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The development of methodological and technological platform for noninvasive estimation of phenolic compounds in leaves and berries

Ph.D. thesis

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This thesis should be cited as:

Šebela D., 2016: The development of methodological and technological platform for noninvasive estimation of phenolic compounds in leaves and berries. PhD thesis-118 pages, University of South Bohemia, Institute of Physics and Biophysics, České Budějovice, Czech Republic.

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List of publications:

1.**Šebela D,** Quinones C, Olejníčková J, Jagadish KSV (2015) Temporal chlorophyll fluorescence signals to track changes in optical properties of maturing rice panicles exposed to high night temperature. Field Crops Research 177: 75-85.

2. **Šebela D,** Olejníčková J, Sotolář R, Vrchotová N, Tříska J (2014) Towards optical detection of *Plamopara viticola* infection in field. Journal of Plant Pathology 96: 309-320.

3. Bellasio C, Olejníčková J, Tesař R, **Šebela D,** Nedbal L (2012) Computer reconstruction of plant growth and chlorophyll fluorescence emission in three spatial dimensions. Sensors 12: 1052-1071.

Peer-reviewed journals without impact factor

1. **Šebela D,** Quinones C, Olejníčková J, Jagadish KSV (2015) High night temperature-induced acceleated maturation of rice panicles can be detected by chlorophyll fluorescence. Global Change: A Complex Challenge, Conference Proceedings 1: 134-137.

2. **Šebela D**, Olejníčková J, Župčanová A, Sotolář R (2012) Response of grapevine leaves to *Plasmopara viticola* infection by means of measurement of reflectance and fluorescence signals. Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis 8: 229-238.

Prepared manuscripts

1. Olejníčková J, **Šebela D**, Vrchotová N, Sotolář R, Tříska J. Pre-syptomatic detection of Plasmopara viticola infection in field grown grapevine leaves using chlorophyll fluorescence imaging. Decrease of photosynthetic activity in infected leaves is associated with production of *trans*-resveratrol, pterostilbene and 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside.

2. **Šebela D**, Turóczy Z, Olejníčková J, Kumšta M, Sotolář R. Effect of ambient sunlight intensity on the temporal phenolic profiles of Vitis vinifera L. cv. `Chardonnay` during ripening season- a field study.

3. **Šebela D**, Turóczy Z, Olejníčková J, Kumšta M, Sotolář R. On-the-go to development of methodological and technological platform for non-invasive UV fluorescence screening of phenolic compounds in grapevine berries.

Prohlášení školitele o rozsahu podílu studenta na publikační činnosti

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1. **Šebela D,** Quinones C, Olejníčková J, Jagadish KSV (2015) Temporal chlorophyll fluorescence signals to track changes in optical properties of maturing rice panicles exposed to high night temperature. Field Crops Research 177: 75-85. **80%**
DŠ performed experiments and participated in experimental work, analyzed the data, wrote and participated in revision of manuscript.

2. **Šebela D,** Olejníčková J, Sotolář R, Vrchotová N, Tříska J (2014) Towards optical detection of Plamopara viticola infection in field. Journal of Plant Pathology 96: 309-320. **60%**
DŠ participated in part of experimental work, analyzed data and participated in writing and revision of the manuscript.

3. Bellasio Ch, Olejníčková J, Tesař R, **Šebela D,** Nedbal L (2012) Computer Reconstruction of Plant Growth and Chlorophyll Fluorescence Emission in 3 Spatial Dimensions. Sensors 12: 1052-1071. **20%**
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DŠ participated in part of experimental work, analyzed data and participated in writing and revision of the manuscript.

Mgr. Julie Olejníčková, Ph.D.

Annotation

Plant optical signals can serve as important source of information about biochemical and physiological processes in plants. These signals are influenced by compounds synthesized by plants during primary or secondary metabolism and thus, can also serve as their qualitative and quantitative indicators. Light reaching plant surface (leaf or fruit) can undergo three main pathways- it can be *(i)* reflected, *(ii)* absorbed or it can *(iii)* transmit through plant material. The probability of these three processes depends on particular wavelength of incident irradiation and on the morphologial characteristics of plant tissues themselves. As such, plant contains various spectrum of photosynthetic pigments and fluorescent compounds which can either reflect, absorb or pass incident irradiation through at specific wavelengths. Biophysical techniques working with these optical properties of plant pigments and/or other compounds have become universal and common tool in basic and applied research. To quote some example, chlorophyll fluorescence imaging, UV induced fluorescence or spectroscopic techniques are on the top of interest thanks to its non-invasive nature, allowing maintain the integrity of measured cells or the whole plant constituents. The main aim of this thesis is to provide a comprehensive study on the possibility of non-invasive monitoring of phenolic compounds in the leaves and fruits.

Anotace

Optické signály rostlinných pletiv mohou sloužit jako významný zdroj informací o biochemických a fyziologických procesech v rostlinách. Tyto signály jsou v zásadě ovlivněny primárním či sekundárním metabolismem - tedy vyzařovány látkami vlastními rostlině, a mohou tak sloužit i jako jejich kvalitativní i kvantitativní indikátory. Při dopadu světelného záření na povrch rostliny (listu či plodu) může dojít v zásadě ke třem hlavním dějům - *(i)* odrazu, *(ii)* pohlcení/absorbci či *(iii)* průchodu daného záření. Pravděpodobnost těchto tří dějů zavisí jak na vlnové délce dopadajícího záření, tak i na vlastnostech samotných rostlinných pletiv. Jako taková jsou rostlinná těla plná pigmentů a fluorescenčních sloučenin, které buď odráží, pohlcují nebo propouští část spektra v různých vlnových délkách daného záření. Biofyzikální techniky pracující s optickými vlastnostmi daných rostlinných pigmentů a sloučenin se staly univerzálním a běžně používaným nástrojem v základním i aplikovaném výzkumu rostlin. Například zobrazování kinetiky fluorescence chlorofylu, měření fluorescence indukované ultrafialovým zářením, nebo měření spektrálních charakteristik odraženého světla se dostaly do poředí zájmu

díky svému neinvazivnímu charakteru, díky němuž zachovávají integritu buněk i celé měřené rostliny. Tato práce se snaží poskytnout ucelenou studii, týkající se možnosti neinvazivního monitoringu fenolických látek v listech a plodech, za pomocí zmíněných optických metod.

Acknowlwdgement

I would like to thank to my supervisor Dr. Julie Olejníčková for her inspiring suggestions, new ideas and perspectives. Her broad view on the subject along with her fruitful comments inspired me in my professional, as well as private life. My special thanks belongs to her holy patience with my writing skills which had (I hope) impact on this thesis. I am also grateful to my colleagues and friends from Nové Hrady (it is not enough space here to mention all of them, but to quote the most important) – Zoltán Turóczy, Kumud B Mishra and Anamika Mishra for their scientific assistance, inspiring talks, motivation and `last, but not least` a lot of fun also in private life.

I would also like to thank to my colleagues and friends whom I met during my scholarship at the International Rice Research Institute, Los Banos, Philippines. Namely, for inspiring talks and profitable collaboration I would like to thank Krishna SV Jagadish; for pleasant time and nice cooperation during `hard-field-work` to Cherryl Quinones. I am also thankful to all of the staff in Institute of Physics and Biophysics, former institute USBE, and Clobal Change Research Institute (CzechGlobe) for taking good care of me and for my pleasant time during my PhD study.

Last but not least, I would like to say special thanks to my family and girlfriend Ruthie for their constant care and patience, and especially for the fact that they are always here to support me in all aspects of my life.

Abbreviations

AL	actinic light
ATP	adenosine-5`- triphosphate
BGF	blue-green fluorescence
CCD	Charge-coupled device
Chl	chlorophyll
Chl-a	chlorophyll a
Chl-b	chlorophyll b
Chl-F	chlorophyll fluorescence
DAS	dark adapted state
ECD	electrochemical detection
GC	gas chromatography
F_0	fluorescence intensity at the minimal level
F_M	fluorescence intensity at the maximal level
F_S	steady state chlorophyll fluorescence level
Fd	ferredoxin
FNR	ferredoxin $NADP^+$ reductase
FR	far-red light
G3P	glyceraldehyde 3-phosphate
HBA	hydroxybenzoic acids
HCA	hydroxycinnamic acids
HPLC	high performance liquid chromatography
LAS	light adapted state
LHC	light harvesting complexes
NADH	nicotineamides

ML	measuring pulsed light
MS	mass spectrometry
OEC	oxygen evolving complex
PAM	pulse amplitude modulation
PC	plastocyanin
PGA	3-phosphoglycerate
PDA	photodiode array
PQ	plastoquinone
PS	photosystem
PSI	photosystem I
PSII	photosystem II
RC	reaction center
RuBisCO	ribulose-1,5-bisphosphate carboxylase
RuBP	ribulose 1,5-bisphosphate
SL	saturation pulse light
UV	ultraviolet
UV/Vis	ultraviolet/visible
Vis	vegetation indices

Chapter`s Overview

This thesis is divided into three chapters. The **first chapter** reviews phenolic compounds. At the beginning of this chapter, these compounds are classified; their presence and role in plants and/or possible ways for their detection in plant material are discussed. In this part also, direct chemical methods and indirect optical methods for phenolic compounds detection are prescribed and discussed.

The **second chapter** describes experimental setup done in the case of particular experiments used in this thesis. Here, plant material, optical methods used and/or chemical analysis are introduced.

The **third chapter** represents the main results of this study. This chapter itself is divided into two main sections, while the first section represents experiments done on the leaf *(3.1.)*, and the second section represents experiments done on the fruit level *(3.2.)*.

In the first section (*3.1.)*, we were trying to explore lab-based experiment done by our group. Here, we tried to demonstrate applicability of not only Chl-F, but also UV induced fluorescence for early detection of *Plasmopara viticola* infection on grapevine leaves. It has been proven that this fungal infection is detectable by means of Chl-F either in susceptible *(3.1.1.)* and/or both susceptible and resistant cultivars *(3.1.2.)*. In both cases, maximum quantum yield of photosystem II was found to be effective to discriminate symptomatic and asymptomatic leaves. Moreover, highly fluorescing phenolic compound synthesized by grapevine leaves under *P. viticola* impact was detectable by means of: *(i)* UV induced fluorescence *(3.1.1.)*, and/or *(ii)* selected chlorophyll fluorescence parameter *(3.1.2.)*. Also, it has been proven that particular Vis were potent to discriminate asymptomatic leaf tissue from those infected by *P. viticola*.

In the second section *(3.2.)*, we tried to follow ripening of both grapevine (*Vitis vinifera* L.) and/or rice (*Oryza sativa* L.), by means of UV induced fluorescence, Chl-F and reflectance measurements, respectively. The potential of UV induced fluorescence for direct detection of phenolic compounds *in vivo* was tested throughout ripening season, while obtained optical signals were subsequently correlated with concentrations obtained from HPLC analysis. We found significant effect of ambient sunlight intensity on the dynamics of several main phenolic compounds for cultivar Chardonnay *(3.2.1.)*. Both for red and white grapevine cultivars we were able to identify specific phenolic compounds *in vivo*, based on the UV induced fluorescence

(3.2.2.). We also proved applicability of handheld prototype `WinePen` for these UV fluorescence readings (*3.2.2.*). Moreover, also Chl-F has been proven to be effective to follow ripening in the case of senescing rice panicles exposed to high night temperature stress (*3.2.3.*).

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1. THEORETICAL BACKGROUND

1.1. Introduction

Grapevine (*Vitis vinifera* L. subsp. *vinifera* Hegi) and rice (*Oryza sativa* L.) are undoubtedly one of the most important plant comodities in the world. Grapevine as well as rice and their products have been essential elements in human life also in the deep history. The earliest evidence of grapevine and/or rice cultivation dates back to 8000 and 2500 B.C., respectively, when their consumptions have been crucial in human life and religions.

Grapevine as well as rice growing nowadays is a global industry, with most of the countries in the world producing either wine and other grapevine products (table fruit, juice and raisins), or rice seeds as basic source of staple food. Interestingly compared to rice, several studies pointed out importance of grapevine leaves as well. In some European countries (e.g. Turkey), not only grapevine berries but also leaves serves traditionally as a food, both in fresh and dried form. Increasing popularity of grapevine and rice products is undoubtedly caused by their health benefit and or importance for human diet. Despite the fact that rice serves as a source of staple food, frequent consumption of grapes/leaves or other grapevine products was associated with low cancer evidence. The observation of low coronary heart disease death rates despite high intake of dietary rich food consumed (Renaud and de Lorgeril, 1992), have formulated so called `French paradox`. This study showed paradoxical epidemiological observation which in principle proved that consumption of grapevine products at a certain forms can serve as a source of nutritional antioxidants and biologically active components in human diet – plant phenolics. In plant kingdom, these phenolics are involved in e.g. defense against aggression by predators, pathogens, parasites, etc.

The synthesis of phenolic compounds is however, connected also with abiotic stress caused by environmental factors, such as e.g. ultraviolet radiation. Geographically, both grapevine and rice are widely cultivated crops exposed to a wide range of environmental challenges and thus, both producing these phenolics as a defense compounds. Global climate change models shows the future scenarios, predicting a much greater variability in many environmental factors. As such, e.g. precipitation patterns (mostly in the form of high

precipitation events; Jones, 2006), changes in the level of ozone layer, and increasing temperature would lead to a drier and warmer climate, together with intensified abiotic stresses as well as pests and disease pressure. All such changes could subsequently affect the global production of both commodities (Wassmann et al., 2009; Fraga et al., 2012; Teixeira et al., 2014; Mozel and Thach, 2014;), since it is a complex relationship between environment and plant, but one that is absolutely necessary to understand. Rapidly increasing demand for higher quality products and/or real time information about the crop physiology is nowadays leading to growing interest in modern technologies. Undoubtedly, one of the most powerful methods is non-invasive optical screening. Such optical methods are popular through their ability to provide synoptic view in two main ways, i.e. their potential is evident: *(i)* by the relationships between optical properties of plant and plant fitness itself, or *(ii)* by the relationships that are known to exist between optical properties of plants and the concentrations of particular plant metabolites.

1.2. Phenolic compounds classification

Metabolites synthesized by plants can be roughly divided into two groups: primary and secondary metabolites. The primary metabolites participate in essential metabolic processes such as growth, development and reproduction. Contrary, secondary metabolites (or sometimes called secondary compounds) are not required for normal growth and development, and are not made through metabolic pathways common to all plants. Bernards (2010) summarized importance of secondary metabolites as being e.g. pigments in plant leaves, flowers and fruits, or as having e.g. defensive role against herbivores and pathogens. Based on the structure and biosynthetic origin, these plant natural products (secondary metabolites) can be divided into three main categories: *(i) alkaloids*, *(ii) terpenoids* and *(iii) phenolics* (Croteau et al., 2000). In general, *(i) alkaloids* are large class of secondary metabolites (Ziegler and Facchini, 2008), including alkaline substances that have nitrogen as part of a ring structure. They have role in chemical-ecological perspective and are interacting with other plants, herbivores or microbes; *(ii) terpenoids*, the dimmers and polymers of 5 carbon precursors called isoprene units, play a crucial role in the interaction of the plant with its environment, i.e. by serving as attractants, herbivore repellents, toxins or antibiotics (Gershenzon and Dudareva, 2007). Despite that, *(iii) plant phenolics* are being the most important secondary metabolites mainly for their benefits and thus, the main attention is focused on them throughout this thesis.

Plant phenolics are usually classified according to their biosynthetic pathways. They are compounds containing one or more aromatic ring, with one or more hydroxyl groups attached. Up to date, there are known more than 8, 000 phenolic structures, widely dispersed throughout plant kingdom. As such, phenolic compounds can be classified by the number and arrangement of their carbon atoms, ranging from simple phenolics to polyphenols groups. Naturally occurring phenolics can be than classified into two groups, the flavonoids and non-flavonoids (Crozier et al., 2009). Though structural diversity of these compounds results in the wide range of phenolics that occur in nature, table 1 shows two main groups of phenolic sompounds, with some of the classes within each group. The non-flavonoids, compared to flavonoids, are more varied and include among others simple phenols, phenolic acids, hydroxycinnamic acids (HCA), coumarins, stilbenes, lignins and tannins. The flavonoids are characterized by a C_6-C_3-C_6 structure and are classified by the type of their heterocycle, i.e. flavonols, flavones, flavanones, flavanols,

anthocyanidins, isoflavones, biflavonoids and condensed tannins. Of these above mentioned basic phenolic compounds however, *(2.1.) phenolic acids (2.2.) tannins* and *(2.3.) flavonoids* are regarded as the main dietary phenolic compounds (King and Young, 1999) and thus, main attention is focused on these particular plant phenolics.

Table 1. Basic structural properties and classification of phenolic compounds

	Carbon skeleton	**Compound class**
Non-flavonoids	C_6	simple phenols
	C_6-C_1	phenolic acids
	C_6-C_3	hydroxycinnamic acids, coumarins
	C_6-C_2-C_6	Stilbenes
	$(C_6$-$C_3)_2$	lignins
	$(C_6$-$C_1)_n$: Glucose	hydrolysable tannins
Flavonoids	C_6-C_3-C_6	flavonols
	C_6-C_3-C_6	flavones
	C_6-C_3-C_6	flavanones
	C_6-C_3-C_6	flavanols
	C_6-C_3-C_6	anthocyanidins
	C_6-C_3-C_6	isoflavones
	$(C_6$-C_3-$C_6)_2$	biflavonoids
	$(C_6$-C_3-$C_6)_n$	condensed tannins

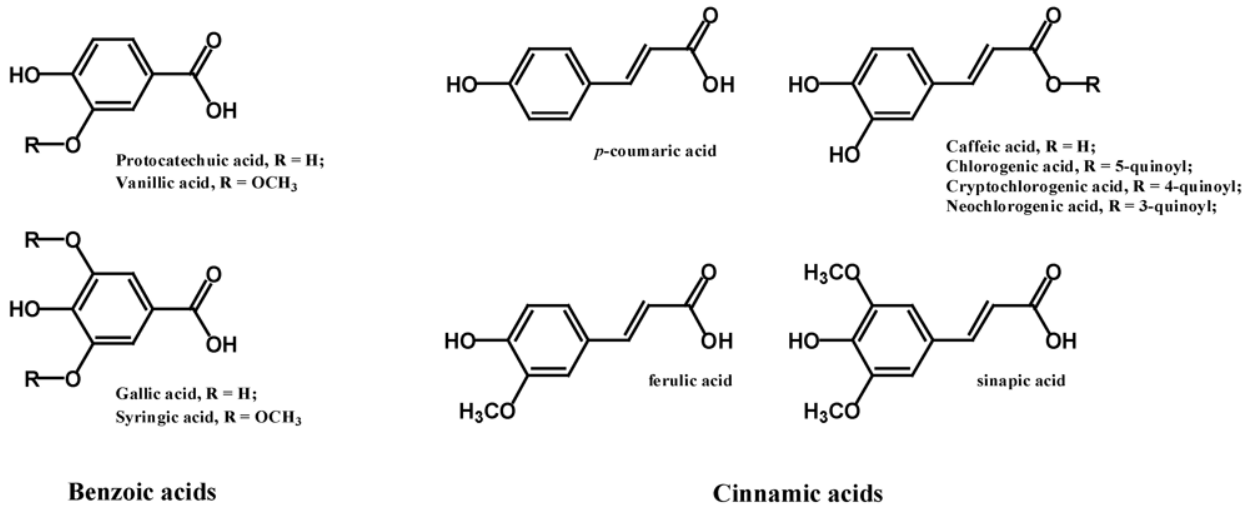
Source: table is inspired by Balasundram et al., (2006)

Phenolic acids

From non-flavonoids group, one of the main compounds of dietary significance are phenolic acids. Phenolic acids are aromatic plant secondary metabolites widely distributed across plant species. Variation in phenolic acids lies in the number and location of hydroxyl groups on the aromatic ring (Pereira et al., 2009). In general, `phenolic acids` term designates phenols that possess one carboxylic acid functionality. Based on C_6-C_1 and C_6-C_3 backbones (Figure 1; Tsao, 2010), there are two main classes of phenolic acids: *(i) derivates of benzoic* and *(ii) derivates of cinnamic acids* (Concalves et al., 2013). Across the derivates of either *hydroxybenzoic (HBA)* or *hydroxycinnamic (HCA) acids*, the basic skeleton remains the same, while the numbers or positions of the hydroxyl groups on the aromatic ring make the difference among them. Variations in the structures of individual *(i) hydroxybenzoic acids* lies on the hydroxylation and methylations of the aromatic ring. As the main phenolic acids from this group presented nearly in

all plants (Robbins, 2003), protocatechuic, vanillic, gallic and syringic acids are the most common (Figure 1). They may be present in soluble form conjugated with sugars or organic acids, as well as bound to cell wall fractions (Rhodes et al., 2002). Four most widely distributed *(ii) hydroxycinnamic acids* are: p-coumaric, caffeic, ferulic and sinapic acids (Figure 1; Robbins, 2003). These acids usually occur in various conjugated forms, esters of hydroxyacids such as chlorogenic, cryptochlorogenic and neochlorogenic acids, as well as their sugar derivatives.

Figure 1. Molecular structures of typical phenolic acids



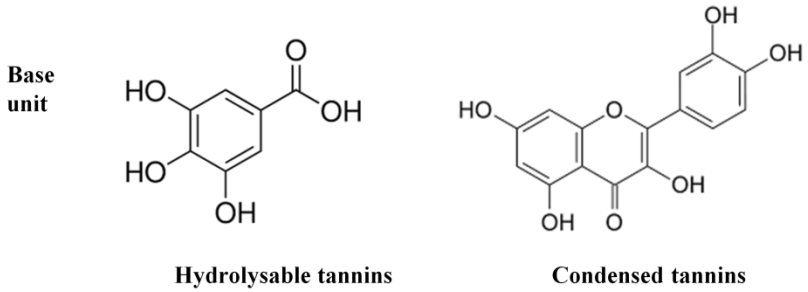
Source: Tsao (2010)

Tannins

Tannins are another major group of polyphenols, in the nature found worldwide, throughout the plant species. Generally, tannins are water soluble phenolic compounds, usually subdivided into two main groups: *(i) hydrolysable tannins* and *(ii) condensed tannins*. The *(i) hydrolysable tannins* (Figure 2, left panel) are readily hydrolyzed by acids; when hydrolyzed, these tannins produce gallic or epigallic acids and sugar. Depending on the nature of the phenolic carboxylic acid, hydrolysable tannins are subdivided into gallotannins and ellagitanins. Hydrolysis of gallotannins yields in gallic acid, while the hydrolysis of ellagitannins yields in hexahydroxydiphenic acid, which is isolated as ellagic acid (Okuda and Ito, 2011). Proanthocyanidins [*(ii) condensed tannins*] are olygomeric or polymeric flavonoids, more widely distributed than *(i) hydrolysable tannins*. The most studied condensed tannins are based on flavan-3-ols, dimers or olygomers of catechin, epicatechin or similar units (Figure 2, right panel),

linked by carbon – carbon bonds not susceptible to cleavage by hydrolysis (e.g. Schofield et al., 2001; Dixon et al., 2005).

