. The concentration in the sub-stomatal cavity also called ammonia compensation point is determined by leaf temperature and ammonium (NH_4^+) concentration and pH of the apoplastic solution. Those two last variables depend on the leaf N metabolism as well as in-fluxes and out-fluxes of various compound to the leaf via the xylem and phloem. Until now, only empirical based models are used to model the ammonia stomatal compensation point.

We designed an experiment to measure the variability of the ammonia stomatal compensation point for leaves of Oilseed rape in a vegetative growth stage with various N nutritions and with dark and light conditions. Two measurement methods were used: apoplast extraction and chamber flux measurements. Both methods show a positive correlation between ammonium concentrations in the nutritive solution and the stomatal compensation point. However, nitrate concentrations have no effect on the compensation point. Plants grown on a mixed ammonium and nitrate nutrition have lower compensation points than plants grown on only ammonium form of N nutrition. Dark and Light compensation were not significantly different from one another although light compensation points had a tendency to be higher than dark compensation points.

The elaborated model is a compartment type model couples to a soil plant atmosphere continuum model. It integrates a photosynthesis model and the major sources and sinks of ammonia in a plant cell. The model points out the high dependence of the compensation point on the water flux and on the ammonium concentration in the xylem.

Key Words: NH_3, NH_4^+, pH, apoplast, xylem, nitrogen fertilization, stomatal exchange.

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Introduction générale

« La chose importante en science n'est pas tellement d'obtenir de nouveaux faits mais de découvrir de nouvelles manières d'y penser. »

William Bragg (Physicien: 1890-1971)

1. Contexte Général

L'azote (N) est un des éléments essentiels à la vie. C'est aussi l'élément le plus abondant dans l'atmosphère, l'hydrosphère et la biosphère du système terrestre. Etonnement, c'est souvent l'élément limitant pour la plupart des organismes et écosystèmes. On distingue l'azote non-réactif sous forme de diazote (N_2) et l'azote réactif qui peut être sous forme d'azote minéral réduit (ammoniac: NH_3 et ammonium: NH_4^+), azote minéral oxydé (nitrate: NO_3^-, oxyde d'azote: NO_x, protoxyde d'azote: N_2O, acide nitrique: HNO_3, …), et l'azote organique (urée, protéines, acides aminées).
L'ammoniac est le composé azoté gazeux le plus lié à l'activité agricole. Environ 80% de l'azote ammoniacal atmosphérique serait d'origine anthropique. Cette production d'ammoniac anthropique a débuté avec la mise en place du processus Haber-Bosch en 1913 qui convertit le diazote atmosphérique en ammoniac et qui sert principalement à la fabrication de fertilisant. L'élaboration d'engrais synthétique a permis une production agricole alimentaire suffisante pour nourrir une population humaine croissante. Le processus Haber-Bosch produirait en moyenne 100 Tg d'azote sous forme d'ammonium par an (Galloway et al. 2003). En France, 97% de l'ammoniac atmosphérique est d'origine agricole (CITEPA, 2001) dont environ 12% sont liés aux plantes cultivées (Bouwman et al. 1997).
Cette production d'ammoniac anthropique place l'ammoniac au centre de la « Nitrogen cascade » (cascade d'azote) décrite par Galloway (1998) illustré en Figure 1. Ainsi une fois le diazote converti en azote réactif, il est rapidement transformé d'une forme à une autre dans les différents compartiments terrestres entraînant des effets non négligeables sur la santé humaine et sur les écosystèmes. L'ammonium, produit par le processus Haber-Bosch, est amené sous forme de fertilisant dans les agro-écosystèmes où il participe au cycle de l'azote du sol et est incorporé par les plantes. A peu près la moitié de l'azote ajouté sur une culture est incorporé dans les plantes récoltées (Smil, 1999), le reste est soit réémis vers l'atmosphère, soit lessivé principalement sous forme de nitrate vers les eaux souterraines.

L'ammoniac gazeux émis vers l'atmosphère contribue à la formation d'aérosols qui ont un effet sur la visibilité et sur la santé humaine. Ces aérosols sont aussi des précurseurs d'oxyde nitreux (N_2O), gaz à effet de serre ayant un indice de réchauffement global de 310 par rapport au dioxyde de carbone (CO_2). Les émissions d'ammoniac entraînent aussi des dépôts d'ammoniac à proximité des sources (dépôts secs) mais aussi des dépôts de dérivés azotés de l'ammoniac sur des surfaces en général naturelles situées jusqu'à des centaines de kilomètres des lieux d'émission (dépôts humides, aérosols) (Asman and Van Jaarsveld, 1992). Ces dépôts provoquent une accélération de

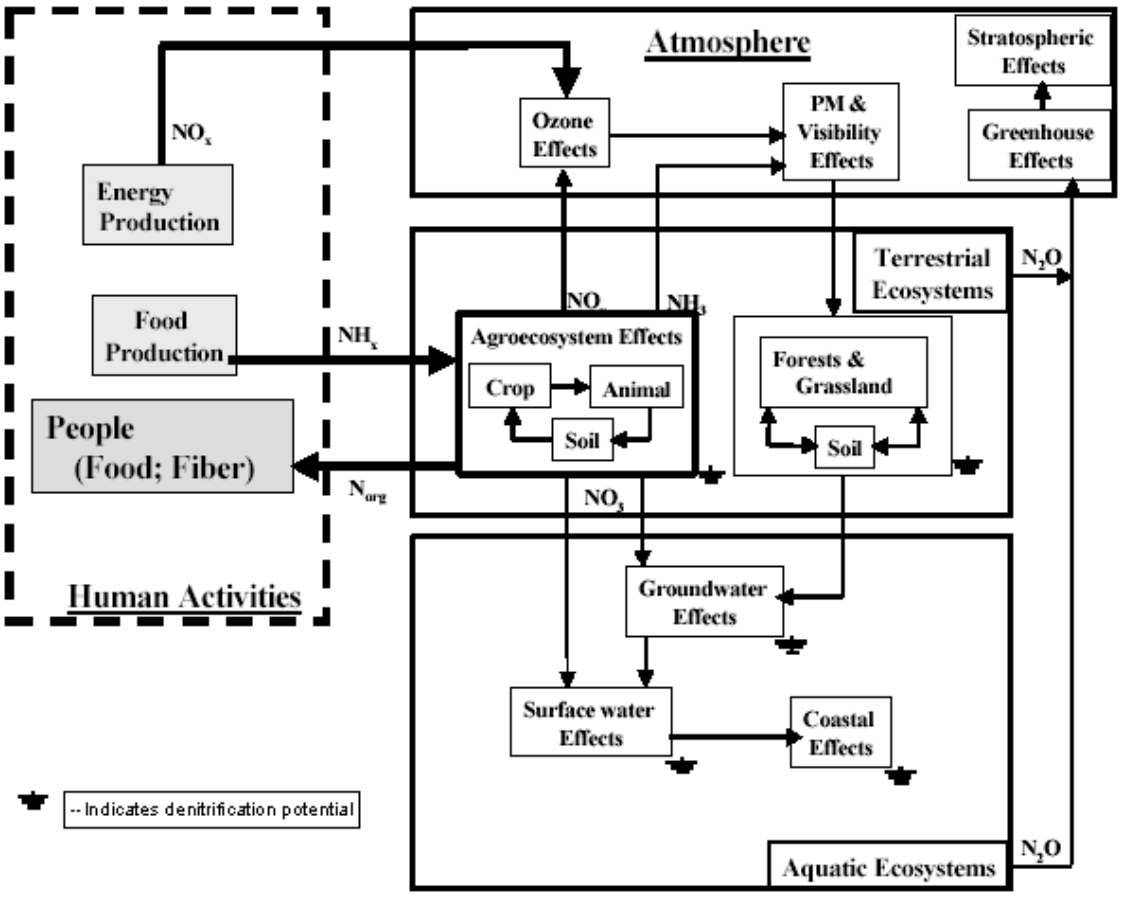

Figure 1: "Nitrogen cascade" selon Galloway et al. (2003)

l'acidification des sols (Dambrine et al. 1998) et sont également à l'origine d'un déséquilibre trophique et d'une modification de la composition des populations végétales ; ils portent donc atteinte à la biodiversité car ils peuvent être une source d'azote pour des écosystèmes limités en azote (Fahey et al. 1999; Landmann, 1995; Skellington and Wilson, 1988).

Les écosystèmes semi naturels et naturels sont donc des puits d'ammoniac alors que les écosystèmes agricoles en sont plutôt des sources. Le sens et l'intensité des échanges d'ammoniac dépendent de la différence de concentration entre l'intérieur du couvert (appelée le point de compensation du couvert: χ_c) et l'air. Le point de compensation du couvert résulte à la fois des flux entre la surface du sol et le couvert et la surface des feuilles (stomates, cuticules) et le couvert. Le flux cuticulaire est essentiellement un flux de dépôt et dépend surtout de l'humidité de l'air, de la température de surface de la feuille et de la texture de surface de la feuille (Flechard et al. 1999; Van Hove et al. 1989). Tandis que le flux stomatique lui est bi-directionnel et dépend de la différence de concentration entre le couvert et la cavité sous-stomatique (appelée le point de compensation stomatique: χ_s) (Farquhar et al. 1980). Le point de compensation stomatique résulte des équilibres thermodynamiques chimiques, des échanges actifs et passifs d'ions, des déséquilibres dans les cycles de l'azote au sein de la cellule, et de l'assimilation et du transport de l'azote dans la plante. Malgré les faibles flux émis par les plantes, les échanges avec la végétation jouent un rôle important dans la régulation des concentrations atmosphériques et dans la détermination du transport à grande échelle (Langford and Fehsenfeld, 1992; Sutton et al. 1994).

2. Problématique

Les échanges de composés azotés entre le sol et l'atmosphère ont été longuement analysés. Il y a cependant plus d'incertitude en ce qui concerne les échanges entre le couvert végétal et l'atmosphère. Le point de compensation du couvert étant mal quantifié pour les grandes cultures et présentant pourtant de fortes variations.

De récentes études réalisées aux Pays-Bas ont mis en évidence l'existence d'un décalage entre les flux d'ammoniac modélisés et les flux mesurés (van Pul et al. 2004) ; les flux modélisés sont environ 30% plus faibles que les flux mesurés. Ce problème qui est en partie expliqué par la transformation du NH_3 en aérosols, pourrait être aussi expliqué par une mauvaise prise en compte des émissions par les plantes cultivées. Les modèles actuels d'estimation des concentrations atmosphériques utilisent un point de compensation stomatique empirique. Ainsi ce point de compensation prescrit ne varie ni en fonction des facteurs du milieu, ni en fonction de l'alimentation azotée ou du stade de croissance de la plante. Or plusieurs études ont montré l'influence de ces facteurs sur

le point de compensation (Francis et al. 1997; Mattsson and Schjoerring, 1996; Mattsson and Schjoerring, 2003; Mattsson et al. 1998; van Hove et al. 2002). A notre connaissance, il n'existe pas à ce jour de modèles mécanistes qui décrivent la dynamique du point de compensation stomatique de l'ammoniac en fonction des facteurs du milieu ou de la nutrition azotée. Riedo et al. (2002) ont couplé un modèle de croissance de prairie avec un modèle d'échanges d'ammoniac basé uniquement sur un système de résistance. Ce modèle n'a cependant pas été appliqué à d'autres types de cultures ou de végétation.

D'autre part, nous savons que les émissions et les dépôts dépendent des métabolismes de l'azote et du carbone dans la plante, cependant cette dépendance est souvent mal quantifiée. Ainsi, une quantification précise du point de compensation stomatique et de sa variation en fonction des conditions environnementales devrait nous permettre de mieux comprendre le déterminisme des échanges entre la végétation et l'atmosphère. Ceci nous permettrait aussi d'obtenir une solide base de données expérimentale sur les variations de χ_s pour différentes espèces végétales, permettant ainsi une meilleure paramétrisation des modèles. Cependant, la mesure de ce point de compensation reste difficile ; il existe deux méthodes permettant sa quantification : la première destructive où l'on extrait l'apoplasme (Husted and Schjoerring, 1995a) et on mesure la concentration d'ammonium et le pH de celui-ci ; la seconde consiste à mesurer les flux échangés dans une chambre et à déduire le point de compensation à partir de la concentration à laquelle le flux s'annule (Husted and Schjoerring, 1995b). Ces deux méthodes sont chacune critiquables et les résultats obtenus ne sont pas toujours directement comparables (Hill et al. 2001). La principale source d'incertitude résulte de la nature chimique de l'ammoniac et des faibles concentrations mesurées.

Ce travail s'articule donc autour du questionnement suivant : Quel est l'impact de la quantité et de la forme de la nutrition azotée et du métabolisme azoté de la plante sur le point de compensation stomatique de l'ammoniac ?

3. Objectifs de Recherche

Ce travail de thèse propose de répondre à cette problématique avec deux objectifs principaux:

1. Mesurer le point de compensation stomatique dans différentes conditions environnementales (obscurité/lumière) et différentes nutritions azotées (ammonium, nitrate et mixte en différentes concentrations). Ces mesures permettront une compréhension plus complète du déterminisme du point de compensation stomatique, de comparer les différentes méthodes de mesures et

de comprendre les causes de leurs divergences. Elles serviront également à tester le modèle élaboré.

2. Elaborer un modèle qui permet d'établir une estimation du point de compensation stomatique des plantes cultivées, en vue de quantifier les flux d'ammoniac entre les feuilles et l'atmosphère. Ce modèle prendra en compte les différents métabolismes de la feuille (photosynthèse, assimilation de l'azote, photoréspiration, etc.), les conditions environnementales présentes (température, rayonnement, humidité, etc.) et les pratiques culturales (apports d'azote, stress hydrique, etc.). Il devra décrire de façon quantitative les compartiments, les puits et les sources d'azote de la feuille susceptibles d'induire ces flux d'ammoniac. Cette approche de modélisation permettra d'étudier les modalités d'action des facteurs climatiques, pédologiques et culturaux sur les flux d'ammoniac.

Nous avons choisi d'utiliser le colza (*Brassica napus* L.) comme plante d'étude (même si le modèle a pour vocation d'être plus générique) pour les raisons suivantes :

- Le colza est une plante de grande culture recevant des apports d'azote non négligeables durant sa croissance (entre 230 a 245 Kg N ha^{-1}) et dont les surfaces de production se voient augmentent notamment ces derniers temps, pour la production de bioénergies.
- Ses feuilles ont une surface importante en comparaison avec d'autres cultures, permettant ainsi de faire des mesures sur des feuilles séparées en ce qui concerne l'extraction et de mettre un nombre limité de plantes dans la chambre à flux.
- Il existe des données dans la littérature sur des mesures de point de compensation de feuilles de colza, nous permettant ainsi de pouvoir faire des comparaisons par rapport à nos mesures.

4. Plan de la thèse

Ce manuscrit s'articule autour de trois axes principaux :

1. Etablir une étude bibliographique approfondie des processus et mécanismes impliqués dans le déterminisme du point de compensation stomatique de l'ammoniac. Elle comprendra l'étude de modèles existants pour la détermination du flux d'ammoniac, des processus d'échanges d'ammoniac et d'ammonium entre les différentes parties de la plante et les différentes parties d'une cellule de mésophylle, et des sources et puits d'azote au sein de la feuille. Cette partie aboutira à l'élaboration d'hypothèses et de simplification nécessaires à la construction du modèle.

2. Mesurer le point de compensation et les flux d'ammoniac sur des plantes de colza en stade de croissance avec différentes nutritions azotées et en conditions de lumière ou d'obscurité. Ces mesures amènent à une comparaison des deux principales méthodes utilisées et à une quantification du point de compensation en relation avec la nutrition azotée et les différentes sources et puits d'ammoniac de la plante (xylème, apoplasme, feuille entière).
3. Construire un modèle de point de compensation stomatique de l'ammoniac prenant en compte le métabolisme azoté et carboné de la plante. Ce modèle permet d'analyser la réponse du point de compensation aux facteurs du milieu et de déterminer à l'aide d'une étude de sensibilité des paramètres et processus dominants dans la determination des flux.

Ainsi ce manuscrit comprendra trois chapitres dont deux ont été acceptés pour publication dans une revue à comité de lecture. Le travail s'achèvera sur une synthèse générale reprenant les principaux résultats. Chaque chapitre sera précédé d'une synthèse en français du contenu de l'article publié.

Chapitre 1: Etat des lieux et approche de modélisation

Relations entre le point de compensation stomatique de l'ammoniac et le métabolisme azoté pour les grandes cultures : états des lieux et approches de modélisation.

"Massad R.S., Loubet B., Tuzet A. and Cellier P., 2008. Relationship between ammonia stomatal compensation point and nitrogen metabolism in arable crops: Current status of knowledge and potential modelling approaches. Environmental Pollution. *154 :390-403.*"

Chapitre 2: Variabilité expérimentale du point de compensation stomatique

Variation du point de compensation stomatique de l'ammoniac pour de jeunes feuilles de colza en fonction de condition lumière/obscurité et pour une nutrition azotée variable.

"Massad R.S., Loubet B, Tuzet A, Autret H. and Cellier P. Ammonia stomatal compensation point of young oilseed rape leaves during light / dark cycles under various nitrogen nutritions." *Accepté dans "Agriculture, Ecosystems and the Environment"*

Chapitre 3: Modèle du point de compensation stomatique de l'ammoniac

Modèle du point de compensation stomatique de l'ammoniac en relation au métabolisme azoté et carboné de la plante.

"Massad R.S. Tuzet A., Loubet B., Perrier A. and Cellier P. Model of STomatal AMmonia compensation Point (StAmP) in relation to the plant nitrogen and carbon metabolisms." *Soumis à "Plant Cell and Environment*"

Chapitre 1 :

Etat des lieux et approche de modélisation

« Rendez les choses aussi simples que possible, mais pas plus simples. »

Albert Einstein (Physicien : 1879-1955)

Ce chapitre établit une étude bibliographique des principaux processus physiques, chimiques et biologiques agissant au niveau d'une feuille dans le déterminisme du point de compensation stomatique de l'ammoniac. De plus il présente une synthèse des principales mesures et modèles existant pouvant servir pour la construction du modèle de point de compensation.

L'échange bi-directionnel d'ammoniac entre la végétation et l'atmosphère a été mis en évidence pour la première fois par Meyer en 1973 et plus tard par Farquhar et al. en 1980 qui a défini le point de compensation pour l'ammoniac. La problématique de l'ammoniac en relation avec la pollution atmosphérique date du début des années 1980 où l'ammoniac atmosphérique a été reconnu comme cause de l'acidification des sols et d'eutrophisation des plans d'eau contribuant à un impact sur la biodiversité (van Breeman et al. 1982 ; Heil et Diemont, 1983). C'est alors que la recherche sur l'ammoniac s'est développée surtout aux Pays-Bas avec les risques croissant de fortes pollutions allant avec les grandes populations d'animaux d'élevage. Ceci s'est évidemment accompagné de développement technique d'instrumentation et de méthodes de mesures. Pour plus d'information se référer à un article récent de Sutton et al (2008) qui retrace l'historique de l'ammoniac en relation aux problèmes environnementaux et à la progression de l'agriculture.

A. Echanges d'ammoniac entre le couvert végétal et l'atmosphère

Les échanges d'ammoniac entre un couvert végétal et l'atmosphère font intervenir plusieurs compartiments et processus. On peut faire la différence entre le dépôt sec qui décrit un processus d'échange bi-directionnel entre la végétation ou le sol et l'atmosphère et un dépôt humide processus selon lequel l'ammoniac se dépose après avoir été incorporé par des gouttelettes de pluie ou de brouillard. Ainsi dans un couvert végétal le sol et les feuilles peuvent être une source ou un puits d'ammoniac. La litière quant à elle est principalement une source. On peut ainsi définir un point de compensation d'ammoniac moyen pour l'ensemble du couvert (χ_c) qui résulte de l'équilibre entre les différents flux à l'intérieur de ce couvert (Figure 2).

Si on se positionne à l'échelle de la feuille, le NH_3 atmosphérique peut être absorbé ou émis par la feuille à travers les stomates. En tant que composé réactif, il se dépose également sur la cuticule et peut être lessivé sur le sol ou réémis. La direction et l'intensité des échanges stomatique d'NH_3 dépendent de la différence de concentration entre l'atmosphère libre et l'intérieur des cavités sous stomatiques; c'est cette concentration à l'intérieur des cavités sous stomatiques qui est appelée point de compensation stomatique (χ_s) (Figure 2). Ce point de

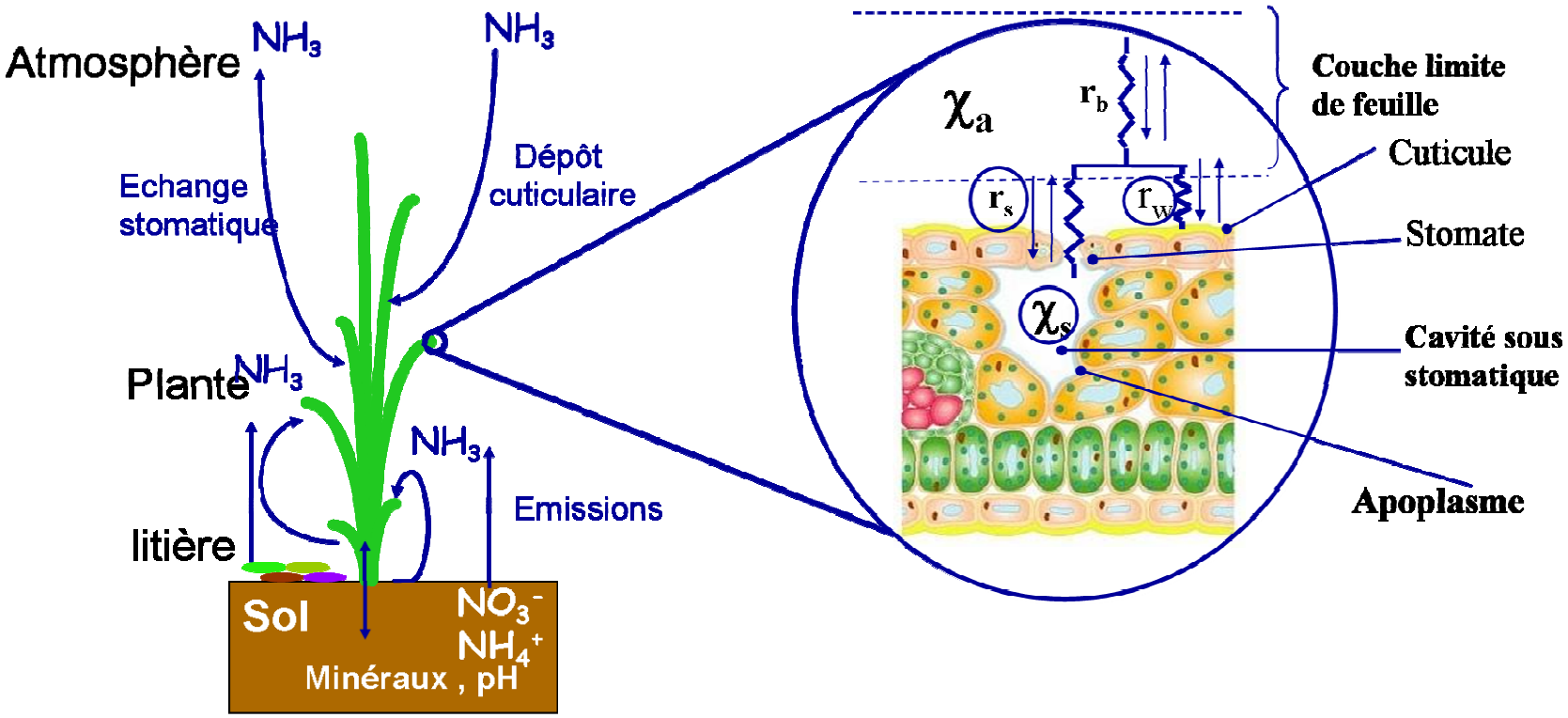

Figure 2: Echanges d'ammoniac entre le couvert végétale et l'atmosphère et entre la surface de la feuille et l'atmosphère.

compensation résulte aussi de l'équilibre thermodynamique existant entre l'ammoniac dissous dans l'apoplasme (fine couche de liquide extracellulaire dans la cavité sous stomatique) et l'ammoniac gazeux contenu dans la cavité sous stomatique.

B. Déterminisme du point de compensation stomatique de l'ammoniac

Le point de compensation stomatique est fortement lié à la concentration en ion ammonium et au pH de l'apoplasme ; il est donc influencé par la nutrition azotée de la plante et les échanges entre les racines et le sol et par le métabolisme azoté et carboné de la plante et par conséquence du stade de croissance de la feuille (croissance, sénescence, …). χ_s est aussi influencé par les conditions environnementales (température et humidité relative de l'air, rayonnement, …). Dans la plante, la concentration en NH_4^+ est déterminée, d'une part, par les échanges minéraux avec le sol [et donc par les caractéristiques physico-chimiques du sol et par le fonctionnement des racines] ainsi que par la circulation dans le xylème, et d'autre part par le métabolisme azoté de la plante. L'apoplasme est quant à lui en étroite relation avec le cytoplasme et donc la concentration en ammonium de celui-ci va influencer la concentration dans l'apoplasme. La relation apoplasme / cytoplasme est fortement déterminée par les mécanismes d'échanges de composés azotés entre les 2 compartiments, alors que la concentration en ammonium dans le cytoplasme est déterminée par les sources et puits cellulaires.

– Les modalités d'échanges à traves la paroi cytoplasmique peuvent être actifs ou passifs. Concernant l'ammoniac dissous, les transferts au niveau de la feuille ne sont pas très bien caractérisés. On considère cependant que le NH_3 dissous diffuse librement à travers la paroi selon un gradient de concentration qui dépend fortement de l'équilibre acide-base et donc du pH, alors que les ions NH_4^+ seraient transportés activement de l'apoplasme vers le cytoplasme.

– Les sources d'ammoniac cellulaire sont nombreuses et dépendent de l'âge de la feuille. Les plus importantes seraient la photorespiration, la réduction du nitrate, la biosynthèse de la lignine et la biodégradation des protéines. Quantitativement, la photorespiration est la source la plus importante, cependant le NH_x produit par ce processus est presque entièrement recyclé et donc réassimilé par le cycle GS/GOGAT

(Glutamine synthetase/Glutamate synthase). La réduction du nitrate en ammonium quand à elle est la seule source d'ammoniac extrinsèque à la plante.

– Le principal puits d'ammoniac cellulaire est le cycle GS/GOGAT qui transforme les ions ammoniac en glutamine qui est par ailleurs transformé en divers acides aminées. Il existe toutefois d'autres puits d'NH_x qui feraient intervenir la GDH (glutamate dehydrogenase).

C. Simplifications et processus essentiels à intégrer dans une modélisation

Cette analyse bibliographique m'a permis d'étudier les principaux processus mis en jeu dans le fonctionnement biologique du cycle de l'azote cellulaire et ainsi j'ai pu sélectionner ceux à intégrer dans la construction d'un modèle de point de compensation stomatique pour l'ammoniac. Ils sont résumés ci-dessous et illustrés dans la Figure 1.3 de ce chapitre.

(1) Le transport de NH_x et NO_3^- entre les racines et les feuilles via le xylème. Nous considérons ce transport principalement régulé par le flux d'eau dans le xylème et donc par l'évapotranspiration et l'absorption racinaire de composés azotés et d'eau. Cette absorption racinaire est bien sûr modulée par la disponibilité de l'azote minéral et de l'eau dans le sol environnant des racines. Cette approche nous permet de prendre en compte la variabilité diurne de l'apport azoté vers les feuilles.

(2) Les équilibres acide–base entre NH_3 et NH_4^+ dans chaque compartiment des cellules de la feuille (vacuole, cytoplasme et apoplasme). Cet équilibre est fortement dépendant du pH de chaque compartiment. Nous savons que le pH du cytoplasme est fortement régulé et présente dans de faibles variations tandis que il existe un fort gradient de pH dans l'apoplasme et dans la vacuole et qui peut avoir un rôle important dans le déterminisme de χ_s.

(3) Les échanges de NH_x et NO_3^- entre l'apoplasme et le cytoplasme. Nous considérons que l'ammonium et le nitrate sont transportés de façon active vers le cytoplasme tandis que l'ammoniac diffuse à travers la membrane cytoplasmique et qui dépend du gradient de concentration entre l'apoplasme et le cytoplasme.

(4) L'équilibre thermodynamique entre la forme aqueuse de l'ammoniac dans l'apoplasme et la forme gazeuse qui est passée dans la cavité sous stomatique. Cet équilibre est fortement dépendant de la température de feuille.

(5) Les échanges stomatiques de l'ammoniac qui se font par diffusion et qui dépendent de (i) la résistance stomatique aux échanges gazeux principalement contrôlée par l'évapotranspiration et (ii) le gradient de concentration d'ammoniac entre la cavité sous stomatique et l'atmosphère avoisinant la feuille.

(6) Les échanges de NH_x (NH_4^+ + NH_3) et NO_3^- entre le cytoplasme et la vacuole. Nous considérons que l'échange de NH_x est passif et donc dépend du gradient de concentration entre la vacuole et le cytoplasme qui fait intervenir le pH mais aussi de la résistance du tonoplaste aux échanges. L'échange de nitrate est principalement actif.

(7) L'assimilation de NH_x. Le cycle GS/GOGAT est considéré comme la principale route d'assimilation de l'ammonium. Pour simplifier, nous regroupons la GS cytoplasmique et chloroplastique en un seul pool. La vitesse d'assimilation du NH_x dépend de la concentration de NH_x et de carbone organique dans le cytoplasme.

(8) La production de NH_x dans le cytoplasme se fait via la photorespiration qui est considérée comme proportionnelle à la photosynthèse pour cause de simplicité.

(9) La production de NH_x se fait via la reduction du nitrate. Nous considérons la réduction comme une équation globale (equation à une seule étape) et donc ignorons le passage sous forme nitrite. Nous considérons de façon simplifiée que cette réduction ne dépend que de la concentration en nitrate dans le cytoplasme.

Relations entre le point de compensation stomatique de l'ammoniac et le métabolisme azotée appliqué aux grandes cultures : états des lieux et approche de modélisation.

"Massad R.S., Loubet B., Tuzet A. and Cellier P., 2008. Relationship between ammonia stomatal compensation point and nitrogen metabolism in arable crops: Current status of knowledge and potential modelling approaches. Environmental Pollution, 154, 390 - 403.

Abstract

The ammonia stomatal compensation point of plants is determined by leaf temperature, ammonium concentration ($[NH_4^+]_{apo}$) and pH of the apoplastic solution. The later two depend on the adjacent cells metabolism and on leaf inputs and outputs through the xylem and phloem. Until now only empirical models have been designed to model the ammonia stomatal compensation point, except the model of Riedo et al. (2002), which represents the exchanges between the plant's nitrogen pools. The first step to model the ammonia stomatal compensation point is to adequately model $[NH_4^+]_{apo}$. This $[NH_4^+]_{apo}$ has been studied experimentally, but there are currently no process-based quantitative models describing its relation to plant metabolism and environmental conditions. This study summarizes the processes involved in determining the ammonia stomatal compensation point at the leaf scale and qualitatively evaluates the ability of existing whole plant N and C models to include a model for $[NH_4^+]_{apo}$.

Capsule: This paper puts together the necessary elements to build up a model for the ammonia stomatal compensation point at the leaf scale and gives an overview of the current knowledge and data available to achieve this goal.

Key Words: NH_3; NH_4^+; pH; apoplast; plant metabolism.

1. Introduction

Ammonia (NH_3) is the most abundant alkaline component in the atmosphere, hence playing a major role in the neutralization of atmospheric acids (Asman & Van Jaarsveld 1992; Erisman et al. 1988). These reactions lead to the formation of ammonium (NH_4^+) aerosols (Finlayson-Pitts & Pitts 1999), which participate in half of the negative radiative forcing of the atmosphere (Adams et al. 2001; Houghton et al. 2001). Ammonium aerosols are also a potential precursor of nitrous oxide (Galbally & Roy 1983), and are known to affect air quality and visibility (Aneja et al. 2001; Erisman et al. 2003; Lefer et al. 1999). Gaseous NH_3 as well as aerosol NH_4^+ deposition contributes to ecosystem acidification, (Hornung et al. 1995; Van Breeman & Van Dijk 1988), aquatic and terrestrial eutrophication (Schulze et al. 1989) and impoverishment of the floral diversity in oligotrophic ecosystems (Pearson & Stewart 1993).

There is increasing evidence that the exchange of ammonia (NH_3) between photolithotrophs and the environment is an important aspect of the global nitrogen cycle (Krupa 2003; Raven et al. 1993). Ammonia flux measurements have been reported over several ecosystems and ranged for example from a deposition flux of 140 ng m^{-2} s^{-1} (Phillips *et al.* 2004) to an emission flux of 4 ng m^{-2} s^{-1} (Wichink Kruit *et al.* 2007) in non managed grasslands. Although the vegetation is not the major source of NH_3, it plays a major role in regulating both NH_3 concentrations and the extent of long-range transport of NH_x (Langford & Fehsenfeld 1992; Sutton et al. 1994).

In the 1990s, extensive NH_3 emission reduction policies in the Netherlands were not matched by an observed reduction of NH_3 concentration and NH_4^+ in rain water (Erisman et al. 1998). This issue was known as the “ammonia gap” in the 1990s. Although the difference between expected and measured trends in concentrations of both NH_3 and NH_4^+ is less pronounced now, there is still a “gap” between the modelled and measured NH_3 concentrations using national NH_3 emission inventories and regional transport models (van Pul et al., 2004). The modelled NH_3 concentration is about 30% lower than the measured one. This gap could partly be explained by emissions from (or reduced deposition to) vegetation (arable cropland or grassland) over the long term.

The canopy can be separated in three compartments: (i) the soil and the (ii) plant, which both can be a sink or a source of NH_3, and (iii) the litter, which is mainly a source of

NH_3 (Denmead et al. 1976; Husted & Schjoerring 1995a; Husted & Schjoerring 1996b; Husted et al. 2000a; Husted et al. 2000b; Nemitz et al. 2000a; Sutton et al. 1993). These three compartments are tightly linked: the plant absorbs ammonia or other N sources from the soil, and is also the main source of litter and finally the litter could give back some NH_3 to the soil. Hence the leaves being at the interface between the atmosphere and the ground, they eventually regulate the atmosphere-surface exchange of NH_3.

At the leaf scale, NH_3 can be absorbed or emitted through the stomata (stomatal exchange) or deposited onto wet surfaces such as dew and subsequently re-emitted when the dew evaporates (Sutton et al., 1995). This cuticular exchange is influenced by the wetness of the surface and its pH (Flechard et al., 1999; Sutton et al. 1995; Van Hove et al. 1989; Wyers & Erisman 1998).

There are a number of studies reporting measured ammonia stomatal compensation points (χ_s) for a range of species, ecosystems, nitrogen supply, age of leaves and environmental conditions. Although the mechanisms underlying changes in χ_s through the season and the day are qualitatively understood (e.g. Loubet et al. 2002; Mattsson & Schjoerring 1996), there are few studies reporting prediction of χ_s through process-based modelling, and for a limited number of ecosystems (Riedo et al. 2002).

Experimentally, two major methods have been developed for assessing the ammonia stomatal compensation point. The first method consists in calculating χ_s from measurements of the ammonia flux either in a cuvette system or by the aerodynamic gradient method and by finding at which concentration the flux is zero (Husted & Schjoerring 1995b; Fléchard and Fowler, 1998). The second method is based on the determination of the leaf apoplastic NH_4^+ concentration and pH to assess χ_s by means of extraction with successive infiltration and centrifugation of leaf segments (Husted & Schjoerring 1995a). These two measurement methods have been reported not to converge (Hill et al. 2001). Possible explanations for such differences might be: (i) spatial variability of pH and $[NH_4^+]$ in the apoplast (Hoffmann & Kosegarten 1995); (ii) possible errors in the measurement of $[NH_4^+]$ and pH in the apoplast by the infiltration/centrifugation technique due to disturbance of the ionic equilibrium between the apoplast and the adjacent leaves (Felle & Hanstein 2002); (iii) possible errors in the aerodynamic measurement technique due to underestimation of the stomatal

conductance of NH_3 (Husted & Schjoerring 1996b) or to soil exchange in cuvette and aerodynamic methods.

Hence developing process-based χ_s models would contribute in a better understanding of the processes determining plant-atmosphere ammonia exchange and its variability as a function of environmental conditions. It will also allow prediction of NH_3 fluxes on a short and long term scale to evaluate several scenarios (agricultural practices, climate change, etc.).

In this paper we present an overview of the biological processes and the existing models for plant-atmosphere ammonia exchange, and how plant and cell metabolism affect χ_s. We then discuss which biological processes should be accounted for to model χ_s and evaluate existing plant metabolism models in the prospect of using them. We will focus on agricultural type of crops in vegetative growth.

2. Compensation point and resistance approach for estimating ammonia fluxes.

At the canopy scale, the potential to emit or absorb ammonia is characterized by the canopy compensation point for NH_3, χ_c, (Sutton et al. 1995), which is defined as the atmospheric NH_3 concentration for which the flux between the surface and the atmosphere switches from emission to deposition (or vice versa) (Farquhar et al. 1980; Flechard 1998). χ_c is a composite parameter expressing the emission and deposition potential of NH_3 from/to the canopy as influenced by the different pathways, including stomatal exchange, NH_3 sorption on leaf surfaces and liberation from degrading litter material or emission from the ground.

At the leaf scale, the stomatal compensation point χ_s is the NH_3 concentration of gaseous NH_3 above the water film (apoplast) in the cell walls of the mesophyll cells (Farquhar et al. 1980). When there is no exchange with the cuticle, NH_3 emission occurs if the NH_3 atmospheric concentration is below χ_s, and conversely. Theoretically, χ_s results from the thermodynamic equilibrium between NH_3 in the gas phase and the liquid phase, and the chemical equilibrium between H^+ and NH_4^+ in the apoplast (Schjoerring 1997).

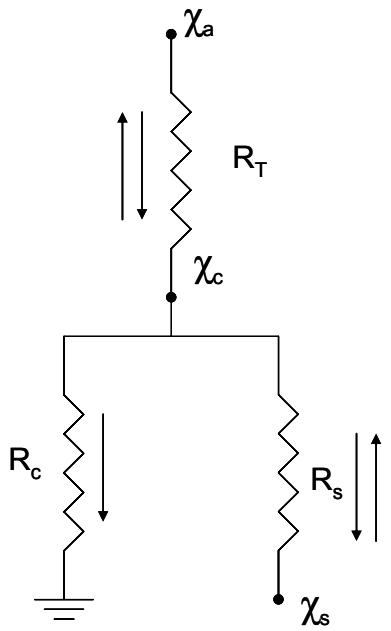

Figure 1. 1: Big leaf two-pathway resistance modal for ammonia

(see e.g. Sutton et al., 1995). The net flux of NH_3 is a result of exchange with the sub-stomatal cavity and deposition on the cuticles. χ_a is the atmospheric ammonia concentration; χ_c, and χ_s, are the ammonia canopy and stomatal compensation points, respectively. R_T is the sum of atmospheric resistances to transfer, R_w the cuticular resistance and R_s the stomatal resistance to NH_3.

