Titre: Title:	In vivo ^{31}P-NMR study of phosphate metabolism for Eschscholtzia californica using a small-scale perfused bioreactor
Auteur: Author:	Jingkui Chen
Date:	2004
Type:	Mémoire ou thèse / Dissertation or Thesis
Référence: Citation:	Chen, J. (2004). In vivo ^{31}P-NMR study of phosphate metabolism for Eschscholtzia californica using a small-scale perfused bioreactor [Mémoire de maîtrise, École Polytechnique de Montréal]. PolyPublie. https://publications.polymtl.ca/7470/

NOTE TO USERS

UNIVERSITÉ DE MONTRÉAL

IN VIVO ^{31}P-NMR STUDY OF PHOSPHATE METABOLISM FOR *ESCHSCHOLTZIA CALIFORNICA* USING A SMALL-SCALE PERFUSED BIOREACTOR

JINGKUI CHEN
DÉPARTEMENT DE GÉNIE CHIMIQUE
ÉCOLE POLYTECHNIQUE DE MONTRÉAL

MÉMOIRE PRÉSENTÉ EN VUE DE L'OBTENTION
DU DIPLÔME DE MAÎTRISE ÈS SCIENCES APPLIQUÉES
(GÉNIE CHIMIQUE)
AUGUST 2004

Your file Votre référence
ISBN: 0-612-97935-0
Our file Notre référence
ISBN: 0-612-97935-0

UNIVERSITÉ DE MONTRÉAL

ÉCOLE POLYTECHNIQUE DE MONTRÉAL

Ce mémoire intitulé:

IN VIVO ^{31}P-NMR STUDY OF PHOSPHATE METABOLISM FOR *ESCHSCHOLTZIA CALIFORNICA* USING A SMALL-SCALE PERFUSED BIOREACTOR

présenté par: CHEN Jingkui

en vue de l'obtention du diplôme de: Maîtrise ès sciences appliquées

a été dûment accepté par le jury d'examen constitué de:

M. LEGROS Robert, Ph.D., président

M. JOLICOEUR Mario, Ph.D., membre et directeur de recherche

M. RIVOAL Jean, Ph.D., membre

ACKNOWLEDGEMENTS

I am very grateful to my Director, Prof. Mario Jolicoeur, for directing me to be thorough and independent and for offering his knowledge and experience during the course of my research.

I would like to thank Canadian Research Chair, Canadian Foundation for Innovation, NSERC and FQRNT for the financial support on this study and the NMR spectrometer.

I would like to acknowledge the technicians, Jean Huard, Carol Paichaud, Robert Delisle and Lionel Valero for their cooperation and kind help on experiments. Additionally, my special thanks go to the people in the Chemical Engineering Department for their understanding and cooperation during my two years study. Thank Dr. Yves Aubin for his kind review of this thesis. Special thanks go to Dr. Zhicheng Xia for his professional suggestion and advisions on NMR technologe.

Many thanks to my colleagues in Bio-P^2 group, Mathieu Cloutier, Dorra Gmati, Maya Klvana, Audrey Fazzi, Caroline De Dobbeleer, Anne Gigout, Steve Hisiger, Virginie McMurray-Beaulieu and Éric B. Boivin, for their kind help and invaluable discussions.

Thanks to my wife for her encouragement and understanding, and to my daughter for her cooperation.

RÉSUMÉ

L'objectif premier visé par ce travail consistait en la validation d'un système bioréacteur à petite échelle développé pour l'étude RMN *in vivo* de la physiologie cellulaire. Le métabolisme du phosphate a été particulièrement étudié pour des cultures cellulaires évoluant dans des environnements ayant des niveaux variés en oxygène dissous. Les étapes ayant mené à la réalisation de ce projet peuvent être regroupées en quatre parties. La première visait la modification d'un bioréacteur à petite échelle conçu au laboratoire afin de permettre la quantification de composés phosphatés et la réalisation d'analyses à long terme. Par la suite, l'étude des réponses physiologiques de cellules soumises à des perturbations en oxygène, du métabolisme du phosphate de cultures cellulaires sous diverses conditions en oxygène ainsi que des taux d'absorption, de stockage et de translocation en phosphate ont respectivement fait l'objet des trois étapes subséquentes.

Les mesures de résonance magnétique nucléaire *in vivo* ont été réalisées à l'aide d'un bioréacteur à petite échelle adapté de façon à pouvoir quantifier et réaliser des expériences devant se dérouler sur un long intervalle de temps. Afin de permettre la quantification de composés par la RMN, une référence phosphatée externe, non invasive et scellée dans un capillaire a été employée. De plus, les constantes de temps T1 et T2 ainsi que le facteur de saturation ont été mesurés pour calibrer les résultats obtenus des mesures de quantification. D'autre part, un humidificateur contenant une solution stérile de D_2O 10% et un condenseur ont été ajoutés au montage pour limiter

l'évaporation et la concentration du milieu de culture possibles pour des systèmes opérant avec de hauts débits d'alimentation en air. L'applicabilité du bioréacteur à petite échelle modifié pour la réalisation des études à long terme a été éprouvée par la culture d'un lit de cellules en mode perfusé avec alimentation de milieu stérile oxygéné, sur une période de 10 jours.

Les résultats obtenus ont démontré que les cellules de *Eschscholtzia californica* sont très sensibles aux stress engendrés par de faibles concentrations en oxygène dissous dans le milieu de culture. Une diminution de la concentration en oxygène dissous de 60% à 25% de la concentration à saturation avec de l'air a entraîné l'acidification du cytoplasme de 0.7 unité de pH. Une réoxygénation du milieu a quant à elle permis de ramener immédiatement le pH cytoplasmique à sa valeur initiale. Des modifications aux comportements cellulaires en réponse à un changement en oxygène dissous ont également été observées lors du transfert des cellules d'un environnement limité en oxygène (culture en flacon) à un milieu riche en oxygène (culture en bioréacteur). Afin de contrer les effets du stress causé par les étapes d'échantillonnage et de transfert des cellules, un délai d'une heure suite à l'inoculation dans le bioréacteur de petite taille a donc été considéré avant l'amorce des mesures de RMN *in vivo*. Ces mesures ont permis d'observer que les cellules cultivées dans un environnement riche en oxygène (culture en bioréacteur) ont un taux d'absorption en phosphate intracellulaire plus élevé que celles évoluant dans un milieu pauvre en oxygène (culture en flacon). D'autre part, les cultures riches en oxygène sont soumises à une limitation en nutriment en fin de

culture, ce qui n'a pas été remarqué pour les cultures limitées en oxygène (culture en flacon).

Un taux d'absorption en phosphate de 1.40 μmol min^{-1} g^{-1}DW a été déterminé à partir des mesures de phosphate total visible sur les spectres de RMN pour les cellules de *Eschscholtzia californica* cultivées en suspension dans un milieu de culture contenant 1.0 mmol Pi L^{-1}. Un transfert continu pour fins d'entreposage du Pi cytoplasmique vers les vacuoles a de plus été remarqué lorsque du Pi était disponible dans le milieu de culture et se poursuivait pour un certain temps après l'épuisement du Pi dans le milieu. Les niveaux d'ATP diminuaient quant à eux conjointement à l'absorption du phosphate par la cellule alors qu'ils augmentaient suite à l'arrêt de l'ajout de phosphate dans le milieu. Ceci suggère que le transport du phosphate est un processus couplé avec l'ATP, tel que décrit dans la littérature. Finalement, tant le processus d'absorption en phosphate que les conditions de faibles concentrations en oxygène ont causé l'acidification du cytoplasme (0.7 unité de pH) ainsi qu'une faible alcalinisation de la vacuole.

Les réponses physiologiques immédiates des cellules soumises à une élicitation ont également été suivies par des mesures *in vivo* de RMN ^{31}P. Une acidification du cytoplasme ainsi qu'une baisse de PME ont été observées dans la première demi-heure après l'élicitation, suivi dans la demi-heure subséquente par une remontée des valeurs tant de pH cytoplasmique que de PME jusqu'à leurs valeurs initiales. Alors qu'une stabilisation du pH a par la suite été notée, le PME a continué à augmenter

significativement pour atteindre une valeur maximale deux heures après le début de l'élicitation. Pour des cellules âgées de trois jours, une valeur de PME 1.8 fois supérieure à sa valeur initiale a été mesurée deux heures après l'amorce de l'élicitation. Des augmentations en ATP, en NADPH et en phosphate cytoplasmique ont également été perçues suite à l'élicitation. L'ensemble bioréacteur perfusé à petite échelle a donc démontré sa capacité à être utilisé pour suivre des processus physiologiques hautement transitoires pour l'étude des cellules végétales.

ABSTRACT

The main goal of this project was the validation of a small-scale bioreactor as a tool to perform *in vivo* NMR monitoring of the physiological state of living cells that submitted to diverse stresses. The cells phosphate metabolism was studied for cells cultures that were under different dissolved oxygen conditions. This research project was divided into four parts, modification of the small-scale bioreactor allowing the quantification of metabolites of interest, study of cells physiological responses to low oxygen stress and under different culture conditions, and finally, the study of phosphate uptake, storage and translocation.

In vivo NMR studies were performed in a small-scale bioreactor, which was modified from a bioreactor previously developed in the laboratory. A noninvasive external phosphorus (2.0 M methanephosphonic acid) reference was sealed in a capillary and installed inside the outlet tube of the bioreactor and used for quantification purposes. T1, T2 and saturation factor were measured for calibrating the quantification results. A gas humidifier containing 10% D_2O in sterile water and a condenser were used to limit medium evaporation and concentration from gas bubbling. The modified small-scale bioreactor showed to perform adequately for long-term study, which was proved by cultivating cell bed for 10 days with perfusion of oxygenated sterile medium.

Eschscholtzia californica cells are very sensitive to oxygen stress. When dissolved oxygen decreased from 60% to 25% air saturation cytoplasm acidified of 0.7 pH unit. Cytoplasmic pH recovered at initial value immediately when medium was re-

oxygenated. Cells behaved under stress during cells transfer. *In vivo* NMR measurements were then suggested to start one hour after inoculation until cell stabilized to new environment. Oxygen-enriched culture (bioreactor culture) consumed intracellular phosphate more rapidly comparing to oxygen-limited culture (flask culture).

Phosphate uptake rate was measured by the NMR visible total phosphate at 1.40 μmol min^{-1} g^{-1}DW for *Eschscholtzia californica* cell suspension with medium Pi of 1.0 mmol L^{-1}. Cytoplasmic Pi was continually transferred into vacuolar for storage when external Pi was available and cytoplasmic Pi was saturated. ATP concentration decreased at sudden phosphate uptake and increased when medium was changed to phosphate free medium. This result suggests that phosphate uptake is an ATP coupling process, which is already described in literature. Both the processes of phosphate uptake and low oxygen stress caused acidification of cytoplasm (0.7 pH unit) and slight alkalinization of vacuole.

Cells physiological responses to elicitors were monitored by *in vivo* ^{31}P NMR. Cytoplasmic acidification and PME decreasing were observed 30 min after elicitation. After another 30 min post elicitation, cytoplasmic pH as well as PME, recovered to initial values. After two hours post elicitation, PME increased significantly to a maximum value. PME of 3-d-old cells in shake flasks reached 1.8 times of initial value in 2 hours elicitation. ATP, NADPH and Cytoplasmic phosphate also increased during elicitation.

CONDENSÉ EN FRANÇAIS

Le développement des techniques de spectroscopie par résonance magnétique nucléaire (RMN) a une histoire récente. Les applications de la RMN aux domaines de la biologie, telles la physiologie, la biochimie ou les études du métabolisme, ont débuté dans les années 70 lorsque Moon (1973) a établi une relation entre le déplacement chimique des métabolites phosphatés et le pH intracellulaire ce qui lui a permis de déterminer de manière non-invasive le pH intracellulaire de cellules de sang de lapin. Moins d'une décennie plus tard, Roberts (1980) effectuait des mesures de pH intracellulaire de racines de maïs en employant la RMN du ^{31}P. Ses travaux l'ont par la suite amené à employer la RMN pour réaliser des études portant sur les réponses des voies de respiration et de fermentation des plantes dans des conditions hypoxiques (Roberts *et al.,* 1982). Plus récemment, la RMN a été utilisée pour l'étude de cellules et de tissus d'origines diverses. Parmi les méthodes d'analyses disponibles, la spectroscopie par résonance magnétique nucléaire se distingue par sa capacité à obtenir des informations uniques sur l'intégration et la régulation du métabolisme des plantes par la combinaison de méthodes de mesure *in vivo* et *in vitro*. Les mesures non-invasives obtenues de la spectroscopie par RMN ont été largement employées pour identifier, quantifier et localiser des métabolites, pour définir l'environnement intracellulaire ou encore pour explorer les voies métaboliques (Ratcliffe *et al.,* 2001). Plusieurs travaux de recherche ont porté sur l'étude du métabolisme et de la nutrition des cellules de plantes (Gerendas *et al.,* 2000; Gout *et al.,* 2001; Summers *et al.,* 2000;

Bligny *et al.,* 1989; Rijhwani *et al.,* 1999; Brodeliust *et al.,* 1985; Sakano *et al.,* 1995); des cellules de mammifères (Gupta *et al.,* 1978), des microorganismes (Grivet *et al.,* 2003; Watts *et al.,* 2002) et des levures (Hesse *et al.,* 2002; Gerlitz *et al.,* 1997).

Le phosphore se classe parmi les éléments essentiels aux organismes vivants. D'une part, des atomes de phosphore sont contenus dans plusieurs composés cellulaires, tels l'ADN, l'ARN, les phospholipides, l'ATP et les sucres phosphatés. D'autre part, des travaux ont démontré que le processus de phosphorylation-déphosphorylation des protéines par le transfert du groupement phosphate est une réaction majeure dans la régulation des fonctions cellulaires (Ranjeva *et al.,* 1987). Le phosphate intracellulaire étant de plus soupçonné d'avoir des effets régulateurs sur le métabolisme des cellules de plantes (Rijhwani *et al.,* 1999), les mesures de phosphates inorganiques intracellulaires s'avèrent fort importantes dans la compréhension des mécanismes cellulaires intervenant dans diverses situations tel que lors de la réaction à des stress.

Les cellules de plantes supérieures montrent des signes de stress lorsqu'elles sont exposées à un environnement limité en oxygène, entre autres. L'activité métabolique de cellules soumises à de telles conditions diminue au fur et à mesure que l'intensité de la perturbation augmente. Tant que la perturbation perdure, les cellules demeurent dans cet état (Netting *et al.,* 2000). L'adaptation des cellules de plantes soumises à des conditions d'oxygène limitant a été observée pour la première fois en 1988 (Saglio *et al.,* 1988), alors qu'il a été démontré que les cellules apicales des racines de maïs étaient plus tolérantes à des conditions d'anoxie si tout le semis était d'abord exposé à des

conditions d'hypoxie (3-4%O_2). Cette découverte a marqué le début d'une succession de travaux majeurs portant sur l'étude des changements physiologiques en réponse à un environnement limité en oxygène (Roberts, 1984; Raven, 1986; Kennedy *et al.,* 1992). Dans cette même optique, des mesures de RMN *in vivo* ont également été réalisées afin d'obtenir des données non-invasives sur les réponses intracellulaires (Roberts *et al.,* 1992). Toutefois, toutes ces études ont en commun l'emploi d'oxygène pur pour créer des conditions de concentration en oxygène dissous variant entre 0 et 100% de la concentration à saturation. Un tel écart entre les conditions limites étudiées est si vaste qu'il s'éloigne largement des conditions en oxygène dissous dans l'environnement réel des cellules cultivées dans la nature ou *in vitro*. Il apparaît aussi important d'être en mesure d'étudier les réponses cellulaires en conditions réelles. En ce qui a trait aux conditions réelles de culture rencontrées en bioprocédés, deux types de culture sont principalement employés, soient les cultures en bioréacteur ou en flacon agité. Au contraire des bioréacteurs qui peuvent offrir un environnement contrôlé par l'ajustement des conditions opératoires telles la vitesse d'agitation et la concentration en oxygène dissous, les cultures en flacon agité ne permettent pas un contrôle rigoureux des conditions de culture.

L'hypothèse ayant servi de prémisse à ce projet tient au fait qu'un suivi de composés phosphatés intracellulaires visibles en RMN permet de suivre les réponses physiologiques cellulaires dans diverses conditions. La validation de cette hypothèse requiert la disponibilité d'un système de culture insérable dans une sonde RMN et

permettant le maintient de la viabilité cellulaire et un contrôle des conditions environnementales de cultures, tel que l'environnement nutritif.

Pour la réalisation de mesures de RMN *in vivo*, de nombreux designs de bioréacteurs à petite échelle ont été réalisés (Rijhwani *et al.,* 1999; Fox *et al.,* 1989). Il s'avère cependant que les systèmes opérés en mode perfusion soient ceux permettant le maintien de cellules et tissus dans des conditions physiologiques définies et contrôlées (Shachar-Hill et Pfeffer, 1996). L'élaboration d'un bioréacteur à petite échelle opérant en mode perfusion et adapté pour les mesures de RMN *in vivo* a été préalablement réalisée dans notre laboratoire (Gmati *et al.,* 2004). Il s'agit de l'unique bioréacteur à petite échelle pour lequel des études hydrodynamiques et des bilans de masses ont été effectués. Son design rend facile le contrôle des conditions de perfusion et le maintien des cellules dans un état stable essentiel à l'étude des flux et des voies métaboliques. De plus, les conditions opératoires peuvent être modifiées aisément afin de permettre l'étude des réponses cellulaires suite à l'imposition d'un stress. Les cellules étudiées dans le cadre de ce projet ont été cultivées en un lit à haute densité cellulaire afin d'améliorer le ratio signal-sur-bruit des mesures de RMN. Bien que les spectres de RMN ^{31}P puissent être enregistrés dans un délai de 10 minutes tout en permettant de percevoir les signaux de PME, de phosphate cytoplasmique et vacuolaire, d'ATP et de NADPH, un délai de 60 minutes s'est avéré préférable pour obtenir un ratio signal-sur-bruit optimal augmentant le degré de précision nécessaire pour quantifier adéquatement ces composés. D'autre part, la RMN a été employée fréquemment pour réaliser des mesures non-invasives de pH intracellulaire. Certains problèmes peuvent par contre

être rencontrés lors de l'emploi ou de l'adaptation d'un bioréacteur à petite échelle pour les études de RMN *in vivo.* Mentionnons un design non approprié pour obtenir des données de quantification, des risques de contamination ainsi que de l'évaporation du milieu de culture perfusé. La quantification par des techniques de RMN est une méthode puissante permettant la mesure non-invasive de métabolites et d'ions, l'étude de flux métaboliques ainsi que l'exploration de voies métaboliques. Un design ne permettant pas d'effectuer de quantification limite toutefois ce type de mesure par RMN *in vivo.* De plus, les risques de contamination et d'évaporation du milieu perfusé confinent l'emploi de la RMN *in vivo* à des expériences se déroulant sur de courts intervalles de temps, suggérés à moins de quatre heures. Or, les études portant sur la compréhension du métabolisme cellulaire exigent des suivis à long terme, pouvant s'étendre sur plusieurs jours. Le bioréacteur à petite échelle a donc dü être modifié pour supporter des études en RMN permettant la quantification de métabolites pour des expériences à long terme.

Ainsi, l'objectif premier visé par ce travail a consisté en l'étude du métabolisme du phosphate, par des techniques de mesures de RMN *in vivo,* pour des cultures cellulaires évoluant dans des environnements ayant des niveaux variés soit en oxygène dissous ou en phosphate. Des « clichés » de l'état cellulaire ont aussi été pris afin de comparer des cellules en culture, soit en flacons agités ou en bioréacteur. Les étapes ayant mené à la réalisation de ce projet peuvent être regroupées en quatre parties. La première a visé la modification d'un bioréacteur à petite échelle afin de permettre la quantification de composés phosphatés et la réalisation d'analyses à long terme. Par la suite, nous avons

procédé à l'étude des réponses physiologiques de cellules soumises à des perturbations en oxygène, au suivi du métabolisme relié au phosphate pour des cultures cellulaires sous diverses conditions de culture, et finalement, des suivis hautement transitoires tels que les taux d'absorption, de stockage et de translocation en phosphate ont été effectués.

Les mesures de résonance magnétique nucléaire *in vivo* ont été réalisées à l'aide d'un bioréacteur à petite échelle adapté de façon à pouvoir quantifier et réaliser des expériences devant se dérouler sur un long intervalle de temps (jours). Afin de permettre la quantification de composés par la RMN, une référence phosphatée externe (2.0 M methanephosphonic acid), non invasive et scellée dans un capillaire a été employée. De plus, les constantes de temps T1 et T2 ainsi que le facteur de saturation ont été mesurés pour calibrer les résultats obtenus des mesures de quantification. D'autre part, un humidificateur contenant une solution stérile de D_2O 10% et un condenseur ont été ajoutés au montage pour limiter l'évaporation et la concentration du milieu de culture pour des systèmes opérant avec de hauts débits d'alimentation en air. L'applicabilité du bioréacteur à petite échelle modifié pour la réalisation des études à long terme a été éprouvée par la culture d'un lit de cellules en mode perfusé avec alimentation de milieu stérile oxygéné, sur une période de 10 jours.

Les résultats obtenus ont démontré que les cellules de *Eschscholtzia californica* sont très sensibles aux stress engendrés par de faibles concentrations en oxygène dissous dans le milieu de culture. Une diminution de la concentration en oxygène dissous de

60% à 25% de la concentration à saturation pour l'air a entraîné l'acidification du cytoplasme de 0.7 unité de pH. Une réoxygénation du milieu a quant à elle permis de ramener immédiatement le pH cytoplasmique à sa valeur initiale. Des modifications aux comportements cellulaires en réponse à un changement en oxygène dissous ont également été observées lors du transfert des cellules d'un environnement limité en oxygène (culture en flacon) à un milieu riche en oxygène (culture en bioréacteur). Afin de contrer les effets de l'adaptation des cellules à leur nouveau milieu, un délai d'une heure suite à l'inoculation a donc été considéré avant l'amorce des mesures de RMN *in vivo*. Ces mesures ont permis d'observer que les cellules cultivées dans un environnement riche en oxygène (culture en bioréacteur) ont un taux d'utilisation en phosphate intracellulaire plus élevé que celles évoluant dans un milieu pauvre en oxygène (culture en flacon). Ainsi, les cultures riches en oxygène sont soumises à une limitation en nutriment en fin de culture, ce qui n'a pas été remarqué pour les cultures limitées en oxygène (culture en flacon).

Un taux d'absorption en phosphate de 1.40 μmol min^{-1} g^{-1}DW a été déterminé à partir des mesures du phosphate total visible sur les spectres de RMN pour les cellules de *Eschscholtzia californica* cultivées en suspension dans un milieu de culture contenant 1.0 mmol Pi L^{-1}. Un transfert continu pour fins d'entreposage du Pi cytoplasmique vers la vacuole a de plus été remarqué lorsque du Pi était disponible dans le milieu de culture et poursuivait pour un certain temps après l'épuisement du Pi dans le milieu. Le niveau d'ATP diminuait conjointement à l'utilisation du phosphate par la cellule alors qu'il augmentait suite à l'arrêt de l'ajout de phosphate dans le milieu. Ceci suggère que le

transport du phosphate est un processus couplé avec l'ATP, tel que décrit dans la littérature. Finalement, le processus de consommation du phosphate a causé l'acidification du cytoplasme (0.7 unité de pH) ainsi qu'une faible alcalinisation de la vacuole.

En complément, un étude des réponses physiologiques immédiates des cellules soumises à une élicitation a également été initiée par des mesures *in vivo* de RMN ^{31}P. Une acidification du cytoplasme ainsi qu'une baisse du PME ont été observées dans la première demi-heure après l'élicitation, suivi dans la demi-heure subséquente par une remontée des valeurs tant du pH cytoplasmique que du PME jusqu'à leurs valeurs initiales. Alors qu'une stabilisation du pH a par la suite été notée, le PME a continué à augmenter significativement pour atteindre une valeur maximale deux heures après le début de l'élicitation. Pour des cellules âgées de trois jours, une valeur du PME 1.8 fois supérieure à sa valeur initiale a été mesurée deux heures après l'amorce de l'élicitation. Des augmentations en ATP, en NADPH et en phosphate cytoplasmique ont également été perçues suite à l'élicitation.

En définitive, les modifications apportées au bioréacteur à petite échelle ont permis d'effectuer avec succès des mesures de quantification ainsi que la réalisation d'expériences menées à long terme par RMN *in vivo*. Les résultats obtenus ont démontré que *Eschscholtzia californica* est très sensible au stress induit par l'oxygène dissous. Les cellules ont démontrées notamment de tels signes de stress lors de leur transfert de flacons agités au bioréacteur perfusé de petite échelle. Pour contrer cet

effet, les mesures de RMN *in vivo* ont par conséquent débuté une heure après l'inoculation des cellules, le temps requis pour une stabilisation physiologique. D'autre part, les résultats de RMN ^{31}P *in vivo* ont montré que le phosphate intracellulaire était consommé rapidement dans les systèmes enrichis en oxygène (culture en bioréacteur) et ont de plus permis d'observer une limitation en nutriments. Les taux d'absorption, de stockage et de translocation du phosphate ont également fait l'objet de ce projet de recherche. Un taux d'absorption en phosphate de 1.40 µmol min^{-1} g^{-1}DW a été déterminé pour un milieu de culture contenant 1.0 mmol Pi L^{-1}, alors que la capacité de stockage en phosphate dans les vacuoles a été établie à 0.322µmol g^{-1} DW.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF APPENDIX

LIST OF SYMBOLS AND ABREVIATIONS

ADP:	Adenosine diphosphate
ATP:	Adenosine triphosphate
B5:	B5 Gamborg medium containing salt, 2, 4-DNP, kinetin, and 30g/L glucose
B5-F1:	B5 medium free of paramagnetic ions
B5-F2:	B5 medium free of paramagnetic ions, with 0.25mmol/L phosphate
B5-F3:	B5 medium free of phosphate and paramagnetic ions
Bo:	The externally applied magnetic field
conc.:	Concentration
d:	Day
D_2O:	Deuterated water
DO:	Dissolved oxygen (% air saturation otherwise specified)
DW:	Dry weight (g)
d1:	Recycle time, the time between repetitions of a pulse sequence (s)
F-6P:	Fructose 6-phosphate
Glc-6P:	Glucose 6-phophate

h:	Plank constant (units)
h:	Hour
I:	Nuclear spin quantum number
I.D.:	Inside diameter
in vivo:	Samples of living cells
in vitro:	Sample of extract
MDP:	methylenediphosphonate
min:	Minute
MPA:	Methyl phosphonate acid
NADP:	Nicotinamide-adenine dinucleotide phosphate;
NDPG:	Nucleotide diphosphate glucoseATP
NMR:	Nuclear magnetic resonance
NOE:	Nuclear Overhauser Effect
O.D.:	Outside diameter
PEEK:	Polyetheretherketone
pH_c:	Cytoplasmic pH
pH_v:	Vacuolar pH
pH_e	External pH

Pi:	Inorganic phosphate;
Pi_c:	Cytoplasmic phosphate (mmol/g-DW)
Pi_v:	Vacuolar phosphate (mmol/g-DW)
Pi_e:	External phosphate (mmol/L)
PME:	Phosphomonesters (Glu-6P, F-6P, etc.)
ppm:	Parts per million- unit of chemical shift
Ptot:	Total NMR detectable intracellular phosphate
γ:	Gyro magnetic ratio
rpm:	Rotations per minute
T1:	The rate constant of longitudinal (spin-lattice) relaxation (s)
T2:	The rate constant of transverse (spin-spin) relaxation (s)
ϕ:	Diameter

CHAPTER 1: INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is a noninvasive and nondestructive measurement that can provide insights into the integration and regulation of plant metabolism by a combination of *in vivo* and *in vitro* measurements. NMR can be used to identify, quantify, localize metabolites, define the intracellular environment, and explore metabolic pathways. The most important is that *in vivo* NMR can provide real-time metabolism of cells or tissues.

Phosphorus is one of the most essential elements for biological organisms. Intracellular phosphate is believed to have important regulatory effects on plant cell metabolism. It has also been established that protein phosphorylation-dephosphorylation by phosphate transfer is the major reaction in the regulation of cellular functions.

Analysis of intracellular phosphate is very important, especially by *in vivo* ^{31}P NMR spectroscopy, which can provide the information of intracellular pH and energy potential, identify and quantify intracellular inorganic phosphate, phosphomonesters (PME) and NADPH, explore phosphate metabolic fluxes and metabolic pathways. In addition, *in vivo* ^{31}P NMR spectroscopy is easy and convenient to study stress physiology by changing medium components, dissolved oxygen, temperature or pH, as well as easy to operate without any extracting.

Oxygen stress has been studied by *in vivo* ^{31}P-NMR spectroscopy to noninvasively get the responses of intracellular environments. But all the studies set the dissolved oxygen range from 100% to 0% pure oxygen saturation, which rarely occurs in the nature or in

bioprocess. However, what is the real response in possible dissolved oxygen conditions? There are two choices for bioprocess, the bioreactor cultures with controlled-dissolved oxygen and flask cultures with limited-dissolved oxygen. There is no report about the cell physiological difference between flask cultures and bioreactor cultures with different dissolved oxygen conditions.

For *in vivo* NMR measurements, a small-scale bioreactor with perfusion system has been designed and developed successfully in our laboratory. It is a perfusion bioreactor that can easily control the perfusion conditions and keep cells in stable state to study the metabolic fluxes and metabolic pathways. In addition, the perfusion conditions can be changed easily to study the stress physiology. But, problems about this small-scale bioreactor are contamination, perfusion medium evaporation and quantification, which limited the applications of *in vivo* NMR studies, such as long-term studies.

1.1. OBJECTIVES

The major objective of this project was to validate the capacity of the small-scale bioreactor to perform *in vivo* ^{31}P-NMR study at steady state and transient conditions. The following sub-objectives had to be addressed: improve the design of the small-scale bioreactor for maintaining long term aseptic conditions, study cell physiology under oxygen stress, compare cells physiological state under different culture conditions, monitor phosphate uptake, storage and translocation.

1.2. STRUCTURE OF THE THESIS

The thesis is divided in 7 chapters. The present chapter is an introduction of the project, witch contains the problem, the objectives and the methodology. A general literature review is presented in chapter 2 where the principle of NMR, the structure of NMR spectrometer and the applications of biological NMR are described and discussed. Chapter 3 defines the materials and methods used and developed. Experimental methods, analytical methods as well as the chemicals and instruments are described. Chapter 4 details the modification and development of the small-scale bioreactor for the purpose of quantification and long-term *in vivo* NMR studies. The limitations of small-scale bioreactors are specifically discussed. Chapter 5 presents a manuscript that has been submitted to the *Journal of Experimental Botany* with the title of the monitoring of *Eschscholtzia californica* cells physiologic state by *in vivo* ^{31}P-NMR. Chapter 6 presents the preliminary results of an *in vivo* ^{31}P-NMR study on cell elicitation. Finally, the chapter 7 groups the general discussion, conclusions and proposes future works.

