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Rôle du métabolisme carboné dans la modulation de l'activité de la source et du puits chez l'érythrone d'Amérique (*Erythronium americanum*)

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Résumé

Les relations entre l'activité de la source et l'activité du puits contrôlent en grande partie la croissance des plantes. Ces activités varient au cours du développement, mais aussi en réponse à des changements des conditions environnementales. Notre étude avait pour but d'identifier le rôle du métabolisme carboné dans la réponse de la croissance d'*Erythronium americanum* à la modulation des activités de la source et du puits. Dans une première partie, l'activité du puits est modulée par la température de croissance. Aux fortes températures, l'activité du puits est plus élevée, alors que sa capacité est réduite. Ces effets, dus à la modulation du métabolisme du saccharose, mènent à une saturation précoce en amidon des cellules du bulbe à forte température. Par la suite, la baisse de la demande en carbone du puits induit un rétrocontrôle négatif de l'activité photosynthétique et finalement, la sénescence foliaire. À l'inverse, l'activité du puits à plus basse température est en rythme avec l'accroissement de la capacité du puits, menant à une biomasse supérieure du bulbe en fin de croissance épigée. Dans une seconde partie, l'activité de la source est modulée en changeant la concentration en CO_2 et en O_3. Malgré la stimulation de la source sous fort CO_2 et son inhibition sous fort O_3, l'accumulation d'amidon et la biomasse du bulbe ne sont pas affectées. En effet, le surplus de carbone parvenant au puits est brûlé par la voie alternative de la respiration, celle-ci étant stimulée par l'activité de l'enzyme malique. La voie alternative de la respiration évite ainsi une saturation hâtive en amidon du bulbe et éventuellement, une sénescence foliaire précoce. Dans une dernière partie, l'activité de la source est modulée par l'irradiance et la photopériode. L'accumulation d'amidon varie en fonction de la photopériode alors que l'irradiance n'a aucun effet. De plus, l'activité photosynthétique est inhibée très précocement sous longue photopériode. Cette inhibition semble due à un déséquilibre entre la quantité totale de carbone fixé par jour et son utilisation suite à son transfert au sein du bulbe. Nous pouvons donc conclure que les régulations du métabolisme carboné permettent d'ajuster l'activité du puits à sa capacité chez l'*Erythronium americanum.*

Mots-clés: amidon, bulbe, *Erythronium americanum*, métabolisme carboné, relations source-puits.

Impact of carbon metabolism in the modulation of the source and sink activities in *Erythronium americanum*.

Abstract

Relationships between source and sink activities largely control the growth of plants. Both activities vary during development as well as in response to changes in environmental conditions. The aim of our study was to identify the role carbon metabolism plays in the response of *Erythronium americanum* growth to changes in source and sink activities. Firstly, sink activity is modulated by changing growth temperature. Sink activity is higher at higher temperatures, whereas sink capacity is more restricted. These effects, due to the modulation of sucrose metabolism, lead to an earlier starch saturation of bulb cells at higher temperature. Thereafter, the reduction in carbon sink demand induces a feedback inhibition of photosynthetic activity and finally, leaf senescence. In contrast, sink activity at low temperature is more in rhythm with the increasing sink capacity, leading to larger bulbs at the end of the epigeous growth season. Secondly, source activity is modulated by changing CO_2 and O_3 concentrations. Despite a stimulation of the source activity under high CO_2 and its inhibition under high O_3, neither plant growth nor starch accumulation was affected. Indeed, excess carbon translocated within the sink is burned by the alternative respiratory pathway. This pathway is stimulated by malic enzyme. Alternative respiratory pathway thus avoids an early starch saturation of the bulb and eventually, an early leaf senescence. Finally, source activity is also modulated by changing irradiance and photoperiod regimes. Starch accumulation changes in response to photoperiod but not to irradiance. Furthermore, photosynthetic activity is inhibited early in the season under long photoperiod. This inhibition seems due to an imbalance between the total amount of carbon fixed per day and its utilisation following translocation to the bulb. We can thus conclude that regulation of carbon metabolism allow to adjust sink activity to sink capacity in *Erythronium americanum*.

Key words: bulb, *Erythronium americanum*, carbon metabolism, source-sink relationships, starch.

Avant-propos

Cette thèse est présentée sous la forme d'articles en vue de publication dans des revues scientifiques (Chapitres 2 à 4) et comporte, en outre, une introduction (Chapitre 1) et une conclusion générale (Chapitre 5). Les contributions respectives sont détaillées, ci-après :

Chapitre 2. Anthony Gandin, Sylvain Gutjahr, Pierre Dizengremel et Line Lapointe sont les co-auteurs de cet article. J'ai réalisé l'ensemble des travaux et des analyses statistiques menant aux résultats présentés dans ce chapitre. J'ai notamment mis au point plusieurs protocoles de mesures d'activités enzymatiques. Ces mesures enzymatiques ont été faites grâce au lecteur de microplaque généreusement prêté par Helga Guderley. Sylvain Gutjahr a effectué les expériences préliminaires à l'origine de ce projet. J'ai effectué la rédaction de cet article sous la supervision de ma directrice de recherche Dr. Line Lapointe et de mon directeur de recherche Dr. Pierre Dizengremel.

Chapitre 3. Anthony Gandin, Line Lapointe et Pierre Dizengremel sont les co-auteurs de cet article. J'ai réalisé l'ensemble des travaux et des analyses statistiques menant aux résultats présentés dans ce chapitre. J'ai effectué la rédaction de l'article sous la supervision de ma directrice de recherche Dr. Line Lapointe et de mon directeur de recherche Dr. Pierre Dizengremel. L'article a été soumis le 28 avril 2009 et accepté le 5 août 2009 dans la revue *Journal of Experimental Botany* (vol. 60(15) : 4235-4248).

Chapitre 4. Anthony Gandin, Pierre Dizengremel et Line Lapointe sont les co-auteurs de cet article. J'ai réalisé l'ensemble des travaux et des analyses statistiques menant aux résultats présentés dans ce chapitre. J'ai effectué la rédaction de l'article sous la supervision de ma directrice de recherche Dr. Line Lapointe et de mon directeur de recherche Dr. Pierre Dizengremel.

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"Toute avancée des connaissances génère autant d'interrogations qu'elle apporte de réponses"
Pierre Joliot

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Liste des abréviations

ADP	adénosine diphosphate
AGPase	ADP-glucose pyrophosphorylase
ANOVA	analyse de variance
ATP	adénosine triphosphate
AOX	alternative oxydase
C	carbone
Ci	concentration intercellulaire de CO_2
CWinv	invertase pariétale
DW	masse sèche (dry weight)
F1,6BPase	fructose-1,6-bisphosphatase
F2,6BP	fructose-2,6-bisphosphate
F6P	fructose-6-phosphate
FAA	formaldéhyde-acide acétique
$FADH_2$	flavine adénine dinucleotide
Fv/Fm	rendement quantique maximale de la photochimie des centres PSII
Fv'/Fm'	rendement quantique de la photochimie des centres ouverts
FW	masse fraîche (fresh weight)
G1P	glucose-1-phosphate
G3P	glycéraldehyde-3-phosphate
Gs	conductance stomatique
HI	forte irradiance
J_{max}	flux d'électron maximal
J_T	flux d'électron total
KCN	cyanure de potassium
LI	faible irradiance
LP	longue photopériode
NADH	nicotinamide adénine dinucléotide, forme réduite
NADPH	nicotinamide adénine dinucléotide phosphate, forme réduite
Ninv	invertase cytoplasmique (neutre)
NPQ	quenching non-photochimique
P	participation de la voie alternative de la respiration
PEPc	phosphoenolpyruvate carboxylase
PhiPS2	rendement quantique du PSII
Pi	phosphate inorganique
PPFD	densité de flux de photons dans le PAR
Pn	taux de photosynthèse net
PR	photorespiration
qN	quenching non-photochimique
qP	quenching photochimique
Rd	respiration à la noirceur
RH	humidité relative
ROS	espèces réactives de l'oxygène
SHAM	acide salicyl- hydroxamique
SP	courte photopériode
SPS	saccharose-phosphate-synthase
Susy	saccharose synthase
T3P	triose phosphate

TPU	utilisation des trioses phosphates
UDP	uridine diphosphate
UTP	uridine triphosphate
v_{alt}	activité de la voie alternative de la respiration
V_{alt}	capacité de la voie alternative de la respiration
Vc_{max}	vitesse de carboxylation maximale
v_{cyt}	activité de la voie cytochromique de la respiration
V_{cyt}	capacité de la voie alternative cytochromique de la respiration
Vinv	invertase vacuolaire
VPD	déficit de pression de vapeur
v_{res}	activité résiduelle de la respiration
V_T	activité totale de la respiration
ρ'	engagement de la voie alternative de la respiration

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Chapitre 1 Introduction

1.1 Les relations source-puits

Les différentes parties d'une plante se divisent en deux catégories distinctes, les organes sources d'une part et les organes puits d'autre part. On associe généralement aux sources les feuilles matures, alors qu'on associe aux puits les feuilles en expansion, les racines, les pièces florales, les fruits et bien entendu, les organes de réserve (bulbe, corme, rhizome et tubercule). Certains organes peuvent alterner d'une fonction de puits à une fonction de source (Turgeon, 1989) et inversement. Le sens dans lequel s'effectue le transport des photoassimilats permet de différencier les organes sources des organes puits (Dickson, 1991). On définit ainsi un organe source lorsque qu'il produit plus de photoassimilats qu'il n'en consomme pour sa croissance et sa maintenance. À l'inverse, un organe qui présente une consommation de photoassimilats nettement supérieure à sa production, est appelé puits. Le puits se caractérise donc par une importation nette de photoassimilats.

D'autre part, l'allocation des ressources entre différents puits en compétition est proportionnelle à la force de chacun des puits. Cette caractéristique représente l'aptitude du puits à importer les photoassimilats (Marcelis, 1996). La force d'un puits se définit selon sa vitesse d'intégration des photoassimilats, nommée l'activité du puits et selon sa capacité maximale à stocker ces photoassimilats, nommée la capacité du puits (Farrar, 1993). L'activité du puits est principalement liée à l'entretien des tissus et à l'incorporation des ressources. La capacité du puits est, quant à elle, déterminée par le nombre de cellules et leur taille. Le transfert des photoassimilats depuis les organes sources vers les organes puits définit, à l'échelle de la plante entière, le patron d'allocation des ressources.

De nombreux facteurs environnementaux sont susceptibles de moduler la force des sources ou celle des puits, modifiant alors le patron d'allocation. Parmi ceux-ci, la concentration en gaz atmosphériques, tels que le dioxyde de carbone et l'ozone, ou encore la lumière constituent des facteurs particulièrement pertinents pour moduler les sources. En effet, une augmentation de la concentration en CO_2 stimule fortement l'activité de la source, en favorisant l'activité carboxylase de la rubisco et donc l'assimilation carbonée (Ainsworth et Long, 2005). À l'inverse, l'ozone inhibe l'activité de la source, en altérant la synthèse des deux sous-unités de la rubisco (Andersen,

2003). Finalement, l'ozone semble aussi contrecarrer les effets positifs d'une forte concentration en CO_2 sur l'activité de la source (Balaguer *et al.*, 1995). De manière similaire, la lumière en apportant l'énergie nécessaire au fonctionnement de la chaîne photosynthétique de transport des électrons, influence fortement l'activité de la source (Kehr *et al.*, 1998). À l'opposé, la température constitue un des facteurs majeurs pour moduler la force des puits. La température affecte ainsi le Q_{10} des réactions telles que celles de la respiration cellulaire, l'apport énergétique permettant une synthèse biologique accrue, ainsi que le cycle cellulaire, en amplifiant le taux de division cellulaire. Suite à une modification des conditions environnementales, un déséquilibre entre les sources et les puits peut alors survenir et affecter le patron d'allocation des ressources et la croissance de la plante. Néanmoins, en cas de déséquilibre, plusieurs mécanismes du métabolisme carboné permettent de rétablir l'équilibre dans les relations source-puits.

1.2 Les régulations du métabolisme carboné au sein de la source

Au sein des organes sources, plusieurs mécanismes permettant de coordonner l'assimilation et la synthèse d'hydrates de carbone avec leur utilisation ont été mis en évidence. Parmi ceux-ci, le fructose-2,6-bisphosphate (F2,6BP) joue un rôle prépondérant en coordonnant l'exportation de trioses phosphates (T3P) et la synthèse de saccharose (Nielsen *et al.*, 2004). La synthèse et la lyse de ce composé sont catalysées par une seule enzyme bifonctionnelle du cytosol : la fructose-6-phosphate 2-kinase-fructose-2,6-bisphosphate phosphatase. L'activité fructose-6-P 2-kinase de cette enzyme catalyse la phosphorylation du fructose-6-phosphate (F6P) cytosolique à l'aide d'ATP et synthétise ainsi le F2,6BP (Fig. 1.1). À l'inverse, la lyse de ce composé est catalysée par l'activité fructose-2,6-bisphospate phosphatase et produit de nouveau du F6P ainsi que du phosphate inorganique (Pi). L'accumulation temporaire de F2,6BP induit donc un coût énergétique non négligeable pour la cellule, qui ne peut être restitué lors de la lyse de ce composé. La synthèse de F2,6BP par cette enzyme bifonctionnelle est stimulée par la présence de phosphate inorganique et de F6P. À l'opposé, la lyse est stimulée par la présence de glycéraldéhyde-3-phosphate (G3P) et dihydroxyacetone-phosphate (T3P). Lorsque l'activité de la source est stimulée, le rapport G3P/Pi augmente ce qui a donc pour effet d'inhiber l'activité fructose-6-P 2-kinase et de réduire l'accumulation de

F2,6BP. Or, le F2,6BP est un inhibiteur de la fructose-1,6-bisphosphatase cytosolique (F1,6BPase), dont l'activité, au sein des organes sources, est fortement corrélée à celle de la saccharose-phosphate-synthase (SPS) et alimente directement cette dernière en F6P (Stitt *et al.*, 1983). Ainsi, une baisse de la teneur en F2,6BP, due à une stimulation de l'activité de la source, stimule la synthèse de saccharose susceptible d'être exporté vers le puits.

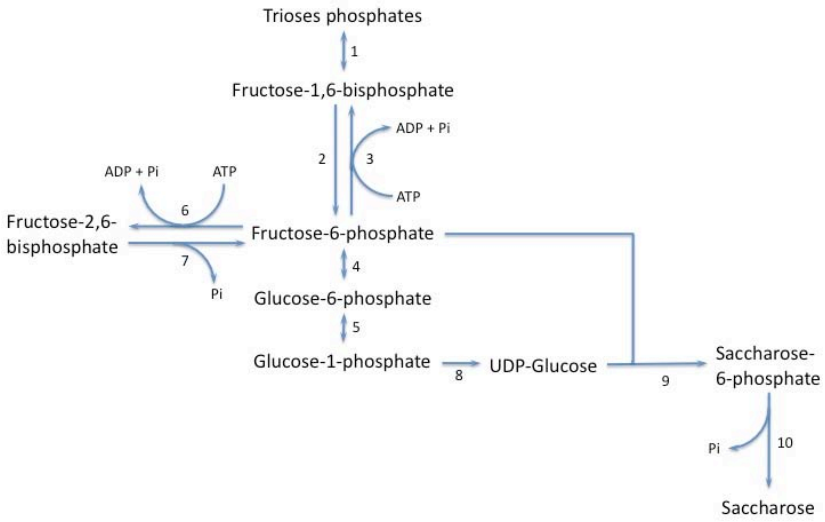

Figure 1.1 Voie métabolique de la synthèse du saccharose à partir de trioses phosphates. 1. Fructose-1,6-bisphosphate aldolase 2. Fructose-1,6-bisphosphatase, 3. Fructose-6-phosphate 1 kinase, 4. Isomerase, 5. Phosphoglucomutase, 6. Fructose-6-phosphate 2-kinase, 7. Fructose-2,6-bisphosphate phosphatase, 8. UDP-glucose pyrophosphorylase, 9. Saccharose-phosphate-synthase, 10. Saccharose-6-phosphate phosphatase.

En outre, les activités de la F1,6BPase et de la saccharose-6-phosphate phosphatase libèrent du Pi dans le cytosol des organes sources. Or, l'exportation, du chloroplaste vers le cytosol, des T3P produits par le cycle de Calvin est due à un complexe protéique, permettant un transport antiport T3P/Pi (Weber *et al.*, 2005). Ce transporteur permet ainsi le retour, dans le chloroplaste, de Pi libéré lors de la synthèse de saccharose et ce, afin d'alimenter la synthèse d'ATP et d'autres métabolites phosphorylés. Ainsi, si la demande en hydrates de carbone du puits diminue, la synthèse et le transfert de saccharose s'en voient affectées, réduisant la libération de Pi dans le cytosol et donc, l'exportation de T3P depuis le chloroplaste (Stitt *et al.*, 1988). L'utilisation des trioses phosphates (TPU) est un paramètre quantifiable à l'aide de courbes de réponse au CO_2

(courbe A-Ci, Fig. 1.2). Ces courbes permettent aussi de quantifier la vitesse de carboxylation maximale (Vc_{max}) et le flux d'électron maximal (J_{max}). Lorsque l'exportation de trioses phosphates diminue, la synthèse et l'accumulation d'amidon dans le chloroplaste sont alors induites, pouvant inhiber éventuellement l'assimilation carbonée par le cycle de Calvin.

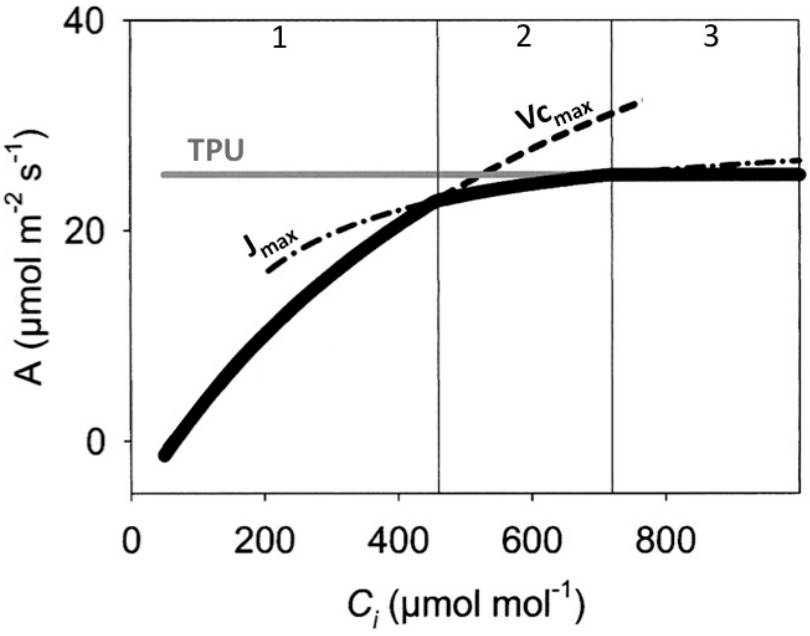

Figure 1.2 Représentation schématique d'une courbe de réponse au CO_2 (courbe A-Ci). Le taux de photosynthèse est limité par l'activité de la rubisco (1), le renouvellement du ribulose-1,5-bisphosphate (2) ou l'utilisation des trioses phosphates et permet de calculer respectivement, la vitesse de carboxylation maximale de la rubisco (Vc_{max}), le flux d'électrons total (J_{max}) et l'exportation des trioses phosphates vers le cytosol (TPU). D'après Long et Bernacchi (2003).

La régulation précedemment décrite permet, en cas de déséquilibre entre la fixation de carbone et la synthèse de saccharose, d'utiliser la synthèse d'amidon au sein du chloroplaste comme un processus "d'overflow". Plusieurs études récentes suggèrent aussi une régulation rédox de l'ADP-glucose pyrophosphorylase (AGPase). Cette enzyme alimente l'amidon synthase en ADP-glucose, constituant ainsi la voie d'entrée de la synthèse d'amidon au sein du chloroplaste. L'AGPase est un hétérotétramère formé de deux sous-unités régulantes et de deux sous-unités catalytiques (Ballicora *et al*.,

2004). La réduction du pont disulfure entre les deux sous-unités catalytiques induit une forte augmentation de l'activité de l'AGPase (Tiessen *et al.*, 2002). Cette réduction peut être modulée par le complexe ferrédoxine-thiorédoxine ou encore, être dépendante de la teneur en pouvoir réducteur (NADPH/NADP). Cette régulation post-transcriptionnelle de l'AGPase permet d'ajuster finement la synthèse d'amidon en fonction de l'assimilation carbonée et de la synthèse de saccharose susceptible d'être exporté vers le puits.

L'accumulation d'hydrates de carbone sous forme d'amidon permet d'éviter, de manière temporaire, une inhibition rapide de la fixation de carbone due à une baisse de la synthèse de saccharose. Toutefois, si le débalancement s'avère trop important ou récurrent, l'activité photosynthétique est inhibée. Certaines espèces, telle que l'érythrone d'Amérique, ne présentent pas d'accumulation d'amidon dans leurs feuilles. Ainsi, ces espèces et certains mutants "starchless" trouvent une alternative possible dans l'accumulation de saccharose ou d'hexose dans la vacuole. Néanmoins, pour ce type de plante, l'étude de la régulation de la photosynthèse par les photoassimilats demeure encore rare dans la littérature.

Différents mécanismes se mettent en place pour maintenir avec précision un équilibre entre la phase biochimique, qui permet l'assimilation du carbone et la phase photochimique, qui assure l'apport énergétique lors du processus photosynthétique. En effet, l'énergie transmise des photons aux molécules de chlorophylle excitées peut être transmise à la chaîne de transport des électrons, contribuant à la synthèse d'énergie chimique (ATP, NADPH) ou encore dissipée sous forme de chaleur ou de fluorescence. En fonction de la quantité de fluorescence émise et de l'apport énergétique initial, on peut mesurer la part d'énergie utilisée pour l'assimilation carbonée. On nomme cette part, le quenching photochimique (qP), par opposition au quenching non-photochimique (qN ou NPQ). Le quenching non-photochimique est représentatif de l'énergie dissipée et donc non utile à l'assimilation carbonée. Cette perte d'énergie se manifeste particulièrement lors d'un rétro-contrôle négatif de la phase biochimique de la photosynthèse, menant généralement à une baisse de la vitesse de carboxylation de la rubisco. Un déséquilibre de l'assimilation carbonée affecte alors le rapport entre l'énergie chimique synthétisée et l'énergie dissipée, modulant par la même le qP et qN. L'activité de la phase photochimique est, de ce fait, modulée en fonction des besoins en

énergie chimique de la phase biochimique. La perte énergétique évite ainsi une surréduction de la chaîne de transport des électrons, phénomène qui induit la formation de composés superoxydes par photoréduction de l'oxygène (Apel et Hirt, 2004). Ces composés, tels que les radicaux libres, possèdent des propriétés cytotoxiques, de par leur forte capacité oxydative, affectant les protéines, l'ADN ou encore les lipides. Dans une certaine mesure, certaines activités détoxifiantes, telles que l'activité de la superoxyde dismutase, permettent alors au chloroplaste de résister à cette attaque oxydative. Ainsi, au sein des organes sources, plusieurs mécanismes permettent de moduler la synthèse et le transfert d'hydrates de carbone en fonction de l'activité de la source et inversement, de moduler l'activité de la source en fonction de la demande du puits. Cependant les mécanismes physiologiques permettant de réduire un déséquilibre entre sources et puits demeurent peu documentés au sein des organes puits.

1.3 Le rôle prédominant du métabolisme du saccharose dans le puits

Le métabolisme du saccharose constitue la voie d'entrée du métabolisme carboné, pour les hydrates de carbone transférés au sein de l'organe de réserve. En effet, le saccharose, formé d'une molécule de glucose et d'une molécule de fructose, constitue la principale forme d'hydrates de carbone transportés par le phloème, de la source vers le puits. Cependant il est à noté que certaines espèces transportent plutôt leshydrates de carbone sous forme de maltose ou encore de sorbitol. L'hydrolyse du saccharose est catalysée par les invertases ou par la saccharose synthase (SuSy). L'activité des invertases génère du glucose et du fructose, alors que l'activité de la SuSy génère du fructose et de l'UDP-glucose (Winter et Huber, 2000). Ces enzymes jouent, de par leurs activités, un rôle crucial dans l'allocation des ressources carbonées, mais aussi, dans l'induction de signaux hexose-dépendants (Wobus et Weber, 1999).

La Susy contribue à la formation de la paroi cellulaire, une association entre cette enzyme et les rosettes du complexe membranaire de la cellulose synthase ayant clairement été mise en évidence (Amor *et al.*, 1995). Cette association permettrait d'approvisionner directement la cellulose synthase en UDP-glucose. Plusieurs mutants déficients en Susy présentent ainsi une baisse de la formation de la paroi cellulaire, caractérisée par une baisse du contenu en cellulose (Tang *et al.*, 1999). D'autre part, il a

aussi été établi que la stimulation de l'activité de la SuSy et l'UDP-glucose produit par cette enzyme sont impliqués dans la stimulation de la synthèse d'amidon au sein des organes de réserve. Deux isoformes de la Susy sont connues dans le maïs (Matic *et al.*, 2004). La première isoforme (SS1) semble contribuer préférentiellement à la synthèse d'amidon, notamment au sein de l'albumen. La seconde (SS2) semble contribuer, quant à elle, à alimenter la glycolyse ou la synthèse pariétale en substrat carboné. Koch *et al.* (1996) suggèrent que la SS1 est inhibée lorsque la teneur en fructose augmente alors que la SS2 est stimulée dans ces conditions. La SuSy influence ainsi l'allocation des ressources carbonées entre d'une part la synthèse de polysaccharides dans les amyloplastes et d'autre part la synthèse polysaccharides dans la paroi cellulaire et l'approvisionnement en substrat de la respiration cellulaire. Des isoformes similaires ont été montrées chez la pomme de terre (Fu et Park, 1995) et la carotte (Sturm *et al.*, 1999). Au sein des organes de réserves, la SuSy est une des rares enzymes dont l'expression est stimulée sous faible concentration d'oxygène (Zeng *et al.*, 1999). En condition anaérobie, cette enzyme peut alors soutenir une synthèse accrue de cellulose ou de callose et ce, d'autant plus que l'activité des invertases est affectée sous ces mêmes conditions.

Les invertases présentent une compartimentation spatiale de leurs activités. Une invertase fonctionne à pH neutre au sein du cytosol et deux à pH acide, soit une au sein de la paroi cellulaire et une dans la vacuole. L'invertase pariétale (CWinv) intervient lorsqu'une partie du transport entre le phloème et les cellules du puits s'effectue de manière apoplastique. Cette contribution s'avère particulièrement importante au cours de la croissance de l'organe de réserve (Sturm et Tang, 1999), au cours du développement de graines (Weschke *et al.*, 2003) ou encore, en réponse à un stress (Herbers et Sonnewald, 1998). D'autre part, l'invertase vacuolaire (Vinv) contribue préférentiellement à la régulation de la turgescence cellulaire et à l'équilibre des concentrations en sucres au sein de puits forts (Sturm, 1999). En effet, l'activité de Vinv double la quantité de solutés osmotiquement actifs et donc, nécessaires à l'expansion cellulaire. Vinv permet ainsi de moduler le taux d'expansion cellulaire en fonction de la quantité d'hydrates de carbone disponibles. Cette enzyme participe de manière évidente à l'importation d'hydrates de carbone et aux signaux cellulaires et ce, particulièrement au cours de la phase de croissance des puits. Par ailleurs, l'invertase cytoplasmique (ou

neutre, Ninv) demeure la plus méconnue tant par ses fonctions que par ses régulations. Elle a souvent été considérée comme une enzyme assurant la maintenance lorsque les activités de la SuSy et de Vinv étaient réduites. Néanmoins, elle est présente dans différents tissus tels que les cotylédons, les racines de *Chichorium* et la racine pivotante de la carotte (Winter et Huber, 2000).

Il semble que l'équilibre entre les activités de la SuSy et des invertases soit impliqué dans le contrôle du développement de la plante. Les activités de ces enzymes seraient elles-mêmes modulées par l'abondance relative des différents sucres solubles. Les invertases contribueraient préférentiellement lors de l'initiation et la croissance du puits alors que la SuSy contribuerait préférentiellement lors des phases de stockage et de maturation du puits (Koch, 2004). Cette transition semble répondre à des changements du rapport hexose/saccharose. Les hexoses produits notamment par l'activité des invertases altèrent la voie de synthèse de plusieurs facteurs de croissance, telles que les cytokinines et la sensibilité de la plante à ces facteurs de croissance (Gibson, 2004). Or, ceux-ci jouent un rôle prépondérant dans la division, la croissance et la maturation cellulaire. Les enzymes hydrolysant le saccharose semblent donc jouer un rôle prépondérant à la fois dans l'allocation du carbone à travers les différentes voies métaboliques du puits, mais aussi dans le développement de ce puits.

1.4 L'accumulation d'amidon au sein du puits

La synthèse d'amidon constitue un des principaux aboutissements du saccharose transféré au sein du puits. En effet, l'amidon constitue la forme majoritaire des hydrates de carbone stockés au sein des organes de réserve. La conversion du saccharose en amidon représente ainsi la voie métabolique dominante au sein de ces organes. Cette voie métabolique a été relativement bien décrite dans la littérature et débute par l'hydrolyse du saccharose en fructose, glucose ou UDP-glucose comme décrit dans la section précédente (Fernie *et al.*, 2002). L'UDP-glucose est converti en glucose-1-phosphate (G1P) par l'UDP-glucose pyrophosphorylase, alors que le glucose et le fructose sont phosphorylés par l'hexokinase et la fructokinase, respectivement (Fig. 1.3). Le glucose-6-phosphate (G6P), produit par l'interconversion des hexose-phosphates, est alors transporté au sein de l'amyloplaste grâce à un transporteur antiport G6P/Pi. Une fois dans l'amyloplaste, le G6P est converti de nouveau en G1P par la

phosphoglucomutase, alimentant ainsi la voie d'entrée de la synthèse de l'amidon: l'ADP-glucose pyrophosphorylase (AGPase). L'amidon est alors formé à partir de l'ADP-glucose par une réaction de polymérisation catalysée par les amidon synthases (starch synthases) et les enzymes de branchement de l'amidon. Les amidon synthases permettent de lier des unités de glucose en chaîne par des liaisons α(1-4), engendrant une organisation en hélice α (Smith *et al.*, 1997). Les enzymes de branchement de l'amidon, quant à elles, lient les unités de glucose par des liaison α(1-6), créant ainsi des ramifications dans la structure de la molécule. Le degré de ramification et de polymérisation permet de distinguer les deux homopolymères constituants l'amidon (Lindeboom *et al.*, 2004). Le premier, l'amylose, est peu ramifié et est formé de plusieurs centaines d'unités de glucose alors que le second, l'amylopectine, est fortement ramifié toutes les 24 à 30 unités et formé de plusieurs milliers d'unités de glucose. L'amylose représente généralement autour de 30% de l'amidon accumulé par les plantes.

Si les acteurs de la voie métabolique menant à la synthèse d'amidon sont bien connus, les régulations de cette voie ont été peu étudiées. De plus, la majorité de ces études porte sur une seule espèce, *Solanum tuberosum*. L'utilisation de mutants a permis de mettre en évidence certains de ces contrôles dans le tubercule de pomme de terre. Ainsi, la modulation des activités de l'UDP-glucose pyrophosphorylase, des hexokinases et des amidon synthases n’induit pas de modulation significative de l'accumulation d'amidon (Zrenner *et al.*, 1993; Marshall *et al.*, 1996). De même, l'altération des enzymes de branchement de l'amidon induit une baisse du rapport amylopectine/amylose, mais sans affecter l'accumulation d'amidon. Par ailleurs, une baisse de l'activité de la Susy, des phosphoglucomutases cytosolique et plastidiale et de l'AGPase n'induit qu'une très légère baisse de l'accumulation d'amidon dans le tubercule de pomme de terre (Zrenner *et al.*, 1995; Fernie *et al.*, 2002; Geigenberger *et al.*, 2004). Il semble en fait que cette dernière enzyme possède une activité largement excédentaire, permettant l'approvisionnement continu de la synthèse d'amidon en ADP-glucose. Cette enzyme présente, par ailleurs, plusieurs isoformes, telle que l'isoforme cytosolique observée dans le grain de blé (Denyer *et al.*, 1996). L'activité de l'AGPase nécessite de l'ATP, importé du cytosol. Si la forte production d'ATP dans les chloroplastes des organes photosynthétiques permet de soutenir

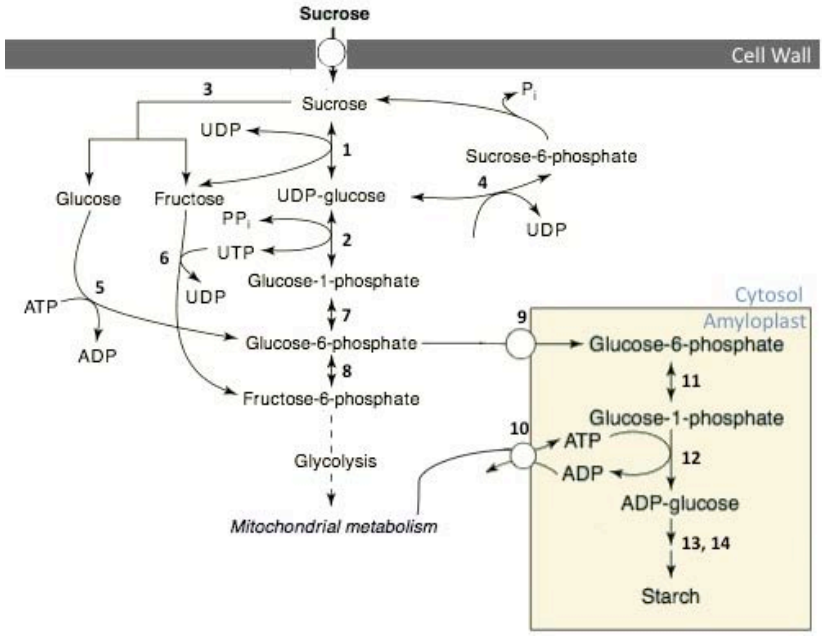

Figure 1.3 Voie métabolique de la conversion du saccharose en amidon au sein d'un tubercule de pomme de terre. 1. Saccharose synthase, 2. UDP-glucose pyrophosphorylase, 3. Invertases, 4. Saccharose-phosphate-synthase, 5. Hexokinase, 6. Fructokinase, 7. Phosphoglucomutase cytosolique, 8. Phosphoglucose isomérase, 9. Transporteur hexose/phosphate, 10. AATPT (amyloplastidial ATP/ADP translocator), 11. Phosphoglucomutase amyloplastique, 12. ADP-glucose pyrophosphorylase, 13. Amidon synthase, 14. Enzyme ramifiante de l'amidon (Starch branching enzyme). D'après Fernie et al. (2002).

la synthèse d'amidon, au sein d'organes hétérotrophes, l'ATP nécessaire à la synthèse d'amidon doit, lui, être importé du cytosol et est principalement produit par la respiration mitochondriale. Certaines études montrent ainsi qu'une altération du transporteur antiport ATP/APD entre l'amyloplaste et le cytosol affecte fortement l'accumulation de l'amidon (Tjaden *et al.*, 1998). La concentration des différents adenylates apparaît comme un des éléments clés modulant l'accumulation d'amidon dans les organes de réserve.