The ratio of χ_s to the concentration in the apoplast $[NH_3]_{aq}$ is temperature dependent, whereas the ratio of $[NH_3]_{aq}$ to $[NH_4^+]$ is pH dependent. χ_s can be expressed, following (Schjoerring, 1997) and (Nemitz et al., 2000b), as:

$$\chi_s = \Gamma \times K_H \times K_d \quad (1)$$

where Γ is the ratio $[NH_4^+] / [H^+]$ in the apoplast, K_H is the Henry equilibrium constant and K_d the dissociation constant for NH_3. Since χ_s can be expressed as a function of $[H^+]$ and $[NH_4^+]$, it may be possible in theory to derive χ_s from measurements or modelling of $[H^+]$ and $[NH_4^+]$ in the apoplast (Husted et al. 1996; Husted et al. 2000a; Riedo et al. 2002).

Modelling surface-atmosphere NH_3 exchange has been based so far on resistance-analogy approaches (eg. Nemitz et al. 2000b; Sutton et al. 1995). These models often take as entry variable either χ_s or Γ, which are both determined empirically. To our knowledge, there is only one model developed by Riedo et al. (2002) which models the dynamics of χ_s (see description paragraph 4.2).

The early NH_3 exchange models considered only exchange through the stomata (Farquhar et al. 1980), or dry deposition onto the cuticle (Erisman et al. 1994; Hicks et al. 1987). Later, two-pathway models (Figure 1) have been introduced that differentiate between stomatal absorption / emission via the stomatal compensation point and whole canopy exchange via the canopy compensation point (Sutton et al. 1993; Sutton et al. 1995). In this model, the net ammonia flux above the canopy (F_T) is calculated by:

$$F_T = \frac{\chi_{c.} - \chi_{a(z)}}{R_{T(z)}} \quad (2)$$

Where $\chi_a(z)$ is the atmospheric ammonia concentration and $R_T(z)$ the sum of atmospheric resistances to transfer between the canopy and height z. χ_c is the canopy compensation point, which depends on the variation of χ_s, the stomatal resistance (R_s), and the cuticular resistance (R_w) (Sutton et al. 1995). To account for the difference in Γ between different growth stages and organs (reproductive organs, senescing leaves), two-layer and three-layer models have been developed (Nemitz et al. 2000b). The one-layer model has been found to reproduce rather well field conditions (Flechard et al. 1999; Sutton et al. 1998; Sutton et al. 1995; Wyers & Erisman 1998). Flechard et al.

(1999) developed a more detailed approach of the leaf water film NH_3 exchange, which accounts for chemistry and pH changes, hence improving the cuticular exchange. The main limitation of these models resides in their empirical parameterisation of Γ, which lacks reproducing the effects of the agricultural practices (nitrogen supply), plant's developmental stage (senescence, protein remobilisation) or plant's carbon and nitrogen cycle (photosynthesis, photorespiration etc.) on Γ.

3. Biological processes at the plant scale involved in the determinism of χ_s.

Leaf apoplastic and xylem ammonium concentrations have been shown to vary with nitrogen supply (NO_3^- or NH_4^+ nutrition), the plant developmental stage, photosynthesis, photorespiration, and glutamine synthetase (GS) activity (Francis et al. 1997; Husted & Schjoerring 1996; Mattsson & Schjoerring 1996; Morgan & Parton 1989; Yin et al. 1996). The ammonium concentration in the leaf apoplast is dependent on import via the xylem, and transport and assimilation into the leaf cells. Table 1 gives a range of measured NH_4^+ concentrations and pH in the apoplast, whole leaf tissue extracts and xylem. Two plant organs, leaves and roots, should be distinguished as well as transport between these two via the xylem and phloem.

3.1. Roots

The importance of roots in water and nutrient transport is well known (Baker & Wraith 1992; Van Noordwijk & de Willigen 1991; Van Noordwijk & van de Geijn 1996). This affects ammonia emission by plants, on one hand, via its effect on the plant water balance and subsequently the functioning of the stomata; on the other, via the effect on the quantity and form of N compounds transported from the soil to the plant and in the plant. The form and quantity of N applied to the soil and subsequently absorbed by the roots changes not only the apoplastic ammonium concentrations but also the pH. Most species utilize NO_3^- or NO_3^- and NH_4^+ in combination without significant effect on accumulation of nitrogen or dry matter (Henry & Raper 1989; Peer & Leeson 1985; Vessey et al. 1990); few species are able to utilize NH_4^+ as soul nitrogen source. Uptake of NH_4^+ and NO_3^- by the roots seems to be regulated by carbohydrate flux from the shoots (Lim et al., 1990) or possibly by amino-nitrogen cycling between the roots and

the shoots (Cooper & Clarkson 1989). The proportionality of uptake between NH_4^+ and NO_3^- varies with: (i) external conditions surrounding the roots such as ratio of ions and pH (Raper et al., 1991; Vessey et al., 1990), (ii) temperature (Macduff & Wild, 1989), (iii) energy availability within the root (Champigny & Talouizte, 1986), and (iv) by distinction in the organ (root or shoot) of assimilation of the N source (Chaillou et al., 1994). It was clearly established that NO_3^- absorption in the roots depends on two transports systems and varies whether the plants were grown in the presence (induced) or absence (non-induced) of nitrate (Forde, 2002). It is currently assumed that all NH_4^+ is assimilated in the roots prior to transport to the shoots as amino acids (Fentem et al. 1983; Givan 1979; Glass & Siddiqi 1995). However concentrations of NH_4^+ of 3–8 mM in cytoplasm of maize root cells (Lee & Ratcliffe 1991), up to 40 mM in the cytoplasm of rice and spruce roots (Kronzucker et al. 1995; Wang et al. 1993), and up to 2 mM in xylem sap of maize and barley plants, (Cramer & Lewis 1993; Mattsson & Schjoerring 1996) were measured indicating that at least a fraction of NH_4^+ might be transported prior to assimilation. Nevertheless, NO_3^- once absorbed by the roots can either be reduced to NH_4^+ in the root cells, stored in the root cells vacuoles, exported via the xylem to the leaves or expelled to the outside of the root (Beevers & Hageman 1969; Pate 1980). Mattsson and Schjoerring, (1996) showed that χ_s generally increases with increasing N supply, and preferentially with NH_4^+ supply to the roots for several plant species (Table 1). However, the relationship between the amount of N absorbed by the roots and the compensation point is not so simple because of a possible masking effect due to apoplastic pH change (Olsen et al., 1995). For *Bromus erectus* (Huds.) for example, NH_4^+ supply, caused an increase in apoplastic pH of 0.24 units in comparison to NO_3^- supply, whereas in *Arrhenatherum elatius* (L.), NH_4^+ uptake was associated with a decline in apoplastic pH (Hanstein & Felle, 1999). Apoplast acidification was also observed with NH_4^+ based nutrition in sunflower (Hoffmann et al., 1992), soybean (Kosegarten & Englisch, 1994) and barley (Mattsson et al., 1998). The apoplastic NH_4^+ and H^+ concentrations usually change within a few hours following a step change in the supply to the roots (Mattsson & Schjoerring 2002). However, the effect of this step change is less known on the mid-term, although for grassland it may be limited to a few days (Loubet et al., 2002).

3.2. Xylem

By definition, the xylem sap is considered to be part of the plant apoplast (Canny 1995). One should distinguish between uptake from the soil solution into the root symplast, subsequent release into the xylem apoplast, and transport to the aerial organs (Engels & Marschner 1992; Pitman 1972; Poirier et al. 1991). There exists a large spatial variance in ion concentrations inside the xylem. According to plants species, time of day, age, location of sampling and nutritional status, the ionic composition of xylem sap varies (Berger et al. 1994; Guzman et al. 1995; Prima-Putra & Botton 1998; Schurr & Schulze 1995). Concentrations of NH_4^+ of up to 2 mM have been measured even under NO_3^- nutrition (Cramer & Lewis 1993; Husted & Schjoerring 1995a) although they are not very common (Glass & Siddiqi, 1995). It has been shown that the NH_4^+ concentration in the xylem sap influences the leaf apoplastic NH_4^+ concentration and therefore the ammonia compensation point (Schjoerring et al., 2002).

3.3. Phloem

By definition the phloem is the tissue forming part of the plant vascular system, responsible for the transport of organic material, from the leaves to the rest of the plant. Phloem differs from xylem in that it is more concentrated in solutes, has a higher pH and contains a lot of important plant metabolites. While movement of water and minerals through the xylem is driven by the gradient in water potential between root, xylem and leaves (negative relative pressures), most of the time, movement through the phloem is driven by positive hydrostatic pressures. Organic molecules such as sugars, amino acids, certain hormones, and even messenger RNAs are transported in the phloem through sieve tube elements (Hayashi et al. 2000; Van Bel 2003). The translocation of carbon and nitrogen assimilates can indirectly influence χ_s via the feedback on photosynthesis (Krapp et al. 1993; Van Oosten & Besford 1995) and ammonia assimilation (Foyer & Noctor 2002; Foyer et al. 2003; Morot-Gaudry et al. 2001a) but also via the remobilization of proteins during leaf senescence. χ_s estimated from measurements of apoplastic concentrations in senescing leaves with high-nitrogen status reached 6–8 nmol mol^{-1} (Mattsson & Schjoerring 2003).

4. Metabolic processes at the leaf scale involved in the determinism of χ_s.

Leaf mesophyll cells are the major site of production of NH_4^+ through photorespiration and assimilation of NH_4^+ throught the GS/GOGAT cycle. Yin et al. (1996) have shown experimentally, using a pH sensitive fluorescence method, that atmospheric ammonia can be assimilated by the leaves if photosynthesis or photorespiration provide the necessary acceptor molecules. Moreover, the exchange of NH_4^+ between the apoplast and the leaf mesophyll cells probably influences apoplastic ammonium concentration. Describing and quantifying cell apoplast exchange and the main sources and sinks of cellular NH_4^+ are essential steps in understanding the mechanisms behind the determination of χ_s.

4.1. Cell-apoplast exchange of NH_4^+

Table 1, shows that measurements of NH_4^+ concentrations in stem xylem sap and in leaf tissue are 6 and 25 times higher than in the leaf apoplastic solution, respectively (Finnemann & Schjoerring 1999). This reveals the presence of an active transport system of NH_4^+ from the apoplast to the leaf cells. Lower NH_4^+ concentrations in the leaf tissue as compared to the stem xylem sap may be attributed either to GS activity in the leaf or to temporary storage in the vacuole.

As for all ions, NH_4^+ is transported to the cell through several pathways:

- Passive diffusion through the cytoplasmic membrane according to the electrochemical gradient on both sides.
- Active transport against an electrochemical gradient, where energy in the form of ATP (adenosine triphosphate) is necessary by more or less specific transporters.

The fact that ammonium is a weak acid implies that its transport into different cellular compartments depends not only on the electrochemical gradient but also on the pH difference across this membrane. High and low affinity systems are responsible for the transport of ammonium to the cytosol (Kronzucker et al. 1996; Wang et al. 1994). However, due to the relatively high concentrations of ammonium found in oil seed rape apoplast, the low affinity system is thought to be privileged in this plant (Nielsen & Schjoerring 1998). The relative contribution of each of these systems to NH_4^+ uptake in vivo is not well determined (Williams & Miller 2001). Pearson et al., (2002) showed

using labelled ^{15}N-NH_4^+ that a high affinity ammonium transporter is present in isolated mesophyll protoplasts of *Brassica napus* L. and that this transporter had physiological similarities with the high affinity NH_4^+ transporters detected in *Arabidopsis thaliana* L. (AtAMT1;3). They suggest that this protein may be regulated by mechanisms similar to GS2 in leaves and that it senses the N status of the cell.

Ammonium efflux from the cytosol to the apoplast is of particular importance when considering the effect of plant metabolism on apoplastic NH_4^+ concentrations. It is still not clear whether this efflux is mediated by membrane transporters or largely accounted for by NH_3 diffusion. Theoretically, a significant proportion of the total NH_3 / NH_4^+ is in the form of NH_3 due to the relatively high pH in the cytosol, and could hence freely diffuse across membranes (von Wiren et al. 2000). Nielsen and Schjoerring (1998) measured that the net uptake of NH_4^+ into the leaf cells of oil seed rape responded linearly to increasing NH_4^+ concentrations between 2 and 10 mM. Measurements of ammonium transport kinetics using ^{15}N-NH_4^+ into isolated protoplasts of *Brassica napus* L. revealed that at external concentrations above 100 µM, linear kinetics is observed. Whereas at concentrations below 100 µM, the transport followed Michaelis-Mentent kinetics with measured Km of 47 µM and Vmax of 20 ng min^{-1} per 3 x 105 cells (Pearson et al., 2002). Ammonium is considered to be transported to the vacuole by passive diffusion. The high concentrations found in the vacuole are a result of a more acidic vacuolar pH (Howitt & Udvardi 2000).

4.2. Cellular NH_4^+

NH_4^+ is constantly generated in the leaf cells by various processes, the most important being nitrate reduction, photorespiration, protein turnover, and lignin biosynthesis (Joy 1988; Leegood et al. 1995) (Figure 2). Assimilation of NH_4^+ in leaf cells mainly occurs via the Glutamine synthetase (GS), Glutamate synthase (GOGAT) cycle (Leegood et al. 1995).

4.2.1. Nitrate reduction

Since ammonium is the reduced N form used by plants for assimilation into amino acids and proteins, nitrate is converted to ammonium by the sequential action of the cytosolic nitrate reductase (NR) and the chloroplastic nitrite reductase (NiR) (Lea & Ireland 1999). Nitrite reductase has a very high affinity for nitrite and therefore prevents it from

accumulating to toxic amounts. The capacity for nitrite reduction in the chloroplasts is much higher than that for nitrate reduction in the cytosol. Therefore all nitrite formed by nitrate reductase can be totally converted to ammonia. This nitrate / nitrite reduction can occur in the roots or in the leaves. The latter would release ammonium in the leaf. The amount of nitrate reduced in the roots changes considerably with plant species (Pate 1973), leading to different potential for ammonium release in leaves. Nitrate reductase activity is induced by nitrate (Wang et al. 2003) and is primarily regulated by light, nitrate, glutamine and sugars among other factors (Campbell 1999; Stitt et al. 2002).

4.2.2. GS/GOGAT cycle

In superior plants, Glutamate synthesis, using the 2 enzymes GS and GOGAT, is the major pathway for ammonium incorporation in metabolism (Lea 1985; Miflin & Lea 1982). Other pathways for ammonium assimilation exist. These routes use the following enzymes: alanine deshydrogenase, aspartate deshydrogenase, aspartase and asparagine synthetase (Morot-Gaudry, 1997).

The role of GS in NH_4^+ assimilation has been clearly demonstrated by experimental studies using methionine sulfomine (MSO), which is a GS inhibitor (Mattsson & Schjoerring 1996; Olsen et al. 1995; Pearson et al. 1998).

Inorganic nitrogen, in the form of ammonia, is assimilated via this GS/GOGAT cycle into the organic nitrogen compounds glutamine and glutamate, which are the nitrogen donors in essentially all biosynthetic reactions involving nitrogen (e.g., amino acids, nucleic acids, and chlorophyll). Primary nitrogen assimilation requires cofactors, reducing equivalents, and carbon skeletons generated during photosynthesis (Lea & Miflin 1974).

In addition to its major role in primary nitrogen assimilation, the GS/GOGAT cycle also plays a crucial role in re-assimilating the large amount of ammonia released during photorespiration as shown in Figure 2 (Kendall et al. 1986; Somerville & Ogren 1980) as well as re-assimilation of large amounts of ammonium ions produced during leaf senescence by protein hydrolysis (Hortensteiner & Feller 2002). Glutamine synthetase exists as multiple isoforms that are either cytosolic (GS1) or plastidic (GS2) (McNally et al. 1983). In leaves of C3 plants, GS1 is expressed in phloem cells and it appears to be involved in the generation of glutamine for nitrogen transport (Kamachi et al. 1992; Pereira et al. 1992). GS2 is expressed in photosynthetic cells where its function is to

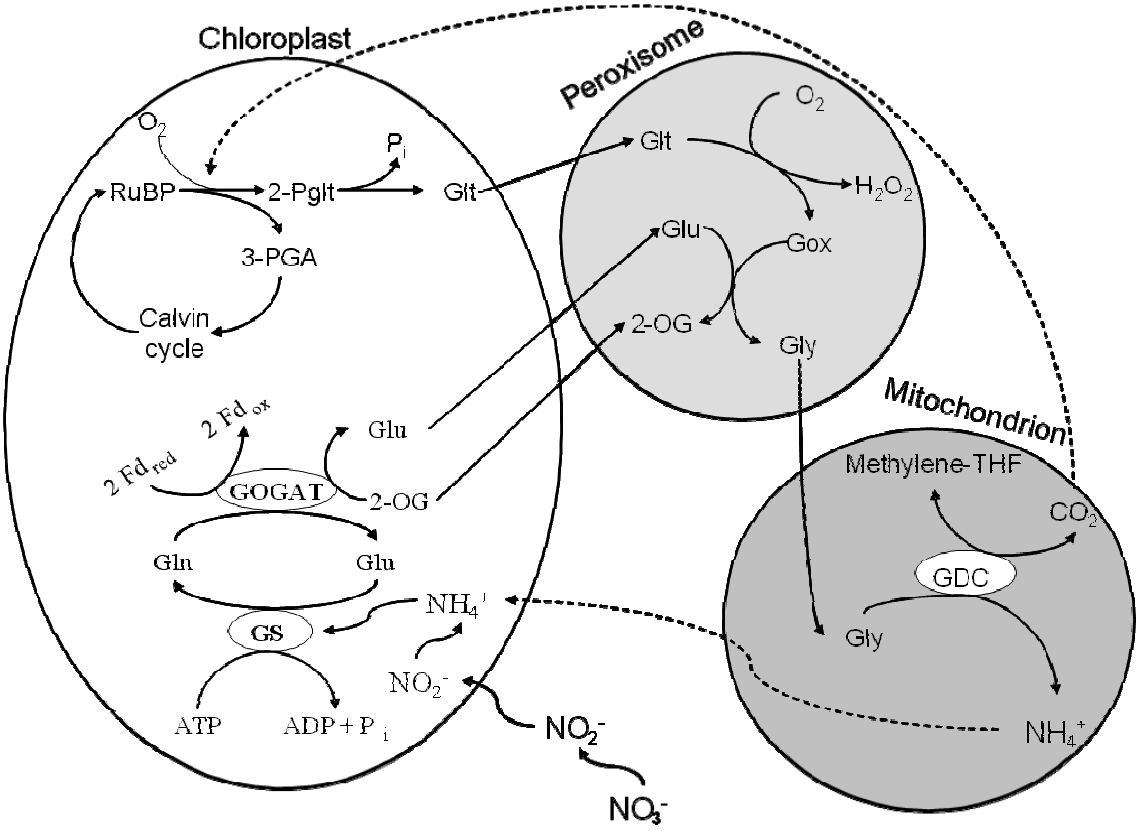

Figure 1.2: Schematic representation of the photorespiratory nitrogen cycle proposed by Keys et al. (1978).

The oxygenation of Ribulose biphosphate (RuBP) leads to the production of 2 phosphoglycolate (2-Pglt) and subsequently to glycolate (Glt). Glycolate is oxidized to glyoxylate (Gox) which is then transformed into glycine (Gly) in the peroxisomes utilizing glutamate (Glu) and forming 2 oxoglutarate (2-OG). Subsequently, glycine is oxidized to ammonia, serine and CO_2 in the mitochondria by glutamate decarboxylase (GDC). The ammonia is reassimilated via the glutamine synthétase/glutamate synthase (GS/GOGAT) pathway generating glutamate.

assimilate ammonium produced from nitrate reduction and photorespiration (Cren & Hirel 1999; Wallsgrove et al. 1987).

For the GS/GOGAT cycle to function there is a strict requirement for N metabolism to interact with C metabolism. Indeed, the GS/GOGAT cycle is considered as one of the main crossroads between the plant's N and C metabolism, this is why there are complex interactions with several aspects of these metabolisms. With respect to N metabolism, the GS/GOGAT cycle is influenced by: the storage and remobilisation of nitrate in different parts of the plant via its action on the induction of the GS gene (Wang et al. 2000), photorespiration since it releases ammonium ions to be recycled (Hirel & Lea 2001), the different pathways of amino-acid biosynthesis and the becoming of these amino acids (Morot-Gaudry et al. 2001b). The proportion of fixed carbon required by NH_4^+ assimilation varies with the plant's developmental stage, N availability and the nature of the products (Lewis et al. 2000).

As for C metabolism, its influence is via the necessary reactions of photosynthesis and respiration to provide the energy, C-skeletons and reductant in the form of 2-oxoglutarate (α-KG) and reduced ferredoxin (Fd_{red}) or NADH, respectively necessary for the various reactions of the GS/GOGAT cycle (Lea et al. 1992; Rhodes et al. 1980).

It is worth noting that GS has a very high affinity for ammonia (K_m = 3-5 μM) and that its functions are optimal for NH_x concentration ranging from 12 to 22 μM (Farquhar et al. 1983; Stewart & Rhodes 1977); whereas K_m values for GOGAT range between 10 and 20 μM (Howitt & Udvardi 2000).

4.2.3. Photorespiration and Photosynthesis

Ammonia assimilation by plants requires carbon skeletons derived from photosynthesized carbohydrates for the synthesis of amino acids (Huppe & Turpin 1994). Furthermore, all metabolic pathways involved in plant N assimilation ranging from nitrate reduction to the GS/GOGAT cycle require energy in the form of ATP. This ATP originates form the breaking down of sugars produced by photosynthesis. In C3 plants, RubisCO (Ribulose 1,5 bisphosphate carboxylase/oxygénase) fixes CO_2 to form ribulose biphosphate (RubP) using light and water. RubisCO also possesses an oxygenase activity, competitor of the carboxylase activity, which permits the oxidization of RubP by oxygen. This process called photorespiration is at the origin of a

biochemical cycle where NH_4^+ is released during glycine decarboxylation in the mitochondrion (Keys et al. 1978).

The partition between carboxylation and oxygenation of RubP is a function of the concentrations of CO_2 and O_2 and temperature in the mesophyll cells (Leegood et al. 1995).

The rate of production of NH_4^+ by photorespiration is the most important release of NH_x in the leaves; it is known to be 10 or more times that of nitrate / nitrite reduction (Keys et al. 1978; Wallsgrove et al. 1983). However, experiments done by Husted et al. (2002) proved that photorespiration does not exert a direct control on χ_s since all the NH_4^+ produced by photorespiration is re-assimilated through a privileged pathway.

4.3. Apoplastic pH

By rearranging eq. (1), it can be seen that χ_s is an exponential function of the pH. The apoplastic pH is thought to be partly regulated by the N nutrition of a species but merely by the regulation of the intracellular pH. Intracellular pH has to be maintained within a narrow range (7.2-7.5) to allow the functioning of all plant metabolisms (Felle, 2001). This regulation involves net H^+ fluxes across the cytoplasmic membrane therefore leading to apoplastic pH changes.

Sub-stomatal apoplastic pH is sensitive to a variety of factors. This is based on two properties: the low passive buffer capacity and the thin aqueous film forming the apoplast. The consequence is that small fluctuations in membrane transport, gas exchange and intercellular exchange of matter influence apoplastic pH (Felle & Hanstein 2002).

The effect of N nutrition on leaf apoplastic pH is still not clear. However, plant species relying on NO_3^- nutrition tend to have high apoplastic pH since nitrogen is assimilated in the shoots. Plants relying on a mixed source of N (NO_3^-, NH_4^+ or organic-N), and which are more likely to favour root rather than shoot assimilation tend to have relatively low apoplastic pH (Pearson et al. 1998).

The acquisition of gas-phase NH_3 by plant shoots will lead to two opposing effects on the acid-base balance of the plant: the dissolution of atmospheric NH_3 in shoot water should increase the shoot pH (production of OH^-), while subsequent metabolism to amino acids should decrease the shoot pH (generation of H^+). Different rates of these

processes would lead to an acid-base imbalance with consequences for the ionic balance and growth of the plant, also depending on the buffer capacity of the apoplast.

5. Priority processes to account for in modelling the ammonia stomatal compensation point.

A number of plant growth and ecosystem functioning models have been developed recently. These models try to explain the plant functioning at different time and space scales. They could make it possible to simulate the variables that determine to a large extent χ_s. Coupling an existing N dynamics model to a resistive scheme for ammonia exchange would allow estimation of the compensation point and ammonia variation with time and external conditions (practices, climate …). However, given the complexity and the variety of the biological processes involved in the determination of the ammonia stomatal compensation point, there is a need to simplify and classify these mechanisms prior to modelling this compensation point. We will discuss the main processes to consider in a realistic modelling approach as well as the advantages and drawbacks of three examples of models.

5.1. Main processes to consider in the determinism of χ_s for a plant in vegetative growth.

Based on the previous literature review, the main processes to consider for building a model for the stomatal ammonia compensation point at the leaf scale are (Figure 3):

(1) NH_x and NO_3^- transport to the leaves via the xylem flow, which is both modulated by evapotranspiration and mineral nitrogen export from the roots. As seen in previous sections, incoming NH_4^+ and NO_3^- in the leaf is modulated by availability of mineral nitrogen in the soil, the root absorption, and the water absorption. In a first order approach, the income of organic nitrogen may be neglected for modelling the compensation point. The diurnal cycle of NH_4^+ and NO_3^- income to the leaves may also be taken into account.

(2) Acid–base equilibrium of NH_3 and NH_4^+ in each compartment (vacuole, cytoplasm and apoplast), which is highly dependent upon the pH. However, the pH tends to be regulated in the cell and can be quite constant there. Nevertheless, in the apoplast, there may be a gradient of pH, which may play a role in the determination of χ_s.

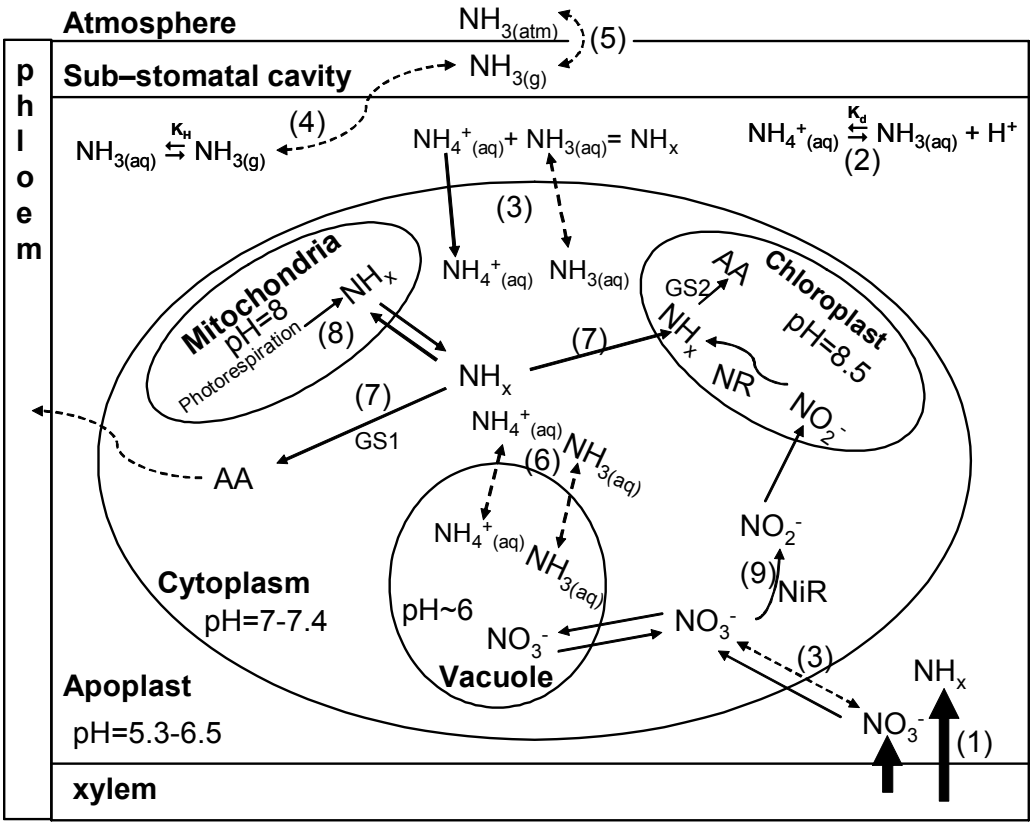

Figure 1.3: Schematic view of the major metabolic processes at the cell level that should be taken into account in the determination of the apoplastic NH_4^+ concentration and the compensation point χ_s

(1) Xylem transfer of NH_x and NO_3^- ; (2) acid/base equilibrium of NH_4^+/NH_3 in the apoplast: (3) and (6) transport process to account for the exchange of NH_3 and NH_4^+ between the cell and the apoplast and the vacuole and the cell; (4) thermodynamic equilibrium of NH_3 between the apoplast and the air in the substomatal cavity; (5) stomatal exchange of NH_3; (7) NH_x assimilation via the GS/GOGAT cycle; (8) photorespiratory production of NH_x; (9) nitrate reduction.

(3) NH_x and NO_3^- exchange between the apoplast and the cytoplasm. NH_4^+ and NO_3^- can be considered as actively transported from the apoplast to the cytoplasm whereas NH_3 may diffuse across the cytoplasmic membrane due to a concentration gradient. The active transport could be modelled with a Michaelis–Menten kinetic reaction. Parameters for transport mechanisms are currently not well known, such as the conductance to diffusion of NH_3 across the membranes.

(4) Thermodynamic equilibrium between the aqueous NH_3 in the apoplast and the gasesous NH_3 in the substomatal cavity, which is a strong function of leaf temperature, and is quite well known.

(5) Stomatal exchange of NH_3 which is a diffusion process dependent on (i) the stomatal resistance controlled by water transfer in the plant and (ii) the concentration gradient of NH_3 between the substomatal cavity and the atmosphere.

(6) Exchange of NH_x (NH_4^+ and NH_3) and NO_3^- between the cytoplasm and the vacuole, which may be considered as a diffusive process dependent on the concentration gradient and the tonoplast resistance to transfer, which is however poorly characterised.

(7) NH_x assimilation. The GS/GOGAT cycle is considered as the major route for NH_x assimilation. For simplicity, cytoplasmic and chloroplastic GS could be considered as a single assimilation process. The rate of NH_x assimilation is dependent on NH_x and organic carbon concentrations in the cytoplasm and could be modelled with a single velocity.

(8) NH_x release in the cytoplasm via photorespiration, which may be considered in a simple way as proportional to photosynthesis.

(9) NH_x production in the cytoplasm by the reduction of nitrate, which can be simplified by ignoring the nitrite stage. Nitrate reduction is currently considered as dependent on $[NO_3^-]$ in the cytoplasm and may be modelled with a Michaelis-Menten process.

5.2. Existing dynamic whole plant N models

Riedo et al. (2002) coupled a two-layer resistance model of ammonia exchange (Nemitz et al. 2001) with a dynamic grassland model: PaSim (Riedo et al. 1998) to simulate the change in χ_s with time, and agricultural practices.

PaSim simulates root and shoot dry matter production. In order to introduce the compensation point in this model, the nitrogen pool was divided into structural and non-structural (symplastic) N to facilitate coupling with the apoplastic compartment. Exchange with the apoplast occurs via the symplastic pool through passive diffusion. Incorporation of apoplastic N into the symplast is modelled as an active flux. Uptake from the roots is incorporated directly into the apoplastic N pool.

The model has been parameterised and tested; it was shown to quantitatively represent the effects of grazing and fertilisation on χ_s. Separating the plant nitrogen pools into symplastic and structural N is an interesting approach to be able to couple with an apoplastic N pool. One of the model's drawbacks is that it does not differentiate between different N sources and was found to underestimate peak emissions. Another gap is that it does not really link to photosynthesis and to water transport inside the plant. Moreover, it was designed for grassland ecosystems and is not well adapted to agricultural crops.

Dewar (1993) developed a model that has the advantage of linking carbon, nitrogen and water flow, an essential point in simulating evapotranspiration and stomatal opening. This model describes: the root–shoot partitioning, the transport of water, reactive nitrogen and carbon through the xylem and the phloem, as well as plant growth. It is divided in root and shoot structural dry matter. The phloem transports organic C and N pools, whereas the xylem transports the inorganic N and C pools. The xylem water potential determines the water status of the plant. The transport rate of phloem from the shoot to the root is proportional to the difference in C substrate or N substrate between the roots and the shoots, whereas the transport rate of N sources through the xylem from the root to the shoot is assumed to be instantaneous; so is the rate of transfer from xylem to phloem. This model deals with plants in vegetative growth and for time steps of the order of 30 minutes which is of great interest for coupling with atmosphere-vegetation exchange models. It describes a simple approach for coupling water and nutrient transport and partitioning at a whole plant level. One of its major features is that it allows the existence of Nitrogen and Carbon gradients between the roots and the shoots (Andrews et al. 2001).

This modelling approach is particularly interesting for modelling whole plant transfers and integrating root absorption of N in determining χ_s. On the other hand, the Dewar

(1993) model has been criticised for not differentiating between N supplies (nitrate/ammonium) (Ericsson 1995; Marschner et al. 1996) and therefore not distinguishing between the sites of N uptake and of N assimilation. Another drawback for modelling χ_s is that it does not have an apoplastic N pool.

The model of Bijlsma and Lambers (2000) and of Bijlsma et al. (2000) was originally designed to asses the interaction between NO_3^- and NH_4^+ on different species' ecology. It has the advantage of differentiating between nitrate and ammonium uptake to the roots, as well as having general non species-specific parameters combined with species-specific parameters. Uptake of ammonium and nitrate to the roots are calculated according to a Michaelis-Menten type equation pondered by root fresh weight, a species specific potential and a substrate status potential which is used as a feed back signal that regulates influx capacities. This signal is considered as a dynamic parameter if the model is coupled to carbon metabolism. Nitrate assimilation in roots is also modelled according to Michaelis-Menten kinetics, whereas nitrate assimilation in the shoots is considered to be proportional to the nitrate translocation rate. Ammonium assimilation in roots is proportional to NH_4^+ uptake and NO_3^- reduction rate. Finally the translocation rate of nitrate form the root to the shoots is considered to be proportional to root NO_3^- concentration and a translocation constant.

This model may be interesting for modelling χ_s as it integrates a mechanistic approach of metabolism accounting for nitrate reduction and ammonia assimilation. The model works on a daily basis and does not account well for water transport and stomatal aperture, which are major drawbacks for modelling atmosphere-surface NH_3 exchange. Another gap in this approach is the inexistence of the apoplastic compartment.

6. Conclusion

Integrating plant metabolism in modelling plant atmosphere ammonia exchange requires the consideration of many biochemical and transport processes. On one hand, whole plant transfers of water and solutes through the roots passing through the xylem and the stomata should be considered. On the other hand, major production and assimilation pathways of ammonium through the different cellular metabolic processes should also be accounted for. This poses a major challenge and implies elaborating a simplified approach. Based on a literature review of the processes and the existing

nitrogen transfer and metabolism models, we have identified a limited number of processes required for modelling χ_s in growing leaves.

Among these processes, the transport mechanisms across the tonoplast and the cell membrane of mesophyll cells are the most uncertain and should be better characterised. The validation of such a model would moreover require to measure in parallel the concentration of NH_x, NO_3^- and the pH of the apoplast, the xylem, the cytoplasm and the vacuole as well as the flows of water (evapotranspiration) and the exchange of NH_3 between the atmosphere and the plant.

In the long term this type of model can offer a tool to evaluate the impact of agricultural practices, especially fertilisation and harvesting, on NH_3 concentrations in the atmosphere at the local and regional scale. Moreover, these models should also help modelling NH_3 deposition around hot-spots (animal housing, manure stores, etc.) (Loubet et al. 2007; Loubet & Cellier 2001; Loubet et al. 2006).