CHAPTER 2: LITERATURE REVIEW

2.1 OVERVIEW OF BIOLOGICAL NMR

The technique of nuclear magnetic resonance (NMR) was invented in 1940s by physicists. Because it can provide information about the chemical structure of matter, NMR became the main useful analytical technique for the chemist involved in synthesis (Veeman, 1997). As the development of NMR technology, high resolution NMR was developed. This made NMR expand to other fields, such as soil science, biology, engineering, medicine, environment, etc.

Most of the interesting biomolecules contain isotopes such as ^{31}P, ^{15}N, ^{13}C and ^{1}H that can be visible by NMR. There are two approaches making NMR a unique analytical tool in biology, *in vivo* NMR, which allows recording NMR data of living samples, and *in vitro* NMR, which allows recording NMR spectra of cells or tissue extracts and purified metabolites such as proteins and diverse biomolecules (Shachar-Hill and Pfeffer. 1996). NMR signal is enhanced by *in vitro* NMR comparing to *in vivo* NMR, because of a better stability and homogeneity of the molecules. Moreover, cell extracts can be concentrated to enhance signal-to-noise ratio. The sensitivity of *in vivo* NMR measurement is limited by the concentration of the molecules in the cells or tissues.

However, *in vivo* NMR measurements can provide information that would not be obtainable by *in vitro*, such as intracellular pH and metabolite compartmentalization. *In vivo* NMR allows the distinct measurement of cytoplasmic pH (pH_c) and vacuolar pH

(pH_v). *In vivo* NMR can be applied to follow metabolism on the same sample rather than from a series of different samples (Shachar-Hill and Pfeffer, 1996).

There are many constrains in performing *in vivo* NMR measurements. The following sections are presenting the basic concepts, the structure of NMR and the applications of NMR on studies of cells metabolism and physiology.

2.2 THE PRINCIPLE OF NMR

The NMR principle in general and the specific applications of *in vivo* NMR have been reviewed (Ratcliffe *et al.,* 2001a; Ratcliffe *et al.,* 2001b; Cohen *et al.,* 1995; Shachar-Hill and Pfeffer, 1996; Veeman, 1997). Ratcliffe (2001a; 2001b) kept eyes on plant cell physiology and metabolisms such as photosynthetic carbon metabolism, non-photosynthetic carbon metabolism, fermentation pathways, one-carbon metabolism, nitrogen metabolism, phosphorlation, mycorrhizal metabolism and metabolic flux. Shachar-Hill (1996) gave more detailed description on why and how to apply NMR technology on studies of cells metabolism and physiology. NMR theory and principle were described clearly by Veeman (1997).

Here, proton NMR is described to introduce the basic concepts of NMR spectroscopy. Atomic nuclei are surrounded by cloud of electrons, which make nuclei behave as small magnets with south and north dipoles. Nuclear magnetic resonance is based on this phenomenon. With the strong external magnetic field Bo, these small magnets of the

nuclei will behave ether parallel or antiparrallel to external magnetic field Bo (as shown in figure 2.1 A and B). The energy difference is

$$\Delta E = \gamma h Bo/2\pi \quad \text{(2-1)}$$

Where h is Plank's constant; Bo is external magnetic field; γ is the gyromagnetic ratio. Since

$$\Delta E = h\nu o \quad \text{(2-2)}$$

The resonant or Larmor frequency νo can be expressed as

$$\nu o = \gamma Bo/2\pi \quad \text{(2-3)}$$

Different nucleus has different gyromagnetic γ and will lead to different observation frequency νo. The behavior of the magnetization in external magnetic field could be described by vector diagrams (figure 2.1 B and C). The bulk magnetization vector **M,** resulting from the differences between spin energy levels, is parallel to external magnetic field Bo without disturbing (figure 2.1 A and B). If a radio frequency ν that equal to the resonant frequency νo ($\nu = \nu o$) is applied in the –Y direction of the nucleus, the bulk magnetization vector **M** will rotate to give a magnetization vector **Mx** in the X-Y plane. This rotation will produce an oscillating current in the NMR coil (figure 2.1D), which is vertical to the Z-axis. The current will then be amplified, digitalized, and processed by Fourier Transform by a computer to express the signal as peak in a NMR spectrum. If the density of electron cloud is always the same around one kind of the nuclei, it will give a unique chemical shift for this kind of nuclei. This makes NMR

useless for chemist or biochemist. Fortunately, the density of electron cloud around a nucleus is affected by other functional groups that connect to this nucleus. For a given external magnetic field, a nucleus that placed in different chemical environments will resonate at different frequencies. This phenomenon is called chemical shift, which can be used to identify different compounds (from their chemical shift). The chemical shift is proportional to the frequency of the applied radio frequency ν, which is up to the magnet strength of NMR spectrometer. Different magnet strength will give different results of chemical shifts (in Hz). So, relative reference with the unit of part per million (ppm) were introduced to standardize chemical shift results on different NMR spectrometers. For example, tetramethylsilane (TMS) was chosen as chemical shift reference at 0 ppm for proton NMR. All the chemical shifts of other proton are relative to TMS. The chemical shift can be obtained by equation 2-4 with the unit of parts per million (ppm).

$$Chemical\ \ shift\ \ in\ \ ppm = \frac{Chemical\ \ shift\ \ from\ \ TMS\ \ in\ \ Hz}{Spectrometer\ \ frequency\ \ in\ \ Hz} \times 10^6 \qquad \ldots 2\text{-}4$$

The signal intensities are the height or the area of peaks. Normally, the heights of peaks are used to calculate signal-to-noise ratio, while the integrated areas are used to for quantification of concentrations. Another concept about NMR is Nuclear Overhauser Effect (NOE). The intensity of a NMR signal can be enhanced with the irradiation of another nucleus during NMR experiment. This phenomenon is called "decoupling". For example, ^{13}C-NMR signal intensity can be enhanced by irradiating proton frequencies

during carbon-13 NMR data acquisition (proton decoupling). The signal-to-noise ratio can be increased under NOE, but quantification will be more complex to perform.

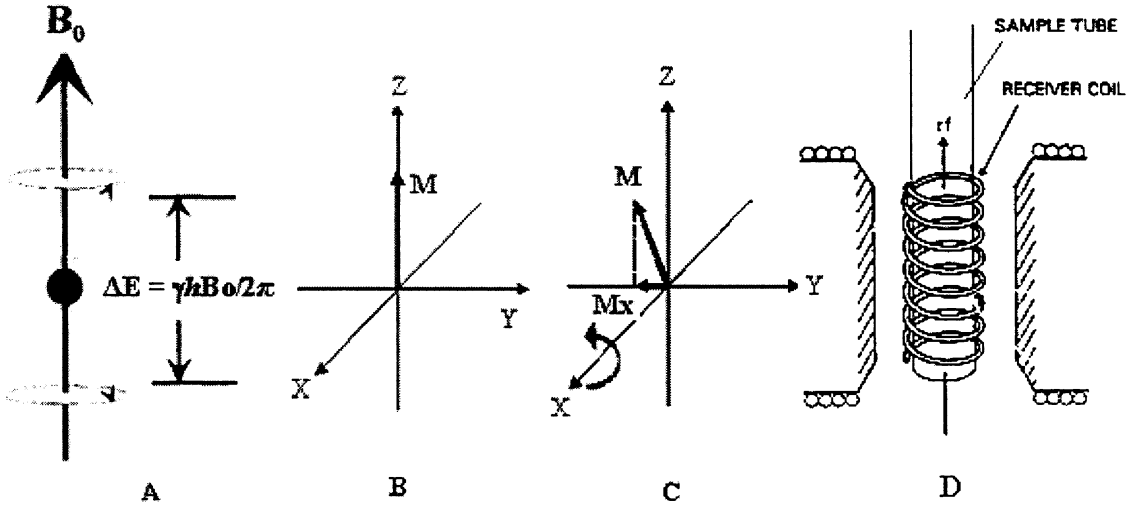


Figure 2.1: The behavior of a proton nucleus in an external magnetic field and the total magnetization

(A): A schematic picture of two orientations (parallel and antiparallel) and the two energy states of a spin with I = 1/2 in a magnetic field Bo. (B): At equilibrium; (c): After perturbation by a pulse (Veeman, 1997; Sanders and Hunter, 1987); (D): The scheme of an NMR coil.

2.3 NMR SPECTROMETER

NMR spectrometer integrates several technologies (physical, electronic, computer) into an analytical system. It contains a magnet, a NMR console, a NMR probe and a computer workstation (figure 2.2). The user selects a nucleus and a pulse sequence by computer workstation. The console accepts the message and sends a raido frequency (special for the selected nucleus) to the probe for irradited the sample. The probe coil receives the week radio frequency that emitted by the sample, when the pulse of radio frequency stops. This week radio frequency is amplified, digitalized by concole, and then sent to computer for Fourier Transform and displaying as a spectrum.

2.3.1 The Magnet

NMR magnet provides a strong, stable, and extremely homogenous magnetic field into which the sample (liquid, solid or animal/plant) is placed. The strength of the NMR spectrometer is typically specified in terms of the resonance frequency for the hydrogen atom expressed in MHz. Typical field strengths on the market today range from 200 MHz to 900 MHz. The magnet used in this research was of 9.4 T at 400 MHz and designed for liquid samples.

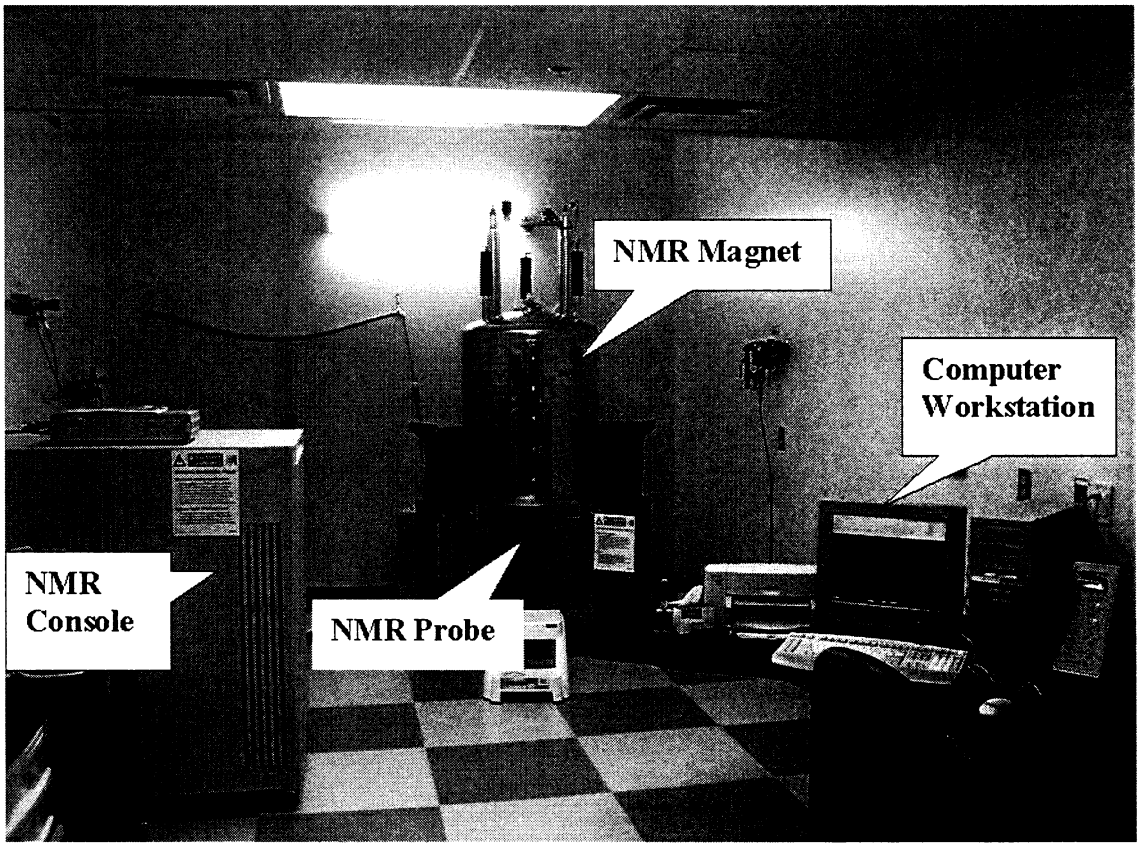


Figure 2.2: The picture of NMR spectrometer

Almost all the NMR spectrometers employ superconducting magnets (Levitt. 2001). For the moment, superconducting materials require to be kept at boiling point of liquid helium (4 K) for being supraconductive (figure 2.3) with no electrical resistance. To save liquid helium, the liquid helium compartment is surrounded by a liquid nitrogen bath. There are two vacume jackets that are applied to mimmium the heat transfor, one is between the liquid helium and liquid nitrogen, another is between liquid nitrogen compartment and surrounding. At the center of NMR magnet, there is a bore for NMR probe and sample tube. In this case the magnet was a narrow bore of 54 mm, thus

limiting the size of the probe. The magnet is energized at installation with a free running current of 63 A which could last for decades if the helium chamber is continuously renewed (3 to 4 times a year). To lower helium evaporation liquid nitrogen has to be also continuously renewed (each 1 to 2 weeks).

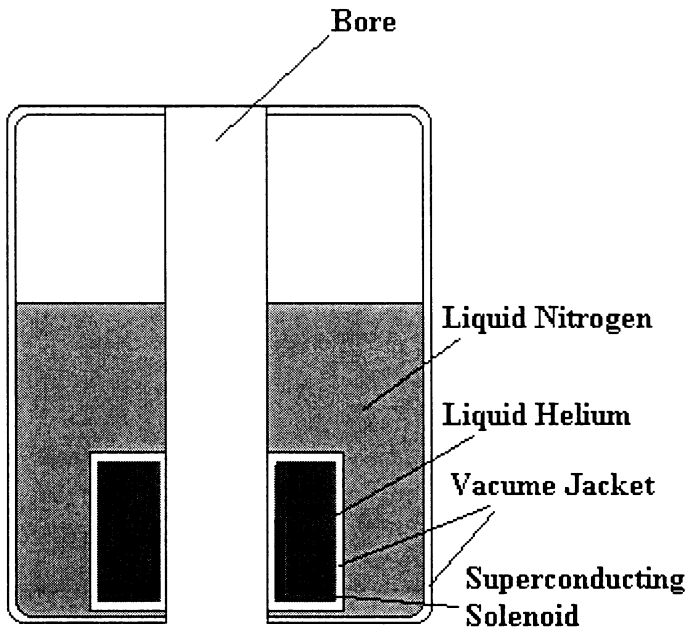


Figure 2.3: The schematic of a NMR magnet.

2.3.2 NMR Probe

NMR probe is designed to hold a NMR tube with liquid sample and placed into the region of homogenous magnetic field. The probe also contains the antennae for irradiating the sample at specific radio frequency and for receiving the very weak radio frequency resonance emitted from the sample (Levitt. 2001). In some cases, the probe

has the function of rotating the sample tube to reduce the width of the NMR peaks and stabilizing the sample temperature by increasing homogeneity of the analysis.

NMR probe (figure 2.4) is a critical part of the NMR spectrometer, and is specifically designed depending of NMR experiments. For example, there are probes that are built for liquid-state NMR experiments or solid-state NMR experiments. For liquid-state NMR, the probe can be designed as a Broadband probe or an indirect probe of different sizes (3mm, 5mm, 10mm, 15mm and 25mm in diameter). Broadband probe is for most of NMR detectable elements, while indirect probe is special for selected nuclei to increase singal-to-noise ratio by converting proton signal to selected nuclei, most of them for carbon for its poor sensitivity.

The basic NMR probe circuit consists of a coil and two capacitors. The inductance of the coil, C1 and C2 (figure 2.4 A) determine the resonance frequency. The capacitors of C1 and C2 are used for tuning the radio frequency to match the observation frequency ν_0 of selected nucleus. The two capcitor knobs on picture of figure 2.4 B are used to adjust C1 and C2 to set the radio frequency. NMR tube can be set to rotate or not. But most of solution NMR is suggested to rotate the sample to nerrow the peak. For *in vivo* NMR measurement, it is impossible to rotate the sample tube since the tube is connected to tubes for medium circulation.

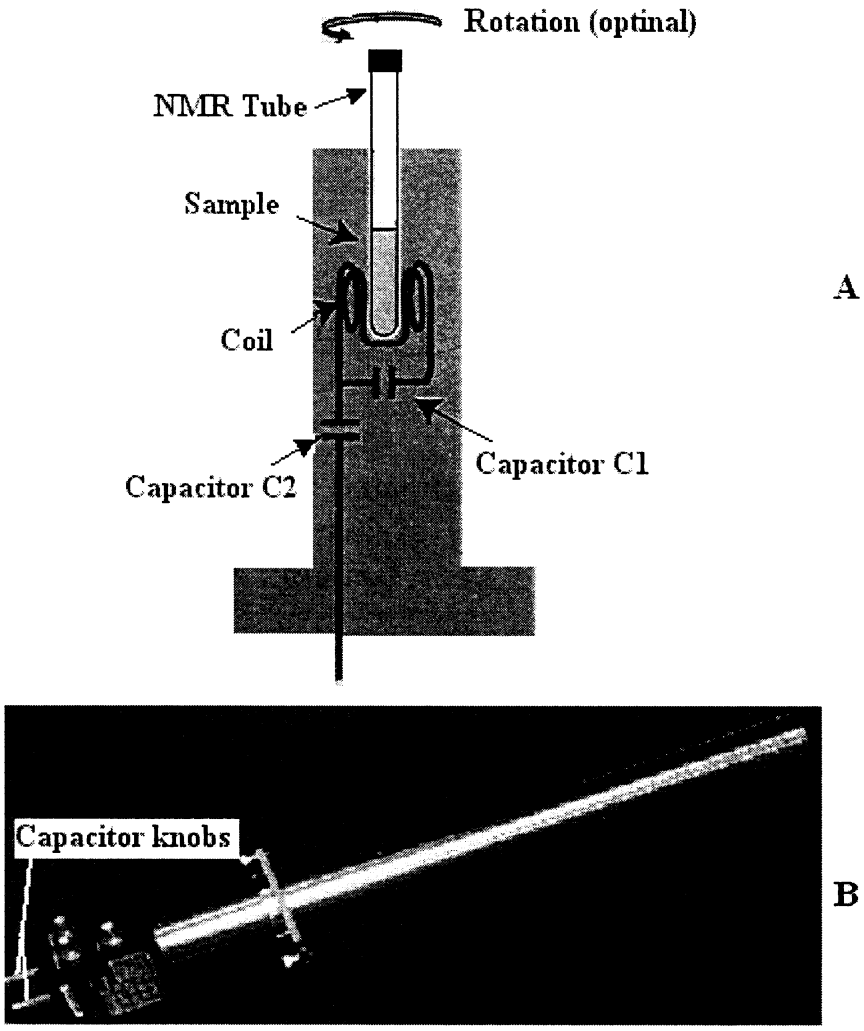


Figure 2.4: NMR probe (B) and the schematic of a single-channel solution-NMR probe (A) (Levitt. 2001)

NMR probes sensitivity has been recently increased dramatically by using cryogenics (i.e. low temperature) technology (cryogen NMR probe). The probe coil can then be maintained at very low temperature to increase the sensivity of 4 times than normal probe. NMR probes can also be coupled to liquid chromatography (LC-NMR) for high-

throughput chemistry analyses. Chemicals in liquid sample are first saperated by liquid chromatography and then each peak passes sequentially into NMR probe for NMR analysis.

2.3.3 NMR Console

The NMR console includes a transmitter, a receiver, and an amplifier. It generates and controls the pulses of high-power radio frequency energy used to excite the sample in the probe (Levitt. 2001). The NMR console (figure 2.2) also receives and detects the very weak signals acquired by the probe, then amplifies them one million times or more that can be read by the computer workstation and further analysed.

2.3.4 Computer Workstations

Today's NMR spectrometers also make heavy use of computers (figure 2.2) with diferent softwares, for control of the various radio frequency pulses, the storage and processing of the NMR data and plot or displaying of NMR spectra. The NMR signals are subjected to complex digital signal processing algorithms including the Fourier Transform to convert the NMR information into a standardized form that is easily interpreted by the end user. The most importance is that the software supports to integrate the area of the peak. These give the possibility of quantification.

2.4 *IN VIVO* NMR

In vivo NMR is a noninvasive measurement capable of recording NMR spectra of living samples as presented previously. The application of *in vivo* NMR has not a long history. In 1973, Moon (1973) reported a relationship between chemical shifts of phosphate metabolites and intracellular pHs, and then intracellular pH of rabbit blood cells was able to noninvasively measured. In the early 1980s, Roberts (1980) measured intracellular pH of maize root tips by ^{31}P-NMR spectroscopy. They subsequently applied this technology to study the response of the plant respiratory and fermentative pathways to hypoxia (Roberts *et al.,* 1982). This led to the burst of the application of *in vivo* NMR to answer the questions that had previously challenged analysis. The work in this area has then expanded quickly since NMR was proved to be a unique noninvasive technique to identify, quantify, localize metabolites in the different cells compartments, define intracellular environments (Ratcliffe *et al.,* 2001b), to explore metabolic pathways and characterize biochemical reaction kinetics. The interesting elements for biologist and biochemical engineers are proton, phosphorus, carbon and nitrogen.

2.4.1 *In vivo* ^{1}H-NMR

In vivo ^{1}H NMR can be used to analyze organic acids, amino acids and carbohydrates (Brown *et al.,* 1977; Fan *et al.,* 1986; Fan *et al.,* 1988). ^{1}H isotope is 100% naturally abundant and its sensitivity is the highest among all the nuclei that can be analyzed by

NMR. However, the application of *in vivo* ^{1}H-NMR is highly problematic because of the strong signal from medium water that is overlapping with other signals.

2.4.2 *In vivo* ^{31}P-NMR

^{31}P is also 100% naturally abundant. The sensitivity of ^{31}P-NMR is second to ^{1}H-NMR. *In vivo* ^{31}P-NMR spectra can be recorded in few minutes from homogeneous and stable cell samples. *In vivo* ^{31}P-NMR spectroscopy has been widely used for studies on cell physiology and metabolism. It can provide information on cellular energy potential and phosphated metabolites' concentrations, phosphate metabolism, intracellular pH, membrane permeability and ions and water distribution. It can also provide an on-line measurement for the real-time monitoring of the phosphate metabolism and the energy potential (Noguchi *et al.,* 2002). Cells and tissues of different origin such as bacteria (Noguchi *et al.,* 2002), fungi (Shachar-Hill and Pfeffer 1996; Rasmussen *et al.,* 2000), yeasts (Rasmussen *et al.,* 2000), plants (Spickett *et al.,* 1993; Gout *et al.,* 2000; Rijhwani *et al.,* 1999) and mammalians (Bergans *et al.,* 2003; Jucker *et al.,* 2000; Cerdan *et al.,* 1990) have been studied by *in vivo* ^{31}P-NMR.

2.4.3 *In vivo* ^{13}C-NMR

In vivo ^{13}C-NMR has been used thoroughly to study diverse organic molecules such as protein structures, protein-protein interactions, as well as cells sugars, metabolites and precursors. ^{13}C-NMR spectroscopy is useful to study both primary metabolism (Mancuso *et al.,* 1994; Mancuso *et al.,* 1998) and secondary metabolism (Hinse *et al.,*

2001; Mancuso *et al.,* 1998), since it can provide information on organic molecules (Kunnecke *et al.,* 2000; Jucker *et al.,* 2000; Cerdan *et al.,* 1990; Pfeffer *et al.,* 1999; Gout *et al.,* 1993). But the natural abundance of carbon-13 is only 1.1% and the sensitivity of ^{31}C-NMR is lower comparing to proton and phosphorus. Carbon-12 is invisible by NMR, because I = 0. Carbon-13 (I = 1/2) labeled nutrients (glucose or other carbon sources) and precursors have then to be added to the culture medium to increase the signal-to-noise ratio.

2.4.4 *In vivo* ^{14}N- and ^{15}N-NMR

The natural abundance of nitrogen-14 is of 99.6% and nitrogen-15 is 0.4%. Although ^{14}N (I=1) has high natural abundance, the presence of quadrupole moment and very low frequency make the signal broaden and difficult for NMR study. ^{15}N (I=1/2) is a better candidate to get sharp signal, but the sensitivity is low with a low abundance and low frequency. ^{15}N labeled nutrients are often used to increase the sensitivity too. However, both *in vivo* ^{14}N and ^{15}N NMR have been applied to quantify and study intracellular nitrate, ammonium (Ford *et al.,* 1994; Gamcsik *et al.,* 1991; Lee *et al.,* 1991).

2.4.5 The limitations of *in vivo* NMR

In vivo NMR is a powerful way to explore intracellular environments and determine the concentrations of metabolites, as well as metabolic pathways as discussed previously. However, there are still important limitations. First of all, not all the metabolites or ions can be detected by NMR. Because *in vivo* NMR remains in liquid NMR technology, it

can only detect isotope of molecules solvable in intracellular environments. Those immobile metabolites or ions due to precipitation or tightly binding to macromolecules can't be detected by liquid NMR. For example, insoluble phosphorous that binds to macromolecules (DNA or RNA) or precipitates out as amorphous calcium phosphate can't be detected by *in vivo* ^{31}P NMR. That is why there is NMR undetectable phosphate pools in cell that are usually called "NMR invisible pools" (Brodeliust et al, 1985). Moreover, *In vivo* NMR can't detect some metabolites or ions at low concentrations (usually 1-2 mM for carbon-13), because NMR is a relatively poor sensitive technology. Secondly, some signals may be biased by overlap with larger signals. In some case, some signals can be broaden or hiden because of ions or metabolites moving, exchanging or the paramagnetic ions in cells. Thirdly, high cost of NMR spectrometer and deuterated solvents and nutrients limit the applications of NMR. For example, carbon-13 labeled glucose is highly expensive ($1000/g) which is increasing significantly the cost of an experiment.

2.5 SMALL-SCALE BIOREACTORS USED FOR *IN VIVO* NMR

The physiological state of cells or tissues can be strongly affected by the culture conditions and then affect the quality of *in vivo* NMR spectra. It is imperative to be able to maintain the living cells in the NMR in desired state, which should satisfy to the requirements of both the physiologist and the NMR spectroscopist. Physiologists require keeping cells or tissues alive in NMR coil for hours or days in a homogeneous

physiologic state among the cells. It should also be possible to change culture conditions to conveniently study the physiological responses during NMR data recording. NMR tube that is used to perform standard *in v*itro measurements can be transformed in a small-scale bioreactor with oxygen and nutrients supplies to satisfy cells requirements. The NMR spectroscopists require packing the cells tightly within the NMR sensitive zone to enhance signal-to-noise ratio. High field magnets with small gaps and conventional probe heads offer rather limited possibilities for the examination of whole plants. Obtention of a compromise between the requirements of both the biologist and the physiologist is often possible (Ratcliffe *et al.*, 2001b).

But problem is that NMR sensitivity is relatively poor. For example, the sensitivity of *in vivo* NMR (BRUKER AMX 400 spectrometer) with a 25 mm probe is around 25-50 uM for phosphate, 1-2 mM for carbon (Schachar-Hill and Pfeffer 1996). So, *in vivo* NMR measurements need to increase signal-to-noise ratio by improving the density of the cell sample in the reading zone of the probe. The maximal amount of cells in the bioreactor is limited by the scale of NMR magnet gap and from the size of NMR probe. Standard NMR probes used in liquid NMR ranged from 3 mm to 25 mm in diameter. Large NMR tubes (ID: 25 mm) allow enhancing signal-to-noise ratio but with a broader signal. However, smaller NMR tubes (ID: 16mm or 10 mm) lead to a lower signal-to-noise ratio but a higher resolution. Smaller NMR tubes such as the 5 mm or 3 mm in diameter could hardly be used as bioreactor because of a lack of room to arrange medium feeding and outlet tubes as well as the cell support devices.

In vivo NMR experiments were first performed with air or oxygen bubbling through or above the cells sample in the NMR tube (Martin *et al.* 1982). A series of small-scale bioreactors showed to be efficient for *in vivo* NMR measurement (figure 2.5), such as air-lift (figure 2.5 A and B), with fixed cells with medium circulation (figure 2.5 C) and perfusion (figure 2.5 D). Since most of *in vivo* NMR studies are performed on tissues and cell suspensions, many authors insist on packing the cells as a cell bed into NMR tube to enhance signal-to-noise ratio. Air-lift small-scale bioreactor configuration (figure 2.5 A and B) is convenient for incubating tissues or cell suspensions with minimum equipment. However, the presence of air (or oxygen) bubbles within the NMR detection zone could significantly affect the homogeneity of the magnetic field and broader the resonance (Shachar-Hill and Pfeffer 1996). Also, it is not suitable for long-term studies, because levels of nonvolatile nutrients and waste products such as CO_2 or lactate will change over time, particularly when high cell densities are used (Shachar-Hill and Pfeffer 1996). Medium circulation systems (figure 2.5 C) are particularly convenient when it is important to maintain a constant extracellular environment. Another system was designed and developed to use *in vivo* NMR as an in-line measurement (Noguchi *et al.,* 2002). Cell culture was pumped directly and circulated between fermentation vessel and NMR tube. The real-time metabolism and energy potential were monitored by this system. But this system is just suitable for bacteria with small cell sizes. It is not suitable for analyzing plant cells and animal cells which have large cell sizes and present sensitivity to shear stress. Perfusion system is the most satisfactory design for maintaining tissues and cells in a defined controlled

physiologic condition (Shachar-Hill and Pfeffer 1996). A small-scale bioreactor with perfusion system has been designed based on literature and used successfully for *in vivo* NMR measurement in our laboratory (Figure 2.5 D and 4.1) (Gmati *et al.,* 2004). To our knowledge, it is the only *in vivo* NMR small-scale bioreactor with adequate hydrodynamic and mass transfer characterization, showing that the cells of the cell bed are homogeneously fed in medium. Cell bed oxygen demand for 4 h perfusion required a minimal medium flow rate of 0.8 mL/min. Residence time distribution assays at 0.8 to 2.6 mL/min showed the medium flowing into cell bed to be perfectly mixed with an axial dispersion coefficient of 0.2 and 1.3 m/s^2, respectively (Gmati *et al.,* 2004). In addition, it is easy to operate and control perfusion conditions, such as temperature, pH, rate of oxygenation, nutrient supply. The small-scale bioreactor that was used in our project is based on Gmati *et al.* (2004), which was modified for long-term experiment and quantification.