Figure 2. Molecular structures of tannins

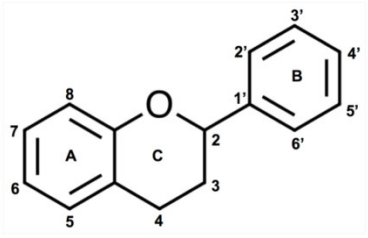


Source: Figure is inspired by Hagerman et al., (1998)

Flavonoids

Flavonoids are polyphenolic compounds; their chemical skeleton is based on 15 carbons with two aromatic rings bound together by three carbon atoms that form an oxygen heterocycle. The basic C_6-C_3-C_6 phenyl-benzopyran flavonoid skeleton can have numerous substituents (e.g. hydroxyl, acetyl, methoxy and methyl groups). Figure 3 shows the general structure of flavonoids and the numbering system used to distinguish the carbon positions around the molecule. The three phenolic rings are referred to as A, B and C (or pyrane) rings. The majority

Figure 3. Molecular structure of flavonoids



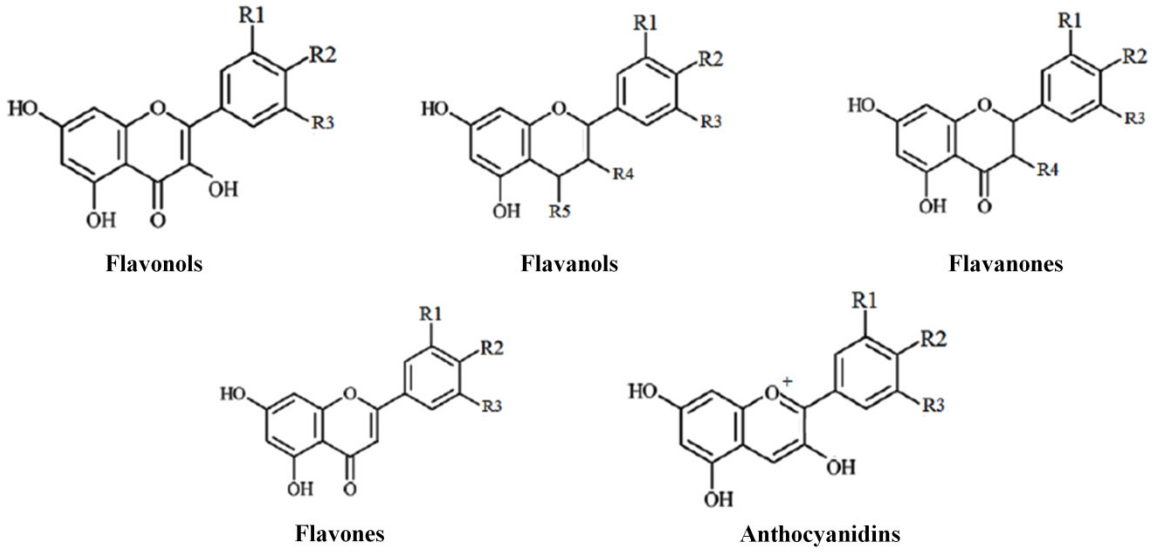
Source: Teixeira et al., (2013)

of the flavonoids exists naturally as glycosides, conjugated to one or several different sugar moieties such as glucose, rhamnose and galactose (Crozier et al., 2009).

Flavonoids are found throughout the plant kingdom and particularly in the epidermis of leaves and in the skin of fruits. The biochemical activities of flavonoids and their metabolites depend on their chemical structure and the relative orientation of various moieties on the molecule. Due to hydroxylation pattern and variations in the chromane ring (Figure 3, C),

flavonoids can be divided into four main groups, namely *major flavonoids*, *isoflavonoids, neoflavonoids* and *stilbenoids* (Harborne and Baxter, 1999), while these major groups can be subsequently divided into several subgroups (Ververidis et al., 2007). As shown in Figure 4, *major flavonoids* can be divided into flavonols, flavanols, flavanones and anthocyanidins sub-

Figure 4. Molecular structure of major flavonoids



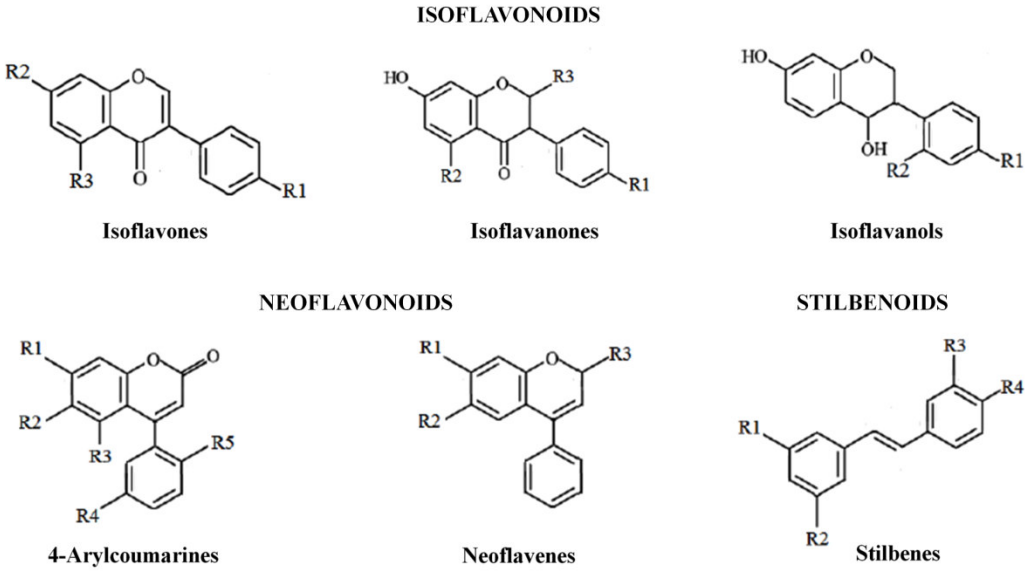
Source: Figure is inspired by Pietta, (2000)

groups. Each subgroup, depending on hydrogenation, hydroxylation, methylation, melonylation, sulphation or glycosilation (Harborne 1988), can than behave as several molecular structures. Based on these structures, main representatives of each subgroup; i.e. flavonols (kaempferol, quercetin and myricetin), flavanols [(+)- catechin, (+)- epicatechin and gallocatechin), flavanones (naringenin, naringin and hesperidin), flavones (apigenin, luteolin and chrysin) and anthocyanidins (cyanidin, delphinidin, pelargonidin and malvidin), were identified, respectively (Stalikas, 2007).

Other three main groups of flavonoids (*isoflavonoids, neoflavonoids* and *stilbenoids*) are represented by Figure 5. As shown, each main group can be devided into subgroups; i.e. *isoflavonoids* (isoflavones, isoflavanones and isoflavanols), *neoflavonoids* (4-arylcoumarines and neoflavenes) and *stilbenoids* (stilbenes), respectively. Depending on the formation of basic backbone in each subgroup, main representatives of *(a) isoflavanoids* are identified as

isoflavones (diadzin, genistin and glycitin), isoflavanones (kieviton), and isoflavanols (phaseollinisoflavan); *(b) neoflavanoids* as 4-arylcoumarines (phenylcoumarin) and neoflavenes (dalbergichromene); and *(c) stilbenenoids* as stilbenes (resveratrol), respectively.

Figure 5. Molecular structure of isoflavonoids, neoflavonoids and stilbenoids



Source: Figure is inspired by Pietta, (2000)

1.3. Presence and role of phenolic compounds in plants

Plant phenolics play a major role in interaction of plants with their environment (Harborne, 1993) and as such, are involved in various biochemicals and physiological processes (Harborne and Williams, 2000). The biosynthesis of the phenolic compounds (both in plant leaves and fruits) has been thoroughly explained and discussed by the expert in the field (e.g. Knaggs, 2003; Tsao and Mc Callum, 2009; Kumar and Pandey, 2013) and thus, only the basic of the biosynthetic pathways is presented here.

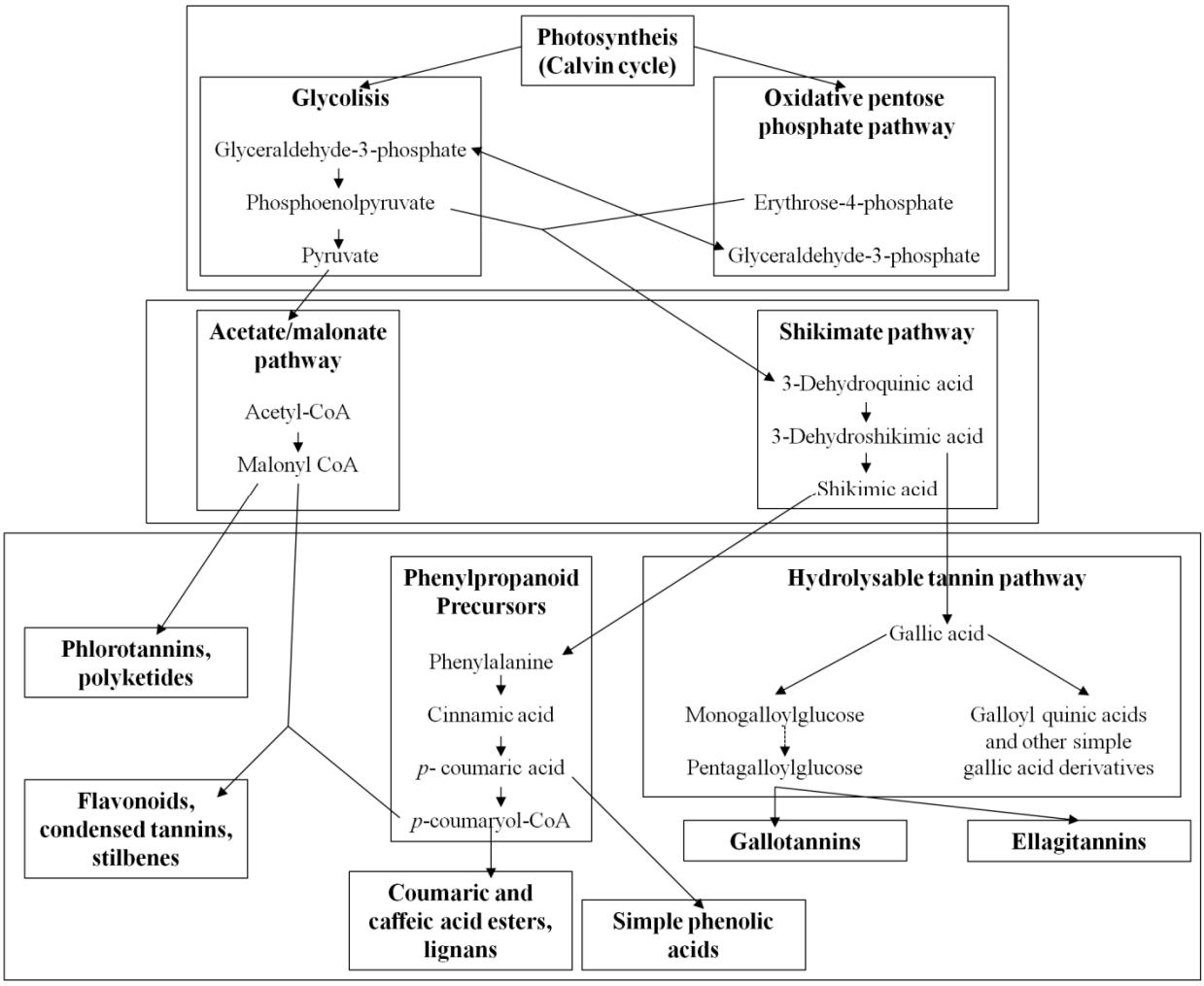
General biosynthetic pathway of phenolic compounds

Plant phenolics, or their immediate building blocks, are derived via two main pathways; while one group of tannins may rely either on one or both of these pathways. In general, plant cells (chloroplast) fix carbon dioxide via the Calvin cycle (for more details see page 28) into glyceraldehyde-3-phosphate, which can be further transformed and accumulated into storage carbohydrates, i.e. sucrose and starch. These can be then degraded (when necessary) into more simple molecules, either by *(i) glycolysis* or throughout the *(ii) oxidative pentose phosphate pathway* (Figure 6). The main products of *(i) glycolisis* are glyceraldehyde-3-phosphate, phosphoenolpyruvate and pyruvate; while the main products of *(ii) oxidative pentose phosphate pathway* are erythrose-4-phosphate and glyceraldehyde-3-phosphate. This glyceraldehyde-3-phosphate has a special role, since it is readily available from the Calvin cycle, and can be portioned between the two carbohydrate-degrading pathways as well, depending on demand (Yaylayan and Keyhani, 2001).

As presented in Figure 6, the first branch occurs between acetate/malonate and shikimate pathways. Both of these pathways are essential for the biosynthesis of flavonoids, coumaric and caffeic acid derivates, and simple phenolic acids; while ellagitannins and gallotannins rely solely on the shikimate pathway (Niemetz and Gross, 2005). If significant levels of glycolitic phosphoenolpyruvate together with erythrose-4-phosphate are directed for shikimate pathway, the production of pyruvate for the needs of acetate-melonate pathway is significantly reduced. This has direct negative effects on flavonoids, condensed tannins and stilbenes biosynthesis, since they would need melonyl CoA as one of their building blocks. However, all plant tannins rely on the efficient function of especially the shikimate pathway; while only phlorotannin producing algae (Jormalainen et al., 2003) need the acetate/malonate pathway for their tannin

synthesis. The second major branch point occurs at 3-dehydroshikimic acid. This is the precursor for the synthesis of gallic acid, the primary building block of all hydrolysable tannins (gallotannins and ellagitannins). Efficient production of gallic acid negatively affects the synthesis of shikimic acids and its products (flavonoids, coumaric and caffeic acids derivates and simple phenolic acids). The hydrolysable tannin pathway contains a third major branch point at pentagollyl glucose, the precursor for both gallotannins and ellagitannins.

Figure 6. General phenolic biosynthesis pathways



Source: Figure is inspired by British Ecological Society, Copyright 2011

Role of phenolic compounds in plants

Contrary to a primary metabolism that refers to either anabolic or catabolic processes, plant secondary metabolism refers to compounds presented in specialized cells. These phenolic compounds are not directly essential for either photosynthetic or respiratory metabolism, but are required for plants survival in the environment. It is well established nowadays that plant phenolics cannot be seen as simply waste products of primary metabolism, accumulating in the plant cells because of the absence of efficient excretory system (Kliebenstein, 2004). In fact, they represent adaptive characters being subjected to natural selection during evolution, since they apparently act as a: *(i) defense compounds*, *(ii) protecting compounds (iii) plant pigments* and *(iv) signal compounds*.

Phenolics as plant defense compounds

In the natural environment, plants encounter numerous pests and pathogens. As an appropriate response to attack from such organisms, plants have developed resistance or tolerance mechanisms that enable them to survive. While the resistance mechanisms usually refer to traits that limit the attack, tolerance strategies do not limit attack but reduce the impact consequences on the plant fitness by adjusting their physiology. As an example, resistance mechanisms include chemical or physical barriers that rapidly clear infection or herbivore, and limit the spread of damage within the host area (e.g. localized cell death). Contrary, tolerance mechanisms often involve some degree of compensation for disease damage (Taylor et al., 2004). Both of these traits however require the relocation of host resources, since the synthesis of defensive chemicals is considered to be costly for the plants itself. One of the possible strategies to avoid this lost is to synthesize defense compounds only after pathogen or insect initial attack. However, this strategy could fail while the initial attack is too rapid or severe and thus, plants exposed to frequent risk of pathogen attack invest to continuous defenses compared to those, that are attacked rarely (Morrissey and Osbourn, 1999).

Phenolics as plant defense against fungal pathogens

Although the relationships of plants and fungi are usually beneficial one (Hartley and Gange, 2009), minority of fungal species have broken this fine balance of mutual relationship.

This small amount of fungi became pathogens for plants mostly because after attack: *(i)* the plant is unable to support nutrient requirements of pathogen and is thus a *non-host*, *(ii)* plants perform physical or chemical barriers that confine successful infections to specialized pathogen species, or *(iii)* after pathogen recognition, plants induce their endogenous multicomponent defense system (Jones and Dangl, 2006). *Non-host* resistance refers to a mechanism that provides resistance against specific parasite or pathogen throughout all the members of plant species (Mysore and Ryu, 2004), contrary to *host* resistance which refers to resistance expressed by plant genotypes within susceptible host species (Thakur and Sohal, 2013).

Survival in plant pathogen interaction is controlled by the efficiency of activation biosynthetic pathways that may lead to resistance of particular plant. The very early activation of the phenolic metabolism in all plants appears to play undoubtedly main role in the expression of disease resistance (e.g. Grayer and Harborne, 1994; Bennett and Wallsgrove, 1994; Harborne, 1995; Treutter, 2006; Mazid et al., 2011; Mithofer and Boland, 2012; Agrawal and Weber, 2015). These pre-formed compounds are ubiquitous in plants and play an important role in *non-host* resistance to fungi. The distribution of pre-formed antifungal phenolics within plant is usually tissue specific; there is tendency of e.g. flavones and flavonols to be located at the plant surface (e.g. in leaf wax) or in the cytoplasmic fraction within the epidermal cells. This is suggesting that these compounds may indeed act as deterrents to pathogens (Furstenberg-Hagg et al., 2013) however, the most of pre-formed antifungal phenolics are sequestered in conjugated form in vacuoles or organelles of healthy plants (Nicholson and Hammerschmidt, 1992), suggesting their defense role.

In summary, the anti fungicidal activity of the phenolics and related compounds has been tested against several fungi and/or pathogens in certain plant species. The top 10 fungal pathogens and their importance in crop plant have been nicely reviewed by Dean et al. (2012) however, the crucial plant-pathogen interaction for this study became *Plasmopara viticola* affecting grapevine leaves (*Vitis vinifera* L subsp. *vinifera* Hegi). For more detailed information see introduction of Šebela et al., 2014 (chapter *3.1.1.*) and Olejníčková et al. (chapter *3.1.2.*).

Phenolics as plant protective compounds

Plants are photoautotrophic organisms, which cannot exist without solar radiation. This solar radiation is necessary for the process called photosynthesis (see more details

`Photosynthesis and Photosynthetic apparatus`, page 24), proceeding with an optimal rate only within a narrow irradiance range (photosynthetically active radiation-PAR). This range is often lower than solar fluxes reaching plants under natural conditions. Therefore, the light energy being absorbed by the photosynthetic apparatus often could not be utilized completely, in the sense of photochemical reactions and thus, the excessive fluxes of solar radiation (harmful for plants) requires protecting mechanism. The common plants response to harmful radiation include: *(i)* increase in xanthophyll-cycle pool size (Kirchgessner et al., 2003), as well as *(ii)* increase in contents of protective compounds. From the protective compounds perspective, plants exposed to the growing environment are endangered mainly by solar ultraviolet-B (UV-B) radiation, ranging from 280-320 nm. In plants, exposure to such UV-B light is potential environmental challenge, which could negatively affect DNA, proteins and membranes and thus, can lead to altered metabolism through the generation of reactive oxygen species (ROS; Jenkins, 2009). In fact, long-term experiments with UV-B radiation have shown limited impact of UV-B on leaf phenolics synthesis (Rozema et al., 2006), while during field surveys, temperature rather than UV-B radiation was found to be possibly the primary driver for leaf phenolic synthesis (Albert et al., 2009).

Despite this fact, it is widely accepted that as a response to the UV-B radiance plants have developed a protective mechanism - phenolic compounds synthesis (Hideg and Vass, 1996). These compounds in fact act as a screen inside epidermal cell layer, by adjusting the antioxidant system at either whole organism or the cell level (Brown et al., 2001; Jansen et al., 2001; Winkel-Shirley, 2002). It has also been proven that the importance of phenolics lies primarily in protection of leaves from photo-oxidative damage, rather than herbivore damage (Ryan et al., 2002; Agati et al., 2009, 2011, 2012; Agati and Tattini, 2010). All these statements are basically reflected by the optical properties of phenolic compounds. Characteristic absorption spectrum of relevant UV screening compounds typically contains two main bands. The first band (peak around 280 nm) appears due to the presence of aromatic rings, and is detected in the absorption spectra of all phenolics. Contrary, the second band (300-360 nm) varies for different classes of phenolics. According to this, it has been proposed that flavonoids with their high absorption in both of these regions act as a good screeners. Activation of these compounds by UV radiance has been shown in number of studies (e.g. Sisa et al., 2010; Ryan et al., 2001; Rozema et al., 2002; Agati and Tattini, 2010; Saito et al., 2013) however, also other phenolic

compounds may be at least as important as flavonoids in UV protection. In principle, such UV effects on secondary metabolism include accumulation not only of flavonoids, but also e.g. cinnamate esters, lignin and tannin (Rozema et al., 1997), differing across the studies. Level of UV tolerance (in principle the ability of plant to synthesize phenolic compounds) differs considerably between genera, species and even closely related species (Sullivan et al., 1992). It is noticeable than tropical and high altitude plants, where UV-B influences are greater, have more pronounced adaptive mechanisms and thus, contain a higher proportion of phenolic compounds than temperate ones do (Sullivan et al., 1992; Nikolova and Ivancheva, 2005).

In summary, the role of phenolic compounds as a protective mechanism of plants against UV light is widely accepted phenomena. Synthesis of these compounds occur both in plant leaves and fruits however, crucial for this study is effect of UV radiation on the synthesis of phenolic compounds in berries of grapevine (*Vitis vinifera* L.). For more detailed informations see introduction part in Šebela et al., (chapters *3.2.1.*, *3.2.2.*).

Phenolics as plant pigments

Despite the above mentioned functions in leaves and fruits, important role of phenolic compounds is also to serve visual signals for animals. Here, plant phenolics act as pigments in flowers and fruits. The main function is to attract pollinators in case of flowers; later to attract animals to eat the fruits and thereby helping in seed dispersal. Flavonoids are among the best-characterized plant secondary metabolites in terms of plant coloration mechanism (Chopra et al., 2006). Variations of hydroxylation pattern of main flavones and flavonols (e.g. apigenin, luteolin, kaempferol, quercetin and myricetin) results in white or yellow structures of the tissues in which they are located. Changes of anthocyanins color depend on the pH. In *vitro*, anthocyanidins are redder and more stable at lower pH, colorless under medium acidic conditions and bluer at high pH. Concerning anthocyanins in *vivo*, several different factors can affect the final color of the fruit or flower. Delphinidin-derrived anthocyanins are known to be responsible for the bluish colors, whereas cyanidin derived anthocyanins are found from light-violet and reddish tissues, respectively.

Phenolics as signal compounds

Several evidences suggested that plant phenolic compounds are potential candidates to serve as signals during mycorrhizal formation (reviewed by Mandal et al., 2010). Phenolic

compounds can directly affect the composition and activity of decomposer communities and thus are influencing the rates of decomposition and nutrient cycling however, this function is above the scope of this thesis and thus, only short evidence about the role of phenolics as a signal compounds is presented.

Presence of phenolic compounds in plants

Phenolic compounds are ubiquitous in the plant kingdom, being found in all parts of the plant. Their quantitative distribution can vary between different organs of the plant (Table 2), and within different populations of the same plants species (e.g. Scalzo et al., 2005).