Tableau 1.1: Free NH4+ and pH measured in the apoplast, xylem and tissue of leaves

Species	Xylem	Apoplast		Leaf tissue NH_4^+	Remark	Reference
	NH_4^+ (µM)	pH	NH_4^+ (µM)			
Grassland						
Bromus erectus		6.1	240		~ 50 kg $N(NO_3^-)$ ha^{-1}	(Hanstein et al. 1999)
Bromus erectus		6.2	200		~ 50 kg $N(NO_3^- + NH_4^+)$ ha^{-1}	(Hanstein et al. 1999)
Bromus erectus		6.4 ± 0.1	33 ± 16	0.7 (µmol g^{-1} f.w.)	3 mM NO_3^-	(Mattsson & Schjoerring 2002)
Bromus erectus		6.6 ± 0.1	45 ± 19	1.3 (µmol g^{-1} f.w.)	3 mM NH_4^+	(Mattsson & Schjoerring 2002)
Bromus erectus		6.5 ± 0.1	369 ± 81	4.3 (µmol g^{-1} f.w.)	6 mM NH_4^+	(Mattsson and Schjoerring, 2002)
Lolium perenne		6.7 ± 0.1	21 ± 5	0.5 (µmol g^{-1} f.w.)	3 mM NO_3^-	(Mattsson and Schjoerring, 2002)
Lolium perenne		6.2 ± 0.1	49 ± 12	0.8 (µmol g^{-1} f.w.)	3 mM NH_4^+	(Mattsson and Schjoerring, 2002)
Lolium perenne		6.3 ± 0.1	170 ± 38	2.9 (µmol g^{-1} f.w.)	6 mM NH_4^+	(Mattsson and Schjoerring, 2002)
Lolium perenne		5.41	93	637 (µM)	grazed grassland	(Loubet et al. 2002)
Lolium perenne		6.4	222	1676 (µM)	After fertilisation	(Loubet et al., 2002)
Arrhenatherrum elatius		5.7	260		~ 50 kg $N(NO_3^- + NH_4^+)$ ha^{-1}	(Hanstein et al., 1999)
Arrhenatherrum elatius		5.9	300		~ 50 kg $N(NO_3^-)$ ha^{-1}	(Hanstein et al., 1999)
Arable crops						
Brassica napus		6.3	350	4 (µmol g^{-1} f.w.)	Lower leaves	(Husted & Schjoerring 1996)
Brassica napus			-	20 (µmol g^{-1} f.w.)	Senescing leaves	(Husted & Schjoerring 1996)
Brassica napus		5.6	250	3 (µmol g^{-1} f.w.)	Upper leaves	(Husted & Schjoerring 1996)
Brassica napus	550		-	300 (µM)	10mM NO_3^-	(Finnemann & Schjoerring 1999)

Species	Xylem	Apoplast		Leaf tissue NH_4^+	Remark	Reference
	NH_4^+ (µM)	pH	NH_4^+ (µM)			
Arable crops						
Brassica napus	700		400	440 (µM)	3mM NO_3^-	(Finnemann & Schjoerring 1999)
Brassica napus	1360		-	500 (µM)	3mM NH_4^+	(Finnemann & Schjoerring 1999)
Brassica napus	350		100	600 (µM)	1.5mM NO_3^-	(Schjoerring et al. 2002)
Brassica napus	650		175	750 (µM)	6mM NO_3^-	(Schjoerring et al. 2002)
Brassica napus	500		150	800 (µM)	3mM NO_3^-	(Schjoerring et al. 2002)
Brassica napus	5000		200	1200 (µM)	10mM NH_4^+	(Finnemann & Schjoerring 1999)
Brassica napus	410		40	300 (µM)	0N	(Finnemann & Schjoerring 1999)
Brassica napus.		5.8	1000		High N	(Husted & Schjoerring 1995a)
Hordeum vulgare				6 (µmol g^{-1} f.w.)		(Schjoerring et al. 1993)
Hordeum vulgare	2200			19 (µmol g^{-1} f.w.)	2 mM NH_4^+	(Mattsson & Schjoerring 1996)
Hordeum vulgare	150		40	300 (µM)	0	(Mattsson et al. 1998)
Hordeum vulgare	350		380	318 (µM)	1mM NH_4^+	(Mattsson et al. 1998)
Hordeum vulgare	420		1040	651 (µM)	2.5mM NH_4^+	(Mattsson et al. 1998)
Hordeum vulgare	2900		2280	1182 (µM)	10mM NH_4^+	(Mattsson et al. 1998)
Hordeum vulgare	1000		1900	1195 (µM)	5mM NH_4^+	(Mattsson et al. 1998)
Hordeum vulgare	120		50	245 (µM)	0.5mM NH_4^+	(Mattsson et al. 1998)
Hordeum vulgare			100 fold inc.	40 (µmol g^{-1} f.w.)	with MSO treatment	(Pearson et al. 1998)
Hordeum vulgare				4 (µmol g^{-1} f.w.)	senescing leaves	(Schjoerring et al. 1993)
Hordeum vulgare	900			12 (µmol g^{-1} f.w.)	2mM NO_3^-	(Mattsson & Schjoerring 1996)

Hordeum vulgare		6.8	Low	0.8 (µmol g^{-1} f.w.)		(Pearson et al. 1998)
Other						
Oryza sativa (roots cytoplasm)				0.2 (µmol g^{-1} f.w.)	low NH_4^+	(Wang et al. 1993)
Oryza sativa (roots cytoplasm)				1.9 (µmol g^{-1} f.w.)	high NH_4^+	(Wang et al., 1993)
Oryza sativa (roots tissue)				2.4 (µmol g^{-1} f.w.)	low NH_4^+	(Wang et al., 1993)
Oryza sativa (roots tissue)				6.8 (µmol g^{-1} f.w.)	high NH_4^+	(Wang et al., 1993)
Oryza sativa (roots vacuole)				2.2 (µmol g^{-1} f.w.)	low NH_4^+	(Wang et al., 1993)
Oryza sativa (roots vacuole)				4.9 (µmol g^{-1} f.w.)	high NH_4^+	(Wang et al., 1993)
Lycopersicon esculentum	1400		150	1200 (µM)	6mM NO_3^-	(Schjoerring et al. 2002)
Lycopersicon esculentum	200		100	1300 (µM)	1.5mM NO_3^-	(Schjoerring et al. 2002)
Lycopersicon esculentum	900		150	950 (µM)	3mM NO_3^-	(Schjoerring et al. 2002)
Wild						
Mercurialis perennis		6.2-6.3	Low	0.08 (µmol g^{-1} f.w.)		(Pearson et al., 1998)
Mercurialis perennis		6.2-6.3	10 fold inc.	10 (µmol g^{-1} f.w.)	with MSO treatement	(Pearson et al., 1998)
Rubus fruticosus		5.5-5.8		0.1 (µmol g^{-1} f.w.)		(Pearson et al., 1998)
Rubus fruticosus				15 (µmol g^{-1} f.w.)	with MSO treatement	(Pearson et al., 1998)
Trientalis europaea		5		0.1 (µmol g^{-1} f.w.)		(Pearson et al., 1998)
Trientalis europaea				9.5 (µmol g^{-1} f.w.)	with MSO treatement	(Pearson et al., 1998)

Apoplast concentrations were measured by using the infiltration/centrifugation technique (Husted & Schjoerring 1995a) except for Pearson et al. (1998)

Chapitre 2 :

Variabilité expérimentale du point de compensation stomatique de l'ammoniac

« L'important, c'est de savoir ce qu'il faut observer. »

Edgar Allan Poe (Romancier : 1809-1949)

L'une des prinicpales problématiques liées au point de compensation stomatique de l'ammoniac reste la quantification de celui-ci en fonction des facteurs du milieu, des pratiques culturales et pour différents types de cultures. Il existe actuellement deux méthodes permettant le calcul du point de compensation à partir de mesures. La première méthode consiste à extraire l'apoplasme foliaire en infiltrant un liquide à l'intérieur des cavités sous stomatiques à travers une série de pression/dépression puis à extraire le liquide infiltré mélangé à l'apoplasme en centrifugant la feuille. On mesure ensuite la concentration en ions ammonium et le pH du liquide extrait. Ceci nous permet de calculer Gamma (rapport entre les concentration en NH_4^+ et en H^+) et de déduire χ_s en mulitpliant par les constantes de Henry et de dissociation. Cette méthode permet l'accès à une valeur moyenne du contenu en ions NH_4^+de l'apoplasme et donc au point de compensation stomatique de la feuille. Cependant elle ne permet pas l'accés à la variabilité temporelle et spatiale des concentrations dans l'apoplasme ; or nous savons qu'il existe un gradient de concentration à la fois intra-foliaire et inter-foliaire. De plus cette méthode est critiquée car elle perturbe fortement le contenu apoplasmique lors de l'infiltration de liquide et pourrait provoquer la contamination de cette apoplasme par du contenu cellulaire lors de la centrifugation. La seconde méthode consiste à déduire le point de compensation du couvert en utilisant des mesures de flux d'ammoniac dans une chambre. Le point de compensation est la concentration dans la chambre à laquelle le flux mesuré est nul. Cette méthode nous permet d'intégrer la variabilité intra et inter-foliaire du point de compensation et nous permet également de suivre sa variation au cours du temps. Cependant, comme on mesure le point de compensation du couvert il faut émettre certaines hypothèses pour calculer le point de compensation stomatique surtout en ce qui concerne le flux cuticulaire. D'autre part, les analyseurs d'ammoniac disponible et la nature chimique de l'ammoniac ne nous permettent pas actuellement d'avoir une précision dans les basses concentrations et d'avoir un seuil de détection fiable. On est donc obligé d'avoir plusieurs plantes dans la chambre et des alimentations azotées fortes.

A. Mesures de concentrations

La mesure de la concentration en ions NH_4^+ en milieu liquide est nécessaire pour analyser le contenu en solutions de sols, d'extrait de matières végétale (xylème, apoplasme, feuilles entières,..) mais aussi de solution ayant servi pour piéger l'ammoniac atmosphérique. L'une des principales contraintes de ces mesures est d'avoir une méthode sélective à l'ammoniac car l'azote réduit sous forme d'amine peut intervenir dans les mesures et fausser les résultats

Figure 3 : Chambre de mesures de flux d'ammoniac avec plantes de colza.

surtout en utilisant la méthode très répandue de mesure par colorimétrie. Husted et al. (2000) évalue expérimentalement plusieurs méthodes de mesures. La méthode qui semble la plus sélective et donc la plus adaptée aux mesures des faibles concentrations est celle faite par conductimétrie après séparation sur membrane semi-perméable (Wyers et al. 1993).

L'extraction des matières végétales pour l'analyse reste un point problématique surtout en ce qui concerne l'apopalsme et le xylème (Schurr, 1998). Lohaus et al (2001) ainsi que Hill et al. (2001) évaluent la méthode d'extraction par infiltration/centrifugation concernant l'apoplasme sachant que pour l'instant il n'existe pas d'autres méthodes permettant d'étudier le contenu en NH_4^+ de l'apoplasme.

Il est essentiel de mentionner ici l'existence de microélectrode permettant de mesurer les concentrations en ions in vivo dans les tissus de la plante. Les microélectrodes sont des petites sondes qu'on place à l'intérieur d'un tissu végétal et qui permettent de mesurer le potentiel électrique entre ce tissu et une référence externe. C'est une technique bien développée pour les mesures de pH et de concentration en nitrate entres autres (Felle & Bertl, 1986 ; Miller et al. 2001). Si cette méthode était applicable aux mesures de concentration en ions ammonium dans l'apoplasme elle permettrait une mesure directe et non destructive. Pour l'instant, les capteurs résineux utilisés pour remplir les microélectrodes ne sont pas assez développés aux ions ammonium et ne permettent donc pas de mesurer de très faibles concentrations. D'autre part, ils ne sont pas assez sélectifs entre les ions potassium et ammonium (voir annexe pour plus de détail).

Concernant l'ammoniac atmosphérique, l'un des principaux problèmes lié aux méthodes de mesures est de pouvoir différencier la forme NH_3 de la forme NH_4^+. La plus grande avancée dans ce domaine est le développement de « dénudeur actifs » qui permettent de piéger l'ammoniac sur une matrice acide et de l'analyser ensuite. Cette méthode a un temps de réponse relativement long et représente une intégration des concentrations sur un temps donné. Des développements sont encore en cours pour permettre des mesures en continu sur plusieurs dénudeurs en même temps.

B. Mesures de flux

La mesure de flux en général est souvent spécifique à l'échelle d'espace considéré. Sur une parcelle agricole, la mesure de flux qui permet le suivi à plus ou moins long terme et qui est applicable pour les concentrations de l'ordre du $\mu g\ m^{-3}$ reste la méthode de gradient (Denmead & Raupach, 1993). Cette méthode assimile les transferts convectifs à un phénomène de diffusion. La mesure de flux par corrélation ne peut pour l'instant encore être

appliquée à l'ammoniac car il n'existe pas de méthodes de mesure de concentration avec une constante de temps adaptée. Pour des échelles d'espaces de l'ordre du 1 m^2 la méthode d'enceinte dynamique ou statique est utilisée (Denmead & Raupach, 1993). L'une des principales contraintes de cette méthode est la réactivité et le dépôt du NH_4^+ sur les surfaces surtout en conditions d'humidité élevée entrainant donc un biais dans les mesures. Cette méthode ne permet pas de différencier les flux en provenance de sol, de la litière ou de la plante. A l'échelle de la plante on peut utiliser la méthode de chambre à flux (Husted & Schjoerring., 1995b) qui repose sur le même principe que l'enceinte dynamique. Cependant l'utilisation de cette chambre en conditions contrôlées permet d'isoler les parties aériennes des parties souterraines de la plante.

C. Mesures expérimentales du point de compensation stomatique

Nous avons conçu une expérimentation (Figures 3) pour mesurer le point de compensation stomatique pour des plantes de colza avec différentes alimentations azotées, dans des conditions de lumière et d'obscurité et en utilisant les deux méthodes de mesure décrites ci dessus. Nous avons utilisé deux méthodes pour déduire le point de compensation dans les mesures par chambre et avons aussi effectué des mesures d'extraction d'apoplasme.

Le point de compensation mesuré par chambre variait entre 0.8 et 12.2 $\mu g\ m^{-3}$ à 20 °C pour des alimentations azotées allant de 10 mM NO_3^- à 10 mM NO_3^- + 5 mM NH_4^+. Il n'y a pas de différences significatives entre les concentrations d' NH_4^+ mesurées par extraction dans des conditions de lumière et d'obscurité. Cependant les concentrations en conditions de lumière ont tendance à être plus élevées que celles en conditions d'obscurité. La concentration d' NH_4^+ variait de 0.1 à 2.1 mM pour l'apoplasme, de 3.9 à 6.6 mM pour l'extrait de feuille entière et de 2.4 à 6.1 mM pour le xylème pour des nutritions azotées allant de 0N à 5mM NH_4^+. Le rapport entre l'augmentation du point de compensation à 20 °C par rapport au traitement 0N et la concentration dans la solution nutritive est entre 0.2 et 0.7 pour les périodes de lumière. Nous notons un rapport similaire pour les concentrations d' NH_4^+ cytoplasmique (0.5). Tandis que pour les concentrations dans le xylème ce rapport est de 1.8 et pour les extraits de feuilles entières il est de 1.4.

Les alimentations mixtes d'NH_4^+ et de NO_3^- avaient un point de compensation plus faible que les alimentations avec du NH_4^+ seul à mêmes concentrations. L'alimentation azotée semble avoir une influence sur la résistance totale aux échanges gazeux probablement à travers la résistance stomatique. Les valeurs de Γ calculés par la méthode d'extraction étaient supérieures aux valeurs de Γ calculées par la méthode de chambre.

Variation du point de compensation stomatique de l'ammoniac pour de jeunes feuilles de colza en fonction de condition lumière/obscurité et pour une nutrition azotée variable.

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Abstract

The plant can be a source or a sink of ammonia (NH_3) depending on its nitrogen fertilisation, metabolism and on background atmospheric concentrations and thus plays a major role in regulating atmospheric NH_3 concentrations. For a better understanding of the driving factors of the NH_3 stomatal compensation point, it is important to analyse the dynamics of leaf NH_3 fluxes in relation to the ammonium and nitrate concentrations in the different leaf compartments as well as to N nutrition and light and dark periods.

We designed an experiment to quantitatively assess leaf-atmosphere NH_3 exchange and the stomatal compensation point and identify the main factors affecting the variation of NH_3 fluxes in oilseed rape. We tested day and night dynamics as well as five nitrogen treatments. Two experimental methods were used: a dynamic open flux chamber and extraction of apoplastic solution. These two methods have been reported not to converge; we therefore compare them and discuss their discrepancies.

Chamber measurements show that there is a good correlation between plant NH_3 fluxes and water fluxes. Compensation points were calculated by two different methods and ranged between 0.8 and 12.2 $\mu g\ m^{-3}$ NH_3 (at 20°C) for the different nitrogen treatments. Apoplastic solution measurements show that there is no significant difference for apoplastic NH_4^+ concentrations ($[NH_4^+]_{apo}$) extracted in dark and light periods for the same nitrogen treatment. Statistical analysis also shows that $[NH_4^+]_{apo}$ is correlated with $[NH_4^+]$ in the nutritive solution and weakly correlated with $[NO_3^-]$. Apoplast NH_4^+ concentrations ranged between 0.1 and 2.1 mM, bulk tissue NH_4^+ concentrations between 3.9 and 6.6 mM and xylem concentrations between 2.4 and 6.1 mM.

Key words: NH_3; pH; apoplast; xylem; flux; chamber; nitrogen metabolism.

1. Introduction

Ammonia (NH_3) participates in the soil and atmosphere nitrogen cycle. It also plays a major role in the plant's nitrogen metabolism. Grasslands and agricultural crops can be either a source or a sink of NH_3, depending on the difference between atmospheric NH_3 concentration and the so-called NH_3 compensation point of the plant. They therefore have a significant impact on the regulation of atmospheric NH_3 concentrations. The stomatal compensation point for NH_3 (χ_s) is defined as the atmospheric NH_3 concentration for which there is no exchange between the leaf and the atmosphere in dry conditions (when the cuticular adsorption / desorption can be neglected) (Flechard 1998). In theory, χ_s is also the gaseous NH_3 concentration in thermodynamic equilibrium with the aqueous NH_3 in the apoplastic solution (solution surrounding the cells) (Farquhar *et al.* 1980).

A better characterisation of the compensation point in relation to nitrogen supply, management practices, and plant species, is crucial to have a better understanding of NH_3 deposition on a local and regional scale. It is also a step forward in the comprehension of the regulation of χ_s by the plant. Moreover, most NH_3 exchange models developed so far do not account for the plant's N metabolism and use prescribed compensation points. For a better understanding of the driving factors of the NH_3 stomatal compensation point, it is important to analyse the dynamics of leaf NH_3 fluxes in relation to the ammonium (NH_4^+) and nitrate (NO_3^-) concentrations in the different leaf compartments as well as to N nutrition and light and dark periods.

Previous studies showed the variability of NH_3 emissions in relation to the plant's development stage (Francis *et al.* 1997; Mattsson and Schjoerring 2003). It was also suggested that these emissions are related to nitrogen reallocation, when nitrogen is transported as NH_4^+ in the xylem sap (Schjoerring *et al.* 1993a, b). Other studies also showed a large response of NH_4^+ concentration in xylem sap and apoplastic NH_4^+ to photosynthesis, respiration and glutamine synthetase (GS) inhibition (Mattsson and Schjoerring 1996a; Yin *et al.* 1996). Apoplastic pH and NH_4^+ concentrations are also directly dependent on the plant's nitrogen nutrition (Mattsson et al. 1998; van Hove et al. 2002)

The canopy NH_3 compensation point (χ_c) can be inferred from measurement of vertical fluxes and concentrations of NH_3, over large fields (e.g. Fléchard. 1998), as well as in cuvettes by finding at which concentration the flux is zero (Husted and Schjoerring, 1995b). Some studies show the effectiveness of these methods to assess the NH_3 compensation point for different species, environmental conditions, and its response to environmental parameters, and plant

nutrition (Mattsson and Schjoerring 1996b; Mattsson and Schjoerring 2003). All these techniques are based on the inference of the stomatal compensation point from fluxes and concentrations in the atmosphere.

Another technique exists; it is based on the determination of leaf apoplastic NH_4^+ concentration and pH to assess χ_s, by means of extraction of the apoplastic fluid with successive infiltration and centrifugation of leaf segments (Husted and Schjoerring 1995a). This technique has been successfully applied to several plants in the field (Husted *et al.* 2000). However, the extraction technique is subject to uncertainties regarding potential regulation of apoplastic pH and NH_4^+ by the plant during the extraction, and buffer effects (Hill *et al.* 2001; Schjoerring *et al.* 2002).

We designed an experiment to quantitatively assess leaf-atmosphere NH_3 exchange in relation to nitrogen nutrition in oilseed rape (*Brassica napus* L.). Oilseed rape is an expanding crop and requires high levels of N fertilization. We based our study on this crop for the environmental issues attached and because it has relatively large leaves making measurements technically easier for both the extraction and the chamber method. We tested dark and light dynamics as well as five nitrogen treatments. Two experimental methods were used: dynamic open flux chamber and apoplast, xylem and whole leaf solution extraction. We try to compare results from the two methods and explain probable reasons for discrepancy.

2. Material and methods

2.1. Plant material

Seeds of oilseed rape (*Brassica napus* L. 'Capvert') were germinated in small green houses for one week and then replanted in aerated nutritive solution. Germinations were spread out on several weeks in a way to have same aged plants for every series of measurement. Plants were grown in phytotrons with average day / night temperatures of 22.0 ± 0.9/18.0 ± 0.6 °C, day length of 12 hours (from 12:00 till 00:00), average photosynthetic photon flux densities (PPFD) of 392 ± 108 µmol m^{-2} s^{-1} and average relative humidity of 74 ± 6 % during the day and 92 ± 4 % during the night. It is to be noted that PPFD variability is due to spatial distribution of light inside the phytotron; plants were weekly rotated inside the phytotron to diminish the effect on growth.

All plants were grown in an aerated nutritive solution with 10 mM NO_3^- and 2 mM NH_4^+ (Lesaint and Coïc 1982) for four weeks before differentiating them into the different nitrogen treatments for one week. Aeration of the nutrient solution was performed using an air pump

delivering approximately 6 L min^{-1}. On average plants had 8 leaves after four weeks and were in a vegetative stage.

The different nitrogen treatments are adaptations of the Coïc-Lesaint solution (Lesaint and Coïc 1982) with the following N concentrations:

- 0 N
- 5mM NH_4^+
- 10 mM NO_3^-
- 5 mM NO_3^- + 1 mM NH_4^+
- 10 mM NO_3^- + 2 mM NH_4^+

For extraction measurements, day extractions were done after 4 hours of illumination, whereas dark extractions were done after 9 hours of darkness.

2.2. Extraction measurements

2.2.1. Extraction of apoplastic solution

A slightly modified version of the vacuum infiltration technique described by Husted and Schjoerring (1995a) was used to determine apoplastic pH and NH_4^+ concentration. The top most developed leaf of the oilseed rape plant was cut. The central petiole was removed. The leaf part was then washed with de-ionized water and blotted dry with laboratory paper. The leaves were then weighed and infiltrated with 50 µM indigo carmine solution (kept at 4°C) in a 60 mL syringe with a series of pressure/vacuum for 5 minutes. Hill et al. (2001) showed that infiltration with indigo carmine instead of sorbitol solution had no significant effect on pH or NH_4^+ measurements. The infiltrated leaves were then blotted dry with clean laboratory paper. Leaves were left to equilibrate for 15 minutes according to Nielsen and Schjoerring (1998). The apoplastic solution was extracted by centrifugation at 2000 gravities for 10 minutes at 4 °C. The duration and centrifugation force were determined after prior tests on oilseed rape leaves for cytoplasm contamination. The contamination tests were done by measuring the activity of malate dehydrogenase (MDH) in apoplast extracts relative to bulk tissue extracts for six repetitions as described by Husted and Schjoerring (1995a). The contamination was less than 3.3 ± 1.5 % of MDH activity in apoplast extract relative to bulk tissue extract. The dilution of the extracted solution was measured using a spectrometer at wavelength 595 nm (iEMS reader, Labsystems). The extracted solution was then frozen in liquid nitrogen and stored at -18 °C before further analysis.

2.2.2. Extraction of bulk tissue solution

Leaf segments were cut into small pieces and ground in liquid nitrogen until we obtained a thin powder. A weighed sample was then put in a 1.5 ml tube together with 1 ml of ultra pure water. The tube was shaken for 15 minutes and then centrifuged at 2000 g for 20 minutes. The supernatant (clear solution on top) was then retrieved, frozen and stored at -18°C for further analysis. The remaining leaf parts were dried and analysed for total nitrogen and carbon content.

2.2.3. Extraction of xylem sap

Xylem sap was collected from the cut surface of the shoot/root junction area under 'root pressure' (Schurr and Schulze 1995). Immediately after detopping the plant 0.5 cm below the shoot stump, it was rinsed with de-ionized water and blotted with absorbent tissue to remove contaminants from cut cells. After discarding approximately 50 mm^3 of sap, each cut surface was blotted again and silicon tubing was fitted over the stump. Sap flowing from the tubing was collected, frozen and stored at -18°C for further analysis.

2.2.4. Sample analysis and calculations

The pH of apoplast and xylem sap extracts was measured with an InLab 423 semi-micro electrode (Mettler Toledo, Udorf, Switzerland). Xylem sap, apoplast and bulk tissue extracts were analysed for NO_3^- using the spectrophotometer absorption method (Griess-illosvay reaction) and for NH_4^+ using the conductivity method after separation on a semi-permeable membrane with a flow injection NH_3 analyser: FloRRia (Mechatronics, the Netherlands). Total nitrogen and carbon content of remaining leaf parts was analysed according to the Dumas method. Apoplastic concentrations were calculated by multiplying the measured concentration with the dilution factor measured by the spectrometer. Bulk tissue extracts are expressed per mass fresh weight and are calculated by multiplying the measured concentration by the ratio of extract volume to leaf sample weight. Gamma (Γ) describes the NH_3 emission potential and is a temperature independent parameter. It can be calculated from extraction measurements as the ratio of NH_4^+ to H^+ ions concentration in the apoplast:

$$\Gamma = \frac{[NH_4^+]_{ap}}{[H^+]_{ap}} \qquad (1)$$

2.3. Chamber measurements

A chamber system was designed. A schematic diagram of the chamber is shown in Figure 1. The plant cuvette had dimensions of 40 x 60 x 40 cm. It allowed pre-treatment of air to have free NH_3, air inside as well as control of NH_3 and water vapour concentrations in the cuvette system. chamber system was designed. A schematic diagram of the chamber is shown in figure 1. .

2.3.1. Pre-treatement of air

Incoming air to the chamber system was sampled in the building underground in order to provide constant CO_2 concentrations. Air was dried by cooling it to 4°C and collecting the condensed water in a metal coil. Air was then filtered for NO, NO_2 and NH_3 by passing through successive purafil (Purafil Inc., Atlanta, GA, USA), granule activated carbon and particle filters. All tubing after the filter system was Teflon PTFE made to minimize NH_3 adsorption. Other material in contact with circulating airflow was stainless steel (connectors).

2.3.2. Flow control

Air was sampled by two pumps (140 L min^{-1}), to provide sufficient pressure and flow. A regulating flowmeter was used to force the air flowrate in a range 40 – 100 ± 0.1 L min^{-1} (F-203AC, Bronkhorst, France). The air flowrate was recorded every 5 seconds in a computer system. The whole system was air tight and we considered that airflow rates at the inlet and at the outlet of the chamber are equal

2.3.3. Plant cuvette

The plant cuvette was entirely made of Teflon material. The lower part was made of a Teflon PTFE board with 2 cm holes for the plant stems. Plants were grown in Teflon PTFE conic shaped caps with 0.5 cm holes. The plant stems after 5 weeks filled entirely the 0.5cm hole and were thus airtight. The upper part was made of transparent Teflon FEP film (50 µm thickness) sealed together by heating. This film was inflated with incoming air. The upper and lower parts were clipped together by forceps. A PTFE Teflon coated axial fan was placed inside the chamber to homogenise the air. Light was provided by four halide lamps (HPI-T 400 W, Philips) placed at about 1 m above the plant cuvette. Measured photosynthetic active

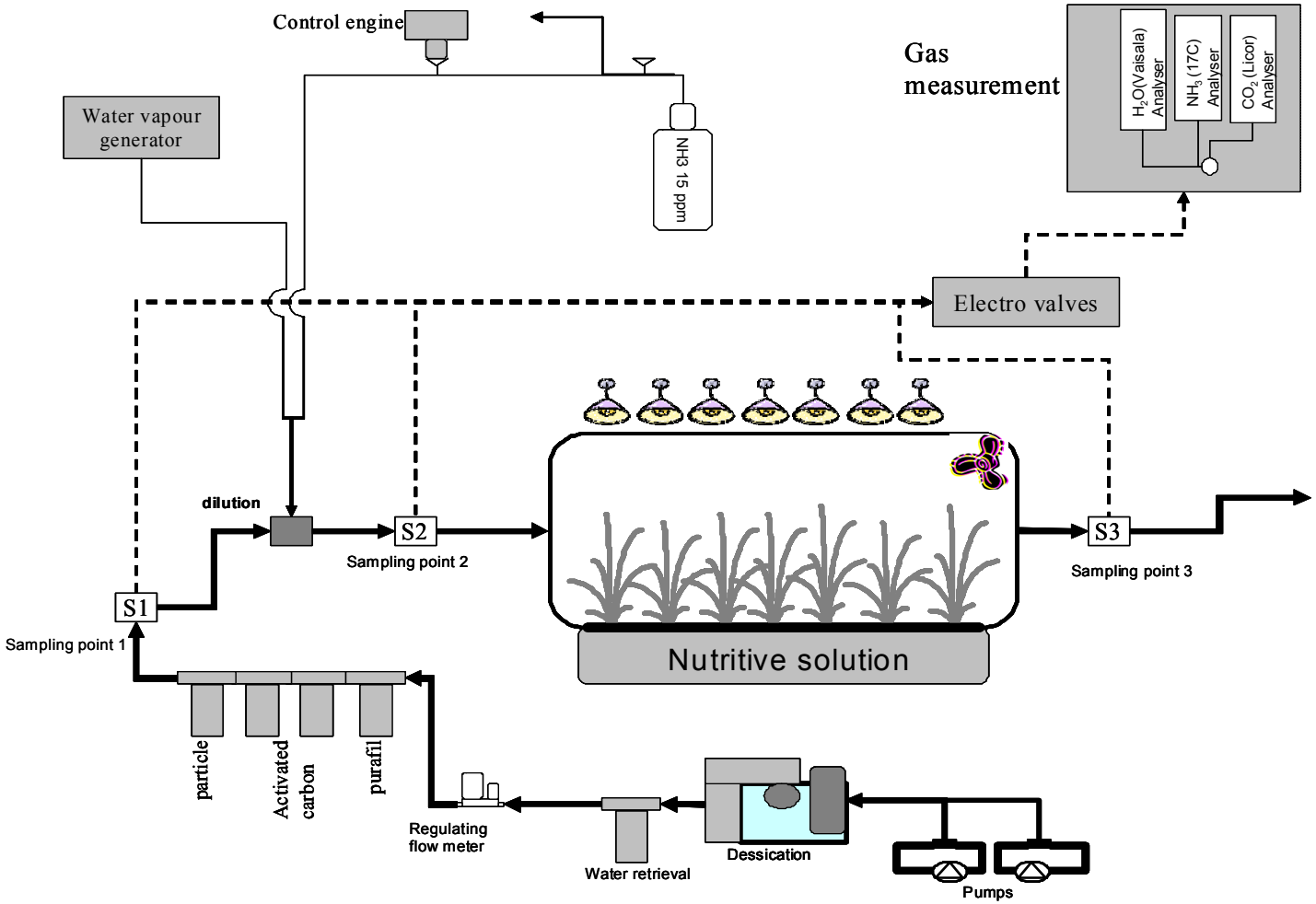

Figure 2.1: Schematic diagram of the flux measurement chamber.

Bold arrows represent the main airflow through the chamber. Dotted arrows represent the sampling flow through the three sampling points. Plain arrows represent water vapour and ammonia injection flows.

radiation (PAR) inside the chamber was 350 µmol m^{-2} s^{-1}. The chamber was placed in an air conditionned room with a temperature ranging between 20°C and 22°C.

2.3.4. Ammonia and water vapor injection

The designed chamber allowed control of water vapour and NH_3 concentrations. Injection was made after the filters. Water vapour was generated by evaporating de-ionized water in a copper tube with 1 mm inside diameter, coiled around an aluminium bar, this bar being heated by a resistance. Water flow through the coil was controlled by a peristaltic pump and thus allowed variation in water injection and thus in air water vapour content at the inlet of the chamber. NH_3 was generated by using a standard NH_3 gas bottle of 14.99 ± 0.3 ppm and by varying air flow out of the bottle and mixing it with zero air. The NH_3 flow coming from the standard bottle was controlled by a needle valve mounted on a stepper computer controlled motor.

2.3.5. Gas sampling and measurement

Gas was sampled at three locations: after the filters, and at the inlet and at the outlet of the plant cuvette. Computer controlled stainless steel electrovalves switched between the sampling locations every 20 minutes. The air sample was passed successively through a LiCOR (Li-820) for CO_2 concentration measurement, a Vaisala (HMP45AC) for water vapour measurement, and in parallel through a 17C chemiluminescence analyser (Thermo Scientific) for NH_3 concentration measurement. There were also thermocouples placed at the inlet and at the outlet to measure air temperatures. Additionnally, four thermocouples were placed on the leaves to measure leaf surface temperature.

The chemiluminescence analyser was calibrated for:

- NO using a dilution system (Thermo scientific 146C model) and a standard NO gaz cylinder (16.81 ± 0.34 ppm),
- NO_x using a NO to NO_x converter system (Thermo scientific 146C model)
- and NH_3 using a dilution system (Environnement SA, VE3M model) with standard permeation tubes delivering 154 ± 1.5 ng NH_3 min^{-1} at 40°C.

The analyser was verified before each set of measurements with a preset NH_3 concentration in the empty chamber. Reported problems concerning incomplete conversion of NO_x to NO by this type of analyser (Schwab et al. 2007) were irrelevant in our case since all measurements were done on filtered air containing theoretically no NO and NO_x.

2.3.6. Chamber data acquisition and calculations

Air flow-rates, CO_2, water vapour and NH_3 concentrations as well as temperatures were averaged over a one minute interval. The electro valves were switched every 20 minutes between the three sampling locations, and the concentration were averaged over the last 10 minutes of the sampling time.

- The chamber flux (F_x) for a compound X is calculated from equation 2:

$$F_X = \left([X]_{outlet} - [X]_{inlet}\right) \times Q_{air\,(inlet)} \quad (2)$$

  Where $[X]_{inlet}$ and $[X]_{outlet}$ are the gaseous (H_2O, CO_2 or NH_3) concentrations at the inlet and outlet of the chamber respectively; $Q_{air(inlet)}$ is the air flow-rate at the inlet. The airflow rate is in $m^3\ s^{-1}$; the water vapour and CO_2 concentrations are in mg m^{-3} whereas the NH_3 concentration is in µg m^{-3} NH_3.
- After chamber gas measurements are done, all plants leaf area were measured with an area meter (LI-3100, LI-COR Inc., Lincoln, Nebraska, USA). These measurements were used for calculating leaf water and NH_3 fluxes by dividing the total flux in the chamber (ng or mg s^{-1}) by the total leaf area (m^2) to get the flux per leaf surface area (ng or mg $m^{-2}\ s^{-1}$).
- We considered that the concentration of a certain compound in the chamber $[X]_{ch}$ is the average of the concentrations measured at the inlet and at the outlet since there is good mixing in the chamber.
- If we consider that exchange of NH_3 with the cuticles and the chamber is negligible, the NH_3 compensation point of the whole can be approximated to χ_s which can be calculated from these chamber measurements using two methods:
  - The regression method consists of calculating the χ_s as the point where F_{NH3} changes sign. Practically, this is done by calculating the intersect of the linear regression between F_{NH3} and $[NH_3]_{ch}$.
  - The resistance analogy method consists of calculating χ_s based on the resistance analogy (equation 3). If we consider that transfer is mainly turbulent in the chamber, then the total resistance to NH_3 exchange is equal to that of water vapour. This assumption is tested below.

$$\chi_s = R_{tot} \times F_{NH3} + [NH_3]_{ch} \quad (3)$$

The system was designed in a way that the exchange with the chamber material is minimized. Therefore, the resistance to water vapour exchange including vegetation (R_{tot}) can be deduced from equation 4 by considering that water vapour concentration in the sub-stomatal cavities $[H_2O]_{sc}$ is at saturation.

$$R_{tot} = \frac{[H_2O]_{sc} - [H_2O]_{ch}}{F_{H2O}} \quad (4)$$

$[H_2O]_{sc}$ is calculated from equation 5; and the saturation vapour pressure of moist air over water (E_{vapsat}) is given by an empirical function (Buck, 1981).

$$[H_2O]_{sc} = \frac{E_{vapsat} \times M_{H2O}}{R \times T_{leaf}} \times 1000 \quad (5)$$

E_{vapsat} is in Pascal, M_{H2O} is the molar mass of water (18 g mol^{-1}), R is the universal gas constant (8.315 J mol^{-1} K^{-1}), T_{leaf} is the leaf temperature in Kelvin and the factor 1000 to convert to mg m^{-3}.

- Gamma (Γ) can also be calculated from chamber measurements using equation 6:

$$\Gamma = \chi_s \cdot \frac{1}{K_H \cdot K_d} \quad (6)$$

χ_s is the NH_3 stomatal compensation point in mol L^{-1}, K_H is the Henry constant and K_d is the dissociation constant. The product $K_d \,.\, K_H$ is defined by the temperature dependent expression below (Sutton et al. 1994):

$$K_H \cdot K_d = \frac{161512}{T_{leaf}} \times 10^{-4507.11/T_{leaf}} \quad (7)$$

2.4. Statistics

Concerning extraction measurements, we had 12 plant replicates per treatment. Bonferroni procedure for multiple tests of significance (Miller, 1981) was done using SPSS 10.0.1. Mean differences were significant at the 0.05 level. Simple correlation tests were also performed on some criteria using two-tailed Pearson correlation test (Plackett, 1983). Correlation was significant at the 0.01 level.

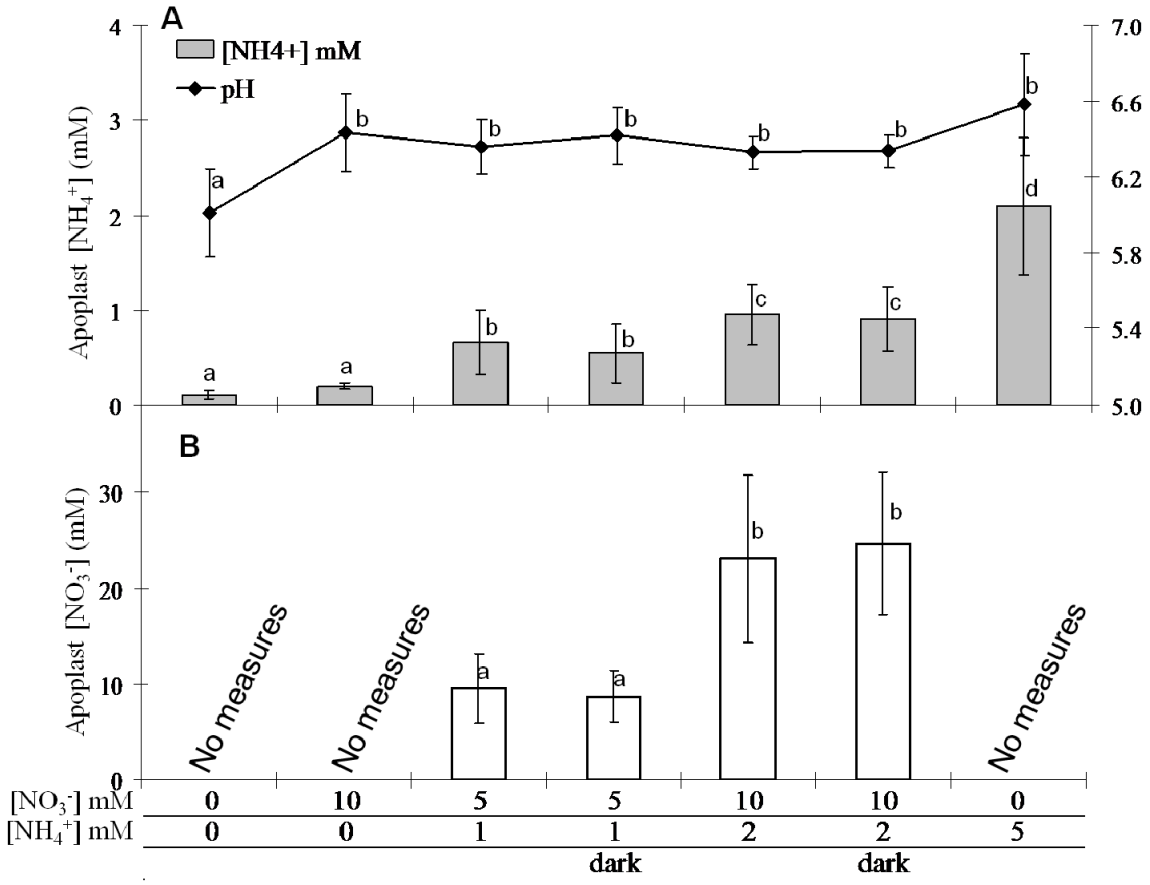

Figure 2.2: Concentrations of NH_4^+ and pH (A) and NO_3^- (B) in leaf apoplastic fluid as affected by N treatment and light and dark extractions.