D'autre part, certains facteurs environnementaux ont été identifiés pour leur capacité à affecter la synthèse d'amidon dans le tubercule de pomme de terre. Ainsi, la conversion des sucres solubles en amidon est réduite lors d'un déficit hydrique des tissus. La SPS est ainsi rapidement activée, en cas de déficit hydrique du tubercule, engendrant une

nouvelle synthèse de saccharose. Cette activation post-transcriptionnelle induit une baisse de l'alimentation en substrat et une inhibition allostérique de l'activité de l'AGPase (Geigenberger *et al.*, 1997). De plus, l'accumulation de sucres solubles dans le cytosol permettrait de rétablir une turgescence cellulaire et ainsi participerait à l'osmorégulation. La SPS exerce ainsi un contrôle important sur la synthèse de saccharose et d'amidon en réponse au stress hydrique. La disponibilité en oxygène constitue un autre des facteurs majeurs, affectant la synthèse d'amidon et le taux de croissance (Geigenberger, 2003). Or, dans les organes de réserve, l'hypoxie est un stress récurrent, induit par l'organe lui-même, de par sa forte activité métabolique et sa grande synthèse d'amidon, dont l'accumulation tend à augmenter la compacité du tissu. De ce fait, ce stress est présent même lorsque la concentration externe d'O_2 est optimale (21% v/v). La concentration en oxygène rencontrée dans un tubercule de pomme de terre avoisine les 8-10% en surface et descend rapidement à 2-5% au centre de celui-ci (Geigenberger *et al.*, 2000). Les concentrations en CO_2 et en oxygène au sein du tubercule de pomme de terre affectent directement la respiration de celui-ci (Dizengremel, 1985). Une faible concentration en oxygène induit une inhibition de la respiration cellulaire ainsi qu'une baisse de la production d'ATP (Geigenberger, 2003). En raison d'un faible statut énergétique de la cellule, la faible teneur en oxygène induit une inhibition globale de la synthèse biologique et en particulier de la synthèse d'amidon. D'autre part, l'augmentation de la teneur en ADP induit un rétrocontrôle négatif des activités de l'hexokinase et de la fructokinase, privilégiant l'hydrolyse du saccharose par la SuSy au dépend des invertases (Geigenberger *et al.*, 2000). En effet, la voie des invertases nécessite de l'ATP et de l'UTP, alors que la voie de la SuSy permet la synthèse d'UTP par l'UDP-glucose pyrophosphorylase. La baisse de la teneur en oxygène induit donc un basculement en faveur d'une voie moins coûteuse en énergie pour l'hydrolyse du saccharose. Il apparaît ainsi que la synthèse d'amidon dans le tubercule de pomme de terre est dépendante à la fois du statut énergétique et de l'approvisionnement en saccharose et en G6P. Néanmoins, ces contrôles ont été bien décrits uniquement lors de la croissance des tubercules de *Solanum tuberosum*. La croissance des tubercules est limitée par l'activité de la source. Il est donc légitime de se demander si des mécanismes similaires ont lieu chez d'autres espèces, au sein d'autres types d'organes de réserve ainsi que lorsque la croissance est limitée par les puits, et dans quelles mesures ces mécanismes influent sur l'accumulation d'amidon.

1.5 Le métabolisme respiratoire et l'oxydase alternative

La respiration aérobique constitue, après la synthèse d'amidon, un des consommateurs de saccharose les plus notables au sein des organes de réserve. La respiration permet de synthétiser de l'ATP à partir de l'énergie des photoassimilats et dans une moindre mesure, de produire des squelettes carbonés. Par ailleurs, celle-ci joue un rôle prédominant sur le bilan de carbone à l'échelle de la plante entière, de par la perte induite de CO_2. Le métabolisme respiratoire se divise en trois étapes majeures : la glycolyse, le cycle de l'acide citrique (ou cycle de Krebs) et la chaîne de transport des électrons (Fernie *et al.*, 2004). De plus, il existe une voie métabolique parallèle à la glycolyse : la voie des pentoses phosphates. La glycolyse se déroule essentiellement au sein du cytosol, alors que le cycle de l'acide citrique et la chaîne de transport des électrons demeurent, respectivement, au sein de la matrice mitochondriale et au sein de la membrane interne de la mitochondrie. La glycolyse implique une cascade de réactions enzymatiques permettant la conversion de saccharose en acide organique, tel que le pyruvate ou le malate. Elle demeure un processus très peu exigeant en ATP et ce, grâce à sa phase conservatrice d'énergie. La consommation en ATP de la glycolyse varie cependant, selon que le saccharose est hydrolysé par la SuSy ou par une invertase, cette dernière impliquant le doublement des besoins en énergie (Fernie *et al.*, 2002). Ainsi, certaines enzymes dépendantes d'énergie, telle que l'hexokinase, ou productrices d'énergie, telle que la pyruvate kinase semblent alors jouer un rôle essentiel dans le contrôle de la glycolyse au sein des organes de réserve (Veramendi *et al.*, 2002). Parallèlement, le G6P produit suite à l'hydrolyse du saccharose, peut alimenter la voie des pentoses phosphates. Cette dernière oxyde alors le G6P en G3P. Cette voie engendre la synthèse de NADPH mais aussi de pentoses, tel que le ribulose-5-phosphate qui est un précurseur à la synthèse des nucléotides et des acides nucléiques. Ensuite, le cycle de l'acide citrique permet d'oxyder complètement le pyruvate produit par la glycolyse, en CO_2, générant 4NADH et 1$FADH_2$. Le pouvoir réducteur produit par ce cycle métabolique et par la glycolyse est alors utilisé par la chaîne de transport des électrons afin de synthétiser de l'ATP.

Cette chaîne permet le transfert des électrons depuis le NADH jusqu'à l'oxygène et ce, au travers de différents complexes protéiques. Cette chaîne de transport des électrons possède une voie dite alternative au travers de laquelle les électrons sont transférés

directement de l'ubiquinone à l'oxygène, dont la réduction en eau est catalysée par l'oxydase alternative (AOX)(Moore et Siedow, 1991). Cette voie contourne deux sites d'efflux de protons (complexes III et IV) et n'encourage donc pas la synthèse d'ATP par l'ATP synthase (complexe V). Plusieurs facteurs sont susceptibles de moduler la répartition des électrons entre la voie cytochromique et la voie alternative, telle que la proportion d'ubiquinone réduite (Wagner *et al.*, 1998), la quantité et la réduction de l'oxydase alternative (Umbach et Siedow, 1993), l'enzyme malique à NAD et le pyruvate qu'elle produit (Millar *et al.*, 1993) et la disponibilité en ADP (Juszczuk *et al.*, 2001). Cette dernière se justifie en partie par le contrôle respiratoire exercé sur la chaîne de transport des électrons par la production d'ATP. La teneur de ces métabolites montre une grande amplitude de variation au cours de la croissance des plantes, en fonction du type de tissu et en fonction des conditions environnementales. Le rôle de la voie alternative de la respiration a suscité de grands intérêts, particulièrement compte tenu de son aptitude à gaspiller l'énergie. Le premier rôle associé à la voie alternative serait celui d'un générateur de chaleur lors du développement de la fleur de certaines Araceae (Wagner *et al.*, 1998). En effet, l'énergie produite lors de la réduction de l'oxygène par l'AOX est dissipée sous forme de chaleur (thermogénèse). Une augmentation de la respiration alternative dans le spadice de *Symplocarpus foetidus* permet ainsi d'atteindre une température de 10°C supérieure à l'environnement. Ce phénomène, appelé la crise respiratoire, permettrait la volatilisation d'amines malodorantes et attractives pour les insectes pollinisateurs. Un rôle plus général suggère que la voie alternative de la respiration dériverait le surplus d'électrons de la chaîne de transport, afin de prévenir une surréduction des molécules d'ubiquinone et d'éviter la formation de radicaux libres (Moller, 2001). Ces molécules induites lors de stress possèdent des propriétés destructrices pour la plante. La voie alternative pourrait être considérée comme un mécanisme de protection contre les stress oxydatifs, au même titre que les superoxides dismutases et les catalases (Maxwell *et al.*, 1999). Enfin, la dernière fonction allouée à la respiration alternative suggère que celle-ci constitue une "voie d'évacuation du trop plein énergétique". En effet, son activité semble être accrue lors d'un apport de glucides. Lambers (1982) a alors suggéré que la voie alternative de la respiration pourrait permettre de consommer les excès de carbone qui ne sont pas utilisés pour le maintien de l'activité métabolique ou le stockage. Néanmoins, à notre connaissance, aucune étude

n'a clairement démontré ce rôle de consommation de carbone excédentaire, qui constituerait alors un élément majeur dans les relations source-puits.

1.6 L'érythrone d'Amérique, une éphémère printanière à bulbe

Le cycle de vie des plantes bulbeuses, aussi appelées géophytes, s'organise autour de l'organe de réserve, qui constitue un puits fort. Celui-ci assure la survie de la plante, de par son aptitude à accumuler les réserves de carbone et de nutriments ainsi qu'à assurer la reproduction clonale par division végétative (De Hertogh et Le Nard, 1993). Les géophytes se distinguent en fonction de leur période de floraison : les floraisons printanières (*Crocus vernus, Tulipa, Erythronium*), les floraisons estivales (*Dahlia, Gladiolus*) et les floraisons automnales (*Colchicum, Nerime*). Notre étude porte sur une des géophytes à floraison printanière, l'érythrone d'Amérique (*Erythronium americanum* Ker-Gawl), la plus commune des érablières nord-américaines.

Cette Liliacée se caractérise par un cycle biologique annuel spécifique (Fig. 1.4). En premier lieu, une levée de la dormance du bulbe est induite par une chute de la température du sol en fin d'été (Risser et Cottam, 1968). On observe alors l'activation des méristèmes et la mise en place d'un nouveau système racinaire et ce, à partir des réserves accumulées (Blodgett, 1910). La baisse de température est ainsi connue pour induire une induction des enzymes chargées de la solubilisation de l'amidon. Les sucres solubles libérés sont alors utilisés pour le maintien et l'entretien des tissus ainsi que pour la morphogénèse foliaire. Dans un second temps, l'érythrone d'Amérique présente une croissance aérienne, dite épigée, qui débute au printemps, dès la fonte des neiges (Muller, 1978).

La plante étend alors sa structure foliaire préformée pendant l'hiver, de sorte d'être rapidement photosynthétiquement active. Cette espèce présente une synthèse rapide des protéines photosynthétiques et un taux de photosynthèse élevé pour une herbacée (Taylor et Pearcy, 1976), afin d'assurer une accumulation de réserves (près de 80-90% d'amidon), la

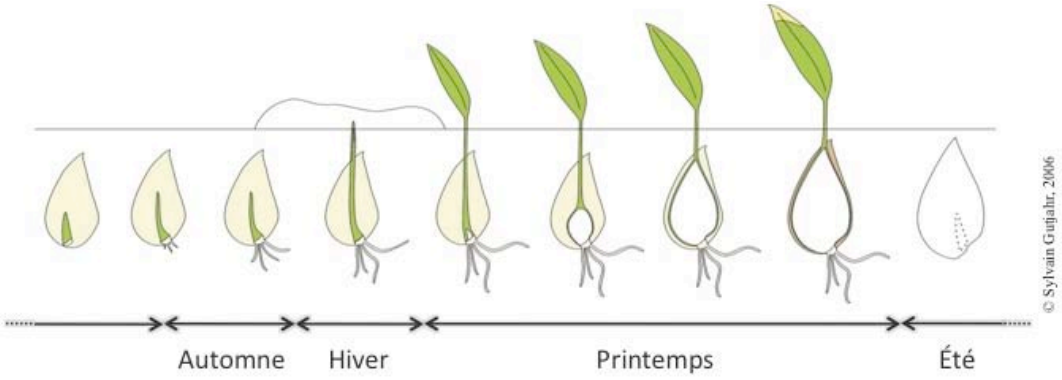

Figure 1.4 Stade de développement du bulbe d'érythrone d'Amérique au cours de son cycle biologique annuel. Illustration de la formation du nouveau bulbe (en blanc) au sein de l'ancien.

plus rapide possible, au sein du nouveau bulbe. En effet, la phase de croissance épigée est courte, entre 40 et 60 jours, et se finalise peu après la fermeture de la canopée. Cette phase assure néanmoins la subsistance de la plante jusqu'au printemps suivant.

Il a rapidement été avancé que la sénescence précoce chez les éphémères de printemps serait induite par la chute de luminosité ayant lieu lors de la fermeture de la canopée (Vezina et Grandtner, 1965). Cette hypothèse s'est vue renforcée par l'idée que les éphémères de printemps, adaptées à des conditions de fortes irradiances, présentent une acclimatation réduite aux faibles irradiances. Cependant, certaines études ultérieures ont indiqué une longévité limitée des parties aériennes alors que les conditions lumineuses demeuraient stables. L'érythrone du Japon présente une durée similaire de la croissance épigée, dans trois milieux forestiers qui diffèrent par leurs conditions d'irradiance (Sawada *et al.*, 1997). Lapointe (2001) a suggéré que l'induction de la sénescence des parties aériennes serait due à une baisse de la demande en carbone des organes de réserve, plutôt que par l'effet direct de facteurs abiotiques. L'activité de la source serait maintenue et la sénescence foliaire retardée tant que la demande en carbone par un puits fort est présente. De récents travaux ont alors indiqué une croissance plus importante de l'organe de réserve à faible température chez l'érythrone d'Amérique (Lapointe et Lerat, 2006) et *Crocus vernus* (Badri *et al.*, 2007). Or, cet effet positif d'une température froide s'accompagne d'une durée de vie de l'appareil foliaire plus importante. L'activité du

puits et la sénescence foliaire apparaissent donc étroitement liées chez les géophytes printanières.

1.7 Problématique

La présente thèse a pour but **d'améliorer notre compréhension des relations source-puits de carbone chez une plante limitée par les puits, l'érythrone d'Amérique**. Cette géophyte constitue un modèle biologique d'intérêt pour cette étude, de par sa morphologie très simple. En effet, les individus immatures présentent une seule feuille et un seul bulbe, soit une seule source et un seul puits. Ceci s'avère alors très proche d'un modèle théorique de relations entre source et puits. D'autre part, le métabolisme carboné et ses régulations ont été bien décrits chez des espèces limitées par les sources. Cependant, la littérature est beaucoup plus limitée chez les espèces limitées par les puits. Dans ces conditions, le rôle de l'organe de réserve est des plus prépondérants. Nous nous sommes ainsi particulièrement intéressés à **approfondir nos connaissances des facteurs intrinsèques contrôlant la force du puits et l'impact de ce puits sur la physiologie et la phénologie de la plante**.

Dans un premier temps, nous avons modulé la force du puits de l'érythrone d'Amérique en faisant varier la température de croissance. Des études antérieures ont indiqué une biomasse finale des bulbes plus importante à 12/8°C qu'à 18/14°C (Lapointe et Lerat, 2006). Une réponse positive de la croissance à de plus faibles températures demeure rare parmi les espèces tempérées. Nous avons tenté d'identifier les mécanismes de **régulation du métabolisme carboné qui affectent la croissance et la capacité de l'organe de réserve**. De plus, cette augmentation de biomasse s'accompagne d'une longévité plus importante de l'appareil foliaire. Afin de confirmer l'hypothèse de Lapointe (2001) à l'effet que la sénescence foliaire chez les éphémères printainières est induite par une baisse de la force du puits, nous avons tenté de **caractériser le lien métabolique entre la baisse de la demande du puits et l'initiation de la sénescence foliaire**. L'accumulation de sucres dans les puits ou les sources semble attendue, mais requiert tout de même d'être vérifiée d'autant plus que l'érythrone n'a pas la capacité d'accumuler d'amidon dans ses feuilles. Finalement, cette étude pourrait permettre **d'identifier certains mécanismes d'adaptation au froid** chez les éphémères printanières, qui leurs assurent une meilleure croissance aux températures froides.

Dans un second temps, nous avons modulé l'activité de la source de l'érythrone d'Amérique, en augmentant la concentration en CO_2 et en ozone atmosphérique durant la croissance épigée. En effet, une augmentation de la concentration en CO_2 stimule l'assimilation carbonée, alors qu'une augmentation de la teneur en ozone l'inhibe. Gutjahr et Lapointe (2008) ont montré qu'une stimulation de l'activité de la source ne permettait pas d'induire une synthèse d'amidon plus importante chez l'érythrone d'Amérique. Nous souhaitons aller plus loin et étudier l'effet d'une modulation de l'activité de la source et de l'assimilation carbonée sur le métabolisme carboné du puits. Ces travaux permettront alors de **comprendre les mécanismes induits par un excès récurrent de ressource carbonée chez une espèce limitée par les puits.**

Dans un troisième temps, nous avons aussi modulé l'activité de la source mais cette fois-ci en faisant varier la quantité de photons disponible pour la plante. Ces traitements diffèrent non seulement par le niveau d'irradiance, mais aussi par la durée de l'irradiance, soit la photopériode. Nous souhaitons ainsi **mettre en évidence une éventuelle dynamique temporelle entre la synthèse d'hydrates de carbone durant la phase photosynthétiquement active et l'utilisation de ces hydrates de carbone autant le jour que la nuit**.

Ces travaux permettront d'améliorer notre compréhension, trop limitée, du métabolisme carboné au sein d'un puits autre que le tubercule de pomme de terre, et de définir son rôle sur l'activité et la croissance du puits mais, aussi sur l'activité de la source et ce, en conditions de limitation par le puits.

Chapitre 2 Low temperature: a way to reduce imbalance between sink capacity to accumulate carbohydrates and sink growth rate in Spring geophytes.

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2.1 Résumé

Chez les printanières à bulbe, une faible température de croissance induit la production d'organes de réserve plus gros et retarde la sénescence foliaire. Néanmoins, la durée de vie des feuilles ne peut expliquer totalement la taille plus importante de la plante. Le métabolisme carboné du bulbe et ses effets, sur l'activité photosynthétique et sur l'initiation de la sénescence, ont été étudiés chez *Erythronium americanum* afin d'identifier certains des mécanismes permettant la production de plus gros organes de réserve à faible température. Les plants ont crû sous trois régimes de température jour/nuit : 18/14°C, 12/8°C et 8/6°C. L'activité et la capacité du puits ont été mesurées tout au long de la saison de croissance via l'analyse d'activités enzymatiques, de l'accroissement cellulaire, de l'accumulation de biomasse et d'hydrates de carbone, alors que l'activité métabolique de la feuille et la sénescence ont été mesurées via l'analyse des échanges gazeux, de la fluorescence chlorophylienne et d'activités enzymatiques. L'accumulation d'amidon dans le bulbe est plus lente aux faibles températures, de par un plus faible taux de photosynthèse net et de par les inductions de l'invertase vacuolaire et de la saccharose-phosphate-synthase dans le puits. Ces inductions ont potentiellement induit un « cycle futile » de synthèse et de dégradation successives du saccharose. De plus, la maturation des cellules du bulbe a été retardée aux faibles températures en raison d'une induction tardive de la saccharose synthase, prolongeant ainsi la phase d'accroissement cellulaire et menant à une plus grande capacité du puits. La plus rapide accumulation de l'amidon et la plus faible capacité du puits observées aux fortes températures ont induit une saturation précoce du bulbe en amidon (88% of MS). Lorsque les bulbes ne pouvait plus accumuler d'amidon, les sucres solubles ont commencé à s'accumuler, induisant vraisemblablement une baisse de l'activité de la fructose-1,6-bisphosphatase et de l'utilisation des trioses phosphates (TPU) dans la feuille. Quelques jours après que ce signal ait été perçu par la feuille, la sénescence a été induite. Une plus longue durée de vie des feuilles et de plus gros bulbes à faible température semblent donc être dus à une plus lente accumulation de l'amidon et à une plus grande capacité du puits, plutôt qu'à des effets directs de la température de croissance sur l'espérance de vie de la feuille.

2.2 Abstract

In spring geophytes, low growth temperature induces the production of larger storage organs and delayed leaf senescence. Yet, leaf life duration cannot totally explain the larger plant size. Bulb carbon metabolism and its effects on leaf activity and on the induction of leaf senescence were investigated in *Erythronium americanum* to identify some of the mechanisms allowing the production of larger storage organs at low temperature. Plants were grown under three day/night temperature regimes: 18/14°C, 12/8°C and 8/6°C. Sink activity and capacity were monitored throughout the growth period via enzymatic activities, cell expansion, biomass and carbohydrate accumulation, whereas leaf activity and senescence were monitored via measurements of gas exchange, chlorophyll fluorescence and enzymatic activities. Starch accumulated more slowly in the bulb at lower temperatures due to a lower net photosynthetic rate and the induction of both vacuolar invertase and sucrose phosphate synthase in the sink, which potentially activated a 'futile cycle' of sucrose synthesis and degradation. Furthermore, bulb cell maturation was delayed at lower temperatures because of delayed induction of sucrose synthase, lengthening the cell expansion phase and leading to a greater sink capacity. The faster rate of starch accumulation and the smaller sink capacity observed at higher temperatures led to early starch saturation of the bulb (88% of DW). Once bulbs could not accumulate more starch, soluble sugars started to accumulate, most likely inducing decreases in fructose-1,6-bisphosphatase activity and triose-phosphate utilization (TPU) in the leaf. A few days after this signal was sensed by the leaf, senescence was initiated. Longer leaf life span and larger bulbs at lower temperature thus appear to be due to slower starch accumulation and larger sink capacity rather than direct effects of growth temperature on leaf life duration.

2.3 Introduction

Low temperatures are well-known to reduce growth rate and final biomass, even for plants well-adapted to low-temperature regimes. Yet, there are exceptions, such as some geophytes where growth is increased at low temperature and a much larger storage organ results (Daymond et al., 1997; Wheeler et al., 2004; Badri et al., 2007). This constitutes a notable case among temperate species. This positive effect on growth of the storage organ is correlated with greater leaf longevity, and therefore, with a longer period of carbon assimilation, which could partly explain higher storage organ biomass. However, in an annual spring ephemeral such as *Floerkea proserpinacoides*, longer leaf life span leads to lower biomass production at low temperature (Houle *et al.*, 2001). Moreover, corm growth and leaf longevity in *Crocus vernus* are more greatly affected by soil temperatures than by air temperatures, supporting the idea that leaf life duration is influenced more by temperature effects on storage organ activity than by direct temperature effects on leaf metabolism (Badri *et al.*, 2007). Lapointe (2001) has suggested that leaf senescence is induced by a reduction in carbohydrate sink demand, once the storage organ is filled with carbohydrates. Low temperature would thus affect the growth of belowground organs, which in return would influence leaf life duration.

Sink activity and capacity define the ability of the sink organ to import carbohydrates, i.e., its sink strength (Marcelis, 1996). In source-limited conditions, sink activity is strongly dependent on the activity of the source and photosynthetic rates should be modulated mainly by abiotic factors. In sink-limited conditions, feedback inhibition of photosynthesis is expected once starch starts to accumulate in great amount in the chloroplasts (Paul and Foyer, 2001), which could eventually lead to leaf senescence. Feedback inhibition of photosynthesis is sensed not only as a decrease in net photosynthetic rate (Pn), but also as a decrease in photochemical quenching and a concurrent increased loss of photochemical energy (i.e., non-photochemical quenching). Sugar-dependent signals appear as a major way to link sink limitation and source activity (Paul and Driscoll, 1997; Sulpice *et al*., 2009).

Sucrose metabolism plays a key role in controlling many metabolic pathways of sink tissues such as cell growth, respiration, starch synthesis, and the fermentation pathway. Starch synthesis and respiratory rate determine sink activity, whereas cell growth

determines sink capacity. Sucrose phosphate synthase (SPS) activity is generally increased at low temperatures (Guy *et al.*, 1992). For example, in potato tuber sweetening, this SPS increase is due to a liberation of hexoses due to amylase and invertase induction by low temperature (Malone *et al.*, 2006). Furthermore, Geigenberger (1998) suggests that high temperature inhibits ADP-glucose pyrophosphorylase (AGPase) activity and starch synthesis due to decrease availability of their substrates, which are used in respiration. Similarly, sucrose synthase (Susy) activity is reduced when potato tubers are subjected to higher growth temperatures, whereas invertase activity is not affected (Lafta and Lorenzen, 1995). Through its effects on different enzymes involved in sucrose metabolism, temperature could thus affect adenylate status and hexose pools, thereby modulating C allocation between the different activities of the sink. The main plant model used so far to study temperature effects on C metabolism at the sink level has been the potato tuber and low temperature effects have been essentially investigated under post-harvest conditions. Temperature could also affect sink capacity, which constitutes one of the two components of sink strength, along with sink activity. Indeed, numerous studies have reported an increase in cell division and cell expansion rates with increasing temperature, as in fruit (Bertin, 2005), in leaves (Tardieu *et al.*, 2000) or in roots (Pritchard, 1994). Changes in both sink activity and capacity may explain the positive response of spring ephemeral plant growth to low temperature.

The present study focuses on changes in both the leaf and bulb at the level of carbon metabolism that could explain the increased low-temperature growth observed in spring geophytes. *Erythronium americanum* Ker Gawl. (trout lily) plants were grown under three temperature regimes: 18°C/14°C day/night, 12°C/8°C, and 8°C/6°C. In addition to responding positively to low temperatures, *E. americanum* provides an interesting biological model for the study of whole-plant carbon allocation, because of its simple morphology; the plant is composed of a single leaf and a single bulb (i.e., one source vs. one sink, Fig. 2.1). Harvests were performed throughout the period of epigeous growth to measure plant growth in relation to phenological status. Gas exchange, chlorophyll fluorescence, plant growth, bulb cell size and carbohydrate concentrations were assessed as measures of source and sink activities. The activities of several sucrose-related enzymes were also assayed to provide insight into the steps regulating carbon

Figure 2.1 Representative illustration of *Erythronium americanum* plants during the different phenological stages of epigeous growth.

metabolism of the source and sink. This work should help unravel some of the mechanisms leading to increased storage organ production of spring ephemerals under low-temperature regimes.

2.4 Material and methods

2.4.1 Plant material and growing conditions

Bulbs of *E. americanum* were collected in autumn in a sugar maple forest near Saint-Augustin-de-Desmaures (QC, Canada; 46°48' N, 71°23' W). Bulbs of similar biomass (0.40-0.45 g fresh weight) were selected and planted in plastic pots containing Turface (Applied Industrial Materials Corp., Buffalo Grove, IL, USA) as substrate and stored in a cold chamber for 4-5 months of cold stratification. Plants were then randomly allocated to growth chambers (PGW36, Conviron Inc., Winnipeg, MB, Canada) under the following light conditions: photoperiod of 14 h and a photon flux density (PPFD) of 400 μmol m^{-2} s^{-1}. *E. americanum* was exposed to three growth temperatures: 18°C/14°C day/night, 12°C/8°C and 8°C/6°C, with relative humidities (RH) of 75%, 65% and 50%, respectively. The two higher temperature regimes (12°C/8°C and 18°C/14°C) correspond to the daily (day and night) mean temperatures encountered at the beginning and end of the *E. americanum* growing season under natural conditions. RH was modulated as a function of growth temperature to maintain a constant vapour pressure deficit (VPD) across chambers. Plants were watered daily and fertilized weekly with 10% Hoagland's solution for optimal growth (Lapointe and Lerat, 2006). The experiment was repeated over two years and treatments were switched among growth chambers between years.

2.4.2 Plant growth measurements

Six plants per chamber were harvested at the following stages: 1) the beginning of the experiment (day 0), 2) initiation of leaf unfolding (day 3, 4 and 5 at 18/14°C, 12/8°C and 8/6°C, respectively), 3) when leaves were completely unfolded (day 5, 7 or 9), 4) the first visual signs of leaf senescence (day 22, 29 or 33), and 5) complete leaf senescence (day 29, 39 or 45, Fig. 2.1). Between leaf unfolding and leaf senescence, harvesting was staggered among the three temperature regimes, i.e., every 2 days for 18/14°C, 3 days for 12/8°C and 4 days for 8/6°C. Leaf area was measured using a Li-

Cor 3100 area meter (Li-Cor Inc, Lincoln, NE, USA). Then, leaf, bulb and roots were lyophilized for 24 h and weighed separately.

2.4.3 Gas exchange and fluorescence measurements

Gas exchange measurements were carried out on the single leaf of five plants per chamber using a Li-Cor 6400 Portable Photosynthesis System (Li-Cor Inc, Lincoln, NE, USA). Measurements were performed on the same plants from leaf unfolding to complete leaf senescence. Measurements were conducted under three light conditions: 0, 400 and 1000 $\mu mol\ m^{-2}\ s^{-1}$. We maintained a background CO_2 concentration of 400 $\mu mol\ mol^{-1}$ and airflow of 200 $\mu mol\ s^{-1}$. Temperature and RH were those of the growth chamber conditions. Pn, stomatal conductance (g_s), intercellular CO_2 concentration (Ci) and dark respiration (Rd) were recorded. Measurements of chlorophyll fluorescence were carried out simultaneous to gas exchange using a Li-Cor 6400-40 pulse-modulated fluorometer. The intensity of the pulse was set at 14000 $\mu mol\ m^{-2}\ s^{-1}$ for 1 sec. Photochemical quenching (qP), non-photochemical quenching (qN), PSII maximum efficiency (Fv/Fm), and electron flux across PSII (PhiPSII) were subsequently estimated. Total electron flux (J_T) was also calculated and used to estimate photorespiratory rates (PR) as: $PR=1/12[J_T-4(Pn+Rd)]$ (Genty *et al.*, 1989). Furthermore, dark respiration measurements were carried out on the bulbs of 5 plants per chamber. The whole bulb fitted into the gas analyzer chamber. Conditions of measurement were similar to those for leaf measurements. All measurements were randomized among treatments between 11 h and 13 h (i.e., the middle of the light period).

Photosynthetic CO_2 response curves (A-Ci curves) were carried out on five plants per treatment at maximum Pn (day 9, 13 and 21 at 18/14°C, 12/8°C and 8/6°C, respectively; see Fig. 2.3A) and a second time, at 17, 25 and 29 days for the three respective temperature regimes (see Fig. 2.3A). Maximum carboxylation rate (Vcmax), maximum electron transport rate (Jmax), and triose phosphate utilization (TPU) were calculated using Photosynthesis software (Li-Cor Inc, Lincoln, NE, USA; Sharkey *et al.*, 2007).

2.4.4 Carbohydrates

Starch, sucrose, and reducing sugar (glucose and fructose) concentrations were determined from the bulbs of three plants per chamber, per harvest date. Bulbs were harvested and flash-frozen in liquid nitrogen. Bulbs were then stored at -80°C until extraction. Frozen tissues were lyophilized for 24 h and weighed before maceration in a solution of methanol, chloroform and water (12:5:3 v/v/v) for 20 min at 65°C (Blakeney and Mutton, 1980). The mixture was dispersed and homogenized with a Polytron (Kinematica, Lucerne, Switzerland) and centrifuged at 3500 rpm for 10 min at 4°C. Starch contained in the pellet was gelatinized in boiling water for 90 min and then hydrolyzed at 55°C for 60 min, in the presence of amyloglucosidase. The supernatant was analyzed before and after invertase digestion to estimate reducing sugars and sucrose concentrations, respectively. Finally, all reducing sugars were quantified colorimetrically at 415 nm after reaction with *p*-hydroxybenzoic acid hydrazide (Sigma Chemical Co., St. Louis, MO, USA).

2.4.5 Enzyme extraction and assays

Soluble proteins were extracted from leaves and bulbs of three plants per chamber, per harvest date. As in the previous assay, the leaf and bulb of each plant were separated, flash-frozen in liquid N_2, and stored at -80°C until extraction. Frozen leaf and bulb tissues (300 mg FW) were ground in liquid nitrogen with a mortar and pestle. Crude enzyme extractions were performed at 4°C in 3 mL 0.1 M HEPES-KOH buffer (pH 7.5), containing 7% w/w polyethylene glycol 20000, 2 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM ethylene glycol-bis-(β-aminoethyl ether)-tetraacetic acid, 10% v/v glycerol, 1 mM phenylmethylsulphonyl fluoride, 9% w/v polyvinylpyrrolidone 25000 (PVP25), 1 µM pepstatine and 1 µM leupeptine. The homogenate was centrifuged at 18000 rpm for 20 min at 4°C. The supernatant was collected and filtered through a Sephadex-G25 column (Pharmacia Company, Uppsala, Sweden). The enzymes were eluted with HEPES-KOH buffer and a volume of 3.5 mL containing the enzymes was collected and used as enzymatic extract for the determination of fructose-1,6-bisphosphatase (F1,6BPase) in the leaf, and neutral invertase (NInv), Susy, vacuolar invertase (VInv), SPS, and AGPase activities in the bulb. The pellet was washed twice with 3 mL of 100 mM HEPES containing 0.5% (m/v) PVP25, 5 mM $MgCl_2$, 5 mM EGTA and 2 mM DTT and centrifuged at 13000 rpm for 20 min at 4°C. The pellet was

then treated with the same medium plus 1 M NaCl overnight and centrifuged at 13000 rpm for 20 min at 4°C. The supernatant was collected and used for the determination of cell wall invertase (CWInv) activity in the bulb. Protein concentrations in the different supernatants was measured according to Bradford (1976).

