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par

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Sujet :

Effets de fongicides anti-*Botrytis* sur les organes végétatifs et reproducteurs de la vigne

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Une dernière phrase pour caractériser sans aucun doute cette thèse : « Un résultat négatif est tout de même un résultat. » : et oui ! comme quoi, on peut tout de même en faire quelque chose !

RESUME

Au vignoble, l'utilisation de fongicides est indispensable pour lutter contre la pourriture grise, causée par le pathogène *Botrytis cinerea*. L'application de **fongicides anti-*Botrytis*** est recommandée à trois stades de développement de la vigne : fin floraison (stade A), fermeture de la grappe (stade B) et début véraison (stade C). Parmi ces fongicides, le **fludioxonil** et le **fenhexamid**, qui appartiennent à des familles chimiques différentes, sont couramment utilisés. Dans un objectif de limitation d'emploi des pesticides, il est nécessaire de mieux connaître les effets de ces produits sur la physiologie de la plante. L'impact de ces fongicides anti-*Botrytis* a donc été évalué à la fois sur les organes végétatifs et reproducteurs de la vigne. En effet, les organes végétatifs, par leur activité photosynthétique, assurent la nutrition nécessaire à la croissance de la plante. Concernant les organes reproducteurs, leur développement va déterminer le rendement. La photosynthèse a donc été utilisée comme paramètre physiologique afin d'étudier le stress provoqué par les fongicides sur les **organes végétatifs**. Les réponses de défense ont également été évaluées puisque le stress chimique, généré par les traitements anti-*Botrytis*, pourrait activer ces réponses. Concernant les effets des fongicides sur les **organes reproducteurs**, des facteurs pouvant influencer leur efficacité ont été évalués : (i) le stade de traitement, (ii) le mode d'action du fongicide, (iii) la pression de sélection exercée par les fongicides sur le pathogène et enfin (iv) les réponses de défense de la vigne.

L'application de fludioxonil et de fenhexamid entraîne au vignoble une réduction faible et temporaire de la **photosynthèse foliaire**. Cette diminution n'est liée ni à une fermeture des stomates, ni à une perturbation de l'activité du photosystème II. En revanche, les fongicides provoquent une répression de l'expression de gènes codant les sous-unités de la Rubisco (*rbcS* et *rbcL*) et une protéine du complexe collecteur de lumière du photosystème I (*cab*). Cette répression pourrait provoquer une réduction de la quantité de Rubisco et de la collecte de l'énergie lumineuse, à l'origine de l'inhibition de la photosynthèse. Une localisation plus précise des sites de perturbation de la photosynthèse a été étudiée sur boutures avec différentes concentrations de fludioxonil. Différents niveaux du processus photosynthétique sont altérés en fonction de la concentration. De plus, un recouvrement du stress est observé pour toutes les concentrations. Ainsi, le suivi de la photosynthèse suggère que les fongicides anti-*Botrytis* provoquent un **stress modéré** des organes végétatifs de la vigne. Cela est confirmé par la non-activation des **réponses de défense** dans les feuilles suite à ces traitements.

L'efficacité des fongicides anti-*Botrytis* est influencée par le **stade de traitement** et le **mode d'action du fongicide**. En effet, le fenhexamid est efficace uniquement lorsqu'il est appliqué au stade A. Le fludioxonil, quant à lui, est efficace à tous les stades de traitement mais son efficacité reste inférieure à celle du fenhexamid au stade A. Par ailleurs, l'étude de la **pression de sélection** des fongicides sur *B. cinerea* a révélé que ce paramètre a peu d'influence sur l'efficacité des traitements. Une activation différentielle des réponses de défense de la vigne suite à l'application des fongicides ne semble pas non plus intervenir sur leur efficacité. En effet, les fongicides activent des **réponses de défense** dans les baies au stade C mais pas dans les organes reproducteurs aux stades A et B. Cela confirme un stress modéré de la vigne suite aux traitements anti-*Botrytis*. L'absence d'activation des défenses dans les fleurs au stade A peut être liée à l'action peu stressante des fongicides ou à la faible sensibilité des fleurs aux stress abiotiques. Pour répondre à ces hypothèses, des **UV-C**, connus pour activer des réponses de défense dans les feuilles et baies de vigne, ont été appliqués sur des inflorescences. Les UV-C activent faiblement les réponses de défense dans les fleurs ce qui signifie que les fleurs ont une faible capacité à activer des réponses de défense suite à un stress abiotique.

Mots-clés : *Botrytis cinerea*, échanges gazeux, expression de gènes, fluorescence chlorophyllienne, fongicides, photosynthèse foliaire, réponses de défense, stress, vigne

ABSTRACT

In the vineyard, the use of fungicides is necessary to control grey mould caused by the phytopathogenic *Botrytis cinerea*. Three applications of **botryticides** are recommended: at the end of flowering (stage A), at bunch closure (stage B), and at the beginning of berry ripening (stage C). **Fludioxonil** and **fenhexamid** belong to two different chemical classes and are commonly used as botryticides. To reduce pesticide use, it's necessary to improve the knowledge about chemical effects on plant physiology. Therefore, effects of these botryticides have been evaluated on grapevine vegetative and reproductive organs. Indeed, photosynthesis performed in **vegetative organs** provides the energy and structural substrates for plant growth. Considering **reproductive organs**, their development is related to the yield. Therefore, the photosynthesis was used as a physiological parameter to study stress caused by fungicides on vegetative organs. Plant defense responses have also been analysed because chemical stress induced by botryticides could activate these responses. Considering the fungicide effects on reproductive organs, various factors which could influence their efficacy were followed: (i) treatment stage, (ii) fungicide mode of action, (iii) selection pressure exerted by fungicides on *B. cinerea* and (iv) grapevine defense responses.

In the vineyard, both fludioxonil and fenhexamid treatments caused a slight and temporary decrease of **foliar photosynthesis**. The inhibition rate was similar with both fungicides and was neither related to stomatal limitation nor to alteration of photosystem II activity. On the contrary, botryticides induced a reduction in the expression of genes encoding small and large subunits of Rubisco (*rbcS* and *rbcL*) and light-harvesting complex protein (*cab*) of photosystem I. Photosynthesis decrease could be linked to a reduction of Rubisco amount and modification of light-harvesting caused by the repression of gene expression. A study was done on cuttings in order to precisely localize the photosynthesis sites of damage following various concentrations of fludioxonil. The sites of alteration were different according to the concentration. Moreover, the stress recovered following all fungicide concentrations. The study of photosynthesis suggests that botryticides cause a **moderate stress** on grapevine vegetative organs. This was confirmed by the fact that **defense responses** were not activated in leaves following botryticide treatments.

Botryticide efficacy was influenced by the **stage of treatment** and **fungicide mode of action**. Indeed, fenhexamid efficacy was only observed when applied at stage A. Considering fludioxonil, it was effective at the three stages but its efficacy was lower than fenhexamid at stage A. In addition, analysis of **selection pressure** by botryticides on *B. cinerea* has shown that this factor had poor influence on treatment efficacy. Similarly, the differences of responsiveness in grapevine reproductive organs following botryticide application seem not to be involved in treatment efficacy. Indeed, **defense responses** were activated in berries after botryticide treatment at stage C but not in reproductive organs at stages A and B. It confirms that botryticides cause a moderate stress on grapevine. Non-activation of defense responses in flowers at stage A may be related either to the weak stress induced by fungicides or to a low responsiveness of these organs to abiotic stresses. **UV-C irradiation**, a stress which efficiently up-regulates defense responses in grapevine leaves and berries, was thus applied on inflorescences to determine their sensitivity to stresses. The defense responses were weakly activated in flowers, which means that flowers have low responsiveness to abiotic stresses.

Keywords: *Botrytis cinerea*, chlorophyll fluorescence, defense responses, foliar photosynthesis, fungicides, gas exchanges, gene expression, grapevine, stress

LISTE DES ABREVIATIONS

Γ*	point de compensation lumineux
Acétyl-CoA	acétyl-coenzyme A
ADN	acide désoxyribonucléique
$AlCl_3$	chlorure d'aluminium
AOC	appellation d'origine contrôlée
APX	ascorbate peroxydase
ARN	acide ribonucléique
ARNm	acide ribonucléique messager
ATP	adénosine triphosphate
BBCH	biologische bundesanstalt, bundessortenamt and chemical industry
BIOGER-CPP	Biologie et gestion des risques en agriculture – champignons pathogènes des plantes
BTH	acide benzo(1,2,3)-thiadiazole-7-carbothioïque-S-méthyl-ester
$C_6H_{12}O_6$	glucose
Ca	concentration en CO_2 ambiant
Cab	chlorophyll a/b binding
CAT	catalase
Ci	concentration interne en CO_2
Ci*	point de compensation pour le CO_2
CIVC	Comité Interprofessionnel du Vin de Champagne
CO_2	dioxyde de carbone
Cyt*b6f*	cytochrome b_6f
e^-	électrons
EAO	espèces activées de l'oxygène
F_O	fluorescence minimale de feuilles adaptées à l'obscurité
F_O'	fluorescence minimale de feuilles adaptées à la lumière
Fdx	ferrédoxine
F_m	fluorescence maximale de feuilles adaptées à l'obscurité
F_m'	fluorescence maximale de feuilles adaptées à la lumière
FNR	ferrédoxine-$NADP^+$ réductase
F_p	maximum de l'émission de fluorescence immédiatement après le passage d'une feuille de l'obscurité à la lumière
F_s	émission de fluorescence à l'état stationnaire sous une lumière actinique

FS	flash de saturation
F_v/F_m	efficacité photochimique maximale du PSII
GPX	glutathion peroxydase
g_S	conductance stomatique
GST	glutathion-S-transférase
H^+	protons
$HO_2^{\cdot}$	radical hydroperoxyl
H_2O_2	peroxyde d'hydrogène
HRGP	hydroxyproline-rich glycoproteins
hv	énergie lumineuse
IBS	inhibiteur de la biosynthèse des stérols
IDM	inhibiteur de la 14α-déméthylation des stérols
LA	lumière actinique
LHC	light harvesting complex
LM	lumière modulée
LOX	lipoxygénase
MDR	multi drug resistant
NADP	nicotinamide adénine dinucléotide phosphate
O_2	dioxygène
$^1O_2$	oxygène singulet
$O_2^{\cdot -}$	ion superoxyde
OEC	oxygen-evolving complex
OGM	organisme génétiquement modifié
$OH^{\cdot}$	radical hydroxyl
PAL	phénylalanine ammmonia-lyase
PC	plastocyanine
PGIP	polygalacturonase-inhibiting protein
PGPR	plant growth-promoting rhizobacteria
Phéo	phéophytine
Pn	photosynthèse nette
Pmax	niveau maximal de photosynthèse
PPFD	photosynthetic photon flux density
PQ	plastoquinone
PQH_2	plastohydroquinone

PR	pathogenesis related
PSI	photosystem I
PSII	photosystem II
ΦCO_2	rendement quantique maximum de fixation du CO_2
Φ_{PSII}	rendement quantique de l'acte photochimique
q_{NP}	quenching non-photochimique
q_P	quenching photochimique
Rd	respiration à l'obscurité
Rl	respiration mitochondriale à la lumière
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygénase
RuBP	ribulose-1,5-bisphosphate
SA	acide salicylique
SOD	superoxyde dismutase
STS	stilbène synthase
TMV	Tobacco Mosaic Virus
UIPP	Union des Industries de la Protection des Plantes
UV	ultra-violet
$V_{c,max}$	efficacité maximale de carboxylation de la Rubisco

LISTE DES PUBLICATIONS ET COMMUNICATIONS

Publications

Publication 1 : **Petit AN, Fontaine F, Clément C & Vaillant-Gaveau N**
Effects of "non-herbicidal" pesticides on photosynthesis – a review. Soumise à *Chemosphere*.

Publication 2 : **Petit AN, Wojnarowiez G, Panon ML, Baillieul F, Clément C, Fontaine F & Vaillant-Gaveau N**
Botryticides affect grapevine leaf photosynthesis without inducing defense mechanisms. *Planta*. Sous presse.

Publication 3 : **Petit AN, Fontaine F, Clément C & Vaillant-Gaveau N** (2008)
Two botryticide effects on leaf photosynthesis grapevine. *In* Columbus F (Ed), Photochemistry Research, Nova Science Publishers, Hauppauge, New York, USA. Sous presse.

Publication 4 : **Petit AN, Fontaine F, Clément C & Vaillant-Gaveau N** (2008)
Photosynthesis limitations of grapevine after treatment with the fungicide fludioxonil. *Journal of Agricultural and Food Chemistry* 56 : 6761-6767.

Publication 5 : **Petit AN, Vaillant-Gaveau N, Walker AS, Leroux P, Baillieul F, Panon ML, Clément C & Fontaine F**
Effects of fungicides on populations of *Botrytis cinerea* and on defense responses of grapevine reproductive organs. Soumise à *Plant Pathology*.

Publication 6 : **Petit AN, Baillieul F, Vaillant-Gaveau N, Jacquens L, Conreux A, Jeandet P, Clément C & Fontaine F**
Low responsiveness of grapevine flowers and berries at fruit set to UV-C irradiation. Soumise à *Journal of Experimental Botany*.

Publication 7 : **Petit AN, Fontaine F, Clément C & Vaillant-Gaveau N** (2008)
(annexe) Gating in grapevine: relationship between application of the fungicide fludioxonil and circadian rhythm on photosynthesis. *Environmental Pollution* doi : 10.1016/j.envpol.2008.07.017.

Communications

Petit AN, Fontaine F, Baillieul F, Clément C & Vaillant-Gaveau N (2008)
Defense responses of grapevine to UV-C exposure during the beginning of reproductive development. *Second International Symposium on Macromolecules and Secondary Metabolites of Grapevine and Wines*, Montpellier, France. Communication orale.

Petit AN, Fontaine F, Clément C & Vaillant-Gaveau N (2008)
Conséquences de l'utilisation des fongicides et insecticides sur la physiologie des plantes cultivées. *XVIII^{ème} congrès du GFP*, Brest, France. Communication orale.

Petit AN, Panon ML, Vaillant-Gaveau N, Mazeyrat-Gourbeyre F, Baillieul F, Clément C & Fontaine F (2007)
Treatment efficacy against grey mould of the grapevine and defence mechanisms activation. *XIVth International Botrytis Symposium*, Cape Town, Afrique du Sud. Communication orale.

Petit AN, Fontaine F, Baillieul F, Clément C & Vaillant-Gaveau N (2007)
Effets de deux fongicides anti-*Botrytis* sur la photosynthèse de la vigne. *XVIIème congrès du GFP*, Bordeaux, France. Communication orale.

Petit AN, Fontaine F, Clément C & Vaillant-Gaveau N (2006)
Effects of two fungicides used against *Botrytis cinerea* on grapevine photosynthesis in vineyard. *XVth FESPB meeting*, Lyon, France. Poster.

INTRODUCTION

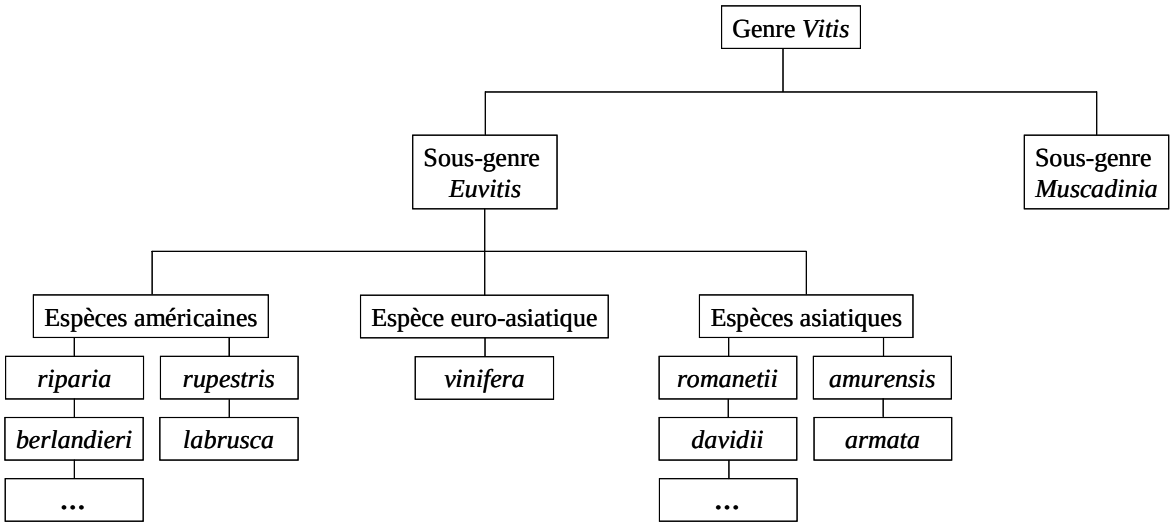


Figure 1. Classification systématique de l'espèce *Vitis vinifera* L. parmi la famille des *Vitaceae* selon Planchon (1887).

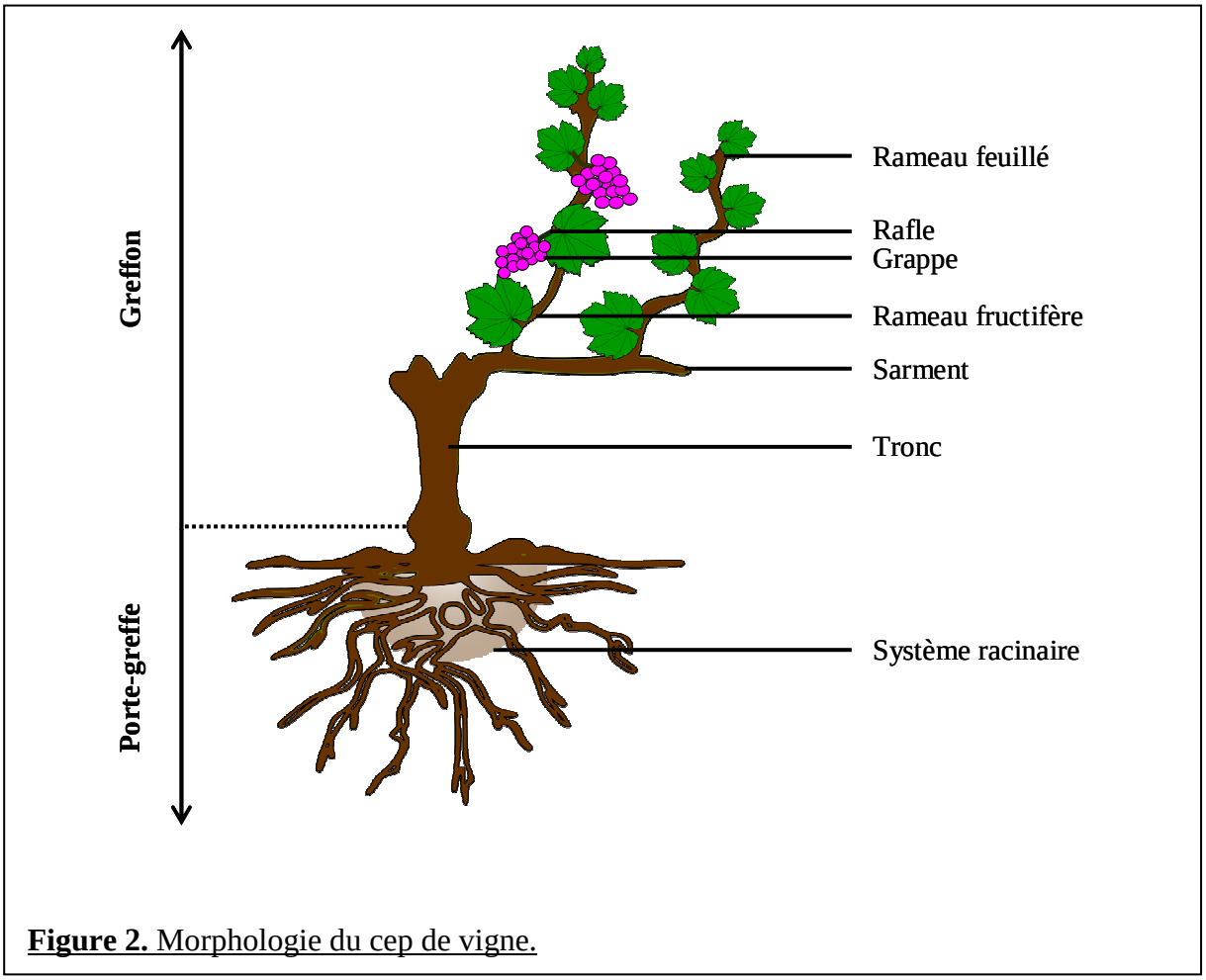


Figure 2. Morphologie du cep de vigne.

I. La vigne

1. Présentation générale de la vigne

a. Présentation botanique

La vigne est une Angiosperme dicotylédone qui appartient à la famille des *Vitaceae*, anciennement appelée *Ampelideae* (Planchon, 1887). Les plantes de cette famille sont des arbrisseaux grimpants, comme des lianes, à tige le plus souvent sarmenteuse mais parfois herbacée, possédant des vrilles opposées aux feuilles. La famille des *Vitaceae* comprend 19 genres dont le genre *Vitis* qui regroupe les vignes cultivées.

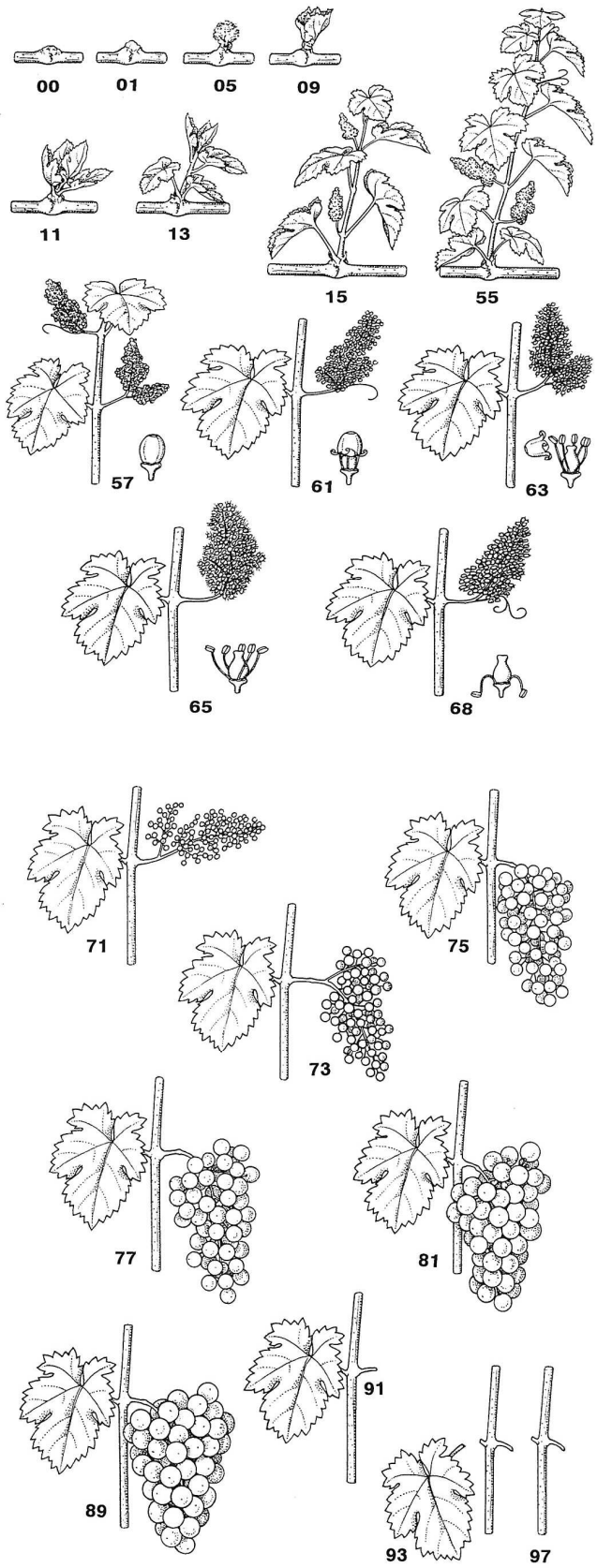
Le genre ***Vitis*** est divisé en deux sous-genres, *Muscadinia* et *Euvitis* (Figure 1). La quasi-totalité des vignes cultivées fait partie de ce dernier, à l'intérieur duquel on distingue 3 groupes : américain, euro-asiatique et asiatique au sens strict. Le premier groupe, présent en Europe et Asie occidentale, ne comporte qu'une seule espèce : ***Vitis vinifera*** (Linné).

Cette espèce se subdivise en milliers de variétés, appelées aussi **cépages** ou cultivars, qui résultent de croisements naturels ou de sélections par les obtenteurs. Ces cépages sont caractérisés, entre autres, par les qualités aromatiques et gustatives de leurs baies et par leurs possibilités d'utilisation : raisin de table, raisin sec, raisin de cuve ou raisin à jus (Reynier, 2003).

A la fin du XIX^{ème} siècle, le phylloxéra, puceron originaire de l'est des Etats-Unis, a failli anéantir les vignobles européens. Afin de surmonter cette invasion phylloxérique, le **greffage** de la vigne a été mis en place en utilisant des porte-greffes issus de plants américains naturellement résistants au phylloxéra (Reynier, 2003). Cette technique a permis d'associer la qualité des cépages français à la résistance des vignes américaines.

Le **greffon**, partie supérieure du cep de vigne, est donc constitué d'un tronc qui se divise en bras portant des bois de taille longs ou courts, appelés sarments (Figure 2). Ces sarments portent des yeux ou ensemble de bourgeons qui donneront naissance à des rameaux feuillés, fructifères ou non. Les rameaux fructifères portent les inflorescences puis les grappes. L'inflorescence comprend un axe principal, ou rafle, sur lequel se développent des ramifications secondaires qui peuvent se ramifier à leur tour pour se terminer par un bouquet de 2 à 5 fleurs. Chaque fleur est fixée sur l'extrémité d'une ramification par le pédicelle. Après fécondation, les fleurs deviennent des baies.

Le **porte-greffe**, ou partie inférieure, produit le système racinaire qui colonise le sol et le sous-sol tout au long de sa vie.



Stade principal 0 : bourgeonnement ou débourrement

00 Dormance : les bourgeons d'hiver sont pointus à arrondis, suivant la variété ils sont brun clair à foncé et les écailles sont plus ou moins appliquées aux bourgeons
01 Début du gonflement des bourgeons : les bourgeons s'allongent à l'intérieur des écailles
03 Fin du gonflement des bourgeons, les bourgeons ne sont pas encore verts
05 « Stade de la bourre » : une protection cotonneuse est nettement visible
07 Début de l'éclatement des bourgeons (débourrement) : l'extrémité verte de la jeune pousse est juste visible
09 Débourrement : l'extrémité verte de la jeune pousse est nettement visible

Stade principal 1 : développement des feuilles

11 Première feuille étalée et écartée de la pousse
12 2 feuilles étalées
13 3 feuilles étalées
1 . Et ainsi de suite...
19 9 ou davantage de feuilles sont étalées

Stade principal 5 : apparition des inflorescences

53 Les grappes (inflorescences) sont nettement visibles
55 Les grappes augmentent de taille, les boutons floraux sont agglomérés
57 Les grappes sont bien développées, les fleurs se séparent

Stade principal 6 : la floraison

60 Les premiers capuchons floraux se séparent du réceptacle
61 Début de la floraison : 10% des capuchons floraux sont tombés
62 20% des capuchons floraux sont tombés
63 Floraison partielle : 30% des capuchons floraux sont tombés
64 40% des capuchons floraux sont tombés
65 Mi-floraison : 50% des capuchons floraux sont tombés
66 60% des capuchons floraux sont tombés
67 70% des capuchons floraux sont tombés
68 La floraison s'achève : 80% des capuchons floraux sont tombés
69 Fin de la floraison

Stade principal 7 : développement des fruits

71 Nouaison : début du développement des fruits, toutes les pièces florales sont tombées
73 Les fruits (baies) ont la grosseur de plombs de chasse, les grappes commencent à s'incliner vers le bas
75 Les baies ont la grosseur de petits pois, les grappes sont en position verticale
77 Début de la fermeture de la grappe (les baies commencent à se toucher)
79 La fermeture de la grappe est complète, les fruits ont fini de grossir

Stade principal 8 : maturation des baies

81 Début de la maturation : les baies commencent à s'éclaircir et/ou à changer de couleur
83 Eclaircissement et/ou changement de couleur en cours
85 Les baies deviennent molles au toucher
89 Les baies sont mûres pour la vendange

Stade principal 9 : sénescence ou début du repos végétatif

91 Après la vendange : l'aoûtement du bois est terminé
92 Début de la coloration des feuilles
93 Début de la chute des feuilles
95 50% des feuilles sont tombées
97 Fin de la chute des feuilles
99 Baies mûres en phase de conservation

Figure 3. Stades phénologiques de la vigne d'après Meier (2001).

La vigne sauvage est une plante dioïque mais la diversité et les mutations ont pu favoriser l'apparition de plantes hermaphrodites.

b. Cycle de développement de la vigne

Le développement de la vigne est une succession de cycles annuels où les bourgeons peuvent se développer selon un cycle végétatif et reproducteur. En 1952, Baggiolini a établi des stades repères dans le développement annuel de la vigne qui servent encore actuellement de base de détermination des **stades phénologiques**. Cette description a ensuite été affinée en subdivisant les stades existants (Eichhorn & Lorenz, 1977). Puis en 2001, Meier a proposé une échelle BBCH (Biologische **B**undesanstalt, **B**undessortenamt and **CH**emical industry) caractérisée par une chronologie discontinue de chiffres allant de 00 à 99, permettant ainsi de préciser certains stades phénologiques intermédiaires (Figure 3). Cette échelle sera utilisée dans ce manuscrit afin de décrire les différents stades de la vigne.

En tant qu'espèce pérenne, le développement de la vigne est interrompu par une période de repos hivernal. Un cycle annuel correspond à la superposition du cycle végétatif et du cycle reproducteur. En Champagne, les cycles végétatif et reproducteur débutent mi-avril avec le débourrement des bourgeons. Le cycle reproducteur est interrompu fin septembre par la vendange alors que le cycle végétatif s'achève mi-novembre (Figure 4).

- ✓ Le cycle végétatif

A la fin de l'hiver, lorsque la température du sol s'élève, le système racinaire rentre en activité. Il se produit une activation de la respiration cellulaire, une reprise de l'absorption de l'eau et des éléments minéraux ainsi qu'une mobilisation des réserves. La conduction de la sève brute dans les vaisseaux ligneux reprend sous l'action des phénomènes osmotiques et provoque un mouvement ascendant de sève, appelé **poussée racinaire** (Huglin, 1986). En absence de végétation, cette sève s'écoule au niveau des plaies de taille : ce sont les **pleurs**.

Vers la mi-avril, les bourgeons commencent à gonfler en écartant les deux écailles protectrices faisant apparaître la bourre. Cette première manifestation de la croissance est appelée **débourrement** et correspond au stade 01 de l'échelle BBCH. Puis l'extrémité verte de la jeune pousse devient visible (BBCH 07) et se poursuit par le développement des feuilles (BBCH 11 à 19).

Pendant leur **croissance**, les feuilles, d'abord **hétérotrophes**, deviennent **autotrophes** vis-à-vis du carbone. En effet, les jeunes feuilles ont une activité photosynthétique trop faible pour assurer leur propre développement. Une fois qu'elles ont atteint la moitié de leur taille

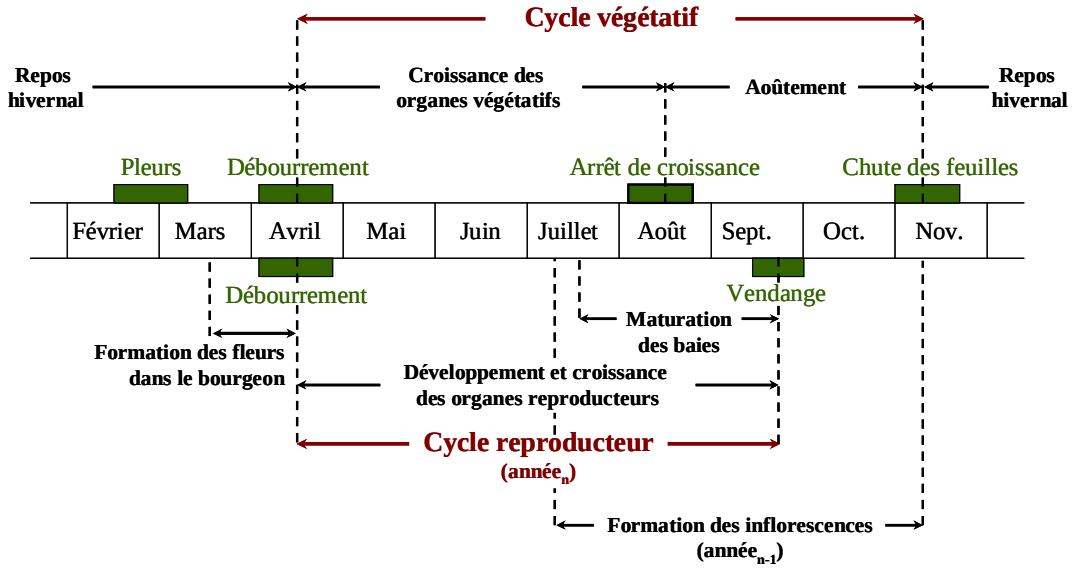


Figure 4. Cycle de développement de la vigne en Champagne d'après Reynier (2003).

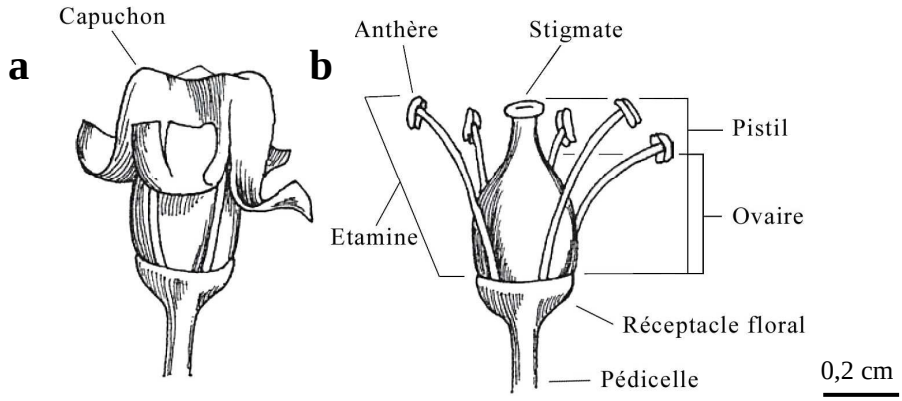


Figure 5. Fleur de vigne à différents stades de la floraison : (a) début de la floraison, le capuchon se détachant de la base de l'ovaire et (b) pleine floraison (Hellman, 2003).

finale, leur activité photosynthétique devient excédentaire (Koblet, 1969). D'organes puits, ou utilisatrices de réserves, elles deviennent alors des organes sources ou exportatrices de glucides. A l'échelle de la plante, le bilan de la nutrition carbonée montre un basculement entre l'hétérotrophie (utilisation des réserves prioritaire) et l'autotrophie (utilisation des photoassimilats) environ 2-3 semaines avant la floraison (Zapata, 1998 ; Zapata *et al*., 2004). En plus d'assurer leur propre nutrition, les feuilles âgées exportent alors des glucides vers les jeunes feuilles, les tiges en croissance, les organes reproducteurs et les racines (Zapata, 1998). Elles contribuent également à la reconstitution des réserves au niveau des parties pérennes du cep que sont les racines, le tronc et les sarments (Zapata, 1998).

Les stades BBCH 53 à 89 caractérisent essentiellement l'évolution de l'appareil reproducteur. Au stade 91, l'**aoûtement**, débuté lors de la maturation des baies, se termine. Les rameaux se lignifient et accumulent des réserves, en particulier sous forme d'amidon (Reynier, 2003). Enfin, courant novembre, c'est la sénescence des feuilles : elles jaunissent puis tombent (BBCH 92 à 97). La plante rentre alors dans la phase de repos végétatif ou **repos hivernal**.

- ✓ Le cycle reproducteur

L'initiation des inflorescences et la formation des fleurs s'effectuent sur deux années successives. En effet, les inflorescences sont initiées durant l'été$_{n-1}$ puis leur développement est stoppé lorsque le bourgeon rentre en dormance (Mullins *et al*., 1992 ; Boss *et al*., 2003). Lors de l'année$_n$, la **formation des fleurs** débute peu avant le débourrement. Après l'étalement des premières feuilles, les inflorescences apparaissent groupées (BBCH 53) puis se séparent lors de l'allongement des entre-nœuds (BBCH 55). Les boutons floraux encore agglomérés se séparent ensuite (BBCH 57) et les pédicelles des fleurs s'allongent, permettant de séparer chaque fleur individuellement.

La **floraison** débute en général mi-juin (BBCH 61) et correspond à l'épanouissement de la fleur par l'ouverture de la corolle, appelée capuchon floral (Figure 5). Les capuchons se détachent progressivement de la base du réceptacle floral puis chutent (BBCH 61 à 69) (Gerrath, 1993 ; Boss *et al*., 2003). La chute du capuchon met à nu l'ovaire et permet aux étamines de s'écarter du pistil et de libérer les grains de pollen, produits par les anthères.

La **nouaison** (BBCH 71) correspond au début du développement de l'ovaire fécondé. Le nombre de fruits mûrs est toujours inférieur au nombre de fleurs développées. En effet, un certain nombre de fleurs non pollinisées et d'ovaires fécondés tombent : c'est la **coulure** (Bessis & Fournioux, 1992). Les déchets floraux (*e.g.* étamines, capuchons) flétrissent et

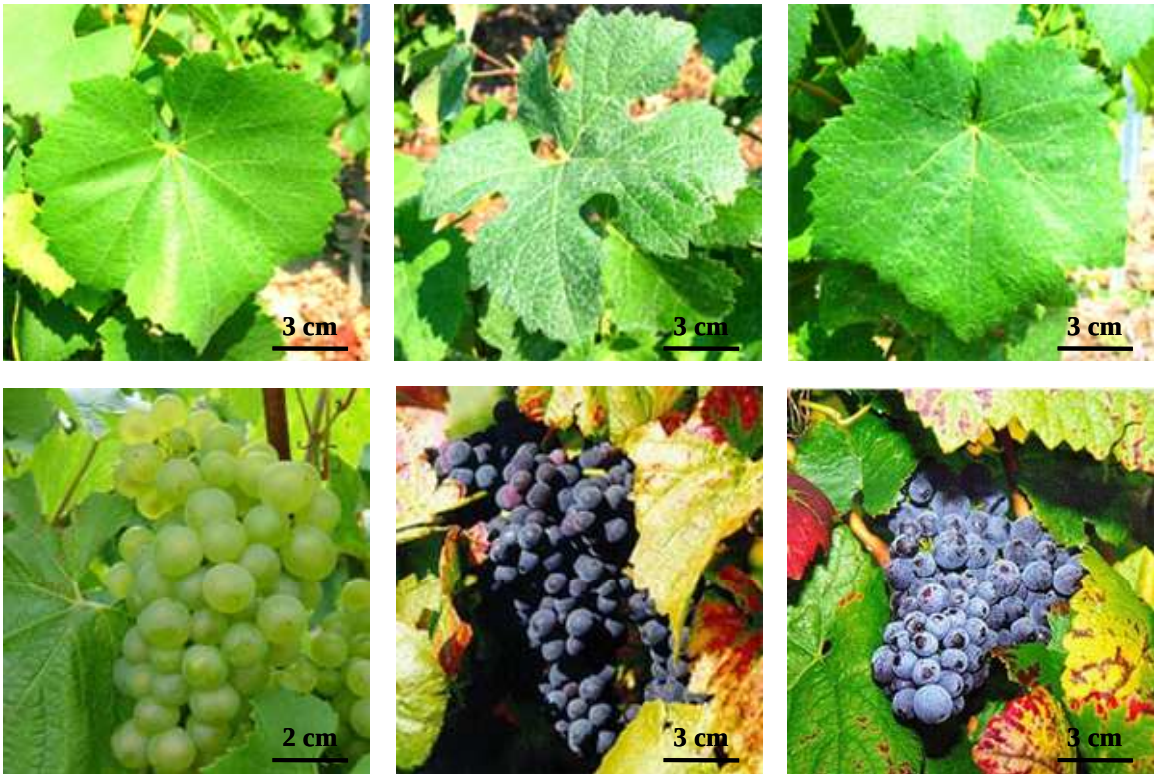


Figure 6. Morphologie des feuilles (en haut) et des grappes (en bas) des principaux cépages de la région champenoise : Chardonnay (a), Pinot Meunier (b) et Pinot noir (c).

tombent, ou restent fixés à leur point d'attache pendant que les jeunes fruits grossissent jusqu'à atteindre la taille de plombs de chasse ou grenailles (BBCH 73) puis de petits pois (BBCH 75). Ensuite, les baies continuent de se développer pour finir par se toucher, ce qui entraîne la **fermeture de la grappe** (BBCH 77).

La **véraison** (BBCH 81) marque le début de la transformation des baies et donc de la maturation. Cette période est caractérisée par une accumulation progressive des glucides solubles, une diminution de l'acidité dans les baies, un changement de couleur et de multiples modifications au niveau de la constitution et de la physiologie de la baie qui se poursuivront lors de la **maturation** (Coombe, 1992 ; Davies & Robinson, 1996 ; Ollat, 1997 ; Davies *et al*., 1999 ; Fillion *et al*., 1999 ; Ageorges *et al*., 2000 ; Terrier *et al*., 2001, 2005). Une accumulation de protéines antifongiques notamment de type « Pathogenesis Related » (PR) est également constatée dans les baies (Robinson *et al*., 1997 ; Tattersall *et al*., 1997 ; Salzman *et al*., 1998). L'ensemble de ces transformations déterminera la qualité finale du fruit et du vin (Kanellis & Roubelakis-Angelakis, 1993). Une fois que les baies ont atteint leur taille maximale et que leur concentration en glucides et leur acidité sont stabilisées, les baies sont à maturité et peuvent être vendangées (BBCH 89).

2. Importance économique de la filière viticole

La vigne couvre de nos jours près de 8 millions d'hectares dans le monde, pour une production annuelle d'environ 300 millions d'hl de vin (Viniflhor, données 2006). La majorité des vignes cultivées est regroupée en Europe (63% du vignoble mondial) et notamment en France, en Italie et en Espagne. En France, la vigne, avec ses 900 000 hectares, occupe 3,2% des surfaces agricoles utilisées mais la viticulture représente 17% de la valeur de la production agricole (Viniflhor, données 2006). La contribution de ce produit à la richesse nationale est donc essentielle.

En **Champagne**, plus de 32 000 hectares sont actuellement consacrés à la culture de la vigne. Le champagne, vin effervescent à appellation d'origine contrôlée (AOC) issu de cette culture, représente actuellement un chiffre d'affaires d'environ 3,4 milliards d'euros pour 300 millions de bouteilles vendues chaque année (www.union-maisons-champagne.fr). Le champagne est élaboré à partir de raisins provenant majoritairement de 3 cépages qui sont le Chardonnay, le Pinot Meunier et le Pinot noir (Figure 6) et qui occupent respectivement 28, 34 et 38% de la superficie du vignoble champenois (www.union-maisons-champagne.fr). Ce

vin représente donc une production d'intérêt économique majeur, où la quantité et la qualité sont des facteurs recherchés.

La vigne est soumise à l'attaque de multiples pathogènes ce qui nécessite l'utilisation de nombreux traitements préventifs pour lutter contre ces stress biotiques. La viticulture est ainsi la première consommatrice de pesticides en France : elle représente quasiment 20% du marché français des pesticides, alors que les vignobles ne constituent que 3,2% de la surface agricole utile (Ministère de l'agriculture et de la pêche, 2005).

Le terme de **pesticide** est devenu au XX^{ème} siècle le terme générique utilisé pour désigner toutes les substances naturelles ou de synthèse capables de contrôler, d'attirer, de repousser, de détruire ou de s'opposer au développement des organismes vivants considérés comme indésirables pour l'agriculture, l'hygiène publique, la santé publique, la santé vétérinaire, ou les surfaces non-agricoles. Dans le domaine de l'agriculture, les pesticides sont utilisés afin de protéger les plantes cultivées et les produits récoltés des attaques de champignons parasites, d'insectes, d'acariens, de rongeurs champêtres ou encore de détruire les adventices ou "mauvaises herbes". Leur mise sur le marché est subordonnée à la détention d'une autorisation publique délivrée par le ministre chargé de l'agriculture, mais aussi d'une évaluation de l'efficacité et de la sélectivité. Tout produit qui, pour un usage donné, ne fait pas l'objet d'une autorisation, est interdit. Ce principe d'évaluation préalable est en vigueur en France depuis 1943. Il a été harmonisé à l'échelon communautaire par la directive 91/414/CEE qui prescrit également un programme ambitieux de réévaluation de l'ensemble.

Les maladies de la vigne sont générées par des parasites divers tels que des virus (court-noué, enroulement…), des bactéries (maladie de Pierce, crown-gall…) ou des champignons (pourriture grise, oïdium, mildiou, esca, eutypiose…). Parmi ces agresseurs, le champignon *Botrytis cinerea* est responsable de la pourriture grise, maladie qui provoque les dégâts les plus importants dans les vignobles du monde entier. En effet, cette maladie occasionne à la fois des dégâts qualitatifs et quantitatifs sur la production des vins (Bulit & Dubos, 1988).

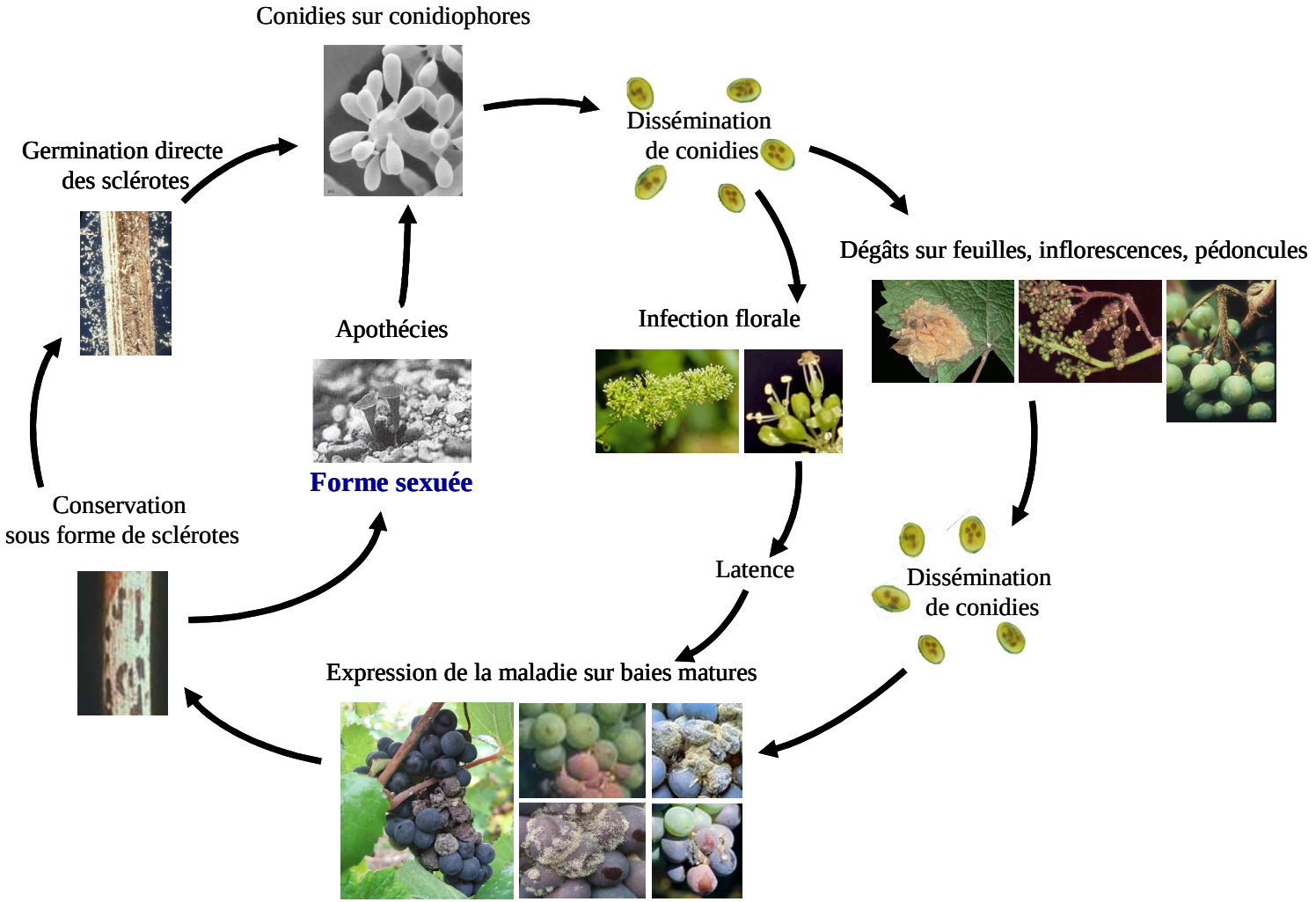


Figure 7. Cycle d'infection de *Botrytis cinerea* chez la vigne.

