UNIVERSITÉ DU QUÉBEC À MONTRÉAL

INVESTIGATION DE L'ACTIVITÉ PHOTOCHIMIQUE DE LA PHOTOSYNTHÈSE ET SON UTILISATION DANS L'ÉVALUATION DES EFFETS TOXIQUES DE XÉNOBIOTIQUES

THÈSE
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AVANT-PROPOS

Durant mes études de Doctorat, les résultats obtenus ont permis de produire huit articles scientifiques qui sont actuellement soumis ou publiés dans des journaux scientifiques internationaux à comité de lecture. Ma contribution à la recherche est majeure dans six rapports dont je figure comme premier auteur. Cette thèse constitue une compilation d'articles scientifiques qui seront présentés dans les chapitres suivants selon les règles de présentation d'une thèse par accumulation d'articles :

CHAPITRE IV : Franck, F., D. Dewez and R. Popovic. 2005. "Changes in the room-temperature emission spectrum of chlorophyll during fast and slow phases of the Kautsky effect in intact leaves". *Photochemistry and Photobiology*, vol. 81, p. 431-436. Dans ce travail, j'ai participé dans l'élaboration des idées, aux expériences en laboratoire et j'ai contribué à l'interprétation des résultats et la rédaction du rapport.

CHAPITRE V : Dewez, D., N. Ait Ali, F. Perreault and R. Popovic. 2006. "Rapid chlorophyll *a* fluorescence transient of *Lemna gibba* leaf as an indication of light and hydroxylamine effect on photosystem II activity". Soumis à *Photosynthesis Research*. Dans ce travail, j'ai contribué à l'élaboration des idées concernant le projet, j'ai effectué des expériences de laboratoire. Pour la contribution des étudiants Nadia Ait Ali et François Perreault, j'ai supervisé leur travail dans la prise et le traitement des données. J'ai contribué à l'interprétation des résultats et j'ai écrit la première version du rapport.

CHAPITRE VI : Dewez, D., P. Eullaffroy, R. Popovic and P. Juneau. 2006. "Rapid chlorophyll *a* fluorescence transients of *Lemna minor* leaves as indication of light and exogenous electron carriers effect on PSII activity". Soumis à *Photochemistry and Photobiology*. Dans ce travail, j'ai contribué à l'élaboration des idées concernant le projet, j'ai effectué la majorité des expériences de laboratoire. J'ai contribué à l'interprétation des résultats et j'ai écrit la première version du rapport.

CHAPITRE VII : Dewez, D., C. Dautremepuits, P. Jeandet, G. Vernet and R. Popovic. 2003. "Effects of methanol on photosynthetic processes and growth of *Lemna gibba*". *Photochemistry and Photobiology*, vol. 78, p. 420-424. Dans ce travail, j'ai contribué à l'élaboration des idées concernant le projet, j'ai effectué la majorité des expériences de laboratoire. Pour la contribution de l'étudiante Claire Dautremepuits, j'ai supervisé son travail dans la prise et le traitement des données. J'ai contribué à l'interprétation des résultats et j'ai écrit la première version du rapport.

CHAPITRE VIII : Geoffroy, L., D. Dewez, G. Vernet and R. Popovic. 2003. "Oxyfluorfen toxic effect on *S. obliquus* evaluated by different photosynthetic and enzymatic biomarkers". *Archives of Environmental Contamination and Toxicology*, vol. 45, p. 445-452. Dans ce travail, j'ai participé à la prise et au traitement des données, et j'ai participé à la rédaction du rapport. L'étudiante Laure Geoffroy a participé à la prise et au traitement des données.

CHAPITRE IX : Dewez, D., L. Geoffroy, G. Vernet and R. Popovic. 2005. "Determination of photosynthetic and enzymatic biomarkers sensitivity used to evaluate toxic effects of copper ions and Fludioxonil in alga *Scenedesmus obliquus*". *Aquatic Toxicology*, vol. 74, p. 150-159. Dans ce travail, j'ai contribué à l'élaboration des idées concernant le projet, j'ai effectué la majorité des expériences de laboratoire. Pour la contribution de l'étudiante Laure Geoffroy, j'ai supervisé son travail dans la prise et le traitement des données. J'ai contribué à l'interprétation des résultats et j'ai écrit la première version du rapport.

CHAPITRE X : Dewez, D., J. Vincent and R. Popovic. 2006. "Validation of photosynthetic-fluorescence parameters as biomarkers for Isoproturon toxic effect on alga *Scenedesmus obliquus*". Soumis à *Environmental Pollution*. Dans ce travail, j'ai contribué à l'élaboration des idées concernant le projet, j'ai effectué la majorité des expériences de laboratoire. Pour la contribution de l'étudiant stagiaire Jonathan Vincent, j'ai supervisé son travail dans la prise et le traitement des données. J'ai contribué à l'interprétation des résultats et j'ai écrit la première version du rapport.

CHAPITRE XI : Dewez, D., N. Boucher, F. Bellemare and R. Popovic. 2006. "Use of different fluorometric systems in the determination of fluorescence parameters from spinach thylakoid membranes being exposed to Atrazine and copper ions". Accepté et sous presse à *Toxicological and Environmental Chemistry, special edition*. Dans ce travail, j'ai contribué à l'élaboration des idées concernant le projet, j'ai participé aux expériences de laboratoire et au traitement des données. J'ai contribué à l'interprétation des résultats et j'ai écrit la première version du rapport. Nathalie Boucher a participé à la prise et au traitement des données.

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ADP	Adénosine diphosphate
APX	Ascorbate peroxydase (L-ascorbate:hydrogène-peroxyde oxydoréductase, EC 1.11.1.11)
ASC	Ascorbate
ATP	Adénosine triphosphate
CAT	Catalase (hydrogène-peroxyde:hydrogène-peroxyde oxydoréductase, EC 1.11.1.6)
CDO	Complexe de dégagement d'oxygène
Chl *a*	Chlorophylle *a*
Chl *b*	Chlorophylle *b*
CO_2	Dioxyde de carbone
CR	Centre réactionnel du photosystème
Cyt b_6f	Cytochrome b_6f
DCMU	3-(3,4-dichlorophényl)-1,1-diméthyl urée
DHA	Déhydroascorbate
DHAR	Déhydroascorbate réductase (glutathion:déhydroascorbate oxydoréductase, EC 1.8.5.1)
DQ	Duroquinone
DQH_2	Duroquinol
EDTA	Éthylène diamine tétraacétique acide
ERO	Espèces réactives de l'oxygène
F	Fluorescence variable induite par une illumination actinique continue
F'_M	Fluorescence maximale induite par un flash saturant sous une illumination actinique continue

F'_O	Fluorescence de base mesurée par une lumière analytique modulée, après une illumination dans le rouge lointain (735 nm)
Fd	Ferrédoxine
F_M	Fluorescence maximale induite par un flash saturant, après une adaptation à l'obscurité
FNR	Ferrédoxine NADP-réductase (ferrédoxine:$NADP^+$ oxydoréductase, EC 1.18.1.2)
F_O	Fluorescence de base mesurée par une lumière analytique modulée, après une adaptation à l'obscurité
GR	Glutathion réductase (glutathion:$NADP^+$ oxydoréductase, EC 1.8.1.7)
GST	Glutathion *S*-transférase (RX:glutathion R-transférase, EC 2.5.1.18, où R peut être un groupement aliphatique, aromatique ou hétérocyclique, et X, un groupement sulfate, nitrile ou halide)
LA	Lumière actinique continue
LHC	Complexes collecteurs de lumière («*Light Harvesting Complexes*»)
LM	Lumière modulée
LS	Lumière saturante
MV	Méthylviologène
NADP	Nicotinamide adénine dinucléotide phosphate sous forme oxydée
NADPH	Nicotinamide adénine dinucléotide phosphate sous réduite
NH_2OH	Hydroxylamine
PAM	«*Pulse Amplitude Modulated*»
PC	Plastocyanine
PEA	«*Plant Efficiency Analyzer*»
Phéo	Phéophytine
Pi	Phosphate inorganique
PQ	Plastoquinone

PQH_2	Plastoquinol
PS	Photosystème
P680	Centre réactionnel du photosystème II
P700	Centre réactionnel du photosystème I
Q_A	Quinone A, accepteur primaire d'électrons du photosystème II
Q_B	Quinone B, accepteur secondaire d'électrons du photosystème II
Q_E	Composant du «*quenching*» non photochimique dépendant de la formation du gradient de protons transthylacoïde
Q_{Emax}	«*Quenching*» dépendant du pH dans le *lumen* du thylacoïde
Q_I	Composant du «*quenching*» non photochimique dépendant des dommages aux complexes du PSII causés par la photoinhibition
Q_N	«*Quenching*» non photochimique
Q_P	«*Quenching*» photochimique
Q_{PQ}	«*Quenching*» associé au «*pool*» de plastoquinones oxydées
Q_T	Composant du «*quenching*» non photochimique dépendant des états de transtions I et II des photosystèmes
RL	Lumière dans le rouge lointain (735 nm)
RUBISCO	Ribulose bisphosphate carboxylase (3-phospho-D-glycérate carboxy-lyase, EC 4.1.1.39)
SOD	Superoxyde dismutase (superoxyde:superoxyde oxydoréductase, EC 1.15.1.1)
Y_Z	Donneur secondaire d'électrons du photosystème II
Φ_{PSII}	Efficacité photochimique maximale du photosystème II pour une plante adaptée à l'obscurité
Φ_{SPSII}	Efficacité photochimique opérationnelle du photosystème II, à l'état stationnaire du transport d'électrons, pour une plante adaptée à une illumination actinique continue

RÉSUMÉ

Dans cette thèse, le projet de recherche concerne l'investigation des effets toxiques des xénobiotiques sur le fonctionnement de l'appareil photosynthétique et l'état physiologique d'un système cellulaire ou d'un organisme multicellulaire. Le changement du rendement et des spectres de la cinétique de la fluorescence chlorophyllienne a été utilisé pour interpréter les mécanismes moléculaires de la photosynthèse lorsque la plante a été exposée à différents xénobiotiques. Les effets des xénobiotiques sur le changement des différents paramètres de fluorescence indiquant le rendement photochimique, la dissipation d'énergie *via* le transport d'électrons ou *via* les voies non photochimiques ont été utilisés pour déterminer des biomarqueurs qui sont indicateurs de toxicité. Dans notre recherche, nos avons utilisé l'algue unicellulaire *Scenedesmus obliquus* et des plantes supérieures (*Lemna sp.* et *Hordeum vulgare*) comme matériel pour les expériences. Des méthodes fluorométriques et spectroscopiques ont été utilisées pour étudier la réaction photochimique primaire et le transport des électrons photosynthétiques. Pour l'étude des réactions photochimiques du photosystème II *in vitro*, les fragments membranaires des thylacoïdes ont été utilisés. L'induction du stress oxydatif a été analysée par l'investigation de l'activité enzymatique de la catalase, l'ascorbate peroxydase, la glutathion réductase et la glutathion *S*-transférase.

Dans la partie concernant l'étude des mécanismes de toxicité des xénobiotiques, nous avons démontré chez *Lemna gibba* que le méthanol inhibe le rendement photochimique et la dissipation d'énergie sous forme non photochimique. Lorsque les concentrations de méthanol étaient au-dessous de 0,5 %, un effet stimulant a été montré sur la formation de la biomasse. Nous avons interprété cet effet comme un mécanisme d'acclimatation de la photosynthèse envers la toxicité du méthanol et ses produits de dégradation.

Dans l'étude de la toxicité de l'oxyfluorfène chez l'algue *Scenedesmus obliquus*, nous avons démontré que ce xénobiotique inhibe la synthèse de la chlorophylle et détériore le transfert d'énergie entre les antennes collectrices et le centre réactionnel du photosystème II. L'augmentation de l'activité des enzymes antioxydantes catalase et glutathion *S*-transférase a été interprétée comme une indication du stress oxydatif induit par l'oxyfluorfène. L'induction du stress oxydatif a également été démontrée comme une conséquence de l'effet toxique du fludioxonyl. Cet effet oxydatif a été indiqué par l'activité des enzymes catalase, ascorbate peroxydase et glutathion *S*-transférase. Il apparaissait que les effets du fludioxonyl sur la photosynthèse étaient négligeables. Cependant, chez la même espèce d'algue, l'inhibition du transport linéaire des électrons du photosystème II a été une conséquence directe des effets toxiques de l'isoproturon. Le changement des paramètres de fluorescence comme l'efficacité opérationnelle du photosystème II, le «*quenching*» photochimique et le ratio entre les antennes collectrices et le centre réactionnel, a montré une forte corrélation avec l'inhibition de la division cellulaire. L'étude des effets toxiques des ions cuivre a montré une forte inhibition de l'activité photochimique du PSII, une augmentation de la dissipation d'énergie sous forme non photochimique et une diminution de la proportion des antennes collectrices par centre réactionnel. L'induction d'un stress oxydatif a aussi été démontrée par le changement de l'activité de la catalase.

Dans la deuxième partie de l'étude, nous avons investigué les effets toxiques des xénobiotiques sur le changement des paramètres de fluorescence utilisés comme des biomarqueurs de toxicité sur la photosynthèse et l'état physiologique d'un système algal

unicellulaire et de l'organisme entier d'une plante. Pour le méthanol, l'atrazine, l'isoproturon, les ions cuivre, l'oxyfluorfène et fludioxonyl, nous avons montré que les paramètres de fluorescence indiquant le rendement photochimique du photosystème II, la dissipation d'énergie *via* le transport d'électrons ou les voies non photochimiques ont été les plus sensibles et fiables indicateurs de leurs effets toxiques sur la photosynthèse et sur l'état physiologique de l'organisme entier. Nous avons également déterminé que les paramètres de fluorescence peuvent montrer un changement structurel qui est induit par les différents xénobiotiques. Pour les xénobiotiques étudiés, nous avons démontré que le ratio entre les antennes collectrices et le centre réactionnel du photosystème II représente un biomarqueur sensible et fiable de leur toxicité. En conclusion, nous avons réussi à déterminer les paramètres les plus sensibles et fiables pouvant être employés comme des biomarqueurs dans les bioessais de toxicité.

INTRODUCTION GÉNÉRALE

L'accumulation de polluants dans l'environnement aquatique peut causer une dégradation des écosystèmes en induisant une toxicité sur de nombreux organismes. En particulier, la productivité primaire des écosystèmes aquatiques peut être affectée par les polluants *via* l'inhibition de la photosynthèse des plantes et des algues qui sont les principaux producteurs de la biomasse. Nous pouvons postuler que la photosynthèse représente un important biomarqueur physiologique parce que la reconnaissance des liaisons entre la photosynthèse et le métabolisme cellulaire peut permettre de détecter les effets toxiques des polluants.

Depuis les 20 dernières années, l'utilisation de la fluorescence chlorophyllienne émise par les plantes a offert une approche originale pour l'étude *in vivo* de l'activité photosynthétique. La mesure de la cinétique de fluorescence offre différents paramètres qui indiquent les réactions photochimiques et biochimiques de la photosynthèse. L'interprétation de ces paramètres à l'échelle moléculaire est complexe et constitue un sujet très actuel. Dans notre étude, la cinétique de la fluorescence chlorophyllienne est utilisée pour interpréter les mécanismes moléculaires de la photosynthèse lorsque des plantes ou des algues unicellulaires sont exposées à des xénobiotiques. Ainsi, l'approfondissement de ces connaissances a permis d'utiliser la fluorescence chlorophyllienne comme un indicateur des effets toxiques de xénobiotiques dans des bioessais. Dans une approche parallèle, les paramètres indiquant la croissance cellulaire et l'activité des enzymes antioxydatifes ont aussi été utilisés pour évaluer les effets toxiques cellulaires par les xénobiotiques. L'étude de la sensibilité des paramètres de fluorescence concernant la photosynthèse, des paramètres de la croissance cellulaire et de l'activité des enzymes antioxydatifes permet de définir des critères méthodologiques pour un bioessai de toxicité utilisant des plantes ou des algues. Dans le chapitre suivant, une introduction aux problèmes de la pollution de l'environnement va être présentée avant d'exposer le projet de recherche au Chapitre II.

CHAPITRE I

LES PROBLÈMES DE LA POLLUTION DE L'ENVIRONNEMENT

1.1 Sources et conséquences de la pollution sur les écosystèmes et les sociétés humaines

L'accroissement de la population a nécessité le développement de technologies associées aux services sociaux, à l'exploitation des ressources naturelles et à leurs transformations en biens de consommation. Cependant, les technologies modernes sont devenues des sources importantes de pollution, causant une altération de l'intégrité des écosystèmes. En majorité, la pollution provient des activités humaines dans les domaines agricoles, du transport, domestiques, militaires, industrielles et minières (voir Tableau 1.1).

Tableau 1.1 Différentes activités humaines génératrices de polluants environnementaux (d'après Manahan, 1997).

Activités humaines	**Groupes de polluants**
Pratiques agricoles	Métaux lourds (ex. Cu), pesticides (organophosphates, organochlorés, carbamates et pyréthrinoïdes) et engrais (nitrates et nitrites).
Moyens de transport	Hydrocarbures aromatiques polycycliques (HAP), métaux lourds (ex. plomb), dioxines, oxydes d'azote, composés soufrés, et produits pétroliers (ex. benzène).
Activités domestiques	Déchets, détergents (phosphates) et HAP.
Activités militaires	Explosifs organiques (ex. TNT) et atomiques.
Activités industrielles et minières	Biphényls polychlorés, dioxines, oxydes d'azote, composés soufrés, composés organométalliques, polymères synthétiques, produits pétroliers, radionucléides, métaux lourds et HAP.

Ces contaminants peuvent se propager sur de longues distances à partir de leur source et persister dans l'environnement pendant de longues périodes (d'après Bliefert et Perraud,

2001). La bioaccumulation des polluants par les organismes et la bioamplification via la chaîne alimentaire sont les principaux processus qui causent une dégradation de la biosphère en affectant la viabilité des espèces biologiques qui composent les différents niveaux trophiques (Newman et Unger, 2003). L'accumulation des pesticides et des métaux lourds dans l'environnement provoque une dégradation de l'intégrité des écosystèmes en modifiant le cycle de la biomasse, en réduisant la biodiversité et en affectant les niches écologiques de différentes espèces (Pimentel et Edwards, 1982; Bernard, 1997). De plus, la contamination de l'air et l'eau peut induire des effets nocifs sur la santé humaine en favorisant à long terme le développement de maladies respiratoires, gastro-intestinales et le cancer (Pimentel *et al.* 1992; Krupa, 1997). D'autre part, l'altération des écosystèmes entraîne indirectement de mauvaises conséquences sur l'économie sociale, car le développement économique des sociétés dépend essentiellement de l'exploitation et de la transformation des diverses ressources naturelles (Daily *et al.*, 1997). Par exemple, la pollution atmosphérique par l'ozone, le dioxyde de soufre, les oxydes d'azote et les métaux lourds peuvent induire des dommages importants sur le rendement des cultures agricoles et forestières (Chappelka et Freer-Smith, 1995). La détérioration des processus écologiques peut occasionner des pertes socio-économiques ou bien des dépenses supplémentaires à long terme dans le domaine agricole, médical ou industriel. Par conséquent, la conservation et la protection de l'intégrité des écosystèmes sont importantes à long terme pour le bien être et la prospérité des populations humaines.

Pour la biosurveillance de la qualité de l'environnement, il existe différentes approches méthodologiques qui permettent d'analyser l'état des écosystèmes. Ces méthodes peuvent être réparties en deux principales catégories :

1. Les méthodes chimiques d'analyse qui permettent d'identifier et de quantifier les polluants dans les milieux physiques et biologiques (eau, sol, sédiments, végétaux, animaux). Par cette approche, il est possible de mesurer la source, l'étendue et l'importance de la contamination de l'environnement (Geerdink *et al.*, 2002; Auersperger *et al.*, 2005).
2. L'évaluation des effets toxiques des polluants sur différents organismes vivants au niveau des individus (unicellulaire et multicellulaire), des populations et/ou des communautés.

Cette approche permet d'estimer le risque de toxicité et l'impact des polluants dans la biosphère (Wenzel *et al.*, 1997; Lytle et Lytle, 2001).

Après de nombreuses années de recherche dans ce domaine, aucune de ces méthodes seules ne permet de fournir des informations fiables et complètes sur l'état de l'environnement. Il a été proposée que seulement une combinaison de ces différentes approches méthodologiques pourrait permettre une évaluation fiable de l'état des milieux et des organismes vivant (Lagadic *et al.*, 1998).

Dans les écosystèmes, les plantes et les algues jouent un rôle important en produisant la matière organique qui sert de source d'énergie et d'éléments nutritifs pour les organismes des niveaux trophiques supérieures (Benenati, 1990). Les végétaux ont aussi une forte capacité de bioaccumulation des polluants, ce qui peut provoquer des conséquences néfastes sur les organismes des niveaux trophiques supérieures (Ribeyre et Boudou, 1990). Il est donc nécessaire d'effectuer des études de phytotoxicité pour déterminer la sensibilité de plusieurs espèces de végétaux (plantes vasculaires et algues) envers différentes classes de polluants (Nitschke *et al.*, 1999).

1.2 Recherche de biomarqueurs dans les études des effets toxiques des polluants

Un biomarqueur est un changement observable et/ou mesurable au niveau moléculaire, biochimique, cellulaire, physiologique ou comportemental d'un organisme, qui indique la présence d'un ou plusieurs substances toxiques dans le milieu (Lagadic *et al.*, 1997). Dans l'état actuel des connaissances en toxicologie de l'environnement, les biomarqueurs peuvent indiquer les effets toxiques de xénobiotiques dans les organismes et la détection d'une pollution dans un milieu (Lagadic et Caquet, 1996). Il est donc important de déterminer des biomarqueurs potentiels et de caractériser leur sensibilité, fiabilité, reproductibilité et portée informative envers la toxicité des xénobiotiques. Pour déterminer la valeur prédictive des biomarqueurs, l'étude des effets des polluants sur des espèces sensibles doit s'effectuer dans des conditions environnementales contrôlées, tels que des biotests au laboratoire, des expérimentations en microcosmes ou en mésocosmes (écosystèmes reconstitués). La

démarche expérimentale doit aussi permettre d'acquérir des connaissances fondamentales concernant les mécanismes de toxicité et les sites d'action des polluants (Figure 1.1) : La toxicité induite dans le système cellulaire dépendra de la concentration, de la réactivité et de la persistance du polluant. De plus, ce type d'étude toxicologique va permettre de déterminer la variation des biomarqueurs causés par la toxicité de différents polluants. Il s'agit de déterminer les biomarqueurs qui permettront un diagnostique précoce de la toxicité des xénobiotiques, par rapport à leur concentration et leur temps d'exposition, bien avant que des effets irréversibles sur la croissance ou le développement du végétal se manifestent.

Pour augmenter la fiabilité des bioessais utilisant les algues et les plantes supérieures, les biomarqueurs utilisés doivent être reliés aux processus biochimiques et physiologiques qui sont essentiels pour le fonctionnement cellulaire (Grimme *et al.*, 1993; Ferrat *et al.*, 2003). Cette approche peut contribuer à la détermination de la sensibilité des paramètres envers les effets toxiques de différents polluants. La division cellulaire représente un biomarqueur intégrateur de toxicité sur la croissance de la biomasse (Nyholm et Källgvist, 1989; Hörnström, 1990). La quantification de la chlorophylle, de l'acide désoxyribonucléique (ADN), des protéines, ou de l'ATP a aussi été utilisée comme indicateur indirect de la biomasse (Van Der Heever et Grobbelaar, 1996). Ces dernières méthodes sont relativement complexes et les résultats obtenus sont difficiles à corréler avec la division cellulaire. De plus, certains polluants peuvent induire un stress oxydatif en causant une altération du système cellulaire. Dans ce cas, les réactions enzymatiques antioxydatifes peuvent être utilisées comme biomarqueurs d'un stress oxydatif induit par des polluants (Tang *et al.*, 1998; Geoffroy *et al.*, 2002). D'autre part, la photosynthèse comporte les processus biochimiques les plus importants en assurant l'énergie essentielle au métabolisme cellulaire et à la croissance de la biomasse. Puisque l'appareil photosynthétique est une cible pour de nombreux polluants, la photosynthèse représente l'un des biomarqueurs les plus prometteurs pour la détection des stress et de leurs effets sur la physiologie des plantes (Lichtenthaler, 1996; Gaspar *et al.*, 2002; Popovic *et al.*, 2003).

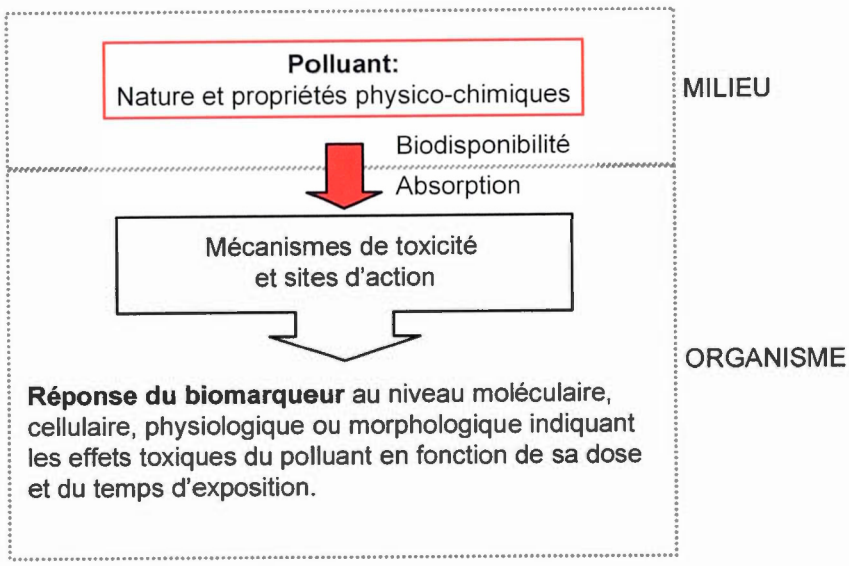

Figure 1.1 Différents aspects considérés dans les études des effets toxiques des polluants sur les organismes vivants (schéma élaboré à partir de Lagadic *et al.*, 1997).

CHAPITRE II

PROJET DE RECHERCHE : INVESTIGATION DES EFFETS TOXIQUES DES XÉNOBIOTIQUES SUR L'ACTIVITÉ PHOTOSYNTHÉTIQUE COMME INDICATEUR DE L'ÉTAT PHYSIOLOGIQUE DES PLANTES

2.1 Modèle général du projet de recherche

La conceptualisation d'un projet de recherche scientifique est essentielle pour définir des problématiques et élaborer une approche méthodologique permettant un progrès des connaissances. Dans cette thèse, le projet de recherche a été élaboré en considérant les concepts fondamentaux qui concernent l'activité photosynthétique à l'échelle moléculaire lorsque la plante est exposée aux effets toxiques des polluants. Les fonctions photosynthétiques ont été étudiées en utilisant les paramètres de la fluorescence chlorophyllienne (Figure 2.1).

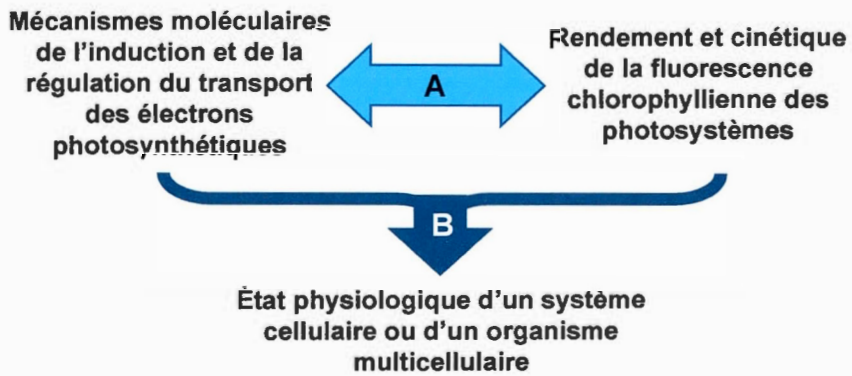

Figure 2.1 Organisation conceptuelle du projet de recherche scientifique concernant l'investigation des effets toxiques des xénobiotiques sur le fonctionnement de l'appareil photosynthétique et l'état physiologique d'un système cellulaire ou d'un organisme multicellulaire.

Deux principales problématiques de recherche ont été définies et indiquées dans ce modèle (Figure 2.1) comme deux composantes complémentaires A et B. A : L'utilisation de

la fluorescence chlorophyllienne dans l'évaluation de l'activité du transport des électrons photosynthétiques affectée par différents paramètres environnementaux, tels que l'intensité lumineuse et la composition chimique du milieu. B : Investigation des paramètres photosynthétiques comme biomarqueurs de l'état physiologique du système cellulaire ou de l'organisme végétal entier. Dans cette étude, l'altération de l'état physiologique est évaluée par le changement de plusieurs biomarqueurs comme des paramètres photosynthétiques de fluorescence, des paramètres de croissance et des paramètres biochimiques.

À partir de ces problématiques, il a été possible de définir différentes hypothèses de travail ainsi que les objectifs qui permettent de les aborder. Ils seront présentés au chapitre III. Avant cela, le contexte théorique qui compose ce projet de recherche sera présenté dans les prochains chapitres.

2.2 Mécanismes moléculaires de l'induction et de la régulation du transport des électrons photosynthétiques

2.2.1 Le site de la photosynthèse

Durant la photosynthèse, les plantes et les algues absorbent l'énergie lumineuse pour la convertir en glucose et en énergie chimique selon l'équation suivante :

$6\ CO_2 + 12\ H_2O + \text{Énergie lumineuse (hv)} \rightarrow C_6H_{12}O_6 + 6\ O_2 + 6\ H_2O$

Ce processus photobiochimique produit de l'oxygène par la photolyse de l'eau permettant la fixation du CO_2 et la biosynthèse de composés organiques tels que les hydrates de carbone (Miller, 1979).

Les mécanismes de la photosynthèse s'effectuent en deux phases : Durant la première phase, les réactions photochimiques, en induisant un transport des électrons entre les photosystèmes II et I, permettent la synthèse de l'ATP et du NADPH. L'ATP et le NADPH sont ensuite utilisés lors de la seconde phase au cours de laquelle une succession de réactions

biochimiques, constituant le cycle de Calvin, permettent la fixation du CO_2 atmosphérique et la biosynthèse des glucides (Ort et Whitmarsh, 2001).

L'ensemble des réactions photochimiques et biochimiques s'effectue dans les organites des cellules végétales que l'on nomme «chloroplaste». Le développement de la microscopie électronique a permis de bien définir la structure du chloroplaste (Malkin et Niyogi, 2000). Cet organite possède deux membranes lipidiques, une interne et une externe, séparant le *stroma* du cytoplasme cellulaire (Figure 2.2). Ces deux membranes permettent les transports gazeux (O_2, CO_2), ioniques (Mg^{2+}, Ca^{2+} et H^+) et moléculaires ($C_6H_{12}O_6$) nécessaires au fonctionnement de la photosynthèse. Les réactions photochimiques s'effectuent par des complexes pigments-protéiques situés dans un système membranaire à l'intérieur du chloroplaste, appelé «thylacoïde». Les enzymes et les cofacteurs intervenant dans les réactions biochimiques de la photosynthèse sont localisés dans le *stroma* du chloroplaste (Figure 2.2).

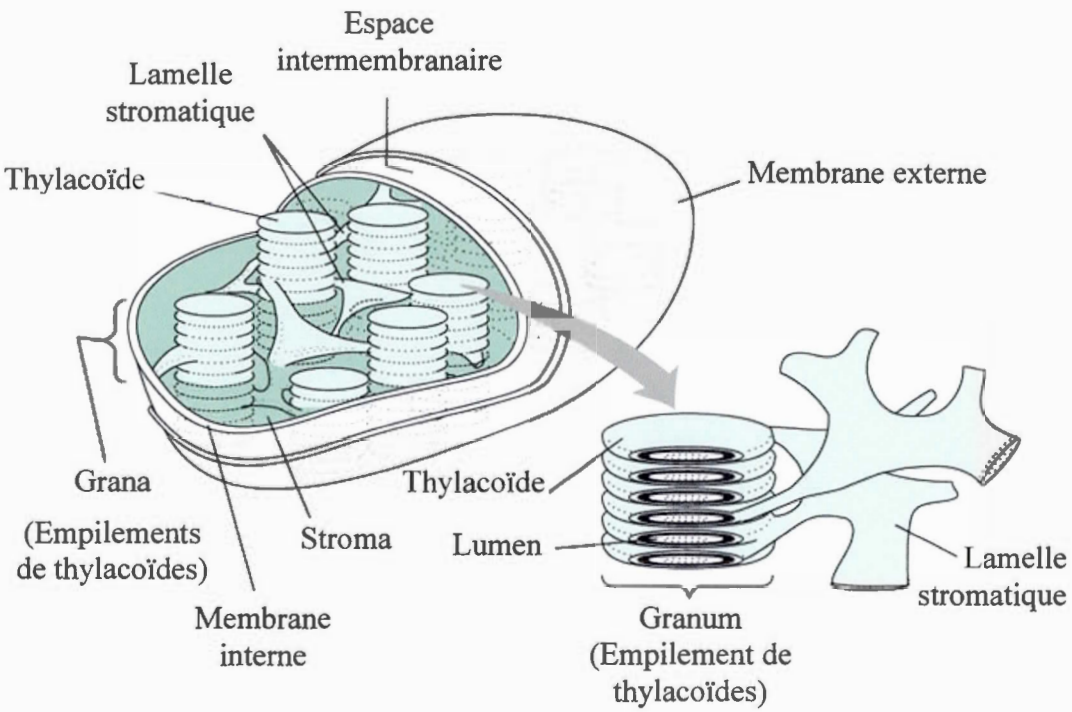

Figure 2.2 Vue tridimensionnelle d'un chloroplaste (schéma d'après Ort, 1986).

Les thylacoïdes sont constitués de vésicules aplaties (les lamelles) dont les empilements forment des *grana* qui sont reliés entre eux par des lamelles stromatiques (Ort et Whitmarsh, 2001). Dans la bicouche phospholipidique de la membrane des thylacoïdes, différents complexes pigments-protéiques qui servent au transport des électrons et protons : Les antennes collectrices de lumière reconnues comme «*Light Harvesting Complex of PSII*» (LHCII) et «*Light Harvesting Complex of PSI*» (LHCI) sont formées de pigments (chlorophylles et caroténoïdes) associés à des complexes protéiques membranaires. Les centres réactionnels des photosystèmes II (PSII) et photosystèmes I (PSI) effectuent les réactions photochimiques primaires en utilisant l'excitation de l'énergie lumineuse transférée par les antennes collectrices de lumière. Les plastoquinones, le complexe cytochrome b_6f et les plastocyanines forment une chaîne de transporteurs des électrons entre le PSII et le PSI. L'ATP synthétase est un système enzymatique transmembranaire qui permet la synthèse de l'ATP (Le fonctionnement est expliqué plus en détails aux Figures 2.11 et 2.12). Les complexes pigments-protéines du PSII et leurs antennes collectrices de lumière se situent dans les régions granaires alors que ceux du PSI, les complexes cytochromes b_6f et l'ATP synthétase sont localisés dans les régions inter-granaires (lamelles stromatiques).

2.2.2 Les aspects structuraux du photosystème II

Le PSII est constitué de complexes pigments-protéiques (comportant environ entre 200 et 300 molécules de chlorophylle et 50 molécules de caroténoïdes), de complexes métallo-protéiques (une vingtaine de polypeptides différents, deux quinones Q_A et Q_B, un ion ferrique, deux molécules de phéophytine, une ou deux molécules de cytochrome b_{559} et un «*cluster*» de manganèse) et des ions chlorure et de calcium servant comme cofacteurs (Whitmarsh et Govindjee, 2002). Ces complexes sont organisés en sous-unités fonctionnelles permettant un fonctionnement efficace du PSII (Figure 2.3) : Les antennes collectrices de lumière assurent le transfert de l'énergie d'excitation absorbée de la lumière vers les centres réactionnels (Owens, 1996).

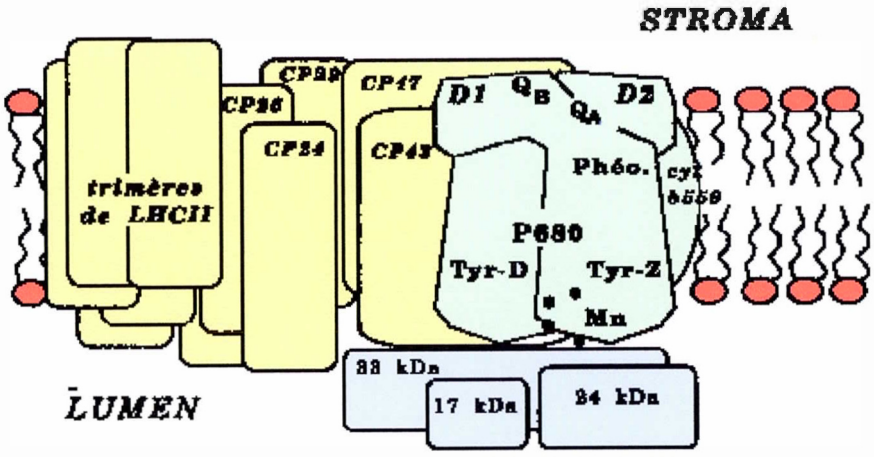

Figure 2.3 Organisation structurale du complexe polypeptidique du PSII (d'après Dau, 1994; Seidler, 1996).

2.2.2.1 Le centre réactionnel du photosystème II

Les protéines D1 et D2 (de 32 et 34 kDa respectivement) forment le centre actif du PSII qui effectue la séparation de charges, c'est-à-dire la réaction photochimique primaire et l'induction du transport des électrons. Ces polypeptides stabilisent les pigments et les transporteurs d'électrons du centre réactionnel. Le centre réactionnel du PSII possède une paire spéciale de chlorophylles jouant un rôle primordial dans la séparation de charges. Le centre réactionnel du PSII est noté P680, l'absorption maximale étant à 680 nm (Jupin et Lamant, 1999).

2.2.2.2 Les antennes collectrices de lumière du photosystème II

Il existe deux types d'antennes qui sont associées aux centres réactionnels des photosystèmes : les antennes internes et les antennes périphériques. Les antennes internes sont les complexes pigments-protéiques : CP24, CP26, CP29, CP43 et CP47 (ayant respectivement une masse moléculaire de 24, 26, 29, 43 et 47 kDa). Environ 40 à 50 molécules de Chl *a* et quelques molécules de β-carotènes sont associées à chacun de ces complexes (Bassi *et al.*, 1990). Les antennes internes contiennent en général moins de

chlorophylle *b* que les antennes périphériques, mais possèdent de la violaxanthine, de la zéaxanthine et des caroténoïdes. Les antennes périphériques, appelées LHCII, contiennent 50 à 60 % de la totalité des pigments chlorophylliens (Thornber *et al.*, 1991). La taille et la composition pigmentaire de ces antennes peuvent varier en fonction des conditions lumineuses (Horton *et al.*, 1996).

2.2.2.3 Le complexe du dégagement d'oxygène (CDO)

Le complexe de dégagement d'oxygène (CDO) est composé de trois polypeptides extrinsèques (du côté du lumen) de 33, 24 et 17 kDa participant dans la photolyse de l'eau (Ghanotakis *et al.*, 1999). Le polypeptide de 33 kDa stabilise le «*cluster*» de manganèse. Le polypeptide de 24 kDa participe directement à la réaction de la photolyse de l'eau durant laquelle interviennent des cofacteurs, les ions Ca^{2+} et Cl^-. Le polypeptide de 17 kDa optimise le rendement du dégagement d'O_2. Les ions chlorure et de calcium jouent aussi un rôle stabilisateur au complexe du dégagement de l'oxygène, car ils interviennent dans la régulation de la protéine de 24 kDa et catalysent la production d'oxygène (Hankamer *et al.*, 1997).

2.2.3 Les aspects fonctionnels du photosystème II

2.2.3.1 L'absorption et le transfert de l'énergie lumineuse jusqu'au centre réactionnel

Les antennes collectrices de lumière absorbent l'énergie lumineuse grâce aux différents pigments, notamment les chlorophylles *a* et *b* (Figure 2.4) et les caroténoïdes. Selon leur configuration moléculaire, chaque pigment absorbe efficacement à des longueurs d'ondes spécifiques. La somme de ces pigments forme un spectre d'absorption qui couvre presque entièrement le spectre d'émission des radiations solaires du visible (Figure 2.5), permettant à l'appareil photosynthétique d'exploiter l'énergie lumineuse avec un rendement optimal (Govindjee et Govindjee, 1974; Horton *et al.*, 1996). L'absorption d'un photon provoque le passage rapide (10^{-15} sec) du pigment photosynthétique de l'état stable à l'état excité. Les excitons générés par les photons absorbés sont transférés de façon aléatoire d'un pigment à

un autre dans le complexe collecteur de lumière jusqu'à la paire spéciale de Chl *a* du centre réactionnel (Figure 2.6).

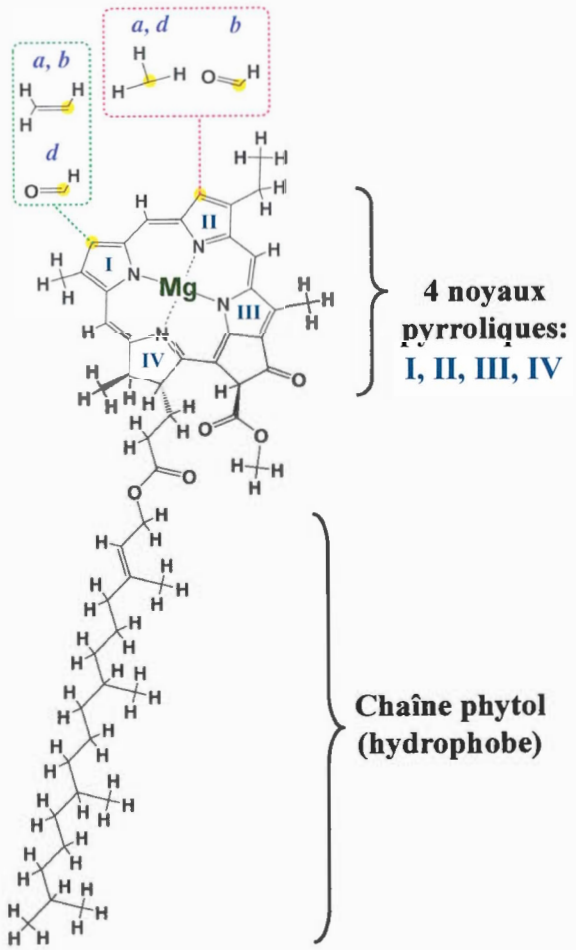

Figure 2.4 Structure chimique des chlorophylles *a*, *b* et *d* (d'après Horton *et al.*, 1996).

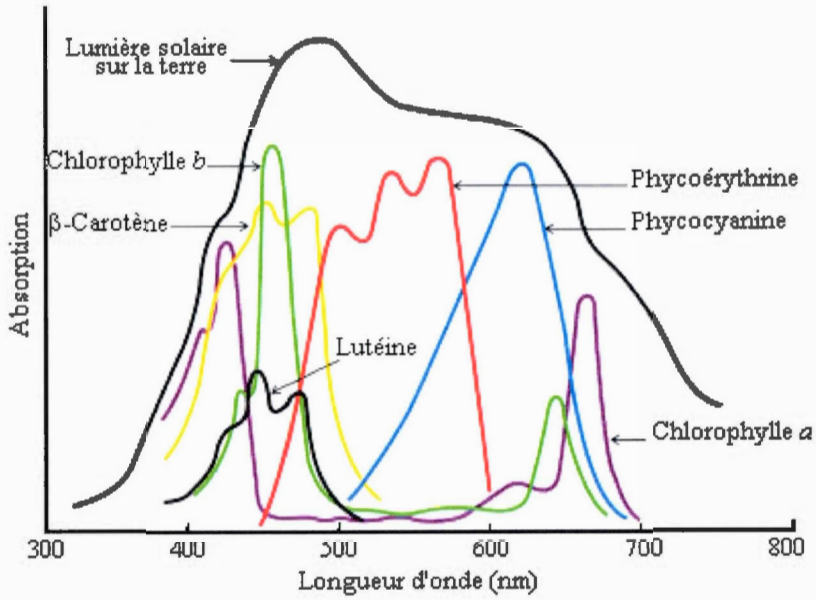

Figure 2.5 Spectre d'absorption des différents pigments présents chez les plantes et les algues (d'après Govindjee et Govindjee, 1974).

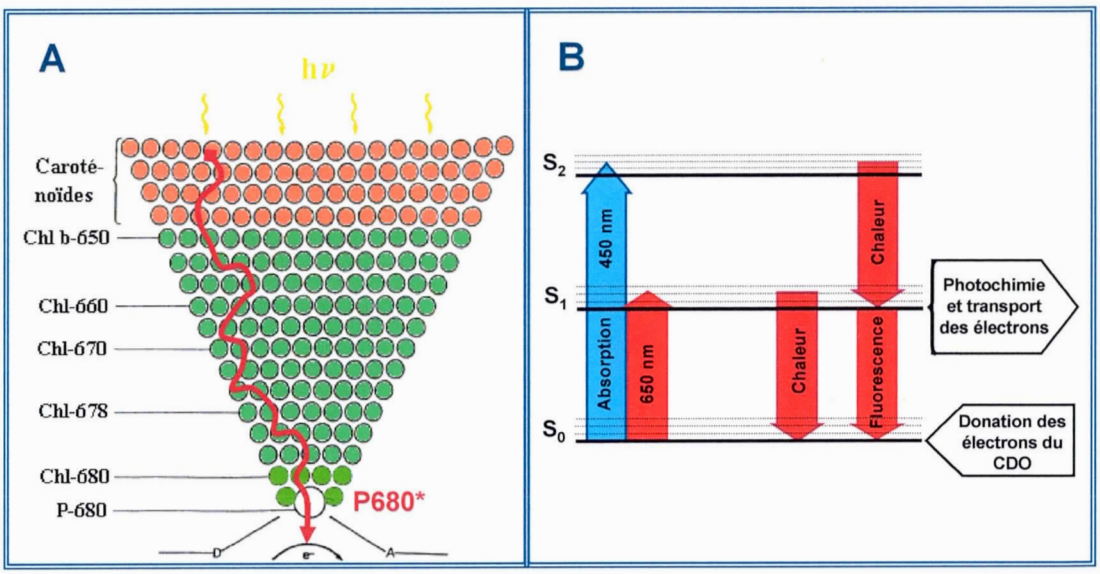

Figure 2.6 A) Schéma de l'organisation des pigments participant au transfert d'énergie dans le PSII. La flèche indique un cheminement aléatoire d'un quanta de lumière parmi les pigments jusqu'au centre réactionnel du PSII (d'après Jupin et Lamant, 1999). B) Niveau d'énergie dans une molécule de chlorophylle et les mécanismes de dissipation d'énergie (d'après Buchanan *et al.*, 2000).

2.2.3.2 La photooxydation de l'eau et la séparation de charges

L'efficacité de la réaction photochimique primaire du PSII dépend du transfer d'électron du centre réactionel P680 à l'état excité (P680*) vers la phéophytine *a* (Phéo). La séparation de charges va induire l'état $P680^{+}$/$Phéo^{-}$ au PSII (Dekker et van Grondelle, 2000). Cette perte d'électron va permettre au $P680^{+}$ de recevoir un électron du résidu tyrosine (Z), le donneur primaire du PSII, qui, dans l'état oxydé, acceptera un électron du complexe de dégagement d'oxygène (CDO) (Figure 2.7). Comme chaque réaction photochimique ne permet la libération que d'une charge positive, la formation d'une molécule d'oxygène exige quatre étapes photochimiques élémentaires (Hall et Rao, 1994; Whitmarsh et Govindjee, 2002).

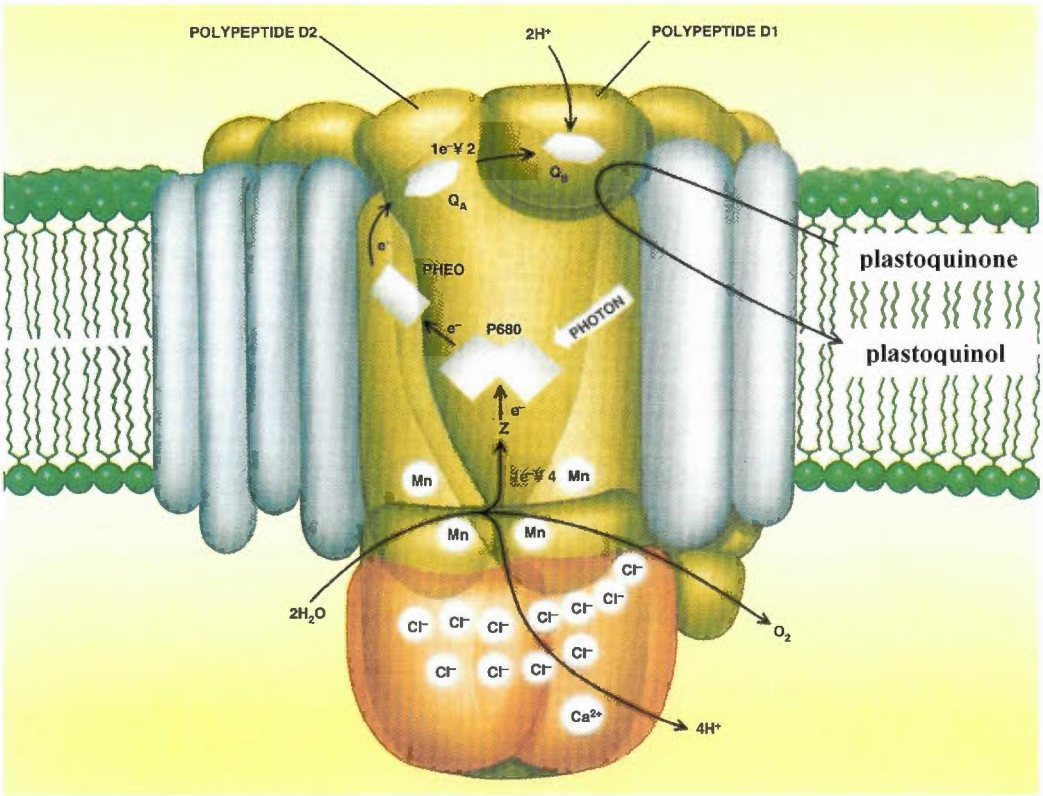

Figure 2.7 Modèle structurel du centre réactionnel du PSII, montrant les deux protéines dominante du PSII (D1 et D2), le centre réactionnel du PSII (P680), le complexe de dégagement d'oxygène (CDO) et son «*cluster*» de manganèse, ainsi que les protéines liant la chl *a*, soit le CP43 et CP47 (d'après Govindjee et Coleman, 2000).

Il a été observé qu'une séquence d'éclairs saturants sur des chloroplastes adaptés à l'obscurité, produisaient de l'oxygène lors d'un cycle de 4 étapes indiqués par les états S0, S1, S2, S3 et S4 (Figures 2.8 et 2.9). Le rendement maximal de dégagement d'oxygène est obtenu après le troisième éclair et par la suite à tous les quatrièmes éclairs. Le modèle des états S permet de comprendre comment la photooxydation de l'eau induit la réaction photochimique. Le «*cluster*» de manganèse du complexe de dégagement d'oxygène (CDO) accumule des charges positives (perte des électrons) fournissant le potentiel nécessaire pour la séparation de deux molécules d'eau en oxygène et 4 protons. Ces protons sont libérés dans le lumen contribuant à la formation d'un gradient transmembranaire de protons nécessaire à la synthèse de l'ATP. Une fois l'état S_4 atteint, le CDO retourne spontanément à l'état S_0 en réagissant avec l'eau pour prendre ses électrons et libérer de l'oxygène (Kok *et al.*, 1970).

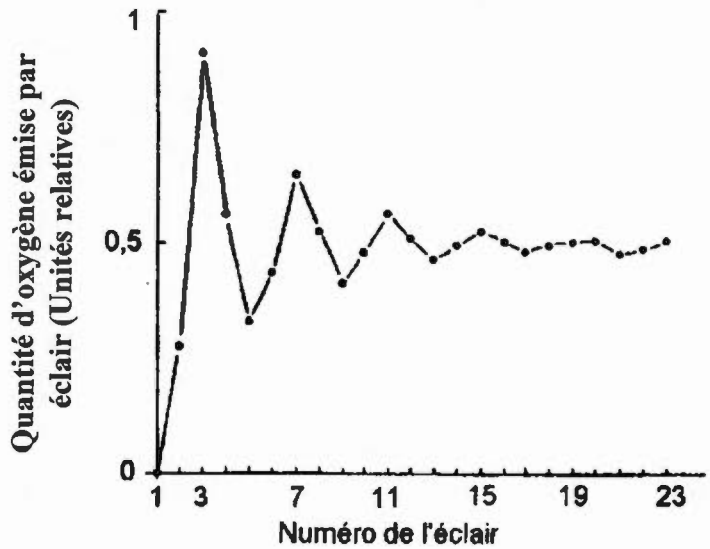

Figure 2.8 Production d'oxygène sous l'effet d'éclairs saturants (Joliot *et al.*, 1969).

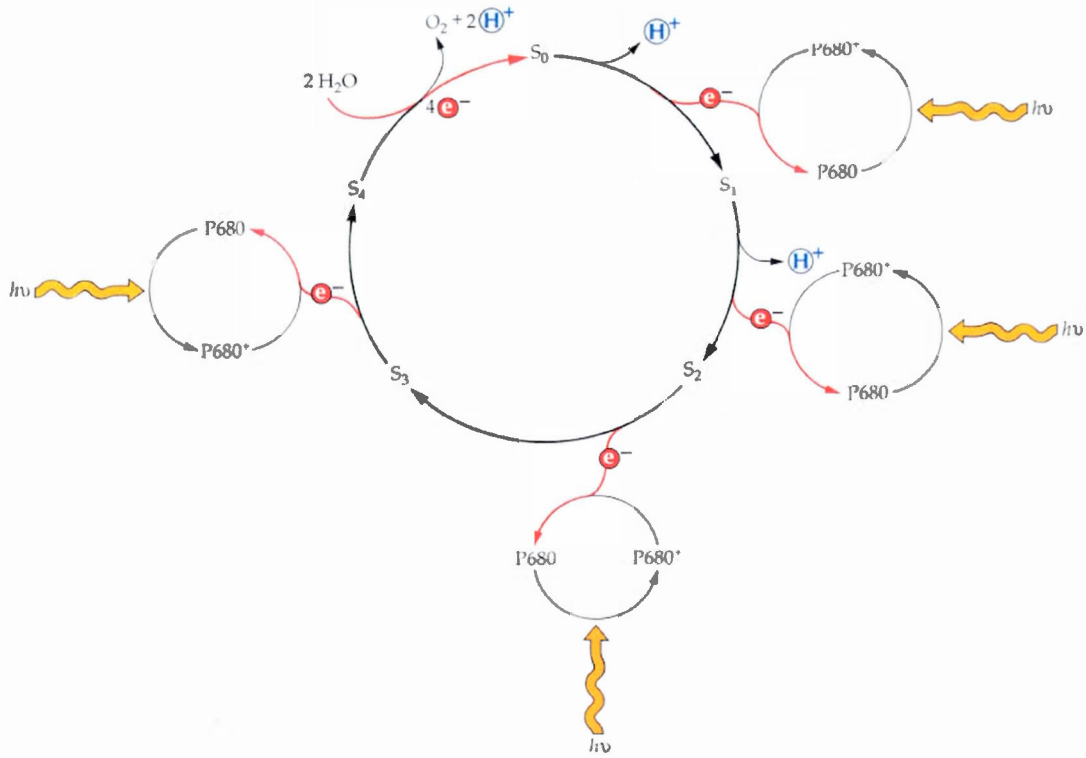

Figure 2.9 Schéma de la formation des états S et la production de protons pendant l'oxydation de l'eau conduisant au dégagement d'O_2. P680 : le centre réactionnel du PSII (Buchanan *et al.*, 2000).