Enzymatic activities were determined spectrophotometrically in a microplate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA). F1,6BPase was assayed according to Vassey and Sharkey (1989), with modifications. The assay medium contained: 200 µL of 50 mM HEPES-NaOH (pH 7), 100 mM KCl, 4 mM $MgCl_2$, 1.2 mM NADP, 3 $U.mL^{-1}$ glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 5 $U.mL^{-1}$ phosphoglucoisomerase (EC 5.3.1.9) and 10 µL of enzymatic extract. Adding 100 µM of fructose-1,6-bisphosphate initiated the reaction. AGPase assay was prepared according to Smith *et al.*, (1989). The assay medium contained: 200 µL of 50 mM HEPES-NaOH (pH 7.8), 5 mM $MgCl_2$, 1.2 mM NADP, 500 µM ADP-glucose, 5 $U.mL^{-1}$ glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 2.5 $U.mL^{-1}$ phosphoglucomutase (EC 5.4.2.2) and 10 µL of enzymatic extract. Adding 3.5 mM of NaPPi initiated the reaction. NInv and Susy were assayed according to Gandin *et al.* (2009). NInv was assayed with 200 µL of 50 mM HEPES-NaOH (pH 7) containing 2.8 mM ATP, 1.2 mM NADP, 12 $U.mL^{-1}$ hexokinase (EC 2.7.1.1), 3.5 $U.mL^{-1}$ phosphoglucoisomerase (EC 5.3.1.9), 1.75 $U.mL^{-1}$ glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 10 µL of enzymatic extract. Adding 100 mM sucrose initiated the reaction. A similar medium with 2 mM UDP was used for the Susy assay. Susy activity was estimated by difference between the reactions described above, both with and without UDP. VInv was assayed according to Tang *et al.*, (1999), with modifications. The assay medium (200 µL) comprised 50 mM sodium citrate (pH 5.6), 10 µL of enzymatic extract and 100 mM sucrose to initiate the reaction. CWInv was assayed with 200 µL of 50 mM sodium citrate (pH 5), 10 µL of enzymatic extract and 150 mM sucrose. Glucose produced in both VInv and CWInv assays was then measured with glucose oxidase-peroxidase reagent (Sigma-Aldrich, St. Louis, MO, USA). SPS was assayed with 200 µL of 50 mM HEPES (pH 7.5), containing 10 mM UDP-glucose, 8 mM fructose-6-phosphate and 32 mM glucose-6-phosphate. Phenyl-ß-glucoside (20 mM) was used to inhibit Susy activity. UDP released by SPS activity was quantified according to Bergmeyer (1974) following NADH consumption by pyruvate

kinase/lactate dehydrogenase activity at 340 nm. Controls without substrates were run for all assays.

2.4.6 Determination of cell size

Cell size was determined on the bulb of three plants per chamber, per harvest. Bulbs were immediately fixed in FAA (formaldehyde-acetic acid-alcohol) solution (Sass, 1958) and then embedded in paraffin. Three transversal thin sections from each bulb were mounted on microscope slides and stained with 0.01% (w/v) toluidine blue. Slides were then observed under light microscope (Olympus, Lake Success, NY, USA). Diameter was measured for each cell along four transects (horizontal, vertical and two diagonals) per thin section for a total of two thin sections per bulb. Cell size in μm^2 was then calculated from the diameter of each cell, assuming that the cells were round.

2.4.7 Statistical analysis

Evolutions of all variables overtime were analyzed by comparing the slope of linear regressions during both increasing and decreasing phases to test temperature effect on the rate of change of these variables through time (Statistix 8.2, Analytical Software, Tallahassee, FL, USA). Furthermore, one-way ANOVAs were carried out on either maximal or minimal values. When either maximal or minimal values formed a single peak, ANOVAs were applied on data from the single harvest composing the peak. When maximal or minimal values formed a plateau, the ANOVA analysis included all data point composing this plateau. Effects were considered significant when $P < 0.05$. *A posteriori* multiple comparisons tests were performed using Fisher's LSD. In order to correct for 'chamber effects', treatments were switched from one growth chamber to another between years. The two years are thus true replicates.

2.5 Results

2.5.1 Plant growth

Erythronium americanum growth was affected by temperature, especially growth kinetics and final biomass of the bulb. Bulb final dry mass was 2.2-fold higher for 12/8°C and 2.7-fold higher for 8/6°C than for the highest growth temperature regime (Fig. 2.2A, Table 2.1). Biomass gain of the bulb ranged from 166% (18/14°C) to 795%

(8/6°C) from leaf unfolding (i.e., day 3, 4 and 5 at 18/14°C, 12/8°C and 8/6°C, respectively) to the first visible signs of leaf senescence. During the first days of growth, the kinetics of biomass accumulation was faster at the highest temperature than under the cooler temperatures (Table 2.2). Afterwards, mean growth rate decreased by 35% at 18/14°C, whereas it increased by 106% and 244% at 12/8°C and 8/6°C, respectively. Growth rate was thus inversely related to growth temperature between state 2 and state 3 (as defined in the Methods). In contrast, leaf life span decreased as growth temperature increased. The initiation of leaf senescence occurred after 22, 29 and 33 days at 18/14°C, 12/8°C, and 8/6°C, respectively, and complete leaf senescence after 29, 39 and 45 days (Table 2.2). Leaf senescence became visible a few days after bulb growth had slowed down. Root biomass kinetics was not affected by the growth temperature regime (Fig. 2.2B). Leaf growth and biomass thus lasted longer at lower temperatures, leading to higher maximum leaf biomass and area at the onset of leaf yellowing (Fig. 2.2C, D).

2.5.2 Gas exchange measurements

Pn measured at 400 μmol m^{-2} s^{-1} (PPFD) – similar to irradiance conditions for growth – exhibited a hyperbolic form through time; the parameters of these hyperbolic forms varied with temperature (Fig. 2.3A). The initial increase of Pn was faster as growth temperature increased, leading to a maximum at 9, 13 and 21 days at 18/14°C, 12/8°C and 8/6°C, respectively (Table 2.1). Maximum Pn was 5% lower at 12/8°C and 34% lower at 8/6°C compared to 18/14°C. Afterwards, Pn decreased more rapidly as temperature increased until complete leaf senescence was attained. Despite a lower maximum Pn, the amount of C fixed during the whole life of the leaf was 59% higher at 12/8°C and 85% higher at 8/6°C compared to 18/14°C, because of the longer photosynthetically active phase at cooler temperatures (Table 2.3). G_s exhibited a higher initial increase and a higher maximum at 18/14°C than at the two cooler temperatures (Fig. 2.3B). Maximum g_s was reached later than the maximum Pn at the two higher temperatures, whereas g_s was synchronized with Pn at 8/6°C. However, Ci was not affected by growth temperature (Fig. 2.3C, Table 2.1).

Table 2.1 Effects of growth temperature on the rates of change through time and on either maximum or minimum values reached for different parameters related to growth, gas exchange, chlorophyll fluorescence, bulb carbohydrates and enzyme activites in *Erythronium americanum*. Rates of change were estimated from the slope of linear regressions. F-values of one-way ANOVAs are presented along with statistical differences: * $P<0.05$, ** $P< 0.01$, *** $P<0.001$. Days at which either a maximum or minimum value was reached are also presented.

Variables	**Comparisons of slopes from linear regressions**		**ANOVAs on maximal or minimal values[1]**	**Days when maxima or minima were reached**		
	Positive slope	Negative slope		18/14°C	12/8°C	8/6°C
Growth parameters						
Bulb DW	70.54 ***	-	444.58 ***	17	29	29
Leaf DW	0.10	1.92	6.37 **	11	13	17
Root DW	-	1.47	0.62	7	10	9
Leaf area	0.65	-	98.47 ***	11	22	29
Bulb cell size	32.44	-	76.21 ***	11	16	25
Gas exchange and fluorescence						
Pn 400	7.03 **	10.31 ***	77.88 ***	9	13	21
g_s 400	7.48 **	9.56 **	8.43 ***	11	19	21
Ci 400	0.34	-	-	-	-	-
Pn 1000	12.29 ***	10.51 ***	23.28 ***	9	13	21
g_s 1000	0.48	7.42	0.86	9	19	29
Ci 1000	0.47	-	-	-	-	-
Rd	-	-	0.39	-	-	-
qP 400	-	37.04 ***	125.20 ***	9	13	29
qN 400	2.75 **	-	2.34 *	9	13	-
Fv/Fm	0.50	8.94 ***	11.79 ***	13	13	24
ΦPS2	6.76 **	9.48 ***	17.12 ***	9	13	21
PR	-	6.39 ***	10.86 ***	7	10	13
Bulb carbohydrates						
Starch	12.02 ***	-	0.169	15	22	29
Sucrose	-	1.17 *	7.49 ***	7	19	29
Reducing sugars	0.54	0.83	0.18	3	4	5
Sucrose (starchless)	7.43*	2.45*	8.61 *	22	22	29
Red. sugars (starchless)	1.76	0.19	23.92 **	15	22	29
Amylose	0.98	0.66	4.30 *	15	25	33

Table 2.1 (suite)

Variables	Comparisons of slopes from linear regressions		ANOVAs on maximal or minimal values[1]	Days when maxima or minima were reached		
	Positive slope	Negative slope		18/14°C	12/8°C	8/6°C
Enzymes						
F1,6BPase	-	0.77	36.70 ***	17	25	33
AGPase	2.05	-	0.63	7	10	17
Susy	89.14 ***	-	3.27 *	13	22	33
Cell wall invertase	-	8.36 ***	0.88	-	-	-
Neutral invertase	4.84 *	1.82	9.87 **	7	10	13
Vacuolar invertase	1.11	0.09	23.59 ***	7	16	29
SPS	0.38	-	32.94 ***	13	22	29
Leaf protein	-	2.98	0.67	-	-	-
Bulb protein	1.62	0.96	1.87	15	22	21

[1]ANOVAs were carried out on maximum values of each variable to compare among growth temperatures, except for the following variables: Root DW, Rd, qN, Sucrose, Reducing sugars and Bulb protein where ANOVAs were carried out on minimum values. When there was no obvious peak or plateau, only comparisons of rates of change were performed and vice versa.

Table 2.2 Growth rate (mg day^{-1}) during two different growth phases (see Fig. 2.1) and leaf longevity of *E. americanum* grown at three growth temperatures: 18/14°C, 12/8°C and 8/6°C.

		Temperature		
	Growth phase	18°C/14°C	12°C/8°C	8°C/6°C
Growth rate	1 to 2	11.9	7.4	7.0
(mg day^{-1})	2 to 3	7.7	13.9	24.1
Leaf longevity (days)		29	39	45

Table 2.3 Whole-plant C budget of *E. americanum* grown at three growth temperatures: 18/14°C, 12/8°C and 8/6°C. Total amount of C fixed in leaf and C respired in leaf and bulb was estimated by integrating, over the whole epigeous growth period, the area under the curve for Pn in leaf (Fig. 2.3A) and for respiration in leaf (Fig. 2.4A) and bulb (data not shown), respectively. Total net amount of C incorporated was estimated as leaf C fixed – (leaf C respirated + bulb C respirated) and then compared to the amount of C accumulated in bulb as starch at the end of leaf senescence. C accumulation efficiency was estimated as the ratio of C accumulated as starch/net C incorporated.

	Temperature		
	18°C/14°C	12°C/8°C	8°C/6°C
C fixed by the leaf (mg)	105	167	194
C respired by the leaf (mg)	40.5	48.4	50.2
C respired by the bulb (mg)	12.4	20.7	29.2
Net C incorporated (mg)	52.1	97.9	114.6
Starch accumulated (mg C)	50.1	107.5	116.3
C accumulation efficiency (%)	96	110	101

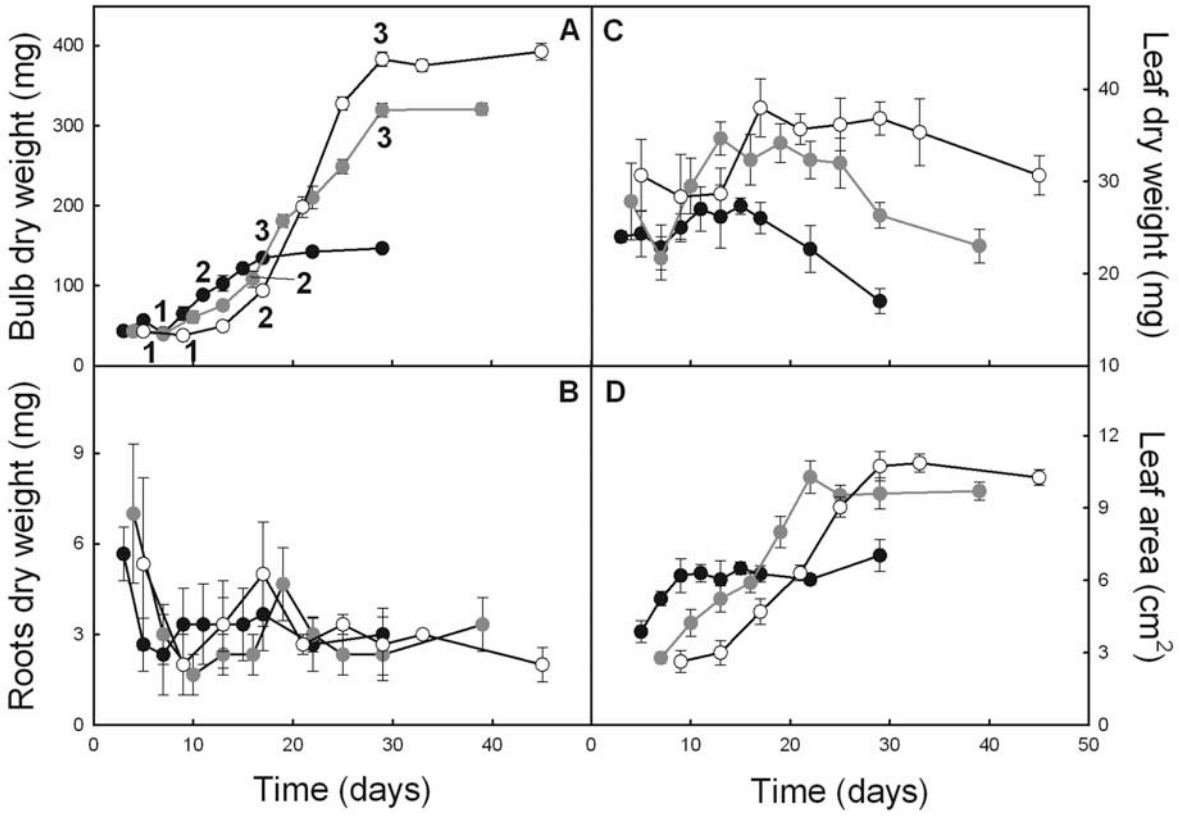

Figure 2.2 Evolution of bulb mass (A), root mass (B), leaf mass (C) and leaf area (D) throughout the epigeous growth period in Erythronium americanum plants grown at 18/14°C (black), 12/8°C (grey) and 8/6°C (white). The first data points correspond to the time at which leaves were completely unfolded. The penultimate data points correspond to the first visual signs of leaf senescence and the last data points correspond to complete leaf senescence. The data points identified 1, 2 and 3 on each curve are the lower and upper thresholds used to estimate two different growth rates (see Table 2.2) during bulb growth. Shown are the means ± SE (N = 2; 6 plants per growing season).

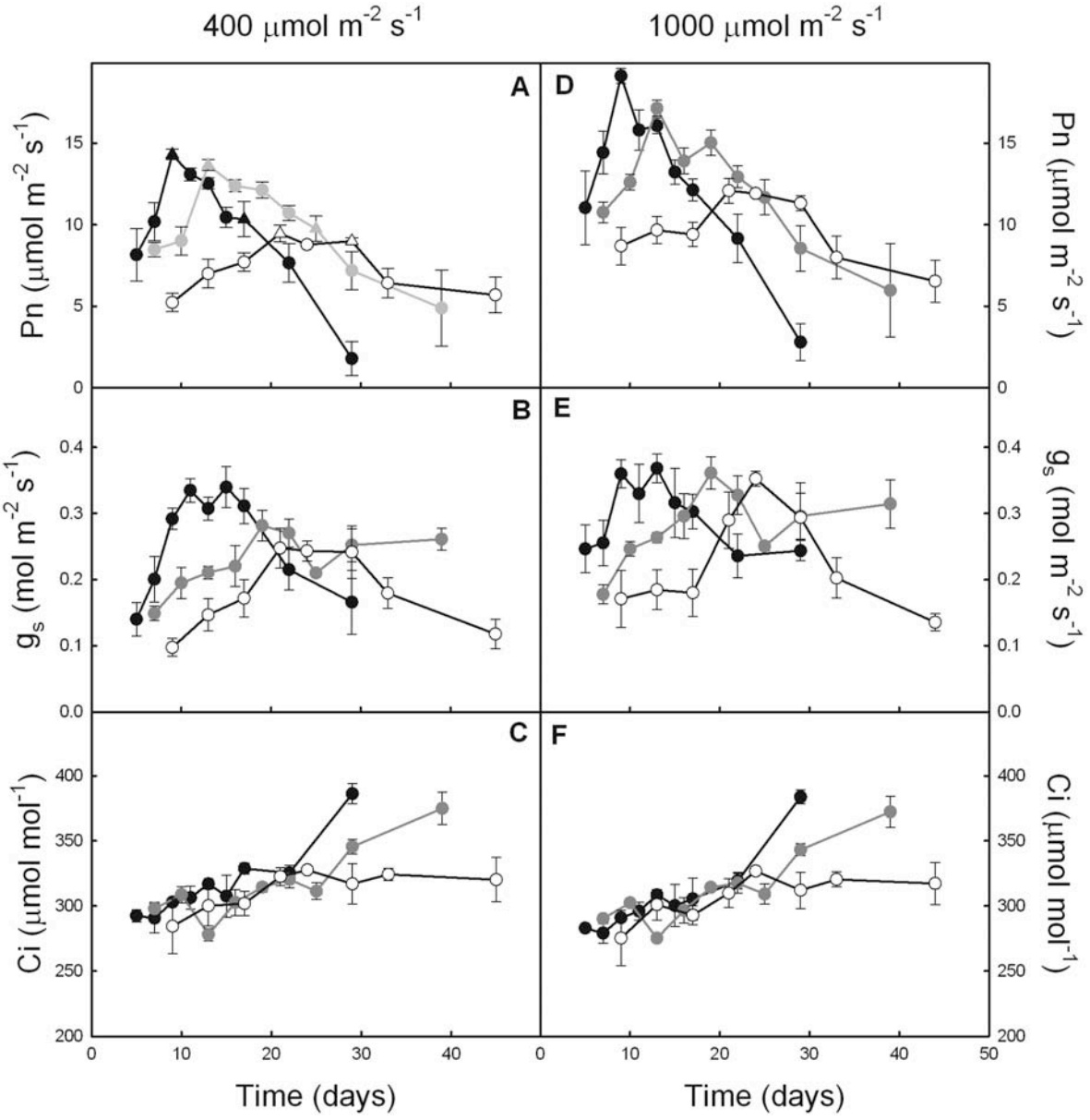

Figure 2.3 Evolution of net photosynthetic rate (A, D), stomatal conductance (B, E) and intercellular CO_2 concentration (C, F) in *Erythronium americanum* plants grown at 18/14°C (black), 12/8°C (grey) and 8/6°C (white). Gas exchange data (mean ± SE) were recorded at both 400 μmol m^{-2} s^{-1} (A, B, C) and at 1000 μmol m^{-2} s^{-1} (D, E, F) (N = 2; 5 plants per growing season). Triangles indicate, for each temperature, the date at which A-Ci curves were carried out.

Pn that was measured under saturating irradiance (i.e. 1000 μmol m^{-2} s^{-1}) exhibited a faster initial increase at warmer temperatures, and a faster decrease after the maximum had been reached (Fig. 2.3D, Table 2.1). Maximum Pn was 10% lower at 12/8°C and 37% lower at 8/6°C than at 18/14°C. Pn was 34% higher under saturating irradiance than under 400 μmol m^{-2} s^{-1} at 18/14°C, whereas Pn was light-stimulated by 25% at 12/8°C and by 27% at 8/6°C. Moreover, the decrease of Pn was 1.3-fold faster under 1000 μmol m^{-2} s^{-1} than under 400 μmol m^{-2} s^{-1} for plants growing at 18/14°C (P<0.041), whereas it was not different between the two PPFDs at the two cooler temperatures (12°C: P=0.185; 8°C: P=0.219). G_s and Ci that were measured under saturating irradiance exhibited a similar evolution over time as under growth irradiance conditions. Growth temperature affected g_s only when measured under growth irradiance conditions; g_s measured under saturating light conditions and Ci under both light conditions were unaffected (Fig. 2.3E, F).

Leaf respiratory rate tended to increase over time at 18/14°C, whereas it decreased continuously at cooler temperatures (Fig. 2.4A). However, respiratory rates did not significantly differ with growth temperature and increased during leaf senescence. The amount of C lost through respiration throughout the epigeous growth period increased both in the leaf and in the bulb as growth temperature decreased (Table 2.3). The amount of C that accumulated in the bulb as starch represented 96%, 110% and 101% of net C incorporated by plants growing at 18/14°C, 12/8°C and 8/6°C, respectively. Thus, almost all net C assimilated by the plant was stored in the bulb as starch. The accumulated amounts of C were thus very similar to the net amounts that were incorporated, suggesting that the punctual gas exchange measurements reported here truly reflect the evolution of plant gas exchange throughout the season.

Photorespiration reached maximum rates soon after leaf unfolding, which was 13% lower at 12/8°C and 47% lower at 8/6°C, compared to 18/14°C (Fig. 2.4B, Table 2.1). Photorespiratory rate then decreased until complete leaf senescence at warmer temperatures, whereas it remained fairly constant at 8/6°C. Photorespiratory rates exhibited changes through time similar to Pn, except at 8/6°C where Pn slowly decreased while photorespiratory rates remained fairly constant.

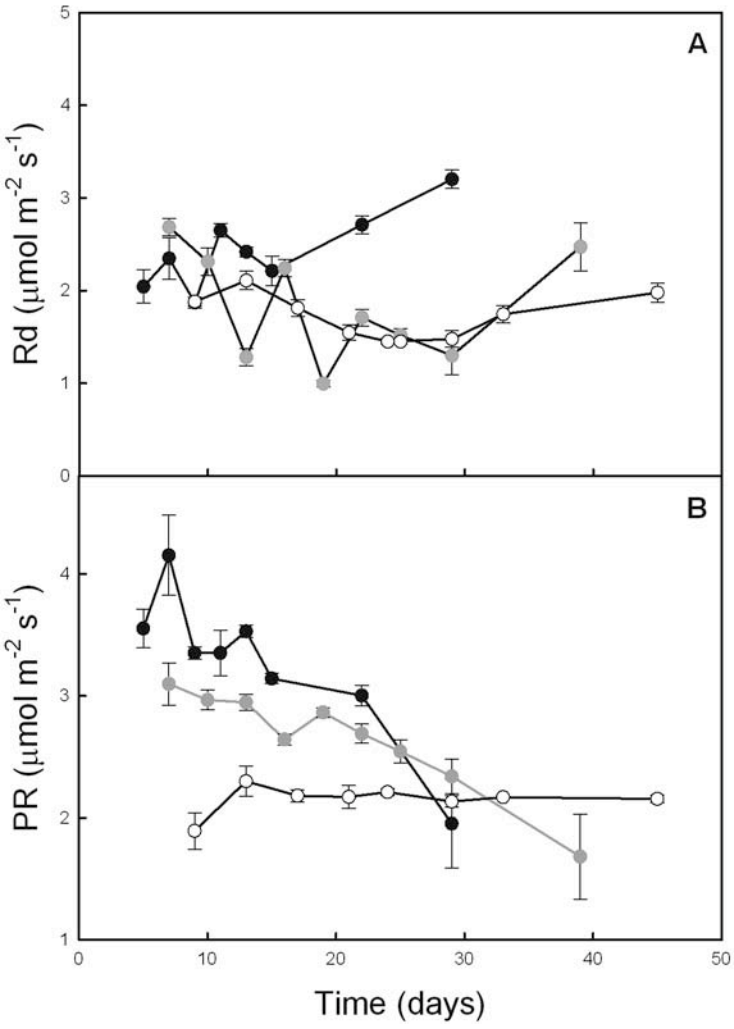

Figure 2.4 Evolution of leaf respiratory (A) and photorespiratory rate (B) over time in *Erythronium americanum* plants grown at 18/14°C (black), 12/8°C (grey) and 8/6°C (white). Photorespiratory rates were estimated from measures taken at 400 µmol m^{-2} s^{-1}. Shown are the means ± SE (N = 2; 5 plants per growing season).

A-Ci curves indicated that Vc_{max}, J_{max} and TPU increased with growth temperature when measured at the time Pn was maximal (Table 2.4). However, a few days prior to the beginning of leaf senescence, Vc_{max} and J_{max} strongly decreased at the two warmer temperatures to the point that both rates were similar among temperature regimes. TPU was even more strongly decreased at warm temperatures than at cold temperature, leading to lower TPU as growth temperature increased.

2.5.3 Chlorophyll fluorescence

At 400 μmol m^{-2} s^{-1}, qP was fairly constant over the first days of growth (Fig. 2.5A), while qP was 8% lower at 12/8°C and 22% lower at 8/6°C than at 18/14°C (Table 2.1). Photochemical quenching then decreased after 9, 13 and 29 days at 18/14°C, 12/8°C and 8/6°C, respectively. Indeed, qP remained fairly constant throughout the growing season until the initiation of leaf senescence in plants growing at 8/6°C, whereas it decreased at the two warmer temperatures and the negative slope was more pronounced at the warmest temperature. In contrast, qN exhibited an earlier and greater increase at 18/14°C than at 12/8°C, whereas at 8/6°C, it remained fairly constant throughout the season and higher than at the two other temperature regimes (Fig. 2.5B). Maximum quantum efficiency of PSII (Fv/Fm) increased during the first few days at the three temperatures (Fig. 2.5C). Maximum Fv/Fm was reached at day 13 at 18/14°C and 12/8°C and at day 24 at 8/6°C, and increased with growth temperature. Thereafter, Fv/Fm decreased faster at higher temperature until complete leaf senescence. Fv/Fm at 8/6° C appeared relatively constant from day 13 onwards. Nevertheless, except in completely senesced leaves, Fv/Fm was always above 0.73, suggesting a fully functional photosynthetic apparatus under all three growth temperature conditions. Electron flux density through PSII (PhiPS2) exhibited a faster increase at 18/14°C than at 12/8°C during the first days, whereas it was stable at 8/6°C until day 29 (Fig. 2.5D). Maximum PhiPS2 was 9% lower at 12/8°C and 35% lower at 8/6°C than 18/14°C. Thereafter, PhiPS2 decreased faster as temperature increased. The reduction in qP and PhiPS2 through time matched the reductions observed with Pn (Fig. 2.2A).

2.5.4 Carbohydrates

Starch concentrations in the bulb decreased at all temperatures during leaf unfolding (Fig. 2.6A). Thereafter, bulb starch concentration increased with time, but the duration

Table 2.4 Parameters estimated from A-Ci curves for *E. americanum* grown at three growth temperatures: 18/14°C, 12/8°C and 8/6°C. Maximum rate of carboxylase activity of Rubisco (Vc_{max}), maximum electron flux (J_{max}) and triose phosphate utilization (TPU) are indicated. Values represent the means of 3 measures ± SE.

	Temperature			**ANOVA**
	18°C/14°C	12°C/8°C	8°C/6°C	*P*-value
A-Ci curves at maximum Pn[1]				
Vc_{max} (µmol m^{-2} s^{-1})	37.3 ± 0.7	27.1 ± 3.8	20.3 ± 2.1	0.004
J_{max} (µmol m^{-2} s^{-1})	123 ± 1	111 ± 1	105 ± 2	0.024
TPU (µmol m^{-2} s^{-1})	9.33 ± 0.11	7.09 ± 1.04	6.22 ± 0.68	<0.001
A-Ci curves before leaf senescence[1]				
Vc_{max} (µmol m^{-2} s^{-1})	21.0 ± 0.9	22.1 ± 1.1	20.6 ± 1.4	0.523
J_{max} (µmol m^{-2} s^{-1})	95.4 ± 1.3	89.9± 0.9	92.5 ± 1.8	0.391
TPU (µmol m^{-2} s^{-1})	4.48 ± 0.52	5.42 ± 0.99	5.99 ± 0.92	0.037

[1]see triangles on Figure 2.2A

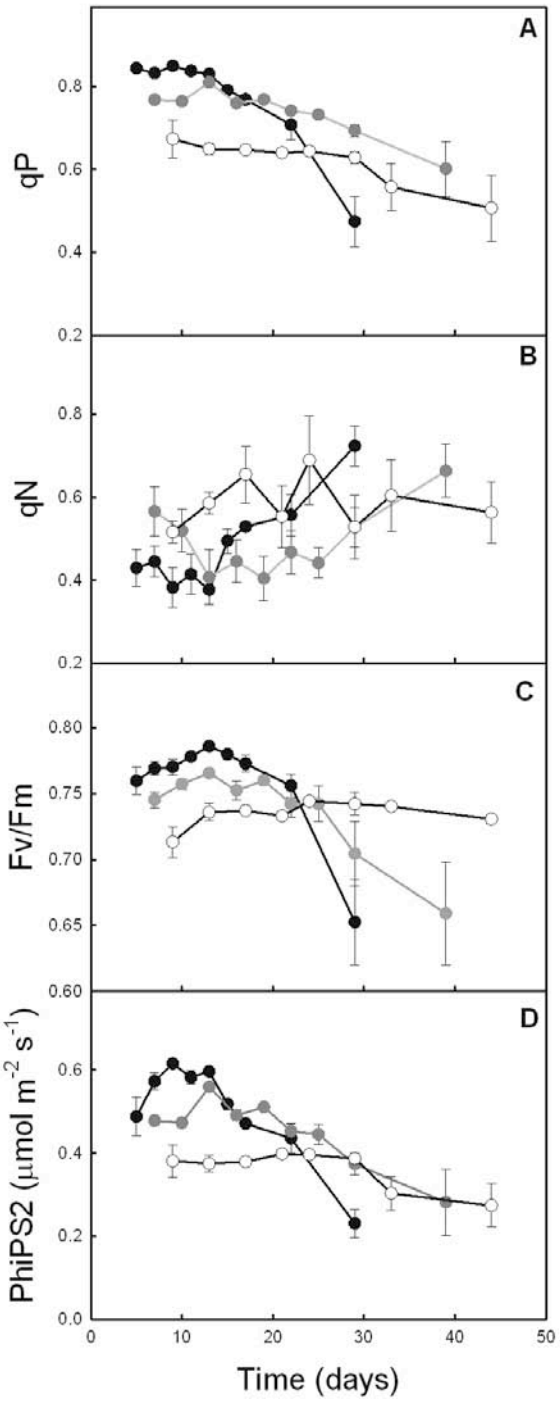

Figure 2.5 Evolution of photochemical quenching (A), non-photochemical quenching (B), PSII maximum efficiency (C) and electron flux across PSII (D) over time in *Erythronium americanum* plants grown at 18/14°C (black), 12/8°C (grey) and 8/6°C (white). Fluorescence data (mean ± SE) were recorded at 400 µmol m^{-2} s^{-1} (N = 2; 5 plants per growing season).

of starch accumulation differed among temperature treatments. Starch accumulated during 12, 18 and 20 days at 18/14°C, 12/8°C and 8/6°C, respectively. Starch accumulation rate increased with temperature, but final starch concentration, around 88% of bulb dry mass, was similar among temperatures (Table 2.1). As shown previously (Lapointe and Lerat, 2006), starch was the main carbohydrate reserve stored in the bulb of *E. americanum.* Starch reached maximum concentration a few days before the initiation of leaf senescence regardless of growth temperature. In contrast, changes in soluble sugar concentration in the bulb – sucrose and reducing sugars – indicated a continuous decline over time in all treatments (Fig. 2.6B, C). The proportion of starch present as amylose increased continuously until leaf senescence, with no significant difference among temperatures in terms of its rate of change (Fig. 2.6D). The increase lasted longer at cooler temperatures, leading to a maximum 7% higher at 12/8°C and 15% higher at 8/6°C before leaf senescence than at 18/14°C. During leaf senescence, amylose decreased at the three growth temperatures and reached similar final content. Given the dilution effect caused by increasing starch concentrations, soluble sugar concentrations were also presented as a function of bulb biomass from which starch accumulation has been subtracted (mg g^{-1}DW-Starch, Fig. 2.6E, F). These values are likely more representative of the concentration that can be sensed by the plant. Starchless soluble sugar indicated a transient increase in concentration at the time starch stopped accumulating. An initial peak was more obvious for reducing sugar than for sucrose and the height of this peak increased with growth temperature. Moreover, high soluble sugar concentrations were maintained until complete leaf senescence at 18/14°C, while they decreased at the two lower temperatures.