II. *Botrytis cinerea*, agent pathogène de la pourriture grise

1. Conséquences économiques de la pourriture grise

La pourriture grise est provoquée par le champignon Ascomycète *Botrytis cinerea* Pers., forme imparfaite du *Botryotinia fuckeliana* (de Bary) Whetzel. *B. cinerea* est un champignon polyphage vivant comme saprophyte sur une multitude de plantes. En effet, *B. cinerea* est également l'agent responsable de la pourriture grise de plusieurs centaines de plantes hôtes incluant des légumes (*e.g.* tomate, concombre, laitue), des plantes ornementales (*e.g.* rose, gerbera), des bulbes (*e.g.* oignon) et des fruits (*e.g.* fraise, kiwi). Les dégâts provoqués par ce champignon ont un impact économique désastreux sur ces cultures. En effet, les pertes provoquées correspondent à 20% des récoltes mondiales pour les cultures concernées et leur coût est estimé entre 10 et 100 milliards d'euros par an (www.genoscope.cns.fr/spip/Botrytis-cinerea-pertes-de-vigne.html). La lutte contre *B. cinerea* nécessite la modification des pratiques culturales, le développement de cépages résistants ou tolérants et l'emploi important de fongicides. Les traitements anti-fongiques contre *B. cinerea* ont coûté environ 540 millions d'euros en 2001, ce qui représente 10% du marché mondial des fongicides (Rapport annuel UIPP, 2002).

Le développement rapide et insidieux de *B. cinerea* engendre chaque année la destruction de centaines d'hectares de cultures viticoles (Bolay & Pezet, 1987). Les pertes estimées pour la vigne en France correspondent à 15-40% des récoltes selon les conditions climatiques. En Champagne, des taux d'infection peuvent atteindre 15 à 25% selon les années (Panon *et al*., 2006). Par ailleurs, la pourriture grise est à l'origine de conséquences graves sur la qualité des vins, provoquant une altération de leurs qualités organoleptiques ainsi qu'une diminution de la moussabilité (Bocquet *et al*., 1995 ; Marchal *et al*., 2001 ; Cilindre *et al*., 2007, 2008).

2. Cycle biologique de *Botrytis cinerea*

L'hiver, le champignon se conserve sous forme de **sclérotes** sur les sarments (Figure 7) (Coley-Smith & Cooke, 1971 ; Coley-Smith, 1980). Au printemps, lorsque les conditions climatiques deviennent favorables, les sclérotes se recouvrent de fructifications asexuées, les **conidiophores**. Les sclérotes peuvent également produire des **apothécies** brunâtres (Coley-Smith & Cooke, 1971 ; Prins *et al*., 2001). Ces fructifications sexuées portent un grand nombre d'asques, dont chacun contient 8 ascospores haploïdes. Après germination, les

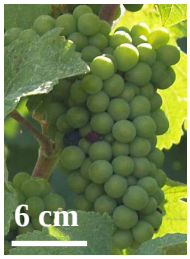
ascospores produisent le mycélium sur lequel se différencient des conidiophores (De Istvanffi, 1903).

Les conidiophores libèrent des spores, ou **conidies**, dont la dissémination est assurée par le vent et la pluie. Les conidies et les filaments mycéliens de *B. cinerea* sont hétérocaryotiques et multinucléés. Ces caractéristiques confèrent au champignon une très large variabilité génétique, lui permettant de s'adapter très rapidement aux conditions du milieu (Hansen & Smith, 1932) et aux fongicides spécifiques (Leroux, 2004). La contamination lors de la germination des conidies nécessite certaines conditions comme la présence d'une pellicule d'eau ou d'une humidité relative d'au moins 90% (Blakeman, 1980). Un substrat nutritif, souvent constitué par des organes ayant perdu leur vitalité tels que des débris d'organes floraux ou des feuilles mortes, est également nécessaire à leur germination (Pezet & Pont, 1986). A partir de ces premiers foyers, la contamination peut se propager à tous les organes de la plante : rameaux, feuilles, pédoncules et baies. Toutefois, les conséquences de l'infection par *B. cinerea* les plus importantes se situent au niveau des fleurs et des baies.

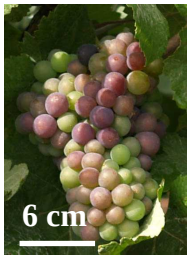
Avant la floraison, les inflorescences sont sensibles à *B. cinerea*. Le pathogène provoque le dessèchement des boutons floraux et la chute d'une partie ou de la totalité de l'inflorescence (Pezet & Pont, 1986). A partir de la floraison, le pathogène est capable de pénétrer dans la fleur sans la détruire. L'**infection florale** est connue comme étant une étape importante dans l'épidémiologie du champignon au niveau des grappes (Nair *et al*., 1995). Le réceptacle de la fleur de vigne semble être le site majeur de pénétration. En effet, la morphologie de cette structure semble très favorable à la collecte de l'inoculum (Pezet & Pont, 1986 ; Holz *et al*., 1997 ; Keller *et al*., 2003 ; Viret *et al*., 2004). Puis jusqu'à la véraison (BBCH 81), le champignon est maintenu dans les baies sans évolution jusqu'à ce stade : c'est la **latence** (McClellan & Hewitt, 1973 ; Hill *et al*., 1981 ; Pezet & Pont, 1986 ; Commenil *et al*., 1997).

Cette latence s'explique par la résistance des jeunes baies qui résulte de la conjugaison de plusieurs processus. Ainsi, des composés constitutifs et induits sont impliqués dans cette résistance. L'acide glycolique (Pezet & Pont, 1988a, b), des composés phénoliques (Goetz *et al*., 1999) et le ptérostilbène, considéré comme la phytoalexine stilbénique la plus toxique de la baie (Langcake *et al*., 1979 ; Pezet & Pont, 1988a, b), sont des composés constitutifs des jeunes baies et présentent une toxicité contre *B. cinerea*. D'autres études ont mis en évidence la biosynthèse de phytoalexines dans les baies infectées par *B. cinerea*, et plus particulièrement de resvératrol (Langcake & Pryce, 1977a ; Pont & Pezet, 1990 ; Jeandet *et*

stade A (BBCH 68)



stade B (BBCH 77)



stade C (BBCH 81)

Figure 8. Stades de traitement de la vigne contre la pourriture grise.

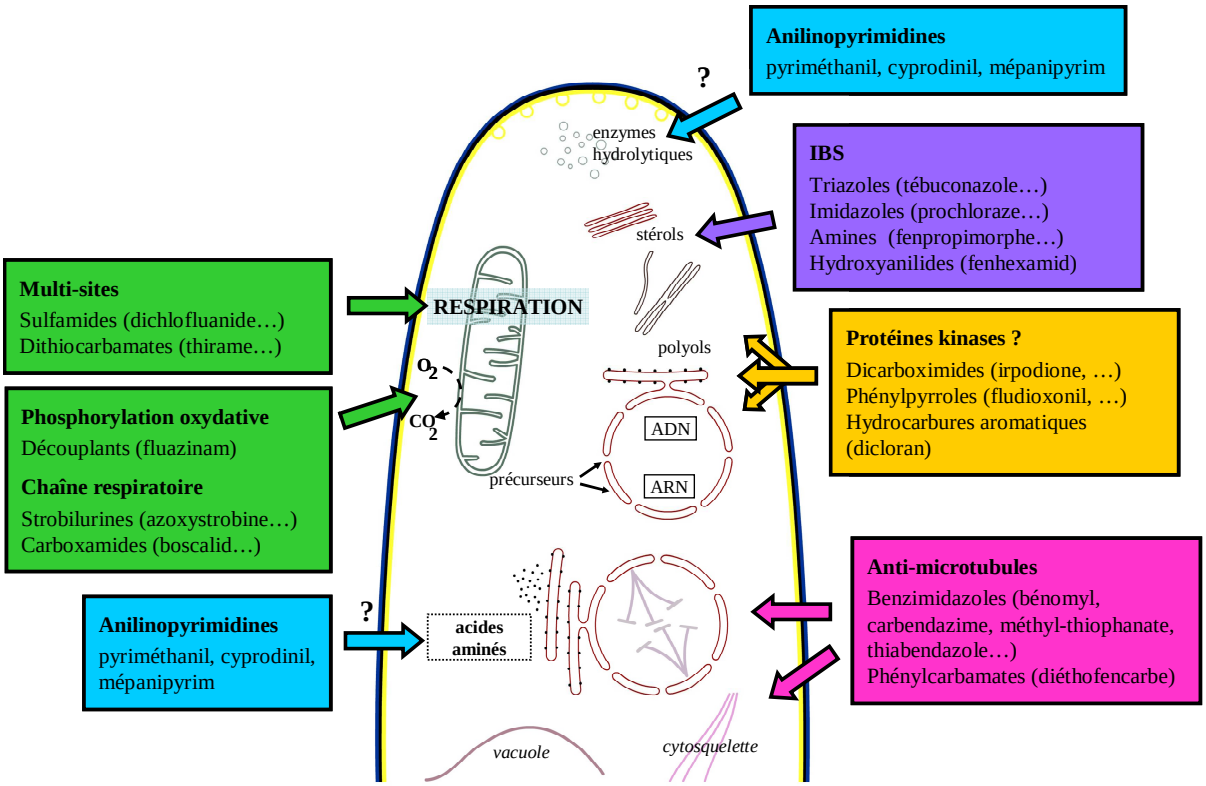


Figure 9. Sites d'action des différentes familles de fongicides anti-*Botrytis*.

al., 1995a ; Bavaresco *et al*., 1997 ; Bais *et al*., 2000). Au vignoble, le rôle déterminant des infections latentes pour le développement ultérieur de la maladie a été démontré par l'efficacité importante de l'application de fongicides à la floraison (Jermini *et al*., 1986 ; Pezet & Pont, 1986). A la véraison, le champignon reprend son développement pathogène alors que les défenses de l'hôte commencent naturellement à décroître ce qui aboutit à l'**expression de la maladie** (Pezet & Pont, 1986 ; Nunan *et al*., 1998 ; Holz *et al*., 2003 ; Pezet *et al*., 2003). La contamination des baies matures se fait également par la pénétration directe des filaments germinatifs issus des conidies ou du mycélium, au niveau de blessures ou en franchissant la cuticule. La contamination s'étend ensuite par simple contact entre baies contaminées et baies saines (Elad *et al*., 2004 ; Pezet *et al*., 2004).

3. Lutte chimique contre la pourriture grise

La pourriture grise conduit régulièrement à des pertes économiques importantes. L'utilisation de fongicides est donc une condition préalable pour produire des récoltes saines. Cette **lutte chimique** vient en complément des **mesures prophylactiques** qui contribuent à réduire l'incidence de la maladie en établissant des conditions environnementales moins favorables à l'infection. Ainsi, réduire la vigueur de la vigne, favoriser l'aération des grappes et limiter les blessures des baies, par une maîtrise correcte des vers de la grappe et de l'oïdium, constituent des mesures préventives primordiales (Panon *et al.*, 2006). En effet, la lutte chimique est très difficile à mettre en œuvre du fait des particularités biologiques du champignon, de son adaptabilité aux conditions extérieures, de son potentiel destructeur à la vendange et du développement de résistances aux fongicides. Par conséquent, cette lutte est préventive et consiste généralement en trois applications effectuées à la fin de la floraison ou **stade A** (BBCH 68), à la fermeture de la grappe ou **stade B** (BBCH 77) et au début de la véraison ou **stade C** (BBCH 81) (Figures 3 et 8). Toutefois, depuis 2006, des stratégies à deux traitements sont recommandées en Champagne du fait des risques de résistance et de résidus (Panon *et al*., 2006). De plus, le nombre de traitements doit être raisonné en fonction de la sensibilité parcellaire et du risque acceptable pour le viticulteur.

Plusieurs familles de fongicides de synthèse sont disponibles pour lutter contre *B. cinerea*. Elles sont classées en 5 catégories, selon leurs modes d'action biochimique sur le pathogène : les fongicides affectant la respiration, le fonctionnement des microtubules, l'osmorégulation, la biosynthèse de méthionine ou des stérols (Figure 9).

a. Les fongicides inhibiteurs de la respiration

Parmi ceux affectant la respiration, les plus anciens sont des fongicides **multi-sites** tels que le dichlofluanide de la famille des sulfamides ou le thirame de la famille des dithiocarbamates. Leur action sur le champignon est principalement due à une forte inhibition de la germination des conidies. Bien que les effets de ces fongicides sur *B. cinerea* soient multi-sites, des cas de résistance ont été observés dans plusieurs pays européens mais jamais dans les vignobles français (Malatrakis, 1989 ; Rewal *et al*., 1991 ; Pollastro *et al*., 1996). Les fongicides inhibiteurs de la respiration plus récents comme le fluazinam, les strobilurines et les carboxamides agissent en bloquant **le fonctionnement des mitochondries**. Le fluazinam exerce une action découplante de la phosphorylation oxydative au niveau des mitochondries (Leroux & Moncomble, 1993). Les strobilurines tels que l'azoxystrobine sont des inhibiteurs du complexe mitochondrial III et les carboxamides comme le boscalid, des inhibiteurs du complexe II (Leroux, 2003 ; Elad *et al*., 2004). Pour le fluazinam et le boscalid, aucune résistance spécifique n'a été décelée à ce jour (Leroux *et al*., 1997 ; Leroux, 2004).

b. Les fongicides anti-microtubules

Il existe deux grandes familles de fongicides anti-microtubules : les benzimidazoles et les phénylcarbamates. Les fongicides de la famille des benzimidazoles, tels que le bénomyl et le carbendazime, ont été développés à la fin des années 1960. Ils représentaient une avancée importante dans le contrôle de *B. cinerea* par leur activité curative (Bolay *et al*., 1974). Ils n'affectent pas la germination des conidies mais inhibent l'élongation du tube germinatif et la croissance mycélienne à faibles concentrations (Leroux *et al*., 1999). Le diéthofencarbe, qui appartient aux phénylcarbamates, est apparu 20 ans après les benzimidazoles. Leurs effets anti-fongiques résultent de l'inhibition de l'assemblage microtubulaire due à la fixation des fongicides sur la **tubuline**, composant majeur des microtubules (Davidse & Ishii, 1995). L'emploi de ces composés anti-microtubules a rapidement provoqué l'apparition de phénomènes de résistance dans les vignobles où les traitements étaient intensifs, ce qui a entraîné l'élimination de ces anti-fongiques dans les régions à haut risque, telles que la Champagne (Leroux & Clerjeau, 1985 ; Smith, 1988).

c. Les fongicides agissant sur l'osmorégulation

Trois familles chimiques appartiennent à ce groupe de fongicides : les dicarboximides, les phénylpyrroles et les hydrocarbures aromatiques.

Les **dicarboximides** ou imides cycliques ont supplanté les benzimidazoles à la fin des années 1970. Toutefois, leur utilisation intensive (jusqu'à 4 à 5 traitements par an dans les vignobles champenois) a conduit à une résistance importante (Leroux & Clerjeau, 1985 ; Leroux & Descotes, 1996). Le **fludioxonil**, qui appartient aux **phénylpyrroles**, a été introduit dans les vignobles européens au milieu des années 1990 en tant que fongicide foliaire, spécifique de *B. cinerea* (Rosslenbroich & Stuebler, 2000). Les **hydrocarbures aromatiques** représentent un groupe ancien et hétérogène de fongicides. A l'intérieur de cette famille, le dicloran est le plus actif contre *Botrytis* spp. Les dicarboximides, les phénylpyrroles et les hydrocarbures aromatiques les plus toxiques comme le dicloran inhibent à la fois la germination des conidies et la croissance mycélienne. De plus, tous ces fongicides produisent des altérations morphologiques similaires au niveau des tubes germinatifs tels que des gonflements, des ramifications anormales et des éclatements cellulaires (Elad *et al*., 2004). Des études biochimiques ont indiqué que ces fongicides affectaient la synthèse de la paroi cellulaire et induisaient l'accumulation de glycérol dans les cellules mycéliennes (Leroux, 1996). Plusieurs recherches suggèrent que leur cible primaire pourrait être des **protéines kinases** impliquées dans la régulation de la biosynthèse des polyols (Orth *et al*., 1995 ; Pillonel & Meyer, 1997 ; Schumacher *et al*., 1997).

Les souches résistantes à la plupart des dicarboximides sont également résistantes aux hydrocarbures aromatiques mais restent sensibles aux phénylpyrroles. Ainsi, même si les modes d'action biochimique des dicarboximides et des phénylpyrroles, ainsi que les mécanismes de résistance ne sont pas pleinement élucidés, il apparaît que dans les vignobles français, les souches résistantes aux dicarboximides sont correctement contrôlées par les phénylpyrroles (Leroux & Descotes, 1996).

d. Les inhibiteurs de la biosynthèse de méthionine

Le pyriméthanil, le cyprodinil et le mépanipyrim appartiennent à la famille des **anilinopyrimidines**. Ils ont été introduits en tant qu'anti-*Botrytis* dans de nombreux pays européens dans le milieu des années 1990. Les anilinopyrimidines n'affectent pas la germination des conidies mais inhibent l'élongation du tube germinatif et la croissance mycélienne de *B. cinerea*, au moins *in vivo* (Rosslenbroich & Stuebler, 2000). Des études concernant leur mode d'action ont montré que ces fongicides pouvaient interférer dans la biosynthèse de plusieurs acides aminés et particulièrement de la **méthionine** (Leroux, 1994 ; Masner *et al*., 1994 ; Fritz *et al*., 1997). Les anilinopyrimidines présentent également la

capacité d'inhiber la sécrétion de protéines extracellulaires, y compris d'**enzymes hydrolytiques** impliquées dans le processus d'infection du champignon (Miura *et al*., 1994 ; Milling & Richardson, 1995). Leur activité antifongique *in vivo* pourrait donc résulter d'une inhibition de la biosynthèse de méthionine dans les cellules fongiques et/ou d'un blocage dans l'excrétion d'enzymes hydrolytiques impliquées dans le processus de pathogénicité (Leroux, 1996). Des souches hautement résistantes aux anilinopyrimidines ont été détectées dans plusieurs vignobles européens dont le vignoble champenois (Hilber & Hilber-Bodmer, 1998 ; Leroux *et al*., 1998, 1999).

e. Les inhibiteurs de la biosynthèse des stérols

Quatre groupes principaux de fongicides inhibiteurs de la biosynthèse des stérols (IBS) peuvent être distingués en fonction de leur site d'action : les inhibiteurs de (1) la squalène époxidase (famille des triazoles), (2) la 14α-déméthylation des stérols appelés aussi IDM (famille des imidazoles), (3) la Δ14-réductase et de la Δ8→Δ7 isomérase (famille des amines) et (4) la C-4 déméthylation (famille des hydroxyanilides) (Elad *et al*., 2004). Ces différents fongicides ne suppriment pas la germination des conidies mais, à faibles concentrations, ils inhibent l'élongation des tubes germinatifs et la croissance mycélienne (Leroux *et al*., 1999). De plus, les tubes germinatifs produits sur des milieux avec ajout de ces fongicides sont déformés par des renflements et leur cytosol présente une apparence granulaire (Leroux *et al*., 1999). Cependant, peu d'entre eux contrôlent efficacement *B. cinerea* au vignoble.

Le plus efficace est le **fenhexamid**. Il a été introduit sur le marché en 2000 du fait de la sélection de souches de *B. cinerea* résistantes à plusieurs fongicides et de la nécessité de développer de nouvelles familles chimiques (Kuck *et al*., 1997 ; Rosslenbroich *et al*., 1998 ; Rosslenbroich & Stuebler, 2000). Le site d'action du fenhexamid semble être différent des autres fongicides anti-*Botrytis* car aucune résistance croisée n'a été décelée avec les autres fongicides (Rosslenbroich & Stuebler, 2000). L'accumulation de plusieurs stérols dans le mycélium traité par le fenhexamid indique que ce fongicide inhiberait la 3-céto réductase impliquée dans la C4-déméthylation (Debieu *et al*., 2001). Des souches de *B. cinerea* résistantes au fenhexamid ont été décelées avant l'introduction sur le marché de cet anti-*Botrytis*.

Les monitorings effectués sur l'ensemble des vignobles français depuis quelques années montrent que quasiment toutes les familles de fongicides anti-*Botrytis* sont désormais

touchées par la résistance. Cette évolution n'est pas corrélée dans l'immédiat avec une perte d'efficacité au champ. Outre la sélection de résistances spécifiques, depuis le milieu des années 1990, des souches résistantes simultanément à tous les modes d'action utilisés ont été détectées : ce sont des souches appelées « **Multi Drug Resistant** » (MDR). Si dans de nombreux vignobles français, les souches de type MDR sont peu nombreuses, la situation en Champagne est bien différente car leur fréquence actuelle est de l'ordre de 50% (Leroux, 2004 ; Panon *et al.*, 2006). Dans ces conditions, même si les niveaux de résistance sont généralement faibles, on ne peut pas exclure que l'efficacité des programmes de lutte anti-*Botrytis* soit affectée en situation de multirésistance généralisée.

Afin de garantir l'efficacité sur le long terme des stratégies anti-*Botrytis,* le nombre de traitements doit donc être réduit au maximum (Delp, 1980). De plus, il est fortement recommandé d'alterner l'emploi des familles chimiques non seulement sur l'année mais aussi pluriannuellement (Dubos, 1999 ; Viret, 1999). Le nombre de familles chimiques disponibles aujourd'hui le permet facilement.

En plus des phénomènes importants de résistance, les pesticides peuvent générer un grand nombre de risques significatifs pour la santé humaine et l'environnement, ce qui nécessite de réduire leur utilisation.

Lors du grenelle de l'environnement, qui s'est déroulé en octobre 2007, un objectif de réduction de moitié des usages de pesticides dans l'agriculture française a été fixé (www.legrenelle-environnement.gouv.fr/grenelle-environnement/spip.php).

En France, le problème de la contamination des milieux aquatiques par les pesticides est depuis plusieurs années particulièrement inquiétant. Un certain nombre de mesures vise à limiter cette contamination. Le décret n°2001-1220 du 20 décembre 2001 (admi.net/jo/20011222/MESX0100156D.html), relatif aux eaux destinées à la consommation humaine, se substitue au décret du 3 janvier 1989. Ces nouvelles dispositions réglementaires visent à renforcer la sécurité sanitaire des eaux de consommation distribuées à la population ou utilisées dans les entreprises alimentaires. Notamment, ce décret détermine des critères de qualité comme des limites de qualité concernant des substances indésirables ou toxiques dont font partie les pesticides. De plus, l'Union européenne a adopté le 23 octobre 2000 une directive cadre (décision n 2455/2001/CE) qui impose aux Etats membres d'atteindre un bon état écologique des eaux souterraines et superficielles en 15 ans (www.vie-publique.fr/actualite/dossier/loi-eau/politique-eau-vers-bonne-qualite-eau-2015.html).

Les risques liés à l'utilisation des pesticides sont particulièrement bien identifiés en viticulture (Ministère de l'agriculture et de la pêche, 2005) sur :

- ✓ la santé des viticulteurs et des travailleurs agricoles : la viticulture est le deuxième secteur le plus exposé en France avec 22% des cas déclarés d'incidents et d'accidents liés à l'utilisation professionnelle des pesticides ;
- ✓ la santé des consommateurs : l'utilisation des pesticides est susceptible d'entraîner leur présence dans les raisins et les vins, même si leur détection reste faible (Flamini & Panighel, 2006) ;
- ✓ la qualité des eaux : la filière viticole est particulièrement exposée aux risques de transfert dans l'environnement des pesticides, aussi bien dans les eaux superficielles que dans les eaux souterraines (Jacobson *et al*., 2005 ; Bony *et al*., 2008) ;
- ✓ la vigne : la pulvérisation de pesticides peut générer un stress sur la plante cultivée, mis en évidence notamment par une perturbation de la photosynthèse foliaire et une induction des mécanismes de défense dans différents organes de la plante (Garcia *et al.*, 2003 ; Saladin & Clément, 2005).

4. Stratégies alternatives contre la pourriture grise

a. Les plantes transgéniques

La **transformation génétique** offre une alternative intéressante puisqu'elle permet l'introduction d'un ou plusieurs gènes dans le génome de la plante tout en maintenant son patrimoine génétique, ses caractères agronomiques et la qualité de ses produits (Yamamoto *et al.*, 2000 ; Vidal *et al.*, 2003 ; Bornhoff *et al.*, 2005 ; Franks *et al.*, 2006).

La transformation génétique est bien maîtrisée chez la vigne. Elle est réalisée aussi bien par coculture avec des souches d'*Agrobacterium tumefaciens* que par biolistique (Colova-Tsolova *et al*., 2001 ; Kikkert *et al*., 2001). Dans la lutte contre *B. cinerea*, les stratégies visent à surexprimer des gènes codant des molécules à fonction antimicrobienne. Ainsi, l'intégration d'un gène codant une stilbène synthase a permis une production plus importante de resvératrol dans les plantes transgéniques (Fan *et al*., 2008) et une meilleure tolérance *in vitro* vis-à-vis de *B. cinerea* (Coutos-Thévenot *et al*., 2001). De la même manière, des vignes transgéniques exprimant un gène codant une protéine inhibitrice de polygalacturonases (PGIP pour polygalacturonase-inhibiting protein) présentent une tolérance plus importante à *B. cinerea* (Agüero *et al.*, 2005). Le rôle des PGIP est de prévenir la dégradation de la paroi

végétale en inhibant les polygalacturonases fongiques, enzymes souvent considérés comme étant des facteurs de virulence (Ten Have *et al*., 1998 ; De Lorenzo *et al*., 2001).

Toutefois, même si la directive européenne 2003/11/CE autorise la préparation de plants transgéniques de vigne, la profession viti-vinicole est très opposée à l'utilisation de vignes transgéniques. De plus, en janvier 2008, le gouvernement français invoque la « clause de sauvegarde » : toute culture d'OGM (organisme génétiquement modifié) commercial est alors interdite en France. Ainsi, d'autres stratégies alternatives ont été envisagées pour lutter contre *B. cinerea*.

b. La lutte biologique

La **lutte biologique** a pour principe d'utiliser des micro-organismes antagonistes afin de réduire la densité de l'inoculum de l'agent pathogène ou d'altérer son activité pathogène. La protection conférée par un agent biologique peut être basée sur un ou plusieurs mécanismes d'action : la compétition pour les éléments nutritifs ou l'espace, le parasitisme, la production de substances toxiques pour le pathogène (antibiose) et/ou la stimulation des défenses de la plante (Thomashow & Weller, 1996 ; Yedida *et al*., 1999 ; Haas *et al*., 2000).

Chez la vigne, certains champignons ou bactéries permettent d'améliorer la résistance à *B. cinerea* (Elmer & Reglinski, 2006). Le champignon le plus largement étudié est le *Trichoderma* spp. Les travaux sur le biocontrôle de *B. cinerea* à l'aide de ce champignon ont débuté il y a 30 ans (Dubos *et al*., 1978, 1982). Des produits à base de *Trichoderma harzianum* et *T. viride* ont été formulés afin d'être commercialisés en tant qu'anti-*Botrytis*. Ils présentent une efficacité parfois équivalente à un programme anti-*Botrytis* de référence à l'aide de fongicides chimiques (Harman *et al*., 1996 ; Latorre *et al*., 1997 ; Sesan *et al*., 1999). Concernant les bactéries, des vignes inoculées ou vaporisées au moyen de bactéries du genre *Bacillus* produisent des phytoalexines et présentent une meilleure résistance à *B. cinerea* (Paul *et al*., 1997, 1998). De plus, des rhizobactéries appelées PGPR pour « plant growth-promoting rhizobacteria » ont montré une efficacité dans la lutte contre *B. cinerea*. La bactérie *Burkholderia phytofirmans* souche PsJN, isolée à partir de racines d'oignon, permet d'augmenter la résistance des vignes *in vitro* vis-à-vis de *B. cinerea* (Ait Barka *et al*., 2000, 2002). Cette bactérie colonise les surfaces racinaires puis se propage de façon systémique dans les parties aériennes de plantules *in vitro*, tout en générant l'accumulation de composés phénoliques chez la plante (Compant *et al*., 2005). Des bactéries des genres *Acinetobacter*,

Pseudomonas, *Bacillus* et *Pantoea*, issues du vignoble champenois, présentent également une efficacité contre *B. cinerea* dans des conditions *in vitro* et sont capables d'activer des réponses de défense au vignoble dans des feuilles et des grappes (Magnin-Robert *et al*., 2007).

c. L'élicitation des défenses naturelles

Une autre stratégie alternative vise à stimuler les défenses naturelles de la vigne par l'utilisation de composés éliciteurs capables de déclencher les mécanismes de defense dont disposent les plantes (Kessmann *et al*., 1994 ; Garcia-Brugger *et al*., 2006). Toute une série de gènes de défense est alors induite et l'activation des réponses de défense aboutit à une protection de la plante contre ses agresseurs.

Chez la vigne, de nombreux éliciteurs d'origine biotique ou abiotique permettent d'activer les mécanismes de défense de la plante, en association avec une résistance à *B. cinerea* dans des suspensions cellulaires, des feuilles et des baies (Elmer & Reglinski, 2006). Parmi ces éliciteurs, l'acide salicylique (SA) est un composé phénolique qui joue un rôle central dans les mécanismes de signalisation intervenant lors de la défense et dans la résistance des plantes aux maladies (Hammerschmidt & Smith-Becker, 1999). Le traitement au SA augmente l'expression de chitinases dans les cellules de vigne (Busam *et al*., 1997b) et stimule l'accumulation de protéines Pathogenesis-Related (PR) dans les racines, les tiges, les feuilles et les grappes de vigne (Renault *et al*., 1996 ; Derckel *et al*., 1996, 1998). L'acide benzo(1,2,3)-thiadiazole-7-carbothioïque-S-méthyl-ester (BTH), analogue du SA, commercialisé sous le nom de Bion® par Syngenta (Kunz *et al*., 1997), induit l'expression de protéines PR dans les cultures cellulaires de vigne ainsi qu'une augmentation de la synthèse de resvératrol et d'anthocyanines dans les baies traitées au vignoble (Busam *et al*., 1997a, b ; Iriti *et al*., 2004). De plus, le BTH a montré au vignoble des résultats prometteurs avec une augmentation de la résistance contre *B. cinerea* (Iriti *et al*., 2004, 2005). Le Synermix®, extrait d'algues marines additionné d'$AlCl_3$ et commercialisé par la société Goëmar, induit *in vivo* la production de resvératrol dans les feuilles de vigne (Jeandet *et al*., 1996, 2000). Des expérimentations au vignoble ont révélé son efficacité, seul ou en association avec un fongicide, dans sa protection contre *B. cinerea* (Jeandet *et al*., 1996, 2000). La société Goëmar a également développé une méthode pour extraire la laminarine à partir d'une algue marine (*Laminaria digitata*). La laminarine induit l'activation de nombreux gènes de défense dans des suspensions cellulaires de vigne et protége les feuilles contre *B. cinerea* (Aziz *et al*.,

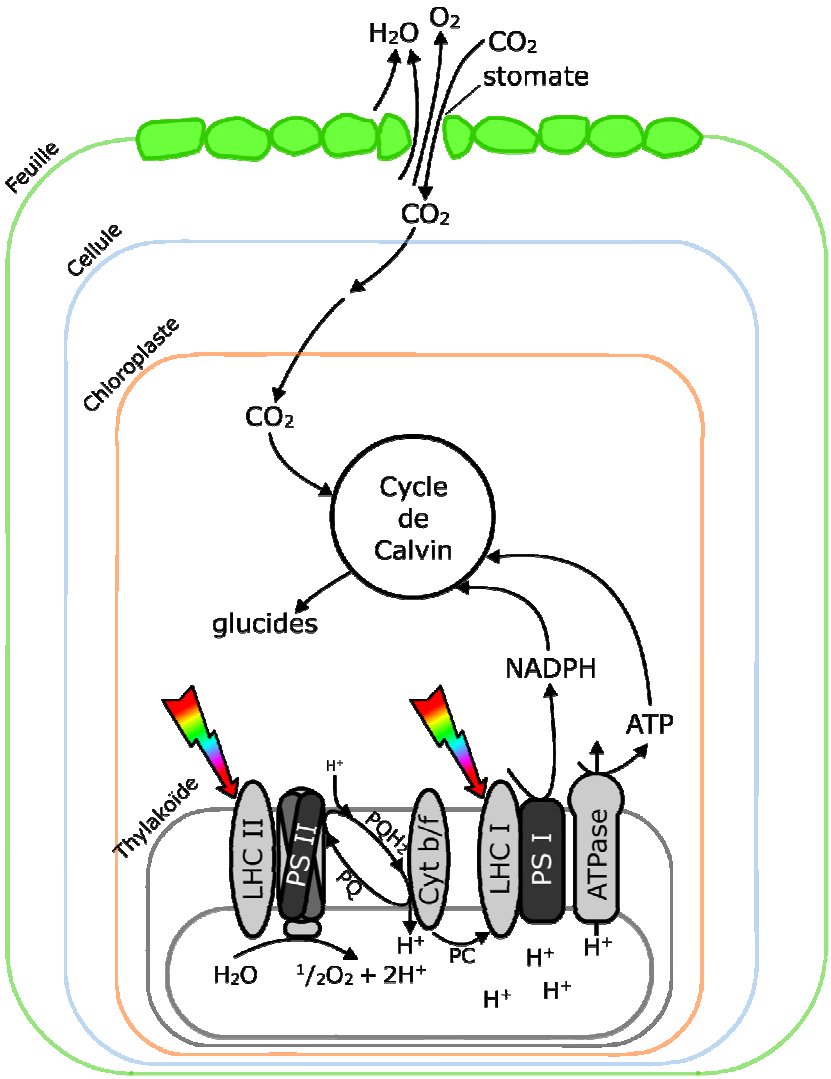


Figure 10. Schéma global du processus photosynthétique (d'après Baker & Rosenqvist, 2004)

2003). Un autre composé, le chitosan, polymère biologique de n-glucosamines obtenu par déacétylation de la chitine, stimule la synthèse de lipoxygénase, de phénylalanine ammonia-lyase, de phytoalexines et de protéines PR dans des feuilles de vigne et augmente la résistance contre *B. cinerea* (Ait Barka *et al*., 2004 ; Aziz *et al*., 2006 ; Trotel-Aziz *et al*., 2006). Au vignoble, sur les baies, le chitosan réduit également l'intensité et la fréquence d'attaque de *B. cinerea* à la fois par sa capacité à stimuler des réactions de défense de la vigne et par un effet fongistatique direct sur *B. cinerea* (Amborabé *et al*., 2004).

Malgré des résultats intéressants, l'utilisation de composés biologiques dans la lutte contre *B. cinerea*, que ce soit dans le cadre de la lutte biologique ou de l'élicitation, présente une contrainte importante pour leur utilisation au vignoble (Fravel, 1999 ; Stewart, 2001 ; Shtienberg & Elad, 2002). La complexité des interactions hôte/pathogène/environnement génère une efficacité très variable de ces stratégies au vignoble. Des approches combinées impliquant plusieurs composants biologiques devront être testées afin d'augmenter l'efficacité de ces stratégies dans la lutte contre *B. cinerea*.

III. Stress et photosynthèse

1. Généralités sur la photosynthèse

La vigne est un organisme phototrophe, c'est-à-dire capable de synthétiser sa propre matière organique à partir d'eau, de sels minéraux et de CO_2 en utilisant l'énergie lumineuse. La transformation de cette énergie lumineuse en énergie chimique, sous la forme de composés organiques, est appelée **photosynthèse** (Figure 10). Elle conduit à la synthèse de glucides et à la production d'O_2 selon l'équation bilan suivante :

$$6\ CO_2 + 6\ H_2O + \text{énergie lumineuse} \longrightarrow C_6H_{12}O_6 + 6\ O_2$$

Le CO_2 de l'atmosphère pénètre dans la plante par les **stomates**. Ces stomates sont formés par deux cellules de garde en regard, laissent entre elles une ouverture ou ostiole qui peut être plus ou moins fermée.

Les **chloroplastes** contiennent la machinerie photosynthétique. Ils renferment un génome complet avec de l'ADN, de l'ARN, des ribosomes et des enzymes nécessaires à la synthèse protéique (Sugiura, 1992). Le génome chloroplastique code pour de nombreux polypeptides qui jouent un rôle important dans la conversion énergétique et le métabolisme du carbone, comme la grosse sous-unité de la ribulose-1,5-bisphosphate carboxylase/oxygénase

(Rubisco) et plusieurs sous-unités du complexe qui synthétise l'ATP (Sugiura, 1992). Les chloroplastes sont le site des réactions primaires et secondaires de la photosynthèse. Ils comportent quatre compartiments structurels : (1) deux membranes formant l'enveloppe, (2) une matrice non structurée, le stroma, (3) un système membranaire interne composé de thylakoïdes et (4) à l'intérieur des thylakoïdes, un lumen.

Les membranes constituant les thylakoïdes renferment les chlorophylles et les caroténoïdes. Le lumen est le site de la photolyse, ou oxydation, de l'eau et par conséquent de la production d'oxygène lors de la photosynthèse. Par ailleurs, il fonctionne aussi comme réservoir de protons qui, au cours du transfert des électrons, seront pompés au travers du thylakoïde et seront utilisés pour synthétiser de l'adénosine triphosphate (ATP), stock d'énergie pour la plante. Ce dernier sera par la suite impliqué dans les réactions du métabolisme photosynthétique primaire. Le stroma est essentiellement composé de protéines. Il contient des enzymes responsables de la réduction photosynthétique du carbone, dont la Rubisco qui fixe le carbone (Sharkey, 1989).

a. Réactions primaires de la photosynthèse

La première phase comprend une série de réactions de transport d'électrons au cours de laquelle l'énergie lumineuse est transformée en énergie chimique stable : le NADPH et l'ATP. Elle se déroule au sein des membranes thylakoïdiennes. Elle comprend deux étapes successives : d'une part, un transfert d'énergie par résonance des chlorophylles au centre réactionnel, puis d'autre part, un transfert d'électrons par oxydo-réductions successives jusqu'à un accepteur final, le $NADP^+$ (Hopkins & Evrard, 2006).

Les pigments assimilateurs, chlorophylles et caroténoïdes, jouent un rôle majeur dans cette première phase. En effet, l'énergie lumineuse est capturée par les pigments au sein des **complexes collecteurs de lumière** (LHC pour light harvesting complex) (Anderson, 1980 ; Thornber *et al*., 1993 ; Van Grondelle *et al*., 1994 ; Grossman *et al*., 1995). Ces complexes associent protéines et chlorophylles dans des orientations bien précises, absorbent l'énergie lumineuse et assurent un transfert efficace de l'énergie captée vers les centres photochimiques (Buchanan *et al*., 2000). Les LHC sont assemblés au sein de deux photosystèmes séparés mais néanmoins biophysiquement liés : les **photosystèmes I et II** (PSI et PSII) (Hillier & Babcock, 2001). Chaque photosystème comprend un **centre réactionnel** et une **antenne** constituée de LHC. La **chlorophylle** intervient d'une part comme collecteur de lumière dans les antennes

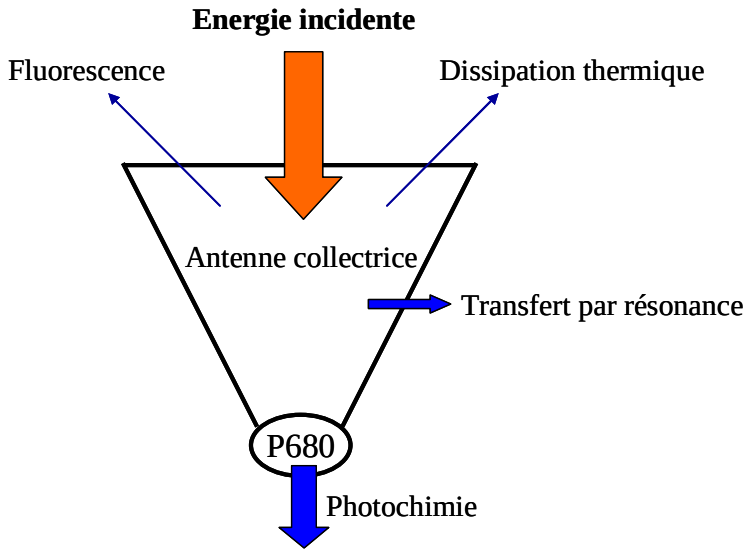


Figure 11. Représentation schématique des différentes voies d'utilisation de l'énergie reçue par le PSII (d'après Dreyer *et al.*, 1995).

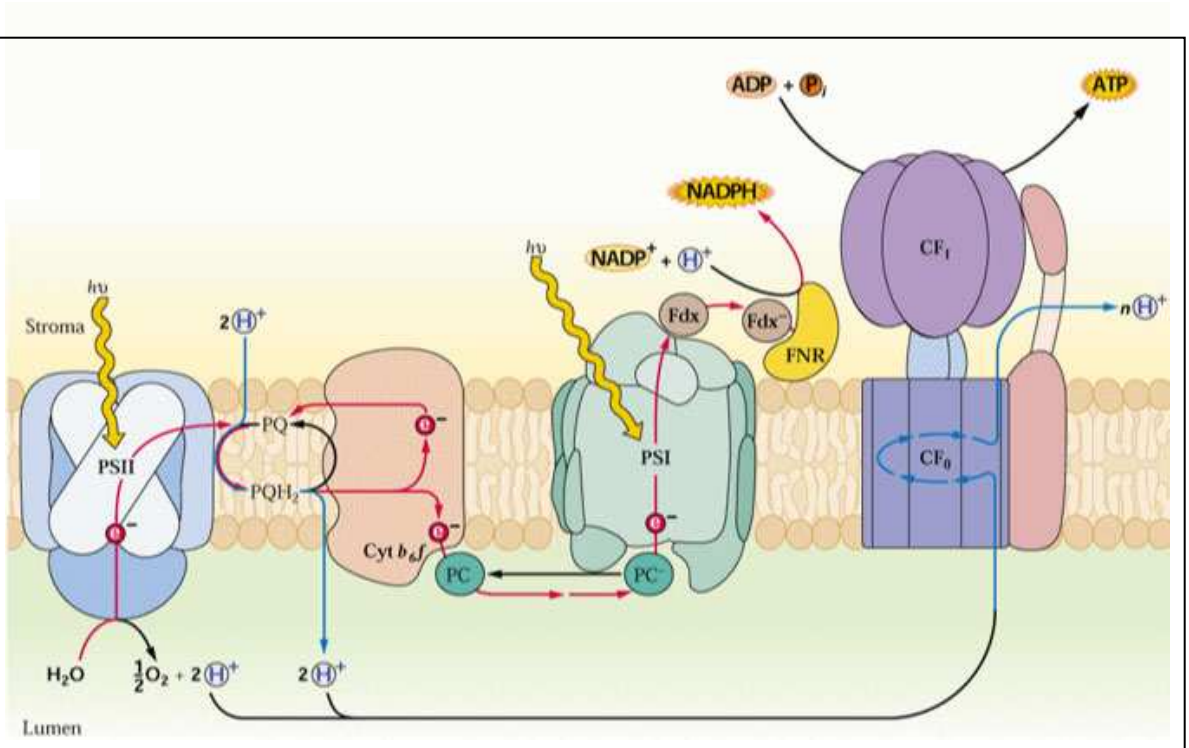


Figure 12. Chaîne de transfert des électrons photosynthétiques (d'après Buchanan *et al.*, 2000).

Organisation membranaire des deux photosystèmes (PSII et PSI), du cytochrome *b6f* (Cyt*b6f*) et de l'ATP synthétase (CF1-CF0) qui participent à la transformation de l'énergie lumineuse (*hv*) perçue en énergie chimique utilisable par la plante. La photolyse de l'eau au niveau du PSII ainsi que l'oxydation de la plastoquinone membranaire (PQH_2) libèrent des protons (H^+) et des électrons (e^-) utilisés au niveau de la ferrédoxine-$NADP^+$ réductase (FNR) et de l'ATP synthétase pour produire du NADPH et de l'ATP (fdx : ferrédoxine ; PC : plastocyanine).

et, d'autre part, comme convertisseur photochimique dans les centres réactionnels des photosystèmes. Elle est présente sous deux formes chez les plantes supérieures : chlorophylle *a* et chlorophylle *b*, qui différent par leur spectre d'absorption. La chlorophylle *a* absorbe fortement à 430 nm et 660 nm alors que la chlorophylle *b* absorbe fortement à 445 nm et 645 nm. Quant aux **caroténoïdes**, en plus d'avoir un rôle de collecteurs de lumière, leur fonction la mieux caractérisée dans la photosynthèse est la photoprotection (Havaux *et al*., 1998). Les caroténoïdes sont capables d'absorber l'énergie lumineuse dans des longueurs d'onde de 420 à 530 nm environ, puis de la transférer aux pigments chlorophylliens. L'énergie lumineuse collectée par les antennes et les complexes LHC est dirigée vers le centre réactionnel. Le centre réactionnel comprend de 4 à 6 molécules de chlorophylle *a* ainsi que des protéines et des cofacteurs qui leur sont associés (Green & Durnford, 1996). Comme le centre réactionnel est le lieu de la première réaction rédox photochimique, il est l'endroit où l'énergie lumineuse est réellement convertie en énergie chimique. Les centres réactionnels de PSI et PSII sont désignés respectivement par les termes de P700 et P680 puisque leur maximum d'absorption se situe respectivement à 700 et 680 nm (Thornber *et al.*, 1993).

Lorsque les molécules de chlorophylles reçoivent des photons, ces derniers passent d'un état fondamental à un niveau énergétique plus élevé dit état excité. Pour revenir ensuite vers l'état fondamental, plusieurs voies de dissipation de cet excès d'énergie sont possibles (Figure 11) (Björkman & Demmig-Adams, 1994 ; Horton *et al*., 1996) :

- ✓ l'énergie peut être transmise entre molécules de pigment par un mécanisme dit de **transfert d'énergie par résonance inductive**, jusqu'à ce que l'excitation atteigne un centre réactionnel photosynthétique ;
- ✓ la désactivation thermique qui se déroule quand une molécule perd l'énergie d'excitation sous forme de **chaleur** ;
- ✓ la **photochimie**, l'énergie de l'état excité permettant à des réactions chimiques de se produire ;
- ✓ la **fluorescence chlorophyllienne** correspondant à l'émission d'un photon par une molécule de chlorophylle excitée. Elle provient essentiellement de l'antenne collectrice du PSII. Cette fluorescence peut être considérée comme un indicateur intrinsèque précis des premières étapes de la photosynthèse. Son intensité est donc directement liée par une relation inverse au rendement photosynthétique de la plante. La mesure de cette fluorescence peut se mesurer *in vivo* de façon non destructrice.

Les principales enzymes impliquées dans le cycle de Calvin sont :

1. rubisco
2. phosphoglycérate kinase
3. glycéraldéhyde 3-phosphate déshydrogénase
4. recyclage du ribulose-1,5-bisphosphate *via* la voie des pentoses phosphates
5. phosphoribulokinase

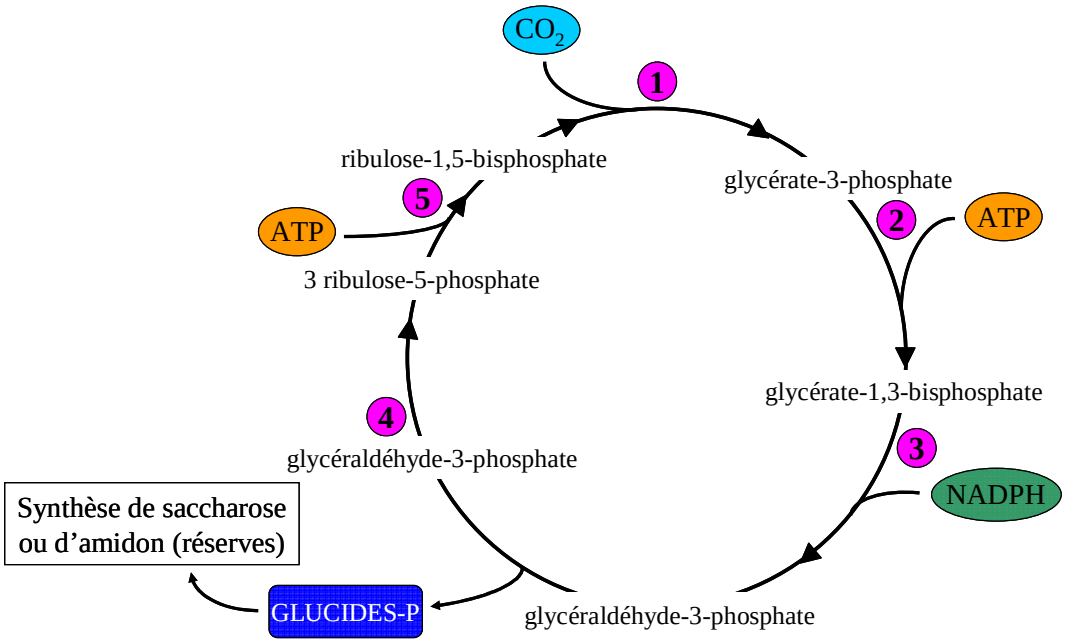


Figure 13. Cycle de Calvin simplifié.