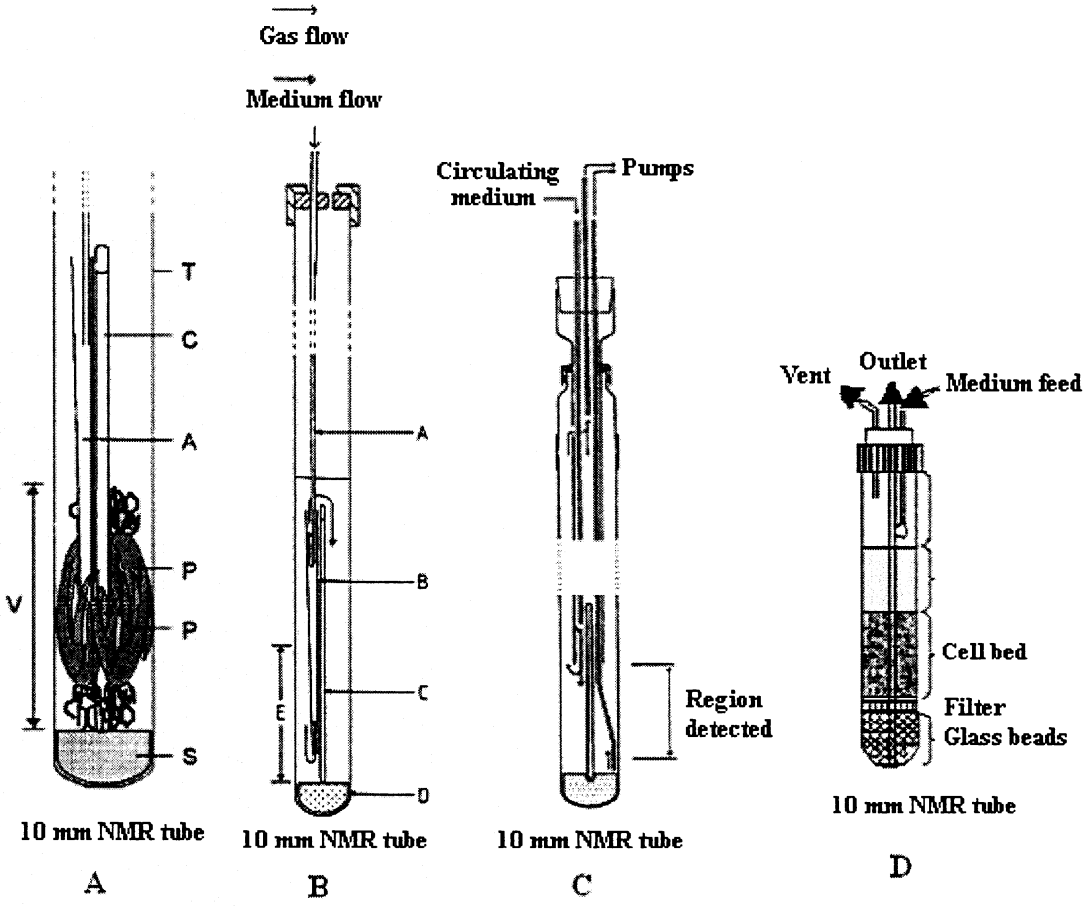


Figure 2.5: Small-scale bioreactors for *in vivo* NMR measurement

A: An experiment for recording *in vivo* NMR spectra from intact *C. intrepidus* plants in a 10mm diameter NMR tube: (A) airlift tube with gas inlet; (C), reference capillary; (P), chamaegigas plant; (S), Teflon spacer; (T), NMR tube containing the suspending medium; and (V), the detectable volume of the sample. (Schiller *et al.,* 1998); **B**: An experimental arrangement for oxygenating an NMR sample using an airlift system: (A), gas inlet; (B), central airlift tube; (C), reference capillary; (D), Teflon spacer and (E), the detectable region of the sample. (Fox *et al.*1989); **C**: An experiment arrangement

for circulating an oxygenated medium through a 10 mm diameter NMR tube. (Ratcliffe *et al.* 2001a); **D**: Small-scale bioreactor was designed to allow oxygenated perfusion medium pass through cell bed from up to bottom of NMR tube and keep cell packed with high density to give high signal-to-noise ratio (Gmati *et al.,* 2004).)

2.6 THE APPLICATION OF *IN VIVO* NMR

2.6.1 Identification and quantification of cells metabolites and nutrients

NMR signals are observed by placing the sample in the magnet of an NMR spectrometer, and the resulting spectrum often contains sufficient information to identify and quantify the metabolites that are present. At least two types of information can be obtained from *in vivo* NMR spectra, chemical shifts and signals intensities. Chemical shift is used to identify metabolites as previously presented. The identification of a nucleus can be done by comparing the chemical shifts of a spectrum with that of known compounds (Bligny *et al.,* 1990) under the same conditions, or with literature database (Shachar-Hill and Pfeffer 1996).

The signal intensities are proportional to the nucleus concentration within the NMR sensitive zone, so metabolite concentrations can be obtained from the absolute intensities in a NMR spectrum. Normally, concentrations of intracellular ions or organic compounds can be easily quantified by comparing the areas of signals to that of a

standard. However, the relationship between area and concentration can differ from peak to peak or from a molecule to another and is sensitive to environmental conditions such as dissolved oxygen, paramagnetic ions and NMR parameters. Quantification is simple when the pulse interval is long enough so that all nuclei are fully relaxed to equilibrium prior to each pulse. But it will take very long time to record a fully relaxed NMR spectrum for the purpose of quantification. Since NMR result is the average of the time during NMR data acquisition, averaged concentrations in metabolites can be obtained for the time of spectra accumulation. But, biologists need to shorten the acquisition time to follow as real-time as possible cells metabolism. The saturated NMR spectra with shorter d1 can satisfy this requirement but make quantification complex. Saturation factor is introduced to convert saturated NMR data to full relaxation data to satisfy quantification and shortening acquisition time. The measurements of saturation factor will be discussed in chapter 5.

The use of standards is necessary for peak quantification. There are two kinds of standards. One is internal standard that is added at known concentrations in the medium. And the concentrations of other metabolites are obtained by comparing the areas to the signal from the standard that appears on the NMR spectrum. However, internal standards have to be carefully selected. It should not damage or being uptaked by the cells. In addition, the signal of an internal standard should be singlet, while the chemical shift should be far away from the signals from cells metabolites. Such as, triethylphosphate can be used as internal standard to quantify cell metabolites (Kirk *et al.,* 1986). Another type of standard is external standard, which is placed within the

NMR measuring volume, not directly contact with the cells. Usually, external standards are sealed in a capillary, which is inserted at the center of NMR tube (Roby *et al.,* 1987; Spickett *et al.,* 1993; Rasmussen *et al.,* 2000). An external standard is noninvasive and can not affect cells behavior. Since there are practically no restrictions for selecting external standards, it is then easier to use a molecule giving a strong signal at a desired chemical shift, far from those of the cells. Usual external standards are MDP (methylenediphosphonate) (Majumday *et al.,* 1999; Osbakken, 1992; Roberts *et al.,* 1997), MPA (methyl phosphonate acid) (Campbell, *et al.,* 1985) for ^{31}P-NMR quantification of animal cells or plant cells, dioaxone (Osbakken, 1992) for ^{13}C-NMR quantification of myocytes cells, 6-fluorotryptophan for ^{19}F NMR quantification of myocytes cells. In addition, there is still a way of quantification by comparing the signals' absolute intensities to a calibration curve determined with standards in the absence of cells (Rager *et al.,* 2000). However, it is virtually impossible to set the NMR spectrometer exactly at the same operating conditions. The sensitivity of NMR coil is changing from experiment to experiment and along a single experiment, unfortunately (Bruch, 1996). A standard has then to be present within the cell sample, for each experiment.

2.6.2 Description of intracellular environment

Intracellular environment of a cell is defined from its content in ions (e.g. nutrients), energy status and redox potential. Moreover and especially for plant cells, changes in the extracellular environment can affect intracellular parameters since the cells are accumulating many nutrients (Lamboursain and Jolicoeur, 2004). As previously mentioned, NMR chemical shift is affected by the intracellular environment (e.g. pH, ionic strength) (Gmati et al., 2004). Therefore, *in vivo* NMR can be used to describe intracellular environment and monitor its evolution. Noninvasive measurement of intracellular pH by ^{31}P-NMR is a good example. The distribution of the two inorganic phosphate molecules ($H_2PO_4^-$ and HPO_4^{2-}) present in cell. The chemical shift of intracellular phosphate is also up to the ratio of these two molecules that is controlled by intracellular pH. Therefore, the changes of chemical shifts of intracellular phosphate will reflect the changes of intracellular pH. Intracellular pH can then be noninvasively measured by *in vivo* ^{31}P-NMR using intracellular phosphate as pH probes. Effect of extracellular pH on intracellular pH was already studied by *in vivo* ^{31}P-NMR (Moon *et al.,* 1973; Gillies *et al.,* 1981; Gerendas *et al.,* 2000; Hesse *et al.,* 2002; Rijhwani *et al.,* 1999). Both cytoplasmic pH and vacuolar pH can be obtained by this noninvasive measurement. Slonczewski (Slonczewski *et al.,* 1981) and coworkers reported that methylphosphonate could be used as intracellular pH probe too. Methylphosphonate is uptaked by the cells and gives both intracellular and extracellular signals in *in vivo* NMR spectrum because of the different pH values between both sides of cell cytoplasm membrane. This invasive measurement was applied on *Escherichia coli*, which has no

vacuole. However, there is no report about how to measure intracellular pH with methylephosphate as intracellular pH probe to study plant cell, which contains a vacuole having its proper pH. In addition, intracellular free magnesium can be determined by ^{31}P-NMR directly (Gupta *et al.*, 1978). Briefly, intracellular free magnesium and the ATP bound magnesium (figure 2.6) are exchanged by the chemical equation (2-5).

$$ATP + Mg^{2+} \xleftrightarrow{K_d} ATP - Mg^{2+} \quad \text{.....2-5}$$

With K_d, the dissociation constant for the ATP-Mg^{2+} complex being given by

$$K_d = \frac{[ATP][Mg^{2+}]}{[ATP - Mg^{2+}]} \quad \text{.....2-6}$$

$$[Mg^{2+}] = K_d \frac{[ATP - Mg^{2+}]}{[ATP]} \quad \text{.....2-7}$$

In this equation, K_d can be detected by saturation transfer that was described in the section of the determination of enzyme kinetics. The concentration in free Mg^{2+} is then estimated in μM for the chemical shift of ATP_β relative to that of ATP_α ($\sigma_{\alpha-\beta}$) with equation 2-8.

$$[Mg^{2+}] = K_d \frac{[10.71 - \sigma_{\alpha-\beta}]}{[\sigma_{\alpha-\beta} - 8.25]} \quad \text{.....2-8}$$

Where 10.71 and 8.25 are the limiting chemical shifts of uncomplexed and fully complexed ATP, respectively. The disadvantage of this method is that the chemical

shift difference between ATP_{α} and ATP_{β} as well as the dissociation constant depends on the pH, which could change under different physiological conditions (Bruch 1996).

Other ions, such as sodium and calcium can be monitored by NMR spectroscopy. Osbakken et al. (1992) studied sodium at different concentration and found that high salt (NaCl) concentration causes to vacuolar alkaliniazton in maize root tips was observed (Spickett *et al.,* 1993). Calcium concentration and calcium flux were monitored by ^{19}F NMR in conjunction with an intracellular calcium-chelating agent 5F-1, 2-bis (2-aminophenoxy) ethane-N, N, N', N'-tertraacetic acid (Osbakken *et al.,* 1992). Nutrients uptake rate and storage rate can also be detected by *in vivo* NMR (Sakano *et al.,* 1995). Results show that *Catharanthus roseus* (L.) G. Don cells absorbed phosphate at a constant rate in the first hour and accumulated into the vacuole supplied with abundant phosphate. The rate of Pi uptake by *Catharanthus roseus* cells was independent of cell density and was constant over a wide range of Pi concentrations (2mM-10μM).

The cellular energy availability can be conveniently measured by comparing the intensities of ATP_{β} to cytoplasmic phosphate (Eskey *et al.,* 1993). Stress such as oxygen stress, salt stress and nutrients stress which causes the change of intracellular environment will be discussed in the section of stress physiology.

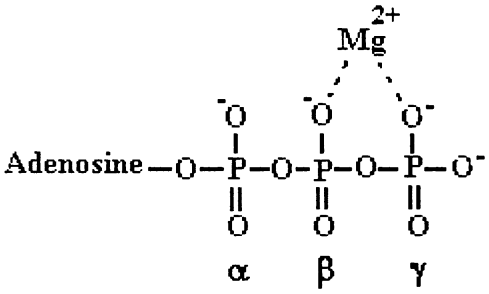


Figure 2.6: The structure of Mg^{2+}-ATP complex

(α,β, γ are used to mark different phosphorous in ATP)

2.6.3 Study of metabolic fluxes and metabolic pathways

Cells can be cultivated in NMR tube for hours to several days with oxygenated sterile medium perfusion. For example, Rijhwani et al. (1999) cultivated hairy roots for 925 h, while Roby and coworkers (1987) have verified that compressed cell bed can survive at least 7 days using a sterile Pi-free nutrient medium.

In vivo NMR spectroscopy has been applied to follow metabolite fluxes and explore metabolic pathways (Ratcliffe, 2001). Here we just give some examples to confirm that NMR spectroscopy is a useful and conveniently technique to perform studies on metabolic fluxes and metabolic pathways.

^{13}C-NMR was used for metabolic studies because it can provide complete information on carbon metabolites. Normally, carbon-13 labeled substrates were added to cultivated cells to increase the sensitivity of the measurement. One carbon tracing is a good example by feeding ^{13}C labeled nutrients or metabolites and following the carbon fate

by *in vivo* or *in vitro* NMR. For example, Rijhwani et al (1999) used 1-^{13}C, 2-^{13}C and 3-^{13}C labeled glucose to perfuse *C. roseus* hairy roots. ^{13}C-NMR was applied to study pentose phosphate pathway, nonphotosynthetic CO_2 fixation and glucan related synthesis pathways. Glucose uptake can be easily observed by the decrease of intensities of 1-C of glucose. Fluxes through glycolysis and the TCA cycle were measured from the detection of labeling in intermediates in the pathway. Synthesis of glucans can be observed by ^{13}C-NMR, indicating a significant diversion of the glucose flux into cell wall and storage products synthesis. When glucose enters the pentose phosphate pathway, the 1-^{13}C label is lost as CO_2 (Figure 2.7) in the first step.

Another example is using ^{13}C labeled methanol to study the metabolism of methanol. The metabolism of methanol in plant cells can be conveniently studied by ^{13}C-NMR. ^{13}C labeled methanol was used to feed plant cells at the concentration of 5 mM, which did not affect cell growth (Gout *et al.,* 2000). The uptake and metabolism of methanol was followed by ^{13}C-NMR to get the results that methanol slowly metabolized to [3-^{13}C]serine, [$^{13}CH_3$]methionine, and [$^{13}CH_3$] phosphatidylcholine as shown in figure 2.8.

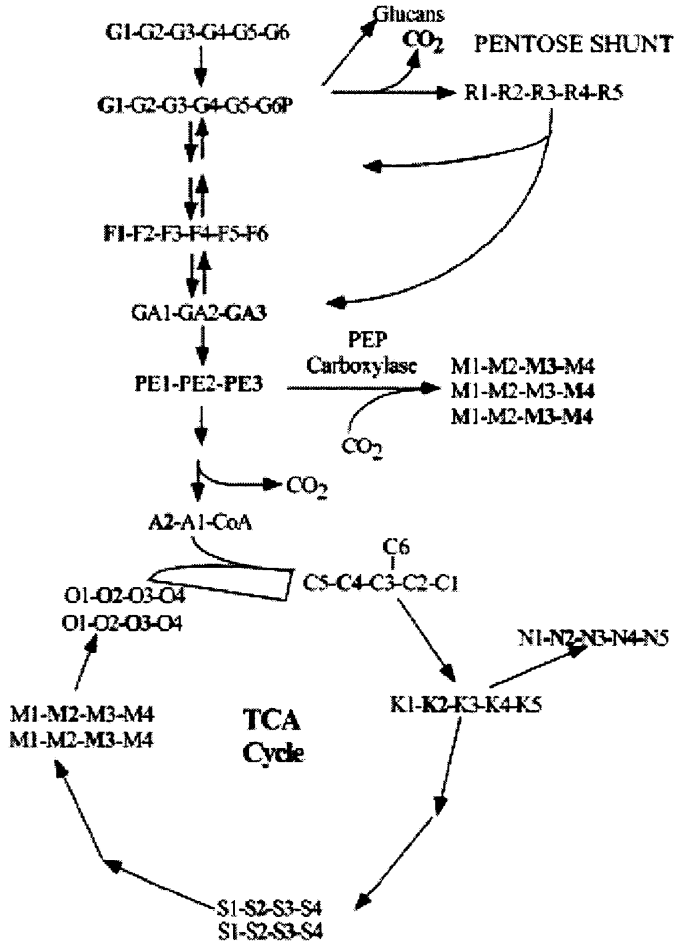


Figure 2.7: Incorporation of label from [1-^{13}C] glucose into PPP pathway, glycolysis, and TCA cycle.

(Intermediates: G, glucose; F, fructose; GA, glyceraldehyde; PE, phosphoenolpyruvate; A, acetyl-CoA; C, citrate; K: α-ketoglutarate; N, glutamine; S, succinate; M, malate; O, oxaloacetate. Bold letters represent ^{13}C label. Labeling in the first turn of TCA cycle has been shown. Similarly, flow of 6-^{13}C and 2-^{13}C labels of glucose in the TCA cycle can be followed. (Rijhwani *et al.,* 1999)).

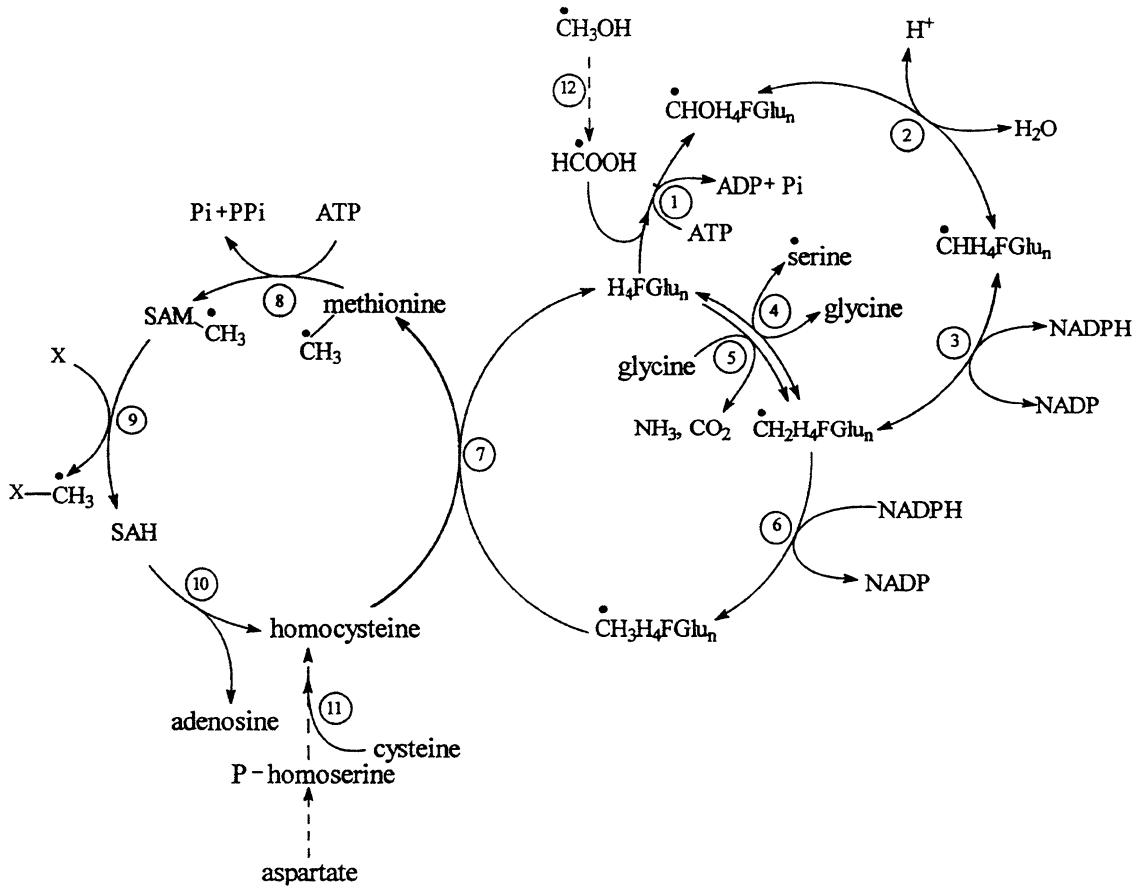


Figure 2.8: Proposed metabolic pathways involved in methanol assimilation by sycamore cells.

(Enzymes: 1, Formyltetrahydrofolate synthetase; 2, methenyl H_4Pte-Glu_n cyclohydrolase; 3, methylenetetrahydrofolate dehydrogenase; 4, Ser hydroxymethyltransferase; 5, Gly decarboxylase; 6, methylenetetrahydrofolate reductase; 7, cobalamin-independent Met synthase; 8, S-adenosyl-Met synthetase; 9, methyltransferase; 10, S-adenosylhomo-Cys hydrolase; 11, cystathionine g-synthase and cystathionine b-lyase; 12, methanol oxidase and formaldehyde dehydrogenase. H_4FGlu_n, 5,6,7,8 tetrahydropteroylpoly-Glu (tetrahydrofolate); $CHOH_4F$-Glu_n, 10-

formyltetrahydrofolate; $CHH_4F\text{-}Glu_n$, 5,10-methenyltetrahydrofolate; $CH_2H_4F\text{-}Glu_n$, 5,10-methylenetetrahydrofolate; $CH_3H_4FGlu_n$, 5-methyltetrahydrofolate; SAM, S-adenosyl-Met; SAH, S-adenosylhomo-Cys. •, (^{13}C) carbon (Gout *et al.,* 2000).)

This technology named one-carbon tracing has been widely applied in yeast (Campbell-Burk *et al.,* 1987) to study the control of glycolysis, in animal cells (Zupke *et al.,* 1995) to investigate metabolic pathways and fluxes through glycolysis, pentose phosphate pathway and tricarboxylic acid cycle, and in plant cells (Dieuaide-Noubhani *et al.,* 1995) to quantify and study the metabolic fluxes of glycolysis and recycling of triose phosphates back to hexose phosphates, the pentose phosphate pathway, the fluxes through malic enzyme, PEP carboxylase and citrate synthesis as well as polysaccharide synthesis.

^{31}P-NMR has been applied to study metabolic fluxes and metabolic pathways as well as the effect of some chemicals or intermediate metabolites. The studies of metabolism include phosphorylation (Bligny *et al.,* 1989), polyphosphate metabolism (Gerlitz *et al.,* 1997), mannose metabolism (Rager *et al.,* 2000; Brouquisse *et al.,* 2001), sucrose deprivation (Roby *et al.,* 1987) and the phosphate metabolism during cell division (Gillies *et al.,* 1981). The effect of intermediate metabolites or chemicals has been also widely studied. For example, the effect of glycerol on plant cell metabolism (Aubert *et al.,* 1994). Moreover, the energy metabolism has been studied by *in vivo* ^{31}P-NMR through the measurement of ATP (Jucker *et al.,* 2000). ATP concentration has been

shown to be highly dynamic and showed to be affected by multiple stresses imposed to the cells (Netting et al., 2000). But with *in vivo* ^{31}P-NMR spectroscopy, ATP can be measured within few minutes (Shachar-Hill and Pfeffer 1996). *In vivo* ^{31}P-NMR was applied often to study ATP synthesis (Jucker *et al.,* 2000), phosphorylation potential and the transmembrane electrical potential during ATP synthesis and hydrolysis (Ogawa *et al.,* 1984) and the energy metabolism during phosphate uptake (Sakano *et al.,* 1995; Montero-Lomeli *et al.,* 1989).

^{14}N- and ^{15}N-NMR is applied to study nitrogen metabolic fluxes and metabolism. There are two nitrogen sources in media used to culture plant cells and tissues, which are nitrate and ammonium. The consumption of these two nitrogen sources by maize roots has been studied by NMR spectroscopy (Lee *et al.,* 1992). *In vivo* ^{15}N-NMR has been used to study secondary metabolism in transformed root cultures of Solanaceae (Ford *et al.,* 1994). Nitrogenous secondary metabolites were detected and information was deduced on the subcellular distribution of nicotine.

2.6.4 Determination of enzyme kinetics

Magnetization transfer in NMR from a nucleus to another is a powerful method to measure enzyme kinetic factors, the fluxes of energy production and utilization (Bruch 1996). Magnetization transfer is the main method for determining kinetics of chemical exchange by perturbating the magnetization of a nucleus in a particular site or sites and following the rate at which magnetic equilibrium is restored. The most common perturbations are saturation transfer and inversion transfer, and the corresponding

techniques are often called 'saturation transfer' and 'selective inversion-recovery' (IUPAC, 1997). Specifically in saturation transfer, one peak is selectively saturated while in inversion transfer, the peak is selectively inverted. Saturation transfer was mainly used on measurement of enzyme kinetic factors by irradiating a nucleus and measuring the spin population transferred to another nucleus by a reaction. ^{31}P-NMR saturation transfer has been performed in a variety of *in vivo* systems to investigate kinetics in a noninvasive manner, such as yeast (Alger *et al.,* 1982), bacteria (Brown *et al.,* 1977), animal cells (Gadian *et al.,* 1981) and plant cells (Roberts *et al.,* 1984a).

The synthesis and the hydrolysis of ATP is an example of kinetic factor measurement by saturation transfer. The exchange reaction of ATP and ADP is showed below (2-9).

$$\mathrm{ADP} + \mathrm{Pi} \underset{\mathrm{Kr}}{\overset{\mathrm{Kf}}{\rightleftharpoons}} \mathrm{ATP} \qquad \text{.....2-9}$$

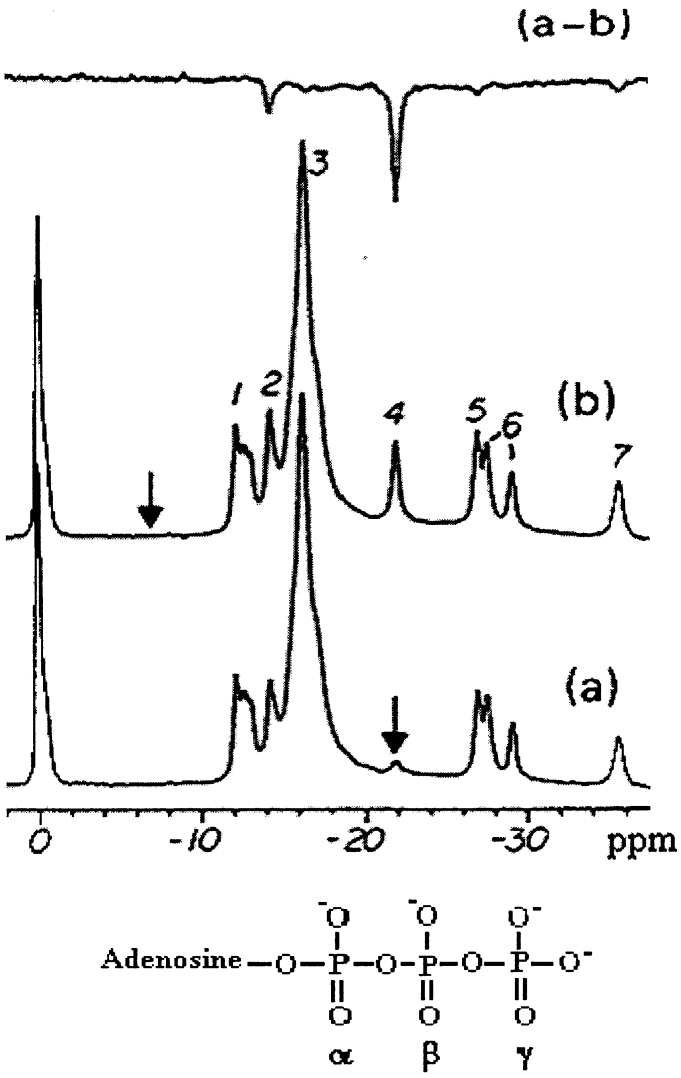


Figure 2.9: *In vivo* ^{31}P-NMR spectra of saturation transfer experiment and the chemical structure of ATP

(Peak assignments: 1, Glucose-6-Phosphate; 2, cytoplasmic Phosphate; 3, vacuolar Phosphate; 4, γ-ATP; 5, α-ATP; 6, UDP-Glucose and nicotinamide adenine nucleotides; 7, β-ATP)

(http://www.plantbiology.ucr.edu/people/faculty/roberts.html)

where K_f and K_r are the first order rate constants for the forward and reverse reactions, respectively.

In vivo ^{31}P-NMR spectrum was shown in figure 2.9b. When phosphorus nuclei of γ-ATP (-21ppm) are selectively irradiated ("saturated") over a period of seconds, the signal at -21 ppm becomes invisible during subsequent observation as shown in Figure 2.9 a. If the saturated phosphorus nuclei move to a different environment (here γ-ATP to Pi), they remain invisible until they return to the ground state via relaxation. Chemical exchange leads to a transfer of saturation. The difference can be seen as a decrease in the intensities of spectral lines other than the irradiated line (figure 2.9 a-b). The magnitude of the decrease in signal height due to saturation transfer reflects, in part, the ATP lifetime in these cells, which is on the order of a few seconds. With this method, Roberts *et al.* (1984) examined directly and simultaneously the relationship between the rate of ATP synthesis and the levels of metabolites such as ATP in maize root tips. Combining with the measurements of oxygen consumption, the P/O ratios in the living tissue and cells could be estimated to around 3 under aerobic conditions and glucose as the carbon source. Campbel *et al.* (1985) used the same techniques working on the yeast *Saccharomyces cerevisiae* to get the P/O ratios of 2.2 and 2.9 for glucose and ethanol feeding, respectively. Saturation transfer techniques were also applied to evaluate creatine kinase kinetics during different pharmacological and physiological interventions (Osbakken *et al.,* 1992). Degani *et al.* (1985) compared the two magnetization transfer methods, inversion transfer and saturation transfer by ^{31}P-NMR. The kinetic parameters obtained by the two methods were the same, whether in solution

or in a perfused heart. However, there are limitations with magnetization transfer. A first problem is that all the magnetization transfer experiment should be done at the time T1. Otherwise, the peaks could be fully relaxed and no magnetization transfer would occured. A second problem is that magnetization transfer is based on two pools, while the metabolites and enzymes could be related to several pools at different rates simultaneously.

2.6.5 Study of stress physiology

Stress physiology has been studied by *in vivo* NMR changing cells environmental conditions. Diverse stresses have been assayed such as changing the dissolved oxygen in the perfusion medium (Roberts, 1984; Raven, 1986; Kennedy *et al.,* 1992); changing one nutrient concentration in perfusion medium (Sakano *et al.,* 1995); adding cryptogein elicitor to study the effect of early events in elicitation process (Pugin *et al.,* 1997), and changing external pH (Gout *et al.,* 1992) or temperature. Netting (2000) reported that the cells attain minimal metabolic activity as a stress intensifies and remain in that state until that stress is relieved.