Grey bars represent NH_4^+ concentrations; white bars NO_3^- concentrations and dots pH. Data are means ± standard deviation of 12 replicates. Significant differences between treatments ($P<0.05$) are indicated by different letters

Concerning chamber measurements, we had 7 plants per set of measurement. For the 10 mM NO_3^- and 5 mM NH_4^+ treatments we had 2 plant sets per treatment with 3 peaks of NH_3 per plant set. As for the 10 mM NO_3^- + 5 mM NH_4^+ and 10 mM NO_3^- + 2 mM NH_4^+ we had 5 plant sets with 3 NH_3 peaks per set. Linear regressions for χ_s determination in the regression method were done with the least square method using SPSS 10.0.1; error was determined with the confidence interval around the mean. The determination of χ_s using the resistance analogy method was done by averaging all obtained χ_s values for a given treatment.

3. Results

3.1. Extraction results

3.1.1. Apoplast

Leaf apoplast NH_4^+ concentrations increased with increasing N supply. Values ranged between 0.1 mM for the 0N treatment to 2.1 mM for the 5 mM NH_4^+ treatment (Figure 2A). The NH_4^+ concentration in the apoplastic solution for the mixed N treatment ranged between 0.5 and 1 mM hence between 2 and 4 times smaller than the 5 mM NH_4^+ treatment. There was no significant difference for NH_4^+ concentrations in the apoplast between the 0N treatment and the 10 mM NO_3^- treatment. However, all the other treatments had a significant effect on apoplastic NH_4^+ concentrations. There was a fair correlation (R^2=0.69) between NH_4^+ concentrations in the apoplast and in the nutritive solutions with $[NH_4^+]_{apo}$ equal 0.83 times the NH_4^+ concentration in the nutritive solution. However there was no correlation between NH_4^+ concentration in the apoplast and NO_3^- concentration in the nutritive solution (R^2=0.01). Although no significant difference is observed, the mean apoplastic NH_4^+ concentration was lower in dark than in light conditions. Concerning apoplastic pH (Figure 2B) we notice no significant difference between the different N treatments except for the 0N, which shows a smaller pH. Although no significant difference is observed, the mean apoplastic H^+ concentration was lower in dark than in light conditions.

NO_3^- concentrations in the apoplast increased with increasing NO_3^- concentration in the nutritive solution. The apoplastic NO_3^- concentration was roughly 2 times larger than the feeding concentration. We observed no significant difference between extractions done after a dark period and extractions done after a light period (Figure 2B). Since dark and light treatments are not significantly different we aggregated the relative data.

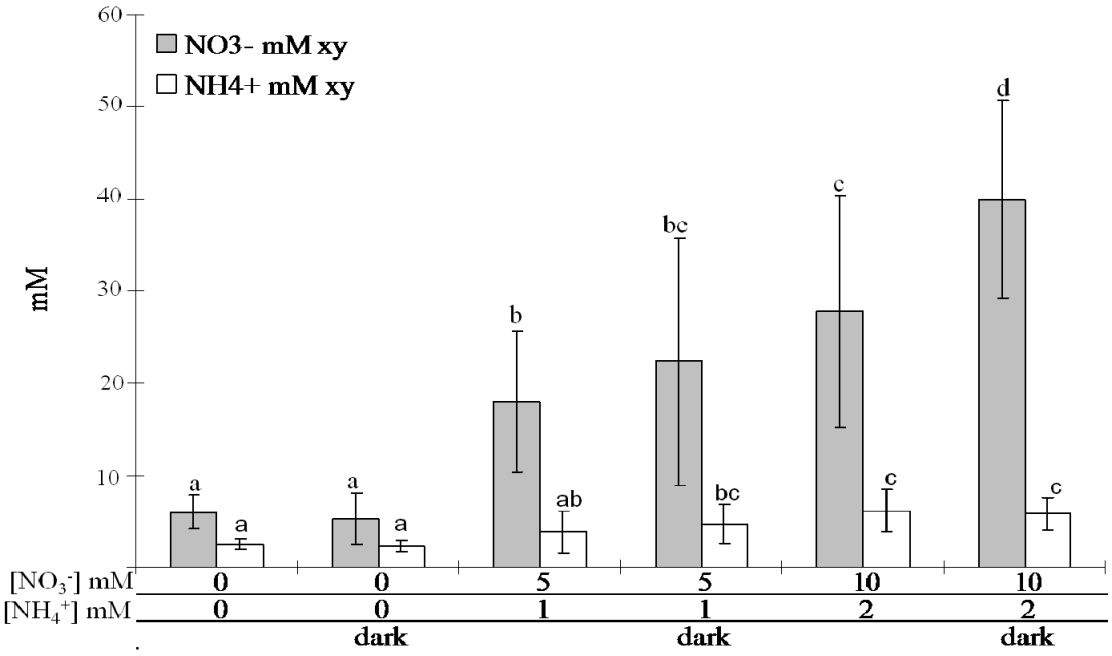

Figure 2.3: Xylem sap concentrations of ammonium and nitrate as affected by N nutrition and light and dark extractions.

White bars represent NH_4^+ concentrations while grey bars NO_3^- concentrations. Data are means ± standard deviation of 12 replicates. Significant differences between treatments ($P<0.05$) are indicated by different letters.

3.1.2. Xylem sap

Measured xylem sap NO_3^- and NH_4^+ concentrations are presented in Figure 3. NO_3^- and NH_4^+ concentrations in the xylem sap increased with increasing NO_3^- and NH_4^+ concentrations in the nutritive solution and ranged between 4.2 and 40 mM for NO_3^- and 2.3 and 6.2 for NH_4^+. Both NO_3^- and NH_4^+ concentrations in the xylem sap are significantly correlated with form and concentration of N nutrition respectively. $[NH_4^+]_{xy}$ is equal to 1.81 times NH_4^+ in the nutritive solution (R^2=0.97) and $[NO_3^-]_{xy}$ was equal to 2.82 times NO_3^- in the nutritive solution (R^2=0.91). There is no significant difference between extractions after light or dark periods on xylem sap concentrations. However, xylem sap NO_3^- concentrations were higher in the dark than in the light. If we aggregate day and night treatments then nutritive solution effect was significant for all treatments. The increase in NO_3^- in xylem sap relative to the 0N treatment was roughly 2 to 3 times the concentration in the nutrient solution. Similarly the increase in NH_4^+ xylem concentration was equal to 1.5 times the nutrient solution.

3.1.3. Bulk tissue

Bulk tissue NH_4^+ concentrations are represented in Figure 4A and range between 6 and 12 µmol g^{-1} FW for the different treatments. If we consider that the leaf fresh tissue density is 1000 g L^{-1} then µmol g^{-1} FW can be approximated to mM. We noticed that $[NH_4^+]_{bulk\ tissue}$ was equal to 1.52 times NH_4^+ in the nutritive solution (R^2=0.42) and $[NO_3^-]_{bulk\ tissue}$ was equal to 1.53 times NO_3^- in the nutritive solution (R^2=0.43). There was no significant difference between extractions following dark and light periods. We also noted no significant difference between the 10 mM NO_3^- + 2 mM NH_4^+, 5 mM NO_3^- + 1 mM NH_4^+ and 10 mM NO_3^- treatments which is contradictory to several other studies which conclude that shoot and root NH_4^+ concentrations increase with increasing NH_4^+ concentrations in the root medium (Mattsson and Schjoerring 1996a; Walker *et al.* 1984). However there was a significant difference with the 5 mM NH_4^+ and the 0N treatments.

NO_3^- bulk tissue concentrations ranged between 47 and 130 µmol g^{-1} FW for the different treatments (Figure 4B). Bulk tissue concentrations were dependent on NO_3^- nutrition and showed no significant difference between light and dark periods.

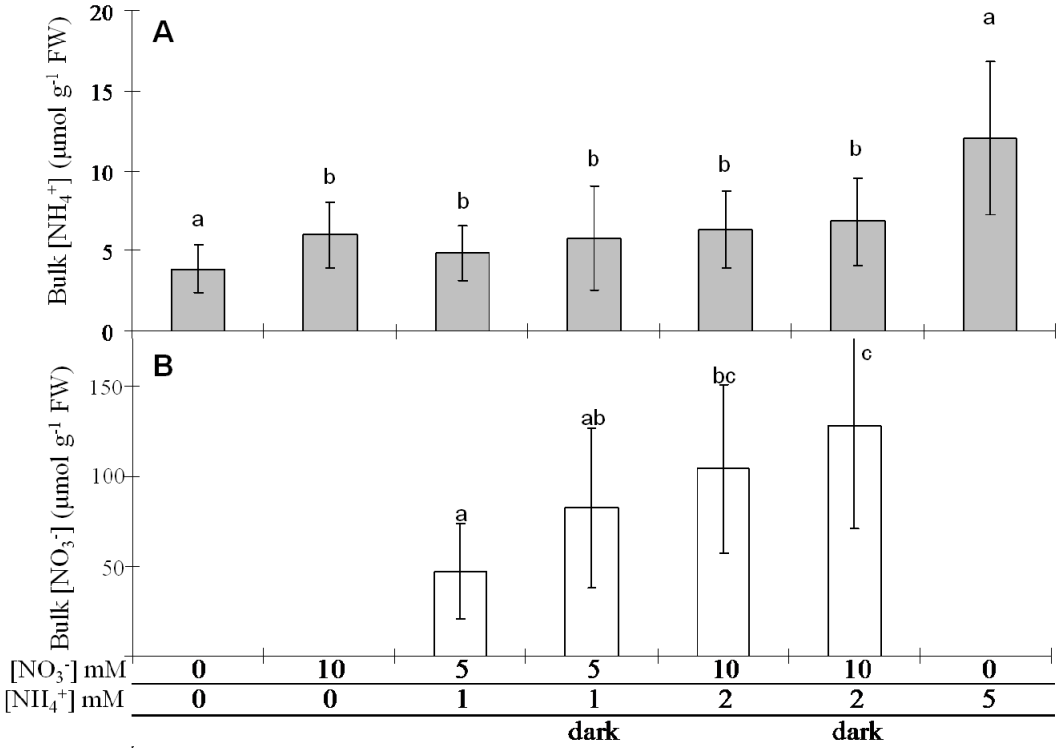

Figure 2.4: Bulk tissue ammonium (A) and nitrate (B) concentrations in leaf extracts of oilseed rape plants grown on different N treatments and for extractions done after a light or dark period..

Grey bars represent NH_4^+ concentrations and white bars NO_3^- concentrations. Results are means ± standard deviation of 12 replicates. Significant differences between treatments ($P<0.05$) are indicated by different letters.

3.2. Chamber measurements

3.2.1. Adsorption by the chamber

Fluxes of NH_3 measured in an empty chamber with a relative humidity of 40% averaged over three repetitions were not significantly different from zero. NH_3 concentrations peaked at 6.5 µg m^{-3} NH_3 for these measurements. However, we distinguished a slight deposition and a long response time of the chamber. The average deposition flux to the chamber estimated before the peak is 0.26 ± 0.25 ng s^{-1} NH_3, which correspond to a flux per chamber surface of 1.1 ng m^{-2} s^{-1} NH_3. The average re-emission after the peak was 0.09 ± 0.09 ng s^{-1}. This corresponds to a flux per chamber surface of 0.2 ng m^{-2} s^{-1}.

3.2.2. Example of dynamic chamber flux measurements

The flux chamber allowed measuring simultaneously fluxes of water vapour, CO_2 and NH_3 while varying NH_3 concentrations at the inlet. Figure 5 shows a typical example of water vapour, CO_2 and NH_3 fluxes. This example is relative to seven plants placed in the chamber with a total leaf area of 1440 cm^2 and fed with 5 mM NH_4^+ in the nutritive solution. The chamber NH_3 concentrations peaked at around 4 µg m^{-3} NH_3. We observe emission fluxes (positive) during episodes of low NH_3 concentrations and deposition fluxes (negative) for episodes of high NH_3 concentrations (Figure 5A). NH_3 emission fluxes were approximately 12 ng m^{-2} s^{-1} NH_3 (per m² leaf) for NH_3 chamber concentrations around 1 µg m^{-3} NH_3 and for 5mM NH_4^+ in nutritive solution. Figure 5B illustrates water vapour and CO_2 fluxes; photosynthetic CO_2 absorption during the light period was summing up to 0.4 mg CO_2 m^{-2} s^{-1} and a respiration rate of about 0.07 mg m^{-2} s^{-1} was observed. We notice that water fluxes are relatively important during the dark period indicating that stomata are not completely closed probably due to high vapour pressure deficit in the chamber and plants being in hydroponics. This explains the NH_3 deposition flux during dark periods and high NH_3 concentrations. Typical chamber temperature and vapour pressure deficit values (VPD) are illustrated in Figure 5C.

3.2.3. NH_3 compensation point

Figure 6 illustrates the relation between NH_3 fluxes (F_{NH3}) and NH_3 concentrations in the chamber ($[NH_3]_{chamber}$). The intersection of the linear regression with the x-axis is the NH_3 compensation point. Whereas the slope is the total conductance to NH_3 exchange in the chamber. Figures 6A, 6C and 6D represent regressions for 3 different N treatments during the

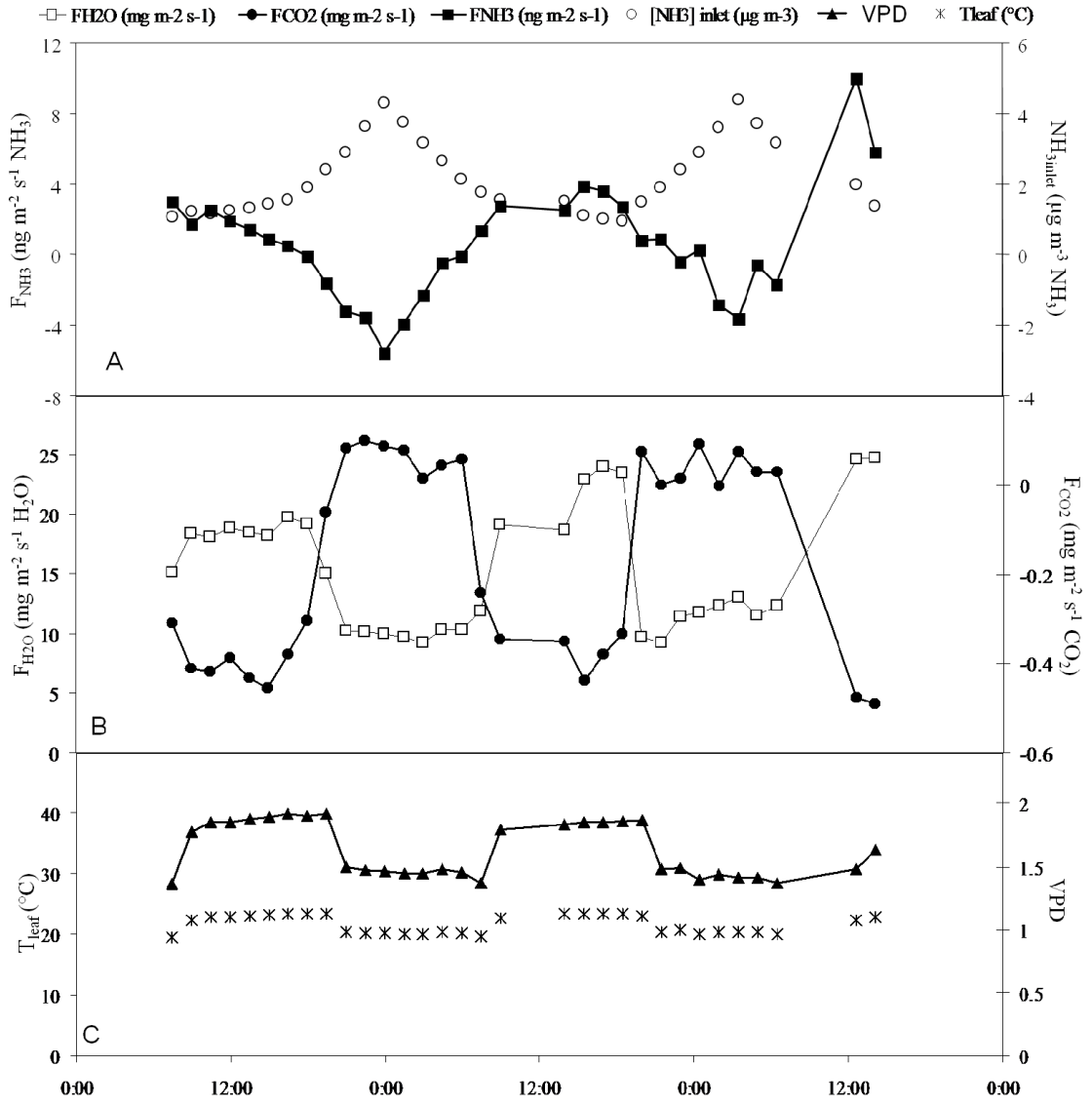

Figure 2.5: Example of typical NH_3, CO_2 and water vapour flux measurements with NH_3 concentrations peaking at around 5 µg m^{-3} NH_3.

Figure A represents NH_3 fluxes in ng m^{-2} s^{-1} on the left scale and NH_3 concentrations at the chamber inlet in µg m^{-3} on the right scale. Figure B represents water vapour fluxes on the left scale and CO_2 fluxes on the right scale in mg m^{-2} s^{-1}. Figure C represents leaf temperature in °C and chamber vapour pressure deficit (VPD). Measured fluxes are integration for 7 plants placed in the chamber with a total leaf area of 1317 cm² and with 10 mM NO_3^- + 2 mM NH_4^+ in the nutritive solution.

light period and Figure 6B represents one N treatment during the dark period. The compensation point increased from 1.6 to 5.6 $\mu g\, m^{-3}\, NH_3$ with increasing NH_4^+ concentrations in the nutritive solution for dark and light measurements. Dark and light compensation points were not significantly different for the same N treatment. Table 1 represents the different compensation points calculated at leaf temperature and extrapolated to a temperature of 20°C for the different N treatments and for light and dark periods.

In Figure 7 we can see calculated values of the NH_3 compensation point for different N treatments during light period using the total resistance to vapour exchange method. All χ_s values were plotted for a single treatment along with the average. Concerning the light period compensation points, there was no significant difference between the 10 mM NO_3^- + 2 mM NH_4^+ and the 10 mM NO_3^- + 5 mM NH_4^+ treatments. However the difference was significant with the other treatments. We notice a high variability of the compensation point; these points are correlated with variability in the resistance to water exchange. Dark period compensation point measurements were all significantly different from one another and had higher values than light period compensation points. These dark period measurements should be treated with caution mainly due to the large standard deviation particularly for the 10 mM NO_3^- + 2 mM NH_4^+ and the 10 mM NO_3^- + 5 mM NH_4^+ treatments but also due to the fact that dark water exchange resistance values are large. The calculated compensation points using the resistance analogy method at leaf temperature and extrapolated to a temperature of 20°C are shown in Table 1.

3.2.4. Cuticular deposition

NH_3 can be deposited on leaf surfaces in non-negligible amounts (Flechard 1998). Deposition depends on leaf surface characteristics, relative humidity and surface chemistry among others (Adema and Heeres 1995; Sutton *et al.* 1995; van Hove *et al.* 1989). We were unable from the chamber measurements to differentiate stomatal from cuticular fluxes of NH_3. Especially since during dark periods, we had non-negligible water fluxes, which led us to think that stomata were not completely closed. We tried to have relatively low water vapour concentrations in the chamber to minimize deposition on the cuticle (RH varied between 30 and 45%), which in turn created a large vapour pressure deficit probably being the cause of the non-zero stomatal flux in the dark periods. In order to evaluate the potential cuticular contribution to the flux, we compared the cuticular resistance (R_{cut}) for NH_3 with the total resistance to water exchange (R_{tot}). We chose the parameterisation of Nemitz *et al.* (2000) as it was fit to NH_3 fluxes over oilseed rape plants. Figure 8 represents these resistance values

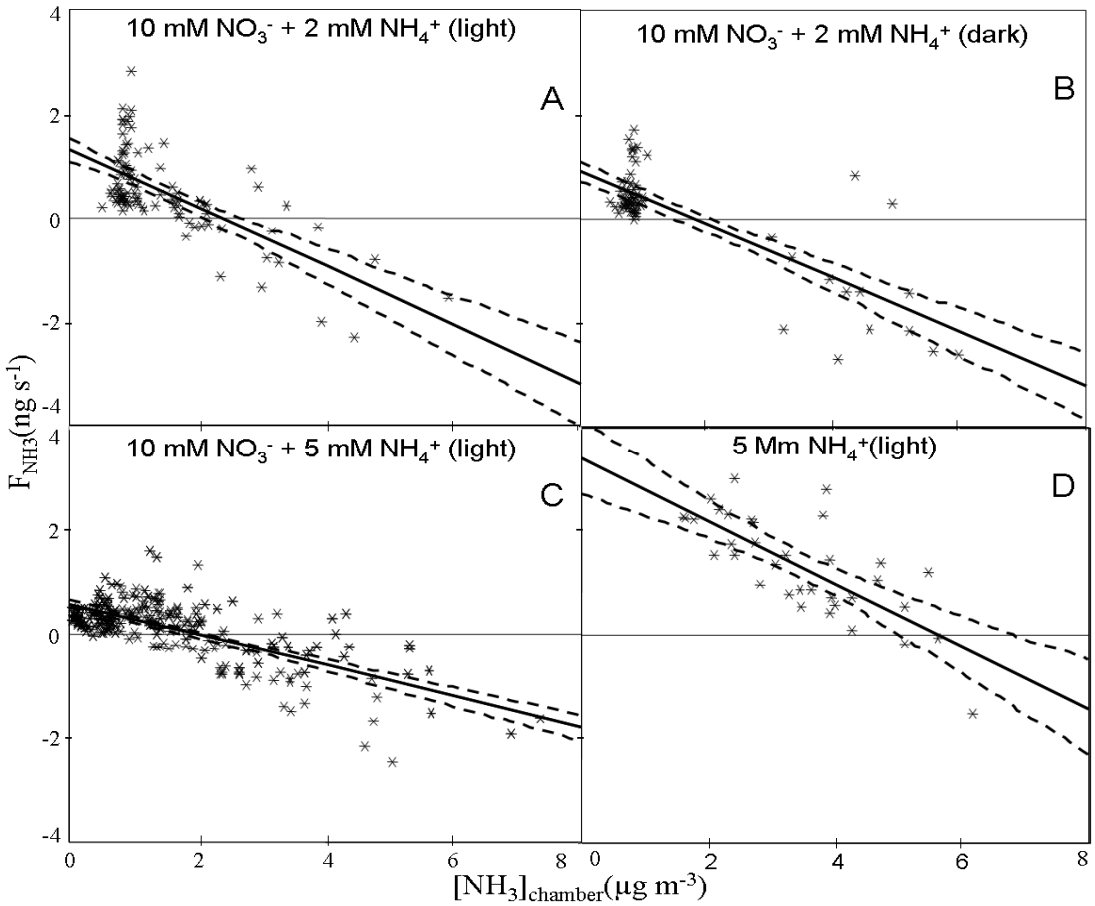

Figure 2.6: Linear regressions between plant ammonia fluxes and NH_3 concentrations in the chamber for different treatments.

Fluxes are in ng s^{-1} NH_3 and refer to the whole chamber system. Full lines represent the linear regression as calculated by SPSS 10.0; whereas dotted lines represent the confidence interval around the mean.

for the different N treatments. R_{tot} values varied between 100 and 2000 s m^{-1} for light periods and between 300 and 5000 s m^{-1} for dark periods. Concerning cuticular resistance it mainly varied with the chamber's relative humidity between 1700 and 29000 s m^{-1}. The minimal interval between R_{cut} and R_{tot} was 1500 s m^{-1} except for a 12 hour dark episode where R_{cut} was smaller than R_{tot}. This episode corresponds to the beginning of a measurement cycle and we considered that plants might have not been fully adapted to the chamber yet and hence was discarded in the dataset. Cuticular exchange of NH_3 is subject not only to the cuticular resistance to NH_3 transfer, but also to boundary layer (R_{bl}) and aerodynamic chamber (R_a) resistances. Given the measured stomatal resistance to water exchange during the light period using a Licor 6400 (~100 s m^{-1}), we can deduce R_a and R_{bl} and therefore have a rough estimate of cuticular fluxes considering that the NH_3 concentration at the canopy surface is zero. The sum of resistances for cuticular NH_3 exchange would range between 2000 and 45 000 s m^{-1} for light periods and between 6000 and 80 000 s m^{-1} for dark periods. The resulting cuticular fluxes would range between -0.04 and -1 ng m^{-2} s^{-1} for light periods and -0.03 and -2 ng m^{-2} s^{-1} for dark periods. Comparing those fluxes to the 'mini' canopy (set of plants in the chamber) fluxes they would induce a median error of 21 ± 20 % in light periods and 24 ± 30 % in dark periods.

3.2.5. NH_3 vs. H_2O resistance to exchange

Calculated resistance values to water exchange from water fluxes and calculated resistance values to NH_3 exchange from the linear regression method during the light period are represented in Figure 9. We notice that there is a significant difference between NH_3 and water vapour resistances. The resistance to water exchange is higher than the resistance to NH_3 exchange except for the 10 mM NO_3^- treatment. This is not surprising as stomatal and leaf boundary layer transfers are diffusive in general and therefore we have a factor of 0.92 between NH_3 and water resistances. We note also a significant difference in resistance values between the different N treatments; plants fed with NH_4^+ having a higher stomatal resistance.

3.3. Comparison between techniques

The calculated increase of the compensation points at 20°C measured by the chamber method relative to the 0 NH_4^+ treatment divided by the NH_4^+ concentration in the nutritive solution ranges between 0.2 and 0.7 for the light period. We notice a similar ratio with extraction measurements for apoplastic concentrations which are roughly 0.5 times the concentrations in the nutritive solution. The ratio of NH_4^+ to H^+ concentration in the apoplast, Γ, can be

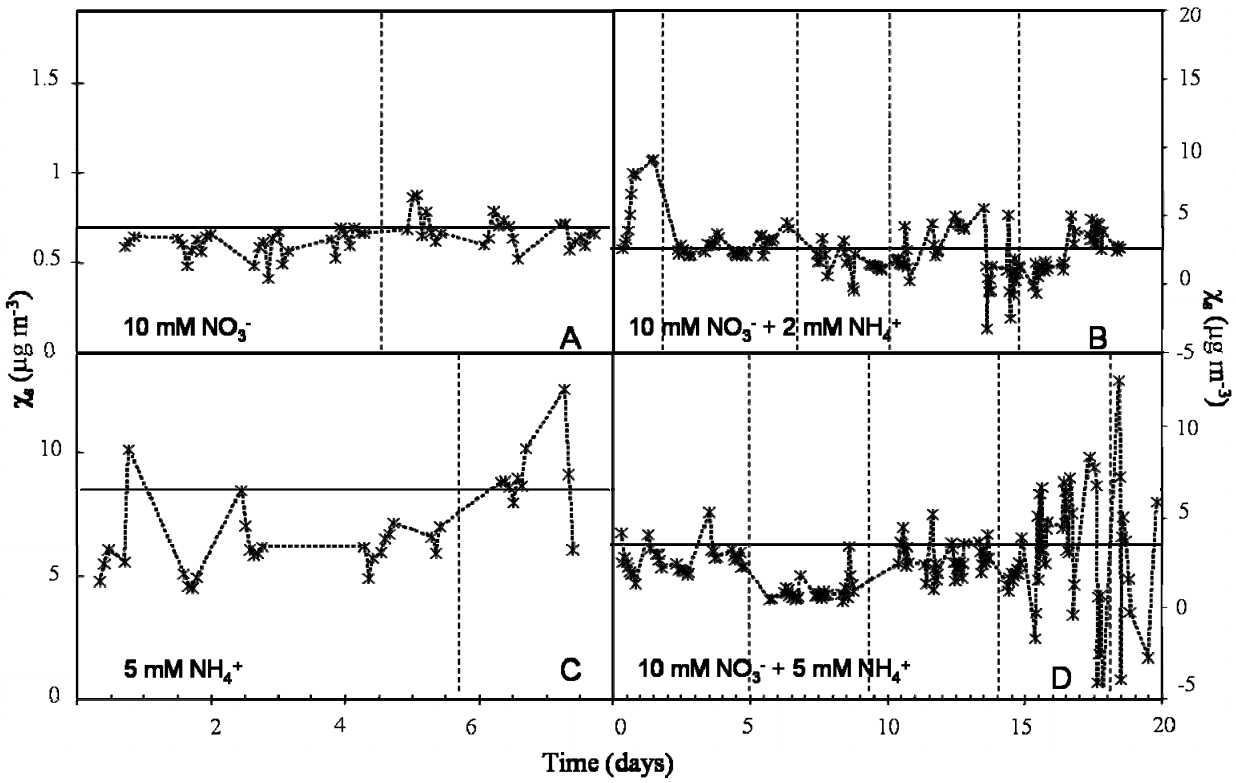

Figure 2.7: Plots of the NH_3 stomatal compensation points for light periods as calculated from total resistance to water exchange and NH_3 chamber fluxes measurements.

Asterisks represent χ_s values, full lines represent means. The four different plots represent four different N treatments; the vertical dotted lines in each plot represent separation between the different repetitions.

calculated from extraction measurements as well as from chamber compensation point measurements. Γ represents a good comparison parameter between the two measurement techniques because it is temperature independent and little assumptions are made to derive it. Figure 10A illustrates a comparison of Γ values calculated from extraction measurements and from chamber measurements for different N treatments and light and dark periods without taking into consideration any errors due to measurement technique. Extraction Γ were clearly larger than chamber Γ in light periods. Hill et al. (2001) calculated higher compensation points with the chamber measurements than with the extraction measurements, mainly because measured compensation points were low. Figure 10B represents recalculated Γ values with the different methods after error correction. Extraction measures were corrected by subtracting 3.3% of the NH_4^+ apoplast concentration; while chamber measures were calculated after correcting for cuticular and chamber fluxes for every measurement point. The discrepancy was clearly diminished after we account for mentioned errors. However, extraction measurements were still over estimated with respect to chamber measurements. (INSERT FIGURE 10)

4. Discussion

Two methods for measuring NH_3 plant fluxes are used nowadays and it is still problematic to evaluate the accuracy of both these methods: the chamber and the infiltration/centrifugation techniques

Apoplast, xylem sap and bulk tissue solution extraction

The infiltration/centrifugation technique was commented by several authors. Felle and Hanstein (2002) evaluated pH changes after flooding of the apoplast. They found an increase of approximately 1.5 units after infiltration; noticing that it took several hours for the pH to come back to the initial values. This could explain the relatively high pH values we measured and the little difference between the N treatments. To our knowledge it is difficult to assess apoplastic NH_4^+ concentrations with a non-invasive technique and therefore the effect of apoplast flooding could not be examined. Lohaus et al. (2001) tested several infiltration solutions, times and centrifugal forces on ion concentrations in the apoplast. They found no difference between the solutions used however they recommend a short infiltration time (<5 minutes) and a centrifugal force less than 1000g. Mattsson and Schjoerring (2002) suggested an equilibration time of 15 minutes after infiltration and before centrifugation. We applied this equilibration time for apoplast extractions. If the extracted pH was systematically larger

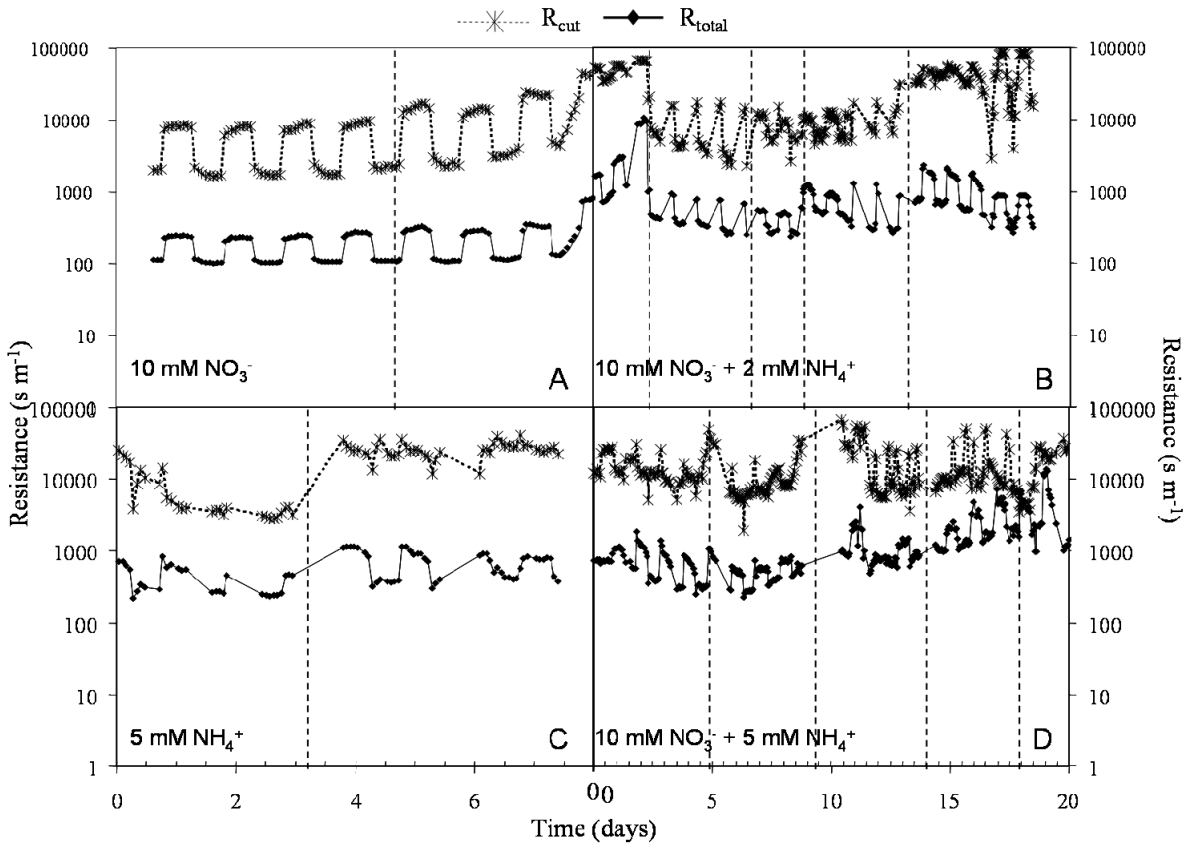

Figure 2.8: Comparison of total resistance to water exchange and cuticular resistance to NH_3 exchange for the different N treatments.

Asterisks represent cuticular resistances in s m^{-1} calculated according to Nemitz et al. (2000); dots represent total resistance to water exchange in s m^{-1} calculated from water fluxes. The four different plots represent four different N treatments; the vertical dotted lines in each plot represent separation between different repetitions.

than the in situ pH, the extraction technique would systematically give larger χ_s than the chamber technique.

Another consideration while dealing with NH_4^+ is the ion analysis technique. Husted et al. (2000) evaluated several methods and concluded that an NH_4^+ selective method is necessary since amino acids and labile amines could interfere if using colorimetric and chromatography methods leading to an over-estimation of NH_4^+ concentrations.

The apoplastic NH_4^+ concentrations we measured for the 0N treatments were higher than those reported in literature since Finnemann and Schjoerring (1999) reported 0.04 mM for oilseed rape grown on 0N and Mattsson et al. (1998) 0.04 mM for barley grown on 0N. On the contrary, apoplastic NH_4^+ concentrations we found for plants grown on NO_3^- or NH_4^+ are similar to the literature: 0.175 mM with 6 mM NO_3^- (Schjoerring *et al.* 2002) and 1 mM with high mixed N nutrition (Husted and Schjoerring 1995a) for oilseed rape and 1.9 mM for barley grown on 5 mM NH_4^+ (Mattsson *et al.* 1998). Mattsson et al. (1998) also reported that barley plants supplied with NO_3^- had a very low NH_3 emission rate compared to plants transferred to NH_4^+ based nutrition, hence in agreement with the low apoplast NH_4^+ concentration found for the 10 mM NO_3^- treatment. Concerning pH values similar results were found by Mühling and Sattelmacher (1995) on field bean and by Dannel et al. (1995) on sunflower leaves. However, Hoffman et al. (1992) measured apoplastic pH values in sunflower to be lower for NH_4^+ grown plants (5.8) compared to NO_3^- grown ones (6.3). Figure 2A shows the opposite behaviour. We had lower pH values for leaves with a mixed N nutrition although not significant. This is probably due to a root rather than shoot assimilation of nitrogen which is also reported by Pearson et al. (1998). For NO_3^- concentrations, measurements were 10 times higher than those reported by Muhling and Sattelmacher (1995) for wild beans and by Cookson et al. (2005) for Arabidopsis; but were similar to those reported by Nikolic and Romheld (2003) for the weak N treatment in sunflower.

The major drawback of the infiltration/centrifugation method is that it lacks to represent spatial variability of the apoplast pH and NH_4^+ concentrations. It was also criticised for changing the equilibrium between the apoplastic solution and the adjacent cytoplasm and having a high possibility of contamination of the extract with cytoplasmic content. We could only estimate the error due to this method by evaluating the contamination of the apoplast by cytoplasmic constituents; the reference used being malate dehydrogenase activity. In our conditions the contamination was around 3.3 %. When we corrected for this error, extraction measurements were in better agreement with chamber measurements.