Table 2. Relative concentration of phenolic compounds in plant tissue

Tissue	Relative concentration
Leaf	flavonols ~ cinnamic acids > catechins ~ anthocyanins
Fruit	cinnamic acids > catechins ~ anthocyanins > flavonols
Wood	catechins ~ anthocyanins > flavanols > cinnamic acids
Bark	the same as for wood but greater concentrations

~approx. same concentration; >higher concentration
Source: Pratt and Hudson, (1990)

In recent years, there is considerable interest in determining total phenolic content of e.g. medicinal plants (Cai et al., 2004; Gan et al., 2010), common vegetables (Kaur and Kapoor, 2002; Chu et al., 2002; Deng et al., 2013) or common fruits (Reddy et al., 2010; Fu et al., 2011). Despite this, the phenolic content of plants constitutes a complex mixture, but only a small number of plant species have been examined systematically for their phenolic content. From the fruit perspective, the main plant phenolics may be classified as shown in Table 3. Phenolic acids are said to be universal, widely distributed throughout the plant species. Fruits represent a significant source of hydroxybenzoic acids (protocatechuic, vanillic, gallic and syringic acids), as well as hydroxycinnamic acids (largely comprised of caffeic and ferrulic acids). Their widespread distribution and high concentration in fruits may be due to their key role which they play in biosynthesis of other, more complicated phenolics. Flavonoids (Table 3) represent most widespread and structurally diverse group of the phenolics. Three of the numerous classes of major flavonoids are widespread and quantitatively dominant: flavanols, flavonols and anthocyanins. The monomeric flavan 3, 4-diols are not the major compounds of fruits however,

Table 3. Different classes of phenolic compounds and characteristic examples in fruits

Class	Common fruit source	Examples
Phenolic acids		
Hydroxybenzoic acids	widely distributed	protocatechuic, vanillic, gallic and syringic acids
Hydroxycinnamic acids	widely distributed	caffeic and ferulic acid
Flavonoids		
Flavanols	apple	(+)- catechin, (-)- epicatechin
	pear	(+)- catechin, (-)- epicatechin
	peach	(+)- catechin, (-)- epicatechin
Flavonols	apple	quercetin, kaempferol
	pear	quercetin, kaempferol
Anthocyanins	apple	cyanidin glycosides including acylated derivatives
	sweet orange	pelargonidin, peonidin, delphinidin, petunidin
	pear	cyanidin glycosides
	peach	cyanidin glycosides
	cherry	cyanidin 3-glucoside and 3-rutinoside
	plum	glycosides of cyaniding, peonidin
Flavones	sweet orange	apigenin, luteolin, tricin
	grapefruit	apigenin, luteolin, tricin
	lemon	luteolin

Source: Table is inspired by Robards et al. (1999)

flavanols are important constituent of fruits in oligomeric or polymeric forms as proanthocyanidins or condensed tannins. Among these, (+)- catechin, (-)- epicatechin, (+)- gallocatechin and (-)- epigallocatechin are found in fruits (table 3) generally in free rather than glycosilated forms which distinguishes them from other flavonoids. The flavonols (quercetin, kaempferol and myricetin are common in many of fruits as presented in table 3. Flavones including apigenin, luteolin and tricin are encountered much less commonly than flavonols and then only in trace amounts. Apart from these compounds, the anthocyanins are the next most abundant and widely distributed flavonoids. These compounds give to most of fruits their red,

violet and blue color, although the red color of some fruits is caused by carotenoid pigments rather than anthocyanins.

Phenolic content of grapes has been more extensively studied than any other fruit with the possible exception of apple. The reason is that these compounds play an important role in the quality of grapes and resulting wines. In grape berries, phenolic compounds are presented mostly in skins and seeds (table 4) however, at certain phenological stages can occur also in flesh.

Table 4. Phenolic compounds produced and accumulated in the grape berry

Compound	Level of synthesis			Location	Phenological scale			
	Skin	Flesh	Seeds		a	b	c	d
Phenolic acids								
HCA[1]	++	+++	++	hypodermal cells and placental cells of the pulp; primarily in the vacuoles of mesocarp cells	+++	+++	+	+
HBA[2]	+	-	++					
Non-flavonoids								
Stilbenes	+++	+	++	berry skin and seeds	-	+	++	+++
Flavonoids								
Flavanols	++	+	+++	dermal cell vacuoles of the skin tissue and cell wall of skin and seeds	+	++	+++	++
Flavonols	++	-	-	specific vacuoles of hypodermal skin cells and seeds coat soft parenquima	++	+	+++	++
Anthocyanins	+++	-*	-	cell layers bellow the epidermis; storage confined to the vacuoles and cytoplasmatic vesicles named anthocyanoplasts	-	-	+	+++

-Level of synthesis: very abundant (+++), abundant (++) to absent (-); berry phenological scale: a (blooming), b (green stage), c (verasion), d (ripening); ([1]) HCA (hydroxycinnamic acids), ([2]) HBA (hydroxybenzoic acids); ([*])Teinturiers (grapes whose flash is red in color) contain anthocyanins also in mesocarp cells.

Source: Table is inspired by Teixeira et al. (2013)

Phenolic acids are commonly accumulated in berry skin, flash and the seeds (HCA, table 4), while HBA are not present in the flesh. The synthesis of HCA occurs mainly before version.

During ripening, their concentration decreases with the increasing fruit size and dilution solutes, though its content per berry remains almost constant. Stilbenes, a non-flavonoid class, are present in grape in trace quantities. These compounds occur naturally mainly in the skin at a mature stage however, can be present also in the seeds and flesh. Grape flavonoids are localized mainly in the berry skin and in the layers of seed coat. Flavanols are located essentially in the seeds, then in the skin and very little in the flash (table 4). Their synthesis occurs at the early stages of fruit development and subsequently, concentration increases towards the verasion. Also, flavonols synthesis occurs primarily during early stages of fruit development and ends around verasion. Flavonols are known to serve as UV protective and co-pigmentation compounds together with anthocyanins and thus, are presented in the grape skin. Anthocyanins in the grape berries are responsible for red, purple and blue pigmentation. Their accumulation in red grape cultivars starts from verasion and reaches its maximum in the latest phases of fruit maturation, when the synthesis is finished (table 4).

1.4. Detection of phenolic compounds in plants

Considerable interest in the accurate detection of phenolic compounds nowadays is reflecting their importance mentioned above. Usually, assays for detection of phenolic compounds in plant samples are carried out in central laboratories using separation techniques. Such situation (in view of huge labor, analytical ballast or time delays associated with centralized laboratory analyses) is showing immediate needs for developing fast and accurate techniques, which would be comparable with these traditional chemical analyses. In this section, traditional chemical analyses *(Direct methods)* and approaches mainly based on non-invasive monitoring of phenolics *(Indirect methods)* are discussed.

Direct methods for phenolic compounds detection

Selection of proper analytical strategy for detection of phenolic compounds in plant depends on the purpose of the study, as well as the nature of the sample and analyte. Assays used for the analysis of phenolic compounds are usually classified as either those which are measuring total phenolic contents, or those which are quantifying specific group or class of phenolics. In both cases, the quantification in plant extract is influenced by the chemical nature of phenolics, as well as assay method, selection of standards and presence of other interfering substances.

Sample preparation and extraction

The key step for every analysis is preparation of sample. Many sample preparation techniques have been developed to determine phenolic compounds in a wide range of plant materials. It is crucial to choose the optimal pretreatment for each plant sample. Simplify, phenolics can be extracted from fresh, frozen or dried plant samples. Solid samples are usually subjected to milling, grinding and homogenization, which may be preceded by air-drying or freeze-drying. It has been proven however, that drying of plant sample can cause undesirable effects on the constituent profiles (Torres et al., 2010) and thus, it is appropriate to select optimal pretreatment according to the structure or properties of analyzed samples. Liquid samples are usually firstly filtered and/or centrifuged, after which they are either directly injected into the separation system or can undergo additional steps using relevant techniques. Subsequent steps in the analysis can be characterized as *(i) extraction* and *(ii) purification of the extract* (Dai and Mumper, 2010).

(i) Extraction

- Extraction by water or aqueous solution of acids (e.g. methanol, ethanol, acetone, ethylacetate etc.)
- Maceration
- Microwave-assisted extraction, ultrasound-assisted extraction
- Pressurized fluid extraction (pressurized liquid/accelerated solvent extraction, subcritical water extraction, supercritical fluid extraction)
- Acid hydrolysis (glycosides → aglycones)
- Alkaline hydrolysis: deacylation of glycosides and catechins
- Enzyme hydrolysis (glycosidases, proteases, amylases)

(ii) Purification of the extract

- Solid phase extraction (sorption and elution)
- Liquid-liquid extraction
- Gel chromatography
- Countercurrent chromatography

Analysis

The principles and/or comparisons of diverse analytical methods are thoroughly reviewed (e.g. Schofield et al., 2001; De Beer et al., 2004; Naczk and Shahidi, 2004; Piljac et al., 2005; Bendini et al., 2007; Stalikas, 2007; Dai and Mumper, 2010; Garcia-Salas et al., 2010; Khoddami et al., 2013; Nayak et al., 2015). To quote the main analytical methods, e.g. *(a)* classical spectrophotometric assays provides simple and fast screening methods to quantify classes of phenolic compounds in plant samples (e.g. Folin-Denis method and Folin-Ciocalteu method); *(b)* modern high-performance liquid chromatography techniques (HPLC) combined with instrumental analysis offer a unique chance to analyze simultaneously all components of interest together with their possible derivatives; *(c)* gas chromatographic (GC) techniques provides accurate methods which are widely used especially for separation and quantification of compound of interest; *(d)* electrochemical detection (ECD); *(e)* mass spectrometry (MS); etc.

Given the chemical properties, phenolic compounds exhibit a higher or lower absoption in ultraviolet/visible (UV/VIS) light. Thus, the most common method of detection are photodiode array (PDA), and UV-fluorescence detection.

Spectrophotometric detection (conventional UV/VIS or PDA detectors), typical absorption maxima of main selected classes of phenolic compounds

- HCA (270-280 nm, 305-330 nm)
- HBA (270-280 nm)
- Coumarines (220-230 nm, 310-350 nm)
- Flavons, flavonols, chalcons (270-280 nm, 310-390 nm)
- Other flavonoids (270-280 nm, 310-350 nm)
- Anthocyanins (500-530 nm)

In summary, sample extraction and selection of the proper analytical methods plays crucial role in each experiment. For more details about selected extraction methods and/or analytical methods see Šebela et al., chapters *3.1.1.*, *3.1.2.*. *3.2.1.* and *3.2.2*.

Indirect methods for phenolic compounds detection

Plant organs (e.g. leaves and fruits) contain a great amount of different pigments, which play a variety of roles. Apart from the function of the phenolic compounds prescribed above, plant contains also pigments that are able to utilize energy from sunlight and transform it to chemical energy in the process called *(`Photosynthesis`)*. These pigments are able to absorb the energy coming from sunlight. Some of them have ability to re-emit such absorbed energy in the form of fluorescence or phosphorescence (*`Fluorophores`*). Knowing the basic optical properties of plant pigments, several methods are commonly used to estimate the content of compound of interest *in vivo*. These possible non-invasive methods for detection are briefly discussed in this section, for more details see Šebela et al., chapter *3.2.2.*

Photosynthesis and photosynthetic apparatus

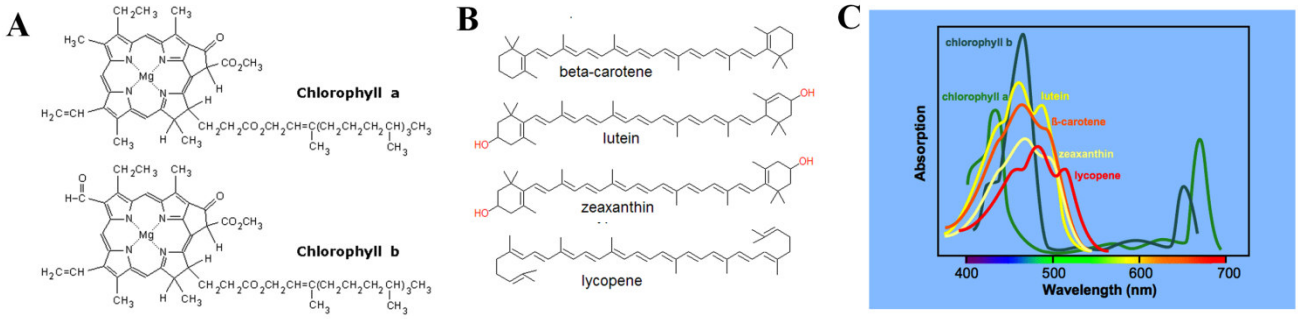
Photosynthesis, an ancient process that gradually accumulated oxygen in the Earth`s atmosphere, is a biphasic process taking place in plants, algae and photosynthetic bacteria (in our

study, we are dealing with C_3 plants). As a biphasic process, photosynthesis can be roughly divided into so called *`light`* and *`dark`* phases. During the *`light`* phase, a set of biochemical processes occur through the action of light. This *`light`* phase takes place in the chloroplasts, specialized subunits in plant and algal cells. Chloroplasts have a diameter of 5-10 μm, depth of 3-4 μm and contain a matrix called thylakoid membranes. In higher plants, thylakoids are organized into so called granum. Grana are connected by non-stacked membranes called stroma lamellae. The fluid compartment that surrounds the thylakoids is known as stroma, while the space inside the thylakoid is called lumen. Light energy during *`light`* phase is captured by antenna pigments located in light harvesting complexes (LHC) of photosystem II (PSII) and photosystem I (PSI), both situated within the thylakoid membranes of chloroplasts. The antenna system of higher plants comprises main *photosynthetic pigments.*

Photosynthetic pigments

The leaves of higher plants contain several types of photosynthetic pigments: *(i) chlorophylls* and *(ii) carotenoids*, a chemical compounds which absorb only certain wavelengths of visible light. *(i) Chlorophylls* are greenish pigments which contain a porphyrin ring (Figure 7A). This is a stable ring shaped molecule, around which electrons are free to migrate. Because the electrons can move freely, the ring has the potential to either gain or lose electrons easily and thus, has the potential to provide energized electrons to other molecules. This is the fundamental process, by which chlorophyll captures the energy from sunlight. There are several kinds of chlorophylls, which differ in some small changes in the ring structure, as well as in different side chains. The most important is chlorophyll a (Chl-a; Figure 7A), which is present in all photosynthesizing plants, algae and cyanobacteria. A second kind of chlorophyll is chlorophyll b (Chl-b; Figure 7A), which is present only in the plants and green algae. A third, common form of chlorophylls, is called chlorophyll c, found only in the photosynthetic members of certain marine algae. Figure 7C shows typical absorption spectra of Chl-a and Chl-b *in vitro*. In principle, there are two distinct absorption bands in blue and red part of the visible spectrum, which position depend on a solvent assayed (they can be shifted towards longer wavelengths with increasing solvent polarity and water content). *(ii) Carotenoids* are usually red, orange or yellow pigments, the most abundant in nature. These compounds are found only in photosynthesizing organisms.

Figure 7. Photosynthetic pigments



Chemical formulas of Chl-a and Chl-b (panel A), main carotenoids (panel B), and absorption spectra of both chlorophylls and carotenoids (panel C).

Source: panel A (http://www.food-info.net/uk/colour/chlorophyll.htm),

panel B (http://en.citizendium.org/wiki/Carotenoid),

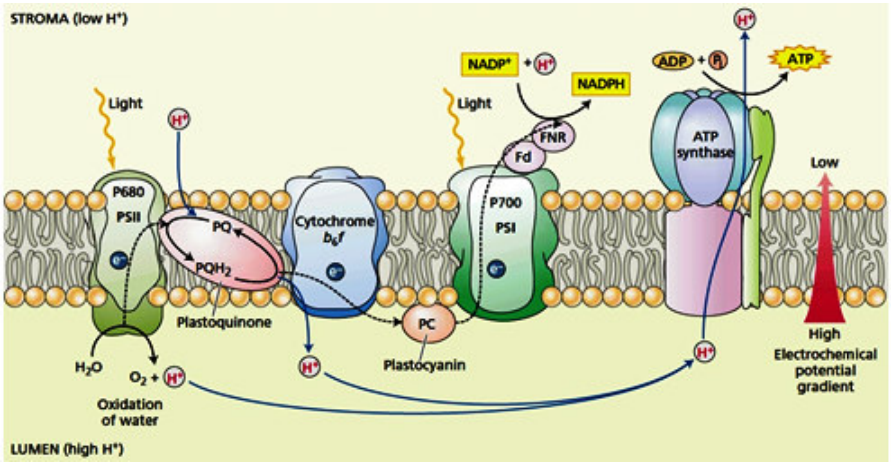
panel C (http://biology.westfield.ma.edu/biol129labs/sites/default/files/Figure%204.gif)

Carotenoids, which are involved in light harvesting process are called `primary`, whereas others, found outside the photosynthetically active tissue, are called `secondary`. The primary ones are present in all photosynthetic pigment-protein complexes. They are essential for: *(i)* proper folding of proteins and stabilization of their structures, *(ii)* they contribute to the efficiency of photosynthesis, since they harvest light of wavelengths where chlorophylls cannot absorb, and/or *(iii)* they provide protection against excessive excitation via de-excitation of chlorophyll directly or in xanthophyll cycle. Carotenoids are chemically derived from terpenoids, made from several isoprene subunits (Figure 7B). Primary carotenoids can be divided into two main groups: *(a)* oxygen free carotenes (α- or β-carotene) or *(b)* oxygenated derivatives, xanthophylls (lutein, zeaxanthin, lycopene). Although carotenoids comprise a lot of compounds, all are characterized by the similar absorption spectrum (Figure 7C). Here, two absorption maxima with one shoulder can be seen for β-carotene, lutein, zeaxanthin and lycopene. The position of these peaks in the UV-blue spectral region (350-500 nm) can be shifted either to longer wavelengths (with increasing extent of conjugation), or to a shorter vawelengths (with increasing amount of oxygen in hydrophilic groups) (Polívka and Sundstorm, 2004).

After the light is being absorbed by LHC, primary electron donor (Chl-a) enters its singlet excited state, and pass the energy by electron resonance transfer towards reaction centers (RC) of both photosystems (PSII and PSI), where they drive a charge separation (Figure 8). *PSII is a unique complex since it can reduce water to molecular oxygen, protons and electrons in the oxygen-evolving complex (OEC). In higher plants, PSII is present mainly as a dimeric structure. The core of PSII contains in total 35 Chl-a molecules, 2 pheophytins a and 8-11 molecules of β-carotene, associated into several proteins. To quote the main, (i) D1 and D2 complexes coordinate the RC, the primary electron donor P680, and all cofactors of electron transport chain, (ii) core antenna complexes CP47 and CP43 coordinate Chl-a and β-carotene molecules and act as an inner antenna, and (iii) several low molecular weight subunits (Dekker and Boekema, 2005). PS I is the second photosystem. It is an integral membrane protein complex that uses light energy to mediate transfer from plastocyanin (PC) to ferredoxin (Fd). The electron transfer components of the reaction center of PSI are a primary electron donor P700.*

The extracted electron from the RC of PSII is transferred through several electron acceptors (Chla, Pheo a, Q_A) and finally reaches Q_B. Here, the electron is further transferred to PSI, via mobile protein situated in the lumen side of the thylakoid membrane [plastocyanin (PC)]. In the core of PSI, electron is again energized (by getting excitation energy from LHCs of PSI). With this additional energy, the electron is transferred to a phylloquinone molecule, and over three protein binding Fe-S cluster containing proteins (located in stromal side of the chloroplast) to ferredoxin (Fd). The last step of this pathway is catalyzed by ferredoxin $NADP^+$ reductase (FNR). Here, FNR transfers electrons to $NADP^+$, yielding NADPH, further consumed in Calvin-Benson cycle. Also, there are other important components of the thylakoid membrane, involved in the primary photochemical processes of photosynthesis (e.g. adenosine triphosphate - ATP synthase). This transmembrane multi-protein structure utilizes protons coming from OEC. Here, the proton gradient between the lumenal and stromal side of the thylakoid membrane is used as a source of energy to synthesize ATP from both ADP and inorganic phosphorus (phosphate group) in a catalytic part of the ATP synthase. This catalytic part is located in the stromal side of the thylakoid membrane. To simplify, during first *`light`* phase of the photosynthesis, the water is broken in the chlorophyll molecule into H^+ and OH^- ions in the presence of light. This phase is resulting in the formation of assimilatory powers such as NADPH and ATP, which are subsequently used in *`dark`* phase.

Figure 8. The scheme of electron transport in oxygenic photosynthesis



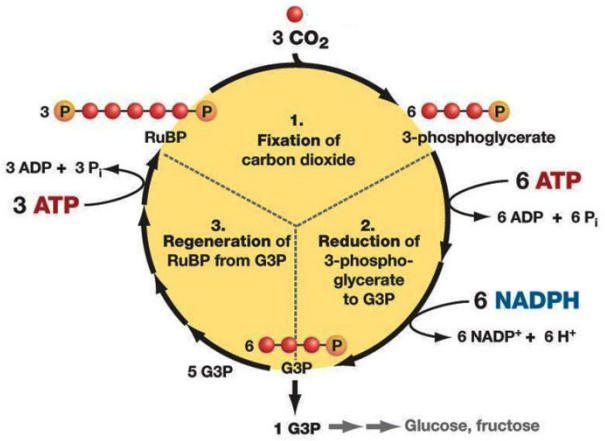
The diagram shows linear electron transport in the pigment-protein complexes (PSII and PSI), large multisubunit protein (Cyt b_6f) and enzyme complex (ATP synthase), which are located in thylakoid membranes of chloroplasts. The energy of absorbed photons is needed to transfer electrons from PSII and PSI donors to their respective acceptors. The electrons are further transferred through several donor/acceptor pairs (in addition to PSII, PSI, Cyt b_6f, ATP synthase and mobile intersystem electron carriers): *(a) PQ* and *(b) PC*. Ad *(a) PQ* connects PSII and Cyt b_6f complex, while *(b) PC* transfers electrons from Cyt b_6f to PSI. Finally, transferred electrons are utilized to reduce a molecule od $NADP^+$ to NADPH. The missing electron is replaced by one being extracted from the molecule of reducing water. As by-product, molecular oxygen is released. In a series of chemical reactions, a proton gradient is build across the thylakoid membrane, due to release of protons into chloroplast lumen after water oxidation, and also due to proton transfer governed by plastoquinone Q_B from stromal to luminal side. This transmembrane proton concentration gradient (ΔpH) powers ADP synthase to ATP production. The co-products from both $NADP^+$ and ADP synthesis (NADPH and ATP) are ultimately utilized in Calvin-Benson cycle, where carbon is assimilated and carbohydrates are synthesized. *Source: Taiz and Zeiger (2010)*

The *`dark`* phase is second step in the mechanism of photosynthesis, occurring independently on light. It takes place in the stroma of the chloroplasts. This phase is purely enzymatic and, in principle, slower than the *`light`* reaction. Two types of cyclic reactions can occur: *(1) Calvin cycle* (or C_3 cycle) and *(2) Hatch Slack* cycle (or C_4). The *(2) Hatch Slack* cycle (C_4 fixation) is an elaborating of the more common C_3 carbon fixation and is believed to have evolved more recently. Since in our study only C_3 plants are investigated, attempt will be focused only on C_3 pathway.

Calvin cycle

The Calvin cycle (C_3 carbon fixation pathway) has three phases (Figure 9). At first, there is carboxylation (carbon fixation), at second there are reduction reactions and finally, ribulose 1,5-bisphosphate (RuBP) regeneration (Blankenship, 2002). In the first phase (fixation of carbon dioxide, Figure 9), a molecule of CO_2 is taken in by the cell and is combined with RuBP to form a six carbon intermediate sugar via enzyme called ribulose-1,5-bisphosphate carboxylase (RuBisCO). The six carbons than breaks down to form two 3-phosphoglycerate (PGA`s), each a three carbons. This is the first stable intermediate compound of Calvin cycle. In subsequent reduction reactions [reduction of 3-phosphoglycerate (PGA) to G3P, Figure 9)], enzyme phosphoglycerate kinase uses ATP and NADPH (those products of light reactions) to catalyses the PGA and produce glycerate-1,3-bisphosphate and ADP as a product. In the subsequent step (regeneration of RuBP from G3P, Figure 9), the enzyme glyceraldehyde 3-phosphate dehydrogenase (G3P) catalyses the reduction of glycerate-1,3-bisphosphate by NADPH and produce glycearldehyde 3-phosphate. During this stage, NADPH get oxidized and becomes $NADP^+$, while regenerate RuBP. Two molecules of NADPH and three molecules of ATP are required for the fixation of each CO_2 molecule. After the regeneration, cycle starts again.