Adaptation of plant cells to low oxygen stress was first observed in 1988 (Aaglio *et al.,* 1988) when it was found that maize root tips were much more tolerant under anoxia if whole seedling were first exposed to hypoxia (3-4% O_2). The acidification of cytoplasm following anoxic conditions is a common phenomenon observed in most organisms including plants (Roberts, 1984; Raven, 1986; Kennedy *et al.,* 1992). Both *in vivo* ^{31}P- and ^{13}C-NMR were used to describe cells response to anoxia measuring

intracellular environment, phosphate and carbon metabolites such as intracellular pH, cytoplasmic and vacuolar phosphate, phosphomonesters (PME), nucleotides, lactate and ethanol (Gout *et al.,* 2001). Cytoplasmic pH decreased 10 min after induction of anoxic conditions (Robert *et al.,* 1992). Studies have also been performed on the effect of medium pH on intracellular pHs. Cytoplasmic and vacuolar pHs were strickly controlled and were shown to be independent from external pH within the range of 4.5-7.5. In contrast, cytoplasmic pH decreased rapidly when external pH was lower than 4.5 and increased progressively when external pH was over 7.5 (Gout *et al.,* 1992). *In vivo* NMR has also been used to study osmotic stress. High ammonium concentration (10 mM) and high external pH also caused intracellular pH change. A small cytoplasmic alkalinization of 0.1-0.2 pH unit and a larger vacuolar alkalinizaton of 0.6 pH unit were reported in maize root tips exposed to high salt concentration (200 mM NaCl) (Spickett *et al.,* 1993). Also during phosphate uptake process, cytoplasmic pH ha been observed to decrease (Sakano *et al.,* 1992). Cytoplasmic pH is thus a very sensitive parameter to probe cells response against diverse stresses.

Elicitation is a natural stress potential for plants when being attacked by pathogens or animals. Plants have developed a number of strategies to defend themselves against kinds of elicitor attack, including synthesis of secondary metabolites, changing physiological state etc. Therefore, elicitors can be used to enhance plant secondary metabolite synthesis (Radman *et al.,* 2003). In fact, the technology of elicitation is also broadly applied *in vitro* to enhance the second metabolism productivity. Similarly as for osmotic and low oxygen stresses, elicitation caused acidification of cytoplasm (Roos *et*

al., 1998) and an increase of cytosolic calcium concentration ("calcium flash") (Muller *et al.,* 2000). It was shown that using a confocal microscope and molecular probes targeting pH that within few minutes elicitor contacts a transient acidification of cytoplasm occurs in parallel with an increase of the vacuolar pH. Cell response to elicitation can also be observed by *in vivo* NMR spectroscopy with a potential for the acquisition of more complete information. Effect of elicitation on the responses of NADPH, ATP and glucose 6-phosphate, cytoplasmic phosphate and vacuolar phosphate has been studied (Pugin *et al.,* 1997).

CHAPTER 3: METHODOLOGY

The author would like to inform the reader that this section is in complement to that was presented in chapter 4, 5 and 6. Please note that all informations were not systematically repeated.

3.1 MATERIAL AND METHODS

3.1.1 Chemicals and instruments

Chemicals: All the chemicals were obtained commercially without any further purification. Methanephosphonic acid 98%: Fisher, Montreal, Canada; Orthophosphonic acid 85%: Fisher, Montreal, Canada; Deuterium oxide: CDN isotopes, Montreal, Canada; Nitrogen gas: Air Canada. pH buffer solutions (pH 4.00 and pH 7.00): Fisher, Ontario, Canada; $MgCl_2$: Sigma; KCl: sigma; KH_2PO_4 : Sigma; HCl: Adrich; NaOH: Aldrich. Chitin: Sigma, Oakville, Canada.

Instruments: NMR spectrometer and 10mm broad-band probe: Varian, USA; 10mm NMR tube: Fisher, Montreal, Canada; Disposable micropipettes (ID=0.80mm and ID=0.199mm): Fisher, Montreal, Canada; Filter: Miracloth, Calbiochem Corp, San Diego, USA; Peristaltic pumps: Masterflex peristaltic pump, Labcor, Montréal, Québec, Canada; Tubes: Masterflex, Montréal, Québec, Canada; Oxygen electrode: Polarographic, Ingold, Urdorf, Switzerland; pH electrode: Accumet electrode, Fisher, Montreal, Canada; In-house 316 SS chamber: Ecole Polytechnique de Montreal,

Montreal, Canada; Oxygen meter: Cole parmer, Italy ; pH meter: Alpha pH 200, Singapore;

3.1.2 External phosphorus reference preparation

0.192 g methanephosphonic acid (98%, Fisher, Montreal, Canada) was dissolved in water and diluted to 1 ml to get 2.0 M solution. 2.0 M methanephosphonic acid solution was sealed and kept in dark and dry environment.

One open end capillary (ID: 0.199 mm; OD: 0.66 mm, Fisher, Montreal, Canada) was immerged into 2.0 M methanephosphonic acid solution. When micro capillary was filled with the solution, both open ends were melted and sealed on flame. All the operation was done in fume hood.

3.1.3 Perfusion medium

Since paramagnetic ions can broaden the NMR signals and should be removed from the culture medium (Shachar-Hill and Pfeffer, 1996), a modified B5 medium containing D_2O (90ml and 10ml respectively) was used as perfusion medium. Three different modified B5 media were used: B5-F1, B5-F2 and B5-F3. B5-F1 is B5 medium free of paramagnetic ions (Fe^{3+}, Co^{2+}, Mn^{2+}) with 1.0 mM phosphate (KH_2PO_4). B5-F2 is B5 medium free of paramagnetic ions with 0.25 mM phosphate. B5-F3 is B5 medium free of paramagnetic ions and phosphate. The preparation of perfusion medium is the same as described in chapter 5.

3.2 ANALYTICAL METHODS

3.2.1 Dry weight measurement

The cells were maintained in shake flasks as described by Lamboursain et al. (2002). Cell dry weight (DW) was obtained by sampling 10 ml of a well-mixed cell suspension cultured in a 500 ml Erlenmeyer flask by a cuted-end 10 ml sterile plastic pipette. Cells were separated by filter under vacuum. After three times washing with deionized water, cells were transferred on an aluminum dish of known weight (Wo). Cells and aluminum dish were placed in an oven for drying at 65°C for 24h. After cooling down, the aluminum dish and cells were weighted (W1). The cells dry weight concentration was then obtained by calculating (W1-Wo) for a cell suspension volume of 10 ml.

3.2.2 Cell bed density measurement

Cell bed was first formed by acting of medium circulation between the reservoir and the small-scale bioreactor. The cell bed length was obtained on the average of two measurements, before and after experiment. The volume of the cell bed was calculated by its length and the inside diameter of NMR tube. After experiment, the cell bed was pumped out of NMR tube by switching inlet and outlet. The cell was collected in a vial, separated on a vacuum filter, washed three times by deionized water, transferred on a known weight aluminum dish and dried in an oven at temperature of 65°C for 24h. The weight of the cell bed was got by the total weight of cell and aluminum dish minus the

weight of aluminum dish. The cell bed density was calculated by the dry weight and the volume of cell bed.

CHAPTER 4: MODIFICATION AND DEVELOPMENT OF SMALL-SCALE BIOREACTOR

4.1 INTRODUCTION

The small-scale bioreactor used on this project was designed and developed previously in our laboratory (figure 4.1) by Gmati *et al.* (2004). The bioreactor showed that it could be used for *in vivo* NMR measurements by cultivation plant cell inside NMR tube. However, the bioreactor design and operation had to be improved to enable sterile experiments as well as limiting water evaporation of the culture medium. Evaporation of D_2O will affect lock of NMR signal. Moreover, capacity for metabolite quantification has to be developed. Original configuration of the small-scale bioreactor is showed in figure 4.1 and the modifications applied are discussed below and can be seen in figure 5.1.

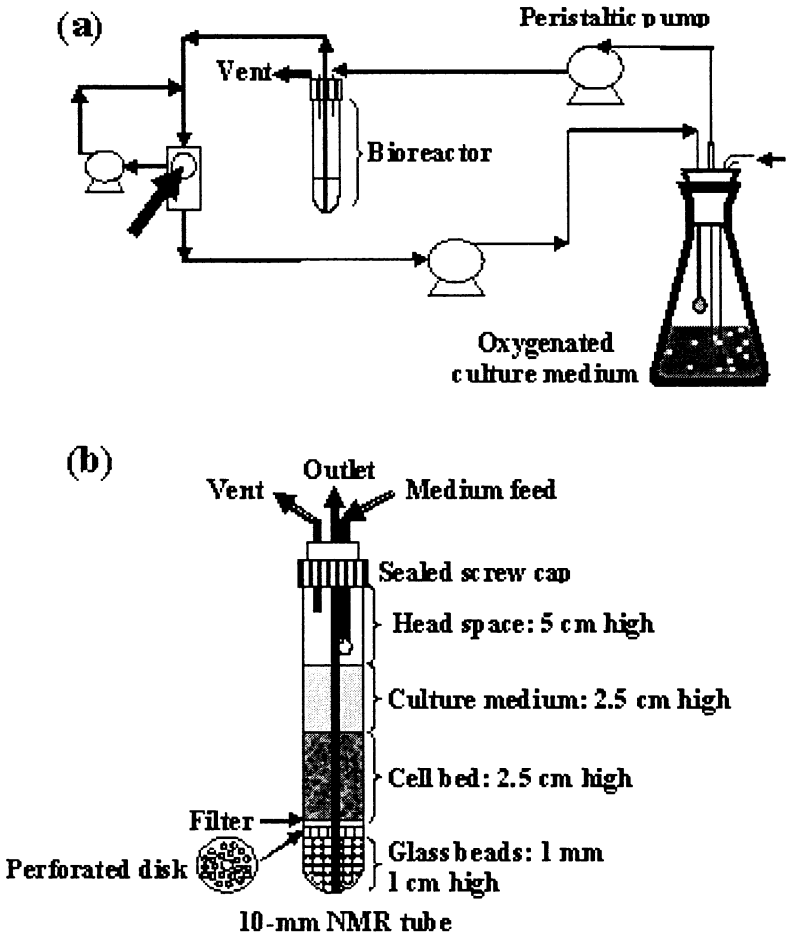


Figure 4.1: The scheme of Small-scale bioreactor

(a) Complete bioreactor system. (b) Detailed outline of the NMR tube modified as a bioreactor. (Gamti et al., 2004)

4.2 MODIFICATION AND CHARACTERIZATION OF SMALL-SCALE BIOREACTOR

4.2.1 Small-scale bioreactor

The small-scale bioreactor is a 10-mm OD screw cap NMR tube (Fisher, Montreal, Quebec, Canada) (Figure 4.1), which contains a filter (Miracloth, Calbiochem Corp.Cat.# 475855, San Diego, USA) supported by glass beads (D=1.0 mm). The small-scale bioreactor with perfusion system has been modified and developed as shown in figure 5.1 and 5.2. First, the open system was modified to a closed system by air filter to avoid contamination from the gas phase. A capillary that contains 2.0 mM methanephosphonic acid was kept at the center of the small-scale bioreactor at the level of NMR coil as a phosphorus concentration reference (figure 5.2). The outlet tube that extends to the bottom of NMR tube was changed to glass capillary with inside diameter of 0.8 mm, which can hold phosphorous reference capillary (OD: 0.66 mm).

4.2.2 Reservoir

A 250 ml Erlenmeyer flask that agitated by a magnetic stirrer and covered with a rubber plug is used as a medium reservoir for perfusion. The cover is connected to four tubes, which are used as inlet, outlet, safety and air supply. Outlet and safety tubes reach the surface of medium, while inlet and air supply extend to the bottom of the reservoir. A gas filter and a gas humidifier that contains 10% D_2O in sterile water are used before

gas bubbling through perfusion medium. A condenser with a gas filter is connected on the reservoir cover to condense the evaporation. Gas supply was connected to a microporous sparger at the bottom of the reservoir.

4.2.3 Medium circulation system

Medium circulation system includes 4 peristaltic pumps, tubes and a vial for collecting waste medium. Peristaltic pumps (Masterflex peristaltic pump, Labcor, Montréal, Québec, Canada) were calibrated before experiment. Three pump heads are mounted on a single pump for the inlet and the outlet tubes to the small-scale bioreactor and medium circulation in a in-house 316 SS chamber (figure 5.2). The in-house 316 SS chamber is modified for measuring adequately on-line dissolved oxygen and medium pH measurements. Another pump is set at higher speed and used as safety pump from the small-scale bioreactor to medium reservoir in case of leaking in NMR coil. According to the experiment, there are four tubes that are connected to the small-scale bioreactor, an outlet, an inlet, a safety tube and air vent. The inlet tube and outlet tube are used to circulate medium between the bioreactor and the reservoir. The safety tube is installed in case of medium overflow and leak into NMR coil, which should be avoided. The forth tube that is used as a vent is connected to an air filter to balance the air pressure in the small-scale bioreactor. Two valves are mounted to direct the medium flow towards waste or circulating between reservoir and small-scale bioreactor.

4.2.4 Phosphorus quantification

A 2.0 mM phosphorus reference solution was sealed in a glass capillary (ID: 0.199 mm, OD: 0.66 mm) and placed inside the outlet tube (OD: 0.80 mm), which was kept at the center and the reading zone of NMR tube to give a chemical shift and concentration reference. Another capillary (OD: 0.66 mm) was installed to block the reference capillary in place in case of entrainment with liquid ascendant low. The concentrations in phosphorus metabolites were obtained by comparing the areas to the area of reference and calibrating by saturation factor. T1 and T2 were analyzed by inverted gate pulse sequence with cell sample, reference and medium perfusion. Saturation factor and phosphorus reference calibration are described in chapter 5.

4.2.5 Detection of intracellular pH

A calibration curve between pH and ^{31}P-NMR chemical shift was obtained with a serial of phosphate solutions in different pH. These solutions were presented by Rijwani et al. (1999) as to minic intracellular environment. Three calibration curves (figure 5.3) were obtained from three different media, medium I: 5.0 mmol/L $MgCl_2$, 0.1 mmol/L KCl; medium II: 5.0 mmol/L $MgCl_2$, 0.2 mmol/L KCl; medium III: B5-F3 medium. Two intracellular pH values were estimated from the curves of serial I and II, and the final estimate was the average of those values. Curve of serial III was applied to identify signal of medium phosphate with set medium pH when medium contains phosphate.

4.3 RESULTS AND DISCUSSION

4.3.1 Small-scale bioreactor was modified for quantification

Quantification is an important issue of NMR measurement as previously stated. The intensity of NMR signals can be used to estimate the concentration of ions or organic acids or other molecules that are NMR visible. NMR signal intensities are proportional to nuclear concentration with the recycle time for the pulse sequence typically longer than 5 times T1 to avoid saturation of the signal (Xia *et al.,* 2001). For *in vivo* ^{31}P-NMR, T1 ranges from about 200 ms to over 6 s depending on the tissue and the magnetic field strength at which the measurements are performed. So, d1 could be of more than 20 s. Because, NMR is a relatively insensitive technology, it is often necessary to accumulate individual spectra over extended time periods (Brodelius *et al.,* 1984). So, collecting unsaturated (full relaxed) data can be a time-consumed process.

Methanephosphonic acid that was seal (figure 4.2) in a capillary (ID: 0.199mm), which was put in outlet tube at the center of NMR tube (figure 4.2) was used as a concentration and chemical shift reference. The chemical shift of 2.0 mol/L methanephosphonic acid is 30.65 ppm down shift to 85% orthophosphornic acid and is far away from the signals of phosphate metabolites. The reference could not damage cell because the chemical and cell were separated by glass. In addition, the reference results in a singlet, which is convenient for its integration. The reference capillary can be autoclaved for sterilization at 121°C.

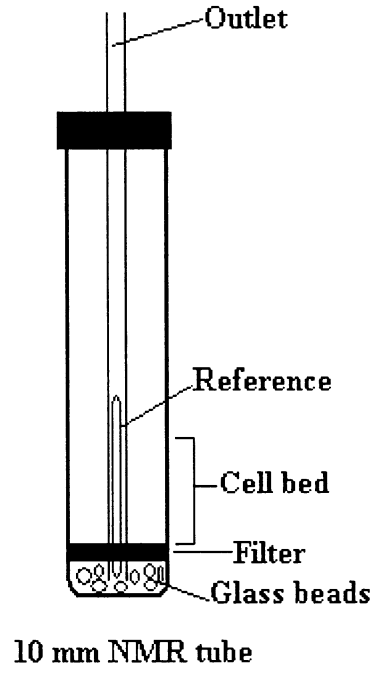


Figure 4.2: The arrangement of phosphorous reference in the small-scale bioreactor

4.3.2 Small-scale bioreactor can be used to detect intracellular pH

The relationship between chemical shift and pH was developed (figure 4.3). The other conditions of series I and II are the same except for different concentration of KCl. Serial III (B5-F3 medium) has much higher ion strength than serial I and II. From figure 4.3, it can be clearly seen that chemical shift increases with the increase of pH. However, there is no significant difference between serial I and II, with different KCl concentration. Comparing to serial I and II, serial III has more down shift due to high ion strength, especially when pH is lower than 6. This suggests that ion strength also affects chemical shift. High ion strength caused a downshift of NMR signals. The

intracellular pH can be evaluated by the chemical shifts of intracellular inorganic phosphate and these calibration curves. The serial III curve can be used to confirm the phosphate medium signal.

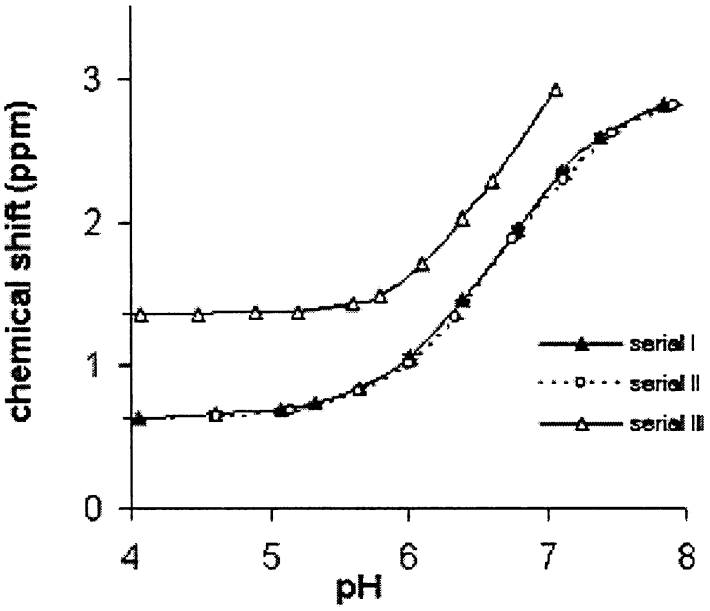


Figure 4.3. The coefficient of pHs and *in vivo* ^{31}P-NMR chemical shifts

(Serial I: 12.5ml 0.1mmol/L $MgCl_2$ [sigma], 0.25ml 0.10mol/L KCl [sigma], 2.5 mL 0.1mol/L KH_2PO_4 and 25 mL D_2O were introduced into a flask and diluted to 250ml. pH were adjusted to different value by 1mol/L HCl and 1mol/L NaOH. Serial II: 12.5ml 0.1mmol/L $MgCl_2$, 0.5ml 0.10mol/L KCl, 2.5 mL 0.1mol/L KH_2PO_4 and 25 mL D_2O were introduced into a flask and diluted to 250ml. pH were adjusted to different value by 1mol/L HCl and 1mol/L NaOH. Serial III: 90ml B5-F3 medium, 10ml D_2O and

0.50g KH_2PO_4 were put into a flask and diluted to 100ml. pH were adjusted to different value by 1.0 mmol/L HCl and 1.0 mmol/L NaOH)

4.3.3 Small-scale bioreactor was modified sucessfully for long-term experiment

As discussed before, the problems of contamination, medium evaporation are main limitations on the applications of the small-scale bioreactor for long term experiments. The problem of contamination was solved by modifying the open system to a closed system using gas filters, sterilizing the bioreactor before each experiment as well as using sterile perfusion medium. The evaporation of medium at high flow rate air bubbling was inhibited by a condenser and pre-humidified gas supply. In addition, the evaporation of D_2O will affect NMR signal lock. Figure 3.4 shows the result of a 10-day experiment with continuous culture in the small-scale bioreactor of *Eschscholtzia californica* cells. During these experiments, the volume of perfusion medium stayed stable. It can be seen clearly that vacuolar phosphate decreased from day 1 to day 6 and increased again at the end of culture (figure 4.4). The signals of PME, cytoplasmic phosphate and vacuolar phosphate can be seen clearly in the 10^{th} day NMR spectrum. These results show that cells can survive in the small-scale bioreactor for 10 days. If cells were dead, the signals of cytoplasmic phosphate and vacuolar phosphate would have merged together and give a single and broad peak.

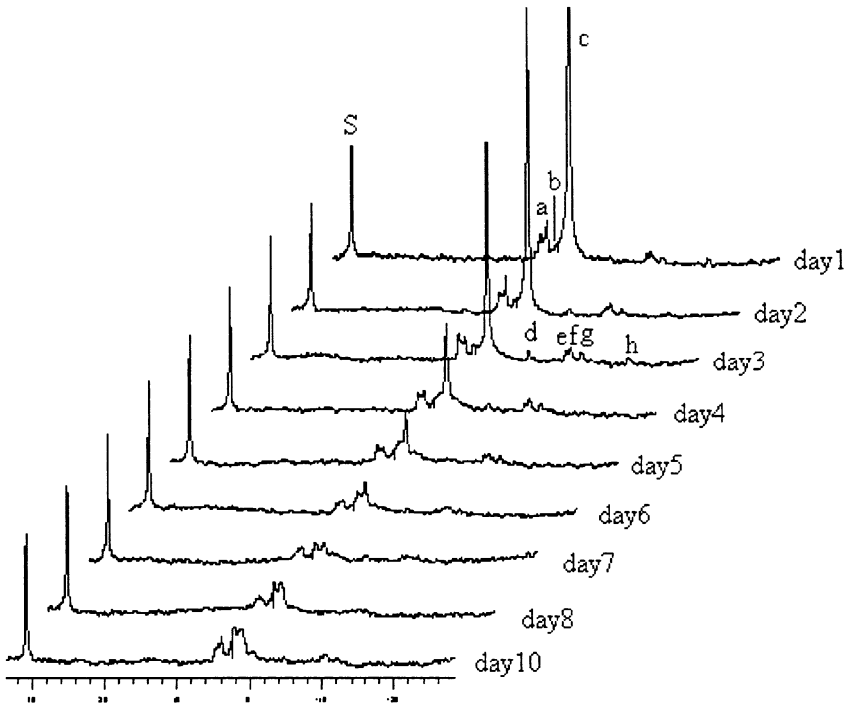


Figure 4.4: *In vivo* ^{31}P-NMR spectra of cell bed continually cultivated in the small-scale bioreactor for 10 days

(Small-scale bioreactor, perfusion medium and humidifying water were put in autoclave for sterilization at temperature of 121°C, 25min liquid cycle. 1-d-old *Eschscholtzia californica* cell sample was taken from flask culture and inoculated into the small-scale bioreactor with 100 ml perfusion medium of B5-F3 and D_2O (9:1 v/v) at flow rate of 1.60 ml/min. Cell bed were cultured at room temperature, laboratory light. Each NMR spectrum was recorded for 10000 scans, corresponding to acquisition time of 3.3 h. Other parameters were described in the section NMR spectroscopy in chapter 5. NMR spectra were recorded every day in 10 days. The signals were assigned as below. S,

Reference; a, PME; b, Pi_c; c, Pi_v; d, ADP_β+ATP_γ; e, ATP_α+ADP_α; f, NADP+NDPG; g, NDPG; h, ATP_β.)

CHAPTER 5: MONITORING OF *ESCHSCHOLTZIA CALIFORNICA* CELLS PHYSIOLOGICAL STATE BY *IN VIVO* ^{31}P NMR

5.1. PRESENTATION OF ARTICLE

A manuscript with the title of the monitoring of *Eschscholtzia californica* cells physiologic state has been submitted to the *Journal of Experimental Botany*. In this manuscript, the effect of oxygen stress was studied by changing the dissolved oxygen in perfusion medium; the cell cultures in different dissolved oxygen conditions were followed by *in vivo* NMR. The oxygen stress of cell transfer was investigated and the way to remove of oxygen stress during *in vivo* NMR measurement was discussed. The phenomenon of phosphate uptake, phosphate storage capacity and phosphate translocation were also discussed in this chapter.

5.2. MONITORING OF *ESCHSCHOLTZIA CALIFORNICA* CELLS PHYSIOLOGICAL STATE BY *IN VIVO* ^{31}P NMR

5.2.1 ABSTRACT

In vivo ^{31}P NMR was applied to the study of oxygen stress, phosphate (Pi) uptake, storage and translocation on *Eschscholtzia californica* cells. A small-scale bioreactor that was previously developed was inserted into a 10 mm NMR probe and continuously perfused with modified B5 medium containing 10% D_2O. The cells were cultivated as cell bed at a high density of 30 $gDW{\cdot}L^{-1}$. Methanephosphonic acid was used as an *in situ* reference. Glc-6P and Fru-6P (grouped as phosphomonoesters or PME), cytoplasmic Pi (Pi_c), vacuolar Pi (Pi_v) and ATP peaks were identified and quantified. The stress induced with cell sampling and transfer from flask culture to the small-scale bioreactor was studied. The cells required 60 min to recover from the stress and stabilizing their cytoplasmic (pH_c) and vacuolar pH (pH_v). Then, reducing dissolved oxygen concentration in the perfused medium from 60% to 25% from air saturation caused a cytoplasmic acidification of 0.7 pH unit, which recovered at initial value after returning at 60% dissolved oxygen. Cells behaviour to highly transient processes such as phosphate uptake were also followed *in vivo* while monitoring pH_c, pH_v, Pi_c, Pi_v, PME and ATP concentrations with time. Finally, cells cultured in shake flasks and in a bioreactor were compared from their respective content in Pi_c, Pi_v, PME and ATP with culture time.

Keywords: Bioreactor, *Eschscholtzia californica, in vivo* NMR, oxygen stress, phosphate uptake, phosphate translocation

Index: ADP: adenosine diphosphate; ATP: adenosine triphosphate; B5-F1: B5 medium free of paramagnetic ions; B5-F2: B5 medium free of paramagnetic ions, with 0.25 mmol/L phosphate; B5-F3: B5 medium free of phosphate and paramagnetic ions; Pi: phosphate; Pi_c: cytoplasmic phosphate; Pi_v: vacuolar phosphate; pH_c: cytoplasmic pH; pH_v: vacuolar pH; PME: phosphomonoesters (Glc-6P, F-6P, etc.); NADP: nicotinamide-adenine dinucleotide phosphate; NDPG: nucleotide diphosphate glucose; qO_2: specific oxygen uptake rate ($mmol \cdot g^{-1}DW \cdot min^{-1}$); $qO_{2,max}$: maximum specific oxygen uptake rate ($mmol \cdot g^{-1}DW \cdot min^{-1}$).

5.2.2 INTRODUCTION

Nuclear magnetic resonance spectroscopy, which was invented in the 1940s (Veeman, 1997) showed to be a unique non-invasive analytical tool in plant physiology and biochemistry (Shachar-Hill and Pfeffer, 1996). Among available non-invasive methods, nuclear magnetic resonance (NMR) spectroscopy is a technique that can provide insights on the regulation of plant metabolism through a combination of *in vivo* and *in vitro* measurements. *In vivo* NMR has been applied to studies on cells and tissues of diverse origin such as plants cells and tissues (Gerendas *et al.,* 2000; Gout *et al.,* 2001; Summers *et al.,* 2000; Bligny *et al.,* 1989; Rijhwani *et al.,* 1999; Brodeliust *et al.,* 1985; Sakano *et al.,* 1995), mammalian cells (Gupta *et al.,* 1978), microbes (Grivet *et al.,* 2003; Watts *et al.,* 2002) and yeasts (Hesse *et al.,* 2002; Gerlitz *et al.,* 1997). In plant

cells, ^{31}P NMR was used for measuring intracellular pH (Moon *et al.,* 1973; Gillies *et al.,* 1981; Gerendas *et al.,* 2000; Hesse *et al.,* 2002; Rijhwani *et al.,* 1999) and for ion flux study (Massonneau *et al.,* 2000), ^{31}P- and ^{13}C-NMR for metabolites quantification (Roby *et al.,* 1987; Spickett *et al.,* 1993; Aubert *et al.,* 1998; Rasmussen *et al.,* 2000), ^{13}C-NMR for the determination of enzyme kinetic factor (Brown *et al.,* 1977) and for the investigation of metabolic pathways (Rijhwani *et al.,* 1999). Thus NMR can be used to identify, quantify and localize metabolites, define the intracellular environment and explore metabolic pathways (Ratcliffe *et al.,* 2001; Shachar-Hill, 2002).

Among the various studies involving *in vivo* NMR, ^{31}P-NMR has shown to provide diverse and rich information on cell behavior. Phosphorus is one of the most essential elements for living organisms as it is involved in the regulation of cellular functions (Ranjeva *et al.,* 1987) and metabolism (Plaxton, 1999, 1998). Analysis of the different intracellular phosphate pools can then enable to study the effect of culture condition on cells physiological state. *In vivo* ^{31}P-NMR can provide important data such as cells cytoplasmic and vacuolar pH, cells energy potential (ATP, ADP, NADH and NADPH), identify, localize and quantify intracellular inorganic phosphate (Pi) and phosphomonesters (PME) such as glucose-6P and fructose-6P.

Different small-scale bioreactors were specifically developed for *in vivo* NMR measurements (Rijhwani *et al.,* 1999; Fox *et al.,* 1989; Roby et al., 1987). Based on the bioreactor configurations found in literature, a perfused small-scale bioreactor system was recently designed and characterized for *in vivo* NMR measurements (Gmati *et al.,*

2004). Hydrodynamic and mass transfer studies showed that the bioreactor maintains a homogeneous nutritional environment at every cell grown into a cell bed. This work thus focuses on the validation of the small-scale bioreactor to perform the monitoring of transient physiological processes by *in vivo* NMR spectroscopy. Suspension cells of *Eschscholtzia californica* were used as model system. The design of the small-scale bioreactor was improved for maintaining long term aseptic conditions, study cell physiology under oxygen stress, compare cells physiological state at different culture conditions, monitor phosphate uptake, storage and translocation.