2.5.5 Soluble protein concentration

The initial concentration of total soluble proteins in the leaf appeared fairly similar among growth temperatures and exhibited a constant decrease over time (Fig. 2.7A). Therefore, reduction of leaf protein did not match with the reduction of Pn over time. In the bulb, protein concentration was maximal soon after leaf unfolding and then decreased until a few days before leaf senescence (Fig. 2.7B). No difference among treatments was observed when bulb protein concentration was at a minimum, at 9 days

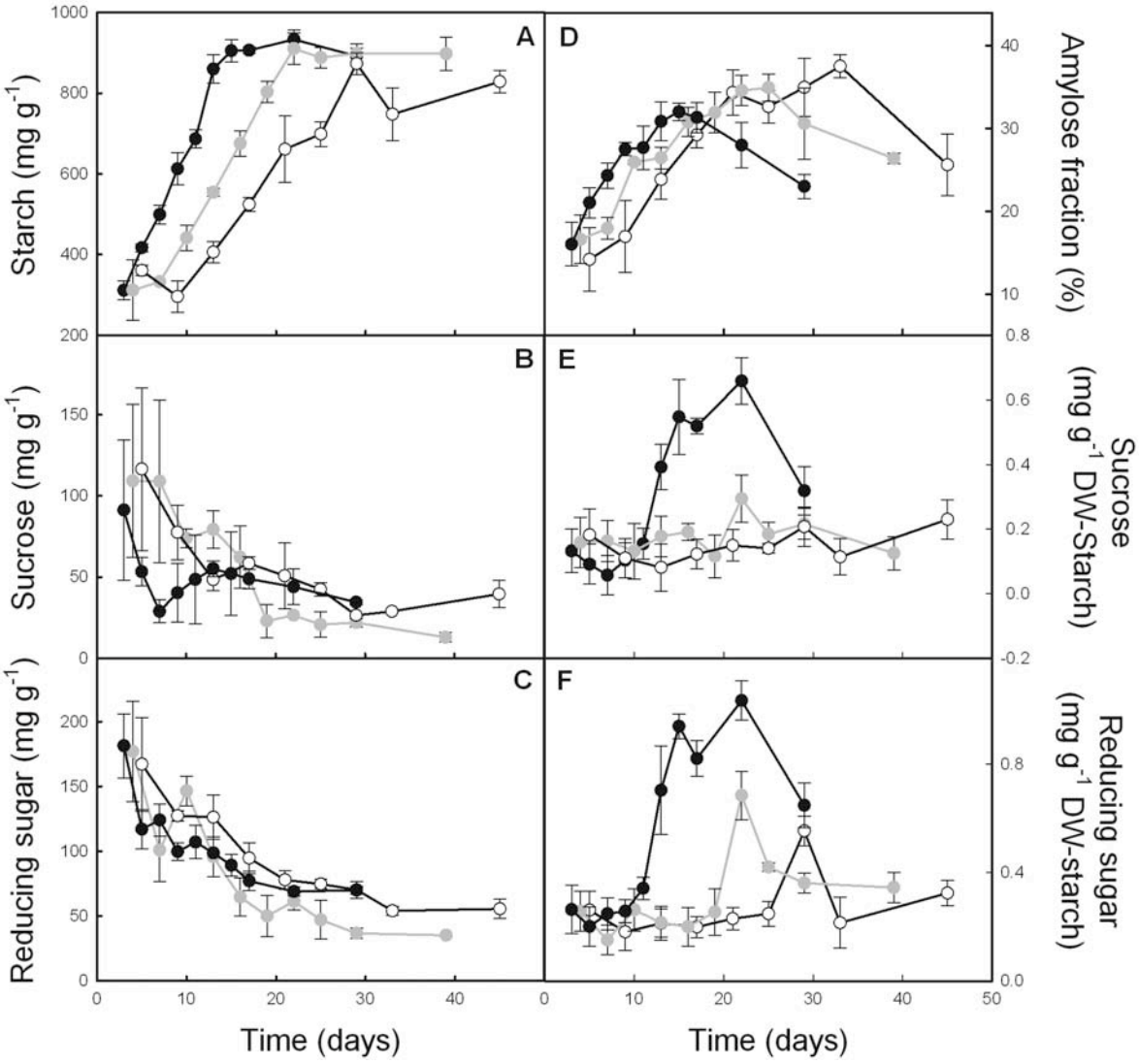

Figure 2.6 Evolution of starch (A), sucrose (B, E) and reducing sugar concentrations (C, F) and fraction of amylose (D) over time in the bulb of *Erythronium americanum* plants grown at 18/14°C (black), 12/8°C (grey) and 8/6°C (white). Concentration of sucrose and reducing sugars are presented as a function of total bulb biomass (mg g^{-1}; B, C) and as a function of bulb biomass other than starch (mg g^{-1}DW-Starch; E, F). Shown are the means ± SE of 3 plants.

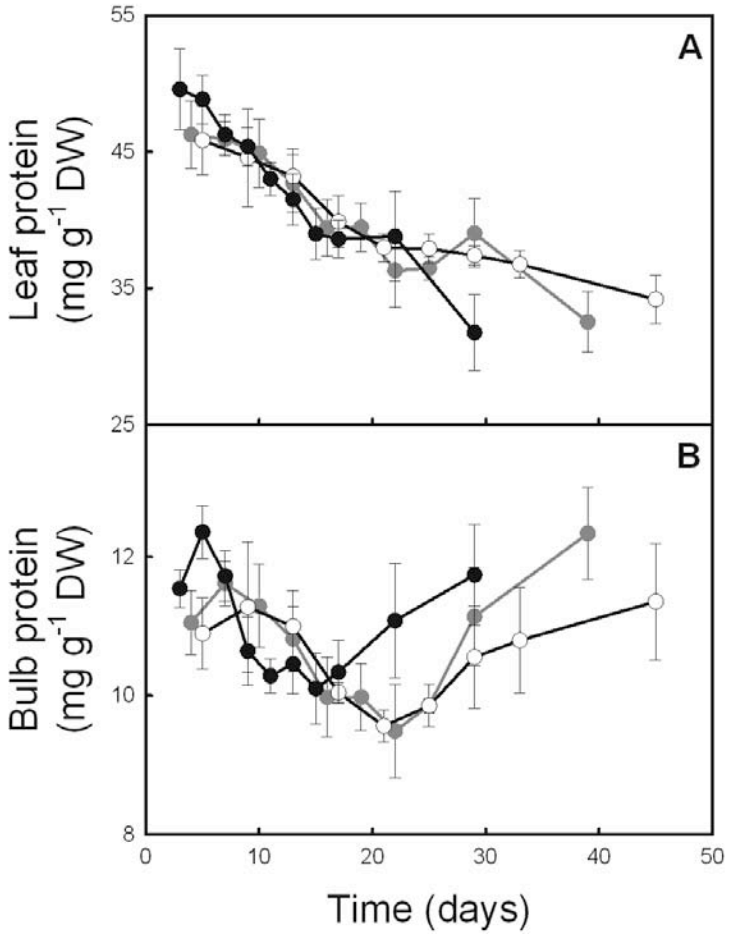

Figure 2.7 Evolution of leaf (A) and bulb (B) protein concentration over time in *Erythronium americanum* plants grown at 18/14°C (black), 12/8°C (grey) and 8/6°C (white). Shown are the means ± SE of 3 plants.

at 18/14°C and after 22 and 21 days at 12/8°C and 8/6°C, respectively. Neither the final increase nor the final concentration of protein was affected thereafter by temperature.

2.5.6 Enzymes of carbon metabolism

In the leaf, F1,6BPase activity exhibited a small increase after leaf unfolding, leading to maximum activity, which was maintained during most of the epigeous growth phase (Fig. 2.8A). Maximum activity of F1,6BPase was 9% lower at 12/8°C and 20% lower at 8/6°C than 18/14°C (Table 2.1). F1,6BPase decreased drastically after 17, 25 and 33 days at 18/14°C, 12/8°C and 8/6°C, respectively, i.e., a few days prior to the first visual sign of leaf senescence. In the bulb, AGPase activity increased during the first few days to reach maximal activity early in the growing season (Fig. 2.8B). The initial increase tended to be faster at warmer temperatures, although there was no significant difference among the treatments ($P = 0.086$). Thereafter, activity remained constant over time until complete leaf senescence, with no difference among growth temperatures. Susy activity increased strongly during the first few days of the growth season and reached a maximum at day 13, 22 and 33 at 18/14°C, 12/8°C and 8/6°C, respectively (Fig. 2.8C). The increase was significantly faster as growth temperature increased. Moreover, maximum Susy activity increased with growth temperature. In contrast, CWInv activity was high at leaf unfolding, with no difference among growth temperatures (Fig. 2.8D). Thereafter, the activity of the CWInv decreased continuously until complete leaf senescence. The decrease occurred faster at warmer temperatures. NInv increased quickly after leaf unfolding, exhibiting a maximum rate at day 7, 10 and 13 at 18/14°C, 12/8°C and 8/6°C, respectively (Fig. 2.8E). The increase was significantly faster and the maximum higher as growth temperature increased. Yet the decrease of NInv activity, which occurred shortly after it had reached maximum values, was not affected by growth temperature. VInv activity increased during the first few days to reach a maximum at day 7, 16 and 29 at 18/14°C, 12/8°C and 8/6°C, respectively (Fig. 2.8F). Maximum activity of VInv was maintained for a longer period of time at 18/14°C than at the two cooler temperatures. Contrary to most other enzymes, the maximum rate attained by VInv decreased with an increase in growth temperature. Neither the initial increase nor the decrease before leaf senescence was affected by growth temperature. Finally, SPS started to increase as soon as plants were moved to the growth cabinets at a similar rate at all three growth temperatures (Fig. 2.8G). SPS activity reached a

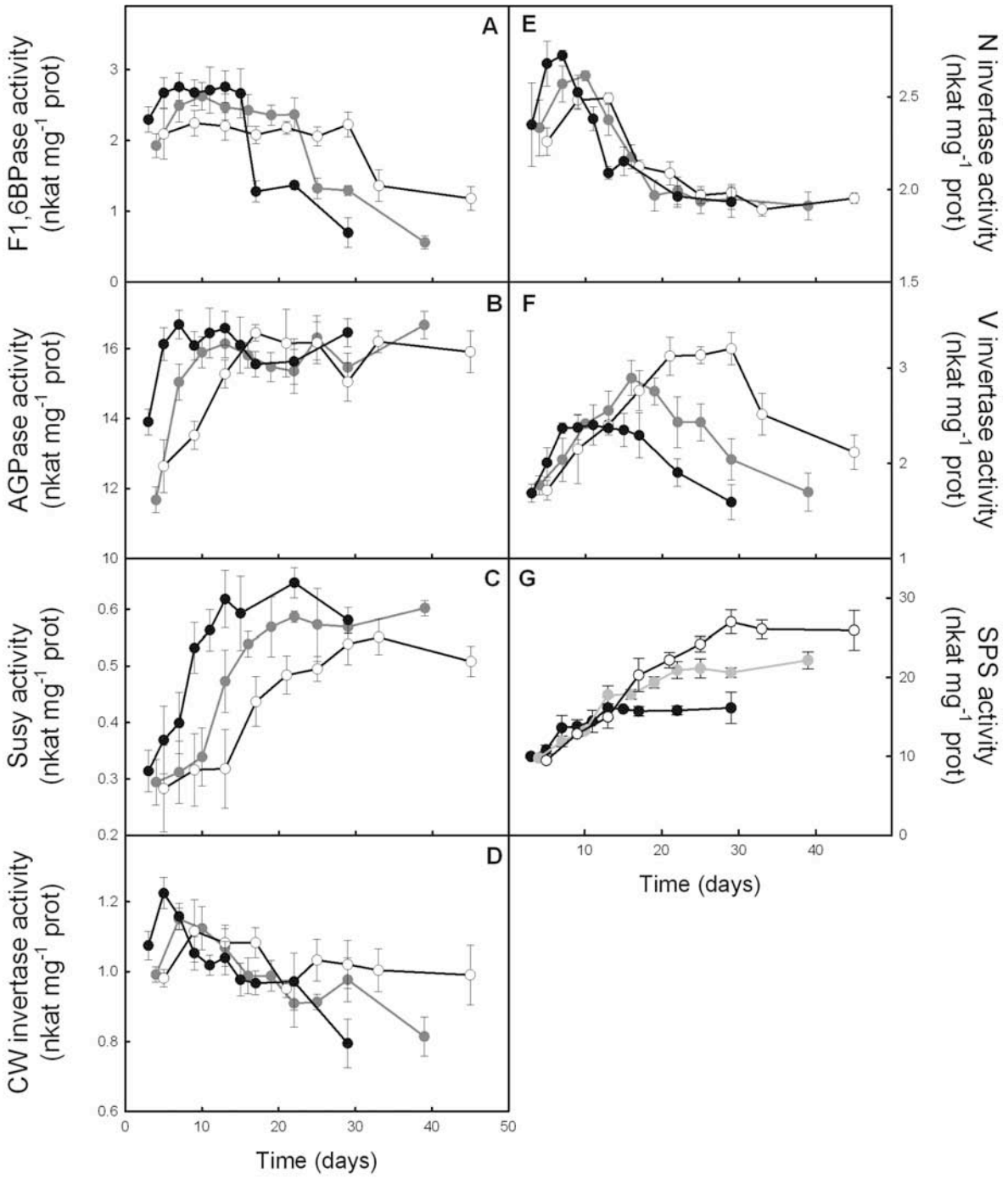

Figure 2.8 Activities of fructose-1,6-bisphosphatase (A) in the leaf and of ADP-glucose pyrophosphorylase (B), sucrose synthase (C), cell wall invertase (D), neutral invertase (E), vacuolar invertase (F) and sucrose phosphate synthase (G) throughout the growth period in the bulb of *Erythronium americanum* plants grown at 18/14°C (black), 12/8°C (grey) and 8/6°C (white). Shown are the means ± SE of 3 plants.

maximum value earlier and was thus lower as growth temperature increased. The maximum activity of SPS was maintained until complete leaf senescence.

The activity of each sucrose hydrolytic enzyme presented a different pattern of activity overtime. CWInv was the first one to be fully activated, replaced a few days later by maximum NInv activity. A few days later, while NInv activity was declining, VInv reached a maximum, which was maintained until a few days before leaf senescence. Once the activity of the VInv started to decline, Susy reached maximum activity, which remained high until the end of the epigeous growth period. Susy activity exhibited the lowest rates among the four sucrose hydrolytic enzymes. Growth temperature stimulated the maximum activity of Susy and NInv but reduced the activity of the VInv and SPS, suggesting that these two latter enzymes might play a role in the overall growth stimulation of the bulb at lower temperature.

2.5.7 Bulb cell growth

Cell growth rate tended to increase with temperature (P = 0.067), but cell expansion stopped much earlier as temperature increased (Fig. 2.9). The final size was thus reached much more rapidly at high temperature, leading to a smaller cell size at the end of the epigeous growth period at the warmer temperatures than at the cooler ones (Table 2.1).

2.6 Discussion

Low growth temperatures strongly increased the final bulb biomass, as well as leaf longevity. Reports of positive effects of low temperature on growth are scarce in temperate zone higher plants and even in arctic herbs. Nevertheless, some high-arctic species exhibit optimal growth at temperatures around 12°C (Heide, 1992; Heide and Gauslaa, 1999), but this has also been shown in some onion cultivars (Daymond *et al.*, 1997). Moreover, positive responses of plant growth to low growth temperatures have been previously reported for *E. americanum* (Lapointe and Lerat, 2006), *Crocus vernus* (Badri *et al.*, 2007) and a few other ornamental spring geophytes (De Hertogh and Le Nard, 1993). Our results thus confirmed the larger bulb size at 12/8°C than at 18/14°C (Lapointe and Lerat, 2006) and in addition, presented evidence that the bulb can even be larger when the plant is grown at 8/6°C. The initial growth rate increased with growth

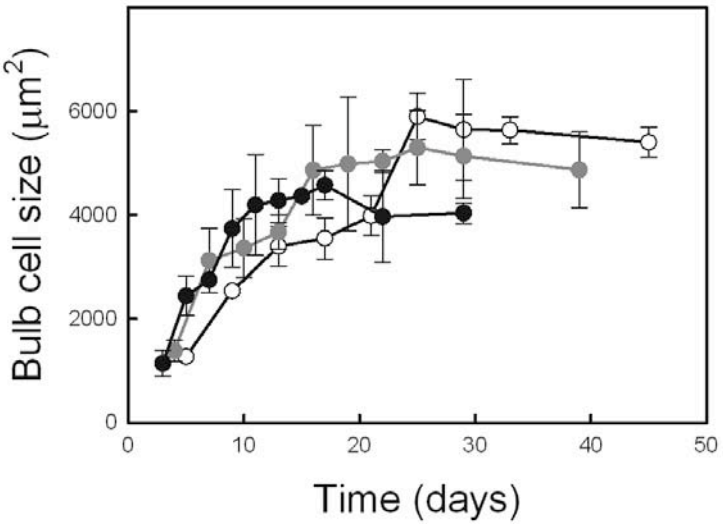

Figure 2.9 Evolution of bulb cell size in *Erythronium americanum* plants grown at 18/14°C (black), 12/8°C (grey) and 8/6°C (white). Shown are the means ± SE of 3 plants.

temperature, but bulb growth rate slowed down earlier at the warmest growth temperature, several days before the first visible signs of leaf senescence. Furthermore, at the time the leaf started to senesce at 18/14°C (22 days), the bulb was already larger at either 12/8°C or 8/6°C than at 18/14°C. These results support the idea that the positive effect of low growth temperature on bulb growth is partially independent of its effect on leaf life duration (Badri *et al.*, 2007).

Pn increased more rapidly and reached a higher maximum at warmer temperatures than at cooler ones. Yet, given the difference in leaf lifespan, there was more C incorporated over the whole epigeous growth period at lower temperatures, despite the greater cumulative losses of C by photorespiration and respiration. This greater net amount of C was thus available for starch synthesis and storage in the bulb. Carbohydrates are apparently more rapidly photosynthesized and available for translocation towards the bulb at 18/14°C than at cooler growth temperatures, but the shorter leaf lifespan reduced the total amount of C that was translocated to the bulb. G_s evolved closely with Pn to maintain fairly constant Ci, not only throughout the season but also across temperatures. High g_s and a high transpiration rate are usual in spring ephemerals (Taylor and Pearcy, 1976) and allow them to maximize C fixation within a limited time frame (Muller, 1978). Stomatal limitation does not occur in *E. americanum* plants in controlled environments, and thus, it appears that factors other than stomatal resistance would explain the modulation of Pn over time.

The present results indicated a sequential induction through time of sucrose-cleaving enzymes, which constitutes the first step in C metabolism at the sink level. Invertase activities peaked at the beginning of the growth period — by order, CWInv, NInv and VInv — while bulb development was initiated and starch accumulated, whereas Susy reached its maximum a few days before leaf senescence, at a time when starch concentrations had reached maximum values and cell growth had strongly slowed down. Invertase contributions are well known to occur during initiation and cell expansion of the sink, whereas Susy contribution occurs during cell maturation (Koch, 2004). Among the sucrose-cleaving enzymes measured, the activity of Susy was activated far earlier at high compared to low growth temperatures, suggesting early maturation of bulb cells. Indeed, cell size measurements indicated a shorter duration of cell expansion and earlier cell maturation as growth temperature increased, whereas the

expansion rate was only slightly affected by temperature. Timing of induction of the different sucrose-cleaving enzymes seems to induce a faster shift from elongation to maturation in *E. americanum* bulb cells growing at warmer temperatures. A similar fast shift was suggested for *Crocus vernus* (Lundmark *et al.*, 2009) in association with an earlier increase in C allocation to cell walls at warmer growth temperature. An increased allocation to cell walls would thus fit with earlier induction of the activity of Susy at warmer temperatures, given the close relation between Susy and cellulose synthase activities (Amor *et al.*, 1995). The early contribution of Susy appears to lead to an overall smaller sink capacity at higher growth temperatures than at lower growth temperatures.

In the bulb, starch was initially consumed to provide C required for new bulb formation and leaf expansion but thereafter, it largely accumulated. Starch accumulated more quickly as growth temperature increased. Starch synthesis is generally stimulated at warmer temperatures, as reported for potato tuber (Geigenberger *et al.*, 1998). The increased activity of VInv as growth temperature decreased suggests sucrose accumulation in the vacuole at lower growth temperatures. VInv is a well-known determinant of sink strength (Tang *et al.*, 1999). SPS activity also increased at lower temperature. Nguyen-Quoc and Foyer (2001) suggest that Vinv and SPS activities may be involved in a futile cycle, inducing sucrose degradation and resynthesis. Induction of such cycle could restrict the availability of the hexose pool for starch synthesis, supporting the slower starch accumulation that we measured at low growth temperatures, despite similar AGPase capacity across temperature treatments. Furthermore, Geigenberger et al. (2004) suggest that starch synthesis is not mainly controlled by the activity of AGPase, but by the ability to import ATP – necessary for ADP-glucose synthesis – from the cytosol into the amyloplast. Mitochondrial respiration, which is the main producer of ATP in heterotrophic storage organs, is certainly stimulated at higher temperatures. The higher availability in ATP due to this stimulation could support the higher rate of starch accumulation at higher temperatures. Thus, the induction of the futile cycle combined with a lower availability in ATP appears to be two key factors explaining the slower starch accumulation in *E. americanum* bulbs, which were growing at lower temperatures. Yet, more rapid starch accumulation at warmer growth temperatures was face to limited sink capacity induced

by early cell maturation. This would explain the earlier cessation of starch accumulation as temperature increased. Moreover, the proportion of amylose decreased at the time that growth in cell size stopped and starch accumulation strongly slowed down, in favour of an increasing proportion of amylopectin. Amylose is interspersed with amylopectin in amorphous regions of starch and depends on the space available within the matrix of amyloplast (Denyer *et al.*, 2001). Changes in the proportion of amylose without changes in starch concentration suggest that starch synthesis continued but in presence of a similar rate of starch degradation. This could explain why AGPase activity remained high up to the end of the epigeous growth despite no further increase in starch concentration.

Under saturating light conditions, Pn decreased more rapidly at the warmest temperatures than under growth light conditions, suggesting an inhibition of the photosynthetic carboxylation phase over time. In the spring ephemeral *Podophyllum peltatum*, the maximum rate of rubisco carboxylation decreases with leaf ageing and as starch concentration in rhizomes increase (Constable *et al.*, 2007). In the present study, Vc_{max} and TPU decreased to a greater extent over time at the warmer temperatures, such that TPU became lower at warm than at cooler temperatures a few days before leaf senescence. A lower TPU is generally due to a reduction of photosynthetic product transport, which induces Pi-limitation of triose-phosphate exportation (Sharkey *et al.*, 2007). The export of photosynthetic product thus appears to be inhibited earlier as growth temperature increased. Moreover, F1,6BPase activity strongly decreased a few days before the initiation of leaf senescence, regardless of growth temperature. F1,6BPase is usually inhibited in response to fructose-2,6-bisphosphate synthesis, which is stimulated when sucrose translocation decreases (Paul and Foyer, 2001). The accumulation of soluble sugars in the bulb, as shown by the starchless concentrations of sucrose and reducing sugars, suggest a reduction in sink strength which could trigger the inhibition of carbohydrate translocation from source to sink. This decrease in the sink strength, once bulb cells were filled with starch (88%), would induce the decrease in F1,6BPase activity and that of TPU, potentially leading to leaf senescence.

Even though leaf protein concentration decreased steadily during the growth cycle, it cannot explain the reduction in Pn since leaf protein concentrations decreased at a similar rate at the three temperatures, whereas the decrease in Pn occurred earlier and

much faster at warmer temperatures. Furthermore, total leaf protein content decreased later in the season (data not shown) suggesting that the initial decrease in protein concentration was due to leaf expansion rather than net protein degradation. Photochemical quenching decreased earlier and faster as temperature increased. A higher proportion of absorbed photons was lost through non-radiative energy dissipation mechanisms, instead of being used to drive photosynthesis (Maxwell and Johnson, 2000). Although the response of qN was not as clear as the response of qP, it also suggested that Pn was down-regulated much sooner at warmer than at cooler temperatures. Moreover, effective quantum yield of PSII (PhiPS2) decreased more rapidly as growth temperature increased. This fast decrease could be due to lower triose phosphate utilisation that induces a large pH gradient known to inhibit electron transport rates (Pammenter *et al.*, 1993). Thus, it appears that the rate of C assimilation at warmer temperatures, which rapidly became too high for the capacity to incorporate C in the sink, led to feedback inhibition of Pn. Maximum quantum yield of PSII (Fv/Fm) remains fairly constant until leaf senescence, suggesting that the decrease of qP and PhiPS2 was not due to an alteration of thylakoid function, which was affected only after senescence had been initiated. In single-rooted sweet potato (*Ipomoea batatas* cv. Koganesengan), Sawada et al. (2003) suggested that photosynthetic activity depends on the development of sink activity, highlighting feedback effects of sink activity on source activity. Early decreases in Pn at warmer temperatures could be a response to an imbalance between source and sink activity. This study provides evidence that the decrease in sink strength, once final sink capacity has been reached, induces an accumulation of soluble sugars in the sink, which in turn triggers leaf senescence. Further studies are necessary to explain the cascade of events leading to the modulation of Pn early in the season and identify the metabolic links between source-sink balance and rate of C assimilation.

In conclusion, the production of a larger storage organ at low temperatures was confirmed in *Erythronium americanum* by including plants grown under the 8/6°C regime. Stimulation of VInv and SPS activities at lower temperatures potentially induce a 'futile cycle' of sucrose degradation and resynthesis in the bulb. This 'futile cycle', when combined with the lower rate of C fixation at lower temperatures, would explain slower bulb starch accumulation at lower temperature, despite similar levels of AGPase

activity at all temperatures. Meanwhile, the delayed induction of sucrose synthase at lower temperatures would lengthen the cell elongation phase in the bulb, leading to a greater sink capacity. Thus, the higher sink activity and the smaller sink capacity observed at higher temperatures would lead to earlier starch saturation of bulb cells. Soluble sugars would then accumulate in the bulb, which could be the signal triggering leaf senescence via a decrease in F1,6BPase activity a few days ahead of the first visual sign of senescence. Starch accumulation during the epigeous growth period was slower at lower temperatures, but this rate was apparently more in rhythm with the evolution of sink capacity, leading to less source-sink imbalance and the production of a larger bulb at the end of the epigeous growth period.

2.7 Acknowledgements

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Chapitre 3 The alternative respiratory pathway allows sink to cope with changes in carbon availability in the sink-limited plant *Erythronium americanum*

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3.1 Résumé

Les mécanismes qui permettent aux plantes de faire face à un surplus récurrent de carbone, lors d'un déséquilibre entre l'activité de la source et l'activité du puits, ont été peu étudiés à ce jour, en conditions de limitation par le puits. La réponse de la croissance et du métabolisme du puits à la modulation de l'activité de la source a été étudiée chez *Erythronium americanum* en soumettant les plantes à une forte concentration en CO_2 et en O_3 durant leur croissance. L'activité du puits a été mesurée via l'analyse du taux de respiration au sein des tissus et des mitochondries, de l'activité d'hydrolyse du saccharose, de l'accumulation d'hydrates de carbone et de biomasse et ce, tout au long de la saison de croissance. L'activité de la source a, quant à elle, été mesurée via l'analyse des échanges gazeux, des activités de la rubisco et de la phosphoenolpyruvate carboxylase, de l'accumulation d'hydrates de carbone et du taux de respiration. La forte concentration en CO_2 a augmenté le taux de photosynthèse net en augmentant la disponibilité en substrat pour la rubisco. La forte concentration en O_3 a diminué le taux de photosynthèse net en réduisant principalement l'activité de la rubisco. Malgré cette modulation de l'activité de la source, ni la croissance de la plante, ni l'accumulation d'amidon n'ont été affectés par les traitements. L'activité de la saccharose synthase dans le puits était plus élevée sous fort CO_2 et plus faible sous fort O_3, modulant par conséquent la quantité d'intermédiaires glycolytiques. La voie alternative de la respiration a été modulée de manière similaire à la saccharose synthase dans le puits, comme montré par l'activité et la capacité de la voie alternative, ainsi que par l'abondance de l'oxydase alternative. Chez cette espèce limitée par le puits, la voie alternative de la respiration semble ajuster l'approvisionnement en carbone de la synthèse d'amidon avec la capacité du puits à stocker cet amidon, empêchant ainsi un rétrocontrôle précoce de la photosynthèse en conditions d'excès de carbone.

3.2 Abstract

Mechanisms that allow plants to cope with a recurrent surplus of carbon in conditions of imbalance between source and sink activity has not received much attention. The response of sink growth and metabolism to the modulation of source activity was investigated using elevated CO_2 and elevated O_3 growth conditions in *Erythronium americanum*. Sink activity was monitored via slice and mitochondrial respiratory rates, sucrose hydrolysis activity, carbohydrates, and biomass accumulation throughout the growth season, while source activity was monitored via gas exchanges, rubisco and phosphoenolpyruvate carboxylase activities, carbohydrates, and respiratory rates. Elevated CO_2 increased the net photosynthetic rate by increasing substrate availability for rubisco. Elevated O_3 decreased the net photosynthetic rate mainly through a reduction in rubisco activity. Despite this modulation of the source activity, neither plant growth nor starch accumulation were affected by the treatments. Sucrose synthase activity was higher in the sink under elevated CO_2 and lower under elevated O_3, thereby modulating the pool of glycolytic intermediates. The alternative respiratory pathway was similarly modulated in the sink, as seen with both the activity and capacity of the pathway, as well as with the alternative oxidase abundance. In this sink-limited species, the alternative respiratory pathway appears to balance carbon availability for starch synthesis with sink capacity to store it, thereby avoiding early feedback-inhibition of photosynthesis in conditions of excess carbon availability.

3.3 Introduction

During growth, source tissues are photosynthetically active and export synthesized carbohydrates to photosynthetically less active or inactive sink tissues, such as roots, fruits, tubers or bulbs (Dickson, 1991). Sinks are thus characterized by a net importation of carbohydrates, which are used for growth, maintenance, and storage. The ability of a sink organ to import assimilates, also referred to as sink strength, is determined by both sink size and sink activity (Farrar, 1993). Strengths of the different sinks define, in turn, carbon (C) allocation patterns at the whole plant level. C allocation patterns change throughout the season, following plant developmental stages, but many abiotic factors can also modify C allocation pattern through changes in the amount of C fixed by the plant.

C allocation in plants with storage organs has often been studied through modulation of the source activity by altering either light levels (Kehr et al., 1998) or gas concentrations (i.e. increasing level of CO_2 and O_3, Balaguer et al., 1995; Andersen, 2003). Stimulation of source activity translates into higher photosynthetic rates, leading to an increased supply of carbohydrates. Excess C is usually allocated to the sinks, leading to more accumulation of reserves in the storage organs (Balaguer et al., 1995). This is the case for source-limited plants. In sink-limited species, photosynthetic rates are modulated more extensively by sink C-demand than by abiotic factors (Sawada et al., 2003), leading to complex physiological and biochemical controls of source activity. A decrease of assimilate utilization by sinks generally leads to the accumulation of sucrose or starch in leaves, which then decreases photosynthesis through feedback inhibition (Paul and Foyer, 2001). However, sugar accumulation in either source or sink organs can also stimulate respiration (Amthor, 1991) and avoid feedback inhibition of different C metabolism pathways. Indeed, as respiration is central to all C metabolism pathways, it could play a key role in C exchanges between source and sink.

Increasing respiratory rates can cause an over-reduction of some of the electron transport chain components (Turrens, 2003). In the terminal step of the respiratory process, electrons can either pass along the phosphorylating cytochrome pathway or the non-phosphorylating alternative oxidase pathway. It has been suggested that the alternative pathway has the ability to use excess ubiquinone electron pools, thereby

avoiding over-reduction of the electron transport chain, which could lead to the synthesis of reactive oxygen species (ROS) (Rich and Bonner, 1978; Moller, 2001). This homeostatic regulation is linked to the hypothesis that the alternative pathway acts as an "energy overflow" conduit for the cytochrome pathway (Lambers, 1982). The main factors that determine electron partitioning between the cytochrome and alternative pathways are the ratio of reduced ubiquinone to total ubiquinone pools (Wagner et al., 1998), the amount and redox state of alternative oxidase (AOX) proteins (Umbach and Siedow, 1993), the presence of α-keto acids (Millar et al., 1993; Umbach et al., 1994), and the availability of ADP and Pi (Juszczuk et al., 2001). The levels of these specific metabolites can vary with developmental stage and environmental conditions. However, modulation of the different respiratory pathways has only been well-documented for source organs (Gonzàlez-Meler et al., 2001; Millenaar and Lambers, 2003). Information is scarce for sink organs, especially in relation to source activity.

The present study attempted to modulate the source activity in *Erythronium americanum* Ker Gawl. (trout lily) and to study its impact on both source and sink C metabolism. Source activity was modulated using different environmental gas conditions: elevated CO_2 to increase C fixation and elevated O_3 to reduce rubisco activity. Due to its simple morphology, *E. americanum* is very close to a theoretical source–sink model and, thus, is an interesting biological model in which to study whole-plant C allocation. Indeed, 90% of individuals in this species have only one leaf and only one bulb (Blodgett, 1910), corresponding to one source and one strong sink. *Erythronium americanum* is an abundant spring geophyte of North American maple forests, whose epigeous development begins early in the spring and takes place over a short period, from snow melt to canopy closure (Taylor and Pearcy, 1976; Muller, 1978). Carbohydrate storage in the bulb is renewed during this period and when completed, appears to induce leaf senescence (Lapointe, 2001). Thus, plant growth becomes rapidly sink-limited as the bulb reaches its final size. Source activity was monitored via gas exchanges, rubisco and phosphoenolpyruvate carboxylase (PEPc) activities, respiratory rates, and sugar concentrations, whereas sink activity was monitored via respiratory rates, sucrose hydrolysis activity, carbohydrate concentrations, and plant biomass. This study should help unravel some of the

regulatory mechanisms that allow sink-limited species to cope with changes in C availability.