Figure 9. Carbon fixation through the Calvin cycle



Source: http://www.uic.edu/classes/bios/bios100/lectures/ps01.htm

Fluorophores in plants

Some of the molecules have ability to re-emit absorbed energy in the form of fluorescence emission and thus, are called fluorophores (Table 5). Such fluorescence emission, when excited by radiation of suitable wavelengths (UV/Vis), can be observed from endogenous fluorescent molecules in living tissue (algae, plant and animal). Plant tissue however, is more strongly fluorescent compared to the animal one.

Table 5. Plant auto-fluorophores

Chemical class		**Examples**
Plant phenolics		
Non-Flavonoids	phenolic acids	
	hydroxycinnamic acids	caffeic acid, ferulic acid, sinapic acid
	stilbenes	resveratrol, derivates of resveratrol
	tannins	ellagic acid
Flavonoids	flavonols	kaempferol, quercetin
	flavanols	catechin, epicatechin
	flavanones	naringenin, naringin, hesperidin
	flavones	apigenin, luteolin, chrysin
	coumarins	
Cyclic tetrapyroles		Chl-a, Chl-b

Source: Table is inspired by Buschmann and Lichtenthaler (1998), Roschchina (2012), Talamond et al. (2015)

UV excitation (340-360 nm) of leaves of higher plants generates two types of fluorescence: *(i) a blue-green fluorescence* (*BGF)* and *(ii) red and far-red fluorescence* (*chlorophyll fluorescence).*

The first type *(i) BGF* is characterized by a wide spectral emission band in the blue region (430-450 nm) and a shoulder in the green region (520-530 nm) (Buschmann et al., 2000; Meyer et al., 2003). The reason of such wide band is that *BGF* combines the emissions of several

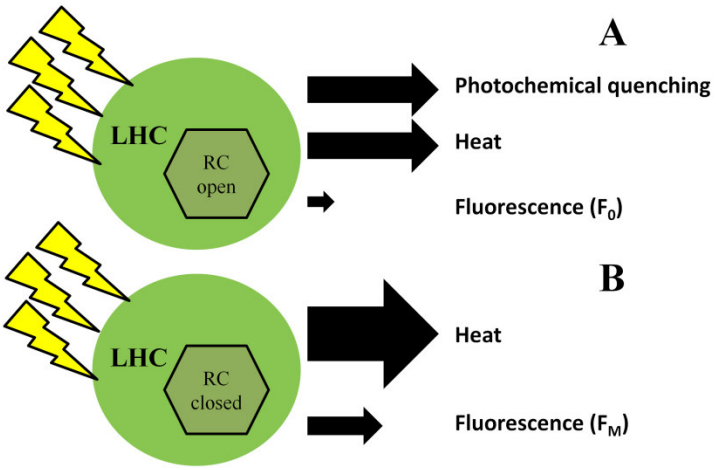
universal cellular fluorophores. These fluorophores include e.g. flavin coenzymes, nicotinamides (NADH), pyridoxal phosphate (vitamine B_6), folic acid (vitamine B_9) however, the most crucial for our study are plant phenolics summarized in table 5. Plant phenolics are, thanks to the π-electron system in aromatic structure, very good absorbers. These compounds are in fact covering majority of UV spectrum (Chapelle et al., 1984; Buschmann and Lichtenthaler, 1998; Cerovic et al., 1999; Buschmann et al., 2000; Ounis et al., 2001; Meyer et al., 2003; Talamond et al., 2015). By simplifying the statement of Cockell and Knowland (1999), `the larger the molecule is the longer wavelength of UV region it can absorb`. Flavonoids, for instance, absorb at longer wavelength (UV-A to blue), while are relatively poor UV-B absorbers (Hofmann et al., 2000). Not only e.g. size of the molecules, but also other factors are affecting *BGF*. For instance, Lichtenthaler and Schweiger (1998) pointed out the effect of pH on the *BGF* of plants. Yet probably, anatomy of plant tissue plays the most crucial role on the resulting *BGF* signal. Fortunately, methods for detecting the tissue-specific distribution of main phenolic compounds have been developed (Hutzler et al., 1998; Cerovic et al., 1999; Agati et al., 2002; Tattini et al., 2004; Polster et al., 2006) and thus, enabled the discrimination of the relative abundance of plant phenolics in different layers of plant tissue.

Contrary to `static` *BGF*, the *(ii) red and far red fluorescence* is highly dynamic process, which could provide insight into molecular processes of photosynthesis. This process (light that is re-emitted after being absorbed by Chl-a molecule of LHC of PSII) is usually called *chlorophyll fluorescence (Chl-F)*. Since in the photosynthetic apparatus, both Chl-b and carotenoids have a role of accessory pigments transferring their energy to Chl-a, also UV radiation can be used to induce Chl-F. To complement the formulation of Chl-F, it is not emitted only from LHC of PSII. There is a small contribution of PSI as well (at far-red region near 735-740 nm), which usually occur at room temperature (Lichtenthaler and Babani, 2004). Despite this, it is widely accepted that the PSII contribution (compared to PSI) is dominating below 700 nm (Butler, 1978; Baker, 2008), even though several studies reported non-negligible contribution of PSI under specific conditions and longer wavelengths over 700 nm (Pfundel, 1998; Agati et al., 2000; Pfundel et al., 2013).

Chl-F is representing one of three pathways that light-induced excitation energy (both UV/Vis) can take during the photosynthetic process. Light energy being absorbed by LHC can be either used *(i)* to drive photosynthesis, its excess energy can be *(ii)* given off as heat, or it can

be *(iii)* re-emitted in the form of light as Chl-F (reviewed by e.g. Krause and Weis, 1991; Maxwell and Johnson, 2000; Adams et al., 2004; Baker, 2008; Murchie and lawson, 2013). These three processes occur in competition (Figure 10) and thus, any increase in the efficiency of one will result in a decrease in the yield of the other two. Typically, Chl-F represents only 1-2% of energy of excitation, while the rest of energy undergoes other two pathways (Maxwell and Johnson, 2000). Despite the fact that Chl-F represents only a small fraction of excitation energy, it can be easily measured by using several techniques, e.g. pulse amplitude modulation (PAM) (Schreiber et al., 1986; Schreiber, 2004; Oxborough, 2004; Murchie and Lawson, 2013).

Figure 10. Schematic diagram of Chl-F



A schematic diagram is showing the interaction between Chl-f (F), photochemical reactions (photochemical quenching) and heat. If the reaction centers (RC) of photosystem II (PSII) are opened (panel A), the incident irradiance (yellow arrows) is absorbed by light harvesting complexes (LHC), where it is dissipated in form of fluorescence (F_0) and/or heat, or is used for photochemical reactions (photochemical quenching). If the reaction ceneters (RC) are closed (panel B), photochemical reactions cannot occur and the dissipation of the energy absorbed by light harvesting complex (LHC) is directed to heat and/or fluorescence exclusively. Area of black arrows represents the probability of energy transfer by the respective pathways (i.e. fluorescence, heat, and photochemical reactions) for energy absorbed by the photosynthetic unit through to charge stabilization in photosystem II (PSII).
Source: Scheme is inspired by Parkhill et al. (2001).

A pulse amplitude modulation (PAM) uses a modulated light source to produce short measuring pulses. The fast detection system of PAM technique is tuned to measure Chl-F only within these pulses and thus allows to indirectly estimate photosynthetic activity and a regulation in plants (e.g. Kraus and Weis, 1991; Nedbal and Koblížek, 2006; Baker, 2008; Brestic and

Zivcak, 2013). Four light sources are typically used for PAM and/or PAM- fluorimeters, respectively. These lights provide quantitatively different radiation which subsequently induces changes in the Chl-F: *(i)* measuring pulsed light (ML), *(ii)* actinic light (AL), *(iii)* saturation pulse light (SL) and *(iv)* far-red light (FR). In fact, the principle of PAM lies on the application of SL in certain phases of sample illumination by ML or AL.

The very first Chl-F transient was observed in 1931 by Kautsky and Hirsch (Govindjee, 1995). Here they witness the situation when the dark adapted sample is exposed to the actinic light; after exposure, there was a fast rise when the Chl-F increased up to 6 times, while with the time of irradiance it slowly declined to a stationary level. This phenomenon became one of the main principles for PAM measurements. The Chl-F transients during PAM measurements comprise of two main phases: *(i)* dark and *(ii)* light phase. When the photosynthetically active sample is dark adapted (DAS), generally all reaction centers of PSII are fully oxidized (RC opened, Figure 10A) and components of the non-photochemical quenching are at their minimum. To measure the minimum level of Chl-F in this pre-darkened state (F_0, Figure 10A), a weak modulated ML is applied. In this *(i)* dark phase, the majority of excitons are used to drive photochemistry or dissipated in the form of heat. Subsequently, single short (ca. 1s) light saturation pulse (SL) is applied, which causes a transient saturation in every RC (RC closed, Figure 10B). Such saturation results in a very fast rise of the yield of Chl-F to its maximum level (F_M, Figure 10B). Both minimum (F_0) and maximum (F_M) Chl-F level determination is essential primarily for non-photochemical processes activated in subsequent *(ii)* light phase. During the light phase, photosynthetic apparatus is gradually moving from DAS to light adapted state (LAS). Here Kautsky effect [the fast rise of Chl-F yield from F_0 to peak Chl-F level (F_P) and subsequent decline to steady state Chl-F level (F_S)], is visible when the AL is switched on. The decline after F_P to F_S can be characterized by a number of inflection points and transient peaks, depending on the light source being applied. In fact, the decline to LAS is affected by multiple factors, including *(i)* photochemical and *(ii)* non-photochemical quenching (e.g. Baker 2008, Roháček et al., 2008). Simplify, the decline caused by *(i)* photochemical quenching is caused by increase in the rate at which electrons are being transferred from PSII and/or due to activation of enzymes involved in stomata opening and carbon metabolism. In contrast to the decline of Chl-F yield caused by photochemical quenching, the decline caused by *(ii)* non-photochemical quenching is resulting from the efficiency, with which energy is converted to heat. The whole

Chl-F transient finishes when AL is switched off. After this step, back-relaxation from LAS occurs, while in a certain time, DAS is restored. To accelerate the transient from LAS to DAS (reoxidation of PSII acceptor side), FR is applied. There are many experimental transients, which can probe photosynthetic apparatus at different time scales (e.g. Nedbal and Koblížek, 2006; Govindjee and Papageorgiou, 2013) however in general, several primary mutually independent Chl-F levels taken from these transients are sufficient for definitions of the most calculated Chl-F parameters. The most convenient parameters giving a relevant insight into the primary photosynthetic processes have been nicely reviewed by (e.g. Roháček, 2002; Roháček et al., 2008; Baker, 2008).

Despite the Chl-F, also other optical techniques are used in this study and introduced in particular subdivisions of chapter 3.

2. MATERIALS AND METHODS

In this chapter, brief explanation of the material and methods used during this study is presented. The detailed descriptions are given in each subdivisions of Chapter 3., i.e. prepared manuscripts and publications, respectively.

2.1. Plant material

Briefly, two model species were used during this study, i.e. grapevine (*Vitis vinifera* L. subsp. *vinifera* Hegi) and rice (*Oryza sativa* L.). For the experimental purposes, selected cultivars from both species were tested. Grapevine cultivars were sampled from *(i)* experimental vineyard (Department of Viticulture and Oenology, Lednice, Czech Republic), *(ii)* vineyard of Winberg Winery Ltd., Mikulov, Czech Republic), while *(iii)* rice genotypes were collected at the International Rice Research Institute (IRRI), Los Banos, Philippines. For more details see chapters *3.1.* and *3.2.*

For the experiments presented in chapter *3.1.*, cultivars differing in their resistance/susceptibility to *Plasmopara viticola* infection were selected and investigated. Namely, susceptible cultivars Cabernet Sauvignon, Pinot Blanc and Pinot Gris were used in experiment presented in chapter *3.1.1.* Both resistant and susceptible cultivars: *(1)* resistant [Cerason, Malverina, Hibernal, Laurot] and *(2)* susceptible [Cabernet Sauvignon, Chardonnay, Muscatel, Saint Laurent, Muller Thurgau, Alibernet] were used in experiment presented in chapter *3.1.2.*

For the experiments presented in chapter 3.2., both grapevine (*Vitis vinifera* L. subsp. *vinifera* Hegi) and rice (*Oryza sativa* L.) were used and tested. In the case of grapevine experiment, cv. Chardonnay was selected and inspected as presented in chapter *3.2.1.* Also, two white [Chardonnay, Riesling] and two red [Pinot Noir, Saint Laurent] cultivars were used as presented in chapter *3.2.2.* During the rice experiment, N22 (highly tolerant) and Gharib (sensitive) genotypes to high night temperature were used.

2.2. Optical measurements

Chlorophyll fluorescence imaging was used in case of *Plasmopara viticola* experiments prescribed in chapters *3.1.1.* and *3.1.2.* In both cases, kinetic imaging fluorometer

(OpenFluorCam 70MF, Photon System Instruments, Ltd., Brno, Czech Republic) was used. Such imaging fluorometer alows to measure sequences of Chl-F images based on pre-defined irradiance and timing protocols (Nedbal et al., 2000). In principle, this fluorometer was equipped with: *(i)* light emitting diod pannels which induced Chl-F and *(ii)* charged-coupled device (CCD) camera which allowed to capture images of resulting Chl-F (512x512 pixel images of 12 bit resolution). Protocol used and Chl-F parameters extracted are prescribed in each chapter (*3.1.1.* and *3.1.2.*), respectively.

Chlorophyll fluorescence measurements were done in the case of grapevine berries and/or rice panicles measurements as prescribed in chapters *3.2.2.* and *3.2.3.*, respectively. In both cases, Chl-F measurements were performed by using prototype of handheld instrument `WinePen` (Photon System Instruments, Ltd., Brno, Czech Republic). Moreover, rice panicles were also measured by using portable fluorometer `FluorPen` (Photon System Instruments, Ltd., Brno, Czech Republic).

UV induced fluorescence measurements both *in vivo* measurements of grapevine leaves and berries (chapters *3.1.1.* and *3.2.2.*), as well as *in vitro* measurements of chemical standards and grape extracts (chapter *3.2.2.*) were recorded with a commercial spectrofluorimeter (Fluormax-P, Jobin Yvon, Horiba Scientific, Japan). Also, *in vivo* UV induced fluorescence was measured by using prototype of handheld instrument `WinePen` (Photon System Instruments, Ltd., Brno, Czech Republic).

Reflectance measurements of the leaves (chapter *3.1.1.*) and berries (chapter *3.2.2.*) were carried out by using commercial optical spectrometer (Spectrometer SM 9000, Photon System Instruments, Ltd., Brno, Czech Republic). In the case of grape berries (chapter *3.2.2.*) and rice panicles (3.2.3.), filed reflectance measurements were performed by using `WinePen` (Photon System Instruments, Ltd., Brno, Czech Republic).

2.3. Chemical analysis

Photosynthetic pigments concentrations of grapevine leaves (chapter *3.1.1.*) and rice panicles (chapter *3.2.3.*) were determined spectroscopically (Lichtenthaler et al., 1987), by using spectrophotometers (Lambda, PerkinElmer, USA) and (Shimadzu UV-1800, Japan), respectively.

HPLC analysis of grapevine leaves and berries were done as follows:

(i) analysis of leaf extracts (chapters *3.1.1.* and *3.2.2.*) were performed by HP 1050 (Ti-series) HPLC instrument (Hewlett Packard, USA), by using 3µm, 150x2 mm, Luna C18 (2) column (Phenomenex, USA). For phenolic compounds detection, HP G1315B diode array detector (DAD, Hewlet-Packard, USA) as well as HP G1315A fluorescence detector (FLD, Hewlet-Packard, USA) were used, respectively.

(ii) analysis of grapevine berries extracts (chapters *3.2.1.* and *3.2.2.*) were performed by using three HPLC systems:

(a) Digital High-Pressure system LC-1A with SCL-10Avp controller (Schimadzu, Japan). For separation, Polymer IEX H form 10mm packing, 250x8 mm+10x8 mm column (Watrex, Czech Republic) was used.

(b) 1200 Infinity Series Instrument (Agilent Technologies, USA) and

(c) Smartline HPLC system (Knauer Instruments, Berlin, germany were used. In both *(b)* and *(c)* cases, Altech Altima C18 3µm particle sized reversed phase column (150x3 mm) was used for the separation and identification of phenolic compounds.

Absorption maxima and the concentrations of total phenolics (chapters *3.2.1.* and *3.2.2.*) were measured by recording absorbance at 280 nm, by using spectrophotometer SPECORD 210 Plus BU (Analytical Jena AG, Germany).

3. RESULTS

3.1. Leaves

This part itself is inspired by laboratory study (Cséfalvay et al., 2009). In fact, it is extension of this study to the field conditions. By monitoring of *Plasmopara viticola* impact on leaf physiology we tried to verified existing knowledge about applicability of Chl-F imaging for pre-symptomatic detection of this fungal infection in naturally infected leaves. Moreover, some new approaches are tested and presented in subsequent chapters (*3.1.1.* and *3.1.2.*)

3.1.1. Towards optical detection of *Plasmopara viticola* infection in the field

Journal of Plant Pathology, 2014

David Šebela, Julie Olejníčková, Radek Sotolář, Naděžda Vrchotová, Jan Tříska

Abstract

Chlorophyll fluorescence, UV-induced fluorescence and reflectance signals of grapevine leaf tissue naturally infected by *Plasmopara viticola* were inspected in three susceptible grapevine cultivars (Cabernet Sauvignon, Pinot Blanc and Pinot Gris). Distribution of F_V/F_M parameter over the leaf was found to be effective to discriminate symptomatic and asymptomatic leaf tissue. Reduction of F_V/F_M parameter ~25% was found in all infected leaf spots. Infected leaves also expressed significantly changed chlorophyll fluorescence induction kinetics expressing much slower electron transport rate on donor and acceptor site of photosystem II. Symptomatic leaves emitted high fluorescence signal under UV excitation 320 nm, centered at 395 nm, emanated mainly from highly fluorescent compound 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside. Increase of this compound was found in all inspected grapevine varieties, while its concentration was strongly dependent on resveratrol concentration. Concentration of *trans*-resveratrol ranged in asymptomatic leaves from 0.1μg · g^{-1} (CS) to 0.2μg · g^{-1} (PB, PG). Highest increase of *trans*-resveratrol in infected leaf tissue was found in PB (4.4μg · g^{-1}), followed by CS (1.6μg · g^{-1}) and PG (1.1μg · g^{-1}). 2,4,6-trihydroxyphenanthrene- 2-*O*-glucoside concentrations varied from 0.41 (PB), 0.75 (PG) to 1.01μg · g^{-1} (CS) in asymptomatic leaves, since its concentration rapidly increase with severity of infection [71.09 (PB), 79.37 (PG), and 26.14 μg · g^{-1} (CS)]. Reflectance vegetation indices SRI, R_{750}/R_{700}, $(R_{780}-R_{710})/(R_{780}-R_{680})$ and CRI_{700} were the most powerful to follow changes in chlorophylls and carotenoids contents under *Plasmopara viticola* infection. Infected leaves also exhibited lower chlorophyll a (~50%) and carotenoids (~70%) content. Combination of these optical signals can be used as an effective, non-invasive tool for an early detection of *Plasmopara viticola* in field.

TOWARDS OPTICAL DETECTION OF *PLASMOPARA VITICOLA* INFECTION IN THE FIELD

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SUMMARY

Chlorophyll fluorescence, UV-induced fluorescence and reflectance signals of grapevine leaf tissue naturally infected by *Plasmopara viticola* were inspected in three susceptible grapevine cultivars (Cabernet Sauvignon, Pinot Blanc and Pinot Gris). Distribution of F_V/F_M parameter over the leaf was found to be effective to discriminate symptomatic and asymptomatic leaf tissue. Reduction of F_V/F_M parameter ~25% was found in all infected leaf spots. Infected leaves also expressed significantly changed chlorophyll fluorescence induction kinetics expressing much slower electron transport rate on donor and acceptor site of photosystem II. Symptomatic leaves emitted high fluorescence signal under UV excitation 320 nm, centered at 395 nm, emanated mainly from highly fluorescent compound 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside. Increase of this compound was found in all inspected grapevine varieties, while its concentration was strongly dependent on resveratrol concentration. Concentration of *trans*-resveratrol ranged in asymptomatic leaves from 0.1$\mu g \cdot g^{-1}$ (CS) to 0.2$\mu g \cdot g^{-1}$ (PB, PG). Highest increase of *trans*-resveratrol in infected leaf tissue was found in PB (4.4$\mu g \cdot g^{-1}$), followed by CS (1.6$\mu g \cdot g^{-1}$) and PG (1.1$\mu g \cdot g^{-1}$). 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside concentrations varied from 0.41 (PB), 0.75 (PG) to 1.01$\mu g \cdot g^{-1}$ (CS) in asymptomatic leaves, since its concentration rapidly increase with severity of infection [71.09 (PB), 79.37 (PG), and 26.14 $\mu g \cdot g^{-1}$ (CS)]. Reflectance vegetation indices SRI R_{750}/R_{700}, $(R_{780}-R_{710})/(R_{780}-R_{680})$ and CRI_{700} were the most powerful to follow changes in chlorophylls and carotenoids contents under *Plasmopara viticola* infection. Infected leaves also exhibited lower chlorophyll a (~50%) and carotenoids (~70%) content. Combination of these optical signals can be used as an effective, non-invasive tool for an early detection of *Plasmopara viticola* in field.

Key words: Downy mildew, grapevine, UV-induced fluorescence, chlorophyll fluorescence, reflectance, *trans*-resveratrol, 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside

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INTRODUCTION

Grapevine (*Vitis vinifera* L. subsp. *vinifera* Hegi) is attacked by many potential pests that can cause the decrease or, in the most serious cases, the complete loss of the yield if no chemical protection is applied. One of the most severe diseases of the grapevine is downy mildew caused by *Plasmopara viticola* [*P. viticola* (Berk. et Curtis ex de Bary) Berlese et de Toni (Kortekamp and Zyprian, 2003)]. *P. viticola* attacks all parts of the plants (e.g. flowers, stems, grapes), but mostly leaves. Symptoms appear as yellowish-green oily spots of different size developing *ca.* seven days after infection (Cséfalvay *et al.*, 2009). As the infection proceeds in wet and cool weather [optimum 20-25°C (Lafon and Clerjeau, 1998)], spots gradually turn brown, become necrotic, are mostly delimited by the main veins and accompanied by profuse sporulation (Lebeda *et al.*, 2008).

The plant-pathogen interaction results in the alternation of the host plant metabolism resulting in changes in photosynthesis, pigment composition and *de novo* synthesis of physical barriers, or synthesis and accumulation of phytoalexins (Moriondo *et al.*, 2005; Mandal *et al.*, 2009; Rolfe and Scholes, 2010; Vrhovsek *et al.*, 2012). Phytoalexins of grapevine belong to group of stilbenes that were first identified by Langcake and Pryce (Langcake and Pryce, 1976; Langcake *et al.*, 1979; Langcake, 1981). One of the major phytoalexin produced by grapevine plants upon *P. viticola* attack is *trans*-resveratrol (3,5,4´-trihydroxystilbene) and its derivative (van Zeller *et al.*, 2011). Tříska *et al.* (2012) have recently identified in infected grapevine leaves a highly fluorescent compound, 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside, deriving from *trans*-resveratrol, which is the main fluorescent natural product arising from *P. viticola* infection.