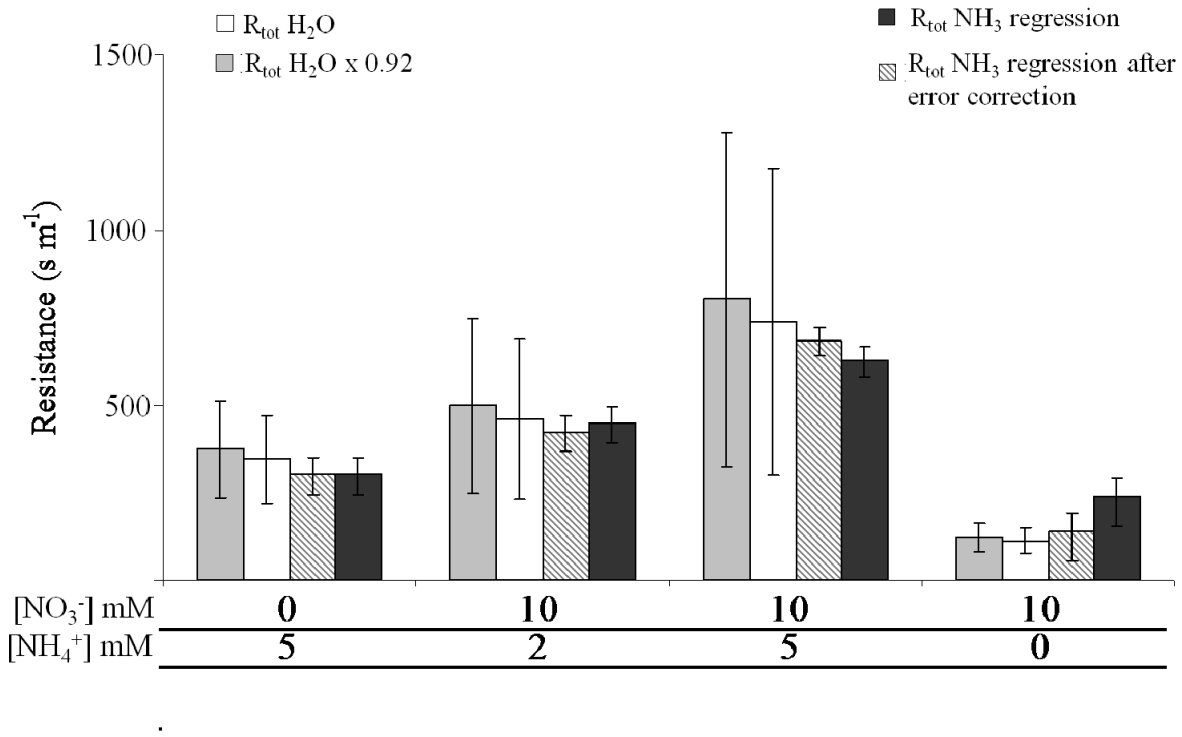

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Figure 2.9: Comparison of total resistance to water exchange and total resistance to NH_3 exchange for the different N treatments.

Black bars represent the total resistance to NH_3 exchange in s m^{-1} calculated from the slope of the linear regression between the plant's NH_3 flux and the NH_3 concentration in the chamber. Whereas striated bars represent the total resistance to NH_3 exchanges calculated in the same manner only after correction for cuticular and chamber adsorption. White bars represent total resistance to water exchange ± standard deviation in s m^{-1} calculated from the plant's water flux measurements and grey bars these same resistances to water exchange multiplied by 0.92 (ratio of H_2O to NH_3 diffusivity in air).

Xylem sap NH_4^+ concentrations were higher than those reported by Schjoerring et al. (2002) and by Finneman and Schjoerring (1999) who had concentrations ranging between 0.3 and 0.6 mM for oilseed rape grown on NO_3^- and concentrations up to 5 mM for oilseed rape grown on 10 mM NH_4^+. Mattsson and Schjoerring (1996a) and Mattsson et al. (1998) measured concentrations for barley plants up to 2.5 mM for plants fed with 2mM NH_4^+ and 3 mM for plants fed with 10 mM NH_4^+. Concerning NO_3^-, concentrations were in the range of those measured by Nikolic and Romheld (2003) who reported 30 mM NO_3^- for sunflower grown with 4 mM NO_3^- and up to 70 mM for sunflower grown on 40 mM NO_3^-.

Bulk tissue NH_4^+ concentrations were in the same range as those reported by Finneman and Schjoerring (1999) and Schjoerring et al. (2002) for oilseed rape. However Schjoerring et al. (2006) reported an increase in bulk NH_4^+ concentrations in wild type tomato plants for extractions after a light period relative to extractions after a dark period possibly due to high rates of photorespiration. The NH_4^+ concentrations started to increase after 4 hours of light and peaked after 8 hours. We did the extractions after only four hours of light which could be too short a period to note a significant difference with the dark period. As for NO_3^-, Schjoerring et al. (2006) reported concentrations in the leaf bulk tissue around 70 µmol g^{-1} FW during light periods and up to 200 µmol g^{-1} FW during dark periods for tomato plants. Cookson et al. (2005) also reported differences in cytosolic NO_3^- concentrations between light and dark periods and explain this by the inactivation of NO_3^- reductase leading to the build up of NO_3^- in the cytosol.

Effect of nitrogen nutrition

Apoplast and xylem NH_4^+ and NO_3^- concentrations are positively correlated to NH_4^+ and NO_3^- concentrations in the nutritive solution. This is explained by the fact that the apoplast and the xylem sap are a continuous fluid (Canny 1995). However, xylem concentrations are 1.8 times larger than concentrations in the nutritive solution and bulk tissue concentrations are 1.4 times larger than concentrations in the nutritive solution irrespective of the nitrogen treatment. Thus upon root absorption, NH_4^+ is concentrated in the xylem sap and transported towards the different plant parts. These results might suggest that NH_4^+ is very actively transported from the apoplast to the cell compartments resulting in low NH_4^+ concentrations in the apoplast. Bulk tissue leaf NH_4^+ concentrations are mainly determined by the interaction with the plant's nitrogen and carbon metabolisms; photorespiration and NO_3^- reduction being the main sources of NH_4^+ and the GS/GOGAT cycle being the main sink of NH_4^+. As a consequence we could see the effect of different N treatments on the plant's total C/N ratio and leaf surface area (results not shown). It is important to distinguish in this scope the different effects

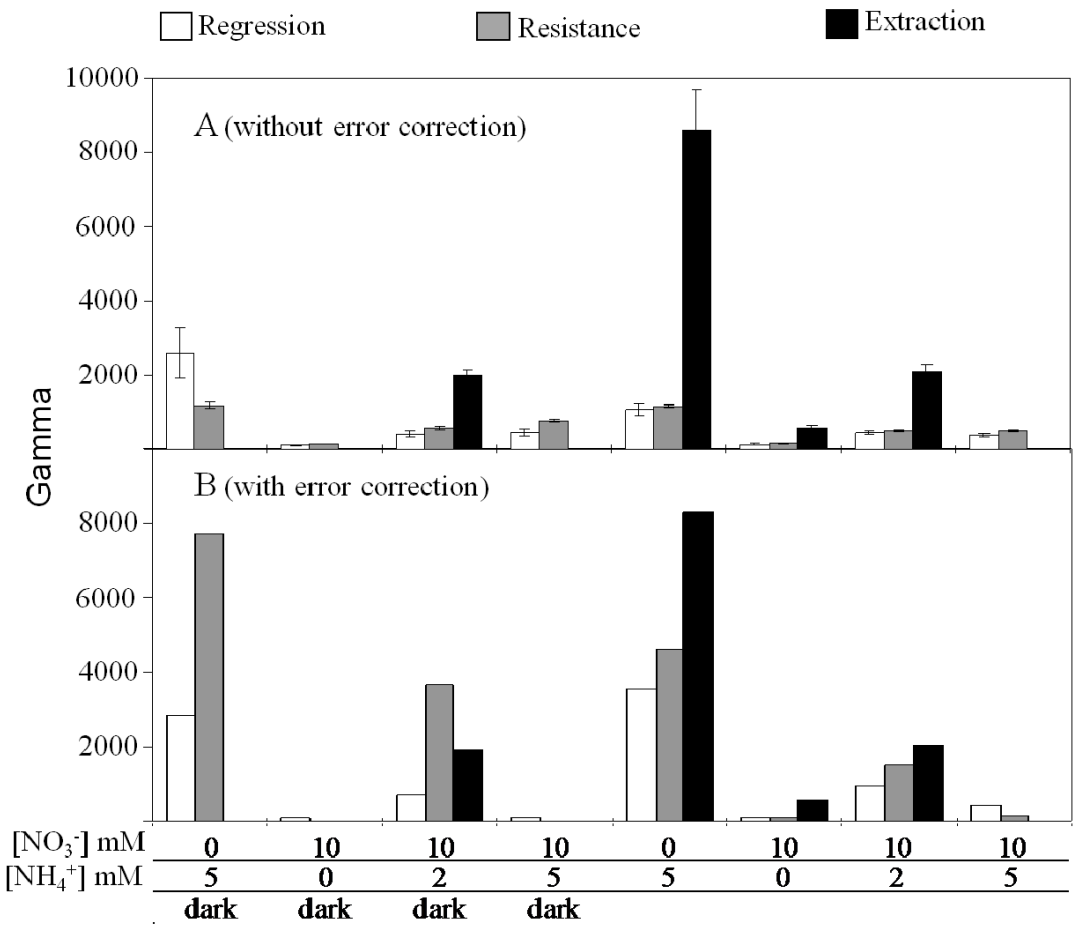

Figure 2.10: Comparison between Γ calculated from extraction measurements, chamber linear regressions and chamber total resistance to water fluxes for the different N and light and dark periods.

Black bars represent extraction Γ ± standard deviation; grey bars represent chamber resistance calculated Γ ± standard deviation and white bars represent regression calculated Γ ± confidence intervals around the mean.

produced by an NH_4^+, NO_3^- or mixed NH_4^+ and NO_3^- nutrition. NH_4^+ as a sole nitrogen source tended to favour plants with low surface areas, high C/N ratios and high compensation points relatively to plants with NO_3^- or mixed nutritive solutions. On the other hand, plants with a mixed nutritive solution even with high NH_4^+ concentrations had lower compensation points. This can be explained by the fact that NO_3^- induces several metabolic processes at the leaf level leading to a better assimilation of NH_4^+ ions. The N form also affected apoplastic pH and therefore the NH_3 compensation point. In general, it was agreed that plants relying on NO_3^- nutrition tend to have high apoplastic pH since nitrogen is assimilated in the shoots whereas plants relying on a mixed source of N (NO_3^-, NH_4^+ or organic-N), and which are more likely to favour root rather than shoot assimilation tend to have relatively low apoplastic pH (Pearson *et al.* 1998).

Effect of dark and light periods

We did not detect any significant difference in apoplast concentrations between light and dark periods. Almost all plant metabolic processes involved in NH_3 assimilation or production such as NO_3^- reduction, photorespiration, and xylem fluxes were reduced if not completely stopped during dark periods, which should normally result in having lower NH_4^+ concentrations which was partly observed although differences were not statistically proven. This aspect deserves to be better assessed as the response of the NH_3 stomatal compensation point to dark periods when plant metabolism is slow could help us determine and quantify important processes involved in the determinism of this compensation point.

Chamber measurements

Chamber measurements provided a non invasive technique for estimating the NH_3 compensation point along with other parameters influencing stomatal NH_3 fluxes. However, the chamber technique is also subject to several errors. We identified three major sources of error in chamber measurements other than errors that might be linked to apparatus precision.

One major consideration was that the NH_3 gas detection limit of the analysers used forced us to have several plants in our chamber and therefore the measured compensation point is an integration for several plants including all the leaves and stems. Husted and Schjoerring (1995a) showed that apoplastic NH_4^+ concentrations in oilseed rape plants were affected by leaf position; upper leaves having higher NH_4^+ concentrations.

Another concern in chamber measurements is the high reactivity of NH_3 gas and its ability to be dry deposited on surfaces. Comparing the empty chamber fluxes to the recorded fluxes from the "mini" canopy the chamber effect ranged from roughly 20 % when we had weak NH_3 concentrations in the chamber to 8% when we had high NH_3 concentrations. There wa

Table 2.1: Ammonia compensation points at leaf temperature and normalized at 20°C calculated by the two methods (resistance analogy and regression) as affected by N treatment and dark and light periods.

NO_3^- (mM)	NH_4^+ (mM)		Average leaf temperature °C	χ_s at leaf temperature ($\mu g\ m^{-3}$)	χ_s normalised at 20°C ($\mu g\ m^{-3}$)	R^2	Calculation method
0	5	dark	20.2 ± 0.1	10.46 ± 2.69	10.24 ± 2.63	0.256	regression
				12.45 ± 2.57	12.19 ± 2.52		resistance
10	0	dark	20.0 ± 0.1	0.53 ± 0.08	0.53 ± 0.08	0.213	regression
				0.80 ± 0.28	0.80 ± 0.28		resistance
10	2	dark	20.7 ± 0.1	1.76 ± 0.33	1.62 ± 0.30	0.637	regression
				7.22 ± 11.94	6.62 ± 10.95		resistance
10	5	dark	20.0 ± 0.1	1.79 ± 0.37	1.80 ± 0.37	0.362	regression
				2.91 ± 7.13	2.92 ± 7.16		resistance
0	5	light	22.5 ± 0.1	5.62 ± 0.83	4.18 ± 0.62	0.562	regression
				9.29 ± 2.35	6.91 ± 1.75		resistance
10	0	light	22.1 ± 0.1	0.61 ± 0.13	0.48 ± 0.10	0.191	regression
				0.81 ± 0.13	0.63 ± 0.10		resistance
10	2	light	22.3 ± 0.1	2.28 ± 0.28	1.74 ± 0.22	0.501	regression
				3.37 ± 1.70	2.57 ± 1.30		resistance
10	5	light	22.0 ± 0.1	1.90 ± 0.20	1.50 ± 0.16	0.501	regression
				2.71 ± 2.67	2.14 ± 2.11		resistance

therefore a non negligible error related to chamber adsorption especially for treatments with a small compensation point. Leaf cuticular deposition is also particularly problematic since leaf cuticles can act as NH_3 sinks or NH_3 capacitors (Flechard *et al.* 1999) thus leading to an underestimation of the NH_3 stomatal compensation point. We tried to evaluate the cuticular NH_3 fluxes by calculating the cuticular resistance. Although calculated resistance values were much bigger than calculated total resistance to water exchange, cuticular fluxes may represent more than half the NH_3 fluxes in the worst conditions. Cuticular resistance calculation remained uncertain due to the complex processes involved and the high variability with leaf type, surface chemistry, and relative humidity. Moreover, even with high cuticular resistance to NH_3 exchange, the flux can be non negligible especially in dark episodes.

Calculation methods of the NH_3 stomatal compensation point could also be questioned. The regression method assumes that χ_s is not dependent on the NH_3 flux, and hence also on the stomatal resistance. Van Hove et al. (1991) and Hanstein and Felle (1999) suggested that NH_3 fluxes between the leaf and its environment have an effect on stomatal resistance, NH_4^+ concentrations and pH values of the apoplast. This potential error was also present in the resistance-analogue method since the water and NH_3 resistances to exchange are considered equal and independent of the NH_3 flux. Moreover, for the calculation of χ_s using the resistance-analogy method we made the assumption that the total resistance to NH_3 exchange in the chamber was equal to the total resistance to water vapour exchange. This assumption is partially true since the total resistance in the chamber consists of the sum of stomatal resistance (R_s), leaf boundary layer resistance (R_{bl}) and chamber aerodynamic resistance (R_a). R_s and R_{bl} are resistances to diffusive transport of molecules and therefore should be different for water vapour and NH_3. In such conditions, R_s and R_{bl} for NH_3 should be multiplied by the ratio of water vapour to NH_3 diffusivity in air 0.92. Concerning R_a, if the transfer in the chamber is turbulent, R_a is independent of the transported gas; if the transfer in the chamber is due to molecular diffusion, then R_a should be multiplied by 0.92. Hanstein et al. (1999) reported values of total resistance to NH_3 exchange calculated from water fluxes to be higher than those calculated from linear regression. They explained it by the fact that NH_3 has a shorter path length for diffusion in the sub-stomatal cavity. This is particularly true since the difference between resistance to NH_3 exchange calculated from the linear regression and resistance to water exchange calculated from water fluxes increased for treatments with high NH_4^+ in the nutritive solution. This difference could also be explained by the error due to chamber and cuticle NH_3 adsorption. Husted and Schjoerring (1996) also compared leaf resistances to transfer relative to NH_3 and water vapour. The ratio they had between the two

values was 0.95 ± 0.08 which is very close to the theoretical value. If we subtract the estimated cuticular flux and the average chamber adsorption flux and recalculate the resistance to NH_3 exchange from the slope of the linear regression between F_{NH3} and $[NH_3]_{ch}$ the disparity between R_{tot} H_2O and R_{tot} NH_3 diminished (Figure 9).

We observed also higher stomatal resistances for plants fed with NH_4^+ than for plants fed with NO_3^- of a mixture of both. Similar responses were observed by Peuke et al. (1994) on *Ricinus communis* L. plants and by Lu et al. (2005) on tobacco plants. These higher stomatal resistances were attributed to higher abscisic acid (ABA) flows between the roots and the shoots in plants having NH_4^+ as nitrogen source. It was argued that increase in pH caused release of ABA which caused stomata to close (Felle and Hanstein 2002; Hartung et al. 1998). This N treatment effect could also be attributed to difference in leaf surface area since plants fed with 10 mM NO_3^- were smaller in general than the other plants thus inducing a smaller aerodynamic resistance in the chamber which is translated in the total resistance to exchange.

Not all sources of error were calculated here and there remains a lot of uncertainties related to each measurement technique which we can not quantitatively asses. Concentration gradients in the apoplast and in the plant cover could not be accounted for with the extraction measurements. Extracting the apoplast for the top most developed leaves could lead to having higher Γ since those leaves would be getting more light than lower leaves and thus having higher rates of photorespiration and NO_3^- reduction. These limitations of both measurement methods lead us to think that corresponding Γ might not be comparable and that adequate measurement technique should be chosen according on the purpose of the results needed. Apoplast extraction are more suitable for leaf and cell scale processes whereas chamber measurements would be more appropriate for flux measurements at the whole plant/canopy scale. Another concern relative to the chamber method is that conditions applied (low relative humidity, hydroponics, PPFD, …) are very different from field conditions and results could not be generalized. However, both techniques have the same response with respect to environmental factors and nitrogen nutrition and allowed us to evaluate the dependency of the NH_3 stomatal compensation point on those factors.

5. Conclusions

Apoplastic NH_4^+ and NO_3^- concentrations of oilseed rape plants grown in controlled conditions seemed to be positively correlated with NH_4^+ and NO_3^- concentrations in the nutritive solution. Extractions done after a dark period and after a light period had non-significantly different NH_4^+ concentrations in the apoplast and in the bulk tissues. Chamber

measurements also showed that the NH_3 stomatal compensation point is correlated with the NH_4^+ concentration in the nutritive solution. Mixed NO_3^- and NH_4^+ treatments had a masking effect on the NH_3 compensation point probably due to increased NH_4^+ assimilation. Total resistance to NH_3 exchange calculated from the linear regression method was significantly different from total resistance calculated from water fluxes. Furthermore the N treatment seemed to be influencing the total resistance to exchange most probably through the effect on stomatal resistance. Calculated Γ from extraction measurements were overestimated with respect to calculated Γ from chamber measurements. After accounting for the errors relative to each measurement technique, discrepancies were diminished, but were still significant. We still do not have another method to assess the NH_3 stomatal compensation point and the choice between these two measurement techniques should be based on scales to which the measurements are to be applied and the processes to study. Further investigations of light and dark effects on the NH_3 stomatal compensation point as well as differences between water and NH_3 stomatal resistances and a better assessment of NH_3 fluxes to the leaf cuticle are necessary.

Chapitre 3 :

Modèle du point de compensation stomatique de l'ammoniac

« La théorie, c'est lorsqu'on sait tout et que rien ne fonctionne. La pratique, c'est lorsque tout fonctionne et que personne ne sait pourquoi. »

Albert Einstein (Physicien : 1879-1955)

En raison de la difficulté des mesures et pour mieux comprendre les interactions entre les différents processus, il est nécessaire de développer des modèles de prédiction des flux d'ammoniac. Les premiers modèles développés sont des modèles classiques d'échanges gazeux entre la végétation et l'atmosphère qui font l'analogie avec la loi d'Ohm et où les flux sont le produit d'un gradient de concentration et de sommes de résistances aux échanges en parallèle ou en série (ex. Monteith & Unsworth, 1990). On distinguera plus tard les échanges dus à la litière et aux sols des échanges avec la végétation ; les échanges stomatiques des échanges cuticulaires (Sutton et al. 1995) ; et la différence entre plusieurs couches de la végétation due à une différence de potentiel d'émission liée à l'âge et à la position de la feuille dans le couvert (Nemitz et al. 2000 ; Nemitz et al. 2001). Ces modèles prennent comme variable de forçage le point de compensation en ammoniac issu des mesures par méthodes de flux (Husted & Schjoerring, 1995b) ou encore la concentration en ions ammonium dans l'apoplasme issue de mesure par méthode d'extraction (Husted & Schjoerring, 1995a). Comme nous l'avons vu dans les précédents chapitres, les différentes mesures démontrent la variabilité du point de compensation en fonction de la nutrition azotée, de l'espèce végétale et de l'âge de la feuille (Massad et al. 2008b ; Mattson et al. 1998 ; Mattson et al. 2003).

Dû à la difficulté de mesures du point de compensation et en l'absence de base de données pour les différentes espèces végétales et dans différentes conditions il y a une nécessité d'intégrer la variabilité du point de compensation dans les modèles d'échanges végétation-atmosphère. Il nous parait intéressant de coupler un modèle d'échange d'ammoniac entre la végétation et l'atmosphère avec un modèle de fonctionnement du couvert faisant ainsi dépendre le point de compensation du métabolisme carboné et azoté de la plante. Riedo et al. (2002) ont ainsi couplé un modèle de fonctionnement de prairie (PaSim) avec un modèle d'échange d'ammoniac. Cette approche n'a cependant pas été adaptée à d'autres types de cultures.

Ce chapitre reprend à la fois les simplifications décrites dans le chapitre 1 et les processus mis en évidence dans le chapitre 2 afin d'aboutir à la construction d'un modèle numérique. Ce modèle couple le métabolisme azoté au métabolisme carboné de la plante et permet de prendre en compte les facteurs du milieu (rayonnement, température, humidité, concentration atmosphérique en ammoniac,…) et la nutrition azotée.

A. Un modèle du point de compensation stomatique de l'ammoniac

Le modèle comprend quatre compartiments : la cavité sous stomatique, l'apoplasme, le cytoplasme et la vacuole. Nous faisons un bilan de masse : au niveau de l'apoplasme des ions ammonium et nitrate, au niveau de la cavité stomatique des molécules d'ammoniac, au niveau du cytoplasme des ions ammonium et nitrate ainsi que des molécules d'azote et de carbone organique et au niveau de la vacuole des ions nitrate et ammonium. Nous considérons les transferts de nitrate, d'ammonium et d'ammoniac entre les différents compartiments ainsi qu'entre la cavité sous stomatique et l'atmosphère et entre le xylème et l'apoplasme. Les sources d'ammoniac dans le cytoplasme sont la réduction du nitrate et la photoréspiration et le puits d'ammoniac se limite à l'assimilation en acides aminés par le cycle GS/GOGAT. C'est ce cycle GS/GOGAT qui couple le métabolisme azoté et carboné car la production d'acides aminés fait intervenir deux substrats : les ions ammonium et les molécules de carbone organique issues de la photosynthèse. La photosynthèse est simulée selon le modèle de Farquhar et al. (1980a).

B. Etude de sensibilité du modèle

Une étude de sensibilité des variables du modèle aux paramètres liés aux compartiments a montré que le point de compensation est surtout conditionné par le pH et le volume de l'apoplasme ainsi que par la vitesse du transport actif d'ammonium entre l'apoplasme et le cytoplasme. Il existe beaucoup de mesures du pH de l'apoplasme et nous remarquons une grande variabilité entre les espèces et les differentes conditions environnementales notamment la nutrition azotée. La gamme de variation s'étend entre 5.8 et 6.6. Le volume apoplastique est lui aussi bien documenté et présente une gamme de variation plus faible que le pH. Il existe par contre très peu d'information sur le transfert actif d'ammonium entre l'apoplasme et le cytoplasme dans les feuilles.

La sensibilité du modèle aux termes sources et puits a aussi été étudiée. Les concentrations en ammonium dans l'apoplasme et le cytoplasme sont très sensibles aux changements de la vitesse maximale de l'assimilation de la GS et du point de compensation du CO_2 sans respiration de nuit (Γ^*). Cependant l'effet est moins marqué sur le point de compensation en ammoniac. Ceci nous amène à constater que le compartiment cytoplasmique et le compartiment apoplasmique sont relativement découplés en conditions de bonne alimentation azotée.

C. Réponse aux variables de forçage

La réponse du modèle aux variables de forçage démontre une grande dépendance du point de compensation au flux d'eau, à la température de feuille et à la concentration en ammonium dans le xylème ; tandis que la concentration en ammoniac atmosphèrique a un effet négligeable sur le point de compensation.

D. Comparaison modèle-mesures

Le point de compensation mesuré par la methode de chambre à flux à été comparé au point de compensation simulé par le modèle en le forçant avec les flux d'eau, le PAR, la température de feuille, et la concentration en ammonium dans le xylème mesuré. On constate que le modèle sous-estime le point de compensation pour les fortes concentrations en ammoniac et qu'une meilleure estimation des paramètres clés notamment le transport actif d'ammoniac entre le cytoplasme et l'apoplasme est nécessaire.

Modèle du point de compensation stomatique de l'ammoniac en relation au métabolisme azoté et carboné de la plante.

"Massad R.S. Tuzet A., Loubet B., Perrier A. and Cellier P. Model of STomatal AMmonia compensation Point (StAmP) in relation to the plant nitrogen and carbon metabolisms. Soumis à " Plant Cell & the Environment."

Abstract

Agricultural crops can be either a source or a sink of ammonia (NH_3). Most NH_3 exchange models developed so far do not account for the plant's nitrogen (N) metabolism and use prescribed compensation points. We present here a leaf-scale simplified NH_3 stomatal compensation point model related to the plant's N and carbon (C) metabolism, for C_3 plants. Five compartments are considered: xylem, cytoplasm, apoplasm, vacuole and sub-stomatal cavity. The main processes accounted for are the transport of NH_4^+, NH_3 and NO_3^- between the different compartments, NH_4^+ production through photorespiration and nitrate reduction, NH_4^+ assimilation, chemical and thermodynamic equilibriums in all the compartments, and stomatal transfer of NH_3.

The simulated compensation point is sensitive to parameters related to the apoplastic compartment: pH, volume and active transport rate. Determining factors are leaf temperature stomatal conductance, and ammonium flux to the leaf. Atmospheric NH_3 concentration seem to have very little effect on the compensation point. Comparison of model outputs to experimental results show that the model underestimates the NH_3 compensation point for high nitrogen fertilisation and that a better parametrisation of sensitive parameters especially active transport rate may be required.

Key words

Ammonia emission; Water flux, Nitrogen fertilisation; Apoplast, Stomatal exchange.

1. Introduction

Ammonia (NH_3) is a major atmospheric pollutant for natural ecosystems. Its depostion causes soil acidification, eutrophication and alters biodiverstiy by favoring nitrophilic species (Fangmeier *et al.* 1994; Hornung *et al.* 1995). It also contributes to the formation of ammonium aerosols, altering therefore the transport of acidic pollutants. Agriculture is the major source of NH_3 (Asman *et al.* 1998; Bouwman *et al.* 1997) and natural vegetation is the major sink. Plants can be either a source or a sink of NH_3 depending on the so-called NH_3 compensation points of the plant, which is the concentration in the sub-stomatal cavity in equilibrium with the atmosphere and the apoplastic solution (Farquhar et al. 1980b).

Modelling surface-atmosphere NH_3 exchange is based on resistance-analogy approaches (eg. Nemitz *et al.* 2000b; Sutton *et al.* 1995). These models often take as entry variable either the concentration of NH_3 in the sub-stomatal cavity (χ_s) also called the stomatal compensation point. Another parameter is often used as being not dependent on temperature: gamma (Γ) It it the ratio of ammonium concentration to pH in the apoplast. Both these variables are determined empirically. Based on a modelling approach, in the Netherlands an "ammonia gap" was identified between the expected reduction in NH_3 concentration following policies to reduce NH_3 emissions and atmospheric monitoring of NH_3 (Erisman *et al.* 1998). The explanation for such a gap is primarily attributed to interactions with atmospheric chemistry (SO_2 and NO_y), fluctuation in weather conditions and interactions with changes in agricultural practices (Loubet *et al.* 2008). However, Sutton et al. (2003) pointed out that the mechanisms of dry exchange of NH_3 between vegetation and the atmosphere with different plant canopies is an issue needing to be addressed for a better understanding of atmospheric NH_x determinism.

The NH_3 compensation point is known to vary with temperature, nitrogen nutrition, plant's developmental stage, and species (Husted *et al.* 1996; Husted & Schjoerring 1996; Mattsson *et al.* 1998; Mattsson & Schjoerring 2003; Nemitz *et al.* 2000a). All these factors are tightly linked with the plants nitrogen and carbon metabolisms (Massad *et al.* 2008a). To our knowledge, there is only one model developed by Riedo et al. (2002) for grasslands which models the dynamics of χ_s in relation to the plants nitrogen nutrition and development stage.

Linking nitrogen metabolism, photosynthesis and respiration with NH_3 exchange and water transfer through the plant will allow a better understanding of the NH_3 stomatal compensation point and its time and space variability. We present here a leaf-scale simplified NH_3 stomatal

compensation point model related to the plant's N and C metabolism, for C_3 plants. We investigate the model's sensitivity to various parameters and forcing variables and we compare the model outputs (NH_3 fluxes and NH_4^+ concentrations in different compartments) to experiments and values reported in the literature

2. Model description

2.1. Model overview

Based on the literature review and simplifications described in Massad et al. (2008a), we elaborated a stomatal leaf-atmosphere exchange model of dry NH_3. This model is intended to simulate NH_3 emission and/or deposition into leaf air space in relation to the leaf's N and C metabolisms and the plant N status and water exchange. This model is intended to represent a single leaf of a C_3 type of plant knowing that photorespiratory pathways are different between C_3 and C_4 type of plant. We also choose to represent a leaf in a vegetative stage of growth knowing that the phenomena of senescence has an important effect on plant NH_3 metabolism (Masclaux *et al.* 2000) thus complicating our preliminary modelling approach.

– **Model compartments**: Leaf structure is divided into three liquid compartments and one air compartment: vacuole, cytoplasm, apoplast and leaf air space respectively. The model is shown schematically in figure 1. We considered that all the reactions taking place in cellular organelles (mitochondria, chloroplasts) take place in the cytoplasm for simplicity reasons. When making mass balance, the apoplastic and leaf air space compartments are considered as one since we only have physical equilibrium between the two for NH_4^+/NH_3 and no transfer.

– **Nitrogen nutrition:** The model considers either ammonia NH_x ($NH_4^+ + NH_3$) based nutrition, nitrate NO_3^- based nutrition or a combination of both. For the time being the root organs are not included in the model and the forcing variables are NO_3^- and NH_x concentrations in the xylem sap. In each compartment we consider a mass balance equation for each nitrogen form (NH_x and NO_3^-).

– **Transfer and equilibrium:** The nitrogen reaches the leaf apoplast via the xylem. In the apoplast and leaf air space, there is an equilibrium between the aqueous ammonia form ($NH_{3(aq)}$), the gaseous ammonia form ($NH_{3(g)}$) and the aqueous ammonium form ($NH_4^+{}_{(aq)}$) and which are temperature and pH dependent. In all other liquid compartments (cytoplasm and vacuole) we have an equilibrium between NH_3 and NH_4^+. NH_4^+ and NO_3^- are actively transported from the apoplast to the cytoplasm whereas $NH_{3(aq)}$ diffuses between the

cytoplasm and apoplast and $NH_{3(g)}$ diffuses between leaf air space and the atmosphere. NH_x and NO_3^- can be temporarily stored in the vacuole. NH_x and NO_3^- diffuse across the tonoplast whereas NO_3^- is actively transported from the cytoplasm to the vacuole.

– **Sources and sinks of NH_x:** We consider that the main sources of NH_x in the cytoplasm are photorespiration and nitrate reduction (Joy 1988) whereas assimilation of NH_x mainly occurs via the Glutamine synthetase (GS), Glutamate synthase (GOGAT) cycle (Leegood *et al.* 1995). Photorespiration is modelled according to Farquhar et al. (1980a).

– **Forcing variables:** The model's forcing parameters are the atmospheric NH_3 ($[NH_3]_{atm}$) and CO_2 concentrations, photosynthetic active radiation (PAR), leaf temperature (T_l), stomatal conductance (g_s), water flow through the xylem (F_{H2O}) and NH_4^+ and NO_3^- concentration in the xylem ($[NH_x]_{xy}$ and $[NO_3^-]_{xy}$). The model is intended to be coupled with the Tuzet et al. (2003) soil-plant-atmosphere water continuum model. For the time being we only take the Tuzet et al (2003) model output parameters (T_l, g_s and F_{H2O}) as input variables for a typical day. StAmP is coupled to the Farquhar et al. (1980a) photosynthesis model for which atmospheric CO_2, PAR, g_s and T_l are common forcing parameters. The Farquhar et al. (1980a) model outputs: CO_2 assimilation rate (A_{CO2}) and dark respiration rate (R_d) are inputs to our model.

Symbols, descriptions, initial values and units of variables and forcing variables are given in Table 1 and those of internal parameters in Table 2. The model functions with a time step of 15 minutes. Fluxes are in mole substrate per square meter leaf surface and per second whereas concentrations are in mole per cubic meter.

2.2. Model equations

We detail here how the different processes, transfers and equilibriums were modelled. For each compartment we give the two general mass balance equations for NH_x and NO_3^- and then detail the equations and parameterization of each flux.

2.2.1. *Apoplast and leaf air space compartment*

In this compartment we consider two compounds, NO_3^- and NH_x. In order to calculate concentrations of NH_x and the stomatal fluxes of NH_3, we considered the balance equation of the apoplast and leaf air space together since there is an equilibrium of $NH_{3(g)}$ and $NH_{3(aq)}$ linking these two compartments. Figure 2 illustrates a flow diagram of the main fluxes and pools of the model compartments. Thus, the NH_x/NH_3 apoplastic/leaf air space pool is filled with input from the xylem ($F_{NHx(xy)}$) and filled/emptied by exchange via the stomata (F_{sNH3})

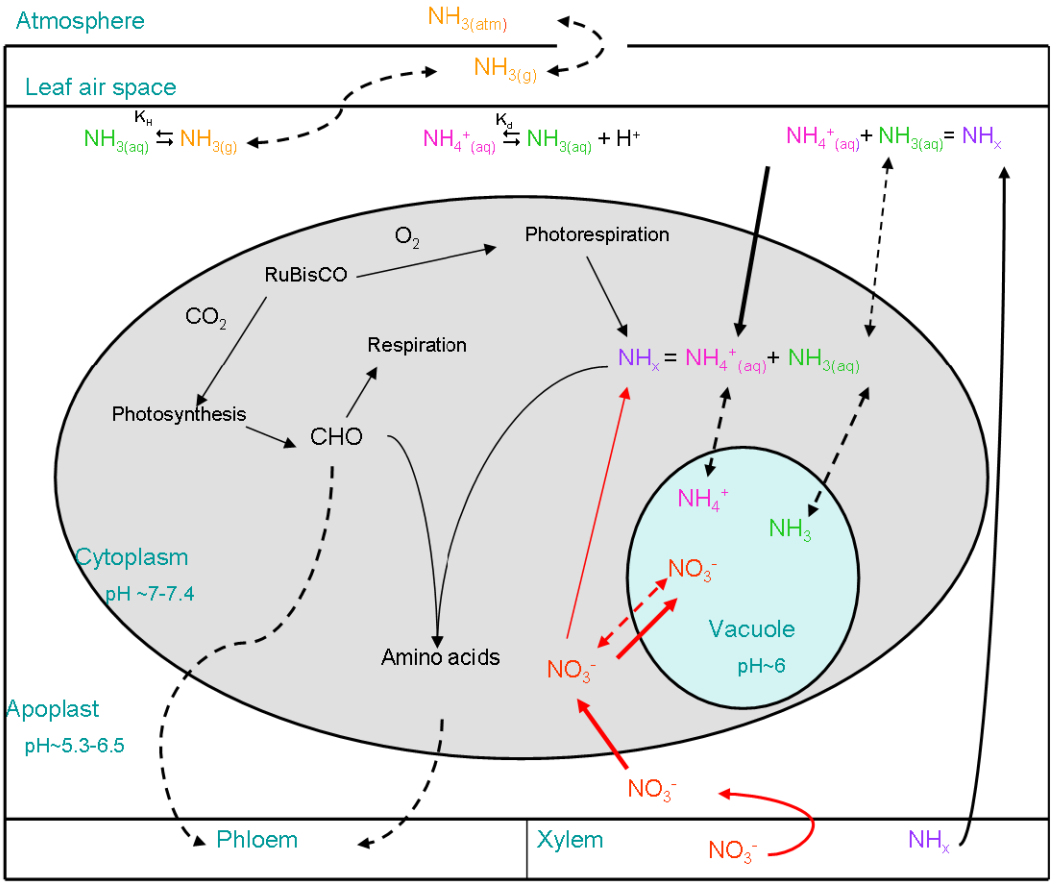

Figure 3.1: Model processes scheme

Scheme of the main processes considered in the stomatal leaf-atmosphere exchange model of dry ammonia. Dotted arrows represent diffusive transport and equilibria while full lines represent chemical transformations and active transport rates.

and with active transport of ammonium ($F_{NH4(ap-cy)}$) and the passive exchange of NH_3 ($F_{NH3(ap-cy)}$) between the apoplast and the adjacent cytoplasm.

Therefore the mass balance of NH_x and $NH_{3(g)}$ in the apoplast/ leaf air space is the following:

$$\frac{d[NH_x]_{(ap)} \times V_{ap} + d[NH_3]_g \times V_{sc}}{dt} = F_{NHx(xyl)} - F_{sNH3} - F_{NH4(ap-cy)} - F_{NH3(ap-cy)} \quad (1)$$

Where $[NH_x]_{ap}$ and $[NH_3]_g$ are the concentrations of NH_x in the apoplast and gaseous NH_3 in the leaf air space respectively. V_{ap} and V_{sc} are the relative volumes of the apoplast and leaf air space compartments per leaf square meter, respectively.