2.2.4 Le transport transmembranaire des électrons

Dans le PSII, l'électron capté par la phéophytine *a* est ensuite transféré par une chaîne de transport composant les quinones (Q_A et Q_B) et les plastoquinones (PQ) (Figure 2.10). Deux électrons sont nécessaires pour réduire une PQ associée au site Q_B. Ces deux réactions d'oxydoréductions successives modifient la configuration stéréochimique de la PQ. Deux protons du *stroma* vont alors neutraliser la molécule. La plastoquinone réduite, PQH_2, diffuse dans la membrane des thylacoïdes et libère le site Q_B qui peut alors accepter une nouvelle PQ membranaire (Whitmarsh et Govindjee, 2002). Les molécules de plastoquinones réduisent les cytochromes b_6f, libérant ainsi deux protons dans le *lumen* du thylacoïde, ce qui contribue à

la formation du gradient transmembranaire de protons nécessaire pour la synthèse de l'ATP. Les cytochromes b_6f transfèrent les électrons à la plastocyanine, puis au $P700^+$ du PSI.

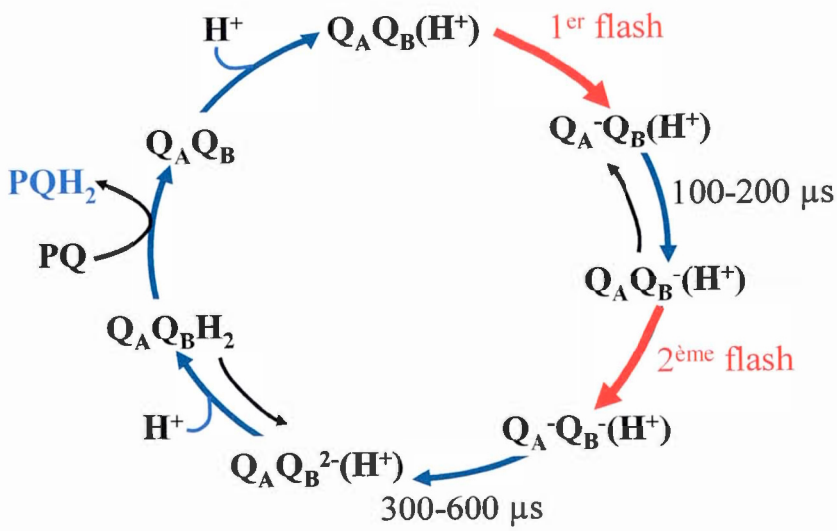

Figure 2.10 Représentation schématique des réactions d'oxydoréduction entre les quinones A (Q_A), B (Q_B) et plastoquinone (PQ) (d'après Whitmarsh et Govindjee, 2002).

La Figure 2.11 résume l'ensemble des réactions s'effectuant durant le transport linéaire des électrons photosynthétiques.

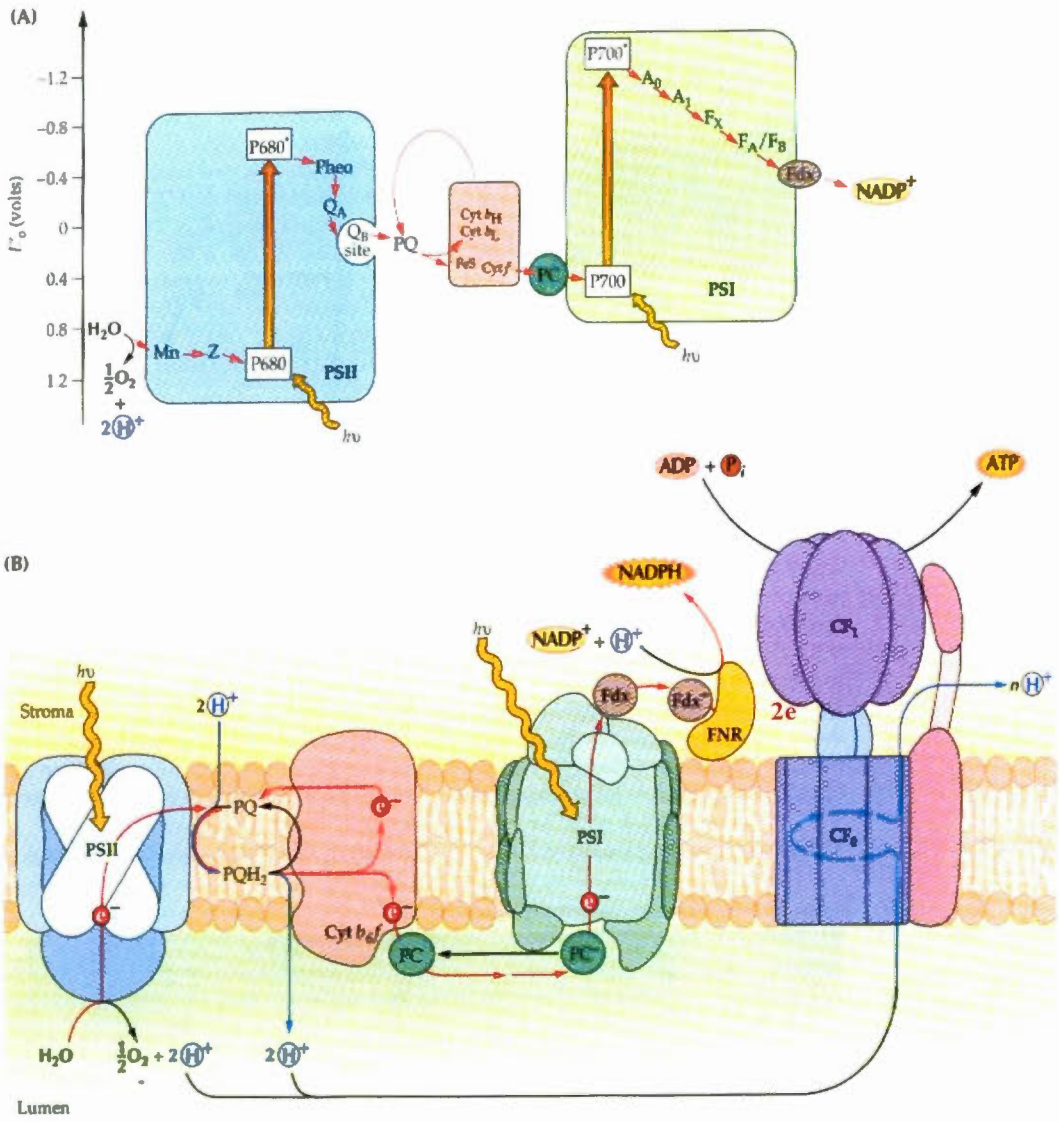

Figure 2.11 (A) Schéma présentant le potentiel redox (les valeurs moyennes de E_m) de chaque transporteur d'électrons transmembranaires. (B) Organisation des transporteurs d'électrons dans la membrane de thylacoïde. Les complexes membranaires sont le photosystème II, le photosystème I, le cytochrome b_{6f} et l'ATP synthétase (CF_1-CF_0). Les flèches indiquent le transfert des électrons (rouge) et la translocation des protons (bleu). PC, plastocyanine; PQ, plastoquinone; Fdx, ferredoxine; FNR, ferrédoxine NADP-réductase (ferrédoxine:$NADP^+$ oxydoréductase, EC 1.18.1.2) (d'après Malkin et Niyogi, 2000).

2.2.5 Aspects structuraux et fonctionnels du photosystème I

Le P700 passe à l'état excité (P700*) lorsque les antennes collectrices de lumière du PSI capturent l'énergie lumineuse. Au centre réactionnel du PSI, la séparation de charges s'effectue rapidement (1-3 ps) entre le P700* et une molécule de Chl *a*, notée A_0. Le $P700^+$ ainsi formé reçoit un électron de la plastocyanine réduite (PC) (Fromme *et al.*, 2001). Les électrons sont transférés *via* une phylloquinone (notée A_1), des centres Fe-S (désignés comme F_X, F_A et F_B) jusqu'à la ferrédoxine (Fdx) (Figure 2.12). La réduction du $NADP^+$ en NADPH est catalysée par la ferrédoxine NADP-réductase (ferrédoxine:$NADP^+$ oxydoréductase, EC 1.18.1.2). Le NADPH est utilisé comme réducteur au cours du cycle de Calvin pour la fixation du CO_2 et la synthèse d'hydrates de carbone (Greenbaum *et al.*, 1995).

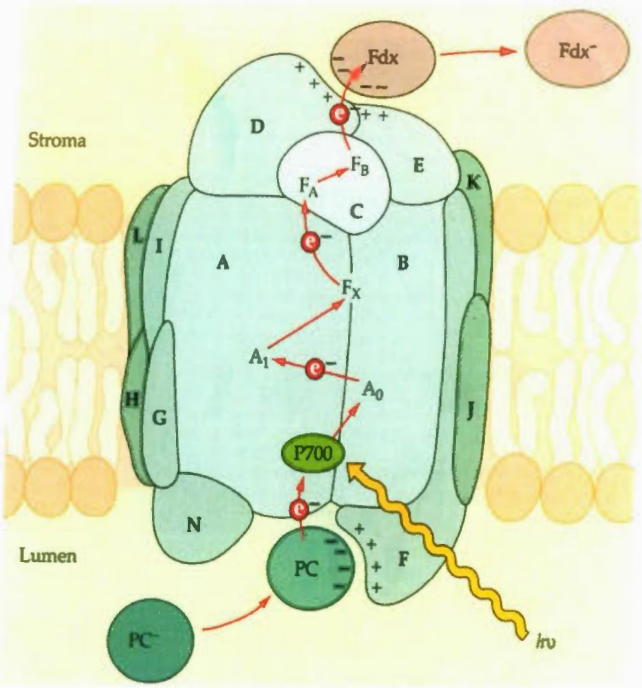

Figure 2.12 Modèle structurel du centre réactionnel du photosystème I présentant l'organisation des sous-unités protéiques (désignées par les lettres) (Malkin et Niyogi, 2000).

2.2.6 La photophosphorylation

Mitchell (1974) a proposé que la synthèse d'ATP soit contrôlée par un couplage «chimioosmotique» entre le transport membranaire des électrons et la formation du gradient de protons transthylakoidiens. Cette hypothèse a été démontrée par de nombreux travaux dont le modèle actuellement accepté est illustré à la Figure 2.13.

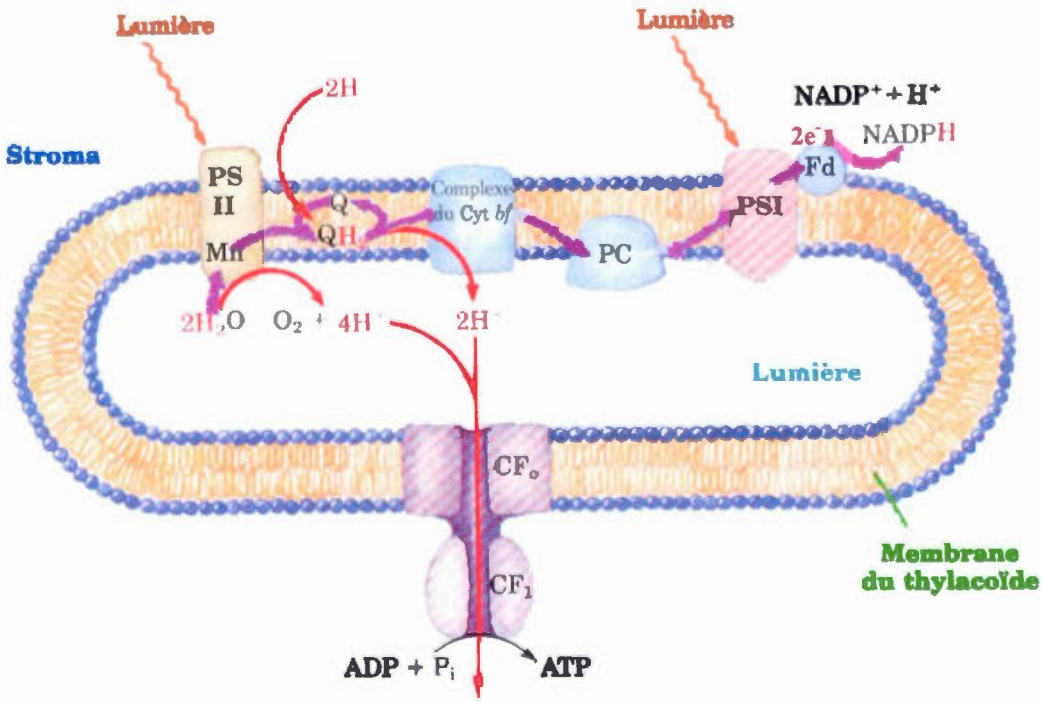

Figure 2.13 Modèle structurel d'un thylacoïde (d'après Lehninger *et al.*, 1994). PSII = photosystème II; Q = Quinone; QH_2 = quinone réduite; Cyt *bf* = cytochrome b_6f; PC = plastocyanine; PSI = Photosystème I; Fd = ferrédoxine; CF_x = facteur de couplage.

Le mécanisme de la synthèse d'ATP peut se résumer selon les étapes suivantes : L'induction du transport linéaire des électrons entre le PSII et le PSI génère un gradient de protons transmembranaire entre le *stroma* et le *lumen* du thylacoïde par l'intermédiaire de la plastoquinone-cytochrome b_6/f. Ce gradient de protons permet la synthèse de l'ATP *via* le fonctionnement de l'ATP synthétase (ATP phosphohydrolase, EC 3.6.3.14) (Boyer, 1993). Présente principalement dans les lamelles du *stroma*, cette enzyme est composée d'une partie

hydrophobe (CF_0) qui permet le passage des protons de l'intérieur vers l'extérieur des thylacoïdes et d'une partie hydrophile (CF_1) qui contient les sites catalytiques responsables de la synthèse de l'ATP à partir de l'adénosine diphosphate (ADP) et du phosphate inorganique (Pi) (Lehninger *et al.*, 1994).

2.2.7 La fixation du CO_2

Dans le *stroma* du chloroplaste, le CO_2 atmosphérique est assimilé et fixé en hydrates de carbone au cours du cycle de Calvin. Les réactions biochimiques de ce cycle nécessitent de l'ATP et du NADPH qui ont été produits par le transport des électrons couplé au transfert transmembranaire de protons (Heineke, 2001). Le cycle de Calvin s'effectue en trois étapes (Figure 2.14) : Durant la première phase, le CO_2 est fixé par la ribulose-1,5-bisphophate carboxylase (3-phospho-D-glycérate carboxylase, EC 4.1.1.39) (Bowles *et al.*, 1971), noté comme Rubisco, dans la carboxylation de la ribulose-1,5-bisphosphate formant le 3-phosphoglycérate. Durant la deuxième phase, le 3-phosphoglycérate est réduit en triose phosphate qui sera exporté dans le cytosol pour être convertie en sucrose. Enfin, la ribulose-1,5-bisphosphate est régénérée durant la dernière phase.

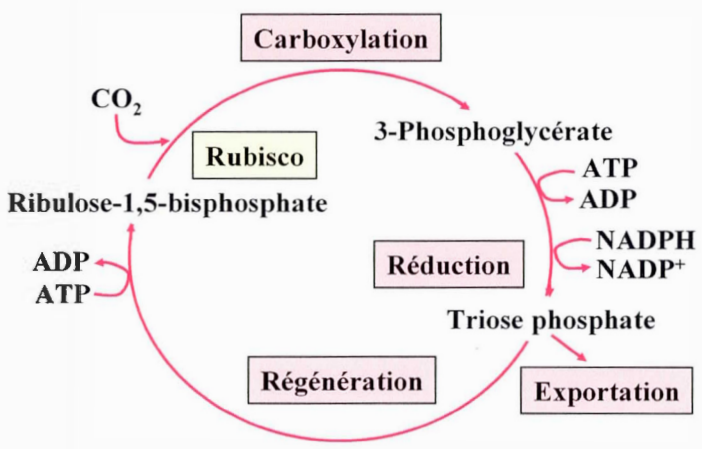

Figure 2.14 Les réactions biochimiques de la fixation du CO_2 lors du cycle de Calvin (d'après Heineke, 2001).

2.2.8 La régulation du transport transmembranaire des électrons

Lorsque les organismes photosynthétiques sont exposés à un ou plusieurs stress environnementaux (ex. : La toxicité des polluants, l'intensité lumineuse et/ou la température en excès), ils subissent une photoinhibition si l'appareil photosynthétique n'arrive pas à s'acclimater. Dans cette situation, la capacité fonctionnelle des photosystèmes est réduite et l'accumulation de l'énergie lumineuse en excès doit être dissipée sous une forme non dommageable pour l'organisme. Pour maintenir un fonctionnement optimal de la photosynthèse, l'appareil photosynthétique utilise un ensemble de mécanismes pour gérer l'énergie d'excitation excessive absorbée par les photosystèmes (Critchley, 1998, 1999). Actuellement, plusieurs mécanismes participent dans la régulation du transport des électrons photosynthétiques, mais les conditions qui induisent la participation de ces mécanismes de régulation restent encore un sujet actuel de recherche (Malkin et Niyogi, 2000).

La dissipation d'énergie sous forme de chaleur par les antennes collectrices de lumière du PSII constitue la principale voie d'évacuation de l'énergie en excès sous une forme non dommageable pour le biosystème (Horton *et al.*, 1994, 1996). Ce mécanisme est induit lorsque la concentration de proton devient trop importante dans le lumen du thylacoïde indiquant un flux excessif des électrons transmembranaires. Cette régulation de l'activité du PSII s'effectue *via* le cycle des xanthophylles (Müller *et al.*, 2001). Durant le cycle des xanthophylles, l'enzyme violaxanthine dé-époxydase (violaxanthine:ascorbate oxydoréductase, EC 1.10.99.3) catalyze la formation de la zéaxanthine en deux étapes (Rockholm et Yamamoto, 1996) :

1) violaxanthine + ascorbate = anthéraxanthine + déhydroascorbate + H_2O

2) anthéraxanthine + ascorbate = zéaxanthine + déhydroascorbate + H_2O

Pour une représentation schématique du cycle des xanthophylles, voir la Figure 2.15. La zéaxanthine s'associe ensuite aux complexes protéiques du PSII permettant la dissipation d'énergie accumulée (dans les LHCIIs) sous forme de chaleur. Il est proposé que cette conformation du PSII n'induise pas un transport linéaire des électrons, mais plutôt un transport cyclique des électrons impliquant le cytochrome b559, une sous-unité périphérique du PSII (Figure 2.16). La zéaxanthine est convertie en violaxanthine par la zéaxanthine

époxydase (zéaxanthine,NAD(P)H:oxygène oxydoréductase, EC 1.14.13.90) en deux étapes (Buch *et al.*, 1995) :

1) zéaxanthine + NAD(P)H + H^+ + O_2 = anthéraxanthine + NA(D)P^+ + H_2O

2) anthéraxanthine + NAD(P)H + H^+ + O_2 = zéaxanthine + NA(D)P^+ + H_2O

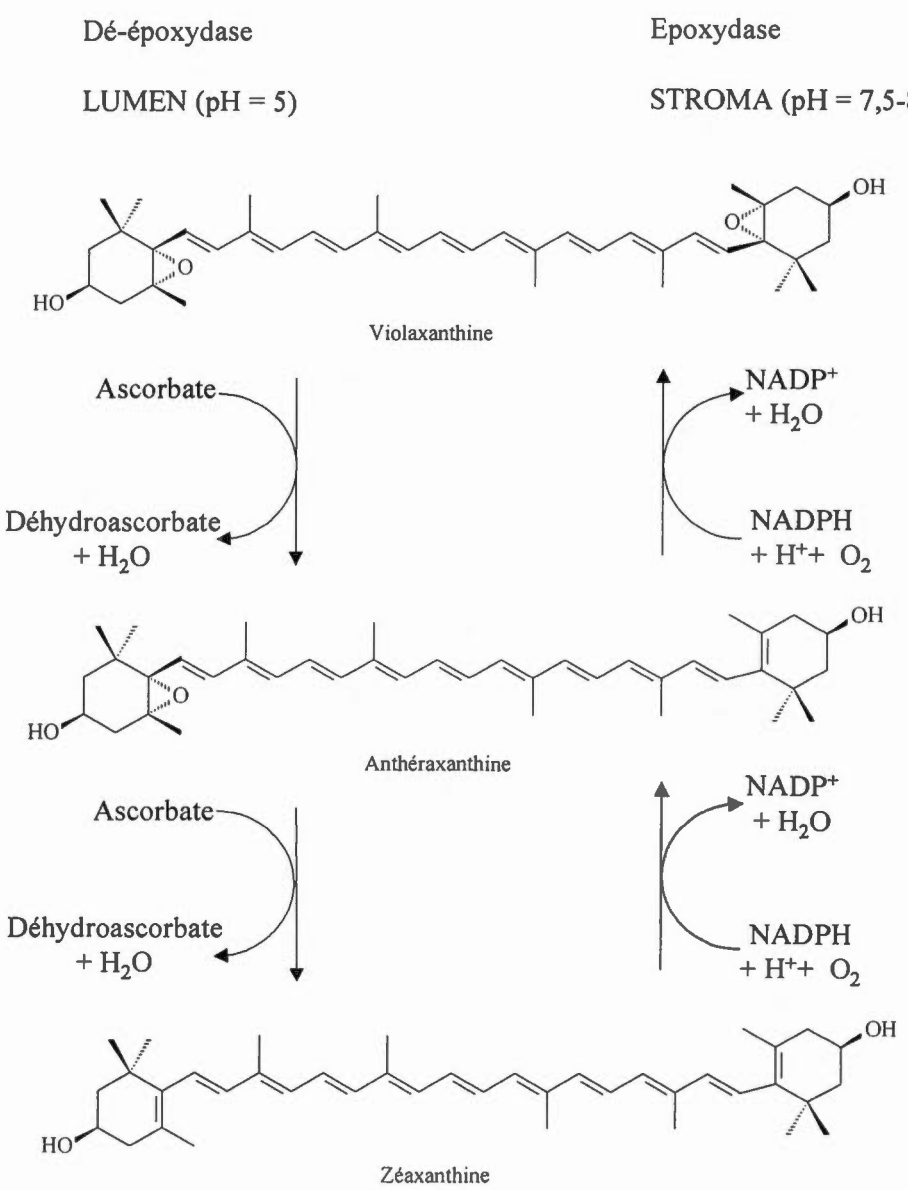

Figure 2.15 Cycle des xanthophylles se produisant dans le chloroplaste (Müller *et al.*, 2001).

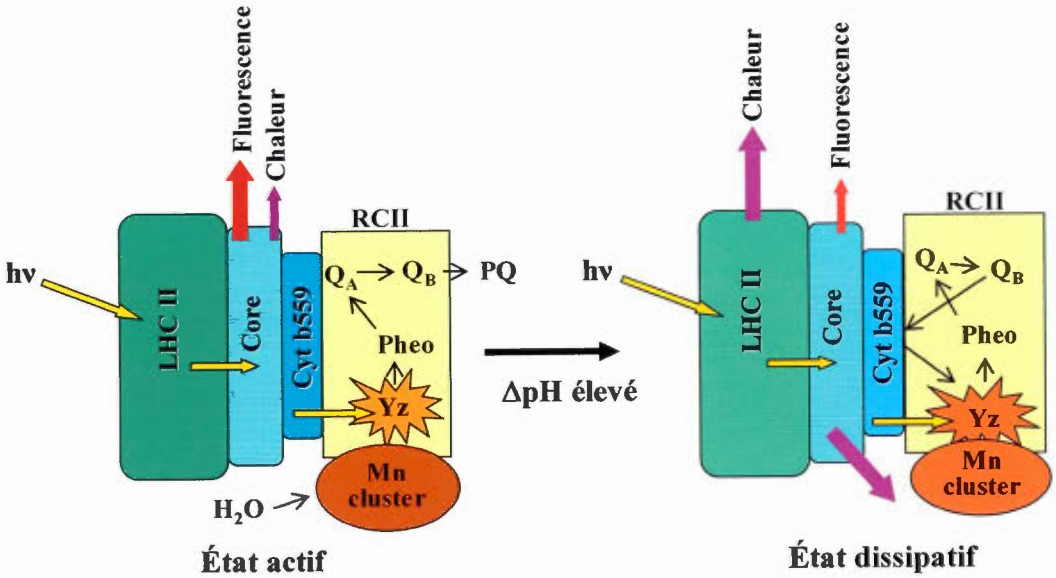

Figure 2.16 Changement structurel et fonctionnel du PSII exposé à des intensités lumineuses excessives (schéma élaboré à partir de Critchley, 1998).

Dans des conditions de stress physiologique créant un déficit en ATP pour le métabolisme cellulaire (ex. : le cycle de Calvin), le système photosynthétique thylacoïdien effectue un transport cyclique des électrons pour conserver un rendement élevé de la synthèse d'ATP (Bendall et Manasse, 1995). Ce transport cyclique des électrons implique le photosystème I, la ferrédoxine-plastoquinone (Fdx-PQ) oxydoréductase et le cytochrome b_6f (Figure 2.17). Comme le PSII n'intervient pas durant ce processus, il n'y a pas de production d'oxygène et de transfert d'électrons sur le $NADP^+$. Le transport cyclique des électrons produit un gradient transthylacoïdien de protons permettant uniquement la synthèse de l'ATP. Après sa réduction par le PSI, la ferrédoxine (Fdx) joue le rôle de cofacteur pour la ferrédoxine-plastoquinone oxydoréductase en transférant des électrons au complexe plastoquinone-cytochrome b_6f. Bien que le transport cyclique des électrons a été démontré *in vitro*, les facteurs qui contrôlent ce mécanisme *in vivo* ne sont pas encore bien compris (Bendall et Manasse, 1995; Malkin et Niyogi, 2000).

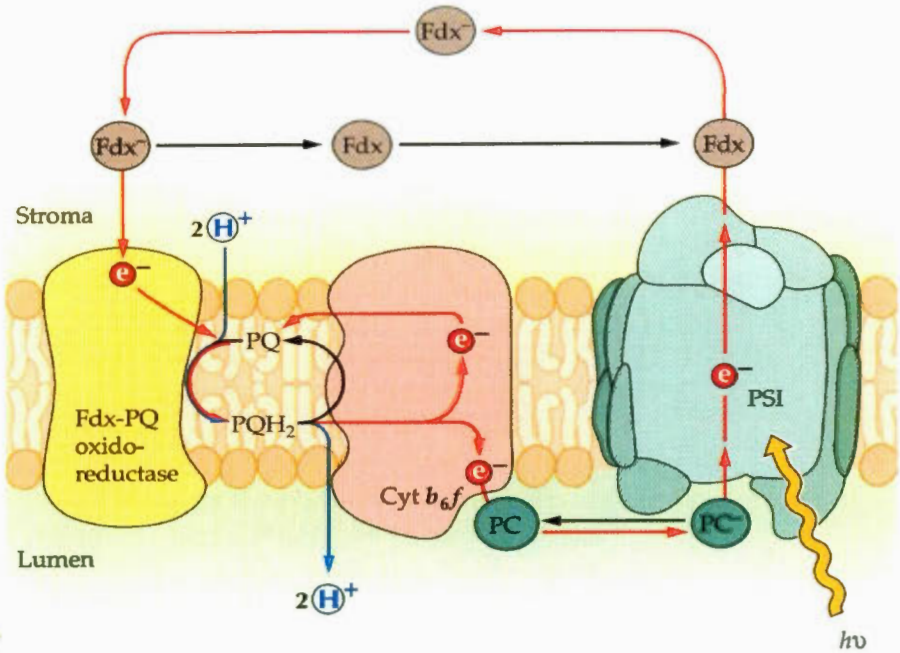

Figure 2.17 Mécanisme du transport cyclique des électrons s'effectuant entre le photosystème I, la ferrédoxine-plastoquinone (Fdx-PQ) oxydoréductase et le cytochrome b_{6f} dans la membrane de thylacoïde (d'après Malkin et Niyogi, 2000).

Chez les plantes supérieures et les algues vertes, les PSII et PSI ont des spectres d'absorption qui se chevauchent, sauf pour certaines longueurs d'ondes qui excitent préférentiellement le PSI. Grâce à la spectroscopie d'absorption différentielle, il est devenu possible de faire fonctionner préférentiellement un des deux photosystèmes par une lumière d'excitation ayant un pic à 650 nm (pour le PSII) ou à 710 nm (pour le PSI). La relation entre le transport d'électrons et la distribution d'énergie lumineuse absorbée des PSII et PSI peut se déséquilibrer, car ces deux photosystèmes placés en série ne fonctionnent pas à la même vitesse. Le phénomène du changement des états I et II permet d'optimiser le transport d'électrons entre les deux photosystèmes par la gestion de la capture de l'énergie lumineuse entre les PSII et PSI (Jupin et Lamant, 1999). Ce mécanisme est induit par la phosphorylation ou la déphosphorylation des antennes. Lorsque l'énergie absorbée par les antennes du PSII (LHCII) provoque un débalancement entre les activités du PSII et du PSI, les LHCIIs sont phosphorylées et se séparent du PSII pour se déplacer vers le PSI (Figure 2.18). Ce

phénomène permet la diminution de la surface d'absorption de l'énergie lumineuse au niveau du PSII et augmente celle du PSI.

La phosphorylation du LHCII du groupement phosphate chargé négativement induit sa séparation avec le PSII chargé aussi négativement. Les LHCII se déplacent alors vers les centres du PSI chargés positivement augmentant la surface d'absorption de l'énergie lumineuse du PSI. À la surface des thylacoïdes granaires, la phosphorylation des LHCII entraîne, par effet électrostatique, un désempilement de *grana* (Barber, 1983).

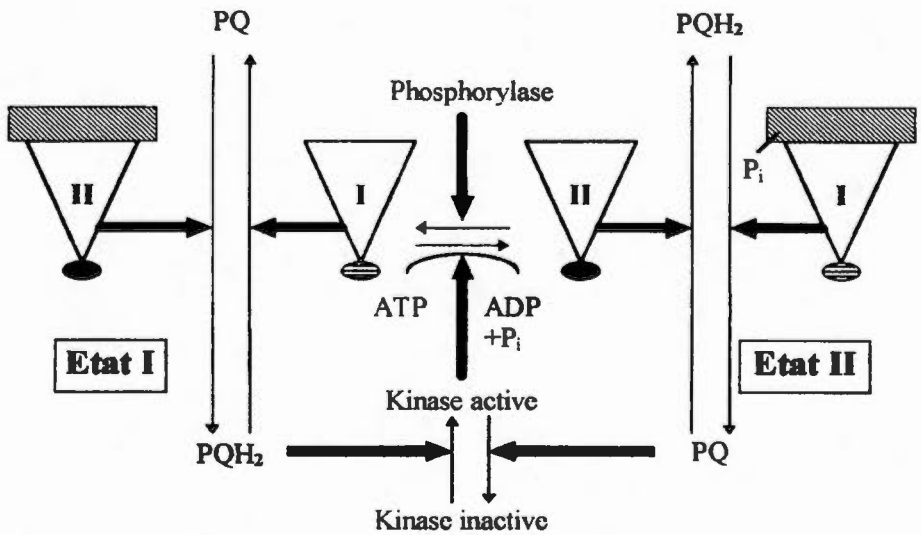

Figure 2.18 Schéma de la régulation des changements d'état I et II par l'induction de la phosphorylation des antennes collectrices de lumière (Jupin et Lamant, 1999).

En résumé, on observe l'état I lorsque le LHCII du PSII s'agrandit alors que celui du PSI diminue; inversement, l'état II indique que la taille des antennes du PSII diminue et celle du PSI augmente. Quand le PSII fonctionne plus rapidement que le PSI, le rapport PQH_2/PQ est grand à cause de l'état réduit de la PQ; inversement, lorsque le PSI fonctionne plus vite, il garde la PQ dans un état plus oxydé et le rapport PQH_2/PQ reste petit. La PQ réduite par le PSII induit l'activation de la kinase qui, en présence d'ATP et de Mg^{2+}, phosphoryle le LHCII (Jupin et Lamant, 1999).

2.3 Le rendement et la cinétique de la fluorescence chlorophyllienne des photosystèmes

Kautsky et Hirsh (1931) ont découvert pour la première fois que des algues vertes, préalablement adaptées à l'obscurité, émettaient de la fluorescence qui variait dans le temps sous une illumination continue. La mesure de la cinétique de la fluorescence variable fut ensuite désignée jusqu'à aujourd'hui sous l'appellation «d'effet Kautsky». Il est maintenant bien établi que la mesure *in vivo* de l'émission de fluorescence de la Chl *a* dépend de l'état d'oxydoréduction de l'accepteur primaire des électrons du PSII, Q_A : le niveau de fluorescence augmente lorsque Q_A se réduit et diminue lorsque Q_A se ré-oxyde (Duysens et Sweers, 1963; Papageorgiou, 1975). L'émission de la fluorescence chlorophyllienne provient principalement de la Chl *a* des antennes collectrices de lumière du PSII. Cependant, la Chl *b* n'est qu'un pigment accessoire servant à transférer l'énergie d'excitation à la Chl *a* (Lazár, 1999).

Le modèle de l'induction et de la cinétique de la fluorescence chlorophyllienne est devenu un modèle de base qui sert à l'interprétation de l'état des réactions photochimiques du PSII et du transport des électrons transmembranaires à l'échelle moléculaire (Figure 2.19) : Lorsqu'une molécule de Chl capte un photon, elle passe d'un état stable à un état instable (niveau de haute énergie d'excitation). Pour retourner à un niveau d'énergie plus faible (état plus stable), cette molécule de Chl excitée peut soit transférer son énergie d'excitation à une molécule voisine de Chl, soit dissiper cette énergie sous forme de chaleur ou émettre un photon sous forme de fluorescence (contribuant à la fluorescence de base, F_O). Le centre réactionnel du PSII reste ouvert et Q_A demeure à l'état oxydée tant que l'énergie d'excitation n'est pas suffisante pour être transférée au centre réactionnel et induire la séparation de charge. Lorsque l'énergie d'excitation collectée est suffisamment importante, le centre réactionnel du PSII devient activé (P680*) et induit la séparation de charge. Ensuite, le centre réactionnel à l'état $P680^+$ est neutralisé par les électrons provenant du complexe enzymatique du dégagement d'oxygène. Le rendement de la fluorescence variable (F_V) émise par les LHCIIs va dépendre de l'état d'oxydoréduction des transporteurs d'électrons entre le PSII et le PSI. En présence d'une lumière saturante, les accepteurs primaires des électrons du centre réactionnel sont réduits. Dans cette condition, la capacité photochimique du PSII atteint son

maximum qui se traduit par un niveau de fluorescence maximal (F_M). Le rendement et la cinétique de la fluorescence chlorophyllienne peuvent donc être utilisés pour analyser l'activité du transport des électrons photosynthétiques associée aux autres processus biochimiques de la photosynthèse (Lazár, 1999, 2006).

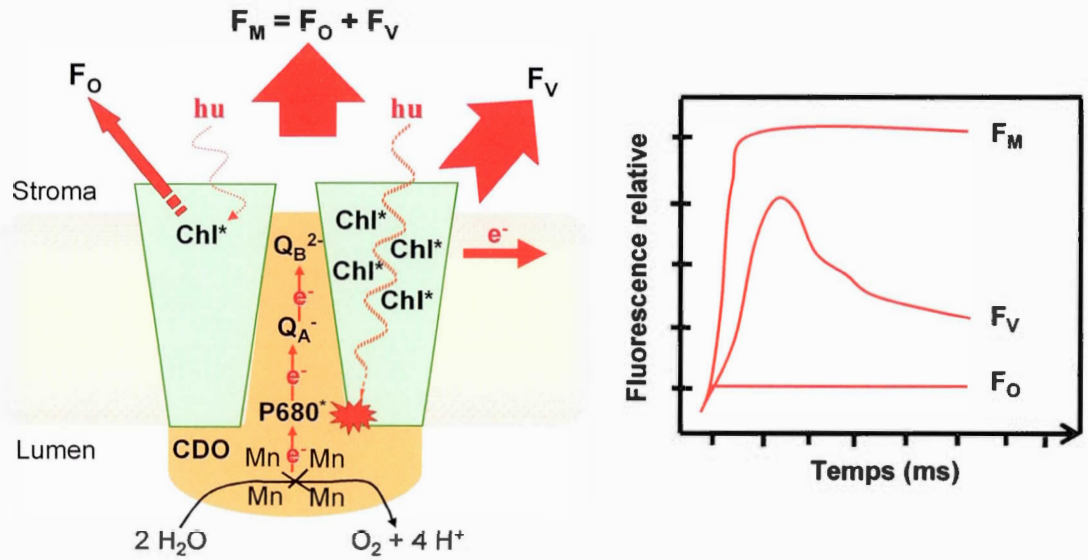

Figure 2.19 Modèle de l'émission de la fluorescence chlorophyllienne du photosystème II (schéma élaboré à partir de Lazár, 1999, 2006).

Dans le modèle présenté à la figure 2.19, l'énergie lumineuse (*hv*) est absorbée par les molécules de Chl composant les antennes collectrices de lumière (en vert). Le groupement protéique (en orange) est formé du complexe de dégagement d'oxygène (CDO), du centre réactionnel P680 ainsi que des accepteurs primaire et secondaire, respectivement Q_A et Q_B. Les flèches en rouge indiquent le transfert d'énergie sous forme électronique (e^-) ou la dissipation d'énergie sous forme de fluorescence lorsque l'énergie incidante est faible (F_O, fluorescence de base), lorsque l'énergie incidante est suffisamment importante pour induire la séparation de charge (F_V, fluorescence variable) et lorsque l'énergie incidante est saturante (F_M, fluorescence maximale).

2.3.1 Principe de la cinétique rapide et polyphasique de la fluorescence chlorophyllienne

La méthode fluorimétrique du «*Plant Efficiency Analyzer*» (PEA) s'est développée pour permettre l'analyse *in vivo* de la cinétique rapide et polyphasique de fluorescence correspondant à l'activité du transport d'électrons du PSII et de la photolyse de l'eau (Strasser *et al.*, 1995; Strasser *et al.*, 2004). Après une adaptation à l'obscurité (environ 30 min), l'induction rapide de la fluorescence chlorophyllienne est obtenue par l'exposition de l'échantillon à un flash de lumière saturante (d'environ 1 sec). Il a été déterminé que le niveau de fluorescence à la transition O, F_O, avait une valeur constante dans les 50 premières microsecondes d'éclairement (Strasser *et al.*, 1995; Sušila *et al.*, 2004). Un changement de ce niveau de fluorescence résulte d'une altération des complexes pigments-protéines formant les LHCIIs (Owens, 1996). Après la transition O, le rendement de fluorescence montre une variation dans le temps indiquant les états des transporteurs d'électrons associés au PSII. Lorsque la cinétique est présentée sur une échelle logarithmique de temps, deux transitions de fluorescence, notées J et I, sont mises en évidence apparaissant avant le niveau maximum de fluorescence (Neubauer et Schreiber, 1987; Strasser et Govindjee, 1991). Durant ces dernières années, la recherche s'est concentrée sur l'analyse des transitions O-J-I-P reflètant les états d'oxydoréduction des accepteurs d'électrons du PSII, Q_A, Q_B et plastoquinone (voir Figure 2.20). Il a été démontré que la transition O-J représente la phase photochimique correspondant à la réduction de Q_A ainsi qu'à la fermeture des centres réactionnels du PSII (Strasser *et al.*, 1995). L'inflexion de la courbe au niveau de la transition J indiquerait le niveau maximum de réduction de Q_A dans les conditions où l'accepteur d'électrons Q_B est limitant. Cependant, la transition J pourrait subir des modifications dépendantes des états de transition S du complexe enzymatique de la photolyse de l'eau : L'inhibition des états S pourrait provoquer l'inactivation du centre réactionnel du PSII et par conséquent l'inhibition du transport d'électrons (Hsu, 1993; Strasser, 1997). L'émission de fluorescence au niveau de la transition I indique une accumulation de $Q_A^-Q_B^-$, se traduisant par la fermeture complète de tous les PSIIs (Strasser *et al.*, 1995; Barthélemy *et al.*, 1997). La diminution de l'intensité de fluorescence après la transition I, appelée «D dip», correspondrait à la régulation du transport d'électrons entre le PSII et le PSI (Hansen *et al.*, 1991). La transition I-P indique une accumulation de $Q_A^-Q_B^{2-}$ et le niveau maximum de fluorescence est atteint lorsque le «*pool*»

de plastoquinones devient complétement réduit (Schreiber et Neubauer, 1987; Strasser *et al.*, 1995). Après la transition P, le taux de réoxydation dépend du transfert d'électrons vers le PSI et de la consommation d'ATP et de NADPH pendant la fixation du CO_2 dans le cycle de Calvin (Krause et Weis, 1991).

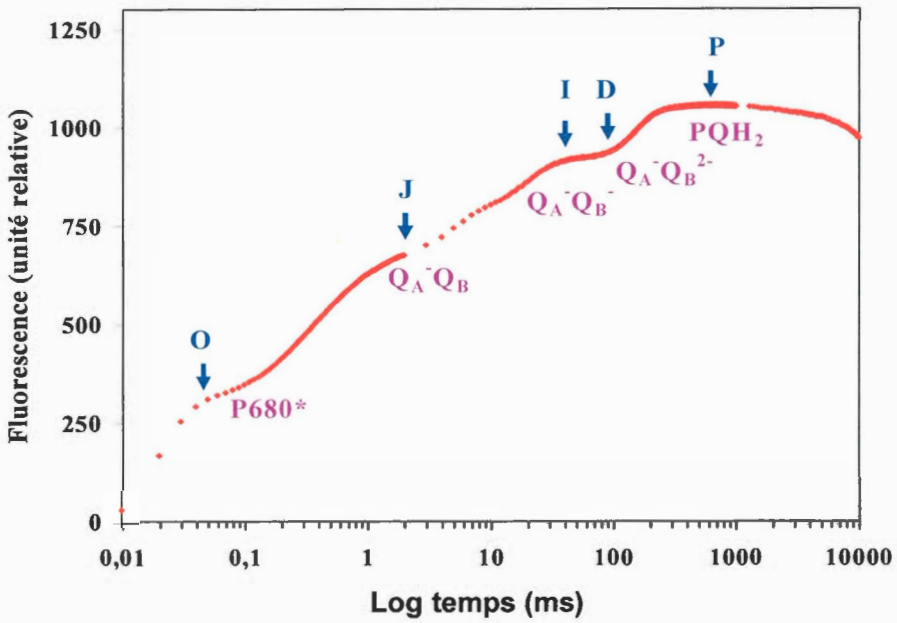

Figure 2.20 Courbe d'induction de fluorescence avec les transitions (O-J-I-D-P) présentées sur une échelle de temps logarithmique. Les transitions O, J, I et P apparaissent respectivement à 50 µs, 2 ms, 30 ms et approximativement à 300 ms (d'après Strasser *et al.*, 1995).

2.3.1.1 Les paramètres photosynthétiques évalués à partir du rendement et de la cinétique rapide de fluorescence

Pour un échantillon adapté à obscurité, l'acquisition des transitions de la cinétique rapide de fluorescence permet d'estimer différents paramètres photosynthétiques associés à l'efficacité photochimique du PSII. La nomenclature des niveaux de fluorescence O-J-I-P est conventionnellement acceptée selon leur présentation à la Figure 2.20 : soit $F_0 = F_{50\mu s}$, $F_J =$

F_{2ms}, $F_I = F_{30ms}$ et $F_P = F_M$ (sous une illumination saturante). Dans les travaux de cette thèse, les paramètres suivants ont été utilisés :

- Le rendement de la réaction photochimique primaire du PSII,

$$\Phi_{Po} = \frac{F_P - F_{50\,\mu s}}{F_P} = \frac{F_V}{F_P}$$

où $F_{50\mu s}$ représente le niveau de fluorescence de base à 50 µs (à la transition O) lorsque les centres réactionnels du PSII sont ouverts et F_M le niveau de fluorescence maximal (à la transition P) lorsque les centres réactionnels du PSII sont fermés. Dans les conditions optimales, la valeur de Φ_{Po} est d'environ 0,8 chez les plantes supérieures (Björkman et Demmig, 1987).

- Le rendement des processus non photochimiques du PSII,

$$\Phi_{Do} = 1 - \Phi_{Po} = \frac{F_{50\mu s}}{F_P}$$ (Strasser *et al.*, 2004).

- Le ratio entre les rendements des processus photochimiques et non photochimiques du PSII,

$$\frac{\Phi_{Po}}{\Phi_{Do}} = \frac{F_V}{F_{50\,\mu s}}$$ (Strasser *et al.*, 2004).

- Le «*quenching*» dépendant des états d'oxydoréduction des plastoquinones,

$$Q_{PQ} = \frac{F_P - F_{30ms}}{F_P - F_{50\mu s}}$$

où F_{30ms} représente le niveau de fluorescence à 30 ms (à la transition I). Ce paramètre estime donc la proportion des plastoquinones réduites (Strasser *et al.*, 1999).

- Le «*quenching*» dépendant de la formation du gradient de proton entre le *stroma* et le *lumen* de la membrane de thylacoïdes,

$$Q_{Emax} = \frac{F_P - F_{6s}}{F_P}$$

où F_{6s} représente le niveau de fluorescence à 6 sec. (Strasser *et al.*, 1999).

- L'absorption de l'énergie lumineuse des LHCIIs par centre réactionnel du PSII,

$$\frac{ABS}{RC} = \frac{M_0}{V_J} \Big/ \Phi_{Po}$$

Le paramètre M_0 indique la vitesse initiale de l'induction de la fluorescence variable dépendent du centre réactionnel du PSII, selon l'équation :

$$M_0 = \frac{F_{300\mu s} - F_{50\mu s}}{\left(F_P - F_{50\mu s}\right) \times 0{,}25}$$

où $F_{300\mu s}$ représente le niveau de fluorescence à 300 µs. Le paramètre V_J indique la fluorescence variable relative à la réduction de Q_A, selon l'équation :

$$V_J = \frac{F_{2ms} - F_{50\mu s}}{F_P - F_{50\mu s}}$$

où F_{2ms} représente le niveau de fluorescence à 2 ms (à la transition J). Le paramètre ABS/RC permet une estimation de la proportion des antennes collectrices par centre réactionnel du PSII (Krüger *et al.*, 1997; Strasser *et al.*, 2004).

- La probabilité (à t = 0) qu'un exciton capté au centre réactionnel du PSII induise un transport d'électrons au-delà Q_A^-,

$\psi_0 = 1 - V_J$ (Strasser *et al.*, 2004).

- Le rendement du transport des électrons induit par le PSII,

$\Phi_{Eo} = \Phi_{Po} \times \psi_0$ (Strasser *et al.*, 2004).

2.3.2 Principe de la cinétique lente de la fluorescence chlorophyllienne

La cinétique lente de la fluorescence chlorophyllienne est mesurée par la méthodologie PAM qui évalue la relation entre le rendement de fluorescence et le fonctionnement du PSII et du PSI (Schreiber *et al.*, 1986; Öquist et Chow, 1992; Schreiber, 2004). Ce système d'analyse est fondé sur l'utilisation de quatre types de sources lumineuses : une lumière analytique modulée (594 nm), une lumière actinique continue (400-700 nm), un flash d'une lumière saturante (400-700 nm) et une lumière dans le rouge lointain (735 nm) (Figure 2.21).

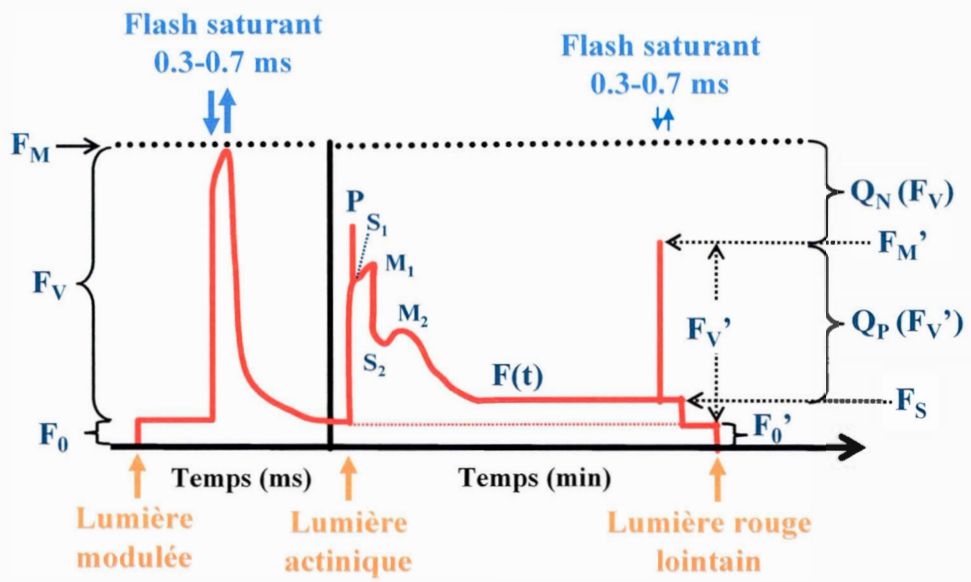

Figure 2.21 Cinétique de fluorescence modulée mesurée à l'aide d'un fluorimètre PAM (schéma élaboré à partir de Schreiber *et al.*, 1986). F_O, fluorescence constante pour une plante adaptée à l'obscurité; F'_O, fluorescence constante pour une plante adaptée à la lumière; F_V, fluorescence variable; F_M, fluorescence maximale pour une plante adaptée à l'obscurité; F'_M, fluorescence maximale pour une plante adaptée à la lumière; Q_N, «*quenching*» non photochimique; Q_P, «*quenching*» photochimique.

Après une adaptation à l'obscurité, la plante est exposée à une lumière analytique modulée de faible intensité, insuffisante pour initier la séparation de charges. Le rendement constant de fluorescence F_O représente la dissipation d'énergie réémise des antennes collectrices quand l'énergie d'excitation n'est pas transférée aux centres réactionnels du PSII qui restent donc à l'état oxydé-ouvert. La lumière saturante (300-700 ms) permet de déterminer le niveau F_M lorsque tous les centres réactionnels sont fermés et l'accepteur primaire d'électrons du PSII, Q_A est complètement réduit. Quand la feuille est soumise à une lumière actinique, les processus photosynthétiques sont déclenchés permettant l'analyse de la cinétique de la fluorescence chlorophyllienne dépendante du transport d'électrons entre le PSII et le PSI. La fluorescence induite par la lumière actinique à l'état stationnaire du transport d'électrons permet d'évaluer la proportion de centres réactionnels fermés. Quand cet état est atteint, la lumière actinique est éteinte et une lumière dans le rouge lointain (qui excite préférentiellement le PSI) est allumée, ce qui permet d'obtenir le rendement de

fluorescence F'_0 correspondant à l'état dans lequel les centres réactionnels sont ouverts. Cette combinaison de lumière permet l'analyse du rendement photochimique des centres réactionnels des PSII ainsi que la dissipation d'énergie sous forme non photochimique (Schreiber *et al.*, 1986; Havaux *et al.*, 1991). Le phénomène de «*quenching*» (expression acceptée par convention) correspond à une diminution de la fluorescence qui peut être lié à la dissipation d'énergie *via* la réaction photochimique (flux d'énergie engagé dans le transport des électrons) et *via* des réactions non photochimiques (flux d'énergie dissipé sous forme de chaleur) (Schreiber, 2004).

2.3.2.1 Les paramètres photosynthétiques évalués à partir de la cinétique de fluorescence à l'état stationnaire

Pour un échantillon adapté à l'obscurité puis à la lumière actinique, la cinétique PAM permet l'estimation de différents paramètres photosynthétiques indiquant l'efficacité du transport des électrons transmembranaires associé au PSII-PSI et la dissipation d'énergie *via* des voies non photochimiques. La nomenclature des niveaux de fluorescence F_0, F_M, F_V, F'_0, F'_M, F'_V et F_S est conventionnellement acceptée selon leur présentation à la Figure 2.21. Dans les travaux de cette thèse, les paramètres suivants ont été utilisés :

- L'efficacité photochimique maximale du PSII pour un échantillon adapté à l'obscurité,

$$\Phi_{PSII} = \frac{F_M - F_0}{F_M} = \frac{F_V}{F_M}$$

où F_0 représente le niveau de fluorescence basal lorsque les centres réactionnels du PSII sont ouverts et F_M le niveau de fluorescence maximal lorsque les centres réactionnels du PSII sont fermés (Bolhàr-Nordenkampf *et al.*, 1989).

- L'efficacité photochimique opérationnelle du PSII pour un échantillon adapté à une illumination actinique continue,

$$\Phi_{sPSII} = \frac{F'_M - F'_0}{F'_M}$$

où F'_0 représente le niveau de fluorescence basal lorsque les centres réactionnels du PSII sont ouverts et F'_M représente le niveau de fluorescence maximal lorsque les centres réactionnels du PSII sont fermés à l'état stationnaire du transport d'électrons (Genty *et al.*, 1989).

- Le «*quenching*» photochimique, Q_P indique la proportion de centres réactionnels du PSII restés ouverts à l'état stationnaire du transport d'électrons. Ce paramètre est évalué selon l'équation :

$$Q_P = \frac{F'_M - F_S}{F'_M - F'_0}$$

où F_S représente le niveau de la fluorescence variable à l'état stationnaire du transport d'électrons. Cette proportion de centres réactionnels du PSII restés ouverts peut participer à la réaction photochimique primaire (Schreiber *et al.*, 1986).