All metabolic alterations caused by infection affect the optical properties of the leaves. Changes in photosynthetic activity are usually measured by means of chlorophyll fluorescence emission (Chl-F) (reviewed by Baker, 2008), changes in pigment composition by means of reflectance vegetation indices (VIs) (Sims and Gamon, 2002), and stilbene location within leaf tissue by means of fluorescence emission excited by UV (Bellow *et al.*, 2012).

Chl-F, emitted by the molecules of chlorophyll-a (Chl-a), is one of the most powerful optical signals of changes in photosynthesis. Light energy absorbed by Chl-a molecules in a photosynthetic systems can undergo three main pathways: it can be used to drive photosynthesis, be dissipated as heat or be re-emitted as Chl-F (Papageorgiou and Govindjee, 2004). Chl-F is not a static variable, for its yield is the highest when photochemistry is the lowest; and vice versa. When a dark-adapted healthy leaf is exposed to actinic light, the induced Chl-F displays characteristic intensity changes, reflecting the induction of photosynthetic activity (Papageorgiou *et al*., 2011). The meaning of Chl-F signals has extensively been reviewed (Maxwell and Johnson, 2000; Roháček, 2002; Papageorgiou and Govinjee, 2004), while its emission has frequently been interpreted as an early signal of biotic stress preceding the appearance of visible symptoms (Barbagallo *et al*., 2003; Nedbal and Whitmarsh, 2004; Cséfalvay *et al*., 2009; Rolfe and Scholes, 2010). Distribution of Ch-F emission in grapevine leaves has successfully been used for the pre-symptomatic detection of *P. viticola* infection in the laboratory (Cséfalvay *et al*., 2009) as well as in the field (J. Olejníčková, unpublished information). Two Chl-F parameters, i.e. maximum (F_V/F_M) and effective (Φ_{PSII}) quantum yield of photosystem II, have been identified as the most sensitive reporters of *P. viticola* infection (Cséfalvay *et al*., 2009).

Stilbenic compounds produced as phytoalexins during fungal attacks emit a violet-blue fluorescence signal under UV excitation light (Hillis and Ishikura, 1968; Below *et al*., 2012). Compared to red fluorescence (Chl-F), which comes predominantly from the chloroplasts of mesophyll cells (Zeiger *et al*., 1980), blue-green fluorescence comes primarily from the vacuoles of epidermis and mesophyll cells, or from the cell walls (Bellow *et al*., 2012). Stilbene-induced fluorescence signals have already been measured *in vivo* (Poutaraud *et al*., 2007, 2010; Bellow *et al*., 2012). Also 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside has been found in infected grapevine leaf tissue thanks to its extremely high UV-excited fluorescence emission (Tříska *et al*., 2012). Thus, we hypothesized that UV fluorescence signal of this phenanthrene derivative is strong enough to be measured *in vivo* at the leaf and/or plant level.

Reflectance signals are predominantly used in remote sensing (Wu *et al*., 2008; Blackburn and Ferwerda, 2008). The spectrum of the light reflected from leaf surfaces is strongly dependent on photosynthetic pigment content i.e. chlorophylls (Chl) and carotenoids (Car) (Merzlyak *et al*., 1997; Ustin *et al*., 2004). Thus, reflectance provides a non-invasive insight to pigment composition of the leaves. The spectrum of reflected light in the visible region (400-800 nm) is characterized primarily by the Chl absorption spectrum, i.e. high reflectance in the green (500-600 nm) and minimal reflectance in the red (660-700 nm) regions. The number of vegetative indices (Vis) could be defined from the variation of the reflected light spectrum to calculate concentrations of particular pigments in the leaves/canopy (Blackburn, 2007). Vis are mathematical transformations of reflectance at specifically selected spectral bands, that maximize sensitivity to target biophysical variables and minimize confounding environmental factors (Myneni *et al*., 1995). The most frequently used Vis for Chl estimation in single leaves are: $(R_{850}-R_{710})/(R_{850}-R_{680})$ (Datt, 1999); SRI R_{750}/R_{700} (Gitelson *et al*., 1996); $(R_{780}-R_{710})/(R_{780}-R_{680})$ (Maccioni *et al*., 2001). Estimation of Car absorbing in the blue region is more complicated since it is affected by an overlapping of their absorption spectra with those of Chl, and by their relatively low concentration in comparison to Chl. Using established values of light absorption for both Chl and Car, and values related only to Chl absorbtion, Car reflectance indices [CRI, (Gitelson *et al*., 2002)] were determined for Car estimation.

In summary, plant optical signals provide a non-invasive insight into plant stress physiology (see among the others Carter, 1993; Carter and Knapp, 2001; Roháček *et al*., 2008). In this study, several optical methods were combined to investigate the changes in the optical properties of grapevine leaves of cvs Cabernet Sauvignon (CS), Pinot Blanc (PB) and Pinot Gris (PG) affected by *P. viticola*, with reference to Chl-F, UV-induced fluorescence, reflectance spectra and particular reflectance indices. We propose that complex measurement of optical signals can lead to early information about crop physiology and that such asymptomatic detection of disease in field conditions can lead to effective strategy of wine producer and the resulting proper management.

MATERIALS AND METHODS

Plant material. Leaves of the three grapevine cultivars (CS, PB, PG) were sampled in an experimental vineyard of the Department of Viticulture and Oenology at Lednice, Czech Republic (48°47'24.16"N; 16°47'53.61"E) on the 16th of July 2010. Symptomless leaves used as control and infected symptomatic leaves were sampled from vines of about the same age. The infection was in the early stage (chlorotic spots on the upper side of the leaves), i.e. 5 to 7 days after inoculation (Cséfalvay *et al*., 2009). To preserve the freshness of sampled leaves, their petioles were coated by wet cellulose, placed in plastic bags, and transported immediately to the laboratory in a portable refrigeration unit at 15°C. In total, 30 samples were collected, five control and five infected leaves from each grapevine cultivar. Chl-F imaging, UV-induced fluorescence, reflectance measurements, as well as chemical analysis were done on each collected leaf.

Chl-F measurements. These were done immediately after collection using a commercial kinetic imaging fluorometer (OpenFluorCam 70MF, Photon System Instruments, Czech Republic) in a laboratory next to the vineyard. Before each measurement, grapevine leaves were kept in the

dark for 20 min. Then, minimum Chl-F in the dark (F_0) was determined using five measuring flashes generated by two panels of light-emitting diodes (LEDs, λ_{MAX} = 635nm). Subsequently, a short saturating flash (1500 mmol photons $m^{-2} \cdot s^{-1}$) was applied to measure maximum Chl-F in the dark (F_M). Saturating light was generated by 250 W white halogen lamp (λ = 400-700 nm). After 15 sec of relaxing dark period , the samples were exposed to actinic light (200 mmol photons $m^{-2} \cdot s^{-1}$) and Chl-F induction was captured, during which the Chl-F level reached a peak after *ca.* 1 sec (F_P) and the Chl-F steady state level after 190 sec of exposure to actinic light was measured. Chl-F induction kinetic was recorded with a CCD camera (Photon System Instruments, Czech Republic) in a series of 512×512 pixel images at 12-bit resolution.

Chl-F parameters F_0 (minimum Chl-F emission in the dark-adapted state), F_M (maximum Chl-F emission in the dark-adapted state), F_P (maximum Cl-F emission measured when the actinic light is switched on, and F_S (steady state of Chl-F emission in the light-adapted state) values were determined (see Rohácek, 2002). Two derived Chl-F parameters were then calculated: maximum quantum yield of PSII photochemistry [F_V/F_M, (Kitajima and Butler, 1975)] according to following formula: $F_V/F_M = (F_M - F_0)/F_M$; $Fd = F_P - F_S$ and time in which Fd/2 was reached. The distribution of Chl-F over the leaves and the mean values of Chl-F parameters of the control and infected leaf tissue were determined.

UV-induced fluorescence measurement. *In vivo* fluorescence emission spectra of control and infected leaf tissues were recorded with a commercial spectrofluorimeter (Fluormax-P, Jobin Yvon, Horiba Scientific, Japan) in dark at room temperature. Excitation wavelength was set to 320 nm which corrsponds to the maximum absorption of *trans*-resveratrol. The slits were adjusted to 1 nm, integration time was set up to 500 ms and each spectrum was recorded with a 2 nm increment. Leaf samples were placed into an external adaptor four mm away from the spectrofluorometer sensor. In total, three spectra were recorder from each control leaf and three spectra from infected leaf tissue.

Reflectance measurement. Hemispherical, adaxial leaf reflectance measurements were carried out directly in the vineyard using a commercial optical spectrometer (Spectrometer SM 9000, Photon System Instruments, Czech Republic). The reflectance spectra (400-800 nm, 1 nm resolution) of control and infected leaf tissues were measured. For each grapevine cultivar 10 reflectance spectra of control and symptomatic leaf tissues were measured in different places over the leaves and averaged. The following vegetation reflectance indices were calculated from the measured reflectance spectra to estimate chlorophyll (Chl) [$(R_{850} - R_{710})/(R_{850} - R_{680})$ (Datt, 1999), $SRI = R_{750}/R_{700}$ (Gitelson *et al*., 1996), $(R_{780} - R_{710})/(R_{780} - R_{680})$ (Maccioni *et al*., 2001)] and carotenoids (Car) [$CRI_{550} = R_{NIR}(1/R_{510} - 1/R_{550})$ (λ_{NIR} = 760-800nm, Gitelson *et al*., 2002) and $CRI_{700} = 1/R_{510} - 1/R_{700}$ (Gitelson *et al*., 2002)] concentration.

Pigment determination. For pigment determination, samples were prepared immediately after optical measurements. Symptomless leaf tissue from control leaves and symptomatic leaf tissue from the infected part of the leaves were cut with a cork borer (diameter 14 mm). Leaf samples were weighted and placed in liquid nitrogen prior to sample homogenization and pigment extraction with 100% methanol. A small amount of MgO (approx. 0.1 mg) was added to prevent chlorophyll (Chl) degradation. Pigment content was determined spectroscopically, measuring absorption spectra at 470 nm, 652.4 nm and 665.2 nm (Spectrophotometer Lambda, PerkinElmer, USA). Chl-a, Chl-b and Car concentrations were calculated according to Lichtenthaler (1987). The pigment content was expressed on a fresh weight basis.

High performance liquid chromatography (HPLC) analysis of stilbenes.

Preparation of samples. Samples for the HPLC analysis were prepared immediately after the optical measurements. Disks 14 mm in diameter were cut with a cork borer from symptomless leaf tissues of control leaves and tissue from the infected part of the leaves. Samples were weighted and extracted for 24 h at room temperature in 0.5 ml of 70% methanol. During extraction, samples were placed in the dark and occasionally shaked. Methanolic extracts were then passed through a glass filter, collected and analyzed with HPLC.

Analysis of samples. Each sample (injection volume 5 µl) was analysed by 3 µm 150×2 mm, Luna C18 (2) column (Phenomenex, USA), using an HP 1050 (Ti-series) HPLC instrument (Hewlett Packard, USA) with water-acetonitrile-*o*-phosphoric acid mobile phase. Mobile phase A used 5% acetonitrile plus 0.1% *o*-phosphoric acid and the mobile phase B used 80% acetonitrile plus 0.1% *o*-phosphoric acid. The gradient was increased from 20% to 80% of B during 20 min and from 80% to 100% of B during 5 min. Flow rate was 0.250 $ml.min^{-1}$ and the column temperature 25°C. For detection a HP G1315B diode array detector (DAD) (Hewlett-Packard, USA) was used with detection wavelengths at 220 and 315 nm, and scanning range 190-600 nm, as well as a HP G1321A fluorescence detector (FLD) (Hewlett-Packard, USA), with excitation wavelength 315 nm, emission wavelength 395 nm, and scanning of emission in the range of 300-600 nm.

Analysis of data. To assess differences of Chl-F, reflectance and pigments data in control and infected leaf tissues, the mean, standard deviation and standard error of the mean were calculated using MS Excel, whereas the significance of Chl-F measurements in the control and infected

leaf tissues was determined by the t-test. Correlations of pigment contents with selected vegetation reflectance indices were also determined and the significance of the correlation coefficients was tested by regression analysis.

RESULTS

Chl-F. The distribution of F_V/F_M, which was used to identify the leaf areas infected by *P. viticola* was irregular (Fig. 1A) showing spots with low a F_V/F_M value, where the photosynthetic activity was impaired by the infection. The leaf spots in which the fungal infection started to propagate expressed similar F_V/F_M value in all three susceptible cultivars. The portion of the leaf that was visually non infected, expressed a high physiological level of F_V/F_M similarly to the symptomless control leaves. In all cultivars similar trend of infection spreading was found (data not shown). In the early phase, *P. viticola* invasion started from many points expressing lower F_V/F_M, followed by an increase in size of these spots. Finally, the infection covered the whole blade and damaged the photosynthetic apparatus of the entire leaf. A comparison of the mean F_V/F_M values from control and infected leaf tissue spots (Fig. 1B) showed that. in the symptomless control leaves, F_V/F_M reached the maximum level (*ca.* 0.83) indicating no damage of photosystem II (PSII). On the contrary, infected spots expressed about 25% lower F_V/F_M values (*ca.* 0.63).

The induction kinetics of Chl-F in dark-adapted leaves provides much more information about the primary photochemistry in control and infected leaf tissue than individual Chl-F parameters. Fig. 2A shows normalized Chl-F induction of control and infected leaf tissue of cv. CS. When the actinic light was switched on, Chl-F started to change, increasing from F_0 to F_P within *ca.* one sec, which represents the electron transport rate on the donor site of PSII under actinic light. Then, Chl-F started to decrease down to F_S, reflecting changes on the acceptor side of PSII, as the electrons are transported from the plastoquinone pool down to photosystem I (PSI). Infected leaf spots expressed slower increase of Chl-F, significantly lower F_P and a much slower decrease from F_P down to F_S (Fig. 2A) compared to control leaf tissues. To evaluate the level of impaired electron transport on the acceptor side of PSII, we have compared the time was compared in which the Chl-F decrease from F_P to F_S reached the half value - Fd/2 (Fig. 2B). Time of the Fd/2 was much longer in infected leaves (32-45 sec) compared to controls (25-32 sec).

Photosynthetic pigments and stilbenes content. The concentrations of photosynthetic plant pigments (Chl-a, total chlorophyll content [Chl-a+Chl-b], Car and stilbenic compounds (*trans*-resveratrol, 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside) are summarized in Table 1. In infected leaf tissue the concentration of Chl-a was significantly lower, which partially explains the much lower F_V/F_M (Fig. 1A, B) and slower electron transport rate (Fig. 2A, B). Decrease of Chl-a concentration (Table 1) in infected leaf tissue was *ca* 56% (CS), 58% (PB) and 45% (PG). Similar results were found in the case of Chl-a+Chl-b (Table 1) and Chl-b content (not shown). One must note that there was a noticeable loss of greenness but no necrosis of infected leaf spots. Chl a/b ratio was found decrease in all infected leaves (Table 1). *P. viticola* infection resulted also in significantly lower Car content in infected leaf tissue compared to control leaves (Table 1). The highest reduction (*ca.* 80%) was observed in infected leaf tissue of cvs CS and PG, the least (*ca.* 70%) in cv. PB.

The concentration of *trans*-resveratrol and 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside (Phenanthrene) was

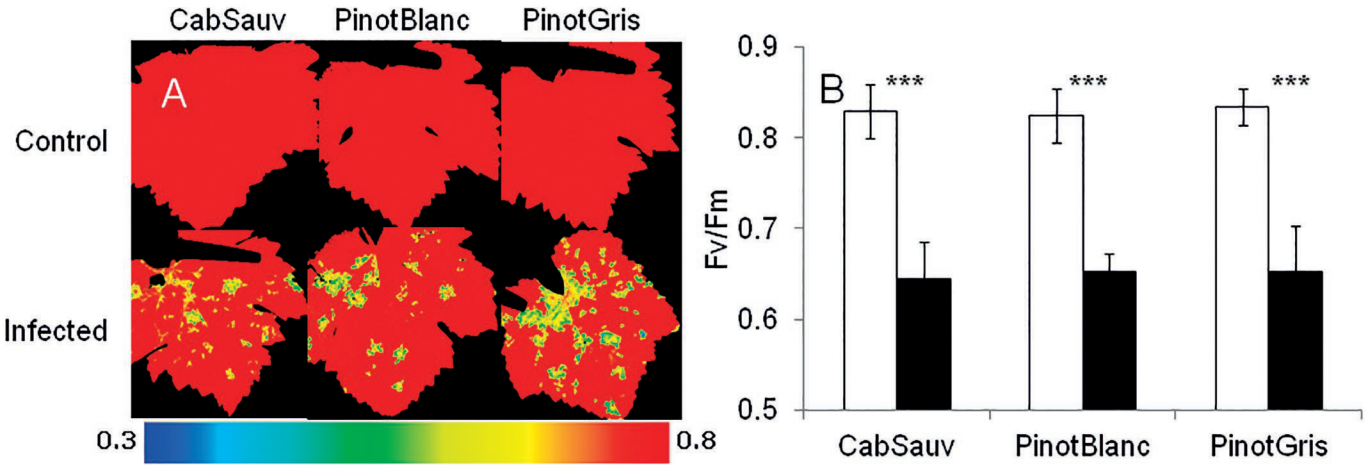

Fig. 1. A. False scale color images of F_V/F_M (maximum quantum yield of photosystem II) distribution over representative control (symptomless, top panel) and infected (bottom panel) leaves of three susceptible grapevine cultivars (Cabernet Sauvignon, Pinot Blanc and Pinot Gris). B. Mean values of maximum quantum yield of photosystem II (F_V/F_M) of control (white columns) and infected (black columns) leaf tissue of three sensitive grapevine cultivars (Cabernet Sauvignon, Pinot Blanc and Pinot Gris). Columns show mean values for 5 controls and 5 infected leaves for each variety. Standard deviation (SD) is shown for control and infected leaves. Asterisks (*, **, ***) indicate significance at 5%, 1% and 0.1%, respectively.

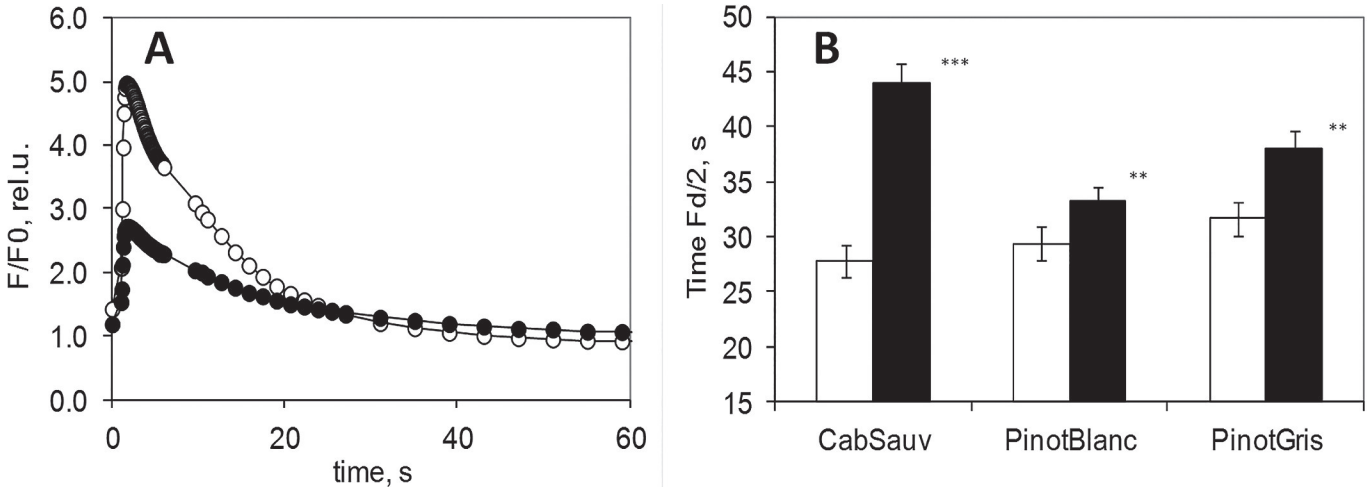

Fig. 2. A. Chl-F induction kinetics of dark adapted cv. Cabernet Sauvignon leaf tissue captured *in vivo* by using OpenFluorCam 70 MF. Chl-F induction was recorded after 20 min of dark adaptation under actinic light [200 mmol(photons).$m^{-2}.s^{-2}$]. Mean values for control (white circles) and infected (black circles) leaf tissue are normalized to the minimum Chl-F level in dark adapted state (F_0), and presented in logarithmic timescale. B. Difference between the time of reaching Fd/2 in dark-adapted leaf tissue when exposed to actinic light [200 mmol(photons)$m^{-2}s^{-1}$]. Control (white columns) and infected (black columns) leaves of three susceptible cultivars (Cabernet Sauvignon, Pinot Blanc, Pinot Gris) were used. Fd/2 is the time, in which Chl-F of the leaves reaches half of the difference between peak fluorescence emission (F_P) and steady state fluorescence emission (F_S) [Fd/2=(F_P–F_S)/2]. Columns represent mean values of five measurements ± standard deviation (SD). Asterisks (*, **, ***) indicate significance at 5%, 1% and 0.1%, respectively.

also analyzed. A significant increase of these compounds was found in all cultivars (Table 1). *Trans*-resveratrol concentration ranged in all control leaf samples from 0.1 (CS) to 0.2 (PB, PG) $\mu g \cdot g^{-1}$. Following infection, concentrations increased several times in infected leaf spots of all cultivars. The highest increase was found in cv. PB (4.4 $\mu g \cdot g^{-1}$), that was approximately 22 times higher than in the control. Significantly lower increase of *trans*-resveratrol content was found in cvs CS (16 fold) and PG (6 fold), respectively. In the case of 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside the highest concentration in the control leaf was also observed in cv. CS (1.01 $\mu g \cdot g^{-1}$). In infected leaf tissue a high phenanthrene content was found in cv. PG (79.37 $\mu g \cdot g^{-1}$), but the highest phenanthrene production was recorded in cv. PB, i.e. *ca.* 173-fold higher phenanthere content in infected leaf tissues compared to controls. *P. viticola* infection resulted also in increased phenanthrene synthesis from *trans*-resveratrol, for phenanthrene concentration was be much higher in infected leaf spots with higher *trans*-resveratrol content (Table 1, cv. PB).

UV-induced fluorescence. Since stilbenic compounds have a high fluorescence signal when they are induced by UV light, the *in vivo* UV-induced fluorescence signal of these compounds in symptomatic and asymptomatic leaf tissue was measured. Fig. 3 shows normalized fluorescence emission spectra of control and infected leaf tissue of cv. CS under excitation at 320 nm. Fluorescence signal showed similar trend in all grapevine leaves (CS, PB, PG). No or very low fluorescence emission was observed in control leaves, whereas infected leaf tissue emitted a high fluorescence signal centered at 395 nm. We assume that most

Table 1. Photosynthetic pigment content (chlorophyll a [Chl a], total chlorophyll [Chl a+b] and total carotenoids [Car]), Chl a/b ratio, *trans*-resveratrol and 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside content in control and infected leaf tissue of three susceptible cultivars (Cabernet Sauvignon, Pinot Blanc and Pinot Gris), naturally infected by *Plasmopara viticola*. Chl-a, Chl a+b are the contents of chlorophyll a and total chlorophyll a, respectively; Car are total carotenoids content (expressed in $mg \cdot g^{-1}$), Chla/b ratio is ratio between Chl-a and Chl-b content; resveratrol and phenanthrene (2,4,6-trihydroxyphenanthrene-2-*O*-glucoside) content expessed in $\mu g \cdot g^{-1}$. Values represent average of 15 measurements ± standard deviation (SD).