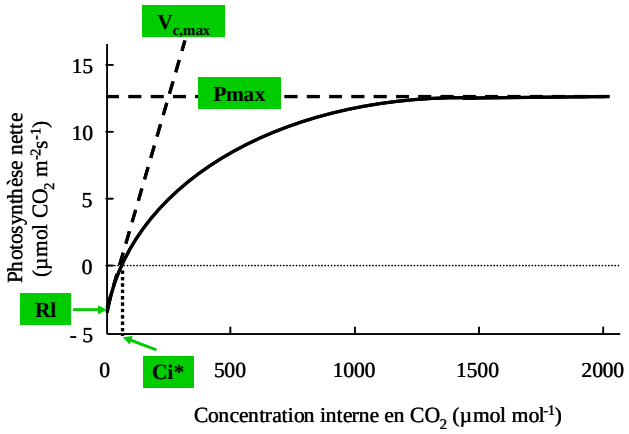
Dans le centre réactionnel, le flux d'électrons est initié par une séparation de charges ou photooxydation (Figure 12). Le **complexe d'émission d'oxygène** (OEC pour oxygen-evolving complex) est responsable de la photolyse de l'eau et de la libération subséquente d'oxygène (McEvoy & Brudvig, 2006). Ce complexe OEC est situé sur la face interne des membranes thylakoïdiennes et est étroitement associé au PSII. Les électrons libérés par la photolyse de l'eau passent dans le PSII. La chlorophylle excitée du centre réactionnel du PSII transfère de l'énergie à la phéophytine (Pheo) qui transmet à son tour des électrons aux **plastoquinones** (PQ) QA et QB. Le complexe **cytochrome *b_6f*** (Cyt*b_6f*), formé de plusieurs protéines enchâssées dans la membrane, intervient alors pour transférer les électrons aux **plastocyanines** (PC) (Kallas, 1994 ; Trumpower & Gennis, 1994). Lors du transfert des électrons entre le PSII et le PSI à travers le cytochrome, une partie de l'énergie est utilisée pour faire passer des protons du stroma vers le lumen (Kallas, 1994 ; Trumpower & Gennis, 1994). Puis, au niveau du PSI, la **ferrédoxine** (Fdx), située dans la membrane sur la face tournée vers le stroma, reçoit des électrons à l'aide de la ferrédoxine-$NADP^+$ réductase (FNR) et réduit le $NADP^+$ en NADPH (Arakaki *et al.*, 1997). Dans le même temps, l'**ATP synthétase** permet la production d'ATP et le retour des protons dans le stroma (Mitchell, 1961).

b. Réactions secondaires de la photosynthèse

Lors de la seconde étape, le NADPH et l'ATP produits sont utilisés afin de réduire le CO_2 en glucides, constituant ainsi les réactions secondaires de la photosynthèse. Cette phase se déroule dans le stroma du chloroplaste au cours du cycle photosynthétique de réduction du carbone ou **cycle de Calvin** (Figure 13) (Calvin, 1962 ; Woodrow & Berry, 1988). Ce cycle fait intervenir 11 enzymes et est initié par une enzyme clé, la Rubisco, qui catalyse l'addition d'une molécule de CO_2 à une molécule d'un accepteur, le ribulose-1,5-bisphosphate (RuBP) (Spreitzer, 1993; Hartman & Harpel 1994). La Rubisco est composée d'une grosse et d'une petite sous-unité. La grosse sous-unité est codée par un gène de l'ADN chloroplastique (*rbcL*) alors que la petite sous-unité est codée par une famille de gènes de l'ADN nucléaire (*rbcS*) (Spreitzer, 1993; Hartman & Harpel 1994).

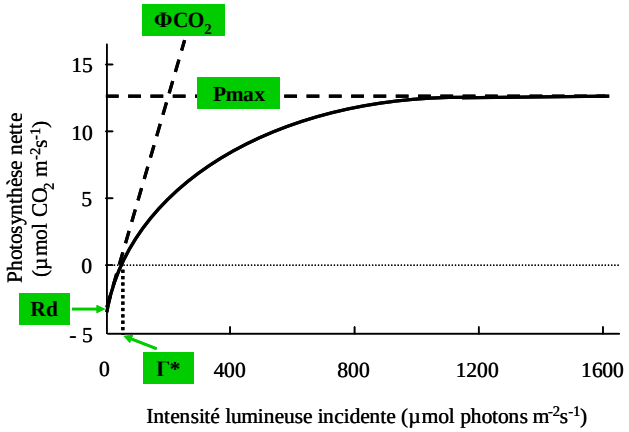
Les courbes de réponse sont obtenues en mesurant de façon simultanée l'activité photosynthétique (ou assimilation en CO_2) à différentes concentrations internes en CO_2 (Ci) ou à différentes intensités lumineuses. Il est alors possible d'ajuster une équation aux données expérimentales et d'en déduire la valeur de différents paramètres.

(1) courbe de réponse de Pn à Ci



Le niveau maximal de photosynthèse (Pmax) obtenu à la lumière et à Ci saturante est limité par l'accepteur de CO_2, le RuBP. La pente de la portion linéaire de la courbe Pn *vs* Ci représente l'efficacité maximale de carboxylation de la rubisco ($V_{c,max}$). Il reflète ainsi le niveau maximal de régénération du RuBP. Ci* représente le point de compensation pour le CO_2, qui est le point où Pn équivaut à 0 et Rl est une estimation de la respiration mitochondriale à la lumière et se situe là où Ci est égale à 0.

(2) courbe de saturation lumineuse



La photosynthèse à éclairement saturant et CO_2 ambiant (Pmax) représente les capacités maximales de la feuille. Le rendement quantique apparent pour la fixation du CO_2 (ΦCO_2) est défini comme la pente de la régression de la courbe de Pn *vs* intensité lumineuse incidente. Γ* correspond à l'intensité lumineuse de compensation c'est-à-dire l'intensité lumineuse à laquelle Pn est égale à la respiration. Rd correspond à la respiration à l'obscurité.

Figure 14. Exemples de courbes de réponse de l'assimilation de CO_2 à la concentration interne en CO_2 et à l'intensité lumineuse incidente (d'après Long & Bernacchi, 2003).

2. Mesures de la photosynthèse

Les mesures d'échanges gazeux et de fluorescence chlorophyllienne sont réalisées afin de caractériser l'activité photosynthétique et de mettre en évidence les effets possibles de stress sur la photosynthèse foliaire des plantes.

a. Les échanges gazeux

Les mesures d'échanges gazeux foliaires à la lumière ont été effectuées grâce à un analyseur à gaz infra-rouge de type LICOR 6400 (LICOR 6400-40; Lincoln, NE, E-U) qui regroupe en une unité portable tous les éléments nécessaires pour la mesure des flux de vapeur d'eau et de CO_2. Ce système comporte :

- ✓ une pince foliaire ventilée munie de capteurs de température à l'intérieur de la chambre et d'un capteur de PPFD (rayonnement photosynthétiquement actif). Cette pince est reliée à une lampe halogène permettant de travailler sous des rayonnements contrôlés ;
- ✓ une entrée de cartouche à CO_2 permettant de travailler sous différentes concentrations de CO_2 ;
- ✓ des microprocesseurs permettant la mesure de la concentration en CO_2 et en H_2O de l'air ;
- ✓ une unité centrale permettant le contrôle des protocoles en plus d'un système d'acquisition et de transfert des résultats sur ordinateur.

La **photosynthèse nette** (Pn), qui équivaut à l'assimilation en CO_2, est déterminée et correspond à la fixation métabolique en CO_2 au niveau du cycle de Calvin utilisant l'énergie lumineuse (von Caemmerer & Farquhar, 1981). La diminution de la photosynthèse peut être occasionnée par une réduction de la pénétration en CO_2, limitée par une fermeture des stomates : c'est la notion de limitation stomatique (Farquhar & Sharkey, 1982). Elle est déterminée grâce aux mesures de **conductance stomatique** (g_S). Cette limitation stomatique peut alors provoquer une diminution de la **concentration interne en CO_2** (Ci) et ainsi une réduction de Pn (Farquhar & Sharkey, 1982).

Par ailleurs, les **courbes de réponses de Pn à Ci** permettent d'étudier la régulation de la photosynthèse par le CO_2, indépendamment de la régulation stomatique (Long & Bernacchi, 2003). Ces courbes sont réalisées en faisant varier la concentration en CO_2 ambiant (Ca) et en déterminant à chaque étape les paramètres Pn et Ci (Figure 14). Les paramètres mesurés

Lorsqu'un végétal est adapté à l'obscurité, toutes les antennes sont connectées et tous les centres réactionnels sont oxydés (ou ouverts).

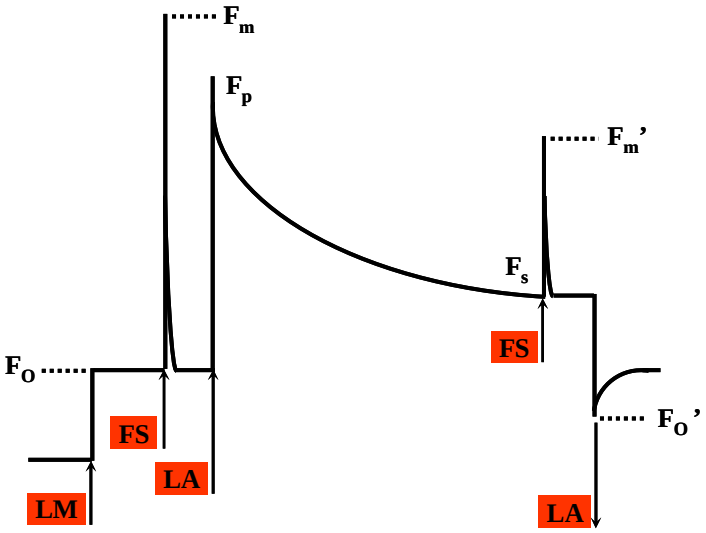
Sous l'action d'un très faible éclairement (**lumière modulée** ou LM), la plupart des photons captés par les antennes vont être transformés en énergie chimique par le centre réactionnel. Les photons non utilisés sont réémis sous forme de fluorescence, qui est alors minimale : $\mathbf{F_O}$.

Si l'on soumet ce végétal à un éclairement intense et bref (**flash de saturation** ou FS), les centres réactionnels vont être rapidement réduits, les capacités d'oxydation des centres sont dépassées. L'énergie des photons est alors réémise sous forme de fluorescence, qui est maximale : $\mathbf{F_m}$. Il est à noter que cet éclairement doit être de courte durée afin d'éviter la déconnexion des antennes, ce qui provoquerait un quenching non photochimique par émission de l'énergie sous forme de chaleur.

Dans le cas où ce végétal est adapté à un éclairement raisonnable (**lumière actinique** ou LA), les photosystèmes sont adaptés afin d'optimiser le transfert d'électrons : une partie des antennes est déconnectée, donc une partie de l'énergie des photons est réémise sous forme de chaleur. Une partie des centres réactionnels sont fermés par la pluie de photons. Tant que ces centres ne sont pas oxydés, ils sont dans l'incapacité de transformer l'énergie lumineuse en énergie chimique. Dans cet intervalle de temps, l'énergie des photons captés par leurs antennes est donc réémise sous forme de fluorescence. Le niveau de fluorescence est alors appelé $\mathbf{F_p}$, supérieur à F_O, qui évolue vers un niveau $\mathbf{F_s}$.

Sous l'action d'un flash de saturation, tous les centres se ferment, l'énergie des photons est réémise sous forme de fluorescence. Cependant comme une partie de l'énergie est dissipée sous forme de chaleur à cause de la déconnexion des antennes, la fluorescence atteint un niveau $\mathbf{F_m'}$, inférieure à F_m.

Suite à cette phase d'éclairement, le végétal est replacé à l'obscurité. Dans un délai assez court, tous les centres vont être réduits mais les antennes restent déconnectées, le niveau de fluorescence atteint une valeur $\mathbf{F_O'}$, inférieure à F_O car il y a moins de centres réactionnels branchés.



Variation de la fluorescence modulée en fonction du temps sur une feuille intacte

Figure 15. Principes des analyses de la fluorescence chlorophyllienne (Maxwell & Johnson, 2000).

peuvent être utilisés comme des indicateurs photosynthétiques et peuvent varier de manière prononcée lorsque la plante subit des stress environnementaux. Le niveau maximal de photosynthèse (Pmax), obtenu à la lumière et à Ci saturante, est limité par l'accepteur de CO_2, le RuBP. Il reflète ainsi le niveau maximal de régénération du RuBP. La pente de la portion linéaire de la courbe représente la capacité maximale de carboxylation de la Rubisco ($V_{c,max}$) (Farquhar *et al.*, 1980). Une diminution de $V_{c,max}$ serait le résultat d'une perte ou d'une inactivation de la Rubisco (Allen *et al.*, 1997). Ce modèle permet également de déterminer le point de compensation pour le CO_2 (Ci*) et d'estimer la respiration mitochondriale à la lumière (Rl). Ci* est la concentration pour laquelle l'absorption de CO_2 au cours de la photosynthèse est équilibrée par la libération de CO_2 à travers la respiration ou tout autre mécanisme.

Un autre outil important en plus des courbes de réponse de Pn à Ci est la **courbe de saturation lumineuse**. Cette courbe est obtenue en faisant varier l'intensité lumineuse incidente. Pmax représente le niveau maximal de photosynthèse à éclairement saturant et CO_2 ambiant. La pente de la portion linéaire de la courbe équivaut au rendement quantique maximum de fixation du CO_2 (ΦCO_2). De plus, le point de compensation lumineux (Γ^*) et la respiration à l'obscurité (Rd) peuvent être calculés à partir des courbes (Long & Hällgren, 1993). Γ^* correspond à un équilibre entre deux processus en compétition : la photosynthèse et la respiration. Lorsque Γ^* est plus faible, cela signifie que la demande en énergie pour l'excitation du PSII est moins importante.

b. La fluorescence chlorophyllienne

La **fluorescence de la chlorophylle *a*** est utilisée comme outil sensible et précoce pour diagnostiquer l'état fonctionnel du PSII en condition de stress chez les végétaux (Maxwell & Johnson, 2000). Ainsi, cette mesure, associée à la mesure d'échanges gazeux, permet une étude plus complète de la photosynthèse foliaire et en particulier des réactions primaires associées au transport des électrons de la chaîne chloroplastique. Cet outil permet de quantifier la part de l'énergie absorbée allouée à la photochimie et au dégagement sous forme de chaleur (Figure 15). De plus, le flux d'électrons passant par le PSII est mesuré : c'est une des premières cibles en cas de stress (Genty *et al.*, 1989). Le **quenching photochimique** (q_P) correspond à $(F_m' - F_s)/(F_m' - F_O')$ et reflète la part de l'énergie atteignant le centre réactionnel, utilisée par la photochimie. Le **quenching non-photochimique** (q_{NP}) est proportionnel à la part d'énergie dissipée sous une autre forme que la fluorescence et la photochimie. Il est égal

à $(F_m-F_m')/F_m'$ (Genty *et al.*, 1989). Les paramètres q_P et q_{NP} sont ainsi couramment utilisés pour l'étude du fonctionnement photosynthétique d'une feuille soumise à des stress environnementaux, en association avec deux autres paramètres : F_v/F_m et Φ_{PSII} (Krause & Weis, 1991 ; Maxwell & Johnson, 2000). Le paramètre $\mathbf{F_v/F_m}$, mesuré pour une feuille adaptée à l'obscurité, correspond à la capacité photochimique maximale du PSII et est égal à $(F_m - F_O/F_m)$. Il représente la part de l'énergie captée par les antennes et non dissipée sous forme de chaleur qui atteint le centre réactionnel. L'efficience photochimique du PSII ou $\mathbf{\Phi_{PSII}}$, mesurée pour une feuille adaptée à la lumière, peut se définir comme étant le rendement quantique de l'acte photochimique et correspond à $(F_m'-F_s)/F_m'$ (Genty *et al.*, 1989).

3. Pesticides et photosynthèse

Les plantes cultivées subissent non seulement l'agression par de nombreux organismes pathogènes mais elles sont également soumises de façon constante à des stress abiotiques divers, qu'ils soient liés aux conditions climatiques, aux pratiques culturales ou à des carences en minéraux. Elles développent alors une variété de symptômes ou signes de stress. Les indicateurs de stress peuvent être visibles comme des perturbations de la croissance et de la morphologie, ou invisibles lors de modifications physiologiques et biochimiques. Parmi ces marqueurs de stress, la photosynthèse des végétaux est influencée par de nombreux stress abiotiques.

L'application de pesticides fait partie de ces conditions stressantes qui sont capables de perturber le processus photosynthétique de la plante. La **publication 1** est une synthèse bibliographique qui concerne les effets de différents pesticides à action non-herbicide sur la photosynthèse des plantes cultivées.

Publication 1

Effects of “non-herbicidal” pesticides on photosynthesis – a review

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Soumise à *Chemosphere*

Effects of "non-herbicidal" pesticides on photosynthesis – a review

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Pesticides have negative effects on photosynthesis

Abstract

Among pesticides, fungicides and insecticides are largely used to control crop diseases. However, it was reported that these pesticides have negative effects on crop physiology, and especially on photosynthesis. Alteration of photosynthesis may lead to a reduction in photoassimilate production resulting in a decrease in both growth and yield. Contact fungicides such as copper inhibit photosynthesis by destructing chloroplasts, affecting PSII activity and chlorophyll biosynthesis. Systemic fungicides like benzimidazoles, anilides and pyrimidine are also phytotoxic whereas azoles stimulate the photosynthesis. A large number of insecticides reduce photosynthesis disturbing stomatal aperture, acting on PSI or PSII activity and ATP formation. This review focuses on the available information about crop photosynthesis after pesticide exposure.

Keywords: Fungicides; Insecticides; Pesticide; Photosynthesis; Stress

1. Introduction

A pesticide is a substance or mixture of substances used to kill a pest. Pests include insects, plant pathogens, weeds, mollusks, birds, mammals, fish, nematodes and microbes that compete with humans for food, destroy property, spread or are a vector for disease or cause a nuisance. Currently, there are 900 pesticide products and 600 active pesticidal ingredients on the market (Hall et al., 2001). A pesticide may be a chemical substance, biological agent (such as virus or bacteria), antimicrobial, disinfectant or device used against any pest. Nevertheless, the main way to control plant diseases is the use of chemicals. Among pesticides, fungicides and insecticides are largely used to raise against direct pathogens or parasitic vectors.

Fungicides remain vital for effective control of plant diseases, which are estimated to cause yield reductions of almost 20% in the major food and cash crops worldwide (Oerke et al., 1994). The great variety of known fungicides can be classified in two main categories: contact and systemic. Contact fungicides, such as copper (Cu) or sulfur (S) have preventive action by killing fungi as spores germinate, before mycelia can grow and develop within the plant tissues (Yuste and Gostincar, 1999). Since their introduction in the 1960s, systemic fungicides have gradually replaced older non-systemic products, establishing higher levels of disease control and developing new fungicide markets (Gullino et al., 2000). Systemic fungicides, known as curative or eradication fungicides, can also kill the fungus when mycelia have penetrated the parenchyma of the plant tissue, stopping the dispersal or infection within the plant (Yuste and Gostincar, 1999). Among systemic fungicides, the benzimidazoles are a group of organic fungicides that are extensively used in agriculture. These types of compounds control a broad range of fungi at relatively low application rates (Delp, 1987).

Without the use of chemical insecticides, dramatic losses in worldwide crop yield would also occur. Indeed, phytophagous insects and mite pests are a major threat to food production for human consumption (Nicholson, 2007). Insecticides are used against insects in all developmental forms. They include ovicides and larvicides used respectively against the eggs and larvae of insects. Insecticides also include substances which kill related forms of insects of animal life such as mites. The classification of insecticides depends on their mode of action and their composition: (1) systemic insecticides are

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incorporated by treated plants and insects ingest the insecticide while feeding on the plants, (2) contact insecticides are toxic to insects brought into direct contact, (3) natural insecticides, such as nicotine and pyrethrum, are produced by plants as defences against insects and (4) inorganic insecticides are manufactured with metals and include arsenates, copper and fluorine compounds, whereas organic insecticides are synthetic chemicals which comprise the largest numbers of pesticides available today (Burgess, 1990).

Although there are benefits to the use of pesticides, there are also drawbacks, such as potential toxicity to humans and other animals. Therefore, the use of chemicals to control plant diseases has stirred public concern in recent years because of the harmful potential of such substances in both environment and food chain, as some chemicals have been demonstrated to be carcinogenic. In addition, it was reported that many protection products still have negative obvious effects on crop physiology such as visible injuries (chlorosis, leaf necrosis, vein discoloration…) and growth reduction (Saladin and Clément, 2005). Several pesticides also generate stress to crops, which is reflected by an alteration of nitrogen and/or carbon metabolism, leading to a lower nutrient availability for plant growth (Saladin and Clément, 2005). Carbon metabolism is of particular importance for crop culture and is reflected by both photosynthetic rate and management of carbohydrate reserves. Indeed, photosynthesis alteration may lead to a decrease in photoassimilate production and thus in plant yield and vigor. Previous works effectively demonstrated that insecticides, methyl parathion, permethrin and methomyl, significantly reduce lettuce photosynthesis and yield (Toscano et al., 1982, Johnson et al., 1983). A negative effect of sulfur containing fungicides (and particularly lime sulfur) was also revealed on apple production by a loss of fruits and a delay in harvest (Palmer et al,. 2003). Another study on the effect of lime sulfur applied at different stages on apple trees reported a leaf phytotoxicity as revealed by necrosis and a reduction of yield in term of first class fruits (Holb et al., 2003).

Various photosynthetic processes can be impaired as stress increases and lead to a decrease in net photosynthesis (Pn), equivalent to CO_2 assimilation (Fig. 1). Stomatal closure, which is reflected by a reduction in stomatal conductance (gs), is often considered as an early physiological response to stress. It results in decreased Pn, through limited CO_2 availability in the mesophyll (Ci) (Cornic, 2000). Fundamental to the photosynthetic process is also the ability of plants to absorb light energy then convert into chemical energy. Light energy is captured by pigments in the light-harvesting complex (LHC) proteins and transferred to the reaction centers of the chloroplast thylakoid membrane. LHC proteins bind chlorophyll (chl) *a*, chl *b*, and carotenoids with weak, non-covalent bonds (Thornber et al., 1993; van

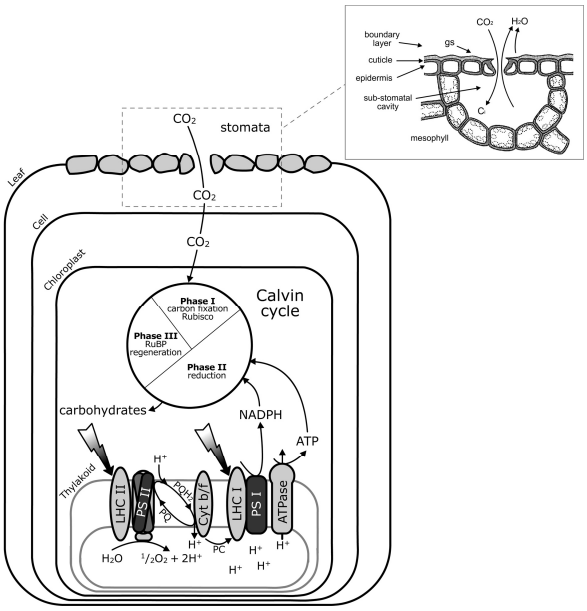

Fig. 1. Schematic representation of the photosynthetic process in higher plants (according to Baker and Rosenqvist, 2004). Light energy is captured by pigments in the light-harvesting antenna complex (LHCI and LHC II) associated with photosystem I (PSI) and photosystem II (PSII) in the chloroplast thylakoid membrane. This drives electron transport from water via a series of electron carriers to NADP, producing reducing power (NADPH) and a H^+ electrochemical potential difference across the membrane. Dissipation of this proton motive force by the passage of H^+ back across the membrane through the ATPase drives the production of ATP. Ribulose 1.5-bisphosphate carboxylase/oxygenase (Rubisco) catalyses the assimilation of CO_2 with ribulose 1.5-bisphosphate (RuBP) in the carboxylation reaction of the Calvin cycle in the stroma of the chloroplast. The diffusion of atmospheric CO_2 into the leaf is regulated by the stomata in the epidermis. Other reactions of the Calvin cycle utilize NADPH and ATP to produce triose phosphates which are required for the synthesis of carbohydrates (Cyt b/f, cytochrome b6/f complex; gs, stomatal conductance; Ci, intercellular CO_2 concentration; PC, plastocyanin; PQ, plastoquinone; PQH2, plastoquinol).

Grondelle et al., 1994; Grossman et al., 1995). LHC are assembled into two separate yet biophysically and photosystem II (PSII) (Hillier and Babcock, 2001). PSII catalyzes light-induced electron transfer from water to plastoquinone. The oxygen-evolving complex (OEC), located on the luminal side of PSII, is responsible for water oxidation and produces molecular oxygen as a by-product, protons and electrons (McEvoy and Brudvig, 2006). The protons intervene in the formation of adenosine triphosphate (ATP), the stock of energy of plants, through the ATPase. The electrons run along a complex transfer chain ending at the formation of another energetic molecule, the nicotinamide adenine dinucleotide phosphate (NADP). Then ferredoxin is reduced by a photochemical reaction at the level of PSI and the ferredoxin-$NADP^+$ reductase enzyme mediates electron transfer from this reduced protein to NADP, with formation of the NADPH necessary for CO_2 fixation (Arakaki *et* al., 1997). An alteration of one of these processes can lead to Pn inhibition. ATP and NADPH are then necessary for the second step, i.e., the energy-consuming reductive conversion of CO_2

into carbohydrates. These latter reactions follow a cyclic sequence, named the Calvin cycle. The key enzyme of the cycle, Ribulose 1.5-bisphosphate carboxylase/oxygenase (Rubisco), is a prerequisite for CO_2 fixation and catalyzes the carboxylation of ribulose 1.5-bisphosphate (RuBP). There is strong evidence that Pn can thus be limited by other biochemical processes occurring in the mesophyll such as Rubisco activity and regeneration RuBP (Farquhar and von Caemmerer, 1982; Harley and Sharkey, 1990; Harley et al., 1992).

The aim of this review is to make an exhaustive account of the currently available information about impacts of pesticides on the photosynthetic process, relating different families of fungicides and insecticides, crop varieties and photosynthetic parameters that are affected.

2. Contact fungicides

There are several chemical compounds used in disease non-systemic control programs, mainly based on copper, sulfur or fludioxonil.

2.1. Copper

Copper is commonly used as pesticide (e.g. Bordeaux mixture) and as algicide in agriculture. Cu is also an essential plant micronutrient as a component of various proteins, particularly those involved in both the photosynthetic (plastocyanin) and the respiratory (cytochrome oxidase) electron transport chain (Baron et al., 1995, for reviews see Droppa and Horváth, 1990). However, excess Cu is strongly phytotoxic for crops. Cu toxicity symptoms were observed in cucumber leaves by a pronounced inhibition of Pn in mature leaves (Vinit-Dunant et al., 2002; Alaoui-Sossé et al., 2004) (Table 1). Although Pn decline in leaves is associated with a parallel decrease in gs, stomatal closure does not account for Pn inhibition (Vinit-Dunant et al., 2002).

Inhibition of photosynthesis in leaves of Cu-stressed plants is more likely a consequence of an altered source-sink relationship, rather than due to toxic effects of copper on photosynthesis. Indeed, growth inhibition decreases carbohydrate export from leaves and leads to both starch and sucrose accumulations and to a feedback inhibition of photosynthesis (Vinit-Dunand et al., 2002; Alaoui-Sossé et al., 2004). Cu toxicity has also a marked effect on chloroplast ultrastructure in Cu-sensitive plants (Baszynski et al., 1988). In addition, chloroplast destruction was observed in Cu-poisoned green algae in parallel with enhanced peroxidative processes (Sandmann and Böger, 1980). Cu alters lipid chloroplast membrane (Szalontai et al., 1999) affecting the light reaction processes, especially those associated with PSII (Maksymiec et al., 1992; Baron et al., 1995). Cu was found to inhibit pigment accumulation and to retard chlorophyll integration into the photosystems of barley (Caspi et al., 1999). Cu inhibits both the synthesis of the chlorophyll precursor delta aminolevulinic acid and the activity of protochlorophyllide reductase, enzyme that catalyzes the reductive formation of chlorophyllide from protochlorophyllide during chlorophyll biosynthesis (Stiborová et al., 1986). Most of the *in vitro* studies using isolated chloroplasts or excised leaves have reported a direct effect of Cu on the photosynthetic electron transport chain (Cedeno-Maldonado and Swader, 1972; Sandmann and Böger, 1980). Another effect of Cu treatment is the suppression of oxygen evolution, indicating a decrease in photosynthetic capacity (Caspi et al., 1999). Cu sensitivity of PSII was confirmed (Ouzounidou et al., 1997; Horváth et al., 1998) with increased PS II inactivation under photoinhibitory conditions (Pätsikkä et al., 1998). Cu action has also been localised within the compounds of the thylakoid membrane such as PSI (Samuelsson

Table 1. Effects of contact fungicides on crop physiology.

Affected parameter	Molecule	References
Inhibition of Pn	copper	Vinit-Dunant et al., 2002; Alaoui-Sossé et al., 2004
	sulfur (kumulus)	Ferree 1979; Ferree et al., 1999; Palmer et al., 2003
	copper sulfate (cuproxat)	Xia et al., 2006
	fludioxonil	Saladin et al., 2003; Petit et al., 2008a,b
Stomatal closure	copper	Vinit-Dunant et al., 2002
	sulfur (kumulus)	Palmer et al., 2003
	copper sulfate (cuproxat)	Xia et al., 2006
Inhibition of Ci	copper sulfate (cuproxat)	Xia et al., 2006
Inhibition of Rubisco	copper	Van Assche and Clijsters, 1990
Decreased of chlorophyll (synthesis or content)	copper	Stiborová et al., 1986
	fludioxonil	Saladin et al., 2003
Modification on chloroplast ultrastructure	copper	Cedeno-Maldonado and Swader, 1972; Sandmann and Böger, 1980; Baszynski et al., 1988; Caspi et al., 1999; Szalontai et al., 1999
PS II inactivation	copper	Maksymiec et al., 1992; Baron et al., 1995; Ouzounidou et al., 1997; Horváth et al., 1998; Caspi et al., 1999 ; Vinit-Dunant et al., 2002
PS I inactivation	copper	Samuelsson and Oquist, 1980
Inhibition of other sites of electron transport chain	copper	Cedeno-Maldonado and Swader, 1972; Shioi et al., 1978; Sandmann and Böger, 1980; Uribe and Stark, 1982; Droppa and Horvath, 1990; Van Assche and Clijsters, 1990; Baron et al., 1995

and Oquist, 1980), the coupling factor (Uribe and Stark, 1982) and the ferredoxin-dependent reactions (Shioi et al., 1978). Finally, a direct inhibition of Rubisco by Mg^{2+} substitution or by interaction with SH-groups of the ATPase may also account for the inhibition of photosynthesis following Cu exposure (van Assche and Clijsters, 1990).

2.2. Sulfur

Sulfur which acts as both plant nutrient and fungicide can also decrease the severity of the disease and improve foliage color and retention (Chastagner, 2002; Stone et al., 2004). In addition, recent studies have revealed that many plants hold sulfur in their xylem as an induced defence response against certain types of fungi (Williams and Cooper, 2003). Therefore, its biological properties make it practically inescapable in modern pesticide formulations. Approximately one-third of all registered pesticides contain at least one sulfur atom (Lamberth, 2004). Despite their efficacy, treatments containing sulfur also cause phytotoxicity on crops (Holb and Schnabel, 2005).

In his review, Ferree (1979) summarised works in the 1930s and 1940s reporting that sulfur and lime sulfur reduce leaf Pn. Later on, Ferree et al. (1999) found that a single spray of sulfur results in a significant decrease in Pn of greenhouse grown apple trees. Similarly, Palmer et al. (2003) reported that leaf photosynthesis of 'Braeburn' apple trees is significantly reduced by all sulphur treatments (lime sulfur or Kumulus) down to almost 50%. The reduction in Pn is always accompanied by smaller gs. The phytotoxicity of cuproxat (copper sulfate) on photosynthesis was investigated in cucumber (Xia et al., 2006): decreased Pn is accompanied by decreased gs and Ci indicating that Pn inhibition is mostly attributed to stomatal factors. However, cuproxat has no significant effects on PSII activity.

2.3. Fludioxonil

Fludioxonil (FDX) is used as fungicide against several pathogens on many crops and for post-harvest management of some fruits and vegetables. To date, this active ingredient is commonly used against grey mould of grapevine. In grapevine, a decrease in Pn was noticed although not associated with reduction in gs (Saladin et al., 2003; Petit et al., 2008a). In parallel, decreases in chlorophylls and carotenoids upon fungicide treatment appeared dose-dependent (Saladin et al., 2003). It was shown that FDX effects on Pn were related neither to inactivation of both

Table 2. Effect of systemic fungicides on crop physiology.

Family	Affected parameter	Active ingredient	References
Anilides	Inhibition of Pn	fenhexamid	Petit et al., 2008b
	Repression of Rubisco genes	fenhexamid	Petit et al., 2008b
Azoles	Inhibition of Pn	cyazofamid	Xia et al., 2006
	Stimulation of Pn	paclobutrazol	Hong et al., 1995
		triadimefon	Kasele et al., 1995; Panneerselvam et al., 1997; Gopi et al., 2005
	Stomatal operture	triadimefon	Kasele et al., 1995
	Stomatal closure	triadimefon	Asare-Boamah et al., 1986; Davis et al., 1986, Fletcher and Hofstra, 1988; Sairam et al,. 1989; Sreedhar, 1991; Panneerselvam et al., 1997; Gopi et al., 2005
	Increased Ci	cyazofamid	Xia et al., 2006
		triadimefon	Kasele et al., 1995
	Increased chlorophyll and chloroplast contents	paclobutrazol	Kishorekumar et al., 2006
		triadimefon	Buchenauer and Rohner, 1981; Gao et al., 1988; Muthukumarasamy and Panneerselvam, 1997b; Gopi et al., 1999
		hexaconazole	Kishorekumar et al., 2006
	Reduced oxygen evolution and electron transport	epoxiconazole	Benton and Cobb, 1997
Benzimidazoles	Inhibition of Pn	dibutylurea	Shilling et al., 1994; Querns et al., 1998
	Chlorophyll declined	carbendazim	Mihuta-Grimm et al., 1990; Garcia et al., 2002
	Carotenoids increased	carbendazim	Garcia et al., 2002
	Disorganization of chloroplasts	dibutylurea	Shilling et al., 1994
	PS II inactivation	dibutylurea	Shilling et al., 1994
	Inhibition of electron transport	dibutylurea	van Iersel and Bugbee, 1996; Querns et al., 1998
Pyrimidines	Inhibition of Pn	pyrimethanil	Saladin et al., 2003
	Modifications of pigment concentrations	pyrimethanil	Saladin et al., 2003

Rubisco and other key Calvin cycle enzymes nor to modification of CO_2 diffusion to Rubisco. Pn recovered 10 days after treatment meaning that FDX had slight deleterious effect on plant photosynthesis or that grapevine has great capacity to overcome this temporary stress (Saladin et al., 2003; Petit et al. 2008a,b).

3. Systemic fungicides

3.1. Benzimidazoles

This group of organic fungicides is widely used in agriculture for pre- and post-harvest protection of crops against many fungal diseases such as anthracnose fruit rot or grey mould (Delp, 1987). However, being fungicides of broad preventive spectrum, they are applied to large proportion of crops that are not even infected by pathogens (Tomlin, 1994). Benomyl is especially effective because it penetrates plants better than carbendazim (methyl 2-benzimidazolecarbamate – MBC), its active metabolite (Upham and Delp, 1973). However, these fungicides can be phytotoxic for crops (Table 2). Moreover, benomyl also breaks down to n-butyl isocyanate (BIC) (Tang et al., 1993) which can subsequently react to produce n-butylamine or N-N'-dibutylurea (DBU). Ingredients of the degradation of benomyl also contribute to the phytotoxicity of the fungicide (Marc et al., 1997).

DBU reduces growth, inhibits Pn of hydrilla (Shilling et al., 1994), petunia and impatiens (van Iersel and Bugbee, 1997) and may be partly responsible for byside benomyl deleterious effects on plant growth. Crops have various sensitivities to DBU. Greenhouse studies revealed that corn is unaffected by DBU whereas it is phytotoxic when applied as a soil drench to cucumber (Shilling et al., 1994). Indeed, physical symptoms in cucumber begin with chlorosis at the leaf margins which extends to the whole leaf and finally leads to necrosis (Shilling et al., 1994). Similarly, Mihuta-Grimm et al. (1990) noted chlorosis and stunting in tomato following application of high benomyl concentrations.

Ultrastructural examination of cucumber chloroplasts of following DBU treatments showed a dilation and a disorganization of granal membranes (Shilling et al., 1994). Moreover, DBU presents similar mode of action to PSII inhibitor. Indeed, it causes elevated chlorophyll fluorescence in cucumber leaf discs and inhibits photosynthetic oxygen evolution in hydrilla. In isolated spinach chloroplasts, DBU also alters oxygen evolution and furthermore reduces photoinduced ferricyanide reduction, ferricyanide reduction by PSII alone, and NADP reduction from a site in the electron transport chain prior to oxidation of plastohydroquinone, explaining Pn reduction (van Iersel and Bugbee, 1996; Querns et al., 1998). Moreover, carbendazim application results to changes in foliar pigment concentrations (Garcia et al., 2002). The application of doses lower than recommended increases carotenoid concentrations

Table 3. Effect of insecticides and others on crop physiology

Family	Affected parameter	Active ingredient	References
Carbamates	Inhibition of Pn	carbaryl	Trumble et al., 1988; Godfrey and Holtzer, 1992; Haile et al., 1999
		formetanate	Lapre et al., 1982
		methomy	Trumble et al., 1988
		methomyl	Abdel-Reheem, 1991; Haile et al., 2000
		trimethacarb	Godfrey and Holtzer, 1992
	Inhibition of electron transport	carbofuran	Krugh and Miles, 1996
Organochlorines	Inhibition of Pn	endosulfan	Haile et al., 2000
		toxaphene	Akbar and Rogers, 1986
	Inhibition of PSII	toxaphene	Akbar and Rogers, 1986
		DDT	Akbar and Rogers, 1985
	Inhibition of electron transport	toxaphene	Akbar and Rogers, 1986
		DDT	Akbar and Rogers, 1985
Organophosphorus	Inhibition of Pn	chlorpyrifos	Krugh and Miles, 1996; Xia et al., 2006
		methidathion	Godfrey and Holtzer, 1992
		methyl parathion	Toscano et al., 1982; LaPre et al., 1982; Jones et al., 1983; Youngman et al., 1990
	Stomatal closure	chlorpyrifos	Krugh and Miles, 1996; Xia et al., 2006
		methidathion	Godfrey and Holtzer, 1992
	Inhibition of PSII	chlorpyrifos	Krugh and Miles, 1996; Xia et al., 2006
	Inhibition of electron transport	fonofos	Krugh and Miles, 1996
Pyrethroids	Inhibition of Pn	permethrin	Toscano et al., 1982; Trumble et al., 1988
		imidacloprid	Xia et al., 2006
	Stomatal closure	imidacloprid	Xia et al., 2006

whereas at higher rate than recommended, carbendazim causes a decrease in all foliar pigments. Garcia et al. (2002) indicated that the treatments with carbendazim may trigger a photoprotection mechanism in the plants *via* the carotenoids, which would interact with light to alleviate damages caused by fungicide application (Demming-Adams and Adams, 1996; Skillman and Osmond, 1998).

3.2. Azoles

The triazole compounds such as triadimefon (TDM), propiconazole (PCZ), hexaconazole (HEX) and paclobutrazol (PBZ), are the largest and most important group of systemic compounds developed for control of crop fungal diseases (Siegel, 1981). These fungicides can present phytotoxicity for crops as well as stimulant effects on plant physiology. Increased Pn after triazole application was reported in rice seedlings (Guirong et al., 1995) and bhendi (Sujatha et al., 1999). Pn increases after PBZ application in apple (Hong et al., 1995) or after TDM application in radish (Panneerselvam et al., 1997) and elephant foot yam (Gopi et al., 2005). On the contrary, cyazofamid inhibits Pn in cucumber but none effect was measured with flusilazole on the same plants (Xia et al., 2006) (Table 2). Gas exchange analysis demonstrated that the suppression of Pn induced by cyazofamid is associated with increased Ci suggesting that Pn alteration is mostly attributed to non-stomatal factors (Xia et al., 2006). TDM increases Pn along with Ci and stomatal conductance in maize (Kasele et al., 1995). On the contrary, TDM treatment induces stomatal closure in bean (Asare-Boamah et al., 1986; Davis et al., 1986, Fletcher and Hofstra, 1988), wheat (Sairam et al., 1989), elephant foot yam (Gopi et al., 2005), radish (Panneerselvam et al., 1997) and mulberry plants (Sreedhar, 1991).

In the mesophyll, the number of cells per unit area in both palisade and spongy layers and chloroplast number per cells in the leaves of Chinese potato increases by the HEX and PBZ treatments (Kishorekumar et al., 2006). Treatment with triazoles increased chlorophyll and carotenoid contents in rice seedlings (Guirong et al., 1995) and bhendi (Sujatha et al., 1999). TDM treatment increases chlorophyll content in leaves of tomato (Buchenauer and Rohner, 1981), radish (Muthukumarasamy and Panneerselvam, 1997b), cowpea (Gopi et al., 1999) and wheat (Gao et al., 1988). Fletcher et al. (2000) reported that triazoles accelerate chloroplast differentiation and chlorophyll production and also protect the integrity of chlorophyll. On the contrary, foliar application of epoxiconazole retards the growth of cleavers (Benton and Cobb, 1997). In addition, seven days after treatment, epoxiconazole clearly reduces oxygen evolution, determined as electron flow from water to ferricyanide, and the associated electron transport capability of isolated thylakoids (Benton and Cobb, 1997).

3.3. Anilide, pyrimidine

Fungicides of the anilide family (pyrimethanil - PMN) and pyrimidine family (fenhexamid - FHD) are new synthesized pesticides notably used for chemical control of *Botrytis cinerea* in viticulture.

Considering PMN, the effects differ according to the studied model. Using in vitro grown plantlets of grapevine, PMN causes a decline in Pn and in photosynthetic pigment concentrations (Saladin et al., 2003). In cuttings, PMN also inhibits Pn but without reduction in chlorophyll concentrations. In the vineyard, differences have been observed according to cultivars. Indeed, PMN increases Pn and pigment concentrations in Chardonnay, whereas a decrease of these parameters is observed in Pinot noir and Pinot Meunier (Saladin et al., 2003).

Petit et al. (2008b) demonstrated a decrease in Pn following FHD treatment in the vineyard. Pn modification is mostly attributed to non-stomatal limitation since the reduction of photosynthesis is associated with few changes in Ci despite gs decline. PSII activity is not affected. Additionally, decreased Pn is coupled with repression of genes encoding Rubisco small and large subunits.

4. Insecticides and others

A large number of insecticides reduce Pn after a single spray application (Table 3). Reductions of Pn were reported in strawberries (LaPre et al., 1982), lemon and oranges (Jones et al., 1983) following application of organophosphate insecticides. An other organophosphorus insecticide, methidathion, significantly reduces both Pn and gs in corn (Godfrey and Holtzer, 1992). Acephate not alters Pn of gerbera (Spiers et al., 2006) whereas it is phytotoxic to lettuce (Haile et al., 2000). Chlorpyrifos decreases Pn and gs in cucumber while Ci increases or remains stable (Xia et al., 2006). For some organophosphorus insecticides, the mode of action has been at least partly characterized. Methyl parathion reduces Pn in cotton (Youngman et al., 1990) and lettuce (Toscano et al., 1982). The latter mechanism has been well documented: methyl parathion degrades to p-dinitrophenol, which has close structure to dinitrophenol, a compound with herbicidal property (Youngman et al., 1989). Youngman et al. (1990) determined that reduced Pn was partly attributed to reduced mesophyll conductance. Another suggested mechanism for reduced Pn following organophosphorus insecticide treatments is the inhibition of electron transport in PSII leading to reduced ATP production (Murthy, 1983). Fonofos affects the photosynthetic electron transport of mung bean leaf cuttings (Krugh and Miles, 1996). This hypothesis was supported by Krugh and Miles (1996) on mung bean, demonstrating the inhibition of chlorophyll fluorescence by some insecticides. PSII activity was significantly reduced by chlorpyrifos which may interact with some component of PSII in mung bean.

The organochlorine pesticides like endosulfan and toxaphene reduces Pn in lettuce (Haile et al., 2000), oat and barley (Akbar and Rogers, 1986). Akbar and Rogers (1986) explained Pn reduction by toxaphene as biochemical inhibition of oxygen evolution in chloroplasts. They speculated that the two sites of photosynthetic electron flow are affected; the first on the oxidising side of PSII and the second being the intermediate electron transport chain between PSI and PSII. The same sites of inhibition were shown in a range of rye varieties with Dichloro-Diphenyl-Trichloroethane (DDT - organochlorine insecticide) (Akbar and Rogers, 1985).

Carbamate pesticides also impair Pn but their mode of action is not fully understood. Formetanate (Lapre et al., 1982) and methomy (Trumble et al., 1988) reduces Pn in strawberries and methomyl in lettuce (Abdel-Reheem et al., 1991; Haile et al., 2000). Carbaryl significantly reduces Pn in corn (Godfrey and Holtzer, 1992), soybean (Haile et al., 1999) and strawberries (Trumble et al., 1988). The soil insecticide, trimethacarb, leads to Pn reduction on corn (Godfrey and Holtzer, 1992). Carbofuran affects the photosynthetic electron transport of mung bean leaf cuttings (Krugh and Miles, 1996).

Considering pyrethroid insecticides, their effect on photosynthesis depends on the crop. Inhibition of Pn with permethrin in strawberries (Trumble et al., 1988) and lettuce (Toscano et al., 1982) has been reported whereas bifenthrin did not alter Pn of gerbera (Spiers et al., 2006). The insecticide imidacloprid inhibited Pn in cucumber (Xia et al., 2006) and this inhibition was accompanied by decrease in gs, while Ci was increased or unaffected. Moreover, imidacloprid does not affect PSII.

5. Adjuvants, surfactants: oil and sapon

Investigations into the morphological and physiological effects of pesticides on plants are relatively limited and often do not include the different effects between the active molecule and the surfactant. Each pesticide contains an active ingredient that is responsible for its pesticidal effect. Nevertheless, the active ingredient must be formulated with other nonpesticidal compounds before it is ready to use. It has been already shown that some surfactants can significantly reduce photosynthesis. Surfactants can be composed of oil or soap. Insecticides and surfactants with an oil-based formulation are more likely to interfere with plant gas exchange compared with other formulations (Ferree, 1979; Haile et al., 2000). Soap and oil have been implicated in plant gas exchange reductions (Olson and Ascerno, 1985; Anderson et al., 1986; Davidson et al., 1990; Hansen et al., 1992). Spiers et al. (2006) determined that Pn and gs were reduced by neem oil in gerbera. Klingeman et al. (2000) confirmed that insectidical soap caused short-term reductions in Pn in azalea.

6. Conclusions and prospects

It was widely demonstrated that non-herbicidal pesticides affect the photosynthetic process. Some pesticides lead to Pn decrease through stomatal closure while others affect the structure and the functioning of chloroplasts. Other molecules have no effect and a few, as the azoles, stimulate Pn. In addition to damage caused by pesticides on crops, all pesticides are potentially harmful for human and environmental health. Moreover, pesticide applicability can be compromised by the emergence of resistant pathogen strains. Thus, there is a need to curtail pesticide use and reduce the environmental impacts of pesticides. In this purpose, there is an increasing demand to develop alternative methods for disease control. One of the potential non-hazardous alternatives is the use of biologically based technologies which are collectively known as “biocontrol” (Chandler et al., 2008). In contrast to chemical pesticides, biocontrol methods are thought to be highly specific, affecting only one or narrowly defined class of organisms. Biocontrol methods are therefore thought to present a greatly reduced risk to the environment and to human health (Pimentel et al., 1992). They can be divided into three sub categories (Copping and Menn, 2000) (i) living organisms that are the “natural enemies” of pest species; (ii) naturally occurring substances, such as plant extracts or insect pheromones; and (iii), as recognised in some countries, such as the United States, genetically modified plants that express introduced genes that confer protection against pests or diseases by enhancing plant resistance. An other alternative strategy commonly involved is the activation of plant defense mechanisms using a variety of biotic and abiotic inducers (Walters et al., 2005). Biotic inducers include infection by necrotizing pathogens and plant-growth promoting rhizobacteria, and treatment with nonpathogens or cell wall fragments. Abiotic inducers include chemicals which act at various points in the signalling pathways involved in disease resistance, as well as water stress, heat shock, and pH stress.

Nevertheless, these alternative methods to chemical pesticides will require further studies. Indeed, induced resistance relies upon a physiological and biochemical response by the plant and so elicitor efficacy may be affected by different climatic and agronomic factors that influence general plant health. Therefore, there is an urgent need for information on, and understanding of, the various factors (such as genotype and environment) that will influence the expression of induced resistance under field conditions. Considering biocontrol, variable performance of these strategies in the field is recognized to constitute a significant constraint for their practical implementation (Stewart, 2001; Shtienberg and Elad, 2002).

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Chez la vigne, les études de la perturbation de la photosynthèse causée par des stress environnementaux concernent principalement des facteurs comme la salinité (Flexas *et al.*, 2004 ; Tattersall *et al*., 2007), le froid (Hendrickson *et al*., 2003, 2004 ; Tattersall *et al*., 2007) ou le stress hydrique (Flexas *et al*., 1998, 1999, 2002, 2004 ; Iacono *et al.*, 1998 ; Escalona *et al.*, 1999 ; Medrano *et al*., 2002 ; Bota *et al*., 2004 ; Bertamini *et al*., 2006, 2007). Toutefois, peu de travaux s'intéressent à l'altération de la photosynthèse causée par l'application de pesticides et plus particulièrement de fongicides. Pourtant, des travaux antérieurs ont montré que l'application de fongicides anti-*Botrytis* peut provoquer un stress chez la vigne (Saladin *et al*., 2003a, b). De plus, la réduction de la photosynthèse occasionnée par les traitements anti-*Botrytis* est dépendante de l'heure d'application au cours de la journée (Annexe : **publication 7**).