- Le «*quenching*» photochimique relatif,

$$Q_{P(rel)} = \frac{F'_M - F_S}{F_M - F'_0}$$

est proportionnel aux nombres de centres réactionnels du PSII restés ouverts par rapport à la fluorescence variable totale lorsque l'échantillon passe d'une adaptation à l'obscurité à une adaptation à la lumière actinique (Buschmann, 1995).

- Le «*quenching*» non photochimique de la fluorescence variable,

$$Q_N = 1 - \frac{F'_M - F'_0}{F_M - F_0}$$

qui est proportionnel au nombre de centres réactionnels du PSII ne participant pas au transport d'électrons (Schreiber *et al.*, 1986).

- Le «*quenching*» non photochimique par rapport à la fluorescence maximale à l'état stationnaire du transport d'électrons,

$$NPQ = \frac{F_M - F'_M}{F'_M}$$ (Bilger et Björkman, 1990).

- Le «*quenching*» non photochimique par rapport à la fluorescence maximale pour un échantillon adapté à l'obscurité,

$$Q_{CN} = \frac{F_M - F'_M}{F_M}$$ (Roháček et Barták, 1999).

- La proportion de centres réactionnels du PSII restés fermés par rapport à la fluorescence variable totale lorsque l'échantillon passe d'une adaptation à l'obscurité à une adaptation à la lumière actinique,

$$UQF_{(rel)} = \frac{F_S - F'_0}{F_M - F'_0}$$ (Juneau *et al.*, 2005).

La variation du «*quenching*» non photochimique dépend de différents mécanismes de régulation du transport d'électrons qui peuvent être séparés en trois composantes (Figure 2.22).

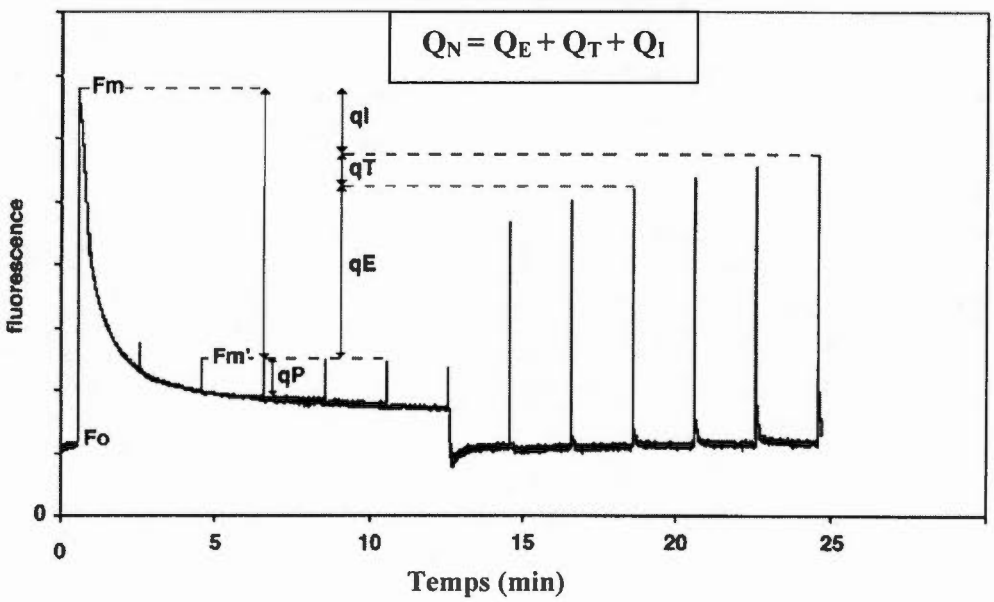

Figure 2.22 Cinétique de fluorescence PAM d'une feuille d'*arabidopsis*. F_O, fluorescence constante pour une plante adaptée à l'obscurité; F_M, fluorescence maximale pour une plante adaptée à l'obscurité; F_M', fluorescence maximale pour une plante adaptée à la lumière; Q_I : «*Quenching*» lié à la photoinhibition; Q_T : «*Quenching*» lié aux états de transition; Q_E : «*Quenching*» dépendant du gradient de protons transthylacoïdien; Q_P : «*Quenching*» photochimique (d'après Müller *et al.*, 2001).

Le quenching de fluorescence Q_E dépendant de la formation du gradient de protons transmembranaire et de l'action de la zéaxanthine (le cycle des xanthophylles) provoquant la dissipation d'énergie sous forme de chaleur (Schreiber *et al.*, 1998), le «*quenching*» de fluorescence Q_T associé au changement des états de transitions I et II (expliqué précédemment au chapitre 2.2.3.6), et le «*quenching*» de fluorescence Q_I comme un indicateur d'altérations structurelles du PSII causés par la photoinhibition (Ruban et Horton, 1995a, 1995b).

2.3.3 Les spectres de fluorescence

La spectrofluorométrie est utilisée comme un outil pour l'analyse de la structure de l'appareil photosynthétique et la distribution d'énergie entre les différents complexes pigment-protéines (Krause et Weis, 1984; Lichtenthaler et Rinderle, 1988). La mesure des spectres de fluorescence à température ambiante (≈24°C) présente trois bandes d'émissions à 685, 695 et 720-740 nm qui sont respectivement attribuées aux LHCs, PSII et PSI (Franck *et al.*, 2002). Dans cette condition de mesure, les spectres respectifs du PSII et du PSI se chevauchent et la forme du spectre de fluorescence indique que la majorité de la fluorescence provient des antennes collectrices de lumière du PSII. L'importance de la contribution du PSI à l'émission de la fluorescence totale est encore ambiguë. Nous supposons que, durant «l'effet Kautsky», le PSI pourrait contribuer à la fluorescence totale de façon plus déterminante.

2.4 L'état physiologique cellulaire ou de l'organisme végétal entier

2.4.1 La photosynthèse comme processus primordial du métabolisme végétal

La photosynthèse représente un processus physiologique primordial pour la cellule végétale en produisant les précurseurs pour le métabolisme des carbohydrates, des acides gras, des acides aminés et nucléiques (Figure 2.23). La dépendence entre la fixation du CO_2 et le rendement photochimique du PSII a déjà été confirmée (Genty *et al.*, 1989). Dans ce système cellulaire, la photosynthèse représente donc un indicateur physiologique lorsque le

métabolisme est altéré par un ou des facteurs de stress environnementaux. En fonction des propriétés du stress environnemental et des caractéristiques biologiques de l'organisme, les réponses physiologiques indiquent un changement métabolique comme un processus d'acclimatation ou de détérioration progressive (Gaspar *et al.*, 2002). Donc, ce type de réponses physiologiques démontre l'état de santé de l'organisme entier. L'interaction entre les facteurs environnementaux et les états physiologiques des plantes offre un avantage très important pour les études en toxicologie environnementale. Le changement de l'activité photosynthétique n'indique pas seulement la santé de l'organisme végétal, mais aussi les effets toxiques des polluants auquels l'organisme végétal est exposé (Popovic *et al.*, 2003).

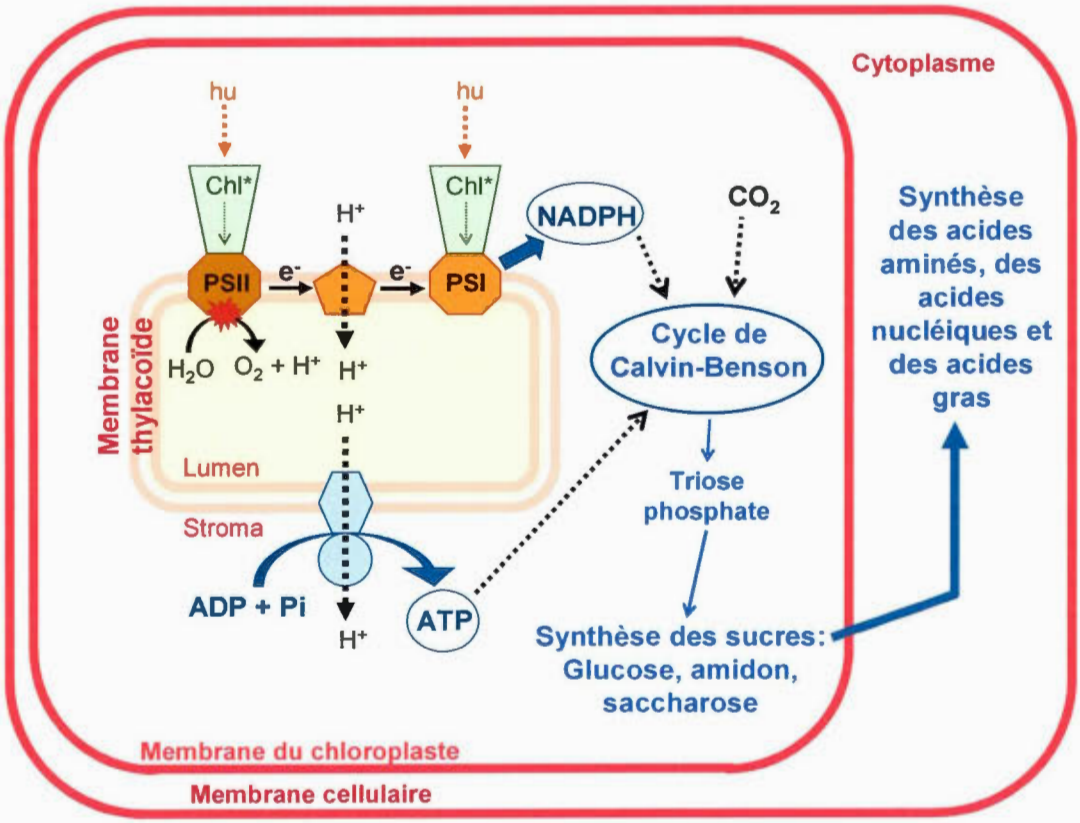

Figure 2.23 Représentation des interconnexions métaboliques entre les réactions photochimiques de la photosynthèse, les réactions biochimiques du chloroplaste et cytoplasme cellulaire (Schéma élaboré à partir de Buchanan *et al.*, 2000; Flügge, 2001).

Durant le processus photosynthétique, la photochimie primaire du PSII est l'étape la plus importante dans le captage de l'énergie solaire et le stockage d'énergie sous forme biochimique nécessaire pour tout le métabolisme cellulaire (Malkin et Niyogi, 2000). Par conséquent, les paramètres photochimiques de la photosynthèse représentent des biomarqueurs potentiels dans l'évaluation de la présence des effets toxiques induits par les xénobiotiques.

2.4.2 L'induction du stress oxydatif de cellules végétales exposées aux xénobiotiques

Le stress oxydatif a été défini par Halliwell (1987) comme un état où la production d'espèces réactives de l'oxygène (ERO) devient dommageable pour l'intégrité cellulaire. Un stress oxydatif dans une cellule se produit lorsque les processus prooxydatifs surpassent les mécanismes des défenses antioxydatifes (Sies, 1991). Le stress oxydatif induit par les xénobiotiques cause un dysfonctionnement de la physiologie cellulaire. Dans ce cas, les EROs possédant une grande réactivité peuvent induire une détérioration des protéines, des lipides membranaires et de l'ADN (Lichtenthaler, 1996; Dat *et al.*, 2000). L'induction des EROs par les xénobiotiques est reconnue comme une réponse commune pour les plantes et les animaux (Hippelli *et al.*, 1999).

2.4.2.1 La chimie des espèces réactives de l'oxygène

Étant biradicalaire, la molécule d'oxygène possède, à son état stable, deux électrons célibataires de spin parallèle sur sa couche électronique externe. Cette configuration rend la molécule d'oxygène non réactive avec les molécules organiques qui possèdent des électrons appariés et de spin opposé. Par contre, les radicaux libres formés à partir de l'oxygène sont des molécules avec un électron célibataire sur la couche électronique externe. Les radicaux libres se forment quand une molécule ayant une paire d'électrons reçoit ou perd un électron (McKersie et Leshem, 1994; Dat *et al.*, 2000). La terminologie «d'espèces réactives de l'oxygène» inclue ici les différentes formes actives de l'oxygène, les hydroperoxydes et les espèces radicalaires (Figure 2.24).

Nom commun	Configuration électronique	Terminologie
Oxygène triplet (état stable)	·O-O·	O_2
Oxygène singulet	O-O:	$^1O_2$
Radical superoxyde	·O-O:	$·O_2^-$
Radical hydroperoxyl	·O-O:H	·OOH
Peroxyde d'hydrogène	H:O-O:H	H_2O_2
Radical hydroxyl	H:O·	·OH
Ion hydroxyl	H:O:	OH^-

Figure 2.24 Nomenclature des différentes formes d'espèces réactives de l'oxygène (McKersie et Leshem, 1994; Dat *et al.*, 2000).

2.4.2.2 Les sites de formation des espèces réactives de l'oxygène

Dans la cellule végétale, la formation des EROs se produit dans les compartiments cellulaires : chloroplaste, mitochondrie, réticulum endoplasmique, peroxysomes et paroi cellulaire (Apel et Hirt, 2004). Le processus de respiration dans les mitochondries utilise l'oxygène comme accepteur terminal des électrons permettant la formation du gradient de protons intermembranaires nécessaire pour la synthèse d'ATP. Cependant, l'altération de ce processus par les xénobiotiques peut induire l'accumulation des EROs (Apel et Hirt, 2004).

Dans la photosynthèse, les xénobiotiques peuvent inhiber le transport transmembranaire des électrons et ainsi détériorer les réactions photochimiques au centre réactionnel des photosystèmes. Dans ces conditions, l'excitation des pigments chlorophylliens non utilisée pour la photochimie primaire peut induire la formation d'EROs. Les photosystèmes peuvent donc représer une source importante de production des EROs (Figure 2.25). Il est reconnu que le PSII, lors de la photolyse de l'eau, contribue à la

formation des EROs et la production des EROs peut aussi être induite *via* la chaîne de transfert des électrons du PSI (Asada, 1994). En inhibant le cycle de Calvin, les xénobiotiques limitent la consommation du NADPH, ce qui peut stimuler la réduction de l'oxygène *via* le PSI et la ferrédoxine (McKersie et Leshem, 1994).

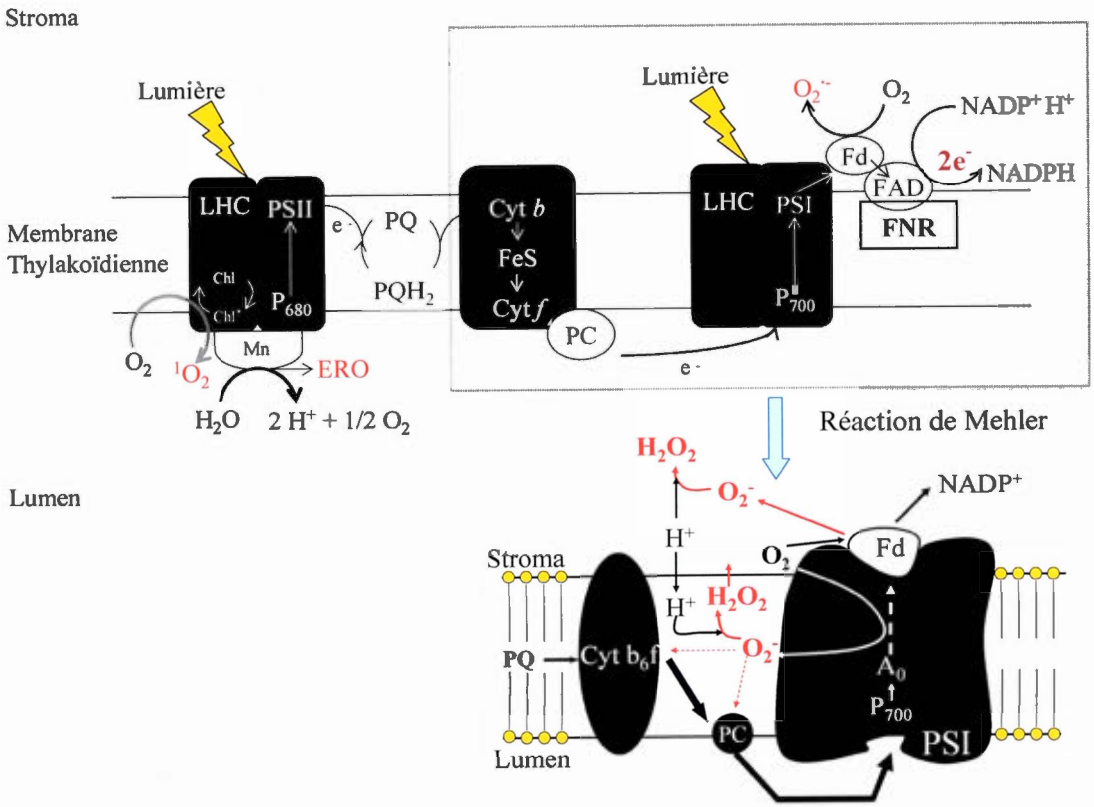

Figure 2.25 Sites de formation d'ERO lors de la photosynthèse. ➡ Transport d'électrons entre les photosystèmes; ⇢ transport d'électrons au sein du PSI. (d'après Asada, 1994; McKersie et Leshem, 1994). FAD : Flavine adénine dinucléotide; FNR : Ferrédoxine $NADP^+$ réductase.

2.4.2.3 Les antioxydatifs cellulaires

L'activation des défenses antioxydatifes par les systèmes enzymatiques comme par exemple la catalase (CAT), l'ascorbate peroxydase (APX), la glutathion réductase (GR) et la

glutathion *S*-transférase (GST) indique la production et l'accumulation des EROs dans les différents compartiments cellulaires (Van Camp *et al.*, 1994; Bowler *et al.*, 1994) :

- La catalase (hydrogène-péroxyde:hydrogène-péroxyde oxydoréductase, EC 1.11.1.6) catabolise le peroxyde d'hydrogène généré dans les peroxysomes par les oxydases (qui participent dans la ß-oxydation des acides gras et le cycle du glyoxalate). La catalase catalyse la réaction suivante : $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$ (Nicholls et Schonbaum, 1963). L'activité de cette enzyme a été détectée dans le cytosol, les peroxysomes et les mitochondries (Chandlee *et al.*, 1983). La grande activité de cette enzyme peut expliquer son rapide «*turn-over*» comparable à celui de la protéine D1 du PSII. Il a été montré que différents facteurs environnementaux peuvent altérer la synthèse et l'activité de cette enzyme (Hertwig *et al.*, 1992; Leipner *et al.*, 2000).
- Dans le chloroplaste, l'ascorbate peroxydase (L-ascorbate:hydrogène-péroxyde oxydoréductase, EC 1.11.1.11) catalyse le catabolisme du peroxyde d'hydrogène selon la réaction suivante : L-ascorbate + H_2O_2 = déhydroascorbate + 2 H_2O (Shigeoka *et al.*, 1980). Par une étude *in vitro*, il a été montré dans le chloroplaste un cycle d'oxydoréduction impliquant le glutathion et l'ascorbate en présence de H_2O_2 (Anderson *et al.*, 1983). L'étude des réactions enzymatiques utilisant le glutathion et l'ascorbate comme substrat a permis de développer le modèle du cycle ascorbate-glutathion, aussi appelé le cycle d'Halliwell-Asada (Figure 2.26) : L'oxydation de l'acide ascorbique produit le monodéhydroascorbate qui va ensuite former le déhydroascorbate et l'ascorbate. Dans les conditions normales, l'ascorbate est à 90 % sous forme réduite. Dans ce modèle, la monodéhydroascorbate réductase (NADH:monodéhydroascorbate oxydoréductase, EC 1.6.5.4) catalyse la réaction : NADH + H^+ + 2 monodéhydroascorbate = NAD^+ + 2 ascorbate, et la déhydroascorbate réductase (glutathion:déhydroascorbate oxydoréductase, EC 1.8.5.1) catalyse la réaction : 2 glutathion + déhydroascorbate = glutathion disulfide + ascorbate (Crook, 1941). La déhydroascorbate réductase représente le lien entre le cycle de l'ascorbate et celui du glutathion.

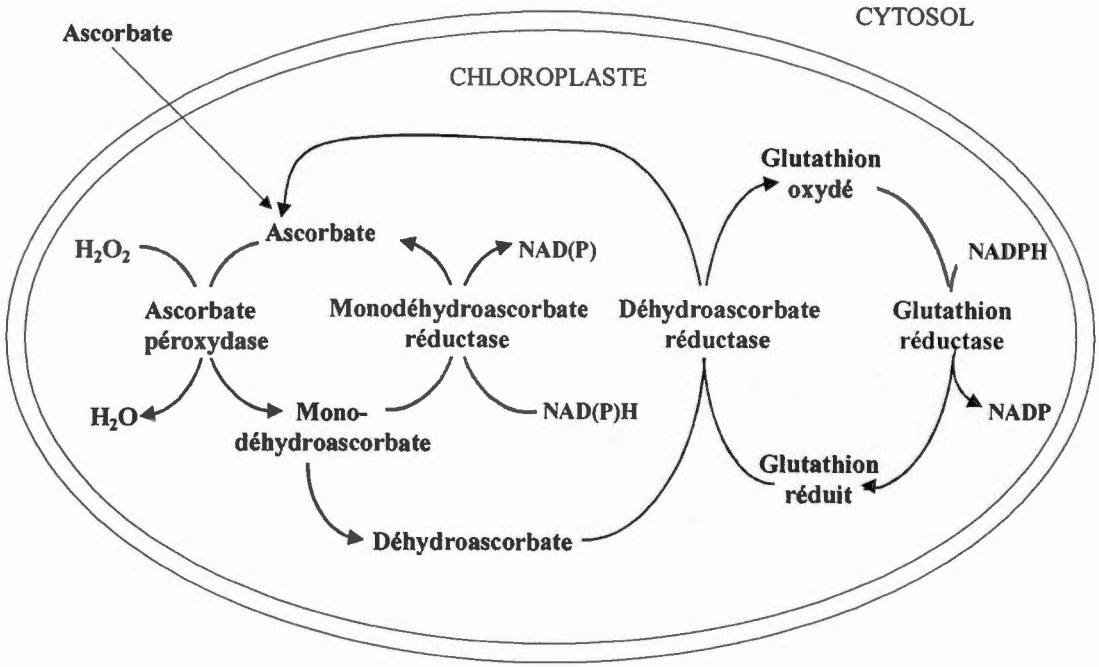

Figure 2.26 Schéma présentant l'élimination du H_2O_2 dans le système enzymatique du cycle ascorbate-glutathion du chloroplaste (d'après Foyer *et al.*, 1994).

- La glutathion réductase (glutathion:$NADP^+$ oxydoréductase, EC 1.8.1.7), comme flavoprotéine située dans le chloroplaste, catalyse la réduction du glutathion disulfide permettant la formation de glutathion (réduit) :

  2 glutathion + $NADP^+$ = glutathion disulfide + NADPH + H^+ (McKersie et Leshem, 1994).

  Il a été montré que 77 % de l'activité de la glutathion réductase s'effectue dans le chloroplaste (Edwards *et al.*, 1990).

- La glutathion *S*-tranférase (RX:glutathion R-transférase, EC 2.5.1.18) catalyse la conjugaison de xénobiotiques avec le glutathion selon réaction suivante :

  RX + glutathion = HX + R-S-glutathion, où R peut être un groupement aliphatique, aromatique ou hétérocyclique, et X, un groupement sulfate, nitrile ou halide (Jakoby, 1985; Sommer et Böger, 1999).

CHAPITRE III

LES PROBLÉMATIQUES SPÉCIFIQUES ET LES OBJECTIFS DE RECHERCHE RÉALISÉS

En première partie de ce travail, la problématique consiste à développer l'interprétation de l'émission de la fluorescence chlorophyllienne comme un indicateur de l'activité du transport transmembranaire des électrons entre le PSII et le PSI. Dans cette problématique, l'appareil photosynthétique a été exposé à des sources (modulée et continue) et des intensités lumineuses différentes dans le but d'analyser les variations du rendement de fluorescence, la capacité photochimique et la dissipation d'énergie sous forme non photochimique. Le changement spectral de la cinétique de fluorescence a été analysé pour déterminer la distribution d'énergie aux antennes collectrices du PSII et du PSI. Dans un premier rapport présenté au chapitre IV, le changement du spectre de fluorescence a été étudié durant «l'effet Kautsky», c'est-à-dire durant le changement de l'émission totale de fluorescence passant des niveaux de la fluorescence basale (F_O) à la fluorescence maximale (F_M) et de F_M à la fluorescence à l'état stationnaire du transport d'électrons (F_T).

Actuellement, la dépendance entre le changement de la cinétique rapide de fluorescence et les états d'oxydoréductions des transporteurs d'électrons du PSII au PSI demande une recherche fondamentale plus approfondie. À notre connaissance, la fluorescence rapide associée à l'activité du PSII n'a pas été étudiée lorsque la plante a été exposée à une illumination continue. L'utilisation de modulateurs exogènes sous forme d'accepteur d'électrons, de donneur d'électrons ou d'inhibiteur va permettre d'induire une altération de différents sites catalytiques associés aux transporteurs d'électrons du PSII et PSI. Les altérations structurelles et fonctionnelles de l'appareil photosynthétique vont se refléter sur le rendement et la cinétique de fluorescence permettant une interprétation des processus photochimiques et du transport des électrons dans la photosynthèse. La réalisation de cette recherche va permettre la compréhension de la relation entre le changement du rendement de la cinétique rapide de fluorescence et les fonctions du PSII dans les états opérationnels. Pour investiguer cette objectif, il a été nécessaire d'étudier le changement des

transitions rapides de fluorescence durant «l'effet Kautsky». Dans le rapport présenté au chapitre V, le rôle du système enzymatique de dégagement d'oxygène a été étudié lorsque la photolyse de l'eau a été progressivement inhibée par différentes concentrations d'hydroxylamine. Dans le rapport présenté au chapitre VI, le changement des transitions rapides de fluorescence a également été analysé durant «l'effet Kautsky» lorsque l'appareil photosynthétique a été exposé à la duroquinone, un accepteur d'électrons du PSII, et au méthyl viologène, un accepteur d'électrons du PSI.

Dans la deuxième partie de ce travail, la problématique a consisté à investiguer et interpréter le changement de l'émission de la fluorescence chlorophyllienne lorsque l'état physiologique de la plante a été altéré par différents xénobiotiques. Dans cette problématique, il a été nécessaire d'étudier les paramètres de fluorescence qui évaluent les différents processus de l'activité photochimique, du transport des électrons et de la biochimie membranaire. Les résultats de cette étude ont permis l'élaboration d'un modèle de différents mécanismes de toxicité pour des xénobiotiques qui induisent une inhibition des réactions photosynthétiques. L'interprétation de la toxicité au niveau de la physiologie de l'organisme entier a été possible par l'investigation de paramètres cytologiques et de l'activité des enzymes antioxydatifes. Le degré de corrélation entre le changement des paramètres photosynthétiques et les paramètres cytologiques et biochimiques a également été étudié lorsque l'organisme végétal ou le système membranaire de chloroplaste ont été exposés à la toxicité des xénobiotiques. Par cette approche méthodologique, il a donc été possible d'estimer l'applicabilité des paramètres photosynthétiques de fluorescence comme des biomarqueurs de l'état physiologique de l'organisme.

Dans l'objectif de recherche, l'utilisation de plusieurs types de xénobiotiques offre l'avantage d'améliorer l'interprétation du changement des paramètres de fluorescence indiquant différents sites et mécanismes d'inhibition. Il a donc été important d'investiguer différents types de pesticides (oxyfluorfène, fludioxonyl, atrazine, isoproturon) et d'autres xénobiotiques comme le méthanol et le sulfate de cuivre. En utilisant les paramètres de fluorescence, il a été possible de distinguer les effets toxiques du méthanol de ses effets stimulants. Dans le chapitre VII, les mécanismes spécifiques concernant la stimulation et les

effets d'inhibition ont été expliqués lorsque la plante *Lemna gibba* a été exposée à différentes concentrations de méthanol. Dans ce contexte, nous avons aussi déterminé les différences dans le mode d'action et les sites d'inhibition des pesticides étudiés. Dans le chapitre VIII, nous avons expliqué les effets de l'oxyfluorfène comme un xénobiotique qui inhibe la synthèse de la chlorophylle et cause un stress oxydatif cellulaire par la formation d'espèces réactives de l'oxygène. Au chapitre IX, nous avons expliqué les effets du sulfate de cuivre, un fongicide qui provoque un dysfonctionnement de la réaction photochimique primaire et cause un stress oxydatif. Le mécanisme d'induction d'un stress oxydatif cellulaire par le fludioxonyl a également été déterminé comme la conséquence de l'effet phytotoxique de ce fongicide. À cause de la différence dans le mode d'action et les sites d'inhibition de ces pesticides, notre étude par une approche multiparamétrique a permis de mieux comprendre la variation de la sensibilité des biomarqueurs utilisés. Dans les chapitres VIII et IX, la dépendance entre les changements des paramètres photosynthétiques, enzymatiques et de croissance a été investiguée pour déterminer la fiabilité de ces paramètres comme biomarqueurs de toxicité cellulaire. En utilisant cette approche, il a été possible d'estimer l'applicabilité de la photosynthèse comme un indicateur de l'état physiologique de l'organisme entier. Dans le chapitre X, la relation entre l'inhibition de la photosynthèse et celle de la division cellulaire a été investiguée lorsque l'algue *Scenedesmus obliquus* a été exposée aux effets toxiques de l'isoproturon. Cette approche a permis de comparer la sensibilité des différents paramètres de fluorescence basés sur la photochimie primaire du PSII avec le développement de la population algale. Les résultats obtenus ont permis la détermination de la validité des paramètres photosynthétiques comme des biomarqueurs de la croissance de la biomasse. Dans le chapitre XI, nous avons aussi examiné la possibilité d'utiliser la réaction photochimique primaire du PSII dans les fragments membranaires de thylacoïdes comme indicateur de toxicité induit par l'atrazine et les ions cuivre. De plus, l'étude de cette problématique a permis de déterminer les critères méthodologiques nécessaires pour un bioessai qui utilise la photosynthèse comme un indicateur de toxicité des xénobiotiques.

CHAPITRE IV

CHANGES IN THE ROOM-TEMPERATURE EMISSION SPECTRUM OF CHLOROPHYLL DURING FAST AND SLOW PHASES OF THE KAUTSKY EFFECT IN INTACT LEAVES

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4.1 Résumé

Le changement des spectres d'émission de fluorescence de la chlorophylle *a* à temperature ambiante de la pièce a été analysé durant «l'effet Kautsky» à l'aide d'un enregistrement rapide par un réseau de photodiodes chez des feuilles d'orge matures et durant leur verdissement. Chez des feuilles matures, la comparaison des spectres de F_O (niveau de fluorescence de base à la transition O) et de F_M (niveau maximum de fluorescence à la transition M) a mise en évidence une amplitude relative de la fluorescence variable maximale à 684 nm pour la bande du photosystème II et minimale à 725 nm pour la bande du photosystème I. Durant le passage du niveau de fluorescence de F_O à F_M, un changement progressif du spectre de la fluorescence variable a été observé à des longueurs d'ondes élevées (rouge). Ce changement indique la présence de différentes cinétiques de fluorescence appartenant aux différentes couches de chloroplastes à l'intérieur de la feuille. Ce phénomène a été vérifié en simulant l'effet du «*screening*» sur le spectre d'émission de fluorescence de chloroplastes isolés et par des expérimentations sur des feuilles en verdissement ayant une faible quantité de chlorophylle. De plus, les expériences effectuées durant le verdissement ont montré que la présence de chlorophylle non couplée aux premières étapes du verdissement et les antennes collectrices de lumière du photosystème II aux dernières étapes du verdissement causaient des effets détectables mais mineurs sur la forme du spectre de fluorescence. Lorsqu'une forte lumière actinique a été appliquée à des feuilles vertes matures, la cinétique de fluorescence lente, qui passe du niveau de fluorescence F_M à F_T (niveau de fluorescence à l'état stationnaire indiqué à la transition T), a été accompagnée d'un changement spectral de la bande du photosystème II à 684 nm vers des longueurs d'ondes plus élevées (rouge) à cause du quenching non-photochimique de la chlorophylle émettant à de courtes longueurs d'ondes attribuée aux antennes collectrices de lumière du photosystème II.

4.2 Abstract

Changes in the room-temperature emission spectrum of chlorophyll (Chl) were analyzed using fast diode-array recordings during the Kautsky effect in mature and in greening barley leaves. In mature leaves, the comparison of F_O (basal level of fluorescence yield at transient O) and F_M (maximum level of fluorescence yield at transient M) spectra showed that the relative amplitude of total variable fluorescence was maximal for the 684 nm Photosystem II (PSII) band and minimal for the 725 nm Photosystem I band. During the increase from F_O to F_M, a progressive redshift of the spectrum of variable fluorescence occurred. This shift reflected the different fluorescence rise kinetics of different layers of chloroplasts inside the leaf. This was verified by simulating the effect of screening on the emission spectrum of isolated chloroplasts and by experiments on greening leaves with low Chl content. In addition, experiments performed at different greening stages showed that the presence of uncoupled Chl at early-greening stages and light harvesting complex II (LHCII) at later stages have detectable but minor effects on the shape of room-temperature emission spectra. When strong actinic light was applied to mature green leaves, the slow fluorescence yield, which declined from F_M to F_T (steady-state level of fluorescence yield at transient T), was accompanied by a slight redshift of the 684 nm PSII band because of non-photochemical quenching of short-wavelength-emitting Chl ascribed to LHCII.

4.3 Introduction

Light-induced variations in chlorophyll (Chl) fluorescence yield, known as the Kautsky effect, are commonly measured to probe photosynthetic characteristics of intact plants at room temperature. The general relationship between photosynthetic electron transport and the variations of fluorescence yield induced by continuous actinic light is well established (reviewed in Refs. Dau, 1994; Govindjee, 1995; Lazár, 1999). As plastoquinones are reduced by Photosystem II (PSII), the fluorescence yield first rises from F_O (basal level of fluorescence yield at transient O) to F_M (maximum level of fluorescence yield at transient M). This process occurs usually within 1 s or less and is referred to as the fast fluorescence rise. It is mainly because of the suppression of photochemical quenching exerted by PSII reaction centers with an oxidized primary electron acceptor quinone A (Q_A). After F_M, the fluorescence yield declines progressively to reach F_T (steady-state level of fluorescence yield at transient T) after several minutes. This decline is caused by partial reoxidation of plastoquinones by Photosystem I (PSI) and by additional quenching processes collectively referred to as non-photochemical quenching (NPQ). Several parameters, which can be derived from a small number of experimentally measured fluorescence yield values (including F_O, F_M and F_T), are used extensively in ecophysiological studies (reviewed in Maxwell and Johnson, 2000).

Although it is known since early studies that several overlapping Chl emission bands exist at room temperature in plants (Lavorel, 1962), kinetic fluorescence measurements are performed usually at one wavelength because it is generally considered that the whole Chl spectrum is affected in a similar manner. Little attention has been paid to the wavelength dependence of different derived parameters such as photochemical efficiency, photochemical quenching or NPQ. The development of diode array detectors over the past 20 years made it possible to record spectra during short time intervals during the Kautsky effect. Some studies have been performed on the spectral changes that occur when intact leaves are exposed to actinic illumination. Two main emission bands (or regions) have been considered in these studies : the 685 nm band, attributed mainly to PSII, and the long-wavelength band at 720-740 nm, supposed to be an unresolved combination of the PSI emission and vibrational bands

of PSII. In intact leaves with high Chl content, the intensity of the 685 nm band is decreased considerably because of self-absorption (Lichtenthaler and Rinderle, 1988). Buschmann and Lichtenthaler (1988) particularly studied the fluorescence decline after F_M and established that the two bands at 685 and 740 nm showed different time courses, with a slower decline for the 740 nm band. More recently, the changes in the 77 K spectrum after illumination at room temperature were studied (Ruban and Horton, 1994, 1995). NPQ was found to preferentially affect distinct components of the low-temperature emission spectrum in the PSII-emission region.

In a previous article (Franck *et al.*, 2002), we have compared the F_O and F_M spectra recorded with a diode array fluorometer in intact green leaves at room temperature, with the purpose of estimating the relative contributions of PSI and PSII in the F_O and F_M spectra. How the spectrum changes progressively during the rise from F_O to F_M was not analyzed, and the fluorescence quenching after F_M was not investigated. These two aspects of the fluorescence induction are studied in this work.

4.4 Materials and methods

4.4.1 Biological material

Green barley leaves were obtained by growing the plants for 7 days in a growth cabinet at a temperature of 23°C and light intensity of 200 μmol m^{-2} s^{-1}. Etiolated plants were grown in darkness and transferred to continuous white light of 45 μmol m^{-2} s^{-1} after 6 days.

4.4.2 Fluorescence measurements

Fluorescence emission spectra from intact leaves were measured at room temperature from the adaxial side. Leaves of greening plants were unrolled before measurements. At least five independent measurements were performed and equivalent spectra were accumulated. The exciting light was provided by a stabilized Oriel light source (Spectra-Physics, Mountain View, CA). It was filtered through a combination of three Corning CS4-96 broad blue filters

(Spectra-Physics) and focused on the leaf sample to produce an actinic light intensity of 660 μmol m^{-2} s^{-1} used for F_M measurements. For the F_O measurements, the exciting light intensity was decreased to 0.4 μmol m^{-2} s^{-1} by neutral filters. Fluorescence was measured at right angles with the excitation-light path using an optical spectrometric multichannel analyzer (Princeton Instruments Inc., Trenton, NJ) equipped with a 1024 channels intensified diode array detector and a SPEX Model 1681 grating monochromator. A high-pass red filter (maximal transmission at 620 nm, Spectra-Physics) was used to protect the detector from blue stray light. The slit width was 0.4 mm, which allowed a spectral resolution of 3 nm. Spectra were corrected for a baseline and for the diode-dependent response of the measuring system, as described (Böddi *et al.*, 2003). The linearity of the detector response as a function of incoming fluorescence intensity was verified using a Chl solution in methanol. Wavelength calibration was performed with an Oriel Neon light source (#6032, Spectra-Physics). Monitoring of the fluorescence yield during spectral measurements was done using a pulse-amplitude-modulated fluorometer (FMS/2S, Hansatech Instruments Ltd., Norfolk, UK).

4.4.3 Chloroplast isolation

Chloroplasts were isolated as in Barthélemy *et al.* (1997). Fluorescence emission spectra and transmission spectra of chloroplast suspensions were recorded using a luminescence spectrometer (LS50-B, PerkinElmer BioSignal Inc., Montreal, PQ, Canada) and a spectrophotometer (Lambda 20, PerkinElmer BioSignal Inc.).

4.4.4 Pigment content

Chl concentration was measured in methanol extracts as in Wellburn (1994).

4.5 Results and discussion

4.5.1 Fluorescence spectra at F_O, F_M, F_T and their derived ratio spectra

Using a sensitive diode array detector with a resolution of 100 ms, we have recorded room-temperature fluorescence emission spectra at different time points of the Kautsky effect under actinic blue exciting light. Because the fluorescence intensity rises very rapidly during the first millisecond after the onset of actinic excitation, the spectrum of the F_O level could only be obtained by averaging 200 scans of the fluorescence emitted under weak analytical light (0.4 μmol m^{-2} s^{-1}). By taking into account the transmission of the neutral filters used for lowering the light intensity, the F_O spectrum could be compared quantitatively with the F_M and F_T spectra recorded by 0.95 s and 5 min with an actinic illumination of 660 μmol m^{-2} s^{-1}. As shown in Figure 4.1A, the three spectra show a main band at 684 nm with a weak shoulder around 700 nm and a broad band around 735-740 nm. As seen, the latter band is clearly heterogeneous, with a visible shoulder around 720 nm. Ratio spectra (Figure 4.1B) were calculated to provide more information on the wavelength dependence of fluorescence quenching at F_O or F_T. The F_M/F_O spectrum had a maximum at the peak position of the main emission band at 684 nm, decreased on both sides of this maximum and shows a minimum at around 720 nm. The F_M/F_T spectrum had a very similar shape beyond 700 nm compared with the F_M/F_O spectrum but differed in its peak position, which was found to be at 679–680 nm instead of 684 nm.

4.5.2 Changes in the fluorescence spectrum during the fast rise from F_O to F_M

The shape of the F_M/F_O ratio spectrum in the near far-red must reflect the overlapping contributions of PSII and PSI to variable fluorescence. PSI can contribute to variable fluorescence because of energy migration from PSII to PSI, a process known as spillover (Butler, 1978). However, because of the low efficiency of spillover in dark-adapted samples, the fluorescence of PSI is expected to increase much less than that of PSII. Hence, the minimum around 720 nm in the F_M/F_O spectrum indicates the region of maximal PSI contribution in the fluorescence spectra, whereas the 740 nm maximum reflects a higher PSII contribution because of vibrational bands (Franck *et al.*, 2002). To determine the position of the PSI fluorescence maximum at room temperature *in vivo*, a difference spectrum of (F_O-F_M) was calculated after normalizing the spectra at 684 nm, a wavelength at which PSI contribution is expected to be the lowest. The difference spectrum (Figure 4.2) shows that

PSI emission is maximal around 725 nm and contributes to the shoulder observed in that region in the total fluorescence spectra. Because of the normalization, this procedure cannot give indications on the possible occurrence of short-wavelength bands, which would overlap strongly with the PSII band at 684 nm. Therefore, the 700 nm band, if it belongs to PSI, cannot be seen clearly in the difference spectrum. The 725 nm maximum for PSI fluorescence and the broadness of the spectrum found in this study are in good agreement with published spectra of PSI particles from higher plants isolated under nondenaturing conditions (Croce *et al.*, 1996). To study the progressive change in the emission spectrum during the fluorescence rise, several spectra were recorded successively between F_O and F_M. The spectra shown in Fig. 3A were obtained by cumulating the fluorescence during 100 ms periods (for instance, the 150 ms spectrum is the sum of the fluorescence emitted between 100 and 200 ms after the onset of actinic light). To obtain sufficient definition in the fluorescence rise, the intensity of the actinic light was decreased to 80 μmol m^{-2} s^{-1}. Under this intensity, the fluorescence maximum found at approximately 1 s was somewhat lower than the true F_M level. F_M spectra of the same leaves were recorded during a 1 s light pulse of 660 μmol m^{-2} s^{-1}, given after 1 min dark adaptation. A careful examination of the spectra shown in Figure 4.3A indicates that the 684 nm band and the long-wavelength region do not behave the same way with time; the 684 nm band increases faster than the long-wavelength fluorescence. More detailed information on the spectral changes was obtained by calculating differences between pairs of spectra recorded during different periods of the fluorescence rise (Figure 4.3B). The shape of these difference spectra showed pronounced differences depending on the considered part of the fluorescence rise. At the beginning of the process, ratio spectra show the expected shape with a maximum at 684 nm (see the "$F_{(150\ ms)}$-F_O" spectrum in Figure 4.3B). Later on, the emission maximum in difference spectra shifted progressively to higher values and the relative intensity of the long-wavelength fluorescence (maximum and around 740 nm) increased markedly. This is shown in Fig. 3B by the "F_M-$F_{(750\ ms)}$" difference spectrum, corresponding to the end of the rise. Such evolution of the difference spectra because of variable fluorescence with time implies that the kinetic of the rise is significantly wavelength dependent. The change in the spectrum of variable fluorescence with illumination time, outlined above, can be explained by taking into consideration screening effects because of the high leaf Chl concentration. Efficient

absorption of the blue exciting light by the upper cell layers results in a strong excitation light gradient inside the leaf (Cui *et al.*, 1991; Vogelman and Han, 2000). Therefore, chloroplasts located deeper in the leaf tissue (in the spongy mesophyll) are exposed to lower light intensity and their fluorescence emission is expected to rise more slowly (Hsu and Leu, 2003). On the other hand, because fluorescence is less reabsorbed at long wavelength than in the region of its maximum, the fluorescence spectrum measured from chloroplasts located deeper in the leaf can be redshifted. We have simulated this effect by calculating the effect of screening on the emission spectrum of isolated chloroplasts on the basis of emission and transmission spectra of a diluted chloroplast suspension. As shown in Figure 4.4, screening of the main fluorescence emission band at 684 nm, because of low transmission in this region, results in a progressive shift of the 684 nm band toward 700 nm and in a relative increase of the contribution of the 740 nm band. This effect is quite similar to the one shown in Figure 4.3B for intact leaves, when difference fluorescence spectra of different parts of the fluorescence rise are compared. We conclude that, as time elapses from the onset of excitation, the measured fluorescence originates from deeper regions of the leaf. This effect must be partly overcome by the adaptation of chloroplasts to different local light intensities, which has been shown to result in larger PSII optical cross section in chloroplasts from spongy mesophyll cells compared with those of palisade cells (Terashima and Inoue, 1984, 1985). The heterogeneous origin of the fluorescence signal as a function of illumination time should not be ignored in attempts to perform mathematical simulations of the fluorescence rise curves. In future, it would be interesting to analyze spectral changes associated with specific subphases of the fluorescence rise (Strasser *et al.*, 1995). This was not possible in this study because of insufficient time resolution. We have studied the effect of Chl accumulation and of PSII assembly on the *in vivo* emission spectrum and its change during the fluorescence rise by using dark-grown leaves subjected to continuous greening for different times. In barley, the development of photosynthetic ability in the presence of light is a rapid process, which has been studied in detail in previous studies. Distinct periods can be considered as far as PSII is concerned. During the first 3-4 h of greening, the composition of PSII is limited to the core and the minor light-harvesting complex II (LHCII) complexes. The assembly of the peripheral LHCII then follows and is completed after approximately 7 h (Dreyfuss and Thornber, 1994). At the same time the Chl *a*/*b* ratio drops to the normal value

of 3. During later greening, Chl continues to accumulate but no marked changes in the pigment-protein stoichiometries of PSII occur. In this study, we have focused our observations on the phase of LHCII accumulation, between 3 and 7 h of greening. At earlier greening times, the phototransformation of sizeable amounts of photoactive protochlorophyllide during an actinic light pulse would have made the spectral changes too complex to analyze (Franck *et al.*, 1993). Small but well-defined changes in the shape of the main emission band around 684 nm were found between 3 and 7 h of greening. In the F_O spectrum, this band showed a 2 nm redshift from 682 to 684 nm in this period (Figure 4.5A). It was then identical to the 684 nm band of normal green leaves (see Figure 4.1A). This indicated the presence of poorly coupled short-wavelength Chl *a* at early-greening stages, which were detected previously as a Chl *a* population with high-fluorescence lifetime and short-wavelength emission maximum in similar experimental conditions (Mysliwa-Kurdziel *et al.*, 1997). Uncoupled Chl *a* has also been detected in thylakoids from fully green spinach (Santabarbara *et al.*, 2001). The redshift of the F_O spectrum shown in this study during greening indicates a decreasing contribution of uncoupled Chl *a* during the assembly of photosystems. At the same time, the relative intensity of long-wavelength emission increased because of increasing self-absorption as Chl accumulates (Figure 4.5A). The spectra of the variable part of the fluorescence (F_M-F_O) showed a different evolution with greening time (Figure 4.5B). Their maxima showed very little change, but a distinct increase in relative intensity was observed in the 640-670 nm region. This change occurred between 4 and 7 h of greening (Figure 4.5C), a period during which the Chl *a*/*b* ratio decreases sharply and massive LHCII assembly occurs (Mysliwa-Kurdziel *et al.*, 1997). Therefore, the slight band broadening between 640 and 670 nm found in this study along with greening in the variable fluorescence spectrum is likely because of an increasing contribution of the fluorescence of Chl *b* associated with LHCII. These results show that the presence of the major LHCII complexes has weak influence on the shape of the main PSII emission band, although most of PSII Chl is located in these complexes. The evolution of the variable fluorescence spectrum along with the $F_O \rightarrow F_M$ transition was also investigated in greening leaves. Leaf samples taken after 3.5 or 7 h of greening already showed a significant relative variable fluorescence (average F_M/F_O values of 0.30 and 0.65, respectively), but the fluorescence rise was slower than in green leaves because of lower antenna size (Barthélemy *et al.*, 1997). However, under

these conditions, it was possible to obtain difference spectra corresponding to different periods of the rise by adjusting the actinic light intensity and the pulse duration. In contrast to green leaves (Figure 4.3B), leaves taken after 3.5 h of greening showed almost constant difference spectra during the fluorescence rise (Figure 4.6, upper panel). Changes similar to those found for mature green leaves were observed after 7 h of greening (Figure 4.6, lower panel), when the Chl *a*/*b* ratio had reached minimal value and Chl concentration was already 45 % of that measured in fully green leaves.

4.5.3 Spectral changes during the slow fluorescence decline from F_M to F_T

As shown by the F_M/F_T spectrum in Figure 4.1B, the fluorescence quenching at the F_T under continuous light shows similar wavelength dependence as the F_M/F_O spectrum at wavelength higher than 700 nm. The minimum around 720 nm found in the F_M/F_T ratio spectrum thus reflects preferential PSII fluorescence quenching compared with PSI. Under conditions of high intensity used in this study (660 μmol m^{-2} s^{-1}), it was found by a parallel pulse-modulated analysis that a high NPQ was established because a saturating light pulse given at F_T had practically no effect on the fluorescence yield. In the shorter wavelength region (around 684 nm), the blueshifted position of the maximum of the F_M/F_T spectrum (see Figure 4.1B) was indicative of a slight shift in the position of the main emission maximum around 684 nm. This shift was evident when comparing the 670-690 nm region of the F_M and F_T spectra normalized at their maximum (Figure 4.7A). Although the emission maximum of this band was the same for F_O and F_M, it was slightly shifted to the red at F_T. This shift was found to be dependent on the light intensity used. Under 80 μmol m^{-2} s^{-1}, it was barely detected (Figure 4.7B). Light intensity also affected the amplitude of NPQ, as already reported (Schreiber *et al.*, 1986). In the experiments leading to the spectra shown in Figure 4.7, the amplitude of NPQ (measured by $NPQ = (F_M\text{-}F_M')/F_M'$) was 3.8 (high-actinic light) and 1.2 (low-actinic light). Therefore, we suggest that the redshift in the PSII emission band is because of NPQ, a conclusion also supported by the fact that photochemical quenching is not associated to any detectable shift (as shown by the F_M/F_O ratio spectrum in Figure 4.1). The slow redshift was not detected at early-greening stages (3.5 h) but was significant after 7 h of greening, although it was less pronounced than in mature green leaves (data not shown).

Thus, the redshift of the PSII emission band during NPQ was related to the presence of LHCII. It indicates that Chl *a* quenching is not homogenous and is the highest in a short-wavelength set of Chl *a* located in LHCII. Judging from the FM/FT spectrum, the room-temperature emission maximum of these Chl is at 680 nm, which is in agreement with the emission maximum of LHCII at room temperature (Hemelrijk *et al.*, 1992; Kirchhoff *et al.*, 2003). Studies on lamellar aggregates of LHCII have shown that lightinduced quenching is associated with a trimer-to-monomer transition in vitro. However, this process was not observed after a short illumination of intact leaves in conditions similar to the ones used in this study (5 min of moderate light) (Garab *et al.*, 2002). The heterogeneous quenching observed in this study is therefore more likely associated with a general quenching of LHCII-associated Chl *a* rather than to a spectral change related to monomerization. Altogether, our observations confirm previous findings of specific effects of NPQ on the 77 K emission spectrum of intact leaves. In the low-temperature spectrum of leaves frozen after induction of NPQ by actinic light, preferential quenching of 77 K emission bands of LHCII was found (Ruban and Horton, 1994). This study shows that continuous recording of fluorescence spectra during the slow part of the Kautsky effect can be used to monitor this effect.

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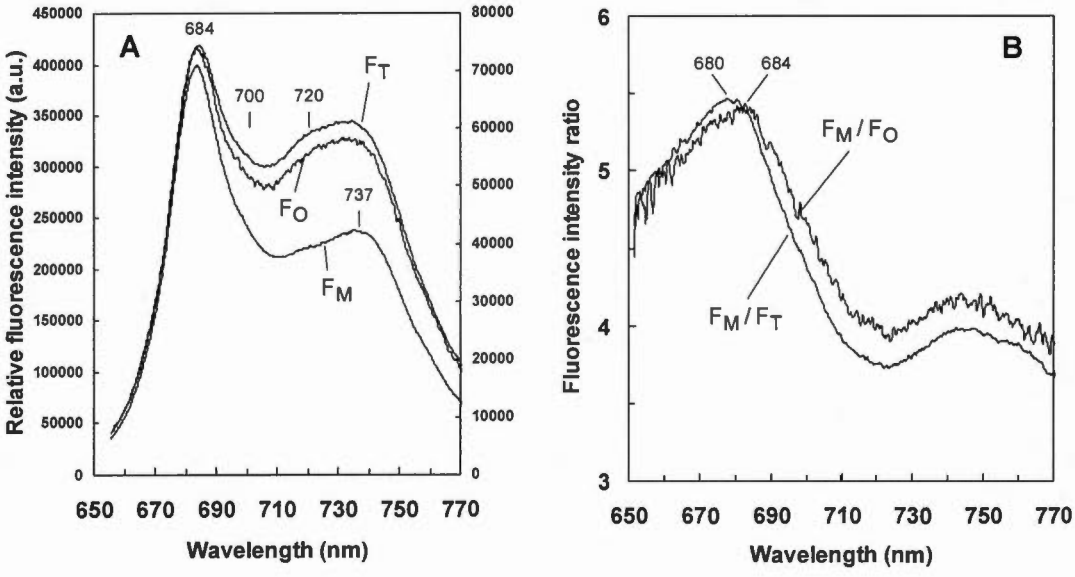

Figure 4.1 A : Room-temperature spectra of the fluorescence emitted by a mature barley leaf at the F_O, F_M and F_T states. F_O was measured under weak analytical light of 0.4 μmol m^{-2} s^{-1}. F_M and F_T were measured under actinic light of 660 μmol m^{-2} s^{-1} with F_M being measured in the time interval 0.6-0.7 s and F_T at 5 min after onset of illumination. Left axis : F_M. Right axis : F_O and F_T. B : F_M/F_O and F_M/F_T ratio spectra.

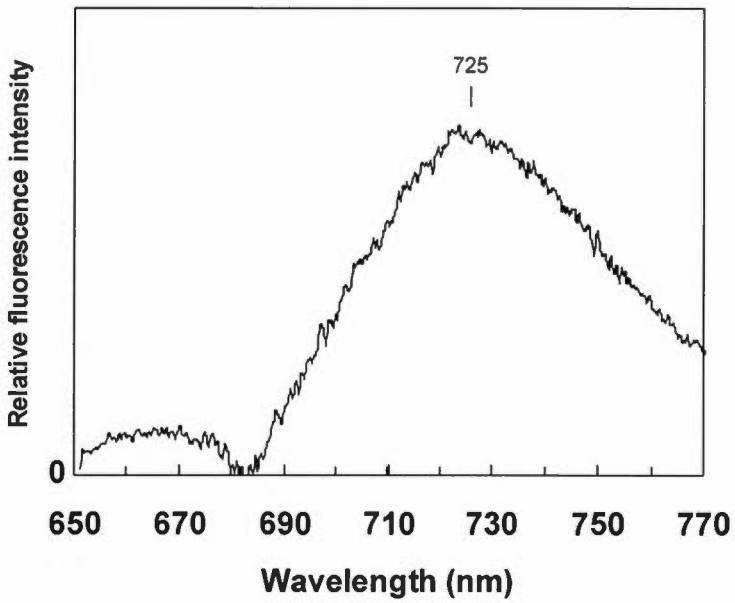

Figure 4.2 "F_O-F_M" difference spectrum obtained after normalization of the F_O and F_M spectra at their maxima.