3.4 Materials and methods

3.4.1 Plant material and growing conditions

Bulbs of *E. americanum* were collected in September in a maple forest near Saint-Augustin-de-Desmaures (QC, Canada; 46°48' N, 71°23' W). Bulbs of similar biomass (0.35–0.40 g fresh weight) were selected and planted in plastic pots containing Turface (calcined clay granules, Applied Industrial Materials Corp., Buffalo Grove, IL, USA) as substrate. Plants were kept in a cold chamber for 5 months of cold stratification at 4 °C and then randomly transferred into eight phytotron chambers. Four different gas treatments were applied to these chambers: control, with 390 μmol mol^{-1} of CO_2 and 0 nmol mol^{-1} of O_3 (charcoal-filtered air); elevated CO_2, with 1000 μmol mol^{-1} of CO_2; elevated O_3, with 80 nmol mol^{-1} of O_3; and elevated CO_2+O_3, with 1000 μmol mol^{-1} of CO_2 and 80 nmol mol^{-1} of O_3. Other growth parameters were held constant: photoperiod, 14 h; air temperature, 18/14°C day/night; relative humidity, 75%; and photon flux density (PPFD), 350 μmol m^{-2} s^{-1}. Ambient air in each chamber was analysed continuously by an ozone analyser (O341M, Environment SA, Paris, France) and a CO_2 analyser (WMA2 PPsystems, Stotfold, UK). Plants were watered daily and fertilized weekly with 10% Hoagland's solution to ensure optimal growth (Lapointe and Lerat, 2006).

3.4.2 Gas exchange measurements

Net CO_2 photosynthetic rate (Pn) was measured under phytotron conditions (CO_2, temperature, and RH) using a Li–Cor 6400 Portable Photosynthesis System (Li–Cor Inc, Lincoln, NE, USA). Light conditions during the measurements were constant at 350 μmol m^{-2} s^{-1}, with air flow at 200 μmol s^{-1}. Measurements were randomized between treatments from 10 h to 13 h. Pn was measured on six plants per treatment (three per chamber) at leaf unfolding (t1, day 5), at the initiation of leaf senescence (t8, day 21), at complete leaf senescence (t9, day 28), and every 2 d from t1 to t8.

3.4.3 Plant growth measurements

Three plants per chamber were harvested on the same days that Pn was measured, plus one extra harvest at the time plants were moved to the chamber (t0). Leaves, bulbs, and roots were dried for 24 h at 70°C and weighed separately.

3.4.4 Enzyme extraction and assays

Measurements were done on four plants per chamber that were harvested 4, 8, 12, 16, and 20 d after transfer to the growth chamber. Leaves and bulbs from each plant were separated and immediately frozen in liquid nitrogen. Tissues were then stored at –80°C until extraction. Frozen leaf and bulb tissues (300 mg FW) were ground in liquid nitrogen with a mortar and pestle. Crude enzyme extractions were performed at 4°C according to Fontaine et al. (1999). Enzymatic activities were determined spectrophotometrically (ELX 808 Microplate reader, Bio-Tek instruments, St-Quentin-en-Yvelines, France) in coupled reactions by monitoring NADH oxidation at 340 nm. Measurements of rubisco and PEPc activities were carried out according to Fontaine et al. (1999). Cytoplasmic invertase was assayed in 200 µl of 50 mM HEPES–NaOH (pH 7) containing 2.8 mM ATP, 1.2 mM NADP, 12 U ml^{-1} hexokinase (EC 2.7.1.1), 3.5 U ml^{-1} phosphoglucoisomerase (EC 5.3.1.9), 1.75 U ml^{-1} glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and 10 µl of enzymatic extract. The reaction was initiated by adding 100 mM sucrose. A similar medium with 2 mM UDP was used for the Susy assay. Susy activity was estimated by difference between the reactions with and without UDP. Controls without substrate addition were run with all assays.

3.4.5 Carbohydrate concentrations

The leaf and bulb of three plants per chamber were analysed for starch, sucrose, and glucose-fructose concentrations at 0, 4, 8, 12, 20, and 24 d after transfer to the growth chambers. Carbohydrates were estimated according to Blakeney and Mutton (1980). Frozen tissues were lyophilized for 24 h and weighed before maceration in a solution of methanol, chloroform, and water (12:5:3 by vol.) for 20 min at 65°C. The mixture was ground with a Polytron (Kinematica, Lucerne, Switzerland) and centrifuged at 3500 rpm for 10 min at 4°C. Starch contained in the pellet was gelatinized in boiling water for 90 min and then hydrolysed at 55°C for 60 min in the presence of amyloglucosidase. The supernatant was analysed before and after invertase digestion to estimate reducing

sugars and sucrose concentrations, respectively. Finally, all reducing sugars were quantified colorimetrically at 415 nm after reaction with p-hydroxybenzoic acid hydrazide (Sigma Chemical Co., St-Louis, MO, USA).

3.4.6 Slice respiration measurements

Respiration was recorded on leaves and bulbs of six plants per treatment at 4, 8, 12, 16, and 20 d following transfer to the growth chambers. A slice of fresh tissue was infiltrated, according to Jolivet et al. (1990), in a medium containing 100 mM mannitol, 10 mM HEPES, 10 mM MES (pH 6.6), and 0.2 mM $CaCl_2$. Slice respiration was measured polarographically in the same medium, using a Clarke-type electrode (Rank Brothers Ltd., Cambridge, England). The alternative pathway was inhibited by adding 10 mM salicylhydroxamic acid (SHAM, resuspended in methoxy-ethanol) and the cytochrome pathway was inhibited by adding 1 mM potassium cyanide (KCN). Addition of SHAM and KCN together was used to record residual respiration (v_{res}). Residual respiration was constant, around 6.1% of the total respiration, for both leaf and bulb tissues, regardless of the treatment or date of harvesting. In accordance with Bahr and Bonner (1973), it is postulated that the cytochrome pathway runs at a saturating rate under the experimental conditions ($V_{cyt} = v_{cyt}$). Total respiratory rate (V_T) was measured in the absence of inhibitors, whereas the activity of the cytochrome pathway (v_{cyt}) was measured in the presence of SHAM as $v_{cyt} = V_{T+SHAM} - v_{res}$, where V_{T+SHAM} corresponds to the respiratory rate when SHAM was added first. The capacity of the alternative pathway (V_{alt}) was measured in the presence of KCN as $V_{alt} = V_{T+KCN} - v_{res}$, where V_{T+KCN} represents the respiratory rate when KCN was added first. The activity of the alternative pathway (v_{alt}) was estimated as $v_{alt} = V_T - v_{cyt} - v_{res}$. The engagement of the alternative pathway (ρ') was determined as the ratio of v_{alt} to V_{alt}. The participation of the alternative pathway (P) was determined as the ratio of v_{alt} to V_T.

3.4.7 Isolation of mitochondria

Fresh bulbs (10 g) were cut in 100 ml of cold extraction medium containing 0.35 M mannitol, 30 mM MOPS buffer (pH 7.4), 7 mM cysteine, 2 mM EGTA, 2.5 mM $MgCl_2$, 0.2% BSA (w/v), and 0.3% PVP (w/v). The bulbs were homogenized for 4×1 s at full speed in a blender (Moulinex, Ecully, France). The homogenate was filtered through a 60 µm nylon net. The bulb fragments retained in the net were reblended in the mixer

with 100 ml of the extraction medium. Homogenization and filtration were repeated as described above. The two successive filtrates were pooled and centrifuged according to Gerard and Dizengremel (1988). The mitochondrial pellet was resuspended in washing medium containing 0.35 mM mannitol, 10 mM MOPS buffer (pH 7.2), and 0.1% BSA w/v. Membrane integrity was measured spectrophotometrically at 550 nm, as the oxidation of reduced cytochrome *c* by washed intact versus burst mitochondria (Krippner et al., 1996). As mitochondrial isolation required much more material (10 g) than respiration on slices of tissue, it was only done for material harvested on day 16 for each treatment.

3.4.8 Mitochondrial respiration

Oxygen uptake was followed polarographically with a Clark-type electrode (Rank Brothers Ltd., Cambridge, England) on isolated mitochondria at 16 d. Respiratory studies were performed in a reaction medium containing 0.35 M mannitol, 5 mM $MgCl_2$, 10 mm KC1, 0.1% BSA (w/v), and 10 mM phosphate buffer (pH 7.2). The oxidation of succinate (20 mM) was measured in the presence of 200 µM ATP at pH 7.2. The oxidation of malate (30 mM) was followed at pH 6.7 in MES buffer, and at pH 7.8 in TRIS-HCl buffer, as these two pHs were optimal for NAD-malic enzyme and malate dehydrogenase activity, respectively. The oxidation of malate at pH 7.8 was carried out in the presence of glutamate (2 mM) and NAD (400 µM) as a co-factor of malate dehydrogenase. The oxidation of NADH (1 mM) was followed at pH 7.2. Mitochondrial protein concentrations were determined according to Bradford (1976). KCN (1 mM) in aqueous solution and SHAM (700 µM) in methoxy-ethanol were used as inhibitors of the cytochrome and alternative pathways, respectively. V_T, v_{cyt}, v_{alt}, ρ', and P were estimated in the same manner as for slice respiration measurements. Moreover, ADP/O ratio was estimated as the molar ratio of ADP added to the mitochondria to oxygen consumed following complete utilization of the ADP. This ratio represents the efficiency of oxidative phosphorylation. Respiratory control (RC) was determined as the ratio of state 3 (i.e. when ADP is in excess) to state 4 (i.e. when ADP is limiting) and represents the control exerted by ATP synthase on the electron transport chain.

3.4.9 AOX immunoblot

The amount of AOX was estimated by the Western blot method on the same isolated mitochondrial extracts used for respiration measurements. Mitochondrial proteins were extracted in 62.5 mM TRIS (pH 6.8) buffer containing 10% v/v glycerol, 2% v/v SDS, 0.005% v/v bromophenol blue, and 28 mM β-mercaptoethanol. The amount of total protein was fixed at 180 µg and separated by SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane using 48 mM TRIS buffer containing 39 mM glycine, 0.04% SDS, and 20% v/v methanol. AOX monoclonal antibodies were used as primary antibodies and anti-mouse IgG fragments conjugated with peroxidase were used as secondary antibodies. The bands were revealed on the autoradiograms using SuperSignal Ultra Chemiluminescent Substrate. Densitometric analysis was performed to quantify the intensities of the bands corrected for the background using ImageJ software (NIH ImageJ, NIH, Bethesda, MD).

3.4.10 Statistical analysis

All variables were analysed by three-way ANOVA (Statistix 8.2, Analytical Software, Tallahassee, FL, USA) testing CO_2 (Elevated versus Low), O_3 (Elevated versus Low), and time (7–10 harvest dates depending on the variable) as fixed effects, except for mitochondrial respiration and AOX immunoblots for which several plants had to be pooled to get enough material for one measurement per treatment. In this case, no ANOVA could be performed and only technical repetitions were carried out. Each of the four treatments was assigned to one of the eight chambers, for a total of two repetitions per treatment (n=2 for most variables). A new series of plants were grown under the same conditions the following year. Plant growth variables were recorded each year to ensure the repeatability of growth conditions over the two years (n=4). Other variables were measured either in the first or the second year due to time constraints and the number of plants per chamber needed for each type of measurements. Significant effects were determined for $P < 0.05$. A posteriori multiple comparisons tests were performed using Fisher's LSD. Pearson product–moment correlations (r) were also carried out on cumulative amount of C fixed per plant, Susy activity and bulb respiratory rate for each harvest date and each treatment.

3.5 Results

3.5.1 Net photosynthetic rate and leaf carboxylase activities

Net photosynthetic rate increased quickly from leaf unfolding (5 d) to reach maximum rates at 9 d, after which Pn decreased continuously until complete leaf senescence at day 28 (Fig. 3.1, Table 3.1). When averaged over the whole life of the leaf, Pn under elevated CO_2 was 59% higher than under control conditions, whereas Pn under elevated O_3 was 29% lower, compared with the control. Under elevated O_3, elevated CO_2 stimulated Pn by 25% until day 9, compared with the control. Subsequently, decreases in Pn under elevated CO_2+O_3 were faster than under ambient air. Thus, Pn values under elevated CO_2+O_3 no longer differed from those of the control after 15 d.

Under ambient air, leaf rubisco activity increased rapidly until day 16 and then decreased before the first visual sign of leaf senescence, which occurs at day 21 (Fig. 3.2A). Leaf rubisco activity tended to increase more rapidly under elevated CO_2 until day 12, but overall activity was not significantly different from that of the control (Table 3.1). O_3 treatment decreased leaf Rubisco activity by 22% from day 12 to 20, regardless of CO_2 concentration. However, leaf PEPc activity responded differently to O_3 stimulation depending on CO_2 concentration (Fig. 3.2B). Between days 12 and 20, O_3-induced increases in PEPc activity were 56% and 38% under ambient and elevated CO_2, respectively. Elevated CO_2 concentrations alone did not affect leaf PEPc activity compared with the control.

3.5.2 Plant biomass

Bulb biomass exhibited a large increase shortly after complete leaf unfolding (i.e. 5 d; Fig. 3.3). The maximum was reached at day 17, a few days before the initiation of leaf senescence (i.e. 21 d), after which time bulb biomass stopped increasing. No treatment significantly affected bulb growth kinetics or its final biomass, compared with the controls (Table 3.1). Bulb biomass represented 83±0.5% of total plant biomass at final harvest. Leaf and root biomass were fairly constant over time and similar among treatments.

Table 3.1 Results of three-way factorial ANOVA testing effects of elevated CO_2 and elevated O_3 treatments on *E. americanum* growth, gas exchanges, metabolites, enzyme activities and respiration over time. *F*-values are presented along with statistical differences: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

	CO_2	**O_3**	**Time**	**CO_2xO_3**	**CO_2xTime**	**O_3xTime**	**CO_2xO_3xTime**
Leaf parameters							
Net photosynthesis	121.19 ***	53.66 ***	45.85 ***	4.49 *	1.96	1.53	0.09
Rubisco activity	1.68	36.07 ***	11.54 ***	0.80	1.06	7.29 ***	1.05
PEPc activity	1.76	77.95 ***	26.75 ***	8.16 *	0.14	6.93 **	1.11
Dry mass	0.03	0.09	20.50 ***	0.01	0.04	0.19	0.02
Sucrose	0.07	4.34	37.43 ***	0.35	0.16 *	2.24	0.41
Reducing sugars	0.12	0.40	0.93	0.25	0.13	0.30	0.50
Slice respiration	0.31	92.76 ***	426.16 ***	3.65	0.62	7.30 ***	0.77
Slice alternative respiration	3.87	152.43 ***	32.27 ***	13.47 **	0.54	6.14 **	0.10
Slice alternative capacity	0.06	342.85 ***	451.84 ***	70.02 ***	5.40 **	25.30 ***	5.87 **
Participation of the alternative pathway	3.87	152.43 ***	32.27 ***	13.47 **	0.54	6.14 **	0.10
Engagement of the alternative pathway	2.41	30.77 ***	-	0.43	-	-	-

Table 3.1 (suite)

	CO_2	O_3	Time	CO_2xO_3	CO_2xTime	O_3xTime	CO_2xO_3xTime
Bulb parameters							
Dry mass	0.07	2.55	84.31 ***	0.22	0.12	0.29	0.33
Susy activity	38.43 ***	38.06 ***	84.97 ***	4.88 *	3.11 *	4.35 *	0.34
Invertase activity	0.15	4.29	375.35 ***	1.22	1.92	0.73	0.38
Starch	4.44	1.38	306.53 ***	0.00	0.62	1.02	0.21
Sucrose	2.19	0.17	165.57 ***	0.07	0.41	0.79	0.43
Reducing sugars	9.58 ***	6.30 *	112.01 ***	3.90	1.43	1.34	0.55
Slice respiration	359.49 ***	190.10 ***	843.79 ***	12.01 **	23.56 ***	19.22 ***	19.30 ***
Slice alternative respiration	305.05 ***	359.87 ***	24.44 ***	94.29 ***	15.70 ***	31.28 ***	20.11 ***
Slice alternative capacity	905.09 ***	796.56 ***	522.52 ***	416.53 ***	78.97 ***	86.45 ***	51.32 ***
Participation of the alternative pathway	305.05 ***	359.87 ***	24.44 ***	94.29 ***	15.70 ***	31.28 ***	20.11 ***
Engagement of the alternative pathway	39.19 **	57.42 **	-	8.24	-	-	-

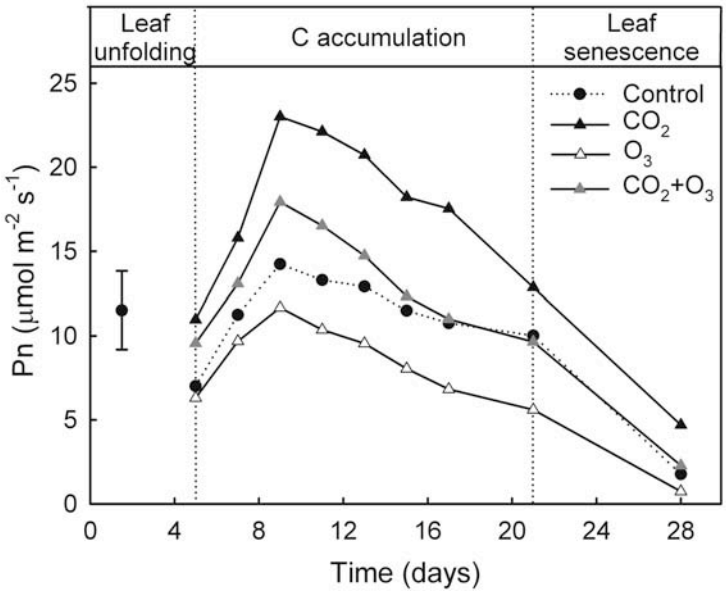

Figure 3.1 Time courses of net photosynthetic rate (Pn) in leaves of *E. americanum* grown under control, elevated CO_2, elevated O_3 and elevated CO_2+O_3 during the epigeous growth period. The last data points (28 days) correspond to complete leaf senescence. The standard error, estimated from MSE term in the ANOVA, is shown with the grand mean (N = 2). The two vertical dotted lines indicate the end of leaf unfolding and the beginning of leaf senescence, respectively.

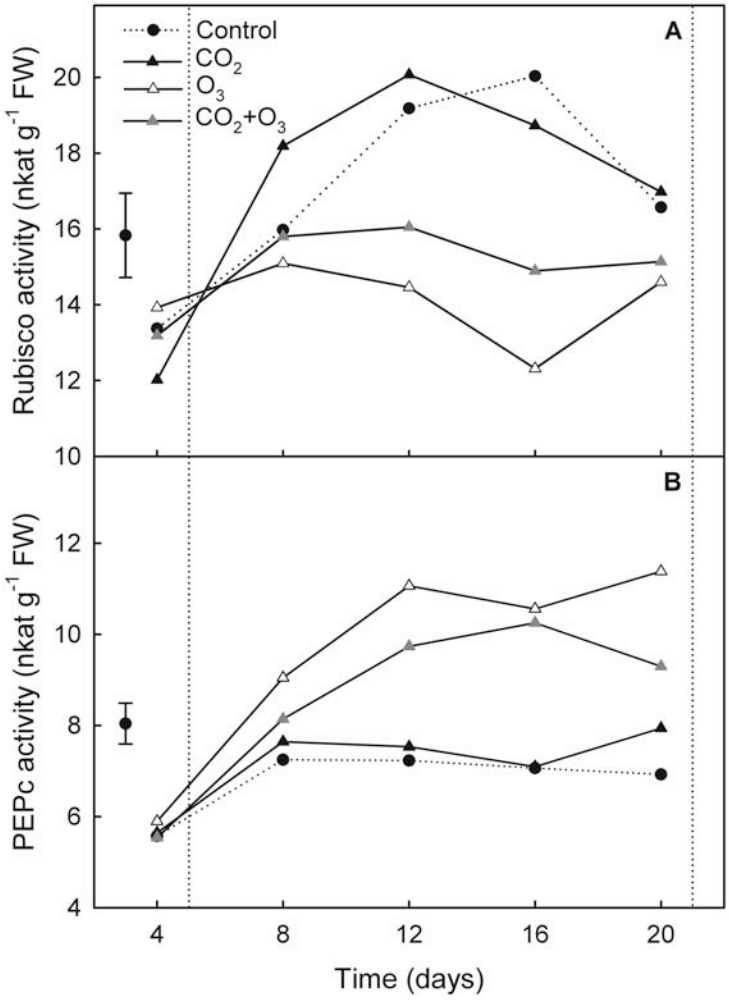

Figure 3.2 Time courses of Rubisco carboxylase activity (A) and PEPc activity (B) in leaves of *E. americanum* grown under control, elevated CO_2, elevated O_3 and elevated CO_2+O_3 during the epigeous growth period. The standard error, estimated from MSE term in the ANOVA, is shown with the grand mean (N = 2). The two vertical dotted lines indicate the end of leaf unfolding and the beginning of leaf senescence, respectively.

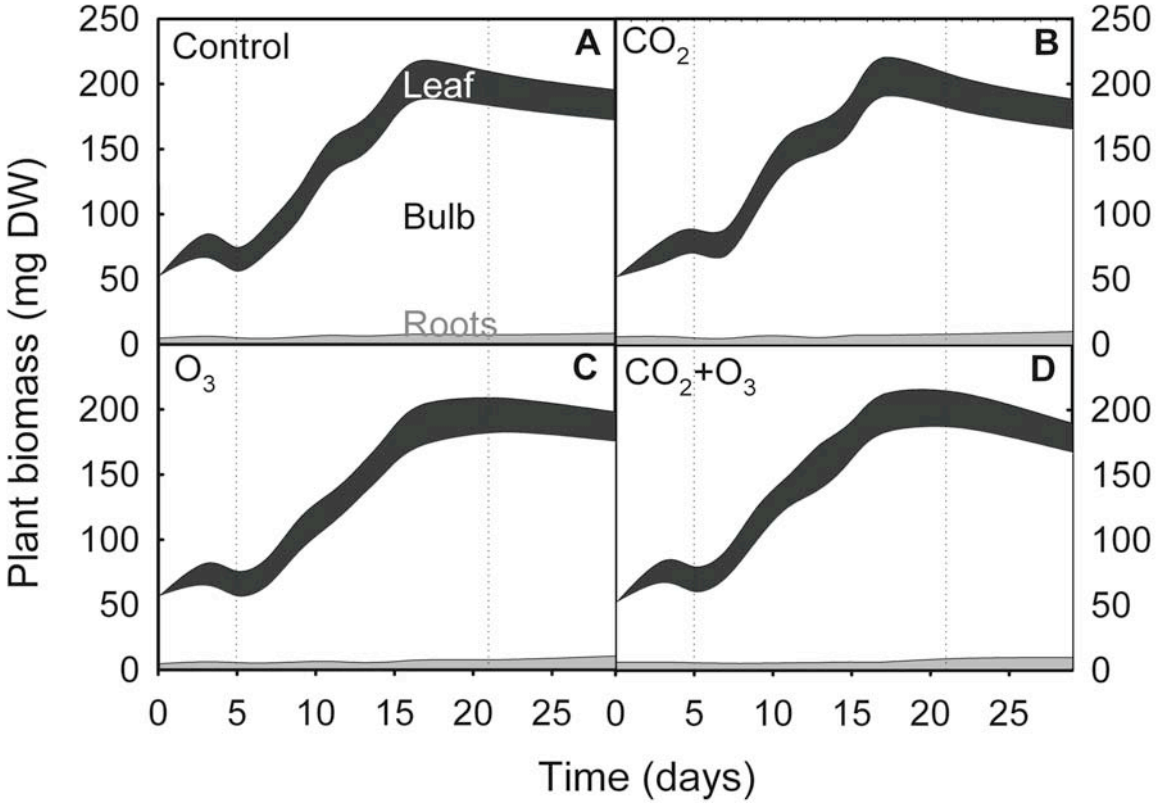

Figure 3.3 Time courses of plant biomass of *E. americanum* grown under control (A), elevated CO_2 (B), elevated O_3 (C) and elevated CO_2+O_3 (D) during the epigeous growth period. The last data points (28 days) correspond to complete leaf senescence. Leaf, bulb and roots are presented in black, white and gray, respectively (N = 4). The two vertical dotted lines indicate the end of leaf unfolding and the beginning of leaf senescence respectively.

3.5.3 Bulb sucrose hydrolysis activities

Invertase activity was high at the beginning of the season up to 8 d in all treatments, while Susy activity was minimum (Fig. 3.4). While invertase decreased progressively thereafter, Susy activity increased to maximum activity at 12 d. At 16 d, invertase activity reached minimum values around 20.1 nkat g^{-1} FW. Invertase activity was greater than Susy activity by 4-fold, suggesting that sucrose was mainly hydrolysed by invertase in the bulb of *E. americanum* (Fig. 3.4A). Elevated CO_2 conditions increased Susy activity by 21% from day 8 to the initiation of senescence, compared with the control, whereas elevated O_3 decreased Susy activity by 10% (Fig. 3.4B, Table 3.1). Susy activity was similar under elevated CO_2+O_3 and control conditions, and presented intermediate values between elevated CO_2 and elevated O_3 treatments. Elevated CO_2 and elevated O_3 did not affect invertase activity.

3.5.4 Sugar accumulation

Starch was absent from the leaf throughout the season in *E. americanum*. Sucrose concentrations in the leaf increased over time (Fig. 3.5A), whereas reducing sugar concentrations were constant, around 25 mg g^{-1} DW (Fig. 3.5B). Neither elevated CO_2 nor elevated O_3 treatments significantly affected leaf sucrose or reducing sugar concentrations (Table 3.1).

In the bulb, starch concentration exhibited a sigmoidal curve similar to that of the bulb mass, suggesting that the main constituent of bulb growth was starch accumulation. This result was confirmed by high final starch concentrations, which reached around 80% in the bulb, irrespective of the treatment (Fig. 3.5C). Starch concentration tended to increase more quickly after 4 d under elevated CO_2 conditions than under the three other growth conditions (P=0.090). Neither elevated O_3 nor elevated CO_2+O_3 conditions affected starch accumulation kinetics. After 16 d, starch concentrations were similar among treatments until leaf senescence. Sucrose exhibited a similar decrease in concentrations under all treatments (Fig. 3.5D). Similarly, reducing sugar concentrations decreased sharply after 4 d (Fig. 3.5E). However, elevated CO_2 conditions maintained a 26% higher concentration from day 8 to complete leaf senescence, whereas elevated O_3 conditions slightly reduced soluble sugar concentrations compared with the control.

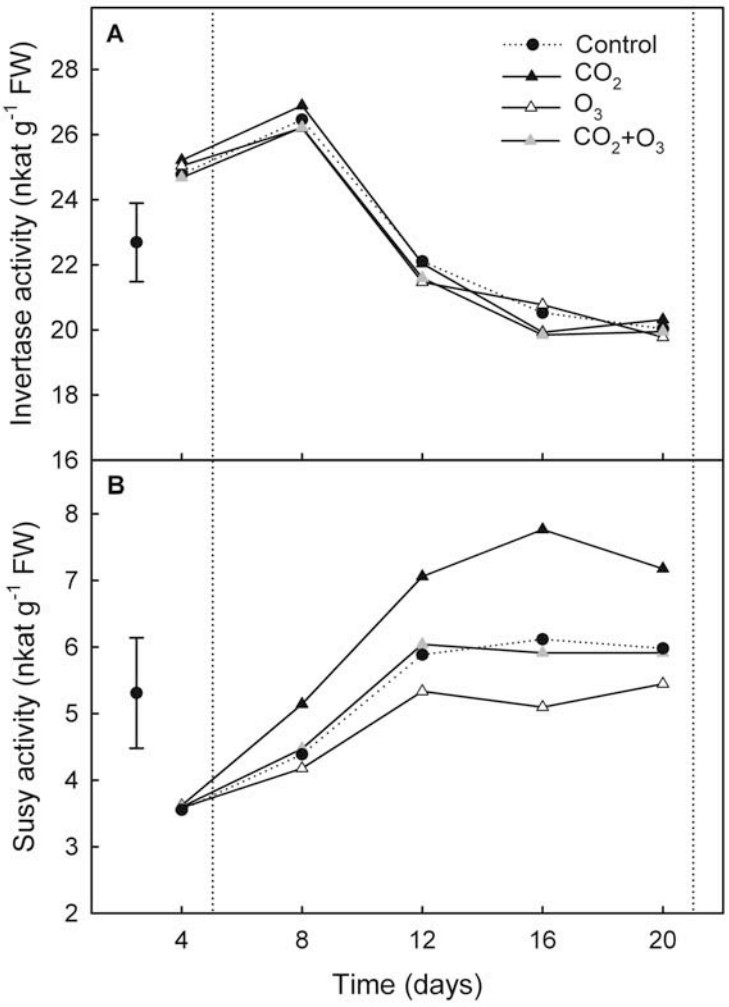

Figure 3.4 Time courses of cytoplasmic invertase activity (A) and sucrose synthase activity (B) in bulbs of *E. americanum* grown under control, elevated CO_2, elevated O_3 and elevated CO_2+O_3 during the epigeous growth period. The standard error, estimated from MSE term in the ANOVA, is shown with the grand mean (N = 2). The two vertical dotted lines indicate the end of leaf unfolding and the beginning of leaf senescence, respectively.

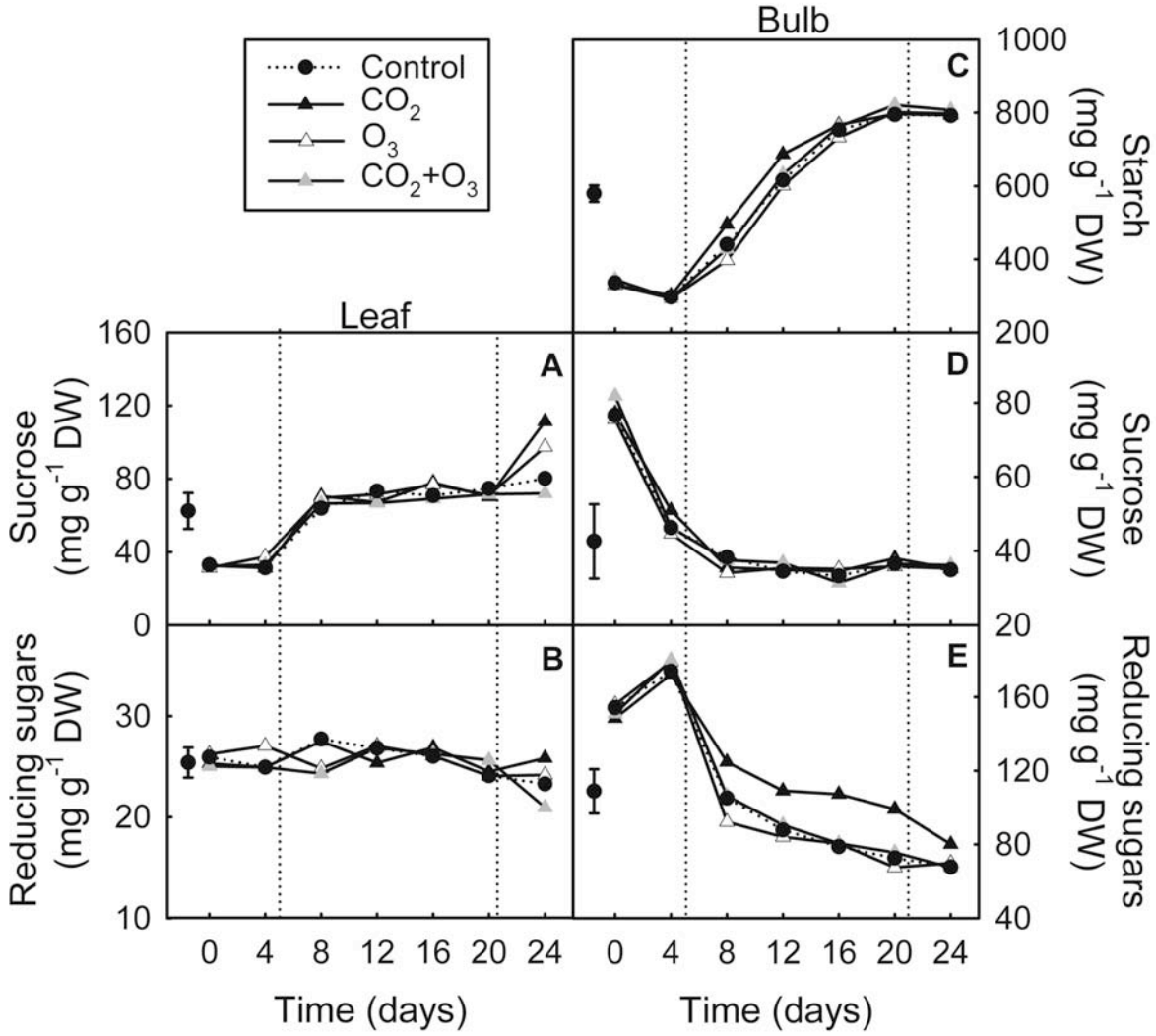

Figure 3.5 Time courses of sucrose (A, D), reducing sugar (B, E) and starch concentrations (C) in leaf (A, B) and bulb (C, D, E) of *E. americanum* grown under control, elevated CO_2, elevated O_3 and elevated CO_2+O_3 during the epigeous growth period. The standard error, estimated from MSE term in the ANOVA, is shown with the grand mean (N = 2). The two vertical dotted lines indicate the end of leaf unfolding and the beginning of leaf senescence, respectively.

3.5.5 Leaf and bulb slice respiration

In the leaf, total O_2 consumption (V_T) increased until 12–16 d and decreased abruptly several days before leaf senescence (Fig. 3.6A). Elevated CO_2 curves were similar to the control curves (Table 3.1), whereas O_3 treatment increased total leaf respiration by 18% between day 8 and day 16 (i.e. during the fastest growth phase), independent of CO_2 concentration. During this period, leaf respiration inhibited by SHAM (v_{alt}) was 2-fold greater in O_3-treated plants than in control plants, leading to an increased participation of the alternative pathway in total respiration (v_{alt}/V_T), from 0.16 in the controls to 0.26 in both O_3 treatments (Fig. 3.6B). Moreover, alternative pathway respiration tended to be higher at elevated CO_2 concentrations than under control conditions, starting from day 8 (P=0.076). Elevated O_3 increased the total capacity of the alternative pathway (V_{alt}) by 59% compared with the controls (Figs 6C, 7A, C). Elevated CO_2 did not affect the capacity of the alternative pathway (Fig. 3.7B). Engagement of the alternative pathway (v_{alt}/V_{alt}) also increased under elevated O_3 compared with the control.