5.2.3 MATERIAL AND METHODS

5.2.3.1 Biological Materials

Shake flask culture

Eschscholtzia californica shake flask cultures were performed as described (Lamboursain *et al.,* 2002). Briefly, 80 g of 10-d-old suspension cells were inoculated into 170 g sterile B5 medium, which contains Gamborg salt (Gamborg *et al.,* 1968), 30 $g.l^{-1}$ of glucose (Sigma, Oakville, Ontario, cat # G-5767), 0.2 $mg.l^{-1}$ 2,4, dichlorophenoxyacetic acid (2,4-D) (Sigma, Oakville, Canada, cat # D7299) and 0.1 $mg.l^{-1}$ kinetin (Sigma, Oakville, Canada, cat # K0753). Medium pH was adjusted to 5.6 using KOH before sterilization. For the experiments, 10-d-old cells were inoculated at 33% of the final volume in 250 mL Erlenmeyer flasks containing 60 mL of B5 medium. Cultures were maintained in the dark on an orbital shaker at 130 rpm, 25°C ± 2°C.

Bioreactor culture

An in-house 3.0-L bioreactor made of a 3-L glass flask (VWR Canlab, cat # 36390.086), equipped with a double helical-ribbon impeller and three surface baffles. The design and geometrical ratio were that of Jolicoeur *et al.* (1992). The top plate made in stainless steel (SS) (grade 316) supports a pH probe (Accumet electrode, Fisher, Montreal, Canada), a dissolved oxygen probe (polarographic : Ingold, Urdorf, Switzerland, Cat. # 11277-284), a gas inlet line and a condenser of the outlet gas. The gas mix was bubbled through a porous (2 µm) sparger installed at the bottom of bioreactor. Air filters (bacterial air vent: Pall-Gelman, VWR Canlab) ensured sterility and a water condenser minimized water losses by evaporation throughout the cultures' duration. Agitation speed was fixed at 60 rpm, and dissolved oxygen was controlled at 60% saturation by a data acquisition system (Virgo, Longueuil, Québec, Canada). All stainless steel parts were cleaned as follows. Parts were treated for 1 hour in 1% (w/v) citric acid, thoroughly rinsed with water, treated for 1 hour in 0.5% (w/v) NaOH and again thoroughly rinsed with water. Culture medium and the bioreactor were steam sterilized separately for 35 min (121°C, 1 bar). Cultures were grown at 25±1°C in the dark. 800 g of 10-d-old *Eschscholtzia californica* suspension cells taken from flask cultures were used as inoculum into 1.7 L of B5 medium. Bioreactor culture was performed at room temperature, in dark for 11 days.

5.2.3.2 Perfusion experiments in the small-scale bioreactor

Paramagnetic ions (Fe^{3+}, Co^{2+}, Mn^{2+}) were removed from the B5 medium since they can broaden the NMR signals (Shachar-Hill et al., 1996). Medium reservoir contained 100 ml of perfusion medium (90 ml B5-F3 medium and 10 ml D_2O (CDN, Montréal, Québec, Canada)), which were stirred and oxygenated.

The small-scale bioreactor used in this study was modified from a system described in previous work (Gmati et al., 2004) (Fig. 4.1). Briefly, the design illustrated in Fig. 5.1 enables sterile culture conditions under medium perfusion. A gas mix was bubbled in a humidifier that contains sterile water at 10% deuterated water (D_2O). Humidified gas was then bubbled into the culture medium reservoir (250 mL Erlenmayer flask). This design with a humidifier avoids water losses from evaporation and thus changes in the concentrations of the medium constituents. The gas mix was bubbled into the liquid B5-F3 medium and 10% D_2O through a porous (2 μm) stainless steel sparger, which generated fine bubbles at a rate of 100 ml/min (1 vvm). Air filters (bacterial air vent: Pall-Gelman, VWR Canlab) ensured sterility and a water condenser minimized water losses by evaporation throughout the cultures' duration. The oxygenated medium was then pumped by a peristaltic pump (Masterflex peristaltic pump, Labcor, Montréal, Québec, Canada, Cat. #77961-00) at a constant flow rate of 1.60 $mL.min^{-1}$ into the small-scale bioreactor. The medium then passes through the cell bed and was pumped at the same rate out of the bioreactor. Liquid outlet then flew into an in-house 316 SS chamber (Fig. 5.2) where dissolved oxygen and medium pH were monitored. The liquid

medium was then returned to the medium reservoir or connected to a waste bottle. The cell suspensions were sampled from a flask using a 10 mL sterile plastic cutted-end pipette and rapidly inoculated into a sterile small-scale bioreactor. Cell bed was obtained by medium perfusion. The first 10 mL medium that flow out of the bioreactor were systematically collected as waste in order to wash the cell bed from paramagnetic ions. Culture medium and bioreactors were steam sterilized separately for 35 min (121°C, 1 bar).

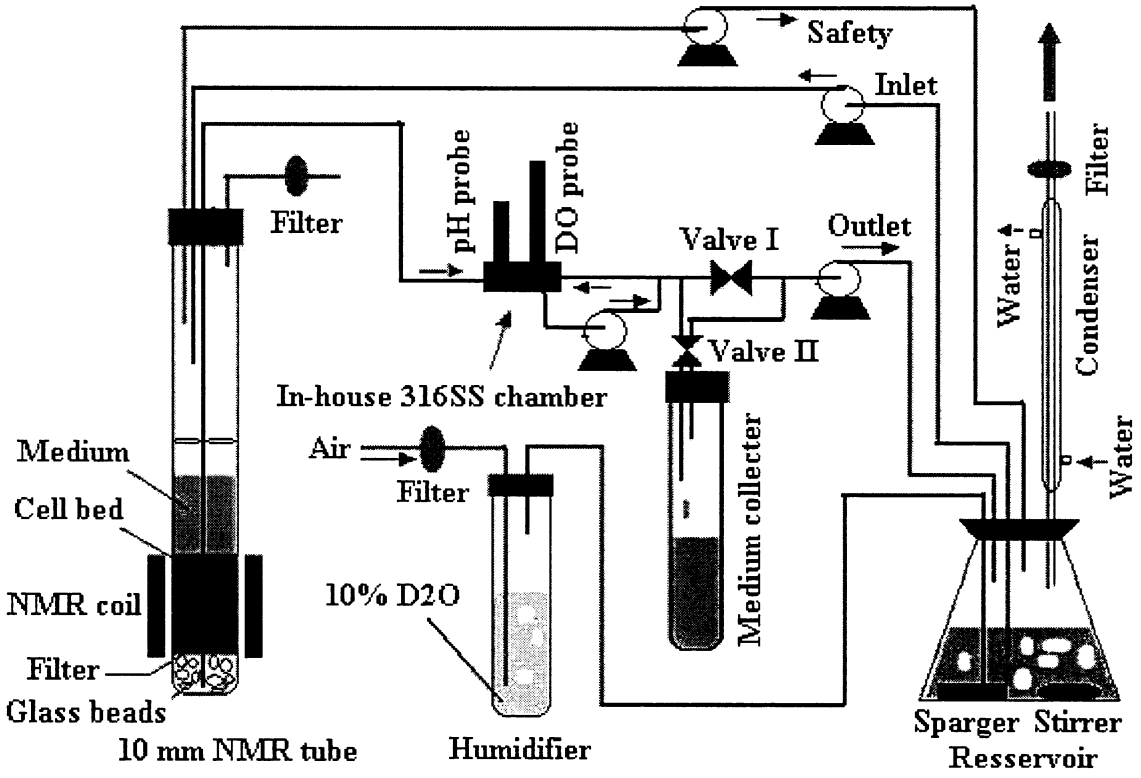


Figure 5.1. The small-scale bioreactor used for *in vivo* NMR experiments.

(See Material and methods section for a detailed description of the bioreactor system.)

5.2.3.3 NMR spectroscopy

In vivo ^{31}P NMR spectroscopy was performed on a Varian Unity Inova 400 MHz NMR spectrometer with a 10-mm Broadband probe. ^{31}P spectra were acquired at 161.839 MHz, with a time delay of 0.5 sec and 45° pulses at temperature of 298.15 K. The acquisition time was set to 0.69 sec and spectra width was 20 kHz. The spectra were acquired with 3000 scans corresponding to a final acquisition time of 60 min, unless otherwise specified. Proton decoupling was continually applied by WALTZ-16 pulse sequence. NMR data were transferred to a personal computer and processed by Mestrec 301 NMR software. The spectra were phased, base line corrected with the line broad of 25 Hz.

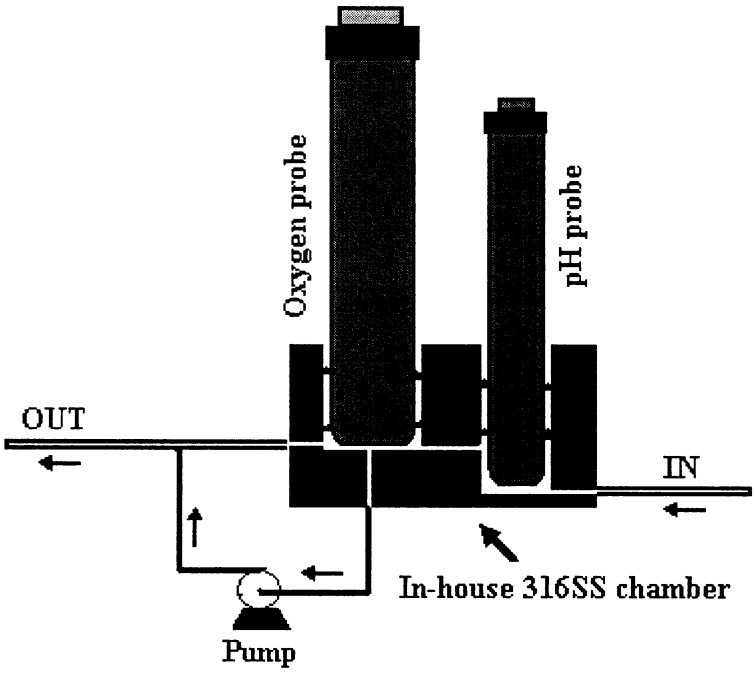


Figure 5.2: The chamber for oxygen and pH on-line measurement.

5.2.3.4 Phosphate quantification

Methanephosphonic acid was used as phosphorus concentration and chemical shift reference (30.60 ppm down shift from 85% orthophosphonic acid (Fisher, Montreal, Canada)). 2 mol L^{-1} Methanephosphonic acid was sealed in a capillary (I.D=0.199 mm, O.D=0.66 mm) (fisher, Montreal, Canada) and put inside the small-scale bioreactor outlet tube (I.D=0.80 mm), which was kept at the center of NMR tube. The reference tube was kept at the NMR coil level. T1 and T2 were analyzed by inverted gate pulse sequence. Saturation factor was analyzed as described by Rijhwani *et al.* (1999). Briefly, the saturation factor was detected in the presence of the reference and under normal cell bed perfusion. ^{31}P *in vivo* NMR spectra were acquired at 161.839 MHz, 298.15K with delay time of 0.5 sec and 21 sec for saturation and fully relaxed spectra, respectively. 45° pulses sequence was applied and the acquisition time was set to 0.69 sec. Spectra width was set at 20 kHz. The spectra were acquired with 2000 scans corresponding to the total acquisition time of 40 min and 12 h for saturated and fully relaxed acquisitions, respectively. The saturated acquisitions were recorded just before and after the fully relaxed acquisition. The saturation factor was calculated as below.

$$\text{saturation factor} = \frac{\text{average of saturated signal intensities}}{\text{relaxed signal intensity}}$$

The concentration in phosphorus is evaluated by comparing the peak area to the reference and calibrated by saturation factor. By this way, the concentration with the unit of millimole phosphorus per liter of cell bed (mmol Pi L^{-1} cell bed) was converted

to millimole phosphorus per gram of dry weight (mmol g^{-1} DW) by cell bed density, which was calculated from the cell bed dry weight and volume. Dry weight is obtained by pumping cell bed out, washing with deionized water twice, drying for 24 h at 65°C and weighting; while the volume of cell bed is obtained from the cell bed length and the inside diameter of NMR tube. The average cell bed density was 30.0 gDW L^{-1}.

^{31}P NMR signals were proportional to the concentration of phosphorus in fully-relaxed spectra, whose delay time should be set to 5 times greater of T1 (Xia *et al.,* 2001). Also, NMR is a relatively insensitive technology. It is often necessary to accumulate individual spectra over extended time periods. Recording a good spectrum for the purpose of quantification can then take a long time. Saturation factor was applied to reduce the total acquisition time. The reference was calibrated by measuring serial of phosphate standards by NMR (Fig. 5. 3). NMR parameters were set to 640 scans with 0.5 sec delay, which corresponded to a total acquisition time of 13 min. The linear range was from 0 to 8 mmol L^{-1} for quantification of phosphorus by comparing the peak area to that of reference and calibrating by saturation factor. Normally, intracellular phosphate of plant cell is within this range.

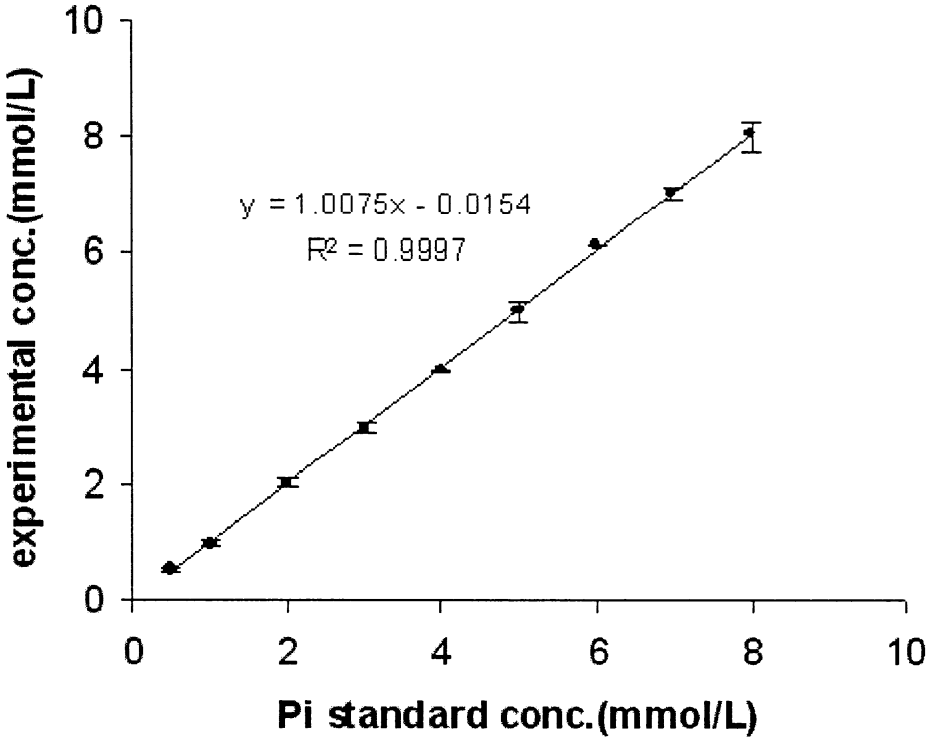


Figure 5.3. Phosphate reference calibration.

(Phosphate reference was calibrated by serial of phosphate standard solutions (0.5, 1.0, 2.0, 3.0, 5.0, 6.0, 7.0, 8.0mM KH_2PO_4) which contained 10% D_2O for locking NMR signal. The standard solutions were introduced into NMR tube with the phosphate reference capillary at the center of NMR tube. Samples were analyzed from low concentration to high concentration. NMR was set to 640 scans with delay time of 0.5s, correspond to total acquisition time is 13 min. Other NMR parameters are the same as material and methods. Error bar is standard deviation with n=3.)

5.2.3.5 Intracellular pH measurements

Intracellular pH was evaluated by the inorganic phosphate chemical shifts according to the relationship between chemical shift and pH established by Gmati et al. (2004).

5.2.3.6 Phosphate uptake study

Phosphate uptake experiment was performed with 5-d-old cells sampled from flask culture. Cell bed was obtained by perfusing 90 ml B5-F3 and 10 ml D_2O at the flow rate of 1.6 mL min^{-1}. After 1 h perfusion, the small-scale bioreactor was inserted into NMR probe to record a control spectrum. Then, the perfusion medium was changed to 90 ml B5-F1 medium and 10ml D_2O, which contains 1.0 $mmol.L^{-1}$ phosphate. NMR spectra were recorded at each hour to follow phosphate uptake. NMR parameters were set as described in NMR spectroscopy. This experiment was performed in duplicate.

5.2.3.7 Phosphate pulse study

Cells and perfusion bioreactor were prepared as described for the phosphate uptake study. After 1 h perfusion of 90ml B5-F3 medium and 10 ml D_2O, the first control spectrum was recorded. Then, the perfusion medium was changed to 90ml B5-F2 medium and 10ml D_2O, which contains 0.25 $mmol.L^{-1}$ phosphate. Two NMR spectra were then recorded (each 60 min accumulation) during 2 h phosphate pulse. Then B5-F2 perfusion medium was changed back to B5-F3 medium. NMR data recording was continued at every hour for 4 hours to follow phosphate translocation. NMR parameters were same as described in NMR spectroscopy.

5.2.3.8 Study of phosphate metabolism in flask and bioreactor cultures

Cells samples were taken from the shake flask culture and the 3.0 L bioreactor each day and were inoculated into the small-scale bioreactor that perfused by 90 ml B5-F3 medium and D_2O at the flow rate of 1.6 mL min^{-1}. NMR spectra were recorded after 1 h perfusion. NMR parameters are set as described in NMR spectroscopy. Experiment was performed in duplicate.

5.2.3.9 Study of oxygen stress

The study of oxygen stress was performed by inoculating 1-d-old cells flask culture into small-scale bioreactor with 100 ml perfusion medium of B5-F3 and D_2O (90:10 v/v) at the flow rate of 2.0 mL min^{-1}. The vent tube of the small-scale bioreactor was connected to the medium reservoir in order to equilibrate the head gas in the bioreactor and the medium reservoir. Perfusion medium was oxygenated by bubbling air or an O_2-N_2 gas mix. NMR parameters were set to 500 scans with 0.5 sec delay, which corresponded to total acquisition time of 10 min. Other parameters are the same as described previously in the section of NMR spectroscopy.

5.2.4 RESULTS AND DISCUSSION

5.2.4.1 Intracellular pH and *in vivo* ^{31}P NMR signals

In vivo ^{31}P NMR spectra were obtained for 1-d-old cells taken from flask culture (Fig. 5.4) after 1 h perfusion of oxygenated medium. Intracellular pH, both cytoplasmic pH (pH_c) and vacuolar pH (pH_v) were evaluated at 7.54 and 5.60, respectively, from a relationship between chemical shifts and pHs established previously (Gmati et al., 2004). The peaks were assigned according to literature (Shachar-Hill *et al.,* 1996) with 2 mM methanephosphoric acid as chemical shift reference at 30.65 ppm, Glc-6P at 4.90 ppm and F-6P at 3.90 ppm (grouped as phosphomonoesters or PME), cytoplasmic Pi (Pi_c) at 2.78 ppm, vacuolar Pi (Pi_v) at 0.78 ppm, ADP_β+ATP_γ at -4.96 ppm, ATP_α+ADP_α at -10.14 ppm, NADP+NDPG at -10.67 ppm, NDPG at -12.26 ppm and ATP_β at -18.66 ppm.

5.2.4.2 Cell sampling and transfer induce a stress that is visible to NMR

Experiments were first conducted looking at cells immediately after their sampling from shake flask culture. Indeed, previous work showed that *E. californica* suspension cells cultured in shake flasks are highly stressed when transferred into the small-scale bioreactor (Gmati et al., 2004). A sudden increase in O_2 consumption rate was observed after transfer and the cells required a minimum of 30 min to recover from sampling and inoculation stresses. Shake flask cultures at high cell density showed to be O_2 limited from day 3 of a 10 days culture (unpublished results). However, even for cells from

2-d-old shake flask culture, which are still in non-limiting O_2 conditions, there is evidence for a stress behavior as shown in Fig. 5.5. Cells pH_c first decreased then increased and stabilized for 60 min, while pH_v has slightly increased. Dissolved oxygen is at ~50% air saturation in shake flasks at day 2 (unpublished results). When these cells are inoculated into the small-scale bioreactor, the dissolve oxygen increases suddenly to 100% air saturation and then decreased to stabilize around 60% in 1 h (Gmati *et al.*, 2004). These results thus suggest perfusing oxygenated medium for 1 h before performing *in vivo* NMR measurements.

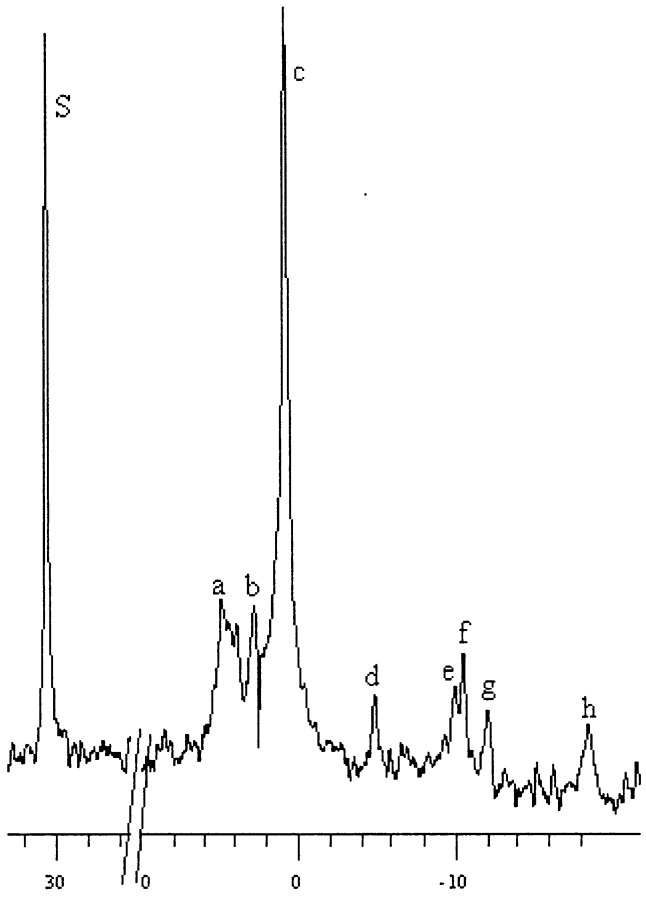


Figure 5.4: *In vivo* ^{31}P NMR spectrum of first day *Eschscholtzia californica* cell.

(NMR parameters were same as described in Material and methods. 1-d-old *Eschscholtzia californica* cell suspension was inoculated into small-scale bioreactor with perfusion medium of 90 ml B5-F3 medium and 10 ml D_2O at flow rate of 1.60 mL min^{-1}. NMR parameters were set as described in NMR spectroscopy. Spectrum was recorded after 1 h perfusion. NMR signals were assigned as S: Reference, a: PME, b: Pi_c, c: Pi_v, d: ADP_β+ATP_γ, e: ATP_α+ADP_α, f: NADP+NDPG, g: NDPG, h: ATP_β.)

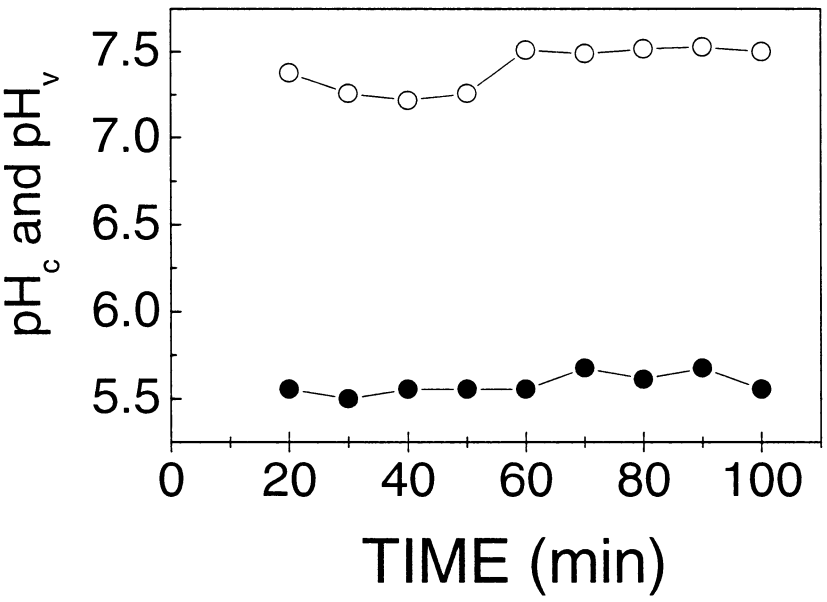


Figure 5.5: The effect of cell sampling and transfer on intracellular pH.

(The ^{31}P NMR spectra were recorded in every 10 min with acquisition of 500 scan and 0.5 s delay. Other NMR parameters are same as described in NMR spectroscopy. Time "0" corresponds to cells transfer into the small-scale bioreactor. ●: vacuolar pH; ○: cytoplasmic pH.)

5.2.4.3 *Eschscholtzia californica* cells are very sensitive to low oxygen stress

Adaptation of plant cells to low oxygen stress was first observed in 1988 (Saglio *et al.*, 1988) and it was found that maize root tips were much more tolerant to anoxia when whole seedlings were first exposed to hypoxia (3-4% O_2). The acidification of cytoplasm following anoxia is a common phenomenon observed in most organisms, including plants (Kennedy *et al.*, 1992). Roberts (1992) reported a pH_c decrease in 10 min following anoxic conditions.

An experiment was thus performed to assess the capacity of the *in vivo* NMR system to monitor effect of low O_2 stress on cell physiology (Fig 5. 6). *E. californica* cells showed a high sensitivity to a low oxygen stress. Cell behaviour was followed changing from initial normoxic conditions (0-30 min) to O_2 limiting conditions (30-110 min) then changed back to initial normoxic conditions (110-140 min). When air feed was changed for a N_2-O_2 gas mix, dissolved oxygen concentration (DO) decreased from 60% to 25% air saturation at the outlet of the small-scale bioreactor. There was a 10 min delay in DO reduction at the outlet of the small-scale bioreactor. This delay corresponds to the time for the medium reservoir to reach equilibrium with N_2-O_2 mix in addition to the time for the medium to flow from the flask and passes through the cell bed. pH_c showed to respond in parallel to DO and decreased form 7.50 to 6.80, and pH_v increased from 5.55 to 5.70. External pH (pH_e) increased of only 0.10 pH unit after 20 min at low O_2 conditions. When N_2-O_2 gas mix was switched back to air only, pH_c and pH_e

recovered at their initial values. Gout (2001) and coworkers reported pH_c decreased from 7.5 to 6.8 while vacuolar pH_v (5.7) and external pH (6.5) remained stable when changing from normoxic to hypoxic culture conditions. Returning back to normoxic culture conditions, pH_c recovered at its initial value (7.5) within 2-3 min, whereas pH_e decreased abruptly. It has been shown that *E. californica* cells sense O_2 limitation at DO below 50% saturation (Lamboursain *et al.,* 2002). At 25% saturation the specific oxygen uptake rate (qO_2) is still at ~90% of $qO_{2,max}$. Similar results for intracellular pH were reported in literature for different plant species. Under anoxic conditions, pH_c has acidified by 0.2 pH unit in rice coleoptiles (Fan *et al.,* 1992), by 0.5-0.6 pH unit in maize root tips (Fox *et al.,* 1995) and by 0.7 pH unit in pea internodes (Summers *et al.,* 2000).

Under O_2 limiting conditions the concentration in PME such as glucose-6-P and fructose-6-P first decreased for ~20 min, stabilized for 20 min and then start to increase ~40 min before the return of normoxic conditions. The first decrease in PME coincided with an increase in ATP concentration level, and the final increase in PME coincided with a second increase in ATP. This result suggests that the cell metabolic network regulation may first pull on PME to generate ATP and then increase glucose uptake to increase fluxes of glycolysis. A sudden increase of glucose uptake results in a net decrease of ATP. Glucose uptake is being performed by proton co-transport, which activates ATPase proton pumps for pH_c control (Brandao et al., 1992; Smith, 2002). Glycolysis is tightly controlled. It was observed that Pi_c only fluctuates slightly and it is thought that intracellular free Pi variations will be buffered by the vacuole, the cell

main free Pi reservoir. This may explain the large variations observed for Pi_v. The return to normoxic conditions resulted in a net decrease in ATP as well as in both Pi_c and Pi_v. Cell reaction to an increase in available O_2 has to be further investigated.

From this work it is clear that the small-scale bioreactor can be an efficient tool to monitor cell physiological state *in vivo*. In this work, 10 min scan accumulation allowed to follow highly transient processes. The signal of ATP was the weakest and may explain partly the fluctuations observed. More acquisition time is thus required allowing to distinguish ATP changes from normal oscillations (Tucker et al., 2004).

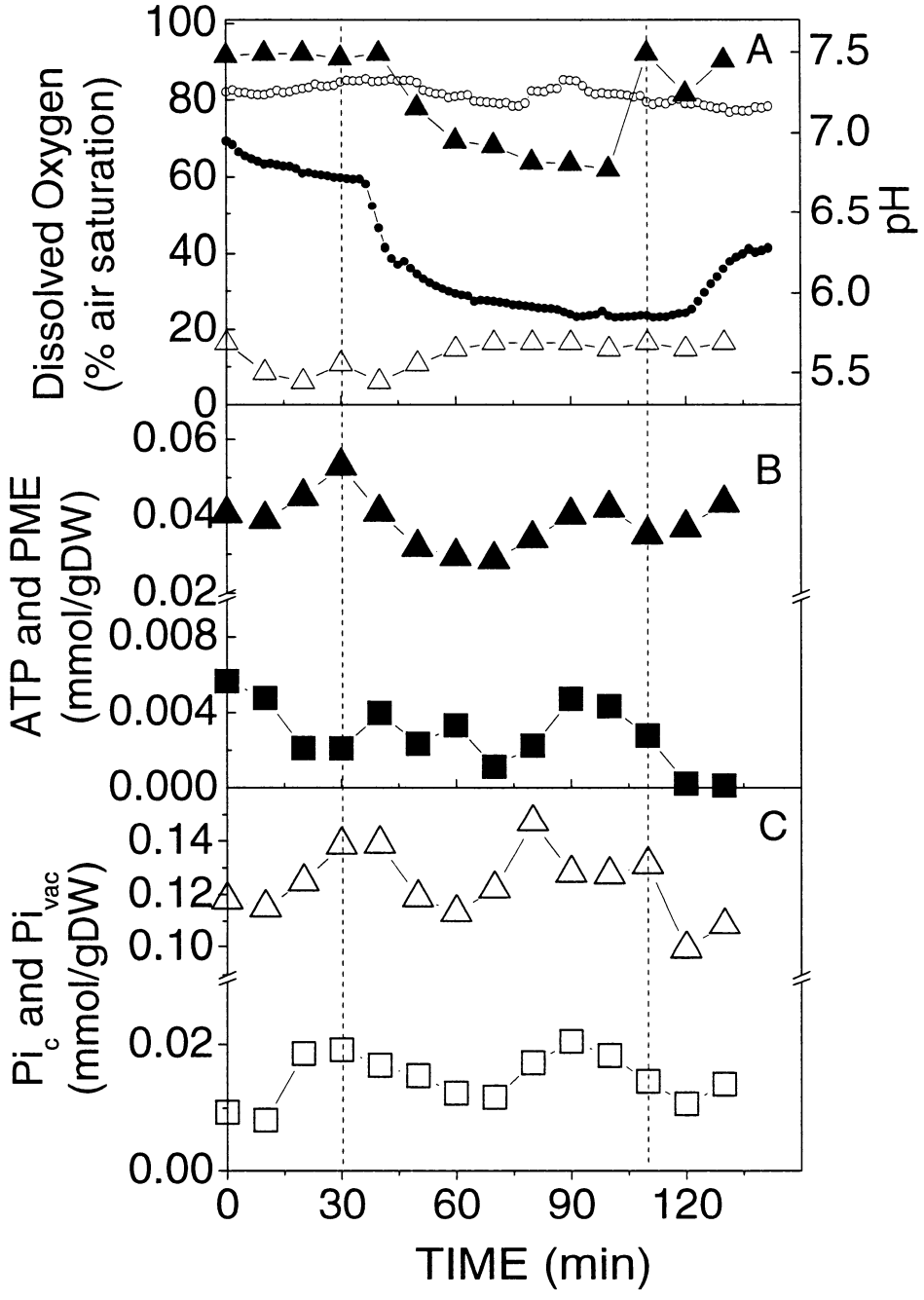


Figure 5.6: The effect of oxygen stress on intracellular pH, external pH and NMR detectable phosphorous compounds.