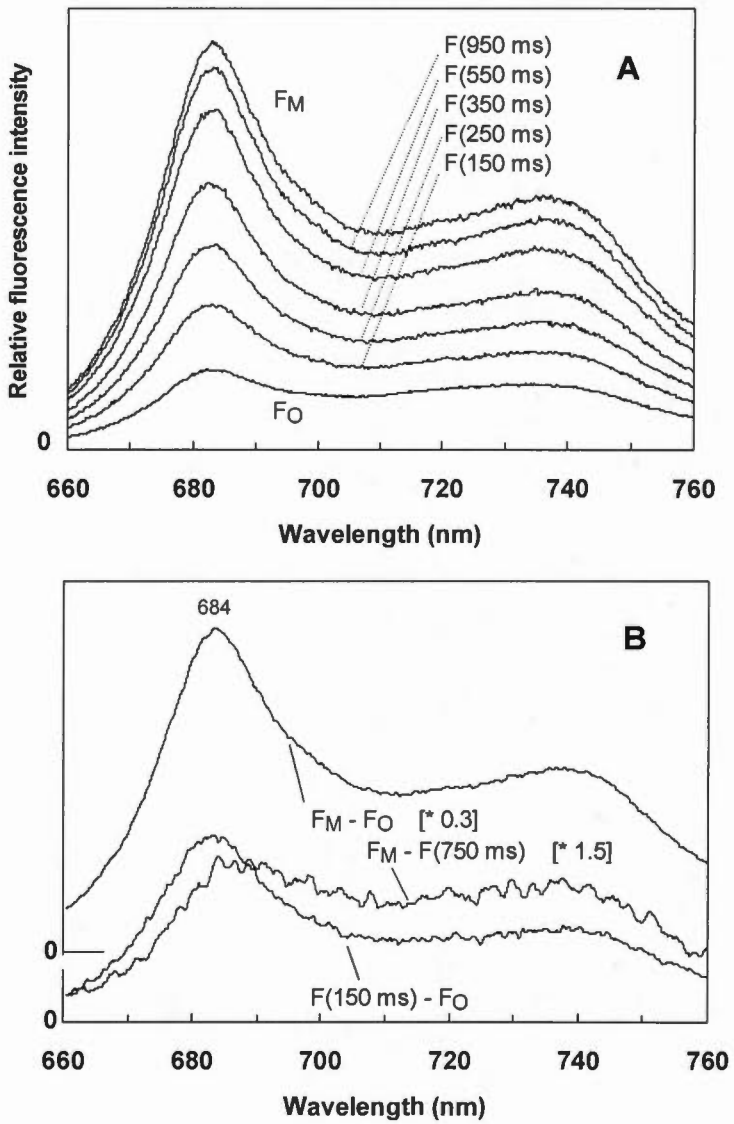

Figure 4.3 A : Emission spectra during the fluorescence rise from F_O to F_M. F_O was first measured under analytical light of 0.4 μmol m^{-2} s^{-1}, then the fluorescence rise was elicited by a 1 s pulse of actinic light of 80 μmol m^{-2} s^{-1} and successive emission spectra of 100 ms were measured. The F_M spectrum was measured during a subsequent 1 s pulse of saturating light (660 μmol m^{-2} s^{-1}) given 1 min after the first pulse. B : Difference spectrum of the total variable fluorescence (F_M-F_O) and difference spectra calculated during the beginning or the end of the fluorescence rise from F_O to F_M.

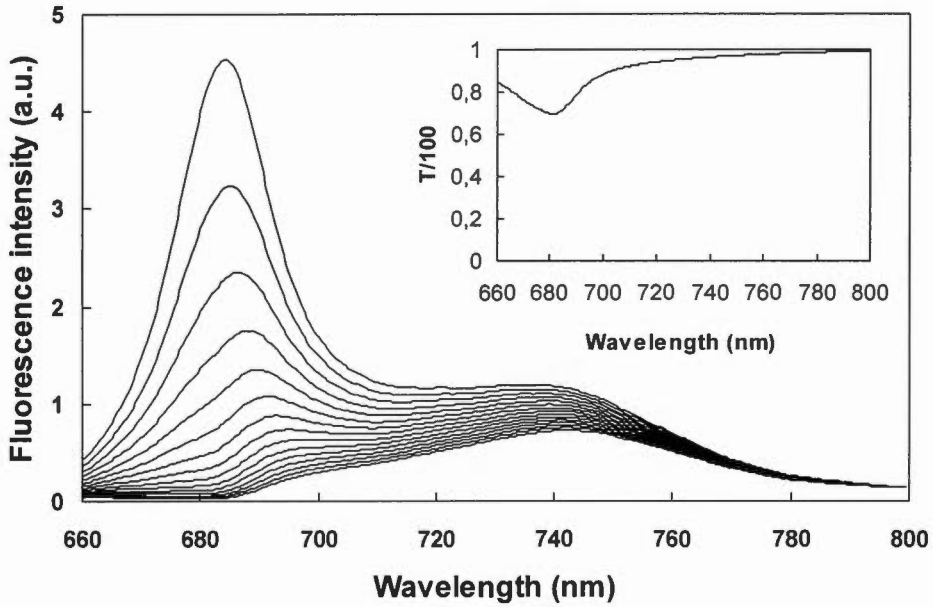

Figure 4.4 Screening effect on the room-temperature emission spectrum of a diluted barley chloroplast suspension (50 μg Chl^{a+b} mL^{-1}). The emission spectrum of the suspension was measured in a thin cuvette (0.5 mm). It was then multiplied several fold by the transmission spectrum (shown in inset) to yield a series of emission spectra simulating the effect of screening by an increasing number of chloroplast layers.

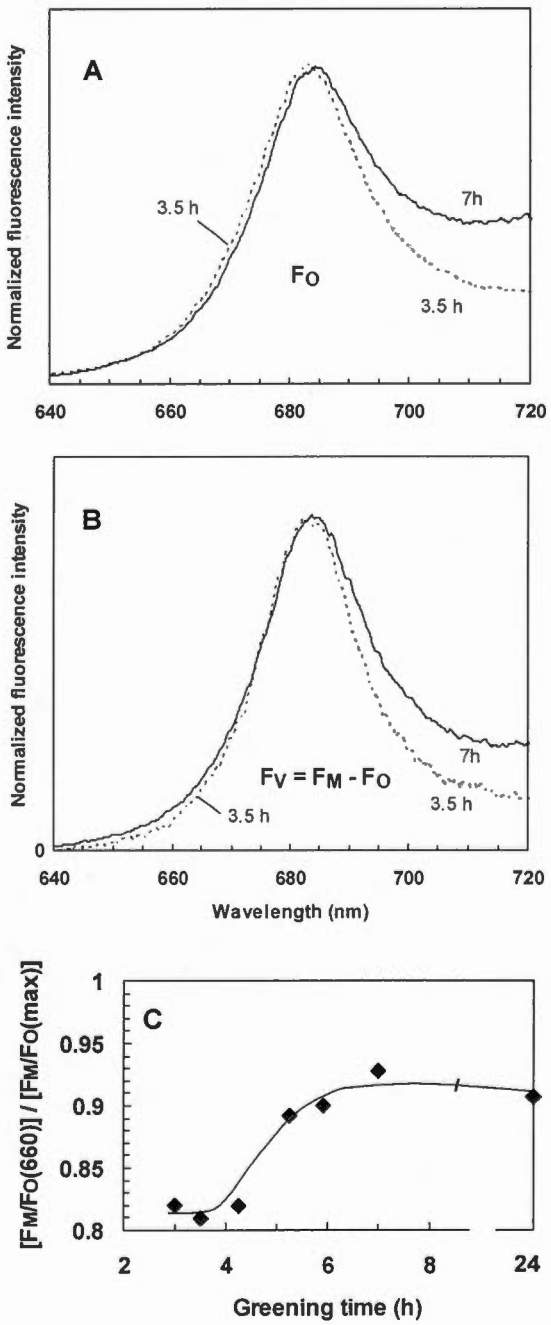

Figure 4.5 A : F_O spectra of dark-grown barley leaves after greening, during 3.5 or 7 h (spectra normalized at their maxima). B : F_V (= F_M-F_O) spectra of the same leaves. C : Dependence on greening time of the relative increase in the short-wavelength region of the variable fluorescence spectrum (calculated as a ratio of the F_M/F_O values measured at 660 nm and at the emission maximum around 683 nm).

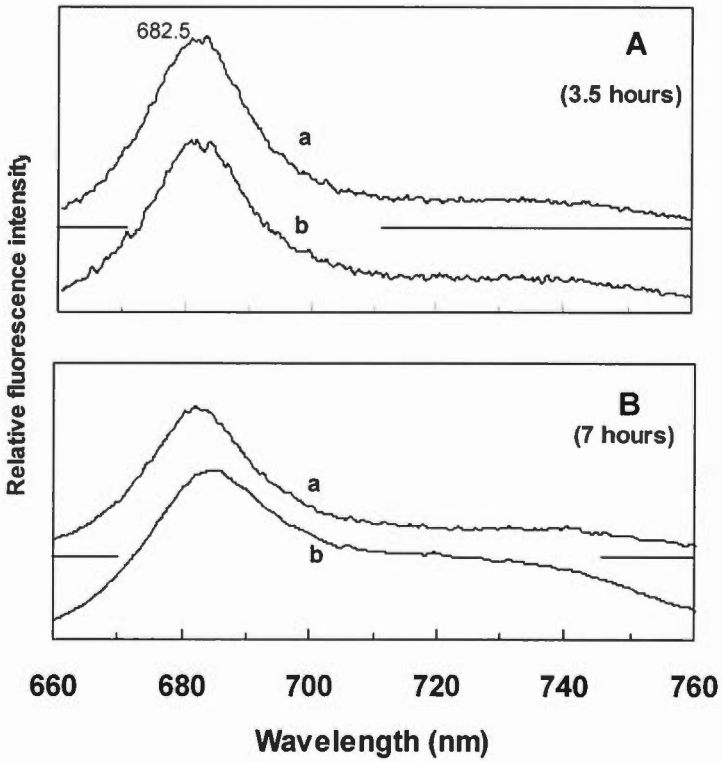

Figure 4.6 Difference spectra calculated during different parts of the fluorescence rise from F_O to F_M in dark-grown barley leaves after greening for 3.5 h (A) or 7 h (B). For comparison, difference spectra were calculated either during the initial part of the rise (covering approximately the first 30 % of the increase at 683 nm) (a) or during the final part of the rise (covering approximately the last 30 % of the increase at 683 nm) (b).

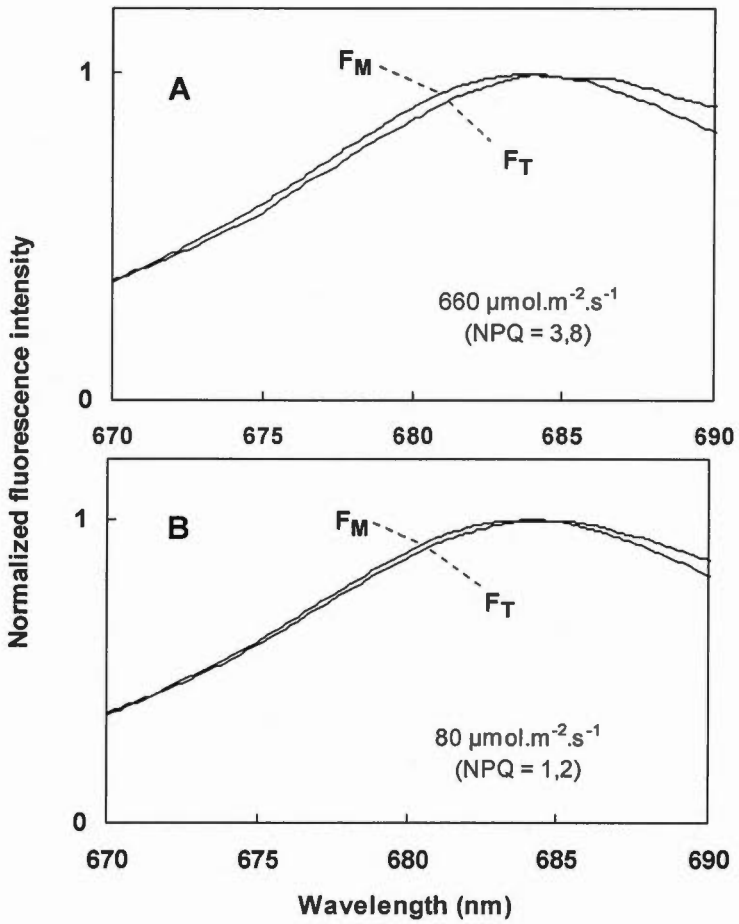

Figure 4.7 The redshift of the PSII emission band during the fluorescence decline from F_M to F_T (spectra were normalized at their maxima). A : Actinic light intensity of 660 µmol m^{-2} s^{-1}. B : Actinic light intensity of 80 µmol m^{-2} s^{-1}. Values of NPQ are shown.

CHAPITRE V

RAPID CHLOROPHYLL *A* FLUORESCENCE TRANSIENT OF *LEMNA GIBBA* LEAF AS AN INDICATION OF LIGHT AND HYDROXYLAMINE EFFECT ON PHOTOSYSTEM II ACTIVITY

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5.1 Résumé

Les transitions rapides de la fluorescence chlorophyllienne induites par un flash saturant (3000 µmoles de photons m^{-2} s^{-1}) ont été investiguées chez la plantule *Lemna gibba* exposée à une illumination continue (100 µmoles de photons m^{-2} s^{-1}) causant «l'effet Kautsky» ou à une faible intensité lumineuse incapable d'induire la réaction photochimique primaire du photosystème II. Les mesures ont été effectuées en utilisant simultanément les fluorimètres «*Pulse Amplitude Modulated*» et «*Plant Efficiency Analyzer*» chez des plantules de *Lemna gibba* non-traitées ou traitées à différentes concentrations d'hydroxylamine (1-50 mM) causant une inhibition graduelle du complexe enzymatique de la photolyse de l'eau. Lorsque les plantules ont été exposées à une illumination continue, les transitions rapides de fluorescence reflétaient les états structuraux et fonctionnels du photosystème II. Dans ces conditions, le changement des temps d'apparition des transitions était associé à un changement drastique des propriétés structurelles et fonctionnelles du photosystème II. La valeur du paramètre de fluorescence qui indiquait la dissipation d'énergie par des voies non-photochimiques variait de façon extrême. Le changement des transitions rapides de fluorescence, induit sous une illumination continue en comparaison avec une faible intensité lumineuse, confirme l'habileté du photosystème II de s'adapter rapidement en deux minutes. Lorsque le complexe enzymatique de la photolyse de l'eau a été inhibé et que des électrons ont été donnés partiellement par l'hydroxylamine, la capacité du photosystème II de s'acclimater aux différentes conditions lumineuses était perdue. Dans cette étude, le changement de la cinétique rapide de fluorescence et du temps d'apparition des transitions ont été une bonne indication du changement des propriétés structurelles et fonctionnelles du photosystème II induit par la lumière ou l'hydroxylamine.

5.2 Abstract

Rapid chlorophyll fluorescence transient induced by saturating flash (3000 μmoles of photons m^{-2} s^{-1}) was investigated when *Lemna gibba* had been exposed to light (100 μmoles of photons m^{-2} s^{-1}) causing the Kautsky effect or in low light intensity unable to trigger PSII photochemistry. Measurements were made by using, simultaneously, Pulse Amplitude Modulated fluorometer and Plant Efficiency Analyzer system, either on non-treated *Lemna gibba* leaf or leaf treated by different concentrations of hydroxylamine (1-50 mM) causing gradual inhibition of the water splitting system. When any leaf was exposed to continuous light during the Kautsky effect, a rapid fluorescence transient may reflect current activity of photosystem II and structural change within photosystem II complex. Under those conditions, variation of transition steps appearing over time was related to a drastic change to the photosystem II functional and structural properties. The value indicating the energy dissipation through non-photochemical pathways was undergoing extreme changes. The change of rapid fluorescence transient, induced under continuous light, when compared to those obtained under very low light intensity, confirmed the ability of photosystem II to have an ability for rapid adaptation lasting only about two minutes. When the water splitting system is inhibited and electron donation partially substituted by hydroxylamine, the adaptation ability of photosystem II to different light conditions was lost. In this study, the change of rapid fluorescence kinetic and transient appearing over time was shown to be a good indication for the change of the functional and structural properties of photosystem II induced either by light or hydroxylamine.

5.3 Introduction

Illuminated leaf, after a dark adaptation, dissipates variable chlorophyll *a* fluorescence, a phenomena known as the Kautsky effect (Kautsky and Hirsch, 1931; Duysens and Sweers, 1963). Fluorescence induction has been shown to have two distinct kinetics in time : One as a rapid fluorescence rise closely related to photosystem II (PSII) photochemistry, and regulated by PSII electron donor and acceptor sides, and an another known as the slow fluorescence decay, lasting for several minutes depending on the energy balance between PSII and PSI and also on the CO_2 fixation process (Papageorgiou, 1975; Lavorel and Etienne, 1977; Walker, 1987). When a dark-adapted plant is exposed to continuous illumination, such slow decay of variable fluorescence yield has been shown to be closely related to the oxido-reduction state of the plastoquinone pool (Neubauer and Schreiber, 1987). On the other hand, when a plant has been exposed to a short saturating flash (300-700 ms), the maximum fluorescence rise has been found to be strongly dependent on the reduction state of PSII primary electron acceptor Q_A and, following on, the electron carriers Q_B and plastoquinones (Schreiber and Neubauer, 1987; Strasser *et al.*, 1995). Kinetics of rapid fluorescence induction *in vivo* has been recognized to indicate the functional state of the water splitting-system, the charge separation at the PSII reaction center and the reduction state of PSII electron acceptor sides (see review Lazár, 2006). The fluorescence yield appearing up to 40-50 μs was considered as the F_0 fluorescence level indicating the state when all PSII reaction centers are open (Strasser *et al.*, 1995; Sušila *et al.*, 2004). The variation of F_0 fluorescence was proposed to be due to the change of the equilibrium transfer between PSII antennae excitation and the PSII reaction center (Owens, 1996). Fluorescence kinetics from F_0 level to the maximum fluorescence yield F_M have different transitions reflecting the functional properties of PSII complex. Fluorescence yield at transition J-I was seen to be dependent on the reduction of Q_A and Q_B electron carriers (Schreiber and Neubauer, 1987; Strasser *et al.*, 1995). It has been also proposed for transition J-I to be dependent on the charge distribution between the water-splitting system and PSII primary Q_A and secondary Q_B electron acceptors (Hsu, 1993). Such interpretation was supported by theoretical simulations of dependency between PSII primary photochemistry and related electron transport process (Lazár, 2003; Zhu *et al.*, 2005). A fluorescence quenching effect during I-P transition, seen as D dip, has been shown to indicate

a dynamic equilibrium between PSII and PSI activity (Hansen *et al.*, 1991). When leaf is exposed to heat treatment, the appearance of an early K step around 200-300 μs has been attributed to be an indication of the water-splitting system inhibition (Guissé *et al.*, 1995; Srivastava *et al.*, 1997; Lazár, 2006). According to this interpretation, loss of J-I-P transitions, when water-splitting system was inhibited, was interpreted to indicate an imbalance between PSII electron flow from acceptor and donor sides causing an accumulation of Y_Z^+ serving as PSII primary electron donor. Therefore, it has been proposed for the K step to indicate the first reduction of Q_A occurring after the light-induced charge separation between P680 and Pheo (Strasser, 1997). However, the quenching effect of the fluorescence yield following the K step was proposed to reflect an oxidation of Q_A^- *via* subsequent electron acceptor (Lazár *et al.*, 1999). On the other hand, when the water splitting system of PSII in pea leaves was inhibited and then PSII electron transport ability partially reconstituted by hydroxylamine, the quenching effect following the K step did not appear (Srivastava *et al.*, 1997). Presently, the most information on the dependency between PSII activity and fluorescence transient is obtained when leaves have been dark-adapted and exposed to short illumination. However, an important question should be posed : how rapid is the fluorescence transient response to PSII activity when a plant is exposed to continuous different illumination conditions. If one may compare such studies with results obtained from a dark-adapted plant, further understanding of how rapid fluorescence transient reflects PSII electron transport activity toward to PSI may be provided for. In this study, when *Lemna gibba* leaves were exposed to different illumination conditions provided by continuous light, a saturating flash was induced periodically in order to investigate the change of rapid fluorescence transient when PSII photochemistry occurred. Furthermore, this study provides valuable information on how the presence of PSII exogenous electron donor as hydroxylamine (NH_2OH) affects *in vivo* (intact leaf) the rapid fluorescence transient when the leaf is exposed to continuous illumination. Such results will provide also for a further understanding of the dependency between the change of rapid fluorescence transient and PSII electron transport during the first term of continuous illumination (Kautsky effect).

5.4 Materials and Methods

5.4.1 Biological material

The duckweed *Lemna gibba* was cultivated in an inorganic autoclaved growth medium (pH 6.5) (Chollet, 1993). Stock culture of *L. gibba* was maintained at 23°C under a continuous illumination of 100 µmoles photons m^{-2} s^{-1} provided by cool white fluorescence lamps (Sylvania GRO-LUX F40/GS/WS, Drummondville, Canada). Triple-fronded *L. gibba* plants in the exponential growth phase were used for experiments.

5.4.2 Rapid fluorescence induction kinetic and related parameters

The measurements of the rapid fluorescence induction from 10 µs to 1 s was done with a PEA fluorometer (Hansatech Ltd., King's Lynn, Norfolk, UK) by using a saturating flash when the photosynthetic apparatus of *L. gibba* was undergoing different illumination conditions (a modulated light of 1 µmole photon m^{-2} s^{-1} and an actinic light of 100 µmoles photon m^{-2} s^{-1}) induced by a PAM fluorometer (Hansatech Ltd., King's Lynn, Norfolk, UK). The PEA saturating flash was provided by an array of six light-emitting diodes giving a maximum emission at 650 nm with an intensity of 3000 µmoles photons m^{-2} s^{-1}. The fluorescence yield at 50 µs was considered as F_0 value ($F_{50\mu s}$) and the maximum fluorescence yield attained as F_M (Strasser *et al.*, 1995; Strasser and Strasser, 1995). The fluorescence induction curves were plotted on a logarithmic time scale allowing for the visualization of transitions. The determination of the 'appearing time' for all rapid fluorescence transients was done by analyzing the first derivative of the fluorescence induction curve (Klinkovsky and Naus, 1994).

Evaluation of photosynthetic parameters related to fluorescence yields or flux ratios within the PSII complex were based on the theory of energy fluxes in biomembranes (Strasser *et al.*, 2004) : The maximum quantum yield of primary photochemistry was determined as $\phi_{Po} = (F_M - F_{50\mu s}) / F_M$; The probability that a trapped exciton moves an electron into electron transport chain beyond Q_A^- was estimated as $\psi_o = 1 - [(F_{2ms} - F_{50\mu s}) / (F_M - F_{50\mu s})]$, where F_{2ms} represents the fluorescence yield at 2 ms; The quantum yield of electron transport was evaluated as $\phi_{Eo} = \phi_{Po} \times \psi_o$; The quantum yield of energy dissipation not used into

primary photochemistry was determined as $\phi_{Do} = 1 - \phi_{Po}$. The complete non-photochemical quenching of chlorophyll fluorescence yield for sample in light adapted state was evaluated as $q_{CN} = 1 - (F_{M(at\ F2,\ F3,\ F4,\ F5\ or\ F6)} / F_{M(at\ F1)})$ (Roháček and Barták, 1999).

5.4.3 Hydroxylamine treatment

The stock solution of NH_2OH was prepared in 100% ethanol. Treatment media was composed of growth medium with addition of NH_2OH from stock solution. The final concentration of ethanol in NH_2OH treated and control samples was 0.15%. At this concentration of ethanol, no inhibition of photosynthetic processes was noticed by comparing control with and without ethanol (data not shown). Six triple-fronded *L. gibba* were exposed to 1, 2, 5, 10 and 50 mM of NH_2OH under static conditions (same as growth conditions) during 30 min in darkness. Treatment of *L. gibba* plant was done in Petri dish containing 50 ml of growth medium and appropriate NH_2OH concentration. Control sample was considered to have the same content, but with no addition of NH_2OH. Each experiment was conducted in triplicate.

5.4.4 Oxygen evolution measurement

For oxygen evolution measurements, three triple-fronded *L. gibba* plants were placed in a gas-phase oxygen electrode chamber (LD2/3, Hansatech Ltd., King's Lynn, Norfolk, UK). The rate of oxygen evolution was monitored by using an oxygen electrode disc known as a Clark type polarographic sensor (Hansatech Ltd., King's Lynn, Norfolk, UK) at a light intensity of 100 μmoles photons m^{-2} s^{-1}. The temperature of the oxygen electrode chamber was controlled at 23°C. Calibration of the chamber and gas-phase measurement of oxygen evolution were done according to standard method (Walker, 1987).

5.4.5 Fluorescence measurements protocol

For the investigation of rapid fluorescence transient during continuous illumination, PAM and PEA fluorometers as an analytical approach were synchronised where all

measurements were done on the same *L. gibba* sample. An optical set up condition such as this was used in order to providing for reproducible and comparable fluorescence yields, by using the same sample in order to avoid the normalization of fluorescence yields during measurements. Under different light conditions provided by the PAM fluorometer, six consecutive PEA saturating flashes distributed as presented in Figure 5.1 were used.

After being adapted for 30 min in darkness, *L. gibba* was exposed to a weak modulated light unable to trigger primary PSII photochemistry for the two min before the first PEA saturating flash was applied (F1). Two min following the first flash the Kautsky effect was induced by PAM actinic light. Second (F2), third (F3) and fourth (F4) flashes were applied at maximal fluorescence emission (F_P), at exponential fluorescence decay and at steady state of fluorescence emission of the Kautsky effect, respectively. The length of exposure between flashes was sufficient to avoid the additional effect of a saturating flash on the change of fluorescence yield under continuous illumination. Following the fourth flash the actinic light was turn off and after two min the fifth saturating flash (F5) was applied. Following two min after the fifth flash the far-red light (735 nm) was on and after two min the sixth flash (F6) was applied. Such flash distribution should provide information on the dependency between the change of fluorescence transient and PSII functional and structural properties when dark adapted leaf was exposed to different continuous illuminations.

5.5 Results and discussion

5.5.1 The change of rapid fluorescence transient when leaf is exposed to different light conditions

In this study, PAM was employed to provide different light conditions, thus permitting the investigation of the structural and functional properties of PSII. Under such conditions, the change of fluorescence transient was obtained from the same leaf sample permitting a direct comparison of fluorescence yields (see Figure 5.1). Kinetics of rapid fluorescence rise and a transient obtained by F1 represents typical fluorescence induction obtained from dark adapted leaf (data not shown) having steps J, I, D and M at maximum fluorescence yield F_M

(Figure 5.2A). The D dip appeared to be evident either for F1 induced after dark adaptation or F5 after relaxation under very low energy modulated light and for F6 when leaf was exposed to far-red light. However, when leaf was exposed to actinic light with an addition of a saturating flash (F2, F3 and F4), the D dip did not appear, showing an imbalance of electron transport in favour of PSII compared to PSI. When a leaf was exposed to continuous actinic light for a few minutes (F3 and F4), some of PSII reaction centers were observed to be converted into inactive and dissipative forms, not contributing to electron transport (Cao and Govindjee, 1990; Critchley, 1998). Therefore, it may be considered that a conformational change of PSII, induced under continuous light, is likely to be the main reason for the decrease of maximum fluorescence yield at step M, which indicated a decrease of PSII quantum yield ϕ_{Po} and an increase of quantum yield of energy dissipation ϕ_{Do} (Figure 5.2A, Table 5.1). We may assume for the increase of F_0 by 40 and 10 % respectively at F2 and F3 to be caused by the same effect. The increase of F_0 value during the initial phase of continuous illumination was reported earlier and this change was interpreted to be due to the conformational change at PSII induced by the dramatic shift of equilibrium between photochemical and non-photochemical processes. Such processes are known to be related to pH gradient formation and feedback by the synthesis of NADPH and ATP (Gerst *et al.*, 1994; Roháček and Barták, 1999). After 2 min exposure to continuous actinic light, the decrease of F_0 level reached its steady state as it was shown for F_0 value at F4 (see Table 5.1). When F2, F3 and F4 were applied (in the presence of actinic light), the change of variable fluorescence yield from J to P step showed active PSII electron transport via Q_B since ψ_o value, as indicator of electron transport beyond Q_A^-, was increased comparing to ψ_o values obtained with F1 and F5 (under omitted actinic light). However, the increase of ψ_o value for F6 (under far-red light) may indicate that far-red light also contributes to PSII as well as PSI electron transport. Therefore, under actinic light, quantum yield of electron transport from Q_A toward to plastoquinone pool was increased, as was indicated by the increase in ϕ_{Eo} value for F2, F3 and F4 (Table 5.1). Under those conditions we may expect that when leaf is exposed to actinic light, the complete non-photochemical quenching, indicated by Q_{CN} value, should be increased according to the proposed model of Roháček and Barták (1999). Indeed, we found that Q_{CN} was increased by seven times (Table 5.1) under actinic light (F3 and F4) as compared, to leaf exposed to low light energy (F1 and F5 under modulated light and F6 under

far-red light). When the ratio between ATP, NADPH synthesis and CO_2 fixation is not in equilibrium (during the Kautsky effect), it is known that proton concentration in the thylakoid lumen is increased and consequently this increases the non-photochemical quenching effect (Krause and Weis, 1991). Therefore, an initial increase of proton concentration in the thylakoid lumen will lead to accumulation of $P680^+$ and its reduction by an alternative way *via* light harvesting complex which will induce a non-photochemical fluorescence quenching event (Horton and Ruban, 1992; Pospíšil, 1997). It was reported for the non-photochemical quenching (NPQ) to reflect a regulatory mechanism involved in energy dissipation of excess absorbed light energy by chlorophyll which is unable to be transferred through the energy storage system. The major part of NPQ was shown to indicate thermal dissipation *via* PSII light harvesting antenna complex (Horton *et al.*, 1996; Horton and Ruban, 2005). This may explain for Q_{CN} value to be negligible or non visible when F1, F5 and F6 were applied (Table 5.1). Since PSII electron transport and energy dissipation were undergoing evident change, it was of interest to investigate the change of fluorescent transient appearing time when flashes were applied. We found that differences in the appearing time were evident when a flash was applied during leaf exposure to actinic light, initiating active electron transport toward to PSI. However, when *L. gibba* leaf was exposed to different light conditions, the appearing time for step J was not affected (see Figure 5.2A and Table 5.1). This should be expected since the appearance of step J was earlier found to be mostly related to the reduction state of Q_A (Strasser *et al.*, 1995; Strasser *et al.*, 2004). On the other hand, it was shown for step I to be strongly affected by plastoquinone pool reoxidation (Strasser, 1997). Therefore, for the non appearing D dip under F2, F3 and F4 (in the presence of actinic light), one may interpret it to be caused by an electron transport activity in favour of PSII compared to PSI. Consequently to those conditions, we may assume for D dip to be masked. On the other hand, if a saturating flash was induced when the leaf was exposed to low light energy (modulated light or far-red light), steps I and D appeared at 30 and 45 ms, respectively. For the delay of step M appearing time (when F2, F3, F4 and F6 were applied), this might be interpreted to be due to the oxidation of plastoquinone pool under actinic or far-red lights. The effect of 50 mM NH_2OH on the change of rapid fluorescence rise induced by six consecutive flashes (Figure 5.2B) will be discussed in the section below concerning NH_2OH as an inhibitor of water splitting system substituting electron donation to PSII.

5.5.2 Rapid fluorescence transient of *Lemna gibba* leaf exposed to hydroxylamine

Exposure of *L. gibba* to 10 mM of NH_2OH during 30 min in the dark was sufficient to induce an inhibition of the water splitting system (Figure 5.3 and Table 5.2). It is known for NH_2OH to liberate manganese from oxygen evolving complex and irreversibly inhibits electron transport between primary electron donor Yz and $P680^+$ (see review Debus, 1992). When different concentrations of NH_2OH from 1-50 mM were applied, a gradual decrease of fluorescence yield induced by saturating flash was observed indicating the inhibition of oxygen evolution, as found by polarographic method measurements (Figure 5.3 and Table 5.2). At 5 mM of NH_2OH, the maximal fluorescence yield was quenched by half compared to the control plant and the disappearance of the J step was followed by the appearance of a K step with a $\tau \approx 300$ µs. The decrease of fluorescence yield at J step may indicate a lower accumulation of Q_A^- due to the limitation of electron supply by the water splitting system. However, a rapid fluorescence rise was found to be present when *L. gibba* was treated with 10 and 50 mM of NH_2OH (Figure 5.3) since PSII electron transport was still active given NH_2OH may serve also as an electron donor to $P680^+$ (Trebst, 1980). Therefore, it may be interpreted that when oxygen evolution in *L. gibba* was inhibited by 10 and 50 mM of NH_2OH, maintenance of rapid fluorescence rise will indicate an electron transport toward to Q_B, although with low efficiency as indicated by the decrease of F_M value by four times and ϕ_{Po} was diminished from 0.78 to 0.48. Moreover, It was reported that NH_2OH is able to support electron transfer *via* Q_A and Q_B by an electron donation to $P680^+$ when water splitting system was inhibited in pea leaf by heat treatment (Srivastava *et al.*, 1997). When the concentration of NH_2OH was gradually increased, we showed for ψ_o and ϕ_{Eo} values to be also gradually decreased indicating a diminished electron transport beyond Q_A, while an indicator of non-photochemical energy dissipation, ϕ_{Do} was increased from 0.22 to 0.52. We interpret the decrease of F_0 value induced by 50 mM of NH_2OH to be caused by a conformational change of PSII light harvesting complex. The decrease of electron transport *via* PSII induced by NH_2OH changed the electron transport balance in favour of PSI, which is shown by the increase of appearing time for step D from 80 to 200 ms and for step M from 200 to 400 ms (Table 5.2).

By using the same experimental protocol and conditions as used for non-treated *L. gibba* (Figure 5.2A), we investigated the change of rapid fluorescence transient under different light regime when *L. gibba* was treated with 50 mM of NH_2OH (Figure 5.2B). Under these conditions, fluorescence rise was induced by F1 showed evident I and M steps. Fluorescence yield at M level was lower compared to I step, indicating for NH_2OH to be a non-efficient electron donor to PSII, since the maximum plastoquinone reduction rate was not still achieved. In this case, lower fluorescence yield at M level is also likely to indicate the rapid reoxidation of plastoquinone probably due to active PSI electron transport. However, when F2, F3, F4 (in the presence of actinic light), F5 (under low energy modulated light) and F6 (under far-red light) were applied, non significant electron transport was initiated (Figure 5.2B). Under those conditions, the presence of transient K indicates that the PSII reaction centers are most likely in the $P680^+$ form (Strasser, 1997).

5.5.3 Conclusion

When leaves are exposed to dark adaptation, rapid fluorescence transient induced by a saturating flash is representative of mainly represent the functional properties of PSII. However, when a typical leaf was exposed to continuous light, inducing the Kautsky effect, the rapid fluorescence transient also indicated, beside the current activity of PSII, a structural change within the PSII complex. When PSII is going through the Kautsky effect, the rapid fluorescence transient indicates the change of the equilibrium transfer between PSII antennae excitation and the PSII reaction center, and also indicates a drastic variation of energy dissipation when PSII is transferred from active to dissipative and inactive forms. At the same time, parameter ψ_o based on the fluorescence yield above step J, indicates an increase of electron transport beyond Q_A electron carriers. Value of Q_{CN}, as a relative indication of energy dissipation through non-photochemical pathways, was ongoing through an extreme change by showing an increase more than seven times. Such drastic change of PSII functional and structural properties was reflected by the change of appearing time for transitions I-D-M. The activity of PSI prolonged the time of appearance causing transition I-D none distinguishable. Under those conditions, the maximal fluorescence yield indicated by step M, was delayed by more than two times, as caused by PSI initiation.

The change of rapid fluorescence transient induced under continuous light, when compared to those obtained under very low light intensity, confirmed the property of PSII to have the ability for rapid adaptation during two minutes. When water splitting system was inhibited and electron donation was partially substituted by NH_2OH, the adaptation ability of PSII to different light conditions was lost. When the water splitting system was inhibited by NH_2OH, the fluorescence yield at step M was decreased four-fold, indicating a poor electron donation of NH_2OH to PSII. Consequently, the PSII quantum yield was twice lower and the non-photochemical dissipation increased by a similar amount. The delay of electron transport via PSII was evident since the speed with which steps D and M appeared was increased by double. The change of the fluorescence transients and their time of appearance reflect the change of functional and structural properties of PSII induced either by continuous illumination or NH_2OH.

5.6 Acknowledgements

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5.7 References

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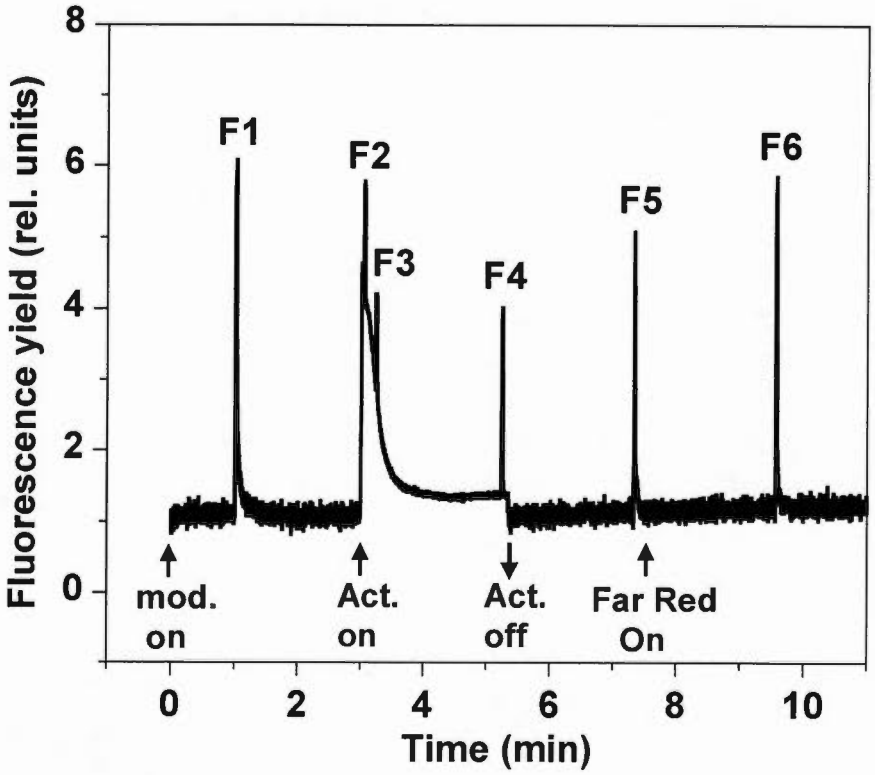

Figure 5.1 Time of PEA fluorescence induction measurements when *Lemna gibba* leaf was dark adapted and exposed to different light conditions. For more details, see materials and methods.

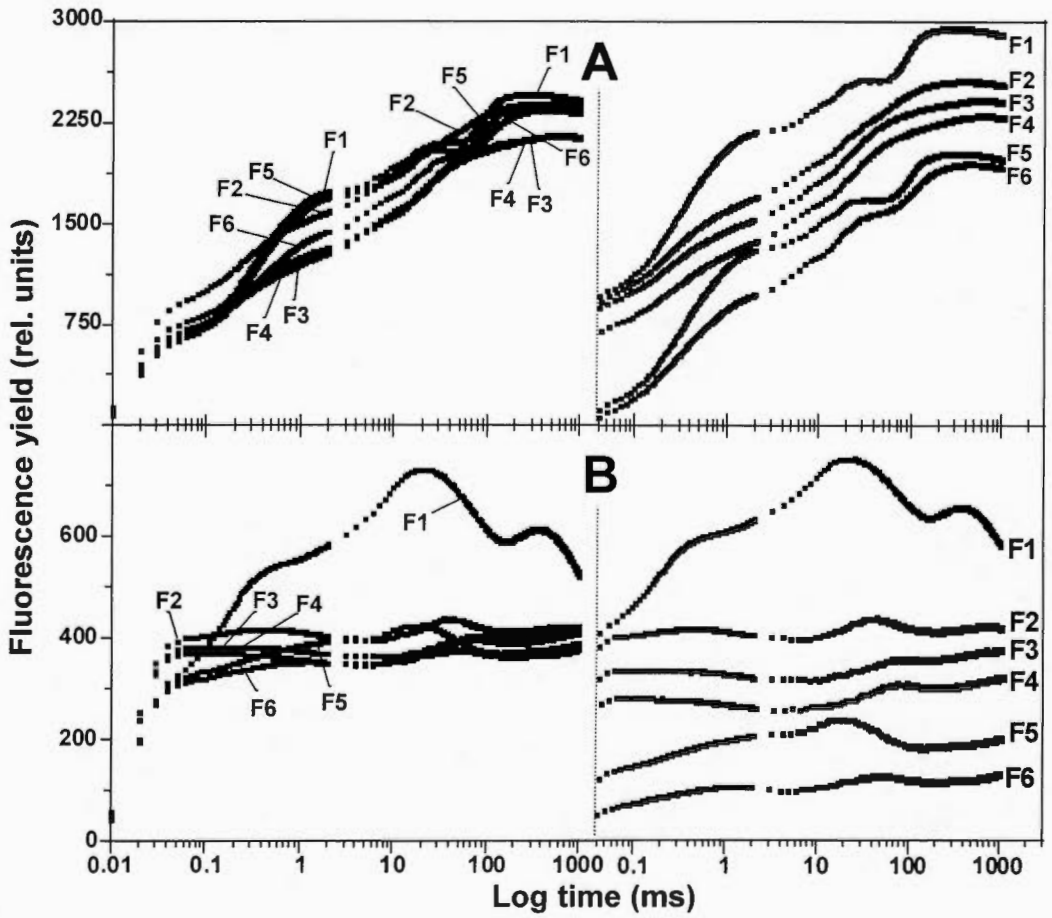

Figure 5.2 Rapid fluorescence inductions of 1 s when *Lemna gibba* leaf was dark adapted and exposed to different light conditions as presented in Figure 5.1. Saturated flash was induced by the following order : F1, under modulated light; F2, F3 and F4, under continuous actinic light; F5, under modulated light; F6, under far-red illumination. A : Control leaf and B : leaf treated with 50 mM NH_2OH. In the left panel, the six fluorescence induction curves are presented on the same scale for comparison. Right panel, the same fluorescence kinetics were separated sufficiently to compare only the form of fluorescence kinetics, therefore the scale order was ignored.

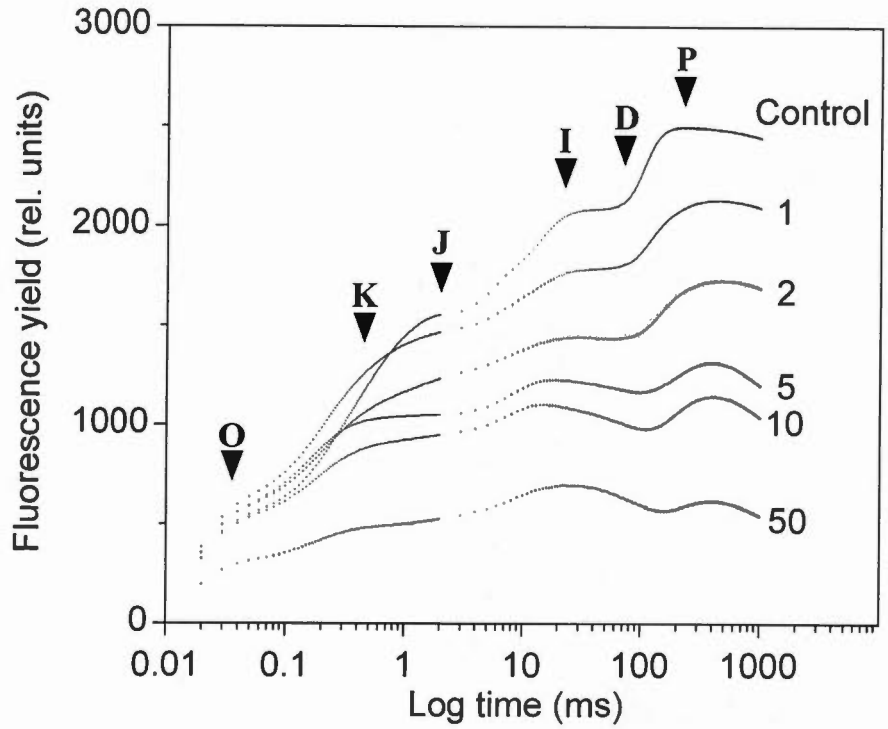

Figure 5.3 Rapid Chl *a* fluorescence transient of *Lemna gibba* leaf treated with 1 to 50 mM NH_2OH during 30 min of dark adaptation priory to measurement. For more details, see materials and methods.

Table 5.1 Parameters based on the rapid rise fluorescence induction measured when *Lemna gibba* was adapted 30 min to darkness. For more details, see materials and methods, and also Figure 5.3.

Fluorescence parameters	PEA flashes after dark or light adaptation					
	F1	F2	F3	F4	F5	F6
F_0	574	820	638	585	579	575
F_M	2568	2525	2299	2276	2521	2525
ϕ_{Po}	0.78	0.68	0.72	0.74	0.77	0.77
ψ_o	0.48	0.63	0.68	0.69	0.48	0.63
ϕ_{Eo}	0.37	0.43	0.49	0.51	0.37	0.49
ϕ_{Do}	0.22	0.33	0.28	0.26	0.23	0.23
q_{CN}	0.00	0.017	0.105	0.114	0.018	0.017
Appearing time τ (ms)						
Step J	2	2-3	2-3	2-3	2	2
Step I	30	n.d.	n.d.	n.d.	30	30
Step D	45	n.d.	n.d.	n.d.	45	45
Step M	200	250	425	425	215	355

n.d. : The appearing time was not distinguishable.

Table 5.2 The change of the rapid rise fluorescence parameters when oxygen evolution of *Lemna gibba* was inhibited by NH_2OH. *Lemna gibba* was treated with NH_2OH during 30 min in the dark priory to measurements of the first 1 sec fluorescence induction.

Fluorescence parameters	[NH_2OH] in mM					
	0	1	2	5	10	50
F_0	549	637	591	596	530	324
F_M	2494	2131	1729	1318	1147	621
ϕ_{Po}	0.78	0.70	0.66	0.55	0.54	0.48
ψ_o	0.48	0.45	0.44	0.37	0.32	0.32
ϕ_{Eo}	0.38	0.31	0.29	0.20	0.17	0.15
ϕ_{Do}	0.22	0.30	0.34	0.45	0.46	0.52
O_2 evolution (nmol/ml/g)	6.2	4.6	1.6	0.7	0.0	0.0
Appearing time τ (ms)						
Step K	n.d.	n.d.	n.d.	0.3	0.3	0.3
Step J	2	2	2	n.d.	n.d.	n.d.
Step I	30	30	30	30	30	30
Step D	80	85	90	100	150	200
Step M	200	380	440	370	370	400

n.d. : The appearing time was not distinguishable.

CHAPITRE VI

RAPID CHLOROPHYLL *A* FLUORESCENCE TRANSIENTS OF *LEMNA MINOR* LEAVES AS INDICATION OF LIGHT AND EXOGENOUS ELECTRON CARRIERS EFFECT ON PHOTOSYSTEM II ACTIVITY

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6.1 Résumé

En utilisant un flash saturant, le changement de la cinétique rapide de la fluorescence chlorophyllienne a été investigué in vivo lorsque Lemna minor a été exposée, sous différentes conditions lumineuses, aux effets de modulateurs exogènes du transport des électrons (les accepteurs d'électrons méthyl viologène et duroquinone; le donneur d'électrons hydroxylamine). L'étude a été effectuée en utilisant une combinaison des systèmes fluorimétriques «*Pulse Amplitude Modulated*» et «*Plant Efficiency Analyzer*», ce qui a permis d'analyzer la cinétique rapide de fluorescence pour différentes conditions lumineuses. Dans ces conditions, les résultats ont montré que le changement de la cinétique rapide était dépendant du transport des électrons de la quinone A à la réduction de la plastoquinone. Cela a été indiqué à la fois par le changement du rendement de la fluorescence et par le temps d'apparition des différentes transitions. Lorsque l'hydoxylamine, le méthyl viologène et la duroquinone ont été employés pour affecter le transport des électrons photosynthétiques, la cinétique rapide de fluorescence a représenté l'état du transport des électrons entre le photosystème II et le photosystème I ainsi que la dissipation d'énergie sous forme non photochimique.

6.2 Abstract

By using saturating flash, we investigated the change of rapid fluorescence rise when *Lemna minor* leaf was exposed to different light conditions and treated with exogenous electron acceptors (methyl viologen and duroquinone) and electron donor (hydroxylamine). Investigation was done by using combined Pulse Amplitude Modulated fluorometer and Plant Efficiency Analyzer system, which were employed simultaneously to provide different light conditions and to induce rapid fluorescence rise respectively. We have shown, when leaf of *Lemna minor* was exposed to different conditions of illumination, that rapid fluorescence rise was greatly influenced by the electron transport functions beyond quinone A-plastoquinone reduction. This was indicated by the change of both fluorescence yield and appearing time of the different transients. When exogenous electron donor (hydroxylamine) and acceptors (methyl viologen and duroquinone) were applied in *in vivo* condition, we showed that rapid fluorescence rise represented a reliable indicator of PSII-PSI electron transport state and energy dissipation process.

6.3 Introduction

Fluorescence induction during Kautsky effect, appearing when dark adapted plant is exposed to continuous illumination, is characterized by the change of the chlorophyll *a* (Chl *a*) fluorescence yield during the first few minutes before fluorescence yield reaches steady state (Govindjee and Papageorgiou, 1971). It is known that fluorescence dissipation mostly arises from Chl *a* associated with photosystem II (PSII) complex (Krause and Weis, 1991). The variation of variable fluorescence yield indicated that PSII quantum yield undergoes through modifications during Kautsky effect (Schreiber *et al.*, 1994; Strasser *et al.*, 2000; Schreiber, 2004). The fluctuation of fluorescence intensity is closely related to the change of PSII reaction centers proportion capable in performing the primary photochemical act supporting electron transport toward photosystem I (PSI) (Duysens and Sweers, 1963; Schreiber and Neubauer, 1987; Neubauer and Schreiber, 1987). The variation of the fluorescence yield is also influenced by the oxido-reduction state of the electron carriers associated with the electron transport chain : water–PSII–PSI–$NADP^+$, showing different transitory states which are related to constant and variable fluorescence yields (Dau, 1994). The first transitory step F_O, the so called constant or dead fluorescence, is considered to be the fluorescence yield when all functional PSII reaction centers are open. It has been postulated that F_O fluorescence emission represents the energy dissipation from excited chlorophyll *a* molecules when PSII antenna is in competition for the excitation energy transfer with PSII reaction center (Mathis and Paillotin, 1981; Owens, 1996). Under some conditions, the increase of F_O yield may occur from an irreversible disconnection of Chl *a* antenna from PSII light harvesting complexes (Briantais *et al.*, 1996). However, the variation of F_O may be also induced by structural reorganization within PSII light harvesting complexes when membrane energization process takes place (Genty *et al.*, 1990; Ruban and Horton, 1994). It was considered for the majority of plant species, being shortly exposed to saturating illumination, that the maximum fluorescence yield, F_M, may be reached between 200 and 1000 milliseconds. At this fluorescence transient, Q_A, Q_B and PQ pools were found to be in reduced state (Schreiber and Neubauer, 1987; Neubauer and Schreiber, 1987). Under continuous illumination, the fluorescence yield from F_M decreased rapidly to the state where quenching effect became constant, therefore showing that PSII-PSI electron transport attained

steady-state (Govindjee and Papageorgiou, 1971). During Kautsky effect, the change of oxido-reduction state within PSII-PSI electron transport chain was found to be associated with processes such as formation of PSII dissipative forms, proton gradient induction across the thylakoid membranes and energy dissipation through non-photochemical quenching pathways (Krause and Weis, 1991). Fluorescence transients J (2-3 ms) and I (30-40 ms) appeared between F_O and F_M levels reflecting functional state of electron transport carriers within PSII complex (Strasser and Govindjee, 1991; Strasser *et al.*, 1995). Extensive research was done on how the O-J-I-M rapid fluorescence transients are related to PSII electron transport activity, and some different hypothetical views have been reported, which in return opened many further questions to be considered (Strasser and Govindjee, 1992; Strasser, 1997; Lazár *et al.*, 1999; Lazár, 2006; Zhu *et al.*, 2006). It was proposed that O-J transient is an indicator of the photochemical phase reflecting the reduction process of primary electron acceptor Q_A, therefore showing the PSII shift from an open to a close state (Strasser and Govindjee, 1992; Strasser *et al.*, 1995). The inflection J may represent oxydo-reduction state of Q_A when there is limitation in electron acceptance by Q_B. However, there are also evidences that water-splitting system function may affect J transient (Lavergne and Leci, 1993; Hsu, 1993). The rise from J to I fluorescence yields was proposed to indicate the closure of the remaining PSII open centers resulting in accumulation of $Q_A^-Q_B^-$ (Strasser *et al.*, 1995, Barthélemy *et al.*, 1997; Samson *et al.*, 1999). The decrease of the fluorescence intensity after the step I, so-called D-dip, has been considered to indicate the electron transport balance between PSII and PSI (Schreiber and Vidaver, 1976; Hansen *et al.*, 1991). It is known that the maximum yield of fluorescence is reached when the PQ pool become reduced and consequently the I-M transition represent an accumulation of $Q_A^-Q_B^{2-}$ (Strasser and Govindjee, 1992; Strasser et al., 1995; Samson *et al.*, 1999). Recently, it was shown that I-M transition for dark-adapted leaf was related to PSI electron transport when PSI acceptor side, as ferredoxin-$NADP^+$-reductase, was altered (Schansker *et al.*, 2005). Therefore, we may assume that rapid transitions of fluorescence should be influenced by the rate of PQ reoxidation depending on electron transfer via PSI. If this assumption is confirmed, it may give new information for interpretation of dependency between PSII-PSI electron transport and the change of rapid fluorescence rise. In this view, investigation of exogenous electron

acceptor effects may offer further information how reconstituted electron transport beyond PSII may affect rapid fluorescence rise.