IV. Stress et mécanismes de défense des plantes

Face à un stress, qu'il soit de nature biotique ou abiotique, les plantes mettent en place des stratégies de défense (Netting, 2002). Trois étapes ont été mises en évidence lors de l'activation des mécanismes de défense : perception, signalisation et réaction. Suite à la perception du stress *via* des **récepteurs** plus ou moins spécifiques, la plante active toute une cascade de signalisation capable de déclencher les défenses. Suite à la transduction du signal d'alerte va alors survenir la **phase « effective » de défense**, incluant des événements cellulaires et moléculaires ainsi que des modifications biochimiques.

Dans la partie qui va suivre seront présentées les principales voies de défense activées suite à un stress abiotique : le renforcement de la paroi pecto-cellulosique, la synthèse de phytoalexines et l'accumulation de protéines PR. Les défenses mises en place suite à l'application de pesticides seront décrites dans une seconde partie.

1. Réponses de défense

a. Le renforcement de la paroi pecto-cellulosique

La composition de la paroi cellulaire peut varier de manière importante en réponse à l'attaque d'un pathogène. Des dépôts de callose, une accumulation de glycoprotéines riches en hydroxyproline (HRGP pour hydroxyproline-rich glycoproteins) et de polymères de composés phénoliques (lignine et subérine) permet un épaississement et un renforcement de la

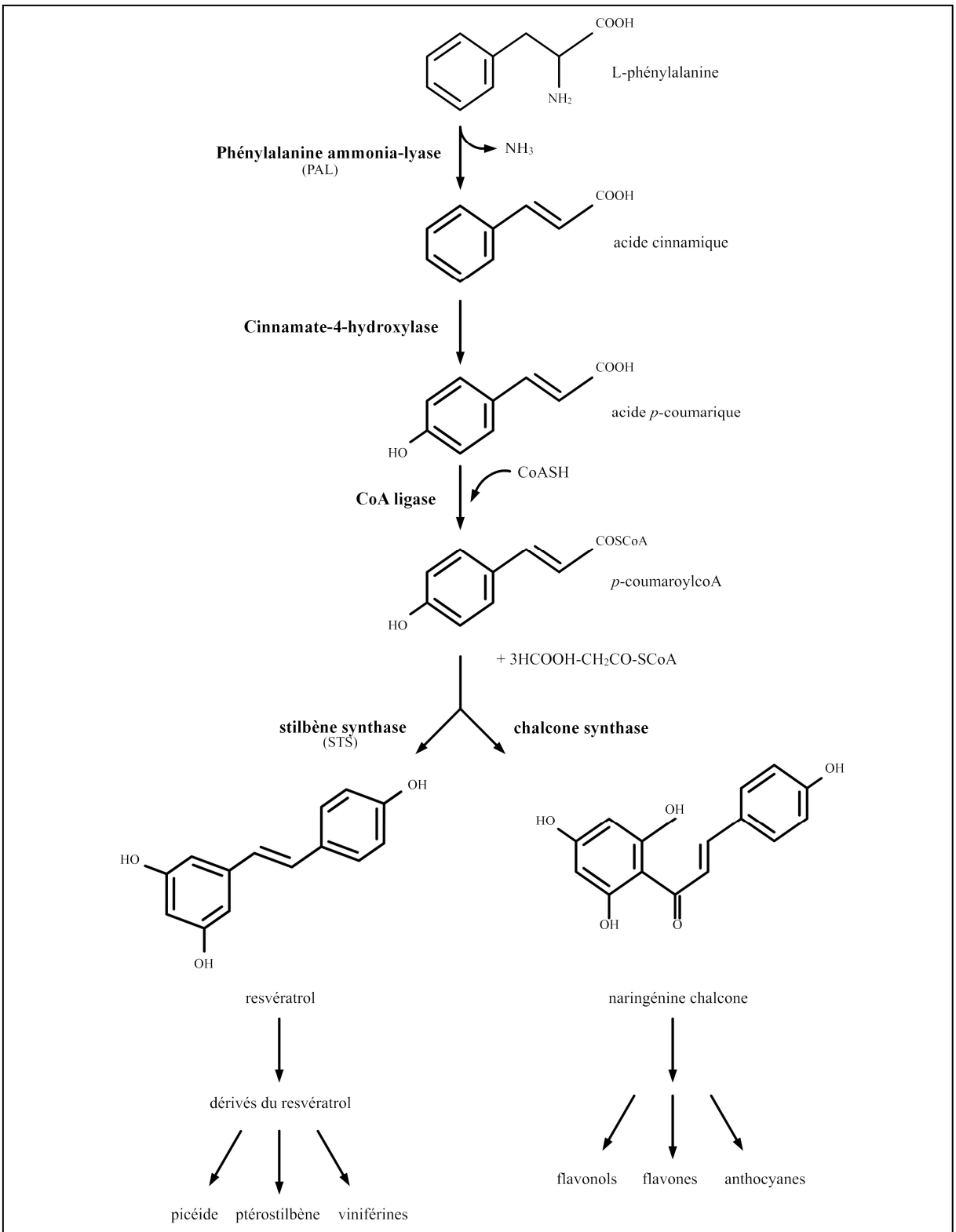

Figure 16. Voie de biosynthèse des stilbènes (d'après Kindl, 1985)

paroi. Ces modifications vont limiter la propagation de l'agent pathogène et la dégradation de la paroi par les enzymes hydrolytiques du pathogène (Benhamou, 1996 ; Hammond-Kosack & Jones, 1996 ; Boudet, 2000). Toutefois, la composition de la paroi peut également être modifiée suite à des stress abiotiques. Ainsi, des modifications de la paroi, identiques à celles occasionnées par un stress biotique, ont été notées suite à des blessures mécaniques (Corbin *et al*., 1987 ; Keller *et al*., 1989 ; De Oliveira *et al*., 1990 ; Bradley *et al*., 1992 ; Boudet, 2000). Le renforcement de la paroi cellulaire a également été observé suite à des chocs thermiques afin de participer à la protection contre le déficit hydrique ou le froid (Rajashekar & Lafta, 1996 ; Gómez *et al*., 1988 ; De Oliveira *et al*., 1990 ; Cho *et al*., 2006 ; Yang *et al*., 2006). D'autres stress comme ceux occasionnés par les métaux lourds, l'application d'un stress UV ou salin sont également capables d'induire le renforcement de la paroi même si le rôle de ce renforcement n'est pas encore élucidé (Degenhardt & Gimmer, 2000 ; Schützendübel & Polle, 2002).

b. La production de phytoalexines

Les phytoalexines sont des petites molécules lipophiles possédant des activités antimicrobiennes. Elles sont synthétisées en réponse à un éliciteur biotique ou abiotique et s'accumulent rapidement autour des sites de nécrose (Kuć *et al*., 1995 ; Smith, 1996). Ces métabolites secondaires peuvent être issus de la voie de l'acétyl-coenzyme A (acétyl-CoA) pour les sesquiterpènes (Essenberg *et al*., 1990) ou provenir de celle de l'acétyl CoA combinée à celle des shikimates pour les stilbènes, les coumarines ou les flavonoïdes (Stafford, 1990 ; Heller & Forkmann, 1993).

Les phytoalexines de la vigne appartiennent à la famille des **stilbènes**. Deux enzymes jouent un rôle central dans le contrôle de la biosynthèse des stilbènes : la **phénylalanine ammmonia-lyase** (PAL), qui catalyse la première réaction de cette grande voie menant à la synthèse des composés phénoliques et la **stilbène synthase** (STS), dernière enzyme permettant l'aiguillage vers la synthèse des stilbènes (Figure 16). L'expression du gène codant la PAL et l'activité de cette enzyme peuvent être modifiées chez la vigne par de nombreux facteurs incluant l'infection par des pathogènes ou des stress abiotiques comme le froid ou une exposition aux UV (Bonomelli *et al*., 2004 ; Bézier *et al*., 2007 ; Sanchez-Ballesta *et al*., 2007). Chez la vigne, les stilbènes, et particulièrement le **resvératrol**, ont beaucoup été étudiés pour leurs activités antifongiques dans le but de lutter contre les maladies au vignoble

Tableau 1. Les différentes familles de protéines PR, d'après Van Loon *et al*. (2006).

Famille	Membre type	Fonction
PR1	PR1a tabac	antifongique
PR2	PR2 tabac	β-1,3-glucanase
PR3	P, Q tabac	chitinase de type I, II, IV, V, VI, VII
PR4	R tabac	chitinase de type I, II
PR5	S tabac	similaire à la thaumatine, antifongique
PR6	inhibiteur I tomate	inhibiteur de protéinase
PR7	P_{69} tomate	endoprotéinase
PR8	chitinase concombre	chitinase de type III
PR9	peroxydase synthétisant des lignines tabac	peroxydase
PR10	PR1 persil	similaire à une ribonucléase
PR11	chitinase de classe V tabac	chitinase de type I
PR12	Rs-AFP3 radis	défensine, antimicrobienne
PR13	THI2.1 *Arabidopsis*	thionine, antimicrobienne
PR14	LTP4 orge	protéine de transfert des lipides, antimicrobienne
PR15	OxOa orge (germine)	oxalate oxydase
PR16	OxOLP orge	similaire à une oxalate oxydase
PR17	PRp27 tabac	inconnue

mais aussi pour leurs activités anti-oxydantes et les bénéfices éventuels pour la santé humaine (Frémont, 2000 ; King *et al.*, 2006 ; Halls & Yu, 2008). Suite à un stress, les stilbènes qui sont accumulés chez la vigne sont le trans-resvératrol, les viniférines, les picéides et le ptérostilbène (Langcake & Pryce, 1977a, b ; Jeandet *et al.*, 1995a ; Waffo Téguo *et al.*, 1996a, b ; Soleas *et al*., 1997). Le resvératrol et ses dérivés sont synthétisés en quelques heures aussi bien suite à un stress biotique que suite à un stress abiotique comme une exposition aux UV, à des sels métalliques ou suite à une blessure au niveau des feuilles et de la pellicule des baies (Jeandet *et al*., 1995b ; Adrian *et al*., 1996, 2000 ; Bavaresco *et al*., 1997 ; Bonomelli *et al*., 2004).

c. La production de protéines « Pathogenesis Related » (PR)

Les premières protéines PR ont été identifiées dès 1970 lors de la réaction hypersensible du tabac au « Tobacco Mosaic Virus » (TMV) (Van Loon & Van Kammen, 1970). Elles ont ensuite été réparties en plusieurs familles selon leur structure et leur fonction. La classification de Van Loon & Van Strien (1999) en répertorie 14, mais aujourd'hui, on compte jusqu'à 17 familles de protéines PR (Van Loon *et al*., 2006) (Tableau 1).

Les protéines PR constituent un grand groupe de protéines très stables en milieu acide et résistantes aux protéases produites par la plante ou par les micro-organismes pathogènes. Ces propriétés leur confèrent une grande stabilité dans les environnements défavorables où elles s'accumulent, notamment dans la vacuole et dans les espaces intercellulaires occupés par les pathogènes (Kauffmann *et al*., 1987). Ces protéines peuvent également être produites lors de la mise en place de mécanismes de défense en réponse à des stress abiotiques tels que les blessures, le froid, un stress salin ou les UV (Stintzi *et al.*, 1993 ; Hammond-Kosack & Jones, 1996 ; Van Loon, 1999 ; Van Loon *et al*., 2006). Enfin, l'expression de ces protéines PR peut être induite lors de la sénescence des feuilles, de la floraison ou par des facteurs de croissance. Dans ce dernier cas, elles peuvent alors être appelées PR-like mais ce terme est peu utilisé du fait de la difficulté à définir des différences entre les protéines PR et les « PR-like » (Van Loon *et al*., 2006).

Les **chitinases** et les **β-1,3-glucanases** représentent les protéines PR les plus étudiées chez la vigne (Derckel *et al*., 1996, 1998 ; Renault *et al*., 1996 ; Busam *et al.*, 1997b ; Robinson *et al*., 1997 ; Kraeva *et al*., 1998 ; Salzman *et al*., 1998). Les conditions d'induction de ces deux familles sont très variables selon les protéines considérées : certaines sont

présentes dans les feuilles saines ou non élicitées, d'autres sont induites dans les feuilles ou les baies par la blessure, l'acide salicylique ou les UV, d'autres exprimées uniquement dans les baies à la véraison et enfin d'autres suite à l'infection par un agent pathogène dans les feuilles ou les baies (*B. cinerea*, *Pseudomonas syringae* ou *Erysiphe necator*) (Renault *et al*., 1996, 2000 ; Derckel *et al*., 1998 ; Jacobs *et al*., 1999 ; Robert *et al*., 2002 ; Bonomelli *et al*., 2004).

2. Pesticides et réponses de défense

Les plantes traitées à l'aide de pesticides subissent un stress chimique mis en évidence par le déclenchement de plusieurs réactions physiologiques, dont l'activation de réactions de défense (Garcia *et al*., 2003 ; Saladin & Clément, 2005). Différentes voies de défense sont stimulées suite aux traitements pesticides, conduisant notamment à la synthèse de composés phénoliques et de protéines PR.

a. Les composés phénoliques

Ces composés sont reliés aux réponses de stress incluant les activités anti-fongiques, les activités anti-oxydantes ainsi que le renforcement de la paroi cellulaire. En réponse aux pesticides, les composés phénoliques peuvent également s'accumuler dans les plantes et contribuer à une résistance plus efficace contre les pathogènes (Daniel *et al*., 1999). Des pesticides génèrent l'accumulation de **phytoalexines** (Kömives & Casida, 1983 ; Guest, 1984 ; Tamogami *et al*., 1995 ; Coulomb *et al*., 1999) ou l'augmentation de l'activité **PAL**, (Scarponi *et al*., 1992 ; Nemat All & Younis, 1995 ; Ruiz *et al*., 1999 ; Garcia *et al*., 2001) provoquant une augmentation de composés phénoliques qui ont un rôle essentiel dans la lignification et la subérisation de la paroi cellulaire.

b. Les protéines PR

Certains fongicides sont capables de stimuler l'expression de gènes codant des hydrolases anti-fongiques telles que des chitinases, des β-1,3-glucanases et d'autres protéines PR ou d'augmenter leur activité (Siefert *et al*., 1996 ; Utriainen *et al*., 1998 ; Herms *et al*., 2002 ; Pasquer *et al*., 2005). La synthèse de protéines PR en réponse aux pesticides, comme la stimulation des composés phénoliques, représente un intérêt majeur pour les plantes cultivées. Ainsi, ces pesticides n'ont pas seulement une action directe sur l'organisme cible (mauvaise herbe, pathogène…) mais également une action indirecte en stimulant les mécanismes de défense des plantes cultivées.

OBJECTIFS DU DOCTORAT

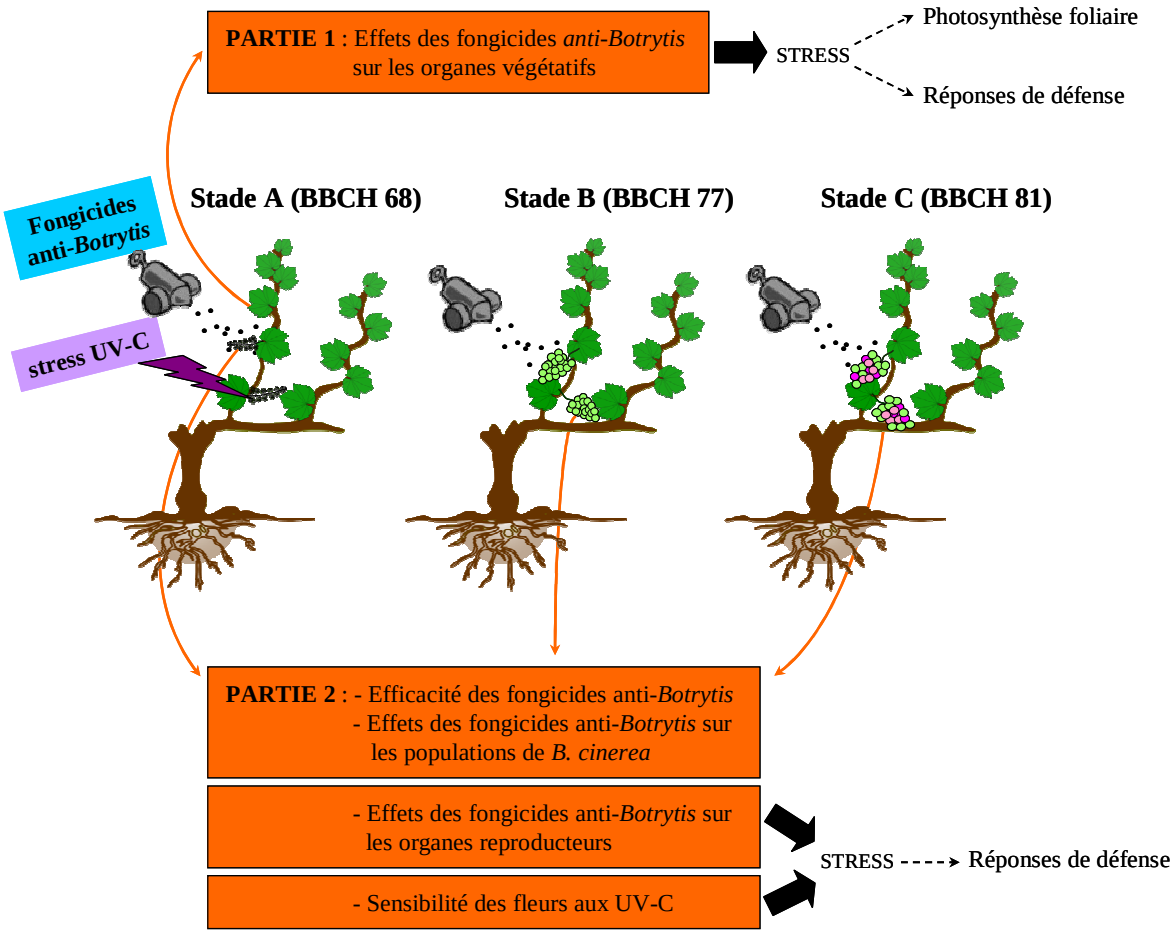

Figure 17. Objectifs des travaux de thèse.

Certains fongicides sont à l'origine d'un stress lorsqu'ils sont appliqués sur les plantes cultivées (Saladin & Clément, 2005). Actuellement, l'application de fongicides anti-*Botrytis* reste indispensable pour limiter le développement de la pourriture grise chez la vigne. Seuls des travaux concernant le stress causé par l'application de deux fongicides anti-*Botrytis*, le fludioxonil et le pyriméthanil, ont été réalisés chez la vigne (Saladin *et al*., 2003a, b). Ces fongicides provoquent une modification de la fixation en CO_2 et de la teneur en pigments photosynthétiques. Un des objectifs de cette thèse a été d'approfondir l'étude des effets du **fludioxonil** sur la physiologie de la vigne afin de déterminer les causes de ces perturbations. De plus, nous avons étudié un autre fongicide anti-*Botrytis* qui appartient à une classe chimique différente, le **fenhexamid**, sur lequel aucune étude n'a été menée. Le choix de ces deux fongicides, fludioxonil et fenhexamid, est justifié par leur utilisation courante au vignoble.

Dans un premier temps, il s'agissait d'étudier l'impact de ces fongicides sur les **organes végétatifs** de la vigne (Figure 17), en utilisant la **photosynthèse** comme paramètre physiologique (PARTIE 1). En effet, par leur activité photosynthétique, les feuilles vont assurer la nutrition nécessaire au développement de la plante (Zapata, 1998). Ainsi, une perturbation de la photosynthèse occasionnée par les fongicides peut conduire à une altération de la nutrition carbonée des organes végétatifs et reproducteurs (Saladin & Clément, 2005) et donc provoquer une diminution du rendement de la plante pour l'année en cours (Lebon *et al.*, 2005). L'altération du processus photosynthétique peut également affecter la reconstitution des réserves pour l'année suivante et donc la vigueur de la plante à long terme (Mullins *et al.*, 1992). En plus de la photosynthèse, l'**activation des défenses** suite aux traitements anti-*Botrytis* a été évaluée dans les organes végétatifs. En effet, l'application de ces fongicides représente potentiellement un stress chimique pour la plante. Lorsque les plantes subissent un stress, elles mettent généralement en place des mécanismes de défense afin de s'adapter aux conditions défavorables (Netting, 2002). Comme le stade A (fin floraison) représente une étape-clé dans le traitement anti-*Botrytis*, les effets des fongicides sur les organes végétatifs ont été évalués à ce stade.

Dans un deuxième temps, les effets des fongicides anti-*Botrytis* ont été évalués sur les **organes reproducteurs** de la vigne aux trois stades de traitement : la fin de la floraison (stade A), la fermeture de la grappe (stade B) et le début de la véraison (stade C) (PARTIE 2). En effet, le développement des organes reproducteurs va déterminer le rendement de la plante. Tout d'abord, l'**efficacité** des fongicides a été évaluée en fonction de leur **stade d'application**. Ensuite, nous avons étudié d'autres facteurs qui pourraient influencer cette

efficacité : la **pression de sélection** exercée par les fongicides sur les souches de *B. cinerea* et les **réponses de défense** au sein des organes reproducteurs suite aux traitements anti-*Botrytis*. En effet, de la même façon que dans les organes végétatifs, le stress généré par l'application des fongicides peut occasionner une activation des réponses de défense. De plus, nous nous sommes intéressés à la **sensibilité des fleurs** aux stress car l'infection florale est une étape cruciale dans l'épidémiologie de la pourriture grise (McClellan & Hewitt, 1973 ; Pezet & Pont, 1986 ; Nair *et al*., 1995). Pour cette rasion, des **UV-C** ont été appliqués sur des organes reproducteurs à des stades encadrant le stade A afin de déterminer leur sensibilité aux stress. En effet, les UV-C sont connus pour induire des réponses de défense dans les feuilles et les baies de vigne (Adrian *et al*., 2000 ; Bonomelli *et al.*, 2004) mais leurs effets n'ont jamais été étudiés dans les fleurs.

PARTIE 1 : effets des fongicides anti-*Botrytis* sur les organes végétatifs

Cette première partie a donc consisté à étudier les effets des fongicides sur la photosynthèse foliaire, biomarqueur de stress fiable de la perturbation des plantes (Krause & Weis, 1991 ; Maxwell & Johnson, 2000). La photosynthèse a été suivie à la fois à l'échelle physiologique, biochimique et moléculaire. De plus, l'activation des réponses de défense suite aux traitements anti-*Botrytis* a été étudiée au sein des organes végétatifs. Cette première partie a fait l'objet de trois articles : deux articles concernant le cépage Pinot noir (publications 2 et 4) et un article sur le Pinot Meunier (publication 3).

Publication 2 : **Petit AN, Wojnarowiez G, Panon ML, Baillieul F, Clément C, Fontaine F & Vaillant-Gaveau N**

Botryticides affect grapevine leaf photosynthesis without inducing defense mechanisms. *Planta*. Sous presse.

Les effets du fludioxonil et du fenhexamid ont été évalués au vignoble sur le cépage **Pinot noir**, à travers l'évolution de la photosynthèse et la mise en place de mécanismes de défense dans les feuilles. Les deux fongicides provoquent une **diminution de la photosynthèse**, principalement due à une limitation non-stomatique. L'activité du PSII est affectée mais n'est pas corrélée à la diminution de photosynthèse. Les fongicides entraînent la **répression de gènes** codant une protéine psbP du PSII du complexe producteur d'oxygène (*psbP1*), une protéine du complexe collecteur de lumière du PSI (*cab*) et une petite sous-unité de la Rubisco (*rbcS*). La réduction de l'expression de ces gènes pourrait en partie expliquer l'altération de la photosynthèse. Ces résultats mettent en évidence un stress subi par la vigne

lors de l'application des fongicides. En parallèle, l'expression de gènes codant des protéines impliquées dans différentes voies de défense a été suivie : une phénylalanine ammonia-lyase (*PAL*), une lipoxygénase (*LOX*) et une chitinase (*Chi4C*). En complément, des dosages d'activité chitinase ont été réalisés. Toutefois, **aucune induction de ces réponses de défenses** par les deux fongicides n'a été détectée dans les feuilles. Les fongicides anti-*Botrytis* conduisent donc à une perturbation de la photosynthèse foliaire sans activer de mécanismes de défense. *In fine*, nos résultats montrent que ces fongicides génèrent **un stress modéré** sur les organes végétatifs de la vigne.

Publication 3 : **Petit AN, Fontaine F, Clément C & Vaillant-Gaveau N** (2008)
Two botryticide effects on leaf photosynthesis grapevine. *In* Columbus F (Ed), Photochemistry Research, Nova Science Publishers, Hauppauge, New York, USA. Sous presse.

Cette étude concerne les effets du fludioxonil et du fenhexamid sur la photosynthèse foliaire au vignoble, sur le cépage **Pinot Meunier**. De la même façon que pour le Pinot noir, la **photosynthèse est altérée** par une limitation essentiellement non-stomatique. Mais contrairement au Pinot noir, l'activité du PSII n'est pas modifiée. L'expression des gènes codant la synthèse de la petite (*rbcS*) et de la grosse (*rbcL*) sous-unité de la Rubisco est diminuée. La **répression de ces gènes** pourrait être impliquée dans la réduction de la quantité de Rubisco et par conséquent dans la diminution de l'activité photosynthétique. Ces résultats confirment les effets négatifs des fongicides anti-*Botrytis* sur la photosynthèse de la vigne et leurs impacts sur l'expression de gènes codant des protéines impliquées dans la photosynthèse.

Publication 4 : **Petit AN, Fontaine F, Clément C & Vaillant-Gaveau N** (2008)
Photosynthesis limitations of grapevine after treatment with the fungicide fludioxonil. *Journal of Agricultural and Food Chemistry* 56 : 6761-6767.

Afin de localiser plus précisément les sites de perturbation de la photosynthèse suite à l'application de **fludioxonil**, cette étude a été réalisée sur des boutures fructifères de **Pinot noir**. Pour cela, des courbes de saturation lumineuse et en CO_2, l'analyse des quenchings par fluorescence chlorophyllienne et des mesures d'activité de réaction de Hill ont été effectuées. Comparé au vignoble, ce modèle nous a permis de faire un suivi sur une cinétique plus longue et de réaliser des pulvérisations à des concentrations différentes (6 mM, concentration recommandée au vignoble, 1,2 mM et 30 mM) afin de déterminer l'impact sur le processus

photosynthétique en fonction de la concentration de fludixonil appliquée. Comme au vignoble, le fludioxonil diminue la photosynthèse sur boutures et cette réduction n'est liée ni à une fermeture des stomates ni à une altération de l'activité du PSII. La concentration la plus faible (1,2 mM) provoque une perturbation rapide de la photosynthèse, causée davantage par l'humidité générée par la pulvérisation du traitement que par la toxicité du fongicide. En effet, une application d'eau provoque les mêmes effets sur la photosynthèse. Les concentrations 6 et 30 mM diminuent la photosynthèse 7 jours après traitement. A 6 mM, la réduction est liée à une augmentation du point de compensation lumineux. A 30 mM, la diminution de photosynthèse est corrélée à une augmentation de la respiration à la lumière. Dix jours après traitement, les plantes ont retrouvé une activité photosynthétique identique aux plantes non-traitées, quelque soit la concentration de fongicide. Cette étude nous a permis de mettre en évidence (i) un **recouvrement** de la photosynthèse suite au traitement anti-*Botrytis* et (ii) des impacts à différents niveaux du processus photosynthétique en fonction de la concentration appliquée.

PARTIE 2 : effets des fongicides anti-*Botrytis* sur les organes reproducteurs et sensibilité des fleurs aux stress

L'efficacité des fongicides anti-*Botrytis* a été déterminée en fonction de leur stade d'application afin de connaître la contribution de chaque stade dans le programme de référence à 3 traitements. Ces résultats ont été mis en relation avec des données concernant les profils de résistance de *B. cinerea* aux fongicides et la stimulation des réponses de défense dans les organes reproducteurs suite aux traitements. L'induction de ces réponses pourrait s'ajouter à l'action directe des fongicides contre le pathogène (Garcia *et al.*, 2003) et permettre une lutte plus efficace.

Suite à l'étude des réponses de défense dans les organes reproducteurs lors des traitements anti-*Botrytis*, nous nous sommes intéressés à la sensibilité des fleurs. En effet, l'infection florale est une étape déterminante dans l'épidémiologie de la pourriture grise. Le stress UV-C a donc été appliqué sur des organes reproducteurs à différents stades encadrant le stade A, puis la mise en place des réponses de défense a été analysée. En effet, aucune étude ne concerne les effets de ce stress, ou d'autres stress abiotiques, sur l'activation des mécanismes de défense dans les fleurs de vigne.

Cette seconde partie a conduit à la rédaction de deux articles : le premier concerne l'efficacité des fongicides anti-*Botrytis* en relation avec les profils de résistance du pathogène et les réponses de défense dans les organes reproducteurs de la vigne (publication 5), le

second porte sur l'étude des mécanismes de défense dans les inflorescences au stade A, suite à l'application du stress UV-C (publication 6).

Publication 5 : **Petit AN, Vaillant-Gaveau N, Walker AS, Leroux P, Baillieul F, Panon ML, Clément C & Fontaine F**

Effects of fungicides on populations of *Botrytis cinerea* and on defense responses of grapevine reproductive organs. Soumise à *Plant Pathology*.

Pendant 6 ans, une collaboration mise en place avec le **Comité Interprofessionnel du Vin de Champagne** (CIVC) a permis d'évaluer la contribution de chaque stade de traitement dans l'efficacité du programme anti-*Botrytis* de référence, qui consiste en trois traitements aux stades A, B et C. Cette étude a été menée sur le cépage **Pinot Meunier** avec les fongicides fludioxonil et fenhexamid. Les résultats ont révélé que le fludioxonil présente la même **efficacité** quelque soit le stade de traitement. Quant au fenhexamid, son application procure une efficacité supérieure au stade A par rapport aux stades B et C. Pour expliquer ces différences d'efficacité, les **profils de résistance** des souches de *B. cinerea* aux fongicides et les réponses de défense de la vigne ont été analysés. Les souches de *B. cinerea* ont été collectées à la vendange puis des tests phénotypiques de résistance aux fongicides ont été réalisés par l'**Unité BIOGER-CPP de l'INRA de Versailles**. Les résultats suggèrent que l'apparition de phénotypes résistants n'est dépendante ni du stade d'application, ni du fongicide utilisé. Les **réponses de défense** ont été évaluées dans les organes reproducteurs de la vigne suite aux applications de fongicides anti-*Botrytis*. Pour cela, l'expression de gènes codant différentes protéines de défense (PAL et plusieurs protéines PR) a été analysée ainsi que les variations de l'activité chitinase. Dans les fleurs au stade A et dans les baies au stade B, ces réponses de défense ne sont pas activées. Au stade C, seule une augmentation de l'activité chitinase a été observée. Une activation différentielle des réponses de défense de la vigne ne peut donc pas expliquer les différences d'efficacité des fongicides. D'autres facteurs pourraient donc intervenir sur cette efficacité, notamment le mode d'action des fongicides sur le pathogène.

Publication 6 : **Petit AN, Baillieul F, Vaillant-Gaveau N, Jacquens L, Conreux A, Jeandet P, Clément C & Fontaine F**

Low responsiveness of grapevine flowers and berries at fruit set to UV-C irradiation. Soumise à *Journal of Experimental Botany*.

Des **UV-C** ont été appliqués sur des inflorescences et des jeunes grappes de boutures fructifères de **Pinot Meunier** afin de connaître la **sensibilité de ces organes aux stress**. En effet, l'application d'UV-C induit chez la vigne de multiples réactions de défense qui se traduisent par une accumulation importante de resvératrol et de protéines PR dans les feuilles et dans les baies (Langcake & Pryce, 1976 ; Douillet-Breuil *et al*., 1999 ; Adrian *et al.*, 2000 ; Bonomelli *et al.*, 2004 ; Borie *et al*,. 2004). Toutefois, cette étude n'a jamais été menée sur fleurs. Différents stades encadrant le stade A (BBCH 57 à 73) ont été étudiés et les défenses ont été suivies à la fois dans les **rafles**, dans les **fleurs** et dans les **jeunes baies**. L'expression de gènes codant des protéines de défense (plusieurs protéines PR, PAL et STS), dont une induction a déjà été observée dans les feuilles et les baies matures suite au stress UV-C, a été analysée ainsi que les variations de l'activité chitinase et l'accumulation de resvératrol. Concernant le taux de base de l'expression des gènes, celui-ci est supérieur dans les fleurs ou baies comparé aux rafles, particulièrement pour un gène codant une glucanase. Après application du stress UV-C, la stimulation des défenses est plus importante dans les rafles que dans les fleurs ou les baies nouées quelque soit le stade. Une fois que les baies ont atteint le stade grenaille (BBCH 73), une activation des réponses de défense est observée. La sensibilité des fleurs vis-à-vis de *B. cinerea* pourrait donc s'expliquer par leur faible capacité à déclencher des réponses de défense.

– Partie 1 –

Effets des fongicides anti-*Botrytis* sur les organes végétatifs

Publication 2

Botryticides affect grapevine leaf photosynthesis without inducing defense mechanisms

Petit AN, Wojnarowiez G, Panon ML, Baillieul F, Clément C, Fontaine F & Vaillant-Gaveau N

Planta

Sous presse

Botryticides affect grapevine leaf photosynthesis without inducing defense mechanisms

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Abstract

The effects of the two botryticides, fludioxonil (fdx) and fenhexamid (fhd), were investigated on grapevine leaves (*Vitis vinifera* L. cv. Pinot noir) following photosynthesis and defense mechanisms. Treatments were carried out in vineyard at the end of flowering. Phytotoxicity of both fungicides was evaluated by measuring variations of leaf photosynthetic parameters and correlated expression of photosynthesis-related genes. Results demonstrated that similar decrease in photosynthesis was caused by fdx and fhd applications. Moreover, the mechanism leading to photosynthesis alteration seems to be the same for both fungicides. Stomatal limitation to photosynthetic gas exchange did not change following treatments indicating that inhibition of photosynthesis was mostly attributed to non-stomatal factors. Nevertheless, fungicides-induced depression of photosynthesis was related neither to a decrease in Rubisco carboxylation efficiency and in the capacity for regeneration of RuBP nor to loss in PSII activity. However, fdx and fhd treatments generated repression of genes encoding proteins involved in the photosynthetic process. Indeed, decreased photosynthesis was coupled with repression of PsbP subunit of photosystem II (*psbP1*), chlorophyll *a/b* binding protein of photosystem I (*cab*) and Rubisco small subunit (*rbcS*) genes. A repression of these genes may participate in the photosynthesis alteration. To our knowledge, this is the first study of photosynthesis-related gene expression following fungicide stress. In the meantime, defense responses were followed by measuring chitinase activity and expression of varied defense-related genes encoding proteins involved in phenylpropanoid synthesis (*PAL*) or octadecanoid synthesis (*LOX*), as well as PR protein (*Chi4C*). No induction of defense was noticed in botryticides treated leaves. To conclude, the photosynthesis is affected without any triggering of plant defense responses.

Keywords: Defense mechanisms, Fungicide, Gene expression, Grapevine, Photosynthesis

Introduction

Grey mould, caused by *Botrytis cinerea* Pers., is a serious fungal disease on grapevine (*Vitis vinifera* L.) culture, which affects both the quantity of harvest and the quality of wine produced from infected grapes (Bulit and Dubos 1988). This disease is mainly controlled in vineyards using chemical fungicides. Three preventive applications are usually recommended: at the end of flowering (BBCH 69), at bunch closure (BBCH 77) and at the beginning of berry ripening (BBCH 81). Fludioxonil (fdx) and fenhexamid (fhd) are commonly used as botryticides (Rosslenbroich and Stuebler 2000). Their modes of action are different suggesting that their by side effects on grapevine leaves may also be different. The phenylpyrrole fdx is a non-systemic molecule which inhibits spore germination, germ-tube elongation and mycelial growth of *B. cinerea*. It increases the glycerol content in fungal cells, leading to a perturbation of the osmoregulation potential (Pillonel and Meyer 1997). Fhd belongs to the new hydroxyanilide category. It is a locosystemic compound which inhibits germ-tube elongation and mycelial growth (Hänβler and Pontzen 1999). Fhd is a sterol biosynthesis inhibitor (SBI) but its target site appears to be different from the ones of other widely used SBI fungicides. It inhibits the 3-keto reductase involved in the enzymatic complex of the sterol C-4 demethylation (Debieu et al. 2001).

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In grapevine, studies of fdx and fhd mostly focused on their modes of action (Hänβler and Pontzen 1999; Rosslenbroich and Stuebler 2000), their residues in grapes and wine (Cabras et al. 1997, 2001) and the induced resistance of *Botrytis* populations to these fungicides (Leroux et al. 2002). Concerning their effects on grapevine physiology, impacts of fdx were already followed by leaf water content, carbohydrate and nitrogenous compounds levels, CO_2 assimilation, O_2 production and photosynthetic pigment contents (Saladin et al. 2003a, 2003b). These studies revealed transient alterations of grapevine physiology after fdx application. Nevertheless, no information is available on the molecular mechanisms that can lead to physiological changes. Concerning fhd, no study has been performed on its by side effects on grapevine at both physiological and molecular levels.

Phytotoxicity effects of fungicides can be examined through leaf photosynthesis which is a key factor in carbon metabolism, involved in grapevine yield and vigour. Effects of fungicides on photosynthesis have been already revealed by modifications of photosynthetic activity, chlorophyll fluorescence, pigment contents or thylakoid function and integrity (Van Iersel and Bugbee 1996; Benton and Cobb 1997; Saladin et al. 2003b; Alaoui-Sossé et al. 2004; Xia et al. 2006). An alteration of photosynthesis may disturb the whole carbon balance of the plant affecting both restitution of reserves to storage organs (Petit et al. 2006) and carbon nutrition in annual leaves and inflorescences (Saladin et al. 2003a, 2003b; Lebon et al. 2005).

In response to stresses, plants can react in complex ways that are reflected by various metabolic responses. It includes both facultative metabolic adaptations that may afford stress protection, and metabolic impairments or injuries. Thus, a decrease of photosynthesis may be a part of plant "adaptive strategy" to overcome environmental stresses (Chapin et al. 1993). In this case, the alteration of photosynthesis is accompanied by the stimulation of plant defense responses such as phenylalanine ammonia-lyase (PAL) activity (Garcia et al. 2003). Several works also report that fungicides can enhance the expression of genes encoding pathogenesis-related (PR) proteins (Garcia et al. 2003). For example, the epoxiconazole fungicide generates an accumulation of chitinase and β-1,3-glucanase proteins in wheat (Siefert et al. 1996). Moreover, copper, which is widely used as a fungicide, enhances the synthesis of a PR10 protein in birch (Utriainen et al. 1998). Lipoxygenase (LOX), an octadecanoid pathway enzyme, was also induced after a morpholine fungicide application in wheat (Pasquer et al. 2005). Thus, in addition to direct effect of fungicides on fungi, the induction of various defense mechanisms may limit the infection by pathogens.

This work reports phytotoxic effects of both fdx and fhd on leaf photosynthesis of vineyard-grown plants (*Vitis vinifera* L. cv. Pinot noir) when applied at the end of flowering. Different aspects of photosynthesis were analyzed such as gas exchange and chlorophyll *a* fluorescence measurements. Indeed, fluorescence combined with gas exchange measurements allows to obtain a full picture of photosynthetic machinery plant response to environmental variations (Roháček 2002). Additionally, chlorophyll *a* fluorescence is a sensitive and early detection tool of damage to photosynthesis apparatus and to the plant physiology resulting from environmental stresses (Maxwell and Johnson 2000). The physiological approach was supplemented with the expression of photosynthesis-related genes. In order to determine whether they could induce defense mechanisms in addition to their antibiotic action against pathogens, we further examined the effects of these two botryticides on the induction of defense responses in leaves by quantifying the expression of defense-related genes and chitinase activity.

Material and Methods

Plant material

Experiments were performed in 2005 and 2006 on Pinot noir grapevines (*Vitis vinifera* L.) planted in 1986, grafted on 41B rootstock, and trained according to the Chablis method (maintening two unpruned canes of the year). The experimental site was located in the Moët et Chandon vineyard in Aÿ (France). The fungicides fdx and fhd (as the formulated products Geoxe® and Teldor®, respectively) were sprayed using the recommended concentration (1 kg ha^{-1} and 1.5 kg ha^{-1} respectively) at the end of flowering (BBCH 69). Control plants in non-treated areas were chosen as perfectly healthy and received none botryticide application. Sunexposed and fully expanded leaves were used for the measurements.

Leaf gas exchange

The net photosynthetic rate (Pn), the stomatal conductance (g_s) and the intercellular CO_2 concentration (Ci) were determined simultaneously on leaves with a portable infrared gas analysis system (Li-Cor Model 6400, Lincoln, NE) using equations developed by Von Caemmerer and Farquhar (1981). Measurements were conducted between 0900 and 1100 daily before the "midday depression" (Chaumont et al. 1994). The gas exchange system was equipped with a clamp-on leaf cuvette that exposed 6 cm^2 of leaf area. Air temperature and humidity were maintained at 25°C and 30%, respectively. Photosynthetically active radiation provided by a red-blue light emitting diode (Li-6400-02, Li-Cor) was fixed at 1,500 µmol m^{-2} s^{-1}. Carbon dioxide concentration was maintained at a constant level of 400 µmol l^{-1} using a LI-6400-01 CO_2 injector with a high-pressure liquid CO_2 cartridge source. Gas exchange measurements were performed on nine leaves of different plants and three times per leaf. The same leaves were used during all the kinetic corresponding to 2 days before fungicide application

and 1, 2, 4 and 8 days after treatment.

Photosynthesis response curves to varying Ci (Pn/Ci) were determined at a saturating photosynthetic photon flux density (PPFD) by step changes of 12 new Ca (carbon dioxide concentration in the air) from 0 to 2,000 µmol l^{-1}. Gas exchange measurements were determined at each step after Pn stabilization. Stomatal limitation to CO_2 diffusion (l_s) was calculated according to Farquhar and Sharkey (1982). The maximum carboxylation rate of Rubisco ($V_{c,max}$) and the maximum electron transport rate contributing to RuBP regeneration (J_{max}) were estimated by fitting the model of Farquhar (Farquhar et al., 1980) to the Rubisco limited portion of the Pn/Ci curves at lower Ci and to the RuBP (Ribulose 1,5-bisphosphate) regeneration limited one at saturated level of Ci, respectively. Curves were done one day after treatment and were obtained from three plants per treatment.

Chlorophyll a fluorescence

Chlorophyll *a* fluorescence was evaluated simultaneously to gas exchange measurement using a pulse-modulated fluorometer (FMS2, Hansatech, King's Lynn, UK) at ambient CO_2 concentration and temperature. Chlorophyll *a* fluorescence reflects the functionality of the photosynthetic apparatus since it results from absorbed light energy that was not used for photosynthetic reactions and heat dissipation (Oxborough 2004). During the whole experiment, measurements were systematically performed on the adaxial side on the central parts of leaf. Leaves were dark adapted for at least 30 min to determine the minimal level of fluorescence (F0) and the maximal fluorescence (Fm) after a saturating flash (1 s, 13,000 µmol m^{-2} s^{-1}). During actinic illumination, chlorophyll fluorescence measurements were taken continuously (Ft). After stabilization, a second saturating flash of 2 s duration was imposed to determine the maximal fluorescence of a light-adapted leaf (Fm′). Removal of the actinic light and the presence of a short period of far-red light allowed measurement of the zero level of fluorescence (F0′). In both dark- and light-adapted states, the fluorescence parameters were calculated according to Schreiber et al. (1986) and Genty et al. (1989): (1) the relative quantum efficiency of PSII electron transport, ΦPSII = (Fm′–Ft)/Fm′; (2) the maximum quantum efficiency of PSII photochemistry, Fv/Fm = (Fm–F0)/Fm; (3) photochemical quenching, q_P = (Fm′–Ft)/(Fm′–F0') calculated according to Van Kooten and Snel (1990). Chlorophyll *a* fluorescence measurements were performed on the same leaves and according to the same kinetic than gas exchange.

RNA extraction

Twelve leaves per treatment were collected 1 day after fungicide spraying. They were immediately frozen in liquid nitrogen then stored at -80°C. The leaves were ground in liquid nitrogen to a fine powder. One hundred mg of powder were used for total RNA extraction and homogenized in extraction buffer (Plant Purification RNA Reagent, Invitrogen, France), according to the manufacturer's instructions. The RNA pellet was resuspended in 20 µL of RNase-free water and quantified by absorbance at 260 nm.

Real-time RT-PCR analysis

One hundred and fifty ng of total RNA were reverse-transcribed using M-MLV reverse-transcriptase (Invitrogen, France) according to the manufacturer's protocol. PCR conditions were described in Bézier et al. (2002). The reaction was

Table 1 Genes analyzed by real-time RT-PCR

Gene	Encoding	Pimer sequence	Accession number
EF1-α	elongation factor 1-alpha	Sense 5' GAA CTG GGT GCT TGA TAG GC 3' Antisense 5' AAC CAA AAT ATC CGG AGT AAA AGA 3'	BQ799343
psbP1	PsbP subunit of photosystem II	Sense 5' GCT GAC GGA GAT GAA GGT GG 3' Antisense 5' AAC CAA AAT ATC CGG AGT AAA AGA 3'	AY222741
Lhca3	light-harvesting chlorophyll-binding protein of photosystem I	Sense 5' GAC ATA CAA CTA CTG GGC AG 3' Antisense 5' TGA GCT TCA GAT CCT TGA GG 3'	AY194366
rbcS	small subunit of ribulose-1,5-bisphosphate carboxylase	Sense 5' GTG CAA TGC ATC GCT TTC ATT 3' Antisense 5' TCC ACA AGG GTC CTA AAC ATG AG 3'	CB340401
LOX	lipoxygenase	Sense 5' CTG GGT GGC TTC TGC TCT C 3' Antisense 5' GAT AAG CCG CAG ATT CAT GC 3'	AY159556
PAL	phenylalalanine ammonia-lyase	Sense 5' TCC TCC CGG AAA ACA GCT G 3' Antisense 5' TCC TCC AAA TGC CTC AAA TCA 3'	X75967
Chi4C	class IV chitinase	Sense 5' TCG AAT GCG ATG GTG GAA A 3' Antisense 5' TCC CCT GTC GAA ACA CCA AG 3'	AY137377

carried out in a GeneAmp 5700 sequence detection system (Applied Biosystems, France) using the following thermal profile: 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension) for 40 cycles. PCR efficiency of the primer sets was calculated by performing real-time PCR on several dilutions. Results were standardized to the EF1α gene expression level (Terrier et al. 2005). Control sample (non-treated leaves) was chosen to represent 1x expression of the genes of interest and treated samples were expressed relative to the corresponding control. Expression of three photosynthesis-related genes and three defense-related genes was tracked (Table 1). The entire experiment, including both the RT and real-time PCR steps, was repeated four times with RNA from different sets of samples.

Chitinase extraction and activity

Twelve leaves per treatment were collected 4 days after treatment for chitinase extraction. Proteins were extracted by homogenizing ground frozen collected leaves (250 mg fresh weight) at 4°C in 1 mL of 50 mM sodium acetate buffer, pH 5.0 containing 1 mM dithiothreitol and 1% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 10,000 *g* for 5 min at 4°C and the clarified supernatant was recovered. Chitinase activity was assayed using a commercial blue enzyme substrate, CM-chitin-RBV solution (Loewe Biochemica, Germany) according to Magnin-Robert et al. (2007). Measurements were conducted in triplicate. Results were expressed mg min^{-1} g^{-1} fresh weight (FW).

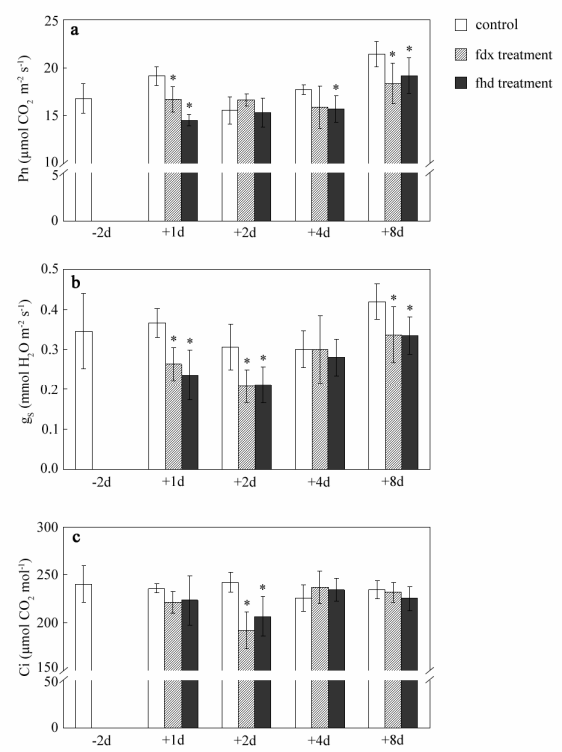

Fig. 1 Changes in (a) net photosynthesis (Pn), (b) stomatal conductance (g_s) and (c) intercellular CO_2 concentration (Ci) in control and fdx or fhd-treated leaves of grapevine. Measurements were made 2 days before treatment (-2d) then 1 (+1d), 2 (+2d), 4 (+4d) and 8 (+8d) days after treatment. Experiment was repeated in 2005 and 2006. Data are the mean ± standard error (n = 27) of a representative experiment. Significant differences at $P < 0.05$ between leaves of control and treated plants are marked by an asterisk

Table 2 Maximum carboxylation rate of Rubisco ($V_{c,max}$), maximum electron transport rate contributing to RuBP regeneration (J_{max}) and stomatal limitation to CO_2 diffusion (l_s) in control and fdx or fhd-treated leaves of grapevine, one day after treatment. Data are the mean ± standard error (n = 3). Means for a considered parameter were not significantly different, when followed by the same letter ($P < 0.05$)

	$V_{c,max}$ (µmol m^{-2} s^{-1})	J_{max} (µmol m^{-2} s^{-1})	l_s (%)
control	35.3 ± 12.9a	72.8 ± 22.2a	39.4 ± 17.0a
fdx treatment	33.4 ± 0.7a	83.2 ± 15.7a	35.3 ± 0.2a
fhd treatment	41.2 ± 11.6a	87.0 ± 27.1a	36.7 ± 8.1a

Statistical analysis

To determine whether values of control plants were significantly different from treated plants, analysis of variance (ANOVA) followed by a Student's *t* test was used. Differences at $P < 0.05$ were considered as significant.