Concerning NO_3^-, we only need to consider the apoplastic compartment since the influx of oxidized forms of nitrogen through the sub-stomatal cavity is not taken into account. Thus, the apoplastic NO_3^- pool is filled from the import via the xylem ($F_{NO3(xy)}$), and emptied by the exchange of NO_3^- with the cytoplasm (F_{NO3ac}). The mass balance equation is the following:

$$\frac{d[NO_3]_{(ap)} \times V_{ap}}{dt} = F_{NO3(xy)} - F_{NO3ac} \quad (2)$$

2.2.1.1. Acid/Base and thermodynamic equilibriums

NH_3 concentrations in the leaf air space are largely dependent on apoplastic pH and therefore on the acid-base equilibrium between $NH_4^+{}_{(aq)}$ and $NH_{3(aq)}$. It is also important to note that ammonium transport into different cellular compartments depends not only on the electrochemical gradient but also on the pH difference across this membrane and that theoretically, a significant proportion of the total NH_3/NH_4^+ is in the form of $NH_{3(aq)}$ due to the relatively high pH in the cytosol, and could hence freely diffuse across membranes (von Wiren *et al.* 2000). It is therefore important to consider the acid base equilibrium of NH_3/NH_4^+ in each of the liquid compartments. There is also a thermodynamic equilibrium between $NH_{3(aq)}$ and $NH_{3(g)}$ in the apoplast/ leaf air space compartments dependent on temperature. For simplicity reasons the pH values in the different compartments are considered to be fixed parameters and not variables although the pH of the apoplast is thought to be partly regulated by the N nutrition of a species (Pearson *et al.* 1998), the NH_3 flux through the sub-stomatal cavity (Felle & Hanstein 2002) and by the regulation of the intracellular pH (Felle 2001).

The acid/base equilibrium between $[NH_3]$ and $[NH_4^+]$ in solution given by:

Tableau 3.1: Symbols, descriptions, units and initial values of variables and forcing parameters

Symbol	Description	Unit	Initial Value
State variables			
$[NH_3]_g$	Gaseous NH_3 concentration in the leaf air space	mol m^{-3}	$3.80x10^{-11}$
$[NH_3]_{ap}$	Aqueous NH_3 concentration in the apoplast	mol m^{-3}	$6.50x^{-5}$
$[NH_4^+]_{ap}$	NH_4^+ concentration in the apoplast	mol m^{-3}	$6.00x10^{-1}$
$[NH_x]_{ap}$	NH_x concentration in the apoplast	mol m^{-3}	$6.00x10^{-1}$
$[NH_3]_{cy}$	NH_3 concentration in the cytoplasm	mol m^{-3}	$9.40x10^{-3}$
$[NH_4^+]_{cy}$	NH_4^+ concentration in the cytoplasm	mol m^{-3}	44
$[NH_x]_{cy}$	NH_x concentration in the cytoplasm	mol m^{-3}	44
$[NH_4^+]_{vac}$	NH_x concentration in the vacuole	mol m^{-3}	19
$[NH_3]_{vac}$	NH_3 concentration in the vacuole	mol m^{-3}	1
$[NO_3^-]_{cy}$	NO_3^- concentration in the cytoplasm	mol m^{-3}	2.8
$[NO_3^-]_{ap}$	NO_3^- concentration in the apoplast	mol m^{-3}	0.3
$[NO_3^-]_{vac}$	NO_3^- concentration in the vacuole	mol m^{-3}	31
$[AA]_{cy}$	Amino acid concentration in the cytoplasm	mol m^{-3}	35
$[C_{org}]_{(cyt)}$	Organic carbon concentration in the cytoplasm	mol m^{-3}	3.3
Rate variables			
F_{sNH3}	Stomatal NH_3 flux	mol m^{-2} s^{-1}	$2.00x10^{-8}$
$F_{NH4(ap-cy)}$	Active NH_4^+ flux from the apoplast to the cytoplasm	mol m^{-2} s^{-1}	$2.00x10^{-7}$
$F_{NH3(ap-cy)}$	Passive NH_3 flux between the apoplast and the cytoplasm	mol m^{-2} s^{-1}	$-2.00x10^{-8}$
$F_{NH3(vac-cy)}$	Passive NH_3 flux between the vacuole and the cytoplasm	mol m^{-2} s^{-1}	$2.80x10^{-6}$
$F_{NH4(vac-cy)}$	Passive NH_4^+ flux between the vacuole and the cytoplasm	mol m^{-2} s^{-1}	$1.00x10^{-1}$
F_{phlc}	organic carbon flux to the phloem	mol m^{-2} s^{-1}	$4.50x10^{-6}$
F_{phlaa}	Amino acid flux to the phloem	mol m^{-2} s^{-1}	$4.50x10^{-6}$
F_{NO3ac}	Active NO_3^- flux from the apoplast to the cytoplasm	mol m^{-2} s^{-1}	$9x10^{-10}$
$F_{NO3(vac-cy)pass}$	Passive NO_3^- flux between the vacuole and the cytoplasm	mol m^{-2} s^{-1}	$6.0x10^{-11}$
$F_{NO3(vac-cy)ac}$	Active NO_3^- flux from the cytoplasm to the vacuole	mol m^{-2} s^{-1}	$15x10^{-10}$
F_{ass}	NH_4^+ assimilation rate	mol m^{-2} s^{-1}	$1.40x10^{-6}$
F_{NR}	Nitrate reduction rate	mol m^{-2} s^{-1}	$2.5x10^{-8}$
F_{pho}	Photorespiratory NH_4^+ production rate	mol m^{-2} s^{-1}	Not necessary
A_c	Enzyme limited photosynthesis rate	mol m^{-2} s^{-1}	Not necessary
A_j	Electron transport limited photosynthesis rate	mol m^{-2} s^{-1}	Not necessary
A_q	Phloem loading limited photosynthesis rate	mol m^{-2} s^{-1}	Not necessary
A_{CO2}	Rate of CO_2 assimilation (photosynthesis)	mol m^{-2} s^{-1}	Not necessary

Symbol	Description	Unit	Initial Value
R_d	Rate of dark respiration	mol m^{-2} s^{-1}	Not necessary
V_o	Rate of RubisCO oxygenation	mol m^{-2} s^{-1}	Not necessary
V_c	Rate of RubisCO carboxylation	mol m^{-2} s^{-1}	Not necessary
Φ	Ratio of RubiCO oxygenation to carboxylation	-	Not necessary
Forcing parameters			
PAR	photosynthetic active radiation	mol m^{-2} s^{-1}	Figure 3
$CO_{2(atm)}$	atmospheric CO_2 concentration	ppm	Figure 3
$[NH_3]_{atm}$	atmospheric NH_3 concentration	µg m^{-3}	1
$[NH_4^+]_{xy}$	xylem NH_4^+ concentration	mol m^{-3}	3
$[NO_3^-]_{xy}$	xylem NO_3^- concentration	mol m^{-3}	8
T_l	leaf temperature	°C	20
g_s	stomatal conductance to H_2O	m s^{-1}	Figure 3
F_{H2O}	H_2O flow through the xylem	m^{-3} s^{-1}	Figure 3

Tableau 3.2: Symbols, descriptions, units and values used of parameters

Symbol	Description	Unit	Value		Reference
H	Henry constant at leaf temperature	M/M	$1.76x10^{3}$	at 26°C	(Farquhar *et al.* 1980b)
K_a	Dissociation constant	mol m^{-3}	$6.10x10^{-7}$	at 26°C	(Farquhar *et al.* 1980b)
$[H^+]_{ap}$	concentration in H^+ ions in the apoplast	mol m^{-3}	$1.0x10^{-3}$		(Husted & Schjoerring 1996)
$[H^+]_{cy}$	concentration in H^+ ions in the cytoplasm	mol m^{-3}	$3.16x10^{-5}$		(Husted & Schjoerring 1996)
$[H^+]_{vac}$	concentration in H^+ ions in the vacuole	mol m^{-3}	$3.20x10^{-3}$		average value
V_{ap}	Volume of apoplast per leaf square meter	m^3 m^{-2}	$28.0x10^{-6}$		calculated from (Husted & Schjoerring 1996)+ leaf thickness 0.2mm from (Stefanowska *et al.* 1999)
V_{sc}	Volume of leaf air space per leaf square meter	m^3 m^{-2}	$54.0x10^{-6}$		
V_{cy}	Volume of cytoplasm per leaf square meter	m^3 m^{-2}	$36.0x10^{-6}$		calculated from (Pfanz *et al.* 1987)+ leaf thickness 0.2mm from (Stefanowska *et al.* 1999)
V_{vac}	Volume of vacuole per leaf square meter	m^3 m^{-2}	$8.2x10^{-6}$		
P_{ap-cy}	NH_3 permeability of the cell membrane relative to leaf square meter	m s^{-1}	$8.00x10^{-8}$	at 25°C	estimate
$P_{NH3(cy-vac)}$	NH_3 permeability of the tonoplast relative to leaf square meter	m s^{-1}	$4.50x10^{-8}$	at 25°C	estimate
$P_{NH4(cy-vac)}$	NH_4^+ permeability of the tonoplast relative to leaf square meter	m s^{-1}	$4.50x10^{-9}$	at 25°C	estimate
$P_{NO3(cy-vac)}$	NO_3^- permeability of the tonoplast relative to leaf square meter	m s^{-1}	$5.2x10^{-7}$	at 25°C	estimate
K_{NO3-cy}	rate parameter of active transport of NO_3^- to the cytoplasm	s^{-1}	$4.0x10^{-9}$	at 25°C	(Sattelmacher & Burkhard 2001)
$K_{NH4ap-cy}$	rate parameter of active transport of NH_4^+ to the cytoplasm	s^{-1}	$5.00x10^{-4}$	at 25°C	(Schjoerring *et al.* 1998)
V_{NRmax}	Maximal rate of nitrate reduction	mol m^{-2} s^{-1}	3.5x10-6		(Yang & Midmore 2005)
K_{mNR}	Michaelis-Menten consant for nitrate reduction	mol m^{-3}	30		(Yang & Midmore 2005)
$V_{maxFNO3}$	Maximal rate of nitrate active transport to the vacuole	mol m^{-2} s^{-1}	$5.2x10^{-7}$		(Martinoia *et al.* 1986)
K_{mFNO3}	Michaelis-Menten consant for nitrate active transport to the vacuole	mol m^{-3}	2.3		(Martinoia *et al.* 1986)

Symbol	Description	Unit	Value		Reference
K_{AA}	Michaelis-Menten consant for amino acid inhibition of assimilation	mol m^{-3}	40.		estimate
K_{phloem}	rate parameter of transport of organic carbon to the phloem	s^{-1}	$4.17x10^{-5}$	at 25°C	(Yang & Midmore 2005)
V_{maxgs}	Maximal rate of NH_x assimilation	mol m^{-2} s^{-1}	$7.5x10^{-5}$	at 25°C	(Yang & Midmore 2005)
K_{NHx}	Michaelis Menten constant for NH_x	mol m^{-3}	4.0	at 25°C	(Yang & Midmore 2005)
K_{Cnst}	Michaelis Menten constant for organic carbon	mol m^{-3}	80.0	at 25°C	(Yang & Midmore 2005)
V_{cmx}	Maximal rate of RubisCO carboxylation	mol m^{-2} s^{-1}	$1.00x10^{-4}$	at 25°C	(Farquhar *et al.* 1980a)
J_{mx}	Maximal rate of electron transport	mol m^{-2} s^{-1}	$2.00x10^{-4}$	at 25°C	(Farquhar *et al.* 1980a)
K_c	Michaelis Menten constant for RubisCO carboxylation	mol mol^{-1}	$2.59x10^{-4}$	at 25°C	(Farquhar *et al.* 1980a)
K_o	Michaelis Menten constant for RubisCO oxygenation	mol mol^{-1}	$1.79x10^{-1}$	at 25°C	(Farquhar *et al.* 1980a)
T_p	transfer rate of organic carbon to the phloem	mol m^{-2} s^{-1}	$1.18x10^{-5}$		(Farquhar *et al.* 1980a)
f	fraction of light not absorbed by chloroplasts	-	$2.30x10^{-1}$		(Farquhar *et al.* 1980a)
θ	empirical curvature factor	Eq m^{-2} s^{-1}	$9.00x10^{-1}$		(Farquhar *et al.* 1980a)
Γ*	CO_2 compensation point	mol m^{-2} s^{-1}	$2.80x10^{-5}$		(Farquhar *et al.* 1980a)

$$[NH_3]_{(aq)} = K_a \frac{[NH_4^+]_{(aq)}}{[H^+]_{(aq)}} \quad (3)$$

Where K_a has a value 6.1×10^{-7} mol m^{-3} at 26°C and varies with temperature according to the following expression (Farquhar *et al.* 1980b) where a = 0.09018 and b = 2729.92:

$$\log_{10}(-K_a) = a + \frac{b}{T(°K)} \quad (4)$$

Since there is a different pH in each of our 3 liquid compartments we apply equation 1 in each of these compartments.

In addition there is a ratio known as the Henry constant (H) between the dissolved concentration of NH_3 in the apoplast and the gaseous concentration of NH_3 in the leaf's airspace in equilibrium with the solution. H is equal to 1762 at 26°C and varies with temperature according to the following expression (Farquhar *et al.* 1980b) where c = -1.6937 and d = 1477.7:

$$\log_{10} H = c + \frac{d}{T(°K)} \quad (5)$$

The Henry constant here is dimensionless since concentrations in liquid and gaseous compartments are both in mol m^{-3}.

Therefore at equilibrium the NH_3 concentration in the leaf's airspace ($[NH_3]_g$) can be calculated from the NH_4^+ concentration in the apoplast ($[NH_4^+]_{ap}$) as follows:

$$[NH_3]_g = \frac{K_a}{H} \cdot \frac{[NH_4^+]_{ap}}{[H^+]_{ap}} \quad (6)$$

2.2.1.2.Stomatal flux of NH_3

The diffusion of NH_3 between the leaf air space and the atmosphere (F_{sNH3}) can be modelled by analogy to electrical resistances, as the difference in concentration between the two heights (the leaf air space $[NH_3]_g$ and the atmosphere $[NH_3]_{atm}$ in this case) impeded by the atmospheric resistances between these heights (stomatal resistance to NH_3 $R_{s(NH3)}$ here) (ex: Monteith & Unsworth 1990). The stomatal resistance to NH_3 is computed from the water stomatal resistance by multiplying by the theoretical ratio of $R_{s(NH3)}/R_{s(H2O)}$ = 1.087 assumed

from the relative diffusivities of NH_3 and H_2O. The stomatal NH_3 flux is therefore modelled as:

$$F_{sNH3} = \frac{[NH_3]_{atm} - [NH_3]_g}{R_{s(NH3)}} \quad (7)$$

2.2.1.3.Xylem fluxes of NH_3 and NO_3^-

As a first approach the NH_x and NO_3^- concentrations in the xylem $[NH_4^+]_{xy}$ and the $[NO_3^-]_{xy}$ respectively are considered as a constant forcing parameter. The NH_3 and NO_3^- fluxes however that come through the xylem vary with the water flux since it is the NH_x/NO_3^- concentration multiplied by the water flux as given below. The water flux is a forcing variable.

$$F_{NHx(xyl)} = [NH_4^+]_{xy} \times F_{H2O(xy)} \quad (8)$$

$$F_{NO3(xyl)} = [NO_3]_{xy} \times F_{H2O(xy)} \quad (9)$$

2.2.1.4.Exchange of NH_3, NH_4^+ and NO_3^- between the apoplast and cytoplasm

There exist two main modes of transfer of solutes between cell compartments: passive and active transports. According to several authors (see Massad *et al.* 2008a), high and low affinity systems are responsible for the transport of NH_x from the apoplast to the cytosol. Furthermore, net uptake of NH_4^+ into the leaf cells of oil seed rape seems to respond linearly to increasing NH_4^+ concentrations in the nutritive solution between 2 and 10 mM (Nielsen & Schjoerring 1998). Therefore the influx of NH_4^+ from the apoplast to the cytoplasm can be modelled as an active process according to the following equation:

$$F_{NH4(ap-cy)} = [NH_4^+]_{ap} \times K_{NH4ap-cy} \times V_{ap} \quad (10)$$

Where $K_{NH4ap-cy}$ is the rate parameter of active transport in s^{-1} and is temperature dependent.
It is still not clear whether this efflux of NH_3 from the cytoplasm to the apoplast is mediated by membrane transporters or largely accounted for by NH_3 diffusion. We consider that NH_3 passively diffuses.

$$F_{NH3(ap-cy)} = \left([NH_3]_{ap} - [NH_3]_{cy}\right) \times P_{ap-cy} \quad (11)$$

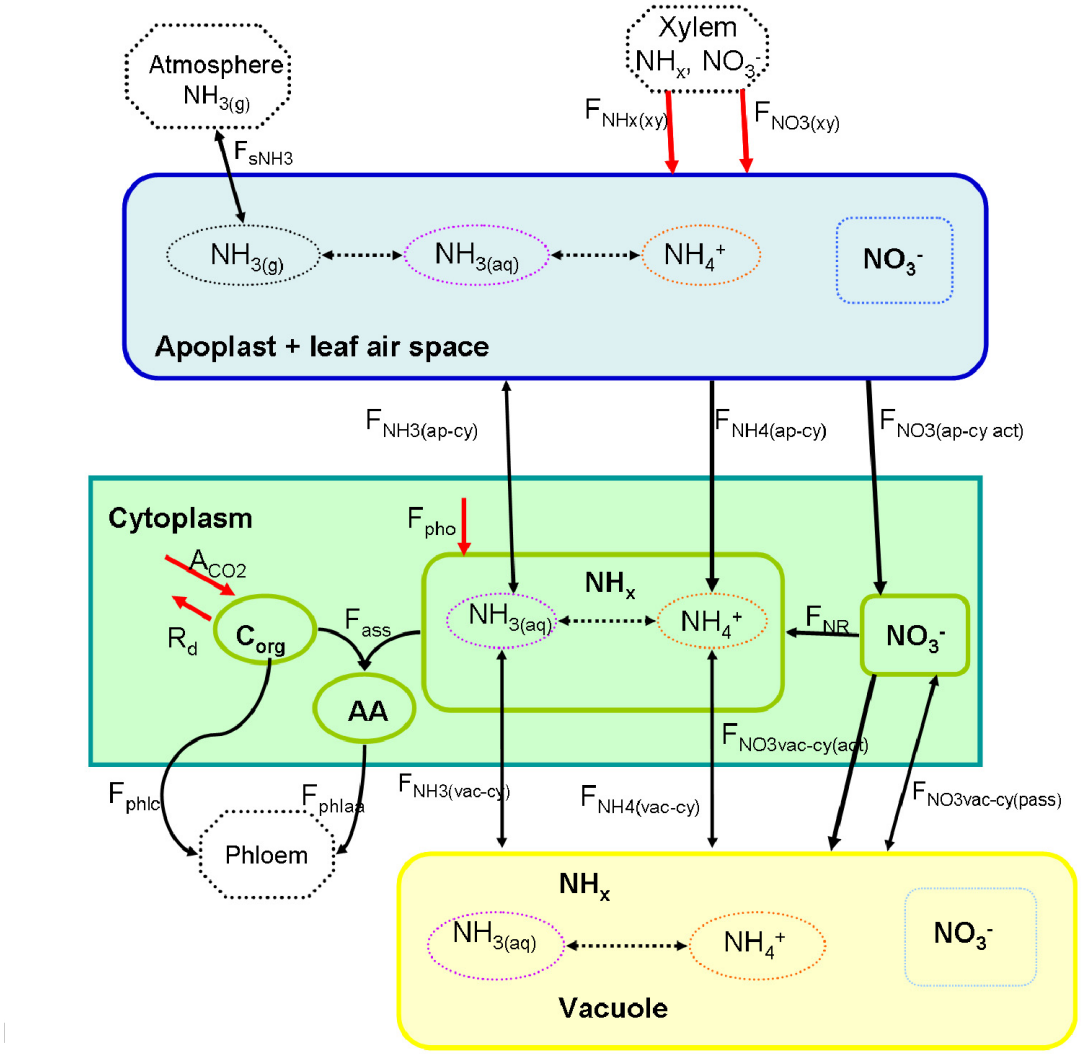

Figure 3.2: Model compartments flow diagram

One way arrows represent active transport, two way arrows represent diffusion, dotted arrows represent equilibriums and red arrows represent forcing parameters.

Where $P_{ap\text{-}cy}$ is the cellular membrane permeability to NH_3 and is temperature dependent, $[NH_3]_{ap}$ and $[NH_3]_{cy}$ are the NH_3 concentrations in the apoplast and cytoplasm respectively.
Concerning NO_3^-, the active transport to the cytoplasm can also be considered as a linear function of the apoplast NO_3^- concentration characterized by a high uptake rate and a low selectivity (Sattelmacher & Burkhard 2001) and can therefore be expressed as:

$$F_{NO3ac} = [NO_3]_{ap} \times K_{NO3ap-cy} \times V_{ap} \quad (12)$$

$K_{NO3ap\text{-}cy}$ is the rate parameter of active transport of NO_3^- in s^{-1} and is temperature dependent and $[NO_3^-]_{ap}$ is the NO_3^- concentration in the apoplast.
We consider that the efflux of NO_3^- and NH_4^+ from the cytoplasm to apoplast is negligible because of the high active rate parameters for these two compounds.
The temperature response equation for all plant parameters used is according to Thornley (1998) as:

$$P_{(T)} = P_{(ref)} \frac{(T_l - T_{273})^2 (T_{318} - T_l)}{(T_{ref} - T_{273})^2 (T_{318} - T_{ref})} \quad (13)$$

Where $P_{(T)}$ is the parameter value at leaf temperature, $P_{(ref)}$ is the parameter value at reference temperature, $T_{(ref)}$ is the reference temperature and T_l is the leaf temperature in °K.

2.2.2. *Cytoplasm compartment*

In the cellular compartment we consider 4 compounds in the mass balance equations: ammonium (NH_x), organic non structural carbon (C_{org}), nitrate (NO_3^-) and amino acids (AA).
The NH_x pool is filled via active transport of NH_4^+ from the apoplast to the cytoplasm ($F_{NH4(ap\text{-}cy)}$), production of NH_x by photorespiration (F_{pho}) and nitrate reduction (F_{NR}). It is emptied by NH_x assimilation into amino acids (F_{ass}) and emptied or filled via the passive diffusion of (i) NH_3 between the apoplast and the cytoplasm ($_{FNH3(ap\text{-}cy)}$), (ii) NH_3 between the cytoplasm and vacuole ($F_{NH3(vac\text{-}cy)}$) and (iii) NH_4^+ between the cytoplasm and vacuole ($F_{NH4(vac\text{-}cy)}$). Therefore the balance equation is the following:

$$\frac{d[NH_x]_{cy}}{dt} \times V_{cy} = F_{NH4(ap-cy)} + F_{pho} - F_{ass} + F_{NH3(ap-cy)} - F_{NH3(vac-cy)} - F_{NH4(vac-cy)} + F_{NR} \quad (14)$$

Where $[NH_x]_{cy}$ is the concentration of NH_x in the cytoplasm and V_{cy} is the relative volume of the cytoplasm per leaf square meter.

Concerning nitrate, the cytoplasm pool is filled by the active transport from the apoplast (F_{NO3ac}) and emptied by the reduction of NO_3^- to NH_4^+ (F_{NR}) and by the active transport of NO_3^- to the vacuole ($F_{NO3(vac\text{-}cy)ac}$) and filled/emptied by the exchange with the vacuole ($F_{NO3(vac\text{-}cy)pass}$):

$$\frac{d[NO_3]_{cy}}{dt} \times V_{cy} = F_{NO3ac} - F_{NO3(vac-cy)ac} - F_{NR} - F_{NO3(vac-cy)pass} \quad (15)$$

2.2.2.1. *Exchange of NH_3, NH_4^+ and NO_3^- between the cytoplasm and adjacent compartments*

Exchange of NO_3^-, NH_4^+ and NH_3 between the cytoplasm and the apoplast are described in section 2.2.3 above.

Exchange with the vacuole is considered to be diffusive for NH_4^+ and NH_3. The vacuole has a storage capacity because of its larger volume relative to the cytoplasm and because of its more acidic pH (Howitt & Udvardi 2000). The diffusive equations are the following where $[NH_3]_{cy}$, $[NH_3]_{vac}$, $[NH_4^+]_{cy}$ and $[NH_4^+]_{vac}$ are the concentrations of NH_3 and ammonium in the cytoplasm and vacuole respectively. $P_{NH3(cy\text{-}vac)}$ and $P_{NH4(cy\text{-}vac)}$ are the permeabilities of the tonoplast to NH_3 and NH_4^+ respectively on a leaf area basis. These permeabilities are also temperature dependent with a temperature response similar to equation 13.

$$F_{NH3(cy-vac)} = \left([NH_3]_{cy} - [NH_3]_{vac}\right) \times P_{NH3(cy-vac)} \quad (16)$$

$$F_{NH4(cy-vac)} = \left([NH_4^+]_{cy} - [NH_4^+]_{vac}\right) \times P_{NH4(cy-vac)} \quad (17)$$

Vacuolar transport of NO_3^- is well documented for roots (Forde 2000; Williams & Miller 2001) however little information is available on vacuole–cytoplasm exchange in leaves. We consider this exchange to be both active and diffusive. The active transport allows storage of NO_3^- in the vacuole whereas diffusion allows efflux from the vacuole to the cytoplasm in conditions of low NO_3^- concentrations in the cytoplasm. Active transport is modelled as a saturated kinetic process represented by a Michaelis-Menten type equation dependent on the concentration of nitrate in the cytoplasm $[NO_3^-]_{cy}$:

$$F_{NO3(vac-cy)ac} = V_{\max FNO3} \frac{[NO_3^-]_{cy}}{[NO_3^-]_{cy} + K_{mFNO3}} \quad (18)$$

Where $V_{maxFNO3}$ is the maximal rate of active nitrate transport to the vacuole and K_{mFNO3} is the Michaelis-Menten constant for NO_3^- active transport. The diffusion of NO_3^- between the cytoplasm and the vacuole is given by the following expression:

$$F_{NO3(vac-cy)pass} = \left([NO_3^-]_{cy} - [NO_3^-]_{vac}\right) \times P_{NO3(cy-vac)} \quad (19)$$

Where $[NO_3^-]_{vac}$ is the NO_3^- concentration in the vacuole and $P_{NO3(cy-vac)}$ is the permeability of the tonoplast to NO_3^- on a leaf area basis and is temperature dependent.

2.2.2.2. Ammonia assimilation into amino acids

We assume that most of the NH_4^+ is assimilated via the glutamine synthetase / glutamate synthase (GS/GOGAT) cycle. The role of GS in NH_4^+ assimilation has been clearly demonstrated by experimental studies using methionine sulfomine (MSO), which is a GS inhibitor (Mattsson & Schjoerring 1996; Olsen et al. 1995; Pearson et al. 1998). We consider that the relative rate of NH_4^+ assimilation into amino acids is mainly determined by the cytoplasmic concentration of organic carbon and NH_4^+. It was also found that NH_4^+ assimilation is inhibted by the accumulation of its products concentration (amino acids $[AA]_{cy}$) (Morot-Gaudry *et al.* 2001). The assimilation rate is modelled according to Michaelis-Menten kinetics with two substrates and with inhibition by amino acids accumulation:

$$F_{ass} = V_{\max gs} \frac{[C_{org}]_{(cy)} \cdot [NH_x]_{(cy)}}{K_{Corg} \cdot [C_{org}]_{(cy)} + K_{NHx} \cdot [NH_x]_{(cy)} + [C_{org}]_{(cy)} \cdot [NH_x]_{(cy)} \cdot \left(1 + {[AA]_{cy}}/{K_{AA}}\right)} \quad (20)$$

Where V_{maxgs} is the maximal rate of NH_x assimilation in mol m^{-2} s^{-1}, $K_{Corg,}$ and K_{NHx} are the Michaelis-Menten constants for organic carbon and NH_4^+ assimilation respectively in mol m^{-3} and K_{AA} is the Michaelis-Menten constant for amino acids inhibition.

2.2.2.3.Photorespiration

Photorespiratory production of NH_4^+ is the result of the oxygenase activity of RubisCO which is at the origin of a biochemical cycle where NH_4^+ is released during glycine decarboxylation in the mitochondrion (Keys *et al.* 1978). Since every two molecules of glycine are converted to one molecule of NH_3, CO_2 and serine. NH_3 is therefore released in amounts equimolar to

CO_2. In the photosynthesis model for C_3 plants, developed by Farquhar et al. (1980a), Φ is defined as the ratio of oxygenation to carboxylation rates of RubisCO, V_o/V_c. Thus for each carboxylation, Φ oxygenations occur which release 0.5 ΦCO_2 thus 0.5 ΦNH_3. Moreover, an equation for Φ given below is deduced by substituting for the kinetic equations of RubisCO and by defining Γ* the CO_2 compensation point in the absence of mitochondrial respiration.

$$\Phi = \frac{V_o}{V_c} = \frac{2 \cdot \Gamma^*}{[CO_2]_{chl}} \tag{21}$$

Where $[CO_2]_{chl}$ is the chloroplastic concentration of CO_2 and is assumed equal to the mesophyll concentration of CO_2.

Therefore the rate of NH_3 production by photorespiration (F_{pho}) can be modelled as the rate of RubiCO carboxylation (V_c) multiplied by 0.5 Φ:

$$F_{pho} = V_c \cdot \frac{\Gamma^*}{[CO_2]_{chl}} \tag{22}$$

V_c is calculated from the Farquhar et al. (1980a) C_3 photosynthesis model as described below and can be either enzyme or electron transport limited; Γ* is a given parameter and is temperature dependent.

2.2.2.4.Nitrate reduction

The primary regulation of NO_3^- reductase activity is considered to be its substrate, NO_3^- (Crawford 1995). Although NO_3^- reduction is also regulated by light, glutamine and sugars among other factors (Campbell 1999; Stitt *et al.* 2002). For simplicity we consider that NO_3^- reduction rate can be modelled by Michaelis-Menten type equation:

$$F_{NR} = V_{NR\max} \frac{[NO_3^-]_{(cy)}}{K_{mNR} + [NO_3^-]_{(cy)}} \tag{23}$$

V_{NRmax} is the maximum rate of nitrate reduction and K_{mNR} is the Michaelis-Menten constant for NO_3^- reduction.

2.2.2.5.Carbon cytoplasm balance

NH_3 assimilation by plants requires carbon skeletons derived from photosynthesized carbohydrates for the synthesis of amino acids (Huppe & Turpin 1994). For this reason, we

found it necessary to do a simple carbon mass balance equation in the cytoplasm. The organic carbon (C_{org}) cytoplasm pool is filled by photosynthesis (A_{CO2}) and emptied by dark respiration (R_d), efflux towards the phloem (F_{phlc}) and assimilation into amino acids (F_{ass}). We consider that carbon substrates used for nitrogen assimilation are mainly constituted of three carbon atoms therefore multiplying the assimilation rate in the organic carbon mass balance by a coefficient $\alpha = 3$:

$$\frac{d[C_{org}]_{cy}}{dt} \times V_{cy} = A_{CO2} - R_d - F_{phlc} - \alpha F_{ass} \quad (24)$$

– Photosynthesis or carbon assimilation as well as dark respiration are calculated according to the model of Farquhar et al. (1980a). This model estimates the rates of CO_2 assimilation by leaves by including the kinetic properties of RubisCO, the requirements of the photosynthetic carbon reduction (PCR) and the photorespiratory carbon oxidation (PCO) cycles for reduced nucleotides and the dependence of electron transport on photon flux.
Photosynthesis is therefore calculated by choosing the minimum between enzyme limited (A_c), electron transport limited (A_j) or phloem loading limited (A_p) assimilation rates:

A_{CO2}=minimum (A_c,A_j,A_q) (25)

– Dark respiration is calculated as being a proportion of the maximal rate of RubisCO carboxylation (V_{cmx})

R_d=0.0089xV_{cmx} (26)

– Nitrogen assimilation is described in paragraph 2.3.2 above.

– Phloem sap flow is assumed to be driven by osmotic pressure gradient between the shoots and the roots; since phloem is mainly constituted of organic carbon substrate, phloem sap flow is considered to be proportional to the organic carbon gradient (Dewar 1993). The organic carbon concentration in the roots is considered to be null as the shoots are the photosynthetic organs. The equation of organic carbon loading to the phloem is therefore given by:

$$F_{phlc} = [C_{org}]_{(cy)} \times K_{phl} \times V_{cy} \quad (27)$$

K_{phl} is the rate parameter of transfer into the phloem in s^{-1} and is temperature dependent.

2.2.2.6.Amino acids cytoplasm balance

We found it necessary to have a simplified amino acid balance equation in order to calculate the concentration of amino acids in the cytoplasm $[AA]_{cy}$ necessary for equation 20. We assume that the amino acid cytoplasm pool is filled by NH_4^+ assimilation (F_{ass}) and emptied by phloem transport (F_{phlaa}). The balance equation therefore becomes:

$$\frac{d[AA]_{cy}}{dt} \times V_{cy} = F_{ass} - F_{phlaa} \quad (28)$$

F_{ass} is calculated as described in equation 20; whereas the rate of amino acids transport by the phloem is considered to be determined by the organic carbon gradient since it is the predominant substrate in the phloem (Dewar 1993) and has therefore the same rate parameter K_{phl}:

$$F_{phlaa} = [AA]_{cy} \times K_{phl} \times V_{cy} \quad (29)$$

2.2.3. *Vacuole compartment*

The vacuole NH_x pool is filled/emptied via the passive diffusion of NH_3 and NH_4^+ between the cytoplasm and the vacuole ($F_{NH3(vac-cy)}$ and $F_{NH4(vac-cy)}$ respectively). Whereas the vacuole NO_3^- pool is filled by the active transport of NO_3^- from the cytoplasm and filled/emptied by the diffusion of NO_3^- between the cytoplasm and vacuole.

We therefore have the following mass balance equations for NH_x and NO_3^-:

$$\frac{d[NH_x]_{vac}}{dt} \times V_{vac} = F_{NH3(vac-cy)} + F_{NH4(vac-cy)} \quad (30)$$

$$\frac{d[NO_3]_{vac}}{dt} \times V_{vac} = F_{NO3(vac-cy)pass} + F_{NO3(vac-cy)ac} \quad (31)$$

Where $[NH_x]_{vac}$ and $[NO_3^-]_{vac}$ are the concentrations of NH_x and NO_3^- in the vacuole respectively, and V_{vac} is the relative volume of the vacuole per unit leaf area.

Exchange equations of NH_x and NO_3^- are described above (paragraph 2.3.1).

2.3. Numerical techniques

The model is coded using Fortran 90. The StAmP and Tuzet et al. (2003) models are run separately but with the same leaf characteristics. The Tuzet et al. (2003) model is run for a

canopy having one layer of leaves and with a leaf area index of one to be as close as possible to a leaf based model. Four outputs of the Tuzet et al. (2003) model (leaf temperature, stomatal conductance, CO_2 concentration in the sub-stomatal cavity and water absorption) are used as forcing parameters for the StAmP model and are represented in Figure 3. Equations are solved numerically using the Powell hybrid method (Powell 1970) in the NAG Fortran library routine (C05NBF).

3. Model sensitivity to parameters

Model parameters used in simulations are listed in table 2. The daily variation of the forcing variables used for these simulations are represented in Figure 3. Atmospheric NH_3 concentration, NO_3^- and NH_4^+ concentrations in the xylem sap as well as leaf temperature were constant and the values used are 1 µg m^{-3}, 3mM, 8mM and 20 °C respectively.

3.1. Model sensitivity to compartment related parameters

Some of the parameters used in the model such as pH and volumes are well documented and we have a good idea of their variability and uncertainty with plant species and conditions. Others however are more uncertain and very few measurements are available. Membrane permeabilities to N compounds as well as active transport rates have been well documented for roots, very little information is found for leaves. Apoplastic pH of oilseed rape leaves measured with the infiltration/centrifugation technique was reported to vary between 5.8 (Husted & Schjoerring 1995a)and 6.6 (Husted & Schjoerring 1996). Whereas apoplastic volumes reported for oilseed rape leaves varied between 8.3 and 14.4 % of total leaf volume (Husted & Schjoerring 1995a) that is approximately between 21 and 36 cm^3 m^{-2} leaf surface. We used these values to test the sensitivity of the model output variables and 25% variation for all other parameters (values shown in Table 3).

In Table 3 we present a summary showing percentage change in model outputs as affected by changes in parameter values. The stomatal NH_3 compensation point and the stomatal NH_3 flux are mostly sensitive to changes in apoplastic pH and volume and to the active transport rate parameter of ammonium between the apoplast and the cytoplasm. Reported apoplast volumes have been shown to vary by around 25% which results in compensation point variation between 22% and 33%. The most uncertain parameter having an important effect on the compensation point is the active transport parameter of ammonium from the apoplast to the cytoplasm. Increasing this parameter by 25% decreases the compensation point by 20%, whereas decreasing it by 25% increases the compensation point by 33%.