In the majority of earlier studies the use of normalized fluorescence yields was necessary to compare results from different experiments, which may introduce some difficulties in interpreting the real change of fluorescence kinetics. To overcome this problem we took advantage of using an adapted optical system combining PEA (Plant Efficiency Analyzer) and PAM (Pulse Amplitude Modulated) fluorometers, permitting simultaneous measurements of rapid fluorescence induction at different stages of Kautsky effect. By exposing dark or light adapted leaf to repetitive saturating flashes every 10 seconds, it has been indicated that rapid fluorescence rise and associated parameters may reflect the effect of induced electron transport (Strasser *et al.*, 2000). In our study, successive saturating flashes were sufficiently distinct within time to avoid additive effects of saturating light on PSII reduction state when plant was exposed to continuous illumination or being dark adapted. The change of the rapid fluorescence transients in *Lemna minor* exposed to continuous illumination was investigated in absence or presence of exogenous electron acceptors (methyl viologen, MV and duroquinone, DQ) and electron donor (hydroxylamine, NH_2OH). We believed that this approach permitted a better understanding of the dependency between the change of O-J-I-M fluorescence transients and PSII-PSI electron transport during Kautsky effect.

6.4 Materials and Methods

The duckweed *Lemna minor* was cultivated in PVC aquaria containing 500 ml of inorganic autoclaved growth medium (pH 6.5) adapted from Chollet (1993). All aquaria were maintained in a growth chamber at 21 °C under continuous illumination (100 µmol photosynthetic active radiation m^{-2} s^{-1}) provided by cool white fluorescent lamps (Sylvania Grolux F 36W). The plants were sub-cultured twice a week.

Stock solutions of DQ, MV and NH_2OH were prepared in 100% ethanol. The treatment media contained growth medium added with these compounds and had a final ethanol

concentration of 0.15 %. At this concentration of ethanol, no inhibition of photosynthetic process was noticed (data not shown). Six colonies of duckweed were exposed to DQ (25, 50 or 100 µM) or MV (100, 200 or 400 µM) without and with 50 mM NH_2OH under static conditions (same as growth conditions) during 30 min in darkness. Plant without added contaminant served as negative control. Treatment was done on Petri dish with a final volume of 50 mL. Each experiment was conducted in triplicate.

Chlorophyll fluorescence emission was measured by employing a combination of PEA and PAM fluorometers (Hansatech®, UK). The change of the rapid fluorescence transient was monitored by triggering a one second PEA saturating flash before, during and after Kautsky fluorescence induction when *Lemna minor* was exposed to continuous actinic light by using PAM fluorometer. The PEA saturating flash was provided by an array of six light-emitting diodes giving a maximum emission at 650 nm with an intensity of 3000 µmoles of photons m^{-2} s^{-1}. The fluorescence induction was measured from 10 µs to 1 s and the fluorescence yield at 50 µs was considered as the real F_O value. The rise of the fluorescence yield was plotted on a logarithmic time scale allowing the visualization of transitions. The fluorescence induction curves were transferred to a personal computer for further analysis. The appearance time of all transients was evaluated by the first derivative of the fluorescence induction curve (Klinkovsky and Naus, 1994). Evaluation of photosynthetic parameters related to fluorescence yields or flux ratios within PSII complex were based on the theory of energy fluxes in biomembranes (Strasser *et al.*, 2004) : The maximum quantum yield of primary photochemistry was determined as $\phi_{Po} = (F_M-F_{50\mu s}) / F_M$; The probability that a trapped exciton moves an electron into electron transport chain beyond Q_A^- was estimated as $\psi_o = 1 - [(F_{2ms} - F_{50\mu s}) / (F_M - F_{50\mu s})]$; The quantum yield of electron transport was evaluated as $\phi_{Eo} = \phi_{Po} \times \psi_o$; The quantum yield of energy dissipation not used into primary photochemistry was determined as $\phi_{Do} = 1- \phi_{Po}$. Adapted from Roháček and Barták (1999) nomenclature, the complete non-photochemical quenching of chlorophyll fluorescence yield for sample in light adapted state was evaluated as $q_{CN} = 1 - (F_{M(at\ F2,\ F3,\ F4,\ F5\ or\ F6)} / F_{M(at\ F1)})$.

Six independent rapid fluorescence inductions were monitored permitting to investigate the influence of Kautsky effect at different stages in comparison with dark adapted plant or

exposed to far red light. Therefore, the following sequence was used (see Figure 6.1) : The first flash was induced after 2 min when plant was exposed to a weak continuous modulated light which did not induce primary photochemical act. Two minutes after the first flash, the PAM continuous actinic light was turned on (100 μmoles of photons m^{-2} s^{-1}) and the second flash was induced at the time when fluorescence induction reached Fp level. The third flash was triggered at the middle of the fluorescence decay following Fp level. The fourth flash was performed at steady state of variable fluorescence (2 min from the third flash). Then, the actinic light was turned off and after 2 min the fifth flash was induced. After the fifth flash, a far-red light (735 nm) was turned on promoting a rapid re-oxidation of the PQ pool and after 2 min the sixth flash was induced.

6.5 Results and discussion

Dark-adapted *Lemna minor* treated with MV or DQ demonstrated evident quenching effect of variable fluorescence which was gradually increased with increasing concentration of those exogenous electron acceptors (Figure 6.2). As seen in table I, this effect did not change the appearance time of the J transient compared to control sample (plant non-treated with MV or DQ). Appearance time of I fluorescence transient was also not modified by the effect of MV, but in presence of 50 or 100 μM DQ the appearance of the I transient was two time shorter compared to control (Figure 6.2, Table 6.1). The quenching effect of the fluorescence showed that both MV and DQ were efficient electron sink for electron transport beyond PSII. Indeed, MV, as a strong electron acceptor at PSI level (Trebst, 1974), maintained electron carriers associated with PSI in an oxidised state, and consequently PQ pool became an efficient quencher of fluorescence as it was indicated by fluorescence yield at M transient. A similar effect was found when dark-adapted pea leaf was treated with methyl viologen and exposed to one saturating flash (Schansker *et al.*, 2005). On the other hand, DQ, known to be an electron acceptor at Q_A (Petrouleas and Diner, 1987), demonstrated complexity in its interaction with PSII (Bukhov *et al.*, 2003). Here, we found that DQ induced a rapid oxidation of PSII electron donor side since fluorescence yields at J and I transients were strongly quenched when DQ concentration was gradually increased. Quenching of J and I fluorescence yields and the appearance of the following D-dip,

observed when 50 and 100 μM DQ was used, may indicate that DQ acts as an intermediate between Q_A and beyond Q_A electron acceptors. The evidence that the appearance time of I transient was shorter compared to control, may support the idea that DQ affected Q_B oxido-reduction state. In DQ treated plant we observed a longer time for appearance of M transient, and this may be explained by the fact that DQ reoxidised quickly Q_A and therefore inducing slower reduction of PQ pool. We observed also that F_O fluorescence yield was quenched in presence of 50 μM and 100 μM DQ (data not shown), showing that DQ quenched excited singlet states of antenna chlorophyll molecules which is in agreement with earlier report (Samson *et al.*, 1999).

When light conditions were different, rapid fluorescence kinetics of the same *L. minor* leaf exposed to saturating flash showed evident differences (Figure 6.3A). Transients J-I-D-M were shown to be consistent when electron transport was not initiated priory to flash, as indicated by fluorescence kinetics induced by F1 and F5. On the other hand, transients J and I were not emphasised clearly when saturating flash was applied on *L. minor* leaf exposed to continuous illumination triggering active PSII-PSI electron transport (during Kautsky effect - F2, F3 and F4). It is worth to mention that kinetics of fluorescence rapid rise induced by F2, F3 and F4 were very similar although the quenching of fluorescence yield was more pronounced at F4. We may interpret this quenching effect to be induced by PSI activity when variable fluorescence indicated that PSII-PSI electron transport was at steady state. Indeed, we noticed an increase of electron transport beyond Q_A^- (ψ_O) and an increase of quantum yield of electron transport (ϕ_{Eo}) found by F4. However, one could not exclude the contribution of other energy dissipation pathways associated with non-photochemical quenching effect (increased q_{CN}) (Table 6.2), which was known to be present at this stage of electron transport (Roháček and Barták, 1999). We may also suppose that the fluorescence kinetics of F2, F3 and F4 reflected conformational change of PSII complex since leaf has been exposed to continuous light for few minutes priory to flash. The increase of F_O value obtained under F2, F3 and F4 flashes and increase of quantum yield of energy dissipation (ϕ_{Do}) may support this interpretation. It is important to mention that appearing time of maximum fluorescence yield, F_M, was two times longer when flash was induced under continuous illumination compared to other conditions. This should be expected since

activation of PSI electron transport under continuous illumination will prevent rapid PQ reduction. When leaf was exposed to far red light priory to flash (F6), we noticed also a quenching effect due to the PSII-PSI electron transport activity triggered by far red illumination since this light source was emitting radiations at 735 nm. It has to be taken in consideration that, for the first few minutes of Kautsky effect, PSII is passing through dramatic change concerning its structural and functional properties, such as energy redistribution between light-harvesting complexes and PSII-PSI, formation of proton gradient in inter-thylakoid space, appearance of PSII dissipative forms, and energy dissipation through non-photochemical pathway (Horton *et al.*, 1996; Critchley, 1998; Horton and Ruban, 2005). When the first flash (F2) was induced at the highest fluorescence yield of Kautsky effect, F_O yield was increased by 40% compared to F_O level obtained for dark-adapted plant (F1, Table 6.2). This may be explained since actinic light induced closure of some PSII reaction centers, therefore not contributing to electron transport but participating in energy redistribution between reaction centers and light harvesting complex. The increase of F_O yield found by F2 may reflect also structural reorganisations within PSII complex induced by continuous illumination. However, when leaf was exposed for few minutes to actinic light (F3 and F4) where PSII reorganisation tended to steady state, F_O level was decreased toward its initial level found at F1. We may assume that this PSII adaptation to continuous light appeared to be also associated with the activation of processes involved in alternative non-photochemical energy dissipation (q_{CN}). The decrease of F_M value was earlier reported to be also due to the membrane energization processes taking place (Schreiber *et al.*, 1994). These changes demonstrated that continuous actinic light, inducing active electron transport, was responsible for modification of rapid fluorescence kinetics compared to those induced by saturating flash following dark adaptation. Such effect has been also noticed by the variation of fluorescence parameters related to energy flux within PSII complex (Table 6.2). As shown, PSII quantum yield (ϕ_{PO}) was decreased when actinic light was applied, which is characteristic for this phase of Kautsky effect (Strasser *et al.*, 2000). These results indicated that kinetic of rapid fluorescence rise, induced by saturating flash when plant was exposed to continuous illumination, served as reliable indicator of PSI electron transport contribution to the variation of energy flux within PSII. When flashes 2, 3 and 4 were applied, the increased time for the appearance of M-step showed also that oxido-reduction state of PQ pool was affected

by PSI activity under actinic light (Table 6.2). According to this interpretation, our results indicated also that electron transport beyond Q_A^- (ψ_O) was increased under continuous illumination (F2, F3 and F4), and consequently the quantum yield of electron transport (ϕ_{EO}) associated with active PSII was also increased. It is expected that formation of dissipative PSII reaction centers and proton gradient will contribute to the increased quantum yield of energy dissipation as we found here (ϕ_{DO}, see table 6.2).

When leaf was treated with MV, as an exogenous electron acceptor sink at PSI, rapid fluorescence kinetics demonstrated some similarities with control sample (Figure 6.3A and B). However, for MV treated leaf strong fluorescence quenching effect was observed at M step for F1 and F5, and consequently fluorescence yield at M level was very close to the one at I transition. This showed that PQ pool was more in oxidised state compared to PQ in control leaf and, as reported recently, that PSI activity may play also a role in the I-M transition obtained from dark-adapted leaf (Schansker *et al.*, 2005). Observed quenching effect at F6 indicated accelerated electron transport via PSI induced by far red light. Under continuous illumination (F2, F3 and F4) similar quenching effect was also detected. We noticed also that fluorescence yield at J and I levels of leaf treated with MV was strongly quenched when flash was induced during Kautsky effect. It appeared that energy dissipation pathways did not seem to change during continuous illumination in MV treated leaf, since MV is a strong electron sink with no limitation for electron transport activity. However, in control plant such limitation was observed (see Table 6.2) according to earlier report when CO_2 fixation was a limiting factor for electron transport at the beginning of continuous illumination (Genty *et al.*, 1989). Indeed, for MV treated plant, quantum yield of electron transport (ϕ_{EO}) and quantum yield of energy dissipation (ϕ_{DO}) were increased at the onset of the actinic light but they were not further changed during the Kautsky effect (Table 6.3). When DQ was used as PSII exogenous electron acceptor we noticed that rapid fluorescence rise induced by saturating flash following dark adaptation (F1) was evidently different compared to those obtained under continuous light (F2, F3 and F4) or at F5 and F6 (Figure 6.3C). We noticed a lack of D-dip in rapid rise of fluorescence for flashes triggered under continuous illumination, which we may interpret to be due to sustained electron transport as it was reported earlier for DQ (Bukhov *et al.*, 2003). We observed a strong quenching of F_O

fluorescence compared to non-treated plant (Table 6.4), which is supported by evidence that DQ quenched efficiently excited singlet states of antenna chlorophyll molecules (Karukstis *et al.*, 1988; Vasil'ev *et al.*, 1998). Other quenching effect was found at F_M which should be considered as result of DQ electron shuttle from PSII to PSI (Bukhov *et al.*, 2003). We showed that DQ induced decrease of ϕ_{PO}, ϕ_{EO} and increase of ϕ_{DO} (Table 6.4). We showed that DQ induced decrease of ϕ_{PO}, ϕ_{EO} and increase of ϕ_{DO} (Table 6.4). It appeared also that the electron transport shuttle from PSII to PSI by DQ maintained PQ pool in a more reduced state indicated by shorter appearing time of maximum fluorescence yield F_M. Therefore, those effects are probably resulted from the complexity of DQ interaction as intermediate of electron transport or quencher of excited states of chlorophyll antenna complex.

When water splitting system, as primary electron donor to PSII, was substituted by 50 mM hydroxylamine, rapid fluorescence rise induced by saturating flash after dark adaptation (F1) depicted the presence of all transients, although strong quenching effect of fluorescence yield was evident compared to control plant not treated with NH_2OH (Insert Figure 6.4A' and Figure 6.3A-F1). Under this condition, fluorescence yield at M level was lower compared to I step, since NH_2OH serves a non efficient electron donor to PSII (Föster and Junge, 1985), therefore the maximum PQ reduction was not achieved. When dark-adapted *L. minor* treated with NH_2OH was exposed to 200 μM MV an important quenching effect of M transient was observed showing that PQ pool was maintained in oxidised state (Figure 6.4A-F1). However, under those conditions, high fluorescence yield found at I transient may indicate that NH_2OH was sufficient to induce Q_B reduction. For the other conditions of illumination (F2-F6), MV was shown to be a very efficient electron sink, since fluorescence yield at all fluorescence transients was mostly quenched resulting in the lack of fluorescence rise with visible transient steps (Figure 6.4A-F2 to F6). However, when PSII donor side was altered by NH_2OH in addition of 50 μM DQ, as electron acceptor, a strong fluorescence quenching was observed and the fluorescence rise showed slightly distinguishable J-I-D-M transients if compared to control plant (Figure 6.4B). No rapid fluorescence rise with evident transients was either observed when flash 1 (F1) was applied. We may interpret these effects to be resulted by the presence of strong exogenous electron acceptor (DQ) at Q_A site (Petrouleas and Diner, 1987) and the non efficient electron donor (NH_2OH) to PSII (Föster and Junge, 1985).

6.6 Concluding remarks

In summary, our data show that when leaf of *L. minor* is exposed to different conditions of illumination, rapid fluorescence rise reflects electron transport functions beyond Q_A-PQ reduction. Therefore, the change of fluorescence yield at transition steps J, I, D and M indicate oxido-reduction state of PSII-PSI electron transport chain. Impact of PSII-PSI electron transport on the change of rapid fluorescence rise under different conditions of illumination can only be seen when the same leaf is exposed to saturating flashes under constant measuring optical setup. Furthermore, we showed that when exogenous electron donor (NH_2OH) and acceptors (MV and DQ) were applied, rapid fluorescence rise under *in vivo* condition was a reliable indicator of PSII-PSI electron transport state and energy dissipation processes taking place.

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6.8 References

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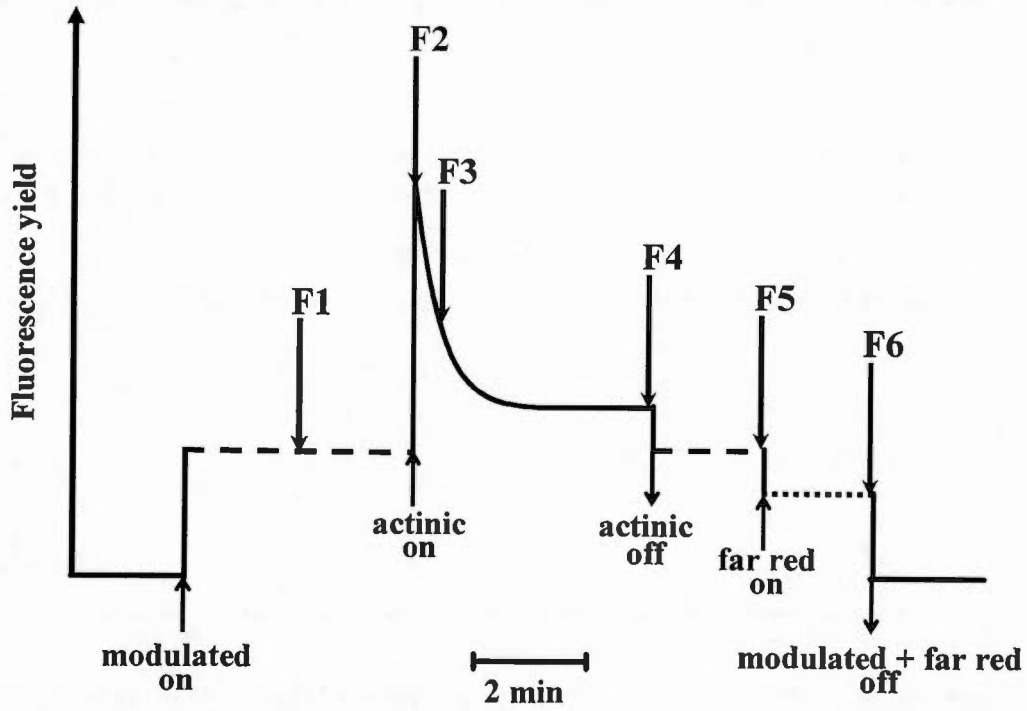

Figure 6.1 PEA flash measurements, noted by numbers, when *Lemna minor* leaf was exposed to different light conditions. Rapid fluorescence induction measured when leaf of *Lemna minor* was exposed to modulated analytical light (F1), to continuous actinic light (F2, F3 and F4), to modulated analytical light (F5) and to far-red illumination (F6). For more details, see materials and methods.

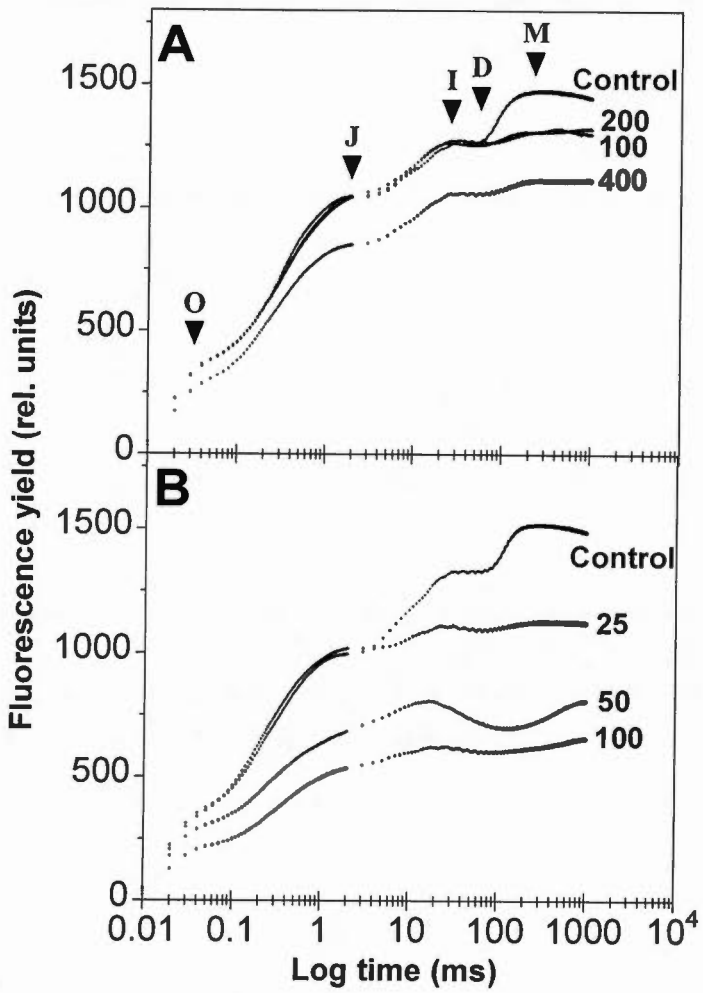

Figure 6.2 Rapid fluorescence induction obtained when leaf of *Lemna minor* was exposed 30 min in the dark to MV (A) and DQ (B). The numbers represent concentration of MV and DQ in μM. Vertical arrows indicate fluorescence transient O, J, I and M.

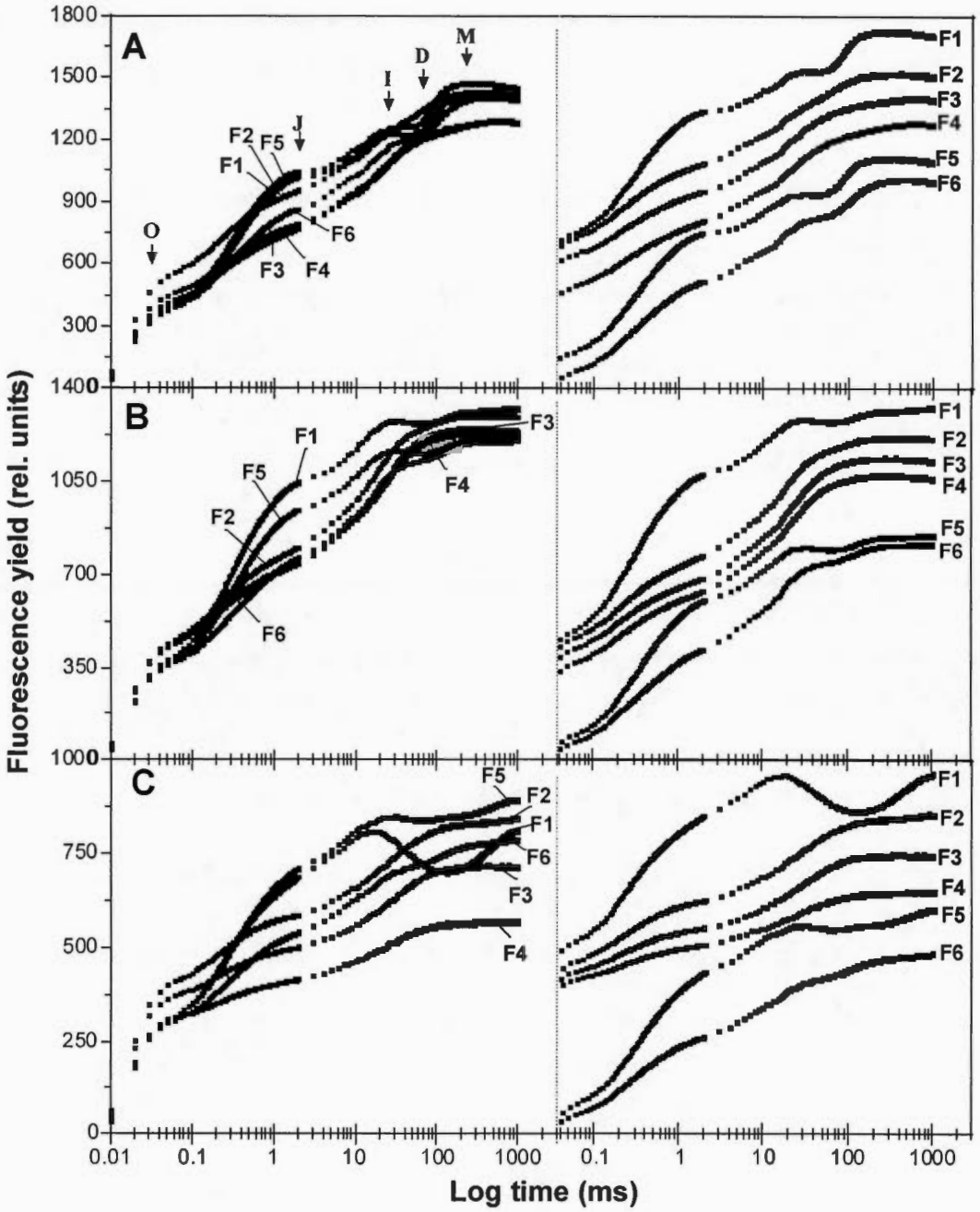

Figure 6.3 Rapid fluorescence induction when leaf of *Lemna minor* was exposed to different light conditions. Numbers represent saturating analytical flashes induced as indicated in figure 6.1. Control measurement (A); 200 μM MV treatment (B) and 50 μM DQ treatment (C). Vertical arrows indicate fluorescence transient O, J, I and M. For a better visual presentation, fluorescence kinetics were separated on the right side of the figures.

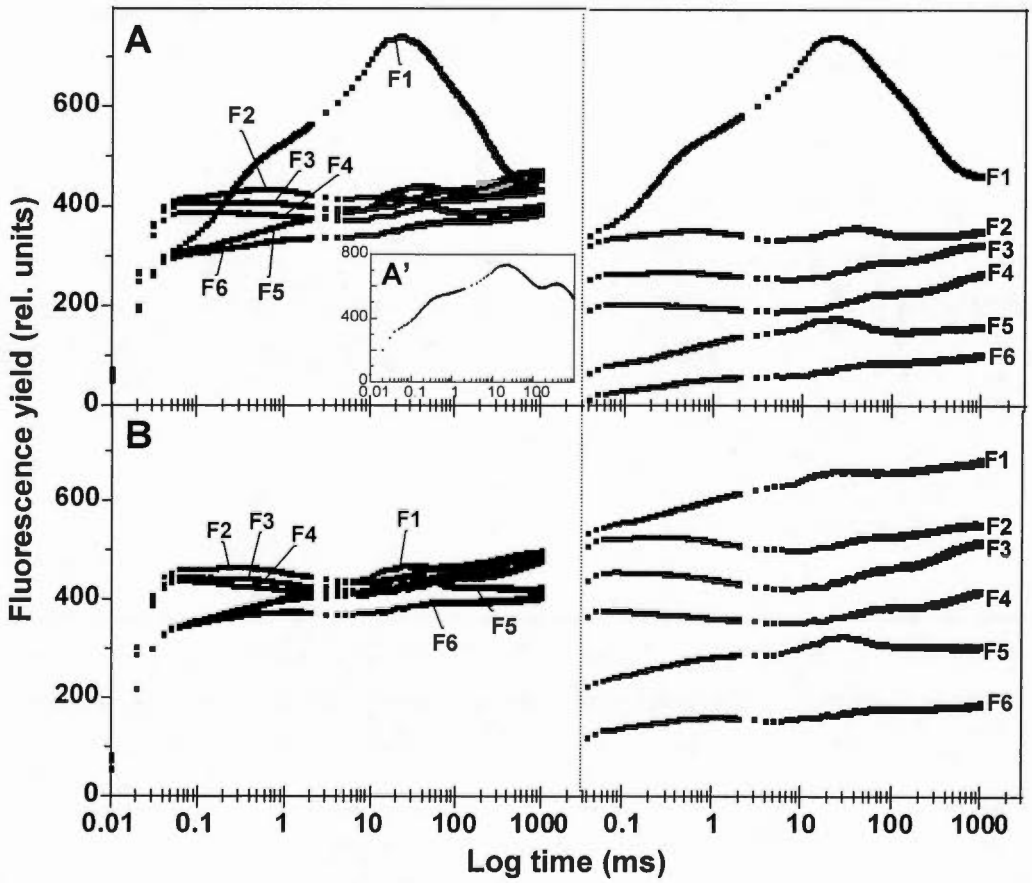

Figure 6.4 Rapid fluorescence induction when leaf of *Lemna minor* was exposed to different light conditions and to treatment 200 μM MV + 50 mM NH_2OH (A), 50 mM NH_2OH (A') or 50 μM DQ + 50 mM NH_2OH (B). Numbers represent saturating analytical flashes induced as indicated in figure 6.1. The insert A' represents the dark-adapted fluorescence kinetic obtained at F1. For a better visual presentation, fluorescence kinetics were separated on the right side of the figures.

Table 6.1 The change in the appearing time τ (ms) of fluorescence transients when *Lemna minor* was exposed to DQ and MV during 30 min in the dark priory to measurement of fluorescence induction.

Appearing time τ (ms)	Control	[MV] in μM			[DQ] in μM		
		100	200	400	25	50	100
Step J	2	2	2	2	2	2	2
Step I	30	30	30	30	25	15	15
Step D	50	70	70	70	75	125	125
Step M	210	400	415	420	281	988	995

Table 6.2 The change of fluorescence parameters from rapid fluorescence transients obtained when leaf of *Lemna minor* (control sample) was exposed to 6 analytical saturating flashes corresponding to different light conditions as described in Figure 6.1.

Fluorescence parameters	PEA flashes					
	F1	F2	F3	F4	F5	F6
F_0	386	539	448	414	379	377
F_M	1476	1434	1293	1291	1422	1408
ϕ_{Po}	0.74	0.62	0.65	0.68	0.73	0.73
ψ_o	0.39	0.54	0.59	0.60	0.39	0.53
ϕ_{Eo}	0.29	0.34	0.39	0.41	0.28	0.39
ϕ_{Do}	0.26	0.38	0.35	0.32	0.27	0.27
q_{CN}	0.00	0.03	0.12	0.13	0.04	0.05
Appearing time τ (ms)						
Step J	2	2	2	2	2	2
Step I	30	n.d.	n.d.	n.d.	30	30
Step D	50	n.d.	n.d.	n.d.	40	40
Step M	210	220	440	440	240	360

n.d. : The appearing time was not distinguishable.

Table 6.3 The change of fluorescence parameters from rapid fluorescence transients obtained when leaf of *Lemna minor* was treated with 200 μM methyl viologen during 30 min in the dark and then exposed to 6 analytical saturating flashes corresponding to different light conditions as described in Figure 6.1.

Fluorescence parameters	PEA flashes					
	F1	F2	F3	F4	F5	F6
F_0	380	442	437	429	367	359
F_M	1324	1296	1248	1228	1215	1204
ϕ_{Po}	0.71	0.66	0.65	0.65	0.70	0.70
ψ_o	0.29	0.58	0.62	0.61	0.32	0.52
ϕ_{Eo}	0.21	0.38	0.40	0.40	0.23	0.37
ϕ_{Do}	0.29	0.34	0.35	0.35	0.30	0.30
q_{CN}	0.00	0.02	0.06	0.07	0.08	0.09
Appearing time τ (ms)						
Step J	2	2	2	2	2	2
Step I	30	n.d.	n.d.	n.d.	30	40
Step D	70	n.d.	n.d.	n.d.	70	n.d.
Step M	386	381	380	380	620	550

n.d. : The appearing time was not distinguishable.

Table 6.4 The change of fluorescence parameters from rapid fluorescence transients obtained when *Lemna minor* was treated with 50 μM duroquinone during 30 min in the dark and then exposed to 6 analytical saturating flashes corresponding to different light conditions as described in Figure 6.1.

Fluorescence parameters	PEA flashes after dark or light adaptation					
	F1	F2	F3	F4	F5	F6
F_0	305	400	363	307	308	299
F_M	810	842	717	570	893	786
ϕ_{Po}	0.62	0.52	0.49	0.46	0.66	0.62
ψ_o	0.25	0.58	0.61	0.59	0.32	0.51
ϕ_{Eo}	0.15	0.30	0.30	0.27	0.21	0.31
ϕ_{Do}	0.38	0.48	0.51	0.54	0.34	0.38
q_{CN}	0	n.d.	0.11	0.30	n.d.	0.03
Appearing time τ (ms)						
Step J	2	2	2	2	2	2
Step I	15	n.d.	n.d.	n.d.	30	40
Step D	125	n.d.	n.d.	n.d.	75	n.d.
Step M	988	953	297	315	958	969

n.d. : The appearing time was not distinguishable.

CHAPITRE VII

EFFECTS OF METHANOL ON PHOTOSYNTHETIC PROCESSES AND GROWTH OF *LEMNA GIBBA*

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7.1 Résumé

Les effets du méthanol sur la croissance et l'activité photosynthétique ont été examinés chez la plantule aquatique *Lemna gibba* exposée à une illumination continue. Cette plante supérieure semble être beaucoup plus sensible à l'effet inhibiteur du méthanol que les algues vertes (Theodoridou *et al.*, 2002, *Biochim. Biophys Acta*, vol. 1573, p. 189-198). Dans ce travail, les résultats montrent que les effets stimulateurs ou inhibiteurs étaient fortement dépendant de la concentration du méthanol et du temps d'exposition. Lorsque *Lemna gibba* a été exposée à 0,2 % de méthanol, le taux de croissance de la biomasse était augmenté de 50 %. Cependant, l'effet stimulateur du méthanol était plus petit lorsque les plantules étaient exposées 48 heures en comparaison à 24 heures. L'accroissement de la biomasse induite par le méthanol n'était pas causée par une augmentation des processus primaires de la photosynthèse mais plutôt par une régulation de la dissipation d'énergie durant la photosynthèse. L'inhibition de la croissance de *Lemna gibba* déjà observée à partir de 0,5 % de méthanol était fortement associée à une augmentation d'une dissipation d'énergie sous forme non photochimique. Le ratio entre la biomasse et la concentration en méthanol semble déterminer l'effet stimulateur ou inhibiteur du méthanol. Quelques explications concernant l'effet stimulateur et inhibiteur seront présentées.

7.2 Abstract

Effects of methanol on growth and photosynthetic activity of *Lemna gibba* exposed under continuous illumination were examined. As a higher plant, *L. gibba* appeared to be much more sensitive to methanol inhibitory effect compared with some algae (Theodoridou *et al.*, 2002, *Biochim. Biophys. Acta*, 1573, 189-198). We found that stimulatory or inhibitory effects were strongly dependent on the methanol concentration and the time of exposure. When the exposure was up to 0.2 % methanol, the growth rate of biomass was improved by 50 %. However, stimulatory effect of methanol appeared to be smaller when plants were exposed for 48 h compared with 24 h. Increase in biomass induced by methanol was not based on the increase in primary photosynthetic process but rather on accommodation of energy dissipation during photosynthesis. Inhibitory effect on the growth of *L. gibba* already observed for 0.5 % methanol was strongly associated with the increase in the non-photochemical energy dissipation. The ratio between biomass and methanol concentration appeared to determine the stimulatory or the inhibitory effect. Suggested explanations for the stimulatory and the inhibitory effects are presented.

7.3 Introduction

Understanding the effects of methanol on plants is still a highly controversial subject because opposed conclusions were obtained concerning the effects on the photosynthetic activity and the increase of biomass. When the unicellular green alga *Scenedesmus obliquus* was exposed to 0.5 % methanol for 48 h, the biomass production and the photosynthetic activity (μmoles 0_2/μg Chl/R) increased over 300 % compared with alga not exposed to methanol (Theodoridou *et al.*, 2002). The stimulatory effect was also induced when higher plant species were sprayed with 10–50 % methanol because the growth was increased from 40 % to 100 % compared with control, showing that methanol improves carbon fixation (Nonomura and Benson, 1992). On the other hand, in toxicity biotests using algal species (*Chlorella vulgaris*, *Selenastrum capricornutum*, *Skeletonema costatum* and *Prorocentrum minimum*) exposed to 0.2 % methanol under continuous illumination for 4 days, the growth was significantly decreased, showing a strong inhibitory effect of methanol (El Jay, 1996a; Okumura *et al.*, 2001). The decrease in photosynthetic activity was also found when some higher plant species (*Prunus avium L.*, *Capsicum annuum L.* and *Cucumis melo L.*) were fumigated with 1% methanol for 4 min (Loreto *et al.*, 1999). One may notice that these controversial results concerning the effects of methanol on photosynthesis and growth are obtained under very different experimental conditions and, in many cases, are hardly comparable with each other (Nonomura and Benson, 1992; Li *et al.*, 1995; El Jay, 1996a; Loreto *et al.*, 1999; Okumura *et al.*, 2001; Theodoridou *et al.*, 2002).

The stimulatory effect of methanol on growth was noticed only under continuous illumination, and damaging effect appeared to be obvious when plants were treated in darkness (Kotzabasis *et al.*, 1999; Theodoridou *et al.*, 2002). It has been found that ^{14}C-methanol undergoes oxidative degradation, resulting in $^{14}CO_2$ production (Cossins, 1964) necessary for the Calvin–Benson cycle (Nonomura and Benson, 1992). If methanol is used as a solvent carrier in the studies of organic pollutants, the lack of consideration for the effects of methanol on the plant cell activity may induce considerable experimental errors in bioassay of toxicity investigation (El Jay, 1996b). For understanding the appearance of the stimulatory or the inhibitory effects of methanol, it is important to consider the ratio between

biomass and methanol concentration when plants have been treated. It is obvious when plants are exposed to methanol fumigation or liquid spray that this criterion could not be applied (Nonomura and Benson, 1992; Li *et al.*, 1995; Loreto *et al.*, 1999). However, we may question whether the stimulatory effect of methanol on the growth rate of biomass is based on the increase in photosynthetic activity or only on an alternative modification of energy dissipation mechanism, which results in efficient use of photosynthesis for energy storage into biomass. At present, there is a lack of understanding on the dependency between the effect of methanol on the growth rate of biomass and the appropriate energy dissipation through the primary process of photosynthesis. We investigated the stimulatory and the inhibitory effects of methanol on growth and photosynthesis when the higher plant species *Lemna gibba* (duckweed) were exposed to different methanol concentrations (biomass/[methanol]). In this study, it was possible to emphasize the relationship between the stimulation or the inhibition of growth and the energy dissipation via photosynthesis when plant has been exposed to the effect of methanol. The higher aquatic plant *L. gibba* is a convenient bioindicator frequently used in toxicity studies because its size is small and it has thin floating leaves, which permit a rapid assimilation of soluble exogenous compounds (Lewis, 1995), thereby providing fast methanol transmission through the plant cellular system and a rapid interaction with photosynthetic processes. In this study, chlorophyll (Chl) fluorescence, oxygen evolution and the growth rate of biomass have been used as valuable indicators for the evaluation of effects of methanol. Under these conditions, the change in the growth of biomass was related to photosynthetic indicators and to associated mechanisms of energy dissipation dependent on photosynthetic primary photochemistry. Therefore, the results obtained may clarify stimulatory or inhibitory effect of methanol seen in higher plant species.

7.4 Materials and methods

L. gibba was cultivated in a growth medium (Chollet, 1993) at 23°C under continuous illumination provided by cool white fluorescence lamps (Sylvania Grolux F 40/GS/WS, Drummondville, Canada), giving light intensity of 100 μmol m^{-2} s^{-1}. Three triple-fronded *L. gibba* plants (aliquot of 60 mg ± 5% biomass) in the exponential growth phase were placed in

petri dishes having 30 mL of growth medium containing initial methanol concentrations of 0.1 %, 0.2 %, 0.5 % and 1 % (vol/vol).

Leaf pigment content solubilized in 80 % acetone was evaluated (Lichtenthaler, 1988), the change in biomass was determined by fresh weight and the rate of oxygen evolution was monitored by using a Clark-type electrode (Walker, 1987).

The measurement of Chl *a* fluorescence induction and parameters, such as the operational photosystem II (PSII) quantum yield Φ_S, the photochemical quenching q_P and the non-photochemical quenching NPQ, was done by using a pulse amplitude-modulated fluorometer (PAM, FMS, Hansatech® Ltd., Norfolk, UK) as described by Rohacek and Bartak (1999). The modulated, actinic and saturating light intensities of 1, 400 and 1450 μmol m^{-2} s^{-1}, respectively, were used. Components of non-photochemical energy dissipation (NPQ), such as the energy-dependent quenching q_E, the state-transition quenching q_T and the photoinhibitory quenching q_I, have been evaluated, as described by Horton and Hague (1988). The yield of Chl *a* fluorescence at transitions of basal fluorescence F_O, variable fluorescence F_V, and maximum fluorescence F_M and related ratios F_O/F_M, F_V/F_O and F_V/F_M were determined by using a plant efficiency analyzer fluorometer (Hansatech®) (Strasser *et al.*, 1995). Before fluorescence measurements, *L. gibba* was adapted to darkness for 30 min.

7.5 Results and discussion

Growth of *L. gibba*, exposed to continuous illumination for 48 h, was affected by the presence of methanol, and this effect was found to have a stimulatory or an inhibitory impact depending on concentration and time of exposure (Figure 7.1). When *L. gibba* was exposed to 0.2 % methanol, the growth rate was improved by 50 % and 25% at 24 and 48 h, respectively. Stimulatory effect of methanol appeared to be smaller at 48 h compared with 24 h of exposure. On the other hand, when *L. gibba* plant was exposed to 0.5 % methanol, the growth rate did not change during 24 h, but it decreased by 30 % at 48 h. However, the growth rate in the presence of 1 % methanol decreased by 50 % and 75 % after 24 and 48 h of treatment, respectively. The growth rate of biomass *vs* different methanol concentrations

showed a biphasic tendency, which indicated a different mechanism of methanol interaction with plant metabolism. It has been reported that for colonial alga *Botryococcus braunii* growth yield improved by 100 % after 10 days when 3 % methanol was initially added to growth media (Nonomura and Benson, 1992). On the other hand, the growth of *Tetraselmis tetrathele*, *Dunaliella tertiolecta*, *Isochrysis galbana*, *Pavlova lutheri*, *Chaetoceros calcitrans*, *Skeletonema costatum*, *Prorocentrum minimum*, *Eutreptiella* and *Heterosigma akashiwo* was strongly inhibited (EC50, effective concentration inhibiting growth by 50 %) by 1-3 % methanol during 4 days of treatment (Okumura *et al.*, 2001). Recently, the inhibitory effect of methanol on algal growth was found for culture having high cell density. It has been suggested that the inhibitory effect of methanol was induced under low light intensity, limiting the photosynthetic activity because low photosynthetic rates in those algal cells did not result in rapid metabolism of methanol (Theodoridou *et al.*, 2002). Variation in responses of these algae or plant species to the effect of methanol appeared to be caused by differences in molecular mechanism concerning interactions of these species with methanol and probably by different experimental conditions. In our experiment, the use of *L. gibba* plant with floating leaves permitted the exposure of leaves to the same light energy during all experiments. When *L. gibba* was exposed to less than 1 % methanol, photosynthetic pigment contents per milligram of fresh weight were not affected, indicating no inhibitory effect on Chl synthesis (Table 7.1). Similarly, no effect on the Chl contents has been found earlier when the higher plant soybean was fumigated with methanol (Li *et al.*, 1995). We emphasize again that the growth rate of *L. gibba* exposed to methanol was stimulated or inhibited depending on methanol concentration and time of exposure. Unfortunately, there is still no information on the effect of methanol on the pigment contents per individual cell. Recently, the packed cell volume of algal culture exposed to methanol was used as an indicator of biomass (Theodoridou *et al.*, 2002), which may permit the comparison of the growth of *L. gibba* based on fresh weight with the change in biomass of the algal culture. The increase in packed cell volume induced by the effect of methanol was interpreted to be caused by the photoregulated assimilation and metabolic processes of methanol, which may result in increase in cellular CO_2 concentration, stimulating rapid growth (Theodoridou *et al.*, 2002). However, when *L. gibba* was exposed to 1 % methanol, the capacity of the photoregulated metabolism of methanol appeared to be insufficient for converting methanol into CO_2

substrate because an inhibitory effect was shown (Figure 7.1). This may indicate that the methanol metabolism capacity of the plant depends on the ratio between cell biomass and methanol concentration. However, no linear correlation was found between growth and photosynthetic activity estimated by oxygen evolution and Chl fluorescence parameters, indicating the complexity of the effect of methanol. Therefore, it was worth to analyze relationship between the stimulatory effect of methanol on growth (0.1 % and 0.2 % methanol effect at 48 h) and the photosynthetic activity parameters. The increase in the growth rate of biomass induced by 0.1 % and 0.2 % methanol was not followed by an increase in the photosynthetic activity! Indeed, when *L. gibba* growth was increased by 50 % (0.2 % methanol), the PSII activity supported by the water splitting system and the oxygen evolution was not changed (Table 7.1). Moreover, for *L. gibba* treated with 0.1 % and 0.2 % methanol, the rapid polyphasic rise of Chl *a* variable fluorescence did not indicate an improvement in the PSII photochemical efficiency compared with control (Figure 7.2). When compared with control, methanol-treated plants (0.1 % and 0.2 %) showed only a slight increase in the size of light-harvesting complexes per PSII reaction center. Moreover, under these conditions, no change was seen for fluorescence parameters concerning the PSII activity such as the PSII quantum yield (F_V/F_M), the basal quantum yield of non-photochemical processes (F_O/F_M), the maximum ratio of photochemical quantum yields and concurrent non-photochemical processes (F_V/F_O), the quenching of the PSII fluorescence by oxidized quinones of the plastoquinone (PQ) pool (q_{PQ}), the functional PSII photochemistry at steady state of electron transport (Φ_S) and the photochemical quenching value (Q_P) (Table 7.1). A change in oxygen evolution was only found when *L. gibba* was treated with 0.5 % or 1 % methanol. At these concentrations of methanol, it appeared that structural organization of light-harvesting complex was affected because Chl *a/b* and ABS/RC ratios were changed (Table 7.1). We found that energy dissipation via non-photochemical processes, indicated by NPQ, was strongly affected by methanol. For *L. gibba* treated with 0.2 % methanol (maximum stimulatory effect), the NPQ was three times lower compared with control (Table 7.1). It is important to note that NPQ consisting of parameters q_E, q_T and q_I had different pattern of change when the concentration of methanol was changed. At low concentration of methanol, the two-fold increase in q_T and two-fold decrease in q_I values may indicate that methanol increases the energy transfer from PSII to PSI and decreases the photoinhibitory

effect. Therefore, the improvement in the growth rate of *L. gibba* treated with 0.1 % and 0.2 % methanol appeared to be related to the alteration in energy dissipation processes associated with the primary photosynthetic activity. We may suppose that in 0.1 % and 0.2 % methanol–treated plants, there was an increase in the availability of CO_2 via the cellular transformation of methanol (Nonomura and Benson, 1992) and consequently a decrease in non-photochemical energy dissipation, which results in efficient nicotinamide adenine dinucleotide phosphate (reduced) and adenosine triphosphate synthesis. However, when *L. gibba* was treated with 1 % methanol for 48 h, the decrease in growth by 60 % was related to the decrease in photosynthetic activity indicated by diminished oxygen evolution rate of 55 % (see Figure 7.1, Table 7.1). Under the same condition, the Chl *a* content per milligram of fresh weight was diminished by 27 % compared with control, whereas contents of Chl *b* and carotenoids did not change. The ratio Chl *a*/*b* was diminished because it was noticed that Chl *a* decreased by 27 % compared with control, whereas carotenoid content did not change. Diminution of PSII photochemical efficiency (F_V/F_M) from 0.82 to 0.48 and oxygen evolution rate associated with an alteration of antenna size (higher ABS/RC ratio) may indicate an alteration of PSII activity (Table 7.1). The diminished fluorescence yield at J transient indicated low reduction rates of the primary PSII electron acceptor Q_A. The yields of fluorescence at transients I and P also progressively decreased when methanol concentration was increased from 0.1 % to 1 %, providing evidence that methanol inhibits electron transport activity from water to PQ (Figure 7.2). For plants treated with 1 % methanol, the basal quantum yield of non-photochemical process in PSII (F_O/F_M) increased more than three times. Under this condition, the ratio F_V/F_O, as the maximal quantum yield of photochemical and concurrent non-photochemical processes of PSII (Rohacek and Bartak, 1999), was decreased by five times. Alteration of the PSII electron transport process is also shown by a diminished variable fluorescence yield, which is induced by a saturating flash (Figure 7.3), and by decreased PSII operational quantum yield (Φ_S, at steady state of electron transport) from 0.71 to 0.35. For *L. gibba* treated with 1 % methanol, it is worth to mention that NPQ decreases by 15 times compared with control (Table 7.1). Therefore, it was evident that the stimulatory or the inhibitory effects of methanol on *L. gibba* exposed to light were strongly dependent on methanol concentration and time of exposure.

7.6 Conclusions

For now, it is unclear why the increase in growth rate of biomass was not linearly correlated with the photosynthetic activity. The effect of methanol seems to change the mechanism of energy distribution during photosynthesis and also the final energy storage into biomass. Some methanol concentrations (0.1-0.2 %) may induce a stimulatory effect on the growth of biomass of *L. gibba* during 24 h of exposure. When the treatment was longer than 24 h, the stimulatory effect was less evident. However, when the concentration of methanol was 0.5 % or higher, the inhibitory effect on photosynthesis and growth was evident, especially when plant cells were exposed for a long period (48 h). Therefore, dual effect of methanol (stimulation or inhibition) on the growth rate was determined by the concentration of methanol or the time of exposure. Because plant species have different structural organization of leaves and photosynthetic tissues, the response to the effect of methanol may also be different. The stimulatory effect of methanol was found to be based on the increase in CO_2 concentration in leaves, which resulted from methanol oxidative degradation in photosynthetic tissues (Cossins, 1964; Nonomura and Benson, 1992). In this case, we may assume that the photosynthetic activity operates under optimal conditions, whereas energy-dissipative processes opposed to growth are diminished. On the other hand, the mechanism of methanol toxicity is not yet well understood. We suppose two possibilities : intensive metabolism of methanol may induce high concentration of CO_2 in photosynthetic tissues, resulting in the inhibitory effect on photosynthetic electron transport and the increase of non-photochemical energy dissipation process if light energy was not increased (Hymus *et al.*, 2001); or when methanol concentration overpasses oxidative degradation capacities of plant cell (Cossins, 1964; Nonomura and Benson, 1992), then accumulated methanol intermediary metabolites may induce a toxic effect. Therefore, the stimulatory effect of methanol on plant growth is worth further investigation. The advantageous use of the stimulatory effect of methanol on the growth rate of biomass demands consideration of the concentration of methanol, the time of exposure and the structural and functional properties of photosynthetic tissues. However, the real effect of methanol shown as an inhibition or a stimulation of plant growth and photosynthesis has to be further investigated at molecular level because it appeared here that the effect of methanol on the higher plant *L. gibba* was a complex of

interactions between energy storage via photosynthesis and non-photochemical energy-dissipative processes, which may change biomass growth.

7.7 Acknowledgments

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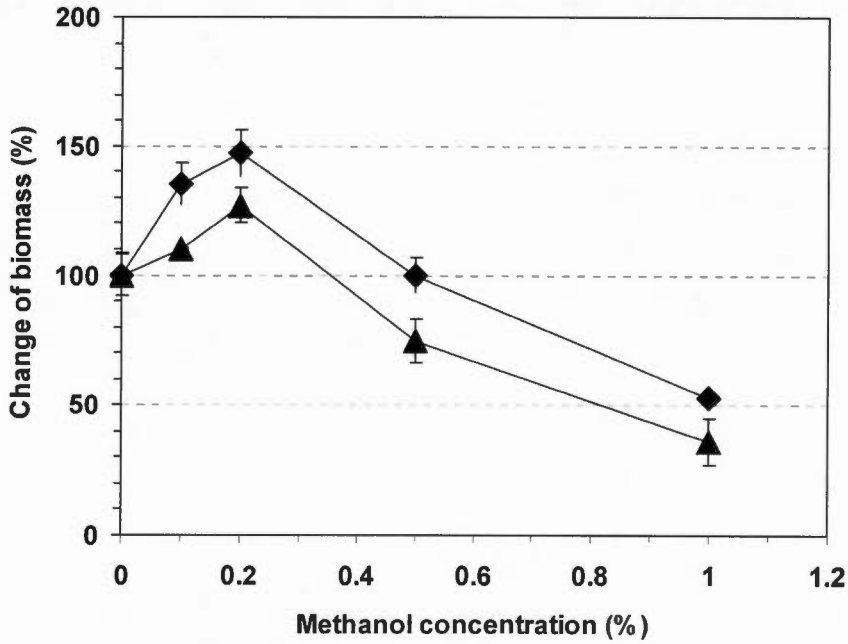

Figure 7.1 The change in growth rate of biomass at 24 (♦) and 48 (▲) h when *L. gibba* was treated with 0.1 %, 0.2 %, 0.5 % and 1 % methanol (vol/vol). The values are expressed in percentage of biomass (mg) compared with untreated control plant (100 %). All data are the mean of at least three experiments.

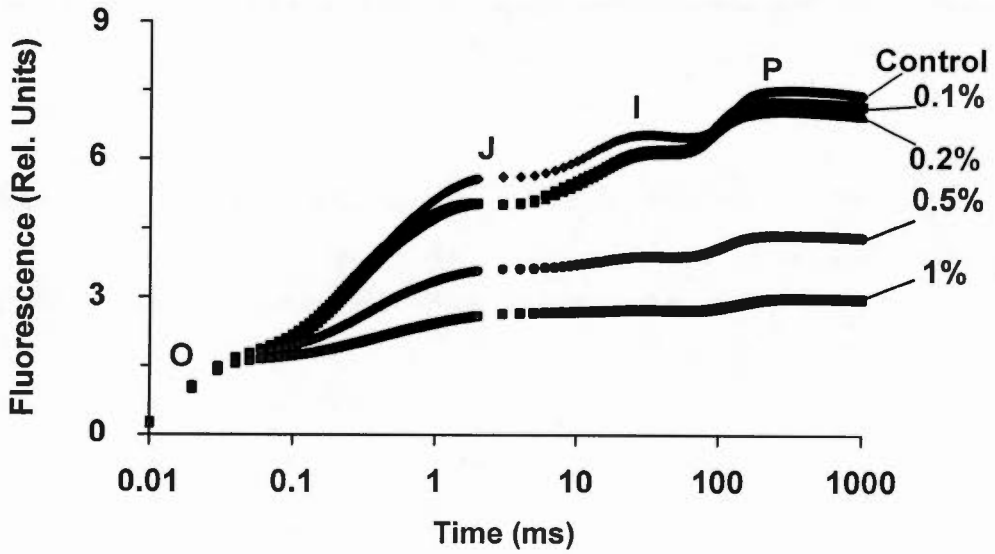

Figure 7.2 Effect of 0.1 %, 0.2 %, 0.5 % and 1 % methanol on *L. gibba* rapid polyphasic rise of Chl *a* fluorescence (after 48 h of exposure). All experimental treatments were performed at least three times.