Cabernet Sauvignon	Control	Infected
Chl a	1.71 ± *0.03*	0.75 ± *0.04*
Chl a+b	2.26 ± *0.04*	1.07 ± *0.06*
Chl a/b	3.07 ± *0.03*	2.37 ± *0.04*
Car	0.15 ± *0.001*	0.03 ± *0.000*
Trans-resveratrol	0.20 ± *0.19*	1.60 ± *0.38*
Phenanthrene	1.01 ± *0.09*	26.14 ± *8.17*
Pinot Blanc	Control	Infected
Chl a	1.29 ± *0.03*	0.64 ± *0.05*
Chl a+b	1.71 ± *0.04*	0.91 ± *0.07*
Chl a/b	3.09 ± *0.05*	2.39 ± *0.05*
Car	0.10 ± *0.006*	0.03 ± *0.003*
Trans-resveratrol	0.20 ± *0.08*	4.40 ± *3.27*
Phenathrene	0.41 ± *0.04*	71.09 ± *25.30*
Pinot Gris	Control	Infected
Chl a	1.57 ± *0.07*	0.85 ± *0.04*
Chl a+b	2.08 ± *0.09*	1.19 ± *0.06*
Chl a/b	3.05 ± *0.03*	2.43 ± *0.03*
Car	0.13 ± *0.014*	0.02 ± *0.005*
Trans-resveratrol	0.10 ± *0.08*	1.1 ± *0.03*
Phenathrene	0.75 ± *0.19*	79.37 ± *43.6*

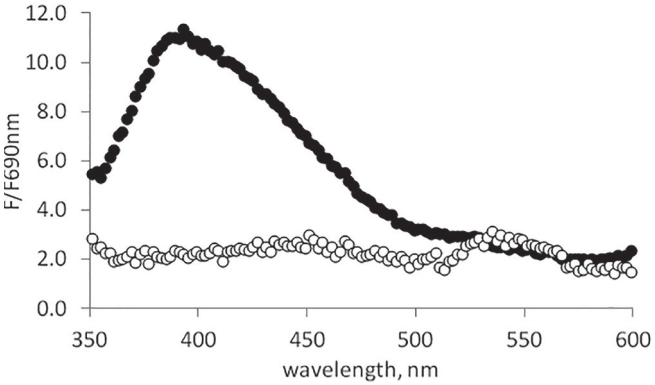

Fig. 3. Fluorescence emission spectrum (excitation 320 nm) of a representative leaf of cv. Cabernet Sauvignon captured *in-vivo* by using spectrofluorimeter Fluormax-P. UV-induced fluorescence was measured using an external adaptor connected with the instrument by an optic fiber bundle. All measurements were done in the dark to prevent inaccuracy caused by incident light. Mean values for control (white circles) and infected (black circles) leaf tissue are normalized to chlorophyll fluorecence level at 690 nm.

of this signal comes from 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside, the newly identified compound in grapevine leaves infected by *P. viticola.* Its concentration was 16- to 72-fold higher than the *trans*-resveratrol content and was abundantly produced in infected leaf tissue (concentration of 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside was 72-fold higher than *trans*-resveratrol in cv. PG compared to controls, 16-fold higher in PB and 16-fold higher CS).

Reflectance measurement. To explore reflectance properties of leaf tissue infected by *P. viticola*, the reflectance spectra in visible light (400-800 nm) was measured on leaves directly in vineyard. Fig. 4A shows a typical reflectance spectrum of control and infected grapevine leaves. Similar results were obtained with all cultivars under study (CS, PG and PB). The reflectance spectra of representative leaves (Fig. 4A) is normalized to 550 nm (maximum reflectance in the green region), where Chl-a and Chl-b absorb minimally, and Car does not absorb at all. Between control and infected leaves, a distinct difference in the 550-700 nm region and over 700 nm was observed. To highlight the disparity between control and infected leaf tissue, percentage differences in reflectance spectra were calculated (Fig. 4B). The difference between reflectance spectra (%) showed the minimum variability in the 400-500 nm region A visible change was first observed in the reflectance range between 505-515 nm, followed by minimal changes in the 515-545 nm region. The next significant difference between control and infected leaf reflectance spectrum was found at 550 nm. In the 550-700 nm region, differences were visible in the whole spectrum, with peak centered at 520, 550, 650 and 680 nm. Lower energy wavelength (700-900 nm) showed the most significant differences at 700 and 750 nm.

From the most contrasting wavelengths of reflectance spectra particular vegetative indices with known physiological interpretation were derived. VIs were selected, which are used for Chl and Car content estimation and their values correlated with the pigments content. In the case of Chl, the highest regression coefficient ($R^2 = 0.8769$, Fig. 5A) was found for VIs (simple ratio index, R_{750}/R_{700}), followed by $(R_{780}–R_{710})/(R_{780}–R_{680})$ ($R^2=0.8318$, Fig. 5B). This VIs clearly discriminated infected leaves with lower Chl content from controls containing higher Chl concentration. Several known reflectance indices for Car content estimation were also analysed to discriminate Car content in control and infected leaves. Among the others, the highest regression coefficient was displayed by CRI_{700} vegetation

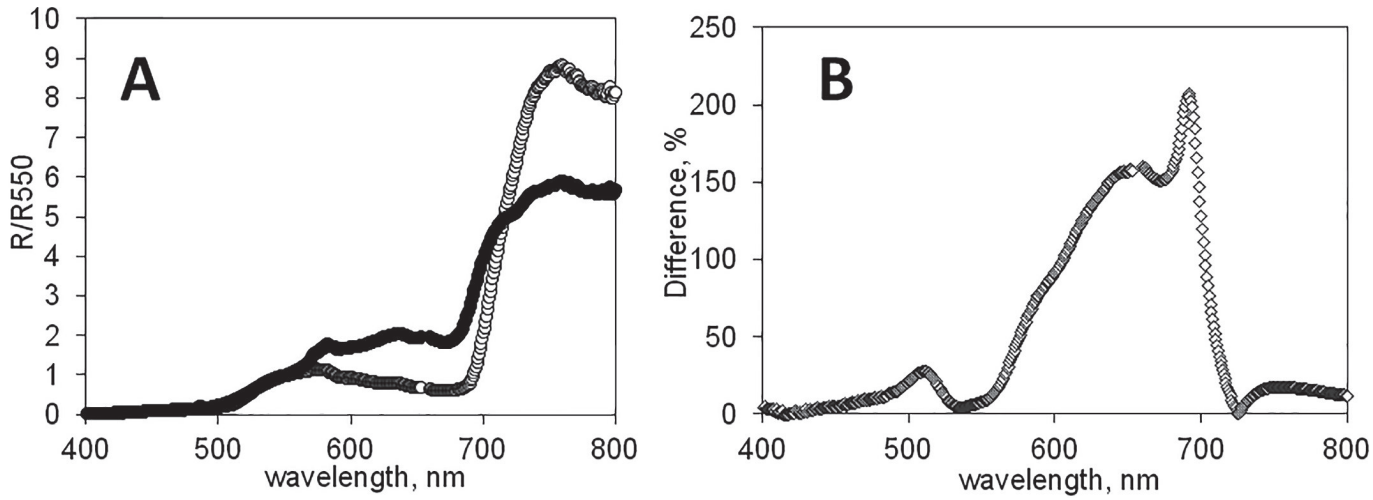

Fig. 4. A. Adaxial reflectance spectra of representative leaves (cv. Cabernet Sauvignon), collected in the field in July 2010, measured spectrometrically. Each line represents the mean of 10 measurements of individual leaf normalized to reflectance at 550 nm (R/R_{550}) for control (whire circles) and infected (black circles) leaf tissue. B. Difference (%) of adaxial reflectance spectra of representative leaves (cv. Cabernet Sauvignon), measured spectrometrically. Each line represents the mean of 10 measurements of a representative leaf. Differences between infected and control leaf tissue (%) were calculated according to the formula: [absolute value of `infected/(control/100)`] for each single wavelength.

index (R^2 = 0.7353, Fig. 5C). Both Chl (SRI (R_{750}/R_{700}); $(R_{780}-R_{710})/(R_{780}-R_{680})$) and CRI_{700} values increased with increased concentration of pigments (Fig. 5A, B, C).

DISCUSSION

Chl-F. Fluorescence and reflectance measurements have broadly been applied to study plant-stress response (Barbagallo *et al.*, 2003; Nedbal and Whitmarsh, 2004; Cséfalvay *et al.*, 2009; Rolfe and Scholes, 2010; Below *et al.*, 2012). In this study, Chl-F, UV-induced fluorescence and reflectance vegetative indices were investigated which, when combined, can give an insight into crop's physiology. This would offer a more effective possibility of detecting a symptomless disease in field conditions, thus allowing the implementation of efficient strategies for vineyard management.

Maximum quantum yield of photosystem II [F_V/F_M (Kitajima and Butler, 1975)] is a frequently used Chl-F parameter for estimating plant-fungal interactions (Scholes and Rolfe, 1996; Soukupová *et al.*, 2003; Berger *et al.*, 2007; Prokopová *et al.*, 2010). This parameter is closely related with the photosynthetic activity of leaf tissue (Kitajima and Butler, 1975). In a previous study the most effective parameter for an early detection of *P. viticola* infection (F_V/F_M) was identified (Cséfalvay *et al.*, 2009). As observed with laboratory (Cséfalvay *et al.*, 2009) and field experiment (Tříska *et al.*, 2012; J. Olejníčková, unpublished information), spreading of *P. viticola* infection shows a trend similar to that observed during this study. Since *P. viticola* does not spread outside the inoculated area, the grapevine cultivars presently studied do not show systemic responses and the Chl-F does not change outside the inoculated spots (Cséfalvay *et al.*, 2009). This finding has been validated during an extensive field study carried out in 2009, in which six sensitive and four resistant grapevine cultivars naturally infected by *P. viticola* were involved (Olejníčková, unpublished infomation). Whereas F_V/F_M value in healthy leaves is 0.83 (Kitajima and Butler, 1975), which indicates no damage of photosystem II (PSII), in this study infected spots expressed *ca.* 25% lower F_V/F_M values, indicating that a damage in the PSII reaction centers had occurred. The irregular distribution of F_V/F_M over the infected leaves was similar to what observed with our previous laboratory (Cséfalvay *et al.*, 2009) and field studies (Tříska *et al.*, 2012; J. Olejníčková, unpublished information). But now a more serious damage of PSII (25%) was observed compared with the findings of previous studies [18% mean decrease in extensive field study in 2009 (J. Olejníčková, unpublished infomation), or *ca.* 10% where only tiny spots were visible (Tříska *et al.*, 2012; Cséfalvay *et al.*, 2009)]. Such differences may be caused by climatic and soil conditions/factors contributing to the spread of *P. viticola* infection, and by different nutritional conditions during the periods of observation.

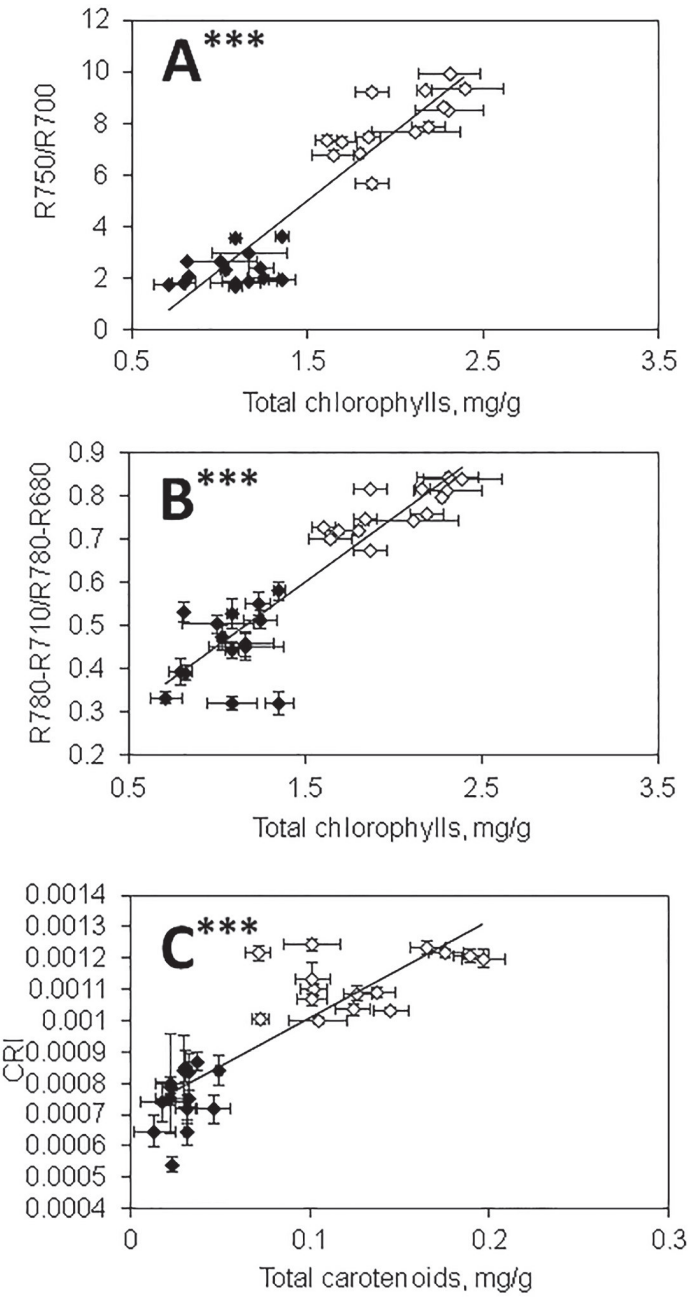

Fig. 5. Linear regression of reflectance vegetation indices and photosynthetic pigment contents: R_{750}/R_{700} vs. total chlorophylls (panel A), $(R_{780}-R_{710})/(R_{780}-R_{680})$ vs. total chlorophylls (panel B) and CRI vs. carotenoids (panel C). Symbols are the mean values of 45 measurements of control symptomless grapevine leaf tissue (white diamonds) and 45 measurements of infected leaf tissue (black diamonds) of three susceptible cultivars (Cabernet Sauvignon, Pinot Blanc and Pinot Gris). Regression points are interleaved by linear trendline with characteristic: Panel A (y=5.3377x-2.9682; R^2=0.8769), panel B (y=0.2951x+0.1576; R^2=0.8318), panel C (y= 0.0031x+0.0007; R^2 =0.7352). Vegetation indices were calculated from the mean reflectance spectrum for each single leaf, and correlated with pigment analysis of each single leaf. Chl a+b and Car content is expressed in $mg \cdot g^{-1}$/fresh weight of the sample. The interpolating line represents a linear regression optimized by MS Excel, error bars represent standard error of the mean (SEM). Asterisks (*, **, ***) indicate significance at 5%, 1% and 0.1%, respectively.

Chl-F induction kinetics of the leaves after dark adaptation (Papageorgiou *et al*., 2011), which provides a more detailed information about the primary photochemistry in the leaves than individual Chl-F parameters (Roháček *et al.* 2008), was also addressed during this study. It was obvious that infected leaf tissue expressed much slower electron transport rate on both donor and acceptor sites of PSII, i.e. much slower photochemistry in infected leaf tissue, indicating damage of thylakoid membranes (Moriondo *et al*., 2005; Nanda *et al*., 2008). One of the reasons could also be the decrease of Chl-a concentration within infected leaf tissue that can also influence the lower F_V/F_M value in infected leaf spots.

Photosynthetic pigments and stilbenes content. It is widely accepted that the concentration of photosynthetic pigments, mostly Chl-a, Chl-b and Car, changes during plant- pathogen interaction. In photosynthesis, Chl-a is the most important pigment for light harvesting process and for the proper function of PSII reaction centers. Similarly to our findings, reduction of Chl content during *P. viticola* infection was observed by Lebeda *et al.* (2008) and Jermini *et al.* (2010) who found a *ca*. 50% decrease of Chl-a+Chl-b. Apart from the Chl-a, Chl-b and total chlorophylls, the ratio of chlorophyll a to b (Chl a/b) has been a key parameter to investigate plant-stress reaction. Depending on the stress, Chl a/b ratio has been reported to either increase (Funayama *et al*., 1997; Nanda and Biswal, 2008) or decrease (Farouk and Osman, 2011) in infected leaves. In principle, decrease in Chl a/b ratio supposes damage to the thylakoid membrane system as suggested also by Moriondo *et al.* (2005) and Nanda *et al.* (2008). Polesani *et al.* (2008) found that *P. viticola* results primarily in the down-regulation of grapevine transcripts in all major functional categories, especially photosynthesis. Genes involved in leaf tissue photosynthesis are down-regulated (Nanda *et al*., 2008; Bilgin *et al*., 2010) and thylakoid membranes are damaged (Moriondo *et al*., 2005; Nanda *et al*., 2008) before the apperance of visible symptoms. All these phenomena contributed to the lower F_V/F_M value observed in infected leaf spots during the present investigation. Carotenoids, which are essential for energy capture from the solar emission spectrum, were significantly reduced under *P. viticola* impact. Such decrease of Car content results in lower photoprotective or light collection capacity of the leaves (Krinsky, 1989; Young, 1991). Also Mahatma *et al.* (2009) observed significant reduction of Car in downy mildew susceptible genotypes of pearl millet. Resistant genotypes contain higher content of Car compared to the sensitive ones, which indicates an antioxidative role of Car under pathogen attack (Krinsky, 1989).

Trans-resveratrol has been identified as one of the main phytoalexins of the grapevine (Jeandet *et al*., 2002) and phenanthrene, which derives from *trans*-resveratrol, has been identified as the main fluorescencing stilbene compound of grapevine leaves infected by *P. viticola* (Tříska *et al*., 2012). The significant increase of these compounds observed during this study is in accordance with the results of our extensive field study in 2009 (J. Olejníčková, unpublished information). Under patogen infection, concentration of phenanthrene increased several times in infected leaf spots of all investigated cultivars, compared with the symptomless leaf tissue. Different increase of phenanthrene concentrations may be caused by the diverse sensitivity of certain cultivars to *P. viticola* (Boso and Kassemeyer, 2008). The highest phenanthrene production following *P. viticola* infection was found in early vegetating cultivars, which confirms our hypothesis that these cultivars, having faster metabolism, can react quickly and more effectively to *P. viticola* than the late cultivars.

As monitored by Langcake and co-workers (Langcake and Pryce, 1976; Langcake *et al*., 1979; Langcake, 1981), biosynthesis of these stilbenes is an defense reaction of grapevines to *P. viticola* infection. Increase of *trans*-resveratrol content was also reported by other authors (Pezet *et al*., 1994; Van Zeller *et al*., 2011), who found that cultivars susceptible to *P. viticola* produce mainly this compound and piceids (glucoside of resveratrol). *P. viticola* infection results also in phenanthrene synthesis from *trans*-resveratrol (Tříska *et al*., 2012), whose concentration was much higher in infected leaves containing higher *trans*-resveratrol content. Therefore, the isomerization process of *trans*-resveratrol to phenanthrene was much more effective in the leaves with a high than in those with a low *trans*-resveratrol content. With a previous extensive field study (Tříska *et al*., 2012; J. Olejníčková, unpublished information) it was also found that both *trans*-resveratrol and 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside contents correlated with decreased photosynthetic activity (F_V/F_M) in grapevine leaf tissue infected by *P. viticola*.

UV-induced fluorescence. High fluorescence signal induced by UV light is emanated by stilbenic compound (Hillis and Ishikura, 1968; Bellow *et al*., 2012). In this study, when infected leaf tissue was excited *in vivo* at 320 nm, it emitted a strong fluorescence signal centered at 395 nm. Such emission peak corresponds to fluorescence emission peak of stilbenes (Poutaraud *et al*., 2007, 2010; Bellow *et al*., 2012) which are involved in plant defense reaction (Chong *et al*., 2009; Allonso-Villaverde *et al*., 2011; Bellow *et al*., 2012). We assume that most of this signal comes from 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside, a newly identified compound in grapevine leaves infected by *Plasmopara viticola* (Tříska *et al*., 2012). The high concentration of this compound combined with its strong fluorescence gave the opportunity of its *in vivo* detection.

Reflectance measurement. The optical properties of vegetation are frequently used in remote sensing (reviewed by Govender *et al*., 2007; Xie *et al*., 2008) and have also been used for detecting chlorosis at the leaf

and canopy level directly in the vineyard (Zarco-Tejada *et al*., 2005). In this study the most contrasting wavelengths were investigated and certain vegetative reflectance indices were used to differentiate and follow the changes between symptomless and infected leaf tissues. The response of grapevine leaf tissues reflectance can be explained by the spectral characteristic of photosynthetic pigments, since *P. viticola* causes stress reactions connected with their changes (Jermini *et al*., 2010). *P. viticola* inhibits Chl production thus inducing reflectance changes detectable first in the red edge region around 700 nm, with weak absorption of Chl-a,-b (Chapelle *et al*., 1992), governed primarily by Chl-a (Carter and Knapp, 2001). Sensitivity to stress-induced reflectance in this region is high (Carter, 1993), similarly to the sensitivity near 550 nm (Buschman and Nagel, 1993; Schepers *et al*., 1996; Gitelson and Merzlyak, 1997) providing close relation with the Chl content (Thomas and Gausman, 1977; Buschmann and Nagel, 1993; Daughtry *et al*., 2000; Gitelson *et al*., 2003). By contrast, the green edge at 520 nm is primarily determined by Car (Merzlyak *et al*., 2003), Chl-a and Chl-b. The maximum for *in-vivo* absorption of Chl-b present in light-harvesting is assumed to be localized at around 650 nm, one of the most contrasting wavelengths. In the near-infrared (*ca.* 750 nm), differences between control and infected leaf tissue are connected with leaf structure (Knipling, 1970) and to changes in leaf anatomy or water content (Sinclair *et al*., 1973).

Several VIs have been developed for remote quantification of leaf chlorophyll content (Chl-a+Chl-b) (Le Maire *et al*., 2004) and/or other biophysical variables (Ustin *et al*., 2004) that are important for the assessment of the health status and functioning of terrestrial ecosystems. Simple ratio index (R_{750}/R_{700}, Gitelson *et al*., 1996) and [(R_{780}-R_{710})/(R_{780}–R_{680}), Maccioni *et al*., 2001] clearly discriminated infected leaves with lower Chl content from controls containing higher Chl concentration. Simple ratio index (SRI), as well as (R_{780}–R_{710})/(R_{780}–R_{680}) are sensitive to Chl concentrations and their changes during different stress progression were investigated in many species (Schuerger *et al*., 2002; Gutierez-Rodriguez *et al*., 2004; Krumov *et al*., 2008; Zhang *et al*., 2011). Compared to SRI, (R_{780}-R_{710})/(R_{780}-R_{680}) enhance absorbtion of Chl by removing interferences caused by variations in leaf scatter. Under *P. viticola* impact, the most reliable index for estimation of infection progress proved to be SRI, signaling positive relationship between R_{750}, leaf anatomy and water content effect (Sinclair *et al*., 1973).

Several known reflectance indices for Car estimation (Chappelle *et al*., 1992; Gamon *et al*., 1992; Penuelas *et al*., 1995; Merzlyak *et al*., 1999) were used to localize symtomless and infected leaf tissues. Among others, the highest regression coefficient displayed CRI_{700} (Gitelson *et al*., 2002), connected with Chl-a (Carter and Knapp, 2001), and reduced during pathogen progress. Car content is closely related to Chl content across a wide range of species (Sims and Gamon, 2002) and thus, proportion of Chl and Car is widely used for diagnosis of physiological state of plants during stresses (Young and Britton, 1990; Lichtenthaler, 1996; D'Arcy-Lameta *et al*., 1996).