Results

Leaf gas exchange

From the first day after fungicide application, significant differences were noticed between control and treated plants (Fig. 1a). Indeed, Pn was inhibited by 12.8 ± 6.9% after fdx spraying. Pn alteration was significantly more drastic with fhd treatment as Pn was reduced by 24.1 ± 3.0%. After 2 days of treatment, the rate of Pn was not significantly different from the control. However, significant differences in Pn were registered 4 days after fhd spraying. Pn was then inhibited by 11.5 ± 7.8% while it was not significantly different with fdx. Nevertheless, fdx and fhd-treated plants had lower Pn than those measured in control plants, 8 days after treatment. The reduction represented 14.2 ± 9.9% and 10.4 ± 8.7% in fdx and fhd treatments, respectively.

Regarding g_s, disruptions were also noticed: one day after treatment, fdx and fhd induced a similar reduction of g_s by 28.0 ± 11.3% and 35.7 ± 17.1%, respectively (Fig. 1b). g_s disturbance was still noticed 2 days after treatment up to 30% in both fdx and fhd-treated plants. Although the value of g_s did not significantly differ between control and treated plants 4 days after treatment, it decreased by about 20% 8 days after fdx and fhd treatments.

Fungicide-treated plants had significantly lower Ci values than those measured in control plants only 2 days after treatment. The reduction represented 20.9 ± 8.0% and 14.8 ± 8.6% in fdx and fhd-treated plants, respectively (Fig. 1c).

Botryticide-induced inhibition of Pn was mostly caused by non-stomatal factors. Indeed, Pn reduction was accompanied by decreased g_s while Ci was unaffected. Ci only declined 2 days after treatment

while Pn was not different from those of control plants at this time point. These results were in agreement with the l_S values. Indeed, there was no effect of fdx of fhd treatments on l_S, estimated from Pn/Ci response curves (Table 2).

Analyses of the Pn/Ci curves of leaves one day following fungicide treatments also allowed calculation of $V_{c,max}$ and J_{max} (Table 2). Both fdx and fhd treatments did not induce any significant change in these photosynthetic parameters.

Chlorophyll a *fluorescence*

Figure 2 shows the effect of fdx and fhd treatments on the chlorophyll characteristic of the leaves. ΦPSII was significantly modified only 2 days after treatment (Fig. 2a), declining by 17.7 ± 13.2% and 23.1 ± 5.3% in fdx and fhd treatments, respectively. This change was associated neither to a reduction in Fv/Fm nor to a decrease in photochemical quenching (q_P) (Fig. 2b, c).

Expression of photosynthesis-related genes

Real-time RT-PCR was used to determine changes in gene expression and thus to identify molecular mechanisms leading to photosynthesis modifications after botryticide treatments. A decline of expression was noticed for the three photosynthesis-related genes whatever the fungicide

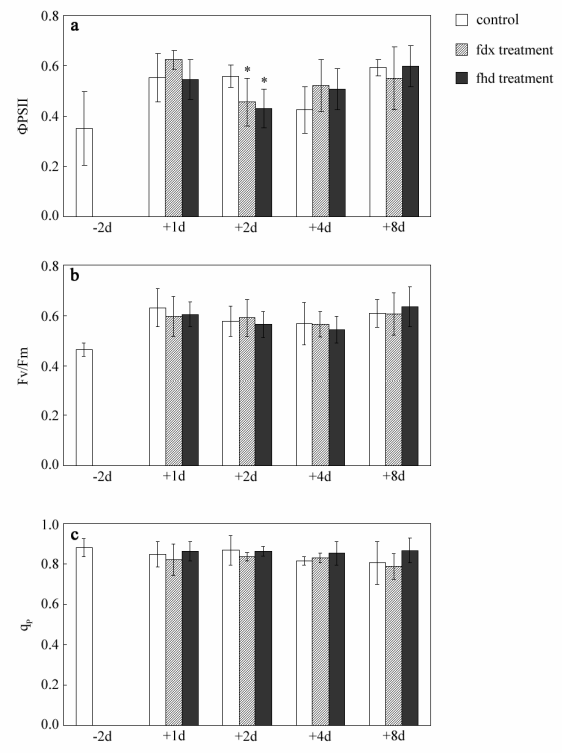

Fig. 2 Changes in (a) effective PSII quantum yield (ΦPSII), (b) maximum efficiency of PSII photochemistry after dark adaptation (Fv/Fm) and (c) photochemical quenching (q_P) in control and fdx or fhd-treated leaves of grapevine. Measurements were made 2 days before treatment (-2d) then 1 (+1d), 2 (+2d), 4 (+4d) and 8 (+8d) days after treatment. Experiment was repeated in 2005 and 2006. Data are the mean ± standard error (n = 9) of a representative experiment. Significant differences at $P < 0.05$ between leaves of control and treated plants are marked by an asterisk

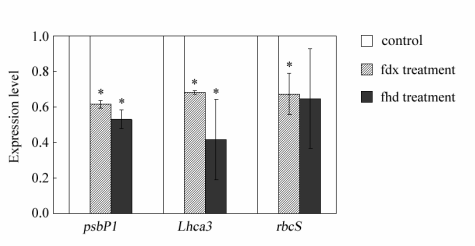

Fig. 3 Expression level of *psbP1*, *Lhca3* and *rbcS* in control and fdx or fhd-treated leaves of grapevine, one day after treatment. Untreated leaves were defined as 1x expression level for each analyzed gene. Data are the mean ± standard error (n = 4). Significant differences at $P < 0.05$ between leaves of control and treated plants are marked by an asterisk

sprayed (Fig. 3). Fdx treatment led to a 30-40% repression of *psbP1*, *Lhca3* and *rbcS* genes. Reduction of *rbcS* expression was similar after fhd application. Nevertheless, effect of fhd was more drastic on *psbP1* and *Lhca3* expression as repression was about 50% and 60%, respectively.

Defense-related genes and chitinase activity

To examine whether fdx and fhd treatments trigger plant defense mechanisms, the expression of defense-related genes was followed (Fig. 4). None of the tested gene was significantly induced. To supplement gene expression study, chitinase activity was followed but no significant difference was observed between control and treated plants (Fig. 5).

Discussion

In leaves of Pinot noir, net photosynthesis was affected after both fdx and fhd treatments, as revealed by modifications of gas exchange and chlorophyll *a* fluorescence. The rate of inhibition was nearly similar with both fungicides. Photosynthesis disruption was detected as early as one day after treatment and until the end of the experiment. In grapevine plantlets grown *in vitro*, fdx has also been shown to cause a decrease of CO_2 fixation and photosynthetic pigment concentrations. On the contrary, fdx stimulated CO_2 assimilation and increased pigment concentration in vineyard (Saladin et al. 2003b). Nevertheless, most of other works reported an inhibitory effect of fungicides on plant photosynthesis. For example, photosynthesis decreased in cucumber plants after copper exposure (Alaoui-Sossé et al. 2004). Cu-stressed leaves accumulate carbohydrates which in turn induce a feedback inhibition of photosynthesis. Other study on cucumber plants treated with cuproxat or cyazofamid fungicides showed a reduction in net photosynthesis attributed to stomatal or non-stomatal factors, respectively (Xia et al. 2006). Benzimidazole fungicides also reduced net photosynthesis in bedding plants (Van Iersel and Bugbee 1996). Similarly the application of the triazole fungicide, epoxiconazole, caused photosynthesis alteration of cleavers, partly due to the modification of phytosterol profiles and thylakoid function (Benton and Cobb 1997). Gas exchange analysis indicates that photosynthesis

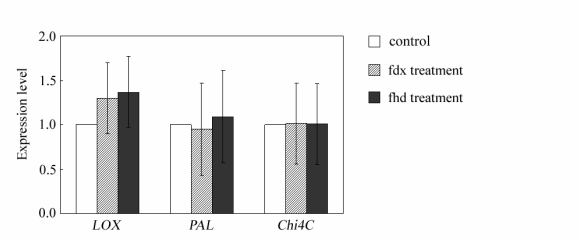

Fig. 4 Expression level of *LOX*, *PAL* and *Chi4C* in control and fdx or fhd-treated leaves of grapevine, one day after treatment. Untreated leaves were defined as 1x expression level for each analyzed gene. Data are the mean ± standard error (n = 4). Significant differences at $P < 0.05$ between leaves of control and treated plants are marked by an asterisk

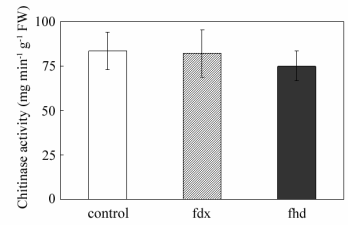

Fig. 5 Chitinase activity in control and fdx or fhd-treated leaves of grapevine, 4 days after treatment. Experiment was repeated in 2005 and 2006. Data are the mean ± standard error (n = 3) of a representative experiment

disturbance following fdx and fhd treatments was mainly attributed to non-stomatal limitation. Nevertheless, no significant changes in $V_{c,max}$ and J_{max} were observed after fdx or fhd treatments, contrary to many abiotic stresses such as drought, salinity stress or UV-B radiation (Allen et al. 1997; Nogués and Baker 2000; Youssef 2007; Nunes et al. 2008). Consequently it can be concluded that neither Rubisco carboxylation efficiency nor the ability to regenerate RuBP are limiting net photosynthesis following fungicide treatments. Inactivation or loss of both Rubisco and other Calvin enzymes did not occur in fdx and fhd-treated plants (Baker *et al.*, 1997). The reduced photosynthesis could be due to lower activity of other enzymes, such as carbonic anhydrase, which catalyzes the conversion of CO_2 to HCO_3, or enzymes involved in the utilization of the photoassimilates (Price et al. 1994; Uehlein et al. 2003).

To further assess non-stomatal limitations to Pn, chlorophyll *a* fluorescence was also analyzed after botryticide application. Indeed, a weakened PSII function can be originate to photosynthesis decrease and PSII function can be assessed by using ΦPSII, especially under environmental stress (van Kooten and Snel 1990). Results of this study show that both fdx and fhd inhibit ΦPSII in agreement with previous works dealing with copper excess (Maksymiec and Baszynski 1999). The reduction in ΦPSII may reveal a decrease in the fraction of open reaction centers and cause a down-regulation of electron transport (Lu and Zhang 2000). However, loss in PSII activity induced by both fungicides seems not to be involved in the decrease of Pn. Indeed, ΦPSII reduction was only observed two days after treatment, whereas Pn was not different between fungicide-treated and control plants. In addition, Fv/Fm and q_P remained stable following fungicide treatments. This indicates that no photodamage to PSII reaction centers and no change in the proportion of closed PSII reaction centers has been induced by fdx and fhd treatments (Genty et al. 1989). The inhibition in Pn, therefore, should be accounted for by other mechanisms other than PSII damage. An alternative sink to Pn for electrons may be oxygen reduction by photorespiration, Mehler ascorbate peroxidase reaction, and/or dark respiration (Lawlor and Cornic 2002).

The expression of three essential photosynthesis-related genes coding proteins involved in PSII (*psbP1*), photosystem I (PSI) light-harvesting complex (*cab*) and Rubisco small subunit (*rbcS*), was repressed one day after fdx and fhd treatments. To our knowledge, it is the first time that the fungicide effects on photosynthesis-related gene expression were evaluated. Thus, fungicide treatments generated repression of genes encoding proteins, involved in different pathways of the photosynthetic process, which could lead to photosynthesis disruption.

PsbP and two other proteins, PsbO and PsbQ are encoded by nuclear genome from higher plants, translated in cytoplasm, translocated into plastids and finally functional in thylakoid lumen. These three proteins are extrinsic components bound to the luminal side of PSII in close proximity to the manganese-calcium cluster (4:1 Mn:Ca). The Mn_4Ca cluster belongs to the oxygen-evolving complex (OEC) where the water splitting and oxygen release take place. The OEC thus plays fundamental role for photosynthesis. It was more precisely demonstrated that PsbP is indispensable and quantitatively related to PSII efficiency and stability (Ifuku et al. 2005). Indeed, PsbP-deficient tobacco plants had chlorotic leaves, unstable Mn_4Ca cluster and suffered from growth retardation. In addition, these plants show severe dysfunctions of PSII. It was also shown that PSII activity was linearly correlated with the total amount of PsbP. It suggests that a certain concentration of PsbP in the lumen should be maintained to support the optimum operation of PSII *in vivo* (Ishihara et al. 2005). In our work, fdx and fhd generated both ΦPSII decline and *psbP* expression decrease. Reduction in ΦPSII was lower than *psbP* repression in agreement with earlier studies indicating that post-transcriptional regulation compensates the low amount of *psbP* mRNAs (Palomares et al. 1993; Ishihara et al. 2005). Thus, study of PSII both at physiological and molecular levels confirms that both botryticides caused PSII alteration.

Fdx and fhd application also caused a reduction of *Lhca3* and *rbcS* expression. The repression of these genes may also participate in the Pn alteration following fdx and fhd applications. The Lhca3 protein is a member of the light-harvesting complex (LHC) with Lhca1, Lhca2 and Lhca4. These proteins are

nuclear-encoded and related to light-harvesting cab binding proteins in the PSI (Green and Durnford 1996). During photosynthesis, light energy is captured by pigments in the LHC and transferred to the reaction centers of the thylakoid membrane. Then, the repression of the *Lhca3* gene by fungicides modifies light-harvesting in the PSI and leads to a decline of Pn. Rubisco is composed of 8 small and 8 large subunits that are respectively encoded by nuclear *rbcS* and chloroplastic *rbcL* genes. It has been shown that a reduction in the amount of Rubisco may be caused by a decrease in the level of endogenous *rbcS* transcripts (Jiang et al. 1994). In tobacco, a nuclear *rbcS* antisense mutant resulted in decreased photosynthetic activity (Zhang et al. 2002). Altogether, these results suggest that the repression of the *rbcS* gene after fdx or fhd treatments is directly involved in the reduction of the Rubisco amount and the subsequent decrease of Pn.

Other environmental factors are known to disrupt photosynthesis and to decrease expression of *cab* genes encoding chlorophyll a/b binding proteins and *rbcS* genes. For example, exposure to ozone (O_3), a stress known to speed up foliar senescence, reduces photosynthetic capacity and efficiency (Nie et al. 1993; Guidi et al. 1997), accelerates loss of Rubisco (Pell and Pearson 1983; Nie et al. 1993) and reduces transcript levels for *cab* and *rbcS* (Bahl and Kahl 1995; Glick et al. 1995). Similarly, ultraviolet-B radiation has been shown to impair the biochemical composition of the chloroplast (Bornman 1989) and to reduce ΦPSII, mainly caused by disruptions in PSII function (Strid et al. 1994). The alteration of the photosynthetic apparatus following UV-B exposure is characterized by (i) a decrease in the content of Rubisco which is a primary cause for the decline in photosynthetic rate (Allen et al. 1997) and (ii) a repression of both the *cab* and *rbcS* genes expression (A-H-Mackerness et al. 1997). Finally, drought and cold stresses also downregulated *rbc*S and *cab* genes (Seki et al. 2002). These various environmental stresses can affect the activities of antioxidative enzymes (Moran et al. 1994; Dai et al. 1997; Baier et al. 2005). Similarly, some fungicides stimulate the activity of antioxidative enzymes (Garcia et al. 2003), suggesting that fdx and fhd may induce oxidative stress on grapevine leaves.

The induction of defense reactions is often associated with disrupted photosynthesis observed under stress conditions. For example, in response to pathogen attack, a downregulation of the *rbcS* gene expression and a simultaneous upregulation of defense genes encoding both PAL and PR proteins have been described (Berger et al. 2004; Bonfig et al. 2006). In addition to *cab* and *rbcS* gene repression, stress inducing oxidative stress also leads to the induction of genes involved in defense responses (A-H-Mackerness et al. 1997; D'Haese et al. 2006). Thus, as plants treated with fdx and fhd have their photosynthesis inhibited, we investigated effects of these fungicides on defense mechanisms. Transcription of the analyzed genes in this work is known to be induced under abiotic stresses conferring oxidative stress (Rizhsky et al. 2002; Bonomelli et al. 2004). Surprisingly, our results show that none of these defense pathways are activated in grapevine after fdx or fhd applications. It means that fdx and fhd inhibit photosynthesis process but do not induce defense reactions in grapevine leaves contrary to other oxidative stress. This could indicate that fungicide stress has not been strong enough to activate defense metabolism. Nevertheless, our results are supported by Pasquer et al. (2005), who described the absence of defense responses in wheat after treatment with two fungicides azoxystrobin or fenpropimorph under field conditions.

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Abbreviations
Ci, Intercellular CO_2 concentration; Fdx, Fludioxonil; Fhd, Fenhexamid; Fv/Fm, Maximum efficiency of PSII photochemistry after dark adaptation; g_s, Stomatal conductance; $J_{max,}$ Maximum electron transport rate contributing to RuBP regeneration; LHC, Light-harvesting complex; LOX, Lipoxygenase; l_s, Stomatal limitation to CO_2 diffusion; PAL, Phenylalanine ammonia-lyase; Pn, Net photosynthesis; PPFD, Photosynthetic photon flux density; PR, Pathogenesis-related; PSI, Photosystem I; PSII, Photosystem II; ΦPSII, Effective PSII quantum yield; q_P, Photochemical quenching; RuBP, Ribulose 1,5-bisphosphate; SBI, Sterol biosynthesis inhibitor; $V_{c,max}$, Maximum carboxylation rate of Rubisco.

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Publication 3

Two botryticide effects on leaf photosynthesis grapevine

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Sous presse

Two botryticide effects on leaf photosynthesis grapevine

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Abstract

The effects of the two botryticides fludioxonil (fdx) and fenhexamid (fhd), were investigated on grapevine leaves (*Vitis vinifera* L. cv. Pinot Meunier) following photosynthesis mechanisms. Treatments were carried out in vineyard at the end of flowering. Phytotoxicity of both fungicides was evaluated by measuring variations of leaf photosynthetic parameters and correlated expression of photosynthesis-related genes. Results demonstrated that similar perturbation of photosynthesis was caused by fdx and fhd applications. Moreover, the mechanism leading to photosynthesis alteration seems to be the same for both fungicides. Photosynthesis modification was mostly attributed to non-stomatal limitation since the reduction of photosynthesis was associated with few changes in intercellular CO_2 concentration despite stomatal conductance decline. Effective PSII quantum yield (ΦPSII) was not affected. Additionally, decreased photosynthesis was coupled with repression of rubisco small subunit (*rbcS*) and rubisco large subunit (*rbcL*) genes. To our knowledge, this is the first study of photosynthesis-related gene expression following fungicide stress.

Keywords: fungicide, gene expression, grapevine, photosynthesis

Introduction

Grey mould, caused by the fungus *Botrytis cinerea*, is a serious disease in grapevine (*Vitis vinifera* L.) culture, which affects both the quantity of harvest and the quality of wine produced from infected grapes (Bulit and Dubos, 1988). This disease is mainly controlled in vineyards using chemical fungicides. Three preventive applications are usually recommended: at the end of flowering (BBCH 69), at bunch closure (BBCH 77) and at the beginning of berry ripening (BBCH 81). Fludioxonil (fdx) and fenhexamid (fhd) are commonly used as botryticides (Rosslenbroich & Stuebler, 2000). Their modes of action are different suggesting that their by side effects on grapevine leaves may also be different. The phenylpyrrole fdx is a non-systemic molecule which inhibits spore germination, germ-tube elongation and mycelium growth of *B. cinerea.* It increases the glycerol content in the fungus, leading to a perturbation of the osmoregulation potential (Pillonel & Meyer, 1997). Fhd belongs to the new hydroxyanilide category. It is a locosystemic compound which inhibits germ-tube elongation and mycelial growth (Hänβler & Pontzen, 1999). Fhd is a sterol biosynthesis inhibitor (SBI) but its target site appears to be different from the ones of other widely used SBI fungicides. It inhibits the 3-keto reductase involved in the enzymatic complex of the sterol C-4 demethylation (Debieu *et al*., 2001).

In grapevine, studies of fdx and fhd mostly focused on their modes of action (Hänβler & Pontzen, 1999; Rosslenbroich & Stuebler, 2000), their residues in grapes and wine (Cabras *et al*., 1997, 2001; Teixeira *et al.*, 2004; Likas *et al*., 2007) and the induced resistance of *Botrytis* populations to these fungicides (Leroux *et al*., 1999, 2002; Baroffio *et al*., 2003). Concerning their effects on grapevine physiology, impacts of fdx were already followed by leaf water content, carbohydrate and nitrogenous compounds levels, CO_2 assimilation, O_2 production and photosynthetic pigment contents (Saladin *et al.*, 2003a, b). These studies revealed transient alterations of grapevine physiology after fdx application. Nevertheless, no information is available on the molecular mechanisms that can lead to physiological changes. Concerning fhd, no study has been performed on its by side effects on grapevine at both physiological and molecular levels.

Phytotoxicity effects of fungicides can be examined through leaf photosynthesis which is a key factor in carbon metabolism and which is involved in

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grapevine yield and vigour. Effects of fungicides on photosynthesis have been already revealed by modifications of photosynthetic activity, chlorophyll fluorescence, pigment contents or thylakoid function and integrity (Lidon *et al.*, 1993; Krugh & Miles, 1996; Van Iersel & Bugbee, 1996; Benton & Cobb, 1997; Saladin *et al.*, 2003b; Tort & Türkyilmaz, 2003; Alaoui-Sossé *et al.*, 2004; Untiedt & Blanke, 2004; Xia *et al.*, 2006). An alteration of photosynthesis may disturb the whole carbon balance of the plant affecting both restitution of reserves to storage organs (Liu & Huang, 2000; Petit *et al.*, 2006) and carbon nutrition in annual leaves and inflorescences (Saladin *et al.*, 2003a, b; Lebon *et al.*, 2005). In response to stresses, plants can react in complex ways that are reflected by various metabolic responses. It includes both facultative metabolic adaptations that may afford stress protection, and metabolic impairments or injuries (Saladin & Clément, 2005). Thus, a decrease of photosynthesis may be a part of plant "adaptive strategy" to overcome environmental stresses (Chapin *et al.*, 1993).

This work reports phytotoxic effects of both fdx and fhd on leaf photosynthesis of vineyard-grown plants (*Vitis vinifera* L. cv. Pinot noir) when applied at the end of flowering. Different aspects of photosynthesis were analysed such as gas exchange and chlorophyll *a* fluorescence measurements. Indeed, fluorescence combined with gas exchange measurements allows to obtain a full picture of photosynthetic machinery plant response to environmental variations (Roháček, 2002). Additionally, chlorophyll *a* fluorescence is a sensitive and early detection tool of damage to photosynthesis apparatus and to the plant physiology resulting from environmental stresses (Maxwell & Johnson, 2000). The physiological approach was supplemented with the expression of photosynthesis-related genes.

Material and Methods

Plant material

Experiments were performed in 2005 and 2006 on Pinot Meunier grapevines (*Vitis vinifera* L.) planted in 1986, grafted on 41B rootstock, and trained according to the Chablis method. The experimental site was located in the CIVC vineyard in Loisy-en-Brie (France). The fungicides fdx and fhd (as the formulated products Geoxe® and Teldor®, respectively) were sprayed using the recommended concentration at the end of flowering (BBCH 69). Control plants in non-treated areas were chosen as perfectly healthy and received none botryticide application. Gas exchange and chlorophyll fluorescence measurements were performed on nine leaves of different plants and twelve leaves were used for real-time RT-PCR analysis. Sunexposed and fully expanded leaves were used for the measurements.

Leaf gas exchange

Net photosynthesis (Pn), stomatal conductance (g_s) and intercellular CO_2 concentration (Ci) were determined simultaneously on leaves with a portable infrared gas analysis system (Li-Cor Model 6400, Lincoln, NE). Measurements were conducted between 9 a.m. and 11 a.m. before the "midday depression" (Chaumont *et al.*, 1994). The gas-exchange system was equipped with a clamp-on leaf cuvette that included 6 cm^2 of leaf area. Environmental conditions during photosynthetic measurements were the following: photosynthetically active radiation = 1500 µmol $m^{-2}s^{-1}$ provided by a red-blue light emitting diode, relative humidity around 30% and chamber temperature at 20°C. Carbon dioxide concentration was maintained at a constant level of 400 µmol l^{-1} using a LI-6400-01 CO_2 injector with a high-pressure liquid CO_2 cartridge source. Gas exchange measurements were performed three times per leaf. The same leaves were used during all the kinetic corresponding to 2 days before fungicide application and 1, 2, 4 and 8 days after treatment.

Chlorophyll a *fluorescence*

Chlorophyll *a* fluorescence was evaluated following each gas exchange measurement using a pulse-modulated fluorometer (FMS2, Hansatech, King's Lynn, UK) at ambient CO_2 concentration and temperature. Chlorophyll *a* fluorescence reflects the functionality of the photosynthetic apparatus since it results from absorbed light energy that was not used for photosynthetic reactions and heat dissipation (Oxborough, 2004). The measuring probe of the fluorometer was placed in the adaxial side in the central part of the leaf. The effective PSII quantum yield (ΦPSII) was computed as (Fm' – Fs)/Fm' according to Genty *et al.* (1989) where Fm' is the maximal fluorescence and Fs is the steady-state fluorescence yield in the light-adapted state (Roháček, 2002). ΦPSII represented the number of electrons transported by a PSII reaction centre per mole of quanta absorbed by PSII. It measures the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry (Maxwell & Jonhson, 2000). Chlorophyll *a* fluorescence measurements were performed on the same leaves and according to the same kinetic than gas exchange.

RNA extraction

Leaves were collected 1 day after fungicide spraying. They were immediately frozen in liquid nitrogen then stored at -80°C. The leaves were ground in liquid nitrogen to a fine powder. One hundred mg of powder were used for total RNA extraction and homogenized in extraction buffer (Plant Purification RNA Reagent, Invitrogen, France), according to the manufacturer's instructions. The RNA pellet was resuspended in 20 µL of RNase-free water and quantified by absorbance at 260 nm.

Table 1. Genes analysed by real-time RT-PCR

Gene	Encoding	Pimer sequence	Accession number
EF1-α	elongation factor 1-alpha	Sense 5' GAA CTG GGT GCT TGA TAG GC Antisense 5' AAC CAA AAT ATC CGG AGT AAA AGA	BQ799343
Lhca3	light-harvesting chlorophyll-binding protein of photosystem I	Sense 5' GAC ATA CAA CTA CTG GGC AG Antisense 5' TGA GCT TCA GAT CCT TGA GG	AY194366
rbcS	small subunit of ribulose-1,5-bisphosphate carboxylase	Sense 5' GTG CAA TGC ATC GCT TTC ATT Antisense 5' TCC ACA AGG GTC CTA AAC ATG AG	CB340401
rbcL	large subunit of ribulose-1,5-bisphosphate carboxylase	Sense 5' AAT TTT TCC TCC ACG GCG ATA Antisense 5' ATC TGC GCC CGC CTT TAT A	AJ635355

Real-time RT-PCR analysis

One hundred and fifty ng of total RNA were reverse-transcribed using M-MLV reverse-transcriptase (Invitrogen, France) according to the manufacturer's protocol. PCR conditions were described in Bézier *et al*. (2002). The reaction was carried out in a GeneAmp 5700 sequence detection system (Applied Biosystems, France) using the following thermal profile: 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension) for 40 cycles. PCR efficiency of the primer sets was calculated by performing real-time PCR on several dilutions. Results were standardized to the EF1α gene expression level (Terrier *et al*., 2005). Control sample (non-treated leaves) was chosen to represent 1x expression of the genes of interest and treated samples were expressed relative to the corresponding control. Expression of three photosynthesis-related genes was tracked (Table 1). The entire experiment, including both the RT and real-time PCR steps, was repeated four times with RNA from different sets of samples.

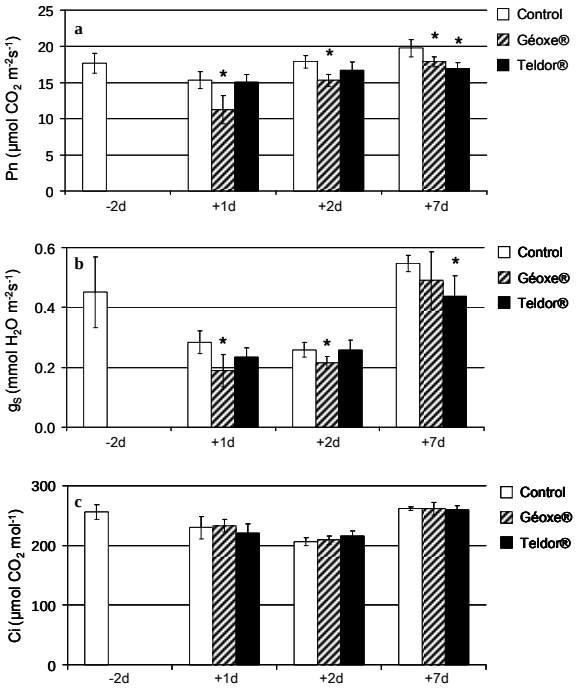

Fig. 1. Changes in (a) net photosynthesis (Pn), (b) stomatal conductance (gs) and (c) intercellular CO2 concentration (Ci) in control and fdx-treated leaves of grapevine. Measurements were made 2 days before treatment (-2d) then 1 (+1d), 2 (+2d) and 7 (+7d)
days after treatment Data are means ± standards errors (n=9). Significant differences at $P < 0.05$ between leaves of control and treated plants are marked by an asterisk.

Statistical analysis

To determine whether values of untreated plants were significantly different from treated plants, analysis of variance (ANOVA) followed by a Student's *t* test was used. Differences at $P < 0.05$ were considered as significant.

Results

Leaf gas exchanges

From the first day to the second day after fungicide application, significant difference was noticed between control and fdx treated plants (Fig. 1a). Pn was inhibited after fdx spraying and remains stable after fhd spraying. Significant differences in Pn were registered 7 days after fhd and fdx spraying. The reduction represented 9.4±3.4% and 14.3±4.0% in fdx and fhd treatments, respectively. Regarding g_s, disruptions were also noticed: one and two day after treatment, fdx induced a similar reduction of g_s by 30.8 ± 19.9% and 16.6 ± 7.7%, respectively (Fig. 1b). The value of g_s decreased by about 21.8 ± 12.1% 7 days after fhd treatments while it remained stable after fdx treatment. Ci was no affected by fungicide treatment (Fig. 1c). Two botryticides induced inhibition of Pn accompanied by decreased g_s while Ci was unaffected. Pn reduction was mostly caused by non-stomatal factors.

Chlorophyll a *fluorescence*

In order to further investigate the non-stomatal factors involved in Pn decrease, ΦPSII was followed in parallel. It was not significantly modified after treatment (Fig. 2).

Expression of photosynthesis-related genes

Real-time RT-PCR was used to determine changes in gene expression and thus to identify molecular mechanisms leading to photosynthesis modifications after botryticide treatments. A decline of expression was noticed for two photosynthesis-related genes whatever the fungicide sprayed (Fig. 3). Fdx and fdh treatment led to a 40-50% repression of *rbcS* and *rbcL* genes. Reduction of *rbcS* and *rbcL* expression were similar after fdx and fhd applications.

Nevertheless, none fdx and fhd effect were noted with *Lhca3* expression.

Discussion

In leaves of Pinot Meunier, net photosynthesis was affected after both fdx and fhd treatments, as revealed by modifications of gas exchange. The time of inhibition was different with both fungicides. Photosynthesis disruption was detected after fdx spraying as early as one day after treatment and until the end of the experiment. After fhd spraying, P*n* was reduced after 7 days. In grapevine plantlets grown *in vitro*, fdx has also been shown to cause a decrease of CO_2 fixation and photosynthetic pigment concentrations. On the contrary, fdx stimulated CO_2 assimilation and increased pigment concentration in vineyard (Saladin *et al.*, 2003b). Nevertheless, most of other works reported an inhibitory effect of fungicides on plant photosynthesis. For example, photosynthesis decreased in cucumber plants after copper exposure (Alaoui-Sossé *et al.*, 2004). Cu-stressed leaves accumulate carbohydrates which in turn induce a feedback inhibition of photosynthesis. Other study on cucumber plants treated with cuproxat or cyazofamid fungicides showed a reduction in net photosynthesis attributed to stomatal or non-stomatal factors, respectively (Xia *et al.*, 2006). Indeed, decreased photosynthesis with curpoxat was accompanied by both decreased stomatal conductance and Ci, thus stomatal closure appeared to be the main factor to depressed photosynthesis. On the contrary, decreased photosynthesis with cyazofamid was associated with increased Ci, which means that cyazofamid-induced inhibition of photosynthesis was mostly attributed to non-stomatal factors. Benzimidazole fungicides also reduced net photosynthesis in bedding plants (Van Iersel & Bugbee, 1996). Similarly the application of the triazole fungicide, epoxiconazole, caused photosynthesis alteration of cleavers, partly due to the modification of phytosterol profiles and thylakoid function (Benton & Cobb, 1997). Gas exchange analysis indicates that photosynthesis disturbance was mostly generated by non-stomatal limitation with both fungicides as revealed by a g_s decrease associated without change in Ci. To further assess biochemical

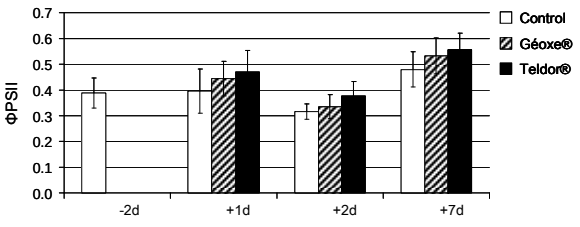

Fig. 2. Changes in relative quantum yield of PSII (ΦPSII) in control and fdx-treated leaves of grapevine. Measurements were made 2 days before treatment (-2d) then 1 (+1d), 2 (+2d) and 7 (+7d) days after treatment Data are means ± standards errors (n=9). Significant differences at $P < 0.05$ between leaves of conrol and treated plants are marked by an asterisk.

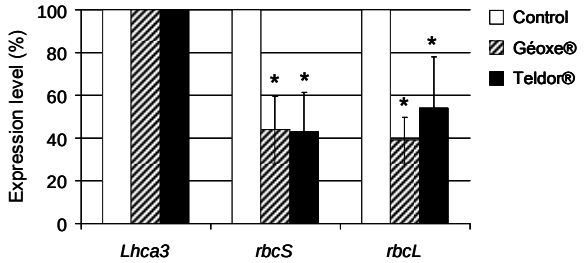

Fig. 3. Expression level of *Lhca3*, *rbcS* and *rbcL* in control and fdx-treated leaves of grapevine, one day after treatment. Expression level for each analysed gene was fixed at 100%. Data are the mean ± standard error (n=3). Significant differences at $P < 0.05$ between leaves of control and treated plants are marked by an asterisk.

limitations to Pn, chlorophyll *a* fluorescence was also analysed after botryticide application. Results of this study show that both fdx and fhd not inhibit ΦPSII. Both fungicides did not modify PSII activity.

The expression of two essential photosynthesis-related genes coding proteins involved in rubisco small subunit (*rbcS*) and large submit (*rbcL)*, was repressed one day after fdx and fhd treatments. These data suggest that photosynthesis was regulated transcriptionally in response to fungicide application. To our knowledge, it is the first time that the fungicide effects on photosynthesis-related gene expression were evaluated. Thus, fungicide treatments generated repression of genes encoding proteins, involved in different pathways of the photosynthetic process, which could lead to photosynthesis disruption. Rubisco is composed of 8 small and 8 large subunits that are respectively encoded by nuclear *rbcS* and chloroplastic *rbcL* genes. It has been shown that a reduction in the amount of rubisco may be caused by a decrease in the level of endogenous *rbcS and rbcL* transcripts (Jiang *et al.*, 1994). In tobacco, a nuclear *rbcS* antisense mutant resulted in decreased photosynthetic activity (Zhang *et al.*, 2002). Altogether, these results suggest that the repression of the *rbcS and the rbcL* genes after fdx or fhd treatments is directly involved in the reduction of the rubisco amount and the subsequent decrease of Pn.

Other environmental factors are known to disrupt photosynthesis and to decrease expression of *rbcS* and *rbcL* genes. For example, exposure to ozone (O_3), a stress known to speed up foliar senescence, reduces photosynthetic capacity and efficiency (Reich, 1983; Held *et al.*, 1991; Nie *et al.*, 1993; Guidi *et al.*, 1997), accelerates loss of rubisco (Pell & Pearson, 1983; Nie *et al.*, 1993; Pell *et al.*, 1997), reduces transcript levels *rbcS* (Bahl & Kahl, 1995; Conklin & Last, 1995; Glick *et al.*, 1995) and *rbcL* (Dizengremel, 2001; Pelloux *et al.*, 2001). Similarly, ultraviolet-B radiation has been shown to impair the biochemical composition of the chloroplast (Bornman, 1989) and to reduce ΦPSII, mainly caused by disruptions in PSII function (Strid *et al.*, 1994). The alteration of the photosynthetic apparatus following UV-B exposure is characterized by (i) a decrease in the content of rubisco which is a primary cause for the decline in

photosynthetic rate (Allen *et al*., 1997) and (ii) a repression of *rbcS* gene expression (A-H-Mackerness *et al*., 1997). Finally, drought, cold or high-salinity stresses also downregulated *rbc*S gene (Seki *et al*., 2002). These various environmental stresses can affect the activities of antioxidative enzymes (Hernández *et al*., 1995; Zhang & Kirkham, 1995; Moran *et al.*, 1994; Baier *et al.*, 2005; Dai *et al.*, 1997). Similarly, some fungicides stimulate the activity of antioxidative enzymes (Wu & von Tiedman, 2002; Garcia *et al*., 2003), suggesting that fdx and fhd may induce oxidative stress on grapevine leaves.

The Lhca3 protein is a member of the light-harvesting complex (LHC) with Lhca1, Lhca2 and Lhca4. These proteins are nuclear-encoded and related to light-harvesting cab binding proteins in the photosystem I (Haworth *et al*., 1983; Knoetzel *et al*., 1992; Green & Durnford 1996; Jansson, 1999). During photosynthesis, light energy is captured by pigments in the LHC and transferred to the reaction centres of the thylakoid membrane. The *Lhca3* gene expression was not modified by fungicides, the light-harvesting in the photosystem I was intact and no leads to a decline of Pn.

Nevertheless, the downregulation of photosynthetic gene expression and the reduced rate of photosynthesis observed after fdx and fhd treatments may lead to a decrease in synthesized sugars. This may induce a perturbation in flower and berry development (Srinivasan & Mullins, 1981; Lebon *et al*., 2005).

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Publication 4

Photosynthesis limitations of grapevine after treatment with the fungicide fludioxonil

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Photosynthesis Limitations of Grapevine after Treatment with the Fungicide Fludioxonil

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The aim of this work was to determine the major limitations to photosynthesis induced by the fungicide fludioxonil (fdx) on nontarget grapevines using cutting as a model. The fdx treatments (1.2, 6, and 30 mM) induced a net photosynthetic rate (P_n) decrease without changes in stomatal conductance, suggesting a nonstomatal limitation. Fdx effects on P_n were related neither to photosynthetic capacity alteration in leaves nor to loss in PSII activity. The mechanism underlying photosynthesis reduction differed according to the concentration. Fdx at 6 mM led to an increase of light requirement for photosynthesis while 30 mM fdx induced an increase in the respiration rate in the light. P_n decrease after 1.2 mM fdx could rather be related to wetness caused by the spraying than to fungicide toxicity. P_n recovered 10 days after treatment, meaning that fdx had little deleterious effect on plant physiology or that grapevine has a great capacity to overcome this temporary stress.

KEYWORDS: Chlorophyll fluorescence; fludioxonil; fungicide; gas exchanges; grapevine; phytotoxicity

INTRODUCTION

In vineyards, productivity requires several pesticide treatments because of the susceptibility of grapevine to a range of diseases. Especially, fungal pathogens are a major problem in the cultivation of grapevine (*Vitis vinifera* L.) around the world. Thus, fungicides represent 80% of all pesticides used in vineyards (*1*). Among them, botryticides are used to control *Botrytis cinerea*, the causal agent of gray mold disease, which causes worldwide yield loss (*2*). Chemical control currently remains the main way to fight this phytopathogenic fungus. Three preventive applications are usually recommended: at the end of flowering (BBCH 69), at bunch closure (BBCH 77), and at the beginning of berry ripening (BBCH 81). Fludioxonil (fdx) [4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1*H*-pyrrole-3-carbonitrile], a phenylpyrrole compound, is commonly used as a botryticide all over the world (*3*). Fdx inhibits spore germination, germ-tube elongation, and the mycelium growth of *B. cinerea* (*4*). It increases the glycerol content in the fungus, leading to a perturbation of the osmoregulation potential (*5*).

Considerable use of pesticides in vineyards generates long-term residues in food and the environment. In addition, some pesticides may also have consequences on crop physiology, such as growth reduction, perturbation of reproductive organ development, alteration of nitrogen, and/or carbon metabolism (*6*). This former physiological trait is fundamental for crop culture and is reflected by both photosynthetic rate and mobilization of carbohydrate reserves. Indeed, as plants rely on their ability to assimilate carbon through photosynthesis for their growth and overall vigor, photosynthesis disruption may decrease yield and vigor. Several works on photosynthesis fluctuations after fungicide application on various crops report modifications of both photosynthetic activity and chlorophyll fluorescence (*7–10*). Photosynthesis alteration was revealed by reduction in net photosynthesis accompanied by changes in stomatal conductance and intercellular CO_2 concentration (*8–10*). Modifications of dark respiration were also noticed after fungicide treatment (*9*). Considering fluorescence, the relative quantum yield of PSII (Φ_{PSII}) and the maximal quantum efficiency of PSII (F_v/F_m) were reduced by some fungicides and were attributed to decrease in photochemical quenching (q_P) (*7, 10*).

Fdx impacts on photosynthesis have already been shown to modify CO_2 fixation and photosynthetic pigment concentration after application on grapevine leaves (*11*). The decrease in CO_2 fixation after fdx application may be attributed to stomatal closure, disruption in capacity of rubisco carboxylation and/or RuBP regeneration, and loss in photosystem II (PSII) activity. The aim of this study was therefore to determine changes in photosynthetic performance and to localize primary sites of damage following fdx application. Different aspects of leaf photosynthesis were thus determined on fruiting cuttings of *V. vinifera* L. (cv. Pinot noir) after fdx treatment at the end of flowering (BBCH 69). At this time, mature leaves are the main sources of carbohydrates for developing leaves, roots, flowers, and berries (*12*). Any perturbation during flower development may thus lead to a decrease in fertilization and yield (*13*). Analyses of gas exchanges with photosynthesis versus inter-

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cellular CO_2 concentration and light response curves allowed evaluation of relative limitations imposed by stomata, carboxylation efficiency, and capacity of RuBP regeneration on leaf photosynthesis (*14–18*). Light respiration has also been investigated to study its putative implication in photosynthesis decrease. Chlorophyll florescence parameters were also analyzed because they are reliable indicators of photosynthetic apparatus state (*19–22*). Finally, Hill reaction activity, which reflects PSII integrity, as well as the efficiency of electron transport, has been evaluated.

MATERIALS AND METHODS

Plant Material. Fruiting cuttings of *V. vinifera* L. (cv. Pinot noir) were used for this experiment. Fruiting cuttings were obtained from canes of grapevine according to the protocol improved by Lebon et al. (*23*). Cuttings were planted in 300 mL pots containing a perlite/sand mixture (1:2, v/v) and transferred to a growth chamber under a temperature of 25 °C (day/night), at a relative humidity of 60% (day/night) and a 16 h photoperiod (400 μmol photons m^{-2} s^{-1}). Plants were irrigated daily with a Coïc and Lesaint medium (*24*). The fdx solution was sprayed after 6 weeks of growth, when cuttings had four leaves and an inflorescence at the end of flowering (BBCH 69). Fdx ($C_{12}H_6F_2N_2O_2$) was obtained from the commercial fungicide Géoxe. The molecular mass of fdx is 248.2 and its water solubility is 1.8 mg L^{-1}. Treatment was performed once with different solutions of fdx in water, 1.2, 6, or 30 mM, corresponding to 0.2, 1, and 5 times the concentration recommended by the manufacturer, respectively. Controls were carried out using untreated plants or plants sprayed with water.

Leaf Gas Exchanges. The net photosynthetic rate (P_n), the stomatal conductance (g_S), and the intercellular CO_2 concentration (C_i) were measured with an open gas exchange system (LI-6400, Li-Cor, Lincoln, NE) using equations developed by Von Caemmerer and Farquhar (*25*). The infrared gas analysis system was equipped with a clamp-on leaf cuvette that exposed 6 cm^2 of leaf area. Air temperature and humidity were maintained at 25 °C and 30%, respectively. Photosynthetically active radiation provided by a red−blue light emitting diode (Li-6400-02, Li-Cor) was fixed at 1500 μmol m^{-2} s^{-1}. Carbon dioxide concentration (C_a) was maintained at a constant level of 400 μmol L^{-1} using a CO_2 injector with a high-pressure liquid CO_2 cartridge source (LI-6400-01, Li-Cor). The same leaves were used during all the kinetic analysis corresponding to a few hours before fungicide application and then 1, 2, 4, 7, 10, and 14 days after treatment. Gas exchange measurements were performed on eight leaves of different plants and three times per leaf. The second leaf from the base of each plant was chosen for measurements.

Photosynthesis response curves to varying C_i (P_n/C_i) were determined at a saturating photosynthetic photon flux density (PPFD) by step changes of 12 new C_a from 0 to 2000 μmol L^{-1}. Gas exchange measurements were determined at each step after P_n stabilization. Measurements and interpretation of the P_n/C_i response have been described previously by Long and Bernacchi (*26*). The in vivo maximum rate of rubisco carboxylation ($V_{c,max}$) was estimated as the slope of the linear portion of CO_2 response curve from the asymptote of the fitted response function (*27*). C_i was regarded as the CO_2 compensation point (C_i*) when P_n was zero, and P_n was estimated as the mitochondrial respiration in the light (R_l) when C_i was zero.

The responses of P_n to step changes in PPFD was measured at a constant C_a of 360 μmol L^{-1}. Twelve PPFDs from 0 to 2000 μmol photons m^{-2} s^{-1} were set. The apparent quantum yield of CO_2 fixation (ΦCO_2) was estimated as the slope of the linear portion of the PPFD response curve. ΦCO_2 is the efficiency of light use in photosynthesis, i.e., the number of moles of CO_2 fixed per mole quantum absorbed by a leaf. The ratio $\Phi_{PSII}/\Phi CO_2$ was also calculated; it represents an estimate of the relationship between the rate of electron transport and carbon fixation. Moreover, the light compensation point (C_i*) and dark respiration (R_d) were calculated from the response curves according to Long and Hällgren (*28*).

Leaf intercellular CO_2 concentration saturated assimilation rate ($Pmax_{(Ci)}$) and PPFD-saturated CO_2 assimilation rate ($Pmax_{(PPFD)}$) were estimated from the irradiance response and CO_2 response curves, respectively. Curves were obtained from three plants per treatment. P_n/C_i and photosynthetic light response curves were done 7 days after treatment.

Chlorophyll *a* Fluorescence. Chlorophyll *a* fluorescence was quantified on attached leaves with a chlorophyll fluorescence imaging system (IMAGING-PAM, Walz, Effeltrich, Germany). The measuring system applied an array of blue-light-emitting diodes (peak wavelength, 470 nm) for saturating light pulses. The frequency of the pulses was adjusted to 10 Hz. Measurements were carried out at a maximal distance between the camera and the leaf, corresponding to a 25 × 34 mm area. The image captured by the charge coupled device (CCD) camera was composed of 640 × 480 pixels. During the whole experiment, measurements were systematically performed on the adaxial side on the central parts of leaves. Leaves were dark adapted for at least 30 min to determine the (F_0) (minimal) level of fluorescence and the maximal fluorescence (F_m) after a saturating flash (1 s, 13 000 μmol m^{-2} s^{-1}). During actinic illumination, chlorophyll fluorescence measurements were taken continuously (F_t). After stabilization, gas exchanges were determined followed by a saturating flash of 2 s duration to measure the maximal fluorescence of a light adapted leaf (F_m'). Removal of the actinic light and the presence of a short period of far-red light allowed measurement of the zero level of fluorescence (F_0'). From these measurements, several fluorescence parameters were calculated according to Schreiber et al. (*29*) and Genty et al. (*22*): $\Phi_{PSII} = F_m' - F_t/F_m'$ and $F_v/F_m = (F_m/F_0)/F_m$. Φ_{PSII} represents the number of electrons transported by a PSII reaction center per mole of quanta absorbed by PSII and F_v/F_m is the ratio of variable to maximal fluorescence. In addition, both photochemical (q_P) and nonphotochemical quenching (q_{NP}) were calculated according to Van Kooten and Snel (*30*): $q_P = (F_m' - F_t)/(F_m' - F_0')$ and $q_{NP} = (F_m - F_m')/F_m'$. q_P reflects the number of open reaction centers. It is an indicator of the capacity of photochemical processes. q_{NP} is linearly related to heat dissipation and is the most common form of protection against excess photons (*19, 20*). On each image, the values of the selected fluorescence parameters were averaged. Images of Φ_{PSII} were displayed with the help of a false color code ranging from 0.000 (black) to 1.000 (pink). Chlorophyll *a* fluorescence measurements were performed on the same leaves and according to the same kinetic as the gas exchanges.