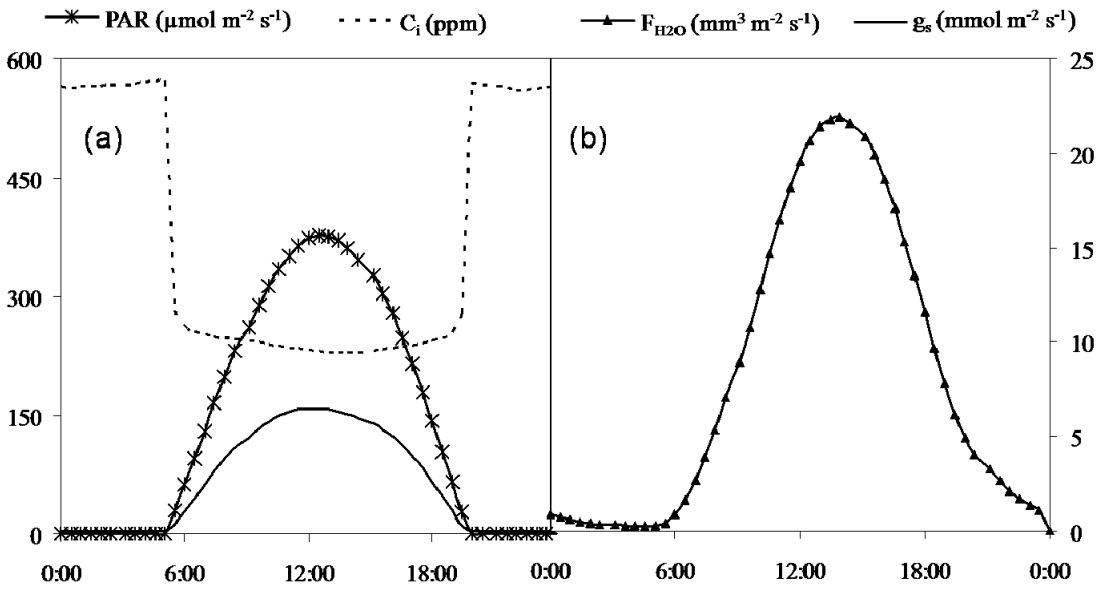

Figure 3.3: Model forcing variables used in simulation

These variables are outputs of the Tuzet et al. (2003) soil-plant-atmosphere continuum model. F_{H2O} is the root absorption of water and is considered as the xylem water flux in our case; g_s is the stomatal conductance to water, PAR is the photosynthetic active radiation at the leaf surface and C_i is the sub-stomatal cavity concentration of CO_2. Xylem concentrations of NO_3^- and NH_x as well as atmospheric NH_3 concentration and leaf temperature are constant during the day with values of 8mM, 3mM, 1µg m^{-3} and 20 °C respectively unless specified otherwise.-

The variation in apoplastic volume is linked to leaf position and is more variable for leaves in a late vegetative growth than leaves in early vegetative growth. This parameter can be determined experimentally with less than 5% error (Husted & Schjoerring 1995a). However variation with the plant's water status should be accounted for. Apoplastic pH has a big effect on the stomatal compensation point and has been shown to vary with nitrogen nutrition (Kosegarten *et al.* 2001), plant water status (Wensuo & William 2007) and atmospheric NH_3 concentrations (Hanstein & Felle 1999) among others. In the model the pH is actually a fixed parameter and can be an important source of variation in estimating the compensation point. It is important to notice that the compensation point is more affected by an increase than by a decrease in pH this can be attributed to the exponential form of the curve linking ammonium/NH_3 equilibrium to pH. The active transport rate of NH_4^+ between the apoplast and cytoplasm gives the greatest uncertainty to our model since we have very few information about it unlike the apoplastic pH and volume.

This sensitivity analysis also points out the relative independence of the apoplastic compartment from the vacuolar and cytoplasmic compartments. Compartment parameters affecting the NH_3 compensation point affect very little concentrations in the cytoplasm and vacuole and vice versa. This can be explained by the big difference in NH_4^+ concentrations between the apoplast and the other compartments and also by the fact the NH_4^+ can only efflux from the cytoplasm to the apoplast whereas it is very actively transported from the apoplast to the cytoplasm. Thus a significant change in concentration in the apoplast is negligible relative to the cytoplasm or vacuole. It should however be noted that no cross-sensitivity effects were analysed, where these may modify the independence of the apoplasm and cytoplasm compartments: for instance, a simultaneous increase in pH_{cy} and $P_{ap\text{-}cy}$. Parameters affecting the vacuolar and cytoplasmic concentrations are shown in Table 3.

3.2. Model sensitivity to parameterisation of sources and sinks of NH_x

We investigate here the model sensitivity to parametrisation of the NH_4^+ sources and sink. The GS/GOGAT cycle is the main NH_4^+ sink in leaves in a vegetative growth stage (Hodges *et al.* 2003). We modelled this sink as a Michaelis-Menten type mechanism having two substrates: NH_4^+ and C_{org} originating from photosynthesis. The parameters related to this equation are the maximal rate of GS activity (V_{maxgs}) and the Michaelis-Menten constants for C_{org} and NH_4^+ (K_{Cnst} and K_{NHX}). We evaluated the effect of each of those three parameters on the $[NH_3]_g$. The model has two cellular sources of NH_x: photorespiration and NO_3^- reduction. We chose to test the effect of photorespiration on the $[NH_3]_g$ through the effect of the CO_2

Tableau 3.3: Model variables response to change in compartment parameter values

Values represent difference between model run with parameter values in table 2 and model run with parameter changed to value in each column multiplied by 100. Blank cases represent values less than 1%.

	Percentage change in model output (%)																			
Variable parameter	$[H^+]_{ap}$ mmol m^{-3}		$[H^+]_{cy}$ mmol m^{-3}		$[H^+]_{vac}$ mmol m^{-3}		$P_{ap\text{-}cy}$ m s^{-1}		$K_{NH4ap\text{-}cy}$ s^{-1}		$P_{NH3cy\text{-}vac}$ m s^{-1}		$P_{NH4cy\text{-}vac}$ m s^{-1}		V_{ap} cm^3 m^{-2}		V_{cy} cm^3 m^{-2}		V_{vac} cm^3 m^{-2}	
Absolute values	1.6	0.3	$2.4x10^{-2}$	$4.0x10^{-2}$	2.4	4.0	$6.0x10^{-8}$	$1.2x10^{-7}$	$3.8x10^{-4}$	$6.3x10^{-4}$	$3.4x10^{-8}$	$5.6x10^{-8}$	$3.4x10^{-9}$	$5.6x10^{-9}$	21	36	27	45	62	103
Percentage change	-50%	92%	-25%	25%	-25%	25%	-25%	25%	-25%	25%	-25%	25%	-25%	25%	-25%	29%	-25%	25%	-25%	25%
$[NH_3]_g$	-37	228	-	-	-	-	-	-	33	-20	-	-	-	-	33	-22	-	-	-	-
$[NH_x]_{ap}$	0.2	-130	-	-	-	-	-	-	33	-20	-	-	-	-	52	-53	-	-	-	-
F_{sNH3}	-96	320	-	-	-	-	-	-	-1	-	-	-	-	-	85	-57	-	-	-	-
$[NH_x]_{cy}$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-2	2	2	-1
$[NH_4^+]_{vac}$	-	-	3	-2	-	-	-	-	-	-	-3	3	4	-2	-	-	-2	2	-	-
$[NH_3]_{vac}$	-	-	3	-2	33	-20	-	-	-	-	-3	3	4	-2	-	-	-2	2	<-100	>100
$[NH_x]_{vac+cy}$	-	-	2	-1	-	-	-	-	-	-	-2	2	3	-2	-	-	-2	2	-	-

compensation point without dark respiration (Γ*) and the effect of NO_3^- reduction through the effect of V_{NRmax}.

We notice that photorespiration has very little effect on apoplastic concentrations (<1%), whereas it has an effect on intra-cellular concentrations (results not shown). Changing Γ* by 15 % resulted in a change in intracellular concentrations of NH_x of approximately 6%. An important thing to note is that an increase in photorespiration rate always leads to an increase in NH_x assimilation rate. Sensitivity analysis of the model to NO_3^- reduction related parameters showed no effect on apoplastic and cellular concentrations of NH_x. A change in 33% in V_{maxgs} resulted also in an insignificant effect on apoplastic NH_x concentrations and in a change of approximately 50% on total intra-cellular NH_x concentrations.

We note that the parameters of the sources and sinks equations have very little effect on the NH_3 compensation point and on the NH_x concentrations in the apoplast in the conditions in which we tested it. However there is a significant effect on the intra cellular concentrations and therefore on the plant's N status which could be useful if the model were to be set up for a plant having different layers or with different growth stages. In this configuration, organic carbon could become limiting for lower leaves and senescing leaves can become sources of NH_x for example leading to a significant effect on apoplastic ammonium concentrations. This sensitivity study also leads us to question our initial hypothesis saying that the plants carbon metabolism influences the NH_3 stomatal compensation point for leaves in a vegetative stage of growth. The effect of NO_3^- reduction parameters are conform to experimental results where it was noted that different NO_3^- fertilizations lead to no significant effect on the NH_3 stomatal compensation point (Massad et al. 2008b; Mattsson *et al.* 1998).

4. Model response to atmospheric forcing variables

Atmospheric forcing parameters are photosynthetic active radiation, temperature and atmospheric NH_3 concentrations. We investigate the effect of each of these variables on the stomatal NH_3 compensation point by having different constant values for each variable while other variables are the same as the values described in Figure 3.

4.1. Photosynthetic Active Radiation (PAR)

Figure 4 shows the effect of different constant values of PAR on ammonium concentrations in the apoplast and in the vacuole and cytoplasm. We notice that the ammonium concentration in the apoplast still shows a diurnal variation and that PAR has very little effect on ammonium concentrations in the apoplast (Figure 4b).

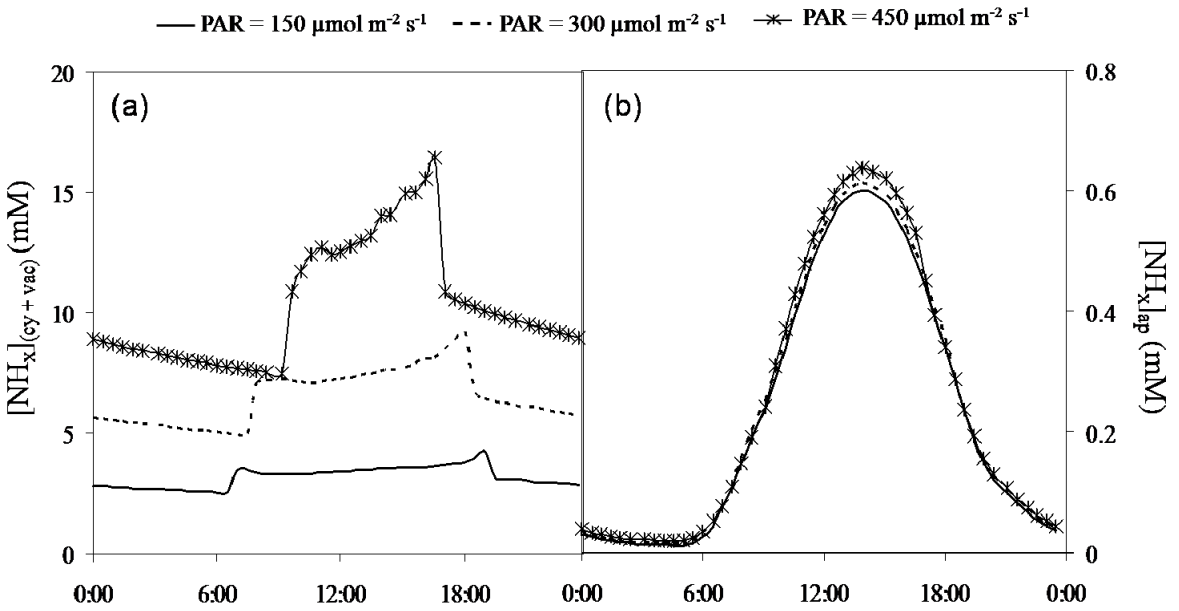

Figure 3.4: **Model's response to different constant photosynthetic active radiation (PAR) values.**

Response of the NH_x concentration in the bulk tissue (cytoplasm + vacuole) (a) and in the apoplast (b) to three different values of PAR. Forcing variables are as described by Figure 3 except for PAR values which are no more variable but take a different constant value for each curve.

Different PAR values however, did affect ammonium concentrations in the vacuole and the cytoplasm (Figure 4a). For PAR values of 150 and 300 µmol m^{-2} s^{-1}, we note a difference between day and night concentrations but a relatively weak variation during these periods (no bell shaped curve).

These results lead us to think that the PAR is not the variable causing the diurnal variation in the stomatal compensation point in the model which is not surprising given the relative independence of the modelled apoplasm and cytoplasm NH_3 concentrations. We know that photosynthetic active radiation affects photosynthesis and photorespiration and we saw that changes in photosynthesis and photorespiration did not have a significant effect on apoplastic ammonium concentrations. The step change in intracellular NH_x concentrations can be explained by the form of the curve of CO_2 concentration in the sub-stomatal cavity (C_i) (figure 3) which results in different photorespiration rates and therefore different NH_x concentrations in the intracellular space. For values of PAR of 150 and 300 µmol m^{-2} s^{-1}, photosynthesis is always limited by electron transport and therefore does not change with changing C_i values. For a PAR value of 450 µmol m^{-2} s^{-1} we notice a steady increase of ammonium concentrations in the vacuole and cytoplasm during the day. This can be caused by the effect of having a higher photorespiration rate than an ammonium assimilation rate. Ammonium assimilation in this case is limited by $[C_{nst}]$ resulting from photosynthesis being limited by RuBisCo carboxylation and not electron transport.

4.2. Leaf temperature

Figure 5 shows the effect of three different leaf temperatures (T_l) on the stomatal NH_3 flux (5a) and on $[NH_3]_g$ (5b). We notice that stomatal NH_3 fluxes increase with increasing T_l (negative flux is deposition and positive flux is emission). This NH_3 flux change meets with the changes in the $[NH_3]_g$, which doubles every 10°C change in T_l.

We know that T_l affects all plant metabolic processes (Leegood & Edwards 1995; Leuning 2002) and therefore could potentially affect $[NH_3]_g$. For example an increase in T_l increases the active transport rate of NH_x from the apoplast to the cytoplasm consequently causing $[NH_3]_g$ to diminish. It should be noted that if T_l only affected K_H and K_d, we should observe a doubling of the compensation point every 5°C change. This is not the case because the NH_3 compensation point reacts in opposite directions to changes of temperature parameterisation. Variation of plant parameters to temperature are modelled according to an empirical formula described by Thornley (1998). This parameterisation however should be considered carefully and more adequate temperature dependence should be investigated.

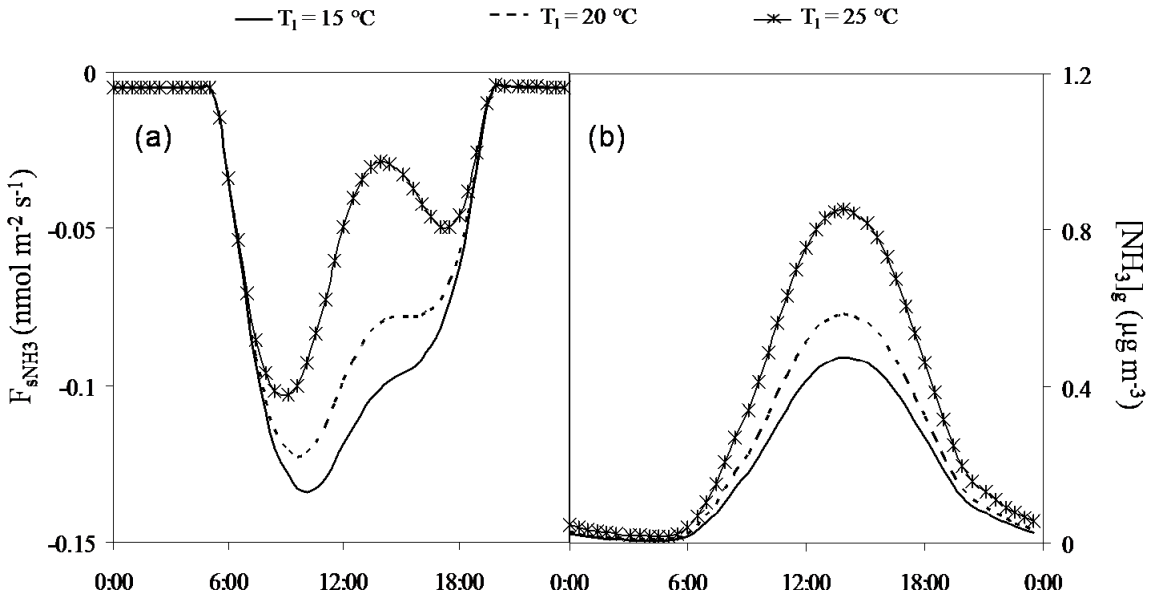

Figure 3.5: Model's response to change in leaf temperature (T_l)

Response of the NH_3 stomatal flux (a) and of the NH_3 compensation point (b) to three different values of leaf temperature. Forcing variables are as described by Figure 3. Positive flux is an emission and negative flux is a deposition.

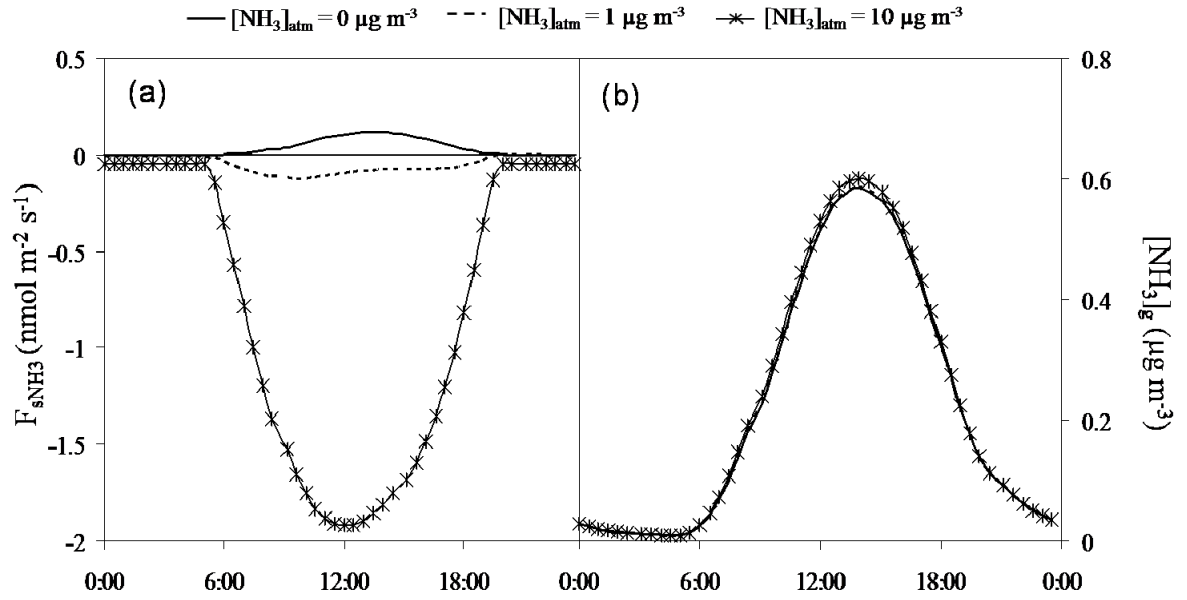

Figure 3.6: Model's response to change in atmospheric ammonia concentration ($[NH_3]_{atm}$).

Response of the NH_3 stomatal flux (a) and of the NH_3 compensation point (b) to three different values of atmospheric NH_3 concentration. Forcing variables are as described by Figure 3 except for the atmospheric NH_3 concentration. Positive flux is an emission and negative flux is a deposition.

4.3. Atmospheric ammonia concentrations

Stomatal NH_3 fluxes depend on atmospheric concentrations since it is a function of the NH_3 gradient between the sub-stomatal cavity and the atmosphere. In Figure 6a we can see the change between an emission flux to a deposition flux as atmospheric NH_3 concentrations change from 0 µg m^{-3} to 10 µg m^{-3}. Atmospheric NH_3 concentrations however have no significant effect on the NH_3 compensation point and consequently on apoplastic concentrations of ammonium (Figure 6b).

This is explained by the fact the stomatal flux in the conditions in which the model was run is negligible relative to the xylem or cellular fluxes of ammonium. Apoplastic extraction measurements (Husted & Schjoerring 1995a) and chamber measurements (Husted & Schjoerring 1995b) hypothesise that $[NH_3]_{atm}$ does not affect $[NH_3]_g$ Although the model suggests that this hypothesis is fulfilled, it may not always be the case since it has been shown that plants with deficient N in the rooting system and high NH_3 in the surrounding atmosphere can feed on atmospheric N (Castro *et al.* 2006).

5. Model response to root forcing variables

Root forcing variables are NO_3^- and NH_x fluxes through the xylem. These variables are modulated in the plant by the conditions around the roots, by root absorption of minerals and by the shoots water status and photosynthesis. In this model the roots are not accounted for. The xylem N concentrations are constant. However the water flux does depend on the shoot water status and photosynthesis. We test here the model response to different constant NH_x and NO_3^- concentrations in the xylem sap while having a variable water flux. We also test the model hypothesis of having a constant N concentration in the xylem sap by examining how the model reacts to a constant N flux through the xylem which is equivalent to saying that N concentration is diluted with more water coming in.

5.1. Nitrogen concentrations in the xylem (N nutrition)

The quantity and form of N nutrition supplied to the plant's root system have been shown to significantly affect the stomatal NH_3 compensation point (Massad *et al.* 2008a; Mattsson *et al.* 1998; Schjoerring 1997). We investigate here the model's response to two different forms of mineral nitrogen applied at different concentrations. Because the model only applies for leaves, we consider that concentrations in the xylem sap are directly correlated to concentrations in the root solution.

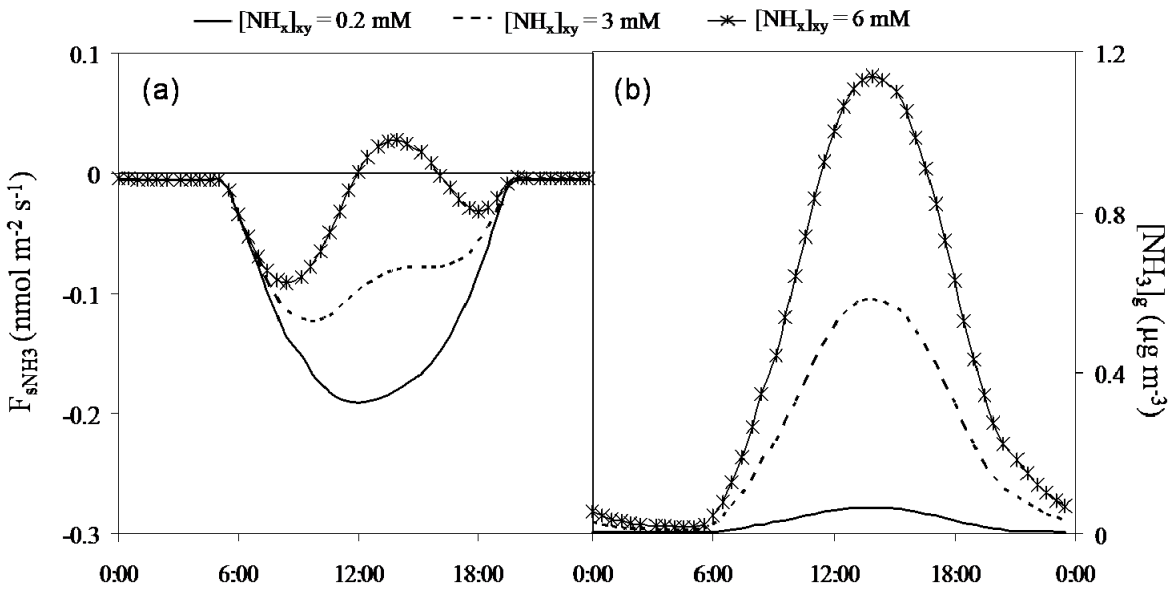

Figure 3.7: Model's response to change in xylem ammonium concentration ($[NH_x]_{xy}$)

Response of the NH_3 stomatal flux (a) and of the NH_3 compensation point (b) to three different values of xylem NH_x concentration. Forcing variables are as described by Figure 3 except for $[NH_x]_{xy}$. Positive NH_3 flux is an emission and negative flux is a deposition.

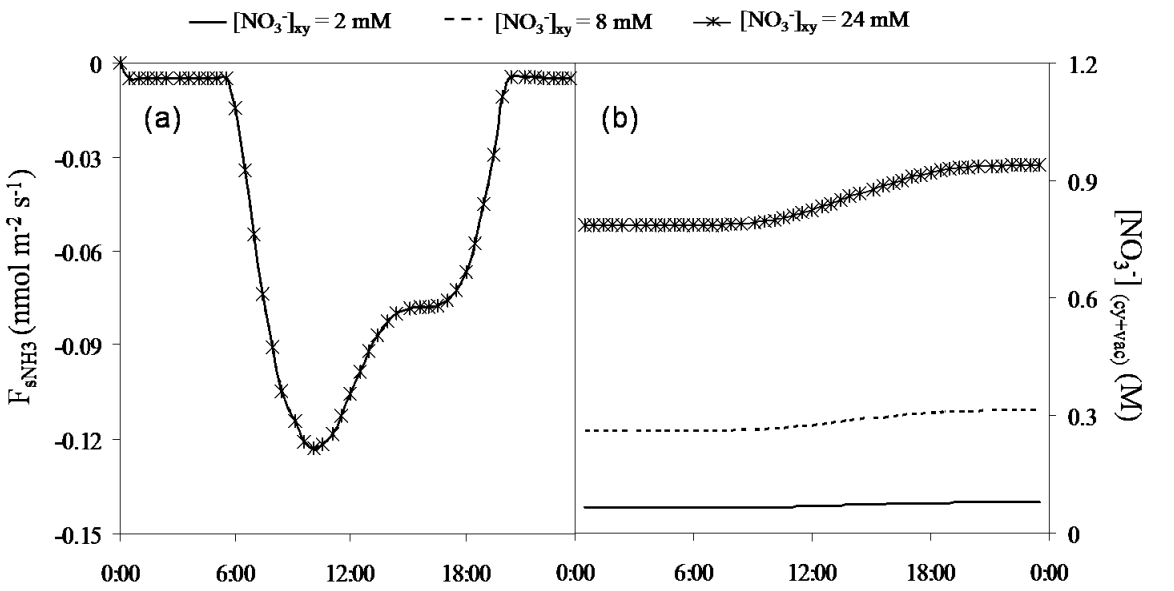

Figure 3.8: Model's response to change in xylem nitrate concentration ($[NO_3^-]_{xy}$)

Response of the NH_3 stomatal flux (a) and of the NO_3^- concentration in the bulk tissue (vacuole + cytoplasm) (b) to three different values of xylem NO_3^- concentration. Forcing variables are as described by Figure 3 except for $[NO_3^-]_{xy}$. Positive NH_3 flux is an emission and negative flux is a deposition.

5.1.1. *NH_x concentration*

Figure 7 shows the response of the stomatal NH_3 flux (7a) and the NH_3 compensation point (7b) to three different concentrations of ammonium in the xylem sap (0.2, 3 and 6 mM). The water flux, PAR, CO_2 concentration in the sub-stomatal cavity and stomatal conductance are as shown in Figure 3. Leaf temperature, NO_3^- concentration in the xylem sap and NH_3 concentration in the atmosphere are constant and fixed to 20°C, 8 mM and 1 μg m^{-3} respectively. We note that the stomatal flux increases with increasing NH_x concentrations in the xylem sap. With the highest NH_x concentrations the flux switches to an emission flux at midday when the water flux is maximum therefore the N flux in the xylem maximal. These fluxes are coherent with the NH_3 compensation point which also increases with increasing NH_x concentrations and surpasses the atmospheric concentration at midday therefore causing the positive stomatal flux.

5.1.2. *NO_3^- concentration*

Figure 8 shows the response of the stomatal flux and the NO_3^- concentration in the cytoplasm and vacuole to different NO_3^- concentrations in the xylem sap. All forcing variables are similar to paragraph 5.2.1 except for the NH_x xylem sap concentration which is fixed to 3 mM and NO_3^- xylem sap concentration which is 2, 8 or 24 mM. We note no significant effect of NO_3^- xylem sap concentrations on NH_3 fluxes (Figure 8a) and consequently on the NH_3 stomatal compensation point. NO_3^- concentrations in the vacuole and cytoplasm increase with increasing nitrate in the xylem sap but show no diurnal variation (Figure 8b).

Such results have been reported in apoplast extraction and chamber measurement experiments (Massad et al. 2008b; Mattsson *et al.* 1998). This absence of diurnal variation is due to the parameterisation of NO_3^- reduction and active transport from the apoplast to the cytoplasm which is rather simplistic and does not account for the NO_3^- reductase circadian oscillation which has been reported and modelled (Yang & Midmore 2005). Another phenomenon which has been reported in literature and which is not represented in the model is the effect of having a mixed NO_3^- and NH_x nutrition on the NH_3 compensation point. It has been noted that plants having a mixed N nutrition tend to have a lower compensation point than plants relying only on NH_x based nutrition (Massad et al. 2008b). This can be explained by root preference in absorption but also by feedbacks on metabolic processes at the leaf level which are not accounted for in this model.

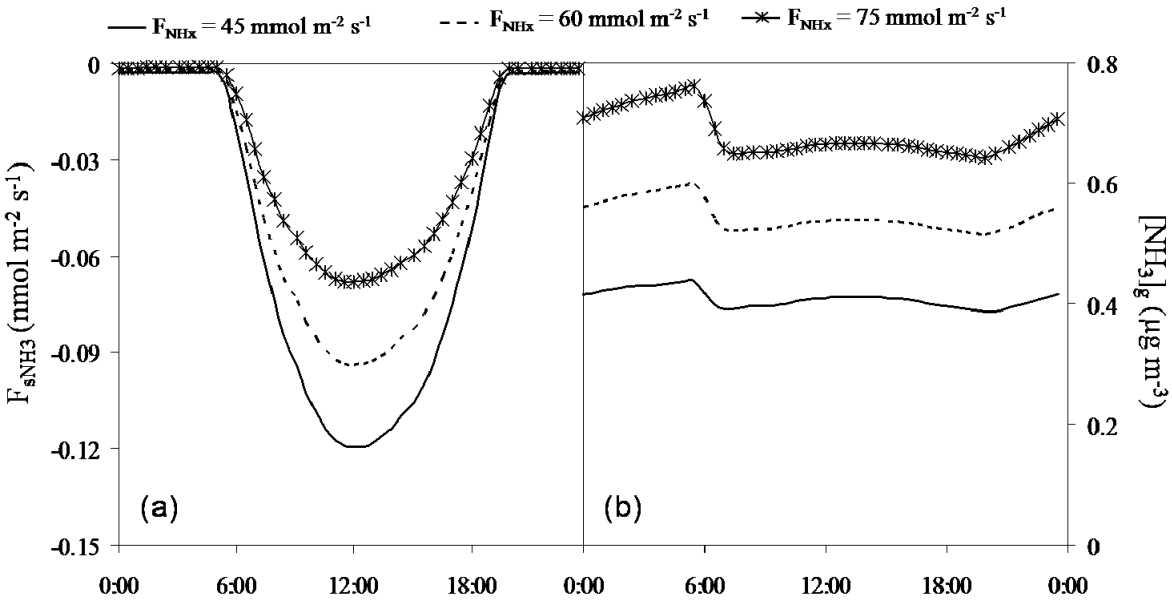

Figure 3.9: Model's response to change in xylem NH_x flux (F_{NHx}).

Response of the NH_3 stomatal flux (a) and of the NH_3 compensation point (b) to three different values of xylem NH_x fluxes. Forcing variables are as described by Figure 3 except for F_{NHX} values which are no more variable but take a different constant value for each curve. Positive NH_3 flux is an emission and negative flux is a deposition.

5.2. N (NH_x and NO_3^-) transport to the leaf

In the model N transport to the leaf is based on having a variable water flux that peaks at mid-day when the leaves stomata are fully open and is minimal during the night (Figure 3). This variable water flux is accompanied by a fixed N (NH_x or NO_3^-) concentration in the xylem. As a result, we have a variable N flux coming to the leaf that is proportional to the water flux. This hypothesis however can be discussed as the N concentration in the xylem can be variable during the day where a dilution occurs when we have a maximal water flux resulting in a constant N flux coming to the leaf. This may also result in a zero N fluxe to the leaf during the night. We tested the response of the model's output variables to three different constant values of N fluxes to the leaf and compared them to a variable daily flux. The model was run with a constant leaf temperature of 20°C, a variable daily stomatal conductance, PAR and CO_2 concentration in the sub-stomatal cavity (Figure 3). In Figure 9 we can see the effect on the stomatal NH_3 flux and on the NH_3 compensation point. NH_x flux values are 45, 60 and 75 mmol m^{-2} s^{-1}. We notice that the stomatal NH_3 flux keeps its diurnal variation as it is mostly regulated by the stomatal conductance (figure 9a). Fluxes are however higher (less deposition) with high N fluxes to the leaf. These higher fluxes meet with higher compensation point values. We also note that the diurnal variation in the NH_3 compensation point is inexistent but that there is a difference between night and day values of the compensation point (Figure 9b).

This leads us to think that the variable N flux was causing the diurnal variation of the compensation point. The difference in compensation point values between day and night might be caused by variable photosynthesis and photorespiration rates leading us to say that cytoplasmic and apoplastic compartments are not completely uncoupled. Experimental results have shown no significant diurnal (light/dark periods) variation of apoplastic NH_x concentrations (Massad et al.2008b; Husted *et al.* 2000). Given that the diurnal variation of the incoming N xylem sap flux is causing the diurnal variation of the apoplastic NH_x concentrations and the compensation point, we have to re-evaluate our hypothesis of having a fixed N concentration in the xylem.

6. Ability of the model to represent response of $[NH_3]_g$ to N nutrition

We present here a comparison between modelled and measured NH_3 compensation points, apoplast NH_x concentrations and intracellular NH_x concentrations. Measurements were done

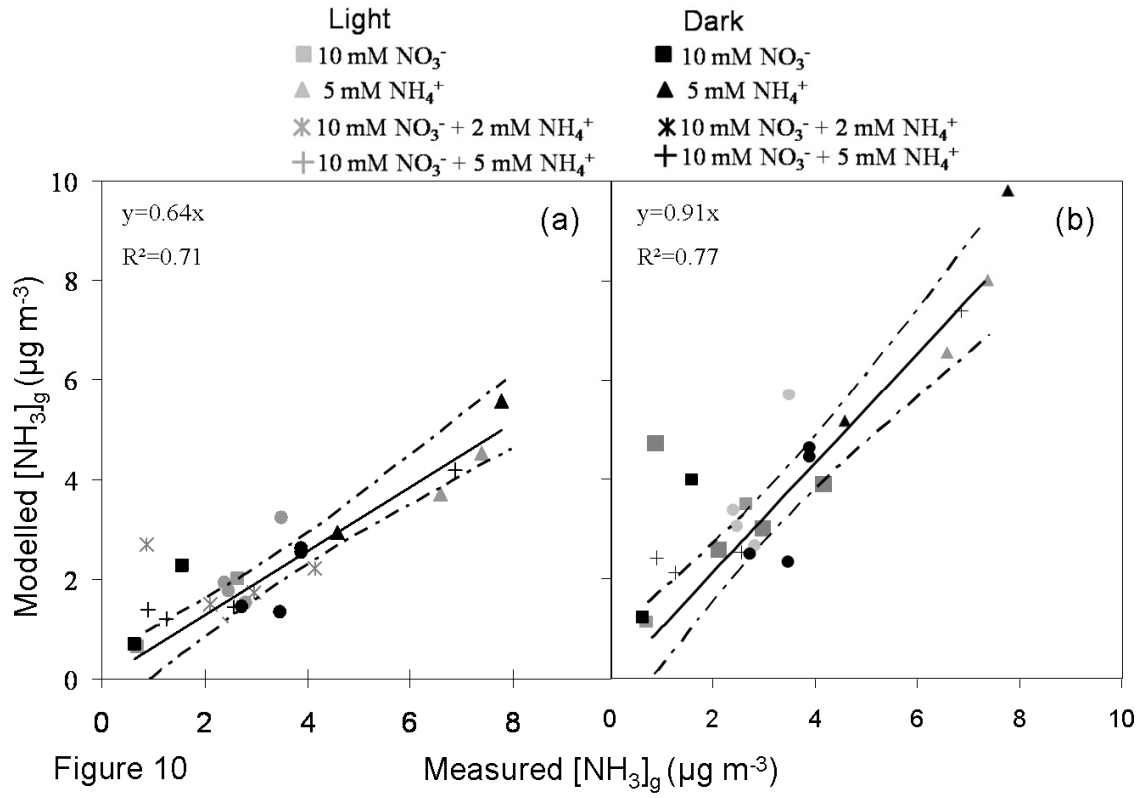

Figure 10

Figure 3.10: Comparison of modelled and measured compensation points $[NH_3]_g$.

Modelled vs measured compensation points (a) with parameter values from table 2, and forcing variables measured from experimental setups. Different symbols represent different N treatments. Black symbols are for dark measurements and grey symbols for light measurements. Figure b represents same graph but with model run with adjusted parameters. Adjusted apoplastic pH was 6.6, apoplastic volume 21 cm^3 m^{-2} and active transport parameter 4 x 10^{-4} s^{-1}. Filled lines are the linear regression for all represented points and dotted lines are the 95% confidence interval for each regression.

in a flux chamber with 7 to 9 oilseed rape plants at 5 weeks of age. Plants were treated with 4 different types of N nutrition and measurements were done for dark and light periods. The chamber system measured leaf temperature, water flux, PAR, photosynthesis rate, and the NH_3 flux. These measurements were used to calculate the stomatal compensation point and the stomatal conductance for water exchange. In parallel we had measurements of xylem sap, apoplast and bulk tissue extracts of NH_x and NO_3^- concentrations in the same conditions (Massad el al., 2008b). The StAmP model was forced with measurements of stomatal conductance, water flux, chamber CO_2 and NH_3 concentrations, PAR, leaf temperature and ammonium and nitrate concentrations in the xylem sap.

6.1. Comparison to chamber measurements

Figure 10a represents comparison between modelled and measured compensation points. As a first attempt the model seems to represent rather well the measured NH_3 compensation point, although high compensation point values are underestimated by the model. This leads to having a linear regression with a slope of 0.65 between measured and modelled compensation points. In an attempt to understand the difference between the measurements and the model for high N treatments, we run the model with different values of parameters that were shown to have an important effect on the modelled N fluxes. The slopes of the linear regressions for the different runs are represented in Table 4. The three pH values chosen were the ones used in the model's sensitivity analysis. We note that for higher pH values the model better represents measured compensation points. In this comparison test the model was not forced with measured pH values, although the measured pH values were around 6.6 which would allow the model to better represent measurements. We notice that a lower apoplastic volume allows a better agreement between modelled and measured compensation points. We varied the active transport parameter by 25% and noticed that a lower active transport would allow us to simulate higher compensation points. However, a 25% variation leads to an overestimation of the model with respect to the measurements. Based on the results shown in Table 4 we run the model again with adjusted values of apoplastic volume, pH and active transport rate. The values chosen were 21 cm^3 m^{-2} for apoplastic volume, 6.6 for apoplastic pH and 4.2 x 10^{-4} s^{-1} for active transport rate parameter. The comparison between modelled compensation points with adjusted parameters and measured compensation points are represented in Figure 10b. We notice a better agreement between modelled and measured compensation points with a slope of 0.91 for the linear regression.

Tableau 3.4: Slopes of the linear regressions between modelled and measured compensation points with changed parameters.