In the bulb, total respiration increased during the first 12 d of growth, after which a maximum value was maintained up to the initiation of leaf senescence (Fig. 3.6D). Respiratory rates were 29% higher under elevated CO_2 from day 12 onwards, whereas elevated O_3 decreased respiration by 10%, compared with ambient air (Table 3.1). Elevated CO_2+O_3 conditions did not significantly affect respiratory rates. Bulb respiration inhibited by SHAM was up to 2.8 times higher under elevated CO_2 conditions from day 12 onwards, compared with the control (Fig. 3.6E). This increase in alternative pathway respiration was responsible for 100% of the increase in total respiration under elevated CO_2 conditions. Elevated O_3 induced a 39% decrease in the activity of the alternative pathway, as averaged over day 12 to day 20, which was responsible for 81% of the decrease in respiratory rates under elevated O_3. Thus, the participation of the bulb alternative pathway was increased from 0.18 in the control to 0.37 under elevated CO_2 and decreased to 0.08 under elevated O_3 at 16 d. Elevated CO_2+O_3 significantly affected neither alternative respiratory pathway rates nor alternative respiratory capacity in the bulb, compared with the control (Figs 6E, F, 7E, H). The alternative pathway capacity (V_{alt}) in the bulb was 2.4 times higher under

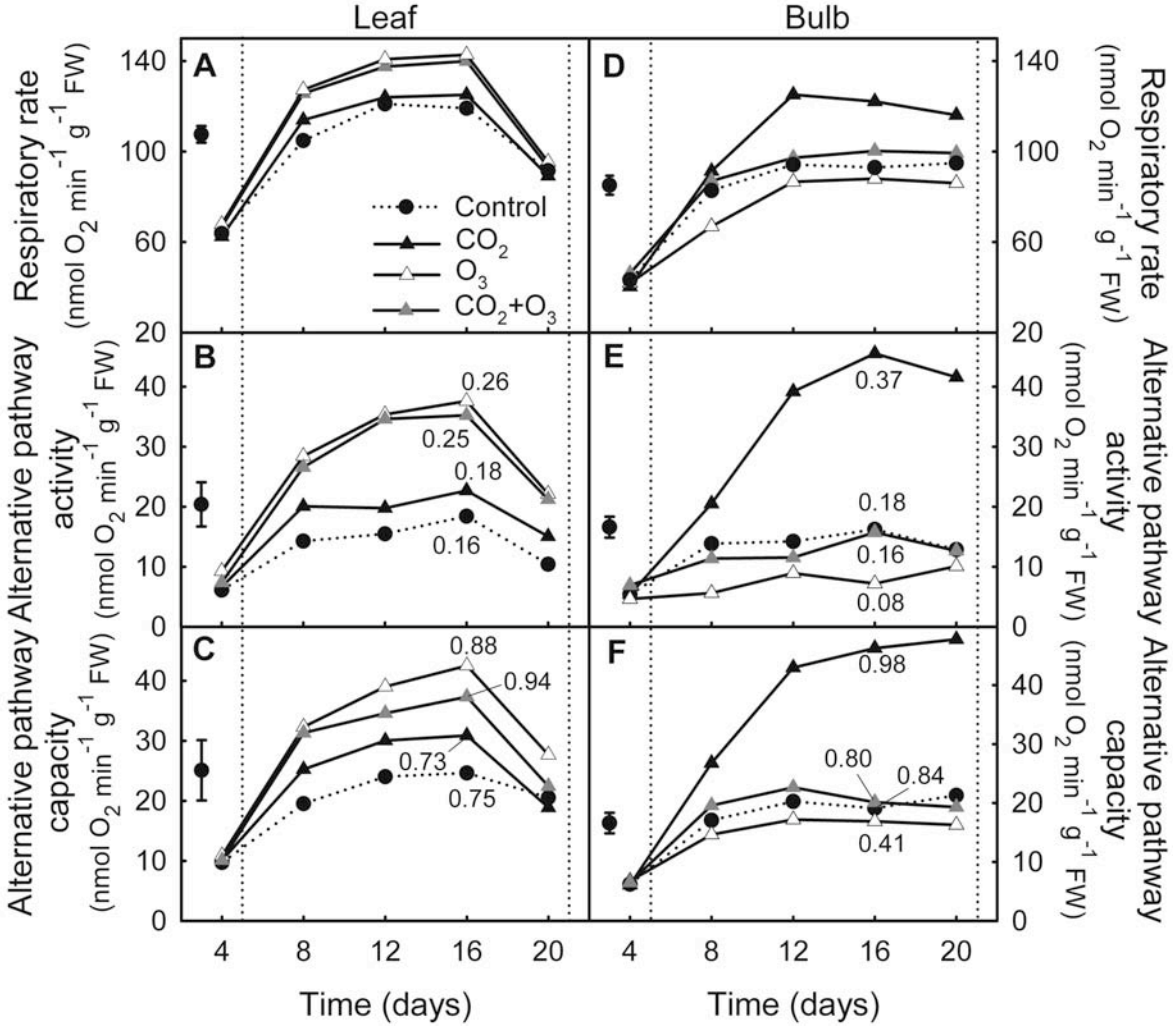

Figure 3.6 Time courses of total respiratory rate (A, D), alternative pathway respiratory rate (B, E) and alternative pathway capacity (C, F) in leaf (A, B, C) and bulb (D, E, F) of *E. americanum* grown under control, elevated CO_2, elevated O_3 and elevated CO_2+O_3 during the epigeous growth period. Participation (B, E) and the engagement of the alternative pathway (C, F) are indicated on the graphs for day 16. The standard error, estimated from MSE term in the ANOVA, is shown with the grand mean (N = 2). The two vertical dotted lines indicate the end of leaf unfolding and the beginning of leaf senescence, respectively.

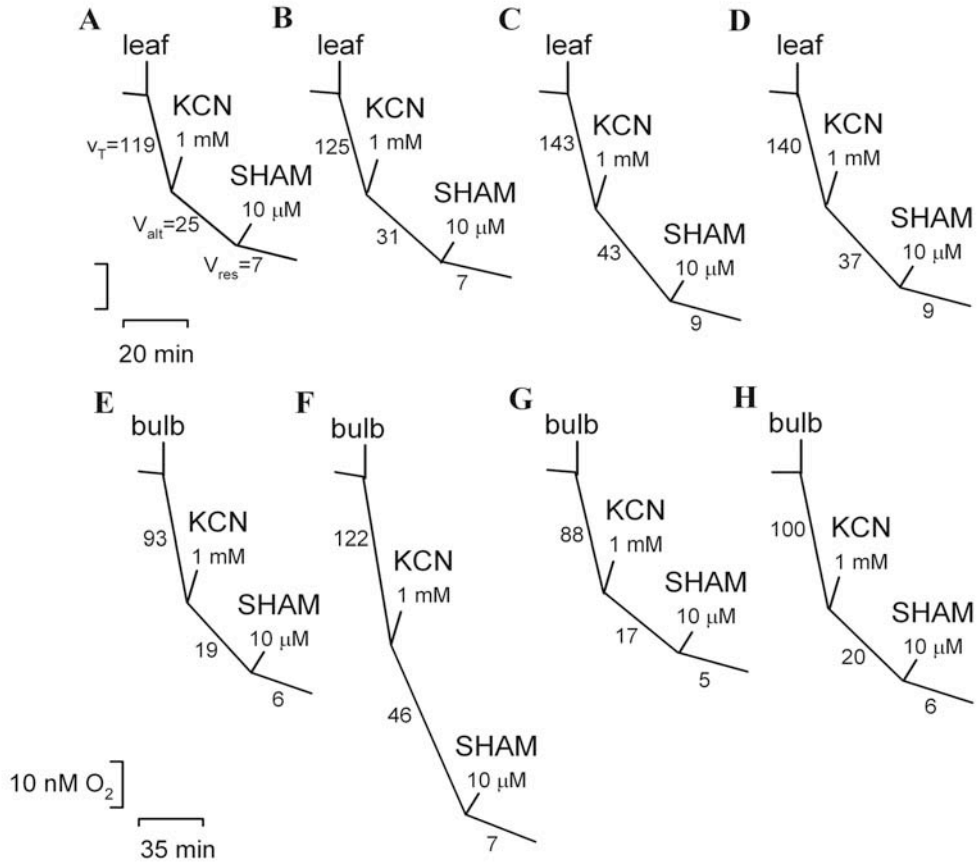

Figure 3.7 Representative traces of respiratory activities of leaf (A, B, C, D) and bulb (E, F, G, H) slices from *E. americanum* grown under control (A, E), elevated CO_2 (B, F), elevated O_3 (C, G) and elevated CO_2+O_3 (D, H) during the epigeous growth period. The oxidation rates, expressed in $nmolO_2\ min^{-1}\ mg^{-1}$ protein, are indicated on the different traces.

elevated CO_2 compared with the control, whereas it was slightly lower than the control under elevated O_3 (Figs 6F, 7F, G). Engagement of the alternative pathway increased from 0.84 in the control to 0.98 under elevated CO_2 and was 2-fold lower under elevated O_3, suggesting a full utilization of the AOX enzyme under elevated CO_2. Engagement of the alternative pathway under elevated CO_2+O_3 conditions (0.80) was similar to that of the control.

3.5.6 Bulb mitochondrial respiration

Mitochondria were extracted from material harvested at 16 d, where the largest differences between treatments were observed for bulb slice respiration (Fig. 3.6). The integrity of washed mitochondrial membrane was high and constant among treatments (78–82%, data not shown). The respiratory rate of the bulb mitochondria was the highest in state 3 (i.e. when ADP is available in excess), with succinate as the initial substrate of the electron transport chain (i.e. 99 nmol O_2 min^{-1} mg^{-1} protein; Fig. 3.8A), and lowest with NADH (i.e. 39 nmol O_2 min^{-1} mg^{-1} protein; Fig. 3.8B). Malate oxidation was 37% higher when malate dehydrogenase activity was induced at pH 7.8 (81 nmol O_2 min^{-1} mg^{-1} protein; Fig. 3.8C) than under malic enzyme activity at pH 6.7 (62 nmol O_2 min^{-1} mg^{-1} protein; Fig. 3.8D). Succinate oxidation was partially inhibited by KCN (1 mM) and cyanide-insensitive respiration, which represented 18% of total respiration, was completely blocked by SHAM (700 µM). Similarly, cyanide-insensitive respiration represented 19% and 24% of the total respiration during malate oxidation at pH 7.8 and pH 6.7, respectively. These results suggest that electron flow was mediated by both the cytochrome and the alternative pathways for all three substrates. On the other hand, KCN almost completely blocked NADH oxidation by the electron transport chain.

Malate oxidation at pH 6.7 exhibited treatment effects, on both respiratory rate and the alternative pathway, that were most similar to the treatment effects observed with slice respiration. In order to approximate the true physiological state and because of the central role played by malate in plant cell metabolism the behaviour of this substrate was therefore presented here to compare the four treatments. When SHAM was first added, it inhibited 15% of malate oxidation at pH 6.7 (i.e. mostly by malic enzyme), suggesting a rate (v_{alt}) of 9 nmol O_2 min^{-1} mg^{-1} protein for the alternative pathway

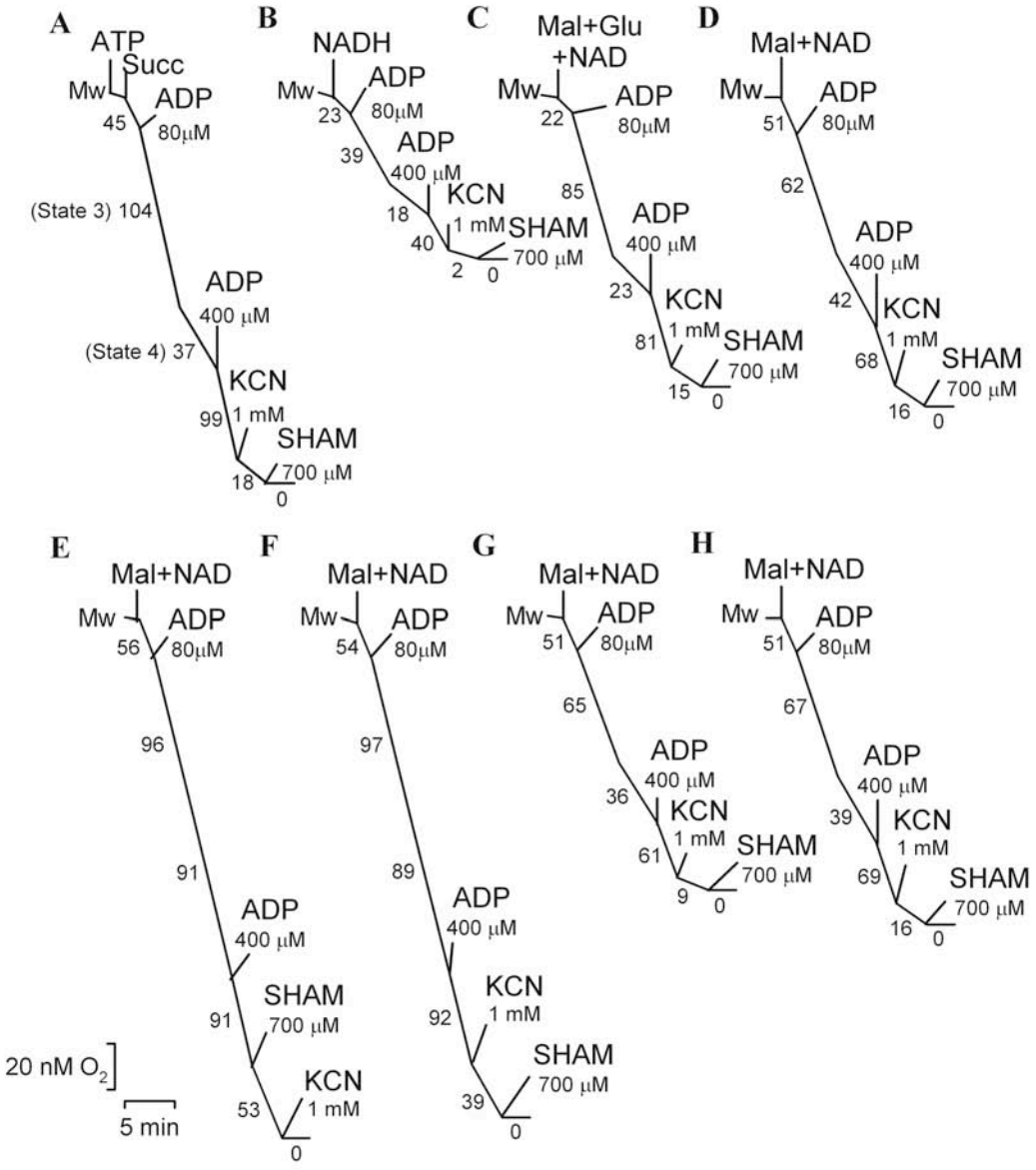

Figure 3.8 Representative traces of respiratory activities of washed mitochondria isolated from *E. americanum* bulbs grown under control (A, B, C, D), elevated CO_2 (E, F), elevated O_3 (G) and elevated CO_2+O_3 (H) during the epigeous growth period. Activities were recorded in presence of succinate (A), NADH (B) and malate (C to H) as substrates. Malate was oxidised in the presence of 2 mM glutamate and 400 µM NAD at pH 7.8 (C) and without glutamate at pH 6.7 (D to H). Respiratory activities with SHAM added first (E) and with KCN added first (F) were presented. Mitochondria preparations used in each series of measures contained 200 µg of proteins. The oxidation rates, expressed in $nmolO_2\ min^{-1}\ mg^{-1}$ protein, are indicated on the different traces.

(Table 3.2). Elevated CO_2 concentrations strongly stimulated malate oxidation from 68 nmol O_2 min^{-1} mg^{-1} to 91 nmol O_2 min^{-1} mg^{-1} protein (V_T). This stimulation was linked to a 4.2-fold higher activity of the cyanide-insensitive pathway (v_{alt}) and a 2.4-fold higher alternative pathway capacity (V_{alt}). Elevated O_3 affected malate oxidation at pH 6.7. The alternative pathway activity was slightly lower, whereas the alternative pathway capacity was reduced by 44% under elevated O_3, compared with the controls. Elevated CO_2+O_3 conditions affected neither malate oxidation at pH 6.7 nor allocation of the mitochondrial electron flux to the alternative pathway or the cytochrome pathway. Moreover, as the activity of the cytochrome pathway was essentially constant under the different treatments (v_{cyt}), modulation of malate oxidation at pH 6.7 can be entirely explained by changes in the activity of the alternative pathway. Thus, the engagement and participation of the alternative pathway was much higher under elevated CO_2 compared with the control. Lower RC and a lower ADP/O ratio were recorded under elevated CO_2 compared to the control, supporting the idea of increased participation of the alternative pathway. Indeed, the alternative pathway reduces the H^+ gradient leading to reduced ATP synthesis. By contrast, RC increased under elevated O_3. RC and the ADP/O ratio were also slightly higher under elevated CO_2+O_3 than under control conditions.

3.5.7 Immunoblotted AOX protein

Western blot analysis of mitochondria isolated from bulbs indicated that, at 16 d, the amount of AOX protein was 1.4 times higher under elevated CO_2 (Fig. 3.9), and 3.4 times lower under elevated O_3, compared with the control. Under elevated CO_2+O_3 conditions, AOX content was slightly lower than in the control plants. On the Western blots, AOX from *E. americanum* appeared as a monomeric reduced form with a molecular mass of 39 kDa. This apparent molecular mass of AOX coincided with the predicted molecular mass of the mature enzyme (39.331 kDa).

3.6 Discussion

3.6.1 Modulation of the source strength

Net photosynthetic rate of *E. americanum* was modulated by CO_2 and O_3 concentrations, as has been shown in numerous other species (Ceulemans and

Table 3.2 Respiratory activities and parameters of *E. americanum* bulb mitochondria isolated at 16 days from control and treated plants. The substrate was malate (30 mM) at pH 6.7. Total respiratory rate (V_T), the activity of the cytochrome pathway (v_{cyt}), the activity of the alternative pathway (v_{alt}), the capacity of the alternative pathway (V_{alt}), ADP/O ratio, the respiratory control (RC), the engagement of the alternative pathway (ρ') and the participation of the alternative pathway (P) are presented (see Material and Methods for description of these variables). Values are the mean of three to four technical repetitions ± SE and expressed in nmol O_2 min^{-1} mg^{-1} protein.

	V_T	v_{cyt}	v_{alt}	V_{alt}	ADP/O	RC	ρ'	P
Control	68 ± 6	58 ± 4	9 ± 3	16 ± 6	2.34 ± 0.3	1.48 ± 0.4	0.57	0.13
Elevated CO_2	91 ± 4	53 ± 1	38 ± 4	39 ± 3	1.98 ± 0.2	1.07 ± 0.2	0.97	0.42
Elevated O_3	61 ± 5	56 ± 6	6 ± 4	9 ± 5	2.21 ± 0.4	1.81 ± 0.1	0.66	0.10
Elevated CO_2+O_3	69 ± 11	59 ± 8	11 ± 6	16 ± 7	2.67 ± 0.3	1.69 ± 0.2	0.69	0.16

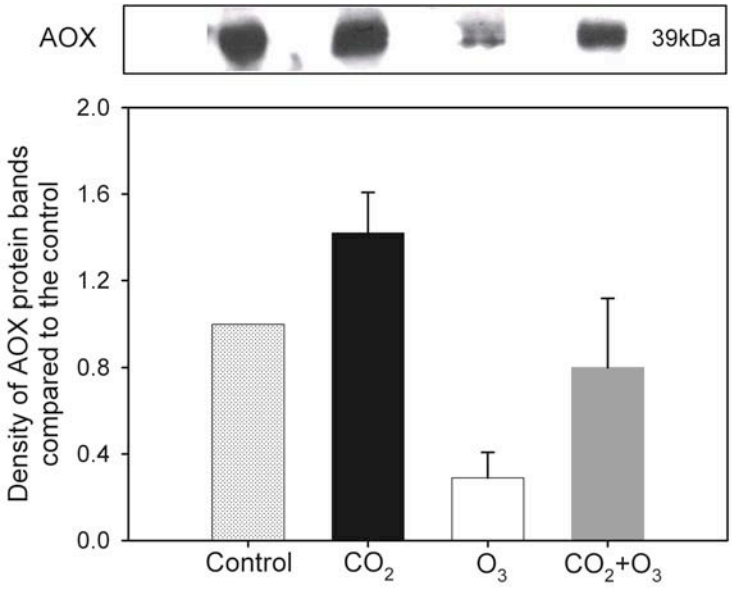

Figure 3.9 A representative western-blot of AOX protein detection (above) and relative amount of AOX protein (below) in mitochondria isolated at 16 days from bulbs of *E. americanum* grown under control, elevated CO_2, elevated O_3 and elevated CO_2+O_3. Each value represents the mean of four technical repetitions ± SE.

Mousseau, 1994; Ainsworth and Long, 2005; Vandermeiren et al., 2005). Pn was stimulated under elevated CO_2 concentrations. It is well known that elevated CO_2 conditions increase CO_2 availability as a substrate for Rubisco, thereby positively affecting the carboxylation/oxygenation ratio. However, the enzyme itself appeared unaffected since the same rates were observed for in vivo Rubisco activity in elevated CO_2 and in the control. By contrast, Rubisco activity was decreased under elevated O_3. Such decreases have previously been observed and appear to be due to an alteration of Rubisco structure and expression (Pelloux et al., 2001). Elevated O_3 stimulated leaf PEPc activity in *E. americanum*. This enzyme supplies the anaplerotic pathway with C skeletons (Dizengremel, 2001). Thus, stimulation of PEPc activity could compensate for C loss induced by Rubisco alteration under elevated O_3. However, this compensation was partial since Pn were lower in O_3-treated plants than in control plants. Thus, we succeeded in both stimulating and inhibiting source activity by increasing the CO_2 and O_3 concentrations, respectively.

O_3 stimulated leaf respiration in *E. americanum*. Eighty per cent of this increased respiratory rate under elevated O_3 can be explained by the increased activity of the alternative pathway. Induction of AOX expression by O_3 has previously been described and has been related to protective mechanisms avoiding ROS production in mitochondria (Tosti et al., 2006). Indeed, AOX protein prevents the over-reduction of the electron transport chain, especially the ubiquinone pool, thus alleviating ozone damage (Maxwell et al., 1999). The higher respiratory rate was partly responsible for the lower leaf Pn in O_3-treated plants. On the other hand, elevated CO_2 did not affect leaf respiration. Respiration is often inhibited under elevated CO_2 concentrations, as demonstrated by Gonzalez-Meler et al. (1996). Moreover, these authors reported a direct inhibition of the cytochrome pathway by elevated CO_2. This inhibition suggests that a portion of the electrons was transferred from the cytochrome pathway to the alternative pathway, which could explain the slightly higher activity of the alternative pathway observed in leaves of *E. americanum* under elevated CO_2, but without any impact on total respiratory rates. Thus, leaf respiration seems to be modulated more by abiotic factors than by source or sink activity.

3.6.2 Source–sink imbalance

Neither starch nor soluble sugar accumulation in the leaf was observed over time under elevated CO_2, nor a reduction in these constituents under elevated O_3. Starch has often been described as a temporary storage sugar in the leaf which contributes to photosynthesis regulation (Goldschmidt and Huber, 1992). Twice-ambient CO_2 often leads to an increase in leaf carbohydrates; for example, Long and Drake (1992) reported an increase of 52% for the soluble sugar content and 160% for starch content. Therefore, it can safely be assumed that more C was translocated to the bulb under elevated CO_2, whereas the opposite occurred under elevated O_3, compared with the controls, since the leaf did modulate its carbohydrate content in response to changes in Pn.

Despite differences in the amount of C translocated to the bulb under the different growth conditions, biomass allocation patterns were similar among treatments. Biomass accumulation in the sink increases steadily during the epigeous growth phase, until it reached a maximum which precedes by a few days the first visual sign of leaf senescence. Numerous reports in the literature have been made regarding positive responses of plant growth to the elevated C supply under elevated CO_2 conditions, including for perennial organs (Daymond et al., 1997; Donnelly et al., 2001). However, a lack of effect on plant growth is possible in sink-limited plants (Arp, 1991; Woodward, 2002). Thus, despite CO_2 stimulation of the source activity, similar rates of biomass accumulation have been reported for onions from bulbing to bulb maturity (Daymond et al., 1997) and for potatoes before tuber initiation (Conn and Cochran, 2006). Thus, storage organ growth cannot increase in sink-limited conditions, whatever the strength of the C source. In these studies, the inability to use the surplus C in the sink organs leads to a down-regulation of Pn, which did not occur in *E. americanum*.

Invertase was strongly activated at the beginning of bulb growth, whereas Susy became activated later in the season. Koch (2004) associated the induction of invertase with cell growth and division and Susy induction with cell differentiation and reserve accumulation. Similar sequential contributions of the different sucrose-cleaving enzymes, as a function of developmental stage, seem to take place in the bulb of *E. americanum*. During invertase induction (4–12 d), sink C-demand was high, as indicated by the kinetics of growth and starch accumulation in the bulb, which were

maximum. This high demand could explain why neutral invertase activity was not affected by treatments. Sink limitation, i.e. growth and starch accumulation slow down, occurred later (from day 12 onward) when Susy induction took over. The rate of sucrose hydrolysis by Susy in the bulb was then stimulated by elevated CO_2 concentrations and inhibited by O_3 exposure. The activity of this enzyme was strongly correlated with the cumulative amount of CO_2 fixed by the leaf (Fig. 3.10A). Among the main carbohydrates stored in the sink, only reducing sugar concentrations were modulated by CO_2 and O_3 concentrations, being higher under elevated CO_2 and slightly lower under elevated O_3. It therefore seems that modulation of Susy activity was sufficient to maintain a constant sucrose concentration in the bulb of *E. americanum*, despite the variable amount of carbohydrates being translocated to the bulb. Susy activity and expression are stimulated by sucrose availability, whereas invertase activity is regulated by the hexose pool (Geigenberger et al., 2004; Koch, 2004). Geigenberger et al. (2004), however, proposed a mechanism to drive carbon into starch synthesis and away from respiration in response to sucrose supply, based on redox-activation of ADP-glucose pyrophosphorylase by sucrose. In the present study, potential surpluses of sucrose under elevated CO_2 did not affect starch accumulation or bulb growth of *E. americanum*. Thus, it appears that the higher amount of C fixed under elevated CO_2 led to a higher flow of sucrose unloaded in the bulb, then to a higher concentration in glucose and fructose through a modulation of Susy. The opposite occurred when the amount of C fixed was lower, as was the case under elevated O_3, thus avoiding in both cases, an accumulation of sucrose in the bulb. Our results suggest that modulation of the activity of Susy avoided AGPase activation and, instead, modulated the pool of glycolytic intermediates available for respiration. Yet, it is also possible that AGPase was already maximal under the O_3 treatment and that, therefore, the surplus of sucrose under all other growth conditions stimulated Susy activity and, sequentially, respiration.

3.6.3 The role of the alternative respiratory pathway

It seems that the bulb of *E. americanum* is unable to modulate its storage capacity in response to changes in the source activity, probably because growth in this species is sink-limited (Lapointe, 2001). Therefore, the different amounts of C translocated to the bulb must induce changes in C metabolism to counterbalance the constant growth rate of the perennial organ. O_2 consumption by the bulb mitochondrial electron transport

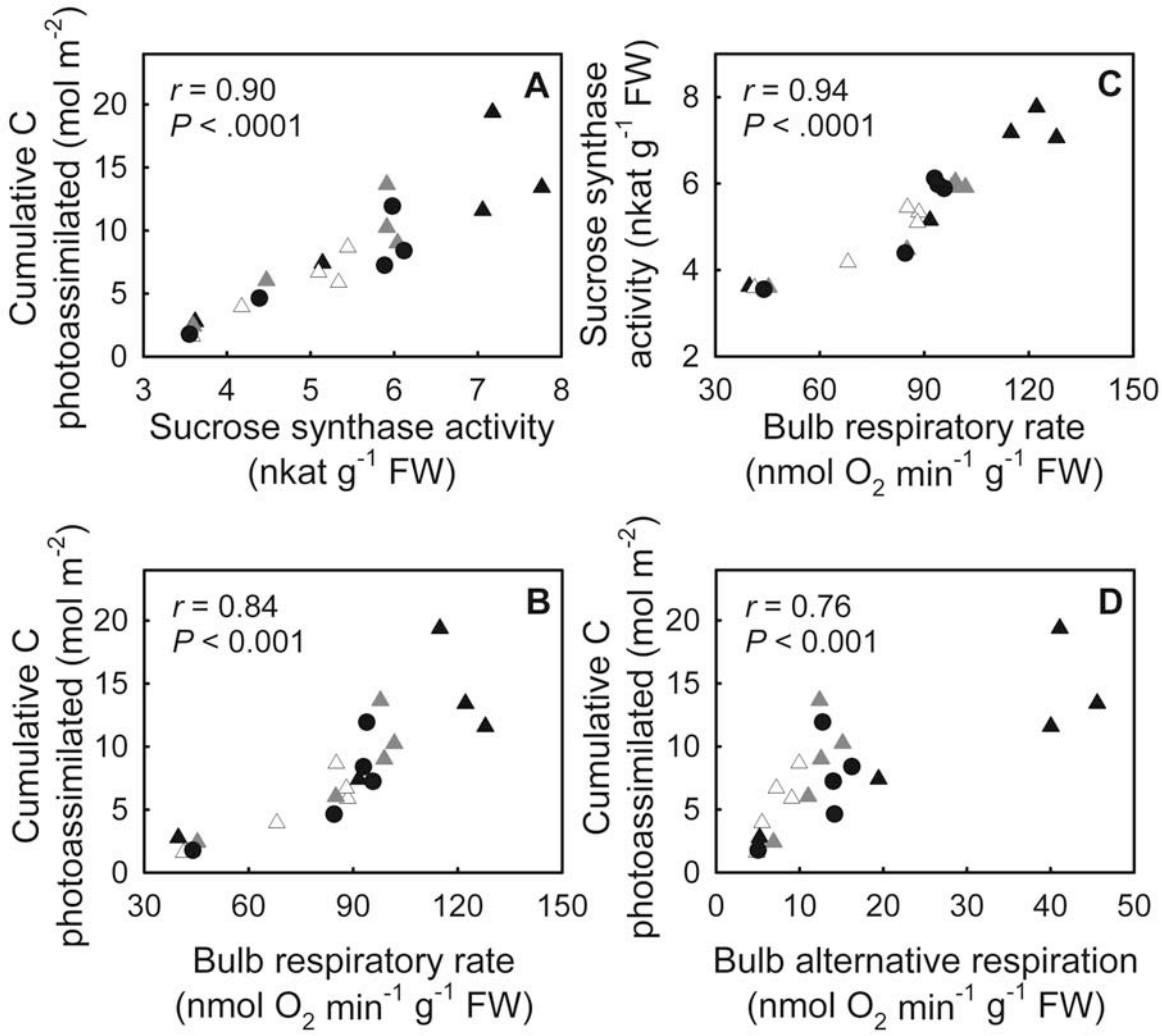

Figure 3.10 Relationship between the cumulative amount of carbon fixed by the leaves and sucrose synthase activity (A), respiratory rate of bulb slice (B), and the alternative respiratory rates of bulb slice (D); and between sucrose synthase activity and respiratory rate in bulb slices (C) of *E. americanum*. Control (black circles), elevated CO_2 (black triangles), elevated O_3 (white triangles) and elevated CO_2+O_3 plants (gray triangles). Pearson correlation coefficients and associated *P*-values are indicated

chain was stimulated under elevated CO_2 and a reduction under elevated O_3 conditions. Bulb respiratory rate is also strongly correlated with the cumulative amount of C fixed in the leaves (Fig. 3.10B) and with Susy activity (Fig. 3.10C). Shugaev and Sokolova (2001) reported a higher respiratory rate in non-differentiated stolons of potato where starch synthesis was low and glucose and fructose concentrations were high, compared with newly formed tubers. Storage organs seem to be able to use the respiratory process to remove the surplus of carbohydrates that cells cannot immediately store. Alternatively, reduced availability of sugars can induce a reduction in respiratory rates without affecting bulb growth rates, as seen under elevated O_3. This result suggests that respiration was already stimulated under control conditions due to the incapacity of the sink to use all sugars for bulb growth.

The use of inhibitors to estimate cytochrome and alternative pathway activities has been criticized (Millar et al., 1995), as it could underestimate the activity of the alternative pathway if the cytochromic pathway is not saturated before the inhibitors are added. Measurements of the capacity of the alternative pathway, on the other hand, are not subject to the same criticism. In the present study, the effects of the treatments on the alternative pathway activity were supported by similar effects on the alternative pathway capacity, in both bulb slices and mitochondrial extracts. Furthermore, the amount of AOX protein in the mitochondrial extracts varied in accordance with the activity and capacity of the alternative pathway. The development of a sink limitation during the season suggests a biological system almost saturated with carbohydrates. The difference between v_{cyt} and V_{cyt} should therefore be minimum or nul. We are confident that, in the present study, the differences observed between treatments for the activity of the alternative pathway were not biased by the use of inhibitors.

It appears that the increase in bulb respiration could be entirely explained by an increased activity of the alternative pathway in elevated CO_2-grown plants. On the other hand, the decrease in respiration induced by the O_3 treatment was partially due to a reduction in the activity of the alternative pathway (80%). This modulation of alternative pathway respiration was also strongly correlated with the cumulative amount of C fixed in the leaves (Fig. 3.10D). In isolated mitochondria, the activity of the alternative pathway also entirely explained the increase in the respiratory rate under elevated CO_2 conditions in response to carbohydrate availability. Numerous studies

have suggested that the alternative pathway could be a mechanism for energy overflow (Lambers, 1982), but few have tried to demonstrate it. The mitochondrial respiration data revealed a strong affinity of the alternative pathway for malate oxidation by NAD-malic enzyme in *E. americanum* bulbs. Moreover, this pathway increased under elevated CO_2 conditions and decreased under elevated O_3 conditions. NAD-malic enzyme may promote the reduction of the disulphide bond of the AOX protein, leading to the more active form of the enzyme (Vanlerberghe et al., 1995). Furthermore, pyruvate produced by NAD-malic enzyme is a well-known activator of the reduced AOX (Millar et al., 1993). Thus, NAD-malic enzyme activity and its subsequent organic acid production could play an important role in stimulating the consumption of excess carbohydrate by induction of the alternative respiratory pathway. In accordance with this hypothesis, both the capacity of the alternative pathway and the abundance of AOX were stimulated under elevated CO_2, and repressed under elevated O_3. Sieger et al. (2005) reported a modulation of AOX abundance and capacity in tobacco cells growing under limiting macronutrient conditions, and concluded that the alternative pathway was stimulated to correct the imbalance between carbohydrate supply and demand, thus controlling anabolism and growth. Stimulation of the alternative pathway would allow consumption of excess C, avoiding early senescence induced by a reduction in C sink demand.