Chloroplasts Isolation and Hill Reaction Measurements. Seven days after treatment, one leaf per plant was collected. The protocol of chloroplasts isolation was modified according to Hernández-Gil and Schaedle (*31*). One gram of leaves was quickly homogenized in a cooled mortar with Fontainebleau sand in 10 mL of extraction medium consisting of 0.35 M sorbitol, 50 mM tricine, pH 7.6, 2 mM EDTA, and 1.5% polyethylene glycol (PEG) 4000 (w/v). The chloroplast homogenate was filtered through fine nylon net. The filtrate was centrifuged for 60 s at 2500*g*. The supernatant fluid was discarded and the pellet containing whole chloroplasts was resuspended in a small volume of homogenizing buffer. At this point, the chloroplast suspension was stored in an ice-cooled glass beaker in the dark. Total chlorophyll (chl) concentration was determined in 80% acetone as described by Lichtenthaler et al. (*32*). The reaction mixture containing chloroplasts equivalent to 20 μg of chl mL^{-1} was used to determine Hill reaction activity.

The rate of the Hill reaction was determined in chloroplast by following the rate of 2,6-dichlorophenol-indophenol (2,6-DCPIP) photoreduction using spectrophotometer at 600 nm. As the rate of oxygen release parallels the rate of DCPIP reduction, results were expressed in mmol O_2 mg of chl^{-1} h^{-1}. Four plants for each treatment were used in this experiment.

Statistical Analysis. To determine whether values of untreated plants were significantly different from treated plants, analysis of variance (ANOVA) followed by a Student's *t* test was used. Differences at $P < 0.05$ were considered significant.

RESULTS

Gas Exchanges. The first day after treatment, significant P_n decrease was observed using water and 1.2 mM fdx treated plants (**Figure 1a**). P_n was inhibited by about 30% after both

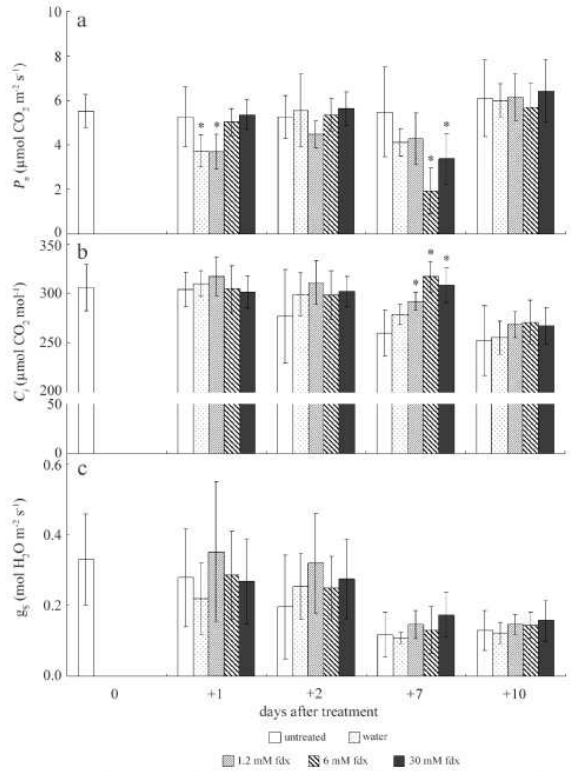

Figure 1. Changes in (**a**) net photosynthesis (P_n), (**b**) intercellular CO_2 concentration (C_i), and (**c**) stomatal conductance (g_S) in untreated, water-treated, and fdx-treated leaves of grapevine. Data are means ± standards errors ($n = 24$). Significant differences at $P < 0.05$ between leaves of untreated and treated plants are marked by an asterisk.

treatments, while it was not significantly different with 6 and 30 mM fdx application. However, g_S and C_i remained stable (**Figure 1b,c**). After 2 and 4 days of treatment (data not shown), the rate of P_n was not significantly different between controls and treated plants. Nevertheless, 6 and 30 mM fdx treated plants had significantly lower P_n than those measured in untreated plants 7 days after treatment. The reduction represented 38 ± 21% of the control values in 30 mM fdx treated plants. P_n decrease was higher with 6 mM fdx since it represented 65 ± 19%. In both cases, P_n reduction was accompanied by increased C_i without modification of g_S. The rise of C_i represented approximately 20% after both treatments. Even if P_n was not modified in 1.2 mM fdx treated plants, C_i increased by 13 ± 4%. Ten days after treatment, gas exchanges recovered in all treated plants and remained stable 14 days after treatment (data not shown).

P_n/C_i and light response curves were used in order to clarify the mechanisms involved in photosynthesis limitations following treatments. This study was performed 7 days after treatment because treated plants presented dramatic photosynthesis disruptions at this time. P_n/C_i curves showed that neither $Pmax_{(Ci)}$ nor $V_{c,max}$ were affected by water or fdx treatments (**Table 1**). Nevertheless, C_i* and R_l strongly increased after water and 30 mM fdx treatments. In detail, C_i* and R_l were enhanced after water treatment by 2.6- and 2.3-fold, respectively. Changes were significantly higher with 30 mM fdx: this treatment generated a 3.3- and 3.5-fold increases of C_i* and R_l, respectively.

In complement, analysis of light response curves was performed. The ΦCO_2 value significantly decreased after all fdx treatments (**Table 2**). ΦCO_2 decline represented approximately 30−45% according to the treatment. In 1.2 mM fdx treated plants, R_d decreased by half. Γ* was affected by 20% in 6 mM fdx treated plants, but no significant differences were registered between untreated and the other treated plants. Considering $Pmax_{(PPFD)}$, no significant changes were detected whatsoever after treatment. The $\Phi_{PSII}/\Phi CO_2$ ratio was inversely correlated to the efficiency of light involvement for carbon fixation. It was significantly higher in all treated plants, indicating that light was less efficient after treatment using either water or fdx. $\Phi_{PSII}/\Phi CO_2$ increase represented from 50 to 90% according to the treatment.

Chlorophyll *a* Fluorescence. All treated plants had lower relative quantum yield of PSII (Φ_{PSII}) values than those measured in untreated plants, only 2 days after treatment (**Figure 2**a). The reduction reached approximately 10% in water, 1.2 mM and 30 mM fdx-treated plants and 7% in 6 mM fdx-treated plants.

Analysis of fluorescence showed a decrease in fluorescence emission, when cuttings were exposed to both water and fdx treatments (**Figure 3**). Modifications appeared throughout the mesophyll for all treatments. The maximum efficiency of PSII photochemistry after dark-adaptation (F_v/F_m), photochemical quenching (q_P), and total nonphotochemical quenching (q_{NP}) were not affected by various treatments during the whole experiment (**Figure 2b−d**).

Hill Reaction Activity. Compared to untreated plants, Hill reaction activity significantly decreased by 36 ± 13% 7 days after 6 mM fdx treatment (**Figure 4**). Using the other concentrations, no significant changes were noticed.

DISCUSSION

The results of the present study showed that photosynthesis was temporarily inhibited after the three fdx concentrations used and also after water application. The impact of fdx on grapevine photosynthesis was different according to the applied concentration. Both water and the lower fdx concentration (1.2 mM fdx) decreased photosynthesis from the first day following treatment. On the contrary, the higher concentrations, 6 and 30 mM, induced P_n decrease after 7 days. Light compensation point and respiration in the light increased 7 days after 6 and 30 mM fdx treatments, respectively. At 10 days after fdx application, recovery of P_n was observed in all treated plants.

As shown by P_n declines, fdx disrupted photosynthesis when applied at the highest concentrations (6 and 30 mM). This information is consistent with previous results obtained in grapevine (*11*) and in other plants with other fungicides (*8, 10*). In addition, fdx phytotoxicity varies according to its applied concentration on grapevine leaves, as observed with other fungicides. For example, the effect of captan on pepper and carbendazim on tobacco revealed that pigment reduction was more pronounced at higher concentrations (*33, 34*). In our experiment, the mechanism underlying photosynthesis inhibition also varies according to fdx concentration. Photosynthesis disruption after both water and 1.2 mM fdx sprayings was similar. P_n was impaired very quickly. Indeed, P_n disruption was detected as early as 1 day after treatment, then P_n recovered from the second day. P_n decrease was not related to changes in both g_S and C_i for both treatments, suggesting a nonstomatal limitation. It seems that loss in PSII activity is not involved in the P_n decrease after water and 1.2 mM fdx. Indeed, Φ_{PSII} reduction was only observed 2

Table 1. Determination of P_n/C_i Response Curves Parameters—$Pmax_{(Ci)}$, $V_{c,max}$, C_i^*, and R_l—in Grapevine Leaves of Untreated, Water-Treated, and Fdx-Treated Plants 7 days after Fdx Application[a]

			fdx-treated		
	untreated	water-treated	1.2 mM	6 mM	30 mM
$Pmax_{(Ci)}$ (μmol CO_2 m^{-2} s^{-1})	11.3 ± 0.8a	10.9 ± 1.3a	11.3 ± 0.1a	11.5 ± 0.9a	12.2 ± 1.1a
$V_{c,max}$ (μmol CO_2 m^{-2} s^{-1})	42.8 ± 3.2a	40.1 ± 10.1a	38.0 ± 3.2a	44.1 ± 2.8a	46.7 ± 5.4a
C_i^* (μmol CO_2 mol^{-1})	74.8 ± 12.7c	190.8 ± 37.7b	84.9 ± 10.3c	82.2 ± 13.9c	267.0 ± 8.5a
R_l (μmol CO_2 m^{-2} s^{-1})	3.2 ± 0.5c	7.5 ± 0.4b	3.2 ± 0.1c	3.6 ± 0.9c	12.6 ± 1.1a

[a] $Pmax_{(Ci)}$, intercellular CO_2 concentration-saturated net CO_2 assimilation rate; $V_{c,max}$, in vivo maximum rate of rubisco carboxylation; C_i^*, CO_2 compensation point; and R_l, estimation of mitochondrial respiration in the light. Data are means ± standards errors ($n = 3$). Means for a considered parameter were not significantly different, when followed by the same letter ($P < 0.05$).

Table 2. Analyses of Photosynthetic Light Response Curve—ΦCO_2, R_d, $Pmax_{(PPFD)}$, and $\Phi_{PSII}/\Phi CO_2$ Ratio—of Grapevine Leaves of Untreated, Water-Treated and Fdx-Treated Plants 7 Days After Fdx Application

			fdx-treated		
	untreated	water-treated	1.2 mM	6 mM	30 mM
ΦCO_2	0.07 ± 0.01a	0.05 ± 0.01a	0.04 ± 0.00b	0.04 ± 0.01b	0.04 ± 0.01b
R_d (μmol CO_2 m^{-2} s^{-1})	1.6 ± 0.0a	1.1 ± 0.1b	0.8 ± 0.0c	1.2 ± 0.1b	1.4 ± 0.2ab
Γ^* (μmol CO_2 mol^{-1})	22.6 ± 3.1cb	26.1 ± 4.1ac	19.8 ± 1.1a	27.7 ± 1.4a	37.0 ± 15.8ac
$Pmax_{(PPFD)}$ (μmol CO_2 m^{-2} s^{-1})	8.9 ± 1.5a	7.7 ± 0.2a	7.7 ± 1.5a	7.6 ± 1.8a	9.4 ± 0.2a
$\Phi_{PSII}/\Phi CO_2$	10.0 ± 1.7b	15.0 ± 2.3a	17.4 ± 0.1a	16.3 ± 2.8a	19.2 ± 5.6a

ΦCO_2, apparent quantum yield of CO_2 fixation; R_d, dark respiration; Γ^*, light compensation point; and $Pmax_{(PPFD)}$, PPFD-saturated net CO_2 assimilation rate. Data are means ± standards errors (n=3). Means for a considered parameter were not significantly different, when followed by the same letter ($P < 0.05$).

days after treatment, whereas P_n was not lower in water and 1.2 mM fdx treated plants compared to untreated plants. Therefore, water and 1.2 mM fdx decreased P_n but did not significantly affect Φ_{PSII} the first day following application, suggesting that the rate of noncyclic electron transport is higher than that required to maintain P_n. An alternative sink to P_n for electrons may be oxygen reduction by photorespiration, Mehler ascorbate peroxidase reaction, mitochondrial respiration in the light, and/or dark respiration (*35*). Nevertheless, R_l was not increased after 1.2 mM fdx treatment, excluding the hypothesis that light respiration increase may contribute to P_n decrease. In addition, analysis of chlorophyll fluorescence was also performed, since it is frequently used to monitor responses of photosynthetic apparatus to environmental stress (*19, 20*). Water and 1.2 mM fdx did not affect q_P and q_{NP}, indicating that energy dissipation was not modified by these treatments. This suggests that the nonstomatal limitation of P_n after water and 1.2 mM fdx may originate from a decrease in CO_2 fixation mediated by rubisco. Indeed, the extent of P_n decrease after both treatments is similar, suggesting that P_n alteration induced by 1.2 mM fdx might be mostly attributed to wetness on leaf surface caused by spraying and not by toxicity of the active ingredient. This hypothesis is supported by previous works. An inhibition of P_n was actually observed in bean leaves in response to wetness (*36*) and was accompanied by a strong degradation of rubisco. Decrease in rubisco amount could thus explain the P_n decrease after 1.2 mM fdx. Our results also indicate that water spraying often used as control in studies of pesticide toxicity (*10, 34, 37*) is not well adapted because of photosynthesis modification caused by water.

The P_n disturbance induced by higher fdx concentrations, 6 and 30 mM fdx, is very different than the one caused by both water and 1.2 mM fdx. At 6 and 30 mM fdx, P_n inhibition at 7 days is associated with increased C_i, indicating a nonstomatal limitation. The strongest effect was registered after a 6 mM fdx treatment. It is likely that PSII is not involved in P_n reduction after both treatments because Φ_{PSII} reduction was only observed 2 days after treatment and recovered rapidly the day after. This may further reflect the ability of treated leaves to maintain high electron transport rates. Many abiotic stresses such as drought, salinity stress, and herbicide application are known to induce decreases in $V_{c,max}$ and $Pmax_{(Ci)}$(*14, 17, 38, 39, 40*). Nevertheless, measurements of P_n versus C_i revealed that these gas exchange parameters were not reduced after fdx treatments. This indicates neither loss nor inactivation of both rubisco and other key Calvin cycle enzymes, which may result in a reduction of carboxylation efficiency and RuBP regeneration rate (*41, 42*). In agreement with these observations, $Pmax_{(PPFD)}$ was also not altered after treatments, suggesting that neither maximal rubisco activity nor RuBP regeneration were modified (*27*). Hence, the photosynthetic capacity of leaves does not seem be affected by fdx application. Reduction in activity of other enzymes, such as carbonic anhydrase, which catalyzes the conversion of CO_2 to HCO_3, or enzymes involved in the utilization of the photoassimilates, may thus cause a decrease in CO_2 fixation. Indeed, carbonic anhydrase (*43, 44*) and some aquaporins are known to be implicated in CO_2 movement (*45–48*) because both proteins act in facilitating the passive CO_2 diffusion process. Our results also demonstrate that decreased photosynthesis after 30 mM fdx treatment is accompanied by respiratory CO_2 (R_l) increasing the CO_2 compensation point (C_i*) and C_i. Thus, the increase of respiration in the light could be partly responsible for the reduction of P_n after 30 mM fdx treatment. Indeed, the lower rate of CO_2 uptake may result from higher rates of CO_2 loss by respiration in the light. Light response curves indicate that light requirements for photosynthesis is altered after 6 mM fdx. Actually, Γ*, which reflects the light conditions required for plant photosynthesis and embodies the ability of a plant to utilize high and low light levels, is increased after 6 mM fdx. Therefore, higher Γ* indicates a higher energy requirement for PSII excitation. In addition, the $\Phi_{PSII}/\Phi CO_2$ ratio, which is an estimate of the relationship between the rate of electron transport and carbon fixation, was approximately 1.5−2-fold higher after fdx treatment, whatever the applied fdx concentration. This implies that more electrons are transported through PSII for each CO_2 molecule assimilated in leaves of treated plants. Therefore, other processes than photosynthesis, such as photorespiration, mitochondrial respiration in the light, N assimilation, and/or

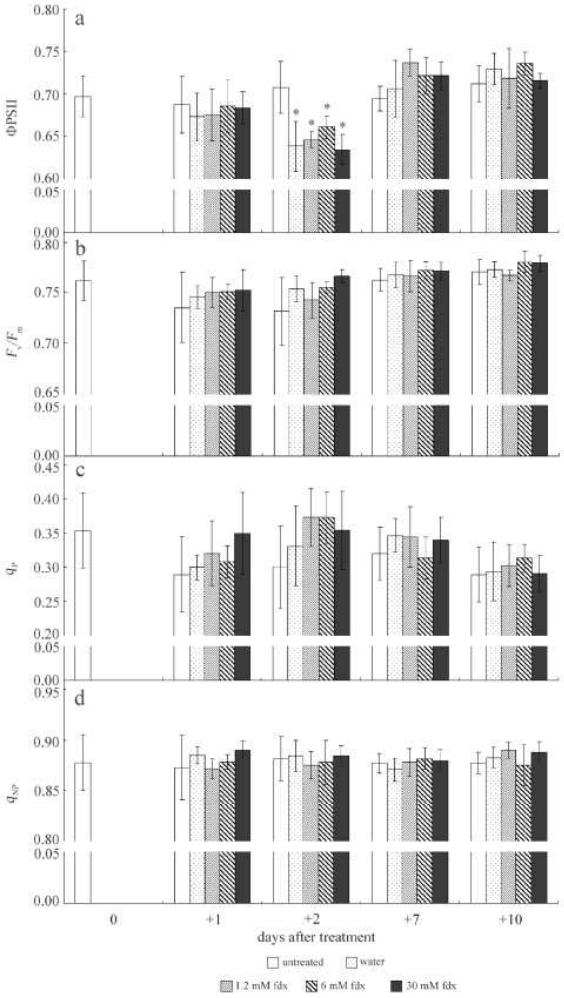

Figure 2. Changes in (**a**) relative quantum yield of PSII (Φ_{PSII}), (**b**) ratio of variable to maximal fluorescence (F_v/F_m), (**c**) photochemical (q_P), and (**d**) total nonphotochemical quenching (q_{NP}) in untreated, water-treated, and fdx-treated leaves of grapevine. Data are means ± standards errors ($n = 24$). Significant differences at $P < 0.05$ between leaves of untreated and treated plants are marked by an asterisk.

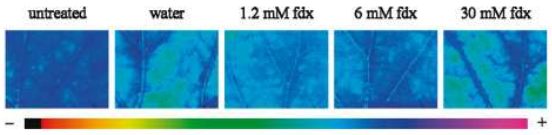

Figure 3. Fluorescence imaging of the abiotic stress induced by fludioxonil. A photograph of relative quantum yield of PSII (Φ_{PSII}) was captured. Data have been mapped to the color palette. The false color code ranges from black (0.000) to pink (1.000), as shown at the bottom of the images.

pseudocyclic electron transport (*22*), may also be operating in fdx-treated leaves. Nevertheless, water application also caused a higher $\Phi_{PSII}/\Phi CO_2$ ratio, indicating that this increase is mostly attributed to the spraying effect.

To locate the possible site of inhibition in the PSII reaction, we also followed DCPIP photoreduction. Oxygen production was inhibited by 6 mM fdx. Nevertheless, since Φ_{PSII} was only affected 2 days after treatment, we can conclude that a decrease in Hill activity does not modify PSII activity.

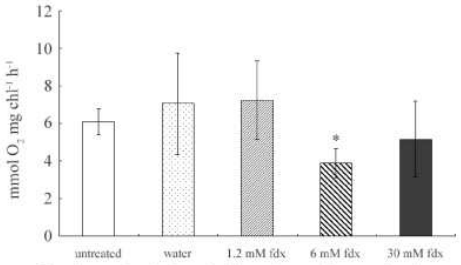

Figure 4. O_2 release by isolated chloroplasts of grapevine leaves from untreated, water-treated, and fdx-treated plants 7 days after fdx application. Data are means ± standards errors ($n = 4$). Significant differences at $P < 0.05$ between leaves of untreated and treated plants are marked by an asterisk.

The recovery of P_n was observed whatever the treatment after 10 days, indicating that fdx did not persistently affect photosynthetic activity. Saladin et al. (*11*) have also noticed that the studied parameters recovered in 10 days for grapevine cuttings treated with fdx. The recovery in P_n may suggest that fdx detoxification occurs in treated leaves.

Each pesticide contains an active ingredient that is responsible for its pesticidal effect. Nevertheless, the active ingredient must be formulated with other nonpesticidal compounds before it is ready to use. In our experiment, fdx was used in a wetting powder in which surfactants can be found. It has been already shown that some surfactants can significantly reduce photosynthesis (*49*). Therefore, the results of this experiment are indicative of the combined phytotoxic effects of both the active ingredient and surfactant.

ABBREVIATIONS USED

Γ*, light compensation point; C_a, carbon dioxide concentration in the air; CCD, charge coupled device; chl, chlorophyll; C_i, intercellular CO_2 concentration; C_i*, CO_2 compensation point; F_0, minimal fluorescence at dark-adapted state; F_0', minimal fluorescence in the light-adapted state; fdx, fludioxonil; F_m, maximal fluorescence in the dark-adapted state; F_m', maximal fluorescence in the light-adapted state; F_t, steady-state fluorescence; F_v/F_m, maximum efficiency of PSII photochemistry after dark adaptation; g_S, stomatal conductance; q_{NP}, nonphotochemical quenching; q_P, photochemical quenching; $Pmax_{(Ci)}$, leaf intercellular CO_2 concentration saturated assimilation rate; $Pmax_{(PPFD)}$, PPFD-saturated CO_2 assimilation rate; P_n, net photosynthetic rate; PPFD, photosynthetic photon flux density; PSII, photosystem II; ΦCO_2, apparent quantum yield of CO_2 fixation; Φ_{PSII}, relative quantum yield of PSII; R_d, dark respiration; R_l, estimation of the mitochondrial respiration in the light; RuBP, ribulose 1,5-bisphosphate; $V_{c,max}$, in vivo maximum rate of rubisco carboxylation.

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Au vignoble, l'application des fongicides fludioxonil et fenhexamid lors du stade A diminue temporairement la photosynthèse foliaire. Les deux fongicides provoquent les mêmes perturbations sur la photosynthèse, bien qu'appartenant à des familles chimiques différentes. Pour les deux cépages testés, le Pinot noir et le Pinot Meunier, la diminution de la photosynthèse est faible (inférieure à 25%). La réduction de la photosynthèse n'a pour origine ni une fermeture des stomates, ni une altération de l'activité du PSII. Cette inhibition de la photosynthèse peut être partiellement causée par une diminution de la quantité de Rubisco et de la collecte de l'énergie lumineuse. En effet, les fongicides provoquent la répression de l'expression de gènes codant une protéine du complexe collecteur de lumière du PSI (*cab*) et la petite sous-unité de la Rubisco (*rbcS*) chez le Pinot noir. Chez le Pinot Meunier, l'expression de gènes codant la synthèse des sous-unités de la Rubisco (*rbcS* et *rbcL*) est réprimée.

La perturbation de la photosynthèse en fonction de la concentration en fludioxonil a ensuite été déterminée sur des boutures fructifères de Pinot noir. Cette étude a révélé que les mécanismes qui conduisent à la réduction de la photosynthèse diffèrent selon la concentration de fongicide. La concentration la plus faible (1,2 mM) diminue la photosynthèse dès le premier jour après traitement, de la même façon que la pulvérisation d'eau. Cette perturbation serait donc davantage liée à l'humidité causée par l'application du traitement qu'à la toxicité du fongicide. Concernant les concentrations les plus fortes, une diminution de la photosynthèse est observée plus tard, soit 7 jours après traitement. La concentration 6 mM conduit à une élévation du point de compensation lumineux alors que la concentration 30 mM augmente la respiration à la lumière. Ces modifications peuvent en partie expliquer la réduction de photosynthèse lors des traitements au fludioxonil. Dix jours après traitement, le recouvrement est atteint pour les trois concentrations de fongicides testées traduisant donc un stress modéré pour la vigne.

La diminution de photosynthèse traduit un stress subi par les organes végétatifs de la vigne lors des traitements anti-*Botrytis*. Nous avons donc déterminé si ce stress conduisait à l'activation de réponses de défense dans ces organes. Nos résultats ont montré que les fongicides anti-*Botrytis* ne causent aucune induction de l'expression des gènes codant une phénylalanine ammonia-lyase (*PAL*), une lipoxygénase (*LOX*) et une chitinase (*Chi4C*). De plus, l'activité chitinase n'augmente pas suite à l'application de ces fongicides. L'ensemble de ces résultats confirme donc un stress modéré pour les organes végétatifs de la vigne lors des traitements anti-*Botrytis*.

Après avoir caractérisé le stress généré par les fongicides fludioxonil et fenhexamid sur les organes végétatifs de la vigne, nous nous sommes intéressés aux effets de ces fongicides sur les organes reproducteurs. En effet, le développement de ces organes est déterminant pour le rendement de la plante.

– Partie 2 –

Effets des fongicides anti-*Botrytis* sur les organes reproducteurs et sensibilité des fleurs aux stress

Publication 5

Effects of fungicides on populations of *Botrytis cinerea* and on defense responses of grapevine reproductive organs

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Soumise à *Plant Pathology*

Effects of fungicides on populations of *Botrytis cinerea* and on defense responses of grapevine reproductive organs

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Abstract

The aim of this work was to determine factors which could influence treatment efficacy against grey mould in the Champagne vineyard. In association with prophylactic treatments, this disease, caused by *Botrytis cinerea*, is controlled by using chemical fungicides at three stages: the end of flowering (stage A), bunch closure (stage B) and the beginning of berry ripening (stage C). Fenhexamid and fludioxonil present a great efficacy against grey mould. In a pluriannual Champagne trial, a single treatment of each fungicide revealed that fenhexamid significantly decreased disease only when applied at stage A. Disease reduction was similar at all stages following fludioxonil but was lower than fenhexamid at stage A. Analysis of resistance profiles of *B. cinerea* to fungicides showed that frequencies of strains resistant to benzimidazoles, anilinopyrimidines, dicarboximides and hydroxyanilides were similar in control and treated plants, whatever the treatment stage or the fungicide. Multi-drug resistant (MDR) strains were strongly selected particularly at stage A after fenhexamid or fludioxonil. However, a single treatment still presented efficacy indicating that selection pressure on *B. cinerea* had poor influence on treatment efficacy in this trial resistance context. Defense responses in grapevine reproductive organs were analyzed after fungicide application. Expression of varied defense-related genes was not induced whatever the treatment stage or the botryticide. Only an increase in chitinase activity was observed at stage C. Therefore, differences in fungicide efficacy can not be explained by differences of grapevine responsiveness. In the case of botryticide treatment, efficacy seems thus to be influenced by many interacting factors.

Keywords: defense responses, fungicide resistance, grapevine, grey mould, treatment efficacy

Introduction

Botrytis cinerea (teleomorph *Botrytonia fuckeliana*) is a widespread fungal pathogen, responsible for the grey mould disease, which causes severe damages in vineyards around the world (Bulit & Dubos, 1988). In the Champagne vineyard, *B. cinerea* is especially feared by wine-growers because of considerable economic losses related to this pathogen. Indeed, depending on the vintage fungal infection rates can reach 15-25% (Panon *et al*., 2006). In addition, wines prepared from infected grapes usually exhibit organoleptic defaults, such as an oxidation of the colour or the appearance of typical aromatic notes (‘‘moldy’’, ‘‘rotten’’), which are not appreciated by consumers, and alteration of foaming properties (Bocquet *et al*., 1995; Marchal *et al*., 2001 ; Cilindre *et al.*, 2007, 2008).

In association with prophylactic treatments, chemical control currently remains the main way to reduce the incidence of grey mould. Several families of anti-*Botrytis* fungicides are available and they can be classified according to their biochemical modes of action: inhibitors of fungal respiration (e.g. boscalid),

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antimicrotubule fungicides, such as benzimidazoles (e.g. benomyl, carbendazim), fungicides affecting osmoregulation**,** such as dicarboximides (e.g. iprodione) and phenylpyrroles (e.g. fludioxonil), methionine biosynthesis inhibitors such as anilinopyrimidines (e.g. pyrimethanil), and sterol bisoynthesis inhibitors (SBIs), such as hydroxyanilides (e.g. fenhexamid) (Leroux *et al*., 2002). Because of the lack of effective methods to predict the occurrence risk for this disease, a reference program consisting in three preventive applications was recommended until 2006 in Champagne: at the end of flowering (stage A), at bunch closure (stage B) and at the beginning of berry ripening (stage C). Fenhexamid and fludioxonil are commonly used as botryticides in vineyards all over the world and are among the most effective fungicides to control grey mould (Rosslenbroich & Stuebler, 2000). In the Champagne vineyard, they currently represent the general basis for the reference program Fenhexamid inhibits germ-tube elongation and mycelial growth of *B. cinerea* (Hänβler & Pontzen, 1999). No cross-resistance with any anti-*Botrytis* fungicide currently on the market was observed indicating that target site of fenhexamid is different from those of other widely used botryticides (Rosslenbroich & Stuebler, 2000). Investigations on the mode of action have suggested that this fungicide inhibits the 3-keto reductase involved in the enzymatic complex of the sterol C-4 demethylation (Debieu *et al.*, 2001). Fludioxonil inhibits spore germination, germ-tube elongation and mycelium growth of *B. cinerea* (Leroux, 1996). It increases the glycerol content in the fungus, leading to a perturbation of the osmoregulation potential (Pillonel & Meyer, 1997; Liu *et al*., 2008).

Fungicide efficacy in vineyard depends on many factors including the selection of pathogen resistance towards fungicide and the crop physiological response. The chemical control of grey mould is impeded in vineyards by the development of resistance to many conventional botryticides. Therefore, since 2006, two treatments are recommended in the Champagne vineyard. Indeed, shortly after the introduction of benzimidazoles and dicarboximides, several cases of resistance were recorded in European vineyards (Leroux & Clerjeau, 1985; Leroux, 1995; Leroux *et al*., 1999, 2002). Anilinopyrimidine-resistant strains were also detected in vineyards of various countries including France but they did not seem to lead to resistance of practical importance (Hilber & Hilber-Bodmer, 1998; Leroux *et al.*, 1998). Considering fenhexamid, natural resistance was detected in *Botrytis* populations in many vineyards, before commercial introduction of this fungicide. Previous authors suggested that *Botrytis* was a complex of at least two species living in sympatry on grapevine and other crops and exhibiting different sensitivity to various fungicides (Fournier *et al*., 2003, 2005; Albertini & Leroux, 2004). Strains highly resistant to fenhexamid before its registration were further characterized as the naturally resistant group I *Botrytis* species (phenotype HydR1) whereas specific resistance to fenhexamid (phenotype HydR3) was later selected after registration of this fungicide in the group II *B. cinerea sensu-stricto* (Leroux *et al.*, 1998; Leroux *et al*., 1999; Albertini & Leroux, 2004; Fournier *et al*., 2005; Fillinger *et al*., 2008 accepted). At last, no specific resistance to fludioxonil was detected in the field in France, whereas such resistance was detected in other fungi such as *Stemphylium vesicarium* (Alberoni *et al*., 2007) and *Alternaria brassicicola* (Avenot *et al*., 2005). In some *B. cinerea* laboratory mutants, cross resistance was observed between phenylpyrroles and dicarboximides but never in the French field resistance isolates (Leroux, 2004; Liu *et al*., 2008). In addition to these specific resistances to a single mode of action, the presence of multi-drug resistant (MDR) strains was detected in French vineyards in the late 1990s, particularly in the Champagne area (Leroux *et al.*, 2006; Panon *et al*., 2006). MDR strains present a simultaneous resistance to a variety of anti-*Botrytis* fungicides which can belong to different families. This kind of resistance is not determined by target gene alteration but by overexpression of drug transporters located in the fungal cell membrane (Leroux, 2004). The development of these resistant strains may play a role in botryticide efficacy.

In addition to their antibiotic action against pathogens, some fungicides can indirectly act by changing plant physiology (Prudet, 1994) or stimulating plant defense responses (Garcia *et al.*, 2003). Indeed, several fungicides stimulate the accumulation of pathogenesis-related (PR) proteins or other defense proteins (Siefert *et al.*, 1996; Ruiz *et* al., 1999; Garcia *et* al., 2001; Wu & von Tiedemann, 2002; Pasquer *et al*., 2005). Therefore, botryticide efficacy may also be related to putative induction of grapevine defense mechanisms which may limit the infection by *B. cinerea*.

Our first objective was thus to determine the assessment of each treatment stage in the three spray-program used against *B. cinerea* in the Champagne vineyard. The second objective was to evaluate some factors which could influence treatment efficacy. For that, selection pressure exerted by fungicides on *B. cinerea* strains and defense responses of grapevine reproductive organs to botryticides were evaluated.

TABLE 1 Phenotypes of sensitivity towards several fungicides described in field populations of *Botrytis*; analysis according to germ tube elongation. Phenotypes were classified according to the resistance levels calculated for the various fungicides (RL = LC_{50} resistant strain/LC_{50} sensitive strain); HS: hypersensitive (RL<0,5); S: sensitive (0,5<RL<2); LR: low resistance (2<RL<10); MR: moderate resistance (10<RL<25); HR: high resistance (RL>25)

	Phenotypes of sensitivity								
Fungicide families	Ben R1	Ben R2	Ani R1	Imi R1	Hyd R1	Hyd R3	MDR 1	MDR 2	MDR 3
Benzimidazoles	HR	HR	/	/	/	/	/	/	/
Phenylcarbamates	HS	HR	/	/	/	/	/	/	/
Dicarboximides	/	/	/	MR	/	/	LR	LR	LR
Phenylpyrroles	/	/	/	S	/	/	**MR**	S/LR	MR
Anilinopyrimidines	/	/	MR/HR	/	/	/	LR/MR	LR/MR	MR
Hydroxyanilides	/	/	/	/	LR	MR/HR	S/LR	**LR/MR**	MR

Material and Methods

Vineyards

Experiments were conducted on experimental vineyard located in Loisy-en-Brie, in the Champagne area (France). High levels of grey mould developed in this vineyard each year. Grapevines (*Vitis vinifera* L. cv. Pinot Meunier), grafted on 41B rootstock and trained according to the Chablis method, were planted in 1986. Individual treatment plots were arranged in a randomized complete block design with four replications. Each replication consisted of at least 36 vine plants. Each replication location was surrounded by two border rows to minimize the drift of fungicide from outside the trial.

All field experiments were conducted using formulated products. Fenhexamid was used from the commercial fungicide Teldor (50% a.i.; Bayer, Monheim, Germany) and fludioxonil was applied from Geoxe (50% a.i.; Syngenta, Basel, Swithzerland). In the spraying program, Teldor was applied at 1.5 kg ha^{-1} rate while Geoxe was used at a rate of 1 kg ha^{-1}. Fenhexamid or fludioxonil were applied at stages A, B or C and each mono-treatment was compared to the reference program. This program consisted of 3 treatments: Teldor (stage A or BBCH 68), Geoxe (stage B or BBCH 77) and Scala (stage C or BBCH 81). Scala (BASF, Ludwigshafen, Germany) contains 400 g l^{-1} of pyrimethanil a.i. and was used at a rate of 2.5 l ha^{-1}. Untreated vines against *B. cinerea* were used as controls.

Treatment efficacy

Infection of grey mould was assessed at harvest from 2002 to 2007, from 100 clusters per replicate. Firstly, the incidence was calculated as the percentage of infected clusters, i.e. showing at least one rotten berry with typical symptoms. Secondly, in each cluster sampled, berries were examined individually to determine the percentage of symptomatic berries (i.e. percentage of the area rotted and/or sporulating), corresponding to disease severity.

Isolates of *Botrytis cinerea*

Field populations of *B. cinerea* were isolated from diseased berries at the harvest (September) from 2002 to 2007. At least twenty infected berries per treatment plot were randomly collected and bulked to produce a spore suspension representative of the plot. Then phenotypes were characterized according to Leroux *et al*. (1999): roughly, the bulk spore suspension was amended on Petri dishes containing discriminating doses of various fungicides. Notations

TABLE 2 Genes analyzed by real-time RT-PCR

Gene	Encoding	Pimer sequence	Accession number
EF1-α	elongation factor 1-alpha	Sense 5' GAA CTG GGT GCT TGA TAG GC 3' Antisense 5' AAC CAA AAT ATC CGG AGT AAA AGA 3'	BQ799343
PAL	phenylalalanine ammonia-lyase	Sense 5' TCC TCC CGG AAA ACA GCT G 3' Antisense 5' TCC TCC AAA TGC CTC AAA TCA 3'	X75967
LOX	lipoxygenase	Sense 5' CTG GGT GGC TTC TGC TCT C 3' Antisense 5' GAT AAG CCG CAG ATT CAT GC 3'	AY159556
Chi4C	class IV chitinase	Sense 5' TCG AAT GCG ATG GTG GAA A 3' Antisense 5' TCC CCT GTC GAA ACA CCA AG 3'	AY137377
GLUC	β-1,3-glucanase	Sense 5' TCA ATG GCT GCA ATG GTG C 3' Antisense 5' CGG TCG ATG TTG CGA GAT TTA 3'	AF239617
PR6	class 6 pathogenesis-related protein	Sense 5' AGT TCA GGG AGA GGT TGC TG 3' Antisense 5' GCA CTA GGG TCC GTG TTT GGG TCG ACG 3'	AY156047

under microscope at 24 h and 48 h enabled to read the percentage of phenotypes corresponding to strains resistant to benzimidazoles (Ben R1 and Ben R2), anilinopyrimidines (Ani R1) or dicarboximides (Imi R1), hydroxyanilides (Hyd R1 and Hyd R3) and MDR strains (phenotypes MDR1 and MDR2, distinguished respectively for their higher resistance to fludioxonil and fenhexamid, and the double mutant MDR3) (Leroux *et al*., 1999; Walker *et al*., 2006) (Table 1).

Grapevine defense responses

RNA extraction

Inflorescences (stage A) or clusters (stages B and C) were collected 1 day after fungicide spraying. They were immediately frozen in liquid nitrogen then stored at -80°C. The flowers and berries were ground in liquid nitrogen to a fine powder. Concerning flowers, 100 mg of powder were used for total RNA extraction and homogenized in extraction buffer (Plant Purification RNA Reagent, Invitrogen, France), according to the manufacturer's instructions. Total RNA was extracted from berries according to Davies and Robinson (1996). The RNA pellet was resuspended in 20 µL of RNase-free water and quantified by absorbance at 260 nm.

Real-time RT-PCR analysis

One hundred and fifty ng of total RNA were reverse-transcribed using M-MLV reverse-transcriptase (Invitrogen, France) according to the manufacturer's protocol. PCR conditions were described in Bézier *et al.* (2002). The reaction was carried out in a GeneAmp 5700 sequence detection system (Applied Biosystems, France) using the following thermal profile: 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension) for 40 cycles. PCR efficiency of the primer sets was calculated by performing real-time PCR on several dilutions. Results were standardized to the EF1α gene expression level (Terrier *et al.*, 2005). Control sample (untreated flowers/berries) was chosen to represent 1x expression of the genes of interest and treated samples were expressed relative to the corresponding control. Expression of five defense-related genes encoding phenylalanine ammonia-lyase (*PAL*), lipoxygenase (*LOX*), class IV chitinase (*Chi4C*), β-1,3-glucanase (*GLUC*) and class 6 pathogenesis-related protein (*PR6*) were tracked (Table 2). The entire experiment, including both the RT and real-time PCR steps, was repeated four times with RNA from different sets of samples.

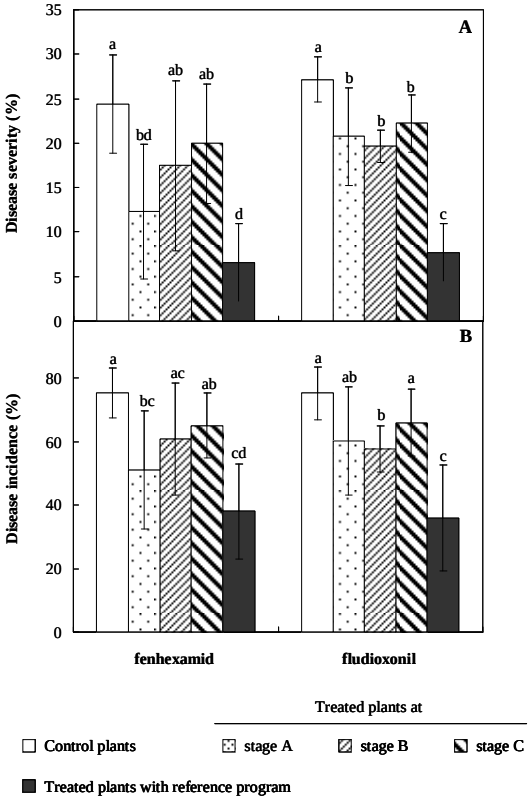

Figure 1 Effects of fungicide treatment on the severity (A) and incidence (B) of grey mould on berries at harvest. Efficacy of fenhexamid of fludioxonil was compared according to the stage of application. Means represent data from 2002 to 2007. Means with the same letter were not significantly different ($P < 0.05$)

Chitinase extraction and activity

Inflorescences or clusters were collected 4 days after treatment for chitinase extraction. Proteins were extracted by homogenizing ground frozen flowers/berries (250 mg fresh weight) at 4°C in 1 mL of 50 mM sodium acetate buffer, pH 5.0 containing 1 mM dithiothreitol and 1% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 10,000 × *g* for 5 min at 4°C and the clarified supernatant was recovered. Chitinase activity was assayed using a commercial blue enzyme substrate, CM-chitin-RBV solution (Loewe Biochemica, Germany) according to Magnin-Robert *et al.* (2007). Measurements were conducted in triplicate on six plants for each treatment. Results were expressed in terms of mg per min per g fresh weight (FW).

Statistical analysis

Results represent means of data from 2002 to 2007, except for grapevine defense responses which were analyzed in 2006 and 2007. For gene expression and chitinase activity, the entire experiment was repeated respectively twice and three times by year. To determine whether values of control plants and plants of treatment plots were significantly different, analysis of variance (ANOVA) followed by a Student's *t* test was used. Differences at $P < 0.05$ were considered as significant.

Results

Efficacy of treatment

Disease severity and incidence were compared between control and treated plants with reference program or with mono-treatment of fenhexamid or fludioxonil either at stages A, B or C (Fig. 1). In control plants, disease severity was about 25% and drastically decreased by 70% when plants were treated with reference program. Fenhexamid single treatment at stage A also strongly reduced severity compared to control plants with a significant

TABLE 3 Percentage of resistance towards fungicides in *B. cinerea* strains, in control plants, in treated plants with fenhexamid or fludioxonil at stages A, B or C, or with reference program. Means represent data from 2002 to 2007. Means for a specific strain were not significantly different, when followed by the same letter ($P < 0.05$)

		fenhexamid-treated at stage				fludioxonil-treated at stage				
Phenotypes	control	A	B	C	reference program	control	A	B	C	reference program
Ben R1	53.3 a	63.2 a	56.7 a	51.7 a	60.0 a	43.3 a	53.3 a	51.7 a	42.5 a	50.0 a
Ben R2	4.2 a	3.3 a	2.5 a	6.7 a	2.0 a	2.5 a	7.5 a	7.5 a	3.3 a	2.0 a
Ani R1	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	10.0 a	0.0 a	2.5 a	0.0 a
Imi R1	15.8 a	8.2 a	11.7 a	14.2 a	10.0 a	19.2 a	5.0 a	20.0 a	6.7 a	10.0 a
Hyd R1	1.7a	9.7a	1.3a	0.0a	2.7a	1.7a	1.7a	5.0a	1.0a	2.7a
Hyd R3	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	10.0 a	0.0 a	0.0 a
MDR1	5.0 a	6.8 a	6.7 a	9.0 a	6.9 a	4.0 a	16.0 a	13.3 a	20.8 a	12.0 a
MDR2	8.0 a	24.5 a	15.4 a	14.5 a	18.1 a	15.0 a	23.3 a	15.0 a	14.2 a	19.2 a
MDR3	0.0 a	0.0 a	0.0 a	7.0 a	0.0 a	0.0 a	3.5 a	15.0 a	0.0 a	0.0 a
∑ MDR	13.0 a	31.3 a	22.1 a	25.3 a	25.1 a	19.0 b	41.7 a	38.3 a	35.0 a	31.2 ab

reduction of approximately 50% (Fig. 1A). At stages B and C, a decrease was also observed after fenhexamid-treatment but it was not significant. Concerning fludioxonil, severity decrease was similar whatever the stage of application and was close to 25% compared to control plants. In control plants, a strong disease incidence was recorded as it was about 75% (Fig. 1B). It was reduced by half after treatment with reference program. After fenhexamid application at stage A, the same trend than these of severity was observed. Indeed, a significant decline of incidence was noticed and represented approximately 30%. On the contrary, fenhexamid-treatment at stages B and C did not lead to a significant incidence reduction. Incidence decrease was not significant when fludioxonil was applied at stages A and C but a decline of about 25% was noticed with fludioxonil-treatment at stage B.

Sensitivity to fungicides

Similar frequencies of benzimidazole (Ben R1 and Ben R2), anilinopyrimidine (Ani R1), dicarboximides (Imi R1) and hydroxyanilides-resistant strains (Hyd R1 and Hyd R3) have been observed between control and treated plants, whatever the stage of treatment and the fungicide applied (Table 3). A high frequency of Ben R1 strains was observed since it varied between 42.5 and 63.2%. Proportion of Ben R2 was largely lower as it reached a maximum of 7.5%. No isolates of Ani R1 were found except after fludioxonil-treatment at stages A (10.0%) and C (2.5%) but frequency was low. Frequency of Imi R1 strains was low and it varied between 5.0 and 20.0%. Finally, frequency of Hyd R1 was also weak as it was always lower than 9.7% while no isolates of Hyd R3 were found except after fludioxonil-treatment at stages B (10.0%).

A higher frequency of MDR after fludioxonil and fenhexamid treatment was observed compared to control plants, even if this difference was not significant in the case of fenhexamid. Similarly, an increase was noticed after reference program application. Frequency of whole MDR strains was lower than 20% in control plants and reached a maximum of 31.3% in fenhexamid and 41.7% in fludioxonil-treated plants at stage A. In fenhexamid-treated plants, the increase of MDR2 was higher than MDR1. Indeed, the increase represented a maximum of 3-fold in MDR2 compared to control plants and 1.8-fold in MDR1. Concerning MDR3, no isolates were found except after treatment at stage C. In fludioxonil-treated plants, MDR1 increase was at maximum with a factor 5.2 compared to control plants whereas the increase was only with a factor 1.5 for MDR2 strains. MDR3 were only detected after fludioxonil-treatments at stages A and B.

Grapevine defense responses

No significant modification in expression of *PAL*, *LOX*, and genes encoding PR proteins (*Chi4C*, *GLUC* and *PR6*) was observed in flowers (stage A) or berries (stages B and C) following fenhexamid or fludioxonil treatments (Fig. 2). Considering chitinase activity, basal level was 1.1 and 1.4 mg min^{-1} g^{-1} FW in control plants at stages A and B, respectively (Fig. 3) and weakly higher in berries at stage C (1.8 mg min^{-1} g^{-1} FW). Following fungicide treatments, only a significant enhancement was noticed after treatment at stage C: chitinase activity increased by approximately 60% after fenhexamid or fludioxonil application.

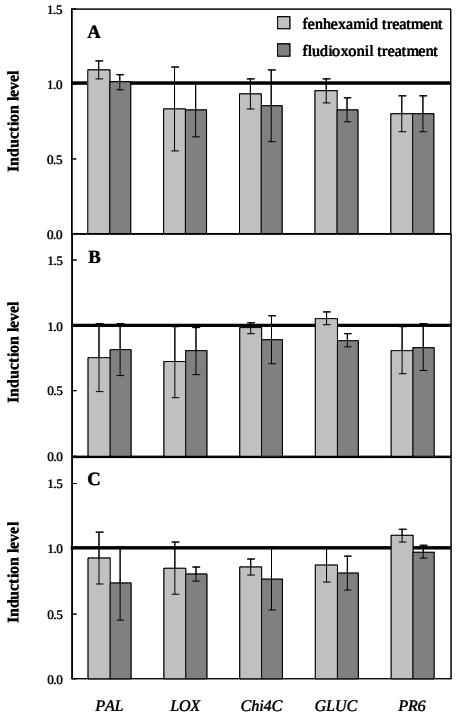

Figure 2 Induction level of *PAL*, *LOX*, *Chi4C*, *GLUC* and *PR6* in fenhexamid and fludioxonil-treated flowers at stage A (A) or berries at stages B (B) or C (C) of grapevine, one day after treatment. Untreated flowers or berries were defined as 1x expression level for each analyzed gene. Data are the mean ± standard error (n = 4)

Discussion

The aim of this work was, first to determine the effect of treatment timing according to botryticide on the efficacy against grey mould disease, and second to identify factors which could influence treatment efficacy. Considering botryticide treatment on subsequent development of grey mould, our results showed a high efficacy of the reference program against the disease. Therefore, this three spray-program ensures the best efficacy against *B. cinerea* development in the Champagne vineyard in comparison with single treatments. In the case of mono-treatment, observations differed according to the fungicide. Considering fenhexamid, efficacy was not identical according to the stage of treatment. Indeed, a significant reduction of both disease severity and incidence was demonstrated when fenhexamid was applied at stage A. On the contrary, no significant reduction of disease was observed when fenhexamid was used at stages B and C, in comparison with control plants. Therefore, the application of fenhexamid at flowering time seems to be decisive against grey mould disease. Similarly, Nair *et al*. (1995) demonstrated that spray application during flowering reduces the predicted risk of berry infection at harvest by 22%. In addition, a better efficacy of botryticide application at full bloom has already been observed compared to treatments at other stages of flowering and at stage B (Jermini *et al*., 1986; Pezet & Pont, 1986). Our results of fludioxonil efficacy were different. Indeed, fludioxonil efficacy was lower than fenhexamid at stage A but was similar between stages, even if slightly better at stage B. Fludioxonil seems thus not to be specifically adapted at a given stage. In addition, fludioxonil seems to act more on disease severity than on disease incidence, and could indicate that fludioxonil may act rather in diminishing the size of fungal foci than in reducing the number of foci. Selection pressure exerted by fungicides on *B. cinerea* strains and defense responses of grapevine to botryticides were then evaluated because they could influence botryticide treatment efficacy and explain differences of efficacy between both fungicides.