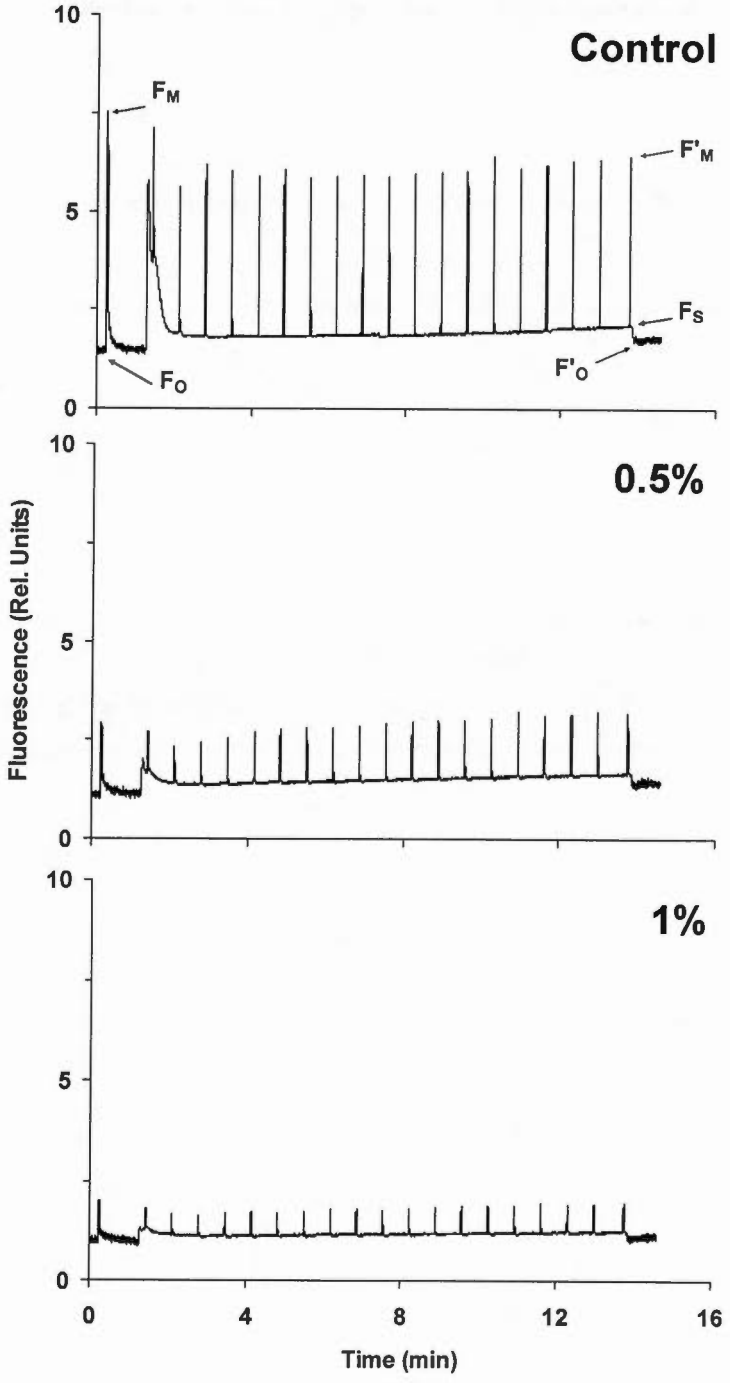

Figure 7.3 Effect of 0.5 % and 1 % methanol on *L. gibba* Chl *a* fluorescence measured by PAM fluorometry (after 48 h of exposure). All experimental treatments measurements were performed at least three times.

Table 7.1 Changes in the growth and photosynthetic parameters (means ± SD of three experiments) in *L. gibba* exposed 48 h to methanol (at concentrations of 0.1 %, 0.2 %, 0.5 % and 1 %). The components q_E, q_T and q_I are expressed in percentage of the total NPQ.

		Methanol concentration (%)			
Parameters	Control	0.1	0.2	0.5	1
O_2 evolution $\mu mol.m^{-2}.s^{-1}/g$ FW	11 ± 0.13	11 ± 0.15	10.5 ± 0.17	5 ± 0.12	5 ± 0.15
Chl *a* µg/mg	0.33 ± 0.01	0.31 ± 0.01	0.33 ± 0.02	0.29 ± 0.01	0.24 ± 0.01
Chl *b* µg/mg	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01
Car µg/mg	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
Chl *a/b*	3.44 ± 0.01	3.67 ± 0.01	3.6 ± 0.01	3.16 ± 0.01	2.83 ± 0.01
ABS/RC	2.38 ± 0.03	2.89 ± 0.03	2.67 ± 0.02	3.01 ± 0.03	3.65 ± 0.02
q_{PO}	0.17 ± 0.02	0.2 ± 0.03	0.18 ± 0.03	0.18 ± 0.03	0.19 ± 0.03
F_O/F_M	0.18 ± 0.02	0.19 ± 0.03	0.19 ± 0.02	0.37 ± 0.04	0.52 ± 0.03
F_V/F_O	4.65 ± 0.03	4.19 ± 0.04	4.2 ± 0.03	1.7 ± 0.02	0.92 ± 0.03
F_V/F_M	0.82 ± 0.02	0.81 ± 0.03	0.81 ± 0.02	0.63 ± 0.03	0.48 ± 0.03
ϕ_S	0.71 ± 0.02	0.71 ± 0.03	0.66 ± 0.04	0.49 ± 0.02	0.35 ± 0.02
q_P	0.94 ± 0.03	0.89 ± 0.03	0.88 ± 0.03	0.82 ± 0.03	0.81 ± 0.03
NPQ	0.21 ± 0.03	0.21 ± 0.03	0.063 ± 0.02	0.016 ± 0.02	0.014 ± 0.02
q_E	57 ± 3	61 ± 6	59 ± 6	57 ± 7	60 ± 5
q_T	11 ± 2	18 ± 2	24 ± 2	26 ± 5	34 ± 5
q_I	32 ± 3	21 ± 6	17 ± 2	17 ± 6	6 ± 5

Car : Carotenoids content

CHAPITRE VIII

OXYFLUORFEN TOXIC EFFECT ON *S. OBLIQUUS* EVALUATED BY DIFFERENT PHOTOSYNTHETIC AND ENZYMATIC BIOMARKERS

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8.1 Résumé

L'effet de différentes concentrations d'oxyfluorfène (7,5, 15 et 22,5 µg L^{-1}) a été investigué chez l'algue verte *Scenedesmus obliquus* pour une exposition de 12, 24 et 48 heures. Les tests ont été effectués en analysant 13 biomarqueurs : La croissance cellulaire, la quantité de chlorophylle, les paramètres photosynthétiques et l'activité des enzymes antioxydatifes. Le changement des 13 biomarqueurs présentait une grande variabilité dans la sensibilité indiquant des différences entre les paramètres concernant leur validité comme biomarqueur lorsque des cultures algales ont été exposées à la toxicité de l'oxyfluorfène. L'ordre de sensibilité entre les biomarqueurs était : La taille des antennes (ABS/RC) > la quantité de chlorophylle > la catalase (CAT) > le rendement photochimique opérationnel du photosystème II (Φ_{SPSII}) > la glutathion *S*-transférase (GST) > la proportion de plastoquinones fonctionnels (Q_{PQ}) > la glutathion réductase (GR) > la croissance cellulaire > le «*quenching*» non photochimique (Q_N) > le «*quenching*» dépendant du gradient de proton (Q_{Emax}) > l'ascorbate peroxidase (APX) > le «*quenching*» photochimique (Q_P) > le rendement photochimique maximum du photosystème II (Φ_{PSII}). L'effet de l'oxyfluorfène sur le changement de ces paramètres a été interprété comme le résultat du mode d'action de l'herbicide à l'échelle moléculaire dans le système cellulaire. Cette étude montre que certains biomarqueurs photosynthétiques et enzymatiques sont d'utiles indicateurs de la toxicité de l'oxyfluorfène chez une espèce d'algue non ciblée. La détermination de l'ordre de sensibilité des biomarqueurs permet de les sélectionner pour une évaluation d'un risque environnemental de la pollution de l'eau.

8.2 Abstract

The effect of Oxyfluorfen was investigated when alga *Scenedesmus obliquus* has been exposed to different concentrations (7.5, 15, and 22.5 $\mu g.L^{-1}$) at 12, 24, and 48 hours of exposure. Toxicity test was done by using 13 biomarkers concerning growth rate, chlorophyll content and indicators of photosynthetic and antioxidant enzyme activities. The change of the 13 parameters showed a great variation of sensitivity indicating differences in parameters' suitability to be used as biomarkers when alga culture was exposed to Oxyfluorfen toxicity. The order of sensitivity between those biomarkers was : Antenna size (ABS/RC) > Chlorophyll content > Catalase (CAT) > Operational PSII quantum yield ($\Phi_{S_{PSII}}$) > Glutathione *S*-transferase (GST) > Functional plastoquinone pool (Q_{PQ}) > Glutathione reductase (GR) > Growth rate > Nonphotochemical quenching (Q_N) > Proton gradient quenching (Q_{Emax}) > Ascorbate peroxidase (APX) > Photochemical quenching (Q_P) > Maximum PSII quantum yield (Φ_{PSII}). The effect of Oxyfluorfen on the changes of those parameters was interpreted as a result of herbicide mode of action at molecular level of alga cellular system. This study indicated for some photosynthetic and enzymatic biomarkers to be useful indicators of toxicity effect induced in non-target alga species. Determination of biomarkers' sensitivity order may facilitate their selection to be used in environmental risk assessment of polluted water.

8.3 Introduction

The herbicide Oxyfluorfen (2-chloro-α,α,α-trifluoro-p-tolyl-3-ethoxy-4-nitrophenyl ether) is commonly used in agriculture to control annual broadleaf and grassy weeds with the recommendation to use 7 kg/ha/year (US EPA, 2002). Dissolved or soil adsorbed Oxyfluorfen may be transported from agriculture areas to aquatic environment by run-off surface water. Therefore Oxyfluorfen may present a risk to aquatic photosynthetic organisms, since it acts as a strong inhibitor of chlorophyll biosynthesis by preventing the transformation of protoporphyrinogen IX into protoporphyrin IX which is a necessary precursor for chlorophyll synthesis (Cobb, 1992; Böger and Sandman, 1998). The inhibition of pigment biosynthesis by Oxyfluorfen may alter the functions of light harvesting complexes (LHC) and energy transfer within photosystem II (PSII) (Karapetyan *et al.*, 1983). The alteration of protoporphyrinogen IX oxidase activity will cause an accumulation of protoporphyrinogen IX (Duke *et al.*, 1991; Scalla, 1991). Non-enzymatic oxidation of protoporphyrinogen IX may induce an accumulation of protoporphyrin IX having a strong photosensitizer property responsible for generation of superoxide as reactive oxygen species (ROS) (Sandmann and Böger, 1988; Aizawa and Brown, 1999). Therefore, superoxide molecules may induce the formation of hydrogen peroxide (H_2O_2) *via* superoxide dismutase (SOD) activity, permitting for hydrogen peroxide to diffuse into different cellular compartments (Bartosz, 1997). The formation of superoxide molecules was found to trigger lipid peroxidation and alteration of the chloroplast membrane fluidity and consequently it may reduce PSII electron transport efficiency (Nakamura *et al.*, 2000; Watanabe *et al.*, 2001). In addition to PSII electron transport inhibition, Oxyfluorfen may alter the proton transport by causing an acidification of the interthylakoid space which further may induce an alteration of LHCII, PSII quantum yield and the activity of the water splitting system (Sharma *et al.*, 1990). The presence of H_2O_2 in photosynthetic cellular system was shown to induce an inhibition of CO_2 fixation causing diminution of plant growth (Asada, 1994). It was found for photosynthetic cellular system to have different enzymatic activities preventing damages by oxidative stress. For superoxide dismutase, it was shown to participate in the reduction of superoxide radical into hydrogen peroxide, therefore protecting cell from oxidative effect. This enzyme appeared to be present in cytosol, mitochondria, chloroplasts, and peroxisomes, therefore playing in those

cellular compartments a scavenging role for superoxide molecules (Van Camp *et al.*, 1994). In chloroplast and cytosol compartments, H_2O_2 is readily broken down by catalase activity (Scandalios, 1994). Others antioxidative enzymes, such as ascorbate peroxidase (APX) and glutathione reductase (GR), are also involved in antioxidative stress system (Asada and Halliwell, 1987; Asada, 1994). In the presence of different pollutants inducing oxidative stress, glutathione *S*-transferase was also found to participate in antioxidative reactions (Knörzer *et al.*, 1999; Teisseire and Vernet, 2001). Recently, some evidence was shown for this enzyme to be a useful biomarker for oxidative stress induced by pollutants (Geoffroy *et al.*, 2002). For Oxyfluorfen, it has been noticed to affect photosynthesis through inhibition of oxygen evolution and PSII electron transport (Sandmann *et al.*, 1984). In photosynthesis, the light energy absorbed by the pigment-protein aggregates induces pigment excitation, which is further transferred *via* Chlorophyll *a* (Chl *a*) to the Photosystem II and I reaction centers (RCII and RCI). The primary charge separation in the reaction centers will then trigger a chain of oxido-reduction events maintaining the electron transport process. The induced PSII-PSI electron transport from water, as a primary electron donor, is coupled with the formation of proton gradient resulting altogether in the reduction of NADP and the biosynthesis of ATP necessary for CO_2 fixation (Krause and Weis, 1991). Therefore, the inhibition of PSII-PSI electron transport by different pollutants may affect other processes related to photosynthesis which further will alter the physiological state of plant (Juneau and Popovic, 1999; Popovic *et al.*, 2003).

According to previous reports, Oxyfluorfen as a peroxidizing herbicide has a complex effect on the functional cellular state shown by destruction of various cellular constituents and functions. Therefore, Oxyfluorfen damaging effect is expected at various sites of cellular metabolism, which may be indicated by different physiological and biochemical biomarkers. In this report, we investigated sensitivity of photosynthetic and enzymatic indicators of alga *Scenedesmus obliquus* exposed to Oxyfluorfen toxic effect. This approach may indicate a new possibility to use some of those parameters as biomarkers for bioassay toxicity tests. Futhermore, determination of biomarker sensitivity may facilitate their use in environmental risk assessment for polluted water.

8.4 Materials and methods

8.4.1 Plant material

The use of unicellular alga *Scenedesmus* as an aquatic model organism for bioassay toxicity test has the advantage of this alga's large distribution in the freshwater phytoplankton community and its frequent use for growth inhibition toxicity tests (Van den Hoek *et al.*, 1995; AFNOR, 1998). *Scenedesmus obliquus* (SAG 276-3a, Göttingen, Germany) was cultivated in a batch culture of 1-L growth medium (Couderchet and Böger, 1993) under continuous illumination (110 ± 10 µmoles of photons.m^{-2}.s^{-1}) provided by white fluorescent lamps (Sylvania Grolux F 36 W) at 28°C ± 1. The alga culture was placed on an orbital shaker (130 rpm) and permanently aerated to provide an optimal concentration of CO_2. Algal cultures used for the experiments were maintained in an exponential growth phase and the stock sample had a cell density of 1.5×10^7 cells.mL^{-1}.

8.4.2 Oxyfluorfen treatment

The algal samples treated with Oxyfluorfen were exposed to the same light intensity and temperature conditions used for growth culture. The algal samples were placed on an orbital shaker during treatment (130 rpm). The stock solution of Oxyfluorfen (provided by Dr. Ehrensdorfer, Augsburg, Germany) was dissolved in methanol and the concentration of methanol in treated samples was 0.05 % (vol/vol) to insure no side effect. The algal cultures were exposed to starting concentrations of 7.5, 15, and 22.5 µg.L^{-1} of Oxyfluorfen at 12, 24, and 48 h, respectively.

8.4.3 Growth measurement

The initial cell density in the treated sample was 2 ± 106 cells.mL^{-1}. The algal cells content was quantified by using a FACScan flow cytometer (Becton Dickinson Instruments) equipped with an argon-ion laser blue light at 480 nm (Franqueira *et al.*, 2000).

8.4.4 Pigment determination

Total chlorophylls were extracted in 100 % methanol at 65°C and quantitative determination was done according to Lichtentaler (1987).

8.4.5 Chlorophyll *a* fluorescence measurements

A Hansatech PEA fluorometer (Hansatech Ltd., King's Lynn, Norfolk, UK) was used to measure the rapid and polyphasic Chl *a* fluorescence related to PSII electron transport activity (Lazar, 1999). To obtain the optimal PSII quantum yield, the used excitation light was 780 µmol of photons.$m^{-2}.s^{-1}$. Prior to these measurements algal cells were concentrated on 13-mm glass fiber filter (Millipore AP20 013 00). The application of algae on the filter (filtration under low pressure) did not induce additional physiological stress which may affect measurements (data not shown). Priories to filtration and fluorescence measurement algal cells were adapted to darkness for 30 min to induce an oxidoreduction equilibrium of PSII-PSI electron transport carriers. During the measurements of rapid fluorescence kinetics, the fluorescence yield at 50 µs was taken as a constant fluorescence value (F_O fluorescence) and the maximum fluorescence yield attained was F_M. Fluorescence yield was measured at 300 µs, 2 ms, 30 ms, and 6 s in order to calculate different fluorescence parameters (Strasser and Strasser 1995). According to Strasser *et al.* (1999), the following fluorescence parameters were evaluated : PSII antenna size, $ABS/RC = ((F300\mu s-F_O)/250\mu s) \times (1/(F_{2ms}-F_O)) \times (F_M/(F_M-F_O))$; Maximum quantum yield of PSII, $\Phi_{PSII} = (F_M-F_O)/F_M$; PSII fluorescence quenching related to oxidized plastoquinone pool, $Q_{PQ} = (F_M-F_{30ms})/(F_M-F_O)$; the value of the pH dependent quenching, $Q_{Emax} = (F_M-F_{6s})/F_M$. Chl *a* fluorescence kinetic was analyzed by using a PAM (Pulse Amplitude Modulated) fluorometer (FMS/2S, Hansatech Ltd.) according to Rohacek and Bartak (1999) (see Figure 2, control) : The fluorescence F_O is measured by using a modulated light (ML) with a low intensity (1 µmol of photons.$m^{-2}.s^{-1}$) to avoid the reduction of the PSII primary electron acceptor, Q_A. The maximal fluorescence yield, F_M, is induced by a short saturating pulse (SP) of white light (2000 µmol of photons.$m^{-2}.s^{-1}$, 0.7 s duration), which triggers the reduction of all Q_A. The fluorescence yield F_S reflects the electron transport under actinic light (AL) (100 µmol of photons.$m^{-2}.s^{-1}$). The maximal

fluorescence yield F_M' is induced by SP given periodically at every 40 s when alga is exposed to continuous AL. At the steady state of electron transport, AL is turned off and a far-red light (FR) is applied to ensure rapid oxidation of Q_A. The fluorescence F_O for light-adapted sample represents the fluorescence yield when all PSII reaction centers are in open state. At steady state of fluorescence yield (10-15 min under continuous actinic light), the operational quantum yield is $\Phi s_{PSII} = (F_M'-F_S)/F_M'$; The photochemical quenching value $Q_P = (F_M'-F_S)/(F_M'-F_O')$, representing the proportion of light excitation energy converted to photochemical act by the active PSII reaction centers, and $Q_N = 1-((F_M'-F_O')/(F_M-F_O))$, representing fluorescence quenching not directly related to photochemistry of PSII (Van Kooten and Snel, 1990).

8.4.6 Enzyme assays

After 12, 24, and 48 hours of exposure to Oxyfluorfen, the algal cultures were collected, centrifuged and pellet was resuspended in 500 µL of 0.1 M sodium phosphate buffer (pH 7) with addition of 500 µL of glass beads (Sigma), and then algal cells were ground for 10 min by using a vortex at 4°C. Enzymes were extracted within 1.0 mL of sodium phosphate buffer and centrifuged at 2300*g* for 20 min at 4°C. For enzyme activity measurements the supernatant was stored at -80°C. Protein content was determined according to Bradford (1976).

Catalase activity (CAT) was evaluated spectrophotometrically by measuring the consumption of H_2O_2 at 240 nm according to Aebi (1984). The testing medium contained in final volume of 750 µL of sodium phosphate buffer (50 mM, pH 7.5), 100 µL of H_2O_2 (200 mM), and 150 µL enzyme extract (10 µg protein) in a final volume of 1 mL.

Ascorbate peroxidase activity (APX) was evaluated by the change of absorbance at 290 nm due to ascorbate oxidation according to Nakano and Asada (1981). The reaction mixture contained in a final volume of 1 mL, 700 µL of sodium phosphate buffer (50 mM, pH 7.5), 50 µL Na-ascorbate (20 mM), 50 µL H_2O_2 (100 mM), and 200 µL enzyme extract (25 µg protein).

Glutathione reductase activity (GR) was measured by the change of absorbance at 412 nm due to the reduction of DTNB (5,5-Dithiobis(2-nitrobenzoic acid)) by GSH (glutathione reduced form) in 2-nitro-5-thiobenzoic acid (Smith *et al.*, 1988). The reaction mixture contained in a final volume of 1 mL, 650 µL sodium phosphate buffer (50 mM, pH 7.5), 50 µL NADPH (2 mM), 50 µL DTNB (15 mM), 50 µL GSSG (glutathione oxidized form) (20 mM), and 200 µL enzyme extract (25 µg protein).

Glutathione *S*-transferase activity (GST) was evaluated by the change of absorbance at 340 nm due to conjugation of GSH to 1-chloro-2,4-dinitrobenzene (CDNB), as described by Drotar *et al.* (1985). The reaction mixture contained in a final volume of 1 mL, 765 µL sodium phosphate buffer (50 mM, pH 7.5), 25 µL GSH (40 mM), 10 µL CDNB (40 mM), and 200 µL enzyme extract (25 µg protein).

8.4.7 Data analysis and statistics

All experiments were repeated at least three times and each sample was analyzed in triplicates. Standard deviation and means were calculated for each treatment. The significance of differences between samples was determined by using Student's *t* test, where *p* value less than 0.05 was considered to be significant.

8.5 Results and discussion

8.5.1 Growth inhibition and chlorophyll content

Oxyfluorfen appeared to be a strong inhibitor of *S. obliquus* cell growth as seen by the strong diminution of cell division at early stage of algal development. When *S. obliquus* was exposed to different concentrations of Oxyfluorfen (from 7.5 to 22.5 $\mu g.L^{-1}$), cell growth was diminished during 48 h. At 48 h of exposure to 22.5 $\mu g.L^{-1}$ Oxyfluorfen cell growth was inhibited by 50% compared to control (Table 8.1). Growth inhibition was related to the inhibition of chlorophyll biosynthesis and to the peroxidative destruction of thylakoid membranes as reported earlier (Wakabayashi and Böger, 1999). We may interpret for this

inhibition to be induced by ROS formation resulted from accumulation of photosensitized of protoporphyrin IX compound (Böger and Sandmann, 1998). Indeed, chlorophyll biosynthesis of *S. obliquus* was strongly inhibited already at 12 h of treatment since the rate of chlorophyll biosynthesis was diminished from 41 to 96 %, respectively, to 7.5 and 22.5 $\mu g.L^{-1}$ Oxyfluorfen compared to control. It has been reported for algal growth inhibition to be measured by the change of chlorophyll content (El Jay, 1996). In this case, when cell growth was compared to chlorophyll biosynthesis, we noticed some discrepancy, for example, at 48 h, algal cells treated with 22.5 $\mu g.L^{-1}$ Oxyfluorfen, the growth was inhibited by 50 % while the rate of chlorophyll biosynthesis was diminished by 96 % compared to control (Table 8.1). This indicates for the measurement of cellular growth by the change of chlorophyll content to be revised and carefully used as indicator of biomass.

8.5.2 Fluorescence parameters related to PSII activity

For the change of Chl *a* fluorescence intensity for *in vivo* condition, it has been shown to be dependent on oxido-reduction state of photosystem II and associated electron carriers, which reflects utilization of excitation energy by light harvesting complex *via* PSII photochemistry (Lazar, 1999). Therefore, by the change of variable fluorescence yield induced by Oxyfluorfen effect, we may indicate the alteration of PSII photochemistry related to plastoquinone oxido-reduction state. We noticed also for rapid rise of fluorescence yield, dependent on Q_A and PQ reduction, to be significantly affected by Oxyfluorfen. For the change of rapid fluorescence transients we may assume to be related to alteration of structural and functional properties of PSII (Strasser *et al.*, 1999). As seen at 48 h of exposure to Oxyfluorfen (Figure 8.1), PSII quantum yield (Φ_{PSII}) of remained PSII functional reaction centers and PQ pool functionally linked to PSII electron transport (Q_{PQ}) were decreased. When *S. obliquus* was treated with Oxyfluorfen (22.5 $\mu g.L^{-1}$) at 48 h, the number of remained PSII active reaction centers per chlorophyll content was decreased since the yield of variable fluorescence, as response to saturating flash, was remarkably decreased (Figure 8.2). For the parameter ABS/RC was found to be indicator of PSII absorption cross-section showing the size of antenna pigment per reaction center (Krüger *et al.*, 1997). For the ratio ABS/RC we found to be increased by the effect of Oxyfluorfen (Figure 8.1). For increased ratio ABS/RC

we may interpret to be induced by diminished active PSII units by Oxyfluorfen which provided larger antenna size per decreased but remained active PSII units (Lavergne and Lecci, 1993). Support to this interpretation is also finding for PSII quantum yield and photochemical quenching of remained active PSII to be slowly changed during 48 h of exposure to Oxyfluorfen (Table 8.1). In algal culture treated with Oxyfluorfen, the quenching effect dependent on the proton gradient (Q_{Emax}) was found to be decreased, probably resulting from the low electron transport flow from PSII towards PSI (Figure 8.1). The parameter Q_{Emax} has been earlier related to efficiency of light energy utilization by PSII photochemistry and electron transport permitting the build-up of the thylakoid ΔpH (Müller *et al.*, 2001). Indeed, when *S. obliquus* was exposed for 48 h to 22.5 $\mu g.L^{-1}$ Oxyfluorfen, the fluorescence yield by saturating pulse under actinic light was negligible, which indicates a strong inhibition of PSII electron transport (see again Figure 8.2). This interpretation is supported also by the decrease of parameters Φs_{PSII} and Q_P in algal culture treated with Oxyfluorfen. We noticed for energy dissipation of PSII *via* non-photochemical pathway to be increased at 24 and 48 h at algal culture exposed to Oxyfluorfen (Table 8.1). Therefore, the increase of non-photochemical energy dissipation dependent on LHC II complex induced by Oxyfluorfen appeared not to be related to formation of the proton gradient, since functional pool of PQ was decreased as seen by the diminution of Q_{Emax} value compared to control (Figure 8.1). We may suppose for this effect to be resulted from photoadaptation process to Oxyfluorfen inhibitory effect. It appeared for the change of photosynthetic-fluorescence parameters to be related to alteration of the photosynthetic apparatus.

8.5.3 Enzyme activity indicating oxidative stress

Earlier, some evidence has been reported for Oxyfluorfen to cause an alteration of the structure integrity of thylakoid membrane (Böhme *et al.*, 1981; Sharma *et al.*, 1990) and recently for this effect it has been proposed to be caused by Oxyfluorfen ROS formation (Böger and Sandmann, 1998). We noticed for antioxidant enzyme activities of CAT, APX, GR, and GST to be increased when *S. obliquus* was exposed to Oxyfluorfen effect (Figure 8.3 and Table 8.1). These findings are in agreement with earlier reported results on antioxidant enzyme activities when cell cultures of soybean, tobacco and bean have been

exposed to peroxidizing type of herbicides (Gullner *et al.*, 1991; Knörzer *et al.*, 1996). Since Oxyfluorfen contributes to ROS formation, one may expect for the antioxidant enzymes to be activated if algal cells are exposed to this herbicide effect. Furthermore, it appeared for glutathione to be the major reductant of H_2O_2 in algal cellular system as similarly it was earlier found in higher plants (Asada, 1994). Our data indicated for glutathione cellular pool to be mostly used for GR and GST activity since APX appeared to be less active compared to GR and GST. For catalase activity of *S. obliquus,* we found to be strongly activated during 48 h, probably caused by diffusion of hydrogen peroxide in all cellular compartments. For catalase activity in higher plant was also found to be one of the main antioxidant enzymes (Knörzer *et al.*, 1996).

8.5.4 Variation of sensitivity for different parameters

As seen, Oxyfluorfen may induce different effects on the cellular functions such as growth, pigment biosynthesis, photosynthetic, and antioxidant enzyme activities. This multi-effect of Oxyfluorfen may offer an advantage in comparing the cellular and functional parameters as biomarkers of toxicity when *S. obliquus* was inhibited. The change of 13 parameters showed a great variation of sensitivity when algal cells were exposed to different concentrations of herbicide at various times of exposure (Table 8.1). At 12 h, the chlorophyll content was the first significant parameter showing the effect of the smallest Oxyfluorfen concentration (7.5 $\mu g.L^{-1}$). For *S. obliquus* treated with 15 $\mu g.L^{-1}$ Oxyfluorfen during 12 h, antioxidant enzyme activities CAT and GR were indicative and only the photochemical parameter Q_{PQ} was significantly changed. These parameters were the most sensitive biomarkers since they indicated Oxyfluorfen toxicity at shortest time of exposure (12 h). However, when initial concentration of Oxyfluorfen was 15 $\mu g.L^{-1}$, the majority of examined parameters were significantly affected after 24 h, with the exception of APX, Φ_{PSII}, and Q_P. For the same time of exposure to 22.5 $\mu g.L^{-1}$ of Oxyfluorfen, variations of all parameters appeared to be significant. When *S. obliquus* was exposed for 48 h to 15 $\mu g.L^{-1}$ Oxyfluorfen, all parameters were significant except Q_P, which became significant only when the concentration of Oxyfluorfen was 22.5 $\mu g.L^{-1}$. Various effects of Oxyfluorfen on the change of parameters may be explained by the herbicide different mode of action at molecular level.

Therefore, this permitted us to establish comparison of sensitivity by using the change of the parameters if they were compared to control. The following order was found to be : ABS/RC > Chlorophyll content > CAT > Φs_{PSII} > GST > Q_{PQ} > GR > Growth rate > Q_N > Q_{Emax} > APX > Q_P > Φ_{PSII}.

8.5.5 Environmental risk assessment

Oxyfluorfen toxicity appeared to be complex since it is a light-dependent peroxidizing herbicide showing especially strong toxic effect in the presence of light. It has been reported from the US EPA (2002) for drinking water assessment concerning chronic and acute Oxyfluorfen concentrations to be 5.1 and 23.4 $\mu g.L^{-1}$, respectively. In the aquatic environment, Oxyfluorfen was found to be stable and persistent component to decomposition process (Humburg, 1994). Biotests with Oxyfluorfen showed strong toxic effect to fish and freshwater invertebrates when exposed either to short- or long-term treatment (US EPA, 1984). However, little information is available concerning Oxyfluorfen toxicity on non-target alga species, preventing new approaches in risk assessment toxicity (Geoffroy *et al.*, 2002). In this study, some biomarkers based on the photosynthetic and antioxidant enzymes activities appeared to be useful tools in environmental risk assessment for peroxidizing herbicides found in aquatic systems. The order of sensitivity between biomarkers shows that the most useful indicators of light harvesting complex size changes, chlorophyll content and the activities of antioxidant enzymes catalase and glutathione *S*-transferase are the most useful for environmental risk assessment. Since the Oxyfluorfen toxicity effect is related to various photosynthetic and biochemical processes, it is of interest to analyze the sensitivity of numerous parameters which may be convenient tools for toxicity assessment in non-target plants. Determined order of sensitivity between those different biomarkers may facilitate the choice of bioassay in assessment of water quality.

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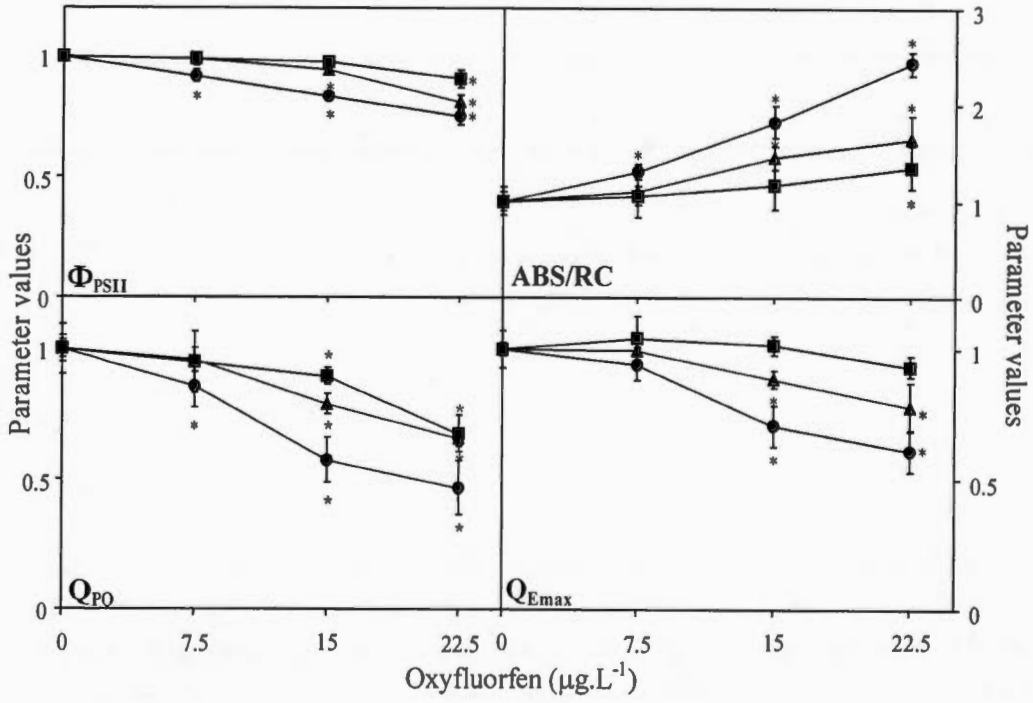

Figure 8.1 Effect of Oxyfluorfen on PSII quantum yield (Φ_{PSII}), antenna size (ABS/RC), functional plastoquinone pool (Q_{PQ}), and proton gradient quenching (Q_{Emax}) after 12 (■), 24 (▲), and 48 (●) hours when *S. obliquus* was treated with different starting concentrations of Oxyfluorfen (*$p < 0.05$).

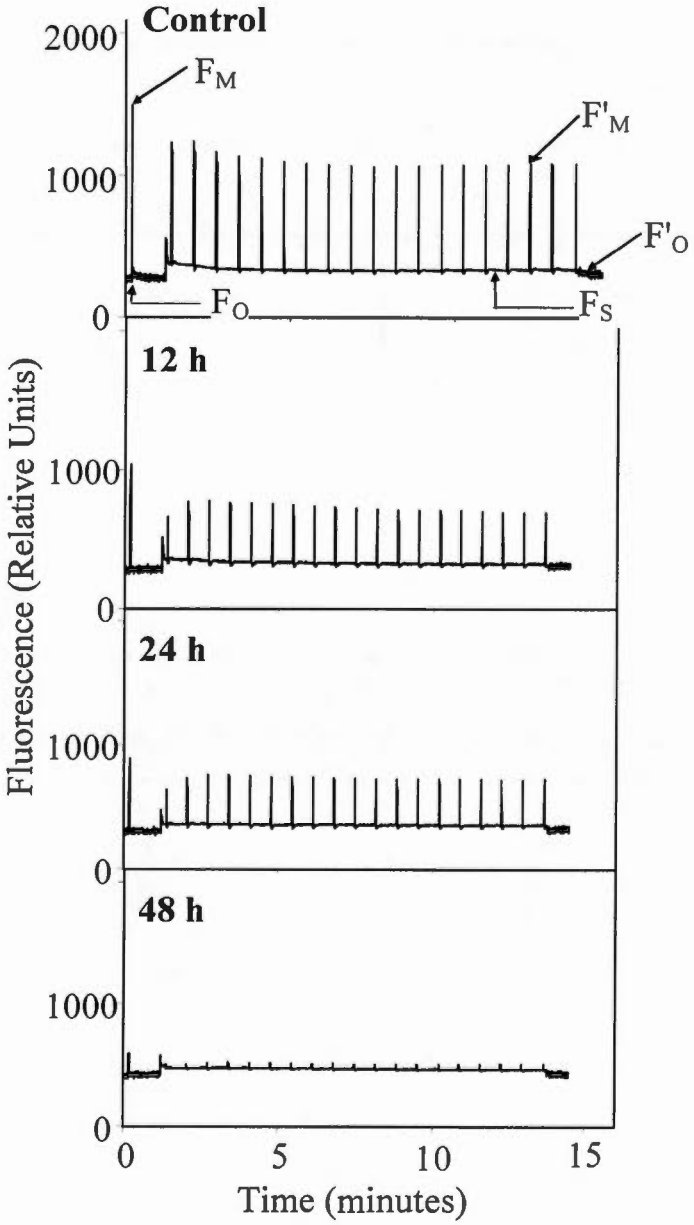

Figure 8.2 Inhibitory effect of 22.5 $\mu g.L^{-1}$ Oxyfluorfen on the PAM chlorophyll fluorescence kinetics when S. obliquus was exposed during 12, 24, and 48 hours.

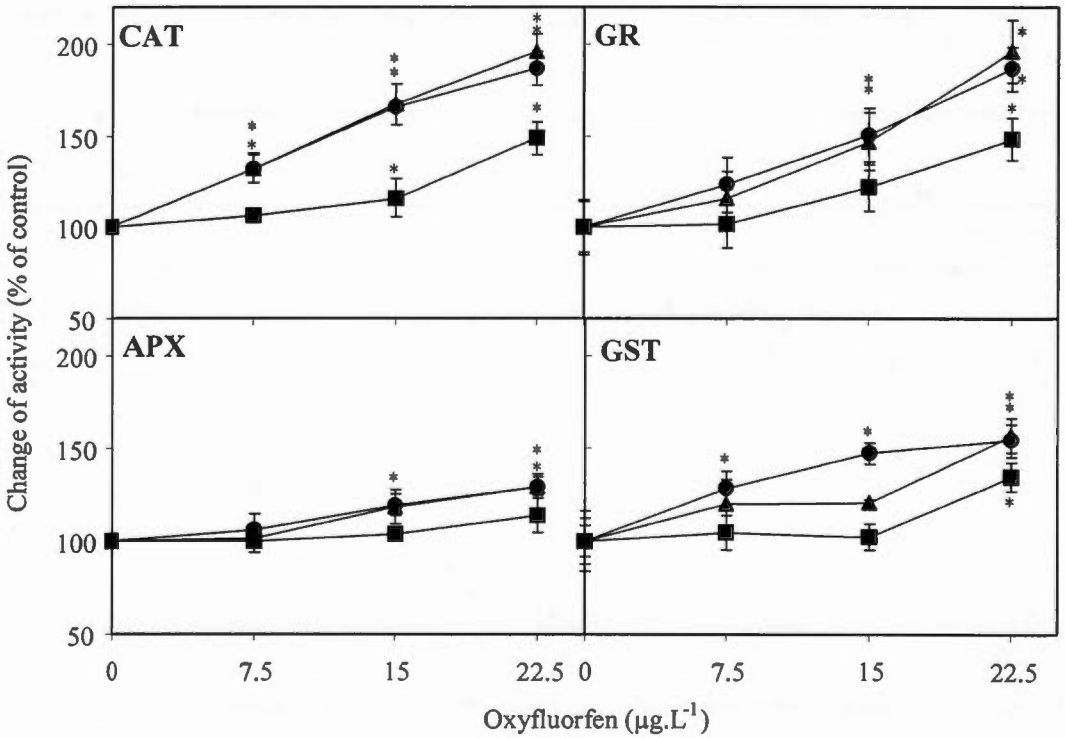

Figure 8.3 Effect of Oxyfluorfen on Catalase (CAT), Glutathione reductase (GR), Ascorbate peroxidase (APX), and Glutathione *S*-transferase (GST) activities at 12 (■), 24 (▲), and 48 (●) hours of exposure to different Oxyfluorfen concentrations (*$p < 0.05$).

Table 8.1 Variations of the 13 parameters at 12, 24 and 48 h when S. obliquus was exposed to different starting concentrations of Oxyfluorfen. The change of values was expressed as % of control. Increase (+) and decrease (-) compared to control (ns : not significant and $p < 0.005$).

Time of exposure	Parameters	Oxyfluorfen ($\mu g.L^{-1}$) 7.5	15	22.5
12 h	Growth rate	ns	ns	ns
	Chl synthesis rate	**- 41 ± 16**	**- 90 ± 16**	**- 96 ± 18**
	CAT	ns	**+ 16 ± 4**	**+ 49 ± 18**
	APX	ns	ns	ns
	GR	ns	**+ 24 ± 12**	**+ 48 ± 11**
	GST	ns	ns	**+ 35 ± 8**
	Φ_{PSII}	ns	ns	**- 10 ± 4**
	ΦS_{PSII}	ns	ns	**- 23 ± 8**
	Q_{PQ}	ns	**- 11 ± 4**	**- 33 ± 15**
	Q_{Emax}	ns	ns	ns
	Q_P	ns	ns	ns
	Q_N	ns	ns	ns
	ABS/RC	ns	ns	ns
24 h	Growth rate	**- 30 ± 8**	**- 55 ± 11**	**- 94 ± 11**
	Chl synthesis rate	**- 14 ± 8**	**- 52 ± 20**	**- 90 ± 13**
	CAT	**+ 33 ± 10**	**+ 67 ± 8**	**+ 96 ± 5**
	APX	ns	ns	**+ 30 ± 6**
	GR	ns	**+ 47 ± 16**	**+ 53 ± 15**
	GST	ns	**+ 21 ± 7**	**+ 57 ± 9**
	Φ_{PSII}	ns	ns	**- 20 ± 8**
	ΦS_{PSII}	ns	**- 15 ± 10**	**- 24 ± 8**
	Q_{PQ}	ns	**- 21 ± 9**	**- 35 ± 12**
	Q_{Emax}	ns	**- 12 ± 2**	**- 22 ± 10**
	Q_P	ns	ns	**- 8 ± 3**
	Q_N	**+ 72 ± 11**	**+ 70 ± 12**	**+ 69 ± 8**
	ABS/RC	ns	**+ 45 ± 8**	**+ 65 ± 14**
48 h	Growth rate	**- 17 ± 8**	**- 31 ± 10**	**- 48 ± 11**
	Chl synthesis rate	**- 25 ± 11**	**- 62 ± 9**	**- 96 ± 12**
	CAT	**+ 32 ± 10**	**+ 66 ± 9**	**+ 87 ± 6**
	APX	ns	**+ 20 ± 5**	**+ 30 ± 7**
	GR	**+ 23 ± 15**	**+ 50 ± 16**	**+ 51 ± 17**
	GST	**+ 29 ± 9**	**+ 48 ± 6**	**+ 55 ± 9**
	Φ_{PSII}	ns	**- 17 ± 6**	**- 25 ± 7**
	ΦS_{PSII}	**- 12 ± 3**	**- 12 ± 7**	**- 56 ± 21**
	Q_{PQ}	**- 14 ± 9**	**- 44 ± 15**	**- 54 ± 17**
	Q_{Emax}	ns	**- 29 ± 11**	**- 39 ± 13**
	Q_P	ns	ns	**- 27 ± 10**
	Q_N	**+ 55 ± 9**	**+ 53 ± 3**	**+ 45 ± 6**
	ABS/RC	**+ 30 ± 6**	**+ 83 ± 9**	**+ 143 ± 5**

CHAPITRE IX

DETERMINATION OF PHOTOSYNTHETIC AND ENZYMATIC BIOMARKERS SENSITIVITY USED TO EVALUATE TOXIC EFFECTS OF COPPER IONS AND FLUDIOXONIL IN ALGA *SCENEDESMUS OBLIQUUS*

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9.1 Résumé

Les méthodes fluorimétriques «*Pulse Amplitude Modulated*» et «*Plant Efficiency Analyzer*» ont été utilisées pour investiguer les paramètres photosynthétiques de fluorescence chez l'algue verte *Scenedesmus obliquus* exposée aux effets inhibiteurs des fongicides sulfate de cuivre et fludioxonyl (*N*-(4-nitrophényl)-*N*'-propyl-urée). Le changement de ces paramètres a été étudié lorsque l'algue *Scenedesmus obliquus* a été exposée à différentes concentrations de ces fongicides (1, 2 et 3 mg L^{-1}). Dans ces conditions expérimentales, les activités enzymatiques de la catalase, de l'acorbate péroxidase, de la glutathion réductase et de la glutathion *S*-transférase ont aussi été investiguées pour évaluer la réponse antioxydatife des effets des fongicides. Le changement de la sensibilité de ces paramètres était dépendant du mode d'action du fongicide, de sa concentration et de son temps d'exposition. Concernant les effets du cuivre, les biomarqeurs photosynthétiques les plus indicatifs étaient le «*quenching*» de fluorescence non photochimique (Q_N), le «*quenching*» de fluorescence induit par le gradient de proton (Q_{Emax}) et la taille des antennes par centre réactionnel du photosystème II (ABS/RC). L'induction d'un stress oxydatif induit par le cuivre a été indiquée par l'augmentation de l'activité de la catalase qui a été le biomarqueur enzymatique le plus sensible et fiable. D'autre part, le changement des paramètres photosynthétiques était très négligeable en présence du fludioxonyl indiquant que ces paramètres ne représentent pas des biomarqueurs valables. Cependant, le fludioxonyl induit une forte activité antioxydatife associée aux enzymes du cytosol comme les activités de la catalase, l'ascorbate peroxidase et la glutathion *S*-transférase. D'après ces résultats, il est suggéré que les activités de ces enzymes antioxydatifes représentent des biomarqueurs sensibles et valables du stress oxydatif induit par le fludioxonyl. La détermination de la sensibilité des biomarqueurs offre un critère réel de leur utilisation dans des études écotoxicologiques.

9.2 Abstract

Modulated PAM fluorometry and Plant Efficiency Analyser methods were used to investigate photosynthetic fluorescence parameters of alga *Scenedesmus obliquus* exposed to inhibitory effect of fungicides copper sulphate and Fludioxonil (*N*-(4-nitrophenyl)-*N'*-propyl-uree). The change of those parameters were studied when alga *S. obliquus* have been exposed during 48 h to different concentrations of fungicides (1, 2 and 3 mg L^{-1}). Under the same condition, enzymatic activities of catalase, ascorbate peroxidase, glutathione reductase and glutathione *S*-transferase were investigated to evaluate antioxidative response to fungicides effects. The change of sensitivity of those parameters was dependent to the mode of fungicide action, their concentration and time of exposure. For copper effects, the most indicative photosynthetic biomarkers were parameters Q_N as non-photochemical fluorescence quenching, Q_{Emax} as the proton induced fluorescence quenching and ABS/RC as the antenna size per photosystem II reaction center. Copper induced oxidative stress was indicated by increased activity of catalase serving as the most sensitive and valuable enzymatic biomarker. On the other hand, Fludioxonil effect on photosynthetic parameters was very negligible and consequently not very useful as biomarkers. However, Fludioxonil induced strong antioxidative activities associated with cytosol enzymes, as we found for catalase, ascorbate peroxidase and glutathione *S*-transferase activities. By obtained results, we may suggest for the activation of those enzymes to be sensitive and valuable biomarkers of oxidative stress induced by Fludioxonil. Determination of biomarkers sensitivity may offer advantages in providing real criteria to use them for ecotoxicological diagnostic studies.

9.3 Introduction

Copper sulphate and Fludioxonil (*N*-(4-nitrophenyl)-*N*'-propyl-uree) are commonly used as fungicides in agricultural practice to protect fruits and vegetable crops (Extension Toxicology Network, 1996; Rosslenbroich and Stuebler, 2000). Excessive amounts of such fungicides dissolved in water or adsorbed on soil particles may be easily transported by run-off surface water to large aquatic reservoirs. It has been found for copper to reach accumulation in lakes, streams and ponds up to 3mg L^{-1} (Lucan-Bouché, 1997). Under this condition, some non-target aquatic plants and algae may be exposed to different toxic effects such as the inhibition of photosynthesis and metabolic processes related to growth (see review Maksymiec, 1997). For Fludioxonil it was found to have a long persistence in aquatic environment since residues of this fungicide were present up to 5 years from its use in grape vineyards region (NRAAVC, 2000), therefore, Fludioxonil may also have additional effects on non-target plant species. For copper and Fludioxonil, it was known to have different mechanism of action when plants have been exposed to their effects. For example, copper was recognized to be a strong inhibitor of photosystem II (PSII) electron transport activity associated to the water splitting system (Samson *et al.*, 1988; Renger *et al.*, 1993). By this effect, copper may alter the energy storage in algae during photosynthesis which was noticed by the change of chlorophyll *a* (Chl *a*) variable fluorescence (Juneau *et al.*, 2002; Mallick and Mohn, 2003). However, Fludioxonil inhibitory effect appeared not to be directly associated with photosynthetic electron transport as seen for copper inhibition, but rather Fludioxonil inhibits enzymatic protein kinase activities causing alteration of proteins phosphorylation process (Pillonel and Meyer, 1997). Anyhow by those toxic effects, both Fludioxonil and copper may provoke a decrease of phytoplankton biomass in aquatic environment (Extension Toxicology Network, 1996; Verdisson *et al.*, 2001). Therefore, it is of interest to detect early toxic effect of those fungicides by using sensitive biomarkers in algal bioassay toxicity. Recently in plant bioassay, different cellular functional biomarkers such as Chl *a* fluorescence, enzymatic activities, stress proteins, immunological markers are available for the determination of toxicity induced by metals, pesticides or other pollutants (see review Ferrat *et al.*, 2003). Chl *a* fluorescence as indicator of the photosynthetic electron transport activity was considered to be very sensitive biomarker when the plant cellular system has

been exposed to herbicides and heavy metals effects (Popovic *et al.*, 2003). The activity of antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione *S*-transferase (GST) were useful biomarkers to evaluate the presence of oxidative stress in plants (Teisseire and Vernet, 2000, 2001; Geoffroy *et al.*, 2002). For SOD it has been reported earlier to participate in the reduction of superoxide radical into hydrogen peroxide (H_2O_2) (Van Camp *et al.*, 1994), which became readily broken down by CAT located in chloroplast and cytosol compartments (Scandalios, 1994). Others enzymes as part of the antioxidative defence system such as APX participates also in the reduction of H_2O_2 and GR is involved in glutathione reduction, important in the regulatory system for the recycling of ascorbate (Asada, 1994). On the other hand, GST was found to participate in the conjugation of pollutants and glutathione in the process of pollutants elimination from cellular system (Marrs, 1996). However, bioassays using antioxidative enzymes activities appeared to need further studies concerning the determination of their sensitivity to specific pollutant.

In the evaluation of copper and Fludioxonil toxic effects, it is useful to use a multiparametric approach concerning photosynthetic and physiological-biochemical biomarkers, since these fungicides have different sites and mechanisms of action in plant cell. One may pose the question does various cellular functional parameters used as biomarkers showed different level of sensitivity depending to their site and mode of action. Determination of biomarkers sensitivity may offer advantages in providing criteria to use them for ecotoxicological diagnostic studies. In this report, we investigated different biomarkers related to photosynthetic and enzymatic activities in order to determine their sensitivity when the alga *Scenedesmus obliquus* was exposed to two different fungicides, copper and Fludioxonil, having different sites and mode of toxicity.

9.4 Materials and methods

9.4.1 Plant material

Alga *S. obliquus* (SAG 276-3a, Göttingen, Germany) was cultivated in a batch culture of 1 L growth medium (Couderchet and Böger, 1993) under continuous illumination (110 ± 10 µmol of photons m^{-2} s^{-1}) provided by white fluorescent lamps (Sylvania® Grolux F 36W) at 28°C ± 1. The alga culture was placed on an orbital shaker (130 rpm) and permanently aerated providing constant concentration of CO_2. Algal cultures used for the experiments were maintained in their exponential growth phase. The stock sample, priory to be used in experiments, had a cell density of 1.5 × 107 cells mL^{-1}.

9.4.2 Copper and Fludioxonil treatments

The algal samples treated with ionic copper (Cu^{2+}) or Fludioxonil were exposed to the same light intensity and temperature conditions used for growth culture. The stock solution of Fludioxonil (provided by Dr. Ehrensdorfer, Augsburg, Germany) was dissolved in acetone having a final concentration of 0.05 % (v/v) in the treated samples. The composition of the media for the control algal sample was similar with no addition of copper or Fludioxonil. During 12, 24 and 48 h, the algal samples were exposed to starting concentrations of 1, 2 and 3 mg L^{-1} of copper or Fludioxonil. During bioassay, experimental samples were also placed on an orbital shaker.

9.4.3 Growth measurement and pigment determination

The initial cell density in the treated sample was 2 × 106 cells mL^{-1}. The algal cells content was quantified by using a FACScan flow cytometer (Becton Dickinson Instruments) equipped with an argon-ion laser blue light at 480 nm (Franqueira *et al.*, 2000). Total chlorophylls were extracted in 100 % methanol at 65°C and quantitative determination was done according to Lichtenthaler (1987).