(The ^{31}P NMR spectra were recorded in every 10 min with acquisition of 500 scan and 0.5 s delay. Other NMR parameters are same as described in NMR spectroscopy. Air feed was replaced with N_2-O_2 mixed gas at 30 min. Air was feed again at 110min. Time "0" corresponds to 30 min after cells transfer into the small-scale bioreactor. (a) ●: Dissolved oxygen; ○: external pH; ▲: cytoplasmic pH; △: vacuolar pH. (b), ▲: PME; ■: ATP. (c) △: Vacuolar phosphate; □: Cytoplasmic phosphate)

5.2.4.4 Cells physiological state differs for shake flasks and bioreactor cultures

In vivo ^{31}P NMR was applied to follow phosphate pools in both shake flask and bioreactor culture (Fig.5.7). Since the inoculation process involves 10-d-old cells that are limited in many nutrients including O_2, Pi uptake showed to be highly dynamic. Pi_c and PME reached their maximum values after only 9 h and showed to be stable within the first 3 days in both cultures. Pi_c then decreased rapidly until day 5 and then slowly until the end of the cultures. The trend for Pi_c with time was similar for both shake flask and bioreactor cultures and seemed to follow cell growth. PME decreased regularly from day 3 to day 7 and then was stable in shake flasks but increased in bioreactor. Pi_v increased at maximum at 9 h (bioreactor) to 1 d (flask) and then decreased close to zero at day 3 for the bioreactor and at day 5 for the flask. Dilution of total intracellular available Pi pools from cell doubling as well as transformation into NMR invisible

pools may explain this decrease of Pi_v (Brodelius *et al.,* 1985). ATP followed a similar trend than Pi_c until day 5 for the bioreactor, stayed stable until day 9 then increased at day 10 to return at a lower value at day 11. In the shake flasks ATP was surprisingly low for the first 3 days and then increased at day 4 to decrease until day 6 to initial level.

Cells in flask and bioreactor reached similar maximum Pi_v concentration. However, Pi_v for the cells in bioreactor decreased 1 to 2 days faster suggesting a higher rate of internal Pi utilisation in the bioreactor. This result suggests a higher metabolic rate for the cells in the bioreactor than that cultured in the shake flask, which can be explained from O_2 limitation in flasks as previously discussed. In both cultures, PME decreased and reached a minimum value at day 7. The cells cultured in the bioreactor showed lower PME concentration than flask culture for the first three days, while ATP is higher than flask culture. This may suggest that ATP production has been reduced under O_2 limitation in shake flask culture. Both Pi_v and PME showed to increase at the end of bioreactor culture. It is thought that the cells may have access to new phosphate sources other than from medium. Since the cells grew faster in the bioreactor than in shake flasks, phosphate may have been released from dead cells and was then uptaken by viable cells. It is also interesting that the ATP level increase at day 10 coincided with this new Pi availability. Cell culture in flask with slower growth did not show this phenomenon.

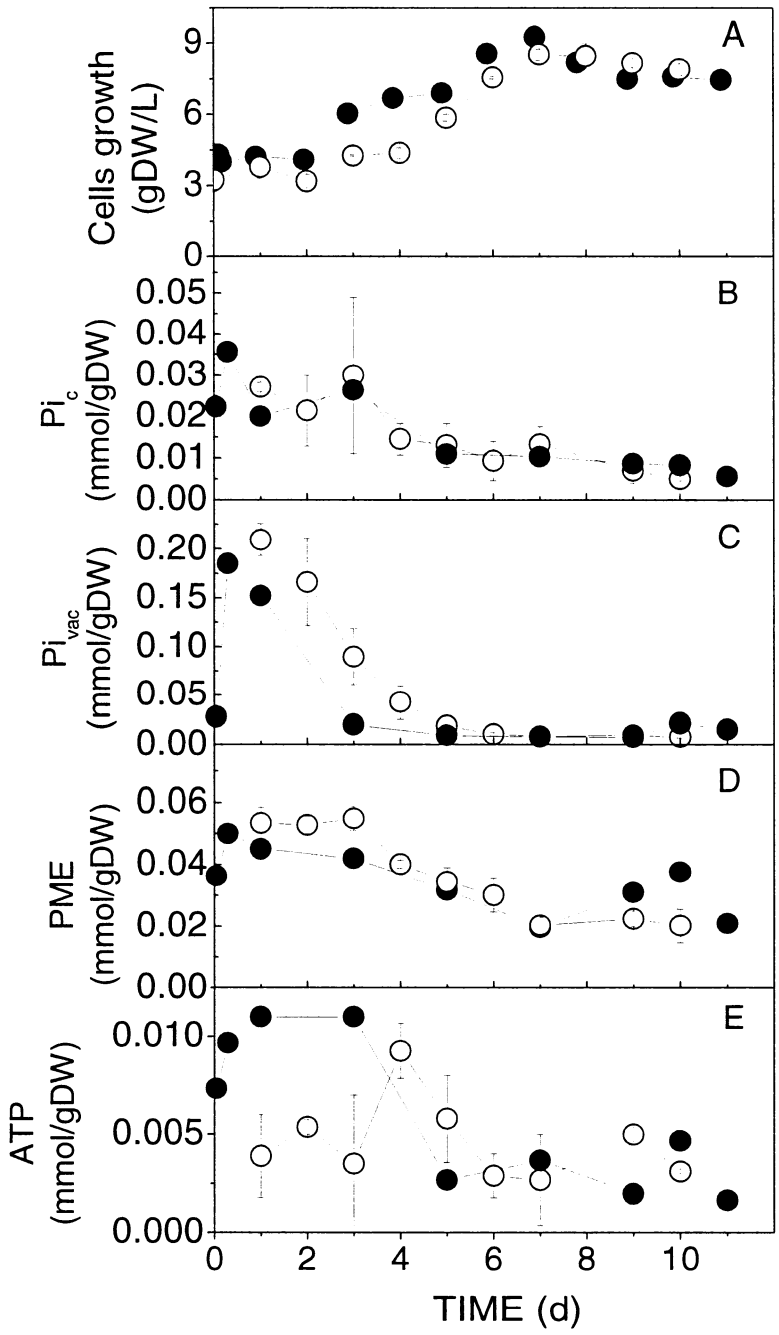


Figure 5.7: Time courses of cell growth and NMR detectable phosphorous compounds in flask and bioreactor cultures.

(○: Flask culture; ●: Bioreactor culture. Time "0" corresponds to 60 min after cells transfer into the small-scale bioreactor. NMR parameters are set as described in NMR spectroscopy. Spectra were obtained for cells samples from the 3-L bioreactor at 1 h and 7 h and then daily. Error bar is standard error with n=2.)

5.2.4.5 Phosphate uptake, storage and translocation can be monitored by *in vivo* ^{31}P-NMR

Since medium phosphate showed to be completely uptaken within the first day in *in vitro* cell culture (Fig. 5.7), two *in vivo* ^{31}P NMR experiments were performed to characterize early cells physiological events occurring in phosphate limiting and non-limiting conditions. The experiments were performed with 5-d-old cells taken from shake flask and transferred into the small-scale bioreactor. These cells were limited in O_2 in flask culture with low Pi_v level (Fig. 5.7).

A cell sample was submitted to a 2 h long pulse with 90ml B5-F2 medium and 10 ml D_2O supplemented at 0.25 mM Pi (Fig. 5.9). After this 2 h Pi pulse perfusion medium was changed to 90 ml B5-F3 medium and 10 ml D_2O, which is Pi-free. Pi_c, Pi_v and PME increased during the 2 h Pi pulse. When changing for Pi-free perfusion medium PME and Pi_c decreased, while Pi_v has continually increased. During Pi uptake ATP has decreased significantly and then increased at initial level after 2.5 h under Pi-free medium (or 4.5 h total). Phosphate uptake, storage and translocation are ATP coupled processes (Plaxton, 1999). The cytoplasm acidified of 0.7 pH unit during this highly

dynamic process of Pi uptake, while pH_v increased of 0.7 unit. After the Pi pulse, pH_v decreased and pH_c was maintained at low value. Co-transport of medium Pi and protons into the cytoplasm will cause a rapid decrease of pH_c and a massive use of ATP to power the H^+-pumps (Massonneau *et al.,* 2000).

A second cell sample was perfused with B5-F1 medium and 10% D_2O supplemented at 1.0 mM Pi (Fig. 5.9). PME increased rapidly while Pi_v increased with a 1 h delay. Pi_c reached a maximal value of 0.059 $mmol \cdot g^{-1}DW$ in less than 2.5 hours. Pi_v increased to a maximum of 0.33 $mmol \cdot g^{-1}DW$ in 8.5 hours, which value was close to 0.23 $mmol \cdot g^{-1}DW$ reported for *Daucus carota* hairy roots (Jolicoeur et al., 2002). Maximum storage capacity of ~0.6 $mmol \cdot g^{-1}DW$ (estimated from 31 $\mu mol\ g^{-1}FW$ of Sakano et al., 1995) was also observed to occur within 2 hours for *Catharanthus roseus* cells. A phosphate uptake rate of 1.40 $\mu mol \cdot min^{-1} \cdot g^{-1}DW$ was calculated from total NMR visible Pi pools (the sum of PME, Pi_c, Pi_v, ATP, ADP, NADP and UDPG). Pi transfer into NMR invisible compounds may introduce a bias to the data but, however, the observed value is similar to 1.50 $\mu mol \cdot min^{-1} \cdot g^{-1}DW$ reported for *Catharanthus roseus* cell suspension (Brodelius *et al.,* 1985). Phosphate uptake rates of 0.40 $\mu mol \cdot min^{-1} \cdot g^{-1}DW$ (Lee et al., 1990) to 2.63 $\mu mol \cdot min^{-1} \cdot g^{-1}DW$ (estimated from 0.33 $\mu mol\ min^{-1}\ g^{-1}FW$ of Sakano et al., 1995) were also reported for *Catharanthus roseus* cells and *Zea mays*, respectively. A wide range is observed in literature between plant species but also within different cell lines. An example of this variability becomes obvious when collecting maximal low affinity Pi uptake rate ($v_{max,II}$) presented in literature. Values of 0.088 $\mu mol \cdot min^{-1} \cdot g^{-}$

^{1}DW (Furihata et al., 1992), 0.288 μmol·min^{-1}·g^{-1}DW (Schmidt et al., 1992) and 2.63 μmol·min^{-1}·g^{-1}DW (Sakano et al., 1995) were reported for *Catharanthus roseus*, from 0.0256 to 1.36 μmol·min^{-1}·g^{-1}DW for *Nicotiana tabacum* (Shimogawara and Usuda, 1995), 0.096 μmol·min^{-1}·g^{-1}DW (Smith, 1966) for *Zea maize* and 0.0021 μmol·min^{-1}·g^{-1}DW (Jolicoeur et al. 2002) for *Daucus carota*.

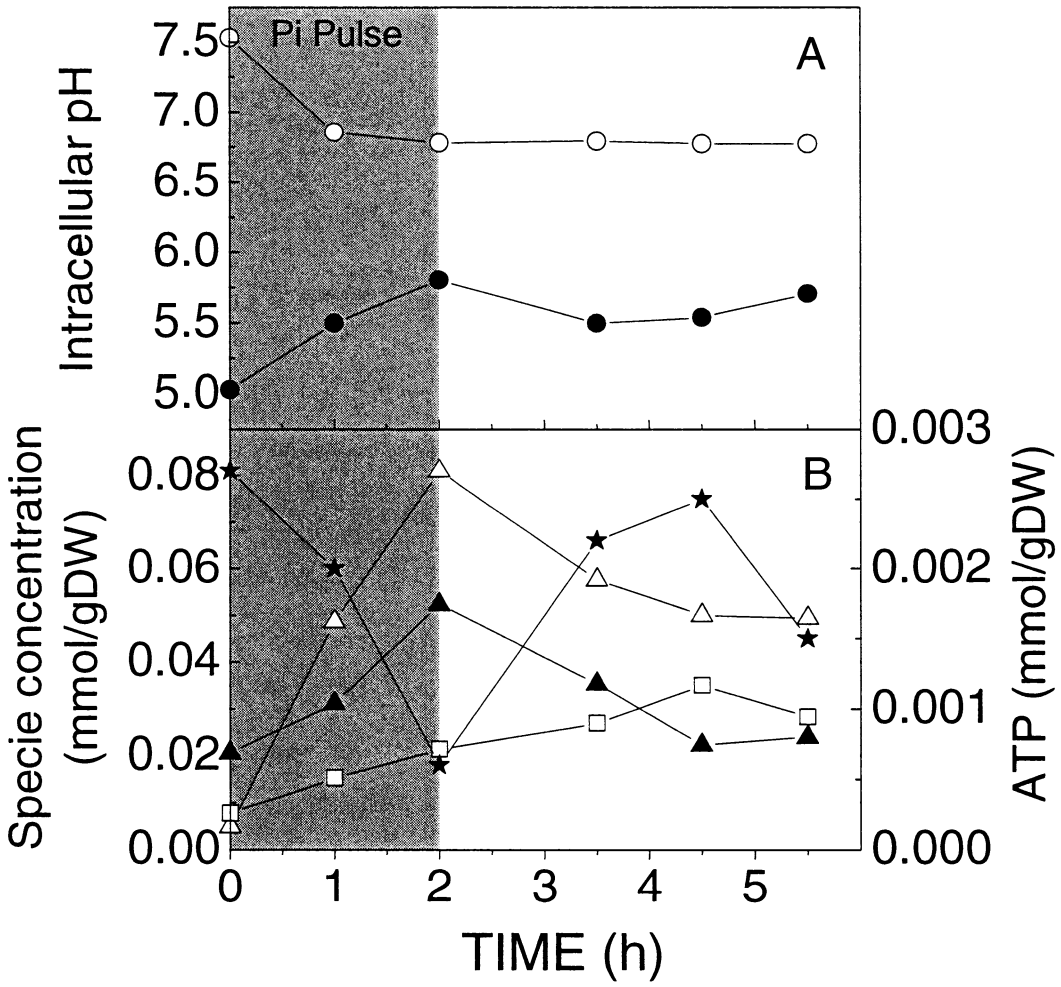


Figure 5.8: The responses of intracellular pH and NMR detectable phosphorous compounds to a phosphate pulse for 5-d-old cells cultured in shake flask.

(○: pH_c; ● : pH_v; △: PME; ▲: Pi_c; □: Pi_v; ★: ATP. NMR parameters are set as described in NMR spectroscopy. Time "0" corresponds to 30 min after cells transfer into the small-scale bioreactor.)

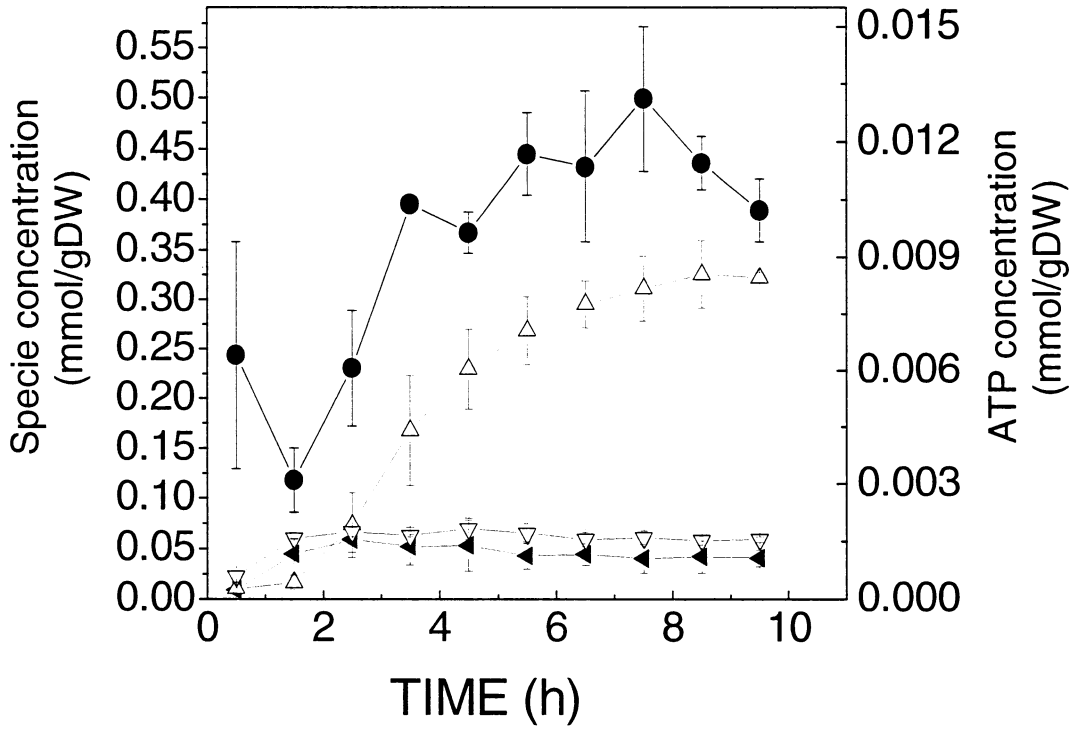


Figure 5.9: ^{31}P NMR detectable compounds during phosphate uptake for 5-d-old cells cultured in shake flask.

(△: Vacuolar Pi; ▽: PME; ▲: Cytoplasmic Pi; ●: ATP. NMR parameters are set as described in NMR spectroscopy. NMR data recording started 60 min after cells transfer into the small-scale bioreactor. The error bar is standard error with n=2.)

5.2.5 CONCLUSION

The small-scale bioreactor showed to be useful for the study of plant cell physiology under diverse stresses and culture conditions. *Eschscholtzia californica* cells showed to be very sensitive to low oxygen stress. Even for dissolved oxygen change from 60% to 25% air saturation cytoplasm was acidified of 0.7 pH unit. NMR detectable compounds such as Pi_c, Pi_v, PME and ATP have also responded to an oxygen stress. Moreover, cells showed to sense a significant stress when transferred from shake flask culture (oxygen-limited system) to the small-scale bioreactor (with perfusion medium at 100% air saturation). The different oxygen conditions in shake flask and bioreactor cultures resulted in different cell physiological states. The small-scale bioreactor has enabled to study transient phenomenon with 10 min spectra accumulation but adequate signal-to-noise ratio for ATP requires 60 min spectra accumulation. The next step will be to use this system to monitor the effect of specific perturbations on viable plant cells. The system will also be adapted to culture mammalian cells.

5.2.6 ACKNOWLEDGMENT

Authors would like to thank Mathieu Cloutier for technical help with the bioreactor preparation. This research project was funded by The Natural Sciences and Engineering Research Council of Canada (NSERC), the Canadian Foundation for Innovation (CFI), the Canada Research Chair Program and the "Fonds Québécois pour la Recherché en Nature et Technologies" (FQRNT).

5.2.7 REFERENCES

Aubert S, Curien G, Bligny R, Gout E, Douce R. 1998. Transport, compartmentation, and metabolism of homoserine in higher plant cells. Carbon-13- and Phosphorus-31-Nuclear magnetic resonance studies. *Plant Physiology* **116:** 547–557

Bligny R, Foray MF, Roby C, Douce R. 1989. Transport and phosphorylation of choline in higher plant cells. *The Journal of Biological Chemistry* **264:** 4888-4895.

Brandao RL, Castro IM, Passos JB, Nicoli JR, Thevelein JM. 1992. Glucose-induced activation of the plasma membrane H+-ATPase in Fusarium oxysporum. *J General Microbiol* **138:** 1579-1586.

Brodeliust P and Vogel HJ. 1985. A phosphorus-31 nuclear magnetic resonance study of phosphate uptake and storgage in cultured Catharanthus Roseus and Daucus carota plant cells. *The Journal of Biological Chemistry.* **260,** 2556-3560.

Brown TR, Ugurbil K, Shulman RG. 1977. Phosphorus-31 nuclear magnetic resonance measurements of ATPase kinetics in aerobic Escherichia coli cells. *Natl. Acad. Sci. USA* **74** (12): 5551-5553.

Fan TWM, Lane AN, Higashi RM. 1992. Hypoxia does not affect rate of ATP synthesis and energy-metabolism in rice shoot tips as measured by ^{31}P-NMR in vivo. *Archives of Biochemistry and Biophysics* **294,** 314-318

Fox GG, Ratcliff RG, Southon TE. 1989. Airlift systems for in vivo NMR spectroscopy of plant tissues. *Journal of Magnetic Resonance* **82:** 360

Fox GG, McCallan NR, Ratcliff RG. 1995. Manipulating cytoplasmic pH under anoxia: a critical test of the role of pH in the switch from aerobic to anaerobic metabolism. *Planta* **195,** 324-330

Furihata T, Suzuki M, Sakurai H. 1992. Kinetic characterization of two phosphate uptake systems with the different affinities in suspension-cultured Catharanthus roseus protoplasts. *Plant Cell Physiology* **33,** 1151-1157

Gerendás J and Ratcliffe RG. 2000. Intracellular pH regulation in maize root tips exposed to ammonium at high external pH. *Journal of Experimental Botany.* **51,** 207-219

Gerlitz TGM and Gerlitz A. 1997. Phosphate uptake and polyphosphate metabolism of mycorrhizal and nonmycorrhizal rots of pine and of suillus bovinux ant varying external pH measured by in vivo 31P-NMR. *Mycorrhiza.* **7,** 101-106

Gillies RJ, Ugurbil K, Hollander JA Shulman RG. 1981, ^{31}P NMR studies of intracellular pH and phosphate metabolism during cell division cycle of saccharomyces cerevisiae, *Natl. Acad. Sci. USA* **78**: 2125-2129

Gmati D, Chen J and Jolicoeur M. 2004. Development of a High-throughput Bioreactor: Application to In Vivo NMR Measurement. *Biotechnology and Bioengineering, i*n press.

Gout E, Boisson AM, Aubert S, Douce R, Bligny R. 2001. Origin of the cytoplasmic pH changes during anaerobic stress in higher plant cells. Carbon-13 and phosphorous-31 nuclear magnetic resonance studies. *Plant Physiology.* **125,** 912-925

Grivet JP et al. 2003. NMR and microbiology: from physiology to metabolomics. *Biochime*. **85,** 823-840

Gupta RK Benovic JL and Rose ZB. 1978. The determination of the free magnesium level in the human red blood cell by ^{31}P NMR. *The Journal of Biological Chemistry*. **253,** 6172-6176

Hesse SJA, Ruijter GJG, Dijkema C, Visser J. 2002, Interacellular pH homeostasis in the filamentour fungus Aspergillus nige,. *European Journal of Biochemistry*. 269: 3485-3494

Kennedy RA, Rumpho ME, Fox TC. 1992. Anaerobic metabolism in plants. *Plant Physiology* **100,** 1-6

Lamboursain L, St-Onge F, Jolicoeur M. 2002. A lab-built respirometer for plant and animal cell culture. *Biotechnology Progress*. **18,** 1377-1386

Lee RB, Ratcliffe RG, Southon TE (1990) ^{31}P NMR measurements of the cytoplasmic and vacuolar Pi content of mature maize roots: relationships with phosphorus status and phosphate fluxes. *Journal of Experimental Botany*. **41,** 1063-1078

Jolicoeur M, Chavarie C, Carreau PJ, Archambault J. 1992. Development of a helical-ribbon impeller bioreactor for high-density plant cell suspension culture. *Biotechnology and Bioengineering*. **39,** 511-521

Massonneau A, Martinoia E, Dietz KJ, Mimura T. 2000. Phosphate uptake across the tonoplast of intact vacuoles isolated from suspension-cultured cells of Catharanthus roseus (L.) G. Don. *Planta,* **211,** 390-395

Moon RB, Richards JH. 1973, Determination of intracellular pH by ^{31}P magnetic resonance, *The Journal of Biological Chemistry* **248:** 7276-7278

Plaxton W. 1999. Metabolic aspects of phosphate starvation in plants. In: Phosphorus in plant biology: Regulatory roles in molecular, cellular, organismic, and ecosystem processes. J.P. Lynch and J. Deikman (Editors), American Society of Plant Physiologists, Rockville, Maryland, Vol **19**, pp. 229-241.

Plaxton WC. 1998. Metabolic aspects of phosphate starvation in plants, In: Deikamn J, Lynch J, eds, Phosphorus in plant biology: Regulatory roles in molecular, cellular, organismic, and ecosystem processes, *American Society of Plant Physiologists,* Rockville, pp 164-176.

Plaxton W. 1996. The organization and regulation of plant glycolysis. Annu Rev Plant *Physiol Plant Mol Biol.* **47,**185-214.

Ranjeva F, Boudet AM. 1987. Phosphorylation of proteins in plants: regulatory effects and potential involvement in stimulus/response coupling. *Annual Review Plant Physiol Plant Mol Biol.* **38,** 73-93.

Rasmussen N, Lloyd DC, Ratcliffe RG, Hansen PE, Jakobsen I. 2000 ^{31}P NMR for the study of P metabolism and translocation in arbuscular mycorrhizal fungi. *Plant and Soil* **226:** 245-253

Ratcliffe R G and Shachar-Hill Y. 2001. Probing plant metabolism with NMR. *Annual Review of Plant Physiology and Plant Molecular Biology* **52:** 499-526

Rijhwani SK, Ho C-H, and Shanks JV. 1999. In vivo 31P and multilable 13C-NMR measurement for evaluation of plant metabolic pathways. *Metabolic Engineering* **1,** 12-25.

Roberts JKM, Hooks MA, Miaullis AP, Edwards S, Webster C. 1992. Contribution of malate and amino acid metabolism to cytoplasmic pH regulation in hypoxic maize root tips studied using nuclear magnetic resonance spectroscopy. *Plant Physiology* **98:** 480-487

Roby C, Martin JB, Bligny R, Douce R. 1987. Biochemical changes during sucrose deprivation in higher plant cells, *The Journal of Biological Chemistry* **262:** 5000-5007

Saglio PH, Drew MC, Pradet A. 1988 Metabolic acclimation to anoxia induced by low (2-4 kPa partial pressure) oxygen pretreatment (hypoxia) in root tips of Zea mays. *Plant Physiology* **86**: 61-66

Sakano K, Yazaki Y, Okihara K, Mimura T, Kiyota S. 1995. Lack of control in inorganic phosphate uptake by Catharanthus roseus (L.) G. Don cells. *Plant physiology.* **108,** 295-302

Shachar-Hill Y. 2002. Nuclear Magnetic Resonance and Plant Metabolic Engineering. *Metabolic Engineering* **4,** 90–97

Shachar-Hill Y and Pfeffer PE. 1996. Nuclear magnetic resonance in plant biology, volume **16**. *American Society of Plant Physiologists USA*

Shimogawara L, Usuda H. 1995. Uptake of inorganic phosphate by suspension-cultured tobacco cells: Kinetics and regulation by Pi starvation. *Plant Cell Physiology* **36,** 341-351

Smith FW. 2002. The phosphate uptake mechanism. *Plant and soil* **245,** 110-114

Spickett CM, Smirnoff N, Ratcliffe RG. 1993. An in vivo nuclear magnetic resonance investigation of ion transport in Maize (Zia mays) and spartina anglica roots during exposure to high salt concentrations. *Plant Physiology* **102:** 629-638

Summers JE Ratcliffe RG, Jackson MB. 2000. Anoxia tolerance in the aquatic monocot potamogeton pectinatus: absence of oxygen stimulates elongation in association with an unusually large Pasteur effect. *Journal of Experimental Botany.* **51,** 1413-1422

Tucker DE, Allen DJ, Ort DR. 2004. Control of nitrate reductase by circadian and diurnal rhythms in tomato. *Planta.* **219,** 277-85

Veeman WS. 1997 Nuclear magnetic resonance, a simple introduction to the principles and applications, *Geoderma* **80,** 225-242**;**

Watts EE, Dean PAW, Martin RR. 2002. 31P nuclear magnetic resonance study of sediment microbial phospholipids. *Canadian Journal of Analytical Sciences and Spectroscopy.* **47,** 127-133

Xia Z, Akim LG, Argyropoulos DS. 2001. Quantitative 13C-NMR Analysis of lignins with internal standards. *Journal of Agric. Food Chem.* 49, 3573-3578

CHAPTER 6: THE STUDIES OF ELICITATION BY ^{31}P-NMR

This work is preliminary and will need to be completed in a near future. We decided to show here the state of this study because we consider it has a high potential to result in original knowledge on cell behavior to elicitation.

6.1 INTRODUCTION

It is well know that *Eschscholtzia californica* cells can produce benzophenthridine alkaloids (sanguinarine, chelerythrine and macarpine), which are pharmaceutically interesting compounds. Elicitors induce biosynthesis of secondary metabolites as a defense against pathogenic organisms in plants. The use of cell suspension cultures in combination with elicitors offer excellent experimental model systems for studies on various elicitor-induced responses of plant tissues. Various elicitors were added to cell suspension culture of *Eschscholtzia californica* to enhance secondary metabolite accumulation, such as yeast extracts (Villegas *et al.,* 1999; Byun *et al.,* 1992; Roos *et al.,* 1998) and chitin (Williams et al., 1992). Radman *et al.* (2003) reviewed the elicitors and the mechanism of elicitation. Cytoplasm acidification and vacuole alkalinization were observed within a few minutes after elicitor contacted the cells (Roos *et al.,* 1998). *In vivo* NMR was applied to investigate the early events in cell responses to elicitation (Ratcliffe *et al.,* 1998; Pugin *et al.,* 1997). Cytoplasmic pH decreased while vacuolar pH increased in the first 30 min of elicitation, which confirmed the results of Roos (1998) from fluorescence technique. In addition, the elicitor has been proved to activate the pentose phosphate pathway, led to a decrease of

glucose 6-phosphate and the accumulation of glyceraldehyde 3-phosphate, 3- and 2-phosphoglyceric acid, and phosphoenolpyruvate. In this chapter, the cells early physiologic events were followed by *in vivo* ^{31}P-NMR spectroscopy with the small-scale bioreactor.