The capacity of the alternative pathway was strongly stimulated by CO_2 and Western blot analysis also revealed a stimulation of AOX abundance, although of smaller amplitude. On the other hand, the capacity of the alternative pathway was slightly inhibited by ozone, whereas AOX abundance was strongly inhibited. Alternative oxidase exists as a covalent and a non-covalent dimer, the former being the less active form of the enzyme (Umbach and Siedow, 1993). The present results suggest that CO_2 mainly promotes the proportion of the more active form (i.e. non-covalent dimer), leading to high capacity of the alternative pathway, whereas O_3 mainly depletes the total amount of AOX protein. It has also been shown that only the more active form is susceptible to pyruvate activation (Umbach et al., 1994). This would agree with the strong stimulation of activity of the alternative pathway by elevated CO_2 and of malate consumption by NAD-malic enzyme that was observed with the mitochondrial

preparations. Both activation and expression of the AOX thus appear to be modulated as a function of C availability within the bulb.

In this study, the response of sink C metabolism to the modulation of source activity was examined in *E. americanum*. Despite the increase in Pn under elevated CO_2 and decrease under elevated O_3, neither bulb growth nor starch accumulation was affected. Susy activity responds to the amount of sucrose translocated to the bulb. This modulation of the enzyme could prevent AGPase activation by sucrose content unless AGPase was already at a maximal rate. In addition, the bulb alternative pathway — activity, capacity, and AOX content — was modulated in response to the amount of C fixed. This modulation was stronger when malate was oxidized as the mitochondrial substrate by the malic enzyme. It is hypothesized that the production of pyruvate by malic enzyme modulates the activity of the AOX, allowing respiration to burn more C in excess conditions and less in more limited C conditions. The regulation of the glycolytic intermediate pool by Susy and of the alternative pathway by pyruvate production adjusts starch accumulation in rhythm with sink growth capacity. In this sink-limited species, sink C metabolism is modulated in response to changes in C availability, whereas source C metabolism mostly appeared to respond to growth conditions. It would be interesting to investigate the kinetics of starch synthesis and remobilization as a function of C availability and sink capacity to determine if other key enzymes are modulated by sink capacity.

3.7 Acknowledgements

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Chapitre 4 Source-sink relationships are dependent upon photoperiod but not irradiance in the sink-limited species *Erythronium americanum*

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4.1 Résumé

L'activité de la source et celle du puits varient durant le developpement des plantes et ce, afin de maintenir une balance entre celles-ci. Lorsque la source est limitante, l'activité du puits est généralement proportionnelle à la quantité d'hydrates de carbone synthétisée par celle-ci. Cependant, la réponse de l'activité du puits face à la modulation de la source demeure encore peu connue lorsque la plante est limitée par le puits. L'activité du puits a été étudiée chez l'*Erythronium americanum*, une espèce limitée par les puits, dont l'activité de la source a été modulée par différents régimes de lumière. Les plants ont été soumis à deux régimes de photoperiode combinés à deux niveaux d'irradiances et ce, afin de mettre en évidence la dynamique journalière ayant lieu entre la synthèse des hydrates de carbone par la source et leurs utlisations par le puits. L'activité de la source a été mesurée via l'analyse des échanges gazeux, et l'activité du puits via l'analyse de la biomasse du bulbe et l'accumulation d'hydrates de carbone. Le taux de photosynthèse net (Pn) était, au départ, plus élevé sous longue photopériode et a ensuite diminué précocemment au cours de la saison de croissance, alors qu'il est demeuré relativement constant jusqu'à quelques jours avant la sénescence foliaire sous courte photopériode. Bien que l'accumulation d'amidon ait été plus rapide au cours des quelques permiers jours sous longue photopériode, le taux de synthèse de l'amidon est devenu similaire sous les deux photopériodes à partir du moment où le Pn a diminué. A l'inverse, ni le Pn ni l'accumulation d'amidon n'ont été affectés par l'irradiance et ce, quelle que soit la photopériode. Pn et l'accumulation d'amidon semblent seulement dépendants de la durée journalière d'assimilation du carbone. Il apparaît alors que la quantité totale d'hydrates de carbone synthetisée sous longue photopériode était en excès par rapport à la capacité du puits à les stocker, induisant ainsi un rétrocontrôle négatif du Pn afin de rétablir un équilibre entre source et puits.

4.2 Abstract

Source and sink activities vary during plant development to remain balanced. Generally, sink activity is modulated as a function of the amount of carbohydrates synthesized by the source in source-limited condition. However, the response of sink activity to source modulation is not well understood in sink-limited plants. Sink activity was investigated in the sink-limited *Erythronium americanum* whose source activity was modulated by light regimes. Plants were subjected to two photoperiod regimes combined with two irradiance levels to characterise the daily dynamics between the synthesis of carbohydrates by the source and utilisation of these carbohydrates by the sink. Source activity was monitored through gas exchange measurements and sink activity was monitored via bulb biomass and carbohydrate accumulation. Net photosynthetic rate (Pn) was initially higher under long photoperiod and decreased early in the growth season, whereas it remained fairly constant until a few days before leaf senescence under short photoperiod. Despite a faster rate of starch accumulation during the first few days under long photoperiod, the rate of starch synthesis became similar once Pn has decreased. In contrast, neither Pn nor starch accumulation were affected by irradiance, regardless of the photoperiod. Pn and starch accumulation seemed to depend only on the daily duration of carbon assimilation. It thus appears that the total amount of carbohydrate synthesized under long photoperiod was in excess compared to the ability of the sink to store them, inducing a feedback inhibition of Pn to restore the source-sink balance.

4.3 Introduction

Spring ephemerals are characterized by a short epigeous growth period, starting quickly after snowmelt and ending during canopy closure (Muller, 1978). During this period, the perennial organ (i.e. rhizome, corm or bulb) is renewed and assumes a storage function for photoassimilates and nutrients. This organ thus plays a major role in plant survival throughout the growth cycle (Whigham, 2004). Spring ephemerals are well adapted to take advantage of their restricted temporal windows. They quickly synthesize photosynthetic proteins and have a high photosynthetic rate among herbaceous (Taylor and Pearcy, 1976), allowing plants to grow, store reserves and reproduce within 40 to 60 days.

It was suggested that leaf senescence in spring ephemerals is induced by light reduction during canopy closure (Vezina and Grandtner, 1965). This hypothesis was supported by the fact that spring ephemerals are well adapted to high light conditions (Sparling, 1967). However, *Erythronium japonicum* exhibits similar leaf life span in three forests differing in light conditions and in tree leaf phenology (Sawada et al., 1997). Moreover, senescence of *Floerkea proserpinacoides* appears to be independent of changes in light conditions (Mokhtar and Houle, 2005). Lapointe (2001) suggested that leaf senescence in spring ephemerals is induced by an imbalance in source-sink relationships following a reduction of carbohydrate sink demand. Indeed, modulation of the source activity of *E. americanum* by high CO_2 and high O_3 conditions did not affect the timing of leaf senescence nor plant growth (Gandin et al., 2009). Growth thus appears to become rapidly sink-limited during the season which would induce the early leaf senescence characteristic of spring ephemerals.

Modulating the source activity is an easy way to affect source-sink relationships. Source activity can be affected either by changing irradiance or by changing photoperiod. Indeed, an increase of PPFD generally leads to a higher absorption of light by chlorophyll and thus, results in an increase in photosynthetic CO_2 fixation (Demmig-Adams and Adams, 1992). In source-limited conditions, this stimulation of the source activity should increase the amount of carbohydrates translocated to the sink and eventually lead to a temporary accumulation of starch in chloroplasts if translocation rates are slower than photosynthetic rates. Photoperiod effects appear more complex. A

longer photoperiod generally induces a higher daily photosynthetic assimilation and can eventually stimulate the net photosynthetic rate (Küppers et al., 1988), leading to higher accumulation of starch in the leaf. Yet, the proportion of photoassimilates allocated to starch in the leaf is lower at longer than at shorter photoperiods (Chatterton and Silvius, 1979). In contrast, some studies indicated that a longer photoperiod could also decrease net carbon assimilation rate, as in potato leaves (Stutte et al., 1996). The authors suggested the possibility of an upper limit to the amount of starch that can be stored in the leaves, beyond which a feedback mechanism regulates carbon assimilation. More recently, it was reported that when night condition is prolonged, a higher proportion of photosynthesized sugars is allocated to leaf starch synthesis during the following photosynthetic assimilation phase (Smith and Stitt, 2007). This adjustment would avoid sugar depletion during the next prolonged night which would otherwise inhibit plant growth. It thus appears that source activity responds to both changes in photoperiod and to the level of starch present in the leaf. Yet, these adjustments of the source activity in response to photoperiod could be further modified in presence of sink limitation, or in species that do not accumulate starch in their leaves.

The present work investigated carbohydrate accumulation in storage organ of *E. americanum* Ker Gawl. (trout lily) in response to the modulation of the source activity by changing light conditions. Potential feedback inhibition of sink limitation on carbon assimilation was also monitored. Source activity was modulated using two photoperiod regimes combined with two levels of irradiance, which produced three different levels of total daily PPFD. *E. americanum* constitutes an interesting biological model to study whole-plant carbon allocation in these conditions, because of its simple morphology composed of a single leaf and a single bulb (i.e. one source vs. one sink, Blodgett, 1910). Furthermore, growth in this species becomes rapidly sink-limited and no starch accumulation occurs in the leaf. Bulb growth, gas exchanges, chlorophyll fluorescence, and carbohydrate concentrations were investigated throughout the epigeous growth period. This work should improve our understanding of the daily dynamics of source-sink relationships in conditions of sink limitation.

4.4 Material and methods

4.4.1 Plant material and growth conditions

This study was carried out on immature plants of *E. americanum*. During spring, bulbs of similar biomass, from 0.35 to 0.40 g fresh weight, were collected in a maple forest near Saint-Augustin-de-Desmaures (QC, Canada; 46°48' N, 71°23' W). They were planted in plastic pots containing Turface (Applied Industrial Materials, Corp., Buffalo Grove, Ill.). Plants were randomly assigned to one of two growth chambers (PGW36, Conviron Inc., Winnipeg MB, Canada). Plants were subjected to four light treatments which differ by irradiance and photoperiod: low irradiance (LI) 200 μmol m^{-2} s^{-1} or high irradiance (HI) 400 μmol m^{-2} s^{-1} and short photoperiod (SP) 7h or long photoperiod (LP) 14h. Both HI-SP and LI-LP treatments supplied the same daily amount of photons (DAP) i.e., 10.1 mol m^{-2} day^{-1}, whereas LI-SP and HI-LP supplied 5.0 mol m^{-2} day^{-1} and 20.2 mol m^{-2} day^{-1}, respectively. Air temperature was maintained at 12/8°C day/night and relative humidity at 65% during the whole epigeous growth period. Plants were watered daily and fertilized weekly with 10% Hoagland's solution for optimal growth (Lapointe and Lerat, 2006).

4.4.2 Harvests

Plants were harvested at the time they were transferred to growth chamber (day 0), at the initiation of leaf unfolding (day 4), when leaf was unfolded (day 7), at the initiation of leaf senescence (tip yellowing) and at complete leaf senescence. Furthermore, samples were harvested every 3 days between complete leaf unfolding (day 7) and the initiation of leaf senescence. Plant growth was measured on 6 plants per treatment per harvest, gas exchange and fluorescence on 5 plants per treatment per date and carbohydrate concentration on 3 plants per treatment per harvest.

4.4.3 Plant growth

Plants were harvested and immediately separated in three parts: leaf, bulb and roots. Leaf area was measured using a Li-Cor 3100 area meter (Li-Cor Inc, Lincoln, NE, USA). Then, leaf, bulb and roots were dried in oven for 24h at 70°C and weighed separately.

4.4.4 Gas exchange and chlorophyll fluorescence

Gas exchange measurements were carried out using a Li-Cor 6400 Portable Photosynthesis System (Li-Cor Inc, Lincoln, NE, USA). During measurements, CO_2 concentration was set at 400 ppm, air flow at 250 µmol s^{-1}, temperature at 12°C and relative humidity around 65%. Measurements were recorded at two different light conditions 400 µmol m^{-2} s^{-1} and 1000 µmol m^{-2} s^{-1} (saturating light) The CO_2 and H_2O gas analyzer allows us to estimate net photosynthetic rate (Pn), stomatal conductance (g_s), and intercellular CO_2 concentration (Ci).

Chlorophyll fluorescence was recorded at the same time as gas exchange using a Li-Cor 6400-40 pulse-modulated fluorometer. A 1-sec pulse of 14000 µmol m^{-2} s^{-1} was applied to estimate photochemical quenching (qP), non-photochemical quenching (NPQ), PSII maximum efficiency (Fv/Fm), photochemical efficiency of open center (Fv'/Fm') and photochemical efficiency of photosynthesis (PhiPS2).

4.4.5 Carbohydrates

Leaf and bulb harvested for carbohydrate analyses were immediately flash-frozen in liquid nitrogen and stored at -80°C. Starch, sucrose and reducing sugar concentrations were determined according to Blakeney and Mutton (1980). Samples were lyophilised for 48h. Dried samples were weighed before maceration in a solution of methanol, chloroform and water, 12:5:3 v/v/v, for 20 min at 65°C. After grinding with a Polytron (Kinematica, Lucerne, Switzerland), the mixture was centrifuged at 3500 rpm for 10 min at 4°C. The pellet containing starch was placed in boiling water for 90 min. Gelatinised starch was then hydrolyzed into glucose at 55°C for 60 min by amyloglucosidase. Glucose was thus quantified colorimetrically at 415 nm after reaction with *p* hydroxybenzoic acid hydrazide (Sigma Chemical Co., St-Louis, MO, USA). Similarly, reducing sugars and sucrose were quantified in the supernatant with *p* hydroxybenzoic acid hydrazide. Sucrose was assayed after digestion of supernatant by invertase then comparing total soluble sugars (after invertase digestion) with reducing sugar concentrations (before invertase digestion).

4.4.6 Statistical analysis

Evolution of all variables overtime was analysed by three-way ANOVAs to test the effects of Irradiance, Photoperiod and Time (SAS 9.1, SAS Institute, Cary, NC, USA). Significant effects were determined for $P < 0.05$. Simple main effect contrasts were analysed when an interaction was significant.

4.5 Results

4.5.1 Gas exchange

When measured at 400 µmol m^{-2} s^{-1}, Pn increased quickly during the first days of the epigeous growth period to reach a maximum at day 13, regardless of the treatment (Fig. 4.1A). The increase was higher when photoperiod was long, leading to a higher Pn at day 13 and 16 (Table 4.1). After day 13, Pn decreased steadily under long photoperiod (LP), whereas it remained constant until day 22 under short photoperiod (SP). Pn thus became lower under LP than under SP at day 22. Thereafter, Pn decreased also under SP until complete leaf senescence. Pn measured at 400 µmol m^{-2} s^{-1} was not affected by growth irradiance. Gs measured under 400 µmol m^{-2} s^{-1} was unaffected by photoperiod or irradiance (Fig. 4.1B). Ci was higher under LP than under SP at day16 and day 28 (Fig. 4.1C). When measured at saturating light conditions (i.e. 1000 µmol m^{-2} s^{-1}), Pn exhibited a similar evolution through time as when measured at 400 µmol m^{-2} s^{-1}, with a higher rate under LP at day 13 than under SP, and a lower rate under LP at day 22 (Fig. 4.1D). Gs measured at 1000 µmol m^{-2} s^{-1} tended to be higher under LP ($P = 0.066$), especially when plant growth was maximal i.e. between day 16 and 22 (Fig. 4.1E). Ci did not exhibit clear treatment effects, but it was higher under LP than under SP at day 22 and 28 (Fig. 4.1F).

4.5.2 Chlorophyll fluorescence

Chlorophyll fluorescence measurements indicated a constant increase of the photochemical efficiency of open centers (Fv'/Fm') until day 13 in all treatment groups (Fig. 4.2A). Thereafter, Fv'/Fm' decreased faster under LP than under SP, leading to a higher Fv'/Fm' under SP from day 16 until complete leaf senescence (Table 4.1). Fv/Fm, measured after dark adaptation, exhibited a quick increase during the first days of the epigeous growth

Table 4.1 Results of two way ANOVA testing effects of photoperiod duration and irradiance treatments on *Erythronium americanum* gas exchanges, chlorophyll fluorescence, bulb carbohydrate and biomass accumulation throughout the epigeous growth period. F-values are presented along with statistical differences: * P <0.05, ** P <0.01, *** P <0.001.

	Photoperiod	**Irradiance**	**PP x Irr**	**Time**	**PP x Time**	**Irr x Time**	**PP x Irr x Time**
Gas exchange							
Pn 400	3.62	1.27	1.89	76.24***	12.67*	7.57	1.89
g_s 400	2.98	5.76	9.77	125.12***	7.39	18.89	21.61
Ci 400	17.23	4.08	3.02	54.20***	2.43**	9.06	2.80
Pn 1000	4.24	0.23	6.69	101.25***	72.7*	0.60	2.83
g_s 1000	3.92	2.15	4.52	98.54***	11.22	9.33	1.81
Ci 1000	7.99	0.30	2.92	27.41***	4.48***	7.29	11.93
Chlorophyll fluorescence							
Fv'/Fm'	49.56***	1.23	3.70	39.19***	17.39**	3.69	0.13
Fv/Fm	17.57	4.38	2.03	56.83***	4.58	2.26	10.09
PhiPS2	5.38	1.09	1.64	67.88***	4.47*	211.83	1.26
qP	7.42	3.56	16.64	48.16***	2.17*	14.11	10.45
NPQ	3.91	4.19	3.65	21.01*	12.60	0.75	0.96
Carbohydrate concentration							
Starch	7.52*	2.25	4.38	84.29***	54.31**	3.04	9.16
Sucrose	0.76	0.11	5.37	93.75**	1.29	5.79	9.24
Red. sugar	1.12	0.64	2.63	7.26**	5.23*	15.52	1.81
Starchless sucrose	1.92	3.89	15.77	2.18**	0.78*	4.01	13.43
Starchless red. sugar	11.91	0.68	0.65	10.31**	2.16***	5.79	19.27
Biomass							
Bulb	8.44*	1.90	7.32	137.39***	87.51	24.12	55.54

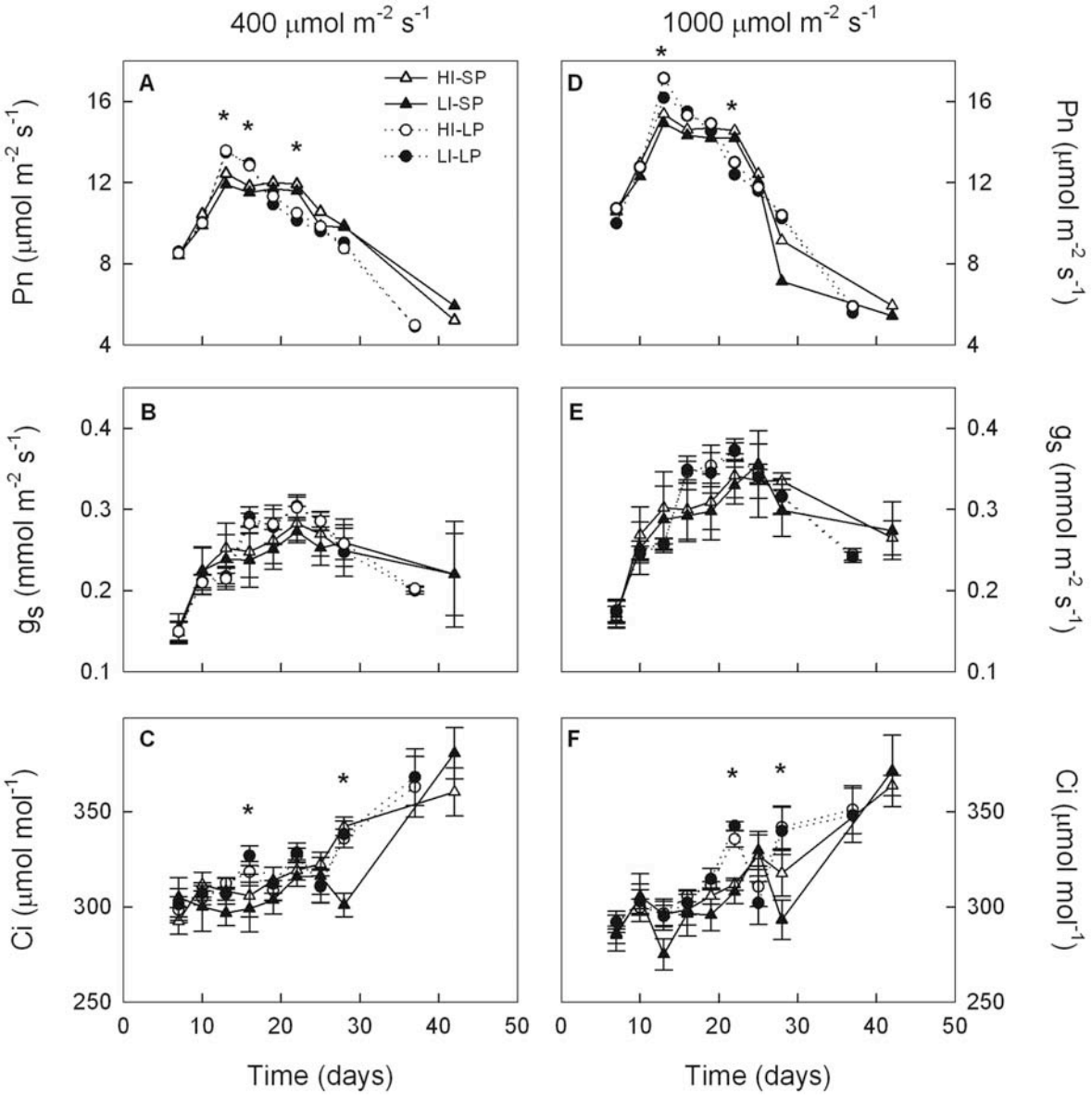

Figure 4.1 Evolution of net photosynthetic rate (A, D), stomatal conductance (B, E) and intercellular CO_2 concentration (C, F) in *Erythronium americanum* plants grown under long (dotted line, circle) or short photoperiod (solid line, triangle) and under high irradiance (black) or low irradiance (white). Gas exchange data (mean ± SE) were recorded at both 400 μmol m^{-2} s^{-1} (A, B, C) and at 1000 μmol m^{-2} s^{-1} (D, E, F) (N = 5). When the interaction Photoperiod × Time was significant, we identified with an asterisk the dates at which photoperiod treatments differed.

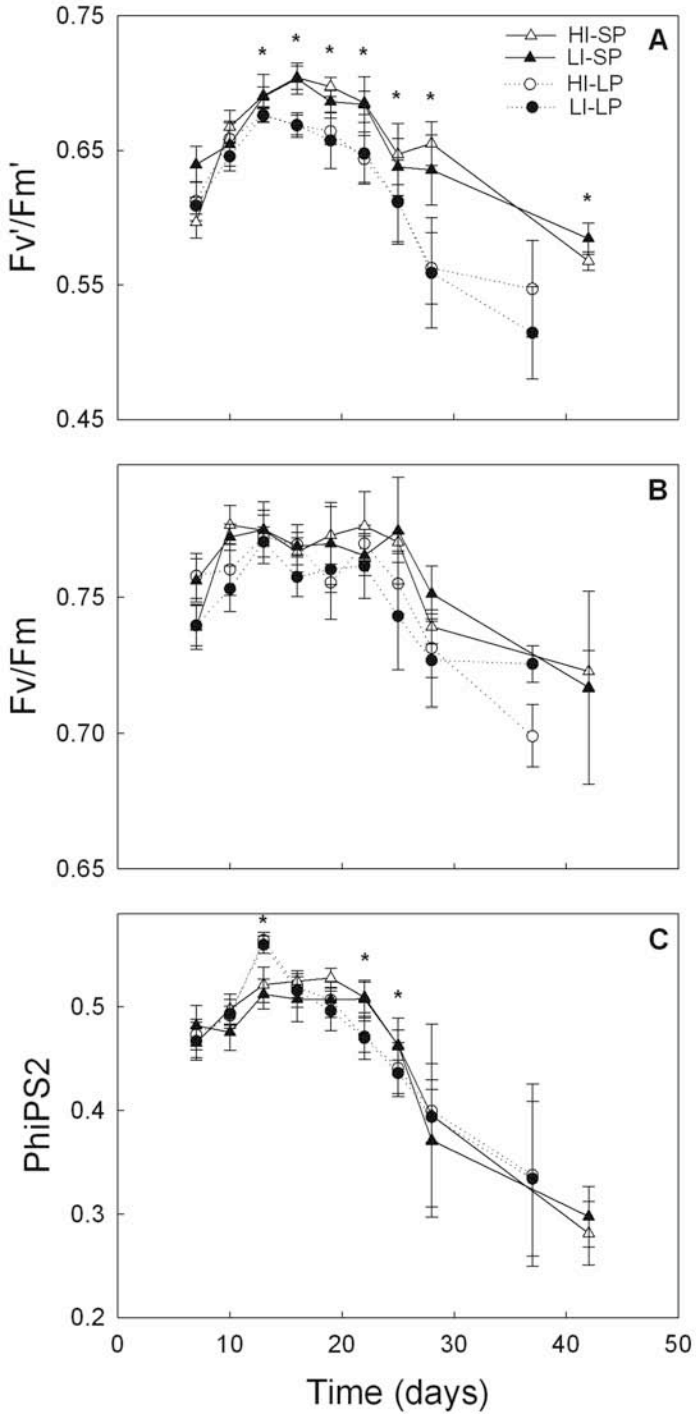

Figure 4.2 Evolution of Fv'/Fm' (A), Fv/Fm (B) and PhiPS2 (C) in *Erythronium americanum* plants grown under long (dotted line, circle) or short photoperiod (solid line, triangle) and under high irradiance (black) or low irradiance (white). Shown are the means ± SE (N = 5). When the interaction Photoperiod × Time was significant, we identified with an asterisk the dates at which photoperiod treatments differed.

period (Fig. 4.2B) and then remained high until a few days before leaf senescence (day 25). Fv/Fm did not differ between photoperiod or irradiance treatments. In contrast, PhiPS2 exhibited a faster increase under LP than under SP until day 13 and then, a faster decrease under LP until the initiation of leaf senescence (Fig. 4.2C). Similarly to Pn, PhiPS2 was thus higher under LP than under SP at day 13 and lower at day 22 and day 25.

Photochemical quenching (qP) exhibited a slow decrease until the beginning of leaf senescence with no difference between photoperiod or irradiance (Table 4.1, Fig. 4.3A). qP was higher under LP at day 13 and at complete leaf senescence. Inversely, NPQ exhibited an initial quick decrease until day 13 under LP, and until day 16 under SP, regardless of the irradiance (Fig. 4.3B). Thereafter, NPQ increased continuously until leaf senescence in all treatments.

4.5.3 Carbohydrates

In the bulb, starch concentration decreased strongly during the first days until day 4. Then it increased faster under LP than under SP (Table 4.1, Fig. 4.4A). After day 13, the rate of increase appeared similar under both photoperiods until day 19 at which point maximum concentration was reached under LP. Starch continued to accumulate until day 28 under SP. Final starch concentration was 13% lower in plants growing under LP than under SP. Starch concentration was not affected by irradiance. Sucrose concentration exhibited a regular decrease during the whole epigeous growth period, regardless of the photoperiod or irradiance (Fig. 4.4B). Reducing sugar concentration decreased more quickly under LP than under SP, leading to lower concentration from day 2 to day 13 (Fig. 4.4C). Thereafter, reducing sugar concentration reached its minimum until complete leaf senescence, with no difference between treatments. Reducing sugar concentration was not affected by irradiance. Sucrose and reducing sugar concentrations were also presented as a function of bulb biomass to which starch biomass had been subtracted (mg g^{-1}DW-Starch; Fig. 4.4D, E), to avoid the dilution effect caused by increasing starch concentrations. These starchless concentrations are likely more representative of the concentration that can be perceived by the plant. Starchless sucrose concentrations were similar among treatments until day 22 after which they strongly increased under SP (Fig. 4.4D). Starchless reducing sugar

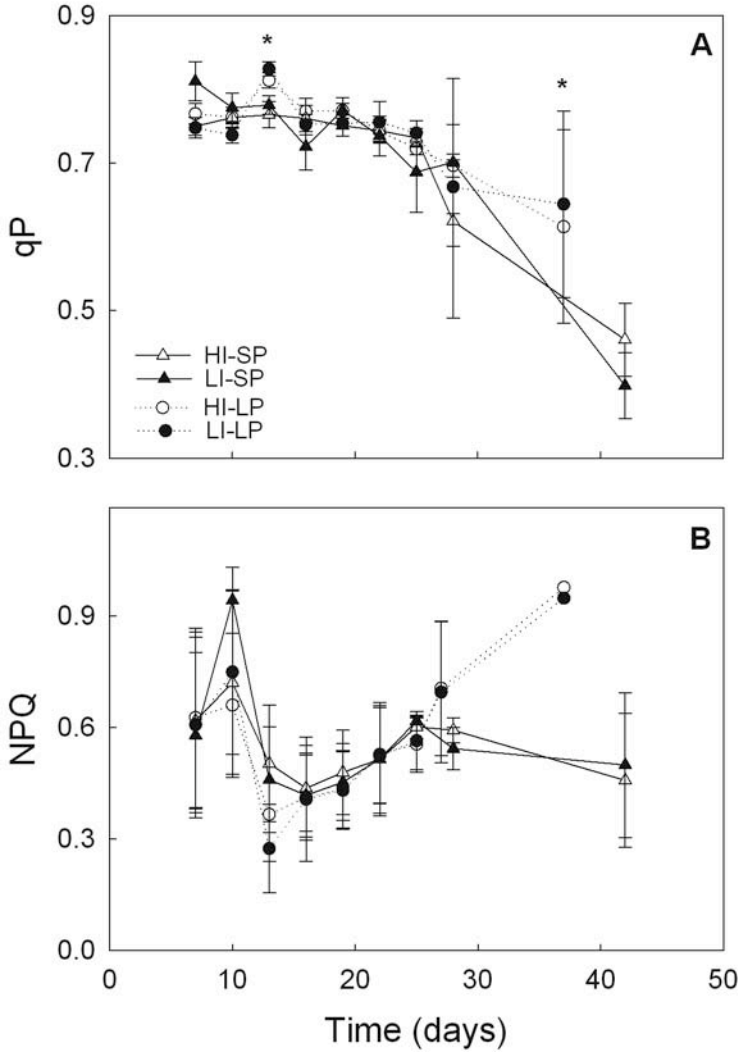

Figure 4.3 Evolution of photochemical quenching (A) and nonphotochemical quenching (B) in *Erythronium americanum* plants grown under long (dotted line, circle) or short photoperiod (solid line, triangle) and under high irradiance (black) or low irradiance (white). Shown are the means ± SE (N = 5). When the interaction Photoperiod × Time was significant, we identified with an asterisk the dates at which photoperiod treatments differed.

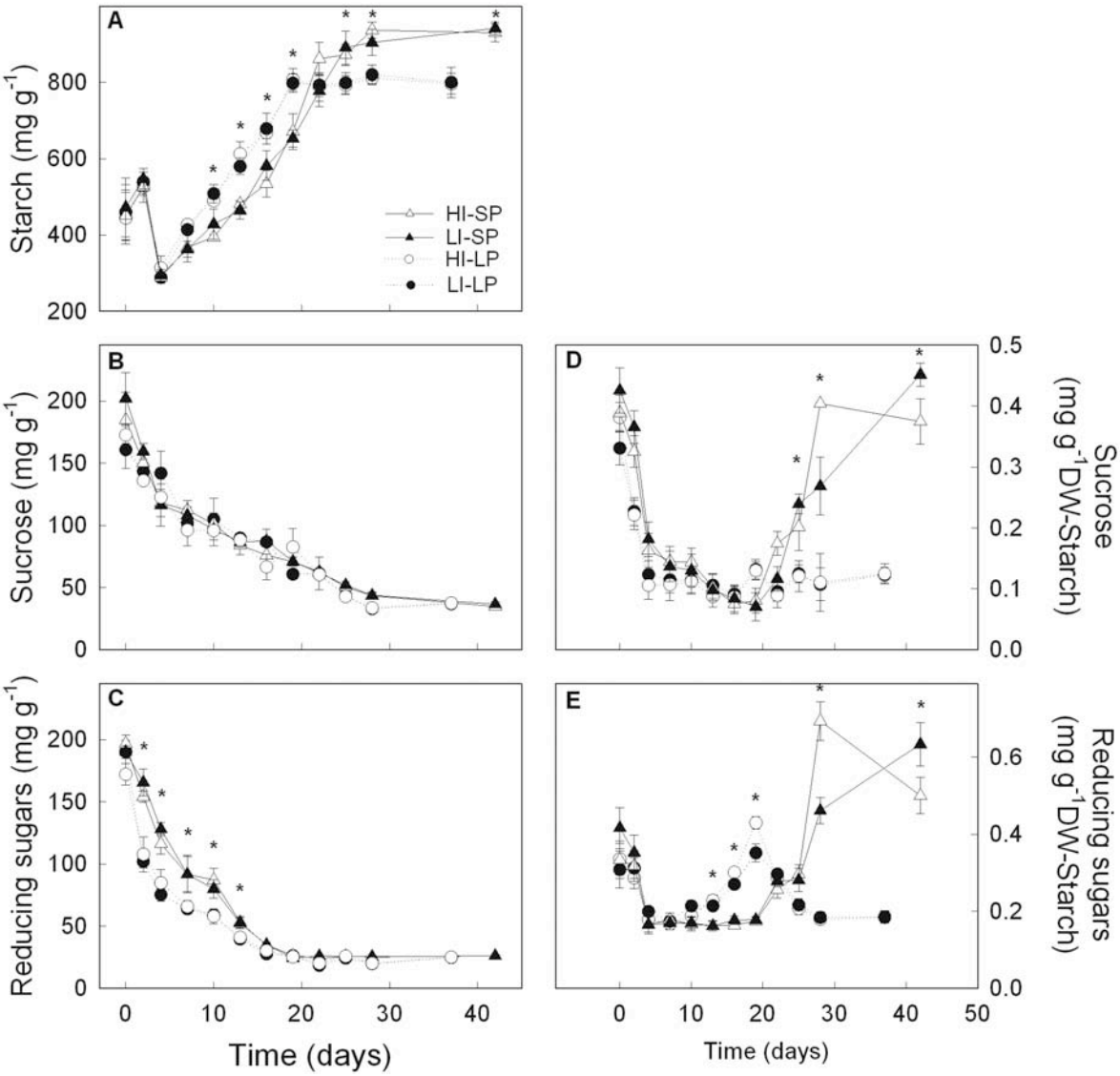

Figure 4.4 Evolution of starch (A), sucrose (B, C) and reducing sugar (C, D) concentration in bulb of *Erythronium americanum* plants grown under long (dotted line, circle) or short photoperiod (solid line, triangle) and under high irradiance (black) or low irradiance (white). Concentration of sucrose and reducing sugars are presented as a function of total bulb biomass (mg g^{-1}; B, C) and as a function of bulb biomass other than starch (mg g^{-1}DW-Starch; E, F). Shown are the means ± SE of 3 plants. When the interaction Photoperiod × Time was significant, we identified with an asterisk the dates at which photoperiod treatments differed.

concentrations were similar in all treatments during the first days (Fig. 4.4E). They increased quickly under LP after day 13 and exhibited a peak at day 20, whereas they remained constant until day 19 under SP. After day 22, starchless reducing sugar concentration strongly increased under SP until complete leaf senescence.