9.4.4 Chlorophyll *a* fluorescence measurements

A Plant Efficiency Analyser fluorometer (PEA, Hansatech Ltd., King's Lynn, Norfolk, UK) was used to measure the rapid and polyphasic chlorophyll *a* fluorescence emission. In

order to obtain optimal PSII quantum yield, the used excitation light was 780 µmol of photons m^{-2} s^{-1}. Before fluorescence measurements algal cells were concentrated on 13 mm glass fiber filter (Millipore #AP20 013 00). The application of algae on the filter (filtration under low pressure) did not induce additional physiological stress, which may affect measurements (data not shown). Priories to filtration and fluorescence measurement algal cells were adapted to darkness for 30 min to induce an oxido-reduction equilibrium of PSII-PSI electron transport carriers. During the measurements of rapid fluorescence kinetics, the fluorescence yield at 50 µs was determined as a constant fluorescence value (F_O fluorescence) and the fluorescence yield attained under saturating pulse (SP) was evaluated as maximum fluorescence yield F_M. In order to calculate fluorescence parameters, the fluorescence yield was monitored up to 6 s (Strasser and Strasser, 1995). According to Strasser *et al.* (1999), following fluorescence parameters were evaluated : PSII antenna size, ABS/RC = $((F_{300\mu s}-F_O)/250\mu s) \times (1/(F_{2ms}-F_O)) \times (F_M/(F_M-F_O))$; Maximum quantum yield of PSII, $\Phi_{PSII} = (F_M-F_O)/F_M$; PSII fluorescence quenching value related to oxidized plastoquinone pool, $Q_{PQ} = (F_M-F_{30\ ms})/(F_M-F_O)$; the value of the pH dependent quenching, $Q_{Emax} = (F_M-F_{6s})/F_M$. Modulated Chl *a* fluorescence kinetic was analyzed by using a Pulse Amplitude Modulated fluorometer during 15 min (PAM, FMS/2S, Hansatech Ltd.) according to Rohacek and Bartak (1999) : The fluorescence F_O is measured by using a modulated light (ML) with a low intensity (1 µmol of photons m^{-2} s^{-1}) to avoid the reduction of the PSII primary electron acceptor, Q_A. The maximal fluorescence yield, F_M, is induced by a short saturating pulse (SP) of white light (2000 µmol of photons m^{-2} s^{-1}, 0.7 s duration), which triggers the reduction of all Q_A. The value of F_S which represents the fluorescence yield at steady state of variable fluorescence under actinic light (AL) (100 µmol of photons m^{-2} s^{-1}) was used to indicate PSII not participating in electron transport (Lazar, 1999). The maximal fluorescence yield F_M' is induced by SP of light given periodically at every 40 s when alga is exposed to continuous AL. At the steady state of electron transport AL is turned off and a far-red light (FR) is applied to ensure complete oxidation of all primary electron acceptor Q_A. Therefore, fluorescence F_O' obtained under this condition represents the fluorescence yield when all PSII reaction centers are in open state. At steady state of fluorescence yield (12 min under continuous actinic light), the operational quantum yield was determined as $\Phi s_{PSII} = (F_M'-F_S)/F_M'$. Under the same condition, the photochemical quenching value was evaluated as

$Q_P = (F_M'-F_S)/(F_M'-F_O')$ which represents the proportion of light excitation energy converted to photochemical act by the active PSII reaction centers. The parameter representing the fluorescence quenching not related to photochemistry of PSII was measured as $Q_N = 1-((F_M'-F_O')/(F_M-F_O))$ according to (Van Kooten and Snel, 1990).

9.4.5 Enzyme assays

After 12, 24 and 48 h of exposure to copper or Fludioxonil, the algal cultures were collected, centrifuged, then pellet was resuspended in 500 µL of 0.1 M sodium phosphate buffer (pH 7) with addition of 500 µL of glass beads (Sigma) and algal cells were ground for 10 min at 4°C by using a vortex. Enzymes were extracted within 1 mL of sodium phosphate buffer and centrifuged at 2300*g* for 20 min at 4°C. For enzyme activity measurements the supernatant was separated and stored at -80°C. Protein content was determined according to Bradford (1976).

CAT activity was evaluated spectrophotometrically by measuring the consumption of H_2O_2 at 240 nm according to Aebi (1984) where the testing medium contained in final volume of 750 µL of sodium phosphate buffer (50 mM, pH 7.5), 100 µL of H_2O_2 (200 mM) and 150 µL enzyme extract (10 µg protein) in a final volume of 1 mL.

APX activity was evaluated by the change of absorbance at 290 nm due to ascorbate oxidation according to Nakano and Asada (1981) where the reaction mixture contained in a final volume of 1 mL, 700 µL of sodium phosphate buffer (50 mM, pH 7.5), 50 µL Na-ascorbate (20 mM), 50 µL H_2O_2 (100 mM) and 200 µL enzyme extract (25 µg protein).

GR activity was measured by the change of absorbance at 412 nm due to the reduction of DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) by glutathione reduced form (GSH) in 2-nitro-5-thiobenzoic acid (Smith *et al.*, 1988) where the reaction mixture contained in a final volume of 1 mL, 650 µL sodium phosphate buffer (50 mM, pH 7.5), 50 µL NADPH (2 mM), 50 µL DTNB(15 mM), 50 µL glutathione oxidized form (GSSG) (20 mM) and 200 µL enzyme extract (25 µg protein).

GST activity was evaluated by the change of absorbance at 340 nm due to conjugation of GSH to CDNB (1-chloro-2,4-dinitrobenzene), in reaction mixture containing in a final volume of 1 mL, 765 µL sodium phosphate buffer (50 mM, pH 7.5), 25 µL GSH (40 mM), 10 µL CDNB (40 mM) and 200 µL enzyme extract (25 µg proteins), as described by Drotar *et al.* (1985).

9.4.6 Data analysis and statistics

All experiments were repeated at least three times and each sample was analyzed in triplicates. Standard deviation and means were calculated for each treatment. The significance of differences between samples was determined by using Student's *t*-test, where *p* value less than 0.05 was considered to be significant.

9.5 Results and discussion

9.5.1 Inhibition of growth and chlorophyll synthesis

Copper is an essential micronutrient for algal growth, but excess concentrations may induce significant toxic effect by altering protein function and consequently enzyme activity. In plant, copper was found to bind Tyr amino acid associated with D1 protein responsible for the electron transport from the water splitting system to photosystem II (Arellano *et al.*, 1995) or to induce the change of oxido-reduction state of cytochrome *b*559 (Burda *et al.*, 2003). Consequently, the photosynthetic electron transport may slow down resulting in the reduction of energy storage via photosynthesis which causes the decrease of biomass growth (Maksymiec, 1997). Copper inhibition of photosynthesis may be also caused by the prevention of chlorophyll to integrate in chloroplast photosynthetic membranes (thylakoids) (Caspi *et al.*, 1999). The change of algal cellular growth rate may be an indicative biomarker, although it represents only a global indicator which integrates effects of copper on different cellular metabolisms. When alga *S. obliquus* was exposed to copper concentrations from 1 to 3 mg L^{-1}, significant inhibition on the growth rate and chlorophyll synthesis was noticed only at 24 and 48 h of treatment (Figure 9.1). This delay of inhibitory effect may be explained by

the presence of low intracellular cop per concentration due to its slow uptake rate. At 48 h, inhibition of cellular growth rate may reach by 50 and 80 % for 1 and 3 mg L^{-1}, respectively. Chlorophyll synthesis at 48 h, in the presence of 3 mg L^{-1} of copper, was decreased also by 80 %. For the diminution of photosynthetic pigment content, it has been proposed to be due to the peroxidative breakdown of pigments and membrane lipids by reactive oxygen species induced by copper (Sandmann and Böger, 1980). It was also found for copper to inhibit the synthesis of δ-aminolevulinic acid and the protochlorophyllide reductase activity, which consequently may inhibit the pigment accumulation and integration into photosystems responsible for photosynthetic electron transport (Stiborová *et al.*, 1986). However, under the same conditions, we found for Fludioxonil to induce a smaller inhibitory effect on the growth rate of *S. obliquus* cells compared to copper (Figure 9.1). No inhibitory effect of Fludioxonil was observed on cellular growth rate of *S. obliquus* treated from 1 to 3 mg L^{-1} up to 24 h. Cellular growth rate and chlorophyll synthesis were only affected at 48 h if algal cells were exposed to 2 and 3 mg L^{-1} of Fludioxonil. For Fludioxonil concentration of 3 mg L^{-1}, growth rate and chlorophyll synthesis were inhibited by 30 and 55 %, respectively. It appeared for this fungicide to have no effect on photosynthesis if algal cells were exposed during shorter period (24 h) and consequently no rapid effect was noticed on biomass growth (data not shown). Since Fludioxonil mode of action is rather related to intermediary metabolism, one may expect a slow effect of Fludioxonil on alga cellular growth and photosynthetic process. However, chlorophyll content affected by Fludioxonil at 48 h of treatment may be used as biomarker of its toxicity.

9.5.2 The change of fluorescence kinetics and parameters related to PSII activity

The effect on the rapid rise of variable fluorescence was examined when alga cells were exposed to copper and Fludioxonil as fungicides. For fluorescence transients J, I and P related to PSII photochemistry and electron transport activity, we found to be changed significantly when copper concentration was gradually increase from 1 to 3 mg L^{-1} (Figure 9.2). Since fluorescence yields at J, I and P transients was evidently quenched, we may assume that PSII electron transport via Q_A, Q_B and the plastoquinone pool was inhibited. It has been proposed earlier for these transients to represent sensitive photosynthetic indicators

when plants have been exposed to the herbicide diuron (Strasser and Strasser, 1995). We noticed for the quenching effect of fluorescence value to be dependent to copper concentration, since 3 mg L^{-1} of copper induced the strongest quenching effect compared to 1 and 2 mg L^{-1} (Figure 9.2). For the quenching effect of the variable fluorescence yield at J, I and P transients, we may interpret to be caused by the deterioration of the water splitting system accordingly to earlier reports (Guissé *et al.*, 1995; Strasser, 1997). On the other hand, Fludioxonil demonstrated no effect on the variable fluorescence yield at J, I and P transients when algal cells were exposed during 48 h (Figure 9.2).

It was of interest to investigate the variation of different photosynthetic fluorescence parameters values related to the variable Chl *a* fluorescence kinetic when alga *S. obliquus* cells were exposed up to 48 h to copper and Fludioxonil effects. We found for copper to affect differently the photosynthetic fluorescence parameters (Figure 9.3) : the values of Φ_{SPSII}, Q_{PQ}, Q_P were decreased while at the same time the values of Q_{Emax}, Q_N and ABS/RC were increased. Compared to the control, those differences for the copper effect were gradually increased when copper concentration and time of exposure were augmented. Decrease of Φ_{SPSII}, Q_{PQ}, Q_P parameters may be interpreted as a consequence of copper inhibition of the total functional PSII photochemistry, the electron transport activity via plastoquinone pool and the real fraction of PSII participating in electron transport activity, respectively shown by those parameters. The maximum quantum yield of PSII, Φ_{PSII} did not show significant variation statistically compared to control concerning the effect of copper and Fludioxonil. One may expect this since PSII photochemistry of the reaction centers non-inhibited by those fungicides was intact. However, proportion of PSII active reaction centers was diminished shown by the decrease of Φ_{SPSII} as the parameter of the total functional PSII photochemistry (Figure 9.3). The increase of the values of Q_{Emax}, Q_N and ABS/RC parameters may indicate for copper effect to alter the energy dissipation via proton membrane flux, the non-photochemical energy dissipation and the change of the structural organization of the PSII light harvesting complex, respectively shown by those parameters. For the photosynthetic fluorescence parameter variations, according their value affected by copper, we may found to be sensitive and valuable biomarkers for copper toxic effects. When the similar investigation was applied to Fludioxonil effect, the variations concerning majority of

those photosynthetic fluorescence parameters were not found except only for Q_{Emax} value shown when algal cells were exposed to 12, 24 and 48 h. However, at 12 h we didn't found variation when algal cells were exposed to 1 and 2 mg L^{-1} of fluodioxonil (Figure 9.3). This may indicate that Fludioxonil affect the proton gradient in thylakoid membranes of chloroplast. However, sensitivity of this variation is much smaller when compared to copper effects. Results presented in Figure 9.3 may suggest that photosynthetic fluorescence parameters may serve as sensitive biomarkers for copper, but not for Fludioxonil effect. These findings are in agreement with earlier reports showing for fluorescence photosynthetic parameters to be sensitive biomarkers of some pesticides toxicity (see review Popovic *et al.*, 2003).

9.5.3 Enzymes activities indicating oxidative stress

Some evidences have been earlier reported for copper to induce in higher plants the production of reactive oxygen species (ROS) which as response may trigger the cellular defense system by activating antioxidative enzymes activities (Weckx and Clijsters, 1996; Teisseire and Vernet, 2000). In wheat grown under excess of copper, the alteration of photosystem II activity may cause the formation of superoxide and hydroxyl radicals. In those plants, antioxidative enzymes activities (SOD, APX) were found to be located in thylakoid membranes containing PSII system (Navari-Izzo *et al.*, 1998). However, the activation of the antioxidative system consisting of CAT, APX, GR and GST activities appeared to be complex, since those enzymes were located at different cellular sites (Foyer *et al.*, 1994; Mittler, 2002); therefore, ROS production induced by toxic effects of copper or Fludioxonil will be dependent to their specific toxic mode of action at different cellular sites.

When algal cells of *S. obliquus* were exposed to copper, increased activities of CAT, APX, GR and GST were depended to copper concentration and time of exposure (Figure 9.4). Enzymatic activation was also found to be induced by Fludioxonil except for GR activity. For each antioxidative enzyme, the increase of specific activity was differently induced by comparing copper to Fludioxonil effects (Figure 9.4). Those results concerning antioxidative enzymes activities indicated a stronger activation of CAT, APX, GST enzymes

in algal cells treated with Fludioxonil compared to those exposed to copper. CAT appeared to be highly activated by copper effects, since CAT activity augmented by 80 % compared to control when algal cells were treated 12 and 24 h with 3 mg L^{-1} of copper. However, CAT activity was less important at 48 h, which may be explained by the alteration of CAT protein integrity by copper effects, as reported earlier in alga *Nitzschia closterium* (Florence and Stauber, 1986). For APX, GR and GST activities, when algal cells were exposed to 3 mg L^{-1} of copper, activation did not over pass 25 % compared to control. On the other hand, Fludioxonil effect at 12 and 24 h induced activation between 40 and 85 % of CAT, APX and GST when algal cells were exposed to 2 and 3 mg L^{-1} (Figure 9.4). The activation was diminished at 48 h, probably due to the deterioration of cellular system functions by Fludioxonil. Since GR activity was not significantly affected by Fludioxonil, we may assume for oxidized glutathione pool to be mostly used by APX and GST activities.

9.5.4 Biomarkers sensitivity related to copper and Fludioxonil effects

Photosynthetic and enzymatic biomarkers as indicators of copper and Fludioxonil effects showed great variation in sensitivity. Evident variation was noticed when copper and Fludioxonil effects were compared either via photosynthetic or enzymatic parameters. Large variation was also evident if one may compare only photosynthetic or enzymatic parameters, probably indicating different mechanisms involved in fungicide effects. These mechanisms may vary also due to time of exposure and fungicide concentrations. Since the copper toxic effect is related to the alteration of various photosynthetic and biochemical processes, it will be interesting to analyze the sensitivity of investigated parameters which may be used as convenient tools for toxicity assessment in non-target plants.

When algal cells were exposed to 2 or 3 mg L^{-1} of copper, PSII electron transport was decreased (see Figure 9.2). Diminished proportion of active PSII may provoke the activation of light energy dissipation via non-photochemical pathway (Müller *et al.*, 2001). Consequently to those alterations, we found for Q_N to be the most sensitive biomarker, since non-photochemical energy dissipation became an important part of the regulatory system in energy transfer when the photosynthetic electron transport system was inhibited by copper.

Since Q_N is dependent to energy flux via PSII reaction center and associated antenna complex (Horton *et al.*, 1994), it is expected for parameter ABS/RC to be also a sensitive biomarker and indeed it appeared to be second in order of sensitivity comparaison. However, Q_{PQ} showed less sensitivity while Q_{Emax} became sensitive only when algal cells were exposed to copper during 48 h (see Figure 9.3 and Table 9.1). On the other hand, photosynthetic fluorescence parameters were not sensitive to Fludioxonil effects, except Q_{Emax} at some less degree. The lack of Fludioxonil effect on photosynthetic fluorescence parameters may be explained by its specific mode of action at cellular level compared to copper effect.

For copper it appeared to have different cellular sites of action besides its inhibitory effect in chloroplast. The increase activity of CAT in alga *S. obliquus* treated with copper (2 and 3 mg L^{-1}) may indicate for copper to produce ROS in cytosol. For CAT activity, it is known to be suited in the cytosol and peroxysomes representing importance sink for H_2O_2 induced by oxidative stress in plant (Chandlee *et al.*, 1983; Willekens *et al.*, 1997). We found for CAT activity as a part of the antioxidative cellular defense system to be the most sensitive indication for copper effect (see Figure 9.4 and Table 9.2). However, we noticed the decrease of CAT activity when algal cells were exposed to copper treatment during 24 and 48 h. We may assume for copper to induce a deterioration effect on CAT protein when alga *S. obliquus* was treated for 24 and 48 h, which is in agreement with earlier findings (Florence and Stauber, 1986). Although enzymes APX, GR and GST are present in cytosol and chloroplast, copper effect on their activities appeared to be by three times less effective compared to CAT activity (see Figure 9.4 and Table 9.2). CAT activity demonstrated to be also the most sensitive when *S. obliquus* was exposed to Fludioxonil effect during 48 h. We noticed for APX activity to be increased at 12 and 24 h when algal cells were exposed to 2 and 3 mg L^{-1} of Fludioxonil concentrations. For now, we don't have explanation why the activity of this enzyme at 48 h was diminished between three and four times. We may only assume for this diminished effect of Fludioxonil to be caused by deficiency of ascorbate as substrate. Since GR was not activated by Fludioxonil effect, we may explain this by the lack of available glutathione oxidized form. Therefore, GR may not serve as an indicative parameter for Fludioxonil effect.

9.6 Conclusion

Sensitivity of investigated biomarkers was highly dependent to fungicides site interactions with cellular functions. The difference between copper and Fludioxonil effects may be explained by their chemical properties associated with organic or ionic forms, which may determine the process of penetration and distribution into different cellular compartments. The sensitivity of biomarkers was also dependent to the change of fungicide concentrations and time of algal exposure. For copper effect, the indicative photosynthetic biomarkers were parameters Q_N, Q_{Emax} and ABS/RC. However, for copper induced oxidative stress, CAT activity appeared to be the most sensitive and valuable indicator. Fludioxonil effect on photosynthetic parameters was very negligible and consequently not very useful as biomarkers. On the other hand, Fludioxonil induced strong antioxidative activities associated with cytosol enzymes, as it has been showed with CAT, APX and GST activities. Consequently, we may suggest for activities of these antioxidative enzymes to be sensitive and valuable biomarkers of oxidative stress induced by Fludioxonil. Further investigation of those biomarkers will offer useful tools for environmental risk assessment toxicity induced by fungicides. In addition, the use of unicellular microalgae have advantage compared to higher plant since alga have bigger surface contact permitting rapid uptake of pollutant. Further advantage is that the unicellular alga *Scenedesmus* is largely distributed in freshwater phytoplankton community and had been used as a model organism for growth inhibition tests (Van den Hoek *et al.*, 1998; AFNOR, 1998).We may assume for biomarkers based on the photosynthetic activity and antioxidative enzyme activities to offer fast and reliable indication in the study of aquatic pollutants toxicity impact.

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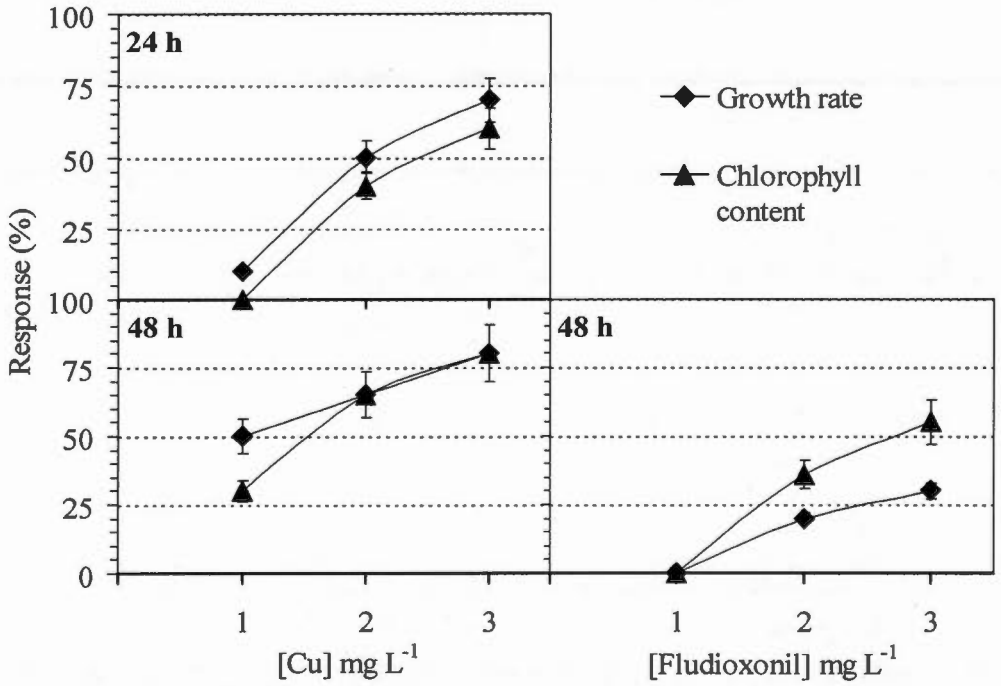

Figure 9.1 Inhibitory effect of copper ions and Fludioxonil on cellular growth rate and chlorophyll synthesis when *S. obliquus* cells were exposed 24 and 48 h. These results are significant changes expressed in percentage (%) from control where $p < 0.005$.

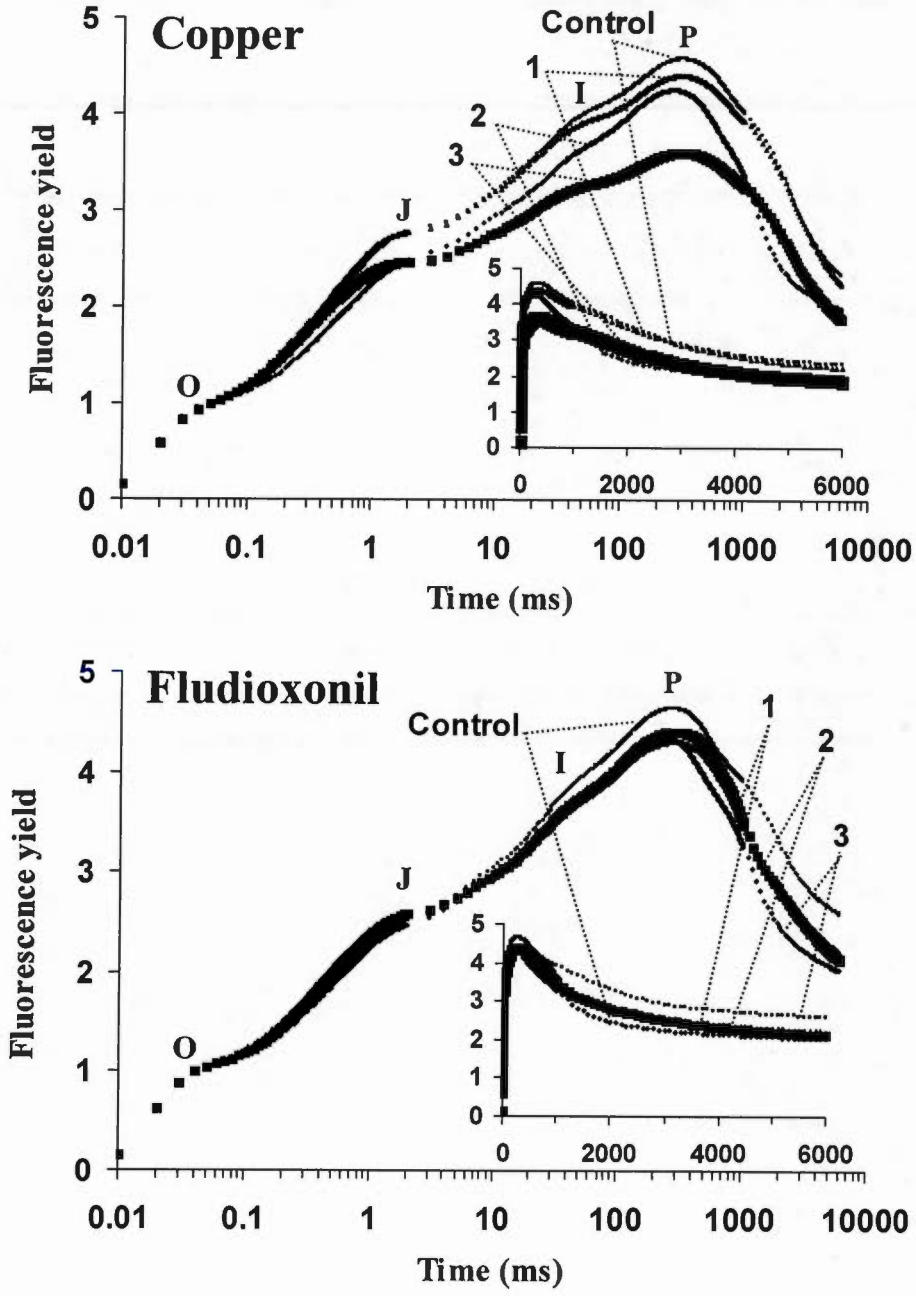

Figure 9.2 Change of the rapid and polyphasic rise of Chl *a* fluorescence kinetics when *S. obliquus* cells were exposed 48 h to copper ions and Fludioxonil. Yield of fluorescence kinetic was normalized at F_O level.

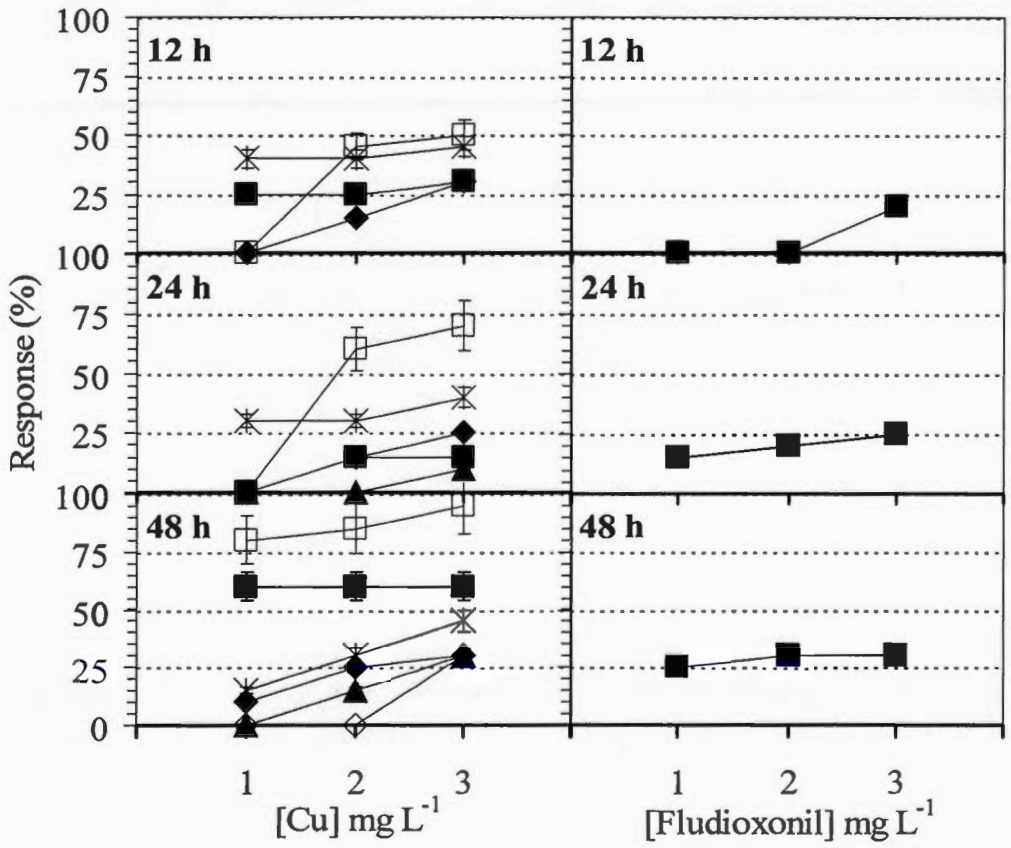

Figure 9.3 Effect of copper ions and Fludioxonil on the change of photosynthetic fluorescence parameters values (indicated as increase : (+) or decrease : (−)) : (−) Φ_{SPSII} (▲), (−) Q_{PQ} (♦), (−) Q_P (◊), (+) Q_{Emax} (■), (+) Q_N (□) and (+) ABS/RC (×) when *S. obliquus* cells were exposed 12, 24 and 48 h. These results are significant changes expressed in percentage (%) from control where $p < 0.005$.

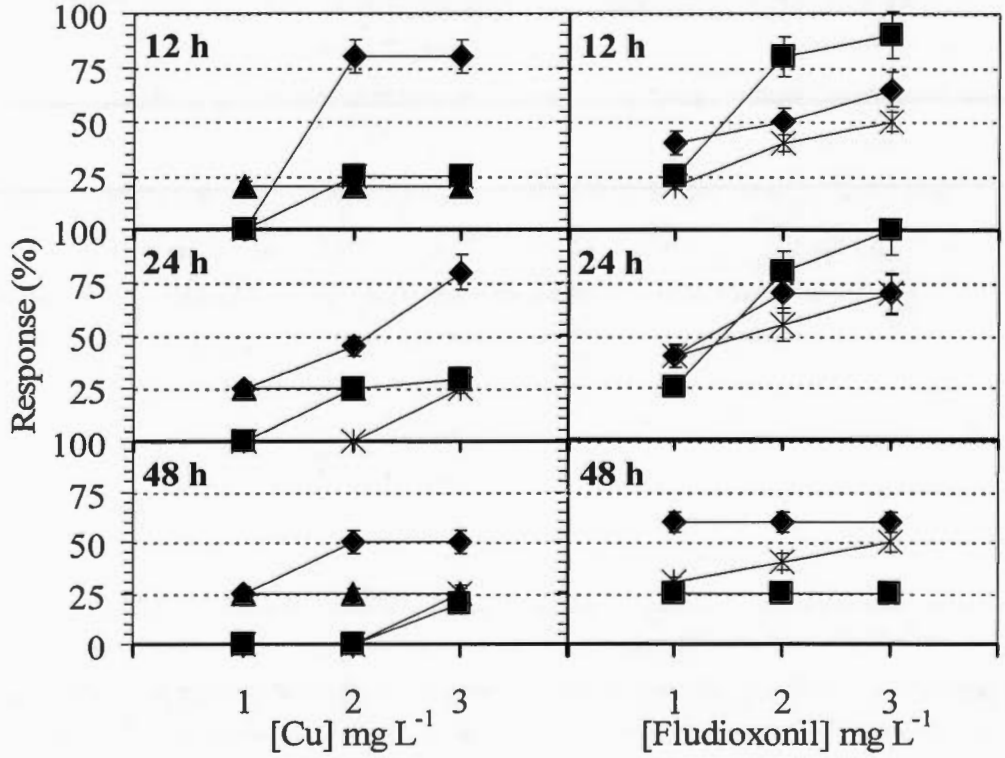

Figure 9.4 Increase of antioxidative enzymes activities of catalase (♦), glutathione reductase (▲), ascorbate peroxidase (■) and glutathione *S*-transferase (×) when *S. obliquus* cells were exposed 12, 24 and 48 h to copper ions and Fludioxonil. These results are significant changes expressed in percentage (%) from control where $p < 0.005$.

Table 9.1 Photosynthetic parameters of fluorescence in alga *S. obliquus* presented by the order of sensitivity (> or =) and concentrations of copper ions and Fludioxonil.

Time of exposure		Fungicides concentrations		
		1 mg L^{-1}	2 mg L^{-1}	3 mg L^{-1}
12 h	Copper	$ABS/RC>Q_{Emax}$	$Q_N>ABS/RC>Q_{Emax}>Q_{PQ}$	$Q_N>ABS/RC>Q_{PQ}=Q_{Emax}$
	Fludioxonil	-	-	Q_{Emax}
24 h	Copper	ABS/RC	$Q_N>ABS/RC>Q_{PQ}=Q_{Emax}$	$Q_N>ABS/RC>Q_{PQ}>Q_{Emax}>\Phi_{sPSII}$
	Fludioxonil	Q_{Emax}	Q_{Emax}	Q_{Emax}
48 h	Copper	$Q_N>Q_{Emax}>ABS/RC>Q_{PQ}$	$Q_N>Q_{Emax}>ABS/RC>Q_{PQ}>\Phi_{SPSII}$	$Q_N>Q_{Emax}>ABS/RC>\Phi_{SPSII}=Q_{PQ}=Q_P$
	Fludioxonil	Q_{Emax}	Q_{Emax}	Q_{Emax}

Table 9.2 Antioxidative enzymatic activities in alga *S. obliquus* presented by the order of sensitivity (> or =) and concentrations of copper ions and Fludioxonil.

Time of exposure		Fungicides concentrations		
		1 mg L^{-1}	2 mg L^{-1}	3 mg L^{-1}
12 h	Copper	GR	CAT>APX=GST>GR	CAT>APX=GST>GR
	Fludioxonil	CAT>APX>GST	APX>CAT>GST	APX>CAT>GST
24 h	Copper	CAT=GR	CAT>APX=GR	CAT>APX=GR>GST
	Fludioxonil	CAT=GST>APX	APX>CAT>GST	APX>GST=CAT
48 h	Copper	CAT=GR	CAT>GR	CAT>GR=GST>APX
	Fludioxonil	CAT>GST>APX	CAT>GST>APX	CAT>GST>APX

CHAPITRE X

VALIDATION OF PHOTOSYNTHETIC-FLUORESCENCE PARAMETERS AS BIOMARKERS FOR ISOPROTURON TOXIC EFFECT ON ALGA *SCENEDESMUS OBLIQUUS*

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10.1 Résumé

Les paramètres photosynthétiques de fluorescence ont été investigués comme biomarqueurs valides de toxicité lorsque l'algue *Scenedesmus obliquus* a été exposée aux effets toxiques de l'herbicide Isoproturon [3-(4-isopropylphenyl)-1, 1-dimethyluree]. Les cellules algales ont été traitées durant 24 heures à des concentrations d'Isoproturon d'ordre 7-500 µg L^{-1} et le taux d'inhibition de croissance attaignait un maximum de 75 % par rapport au témoin. L'induction de la fluorescence chlorophyllienne de la chlorophylle *a* chez des cellules algales traitées à l'Isoproturon a indiqué une inactivation des centres réactionnels du photosystème II et donc une forte inhibition du transport des électrons. Une corrélation linéaire a été trouvée ($R^2 \geq 0.861$) entre la variation du taux de division cellulaire induit par l'Isoproturon et le changement des paramètres photosynthétiques de fluorescence suivants : Le rendement photochimique opérationnel du photosystème II (Φ'_M), le «*quenching*» photochimique (q_P) et le «*quenching*» photochimique relatif ($q_{P(rel)}$). La variation du taux de division cellulaire était aussi linéairement dépendent ($R^2 = 0.838$) au paramètre de fluorescence $UQF_{(rel)}$. Une corrélation non linéaire a été trouvée ($R^2 = 0.937$) uniquement entre la variation du taux de division cellulaire et l'efficacité du transfert de l'énergie absorbée au centre réactionel du photosystem II (ABS/RC). Par conséquent, les fortes corrélations entre le changement du taux de division cellulaire et les paramètres photosynthétiques de fluorescence investigués offre une évidence qui supporte l'utilisation de ces paramètres dans les bioessais algaux comme biomarqueurs sensibles et fiables des effets toxiques de l'Isoproturon.

10.2 Abstract

Photosynthetic-fluorescence parameters were investigated in order to be used as valid biomarkers of toxicity when green alga *Scenedesmus obliquus* was exposed to the herbicide Isoproturon [3-(4-isopropylphenyl)-1, 1-dimethylurea] effect. Algal cells were treated during 24 h with Isoproturon from 7 to 500 µg L^{-1} and maximal growth inhibition was 75 % compared to control. Chlorophyll *a* fluorescence induction of algal cells treated with Isoproturon showed an inactivation of photosystem II (PSII) reaction centers and a strong inhibition of PSII electron transport. A linear correlation was found ($R^2 \geq 0.861$) between the variation of cellular division induced by Isoproturon and the change of photosynthetic-fluorescence parameters : effective PSII quantum yield (Φ'_M), photochemical quenching (q_P) and relative photochemical quenching ($q_{P(rel)}$). The variation of cellular division was also linearly dependent ($R^2 = 0.838$) on the relative unquenched fluorescence parameter ($UQF_{(rel)}$). Non-linear correlation was found ($R^2 = 0.937$) only between cellular division variation and the energy transfer efficiency from absorbed light to PSII reaction center (ABS/RC). Therefore, high correlations between the change of cellular division and investigated photosynthetic-fluorescence parameters provide supporting evidence to use these parameters in algal bioassay as sensitive and reliable biomarkers of Isoproturon toxic effect.

10.3 Introduction

Isoproturon, 3-(4-isopropylphenyl)-1,1-dimethylurea, has been widely used as herbicide in agriculture practice. In contaminated agricultural areas, Isoproturon was transferred from treated soil by runoff water to large aquatic reservoirs (Nitschke and Schüssler, 1998). For this herbicide it has been shown to be one of the most commonly found herbicide in surface or groundwater (Environmental Agency, 1999) and consequently, its accumulation in aquatic environment may induce unwanted toxic effects on different non-target aquatic plant species. Isoproturon, as a phenylurea type of herbicide, induces alteration of photosynthesis because it inhibits photosystem II (PSII) activity due to herbicide binding process with D1 protein of PSII reaction center (Naber and Van Rensen, 1991). Due to PSII inhibition, it has been suggested for Isoproturon toxicity to diminish primary production of biomass in freshwater ecosystems (Bérard and Pelte, 1999). By using photometric bioassay methods, the inhibition of algal growth by Isoproturon has been shown for green alga *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa*, *Scenedesmus obliquus* and *Chlorella vulgaris* (Ma, 2002; Reboud, 2002; Rioboo *et al.*, 2002). Current use of algae in the study of herbicide toxic effects on photosynthesis seems to be a convenient approach for fundamental and applied toxicology research. The change of photochemical acts of photosynthesis directly affects the synthesis of ATP and NADPH resulting in the alteration of the whole photosynthetic process and consequently cellular growth, which is readily indicated by PSII dependent fluorescence emission (Seaton and Walker, 1990; Gerst *et al.*, 1994). It is advantageous to use chlorophyll *a* (Chl *a*) fluorescence measurements as an indicator of PSII primary photochemistry and associated photosynthetic electron transport since this approach may provide some information on the interaction of herbicides with photosynthesis at the molecular level. Since the biomass growth of plant is highly dependent on photosynthesis, the measurement of the photosynthetic process may become an important monitoring method to determine rapidly plant physiological state and growth (El Jay *et al.*, 1997). Presently, measurements of Chl *a* fluorescence by Pulse Amplitude Modulated (PAM) fluorometer and Plant Efficiency Analyser (PEA) are used as non-destructive, rapid and efficient methods for *in vivo* study of photosynthetic activity when plant has been exposed to different environmental stress (Schreiber, 2004; Strasser *et al.*, 2004). Therefore, by using Chl *a*

fluorescence measurements, we may provide an advantage for bioassay toxicity testing, since it represents a rapid and sensitive biomarker when plant has been exposed to herbicides toxicity (Popovic *et al.*, 2003). However, further knowledge on the correlation between algal cell growth and photosynthetic-fluorescence parameters may facilitate the validation of those photosynthetic-fluorescence parameters to be used as convenient toxicity biomarkers. Great numbers of photosynthetic-fluorescence parameters are used in photosynthesis research (Roháček, 2002; Force *et al.*, 2003), but we are still missing their validation for toxicology studies. It should be admitted that some photosynthetic-fluorescence parameters are not indicative for toxicity tests probably resulting by complexity of interaction between pollutant and cellular system or by their methodological formulation (Juneau *et al.*, 2005).

In this report, we investigated the sensitivity of some photosynthetic-fluorescence parameters dependent to PSII primary photochemistry and associated electron transport in relation to the Isoproturon toxic effect on alga *Scenedesmus obliquus*. We attempt to validate these photosynthetic-fluorescence parameters as reliable toxicity biomarkers of Isoproturon by determining their correlation with cellular division rate. Therefore, a gradual growth inhibition of algal cells induced by different concentration of Isoproturon was used as a reference for the validity of photosynthetic-fluorescence parameters.

10.4 Experimental section

10.4.1 Plant material

Alga *Scenedesmus obliquus* (SAG 276-3a, Göttingen, Germany) was cultivated in a batch culture of 1 L growth medium (Bishop and Senger, 1971) under continuous illumination (100 ± 10 µmoles photons m^{-2} s^{-1}) provided by white fluorescent lamps (Sylvania® Grolux F 36 W) at 28°C ± 1. The algal culture was placed on an orbital shaker (130 rpm) and permanently aerated to obtain constant CO_2 concentration in the growing medium. When the algal culture was in the exponential growth phase, a sample having a cell density of 5×10^7 cells ml^{-1} was used for this study.

10.4.2 Isoproturon treatment

The stock solution of Isoproturon was made with pure Isoproturon compound (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) dissolved in 100% methanol to obtain 4.8 mM of stock solution. For investigation of Isoproturon toxicity, treated algal samples were prepared with growth medium in a final volume of 50 ml where methanol concentration did not exceed 0.05 % (v/v). The algal samples were exposed 24 h to nominal concentrations 7, 15, 30, 60, 125, 250 and 500 $\mu g\ L^{-1}$ of Isoproturon corresponding respectively to 37, 75, 150, 300, 600, 1200 and 2400 nM. During Isoproturon treatment, samples were placed on an orbital shaker where light intensity and temperature conditions were the same used for growth culture.

10.4.3 Determination of Isoproturon effect on cellular division

Algal cell division was determined by FACScan flow cytometry (Becton Dickinson Instruments) in which fluorescent carboxylate microspheres were used as the counting reference (Haugland, 1996).

10.4.4 Chlorophyll *a* fluorescence measurements

Prior to fluorescence measurement, treated algal samples were adapted 30 min to darkness in order to obtain an oxidized steady state of PSII (Horton and Hague, 1988). Algal cells were concentrated on a 13 mm glass fibre filter (Millipore #AP20 013 00) by using a low pressure filtration unit which provided to form uniform layer of algal cells necessary for reproducible measurement of fluorescence. This application did not induce a physiological stress on algal cells affecting fluorescence measurements (data not shown).

A Handy-Plant Efficiency Analyser fluorometer (Hansatech® Ltd., King's Lynn, Norfolk, UK) was used to measure the rapid and polyphasic chlorophyll *a* fluorescence kinetic during 1 sec induced by a light intensity 2500 µmol photons $m^{-2}\ s^{-1}$. Fluorescence yield (F) at investigated fluorescence transients were determined according to Strasser and

Strasser (1995). Fluorescence yields were evaluated at 50 μs, 300 μs and 2 ms. The maximal fluorescence yield, F_M was determined at P transient. In analytical approach to fluorescence parameters, the fluorescence yield at 50 μs was considered to be F_O value ($F_{50\mu s}$). Parameter indicating the transfer efficiency of absorbed light energy (ABS) to PSII reaction center (RC) was evaluated by the ratio ABS/RC = $(M_o/V_J)/\phi_{PO}$. The M_o value was obtained as the ratio M_o = $(F_{300\mu s}-F_{50\mu s})$ / $((F_M-F_{50\mu s})$ x 0.25) indicating the inhibition state of the water splitting system according to (Strasser and Strasser, 1995). The fluorescence quantum yield at J transient was evaluated as V_J = $(F_{2ms}-F_{50\mu s})/(F_M-F_{50\mu s})$ indicating the efficiency of PSII primary photochemistry by the reduction of Q_A as PSII primary electron acceptor. The quantum yield at P transient was evaluated as ϕ_{Po} = $(F_M - F_{50\mu s})/F_M$ showing PSII efficiency in reduction of plastoquinone pool (Strasser *et al.*, 2000).

Analysis of the fluorescence induction of Chl *a* was done by using a PAM (FMS/2S, Hansatech® Ltd., King's Lynn, Norfolk, UK) having an analytical modulated light (0.02 μmoles photons m^{-2} s^{-1}), a saturating white light pulses (1100 μmoles photons m^{-2} s^{-1}, duration of 0.3 sec) and an actinic light (70 μmoles photons m^{-2} s^{-1}). The nomenclature for the investigated PAM parameters was used according to Van Kooten and Snel (1990). The variable fluorescence $F_V = F_M-F_O$ of dark adapted plant was evaluated by using the maximal fluorescence yield F_M induced by a saturating light pulse and the constant fluorescence F_O was evaluated under analytical low energy modulated light. The variable fluorescence level at steady state F_S was obtained 12 min after the continuous actinic light has been triggered. At steady state of variable fluorescence, the effective quantum yield of PSII was evaluated as $\phi'_M = (F'_M-F_S)/F'_M$ where F'_M represents the maximum fluorescence yield obtained by a saturating flash when algal cells were adapted to actinic light (Genty *et al.*, 1989). Dissipation of absorbed light energy through photochemical process was estimated as $q_P = (F'_M-F_S)/(F'_M-F'_O)$, where F'_O represents F_O fluorescence measured in the presence of far-red light permitting the oxidation of PSII-PSI electron transport carriers (Schreiber *et al.*, 1986). The relative energy dissipation through PSII photochemical process was estimated as $q_{P(rel)} = (F'_M-F_S)/(F_M-F'_O)$ according to (Buschmann, 1995) and the relative unquenched fluorescence as $UQF_{(rel)} = (F_S-F'_O)/(F_M-F'_O)$ indicating PSII in closed state not contributing to electron transport (Juneau *et al.*, 2005).

10.4.5 Data analysis and statistics

All experiments were performed at least three times and each algal sample was analyzed in triplicate. Means and standard deviation were calculated for each treatment. The significance of differences between samples was determined by using Student's *t* test, where *p* value less than 0.05 was considered to be significant. A logistic response curve as the trend of the data was determined for correlation analysis by using a least-square fitting method in software ORIGIN 6.0 (Microcal Software, USA) based on the Levenberg-Marquardt algorithm.

10.5 Results and discussion

In bioassays using different algal species, the change of cellular growth has been used as the most valid and common biomarker of pesticides toxicity because it represents an integrating parameter showing the pesticide inhibitory effect on all cellular metabolism (Peterson *et al.*, 1994; Ma, 2002; Gómez de Barreda Ferraz, 2004). In this study, cellular division was used as a biomarker of reference for comparison with photosynthetic-fluorescence parameters when *S. obliquus* was exposed 24 h to different Isoproturon concentration. When *S. obliquus* was exposed to a low concentration of Isoproturon (7 $\mu g\ L^{-1}$), the cellular division was increased by 50% compared to control sample. Some stimulatory effects of low pesticide concentrations on algal growth have also been reported earlier, and were interpreted to be caused by a physiological acclimation (El Dib *et al.*, 1991; Franqueira *et al.*, 1999; Rioboo *et al.*, 2002). However, when *S. obliquus* was exposed to Isoproturon concentration higher than 15 $\mu g\ L^{-1}$, the cellular division was diminished compared to control (Figure 10.1). Gradual increase of Isoproturon concentration, from 30 to 500 $\mu g\ L^{-1}$, induced a strong trend of inhibitory effect on algal cell division. As shown, when *S. obliquus* was treated with 30 $\mu g\ L^{-1}$ of Isoproturon, the cellular division decreased by 25% and reached 75% at the highest concentration (500 $\mu g\ L^{-1}$) when compared to control (Figure 10.1). The found algistatic effect is in agreement with previous reports showing the decrease of growth by 50% when different algal species were exposed 96 h to 20-25 $\mu g\ L^{-1}$ of Isoproturon (Anton *et al.*, 1993; Kirby and Sheahan, 1994; Rioboo *et al.*, 2002). In those studies, the

Isoproturon induced inhibition of cell division was shown to be dependent on exposure period and the algal species. Such decrease of algal growth in the presence of Isoproturon was caused by photosynthesis deterioration. However, correlation between growth rate and the change of photosynthetic indicators were not investigated.

As mentioned previously, Isoproturon inhibits PSII electron transport by its interaction with D1 protein having the main regulatory role in PSII primary photochemistry (Zer and Ohad, 1995). The alteration of D1 protein function will induce a modification of Chl *a* fluorescence associated to PSII activity. The increase of fluorescence quantum yield at J transient has been interpreted as an indication of PSII electron transport inhibition (Strasser *et al.*, 1995; Lazár *et al.*, 1998). Therefore, for this increase it has been suggested to reflect a light-induced accumulation of Q_A^- while Q_B was maintained in oxidized state (Lazár, 2006). Indeed, when we treated *S. obliquus* with 30 $\mu g\ L^{-1}$ of Isoproturon, the fluorescence yield at J transient was increased showing an inhibition of electron transport at the reducing side of PSII. However, when *S. obliquus* was exposed to higher concentrations than 30 $\mu g\ L^{-1}$ of Isoproturon, a decrease of the fluorescence yield at P transient was found. This may indicate for this herbicide to induce also an inhibition at the water splitting system (Figure 10.2). This interpretation is in agreement with evidence found earlier for the change of fluorescence yields at J-P transients to be induced by the inhibition of the PSII water splitting system (Pospíšil and Dau, 2000). By the increase of M_o parameter, we showed additional evidence for Isoproturon to induce an inhibition of the water splitting system of *S. obliquus* (Figure 10.3) which was also shown earlier by the increase of the fluorescence yield at 300 μs known as K transient (Strasser, 1997). This interpretation is in agreement with our results showing for Isoproturon to increase quantum yield at J transient (V_J) and the decrease of quantum yield at P transient (Φ_{Po}) (Figure 10.3). Such an Isoproturon induced modification of M_o, V_J and Φ_{Po} parameter values may serve as sensitive and rapid indication for alteration of PSII primary photochemistry.

The PAM fluorescence induction of *S. obliquus* treated with Isoproturon indicated a strong decrease of PSII electron transport activity, since variable fluorescence yield induced by a saturating flash for either dark or light-adapted algal cells was highly diminished (Figure

10.4). Under the same conditions, the F_S level was relatively increased compared to F_M obtained from dark adapted algal cells. When *S. obliquus* was exposed to high concentration of Isoproturon (500 $\mu g\ L^{-1}$), most of PSII functional reaction centers were abolished indicated by no fluorescence response to saturating flash, while some PSII reaction centers remained in closed state as seen by high yield of F_S level according to (Krause and Weis, 1991). Therefore, from the change of F_M, F'_M and F_S values, we may conclude for Isoproturon to decrease electron transport from PSII toward to photosystem I and to increase closed PSII reaction centers not participating in electron transport (see Figure 10.4).

To investigate the reliability of photosynthetic-fluorescence parameters as indicators of Isoproturon toxicity, we studied the dependency between the change of photosynthetic-fluorescence parameters and Isoproturon concentrations. As shown in Figure 10.5, we found for parameters Φ'_M, q_P, $q_{P(rel)}$, $UQF_{(rel)}$ and ABS/RC to be very dependent on the Isoproturon concentration by showing a high degree of correlation ($R^2 > 0.9$). Values of parameters Φ'_M, q_P and $q_{P(rel)}$ decreased exponentially when the Isoproturon concentration was gradually increased. However, under the same condition, the variation of ABS/RC values showed an increase having a sigmoidal form. This increase was expected since Isoproturon inhibited some PSII reaction centers. The value of $UQF_{(rel)}$ as an indication of PSII closed state, not contributing to electron transport, was exponentially increased. The relative increase of the size of light harvesting complex per reaction center induced by Isoproturon effect, we may interpret to be caused by the decrease of active PSII reaction centers and consequently light harvesting complex transferred excitation energy to a smaller number of PSII, according to (Lavergne and Lecci, 1993).

Further investigation was done on the dependency between the alteration of the photosynthetic activity by Isoproturon effect and the inhibition of algal cell division. Since the exposure of *S. obliquus* to Isoproturon was sufficient to induce a visible inhibitory effect on cellular division and photosynthetic activity, we investigated correlation between cellular growth and the change of photosynthetic-fluorescence parameters. As shown in Figure 10.6, we found a linear correlation ($R^2 \geq 0.861$) between the change of cell division induced by Isoproturon toxic effect and the change of photosynthetic-fluorescence parameters Φ'_M, q_P

and $q_{P(rel)}$. The variation of cellular division was also linearly dependent ($R^2 = 0.838$) to the parameter $UQF_{(rel)}$. On the other hand, the correlation between cellular division variation and the parameter ABS/RC was non-linearly dependent (Figure 10.7), probably resulted from the non-linear change of the size of total light harvesting complex.

The measurements of Chl *a* fluorescence induction of alga *S. obliquus* cells may provide rapid evaluation of plant physiological state shown by different photosynthetic-fluorescence parameters. In this study, we indicated that the change of those parameters was highly correlated with cellular division as an indication of biomass growth. Therefore, such parameters as Φ'_M, q_P, $q_{P(rel)}$, $UQF_{(rel)}$, and ABS/RC may also readily provide information on state of cellular growth. Such bioassay of Isoproturon toxicity test may give valid evaluation and approach in using photosynthetic-fluorescence parameters as toxicity biomarker for pollutants interaction with plant. Furthermore, the application of those biomarkers may offer new methodological advance for algal bioassay toxicity testing when PAM and PEA fluorometric methods are used.

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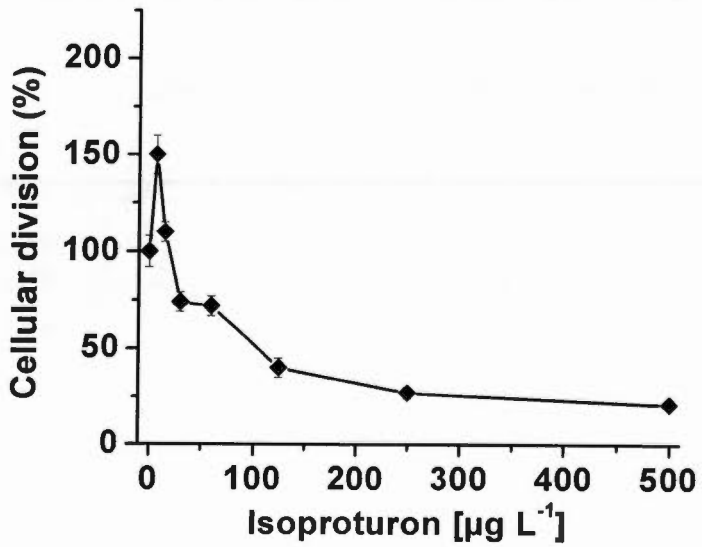

Figure 10.1 Cellular division of alga *S. obliquus* exposed 24 h to Isoproturon.