6.2 MATERIALS AND METHODS

6.2.1 Elicitor preparation

A 2% chitin extract in B5-F3 medium (10% D_2O) was prepared as described below. 2.0 g of chitin (Sigma, Oakville, Canada) and 10 ml B5-F3 medium with 10% D_2O were added in a mortar. Chitin was grinded by pestle for 10 min then another 10 ml of B5-F3 medium with 10% D_2O were added. Chitin was continuously grinded for another 10 min. After repeating once, the suspension was transferred into a 250 ml Erlenmeyer flask that contained a magnetic stirrer. The mortar and pestle were washed three times by B5 medium (10% D_2O) with the total volume of B5-F3 medium (10% D_2O) of 100 ml. Then, the flask was covered by double layer aluminum foil and put into autoclave for sterilization at temperature of 121°C for 25 min at liquid cycle. When cooling down, the suspension was continuously stirred on a magnet plate for over night. Then the extract was vacuum filtered, transferred into another clean flask with double layer aluminum, covered and sterilized in autoclave at temperature of 121°C for 25 min at liquid cycle. When it cooled down, the 2% chitin extract was kept for *in vivo* ^{31}P-NMR elicitation studies.

6.2.2 NMR spectroscopy

In vivo ^{31}P-NMR spectroscopy was performed on a Varian Unity Inova 400 MHz NMR spectrometer with a 10-mm Broadband probe as descried in the section of NMR spectroscopy in chapter 5. In the first hour of elicitation, NMR spectra were accumulated and recorded at every 500 scans, corresponding to an acquisition time of 10 min. 6 accumulative NMR spectra were recorded in the first hour of elicitation. Then, NMR spectroscopy was applied to follow the cells at each hour for 5 hours with 3000 scans, corresponding to an acquisition time of 1 h for each spectrum. Before elicitation, *in vivo* ^{31}P-NMR spectra were recorded as controls also with 3000 scans. The intracellular pH was evaluated by intracellular phosphate chemical shift. The concentrations of metabolites were obtained by comparing the areas of peaks to that of reference as described before.

6.2.3 The study of elicitation by *in vivo* ^{31}P-NMR

The study of elicitation was performed by *in vivo* ^{31}P-NMR spectroscopy with medium perfusion and phosphate reference. Cells sample was taken from 1-d-old and 3-d-old flask cultures and inoculated in the small-scale bioreactor as previously described. 80 ml B5-F3 medium and 8 ml D_2O were used as perfusion medium at a flow rate of 1.6 ml/min. After 1 hour perfusion to stabilize dissolved oxygen, two hours of medium perfusion and NMR data acquisition were performed to obtain the control spectrum.

Then 8 ml 2% chitin extract was introduced into the medium reservoir to obtain a concentration of 0.2% chitin extract.

6.3 RESULTS AND DISCUSSION

6.3.1 Cytoplasmic acidification

The effect of elicitation on intracellular pH is showed in Figure 6.1. Cytoplasm acidified in 10 min of 0.3 and 0.7 pH unit for 1-d-old and 3-d-old *Eschscholtzia californica* cells, respectively. These confirmed those of Roos (1998), Ratcliffe (1998) and Pugin (1997). Cytoplasmic pH then slowly recovered to initial value in 2 hours for 1-d-old cell. For 3-d-old cell, after half an hour cytoplasmic pH increased to initial value. Vacuole acidified of 0.5 pH unit for 1-d-old cells, while it is stable for 3-d-old cells. Vacuole alkalinization (Roos *et al.,* 1998) was not observed in these treatments.

6.3.2 PME concentration is sensitive to elicitation

The results of effect of elicitation on PME and cytoplasmic phosphate are summarized in figure 6.2. Elicitor caused a decrease of PME in first 30 min of elicitation for both 1-d-old and 3-d-old cells (data not shown). In 60 min, PME increased back to its initial value. In the second hour post-elicitation, PME increased to a maximum value. The effect of elicitation on 1-d-old cells is not as significant as or the 3-d-old cells. In two hours elicitation, PME in 3-d-old cells reached 1.8 times fold of its initial value (Figure 6.2). Cytoplasmic phosphate has also increased, and then recovered to its initial value

after 3 hours elicitation. Both ATP and NADPH increased compared to non elicited cultures (figure6.3).

6.4 CONCLUSION

The early events in cells responses to elicitation were monitored by *in vivo* ^{31}P-NMR. Cell cytoplasm acidified within the first 30 min and return at its initial value in an hour. PME decreased and then increased to 1.8 times its initial value in 2 hours elicitation. ATP, NADPH and cytoplasmic phosphate have responses due to elicitation.

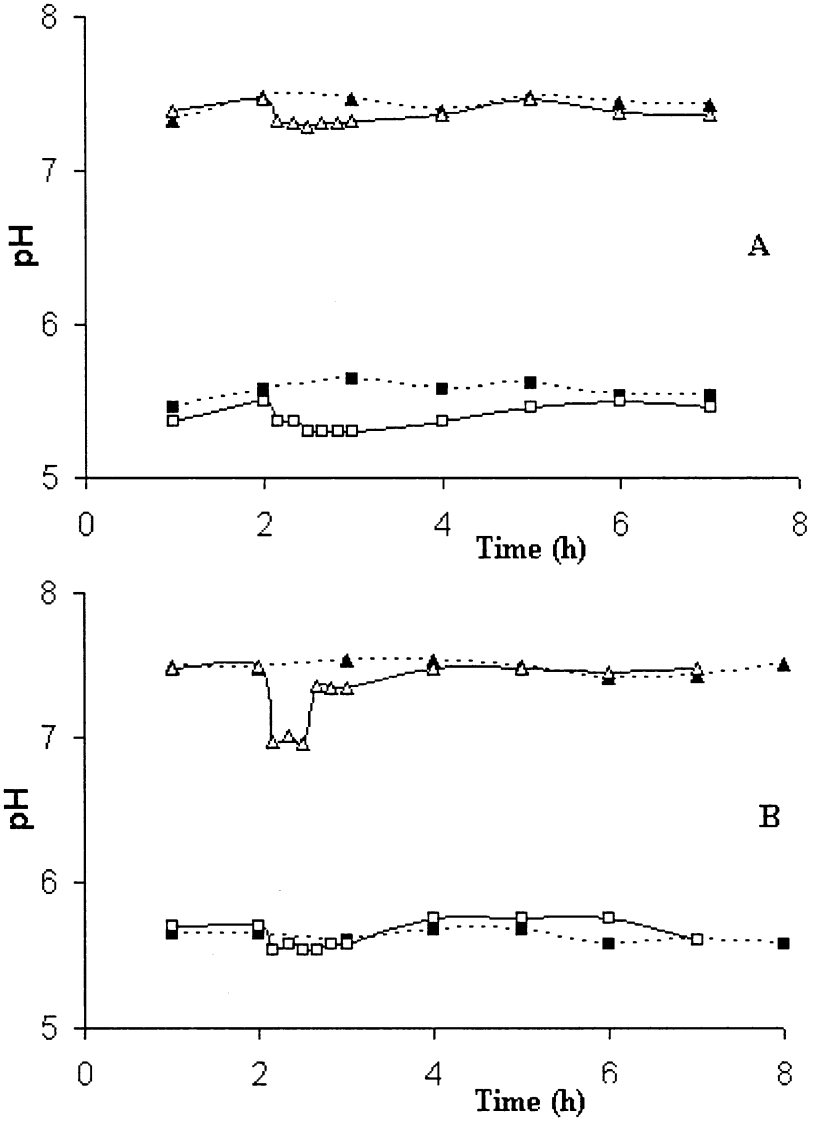


Figure 6.1: The effect of elicitation on intracellular pH

(A: 1-d-old cell; B: 3-d-old cell. △: Cytoplasmic pH of elicitation; ▲: Cytoplasmic pH of control; □: Vacuolar pH of elicitation; ■: Vacuolar pH of control. Cell was elicited at time 2 hours.)

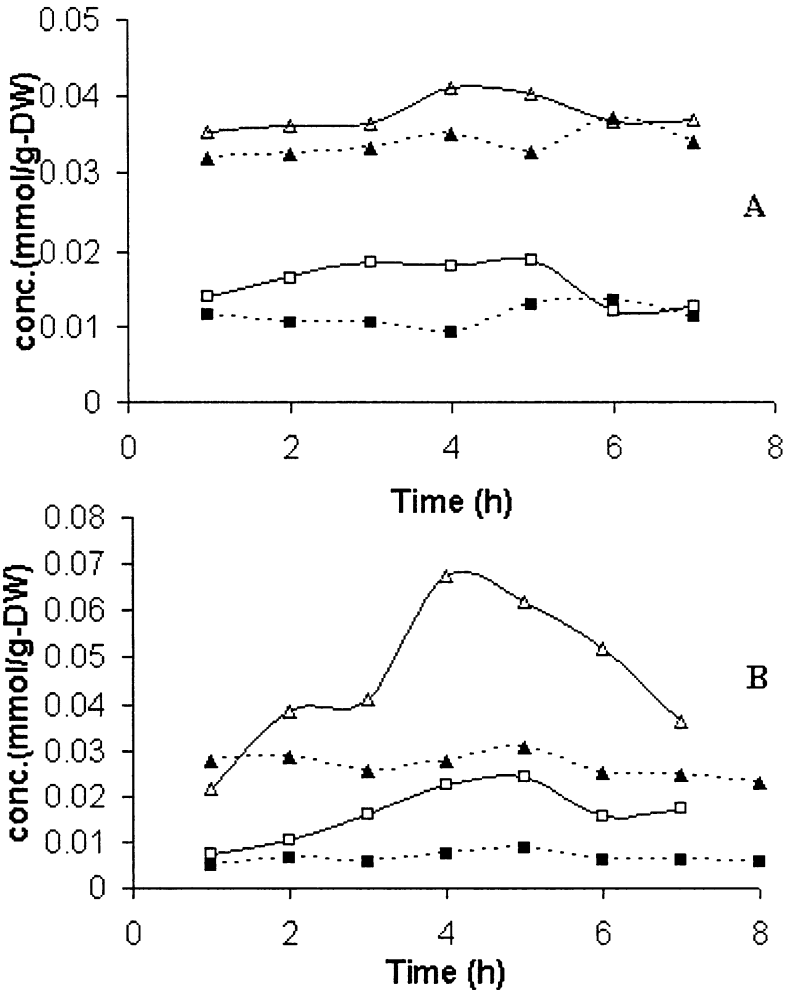


Figure 6.2: The effect of elicitation on PME and cytoplasmic phosphate

(A: 1-d-old cell; B: 3-d-old cell. △: PME of elicitation; ▲: PME of control; □: Cytoplasmic Pi of elicitation; ■: Cytoplasmic Pi of control. Cell was elicited at time 2 hours.)

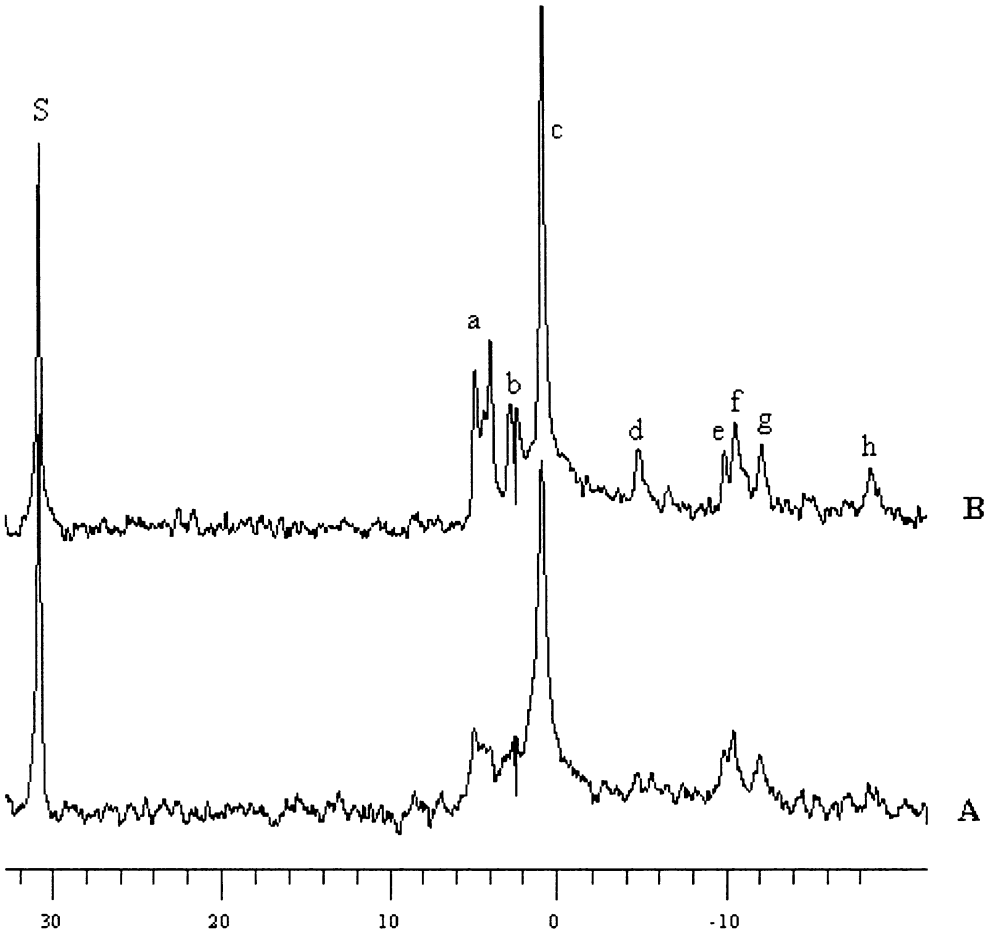


Figure 6.3: *In vivo* ^{31}P-NMR spectra of 3-d-old cells before (A) and 2 hours after (B) elicitation

(NMR resonance assignment: S, reference; a, PME; b, Cytoplasmic Pi; c, Vacuole Pi; d, ADP_β+ATP_γ; e, ATP_α+ADP_α; f, NADP+NDPG; g, NDPG; h, ATP_β)

CHAPTER 7: GENERAL DISCUSSION, CONCLUSION AND FURTURE WORK

7.1 GENERAL DISCUSSION

In this study, the small-scale bioreactor was successfully modified and developed for quantification and long-term study. The study of metabolism by *in vivo* NMR is possible with this modified small-scale bioreactor. Cells physiological responses to low oxygen stress, phosphate metabolism of cell cultures in different dissolved oxygen conditions were studied by *in vivo* ^{31}P NMR spectroscopy. In additon, phosphate uptake, storage and translocation were monitored.

In vivo NMR studies were performed in a small-scale bioreactor, which was successfully modified and developed based on our previous work for quantification and long-term experiment. Noninvasive external phosphorus reference sealed in capillary was prepared for quantification. T1, T2 and saturation factor were measured for calibrating the quantification results. The humidifier with 10% D_2O in sterile water and a condenser were applied to inhibit medium evaporation and concentration under high flow rate of air bubbling. The modified small-scale bioreactor can be used for long-term study, which was proved by cultivating cell bed for 10 days with oxygenated sterile medium perfusion.

Low oxygen stress and cell physiological state diference under different oxygen conditions were studied by *in vivo* ^{31}P NMR spectroscopy with the modified small-scale

bioreactor. The results showed that *Eschscholtzia californica* cells are very sensitive to low oxygen stress. When dissolved oxygen decreased from 60% to 25% saturated by air, cytoplasm was acidified 0.7 pH unit. Cytoplasmic pH recovered back to initial value immediately with reoyxgnated medium. Cells behave under stress during cell transfer. *In vivo* NMR measurements were suggested to start one hour after inoculation until cell stabilized to new environment. Oxygen-enriched culture (bioreactor culture) consumed intracellular phosphate quickly comparing to oxygen-limited culture (flask culture). Oxygen enriched culture behaved nutrients limitation at the end of culture, which wasn't observed in oxygen-limited culture (flask culture).

Phosphate uptake and translocation were studied by *in vivo* ^{31}P NMR spectroscopy with Pi continutally feeding and pulse feeding. Phosphate uptake rate was measured by the NMR visible total phosphate at the value of 1.40 μmol min^{-1} g^{-1}DW for *Eschscholtzia californica* cell suspension at medium Pi of 1.0 mmol L^{-1}. Cytoplasmic Pi was continually transferred into vacuolar for storage when external Pi is available and in the early event when external Pi was off. ATP decreased during phosphate uptake and increased when external phosphate was off. These suggest that phosphate uptake is an ATP coupling process. Both the processes of phosphate uptake and low oxygen stress caused acidification of cytoplasm (0.7 pH unit) and slight alkalinization of vacuole.

Cells early events physiological responses to elicitors were monitored by *in vivo* ^{31}P-NMR. Cytoplasm acidification and PME decreasing was found in first half hour of elicitation. After another half elicitation, cytoplasmic pH as well as PME, recovered to

initial value. In another hour elicitation, PME increased significantly to maximum value. The responses of 3-d-old cell PME reached 1.8 times of initial value in 3 hours elicitation. ATP, NADPH and Cytoplasmic phosphate also increased during the early event of elicitation.

In short, the objectives of this project were achieved by modifying the small-scale bioreactor to perform low oxygen stress and phosphate uptake studies. The small-scale bioreactor was proved that it can be used to monitor oxygen stress by 10 min data recording. NMR spectrum can be enveluated to yield the information of introcellular pH and the concentrations of phosphorous metabolites that was used to follow the cell physiological responses to kinds of stress. The phenominium of phosphate uptake, storage and translocation was studied, which can be used to set up metabolism model.

7.2 CONCLUSION

In this thesis, the phosphate metabolism, the physiological responses of low oxygen stress, phosphate uptake, storage and translocation were studied. Current results of *in vivo* ^{31}P-NMR study of elicitation were also discussed. The small-scale bioreactor was modified and developed successfully for quantification and long-term *in vivo* NMR studies.

First, small-scale bioreactor was modified successfully for quantification, long-term experiment. Noninvasive external phosphorus reference that sealed in capillary was prepared for quantification, while the humidifier with 10% D_2O in sterile water and a condenser were applied to remove the effect of medium evaporation and concentration under high flow rate of air bubbling. Cell bed has already been successfully cultivated in this small-scale bioreactor for 10 days with oxygenated sterile medium perfusion.

Second, *Eschscholtzia californica* cells are very sensitive to low oxygen stress. When dissolved oxygen decreased from 60% to 25% saturation by air, cytoplasm was acidified 0.7pH unit.

Third, cells behave under stress during cell transfer. *In vivo* NMR measurements were suggested to start one hour after inoculation until cells were stabilized in new environment.

Forth, Oxygen-enriched culture (bioreactor culture) consumed intracellular phosphate more quickly comparing to oxygen-limited culture (flask culture). Oxygen-enriched

culture behaved nutrients limitation at the end of culture, which wasn't observed in oxygen-limited culture (flask culture).

Fifth, phosphate uptake rate and the capacity of phosphate storage in vacuole were studied. Phosphate uptake rate is 1.40 μmol min^{-1} g^{-1}DW, while the maximum capacity of phosphate storage in vacuole is 0.322 mmol g^{-1}DW for *Eschscholtzia californica* cell suspension at medium Pi of 1.0 mmol L^{-1}. Phosphate uptake is an ATP coupling process and caused cytoplasm acidification.

Sixth, cell early events physiological responses to elicitors were monitored by *in vivo* ^{31}P-NMR. Cytoplasm acidification and PME decreased in first 30 min and recovered to initial value in an hour. PME increased to maximum value of 1.8 times of initial value in two hours elicitation. After that it slowly recovers back to initial value.

7.3 FUTURE WORK

One of the future works is that the small-scale bioreactor will be further modified. The small-scale bioreactor was already modified to support quantification and long-term *in vivo* NMR studies. But there are still some problems that limit the application of *in vivo* NMR studies. One is that dissolved oxygen is not fully controlled in this bioreactor. For example, when cells contacted elicitor, the oxygen consumption increased and dissolved oxygen decrease to around 30% from air saturation. Another is that the in-house 316SS chamber, which was used to measure on-line dissolved oxygen and external pH still need to be modified. The chamber holds too much medium (around 3 ml) inside. The retention time is 2 min at the flow rate of 1.60 ml/min. This causes a delay in the measurement of external pH and dissolves oxygen. In addition, the entrainment of gas bubbles inside the chamber can perturb the quality of the dissolved oxygen and medium pH data.

Another goal for future of works is continuing the project of elicitation. The effect of elicitation on cells early events was only performed with 1-d-old and 3-d-old cells. The next step of this project will be to study the relationship between the cells early responses with the ultimate cells productivity using both *in vivo* and *in vitro* ^{31}P-NMR combined to HPLC and LC-MS. In addition, the cells response of pentose phosphate pathway and redox potential should be studied by *in vitro* ^{31}P-NMR and ^{13}C-NMR with ^{13}C labeled nutrients pulse feeding.

The third part of future work will consist in extrapolating the work performed with plant cells to CHO (Chinese Hamster Ovary) cells. The small-scale bioreactor will have to be modified for mammalian cells.

REFERENCES

Alger, J. R., Hollander, J. A. and Shulman, R. G. (1982). *In vivo* phosphorus-31 nuclear magnetic resonance saturation transfer studies of adenosinetriphosphatase kinetics in *Saccharomyces cerevisiae*. Biochemistry **21,** 2957-2963

Aubert, S., Gout, E., Bligny, R. and Douce, R. (1994). Multiple effects of glycerol on plant cell metabolism. The Journal of Biological Chemistry **269,** 21420-21427

Bergans, N., Dresselaers, T., Vanhamme, L., Hecke, P. V., Huffel, S. V. and Vanstapel, F. (2003). Quantification of the glycogen ^{13}C-NMR signal during glycogen synthesis in perfused rat liver, NMR in Biomedicine **16,** 36-46

Bligny, R., Foray, M., Roby, C. and Douce, R. (1989). Transport and phosphorylation of choline in higher plant cells. The Journal of Biological Chemistry **264,** 4888-4895

Bligny, R., Gardestrom P., Roby, C. and Douce, R. (1990). ^{31}P-NMR studies of spinach leaves and their chloroplasts. The Journal of Biological Chemistry **265**, 1319-1326

Brodeliust, P. and Vogel, H. J. (1985). A phosphorus-31 nuclear magnetic resonance study of phosphate uptake and storage in cultured *Catharanthus roseus* and Daucus carota plant cells. The journal of biological chemistry **260,** March 25, 2556-3560.

Brouquisse, R., Evrard, A., Rolin, D., Raymond, P. and Roby, C. (2001). Regulation of protein degradation and protease expression by mannose in Maize Root Tips. Pi sequestration by mannose my hinder the study of its signaling properties. Plant Physiology **125,** 1485-1495

Brown, F. F., Campbell, I. D. Kuchel, P. W. and Rabenstein, D. C. (1977). Human erythrocyte metabolism studies by ^{1}H spin echo NMR, FEBS Letters **82,** 12-16

Brown, T. R., Ugurbil, K. and Shulman, R. G. (1977). ^{31}P nuclear magnetic resonance measurements of kinetics in aerobic *Escherichia coli* cells. Natl. Acad. Sci. USA, **74,** 5551-5553

Bruch, M. D. (1996). NMR spectroscopy techniques, 2nd edition. New York.

Byun, Y. S., Kim, C. and Pedersen, H. (1992). Elicitation of alkaloid production at different growth stages in cell suspensions of Eschscholtzia californica. Plant tissue culture letters **9** (3), 164-168

Campbell, S. L., Jones, K. A. and Shulman, R. G. (1985). *In vivo* ^{31}P nuclear magnetic resonance saturation transfer measurements of phosphate exchange reactions in the yeast saccharomyces cerevisiae. FEBS **193,** 189-193

Campbell-Burk, S. L., and Shulman, R. G. (1987). High-resolution NMR studies of Saccharomyces cerevisiae. Ann. Rev. Microbiol **41,** 595-616

Cerdan, S., Künnecke, B. and Seelig, J. (1990). Cerebral metabolism of [1, 2-$^{13}C_2$] acetate as detected by *in vivo* and *in vitro* ^{13}C-NMR, The Journal of Biological Chemistry, **265**(22): 12916-12926

Cohen, J. S., Jaroszewaski, J. W., Kaplan, O., Ruiz-Cabello, J. and Collier, S. W. (1995). A history of biological applications of NMR spectroscopy. Process in Nuclear Magnetic Spectroscopy **28,** 53-85

Degani, H., Laughlin, M., Campbell, S. and Shulman, R. G. (1985). Kinetics of creatine kinase in heart: a ^{31}P-NMR saturation- and inversion-transfer study. Biochemistry **24,** 5510-5516

Dieuaide-Noubhani, M., Raffard, G., Canioni, P., Pradet, A. and Raymond, P. (1995). Quantification of compartimented metabolic fluxes in maize root tips using isotope distribution form ^{13}C and ^{14}C-labeled glucose. Journal of Biological Chemistry **270,** 13147-13159

Eskey, C. J., Koretsky, A. P., Domach, M. M. and Jain, R. K. (1993). Role of oxygen vs. glucose in energy metabolism in a mammary carcinoma perfused *ex vivo*: Direct measurement by ^{31}P-NMR. Proc. Natl. Acad. Sci. USA **90,** 2646-2650

Fan, T. W. M., Higashi, R. M. and Lane, A. N. (1986). Monitoring of hypoxic metabolism in superfused plant tissues by *in vivo* proton NMR, Archives of Biochemistry and Biophysics, **251,** 674-687

Fan, T. W. M., Higashi, R. M. and Lane, A. N. (1988). An *in vivo* proton and phosphaorus-31 NMR investigation of the effect of nitrate on hypoxic metabolism in maize roots, Archives of Biochemistry and Biophysics, **266,** 592-606

Fonseca, L., Alves, P. M., Carrondo, M. J. T. and Santos, H. (2001). Effect of ethanol on the metabolism of primary astrocytes studied by ^{13}C- and ^{31}P-NMR spectroscopy. Journal of Neuroscience Research **66,** 803-811

Ford, Y., Fox, G. G., Ratcliffe, R. G. and Robins, R. J. (1994). *In vivo* ^{15}N NMR studies of secondary metabolism in transformed root cultures of Datura stramonium and Nicotiana tabacum. Photochemistry **36,** 333-339

Fox, G. G., Ratcliffe, R. G. and Southon, T. E. (1989). Airlift systems for *in vivo* NMR spectroscopy of plant tissues. Journal of Magnetic Resonance **82,** 360

Gadian, D. G., Radda, G. K., Brown, T. R., Chance, E. M., Dawson, M. J. and Wilkie, D. R. (1981). The activity of creatine kinase in frog skeletal muscle studied by saturation-transfer nuclear magnetic resonance. Biochemical Journal **194,** 215-228

Gamcsik, M. P., Constantinidis, I. and Glickson, J. D. (1991). *In vivo* nitrogen-14 nuclear magnetic resonance spectroscopy of tumors: detection of ammonium and trimethylamine metabolites in the murine radiation induced fibrosarcoma 1, Cancer Research 51, 3378-3383

Gerendás, J. and Ratcliffe, R. G. (2000). Intracellular pH regulation in maize root tips exposed to ammonium at high external pH. Journal of Experimental Botany **51**,207-219

Gerlitz, T. G. M. and Gerlitz, A. (1997). Phosphate uptake and polyphosphate metabolism of mycorrhizal and nonmycorrhizal roots of pine and of suillus bovines at varying external pH measured by *in vivo* ^{31}P-NMR. Mycorrhiza **7**, 101-106

Gillies, R. J., Ugurbil, K., Hollander, J. A. and Shulman, R. G. (1981). ^{31}P-NMR studies of intracellular pH and phosphate metabolism during cell division cycle of *saccharomyces cerevisiae*, Natl. Acad. Sci. USA, **78**, 2125-2129

Gmati, D., Chen, J. and Jolicoeur, M. (2004). Development of a Small-scale bioreactor: Application to *in vivo* NMR Measurement. Biotechnology and Bioengineering, (in press)

Gout, E., Bligny, R. and Douce, R. (1992). Regulation of intracellular pH values in higher plant cells. The Journal of Biological Chemistry **267,** 13903-13909

Gout, E., Bligny, R., Pascal, N. and Douce, R. (1993). ^{13}C Nuclear magnetic resonance studies of malate and citrate synthesis and compartmentation in higher plant cells, The Journal of Biological Chemistry **268,** 3986-3992

Gout, E., Aubert, S., Bligny, R., Rébeillé, F., Nonomura, A. R. Benson, A. A. and Douce, R. (2000). Metabolism of methanol in plant cells. Carbon-13 nuclear magnetic resonance studies. Plant Physiology **123,** 287-296

Gout, E., Boisson, A. B., Aubert, S., Douce, R. and Bligny, R. (2001). Origin of the cytoplasmic pH changes during anaerobic stress in higher plant cells. Carbon-13 and phosphorous-31 nuclear magnetic resonance studies. Plant Physiology **125,** 912-925

Gupta, R. K., Benovic, J. L. and Rose, Z. B. (1978). The determination of the free magnesium level in the human red blood cell by ^{31}P-NMR, The Journal of Biological Chemistry, **253:** 6172-6176

Hesse, S. J. A., Ruijter, G. J. G., Dijkema, C and Visser, J. (2002). Interacellular pH homeostasis in the filamentour fungus *Aspergillus nige,*. Euro. J. Biochem. **269,** 3485-3494

Hinse, C. and Sheludko, Y. V. (2001). *In vivo* NMR at 800MHz to monitor alkaloid metabolism in plant cell cultures without tracer labeling, Journal of American Chemistry Society **123,** 5118-5119

IUPAC. (1997). IUPAC Compendium of chemical technology, 2nd edition.