Both Chl [SRI (R_{750}/R_{700}); (R_{780}–R_{710})/(R_{780}–R_{680})] and CRI_{700} values increased with increased concentration of pigments. Hence they were able to discriminate asymptomatic leaf tissue with higher pigment content and symptomatic leaf tissue with lower pigment content.

In conclusion, *P. viticola* infection causes decreases of photosynthetic activity and pigment concentrations, and increases production of *trans*-resveratrol and 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside. All these changes are reflected in the optical properties of infected leaf tissues. These, have impaired function of photosynthetic apparatus resulting in decrease of chlorophyll fluorescence emission, i.e. lower F_V/F_M and F_P values, and longer time in which Fd/2 is reached. F_V/F_M parameter was found effective for discriminating symptomless and naturally infected leaf tissue in three susceptible cultivars (CS, PB and PG). Increase of UV-induced fluorescence (exc. 320 nm) with maximum emission centered at 395 nm can be used to follow changes of *trans*-resveratrol and 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside production in infected leaf tissue *in vivo*. Reflectance vegetation indices SRI R_{750}/R_{700}, (R_{780}–R_{710})/(R_{780}–R_{680}) and CRI were the strongest detectable changes in chlorophylls and carotenoids content, respectively. We can conclude that combination of UV-induced fluorescence, Chl-F and reflectance can be used as an effective tool for non-invasive, asymtptomatic detection of *P. viticola* infection in field, as well as a remote sensing tool to discriminate symptomless from infected leaves. Since during this study only susceptible grapevine varieties were tested, more work should be done to test the algorithm in resistant grapevine cultivars, e.g. Hibernal, Malverina and Laurot to quote a few.

ACKNOWLEDGEMENTS

Study funded by the Grant Agency of the Czech Republic, grant number 525/09/0365, and by CzechGlobe-Centre for Global Climate Change Impacts Studies, Reg. No. CZ.1.05/1.1.00/02.0073. Authors wish to thank M. Kumšta and J, Kubásek for their help with HPLC analysis and fluorescence measurements.

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Received October 25, 2013
Accepted January 21, 2014

3.1.2. Pre-symptomatic detection of *Plasmopara viticola* infection in field grown grapevine leaves using chlorophyll fluorescence imaging. Decrease of photosynthetic activity in infected leaves is associated with production of *trans*-resveratrol, pterostilbene and 2,4,6-trihydroxyphenanthrene-2-*o*-glucoside

Prepared manuscript

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Abstract

Plasmopara viticola is devastating pathogen of grapevine (*Vitis vinifera* L.) grown in relatively warm and humid climates. Early information about its occurrence can improve fungicide treatment and thus, can reduce economical loss to minimum. Chlorophyll fluorescence measurements were used to expand our lab-based study for *(i)* six sensitive [Cabernet Sauvignon, Chardonnay, Muscatel, Saint Laurent, Müller Thurgau, Alibernet] and *(ii)* four resistant [Cerason, Malverina, Hibernal Laurot]. In accordance to lab-based experiment, maximum quantum yield of photosystem II (F_V/F_M) was found to be effective to discriminate asymptomatic and/or leaf tissue naturally infected by *P. viticola*. Contrary to resistant cultivars, susceptible ones were not able to localize spread of infection however, no significant differences of F_V/F_M in infected leaf spots were found between both susceptible and resistant cultivars. Also, no clear difference between susceptible and resistant cultivars were found in case of *trans*-resveratrol, pterostilbene, or 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside synthesis under *P. viticola* impact. The particular concentrations however, were well correlating with F_V/F_M [R^2=0.74 for *trans*-resveratrol and R^2=0.78 for 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside, respectively], while in both cases we were able to differ between the groups of healthy and naturally infected leaf tissue. Our results indicate that F_V/F_M can be used not only for early detection of *P. viticola*, but can also be used as *non-invasive* tool for estimation of stilbene content in both susceptible and resistant grapevine leaves.

Pre-symptomatic Detection of *Plasmopara Viticola* Infection in Field Grown Grapevine Leaves Using Chlorophyll Fluorescence Imaging. Decrease of Photosynthetic Activity in Infected Leaves is Associated With Production of *trans*-resveratrol, pterostilbene and 2,4,6-trihydroxyphenanthrene-2-*O*-Glucoside

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***Key words*—Downy mildew, chlorophyll fluorescence, resistant, susceptible, Vitis vinifera L.**

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Introduction

Grapevine (*Vitis vinifera* L.,1753 subsp. *vinifera*) is endangered by many potential pests that may cause decreases, or yield destruction if no chemicals are applied. One of the most significant diseases of grapevine is downy mildew caused by fungus *Plasmopara viticola* (Berk. & Curtis ex de Bary; e.g. Kortekamp and Zyprian, 2003). *P. viticola* attacks all green parts of grapevine, impairs leaf physiology soon after the onset of infection and decreases the grape yield (Polesani et al., 2008). First symptoms of infection typically appear as areas of yellowish - green oily spots in different size, ca 5 - 7 days after the infection (e.g., Cséfalvay *et* al., 2009).

Usually, biological interaction between plant and pathogen results in changes of photosynthesis, followed by changes in pigment composition (e.g. Moriondo et al., 2005; Mandal et al., 2009; Cséfalvay et al., 2009; Rolfe and Scholes, 2010; Šebela et al., 2012, 2014). In parallel, a complex array of defense responses is induced, as a *de novo* synthesis of physical barriers, or synthesis and accumulation of

phytoalexins (Renault et al., 1996; reviewed by e.g. Kuc, 1995; Hammerschmidt, 1999).

Changes in primary metabolism of infected leaf tissue can be followed by means of chlorophyll fluorescence (Chl-F) measurements (e.g. Scholes and Rolfe, 1996; Chou et al., 2000; Nedbal and Whitmarsh, 2004; Swarbrick et al., 2006; Cséfalvay et al., 2009), thanks to its close relationship with photosynthesis (e.g. Roháček et al., 2008; Bjorn et al., 2009; Papageorgiou and Govindjee, 2011; Papageorgiou and Govindjee, 2011). Chl-F emission is one of the three pathways of light energy de-excitation and hence, it is inversely correlated with photochemistry, which leads to CO_2 assimilation and heat dissipation (reviewed by e.g., Papageorgiou and Govindjee 2004). Chl-F has been frequently used for an early detection of the various infections before the occurrence of visible symptoms (e.g. Chaerle and Van Der Straeten, 2001; Barbagallo et al., 2003; Nedbal and Whitmarsh, 2004; Cséfalvay et al., 2009; Rolfe and Scholes, 2010). In our previous lab-based study we proved that distribution of Ch-F emission over the grapevine leaves can be successfully used for pre-symptomatic detection of *Plasmopara viticola* infection (Cséfalvay et al., 2009). Here, as the most sensitive reporters of *P. viticola* infection were identified Chl-F parameters relating to photosynthetic activity - maximum (F_V/F_M; Kitijama and Butler, 1975) and effective (Φ_{PSII}; Genty et al., 1989) quantum yield of photosystem II.

Grapevine phytoalexins belongs to group of stilbenes, secondary metabolites with antifungal activities. Langcake as a first author identified stilbenes as phytoalexins of grapevine (Langcake and Pryce, 1976; Langcake et al., 1979; Langcake, 1981). Under *P. viticola* attack, trans-resveratrol (3,5,4` - trihydroxystilbene), trans- and cis-piceid (3-O-β-D-glucoside of resveratrol), trans-pterostilbene (3,5-dimethoxy-4`-hydroxystilbene) and viniferins (cyclic dehydrodimers of resveratrol) are produced (Pezet et al., 1994; Langcake, 1981; Pezet et al., 2003; Babíková et al., 2008). Bound forms of resveratrol (t*rans* and *cis*-piceids) were found to accumulate also in grapecine berries infected by *P. viticola* (Romero-Perez et al. 2001). Recently, highly fluorescing compound in grapevine leaves infected by *Plasmopara viticola* was identified (Tříska et al., 2012). Chemically, this compound is a 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside, resulting from the isomerization reaction of *trans*-resveratrol to *cis*-resveratrol. This phenanthrene derivative was identified for the first time as a main fluorescent natural product arising in relation to *P. viticola* impact on the vine leaves.

In this study we have expand our lab-based study (Cséfalvay et al., 2009) into the field. We have aimed at verification that Chl-F distribution over naturally infected leaves can be also used for pre-symptomatic detection of *P. viticola* infection in field. Here we used leaves of six sensitive (Cabernet Sauvignon, Chardonnay, Muscatel, Saint Laurent, Müller Thurgau, Alibernet) and four resistant (Cerason, Malverina, Hibernal Laurot) grapevine cultivars. In parallel, we analyzed content of two major phytoalexins (*trans*-resveratrol and pterostilbene) and the newly identified 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside in infected leaf tissue of all ten cultivars. This phenanthrene derivative was analyzed in leaves of both susceptible and resistant cultivars naturally infected by *P. viticola* (Tříska et al., 2012). Concentrations of these phytoalexins were correlated with decrease of photosynthetic activity in the leaf tissue and with degree of resistance in individual grapevine cultivars.

Materials and methods

Plant material

Leaves of six susceptible (Cabernet Sauvignon, Chardonnay, Muscatel, Saint Laurent, Müller Thurgau, Alibernet) and four resistant grapevine cultivars (Cerason, Malverina, Hibernal, Laurot) have been sampled in experimental vineyard (Department of Viticulture and Oenology, Lednice, Czech Republic, 48°47'24.16"S; 16°47'53.61"V), on 16th July 2010. Healthy and infected leaves were collected both from sun exposed and shade part of the plants. The infection was identified as the fungal infection by *Plasmopara viticola*. It was in the state of beginning oily spots formation on the upper side of the leaves, i.e. ca 5 - 7 days after inoculation (according to Cséfalvay *et al*. 2009). After samples collection in vineyard, each petiole was coated by wet cellulose to avoid drying, and transported immediately to the laboratory in plastic bags by using portable refrigeration unit added to 15ºC. In total, 200 leaves have been collected, 10 healthy and 10 infected leaves from each grapevine variety.

Chlorophyll fluorescence imaging measurements were done on each collected leaf. HPLC analysis was done on 60 collected leaves in total, 3 repetition of each cultivar.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence (Chl-F) measurements were done by using commercial kinetic imaging fluorometer (OpenFluorCam 70MF, Photon System Instruments, Ltd., Brno, Czech Republic; Nedbal et al., 2000), in laboratory situated directly in the vineyard. Grapevine leaves were dark adapted for 20min before each measurement. Chl-F emission from leaves was excited by two panels of light emitting diodes (LEDs, $\lambda_{max} \approx 635$ nm), that generate short measuring flashes (10 μs) and actinic light. Saturating light pulses (1500 μmol (photons) $m^{-2} \cdot s^{-1}$, 1s) were generated by a 250W halogen lamp (λ = 400−700 nm). Chl-F emission from the leaves was recorded by CCD camera in series of 512x512 pixel images of 12-bit resolution.

First, minimum Chl-F in dark (F_0) was determined using five measuring flashes generated by LED panels. Subsequently, short saturating flash was applied to measure maximum Chl-F in dark (F_M). Chl-F parameters F_0 and F_M values were determined. Then, F_V/F_M parameter (maximum quantum yield of PSII photochemistry; F_V/F_M; Kitajima and Butler, 1975) was calculated according to following formula: $F_V/F_M = (F_M-F_0)/F_M$. Images of F_V/F_M distribution over the grapevine leaves were inspected in order to display differences between healthy and infected leaf areas. Values of F_V/F_M in healthy and infected leaf tissue have been calculated to quantify the level of photosynthetic activity decrease. Comparison between control and infected leaf tissue was done

using healthy or infected leaf segments selected in each leaf (6 from each susceptible, 3 from each resistant leaf variety).

HPLC analysis

Sample preparation Based on F_V/F_M distribution over measured leaves, three samples of *(a)* leaf tissue from the center of infection in the infected leaves, and *(b)* healthy tissue from control leaves from each measured leaf were taken as cuts of circular shape using a cork borer. Diameter of these cuts was 14 mm and the area 154 mm^2. Leaf tissue samples were weighed and extracted in 0.5 ml of 70% methanol for 24 h at room temperature in dark, with occasional shaking. Then, the methanolic extract was collected, passed through a glass filter, and analyzed using HPLC.

Sample analysis The samples were analyzed using an HP 1050 (Ti-series) HPLC instrument (Hewlett Packard, U.S.A.) on a 3 µm, 150 mm x 2 mm, Luna C18(2) column (Phenomenex, U.S.A.) with water-acetonitrile-*o*-phosphoric acid mobile phase. Mobile phase A used 5% of acetonitrile + 0.1% of *o*-phosphoric acid; mobile phase B used 80% of acetonitrile + 0.1% of *o*-phosphoric acid. The gradient was increased from 20% of B to 80% of B during 20 min and from 80% of B to 100% of B during 5 min. Flow rate was 0.250 $ml.min^{-1}$ and column temperature 25 °C. Injection volume was 5 µl. Also used was HP G1315B diode array detector (DAD) (Hewlett-Packard, U.S.A.), with detection wavelengths at 220 and 315 nm, and scanning range 190–600 nm, as well as an HP G1321A fluorescence detector (FLD) (Hewlett-Packard, U.S.A.), with excitation wavelength 315 nm, emission wavelength 395 nm, and scanning of emission in the range of 300–600 nm.

Data analysis

Mean and standard deviation were calculated to assess differences of Chl-F by using MS Excel. Correlations of Chl-F with particular stilbenes concentration was also determined, the significance of regression analysis was tested by MS Excel, too.

Results

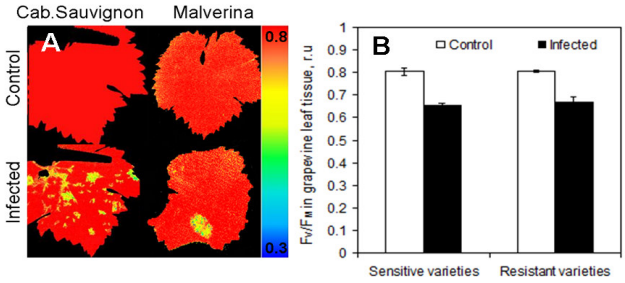
Chlorophyll fluorescence signatures of *P. viticola*

Imaging of F_V/F_M parameters According to our previous study, in which we proved that Chl-F parameters referring to photosynthetic activity (maximum and effective quantum yield of photosystem II [F_V/F_M, Φ_{II}]; Cséfalvay et al., 2009) were the most effective to discriminate the infected and healthy tissue in the early infection stages, we used F_V/F_M in this study to verify these findings on various grapevine cultivars in field.

Fig.1A shows Chl-F images of the representative control (top panel) and infected (bottom panel) leaves of susceptible (Cabernet Sauvignon) and resistant (Malverina) cultivars. Control leaves expressed homogeneous distribution of F_V/F_M, while infected leaves where the fungal infection started to precede contained leaf spots with significantly lower F_V/F_M values. Distribution of these spots differed between susceptible and resistant cultivars. In susceptible cultivars, the infection started from many infected points on the leaf with lower F_V/F_M, from which they were spreading and finally covered the whole leaf. In resistant cultivars, the infection was localized to one or maximally two big spots with reduced F_V/F_M. No expansion of these affected areas was observed. Similar observations were done for all measured susceptible and resistant cultivars.

To compare to which extend is F_V/F_M decreased in the infected leaf tissue, we have extracted Ch-F from the infected leaf spots and compared them with those from controls. Fig. 1B shows the comparison of F_V/F_M from the healthy (white columns) leaves and infected (black columns) spots. In all control healthy leaves F_V/F_M reached the maximum level, which is around 0.8. Contrary, infected spots expressed ca 18% lower F_V/F_M value (~ 0.65), which indicate damage of PSII reaction centers. Typically, F_0 levels were higher while F_M levels were lower in infected leaf spots (data not shown).

Figure 1. Graphical interpretation of F_V/F_M parameter



[A] False color scale images of maximum quantum yield of photosystem II (F_V/F_M) distribution over representative healthy grapevine leaves (top panel) and leaves infected by *P. viticola* infection (bottom panel) of susceptible (Cabernet Sauvignon) and resistant (Malverina) cultivar. Plants were grown and infected naturally under field conditions. [B] Mean values of F_V/F_M in control (white columns) and infected (black columns) grapevine leaf tissue. Each data point represents the average of 20 - 30 measurements. Bars represent standard deviations.

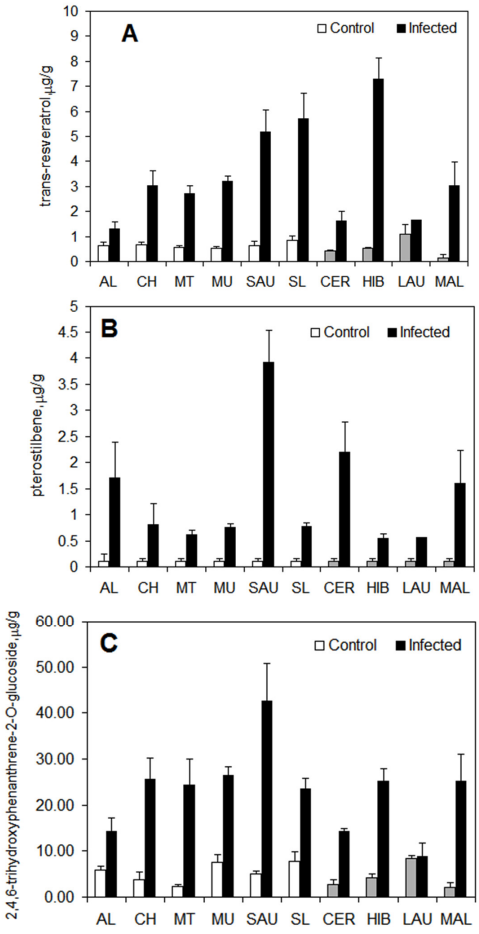
Samples for phenolic compounds and photosynthetic pigments determining were taken in the areas with reduced F_V/F_M. Two well known stilbenes acting like phytoalexins (*trans*-resveratrol and pterostilbene) were analyzed. In addition, concentration of one newly discovered, highly fluorescing compound 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside that is derived form of trans-resveratrol was analyzed, too. Significant increase of all three phenolic compounds was found in infected leaf tissue of all measured grapevine cultivars as shown in Fig.2. No clear difference between susceptible and resistant cultivars was observed.

Trans-resveratrol (Fig.2A) occured in all control leaf samples; varied in concentration between 0.15 (Malverina) – 1.1 mg/g (Laurot). Under infection, *trans*-resveratrol content increased several times in the grapevine leaf tissue. The highest increase was found in the case of Hibernal cultivar (7.3 mg/g), being approx. 13 times more than in control leaf tissue. Laurot cultivar (resistant to *P. viticola*) expressed no

significant difference between healthy and infected leaf tissue. In case of pterostilbene, control leaf tissues did not contain its detectable content. However, under the impact of *P. viticola*, pterostilbene concentration significantly increased in all grapevine cultivars (Fig.2B). The highest concentration was observed in Cabernet Sauvignon (3.9 mg/g), the lowest concentrations were measured in Laurot and Hibernal cultivars (0.5 and 0.56 mg/g, respectively).

Also, 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside concentration increased in grapevine leaf tissue under infection of *P. viticola* (Fig. 2C). The highest content of this compound was found in Cabernet Sauvignon leaves (42 mg/g); the lowest and comparable concentration with that in control was detected in Laurot (8.9 mg/g). However, one can note that neither *trans*-resveratrol, pterostilbene, nor 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside concentrations were not reflecting susceptibility and/or resistance to fungal disease of the grapevine cultivars.

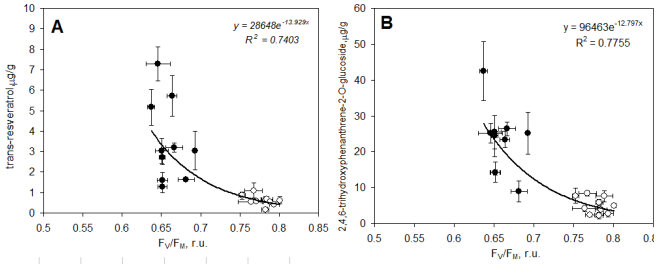
Figure 2. Mean values of main stilbenes



Mean values of trans-resveratrol [A], pterostilbene [B] and 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside [C] concentrations in control susceptible (white columns), control resistant (grey columns) and infected (black columns) grapevine leaf tissue. Samples of leaf tissue were collected from plants cultivated in field, that were naturally infected by *P. viticola*. Each data point represents the average of 3 measurements. Bars represent standard deviations.

Since 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside is derived from *trans*-resveratrol, we have correlated their concentrations with photosynthetic activity in control (open symbols) and infected (closed symbols) leaf spots of leaves as shown in Fig.3. In both correlation graphs, we were able to differ between the groups of healthy leaf tissue with high photosynthetic activity and low concentration of polyphenols (open symbols), from infected leaf tissue with low photosynthetic activity and higher concentration of polyphenols (closed symbols). The correlation between both polyphenols (*trans*-resveratrol and 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside) and F_V/F_M value (Fig. 3A, B, respectively) was at a similar level (R^2 = 0.74 and 0.77, respectively).

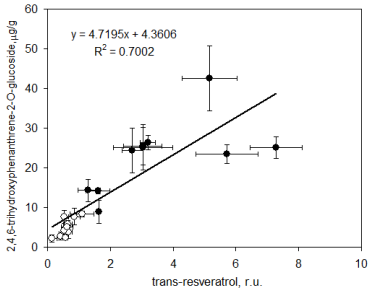
Figure 3. Correlation of F_V/F_M and particular stilbenes concentration



Correlation of *trans*-resveratrol [A] and 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside [B] content with maximum quantum yield of photosystem II (F_V/F_M), measured in healthy (white symbols) and infected (black symbols) grapevine leaf tissue by *P. viticola*. Each data point represents average of 10 measurements. Error bars represent standard deviations. The interpolating line represents an exponential regression optimized by MS Excel.

Hence, we tried to correlate *trans*-resveratrol concentration in grapevine leaf tissues with 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside content as shown in Fig.4. We found linear relationship between these two related polyphenols in grapevine leaf tissue. Similarly to F_V/F_M correlation graphs with these two related polyphenols (Fig.3A,B), group of healthy leaf tissue with low content of *trans*-resveratrol and 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside (open symbols) can be distinguished from infected leaf tissue producing both polyphenols in higher value.

Figure 4. Correlation between *trans*-resveratrol and the derivative content



Correlation of 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside concentration with *trans*-resveratrol content in healthy (white symbols) and infected (black symbols) grapevine leaf tissue by *P. viticola*. Each data point represents average of 10 measurements. Error bars represent standard deviations. The interpolating line represents a linear regression optimized by MS Excel.

Discussion

Chl-F emission has been broadly applied to study biotic stress in plants (reviewed by e.g. Maxwell and Johnson, 2000; Baker, 2008), since it tracks the physiological alterations induced in infected plants (Lichtenthaler and Miehé, 1997; Berger et al., 2007b; Atkinson and Urwin, 2012). Among other Chl-F parameters, maximum quantum yield of photosystem II (F_V/F_M) is one of the most frequently used Chl-F parameter for plant-fungal interactions estimation (e.g. Scholes and Rolfe, 1996; Soukupová et al., 2003; Berger et al., 2007a; Cséfalvay et al., 2009; Prokopová et al., 2010; Tung et al., 2013). In our previous study (Cséfalvay et al. 2009), both maximum and effective quantum yield of PSII (F_V/F_M and Φ_{PSII}, respectively; Kitajima and Butler, 1975; Genty et al., 1989), were identified as the most potent to detect the early phases of *Plasmopara viticola* infection in grapevine leaves. These Chl-F parameters reported on local perturbations in the photosynthetic efficiency of photosystem II that were specifically associated with the spots in which the disease was developing.