Firstly, fungicide resistance profiles have been compared between control and treated plants according to the fungicide and the stage of treatment. As field populations of *B. cinerea* were isolated from relatively small plots, it only allowed describing a trend. Frequencies of strains resistant to benzimidazoles (Ben R1 and Ben R2), anilinopyrimidines (Ani R1), dicarboximides (Imi R1) and hydroxyanilides (Hyd R1 and Hyd R3) were similar after both fenhexamid and fludioxonil treatments at all stages, and between control and treated plants. High frequencies of Ben R1 strains were recorded despite the absence of selection pressure. Indeed, benzimidazoles have been developed at the end of 1960's and the use of these compounds has rapidly induced development of highly resistant strains, particularly in locations of intensive treatments such as in the Champagne vineyard (Leroux & Clerjeau, 1985). These strains exhibited the mutation E198A in the gene encoding β-tubulin. These high proportions of BenR1 strains in absence of contemporary selection pressure may indicate null of low fitness penalty induced by this mutation (Leroux & Clerjeau, 1985). Frequency of Ben R2 strains (mutation F200Y in the β-tubulin target gene), simultaneously resistant to benzimidazoles and phenlycarbamates, was very low and even null in some cases. This genotype was selected by the mixture of the active ingredients carbendazim and diethofencarb in the Champagne vineyard (Leroux *et al*., 2006) which has very few usages against grey mould nowadays. Low

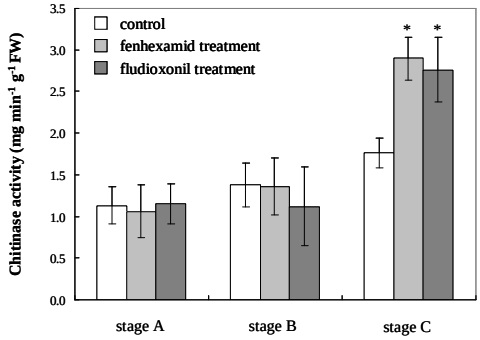

Figure 3 Chitinase activity in control and fenhexamid or fludioxonil-treated flowers (stage A) or berries (stages B and C) of grapevine, four days after treatment. Data are the mean ± standard error (n = 6). Significant differences at $P < 0.05$ between control and treated plants are marked by an asterisk

frequencies of this phenotype in the trial may indicate that the mutation F200Y induces high fitness penalty. AniR1 frequency was low and even null in most cases including the reference program. It confirms that this resistance was not dramatically selected by the pyrimethanil treatment at stage C. This resistance, whose mechanism is still unclear (Fritz *et al*., 2003) was often described as unstable, probably because of fitness penalty (Leroux *et al*., 2004). Frequency of Imi R1 strains was relatively low though slightly higher than Ben R2 and Imi R1. Since the end of the 1990's, the use of dicarboximides is reduced and is correlated with decrease in frequency of these strains (Leroux *et al*., 2006). Many alterations have been described within the *Bos1* gene, conferring resistance to dicarboximides but the most frequent mutation is I365R/S/G (Leroux *et al*., 2002; Cui *et al*., 2004, Ma *et al*., 2007, Liu *et al*., 2008). Low frequencies of Imi R1 strains in the populations, in absence of selective pressure should indicate low fitness for this phenotype. Considering resistance to hydroxyanilides, frequency of natural resistance (Hyd R1) was very low and specific resistance (Hyd R3) was null for almost all treatments. The rarity of Hyd R3 explains that no practical resistance has been recorded with fenhexamid (Leroux *et al*., 2002). MDR strains were selected after fludioxonil and fenhexamid treatments. After application of fenhexamid, levels of MDR2 were higher than MDR1. Indeed, Walker *et al*. (2006) described that MDR2 strains were rather selected by fenhexamid. Higher levels of resistance against fenhexamid are encountered among MDR2 phenotype (Table 1). On the contrary, MDR1 increase was higher than MDR2 in fludioxonil-treated plants. MDR1 stains exhibit higher levels of resistance to fludioxonil (Table 1) and should be more likely selected by fludioxonil, as already observed by Walker *et al*. (2006). This difference in the qualitative selection pressure exerted by both botryticides may be related to the kind of transporters that are overexpressed in each phenotype, and that can be more specific to fenhexamid in the case of MDR2 and to fludioxonil in the case of MDR1. MDR3 phenotype, described as a natural hybrid between MDR1 and MDR2 and cumulating the biological characteristics of both phenotypes, is still relatively rare on this plot and its selection is not so clear. Nevertheless, even if the highest frequencies of MDR phenotypes have been recorded after fludioxonil or fenhexamid application at stage A (respectively 41.7 and 31.3 % of MDR), efficacy of both fungicides is below the one achieved with the reference program but is still better than the control. This indicates that a single treatment still has a bit of efficacy when resistance occurs at these frequencies in the *Botrytis* populations, especially for fenhexamid that exhibits the best efficacies in these conditions at stage A.

Secondly, defense responses of grapevine reproductive organs have been evaluated following fenhexamid or fludioxonil application at the three tested stages. Our results showed that expression of defense-related genes was not induced following fenhexamid or fludioxonil treatment, whatever the stage of application. Only an increase in chitinase activity was noticed in berries at stage C after either fenhexamid or fludioxonil treatment. However, fungicides did not exhibit a better efficacy at this stage. It suggests that differences in fungicide efficacy seem not to be explained by a different activation of defense reactions in reproductive organs following botryticide spraying. Moreover, stress following these treatments is low for grapevine reproductive organs contrary to other fungicides on crops. Indeed, stimulation of defense responses by fungicide application has already been observed in various crops (Garcia *et al.*, 2003; Saladin & Clément, 2005). The azoxystrobin strobilurin fungicide increased the expression of PR genes in wheat (Pasquer *et al.*, 2005) and also induced some antioxidant activity in barley (Wu & von Tiedemann, 2002). A systemic triazole fungicide generated an accumulation of chitinase and β-1,3-glucanase proteins in wheat (Siefert *et al.*, 1996). Copper, which is widely used as a fungicide, enhanced the synthesis of a PR10 protein in birch (Utriainen *et al.*, 1998) and the synthesis of total phenolic compounds in berry pellicles (Coulomb *et al.*, 1999). Garcia *et al.* (2001) found an increase in leaf PAL activity as well as the accumulation of phenolics in tobacco plants treated with a benzimidazole fungicide. In grapevine, application of pyrimethanil botryticide on cuttings increased proline content, a stress indicator (Saladin *et al*., 2003). Moreover, it was observed that application of a dicarboximide fungicide in vieyard indirectly acted against *B. cinerea* by changing plant physiology (Prudet, 1994). Indeed, it was acting positively on conservation of berry inhibition ability against *B. cinerea* and delayed skin destructuring. These modifications led to a better protection of grapevine against *B. cinerea*. On the contrary, our results did not show modifications of grapevine physiology by botryticides which could indirectly act against pathogen infection.

Both factors studied on this experiment, resistance profiles of *B. cinerea* strains and physiological response of grapevine, do not explain differences of efficacy between stages of treatment and fungicides. It suggests that other parameters may influence botryticide efficacy. Firstly, efficacy of chemical control is very fluctuating according to the years. Indeed, we observed high differences of program reference efficacy between years. It means that conditions of the year highly influence treatment efficacy in vineyard. In addition, fungicide modes of action have consequences on efficacy. Fenhexamid is characterized by a high preventive activity against grey mould (Suty *et al.*, 1997) and showed a better efficacy at stage A. This stage corresponds to the end of flowering, known to be the key-step in the epidemiology of *B. cinerea* in grape (McClellan &

Hewitt, 1973; Nair *et al*., 1995; Keller *et al*., 2003; Pezet *et al.*, 2003; Viret *et al.*, 2004). Therefore, fenhexamid probably prevents flower infection and thus slow down the epidemiology of the disease in an important way. At stages B and C, efficacy of fenhexamid was largely reduced compared to stage A. At these stages, *B. cinerea* is present (quiescent or latent) inside the berry without causing disease symptoms (McClellan & Hewitt, 1973; Pezet & Pont, 1986; Nair *et al*., 1995). It means that fenhexamid seems not to be able to suppress the fungus already present inside berries which is coherent with the preventive activity of this fungicide. Therefore, in vineyard, fenhexamid is particularly well adapted for reduction of flower infection by *B. cinerea* whereas at later stages, once the fungus is present inside berries, efficacy of fenhexamid is low. On the contrary, similar efficacy of fludioxonil whatever the stage of treatment suggests a lower preventive action of this fungicide on *B. cinerea*. It means that the use of fludioxonil is less dependent on the developmental stage of grapevine.

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Publication 6

Low responsiveness of grapevine flowers and berries at fruit set to UV-C irradiation

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Low responsiveness of grapevine flowers and berries at fruit set to UV-C irradiation

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Abstract

In grapevine, stimulation of defense responses has been demonstrated using various types of abiotic stresses in both leaves and berries, as revealed by the increasing expression of genes encoding defense-related proteins or the stimulation of their corresponding activities. However, the capability of inflorescences to respond to abiotic stresses has never been investigated. Therefore, plant defense reactions in response to UV-C irradiation were followed in inflorescences and young clusters focusing on both bunchstems and developing flowers/berries from separated floral buds stage (BBCH 57) to groat-sized berries stage (BBCH 73). In this purpose, the expression of various defense-related genes encoding pathogenesis-related (PR) proteins (class I and III chitinases, *Chi1b* and *CH3*, β-1,3-glucanase, *GLUC*), phenylalanine ammonia-lyase (*PAL*) and stilbene synthase (*STS*) was analyzed in parallel with variations of chitinase activity and the accumulation of the phytoalexin resveratrol.

Multiple defense responses were induced in bunchstems of both inflorescences and clusters following UV-C treatment. Firstly, expression of genes encoding PR proteins was stimulated and chitinase activity was enhanced. Secondly, *PAL* and *STS* expression increased in association with resveratrol accumulation. Amazingly, no defense mechanism was significantly induced in grapevine flowers following UV-C exposure, whatever the stage analyzed. Similarly, in berries at fruit set, induction of gene expression was weak and neither increase in chitinase activity nor resveratrol synthesis was noticed. However, in groat-sized berries, responsiveness to UV-C increased, as revealed by the induction of *CH3*, *PAL* and *STS* expression, together with resveratrol accumulation. The differential responsiveness between bunchstems, flowers and berries is discussed.

Key words: defense responses, grapevine, inflorescences, PR proteins, resveratrol, UV-C irradiation

Introduction

In their natural environment, plants are exposed to a variety of biotic (fungi, viruses) and abiotic (drought, extreme temperatures, salinity) stresses which lead them to respond by inducing defense responses (Cheong *et al.*, 2002; Mithöfer *et al.*, 2004; Fujita *et al.*, 2006). In grapevine, two main induced defense mechanisms have been well characterized in both leaves and berries: the accumulation of pathogenesis-related (PR) proteins and the synthesis of stilbene phytoalexins. PR proteins, including chitinases and β-1,3-glucanases, have been detected following (i) abiotic stresses such as wounding, herbicide stress, UV-C irradiation or elicitor treatment (Renault *et al.*, 1996; Busam *et al.*, 1997; Jacobs *et al.*, 1999; Bonomelli *et al.*, 2004; Castro *et al.*, 2005; Belhadj *et al.*, 2006; Trotel-Aziz *et al.*, 2006) and (ii) after exposure to pathogens such as *Botrytis* cinerea, *Erysiphe necator* or *Plasmopara viticola* (Renault *et al.*, 1996; Busam *et al.*, 1997; Derckel *et al.*, 1998; Giannikis *et al.*, 1998; Jacobs *et al.*, 1999; Bézier *et*

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al., 2002; Robert *et al.*, 2002; Magnin-Robert *et al.*, 2007) in both leaves and berries. Stilbene phytoalexins also accumulate in leaves (Langcake and Pryce, 1976, 1977; Dercks and Creasy, 1989; Sbaghi *et al.*, 1995; Adrian *et al.*, 1996; Douillet-Breuil *et al.*, 1999; Bonomelli *et al.*, 2004; Borie *et al.*, 2004) and in berries (Jeandet *et al.*, 1992, 1995; Adrian *et al.*, 2000; Iriti *et al.*, 2004) following fungal infection (*B. cinerea* or *P. viticola*) or abiotic stresses (aluminium chloride, wounding, UV-C light or benzothiadiazole). Among stilbenes, resveratrol is quantitatively the major component of the grapevine response to infection or stress and its accumulation is correlated with grapevine tolerance to *B. cinerea* in both leaves and berries (Adrian *et al.*, 1997; Jeandet *et al.*, 2002). Resveratrol and its derivatives are formed *via* the phenylpropanoid/polymalonate pathway, the first step being catalyzed by phenylalanine ammonia-lyase (PAL) and the last step by stilbene synthase (STS). Genes encoding both enzymes are up-regulated in response to methyl jasmonate treatment in leaves (Belhadj *et al.*, 2006) and to *B. cinerea* infection in leaves and berries (Bézier *et al.*, 2002).

Considering flowers, defense responses are poorly documented compared with those of grapevine leaves and berries although flowering represents a key stage in infection for some pathogens such as *B. cinerea*. To date, only stilbene accumulation, especially resveratrol, has been measured in flowers after inoculation by the pathogen *B. cinerea* (Keller *et al.*, 2003). However, stilbene synthesis was not systematic in inoculated flowers and had a limited role in the inhibition of flower infection (Keller *et al.*, 2003). Infection of grapevine flowers is an important stage in the epidemiology of *B. cinerea* (Pezet and Pont, 1986; Nair *et al.*, 1995) and, therefore, preventive application of chemical fungicides is used at the end of flowering (BBCH stage 68) to impede flower infection. Considering the defense responses to abiotic stresses, no information is currently available about grapevine flowers, as well as in floral organs of other plants. However, chitinases and β-1,3-glucanases are known to be developmentally expressed in floral organs of various plants, especially in pollen tube and pistils (Lotan *et al.*, 1990; Takeda *et al.*, 2004; Liljeroth *et al.*, 2005). They may thus be involved in the process of reproduction rather than in the defense mechanisms.

Therefore, our objective was to assay defense regulation in grapevine inflorescences in response to UV-C irradiation, an abiotic stress that efficiently up-regulates defense responses in grapevine leaves and berries (Adrian *et al.*, 2000; Bonomelli *et al.*, 2004). Particular attention was paid to the end of flowering (BBCH stage 68), a key stage in the *B. cinerea* epidemiology and on the surrounding stages from BBCH stage 57 (separated floral buds) to 73 (groat-sized berries), using fruiting cuttings of Pinot Meunier (*Vitis vinifera* L). Defense mechanisms were measured both in bunchstems and flowers/berries of inflorescences/clusters, focusing on (i) expression of various defense-related genes coding for PR proteins and proteins related to resveratrol synthesis, (ii) chitinase activity and (iii) resveratrol contents.

Material and Methods

Plant material

Fruiting cuttings of *Vitis vinifera* L. (cv. Pinot Meunier) were obtained from canes of grapevine according to the protocol improved by Lebon *et al.* (2005). Cuttings were planted in 300 ml pots containing a perlite/sand mixture (1:2, v/v) and transferred to a growth chamber under a temperature of 25°C (day/night), at a relative humidity of 60% (day/night) and a 16 h photoperiod (400 µmol photons m^{-2} s^{-1}). Plants were daily irrigated with a complete mineral nutrient solution containing nitrate and ammonium (Coïc and Lesaint, 1971). Five stages of development were identified according to the BBCH scale (Meier, 2001) and surrounding the BBCH stage 68: separated floral buds (57), 20% of caps fallen (62), 80% of caps fallen (68), fruit set (71) and groat-sized berries stages (73). Defense mechanisms were determined in the bunchstem tissue, flowers and small berries. Bunchstem corresponds to both the peduncle, which is the stem of the panicle, including the central axis and the stem of the laterals, and the pedicels (Jackson and Coombe, 1995).

UV-C irradiation

Grapevine inflorescences or clusters were irradiated using a UV-C lamp (254 nm, Vilber Lourmat, Model VL-6.C, output 7.1 $W.cm^{-2}$, 15 cm distant) for 6 min (3 min on each side of the inflorescence/cluster). Then cuttings were maintained in growth chamber until sampling. Controls consisted in non-irradiated plants.

RNA extraction

Inflorescences or clusters were collected 24 h after UV-C irradiation. Bunchstem was immediately separated from flowers/berries then frozen in liquid nitrogen and stored at -80°C. Samples (8 pooled plants) were ground in liquid nitrogen to a fine powder. One hundred mg of powder were used for total RNA extraction and homogenized in extraction buffer (Plant Purification RNA Reagent, Invitrogen, France), according to the manufacturer's instructions. The RNA pellet was resuspended in 20 µl of RNase-free water and quantified by absorbance at 260 nm.

Real-time RT-PCR analysis

One hundred and fifty ng of total RNA were reverse-transcribed using M-MLV reverse-transcriptase (Invitrogen, France) according to the manufacturer's protocol. PCR conditions were described in Bézier *et al.* (2002). The reaction was carried out in duplicate data in a GeneAmp 5700 sequence detection system (Applied Biosystems,

France) using the following thermal profile: 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension) for 40 cycles. To calculate the copy number for each sample, standard curves were generated by performing real-time PCR on serial dilutions of specific purified DNA. The standard curves were then constructed by plotting the Ct (threshold cycle) values *vs* the logarithm of the copy number of purified PCR products. The mRNA copy number of each sample was calculated from the standard curve using its Ct value and corrected by normalisation against *EF1α* mRNA (Terrier *et al.*, 2005). The results were expressed as mRNA copy number/10,000 *EF1α* mRNA. In addition, induction factor following UV-C stress was calculated: control sample (non-irradiated inflorescence or cluster) was chosen to represent 1x expression of genes. Expression of five defense-related genes encoding class I (*Chi1b*) and class III (*CH3*) chitinases, a β-1,3-glucanase (*GLUC*), a phenylalanine ammonia-lyase (*PAL*), and a stilbene synthase (*STS*) were tracked (Bonomelli *et al.*, 2004).

Chitinase extraction and activity

Inflorescences/clusters of 8 different non-pooled plants were collected 96 h after UV-C irradiation and bunchstems were separated from flowers/berries. Proteins were extracted from each sample (independent bunchstems and flowers/berries) by homogenizing ground frozen collected samples (250 mg fresh weight) at 4°C in 1 ml of 50 mM sodium acetate buffer, pH 5.0 containing 1 mM dithiothreitol and 1% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 10,000 × *g* for 5 min at 4°C and the clarified supernatant was recovered. Chitinase activity was assayed using a commercial blue enzyme substrate, CM-chitin-RBV solution (Loewe Biochemica, Germany) according to Magnin-Robert *et al.* (2007). Measurements were conducted in triplicate. Results were expressed in mg min^{-1} g^{-1} fresh weight (FW) in both bunchstems and flowers/berries.

Identification and quantification of stilbenes

Twenty-four hours following UV-C exposure, inflorescences/clusters were examined under long wavelength UV-light. Resveratrol and the biosynthetically related oligomers viniferins are very easily detected at 365 nm since they give characteristic bright blue fluorescence (Langcake and Pryce, 1976). This method thus provides a rapid qualitative assessment of the inflorescence/cluster response to induction. Quantification of stilbenes was performed by high performance liquid chromatography (HPLC) (Jeandet *et al.*, 1997). Briefly, bunchstem and flowers/berries were separated then ground individually in a mortar with sand and 10 ml of methanol-water (8:2, v/v). Extracts were prepared according to Jeandet *et al.* (1991). Each extract was filtered and kept at -80°C until HPLC analysis.

Statistical analysis

All the results were obtained from two completely replicated experiments. Results presented are data obtained from one experiment.

To determine whether chitinase activity and resveratrol accumulation of control plants were significantly different from UV-C treated plants, analysis of variance (ANOVA) followed by a Student's *t* test were used. Differences at $P < 0.05$ were considered as significant.

Results

Expression of grapevine defense-related genes

The basal level of gene expression fluctuated both in bunchstems and flowers/berries along the development from separated floral buds (57) to groat-sized berries (73) stage (Fig. 1, control) but was generally higher in flowers and berries than in bunchstems, especially at stages 62 and 68. For example, at stage 68, basal levels of gene expression were 7- to 13-fold higher in flowers than in bunchstems, except for *GLUC* which exhibited a strong discrepancy (up to 340-fold) between both organs. In bunchstems, the lowest basal level was noticed at stage 68 whatever the gene analyzed and the highest basal level was recorded at stage 71, except for *GLUC* which was maximum at stage 73. The variation between the lowest and the highest level of gene expression fluctuated from 3- to 17-fold, except for *GLUC* (130-fold). In flowers/berries, basal level fluctuation depended on the gene. Basal level of *Chit1b* reached a 6-fold higher expression at stage 62 and then decreased 2 fold at stage 73. The *CH*3 expression profile remained constant from stage 57 to 71 and then decreased 2 fold at stage 73. Considering *GLUC*, a steady 20-fold increase of expression was observed from stages 57 to 71 and was followed by a 7-fold decrease at stage 73. On the contrary, basal level of *PAL* and *STS* expression peaked 6-7 fold at stage 62 and then progressively decreased until stage 73 corresponding to a level, respectively, 4- and 2-fold lower compared with the level measured at stage 57.

Following UV-C exposure, highest transcript accumulations were measured in bunchstems, except for *GLUC* which were in flowers/berries (Fig. 1, UV). A strong discrepancy in the induction of gene expression between bunchstems and flowers/berries was observed (Fig. 2). In bunchstems, levels of gene expression increased to reach a maximal induction at stage 68, except for *PAL* (stage 62, Fig. 2A). The highest induction was observed for *STS* and was 209-fold over the basal level while the lowest induction was measured for *Chi1b*, though it reached 25-fold compared to the basal level. Then levels of gene expression decreased until stage 73 for *Chit1b* and

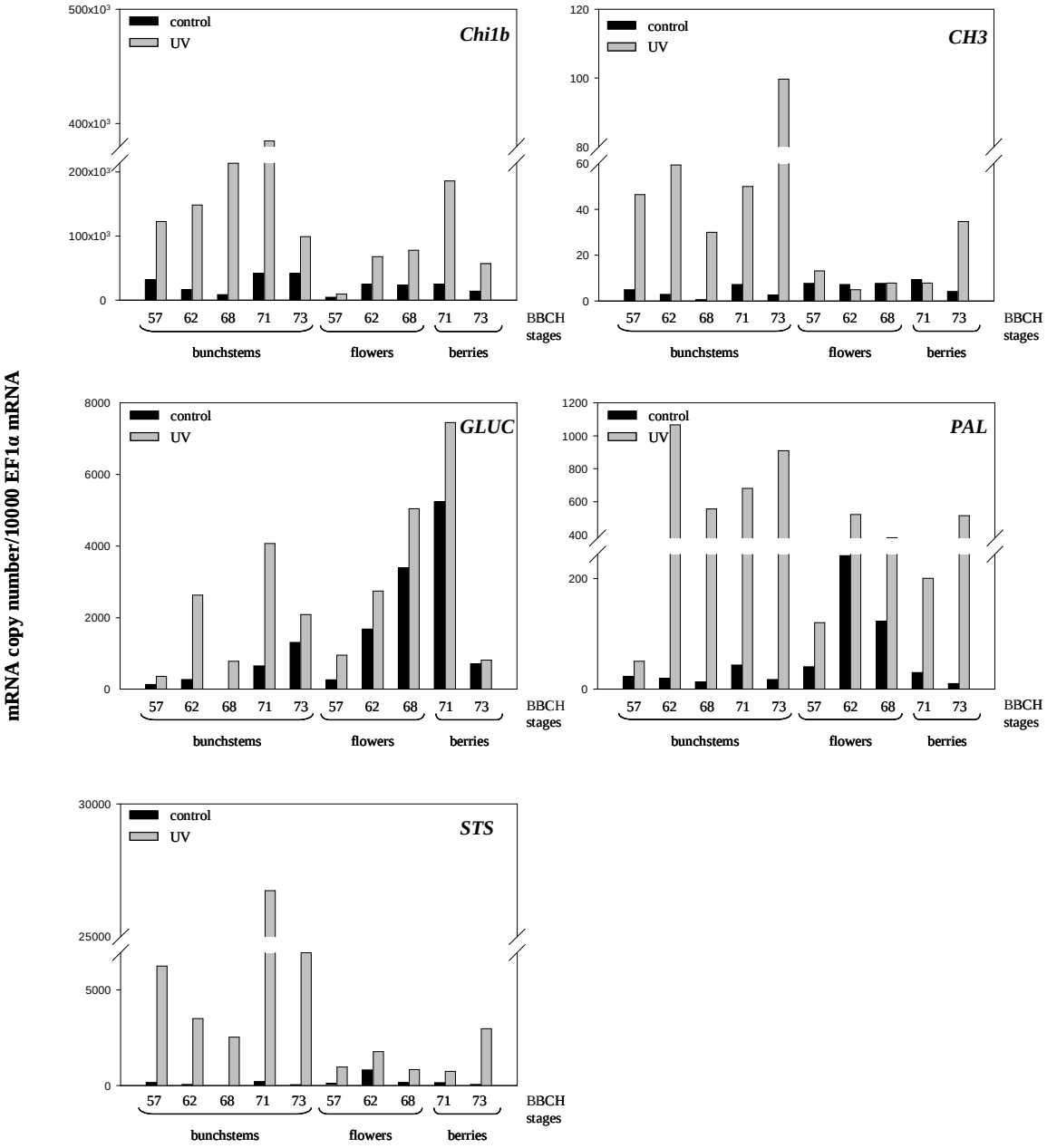

Fig. 1. Accumulation of *Chi1b*, *CH3*, *GLUC*, *PAL* and *STS* transcripts in bunchstems and flowers/berries of control and UV-C treated inflorescences/clusters from separated floral buds stage (BBCH 57) to groat-sized berries stage (BBCH 73), 24 h after treatment. Results are means of duplicate data of one representative experiment out of two.

GLUC. On the contrary, considering *CH3*, *PAL* and *STS* expression, a decrease was observed at stage 71 but was followed by an increase at stage 73. Considering flowers and berries at fruit set (Fig. 2B, stages 57 to 71), no significant induction of gene expression was globally measured compared to the basal level, except for *Chit1b*, *PAL* and *STS* expression for which a low induction of 6-8 fold was measured at stages 57 or 71. On the contrary, in groat-sized berries (Fig. 2B, stage 73), a strong induction of expression was recorded for *PAL* and *STS* corresponding respectively to 50- and 61-fold compared to the basal level, while *CH3* expression increased 8-fold.

Chitinase activity

A similar basal chitinase activity wavering between 0.4 and 1.4 mg min^{-1} g^{-1} FW was measured in control plants whatever the organ and developmental stage (Fig. 3, control). After UV-C exposure, chitinase activity increased from 4 to 13-fold in bunchstems (Fig. 3, UV, lanes S), which was coherent with induction of *Chi1b* and *CH3* expression in UV-C treated bunchstems (Fig. 2A). In flowers and berries, the basal chitinase activity remained constant following UV-C exposure (Fig. 3, UV, lanes F and B)

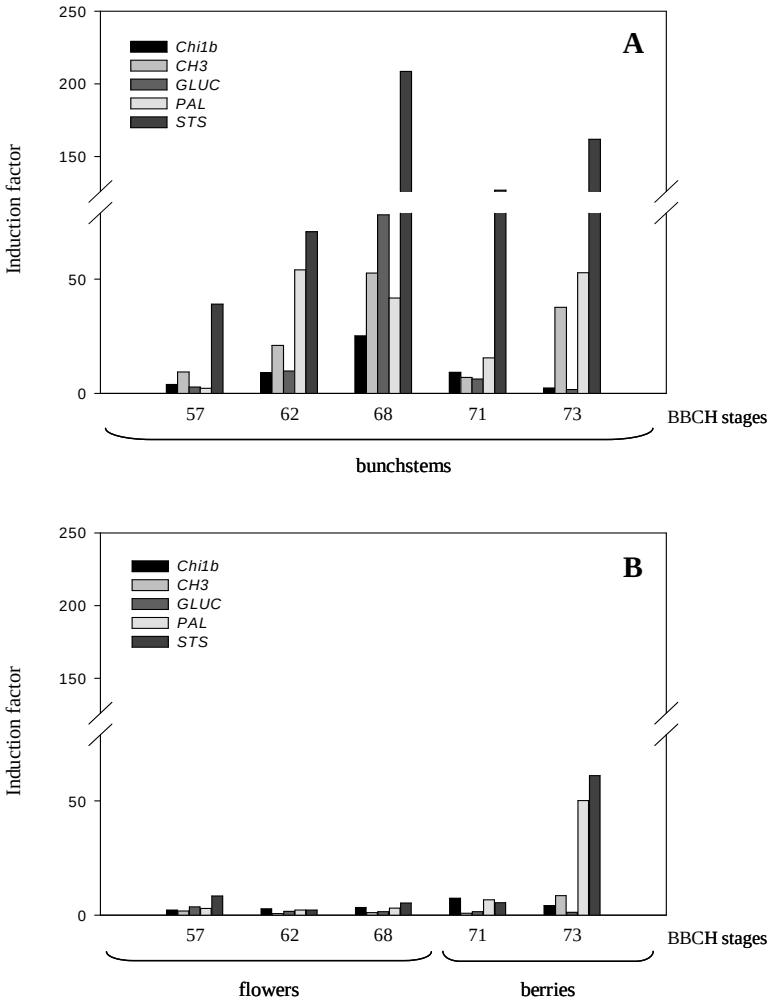

Fig. 2. Induction factor of *Chi1b*, *CH3*, *GLUC*, *PAL* and *STS* in bunchstems (A) and flowers/berries (B) of UV-C treated inflorescences/clusters from separated floral buds stage (BBCH 57) to groat-sized berries stage (BBCH 73), 24 h after treatment. Bunchstems and flowers/berries of control inflorescences/clusters were defined as 1x expression level for each analyzed gene. Results are means of duplicate data of one representative experiment out of two.

despite a low induction of *Chi1b* or *CH3* expression (Fig. 2B).

Resveratrol accumulation

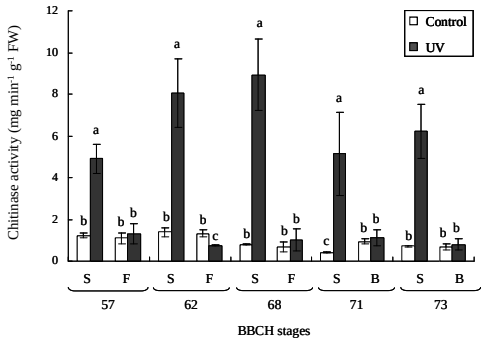

Fig. 3. Chitinase activity in bunchstems (S) or flowers/berries (F/B) of control and UV-C treated inflorescences/clusters from separated floral buds stage (BBCH 57) to groat-sized berries stage (BBCH 73), 96 h after treatment. Data are means from two independent measurements, each consisting of eight replicate. At each BBCH stage, means with the same letter were not significantly different ($P < 0.05$).

When observed under long UV, inflorescences showed a slight natural fluorescence (Fig. 4A) which was not visible in clusters at the groat-sized berries stage (Fig. 4C). When analyzed by HPLC, no compound with retention time similar to resveratrol or viniferins was identified in extracts from control flowers, young berries or bunchstems (data not shown).

Exposure to UV-C light induced phenolic compound accumulation. When observed under long UV, bunchstems of UV-C treated inflorescences or clusters exhibited a bright deep blue fluorescence (Figs. 4B, D). This fluorescence was also clearly detected in groat-sized berries (Fig. 4D) whereas it was low in flowers (Fig. 4B). When analyzed by HPLC, resveratrol was measured in UV-C treated bunchstems and groat-sized berries but was undetectable in flowers and berries at fruit set. No viniferin was identified in any organ, whatever the considered developmental stage. Resveratrol accumulation in UV-C treated bunchstems varied between 6 and 16 µg g^{-1} FW according to the stage of

Table 1. Resveratrol accumulation (µg g^{-1} FW) in bunchstems and flowers/berries of control and UV-C treated inflorescences/clusters (24 h after treatment) from separated floral buds stage (BBCH 57) to groat-sized berries stage (BBCH 73). Data are means from two independent measurements, each consisting of three replicate. Means were not significantly different, when followed by the same letter ($P < 0.05$).

BBCH stages	bunchstems		flowers/berries	
	control	UV	control	UV
57	0.0^{b}	15.5^{a}	0.0^{b}	0.0^{b}
62	0.0^{b}	8.5^{a}	0.0^{b}	0.0^{b}
68	0.0^{b}	13.6^{a}	0.0^{b}	0.0^{b}
71	0.0^{b}	6.1^{a}	0.0^{b}	0.0^{b}
73	0.0^{b}	12.9^{a}	0.0^{b}	15.2^{a}

development which was similar to the accumulation measured in treated berries at stage 73 (Table 1). These results are coherent with expression of *PAL* and *STS* whose induction was observed in bunchstems and young berries contrary to flowers (Fig. 2).

Discussion

UV-C exposure is long known as an inductor of defense responses in grapevine leaves (Langcake and Pryce, 1977; Jeandet *et al.*, 1992; Sbaghi *et al.*, 1995; Douillet-Breuil *et al.*, 1999; Bonomelli *et al.*, 2004; Borie *et al.*, 2004) and berries (Adrian *et al.*, 2000; Bais *et al.*, 2000), but no information is available concerning inflorescence responsiveness. Our study clearly demonstrates that flowers and berries at fruit set do not respond significantly to UV-C exposure by enhancement of defense mechanisms, contrary to bunchstems and groat-sized berries.

Considering healthy non-treated plants, results showed that all defense-related genes exhibited a basal level of expression which fluctuated both in bunchstems and flowers/berries from separated floral buds (57) to the groat-sized berries (73), and was generally higher in flowers and berries. Previous studies on grapevine also reported that a class IV chitinase was expressed in a flower- and berry-specific manner (Robinson *et al.*, 1997) and that *PAL* maximum expression occurred in flowers and then decreased in berries (Boss *et al.*, 1996). Otherwise, no information is available concerning the basal level of defense-related genes in grapevine floral organs. *PAL* basal level of expression correlated with those measured by Boss *et al.* (1996) in flowers and young berries. Considering *GLUC*, basal level of expression gradually increased in flowers to reach a high level, much more greater than in bunchstems. Increase was shown to be less dramatic when considering *Chit1b* whereas basal level of *CH3* expression remained stable. In other plants, previous studies have revealed that chitinases and β-1,3-glucanases are highly expressed in floral organs such as pistils or stamens of healthy plants and may constitute a natural defense system against pathogen infection at flowering (Harikrishna *et al.*, 1996; Takakura *et al.*, 2000; Liljeroth *et al.*, 2005). Regarding the level of transcript accumulation, the glucanase analysed in this study could have such a role in grapevine flowers. It is also interesting to notice that the basal level of *GLUC* expression still increased in berries at fruit set to reach its maximal level and then suddenly dropped in groat-sized berries. Thus, glucanase could also have a protective role for the very young berry. Although chitinases and glucanases were primarily thought to play a role in the protection of flowers, they might also have non-defense functions (Lotan *et al.*, 1989; Ori *et al.*, 1990; Harikrishna *et al.*, 1996; Takakura *et al.*, 2000; Takeda *et al.*, 2004; Liljeroth *et al.*, 2005). β-1,3-glucanases may notably be involved in facilitating pollen tube extension by their cell wall hydrolyzing activities in the female tissue when the pollen tube grows (Lotan *et al.*, 1989; Ori *et al.*, 1990; Harikrishna *et al.*, 1996). Since gene expression was measured in whole flowers, cellular localization of the glucanase transcript during the whole flower development (stages 53 to 68) could determine spatio-temporal expression of this gene and clarify a putative role of glucanase in grapevine reproductive development.

Following UV-C exposure, an induction of genes encoding chitinases (*Chi1b*, *CH3*), a glucanase

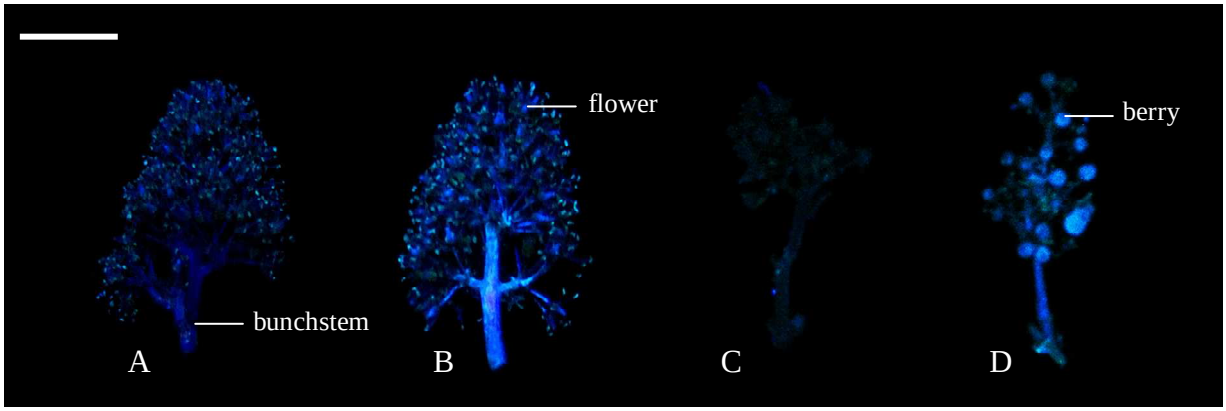

Fig. 4. Inflorescence (a, b) (BBCH 68) and cluster (c, d) (BBCH 73) observed under UV-light (365 nm) 24 h after UV-C irradiation at 254 nm for 6 min (scale bar = 1.3 cm). Controls consisted in non-irradiated inflorescences (a) or clusters (c). A bright blue fluorescence characteristic of stilbenes was observed in bunchstems (B, D) and berries (D) after UV-C treatment. Sixteen irradiated and non-irradiated plants were observed..

(*GLUC*), *PAL* and *STS*, as well as an increase in chitinase activity and resveratrol accumulation was observed in bunchstems. Bunchstems seem to behave like leaves, vegetative organs. Indeed, previous studies reported resveratrol accumulation which correlated with *STS* mRNA production, as well as induction of PR gene expression, chitinase and β-1,3-glucanase enzyme activities in grapevine UV-C treated leaves (Bonomelli *et al.*, 2004; Borie *et al.*, 2004). Contrary to these organs, no significant defense induction (changes in the level of gene expression, chitinase activity or resveratrol accumulation) was observed in UV-C treated flowers. Nevertheless, both control and UV-C treated flowers exhibited a low fluorescence when observed under long wavelength UV-light. Such fluorescence could be explained by the autofluorescence of some floral parts such as pollen exine (Abreu and Oliveira, 2004) and may not be related to defense responses. Higher basal level of gene expression was noticed in flowers compared to bunchstems. However, this may not account for the differences of responsiveness observed between these two organs, because after UV-C stress, the accumulation of transcripts was much higher in bunchstems than in flowers, except for *GLUC*. Considering the particular basal level regulation of *GLUC*, it can be hypothesized that the glucanase behaves like a PR-like protein in flowers and therefore is not significantly regulated by stress (van Loon *et al*., 2006). No study has investigated the regulation of defenses in floral tissues of grapevine or other plants following abiotic stresses. Nevertheless, Keller *et al*. (2003) reported a low accumulation of stilbenes, especially resveratrol, in grapevine flowers following *B. cinerea* infection. However, resveratrol accumulation was not systematic and many inoculated flowers failed to produce stilbenes. The authors hypothesized that the high susceptibility of grape flowers to *B. cinerea* may be partially related to their poor ability for stilbene synthesis. Our results confirm that flowers also exhibit a poor capacity to induce their different defense mechanisms in response to an abiotic stress. Low responsiveness of flowers could be explained by activation of other mechanisms than defense functions. Indeed, formation of reproductive organs, pollination and fertilization are complex processes characterized by various peculiar events such as organogenesis, differential cell division, and variations in gene expression (Gasser, 1991).

Like in flowers, we observed that no defense mechanisms were significantly induced in berries at fruit set (stage 71) following UV-C stress. On the contrary, groat-sized berries (stage 73) became sensitive to UV-C, as revealed by an induction of some defense responses. Differences of responsiveness between berries at stages 71 and 73 may be explained by changes in biosynthetic pathways such as stilbene and flavonoid pathways. Indeed, resveratrol derives from the same pathway and shares common precursors with flavonoids. Besides it was suggested that stilbene synthase may compete with chalcone synthase, the key enzyme of flavonoid biosynthesis (Hrazdina and Wagner, 1985). Therefore, the increase in resveratrol accumulation in berries after stage 71, as reported here, may be related to a decrease in the concentration of flavonols, as previously descried by Downey *et al.* (2003). In groat-sized berries, a weak *CH3* induction was observed but was not related to any increase in chitinase activity. In grapevine, chitinases exist as multiple isoforms (Derckel *et al.*, 1996; Jacobs *et al.*, 1999; Robert *et al.*, 2002 ; Bézier *et al.*, 2007). Measurement of chitinase activity in a crude protein extract that corresponds to the measure of all chitinase activities could explain the lack of correlation observed between *CH3* gene expression and chitinase activity. In addition to *CH3* induction, a stimulation of *PAL* and *STS* expression and resveratrol synthesis was recorded in berries at stage 73. Resveratrol accumulation has previously been characterized in berries exposed to UV-C at later stages (Jeandet *et al.*, 1991; Bais *et al.*, 2000). The stilbene phytoalexin content was shown to fluctuate along berry development as resveratrol synthesis increased during the first weeks of berry development and then dramatically declined at maturity (Jeandet *et al.*, 1991; Bais *et al.*, 2000).

To conclude, differences in the responsiveness to UV-C irradiation have been observed between bunchstems and flowers of grapevine inflorescences. Indeed, no significant induction of defenses was observed in flowers whereas a high induction was noticed in bunchstems. It could be interesting to study other biotic and abiotic stresses to determine whether flowers are finally no responsive organs whatever the stress considered. The low responsiveness of ripened berries (Jeandet *et al.*, 1991) and flowers to stresses may be one of factors contributing to the high susceptibility of these organs to *B. cinerea*.

Abbreviations: CH3, class III chitinase; Chi1b, class I chitinase; Ct, threshold cycle; FW, fresh weight; GLUC, β-1,3-glucanase; HPLC, high performance liquid chromatography; PAL phenylalanine ammonia-lyase; PR, pathogenesis-related; STS stilbene synthase.

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Différents facteurs pouvant expliquer les différences d'efficacité des fongicides anti-*Botrytis* au vignoble ont été analysés :

- facteur 1 : le fongicide utilisé ;
- facteur 2 : le stade de traitement ;
- facteur 3 : la résistance du pathogène aux fongicides ;
- facteur 4 : l'effet éliciteur des fongicides.

Pour étudier le premier facteur, l'efficacité de deux fongicides, le fludioxonil et le fenhexamid, a été évaluée. Ces fongicides appartiennent à deux familles chimiques différentes et leur mode d'action sur *B. cinerea* est également différent. En parallèle, ces fongicides ont été appliqués aux stades A, B ou C afin de déterminer la contribution de chaque stade de traitement dans le programme de référence à trois traitements, ce qui correspond au second facteur. Les résultats obtenus sont différents selon le fongicide utilisé. Le fenhexamid provoque une réduction significative de l'intensité et de la fréquence de la maladie uniquement lorsqu'il est appliqué au stade A. Pour le fludioxonil, une diminution similaire de la maladie est observée à tous les stades de traitement mais son efficacité est inférieure à celle du fenhexamid au stade A. Ainsi, le mode d'action du fongicide et le stade de traitement interviennent dans l'efficacité du traitement anti-*Botrytis*.

Afin d'étudier le troisième facteur, les profils de résistance de *B. cinerea* aux fongicides ont été déterminés. Cette étude a montré que les fréquences de souches résistantes aux benzimidazoles, anilinopyrimidines et dicarboximides sont identiques pour les plantes traitées et non-traitées. De plus, il n'y aucune différence en fonction du fongicide et du stade de traitement. Pour les souches MDR, une plus grande fréquence est notée suite aux traitements anti-*Botrytis*, particulièrement au stade A. Pourtant, la présence importante de ces souches n'empêche pas une certaine efficacité des fongicides. Ces résultats montrent que la pression de sélection exercée par les traitements sur *B. cinerea* influence faiblement l'efficacité des fongicides.

Le dernier facteur évalué est l'effet éliciteur des fongicides sur la vigne. En effet, la différence d'efficacité des fongicides pourrait ainsi être liée à une capacité différente des organes reproducteurs à activer des réponses de défense suite aux traitements. L'expression de gènes intervenant dans différentes réponses de défense (*PAL*, *LOX*, *Chi4C*, *GLUC* et *PR6*) n'est pas induite quelque soit le stade de traitement ou le fongicide. Seule une augmentation de l'activité chitinase est observée au stade C pour les deux fongicides. L'efficacité des fongicides ne semble donc pas être liée à une activation différentielle des réponses de défense de la plante selon le stade de traitement ou le fongicide utilisé. De plus, ces résultats montrent

que le stress causé par l'application des fongicides anti-*Botrytis* sur les organes reproducteurs est faible.

Les fongicides anti-*Botrytis* n'activent pas de réponses de défense dans les fleurs au stade A. Afin de déterminer si cela était dû uniquement à l'action peu stressante des fongicides ou à la faible sensibilité des fleurs aux stress abiotiques, nous avons appliqué des UV-C sur des inflorescences. En effet, les UV-C sont connus pour induire des réponses de défense à la fois dans les feuilles et les baies de vigne (Adrian *et al.*, 2000 ; Bonomelli *et al.*, 2004). Différentes réponses de défense ont été analysées à plusieurs stades de développement encadrant le stade A (stades BBCH 57 à 73), à la fois dans les rafles et les fleurs ou jeunes baies. L'expression de gènes codant plusieurs protéines PR (des chitinases, *Chi1b* et *CH3*, une β-1,3-glucanase, *GLUC*), une phénylalanine ammonia-lyase (*PAL*) et une stilbène synthase (*STS*) a été analysée en parallèle des variations de l'activité chitinase et de l'accumulation de resvératrol. Dans les fleurs et les baies, le taux de base de l'expression des gènes est généralement plus élevé que dans les rafles, particulièrement pour le gène *GLUC*. Suite à l'application des UV-C, l'induction des réponses de défense est nettement supérieure dans les rafles comparée aux fleurs et aux baies nouées (stades 57 à 71). Quand les baies atteignent le stade grenaille (73), une augmentation de l'expression des gènes *CH3*, *PAL* et *STS* est observée, en relation avec une accumulation de resvératrol. Contrairement aux autres organes de la vigne, les fleurs présentent donc une faible sensibilité aux UV-C ce qui suggère que les fleurs ont une capacité réduite à activer des réponses de défense suite aux stress abiotiques.