Jucker, B. M., Dufour, S., Ren, J., Cao, X., Previs, S. F., Underhill, B. et *al.* (2000). Assessment of mitochondrial energy coupling *in vivo* by $^{13}C/^{31}P$-NMR, Proc. Natl. Acad. Sci. USA, **97**(12), 6880-6884

Kennedy, R. A., Rumpho, M. E. and Fox, T. C. (1992). Anaerobic metabolism in plants. Plant Physiology **100,** 1-6

Kirk, K., Raftos, J. E. and Kuchel, P. W. (1986). Triethyl phosphate as an internal ^{31}P-NMR reference in biological samples. Journal of Magnetic Resonance **70**, 484-487

Künnecke, B., Küstermann, E. and Seelig, J. (2000). Simultaneous *in vivo* monitoring of hepatic glucose and glucose-6-phosphate by ^{13}C-NMR spectroscopy. Magnetic Resonance in Medicine **44,** 556-562

Lamboursain, L. and Jolicoeur, M. (2004). Critical influence of *Eschscholtzia californica* cells nutritional state on secondary metabolite production. Submitted to Biotechnology and. Bioengineering

Lee, R. B. and Ratcliffe, R. G. (1991). Observations on the subcellular distribution on the ammonium ion in maize root tissue using *in vivo* nitrogen-14 nuclear magnetic resonance spectroscopy, Planta **183,** 359-367

Lee, R. B., Purves, J. V., Ratcliffe, R. G. and Shaker, L. R. (1992). Nitrogen assimilation and the control of ammonium and nitrate absorption by maize roots. Journal Experimental Botany **43,** 1385-1396

Levitt, M. H. (2001). Spin dynamics, basics of nuclear magnetic resonance. John Wiley & Sons, Ltd.

Lyngstad, M. and Grasdalen, Hans. (1993). A new NMR airlift bioreactor used in ^{31}P_NMR studies of itaconic acid producing Aspergillus terreus, Journal of Biochemical and Biophysical Methods **27,**105-116

Majumdar, K. C., Nasaruddin, K. and Ravinder, K., Sundaram, C. S., Manickam, P. and Shivaji, S. (1999). ^{31}P Nuclear Magnetic Resonance Studies on the Phosphorus-containing Metabolites of the Developing Embryos of a Freshwater Catfish, Clariasbatrachus (L.). Marine Biotehcnology **1,** 221-229

Mancuso, A., Sharfstein, S. T., Tucker, S. N., Clark, D. S. and Blanch, H. W. (1994). Examination of primary metabolic pathways in a murine hybridoma with carbon-13 nuclear magnetic resonance spectroscopy, Biotechnology and Bioengineering, **44,** 563-585

Mancuso, A., Sharfstein, S. T., Fernandez, E. J., Clark, D. S. and Blanch, H. W. (1998). Effect of extracellular glutamine concentration on primary and secondary metabolism of a muring hybridoma: an *in vivo* ^{13}C Nuclear magnetic resonance study, Biotechnology and Bioengineering, **57,** 175-186

Martin, J. B., Bligny, R., Rébeillé, F., Douce, R., Leguay, Y., Mathieu, Y., and Guern, J. (1982). A ^{31}P nuclear magnetic resonance study of intracellular pH of plant cells cultivated in liquid medium, Plant Physiology **70,** 1156-1161

Montero-Lomeli, M., Martins, O. B. and Dreyfus, G. (1989). Coupling of ATP hydrolysis to phosphate uptake in *Rhodospirillum rubrum* chormatophores under the influence of Ca^{2+} and Mg^{2+}. The Journal of Biological Chemistry **264,** 21014-21017

Moon, R. B. and Richards, J. H. (1973). Determination of intracellular pH by 31P magnetic resonance, The Journal of Biological Chemistry **248,** 7276-7278

Müller, J., Staehelin, C., Xie, Z., Neuhaus-Url, G. and Boller, T. (2000). Nod factors and chitooligomers elicit and increase in cytosolic calcium in aequorin-expressing soybean cells. Plant Physiology **124,** 733-739

Netting, A. G. (2000). pH, abscisic acid and the integration of metabolism in plants under stressed and non-stressed conditions: cellular responses to stress and their implication for plant water relations, Journal of Experimental Botany **51,** 147-158

Noguchi, Y., Shimba, N., Toysaki, H., Ebisawa, K., Kawahara, Y., Suzuki, E. *et al.,* (2002). *in vivo* NMR system for evaluation oxygen-dependent metabolic status in microbial culture, Journal of Microbiological Methods **51,** 73-82

Ogawa, S. and Lee, T. M. (1984). The relation between the internal phosphorylation potential and the proton motive force in mitochondria during ATP synthesis and hydrolysis. The Journal of Biological Chemistry **259,** 10004-10011

Osbakken, M., Ivanics, T., Zhang, D., Mitra, R. and Blum, H. (1992). Isolated cardiomyocytes in conjunction with NMR spectroscopy techniques to study metabolism dan ion flux. The Journal of Biological Chemistry **267,** 15340-15347

Pfeffer, P. E., Dounds Jr., D. D., Bécard, G. and Shachar-Hill, Y. (1999), Carbon uptake and metabolism and transport of lipids in an *Arbuscular Mycorrhiza*, Plant Physiology **120,** 587-598

Pugin, A., Frachisse, J., Tavernier, E., Bligny, R., Gout, E., Douce, R. and Guern, J. (1997). Early events induced by the elicitor cryptogein in tobacco cells: involvement of a plasma membrane NADPH oxidase and activation of glycolysis and the pentose phosphate pathway. The Plant Cell **9,** 2077-2091

Radman, R., Saez, T., Bucke, C. and Keshavarz, T. (2003). Elicitation of plants and microbial call systems. Biotechnology and Applied Biochemistry **37,** 91-102

Rager, M. N., Binet, M. R. B., Ionescu, G. and Bouvet, O. M. M. (2000). ^{31}P-NMR and ^{13}**C-NMR** studies of mannose metabolism in plesiomonas shigelloides- toxic effect of mannose on growth, Eur. J. Biochem. **267,** 5136-5141

Rasmussen, N., Lloyd, D. C., Ratcliffe, R. G., Hansen, P. E. and Jakobsen, I. (2000). ^{31}P-NMR for the study of P metabolism and translocation in arbuscular mycorrhizal fungi. Plant and Soil **226,** 245-253

Ratcliffe, R. G., and Roscher, A. (1998). Prospects for *in vivo* NMR methods in xenobiotic research in plants. Biodegradation **9,** 411-422

Ratcliffe, R. G., Roscher, A. and Shachar-Hill, Y. (2001a). Plant NMR spectroscopy. Progress in Nuclear Magnetic Resonance Spectroscopy **39,** 267-300

Ratcliffe, R. G. and Shachar-Hill, Y. (2001b). Probing plant metabolism with NMR. Annual Review of Plant Physiology and Plant Molecular Biology **52,** 499-526

Raven, J. A. (1986). Biochemical disposal of excess H^+ in growing plants? New Physiologist **104,**175-206

Richey, B., Cayley, D. S., Mossing, M. C., Kolka, C., Anderson, C. F. and Record Jr, M. T. (1987) Variability of the intracellular ionic environment of *Escherichia coli.* Differences between *in vitro* and *in vivo* effects of ion concentrations on protein-DNA interactions and gene expression, Journal of Biological Chemistry **262,** 7157-7164

Rijhwani, S. K., Ho, C. and Shanks, J. V. (1999). *In vivo* ^{31}P and multilabel ^{13}C-**NMR** measurements for evaluation of plant metabolic pathways, Metabolic Engineering, **1,** 12-25

Roberts, J. K. M., Ray, P. M., Norma, W. and Oleg, J. (1980). Estimation of cytoplasmic and vacuolar pH in higher plant cells by phosphorus -31 NMR, Nature **283,** 870-872

Roberts, J. K. M., Wemmer, D., Ray, P. M. and Oleg, J. (1982), Regulation of cytoplsmic and vacuolar pH in maize root tips under different experimental conditions, Plant Physiology **69,** 1344-1347

Roberts, J. K. M., Wemmer, D. and Oleg, J. (1984a). Measurement of mitochondrial ATPase activity in maize root tips by saturation transfer ^{31}P nuclear magnetic resonance. Plant Physiology **74,** 632-639

Roberts, J. K. M. (1984b). Study of plant metabolism *in vivo* using NMR spectroscopy. Annual Review of Plant Physiology **35,** 375-386

Roberts, J. K. M. Hooks, M. A., Miaullis, A. P., Edwards, S and Cecelia, W. (1992a). Contribution of malate and amino acid metabolism to cytoplasmic pH regulation in hypoxic maize root tips studied using nuclear magnetic resonance spectroscopy. Plant Physiology **98,** 480-487

Roberts, J. K. M. and Pang, M. K. L. *et al.* (1992b). Estimation of ammonium ion distribution between cytoplasm and vacuole using nuclear magnetic resonance spectroscopy. Plant Physiology **100,** 1571-1574

Roberts, J. K. M., Aubert, S., Gout, E., Bligny, R. and Douce, R. (1997). Cooperation and competition between adenylate kinase, nucleoside diphosphokinase, electron transport, and ATP synthase in plant mitochondria studied by ^{31}P-nuclear magnetic resonance. Plant Physiology **113:** 191-199

Roby, C., Martin, J., Bligny, R. and Douce, R. (1987). Biochemical changes during sucrose deprivation in higher plant cells, The Journal of Biological Chemistry, **262,** 5000-5007

Roos, W., Evers, S., Hieke, M., Tschöpe, M. and Schumann, B. (1998). Shifts of intracellular pH distribution as a part of the signal mechanism leading to the elicitation of benzophenanthridine alkaloids. Plant Physiology **118,** 349-364

Saglio, P. H., Drew, M. C. and Pradet, A. (1988). Metabolic acclimation to anoxia induced by low (2-4 kPa partial pressure) oxygen pretreatment (hypoxia) in root tips of *Zea mays*. Plant Physiology **86**, 61-66

Sakano, K., Yazaki, Y. and Mimura, T. (1992). Cytoplasmic acidification induced by inorganic phosphate uptake in suspension cultured *Catharanthus roseus* cells. Plant Physiology **99,** 672-680

Sakano, K., Yazaki, Y., Okihara, K., Mimura, T. and Kiyota, S. (1995). Lake of control in inorganic phosphate uptake by *Catharanthus roseus* (L.) G. Don cells. Plant Physiology **108,** 295-302

Sanders, J. K. M. and Hunter, B. K. (1987). Modern NMR spectroscopy, Oxfrod New York Toronto

Schiller, P., Hartung, W. and Ratcliffe, R. G. (1998). Intracellular pH stability in the aquatic resurrection plant chamaegigas intrepidus in the extreme environmental conditions that characterize its natural habitat. New Phytologist **140,** 1-7

Shachar-Hill, Y. and Pfeffer, P. (1996). Nuclear magnetic resonance in plant biology, current topics in plant physiology: an American society of plant physiologists series, volume **16**

Slonczewski, J. I., Rosen, B. P., Alger, J. R. and Macnab, B. M. (1981). PH homeostasis in Escherichia coli: measurement by ^{31}P nuclear magnetic resonance of methlphospahte and phosphate, Proc. Natl. Acad. Sci. USA, **78,** 6271-6275

Spickett, C.M., Smirnoff, N. and Ratcliffe, R. G. (1993). An *in vivo* nuclear magnetic resonance investigation of ion transport in Maize (*Zia mays*) and spartina anglica roots during exposure to high salt concentrations, Plant Physiology **102**, 629-638

Veeman, W. S. (1997) Nuclear magnetic resonance, a simple introduction to the principles and applications, Geoderma **80,** 225-242;

Villegas, M., León, R. and Brodelius, P. E. (1999). Effects of alginate and immobilization by entrapment in alginate on benzophenanthridine alkaloid production in cell suspension cultures of *Eschscholtzia californica*. Biotechnology Letters **21**, 49-51

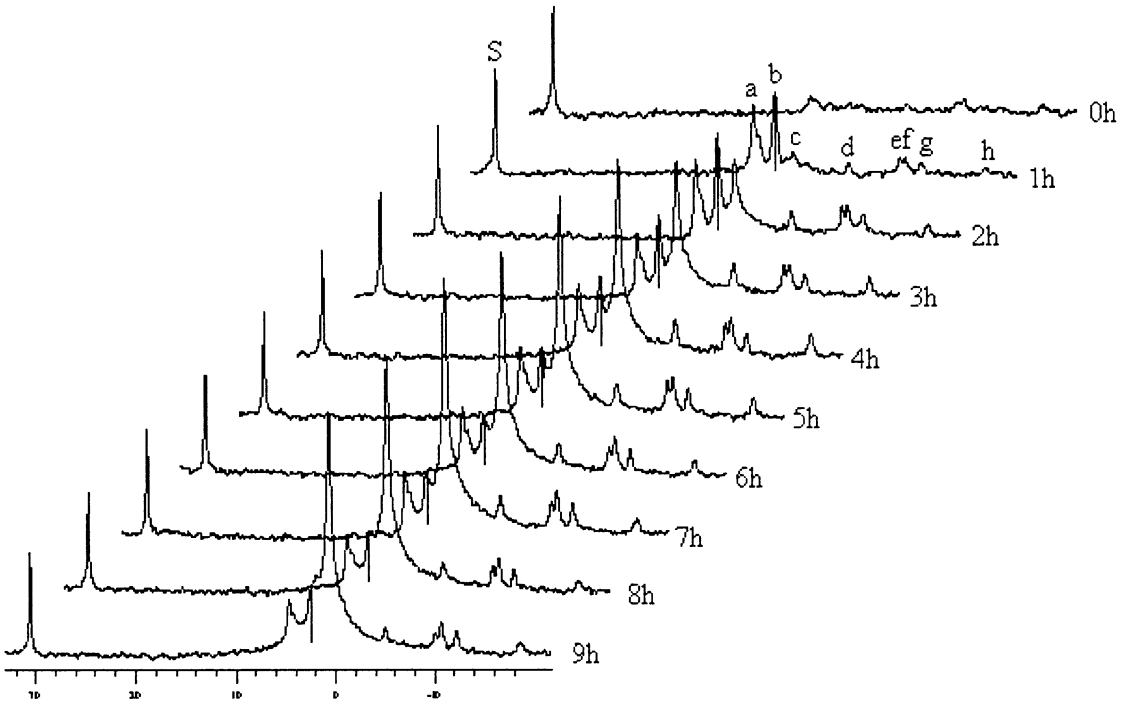
Williams, R. D., Bédard, C., Chavarie, C. and Archambault, J. (1992). Effect of polymeric adsorbents on the production of sanguinarine by papaver somniferum cell cultures. Biotechnology and bioengineering, **40,** 971-977

Xia, Z. Akim, L. G. and Argyropoulos, D. S. (2001). Quantitative ^{13}C NMR Analysis of lignins with internal standards. Journal of Agric. Food Chem. **49,** 3573-3578

Zupke, G. and Stephanopoulos, G. (1995). Nuclear magnetic resonance analysis of cell metabolism. Current Opinion in Biotechnology **6,** 192-197

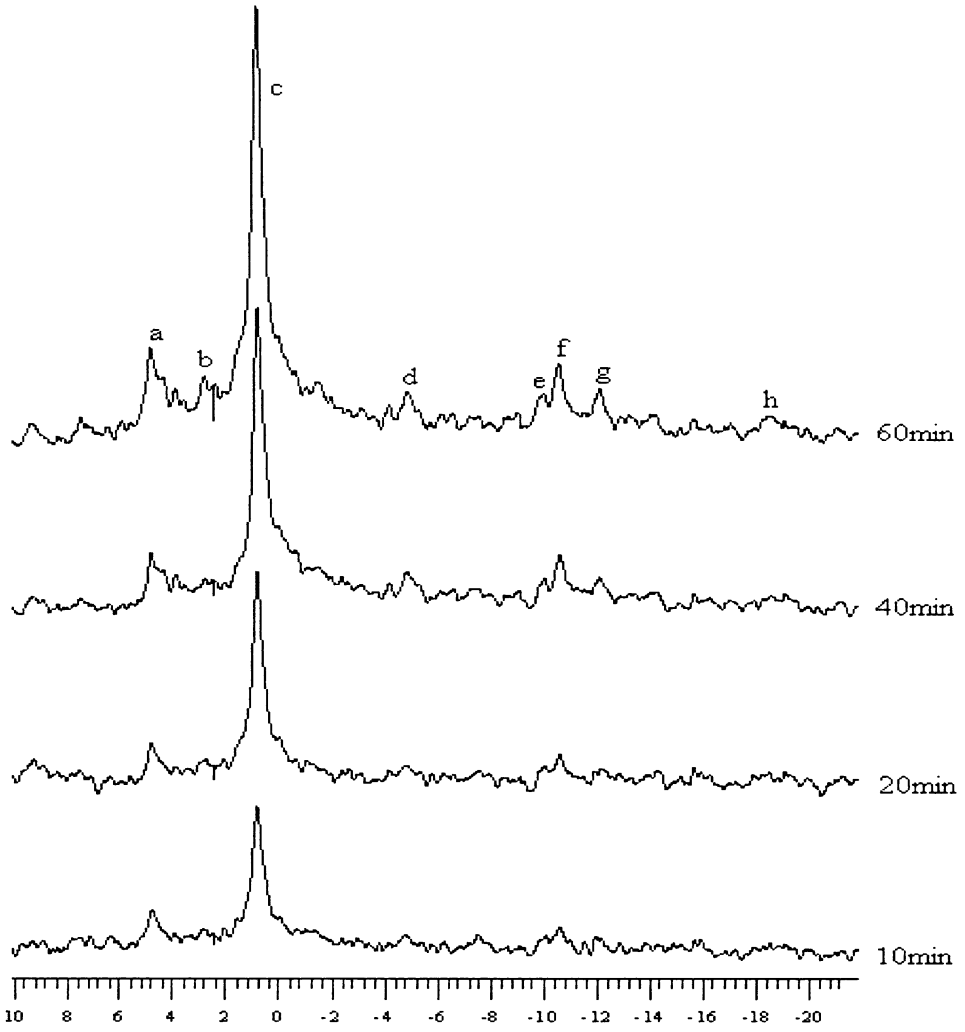
APPENDIX

APPENDIX.1. IN VIVO ^{31}P-NMR SPECTRA OF PHOSPHATE UPTAKE



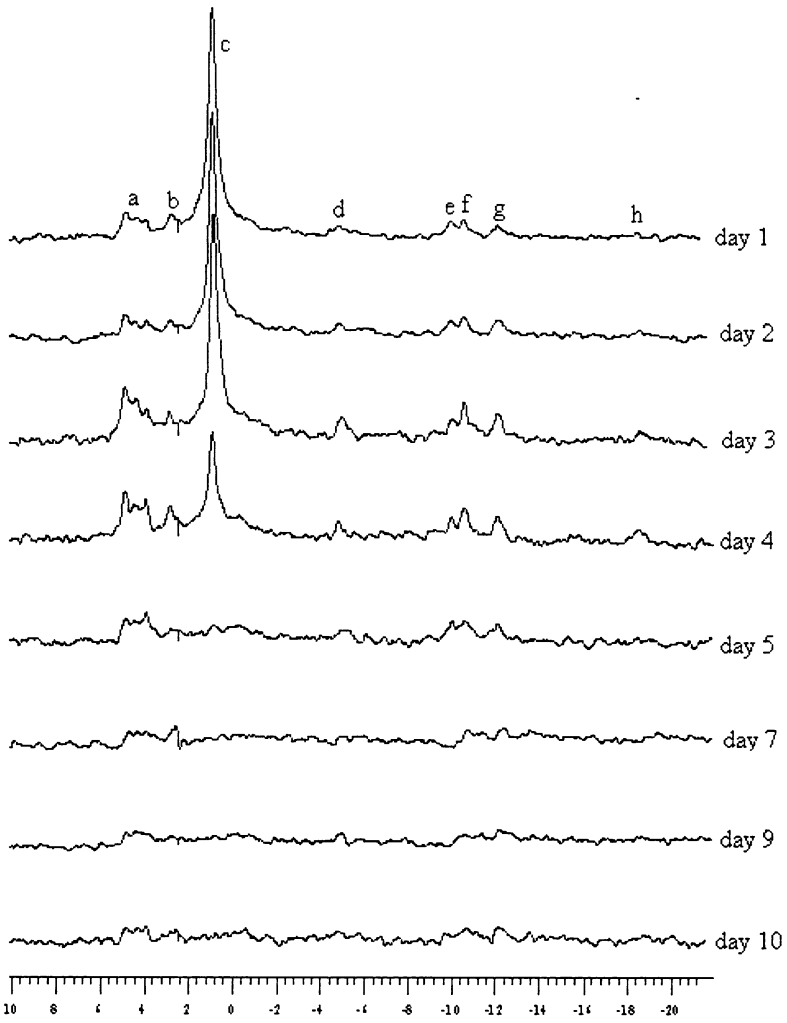
(NMR resonance assignment: a, PME; b, Cytoplasmic Pi; c, Vacuole Pi; d, $ADP_\beta+ATP_\gamma$; e, $ATP_\alpha+ADP_\alpha$; f, NADP+NDPG; g, NDPG; h, ATP_β)

APPENDIX.2. IN VIVO 31P-NMR ACCUMULATIVE SPECTRA OF FIRST DAY CELL



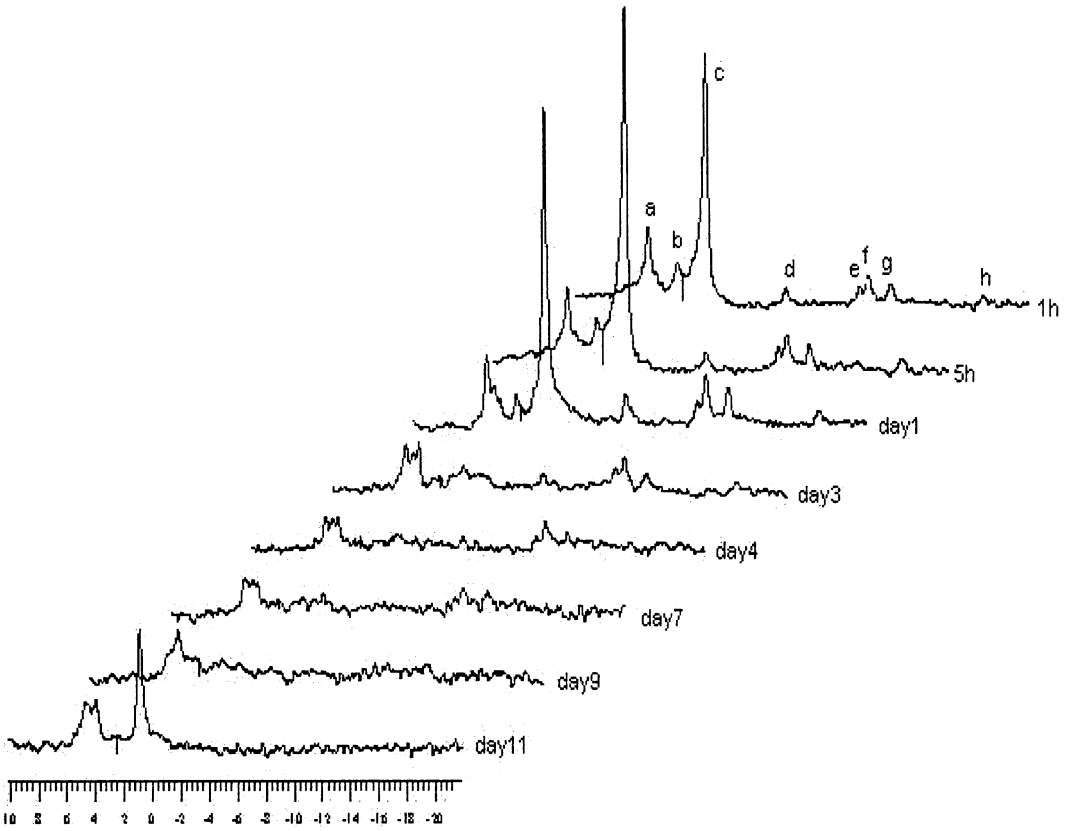
(NMR resonance assignment: a, PME; b, Cytoplasmic Pi; c, Vacuole Pi; d, $ADP_{\beta}+ATP_{\gamma}$; e, $ATP_{\alpha}+ADP_{\alpha}$; f, NADP+NDPG; g, NDPG; h, ATP_{β})

APPENDIX.3. IN VIVO 31P-NMR SPECTRA OF FLASK CULTURE IN 10-DAY CYCLE



(NMR resonance assignment: a, PME; b, Cytoplasmic Pi; c, Vacuole Pi; d, $ADP_{\beta}+ATP_{\gamma}$; e, $ATP_{\alpha}+ADP_{\alpha}$; f, NADP+NDPG; g, NDPG; h, ATP_{β})

APPENDIX.4. IN VIVO 31P-NMR SPECTRA OF BIOREACTOR CULTURE IN 11-DAY CYCLE



(NMR resonance assignment: a, PME; b, Cytoplasmic Pi; c, Vacuole Pi; d, $ADP_{\beta}+ATP_{\gamma}$; e, $ATP_{\alpha}+ADP_{\alpha}$; f, NADP+NDPG; g, NDPG; h, ATP_{β})

APPENDIX.5. THE PROCESS OF AN *IN VIVO* ^{31}P NMR EXPERIMENT

BIO-P ^{2} École Polytechnique	Standard Operating Procedure	Page 1 of 9
SOP N°: NMR1-2004	Date: Sept. 01, 2004	Review n°: new
Written by: Jingkui Chen		Last review by: ∅
TITLE:	The process of in *vivo* ^{31}P-NMR experiment	

1. OBJECTIVE

To descrip the process of *in vivo* ^{31}P NMR measurement with the small-scale bioreactor. The goal of this experiment is to study the metabolism or cell physiology by *in vivo* NMR spctroscopy.

2. RESPONSIBILITIES

This standard operating procedure has to be followed by everybody who wants to perform this experimentation.

BIO-P 2 École Polytechnique	Standard Operating Procedure	Page 2 of 9
SOP N°: NMR1-2004	Date: Sept. 01, 2004	Review n°: new
Written by: Jingkui Chen		Last review by: ∅
TITLE:	The process of in *vivo* ^{31}P-NMR experiment	

3. PRINCIPLE

To subculture the plant cells in small-scael bioreactor to with B5-F3 medium perfusion and NMR data recording. NMR data will be processed to yield the information of intracellular pH and the concentration of metabolits.

4. EQUIPMENT, MATERIAL AND CHEMICALS

- Flask culture or bioreactor culture (EC6 cell suspensition)
- NMR spectrometer and 10 mm broadband probe (Varian, USA)
- Small-scale bioreactor with phosphorous reference capillary
- Oxygen electrode
- pH electrode
- Autoclave
- Pipettes of 10 ml

- Propipette
- Balance
- Ethanol 70% V/V
- B5-F3 medium (B5 medium free of phosphate and parameter ions: Fe^{2+}, Co^{2+} and Mn^{2+})
- D_2O
- Water
- Oven (65°C)
- Vial (50 ml)

BIO-P 2 École Polytechnique	Standard Operating Procedure	Page 4 of 9
SOP N°: NMR1-2004	Date: Sept. 01, 2004	Review n°: new
Written by: Jingkui Chen		Last review by: ∅
TITLE:	The process of in *vivo* ^{31}P-NMR experiment	

5. EXPERIMENTATION PROCEDURE

Sampling and inoculating

Cell was sample from flask or bioreactor culture by cut end 10 ml pipette, inoculated into small-scale bioreactor (figure 5.1) and perfused by 100 ml B5-F3 medium and D_2O (90:10 v/v) to get a high density cell bed. After 1-hour perfusion for stabilization of oxygen stress, the small-scale bioreactor was put into NMR gap to keep cell bed and phosphorous reference at NMR sensitive zoon.

Tuning

- Log in NMR spectrometer with user name and password and load VNMR by click the button at the bottom of screen.
- Disconnect the X cable from probe and connect it to tune interface.
- In VNMR window, type 'tn=P31 dn=H1 su'

- Set tune channel to 1, the sensitivity level to 8, and adjust the capacitor knobs at the bottom of probe (figure 2.4 B) till the number near to zero. Then connect the X cable back to probe,
- Disconnect the proton cable from probe and connected to tune interface. Change the tune channel to 2 and keep the sensitivity level at 8.
- Adjust the capacitor knobs at the bottom of probe till the number around zero (normally <20). Connect the proton cable back to probe. Tuning is ready.

Lock

- Type 'acqi' to get the acquisition window.
- Set lock and spin to 'off' in lock window.
- Adjust 'Z0' to reach on resonance condition, when on resonance condition is met, a well-shaped plateau will be observed instead of sine waves.

BIO-P 2 École Polytechnique	Standard Operating Procedure	Page 6 of 9
SOP N°: NMR1-2004	Date: Sept. 01, 2004	Review n°: new
Written by: Jingkui Chen		Last review by: ∅
TITLE:	The process of in *vivo* ^{31}P-NMR experiment	

- Set lock gain and lock power, adjust lock phase to get lock level around 20-40.
- Lock the deuterium signal by clicking 'ON'.

Shimming

- In lock window, click 'Shim' and select 'manual'.
- Adjust with 'Z1' and 'Z2' and all other button to maximize the lock level. If the lock level is too high, go back to lock window to decrease the lock gain and lock power to keep the lock level between 20 and 40.
- When shimming is done, lock window is closed and back to VNMR window.
- In VNMR window, click setup button to choose nuclei and solvent. Then set delay time, pulse sequence and acquisition time.
- Type 'go' to record NMR spectrum.

BIO-P 2 École Polytechnique	Standard Operating Procedure	Page 7 of 9
SOP N°: NMR1-2004	Date: Sept. 01, 2004	Review n°: new
Written by: Jingkui Chen		Last review by: ∅
TITLE:	The process of in *vivo* ^{31}P-NMR experiment	

Data process

- When NMR spectrum acquisition is done, type 'wft' to display NMR spectrum.
- Type 'aph' for automatic phasing, 'dscale' to display scale.
- Click 'part integral' and 'reset' to set intergration of pick areas.
- Select the peak of phosphorous reference and click 'set int' to set the concentration of reference (in this project is 1.03 mmol/L).
- Type 'dli' to display all the phosphate metabolites concentrations.
- The intracellular pH can be evaluated by the intracellular inorganic phosphate chemical shifts with the relationship of pH and chemical shift.
- When the experiment is done, take the small-scale bioreactor out of NMR gap and type 'exit' to quit VNMR program. Right click mouse and pull down to logout.

Cell bed density analysis

- When NMR measurement is done, the cell was pumped out of NMR tube by switching the inlet and outlet.
- The cell was collected in a vial, separated on vacuum filter, and dried in oven at temperature of 65°C for 24h to get dry weight.
- The cell bed length and the diameter of NMR tube is used to calculate cell bed volume.
- The cell bed density is obtained by the cell bed dry weight and volume.

6. INSTRUCTIONS IN CASE OF PROBLEMS

Call Jingkui Chen or call Varian directly.

7. REFERENCES

Varian. 2000. Getting started, Varian NMR Spectrometer Systems with VNMR 6.1 C Software, Pub. No. 01-999160-00, Rev. A0800.

Varian. 2000. System Administration, Varian NMR Spectrometer Systems with VNMR 6.1 C Software, Pub. No. 01-999160-00, Rev. A0800.

Varian. 2000. User Guide: Liquid NMR, Varian NMR Spectrometer Systems with VNMR 6.1 C Software, Pub. No. 01-999160-00, Rev. A0800.

Varian. 2000. VNMR Command and parameter Reference, Varian NMR Spectrometer Systems with VNMR 6.1 C Software, Pub. No. 01-999160-00, Rev. A0800.