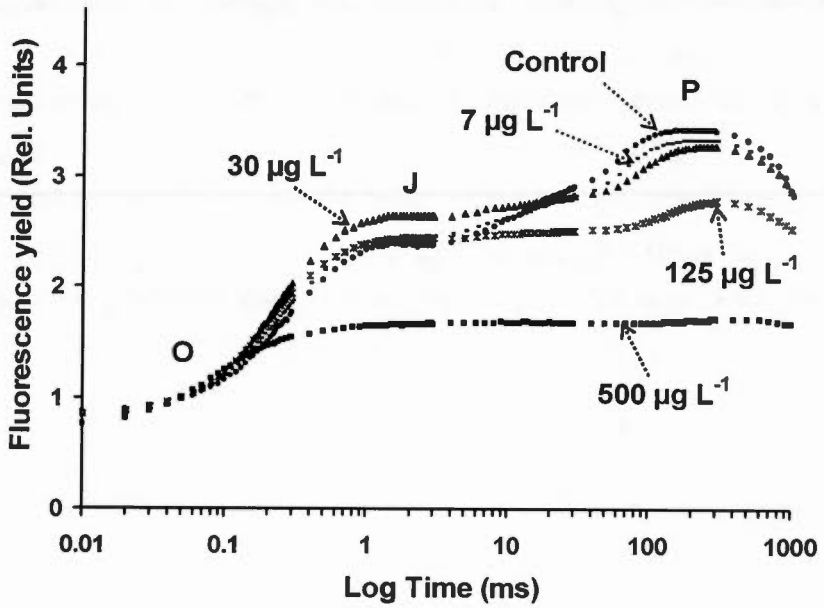

Figure 10.2 The change of rapid polyphasic kinetics of Chl *a* fluorescence when *S. obliquus* cells were exposed for 24 h to Isoproturon. Fluorescence yield was normalized at F_O level.

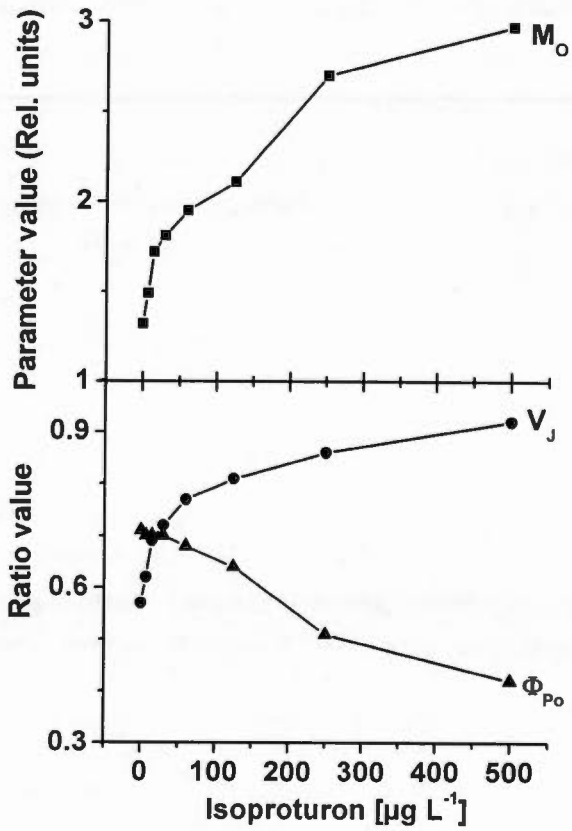

Figure 10.3 The change of fluorescence parameters value for M_O, V_J and Φ_{Po} when *S. obliquus* was exposed for 24 h to Isoproturon. For more details, see material and methods.

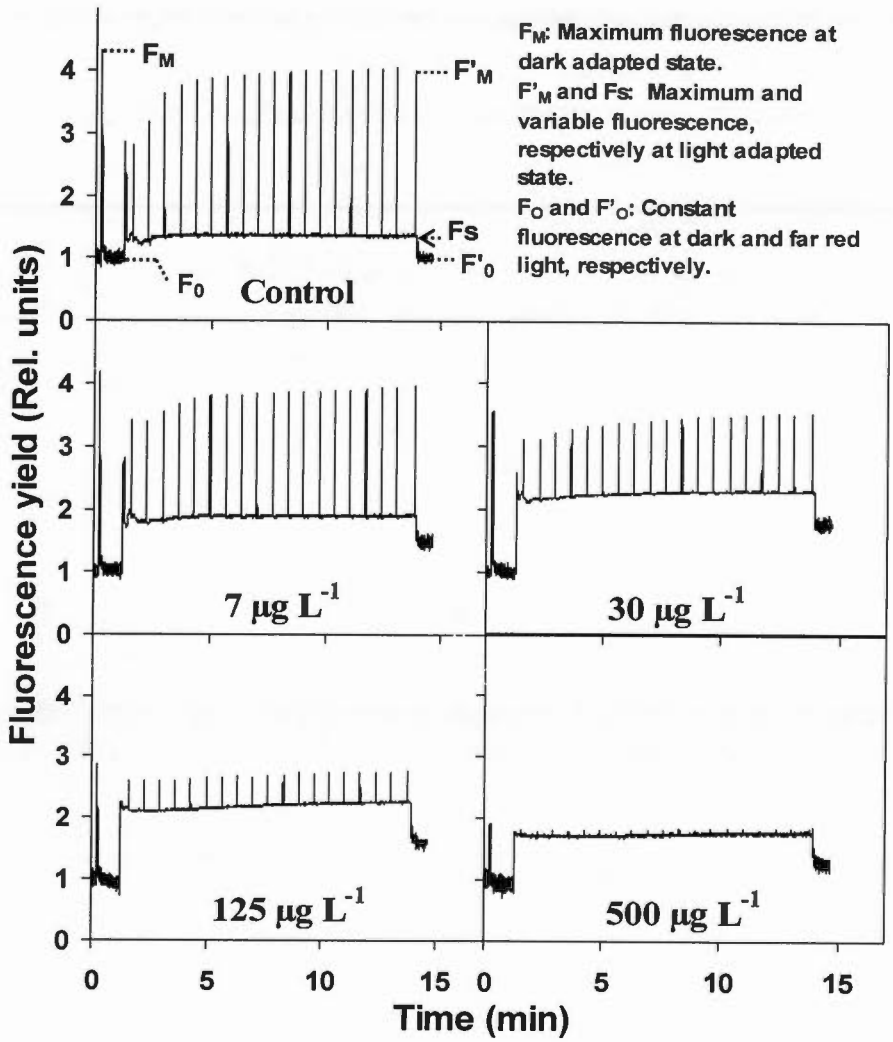

Figure 10.4 PAM fluorescence induction measured during 15 min when *S. obliquus* was previously exposed 24 h to Isoproturon. For all fluorescence kinetics, fluorescence yield was normalized at F_O level.

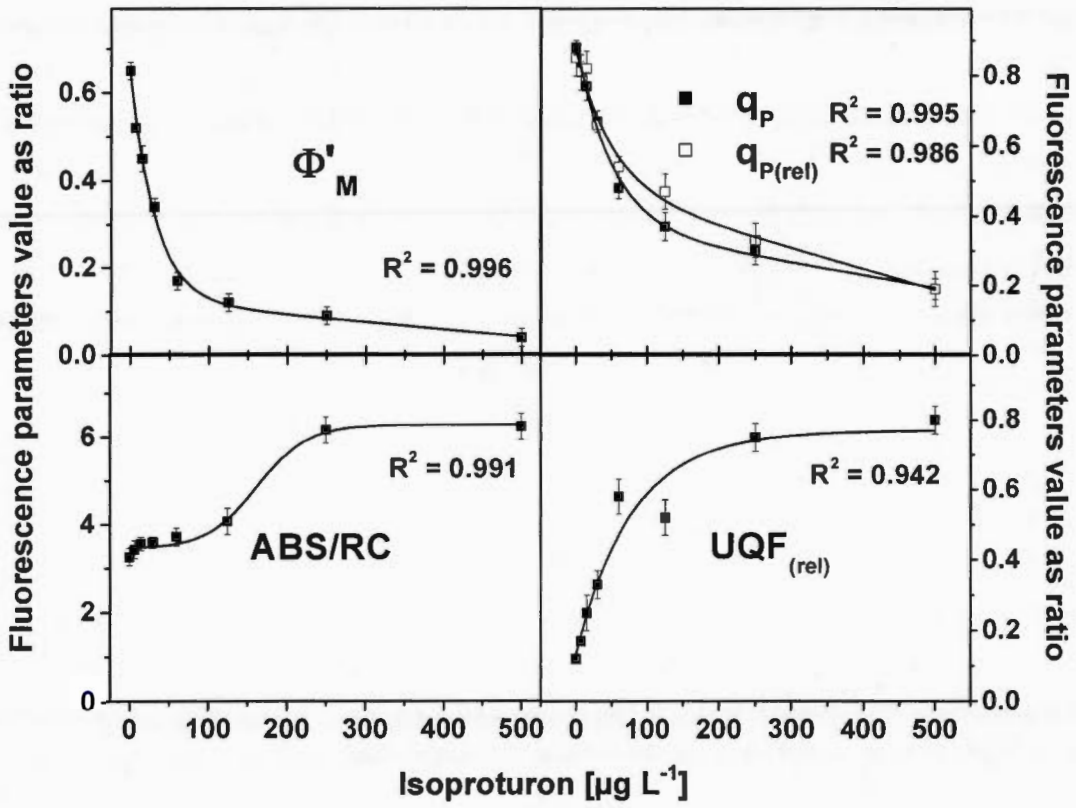

Figure 10.5 The change of fluorescence parameters corresponding to the effective PSII quantum yield (Φ'_M), the dissipation of absorbed light energy through photochemical process (q_P), the relative energy dissipation through photochemical process ($q_{P(rel)}$), the transfer efficiency of absorbed light energy to PSII reaction center (ABS/RC) and the relative unquenched fluorescence ($UQF_{(rel)}$) when *S. obliquus* was exposed 24 h to Isoproturon. Non-linear regressions were performed for the determination of the correlation between Isoproturon concentration and photosynthetic-fluorescence parameters response.

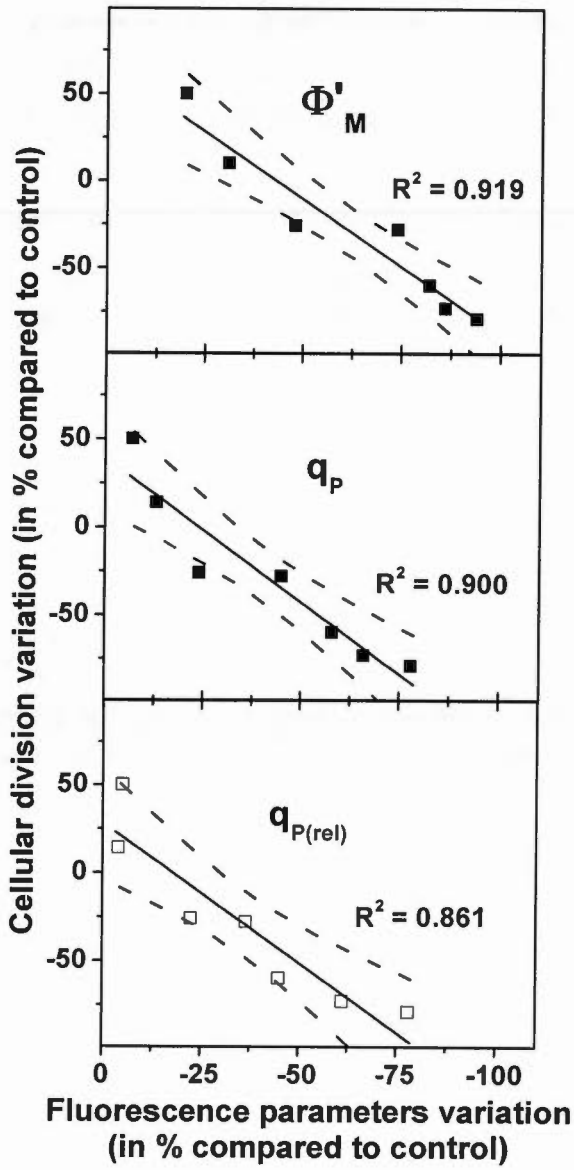

Figure 10.6 Correlation between the cell density variation and the change of the effective PSII quantum yield (Φ'_M), the dissipation of absorbed light energy through photochemical process (q_P) and the relative energy dissipation through photochemical process ($q_{P(rel)}$) when *S. obliquus* was exposed 24 h to Isoproturon. Linear regressions were performed where intervals of dash lines indicated confidence intervals (95%).

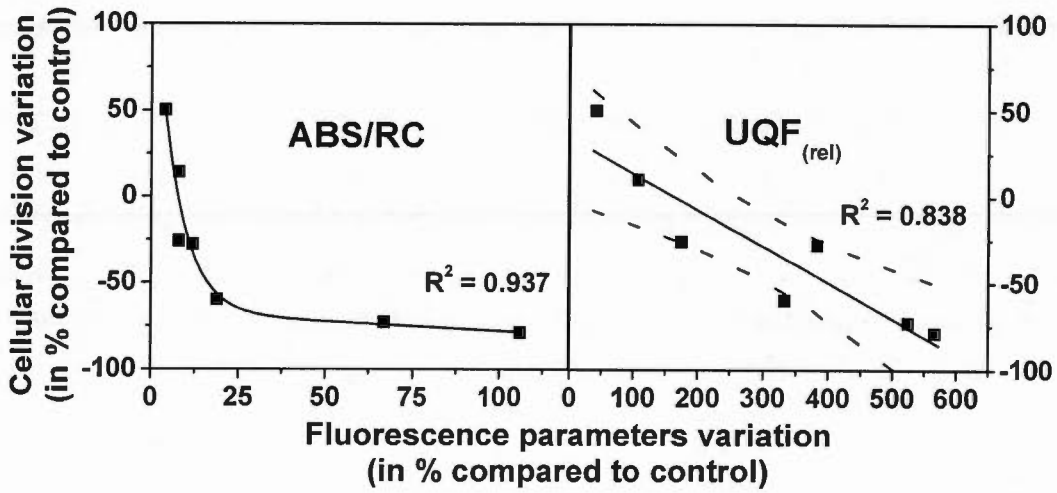

Figure 10.7 Correlation between the cell density variation and the change of the energy transfer efficiency of absorbed light energy to PSII reaction center (ABS/RC) and fluorescence energy dissipation from closed PSII reaction center noted as the relative unquenched fluorescence ($UQF_{(rel)}$) when *S. obliquus* was exposed 24 h to Isoproturon. Linear and nonlinear regressions were performed where dash lines indicated confidence intervals (95%).

CHAPITRE XI

USE OF DIFFERENT FLUOROMETRIC SYSTEMS IN THE DETERMINATION OF FLUORESCENCE PARAMETERS FROM SPINACH THYLAKOID MEMBRANES BEING EXPOSED TO ATRAZINE AND COPPER IONS

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11.1 Résumé

La sensibilité des paramètres de fluorescence du photosystème II, comme indicateurs des effets inhibiteurs de l'Atrazine et du cuivre, ont été investiguées en utilisant des systèmes fluorométriques LuminoTox, «*Pulse Amplitude Modulated*» (PAM) et «*Plant Efficiency Analyzer*» (PEA). L'inhibition du photosystème II a été induite lorsque des échantillons de membranes de thylacoïdes d'épinard ont été traités à l'Atrazine et au sulphate de cuivre durant 15 min. Dans ces échantillons, la variation des paramètres de fluorescence suivant a été investiguée : Pour le PAM et le PEA, la fluorescence variable maximale, F_V, le rendement des processus non photochimiques du PSII, F_O/F_M, le rapport entre les processus photochimique et non photochimique du PSII, F_V/F_O, et le rendement maximale de la réaction photochimique primaire, F_V/F_M. Pour le LuminoTox, l'évaluation des paramètres F_2-F_1, F_2/F_1, $(F_2-F_1)/F_1$ et $(F_2-F_1)/F_2$ a été basée sur les niveaux de fluorescence F_1 et F_2 mesurés respectivement à une faible et à une forte intensité lumineuse. Il a été montré que le changement de la sensibilité des réponses d'inhibition (Ir) était causé par le système optique du fluorimètre ou par une différence dans les mécanismes d'inhibition entre l'Atrazine et le cuivre. L'ordre de sensibilité de la EC-50% était pour l'Atrazine : $Ir_{(F2-F1)/F1}$ (LuminoTox) > $Ir_{FO/FM}$ (PAM) > $Ir_{F1/F2}$ (LuminoTox) > Ir_{F2-F1} (LuminoTox) > $Ir_{FV/FO}$ (PAM) > $Ir_{(F2-F1)/F2}$ (LuminoTox) > $Ir_{FV/FO}$ (PEA) > $Ir_{FO/FM}$ (PEA) > $Ir_{FV/FM}$ (PEA) > $Ir_{FV/FM}$ (PAM) et pour le cuivre : Ir_{F2-F1} (LuminoTox) > $Ir_{FO/FM}$ (PEA) = Ir_{FV} (PEA) > $Ir_{FV/FO}$ (PEA) > $Ir_{F1/F2}$ (LuminoTox) > $Ir_{(F2-F1)/F1}$ (LuminoTox) > Ir_{FV} (PAM) > $Ir_{FO/FM}$ (PAM) > $Ir_{FV/FO}$ (PAM) > $Ir_{FV/FM}$ (PEA) > $Ir_{(F2-F1)/F2}$ (LuminoTox). L'étude de ces Ir basée sur les paramètres de fluorescence apporte une indication utile pour une approche pratique de l'évaluation de la phytotoxicité de l'Atrazine et du cuivre.

11.2 Abstract

Sensitivity of photosystem II fluorescence parameters, as indicators of Atrazine and copper inhibitory effects, were investigated by using LuminoTox, Pulse Amplitude Modulated (PAM) and Plant Efficiency Analyzer (PEA) fluorometric systems. Inhibition of Photosystem II was induced when sample of spinach thylakoid membranes was treated with Atrazine or copper sulphate during 15 min. In those samples we investigated the change of following fluorescence parameters : For PAM and PEA, the maximal variable fluorescence as F_V, the basal quantum yield of non photochemical processes in PSII as the ratio F_O/F_M, the relationship between photochemical and concurrent non photochemical processes within PSII as the ratio F_V/F_O and the maximum quantum yield as the ratio F_V/F_M. For the LuminoTox, the evaluation of the parameters [F_2-F_1, F_2/F_1, (F_2-F_1)/F_1 and (F_2-F_1)/F_2] was based on two fluorescence yields F_1 and F_2 measured under low and high light intensities, respectively. We found for the change in sensitivity of the inhibitory response (Ir) to be caused either by the specific optical mode of the fluorometric system or by differences in inhibitory mechanism between Atrazine and copper. The sensitivity order of EC-50% of Ir was for Atrazine : $Ir_{(F2-F1)/F1}$ (LuminoTox) > $Ir_{FO/FM}$ (PAM) > $Ir_{F1/F2}$ (LuminoTox) > Ir_{F2-F1} (LuminoTox) > $Ir_{FV/FO}$ (PAM) > $Ir_{(F2-F1)/F2}$ (LuminoTox) > $Ir_{FV/FO}$ (PEA) > $Ir_{FO/FM}$ (PEA) > $Ir_{FV/FM}$ (PEA) > $Ir_{FV/FM}$ (PAM) and for copper : Ir_{F2-F1} (LuminoTox) > $Ir_{FO/FM}$ (PEA) = Ir_{FV} (PEA) > $Ir_{FV/FO}$ (PEA) > $Ir_{F1/F2}$ (LuminoTox) > $Ir_{(F2-F1)/F1}$ (LuminoTox) > Ir_{FV} (PAM) > $Ir_{FO/FM}$ (PAM) > $Ir_{FV/FO}$ (PAM) > $Ir_{FV/FM}$ (PEA) > $Ir_{(F2-F1)/F2}$ (LuminoTox). Study of those Ir based on fluorescence parameters may provide useful indication for the practical approach in the evaluation of Atrazine and copper phytotoxicity.

11.3 Introduction

The risk of water quality deterioration is an important problem since pollution of aquatic environment is frequently induced by heavy metals and herbicides coming from different domains of human activities (Manahan, 1997). Therefore, there is a need to use efficient monitoring systems for the evaluation of toxicity risk. For monitoring system used in the detection of pollutant toxicity, it is necessary to demonstrate high sensitivity of measurements.

In the past, one of the most frequently employed herbicide in agriculture practice was Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) used in the control of broadleaf weeds (Solomon *et al.*, 1994). It is known for Atrazine to interact with quinone binding site at the D1 protein of photosystem II (PSII) causing an inhibition of photosynthetic electron transport (Fuerst and Norman, 1991). Copper sulphate is also commonly used as fungicides in agricultural practice to protect fruits and vegetable crops (Extension Toxicology Network, 1996; Rosslenbroich and Stuebler, 2000). For copper effect it was determined to inhibit PSII primary photochemistry and electron transport by interacting at the PSII water-splitting system (Samson *et al.*, 1988; Renger *et al.*, 1993). The site of this interaction was related to the alteration of 17 and 23 KDa proteins of the oxygen-evolving system (Pätsikkä *et al.*, 2001). Therefore, response of PSII to the toxic effect of Atrazine and copper may provide a useful biosensor for detection of toxicity induced by pollutants having similar mechanism of interaction. The use of biosensors based on the photochemical activity of PSII represents a useful approach for the evaluation of pollutants toxicity risk in aquatic ecosystems (Giardi *et al.*, 2001). The use of thylakoid membranes as biosensor containing active PSII may offer advantage by having higher sensitivity and reproducibility of measurements compared to intact plant. Isolated thylakoid membranes, having all electron donors and acceptors functionally linked to PSII photochemistry (Izawa, 1980), may represent sensitive indicator of pollutant inhibitory effect since the pollutants may interact promptly with exposed target sites, therefore provoking a rapid response to toxic effect (Piletskaya *et al.*, 1999). High sensitivity of such bioanalytical device is based on chlorophyll

a fluorescence measurement as indicator of PSII primary photochemistry and associated electron transport activity (Karukstis, 1991).

Thylakoid membranes, as a fluorometric analytical biosensor in the detection of herbicides and heavy metals toxicity, have been considered to be a convenient approach in applied toxicology research (Boucher and Carpentier, 1999; Laberge *et al.*, 2000). Presently, different fluorometric instruments have been used as efficient systems for monitoring PSII photochemistry when the photosynthetic apparatus has been exposed to different environmental effects (Strasser and Strasser, 1995; Juneau and Popovic, 1999; Boucher *et al.*, 2005; Bengtson Nash *et al.*, 2005). In this study, we investigated the sensitivity of fluorescence measurements by using the LuminoTox Analyser (Lab-Bell Inc., Shawinigan, Quebec, Canada), the Pulse Amplitude Modulated (PAM), and the Plant Efficiency Analyser (PEA) (both : Hansatech® Ltd., King's Lynn, Norfolk, UK) when samples of isolated thylakoid membranes were exposed to Atrazine or copper inhibitory effect.

11.4 Material and methods

11.4.1 Biomaterial

Thylakoid membranes were isolated from spinach leaves as described previously (Goetze and Carpentier, 1990). Thylakoid sample was resuspended in 50 mM HEPES-NaOH pH 7.5, 330 mM sorbitol and 2 mM $MgCl_2$. The determination of chlorophyll concentration was done according to Porra *et al.* (1989). For experiments, stock of thylakoid membranes was stabilized and conserved under vacuum condition (Wiggenhorn *et al.*, 2005).

11.4.2 Atrazine and copper stock solutions

Atrazine (Chem Service) stock solution was prepared in methanol at a concentration of 1 mg mL^{-1} and diluted in filtered water (NANOpur II water purification system, Barnstead) to obtain concentrations used for treatments. Copper stock solution, calculated as ionic Cu^{2+}, was prepared as copper sulphate (Aldrich Chemical) at a concentration of 1 mg mL^{-1}.

11.4.3 Instruments used for fluorescence measurements

By using the PAM fluorometer (FMS/2S, Hansatech® Ltd.), F_O fluorescence level was obtained under an analytical light of 1 µmol photons m^{-2} s^{-1} provided by 594 nm amber light emitting diodes (LED). This light was modulated and synchronized with photodiode detector used to measure the basal fluorescence yield, F_O at wavelength over 700 nm. The PSII quantum yield value as $F_V / F_M = (F_M - F_O) / F_M$ was used to determine the intensity of the saturating pulse. Therefore, a short (0.7 sec duration) saturating pulse of white light having 500 µmol photons m^{-2} s^{-1} permitted to measure the maximal fluorescence yield, F_M and evaluate the maximal variable fluorescence yield as $F_V = F_M - F_O$.

The Handy-PEA fluorometer (Hansatech® Ltd.) used three ultra-bright red LED's providing excitation light with a maximum emission at 650 nm (spectral line half width of 22 nm). Fluorescence emission was detected over 700 nm. The maximal variable fluorescence yield was evaluated as $F_V = F_M - F_O$, where F_O was considered to be the fluorescence yield at 50 µs and F_M the maximum fluorescence yield obtained by using an excitation light of 3000 µmol photons m^{-2} s^{-1} during 2 sec according to Strasser and Strasser (1995). For this optical set up, if a lower excitation light energy was used, the maximum PSII quantum yield was not attained (At a light intensity of 500 µmol photons m^{-2} s^{-1}, the PSII quantum yield for not treated sample was only 0.4).

The fluorometer LuminoTox Analyzer (Lab-Bell Inc.) had an excitation light at 470 nm displayed by three LEDs. For fluorescence detection was used a photodiode protected by a filter HB700 (Optima, cut off filter having 5% of transmittance at 680 nm, 25% at 690 nm and 50% at 700 nm). Two levels of fluorescence yield, F_1 and F_2 were determined according to Bellemare *et al.* (2001) in the following sequence : 1- The background level was evaluated at base line of fluorescence emission. 2- A low light intensity (20 µmol photons m^{-2} s^{-1}) was triggered during 2 sec. The fluorescence yield measured under those conditions was subtracted with the base line fluorescence emission, therefore giving F_1 fluorescence. This fluorescence value did not vary for control samples. 3- Following exposure to the low light intensity being interrupted after 2 sec, the sample of thylakoids membrane was promptly

exposed to a flash of 500 µmol photons m^{-2} s^{-1} (0.7 sec duration) triggering the fluorescence yield F_2. This fluorescence yield was also subtracted with the base line of fluorescence emission. Difference between fluorescence yields evaluated as F_2-F_1 was used as an indication for the change of variable fluorescence.

11.4.4 Bioassay procedure

Before being used for treatment the sample of stabilized thylakoid membranes was resuspended in 5 mL to obtain 50 µg mL^{-1} by using reactive buffer containing 50 mM Hepes-NaOH pH 7.5, 330 mM sorbitol, 2 mM $MgCl_2$ and 1 mM NH_4Cl. Preparation and conservation of sample was done at 4°C in darkness. The sample of thylakoid membranes used for treatments contained 5 µg Chl mL^{-1}. Priory to measurement sample was treated at room temperature during 15 min in the dark with Atrazine concentration of 1, 6, 10, 40, 200 and 600 µg L^{-1} or with a copper concentration of 10, 50, 100, 250, 500 and 1000 µg L^{-1}.

By using PAM and PEA, fluorescence parameters were measured according to Rohacek (2002). Following fluorescence parameters were investigated : 1- The maximal variable fluorescence as $F_V = F_M$-F_O; 2- The basal quantum yield of non-photochemical processes in PSII as the ratio F_O/F_M; 3- The ratio between photochemical and concurrent non-photochemical processes within PSII determined as F_V/F_O; 4- The maximum quantum yield measured by the ratio F_V/F_M. For the LuminoTox the evaluation of the parameters F_2-F_1, F_2/F_1, $(F_2$-$F_1)/F_1$ and $(F_2$-$F_1)/F_2$ was based on two fluorescence yields F_1 and F_2 measured under low and high light intensities, respectively (as described in the section Instruments used for fluorescence measurements). When control samples (not treated thylakoids) were used, the measurements of investigated fluorescence parameters by using PAM, PEA and LuminoTox gave large variation if instrumental systems were compared (Table 11.1). The PEA F_V/F_M parameter was evidently smaller compared to PAM F_V/F_M and LuminoTox $(F_2$-$F_1)/F_2$ measurements. The PEA measurement of F_O was evaluated by definition as fluorescence yield at 50µs according to Strasser and Strasser (1995). Under saturating light this yield of fluorescence should contain also a contribution of variable fluorescence for thylakoid membranes. Therefore, overestimation of F_O value may result in decrease of

variable fluorescence F_V evaluation. This discrepancy may logically induce the decrease of the ratio F_V/F_M and F_V/F_O, and an increase of F_O/F_M ratio. In order to determine the sensitivity of the fluorometric systems, we had to investigate the change of the inhibitory response for studied parameters. For each fluorescence parameter, the inhibitory response (Ir) induced by Atrazine or copper treatment was shown as the change in percentage compared to control where $Ir_{parameter} = [parameter^C - parameter^T] / parameter^C$, (C and T represent values from control and treated samples, respectively).

11.4.5 Data analysis and statistics

The experiments were performed at least three times and each thylakoids sample was analyzed in triplicates. The dose-response curves for all fluorescence parameters were obtained from fits of the data by using a four-parameter logistic equation : $y = a_1 e^{-x/a2} + a_3 + a_4 x$. The data were fitted to this logistic response relationship using a non-linear least-square fitting method in software ORIGIN 6.0 (Microcal Software, USA) based on the Levenberg–Marquardt algorithm. The effective concentration for 50% of the inhibitory response (EC-50%) of all fluorescence parameters was also determined from the non-linear least-square fits.

11.5 Results and discussion

In this study we investigated the effect of Atrazine and copper on the fluorescence yields measured by PEA and PAM (F_O and F_M) and by LuminoTox (F_1 and F_2) (Figure 11.1). We found for PAM and PEA F_O fluorescence yields of Atrazine treated thylakoids to be increased by 60 and 25 %, respectively. Under the same conditions, the LuminoTox F_1 fluorescence yield was also increased by 25 %. However, the change of F_1 fluorescence yield induced by copper was very sensitive since F_1 value for 500 $\mu g\ L^{-1}$ of copper was diminished by 60 %. The PEA maximal fluorescence yield, F_M, was not changed by Atrazine while the PAM maximal fluorescence yield, F_M, was decreased only by 10% compared to control. No relationship was found between Atrazine concentration and the change of F_M fluorescence yields. However, under the same conditions, the LuminoTox F_2 fluorescence yield was

strongly decreased by Atrazine effect. This decrease was dependent to Atrazine concentration. The F_2 fluorescence yield induced by high light energy was measured in previously illuminated thylakoids (by 20 μmol photons m^{-2} s^{-1}), therefore having partially reduced pool of Q_B, secondary electron acceptor of PSII. For the decrease of F_2 fluorescence yield we may suppose to be caused by a photoinhibitory effect since PSII electron transport was inhibited by Atrazine. This interpretation is supported by earlier found evidence for photoinhibition to be induced in plant treated with herbicide inhibiting PSII and being exposed to light (Strasser, 1997; El Jay *et al.*, 1997). On the other hand, the decrease of the LuminoTox F_2 fluorescence yield was strongly related to copper concentration and was more sensitive compared to the change of PAM and PEA F_M fluorescence yields. The decrease of LuminoTox F_2, PAM and PEA F_M fluorescence yields was expected since the inhibition of the water-splitting system by copper may increase the photoinhibitory effect (Samson *et al.*, 1988; Hakala *et al.*, 2005). Under the same conditions, pre-illumination treatment used in LuminoTox measurements may further increase the photoinhibitory effect since F_2 value was decreased even by 80%.

The inhibitory response based on variable fluorescence ($Ir_{F2\text{-}F1}$ and Ir_{FV}) was changed differently by the effect of Atrazine and copper when measurements were done with LuminoTox, PAM and PEA systems (Figure 11.2). For LuminoTox $Ir_{F2\text{-}F1}$ it was found to be the most sensitive and the decrease was dependent to Atrazine and copper concentrations if compared to PAM and PEA measurements. However, when PSII acceptor side was gradually inhibited by Atrazine (Figure 11.3), the basal quantum yield of non photochemical processes in PSII (F_O/F_M) measured by PAM and the LuminoTox parameter F_1/F_2 were highly increased as indicated by inhibitory response. On the other hand, F_O/F_M measured by PEA was also increased but at less extent compared to PAM F_O/F_M and LuminoTox F_1/F_2. We may interpret for this increase to be closely related to the decrease of PSII functional electron transport since it was earlier found that the decrease of basal quantum yield of non photochemical processes of PSII was proportional to the decrease of PSII electron transport (Bilger *et al.*, 1987). In the case of Atrazine inhibitory effect, it was shown for $Ir_{F1/F2}$ (LuminoTox) to be the most indicative compared to $Ir_{FO/FM}$ of PAM and PEA. However, for copper effect we did not found evident differences in sensitivity when LuminoTox, PAM and

PEA systems were compared (see again Figure 11.3). In this study we also noticed for the decrease of F_V/F_O value, as the ratio between photochemical and non photochemical processes in PSII, and the value of $(F_2-F_1)/F_1$ to be dependent to Atrazine and copper concentrations as indicated by the change of inhibitory responses (Figure 11.4). We may interpret for the decrease of the ratio between photochemical and non photochemical processes in PSII to be caused by the deterioration of PSII photochemistry according to Babani and Lichtenthaler (1996). For Atrazine effect, the change of Ir value was the most indicative by the LuminoTox measurement ($Ir_{(F2-F1)/F1}$) compared to $Ir_{FV/FO}$ of PAM and PEA. However, for copper effect LuminoTox $Ir_{(F2-F1)/F1}$ was the least sensitive as an indicator (Figure 11.4). We further examined the sensitivity of the inhibitory response which was related to quantum yield of PSII photochemistry. We noticed, when thylakoid membranes have been exposed to Atrazine effect, that LuminoTox $Ir_{(F2-F1)/F2}$ was the most sensitive compared to $Ir_{FV/FM}$ of PAM and PEA. On the other hand, for copper effect $Ir_{FV/FM}$ of PEA was the most sensitive compared to PAM and LuminoTox measurements (Figure 11.5).

By using thylakoid membranes as biosensor, PAM, PEA and LuminoTox fluorescence parameters showed evident differences in sensitivity which were caused by the specific Atrazine or copper inhibitory effect and by different optical set up related to those fluorometric systems. Analysis of the level of effective concentration (EC-50%) for Atrazine and copper inhibition may indicate the level of sensitivity for those parameters. Therefore, investigation of sensitivity order for those parameters may be useful for bioassay toxicity testing. We should indicate that the variation of sensitivity between different parameters evaluated by the same fluorometric system was due to the specificity of Atrazine and copper toxicity mechanism (Popovic *et al.*, 2003). In this study we found for the values of EC-50% of PAM and PEA inhibitory response Ir_{FV}, $Ir_{FO/FM}$, $Ir_{FV/FO}$ and $Ir_{FV/FM}$ and of LuminoTox Ir_{F2-F1}, $Ir_{F1/F2}$, $Ir_{(F2-F1)/F1}$ and $Ir_{(F2-F1)/F2}$ to show a large variation (Table 11.2). The order of EC-50% sensitivity related to the inhibitory response for Atrazine effect was $Ir_{(F2-F1)/F1}$ (LuminoTox) > $Ir_{FO/FM}$ (PAM) > $Ir_{F1/F2}$ (LuminoTox) > Ir_{F2-F1} (LuminoTox) > $Ir_{FV/FO}$ (PAM) > $Ir_{(F2-F1)/F2}$ (LuminoTox) > $Ir_{FV/FO}$ (PEA) > $Ir_{FO/FM}$ (PEA) > $Ir_{FV/FM}$ (PEA) > $Ir_{FV/FM}$ (PAM). For copper effect, the order of sensitivity was Ir_{F2-F1} (LuminoTox) > $Ir_{FO/FM}$ (PEA) = Ir_{FV} (PEA) > $Ir_{FV/FO}$ (PEA) > $Ir_{F1/F2}$ (LuminoTox) > $Ir_{(F2-F1)/F1}$ (LuminoTox) > Ir_{FV} (PAM) > $Ir_{FO/FM}$ (PAM) > $Ir_{FV/FO}$

(PAM) > $Ir_{FV/FM}$ (PEA) > $Ir_{(F2-F1)/F2}$ (LuminoTox). We may conclude that the inhibitory responses of those fluorescence parameters may provide useful indication for the practical approach in the evaluation of Atrazine and copper phytotoxicity.

11.6 Acknowledgments

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11.7 References

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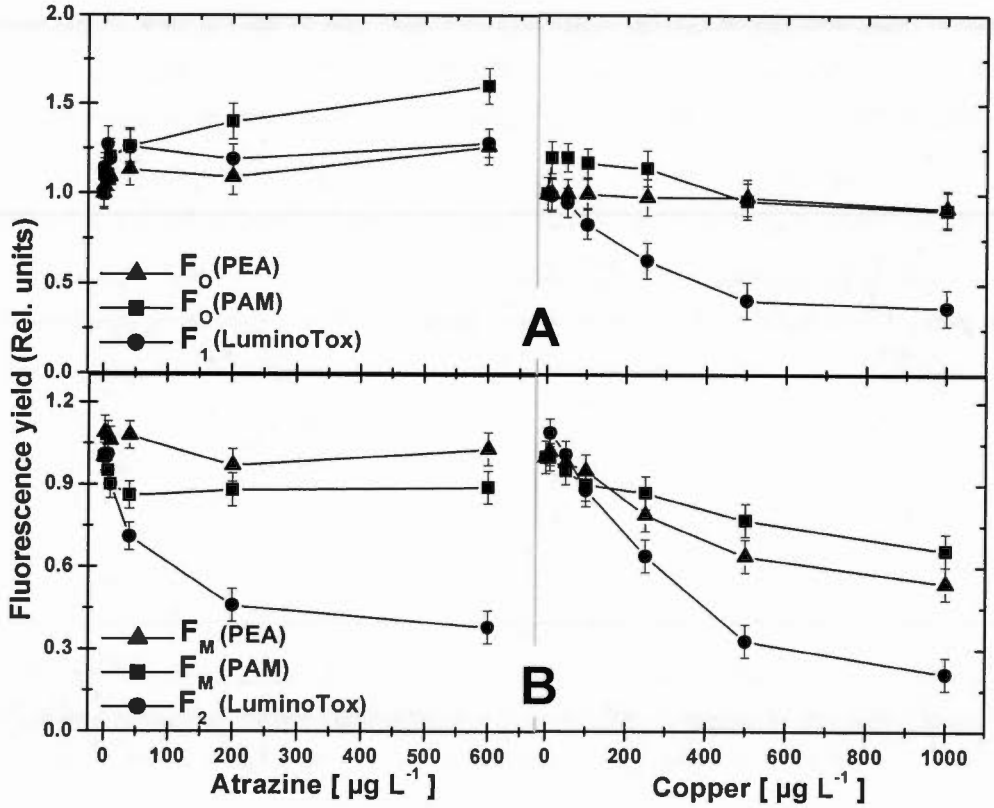

Figure 11.1 Change of fluorescence yields obtained by PAM, PEA and LuminoTox measurements. The fluorescence yield was normalized to control (not treated sample) as value one.

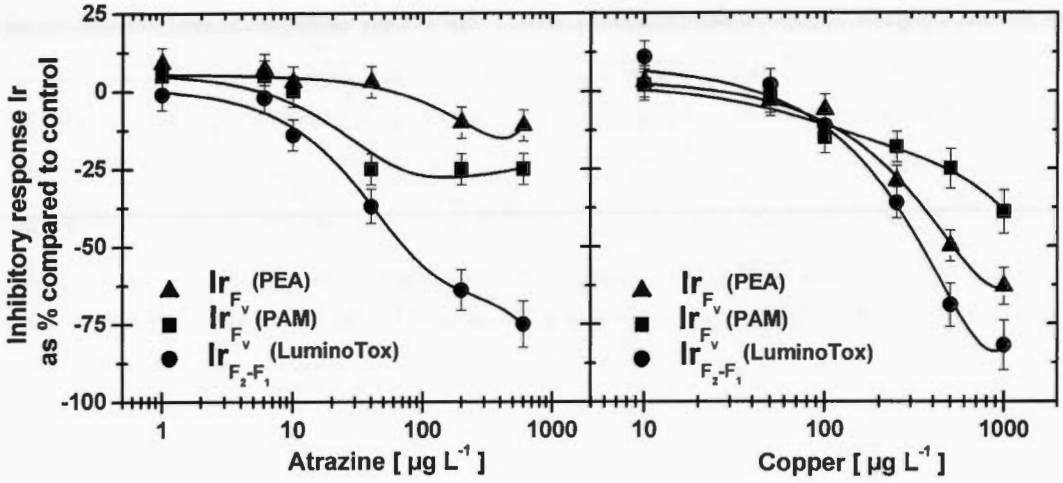

Figure 11.2 Variation of the inhibitory response (Ir) evaluated by PAM, PEA and LuminoTox fluorometers when thylakoid samples were exposed to Atrazine and copper ions effect.

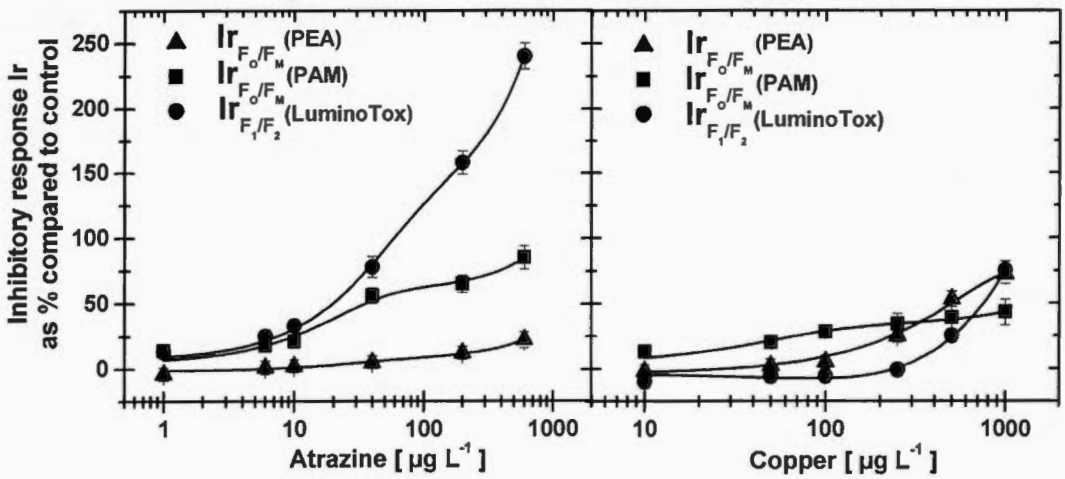

Figure 11.3 Variation of the inhibitory response (Ir) evaluated by PAM, PEA and LuminoTox fluorometers when thylakoid samples were exposed to Atrazine and copper ions effect.

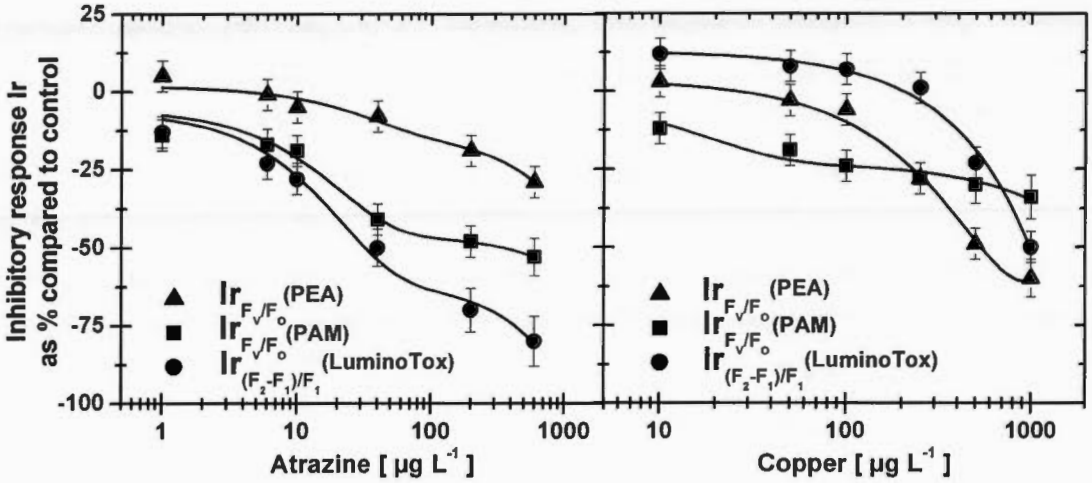

Figure 11.4 Variation of the inhibitory response (Ir) evaluated by PAM, PEA and LuminoTox fluorometers when thylakoid samples were exposed to Atrazine and copper ions effect.

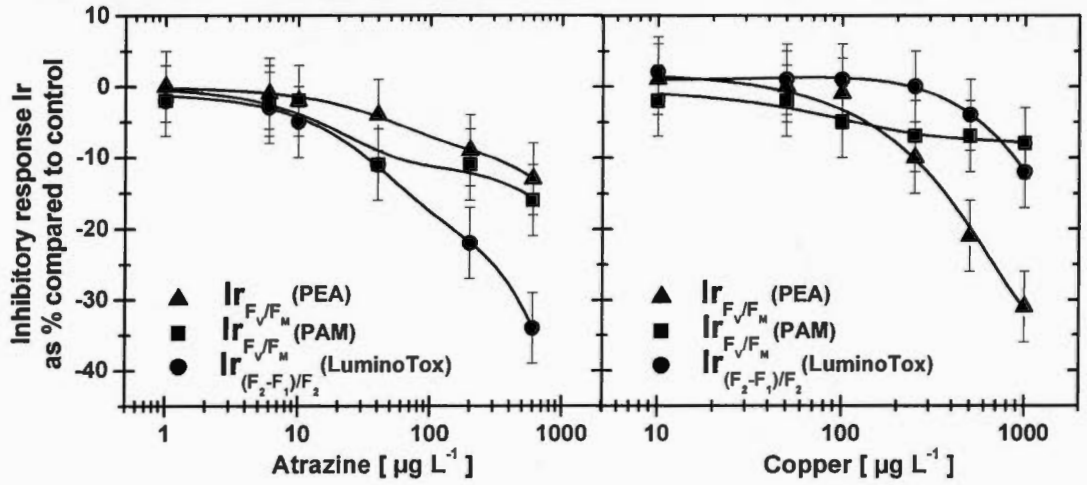

Figure 11.5 Variation of the inhibitory response (Ir) evaluated by PAM, PEA and LuminoTox fluorometers when thylakoid samples were exposed to Atrazine and copper ions effect.

Table 11.1 Response of fluorescence parameters evaluated by PAM, PEA and LuminoTox systems by using not treated thylakoid membranes. Coefficient of variation is presented in %.

	Photosynthetic parameters		
Fluorometers	F_O/F_M	F_V/F_O	F_V/F_M
PAM	**0.16** ± 9%	**5.09** ± 10%	**0.84** ± 2%
PEA	**0.33** ± 9%	**2.07** ± 9%	**0.68** ± 3%
	F_1/F_2	$(F_2-F_1)/F_1$	$(F_2-F_1)/F_2$
LuminoTox	**0.13** ± 7%	**6.71** ± 9%	**0.87** ± 2%

Table 11.2 Atrazine and copper ions concentration inducing 50% of the inhibitory response (EC-50%) based on fluorescence measurements by PAM, PEA and LuminoTox fluorometers.

Fluorometers	EC-50% in µg L^{-1}							
	Ir_{FV}		$Ir_{FO/FM}$		$Ir_{FV/FO}$		$Ir_{FV/FM}$	
	Atrazine	**copper**	**Atrazine**	**copper**	**Atrazine**	**copper**	**Atrazine**	**copper**
PAM	n.r.[a]	1443[b]	36	1519[b]	349	2263[b]	4512[b]	n.r.[a]
PEA	n.r.[a]	515	1669[b]	515	1398[b]	535	4388[b]	2267[b]
	Ir_{F2-F1}		$Ir_{F1/F2}$		$Ir_{(F2-F1)/F1}$		$Ir_{(F2-F1)/F2}$	
	Atrazine	**copper**	**Atrazine**	**copper**	**Atrazine**	**copper**	**Atrazine**	**copper**
LuminoTox	73	349	40	762	32	980	1137[b]	3330[b]

[a]n.r. : No response.

[b]values were extrapolated from the dose-response curves.

CONCLUSIONS

Le travail présenté dans cette thèse contribue à l'avancement des connaissances en abordant deux principales problématiques qui concernent : 1- Les effets toxiques des xénobiotiques sur le fonctionnement de l'appareil photosynthétique; 2- Les effets des xénobiotiques sur le changement des paramètres photosynthétiques utilisés comme biomarqueurs de toxicité cellulaire. Dans la première partie, les résultats ont contribué à l'interprétation des mécanismes moléculaires de l'induction et de la régulation du transport des électrons photosynthétiques par l'analyse du changement spectral et du rendement de la cinétique de fluorescence. Dans la deuxième partie, les résultats obtenus ont permis la détermination de la sensibilité des paramètres photosynthétiques de fluorescence lorsque des plantes ou des algues ont été exposées à des contaminants métalliques ou organiques. Cette variation dans la sensibilité des paramètres informe sur leur applicabilité comme biomarqueur de toxicité cellulaire.

Le photosystème II et le photosystème I contribuent ensemble au transport transmembranaire des électrons qui permet la réduction du $NADP^+$ et la formation du gradient de proton induisant la synthèse d'ATP. Par notre étude du changement spectral de fluorescence pendant «l'effet Kautsky», nous avons conclu que le changement de la distribution de l'énergie d'excitation entre le PSII et le PSI était affecté par la dissipation d'énergie *via* les voies non photochimiques. Notre conclusion envisage que ce phénomène doit être considéré dans l'avenir concernant l'utilisation de la fluorescence chlorophyllienne dans l'étude des mécanismes d'inhibition induits par les xénobiotiques sur la régulation du transfert d'énergie entre le PSII et le PSI.

Sur la base de l'analyse de la cinétique rapide de fluorescence caractérisée par les transitions O-J-I-D-M, nous avons conclu que l'hydroxylamine, comme modulateur exogène, peut inhiber (dépendant de sa concentration) complètement le système enzymatique du dégagement d'oxygène et servir comme un donneur d'électrons. Nous avons retrouvé que son efficacité comme donneur d'électrons n'a pas été suffisante pour maintenir la plastoquinone à l'état réduit. Dans ces conditions, nous avons détecté un important effet «*quenching*» de la

fluorescence variable. De plus, l'apparition de la transition K a été déterminée comme la conséquence de l'inhibition du dégagement d'oxygène et l'inactivation du centre réactionnel du PSII. Cependant, l'utilisation de la duroquinone et du méthyl viologène comme accepteurs exogènes d'électrons nous a permis de déterminer que l'effet «*quenching*» sur toutes les transitions de la cinétique rapide de fluorescence était la conséquence d'une accélération du transport des électrons. De plus, nous avons conclu que l'utilisation du méthyl viologène, comme herbicide (paraquat), causait une forte compétition avec le $NADP^+$, l'accepteur terminal des électrons. Dans ces conditions, la photosynthèse était détériorée par l'inhibition de la formation du NADPH qui est nécessaire pour la fixation et la réduction du CO_2.

Nous avons également retrouvé que le méthanol, comme modulateur exogène, induisait deux effets différents : un effet stimulateur et un effet inhibiteur qui dépend de la concentration de méthanol. Concernant l'effet stimulateur à des concentrations inférieures ou égales à 0,2 % de méthanol, notre recherche supporte l'interprétation que le méthanol peut être une source de carbone *via* son processus de dégradation et, par conséquent, il va stimuler les processus de fixation du CO_2 dans la photosynthèse et le métabolisme des sucres. Cette interprétation a été confirmée par la découverte d'une très forte stimulation de la croissance de la biomasse par le méthanol. Cependant, une concentration de méthanol qui dépasse 0,5 % inhibe fortement les réactions photochimiques, le transport des électrons et les processus de régulation de la dissipation d'énergie *via* les voies non photochimiques. Dans ces conditions, nous avons retrouvé que le nombre de centres réactionnels actifs était diminué induisant une inhibition du transport linéaire des électrons, de la fixation du CO_2 et de la formation de la biomasse.

Nous avons également confirmé que le site d'inhibition de l'isoproturon était au niveau du PSII. Le mode d'action toxique de l'isoproturon s'est manifesté en fonction de sa concentration selon deux mécanismes : À faible concentration d'isoproturon (7-30 $\mu g\ L^{-1}$), l'inhibition du transport des électrons est induite au niveau de Q_A, l'accepteur primaire du PSII, et à plus forte concentration (125-500 $\mu g\ L^{-1}$), l'inhibition du côté donneur du PSII (centre réactionnel). Ce dernier mode d'inhibition a causé une diminution du nombre de centres réactionnels du PSII apte à effectuer la réaction photochimique primaire. Nous avons

aussi démontré que les ions cuivre, comme un inhibiteur du transport des électrons du PSII, induit un stress oxydatif qui altère la réaction photochimique primaire. L'oxyfluorfène induit également un très fort stress oxydatif. Nos résultats confirment que cet herbicide produit une accumulation de protoporphyrine IX qui est responsable de la formation de superoxide. Nous avons déterminé que l'inhibition du fonctionnement de l'appareil photosynthétique était une conséquence de l'altération de la synthèse des pigments et de leur association avec les protéines membranaires. Nous avons conclu que l'inhibition de la croissance cellulaire était causée par l'altération de l'activité photosynthétique et l'induction du stress oxydatif. D'autre part, nous avons conclu que le fludioxonyl induisait un fort stress oxydatif dans le cytosol cellulaire causant une inhibition de la croissance cellulaire sans altérer la réaction photochimique primaire du PSII.

Dans notre étude, nous avons conclu que les paramètres de fluorescence utilisés comme biomarqueurs de l'activité photosynthétique ne montraient pas la même sensibilité en présence des différents xénobiotiques. La sensibilité des paramètres de fluorescence a été dépendante du site d'interaction du xénobiotique avec le transport transmembranaire des électrons et de sa concentration. L'ordre de sensibilité des paramètres a donc été déterminé par le mode d'action toxique du xénobiotique à l'échelle moléculaire. Parmis les biomarqueurs de fluorescence étudiés, les plus sensibles étaient : Le ratio entre la proportion des antennes collectrices par centre réactionnel du PSII (ABS/RC), l'efficacité photochimique opérationnel du PSII (Φ'_M), le «*quenching*» non photochimique (NPQ ou Q_N) et le «*quenching*» photochimique (Q_P). Par conséquent, nous avons pu constater que ces paramètres de fluorescence représentaient des biomarqueurs caractérisés par une grande sensibilité et fiabilité dans l'évaluation de la toxicité cellulaire.

AUTRES CONTRIBUTIONS

Dewez, D., P. Le Lay, D. Kovacevic, and R. Popovic. 2006. "Evidence of an acclimation process to drought-stress in barley plants at early stage of development : Chlorophyll-protein complexes formation and electron transport activity of photosystem II". *Physiologiae Plantarum* (soumis).

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