4.5.4 Biomass allocation

Bulb biomass exhibited a slight decrease until day 7 in all treatments (Fig. 4.5). Between day 7 and day 13, bulb biomass increased faster under LP than under SP, regardless of the irradiance (Table 4.1). Bulb growth then increased at a similar rate under both photoperiods until day 25. Bulb biomass stopped increasing a few days earlier under LP than under SP, and final bulb biomass was similar among treatments.

4.6 Discussion

Neither irradiance nor daily amount of photons appears to affect net photosynthetic rate, measured at either 400 $\mu mol\ m^{-2}\ s^{-1}$ or at saturating light conditions. Growth irradiance generally induces important changes within the leaf, which results in increasing photosynthesis in high-light-grown plants especially when measured near light saturating conditions (Evans and Poorter, 2001). Our results suggest that there is no low-light acclimation in plants growing under 200 $\mu mol\ m^{-2}\ s^{-1}$. Eickmeier and Schussler (1993) also showed that *Claytonia virginica*, another spring ephemeral, do not present acclimation to low irradiance. In *E. americanum*, Pn was initially higher in plant growing in LP, supported by a momentarily higher qP and higher PSII efficiency of open center (Fv'/Fm'). But, Pn decreased early in plants growing under LP whereas it was maintained fairly constant until a few days before leaf senescence under SP. This is consistent with the data reported on potato (Stutte et al., 1996) where Pn decreased over time under LP due to starch accumulation in the leaves. The early decrease of Pn was accompanied by a similar decrease of PhiPS2 and Fv'/Fm' under LP after day 13, supporting the idea that Pn was inhibited early in plants growing under LP. Given that *E. americanum* do not accumulate starch in its leaf, it is possible that the early decrease of Pn under LP could be due to an excess of sucrose or hexose accumulation. Yet, previous studies fail to identify which sugar accumulates in the leaf at the time Pn starts to decrease under LP (unpublished data).

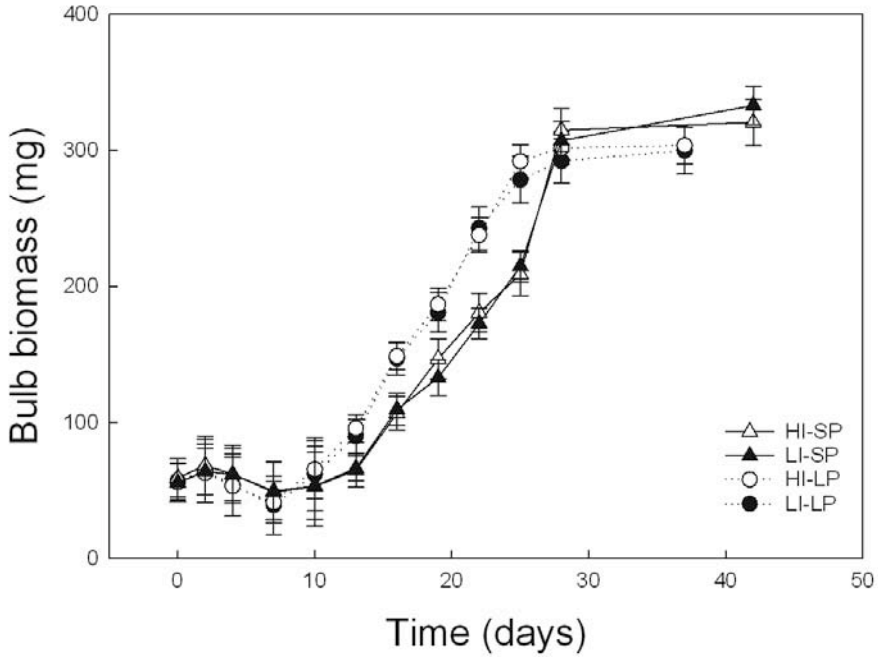

Figure 4.5 Evolution of bulb biomass (A) of *Erythronium americanum* plants grown under long (dotted line, circle) or short photoperiod (solid line, triangle) and under high irradiance (black) or low irradiance (white). Shown are the means ± SE (N = 6).

Other potential factors that could induce a reduction in Pn under LP have also been investigated. The lack of effect of the photoperiod on g_s suggests that stomatal closure was not responsible for the faster decrease of Pn under LP. The fact that Pn was higher when measured under 1000 µmol m^{-2} s^{-1} than under 400 µmol m^{-2} s^{-1} even for LP plants suggest that the photosynthetic capacity was not limiting Pn under the growth light conditions used. Indeed, Sparling (1967) indicated that spring ephemerals are well adapted to high irradiance conditions, allowing them to sustain high carbon assimilation rates. We reported a high and fairly constant Fv/Fm until a few days before the initiation of leaf senescence, regardless of the treatments. Low Fv/Fm values are associated with photoinhibition and alteration of PSII reaction center (Demming-Adams and Adams, 1992). Therefore, the early decrease of Pn under LP was not due to photoinhibition or excess irradiance, but probably to feedback inhibition. However, qP did not exhibit a clear effect of photoperiod or irradiance and NPQ increased continuously until leaf senescence. The evolution of quenching indicated a lower loss of energy under LP when Pn reached its maximum (Roháček, 2002), but did not explain the rapid inhibition of Pn thereafter. Thus, given the lack of effect of photoperiod on quenching, the early inhibition of photosynthesis under LP does not seem to be due to a feedback control from the biochemical phase of photosynthesis. Further studies are needed to identify the triggering signal inducing this early feedback inhibition of photosynthesis under LP.

The decreasing starch concentration in the bulb at the beginning of the epigeous growth period was probably due to the action of amylase allowing to maintain a high pool of soluble sugars available for leaf expansion and growth (Smith et al., 2005). Shortly after, starch started to accumulate, with a faster rate under LP than under SP, until day 13. This faster accumulation could be related to the higher rates of carbon fixation under LP until day 13. After day 13, kinetic of starch accumulation became similar under both photoperiods, probably due to the early inhibition of Pn under LP. In contrast, neither irradiance nor daily amount of photons affected starch concentration. Starch accumulation thus seems to be independent of the amount of energy available and of the daily amount of carbon assimilated, but strongly dependent on the daily duration of carbon assimilation.

Smith and Stitt (2007) suggested the presence of a control mechanism that modulates starch synthesis in the leaf during the day as a function of the ability to use it for plant growth during the night. Starch accumulation in the leaf would thus act as a buffer to either avoid carbon starvation at the end of the night under SP, or when the rate of carbon fixation is higher than the rate of carbon translocation towards the sink. Similarly, we postulate that early in the season, *E. americanum* plants grown under SP have very limited amount of sugar in the leaf at the end of the night, whereas under LP, leaves start the photoperiod with some sugars left. However, the incapacity of *E. americanum* to synthesize starch in its leaves could explain the early reduction in Pn observed under LP due to the absence of this metabolic pathway to divert the extra daily C. Under SP, there would be a closer equilibrium between the total amount of carbon fixed per day and its use during the 24h period through carbon translocation and bulb starch synthesis, explaining the constant Pn over time until sink-limitation builds up at the end of the growth season and induces leaf senescence.

Starchless reducing sugar concentration (mg g^{-1} DW-Starch) increased strongly in the bulb under LP and reached a maximum at the time starch stopped to accumulate. Hexose accumulation generally induces a decrease of sink strength and a slowdown of carbohydrate translocation (Herbers and Sonnewald, 1998). This decrease of sink strength could be responsible for the induction of leaf senescence a few days later. Thus sink activity changes during the season and would eventually lead to feedback inhibition of Pn under both SP and LP a few days before the first sign of leaf senescence becomes visible.

In summary, photoperiod had more influence on the source-sink relations in *E. americanum* than irradiance or total daily amount of light. Daily source activity appears to be in equilibrium with sink activity under SP, whereas under LP, there appears to be a slight desequilibrum that, with time, would explain the reduction in Pn. Overall, both plant biomass and starch accumulation were unaffected by the treatments, probably due to fine regulatory controls that are activated under LP. Early slowdown of Pn under LP most probably avoids the induction of precocious leaf senescence. However, under both photoperiods, reducing sugars eventually accumulates in the sink, as sink strength decreases, inducing leaf senescence. Therefore, different regulatory steps are activated throughout the growth period to maintain the balance between source and sink activity.

Further work will focus on the fine regulatory mechanisms that take place early in the season in species such as *E. americanum* that do not accumulate starch in their leaves.

4.7 Acknowledgements

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Chapitre 5 Conclusion générale

Cette étude a permis de mettre en évidence plusieurs aspects des relations source-puits de carbone chez une géophyte printanière, l'érythrone d'Amérique. À cette fin, nous avons utilisé plusieurs facteurs abiotiques modulant tour à tour l'activité du puits et celle de la source, soit respectivement la température de croissance, la concentration en CO_2 et O_3 et la lumière. Nous avons concentré notre attention sur la réponse du métabolisme de l'organe de réserve qui représente le puits fort chez cette espèce, à un déséquilibre entre l'activité de la source et celle du puits. Nous nous sommes également intéressés à l'impact de cette réponse de l'organe de réserve sur l'activité de la source, qui est représentée par une unique feuille chez cette espèce. Nous avons ainsi tenté d'identifier l'existence d'un éventuel rétrocontrôle du puits visant à rétablir l'équilibre entre source et puits.

5.1 L'accumulation d'amidon dans l'organe de réserve

Au cours de nos travaux, nous avons pu observer un patron commun d'évolution de la concentration en l'amidon. Ce patron présente, au cours des premiers jours, une consommation d'amidon. Cette hydrolyse des réserves permet d'alimenter en hydrates de carbone l'expansion de l'appareil foliaire et du nouveau bulbe. Grâce à l'expansion rapide de l'appareil foliaire, celui-ci devient rapidement photosynthétiquement actif permettant à l'activité de la source de prendre son essor. L'accumulation d'amidon dans l'organe de réserve débute alors et se poursuit durant la majeure partie de la croissance épigée. Cette accumulation est rapide, régulière et massive, menant à une quasi-saturation de l'organe de réserve (80-90%). Bien que ce patron d'accumulation semble immuable chez cette espèce et ce, quel que soit le traitement, les paramètres et la cinétique de celui-ci varient en réponse aux facteurs abiotiques utilisés.

Nous avons montré que la vitesse d'accumulation d'amidon augmentait avec la température. Ce phénomène semble en partie dû à la présence d'un «cycle futile» de dégradation et de re-synthèse du saccharose à température froide. Ce cycle est induit par une stimulation, aux températures froides, des activités de l'invertase vacuolaire et de la saccharose-phosphate-synthase. Cette stimulation limite ainsi l'approvisionnement en hexoses de l'ADP-glucose pyrophosphorylase. A l'inverse, l'activité de ce «cycle futile» est réduite à forte température et la disponibilité des hexoses est donc plus importante pour la synthèse d'amidon (Fig 5.1). La vitesse d'accumulation de l'amidon est aussi

dépendante de la quantité d'hydrates de carbone transférés au sein de l'organe de réserve. Cette quantité est vraisemblablement plus importante à forte température, compte tenu du plus fort taux d'assimilation net au sein de la feuille, davantage à même d'alimenter la synthèse d'amidon, mais aussi la respiration cellulaire. Il apparaît par ailleurs vraisemblable que la respiration du bulbe augmente avec la température. La respiration aérobie constitue le principal producteur d'ATP au sein d'un organe hétérotrophe. De plus, l'activité de l'ADP-glucose pyrophosphorylase est très dépendante en énergie. Un fort taux de respiration pourrait permettre de stimuler, par un apport accru en énergie, la synthèse d'amidon. Ainsi, le puits semble pouvoir tirer partie d'une quantité plus importante d'hydrates de carbone disponibles à forte température, en alimentant avantageusement, à la fois en substrat mais aussi en énergie, la synthèse d'amidon.

Néanmoins, l'approvisionnement en hydrates de carbone et le cycle futile ne peuvent expliquer complètement la vitesse d'accumulation d'amidon. En effet, lorsque nous avons stimulé la source par une concentration élevée en CO_2, l'accumulation d'amidon ne fut pas pour autant accélérée et ce, malgré une quantité accrue d'hydrates de carbone synthétisés et disponibles. De la même manière, l'inhibition de l'activité de la source sous fort O_3 n'induit pas d'effet sur l'accumulation de biomasse au sein du puits. Ces modulations ayant été effectuées sur des plants d'érythrone d'Amérique poussant à une température de croissance de 18/14°C, l'induction du cycle futile est donc vraisemblablement limité. Nos travaux ont montré que les excès de carbone dus à la stimulation de la source sont brûlés au cours de la respiration de l'organe de réserve. La stimulation de la respiration est alors assumée en majeure partie par une augmentation de l'activité de la voie alternative et ne permet donc pas de bénéficier d'une synthèse accrue d'énergie. Il apparaît alors vraisemblable, dans ce cas, que l'absence d'augmentation de la synthèse d'ATP explique l'absence de stimulation de la synthèse d'amidon en condition d'excès de carbone. Le statut énergétique de la cellule (ATP/ADP) semble ainsi jouer un rôle prépondérant sur la vitesse d'accumulation de l'amidon au sein du bulbe d'érythrone d'Amérique, que ce soit en réponse à une modulation de l'activité de la source par la concentration en CO_2 et en O_3 ou bien, à une modulation de l'activité du puits par la température de croissance. Il s'avèrerait particulièrement

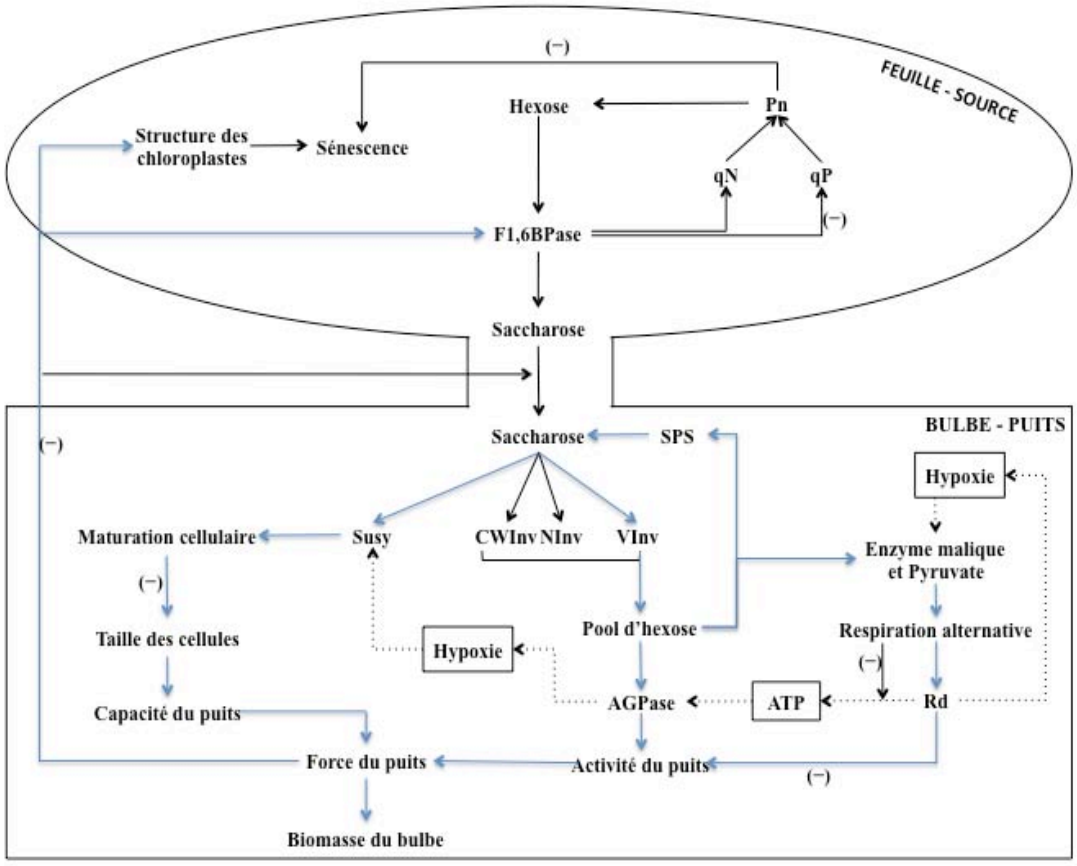

Figure 5.1 Synthèse schématique du rôle du métabolisme carboné dans les relations source-puits chez l'éryhthrone d'Amérique. Les traits bleus indiquent les relations entre deux facteurs mises en évidence dans ce mémoire. Les traits noirs indiquent les relations entre deux facteurs connues de la littérature. Les traits pointillés indiquent les hypothèses misent en évidence dans la discussion. (-) indique une relation négative entre deux facteurs.

intéressant, au cours des prochaines expériences, de mesurer l'évolution de la concentration des adénylates au sein des cellules du bulbe, afin de mieux cerner leurs impacts sur la physiologie de l'érythrone d'Amérique.

D'autre part, la production d'énergie par la respiration dépend fortement de la disponibilité en pouvoir réducteur, mais aussi en oxygène, qui constitue le dernier accepteur d'électrons. Or, la disponibilité en oxygène est un problème récurrent au sein des organes de réserve (Geigenberger *et al.,* 2000). Nous avons pu observer que le bulbe d'érythrone d'Amérique accumulait jusqu'à 80-90% de sa biomasse totale en amidon, créant ainsi un tissu très compact. L'accumulation plus rapide d'amidon observée à forte température pourrait contribuer à induire une condition d'hypoxie précoce. Cet état d'hypoxie semble étayé par une induction tout aussi précoce de la saccharose synthase à forte température, induisant une plus faible capacité du puits. En effet, il est connu que la saccharose synthase est stimulée en cas d'hypoxie, alors que les invertases présentent une inhibition (Zeng *et al.,* 1999). De manière similaire, l'activité de la saccharose synthase a augmenté lorsque la respiration du bulbe, et donc la consommation d'oxygène, sont fortement stimulées, suite à la stimulation de l'activité de la source par une concentration élevée en CO_2. Cette augmentation de la respiration serait susceptible d'amplifier l'hypoxie induite par une forte accumulation d'amidon au sein du bulbe. Ainsi, une forte synthèse d'amidon pourrait affecter la diffusion et l'approvisionnement en oxygène, tandis qu'un fort taux de respiration réduira l'oxygène présent au sein de l'organe de réserve. La combinaison de ces deux facteurs est susceptible de stimuler, via l'émergence d'une hypoxie, l'hydrolyse du saccharose par la saccharose synthase au détriment des invertases, modulant ainsi les signaux basés sur les hexoses. D'autre part, il a été montré qu'une faible concentration en oxygène induit une induction de l'enzyme malique à NAD (Roberts *et al.,* 1992). Nos travaux ont indiqué que l'augmentation de la respiration sous fort CO_2, due à une augmentation de la voie alternative, est probablement activée par le pyruvate et l'enzyme malique à NAD. Aussi, l'hypoxie pourrait justifier l'induction de cette enzyme et par conséquent, la voie alternative de la respiration. Comme mentionné dans le paragraphe précédent, la stimulation de la respiration alternative permet de maintenir une synthèse constante d'amidon dans le bulbe quelle que soit l'activité de la source, tout en consommant les excès de carbone. Donc, de par ses effets sur les activités de la saccharose synthase et

de l'enzyme malique à NAD, la concentration en oxygène pourrait jouer un rôle clé au sein de l'organe de réserve, liant l'approvisionnement de celui-ci en hydrates de carbone, la respiration cellulaire et la quantité d'énergie produite par celle-ci, et la synthèse d'amidon. À l'avenir, il apparaît des plus pertinents d'investiguer l'évolution de la teneur en oxygène du bulbe d'érythrone d'Amérique et ce, en parallèle avec l'accumulation d'amidon et le taux de respiration.

5.2 Le contrôle du puits sur l'activité de la source et l'initiation de la sénescence foliaire

La sénescence foliaire est un processus complexe qui a fait l'objet de nombreuses études et articles de revue (Buchanan-Wollaston *et al.*, 2003; Lim *et al.*, 2007). Nous avons confirmé que la température de croissance module la durée de vie de la feuille. La sénescence chez l'érythrone d'Amérique est retardée à mesure que la température baisse. Au cours de chacune des expériences (chapitre 2 à 4), la sénescence est initiée quelques jours après l'arrêt de l'accumulation d'amidon et de la croissance du bulbe. La sénescence des éphémères printanières semble donc induite lorsque que la demande en carbone du puits diminue (Fig 5.1). Par ailleurs, nous avons pu mettre en évidence l'accumulation de sucres solubles, principalement du glucose et du fructose, suite à l'arrêt de la synthèse d'amidon. Nous pouvons supposer que cette accumulation mène, via une baisse de la force du puits, à une diminution du taux de transfert des hydrates de carbone vers le bulbe. Cette diminution précède ainsi la chute de l'activité de la fructose-1,6-bisphosphatase, l'altération des chloroplastes (Fv/Fm) et la sénescence subséquente. La sénescence foliaire chez l'érythrone d'Amérique semble effectivement être initiée dès lors que la demande en carbone du puits diminue, utilisant l'accumulation de sucres solubles comme signal entre puits et source.

Nos résultats ont aussi indiqué une variation du taux de photosynthèse net au cours du temps. Si l'augmentation de celui-ci durant les premiers jours se justifie facilement par la mise en place de l'appareil foliaire et de la synthèse protéique, la chute qui s'ensuit est plus difficilement explicable. Cette chute est néanmoins présente quel que soit le traitement appliqué, excepté lorsque les plants croissent sous photopériode courte. D'autre part, la chute du taux de photosynthèse net survient de manière trop précoce pour être entièrement imputable à l'accumulation des sucres solubles ou à l'arrêt de

l'accumulation d'amidon, ni même au processus de sénescence. De plus, le Fv/Fm ne suggère pas d'altération des chloroplastes écartant l'idée d'un signal précoce de sénescence. Au delà du contrôle grossier exercé par le puits sur la sénescence foliaire, un second contrôle, plus fin et continu, semble réguler le taux d'assimilation net et ce, très tôt dans la saison de croissance. De plus ce contrôle précoce semble dépendant de la durée de l'assimilation photosynthétique, alors que le taux de photosynthèse net instantané n'influe aucunement sur ce contrôle. En effet, le Pn présente la même diminution précoce lorsque l'activité de la source est modulée, que ce soit par la concentration en CO_2, en O_3 ou encore par l'irradiance. Seule une baisse de l'activité du puits à faible température altère ce contrôle précoce du Pn. Il semble alors qu'à 8/6°C, Pn, qui est plus faible, est davantage en équilibre avec la force du puits. A l'inverse, l'activité de la source à forte température est en déséquilibre avec celle du puits. Ce déséquilibre est par ailleurs amplifié sous fort CO_2. La baisse de la température de croissance et de la durée de la photopériode semble induire un meilleur équilibre entre source et puits qui s'accompagne de l'absence d'une baisse précoce du Pn. Le contrôle précoce du Pn semble, selon toute vraisemblance, être lié à l'activité du puits et donc aux taux d'utilisation des hydrates de carbone photosynthétisés.

Il a récemment été mis en évidence que le tréhalose (et trehalose-6-phosphate), connu pour son rôle de sucre de stockage, pouvait jouer un rôle majeur dans la feuille et alternatif aux photoassimilats dans le contrôle exercé par les sucres sur la photosynthèse (Eastmond *et al.*, 2003). Ainsi, chez l'*Arabidopsis thaliana*, une synthèse accrue de tréhalose dans les feuilles annule complètement l'inhibition induite par un excès de glucose sur l'activité photosynthétique (Avonce *et al.*, 2004). Or, la synthèse de tréhalose interagit aussi avec la synthèse temporaire d'amidon dans la feuille, notamment en partageant un substrat commun, le glucose-6-phosphate. La synthèse de tréhalose et son interaction avec le glucose n'ont cependant pas été montrées au sein d'un organe de réserve. Nous pouvons, toutefois, émettre l'hypothèse qu'à mesure que la synthèse d'amidon s'intensifie dans le bulbe d'érythrone d'Amérique, la synthèse de tréhalose pourrait diminuer par manque de substrat, levant ainsi progressivement l'influence du tréhalose sur le rétrocontrôle induit depuis le puits, par le glucose. Cette intensification du rétrocontrôle à mesure que la synthèse d'amidon s'intensifie est alors susceptible d'expliquer l'inhibition précoce de l'activité photosynthétique. À l'inverse,

lorsque l'activité du puits est faible (courte photopériode, température froide), la synthèse de tréhalose pourrait être élevée et donc l'altération induite par celui-ci sur le rétrocontrôle serait importante.

Nos travaux ont donc mis en évidence un contrôle fort de la photosynthèse et de la sénescence lorsque l'accumulation d'amidon s'arrête, ainsi qu'un second contrôle plus fin du Pn, l'ajustant à l'activité du puits tout au long de la croissance de l'organe de réserve. Toutefois, des travaux supplémentaires seront nécessaires pour mieux comprendre et identifier les acteurs de ce contrôle régulier et proportionné du taux d'assimilation net. Il sera entre autres propice d'étudier le métabolisme du tréhalose afin de cerner un rôle éventuel de ce métabolite.

Notre étude a permis d'identifier différents mécanismes de régulation du métabolisme carboné au sein du puits, qui permettent d'ajuster l'activité du puits à l'accroissement de sa capacité à stocker le carbone et ce, en modulant l'activité des enzymes du métabolisme du saccharose et la respiration mitochondriale. D'autre part, la saturation du bulbe en amidon et l'arrêt de la synthèse de l'amidon semblent induire la sénescence foliaire. Nous avons pu mettre en évidence, tout au long de ces travaux, le rôle prédominant de l'organe de réserve sur la physiologie mais aussi sur la phénologie de la plante et ce, particulièrement dans des conditions où la croissance est limitée par les puits. Il apparait prépondérant de continuer d'étudier l'impact de cette limitation de la croissance par les puits afin de mieux caractériser les mécanismes de contrôle impliqués et ce d'autant plus que la littérature est limitée à ce sujet malgré le fait que plusieurs études suggèrent qu'il s'agit d'un phénomène fréquent chez les espèces indigènes. Par ailleurs, ayant observé une accumulation de biomasse inférieure aux fortes températures, certaines questions se posent quant à la survie de ce type de plante dans un contexte de réchauffement climatique. Bien que possédant une aire de répartition étendue qui atteintle sud des États-Unis, l'érythrone d'Amérique semble y coloniser plutôt les milieux alpins ou subalpins. La température moyenne printanière y est ainsi plus basse, supportant l'idée d'une faible adaptation de cette espèce à des températures plus élévées. Nous pouvons ainsi supposer qu'une augmentation future de la température printanière viendrait repousser son aire de répartition vers des zones plus nordiques ou vers des altitudes plus élevées. Cependant, elle est inféodée aux forêts décidues; elle suivra donc la migration future de ces forêts. D'autre part, les modèles

climatiques indiquent une augmentation de la concentration en CO_2 atmosphériques. Or, cette espèce ne semble pas tirer avantage d'un apport accru en CO_2, du moins tant que l'on n’augmente pas la capacité du puits. Des études ultérieures permettront d’affiner notre compréhension de la physiologie des éphémères printanières et ainsi mieux prédire comment et dans quelle mesure elles pourront s’adapter aux changements climatiques.

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Annexe

Liste des publications

Low temperature: a way to reduce imbalance between sink capacity to accumulate carbohydrates and sink growth rate in spring geophytes.

Anthony Gandin, Sylvain Gutjahr, Pierre Dizengremel et Line Lapointe.

(Soumis) Plant Cell and Environment

The alternative respiratory pathway allows sink to cope with changes in carbon availability in the sink-limited plant *Erythronium americanum*

Anthony Gandin, Line Lapointe et Pierre Dizengremel

(2009) *Journal of Experimental Botany*, 60 (15): 4235-4248.

Source-sink relationships are dependent upon photoperiod but not irradiance in the sink-limited species *Erythronium americanum*

Anthony Gandin, Pierre Dizengremel et Line Lapointe

(À soumettre)

Rôle du métabolisme carboné dans la modulation de l'activité de la source et du puits chez l'érythrone d'Amérique (*Erythronium americanum*)

Résumé: Les relations entre l'activité de la source et l'activité du puits contrôlent en grande partie la croissance des plantes. Ces activités varient au cours du développement, mais aussi en réponse à des changements des conditions environnementales. Notre étude avait pour but d'identifier le rôle du métabolisme carboné dans la réponse de la croissance d'*E. americanum* à la modulation des activités de la source et du puits. Dans une première partie, l'activité du puits est modulée par la température de croissance. Aux fortes températures, l'activité du puits est plus élevée, alors que sa capacité est réduite. Ces effets, dus à la modulation du métabolisme du saccharose, mènent à une saturation précoce en amidon des bulbes à forte température. Par la suite, la baisse de la demande en carbone du puits induit un rétrocontrôle négatif de l'activité photosynthétique et finalement, la sénescence foliaire. À l'inverse, l'activité du puits à faible température est en rythme avec l'accroissement de la capacité, menant à une biomasse supérieure du bulbe en fin de croissance épigée. Dans une seconde partie, l'activité de la source est modulée en changeant la concentration en CO_2 et en O_3. Malgré la stimulation de la source sous fort CO_2 et son inhibition sous fort O_3, l'accumulation d'amidon et la biomasse du bulbe ne sont pas affectées. En effet, le surplus de carbone parvenant au puits est brûlé par la voie alternative de la respiration, celle-ci étant stimulée par l'activité de l'enzyme malique. La voie alternative de la respiration évite ainsi une saturation hâtive en amidon et éventuellement, une sénescence foliaire précoce. Dans une dernière partie, l'activité de la source est modulée par l'irradiance et la photopériode. L'accumulation d'amidon varie en fonction de la photopériode alors que l'irradiance n'a aucun effet. De plus, l'activité photosynthétique est inhibée très précocement sous longue photopériode. Cette inhibition semble due à un déséquilibre entre la quantité totale de carbone fixé par jour et son utilisation suite à son transfert au sein du bulbe. Nous pouvons donc conclure que les régulations du métabolisme carboné permettent d'ajuster l'activité du puits à la capacité de celui-ci chez l'*E. americanum.*

Mots-clés: amidon, bulbe, *Erythronium americanum*, métabolisme carboné, relations source-puits.

Impact of carbon metabolism in the modulation of the source and sink activities in *Erythronium americanum*.

Abstract : Relationships between source and sink activities largely control the growth of plants. Both activities vary during development as well as in response to changes in environmental conditions. The aim of our study was to identify the role carbon metabolism plays in the response of *E. americanum* growth to changes in source and sink activities. Firstly, sink activity is modulated by changing growth temperature. Sink activity is higher at higher temperatures, whereas sink capacity is more restricted. These effects, due to the modulation of sucrose metabolism, lead to an earlier starch saturation of bulbs at higher temperature. Thereafter, the reduction in carbon sink demand induces a feedback inhibition of photosynthetic activity and finally, leaf senescence. In contrast, sink activity at low temperature is more in rhythm with the increasing sink capacity, leading to larger bulbs at the end of the epigeous growth season. Secondly, source activity is modulated by changing CO_2 and O_3 concentrations. Despite a stimulation of the source activity under high CO_2 and its inhibition under high O_3, neither plant growth nor starch accumulation was affected. Indeed, excess carbon translocated within the sink is burned by the alternative respiratory pathway. This pathway is stimulated by malic enzyme. Alternative respiratory pathway thus avoids an early starch saturation of the bulb and eventually, an early leaf senescence. Finally, source activity is also modulated by changing irradiance and photoperiod regimes. Starch accumulation changes in response to photoperiod but not to irradiance. Furthermore, photosynthetic activity is inhibited early in the season under long photoperiod. This inhibition seems due to an imbalance between the total amount of carbon fixed per day and its utilisation following translocation to the bulb. We can thus conclude that regulation of carbon metabolism allow to adjust sink activity to sink capacity in *E. americanum*.

Key words: bulb, *Erythronium americanum*, carbon metabolism, source-sink relationships, starch.