CONCLUSIONS

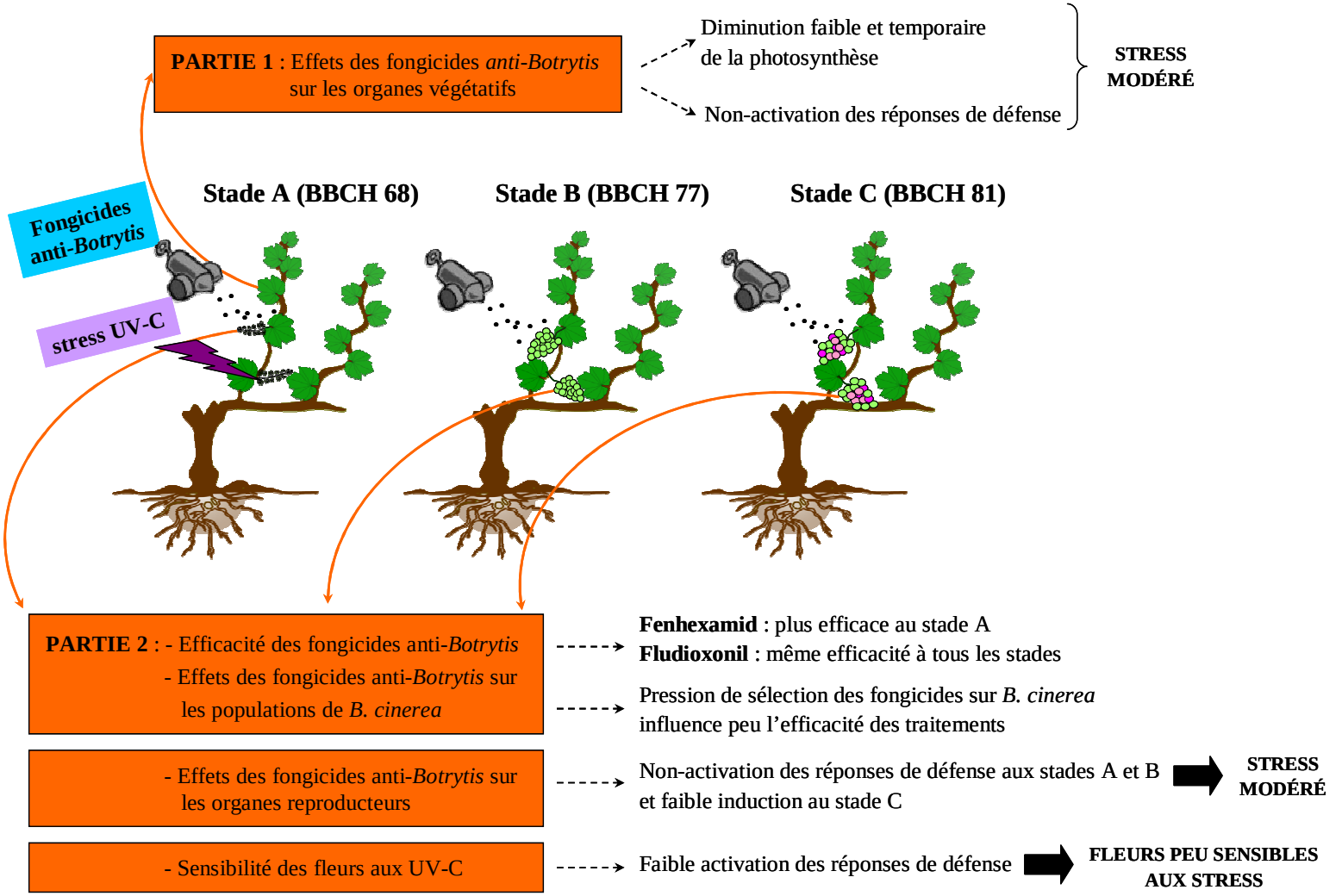

Figure 18. Effets des fongicides anti-*Botrytis* sur les organes végétatifs et reproducteurs de la vigne et sensibilité des fleurs.

Notre étude a révélé que les fongicides anti-*Botrytis*, le fludioxonil et le fenhexamid, couramment utilisés en viticulture, provoquent un stress modéré pour la vigne (Figure 18). En effet, ces fongicides génèrent une diminution faible et temporaire de la photosynthèse foliaire dans les organes végétatifs (stade A), sans activer de mécanismes de défense. Dans les organes reproducteurs, les réponses de défense ne sont pas activées lors des traitements aux stades A et B mais sont faiblement induites au stade C. L'application d'UV-C sur fleurs au stade A a montré en outre que ces organes sont peu sensibles aux stress.

PARTIE 1 : effets des fongicides anti-*Botrytis* sur les organes végétatifs

1. Diminution de la photosynthèse foliaire suite aux traitements

Les deux fongicides anti-*Botrytis*, le fludioxonil et le fenhexamid, diminuent la photosynthèse foliaire lors de leur application au stade A. Bien qu'appartenant à des familles chimiques différentes, ces fongicides ont altéré la photosynthèse de façon similaire.

Au vignoble, les deux fongicides provoquent une **réduction temporaire de la photosynthèse nette** des cépages Pinot noir et Pinot Meunier (publications 2 et 3). Cette réduction est comparable pour les deux cépages, toujours inférieure à 25% et attribuée principalement à une limitation non-stomatique. De précédents travaux ont déjà montré une diminution de la photosynthèse des plantes cultivées lors de l'application de fongicides appartenant à d'autres familles chimiques (Benton & Cobb, 1997 ; Alaoui-Sossé *et al.*, 2004 ; Untiedt & Blanke, 2004 ; Xia *et al.*, 2006). Une altération de l'activité du PSII n'est pas impliquée dans la diminution de photosynthèse suite aux traitements anti-*Botrytis*. Une diminution de cette activité, à l'origine d'une réduction de la photosynthèse, a pourtant déjà été observée lors d'autres traitements fongicides (Krugh & Miles, 1996 ; Xia *et al.*, 2006).

Les fongicides fludioxonil et fenhexamid provoquent la **répression de certains gènes codant des protéines impliquées dans la photosynthèse**, ce qui n'a jamais été évalué auparavant. Concernant le Pinot noir, une répression de gènes codant une protéine psbP du PSII du complexe producteur d'oxygène (*PsbP1*), une protéine située dans le complexe collecteur de lumière du PSI (*cab*) et la petite sous-unité de la Rubisco (*rbcS*) a été observée. La répression du gène *psbP1* est bien corrélée à l'altération du PSII, comme précédemment décrit par Ifuku *et al*. (2005) mais cette altération n'a pas d'impact direct sur la photosynthèse. Toutefois, la répression des gènes *cab* et *rbcS* pourrait conduire à une diminution dans la collecte de l'énergie lumineuse et de la quantité de Rubisco, à l'origine d'une diminution de la photosynthèse. En effet, une répression de ces gènes reliée à une diminution de la

photosynthèse nette a déjà été mise en évidence lors de l'application de divers stress abiotiques (Bahl & Kahl, 1995 ; Conklin & Last 1995 ; Glick *et al*., 1995 ; A-H Mackerness *et al.*, 1997 ; Seki *et al.*, 2002). Pour le Pinot Meunier, les gènes codant la synthèse de la petite et de la grosse sous-unité de la Rubisco sont réprimés, ce qui provoque une réduction de la quantité de Rubisco et donc de la photosynthèse.

Les effets de différentes concentrations de fludioxonil sur la photosynthèse du Pinot noir a révélé que les **impacts sur le processus photosynthétique différent selon la concentration de fongicide** (publication 4). Cela a déjà été observé pour d'autres fongicides (Garcia *et al*., 2002 ; Tort & Türkyilmaz, 2003). La concentration la plus faible (1,2 mM) diminue la photosynthèse d'environ 30% dès le premier jour après traitement. La pulvérisation d'eau induit les mêmes effets. Ainsi, cette réduction serait davantage liée à l'humidité provoquée par l'application du fongicide que par la toxicité de la matière active (Hanba *et al*., 2004). Sept jours après traitement, les concentrations 6 et 30 mM réduisent la photosynthèse de respectivement 38 et 65%. Pour la concentration appliquée au vignoble (6 mM), cette réduction est corrélée à une augmentation du point de compensation lumineux. Ce dernier correspond à l'intensité lumineuse minimale requise afin que la photosynthèse apparente devienne positive et permette à la plante d'assurer sa croissance. Pour la concentration la plus forte (30 mM), la diminution de photosynthèse est liée à une augmentation de la respiration à la lumière. Toutefois, les modifications de la photosynthèse ne sont que ponctuelles et le **recouvrement** est atteint dès le 10ème jour après traitement, quelque soit la concentration de fludioxonil testée, traduisant une faible toxicité du fongicide sur la vigne.

2. Réponses de défense non activées dans les feuilles

Ni l'expression de gènes de défense, ni l'activité chitinase ne sont modifiées suite aux traitements anti-*Botrytis* dans les feuilles de Pinot noir (publication 2). Une diminution de la photosynthèse associée à une activation des réponses de défense a pourtant déjà été observée chez des plantes en conditions de stress (A-H-Mackerness *et al.*, 1997 ; Berger *et al.*, 2004 ; Bonfig *et al.*, 2006). De plus, certains fongicides sont connus pour activer des réponses de défense chez les plantes cultivées (Siefert *et al.*, 1996 ; Wu & von Tiedemann, 2002 ; Pasquer *et al*., 2005). L'ensemble de ces données suggère une **faible sensibilité de la vigne aux fongicides anti-*Botrytis***.

Nos résultats ont donc montré que l'application de fludioxonil et de fenhexamid au stade A provoque une diminution de la photosynthèse foliaire. Bien que les deux fongicides testés

appartiennent à des familles chimiques différentes, les effets sur la photosynthèse de la vigne sont similaires. La perturbation de la photosynthèse peut en partie être expliquée par la répression de certains gènes codant des protéines impliquées dans la photosynthèse. Notre étude a également révélé que les mécanismes qui conduisent à la réduction de photosynthèse diffèrent selon la concentration de fongicide. **Enfin, l'ensemble de nos résultats suggère que l'application des fongicides anti-*Botrytis* provoque un stress modéré pour la vigne. En effet, la photosynthèse diminue faiblement et de façon temporaire et les réponses de défense ne sont pas activées.**

PARTIE 2 : effets des fongicides anti-*Botrytis* sur les organes reproducteurs et sensibilité des fleurs aux stress

1. Effets des fongicides anti-*Botrytis* sur les populations de *B. cinerea* et sur les réponses de défense de la vigne

L'étude sur le Pinot Meunier de **l'efficacité du fludioxonil et du fenhexamid en fonction du stade d'application a révélé des différences selon le fongicide utilisé** (publication 5). Le fenhexamid présente une efficacité importante lorsqu'il est appliqué au stade A, conduisant à une diminution de l'intensité et de la fréquence de la maladie de respectivement 50 et 30%. Toutefois, aucune diminution significative de ces paramètres n'est observée lors des traitements au fenhexamid aux stades B et C. Concernant le fludioxonil, il diminue l'intensité de la maladie de 25% à tous les stades de traitement et seul le traitement au stade B permet une diminution de la fréquence de 25%. En résumé, le fenhexamid semble donc procurer une meilleure efficacité contre la pourriture grise mais uniquement lorsqu'il est utilisé au stade A.

L'étude des profils de résistance des souches de *B. cinerea* aux fongicides n'a pas permis d'interpréter les différences d'efficacité. En effet, **les profils de résistance sont semblables quelque soient les fongicides ou les stades de traitement**. L'expression de gènes de défense codant la PAL et plusieurs protéines PR a été suivie dans les organes reproducteurs suite aux traitements anti-*Botrytis* aux trois stades. **Aucun des gènes analysés n'a présenté une augmentation de son expression, quelque soient le stade et le fongicide appliqué**. Une induction de l'expression de gènes codant des protéines PR et une augmentation de l'activité de ces protéines et de la PAL ont pourtant été identifiées lors de l'utilisation de fongicides sur des plantes cultivées (Sierfert *et al*., 1996 ; Utriainen *et al*.,

1998 ; Garcia *et al*., 2001 ; Pasquer *et al*., 2005). Toutefois, nous avons observé au stade C une augmentation de l'activité chitinase dans les baies, après traitements au fludioxonil et au fenhexamid. Les baies au stade C semblent donc être plus sensibles aux fongicides anti-*Botrytis* que les fleurs et les baies aux stades A et B. Néanmoins, comme l'expression d'aucun gène de défense suivi n'a été induite, cela traduit encore une fois un **stress modéré suite à l'application des fongicides anti-*Botrytis*.**

D'autres facteurs doivent donc être à l'origine des différences d'efficacité observées. La spécificité des fongicides pourrait notamment expliquer ces différences. En effet, le fenhexamid est caractérisé par une action préventive importante contre la pourriture grise (Suty *et al.*, 1997). Ce fongicide est très efficace au stade A, qui correspond à la fin floraison, stade particulièrement important dans l'épidémiologie de la maladie (McClellan & Hewitt, 1973 ; Nair *et al*., 1995 ; Keller *et al*., 2003 ; Pezet *et al.*, 2003 ; Viret *et al*., 2004). Ainsi, le fenhexamid agirait davantage sur l'infection florale par *B. cinerea* et serait moins efficace à des stades plus tardifs. Au contraire, le fludioxonil aurait une action moins préventive et agirait davantage sur le champignon développé, ce qui expliquerait que son positionnement soit moins important.

Dans les fleurs au stade A, les fongicides anti-*Botrytis* ne stimulent pas les réponses de défense. Or, l'infection florale est une étape cruciale dans l'infection par *B. cinerea*. Nous avons donc voulu savoir si cette non-activation des défenses était liée à l'action peu stressante des fongicides ou à la faible sensibilité des fleurs de vigne aux stress en général. Pour répondre à ces hypothèses, la sensibilité des fleurs a été évaluée en utilisant un stress bien identifié chez la vigne : le stress UV-C. En effet, les UV-C sont connus pour induire des réponses de défense à la fois dans les feuilles et les baies de vigne (Langcake & Pryce, 1976 ; Douillet-Breuil *et al*., 1999 ; Adrian *et al.*, 2000 ; Bonomelli *et al.*, 2004 ; Borie *et al*., 2004).

2. Sensibilité des fleurs de vigne aux UV-C

Les mécanismes de défense sont peu ou pas activés dans les fleurs et les baies nouées (BBCH 57 à 71) suite au stress UV-C (publication 6). Une fois que les baies ont atteint le stade grenaille (BBCH 73), les réponses de défense sont induites comme cela a déjà été observé dans les baies à différents stades de développement (Jeandet *et al.*, 1991; Bais *et al.*, 2000). Cela peut s'expliquer par d'importants changements dans la composition des organes reproducteurs, entre les stades BBCH 71 et 73, à l'origine d'une capacité différente à répondre aux UV-C. Dans les rafles, quelque soit le stade suivi (BBCH 57 à 73), la sensibilité aux UV-C est supérieure aux fleurs. Une activation similaire des réponses de défense a déjà

été observée dans les feuilles de vigne suite à l'application des UV-C (Jeandet *et al.*, 1991; Jeandet *et al.*, 1992; Sbaghi *et al.*, 1995; Bonomelli *et al.*, 2004; Borie *et al.*, 2004). Cela suggère que les organes végétatifs de la vigne présentent les mêmes capacités à répondre suite à un stress abiotique.

Les fleurs présentent donc une faible sensibilité au stress UV-C contrairement aux autres organes de la vigne. Dans le cas du gène *GLUC*, le taux de base est nettement plus élevé dans les fleurs témoins que dans les rafles. Cela peut expliquer que le stress UV-C stimule peu l'expression de ce gène dans les fleurs. La β-1,3-glucanase, codée par le gène *GLUC*, ne semble pas avoir un rôle uniquement dans les réponses de défense mais pourrait intervenir dans le processus de reproduction, notamment dans la croissance du tube pollinique (Harikrishna *et al.*, 1996 ; Liljeroth *et al.*, 2005).

L'étude des réponses de défense dans les organes reproducteurs de la vigne a montré que les fongicides anti-*Botrytis*, fludioxonil et fenhexamid, ne génèrent pas de réponses de défense dans les fleurs et les baies aux stades A et B. Seules les baies au stade C ont montré une augmentation de leur activité chitinase suite aux traitements fongicides mais sans induction de l'expression des gènes de défense. Comme pour les organes végétatifs, **le stress subi par les organes reproducteurs lors de l'application de fongicides semble donc faible.** L'application d'un stress UV-C sur les inflorescences au stade A a confirmé l'absence d'activation de réponses de défense dans les fleurs mais a révélé une induction dans les rafles. Les **fleurs semblent donc être peu sensibles** aux stress. Cela peut s'expliquer par l'activation lors de la floraison d'autres mécanismes que les réponses de défense. En effet, la formation des organes reproducteurs, la pollinisation et la fécondation sont des processus complexes caractérisés par des événements spécifiques comme l'organogenèse ou des modifications dans l'expression des gènes (Gasser *et al.*, 1991).

Dans le cadre de stratégies alternatives à l'utilisation de produits chimiques, un des procédés vise à stimuler les défenses naturelles de la vigne par l'utilisation de composés éliciteurs (Kessmann *et al.*, 1994 ; Garcia-Brugger *et al.*, 2006). Cela génère une induction de différents gènes de défense et les défenses activées aboutissent à une protection de la plante contre les maladies, les ravageurs et améliorent dans certains cas la tolérance aux stress climatiques. Les éliciteurs se caractérisent par une toxicité nulle pour l'utilisateur et l'environnement aux doses préconisées et appliquées et par un mode d'action non direct sur les pathogènes contrairement aux fongicides conventionnels (Walters *et al.*, 2005). Malgré les atouts de cette approche, nos résultats ont montré qu'il semble difficile d'activer les réponses de défense dans les fleurs de vigne. L'utilisation de fongicides chimiques à ce stade semble

donc être la technique la mieux adaptée pour limiter l'infection florale contre *B. cinerea*. A l'inverse, à des stades plus tardifs comme le stade C, au cours duquel les réponses de défense sont activées aussi bien par les UV-C que par les fongicides anti-*Botrytis*, l'utilisation d'éliciteurs pourrait être prometteuse.

PERSPECTIVES

Accepteur d'électrons :

a1 benzoquinone

a2 ferricyanure de potassium, méthylviologène (paraquat)

Donneurs d'électrons :

d catéchol, acide ascorbique

Inhibiteur d'électrons :

in diuron

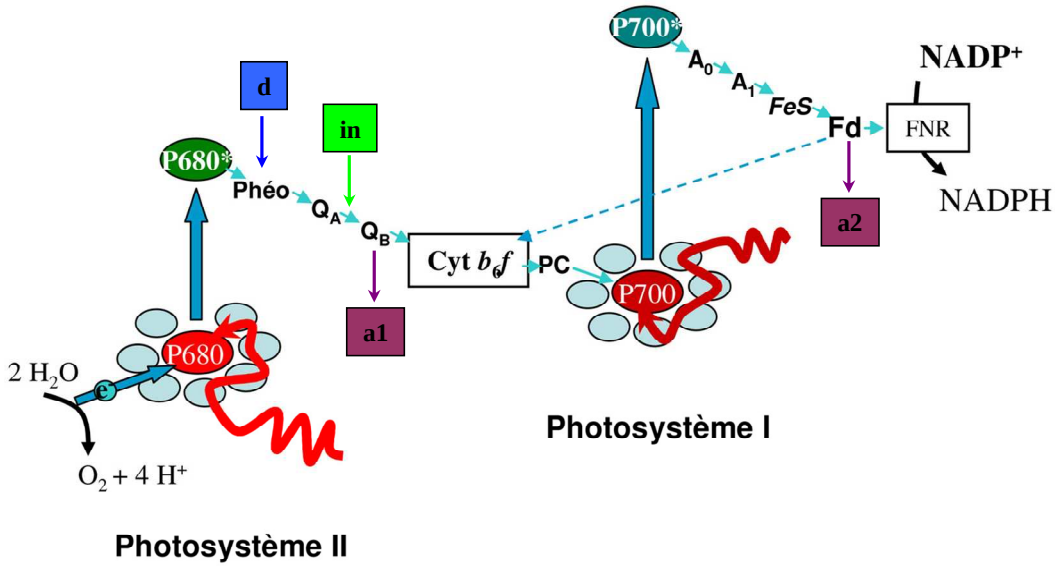

Figure 19. Les sites d'action de donneurs, accepteurs ou inhibiteurs couramment utilisés dans l'étude de la chaîne de transfert d'électrons photosynthétique (Murty & Rjagopal, 1995).

P680 : centre réactionnel du PSII
Phéo : phéophytine
QA et QB : plastoquinones
Cytb6f : cytochrome *b6f*
PC : plastocyanine
P700 : centre réactionnel du PSI
Fd : ferrédoxine
FNR : ferrédoxine-$NADP^+$ réductase

I. Effets dissociés de la matière active et du formulant sur la photosynthèse

Nous avons montré que la photosynthèse foliaire est altérée par le fludioxonil et le fenhexamid. Toutefois, n'ayant testé que le produit commercial, les résultats de notre étude ne permettent pas de dissocier l'impact de la **matière active** et du **formulant**. Or, il a déjà été montré que les formulants seuls peuvent altérer la photosynthèse (Haile *et al*., 2000). Il serait donc intéressant de tester séparément le formulant et la matière active.

Afin de préciser le site d'action des fongicides sur la photosynthèse, nous pourrions analyser le **transfert d'électrons** au niveau de la chaîne photosynthétique. En effet, les fongicides peuvent altérer ce processus (Droppa & Horváth, 1990 ; Baron *et al.*, 1995). Pour cette étude, il est nécessaire d'obtenir des **chloroplastes isolés** et à enveloppe brisée. Ces chloroplastes sont alors inaptes à fixer le CO_2 et donc à synthétiser l'accepteur naturel d'électrons, le glycérate-3-phosphate, produit par la fixation de CO_2 dans le cycle de Calvin. L'utilisation de donneurs, d'accepteurs et d'inhibiteurs d'électrons artificiels permet alors d'étudier à quel niveau les fonctions des chloroplastes sont altérées (Murthy & Rajagopal, 1995). Des accepteurs d'électrons artificiels comme la benzoquinone, le ferricyanure de potassium ou le méthylviologène ou paraquat, des donneurs comme le catéchol ou l'acide ascorbique et enfin des inhibiteurs tels que le diuron sont couramment utilisés pour étudier les perturbations de la chaîne d'électrons photosynthétique (Figure 19). Cela permettrait ainsi d'identifier de façon précise les sites d'action des fongicides, du formulant ou de la matière active au niveau des photosystèmes I et II.

II. Défenses anti-oxydantes dans les feuilles suite aux traitements anti-*Botrytis*

Nos résultats ont montré que la photosynthèse diminue lors de l'application de fludioxonil et de fenhexamid sur les feuilles de vigne. De plus, une répression de gènes codant des protéines impliquées dans la photosynthèse a été observée suite aux traitements anti-*Botrytis*. Une diminution de la photosynthèse liée à la répression de ces gènes a déjà été identifiée lors de l'application d'autres stress abiotiques comme l'exposition aux UV-B, le stress salin ou la sécheresse qui engendrent un **stress oxydatif** chez les plantes (Bahl & Kahl, 1995 ; Conklin & Last, 1995 ; A-H Mackerness *et al.*, 1997 ; Seki *et al*., 2002). De la même façon, un stress oxydatif pourrait donc être généré par l'application des fongicides anti-*Botrytis*. Le stress oxydatif génère la production d'espèces activées de l'oxygène (EAO) de façon rapide et massive (Dat *et al.*, 2000 ; Wohlgemuth *et al*., 2002 ; Apel & Hirt, 2004 ;

SOD : superoxyde dismutase

1 catalase / **2** peroxydases / **3** enzymes du cycle ascorbate-glutathion

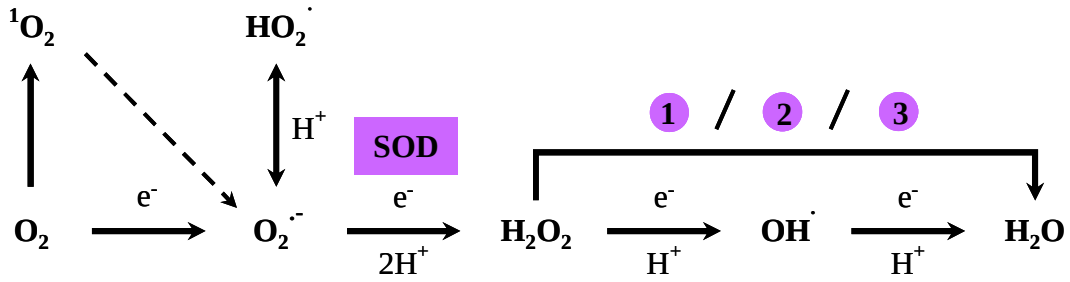

Figure 20. Mécanismes de production des ROS et systèmes enzymatiques de détoxication (d'après Vranova *et al.*, 2002).

O_2 : oxygène moléculaire
$^1O_2$: oxygène singulet
$O_2^{\cdot -}$: ion superoxyde
$HO_2^{\cdot}$: radical hydroperoxyl
H_2O_2 : peroxyde d'hydrogène
$OH^{\cdot}$: radical hydroxyl

Chinnusamy *et al*., 2006). Ces EAO ont comme rôle principal l'activation de la réponse aux stress et la mise en place des mécanismes de défense (Dalton *et al*., 1999 ; Knight & Knight, 2001 ; Mittler, 2002 ; Apel & Hirt, 2004 ; Mori & Schroeder, 2004).

Afin de se protéger des dommages causés par le stress oxydatif, les cellules végétales possèdent un ensemble de **mécanismes antioxydants** (Figure 20) (Apel & Hirt, 2004). Les mécanismes enzymatiques de détoxication incluent l'action de différentes enzymes telles que (i) des superoxydes dismutases (SOD) qui agissent en première ligne en dismutant l'ion superoxyde en H_2O_2 (ii) des peroxydases (ascorbate peroxydase ou APX, glutathion peroxydase ou GPX) et (iii) des catalases (CAT) qui réduisent l'H_2O_2 en eau (Dat *et al*., 2000 ; Orozco-Cardenas *et al*., 2001 ; Mittler, 2002). Parallèlement, les glutathion-S-transférases (GST) détoxifient les produits endogènes toxiques générés lors du stress oxydatif tels que les peroxydes de lipides, en catalysant la conjugaison du glutathion avec ces produits endogènes (Marrs, 1996). La synthèse de ces différentes enzymes, couplée à la production de composés anti-oxydants tels que des vitamines C et E, des flavonoïdes et des caroténoïdes induites par les EAO, contribue à renforcer la protection cellulaire durant le stress (Larson, 1995). Comme les fongicides anti-*Botrytis* pourraient être à l'origine d'un stress oxydant, l'étude des mécanismes de détoxication dans les feuilles de vigne apporterait des informations sur la capacité de la vigne à s'adapter au stress provoqué par l'application de ces fongicides. En effet, une augmentation de la synthèse d'enzymes de détoxication (*e.g.* SOD, CAT ou APX) a déjà été observée suite à l'utilisation de fongicides chez l'orge (Wu & von Tiedemann, 2002). De plus, une augmentation de la teneur en caroténoïdes suite à des traitements anti-*Botrytis*, fludioxonil et pyriméthanil, a déjà été observée chez la vigne (Saladin *et al*., 2003a).

III. Réponses de défense dans les fleurs de vigne

Notre étude sur la réponse des inflorescences de vigne aux UV-C a montré que les mécanismes de défense étaient faiblement activées dans les fleurs, entre les stades BBCH 57 et 68. Toutefois, le développement des fleurs de vigne débute à un **stade plus précoce** qui est le stade BBCH 53 (inflorescence visible). Il est possible que la sensibilité des fleurs aux stress varie au cours de leur développement. Il serait donc intéressant d'étudier les réponses de défense à partir de ce stade afin de déterminer la sensibilité des fleurs aux stress tout au long de leur développement.

La localisation des lieux de synthèse des ARNm par la technique d'hybridation *in situ* permettrait de comprendre la **dynamique spatiale et temporelle** des défenses induites lors

d'un stress dans les fleurs au cours de leur développement. En effet, notre étude des réponses de défense suite au stress UV-C a concerné des fleurs entières. Or, il a été montré que certains tissus floraux, tels que le style ou les anthères, expriment les gènes de défense de façon spécifique (Liljeroth *et al*., 2005). En complément de la technique d'hybridation *in situ*, une étude immunocytochimique permettrait de déterminer la distribution des protéines au niveau des tissus floraux.

Dans un premier temps, l'étude de la **β-1,3-glucanase**, enzyme hydrolytique impliquée dans les réponses de défense des plantes, paraît la plus pertinente. Chez la vigne, l'induction de l'expression des gènes codant des β-1,3-glucanases et l'accumulation de ces protéines ont ont déjà été observées lors de stress biotiques et abiotiques dans les feuilles (Bonomelli *et al.*, 2004 ; Bézier *et al.,* 2007) et les baies (Derckel *et al*., 1998). Dans les fleurs, aux stades que nous avons suivis (BBCH 57 à 68), une variation de l'expression du gène codant une β-1,3-glucanase a été observée dans les fleurs témoins, avec un niveau de base nettement supérieur à celui des rafles. Chez l'orge, une variation de l'expression des β-1,3-glucanases a également été montrée au cours du développement floral (Liljeroth *et al*., 2005). En plus de leur action de défense contre les pathogènes, les glucanases, synthétisées au niveau du pistil ou des grains de pollen, pourraient ainsi jouer un rôle dans le développement de la plante. Notamment, ces enzymes en hydrolysant les tissus femelles, stigmate et style, permettraient au tube pollinique de se développer (Liljeroth *et al.*, 2005). L'étude de l'expression de la β-1,3-glucanase, tout au long du développement floral de la vigne et dans les différents organes reproducteurs, permettrait de comprendre avec plus de précision l'expression spatio-temporelle de ce gène et d'en déduire son implication potentielle dans le processus de reproduction. De plus, une étude de la régulation de son expression suite à l'application d'un stress permettrait de connaître son implication dans la protection des tissus floraux contre les pathogènes. Dans la même optique, d'autres protéines de défense, comme les **chitinases**, pourraient être étudiées. En effet, les chitinases sont également régulées au cours du développement floral (del Campillo & Lewis, 1992 ; Liljeroth *et al.*, 2005) et connues pour être induites suite à des stress biotiques et abiotiques dans les feuilles (Bonomelli *et al*. 2004) et les baies de vigne (Robert *et al*., 2002).

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ANNEXE

Publication 7

Gating in grapevine: relationship between application of the fungicide fludioxonil and circadian rhythm on photosynthesis

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Gating in grapevine: Relationship between application of the fungicide fludioxonil and circadian rhythm on photosynthesis

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The period of fdx spraying was an important parameter in stress response: the midday fdx treatment is more suitable to treat grapevine with fdx.

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ABSTRACT

The aim of this study was to determine the impact of the fludioxonil (fdx) fungicide on the diurnal fluctuation in grapevine photosynthesis. Therefore, fdx treatment was performed at the end of flowering, at 8 am, 12 am or 7 pm. The study was performed in experimental field and several photosynthesis parameters were followed one day after treatment. Morning fdx treatment induced (i) a significant and simultaneous drop of both photosynthesis (Pn) and stomatal conductance between 8 am and 4 pm and (ii) an increase of intercellular CO_2 concentration when compared to control plants. On the contrary, evening fdx treatment did not affect Pn whereas midday treatment caused Pn increase after 4 pm. These data suggest that (i) morning fdx treatment results in a non-stomatal limitation of Pn, (ii) midday treatment is more suitable to treat grapevine with fdx and (iii) a phenomenon of gating was noticed.

1. Introduction

Grey mould, caused by the fungus *Botrytis cinerea*, is a serious disease in grapevine (*Vitis vinifera* L.) culture, affecting both the quantity of harvest and the quality of wine produced from infected grapes (Bulit and Dubos, 1988). Up to now, this disease is exclusively controlled in vineyards by chemical fungicides. Three preventive applications are generally recommended: at the end of flowering (BBCH 69), at the bunch closure (BBCH 77) and at the beginning of berry ripening (BBCH 81). Fludioxonil (fdx) [4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile] is a contact fungicide recommended for the control of *B. cinerea* and is widely used against this phytopathogen (Rosslenbroich and Stuebler, 2000). Fdx is a non-systemic molecule, which inhibits spore germination, germ-tube elongation and mycelium growth of *B. cinerea* (Leroux, 1996). Moreover, fdx increases the glycerol content in the fungus, leading to a perturbation of its osmoregulation potential (Pillonel and Meyer, 1997).

In grapevine, pesticides significantly affect plant physiology (for review see Ref. Saladin and Clément, 2005). Fdx affects several physiological parameters including leaf water and nitrogenous contents. The photosynthetic apparatus is also temporarily affected as revealed by fluctuation of CO_2 assimilation and photosynthetic pigment contents (Saladin et al., 2003a,b). This may alter the whole plant physiology since carbon metabolism is involved in grapevine yield and vigour. Fdx applied during flower and berry development, may thus have significant consequences on berry yield. Indeed, during berry ripening, there is a strong competition for sugar nutrients between the reserve restitution in the woody part of the plant and berries during maturation (Zapata et al., 2004). The use of reserves to counteract the photosynthesis decrease, fungicide-induced effects, may alter berry development and thus, affect grapevine growth and yield.

In plants, there is an important interaction between carbohydrate metabolism and circadian rhythm (Bläsing et al., 2005). Plants have endogenous biological rhythms that enable them to daily organize their physiological, metabolic and developmental processes such as germination, growth, enzyme activity, flower opening, fragrance emission (Hotta et al., 2007) and most parameters of photosynthesis including stomatal movement, gas exchange and CO_2 fixation (Yakir et al., 2007). In addition, recent data reveal that the circadian rhythm modulates the ability to respond to abiotic stresses such as cold (Fowler et al., 2005), mechanical stimulation (Anderson-Bernadas et al., 1997) and wind (Gaal and Erwin, 2005).

The aim of the study was to determine whether circadian rhythm modulates grapevine ability to respond to fdx chemical stress. The impact of fdx on the diurnal fluctuation in grapevine

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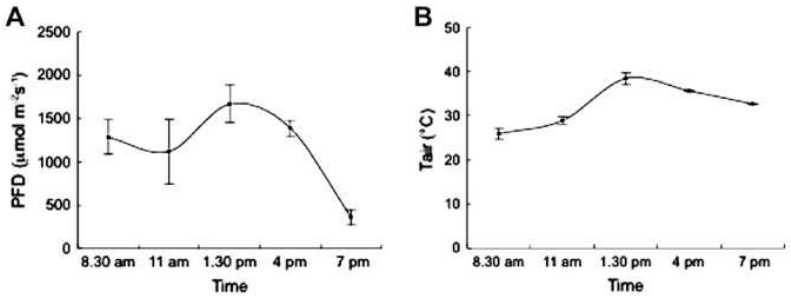

Fig. 1. Photon flux density, PFD (A) and air temperature, Tair (B) over the photoperiod. The values given are the means ± SD ($n = 8$).

photosynthesis was evaluated by following different aspects of gas exchange in experimental vineyards (*V. vinifera* L. cv. Chardonnay) at the end of flowering (BBCH 69).

2. Materials and methods

2.1. Plant material and treatment

Experiments were performed on Chardonnay grapevines (*V. vinifera* L.) grafted on 41B rootstock on the experimental vineyard in Reims (France). Plants were grown during 3 years in PVC containers with 100 L of compost.

The fdx fungicide (under formulated products Geoxe®) was sprayed using the recommended concentration (4 mM) at the end of flowering (BBCH 69). During our experiment the sun rose around 6 am local time. The photoperiod was 15 h day/9 h night. Eight plants were sprayed in the morning (8 am), 8 in the midday (12 am) and 8 in the evening (7 pm). Eight control plants in non-treated areas were chosen as perfectly healthy and received none botryticide application.

2.2. Gas exchanges

The net photosynthesis (Pn), the stomatal conductance (gs), the intercellular CO_2 concentration (Ci), the transpiration rate (T), the incident photon flux density (PFD) and the air temperature (Tair) were measured simultaneously using a portable infrared gas analyser (LI-Cor Model 6400, Lincoln, NE, USA). The infrared gas analysis system was equipped with a clamp-on leaf cuvette that exposed 6 cm^2 of leaf area. Humidity was fixed at 30%. CO_2 concentration was maintained at a constant level of 360 µmol l^{-1} using a LI-6400-01 CO_2 injector with a high-pressure liquid CO_2 cartridge source. Gas exchanges measurements were performed on mature sun-exposed leaves throughout the natural photoperiod. Three replicate measurements per plant were conducted one day after treatment between 8.30 am and 7 pm.

Regression associated to Pn response to incident natural PFD was represented. The apparent quantum yield of CO_2 fixation (ΦCO_2) was calculated as the slope of the linear portion of the response curves between 0 and 300 µmol photons m^{-2} s^{-1}. Dark respiration (Rd) was calculated for $x = 0$ and compensation point (Γ) for $y = 0$. Using high PFD (>1200 µmol m^{-2} s^{-1}), the slope of curves was calculated (Bigot et al., 2007). Moreover, response of gs, Ci and T to incident natural PFD was represented.

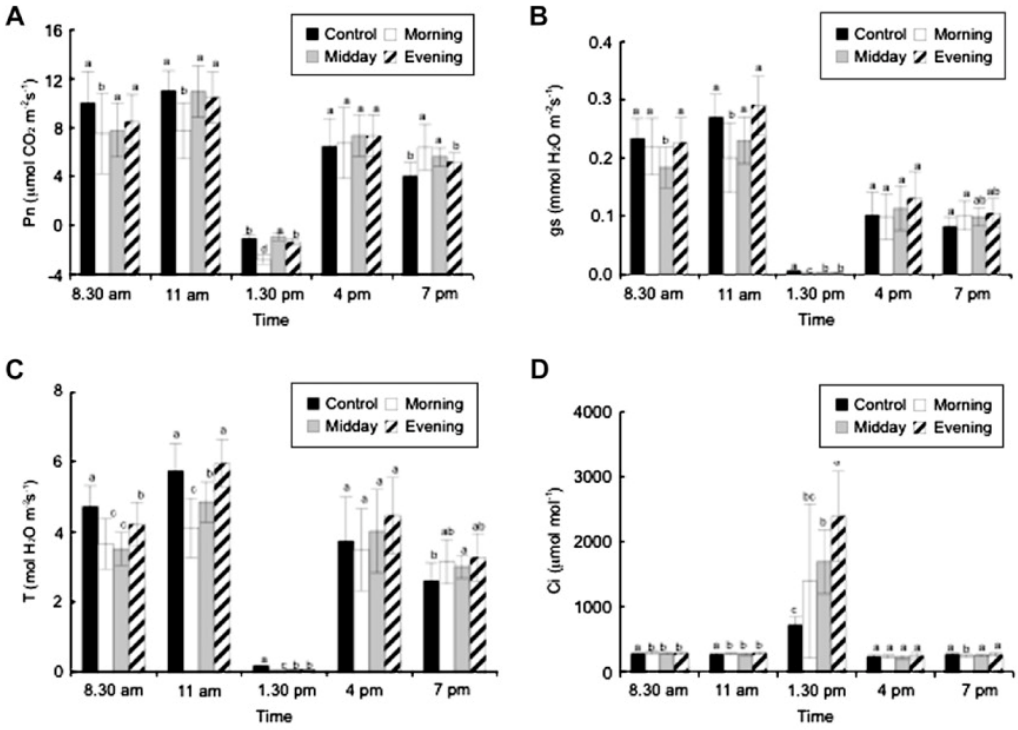

Fig. 2. Net photosynthesis (A), stomatal conductance (B), transpiration rate (C) and intercellular CO_2 concentration (D) in leaves after fdx spraying at various moments of photoperiod (morning, midday, evening). The control represents plants without fdx treatment. The values given are the means ± SD ($n = 8$). Means for a considered parameter were not significantly different when marked by the same letter.

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2.3. Statistical analysis

Eight replicate plants per treatment were carried out ($n = 8$). All data were analyzed using the Mann and Whitney test at the 0.05 probability level.

3. Results

3.1. Gas exchanges

PFD and air temperature were presented in Fig. 1. By 8.30 am, PFD was about 1300 μmol m^{-2} s^{-1}, peaking at 1700 μmol m^{-2} s^{-1} at 1.30 pm (Fig. 1A). PFD decreased thereafter below 350 μmol m^{-2} s^{-1} after 7 pm. Tair increased along with PFD to reach 38 °C at 1.30 pm (Fig. 1B). By 4 pm, Tair dropped to 35 °C and continued to decrease down to 32 °C at 7 pm.

Between 8.30 am and 11 am, Pn, gs and T were maximal in control plants (Fig. 2A–C). They decreased until 1.30 pm and increased again from 1.30 pm to 4 pm. Morning fdx treatments induced significant drop of Pn between 8 am and 4 pm when compared to control plants and a significant increase at 7 pm (Fig. 2A). This Pn reduction was simultaneous to either a Ci increase (at 8 am, Fig. 2D) or a gs decrease (at 1.30 pm) or both (at 11 am). These data suggest that morning fdx treatment results in a non-stomatal limitation of Pn. In opposite, at 7 pm, Pn increase was associated with Ci decrease and stable gs, suggesting a stomatal regulation compared to control plants. Midday fdx treatment only induced Pn and gs increase after 4 pm while Ci remained stable. Therefore, Pn regulation was stomatal after midday treatment. On the contrary, fdx treatment applied in the evening did not affect Pn at none time of the day after treatment.

3.2. Light response curves

Regression associated to responses of Pn, gs, Ci and T to PFD was represented in Fig. 3. With higher light intensity Pn, gs and T increased until 900 μmol m^{-2} s^{-1} then declined while Ci increased whatever the period of fdx treatment. Treated leaves had a lower photosynthetic capacity than control leaves. Treated plants at the morning responded in a lower extent to the light than control plants. In morning treated leaves, Pn max was strongly inhibited compared to the control (Fig. 3A, Table 1). Pn max of leaves treated at the midday and the evening has little inhibition. Apparent quantum yield of CO_2 fixation (ΦCO_2) showed a significant decrease with fdx treatment (Table 1). The strongest inhibition was measured in treated plants in the evening while the lowest reduction was emphasized following the midday treatment.

Dark respiration (Rd) increased according to the period of treatment. Higher Rd increase was noted following the morning treatment. Lowest modifications occurred after the midday treatment. Compensation point declined after fdx treatment whatever application period, especially after evening treatment. Using the high PFD (>1200 μmol m^{-2} s^{-1}), all the slopes of curves were negative (Fig. 3, Table 1), meaning that there was photoinhibition. However, higher inhibition was measured in midday treated plants.

4. Discussion

Our results provide new insights into the dynamic approach of fdx possible effects on grapevines photosynthetic activity and importance of the time of fungicide application. Fdx induced

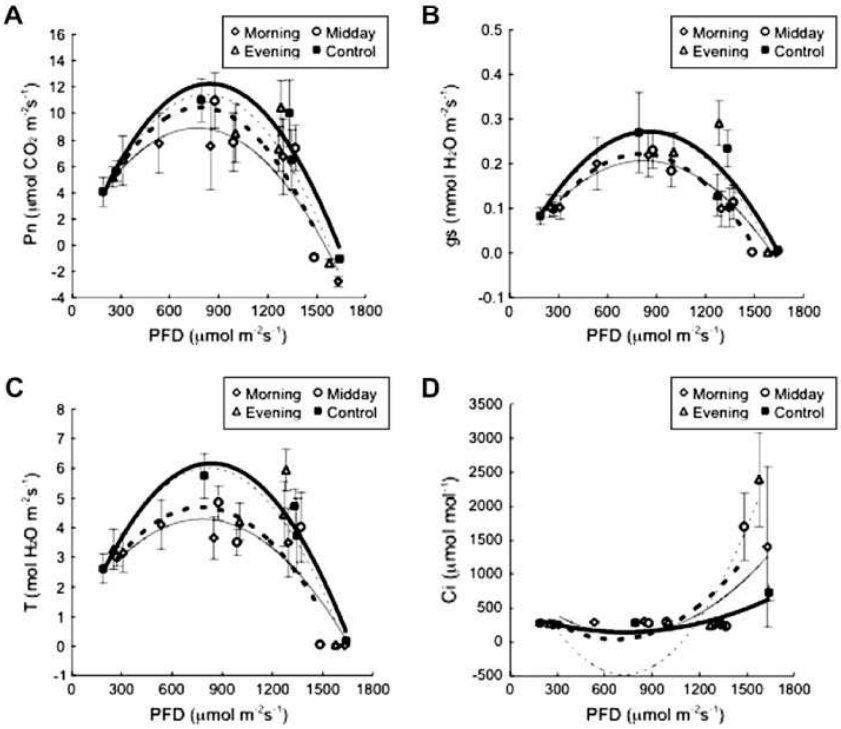

Fig. 3. Net photosynthesis (A), stomatal conductance (B), transpiration rate (C) and intercellular CO_2 concentration (D) in leaves during the day after spraying of fdx at morning, midday, or evening, exposed under natural PFD. The control represents plants without fdx treatment. The values given are the means ± SD ($n = 8$). ———— Indicate a polynomial regression for the morning values ($r^2 = 0.9078$), ▬ ▬ for the midday values ($r^2 = 0.9221$), – – – – for the evening values ($r^2 = 0.7449$) and ▬▬ for the control values ($r^2 = 0.7857$).

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Table 1
Analyses of photosynthetic light response curves: maximal photosynthesis (Pn max), the apparent quantum yield of CO_2 fixation (ΦCO_2), dark respiration (Rd), compensation point (Γ), and slope with PFD > 1200 μmol m^{-2} s^{-1} of grapevine one day after treatment

Period of fdx application	Pn max (μmol m^{-2} s^{-1})	ΦCO_2	Rd (μmol m^{-2} s^{-1})	Γ (μmol m^{-2} s^{-1})	Slope with PFD > 1200 μmol m^{-2} s^{-1}
Control	12.2^{a}	0.0115^{a}	1.8867^{d}	-164.06^{a}	-0.0256^{a}
Morning	11.6^{b}	0.0059^{c}	4.5793^{a}	-776.15^{d}	-0.0281^{b}
Midday	10.2^{c}	0.0088^{b}	3.2206^{c}	-365.97^{b}	-0.0720^{c}
Evening	8.5^{d}	0.0044^{d}	4.0991^{b}	-931.61^{c}	-0.0281^{b}

Means for a considered parameter were not significantly different when marked by the same letter ($n = 8$).

a strong Pn inhibition accompanied by gs, T, ΦCO_2 and Rd declines. The greater accumulated carbon gain was by the grapevine treated in the midday. It was +3%, +14% and +14% compared to control plants and to plants treated at the morning and at the evening, respectively. These data complement preliminary information on fdx effects on grapevine physiology. Saladin et al. (2003a,b) showed that, in vitro, both water content and osmotic potential decreased in fdx treated leaves. Carbohydrate accumulated, suggesting that plantlets could react to the stress through an active osmoregulation process by uptaking sugars from the medium. Moreover, in vineyard, fdx modified leaf water content and carbohydrate levels, whereas nitrogenous compounds accumulated transiently. Effects of fungicides on photosynthesis have been already revealed by modifications of photosynthetic activity, chlorophyll fluorescence, and pigment contents (Van Iersel and Bugbee, 1996; Benton and Cobb, 1997; Tort and Türkyilmaz, 2003; Untiedt and Blanke, 2004; Xia et al., 2006). In vitro studies using isolated chloroplasts or excised leaves have reported a direct effect of copper on the photosynthetic electron transport chain, lipid peroxidation of thylakoid membranes, or with the alteration of lipid chloroplast membrane, affecting the light reaction processes, especially those associated with PSII (Alaoui-Sossé et al., 2004). Krugh and Miles (1996) showed that the fungicide tributyltin chloride hinders PSI electron transport or in some other way inhibits the oxidation of the PSII electron transport components.

4.1. Stress responses and circadian rhythm

We have measured one variation in grapevine responses to the same fdx spraying applied at different times of the day. Morning fdx spraying induced depression of carbon fixation between 8 am and 1.30 pm whereas midday fdx spraying stimulated Pn after 7 pm. This phenomenon is so-called gating. Hotta et al. (2007) have reported that external stimuli of equal strength applied at different times of the day can result in different intensities of response. One example of gating is the diurnal variation in the inhibition of stem elongation by wind (Gaal and Erwin, 2005). When wind perturbation was given to *Cosmos bipinnatus* at different times of the day, the most intense effect on growth was observed when wind was applied during the day (Gaal and Erwin, 2005). Similarly, inhibition of stem growth in the legume *Phaseolus vulgaris* by mechanical stimulation was also greatest at the beginning of the day (Anderson-Bernadas et al., 1997).

4.2. Photosynthesis and circadian rhythm

Between 8 am and 1.30 pm, Pn reduction one day after the morning fdx spraying was attributed to a non-stomatal limitation. On the contrary, Pn increases at 7 pm seems to be linked to a stomatal regulation. Similarly, Maroco et al. (2002) have shown that grapevine photosynthesis can be regulated via both stomatal and non-stomatal processes in drought conditions.

In our work, stomatal regulation was observed. Plants, such as grapevine, promote stomatal opening, allowing CO_2 uptake and fixation, as soon as sufficient light is available to drive photosynthesis. It is known that stomata close around midday and start closing long before dusk (Webb, 1998). These responses were traditionally considered as a consequence of the water status of the leaf, but recent works demonstrated that, at least in well-watered plants, they are due to endogenous circadian control of the guard cell (Dodd et al., 2005). Regulation of stomatal pore size, during favourable environmental conditions optimizes CO_2 uptake against water loss. However, under stress conditions, stomatal closure prevents water loss. Guard cells are able to integrate many internal and external signals and produce an appropriate turgor response that results in guard cell movements allowing to control stomatal aperture (Hetherington and Woodward, 2003).

Non-stomatal limitation noticed after fdx treatment can be explained by stimulation of photorespiration and day-respiration at higher temperatures, for example, at 1.30 pm (Mohotti and Lawlor, 2002). Short-term temperature increases stimulate photosynthesis until the temperature optimum is reached (Saxe et al., 2001). Increasing air temperature decreases Rubisco specificity for and solubility of CO_2 more than O_2, which stimulates photorespiration and reduces photosynthesis (Saxe et al., 2001). In addition, following sub-saturating PFD (100–300 μmol m^{-2} s^{-1}), Pn was strongly limited by electron transport (ΦCO_2 inhibition) for treated plants in the morning and in the evening. This phenomenon has been previously reported as a result of a reduced RuBP regeneration, which may be limited when (i) light-harvesting and electron transport produce ATP and NADPH; (ii) the stromal bisphophatases regenerate RuBP in the photosynthetic carbon reduction cycle and (iii) end-product synthesis consumes triose-phosphates and regenerates inorganic phosphate for photophosphorylation (Allen and Ort, 2001).

5. Conclusion

Interpreting the effect of fdx spraying on grapevine photosynthesis, we showed for the first time, a phenomenon of gating. Our work strongly suggests that the period of fdx spraying was an important parameter in stress response. These data suggest that morning fdx treatment results in a non-stomatal limitation of Pn. Midday treatment is more suitable to treat grapevine with fdx because it is the treatment which disrupts least the photosynthesis.

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