Changed Parameter	Slope	R^2
$pH_{ap} = 6.6$	0.78	0.71
$pH_{ap} = 5.8$	0.43	0.71
$V_{ap} = 21\ cm^3\ m^{-2}$	0.77	0.74
$V_{ap} = 36\ cm^3\ m^{-2}$	0.43	0.71
$K_{NH4ap\text{-}cy} = 3.8 \times 10^{-4}\ s^{-1}$	1.86	0.44
$K_{NH4ap\text{-}cy} = 6.3 \times 10^{-4}\ s^{-1}$	0.41	0.71

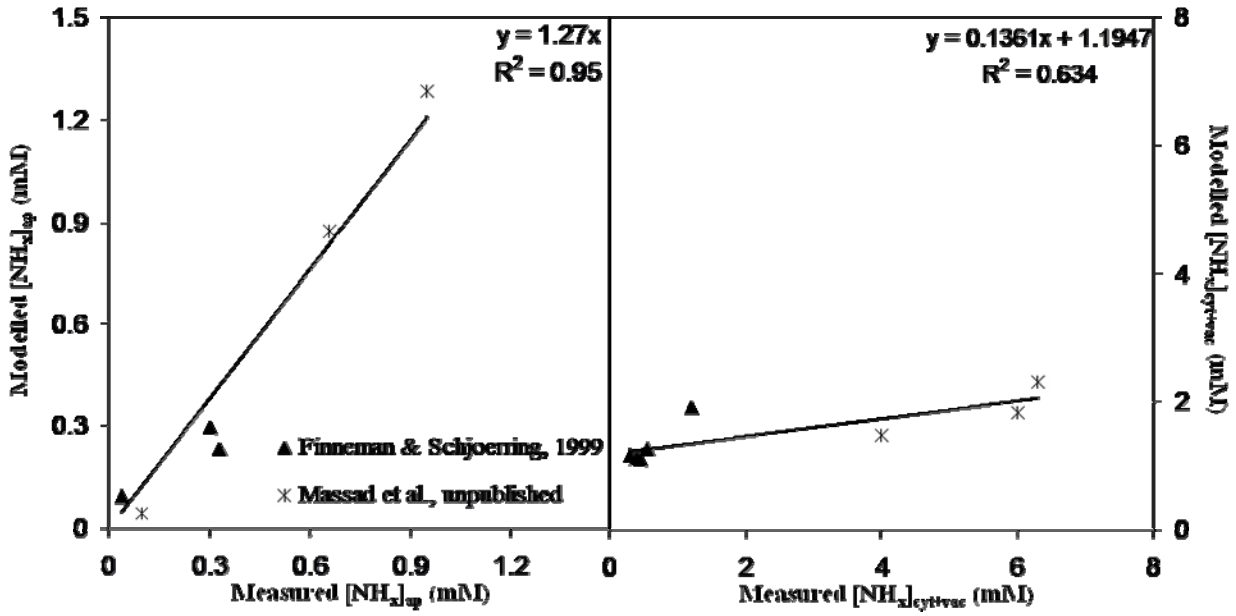

Figure 3.11: Comparison of modelled and measured apoplastic and intracellular NH_x concentrations.

Modelled vs measured apoplastic NH_x concentrations (a) and bulk tissue concentrations (vacuole + cytoplasm) (b) with parameter values from table 2, and forcing variables as simulated by the Tuzet et al. (2003) continuum model. Grey squares are for measurements from Massad et al. (2008b) and black triangles are from Finneman and Schjoerring (1999).

Measured compensation points with the chamber setup are subject to error linked to chamber and cuticular adsorption and to method of estimation of the compensation point (see Massad et al. 2008b for a discussion). Therefore comparison should be considered carefully. However, this comparison points out the importance and sensitivity of the model to apoplastic pH, volume and active transport rate. A good determination of these three parameters implies a good prediction of the compensation point value.

6.2. Comparison to extraction measurements

Figure 11 represents a comparison between modelled and measured apoplastic (a) and bulk tissue (b) ammonium concentrations. Forcing variables were PAR, ambient NH_3 concentration and xylem sap NH_x and NO_3^- concentrations along with the Tuzet et al. (2003) model outputs: stomatal conductance, xylem water flux, leaf temperature and CO_2 concentration in the sub-stomatal cavity. We compared measurements obtained by Finneman and Schjoerring (1999) and Massad et al. (2008b). We note that the model in this case overestimates measured ammonium concentrations in the apoplast but underestimates concentrations in the leaf bulk tissue extract. More precisely, the model does not show large changes in cytoplasmic NH4+ with increasing N supply as opposed to the measurements. This is clearly shown by the linear regression between measurements and modelled which gives a slope of 1.27 for apoplastic concentrations and 0.14 for bulk tissue extracts.

Extraction measurements are also subject to error (Massad el al. 2008b) which would probably lead to over-estimating the measured apoplastic NH_x concentrations. Noting that the chamber and the extraction comparisons were obtained from the same model run, one could think that a weak active transport rate from the apoplast to the cytoplasm could lead to an overestimation of apoplastic concentrations and underestimation of bulk tissue concentrations. Comparison of bulk tissue NH_x concentrations would also suggest that the apoplast-cytoplasm transport rate is too small.

This picture may however be not as simple since the NH_x sink (NH_x assimilation) in the cytoplasm in our case is always limited by NH_x availability and therefore is always in a very close range to the major NH_x source (photorespiration). This causes all the NH_x present in the cytoplasm to be directly assimilated and never to be able to accumulate and thus have an increase in cytoplasmic concentrations.

7. Conclusion

The leaf scale process based model presented here allows a coupling of water transfer, carbon and nitrogen metabolisms in a purpose to simulate the stomatal NH_3 compensation point. The model can be a useful tool in understanding the determinism of the compensation point in relation to environmental conditions, plant metabolism and N fertilisation.

The modelled compensation point is mostly sensitive to apoplast related parameters: volume, pH and active transport rate from the apoplast to the cytoplasm.

Volume and pH are well documented however active transport rate remains a major uncertainty in this modelling approach.

Atmospheric NH_3 concentration does not affect the stomatal compensation point in the conditions tested here.

Different NO_3^- concentrations in the xylem sap do not seem to generate different compensation point values.

The most important environmental factors determining the stomatal NH_3 compensation point are leaf temperature, and NH_x flux coming to the leaf. However the effect of temperature is found to be less pronounced than expected mainly because of the opposite evolution of the compensation in response to the temperature parameterisation of plant processes.

The apoplast and the cellular compartments seem to be uncoupled and the leave's N and C metabolisms seem to have little effect on the stomatal compensation point. However, a change in the apoplast-cytoplasm transfer rate as well as a modification of the NH_x diurnal flux to the leaf may modify the picture slightly; as in such case the diurnal apoplastic variation is mainly due to the NH_x transfer rate from the apoplast to the cytoplasm.

Moreover, the comparison of measured and modelled NH_x concentrations in the apoplast and leaf bulk tissue suggests that the model should have a more efficient active transport between the apoplast and the cytoplasm.

Based on this modelling approach a better understanding of the NH_3 stomatal compensation point should pass by a better characterisation of the transport processes of NH_3 and NH_4^+ between the apoplast and the cytoplasm and a better understanding of the determinism of apoplastic pH.

This modelling approach also suggest the relative uncoupled behaviour between the apoplastic and cytoplasmic compartments with respect to NH_x concentrations for a single layer canopy in a vegetative stage of growth. This behaviour however should be different for

plants having senescent leaves and in multi-layered canopies. This aspect requires further investigation.

However, intra-cellular concentrations do determine N leaf to leaf fluxes especially in multi-layered plants and would therefore be important in determining the NH_3 stomatal compensation point. This would be an interesting approach in simulating the NH_3 compensation point for senescing leaves and would suggest integrating a root compartment in the actual model as well as extending it to cover several plant layers.

Discussion générale

« Douter de tout ou tout croire sont deux solutions également commodes, qui l'une et l'autre nous dispensent de réfléchir »

Henri Poincaré (Mathématicien 1854-1912)

Les échanges d'ammoniac entre la végétation et l'atmosphère bien que très faibles en terme de flux par rapport aux émissions des bâtiments d'élevage ou bien du sol restent un point critique dans le bilan global des composés azotés réactifs. Ceci à cause :

– du rôle important que joue la végétation dans la régulation des concentrations atmosphériques et dans le transport à grandes échelles ;

– des incertitudes liées à la quantification des échanges entre la végétation et l'atmosphère ;

– et du manque de connaissance sur le déterminisme du potentiel d'émission du couvert végétal.

L'objectif de la thèse est de comprendre le déterminisme des échanges stomatiques d'ammoniac entre la végétation et l'atmosphère en relation au métabolisme azoté et carboné de la plante afin de mieux quantifier les échanges végétation –atmosphère.

Ce travail a abouti à la construction d'un modèle permettant de simuler le point de compensation stomatique de la feuille en relation au métabolismes azoté et carboné. Il permet également de prendre en compte dans cette determination du point de compensation l'état hydrique de la feuille à travers la conductance stomatique et le flux d'eau dans le xylème. Ce modèle a permis d'évaluer l'impact des différentes nutritions azotées sur le point de compensation ainsi que les différentes conditions environnementales (température de feuille, PAR, …). Les résultats obtenus par le modèle dépendent des hypothèses considérées pour sa construction. Une première comparaison entre sorties du modèle et résultats expérimentaux donne des résultats assez satisfaisants ce qui nous réconforte dans les hypothèses choisies. La plupart des hypothèses sont basées sur des données et des résultats de la littérature. Cependant certaines hypothèses sont moins sures et remettent en question les résultats du modèle.

Les mesures des points de compensation en chambre à flux et par extraction d'apoplasme ont permis la caractérisation de celui-ci en conditions jour et nuit et avec différentes nutritions azotées. Ces expérimentations ont aussi permis d'évaluer les deux méthodes de mesure et de comprendre les raisons de leurs divergences.

Dans cette partie je reviens sur les questions auxquelles cette thèse prétend répondre et je soulèverai également les points à approfondir.

1. Relation entre les différentes nutritions azotées et le point de compensation stomatique de l'ammoniac

Plusieurs auteurs ont étudié la variation du point de compensation en ammoniac avec la nutrition azotée de la plante (Mattsson et al. 1998 ; Mattsson & Schjoerring, 2002). Ils ont

ainsi constaté la relation linéaire entre la concentration en ammonium dans la solution nutritive et le point de compensation. Par contre une alimentation azotée sous forme de nitrate avait très peu d'effet sur le point de compensation. Lors de nos expérimentations en chambre à flux mais aussi avec la méthode d'extraction d'apoplasme nous avons les mêmes résultats. Nous avons aussi constaté qu'une alimentation mixte en ammonium et nitrate à même concentration en ammonium qu'une alimentation en ammonium seule n'engendre pas le même point de compensation. Ainsi avec une alimentation mixte on a un point de compensation plus faible. Ceci peut être expliqué à la fois par le fait que les racines des plantes de colza absorbent préférentiellement le nitrate et aussi parce que le nitrate induirait une vitesse d'assimilation plus forte de l'ammonium dans les cellules d'où un point de compensation plus faible.

Les simulations avec le modèle démontrent aussi une forte dépendance du point de compensation de la concentration en ammonium dans le xylème et une faible dépendance sur la concentration en nitrate. Le modèle est pour l'instant appliqué à l'échelle de la feuille, la concentration d'ammonium dans le xylème est une variable de forçage. L'ammonium dans le xylème provient soit de l'absorption racinaire soit de la redistribution d'azote au sein de la plante. Il est donc important d'examiner en détail le compartiment racinaire et de pouvoir déterminer la concentration en ammonium dans le xylème en relation avec l'absorption racinaire et la distribution d'azote au sein de la plante.

2. Mesures des échanges en condition de jour et de nuit

Il est évident que les flux d'ammoniac entre la végétation et l'atmosphère sont différents entre le jour et la nuit et ceci principalement à cause de la fermeture des stomates. Le point de compensation stomatique et la concentration dans le liquide apoplastique sont-ils différents entre le jour et la nuit ? Le modèle développé simule un point de compensation plus faible la nuit que le jour. Ceci peut être expliqué par le fait que les deux principales sources d'ammoniac du modèle : la photorespiration et le flux de xylème sont très faibles en conditions de nuit. Deux expérimentations dans la littérature essaient de mesurer des points de compensation en conditions de jour et de nuit (Husted & Schjoerring, 1996 ; Mattsson & Schjoerring, 1996). La variation mesurée est très faible. Nous avons fait des mesures en conditions de jour et de nuit dans nos expérimentations. Nous avons ainsi remarqué une différence entre les mesures faites de jour et de nuit. Cependant due à la dispersion des mesures cette différence n'est pas significative. Des résultats non publiés encore de concentrations en ammonium dans l'apoplasme (Herrmann et al. 2008 ; Mattsson et al. 2008)

indiquent aussi une variabilité du point de compensation entre jour et nuit sur une prairie naturelle qui s'accentue après fertilisation.

La différence entre les conditions jour et nuit est principalement liée au métabolisme carboné de la plante. La photosynthèse et la photoréspiration étant les principaux processus foliaire affectés par le PAR. A travers l'analyse de sensibilité du modèle nous avons établi que le point de compensation n'est pas sensible aux paramètres de la photosynthèse. Ceci peut en partie expliquer les points de compensation similaires mesurés de jour et de nuit. Nous ne savons pas encore comment le métabolisme carboné peut influencer le point de compensation à plus long terme. Nous savons que les feuilles sénescentes ont un point de compensation plus élevé que les feuilles en croissance et que la sénescence résulte d'une modification des métabolismes carboné et azoté de la plante.

3. Mise en évidence de l'importance du flux d'eau dans le xylème et de la concentration en ammonium

Le modèle a permis de tester la réponse du point de compensation de l'ammoniac aux facteurs du milieu et aux termes sources et puits cellulaires. Ainsi avec les hypothèses considérées pour la construction du modèle on a pu conclure que le point de compensation stomatique était surtout dépendant de la température de feuille, du flux d'eau dans la plante et de la concentration en ammonium dans le xylème. Une partie des ions ammonium pourrait donc après une forte fertilisation passer directement du xylème vers l'apoplasme et vers l'atmosphère sans passer par la cellule et donc ne serait pas influencée par le métabolisme azoté. D'autre part, on a constaté que c'est le flux d'eau et donc l'apport des ions azotés qui provoque les variations diurnes dans le modèle du point de compensation. Cette hypothèse pourrait expliquer les résultats de Herrmann et al. (2008) qui montrent une variation diurne plus importante après une fertilisation azotée.

Il est donc important de bien caractériser le flux d'eau dans la plante à la fois pour caractériser l'ouverture stomatique et aussi pour caractériser le flux du xylème. Le couplage du modèle de point de compensation avec le modèle de continuum sol-plante-atmosphère nous permettrait de mieux comprendre la relation du point de compensation au flux d'eau et aux réservoirs d'eau de la plante.

4. Compartimentation de l'ammoniac et relation au point de compensation de l'ammoniac

L'analyse de sensibilité du modèle a montré une très faible dépendance de la concentration en ammonium dans l'apoplasme de celle du cytoplasme à des échelles de temps très courtes (quelques journées) après une fertilisation. A l'échelle de la journée, la concentration en ammonium dans l'apoplasme est indépendante de la concentration et donc des processus ayant lieu au sein du cytoplasme. Une probable piste de simplification du modèle serait donc l'élimination du compartiment cellulaire. Ceci s'applique dans des conditions de forte alimentation azotée. Si on observe la dynamique des concentrations en ammonium dans l'apoplasme et le cytoplasme sur plusieurs jours et en conditions de faible alimentation azotée, on remarque une influence des concentrations dans le cytoplasme et la vacuole sur l'apoplasme. Cette dépendance peut être expliquée par le temps de réponse des différents compartiments aux changements de la nutrition azotée. Le temps de réponse de l'apoplasme étant de 2 heures approximativement alors que celui de la vacuole est de 20 heures.

Une autre hypothèse discutable est le transfert actif d'ammonium entre l'apoplasme et le cytoplasme. Je considère que ce transfert est proportionnel à la concentration en ammonium dans l'apoplasme et à une vitesse de transfert fixée à une valeur constante. Cette hypothèse est partiellement justifiée par des mesures démontrant un rapport fixe entre concentration en ammonium dans l'apoplasme et le cytoplasme (Nielsen & Schjoerring, 1998). De plus l'analyse de sensibilité a montré une très grande sensibilité du point de compensation simulé à la vitesse de transfert actif fixé. Cette vitesse de transfert actif est mal définie et il n'existe presque pas de valeurs dans la littérature.

5. Comparaison de mesures par extraction d'apoplasme et par chambre à flux

Comme décrit dans la partie 1 de cette discussion, il n'existe pas actuellement de méthode évidente permettant de mesurer le point de compensation stomatique de l'ammoniac ou la concentration en ammonium dans l'apoplasme. Le système de mesure par chambre génère une erreur totale entre 10 et 60% qui serait principalement due au flux cuticulaire et au flux sur la surface de la chambre elle-même. Il faut y ajouter les erreurs dues aux différents appareils de mesures connecté à la chambre (analyseur d'NH_3, débitmètre massique, capteur d'humidité, …). Quand aux mesures par extraction j'ai mesuré une erreur due à la contamination par du matériel cytoplasmique de 3.3%. Il faut aussi pour ce type de mesures considérer les limites de la méthode due à l'infiltration d'un liquide dans la cavité sous stomatique et qui pourrait influencer les flux entre apoplasme et cytoplasme et donc fausser

les résultats. Gardant ces facteurs en tête les résultats obtenus ne sont pas différents à d'autres mesures citées dans la littérature. Ainsi la gamme de variation du point de compensation mesuré par les deux méthodes n'est pas différente de la gamme de variation rapportées dans la littérature et les variables influençant le point de compensation (concentration en ammonium et en nitrate, conditions nuit et jour) ont été rapportées précédemment. Cependant la comparaison entre la méthode d'extraction et la méthode de chambre à flux montre un point de compensation plus élevé mesuré par la méthode d'extraction et qui est d'ordre inverse à ceux rapporté par Hill et al. (2001) qui eux mesurent un point de compensation plus fort par chambre à flux. Ces mesures doivent donc être considérées avec précaution et toujours en prenant en compte les sources d'erreurs possibles.

Il est certes difficile de comparer ces deux méthodes pour les raisons suivantes :

- Elles ne s'appliquent pas aux mêmes échelles. L'extraction d'apoplasme mesure la concentration moyenne de l'apoplasme d'une feuille sachant qu'il y a un gradient dans l'apoplasme d'une même feuille. Alors que la méthode de chambre à flux permet le calcul du point de compensation moyen pour toutes les plantes interposées dans la chambre en intégrant le flux de toutes les feuilles.
- Elles ne mesurent pas les mêmes variables. La méthode d'extraction permet de recalculer un point de compensation stomatique connaissant la température de la feuille et le pH et en supposant une concentration en ammoniac homogène au sein de la chambre. La méthode de chambre permet de recalculer un point de compensation couvert. Pour l'instant il est difficile de différencier le flux cuticulaire du flux stomatique. La seule solution existante est d'essayer de minimiser le flux cuticulaire en forçant une faible humidité relative.
- Il est difficile d'obtenir exactement les mêmes conditions étant donné que la méthode d'extraction permet une mesure ponctuelle dans le temps alors que la méthode de chambre est une mesure dynamique.

Les mesures avec les deux méthodes montrent une même dynamique de changement en fonction de la nutrition azotée et des conditions jour et nuit. Cependant les valeurs absolues sont significativement différentes entre ces deux méthodes. La méthode de flux donne des valeurs plus faibles par rapport à la méthode d'extraction ce qui est contraire aux résultats de Hill et al. (2001) qui ont des points de compensation plus forts avec la méthode des flux.

Conclusion & perspectives

« La Science est toujours erronée.
Elle ne résout jamais un problème
sans créer dix d'avantage »

George Bernard Shaw (Romancier: 1856-1950)

La question essentielle à laquelle ce travail de thèse essaye de répondre est la suivante :
« Quel est l'impact de la quantité et de la forme de la nutrition azoté et du métabolisme azoté de la plante sur le point de compensation stomatique de l'ammoniac ? »
J'ai tenté d'y répondre à travers :

1. Une analyse bibliographique de la littérature sur le sujet en vue d'extraire les principaux processus agissant sur le déterminisme du point de compensation stomatique de l'ammoniac.
2. La conception d'expérimentation utilisant deux méthodes de mesures (extraction d'apoplasme et chambre à flux). Ces expérimentations ont permis l'accès au point de compensation (afin de déterminer l'influence de la nutrition azotée et de conditions jour et nuit sur le point de compensation). Les données de ces expérimentations avaient aussi pour but d'être utilisées pour la validation du modèle.
3. La construction d'un modèle en utilisant les simplifications faites à partir de l'étude bibliographique, qui permet de simuler le point de compensation stomatique de l'ammoniac. L'étude de l'influence des facteurs du milieu et de la fertilisation azotée sur le point compensation de l'ammoniac à travers différentes simulations du modèle.

Les principaux résultats obtenus peuvent être résumés dans les points ci-dessous :

– La forte dépendance du point de compensation stomatique de l'ammoniac de la concentration en ions ammonium dans la nutrition azotée.

– La faible dépendance du point de compensation stomatique de la concentration en nitrate dans la nutrition azotée.

– Une nutrition azoté mixte ammonium et nitrate minimise la dépendance du point de compensation de la concentration en ions ammonium dans le cas du colza.

– Les conditions de jour auraient tendance à induire un point de compensation plus fort que les conditions de nuit bien que ce résultat ne soit pas statistiquement avéré.

– Une forte dépendance du point de compensation en ammoniac du flux d'eau dans la plante et de la concentration en ions ammonium dans le xylème.

– Une indépendance du point de compensation stomatique de la concentration en ammoniac de l'atmosphère.

Outre ces résultats, ce travail a permis de développer un système de chambre à flux qui s'inspire de modèles déjà existants et qui permet entre autre de calculer le point de

compensation stomatique de l'ammoniac. A travers mes expérimentations j'ai pu me rendre compte des limites de ce système de mesure. Les limites sont principalement causées par la nature chimique de l'ammoniac et par l'absence d'analyseur permettant une mesure précise et fiable des concentrations dans l'air. Ainsi un des principaux problèmes rencontrés était de pouvoir séparer dans les mesures par chambre le flux stomatique du flux cuticulaire. Ce flux cuticulaire est mal caractérisé pour l'instant et nous avons peu de données là-dessus. Un autre problème rencontré était la limite de détection des concentrations de l'analyseur utilisé. Ceci nous a obligé à faire des mesures avec de fortes concentration en ions ammonium dans la solution nutritive afin d'avoir des flux et donc des concentrations en ammoniac dans la chambre relativement élevées.

Une autre réalisation de cette thèse fut le développement d'un modèle de point de compensation stomatique de l'ammoniac en relation au flux d'eau et au métabolisme azotée et carboné de la plante. Ce modèle pour l'instant est un modèle statique qui s'applique pour une feuille de colza dans un stade végétatif de croissance. Les résultats du modèle ont été comparés aux mesures par chambre et par extraction mais ceci mériterait une comparaison plus rigoureuse et dans différentes conditions afin de pouvoir valider le modèle. D'autre part ce modèle a aussi ses limites. L'étude de sensibilité montre une forte dépendance du volume et du pH de l'apoplasme ainsi que de la vitesse de transport actif de l'ammonium entre l'apoplasme et le cytoplasme. Nous avons très peu de données sur cette vitesse de transport et avons difficilement accès à travers des mesures à sa valeur.

Les perspectives de ce travail sont nombreuses et variées. Du point de vue mesure, il serait intéressant d'effectuer des mesures en chambre avec de l'azote marqué qui permettrait peut être de différencier le flux cuticulaire du flux stomatique. Une autre piste intéressante serait l'utilisation de plantes mutantes dont la caractéristique est d'avoir des stomates ouverts en l'absence de lumière. Cela permettrait par exemple de tester expérimentalement la dépendance du point de compensation en ammoniac du flux d'eau et non du PAR en utilisant une variété mutante de la plante Arabidopsis dont la caractéristique est d'avoir des stomates ouvertes en absence de lumière. Tout ceci dépend bien sur de l'adaptabilité des analyseurs et des méthodes de mesures où il y a beaucoup à améliorer.

D'un point de vue modélisation les perspectives à court terme seraient d'étendre le modèle à l'échelle de la plante et d'inclure un compartiment racine. Ceci permettrait de mieux valider le modèle avec des données obtenues aux champs et pour des conditions environnementales plus réalistes et plus variées. Une autre étape intéressante serait d'étendre le modèle à toute une durée culturale de la croissance à la sénescence. Un aspect crucial serait d'inclure les

processus de sénescences car on sait que les feuilles sénescentes sont une source importante d'ammoniac. Cette étape peut se faire soit en incluant le modèle du point de compensation d'ammoniac dans un modèle de cultures (CERES, ou autres) soit en développant un module de croissance. Ce modèle s'applique au colza, il serait intéressant également de l'étendre à d'autres espèces agronomique de type C_3 ou C_4 et de le tester.

Pour conclure, j'énumère quelques questions que ce travail a pu soulever et qui seraient intéressantes à explorer dans le futur pour mieux comprendre et caractériser les échanges d'ammoniac entre la végétation et l'atmosphère.

- Quelle est la part du flux cuticulaire dans les échanges d'ammoniac entre la végétation et l'atmosphère et comment la caractériser ?
- Le transport actif entre l'apoplasme et le cytoplasme est il déterminant dans le point de compensation stomatique de l'ammoniac et comment le caractériser ?
- Quels sont les paramètres génériques du modèle et lesquelles sont spécifiques à l'espèce ; et est-ce que nous arriverons en changeant certains paramètres à reproduire la variabilité interspécifique du point de compensation ?
- Le nitrate dans l'apoplasme se transforme-t-il en NO_2 et existe-t-il un point de compensation stomatique pour le NO_2 ?

Annexe

Rapport suite à la visite au Professeur Hubert Felle au « Botanic Institue » à Justus Liebig University afin de se familiariser avec la méthode de mesures par Micro-éléctrodes et d'étudier leurs applicabilités aux mesures des concentrations en ammonium dans l'apoplaste des feuilles. Cette visite à été financer par le programme « Nitrogen in Europe (NinE)» du « European Science Foundation (ESF) ».

Visit to Professor Hubert Felle
Botanic Institute
Justus Liebig University
13-16 June, 2006

Ion selective micro electrode technique

1. Principle

The principle of measurement in microelectrodes is based on potential difference. Potentiometric microsensors are based on charge separation of ions across a membrane. An electrical potential difference is hereby generated according to the Nernst equation:

$$\Delta E = \frac{RT}{zF} \ln\left(\frac{a_i}{a_e}\right)$$

where R is the gas constant, T the absolute temperature, z the charge of the ion, F the Faraday constant, a_i and a_e the ion activity, in the sample and in the electrolyte solution, respectively. As the activity in the electrolyte solution (a_e) may be considered constant, the electrical potential difference across the membrane becomes proportional to the logarithm of the ion activity in the sample. The formula can then be simplified to the following:

$$\Delta E = E_0 + k.\log(a_i)$$

introducing a constant, k, and an offset potential, E_0.

There are three major types of potentiometric microsensors: full glass, Ag/AgCl half cell and liquid ion-exchange (LIX) based microsensors. The microsensor used in our case is the Liquid ion-exchange (LIX) based microsensor. The principle of the LIX microsensor is the same as the other potentiometric microsensors. The ion selective membrane is in this case a liquid ion exchanger. Liquid ion exchangers are commercially available (e.g. from Fluka). To seal the capillary tip of the microsensor with the hydrophobic membrane the glass has to be silanized

(Ammann, 1986). The LIX membrane can be solidified in the tip with PVC. In *ion selective electrodes* (ISE) the dissociation of groups contained in the sensitive membrane or the liquid in the LIX type microsensor causes alterations of the charge density and thus changes of the potential. The activity and the potential are logarithmically related. To account for the influence of disturbing ions, e.g., of a substance P, a selectivity coefficient, k_{sp} is introduced. The general shape of the typical response curve is a third degree polynomial, with a linear portion generally in the range 10^{-4}-10^{-2} M, where deviations at low concentrations (< 10^{-4} M) indicate the detection limit of the potentiometric electrode, and at high concentrations are due to saturation of the enzyme in the sensor liquid. The activity of the enzyme influences the response time of the electrode and this is also affected by the thickness and permeability of the enzyme layer or liquid sensor. Optimum response times are achieved with thin highly permeable layers using as high an enzyme activity as possible. In practice however more durable, thicker membranes, with a slower response time, are generally employed in order to prolong the lifetime of the sensor.

The ion selective microelectrodes gives the whole potential difference between the sample and the electrolyte solution, to be able to differentiate between the potential difference due to the ion in question and the voltage change in the sample due to other fluctuations; one should measure in the same time the voltage change in the sample by placing a voltage reference micro electrode.

2. Building technique

Ion selective micro electrodes are slightly different in building than voltage reference electrodes. During my stay I prepared both kinds of electrodes. The main steps for building are the following:

a. Pipette pulling

Micro electrodes are built from thin diameter glass rods. This allows us to have electrodes with variable tip sizes depending on the type of use. The glass rod should have a solid filament in it to allow filling (see step c).

Micro electrodes for apoplastic use should be blunt with a diameter tip of 4 to 5 µm. This prevents cell injury during placement and therefore facilitates it. For intracellular use however, micro electrodes should be sharp and much thinner to be able to penetrate the

cell membrane. It should be noted that microelectrodes for intracellular use should also be shielded to prevent the pushing and the loss of the sensor when the electrode is inserted in the cell due to the cell turgor.

Blunt electrodes are pulled in two steps using a Patch-Clamp puller. Time and intensity of the puller are to be adjusted to obtain 4 or 5 µm thick tips.

b. Silanization

Silanization is an important step in building. It only applies for ion selective microelectrodes, voltage reference electrodes should not be silanized. This step consists of rendering the electrode tip hydrophobic so that the sensor fills it to the top and is well fixed. The silanizing mixture consists of 0.2% TributylSilane dissolved in chloroform. Once pulled electrodes are heated in an oven at 200°C for 1 hour and are then dipped through the rear end in the silanizing solution. The solution migrates to the top of the electrode along the solid filament. Electrodes are then baked again for approximately 1 hour. This operation is repeated 2 times to make sure the tip is well silanized.

c. Filling

Depending on the use of the electrode, the filling can either be with the resin cocktail or sensor at the tip followed by the reference solution (1 M KCl) for ion selective electrodes or with a 0.5M KCl solution mixed with 1% agar for voltage electrodes.

- Filling procedure for voltage electrodes

  Mix 1% agar in 0.5M solution KCl and heat. Once the agar is dissolved, place the whole electrode in the hot mixture, leave for 2 minutes (air bubbles appear at the rear end indicating that the solution is getting in), remove the electrode, and place it in a 0.5M KCl solution with no agar. Once cooled the electrode is totally filled with the reference solution using a needle syringe. No air bubbles should be present inside the electrode.

- Filling procedure for the ion sensitive electrode

  There are two ways for filling the ion selective micro-electrode. The same result however is obtained; the resin cocktail (consisting of 200 mg PVC (Fluka) in 5 ml tetrahydrofuran (Fluka) 70% volume mixed with 30 % Fluka resin of the selected sensor) being at the tip of the electrode (1 mm) topped by a thin layer of sensor alone (no PVC) to prevent further evaporation finally followed by the reference solution (1M KCl).

The first technique of filling consists of:

- Pulling a filling pipette suited to reach into the silanized micro electrode tip.
- Sucking the resin cocktail into the filling pipette with the help of a syringe (front filling).
- Inserting the filling pipette into the silanized electrode as far as possible and gently pressing a small amount of cocktail into the tip.
- The resin normally migrates alone to the top of the electrode along the internal solid filament leaving no air bubbles, if air bubbles are there try removing them by sucking through the filling pipette.
- Fill to approximately 1 mm and leave so that half of the resin evaporates.
- With the help of the filling pipette top again with pure sensor to prevent further evaporation.
- Fill the rest of the electrode with the reference solution using a filling pipette at the beginning and then using a needle end syringe. Make sure no air bubbles are stuck in the electrode.

The second filling technique consists of placing the electrode upright in the resin cocktail (sensor + PVC) and waiting for the spontaneous filling (approximately 30 minutes). The rest of the procedure is as the first one (letting evaporate till 50%, topping with pure sensor with the help of a filling pipette and then with the reference solution).

Filled micro electrodes give a better performance if left to equilibrate overnight. Micro electrodes should be stored in a dry, cool and dark place.

3. Placing technique

Micro electrodes are connected to a high impedance (10^{15} Ohm) amplifier. It should be a double channel amplifier to be able to have the difference between the two electrodes (voltage and ion selective) the amplifier is connected to a recorder or printer as output.

The two electrodes are mounted on 3 dimensional movement micro manipulators to facilitate placing inside the sub-stomatal cavities.

The placement is done under a microscope with a moving main body tube (and not stage). A cold light source is favorable.

The plant sample should be well fixed and should be connected to a reference solution (usually with conditions similar to as those of the apoplast) which is grounded so that we have

a closed electrical circuit. This solution can also serve to control or to change ionic concentrations in the apoplast.

The whole assembly should be mounted on a vibration absorbing table (to minimize noise due to vibrations and to prevent displacement of the electrode inserted in the sub-stomatal cavity). The system should also be enclosed in a Faraday cage to prevent perturbations due to static electricity.

Once the plant mounted, we should wait for the stomata to open before placing the electrodes in place.

Once the stomata open the following steps are to be followed:

- Have the stomata in focus under the microscope.
- Connect the 2 microelectrodes to the pre-amplifiers and approach them as much as possible to the leaf surface.
- The angle of the electrodes with respect to the leaf should be as close as possible to the perpendicular to the leaf surface without interfering with the microscope objective (45° approximately).
- Now place the electrodes in focus and try to adjust their place just above a stomatal opening each by going to and from a focus on the stomata and the microelectrodes.
- Get the electrodes closer to the leaf surface by first descending the microscope objective a little and then the electrodes so that they are back in focus (in order to avoid breaking the electrodes or injuring the plant by hitting the electrodes with it).
- Once the electrodes and the stomata are almost in focus, adjust with the fine adjustments buttons the horizontal placement above the stomatal opening.
- Start the amplifier and while observing the voltage reading, with the fine adjustment button, descend the microelectrodes so that there is a voltage reading (the electrical circuit closes).
- Make sure the reading is relatively stable, close the faraday cage and now turn on the output recorder.

4. Calibration and tests

During the visit, several calibration tests were done. Few tests were made on plants, but several placements were done to get familiar with the technique.

a. pH electrode calibration and test

The calibration should be done with a continuous flow of the calibration solution to insure that there is no ion accumulation. The pH selective micro electrode was calibrated with

standard buffer solutions with the following pH values: 4.1, 5.0, 5.9, 7.3, and 8.5. The calibration was also done with buffer solutions containing 1 M KCl to test the selectivity of the electrode. The results showed no difference between solutions with or without KCl, indicating that the pH sensitive electrode is not influenced by K^+ ions. Upon plotting the pH against the voltages obtained, we obtain a straight line with a slope of 55.2 (mV/pH) which is in the normal range as compared to other pH microelectrodes (Felle, 2002).

We also tested the pH electrode in the apoplast of barley leaves. We notice that the pH varies with lighting conditions drastically at the beginning and then tends to come back to its initial value. We also recorded that the pH changed upon changing the reference solution in which the tip of the leaf bathes. This change is particularly important as it indicates that pH values measured in the apoplast by the infiltration/centrifugation technique could be altered by the infiltration process.

b. NH_4^+ electrode calibration and test

As a first step we built an ammonium selective micro electrode with a PVC mixed tip. The electrode was calibrated with ammonium solutions of the following concentrations: 100, 10, 1, 0.1, and 0.01 mM in a buffered pH 5 solution.

The electrode showed a linear response to concentrations above 1 mM NH_4Cl and a non linear response for inferior concentrations.

Given that the ammonium cocktail sensor used in not very selective with respect to KCl and to pH (see Appendix) we had to test the effect of different pH values and KCl concentrations on the electrode's response. The values chosen were the ones likely to be expected in the apoplast (pH 5 and 6; KCl concentrations of 1 and 10 mM).

- pH effect

  Testing the electrode with two buffer solutions one of pH 5 and the other of pH 6 for different ammonium concentrations showed no significant difference between the 2 pH values. According to the documents provided by Fluka (Appendix) concerning the ammonium selective cocktail, there should be a pH effect. We probably would have detected this effect in other ranges of pH, but it wouldn't affect measures in the apoplast since the pH only varies between 5 and 6.

- KCl effect

  We tested 2 KCl concentrations (1 and 10 mM) in a buffer solution of pH 5 and with ammonium concentrations ranging from 0.01 to 100 mM. The electrode was not sensitive for ammonium concentrations above 10 mM but revealed to be sensitive as compared to the calibration done with no KCl for concentrations of ammonium

inferior to 10 mM. The difference in response is not really a problem since by adding 1 mM KCl to the reference solution in which the plant lies we can control the concentration in the apoplast and thus can calibrate the electrode with a 1 mM KCl solution. However, the electrode did not respond at all to concentrations of ammonium inferior to 0.1 NH_4Cl for the solution containing 1 mM KCl and inferior to 1 mM NH_4Cl for the solution containing 10 mM KCl. This is a little more problematic since the concentrations of ammonium in the apoplast are normally below 1 mM.

We tried to build an electrode with no PVC mixed with the sensor and with a smaller tip end to prevent the sensor from flowing out. With this electrode we were able to detect a response between 0.1 an 1 mM NH4CL with 1 mM KCl. This response was not linear but with further modifications and a rigorous calibration it should be able to fit our purpose.

c. Voltage electrode testing

In order to have some indications on how electrodes work on plants, we tried to measure voltage differences in barley plants in the apoplast and inside mesophyllic cells. The voltage value is not quantifiable by itself. It varies a lot from cell to cell and from electrode to electrode. It gives however an indication on the polarization or depolarization of the apoplast and the cell.

5. Conclusion

The ion selective micro electrode technique would be interesting as a tool for measuring ammonia exchange between the sub-stomatal cavities and the atmosphere. It is particularly interesting to measure pH values since the pH selective micro electrode has been widely used and is fairly selective. Further investigations have to be done concerning the ammonium selective electrode, especially concerning the selectivity with respect to KCl. Electrodes with a better performance that the one sited here were obtained before as indicated by Hanstein and Felle (1999). Another interesting feature to investigate would be building micro electrodes for gas measurements inside the sub-stomatal cavity similar in principle to the electrode build for CO_2 measurement (Hanstein et al., 2001).

An approximate budget for acquiring the material necessary for this technique is detailed in the table below:

Description	**Cost (€)**
High impedance Amplifier (input resistance 10^{15} Ohm) with 2 channels, Differential amplifier	6000
2 Micro manipulators	4000 x 2
Microscope	3000
Chart recorder or recording output	3500
Vibration absorbing table	Home built
Faraday cage	Home built
Puller	8000
Consumables (glass rods, sensors, electrode holders, …)	1000
Total	**~ 30 500**

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