To verify F_V/F_M effectiveness for pre-symptomatic detection of *P. viticola* in field, we have measured 200 grapevine leaves (100 healthy and 100 infected). The infection was in the state of visual symptoms beginning (oily spots), referring to ca 50% of the incubation period after 5 = 7 days (Unger et al., 2007; Cséfalvay et al., 2009). All infected leaves expressed heterogeneity in F_V/F_M distribution. This F_V/F_M distribution over infected leaves (Fig. 1A) clearly indicated impaired leaf spots with lower F_V/F_M, in accordance with the lab-based study (Cséfalvay et al., 2009). Although the level of F_V/F_M decrease was similar in infected leaf spots of both susceptible and resistant cultivars (ca 18 %, Fig. 1B), their distribution over the leaves differed (Fig. 1A). We propose that it is associated with the response of resistant leaves (Malacarne et al., 2011). Resistant cultivars (Cerason, Hibernal, Laurot, Malverina) enclosed the infection in few big spots from which the infection did not proceed, while also biosynthesis of phytoalexins was activated. In sensitive cultivars (Alibernet, Chardonnay, Cabernet Sauvignon, Müller Thurgau, Muscatel) the number as well as the area of spots with reduced photosynthetic activity continuously increased as the infection progressed (see also Tříska et al. 2012). Šebela et al. 2012 reported that leaf tissue infected by P. viticola revealed lower content of photosynthetic pigments (chlorophylls, carotenoids) and much slower electron transport rate on PSII donor and acceptor side compared to control leaves that contributes to lower photosynthetic activity. This field study confirms the results from our previous study (Cséfalvay et al. 2009) that measurement of F_V/F_M distribution over grapevine leaves can reveal the infection of *P. viticola* several days before the infection significantly affect the whole leaf performance.

The biosynthesis of stilbenes belongs to active defense response of grapevine plants under fungal attack including *P. viticola* infection (e.g., Langcake and Pryce, 1976; Langcake et al., 1979; Langcake, 1981; Dercks and Creasy, 1989; Pezet and Pont, 1995; Schouten et al., 2002; Aziz et al., 2003; Pezet et al., 2004; Schmidlin et al., 2008; Trouvelot et al., 2008; Alonso-Villaverde et al., 2011; Macedo Basto et al., 2011; Gauthier et al., 2014; Duan et al., 2015). In our study, the samples for determining main stilbenes and their derivatives (*trans*-resveratrol, pterostilbene and 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside) were taken in the areas with reduced F_V/F_M. Briefly, 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside has been recently found and for the first time identified as a main fluorescent natural product arising in relation to *P. viticola* impact on the vine leaves (Tříska et al., 2012). Concentrations of all three compounds were significantly higher (even several times) in infected leaf tissue (Fig. 2). Surprisingly, no clear difference between groups of susceptible and resistant cultivars was observed in concentrations of all three polyphenols. Generally, *trans*–resveratrol was produced to a greater extent than pterostilbene in infected leaves, while also both groups (susceptible and resistant cultivars) contained comparable amounts of *trans*–resveratrol. This finding is in accordance with other authors who observed that susceptible grapevine cultivars infected by *P. viticola* mainly produce *trans*-resveratrol and piceids (glucoside of resveratrol) (Pezet et al., 1994), whereas resistant ones produce *trans*-resveratrol, *trans*-pterostilbene and cyclic dehydrodimers of resveratrol (viniferins) (Langcake, 1981; Pezet et al., 2003).

In this field study, *trans*-resveratrol was identified as the main phytoalexin. On the leaf level and at the beginning of *P. viticola* infection is the *trans*-resveratrol production restricted to invaded leaf spots (Dercks et al., 1989; Malacarne et al., 2011; Vrhovsek et al., 2012). 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside is derived from *trans*-resveratrol in relation to *P. viticola* impact on the vine leaves (Tříska et al., 2012), and during this field study was isolated from all measured leaves of both susceptible and resistant cultivars (Fig. 2C). Concentration of this phenanthrene derivative was much higher than that of *trans*-resveratrol (Fig. 2B). In addition, it seems that the isomerization process from *trans*–resveratrol to 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside is more effective in cultivars having the higher *trans*-resveratrol production (Šebela et al., 2014). We propose that one of the reasons can be prescribed as the efficiency of *trans*-resveratrol transformation pathways under solar light exposure (Rodríguez-Cabo et al., 2015).

Both *trans*-resveratrol and 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside concentrations correlate well and similarly with maximum quantum yield of PSII (F_V/F_M) in grapevine leaves (R^2 = 0.74 and 0.77, respectively, Fig. 3). It is obvious that secondary metabolism has been activated in tissue invaded by *P. viticola*, that expressed lower photosynthetic activity with impaired electron transport rate in photostystem II and lower chlorophyll and carotenoids content (Šebela et al., 2012). The biotic stress-induced decrease of Chl-F signal have been investigated in several studies (reviewed by e.g. Maxwell and Johnson, 2000; Roháček et al., 2008; Baker, 2008) however, correlation of the signal with direct concentrations of particular stilbenes under fungal attack is a novelty, which undoubtedly gives advantage

for pre-symptomatic detection of *P. viticola* in field conditions, presented in this study.

Since recently more than 3.5 million ha of vineyards in European Union (EU) are planted by cultivars of *Vitis vinifera* that are susceptible to downy mildew disease (Eurostat report 2007), such early information of crop physiology and detection of disease directly in field conditions can lead to effective strategy of wine producer and the resulting proper management. Moreover, measurement of Chl-F could also help in large-scale screening of quantitative resistance to *P. viticola*. Also UV fluorescence signal measuring *in vivo* can help in resistance screening. Stilbenes that are highly implicated in the defence reaction and resistance of grapevines to fungal infections (e.g., Chong et al., 2009; Allonso –Villaverde et al., 2011; Bellow et al., 2012), emit violet-blue fluorescence under UV light (Hillis and Ishikura, 1968). There are many studies using their fluorescence signal to localize naturally occurring stilbenes *in vivo* (e.g. Poutaraud et al., 2007; Poutaraud et al., 2010; Bellow et al., 2012). Bellow et al. (2012) showed that there are differences in stilbenes localization *in vivo* in leaf tissue of sensitive and resistant grapevine cultivars. Also 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside, recently identified as highly fluorescing compounds (Tříska et al. 2012), have been proven to be detectable *in vivo* in grapevine leaves infected by *P. viticola* (Šebela et al., 2014). Compraed to our study however, authors reported just three cultivars sensitive to *P. viticola*. Here we present broader study and thus, our approach leads to more complex information about the responses of both sensitive and resistant cultivars.

Conclusions

We propose that high potential of Chl-F imaging in early detection of *P. viticola* infection in field would be increased by UV in-vivo fluorescence of 2,4,6-trihydroxyphenanthrene-2-*O*-glucoside. Such information can be used for high-throughput screens, to precisely estimate the dynamics and/or impact of *P. viticola*, in both resistant and susceptible cultivars. The novel approach presented here, with appropriate scientific adjustment, can potentially be extended on a large scale of grapevine cultivars in an attempt to quantify genetic diversity between resistant and sensitive cultivars.

Acknowledgements

This study was funded by the Grant Agency of the Czech Republic, grant number 525/09/0365, and by CzechGlobe – Centre for Global Climate Change Impacts Studies, Reg. No. CZ.1.05/1.1.00/02.0073.

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3. RESULTS

3.2. Fruits

This part is consists of three independent experiments. In first (*3.2.1.*), we tried to define if there is some influence of ambient sunlight intensity on phenolic compounds synthesis during ripening. During the second part (3.2.2.) we were trying to develop non-ivasive method for UV fluorescence screening of phenolic compounds in grape berries and finally, in third part (*3.2.3.*) we were trying to find optical parameter to follow accelerated panicle senescence under high-night temperature impact.

3.2.1. Effect of ambient sunlight intensity on the temporal phenolic profiles of *Vitis vinifera* L. cv. `Chardonnay` during ripening season-a field study

Prepared manuscript

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Abstract

Grape phenolics are considered to have significant impact on wine quality, their quantity and dynamics being strongly influenced by environmental conditions. We investigated the effect of ambient sunlight on the temporal dynamics of phenolics in cv. `Chardonnay` during ripening season 2012, from verasion till harvest in field conditions. Phenolics profiles were temporally monitored at pre-defined time intervals, by using high pressure liquid chromatography (HPLC) and spectrophotometric (OD) analysis. The obtained concentrations were correlated with the average sunlight intensities preceeding the sampling. No significant correlations between the hydroxycinnamic acids, hydroxybenzoic acids and stilbenes content were in contrast with the strong relationships found between; *(i)* $OD_{280\ nm}$ *(ii)* catechin, *(iii)* flavanols and *(iv)*total polyphenols and the fluctuating dose of ambient sunlight, reaching the grapes throughout the ripening season. The light dependent dynamics of several main phenolic compounds in cv. `Chardonnay` during the ripening help to establish correlation models that could increase the applicability of meteorological data in the assessment of optimal phenolic ripeness in modern viticulture.

Effect of Ambient Sunlight Intensity on the Temporal Phenolic Profiles of *Vitis vinifera* L. cv. `Chardonnay` During Ripening Season-a Field Study

David Šebela, Zoltán Turóczy, Julie Olejníčková, Michal Kumšta, Radek Sotolář

***Abstract*— Grape phenolics are considered to have significant impact on wine quality, their quantity and dynamics being strongly influenced by environmental conditions. We investigated the effect of ambient sunlight on the temporal dynamics of phenolics in cv. `Chardonnay` during ripening season 2012, from verasion till harvest, in field conditions. Phenolics profiles were temporally monitored at pre-defined time intervals, by using high pressure liquid chromatography (HPLC) and spectrophotometric (OD) analysis. The obtained concentrations were correlated with the average sunlight intensities preceeding the sampling. No significant correlations between the hydroxycinnamic acids and stilbenes content were in contrast with the strong relationships found between; *(i)* $OD_{280\ nm}$ *(ii)* catechin, *(iii)* sum of catechin and epicatechin, *(iv)* flavanols and *(v)* total polyphenols and the fluctuating dose of ambient sunlight, reaching the grapes throughout the ripening season. The light dependent dynamics of several main phenolic compounds in cv. Chardonnay during the ripening help to establish correlation models that could increase the applicability of meteorological data in the assessment of optimal phenolic ripeness in modern viticulture.**

***Key words*—abiotic stress, catechin, chardonnay, grape berries, UV light, viticulture**

Introduction

Viticulture worldwide is influenced by constant and sometimes drastic changes in many environmental factors (Fraga et al., 2012). Therefore, a major challenge for wine producers nowadays is to constantly cope with the global alterations in the environmental conditions, of both biotic and abiotic origin. Viticulture and climate change are interrelated processes, taking place on a global scale, in which temperature, precipitation and solar radiation are the main drivers (Abou-Hussein, 2012). Overexposure or the lack of any of these factors may influence yield and wine quality, meaning that the delicate equilibrium between these environmental components has a great effect on the final productivity.

Sunlight is composed mostly of infrared wavelengths, but contains also a small amount (approx. 8-9%; Frederick, 1993) of ultraviolet (UV) light harmful to life on Earth (reviewed by Hollósy, 2002). UV light can be divided into three categories, based on their spectral properties: UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (100-280 nm). UV-A radiation is not absorbed by the ozone layer, compared to complete absorption of UV-C. Intensity of UV-B light (potentially harmful to plants) reaching the Earth surface is strongly dependent on the thickness of ozone layer. Thus, due to decrease of stratospheric ozone concentrations, there is an increase of ambient UV radiation reaching the surface of the Earth (e.g. McKenzie et al., 2003). The amount of ambient UV light however, is also strongly dependent upon spatial and temporal factors, such as season of the year and time of the day (Roderick, 1999); cloud cover (Estupiñán et al., 1996); canopy coverage (Théry, 2001), etc.

Effect of ambient and elevated UV-B light on terrestrial plants has been examined in many studies. Under realistic spectral regimes in the field experiments there is a species-specific plant response, including alterations in plant morphology and architecture (Kakani et al., 2003), DNA-damage and antioxidant response (Hollósy, 2002;) and effect on photosynthetic efficiency (Burger and Edwards, 1996). Still, perhaps the most common plant response to ambient and elevated UV-B radiation is the production of UV absorbing compounds, such as phenolic compounds (Meijkamp et al., 2001; Caldwell et al., 2003; Fedina et al., 2007; Agati et al., 2009, 2011).

Grapevine (Vitis *vinifera* L. subsp. *vinifera* Hegi, 1753) phenolics are important quality components, contributing not only to the taste and mouthfeel properties of wines, but also to the color. Grape composition is strongly variety dependent (Singleton and Trousdale, 1983; Gatto et al., 2008), but can also be influenced by environmental conditions (Dokoozlian and Kliewer, 1995; Teixeira et al., 2013; Kuhn et

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al., 2014). Such grapevine phenolics are divided into two groups: non-flavonoid compounds and flavonoids. From a wine quality perspective, the major non-flavonoid compounds are hydroxycinnamic acids and stilbenes. Flavonoids represent source of antioxidants in grapevine (Conde et al., 2007) and include classes of *(i)* flavanols (catechin and epicatechin), particularly relevant for astringency in final product (Kallithraka et al., 1998), *(ii)* flavonols (quercitin) that may influence bitterness (Preys et al., 2006), and *(iii)* anthocyanins (e.g malvidin-3-*O*-glucoside), that separate red varieties from white ones. From medical perspective, several of these phenolic compounds are also known for their health benefits (Yao et al., 2004). A variety of experiments have shown, that selected flavonoids posesses antiallergic, anti-inflammatory, antiviral and antioxidant activities (reviewed by Pal and Verma, 2013). Some flavonoids also display a wide spectrum of biological activities, including ones that might be able to influence metabolic processes affected during cancer development (reviewed by Pandey and Rizvi, 2009). Moreover, the importance of some phenolic compounds and their protective role against e.g. grapevine yellows (Rusjan et al., 2012) or downy mildew (Šebela et al., 2014) has also been proven.

Grapevines have to cope with relatively great doses of ambient radiation in the field during their life cycle and throughout each ripening season. At the same time, irradiance reaching each grape berry is affected by fluctuations of intensity, spectral quality and periodicity of incoming light (Frankhauser and Staiger, 2002). Such imbalance in incident light can cause changes in synthesis of phenolic compounds and the resulting fluctuating trend of their concentrations during ripening season. Based on the photo-protective role of phenolic compounds in grape berries (Mira de Orduna, 2010), the light dependency on the synthesis of phenolic compounds has been tested across the grapevine varieties by two classical approaches, i.e. by the shading (Price et al., 1995; Spayd et al., 2002; Downey et al., 2004; Adams, 2006) and by UV light filtering (Keller et al., 2004; Berli et al., 2008) experiments. Both attempts showed the significance of UV radiation in these processes, however very few studies have reported about the possible effects of both UV and visible light on the synthesis of these compounds (Schultz, 2000; Kolb et al., 2001; Schreiner et al., 2012), or about their seasonal-temporal characteristics. Even though the dependence of the phenolics content on the grape developmental stages has been well documented (Downey et al., 2003; Doshi et al., 2006; Conde et al., 2007), real-time, on-site data, concerning the relationship between ambient sunlight intensity, phenolic profile and the berry development throughout the ripening process has been scarce. Thus, the objective of this study was to determine the effect of ambient sunlight intensity on the maturation and subsequent phenolic profiles of grapevine berries in the common white variety Chardonnay, during ripening season 2012.

Materials and methods

Plant material

Study was carried out in the experimental vineyard of the Department of Viticulture and Oenology, located in Lednice, Czech Republic (48°47'24.16"N; 16°47'53.61"E), during ripening season 2012. The time course of the monitoring process has been selected according to Coombe (1992), i.e. early ripening season corresponded to `verasion` and maturity to `harvest` phase. Chardonnay grapevine variety was sampled at pre-defined time intervals, i.e. alternatively each second and fifth day, in the period between August and October. Each date, samples were collected at the same time (08:00 AM) in the vineyard and immediately transported to laboratory. To attenuate environmental differences other than influence of ambient sunlight intensity, clusters were always taken from the first fruit-bearing shoot of south east orientation. To prevent sample dry out, each cluster was placed into a plastic bag and transported at controlled temperature of 15°C.

Extraction of polyphenols

For the chemical analysis, one cluster was selected for each sampling date, from which 18 berries were randomly cut, taking into account that they were equally distributed from the whole cluster. Extracts from the whole grape berries (i.e. including skin, flash and seeds) were done as follows: Out of the 18 berries sampled, six groups containing three random berries were extracted, i.e. smashed in a solution containing 90% methanol (MeOH) and 1mM potassium metabisulfite ($K_2H_2O_5$) in 1:1 ratio (mg berry/ml MeOH+ $K_2H_2O_5$; Kumšta *et al.*, 2012), and left in the dark at room temperature for 2 weeks. The resulting six extracts were used to calculate the average concentrations for each sampling date. Following extraction, the homogenate was centrifuged at 15.000 rpm for 10 min. Supernatants were than filtered through 0.45 μm Millipore filters (Merck Millipore, Prague, Czech Republic) and stored at -20°C until the analysis.

HPLC analysis of polyphenols

In order to increase the reliability of data, two similar HPLC methods were used:

1. Percentage ratio and concentrations of total polyphenols HPLC analysis was performed using Digital High-Pressure system LC-1A with system controller SCL-10Avp (Schimadzu, Japan). For the separation, Polymer IEX H form 10 mm packing, 250x8mm + 10x8 mm column (Watrex, Czech Republic) was used. To shorten the analysis time and to facilitate the separation, the column was kept in 60ºC during analysis, using column thermostat with manual spraying valve Rheodyne CTO-10Acvp (Shimadzu, Japan). Isocratic elution was used with 2mM H_2SO_4 composition of mobile phase. The applied flow rate, provided by pump (LC-10 Advp) with two channels, was 0.75 ml/min.; the injected sample volume was 20μl.

2. Time trend of total polyphenols analysis was performed by 1200 Infinity Series Instrument (Agilent Technologies, USA), equipped with thermostat and auto sampler. For the separation, Alltech Alltima C18 3μm particle size reversed

phase column 150 x 3mm (Fisher Scientific, USA) was used with the following mobile phase: 50% acetonitrile, 15 mM $HClO_4$, 10 % methanol as solvent A, and 15 mM $HClO_4$ as solvent B, with an equal flow rate of 0.5 ml min^{-1}. The gradient employed was as follows: 0min, 2 % A, 20min, 26 % A, 30min, 45% A, 35min, 70% A, 37min, 100% A, 39 min 0% A, 50 min, 2% A. During the analysis, the column was kept at 60°C to shorten the separation time. The detection wavelengths were following: flavan-3-ols (catechin and epicatechin) were detected at 280 nm, HCA (caftaric acid) at 315 nm and flavonols (e.g quercetin) at 360 nm, respectively. The different phenolic compounds were identified based on their retention times and the UV-vis spectra by comparison with the pure standards. HPLC pure standards for the phenolics compounds of interest (catechin, epicatechin, caftaric acid) were purchased (PhytoPlan, Germany).

Reagents and solvents

HPLC grade standards (PhytoPlan, Heidelberg, Germany); reagents constituting the mobile phase: methanol (Chromservis, Czech Republic); acetonitrile (Chem-Lab NV, Belgium); and perchloric acid (Acros Organics, Czech Republic). All these reagents were HPLC pure.

Spectroscopic analysis

Spectroscopic analysis for each extract was carried out by using spectrophotometer SPECORD 210 Plus BU (Analytic Jena AG, Germany). Concentration of total phenolics was obtained by recording absorbance at 280nm (OD_{280}, Pirie and Mullins, 1976; Somers and Evans, 1977), providing fast and accurate estimation in both red and white varieties (Jensen et al., 2008; Oberholster et al., 2010).

Light intensity measurements

The amount of sunshine at experimental field was recorded by a standard Campbell-Stokes sunshine recorder (heliograph). To maintain uniformity of the light measurements throughout the experiment, instrument was placed 1m above ground, at a circa 50m distance from the experimental field, where the shading of the sphere by the entire canopy was not limiting factor. The device itself consisted of the sphere made from annealed optical glass and the recording sheet. Sun intensity was continuously recorded by the sheet, through a sun beam coming from the sphere; the record itself was separated to the 30 minutes blocks. Light intensity was than calculated as an average of sunlight intensity of the whole day (W/m^2), from these 30 min blocks. For the experimental purposes, daily average light intensity was calculated at several pre-defined time intervals, (i.e. day of experiment (DE)) and as an average from the daily means up to 6 days before experiment (6DE).

Data analysis

Open LAB CDS ChemStation software (Agilent Technologies, USA) and LCsolution software (Shimadzu, Japan) were used to integrate chromatographic peaks, to create standard calibration curves and to calculate the real concentrations of selected phenolic compounds. The significance of the differences in the concentrations between `verasion` and harvest was determined by t-test; mean, standard error and correlations were determined; significance of the correlation coefficients was tested by regression analysis, ANOVA, MS Excel.

Results and Discussion

With the onset of veraison, several biochemical changes begin to happen in the grape berries; increase of the berry size and sugar accumulation, followed by decrease in acid levels being perhaps the most important and trivial. However, the optimal physiological ripeness is a far more complex phenomena, involving changes in the concentrations of several secondary metabolites, significantly impacting the resulting wine quality. As the harvest was gradually approaching, the modifications in phenolic content were registered. The changes in the main groups of phenolic compounds are graphically interpreted by means of *(i)* percentage ratio to total polyphenol content (Figure 1A) and *(ii)* the real concentrations (Figure 1B), at the `verasion` stage and `before harvest`. Since the extraction procedure (i.e. effect of the solvent concentration and/or contact time) can largely influence the determination of phenolics in grape samples (Gambuti et al., 2009), method for the whole berry extraction commonly used in our lab (see materials and methods, Kumšta et al., 2012) has been used.

Several reports have shown how the phenolics profiles of grapes are changing during the ripening (e.g. Pirrie and Mullins, 1977; Kennedy et al., 2000, 2002; Adams, 2006) and/or how UV light can influence the synthesis and the production of these compounds (e.g. Versari et al., 2001; Kolb et al., 2003; Berli et al., 2008), that are important for wine quality. In the present study, total polyphenols content decreased significantly from ca. 770 $mg \cdot kg^{-1}$ to approx. 540 $mg \cdot kg^{-1}$ fresh weight. This over 25% ($P<0.001$) decrease in the percentage ratio of total polyphenol content is in a good agreement with the results described by Fernandéz de Simón et al. (1993), Kennedy et al., (2000), Jordão et al. (2001), Pena-Neira et al. (2004), Ivanova et al. (2011) and Andjelkovic et al. (2013). The recorded, general trend presented in the literature is the decrease and later on the stabilization of flavanols content in grapes, which agrees with our observations. It's worth mentioning, that at the early ripening season (`verasion`), almost 90% of total polyphenols [stored in the skin and seeds (Hollecker et al., 2009; Fanzone et al., 2011)] are represented by flavanols - catechin and epicatechin. However, during ripening there is evidence for decrease in the level of free flavanols, together with an increase in the level of other derivates (Perez-Magarino et al., 2004), that corresponds to a ca. 25% ($P<0.001$) increase of flavonoids and HCA+stilbenes, non-flavonoid compounds content found in this study (Figure1A).

Increase in flavonoids content from 40 to 160 $mg \cdot kg^{-1}$ observed during this experiment (approx. 23%, $P<0.001$, of total polyphenols ratio, Figure 1A) corresponds to increased flavonoid biosynthetic pathway in grapevine during ripening process. This pathway has been well characterized in berries skin and seeds (Boss et al., 1996; Bogs et al., 2006). In total,

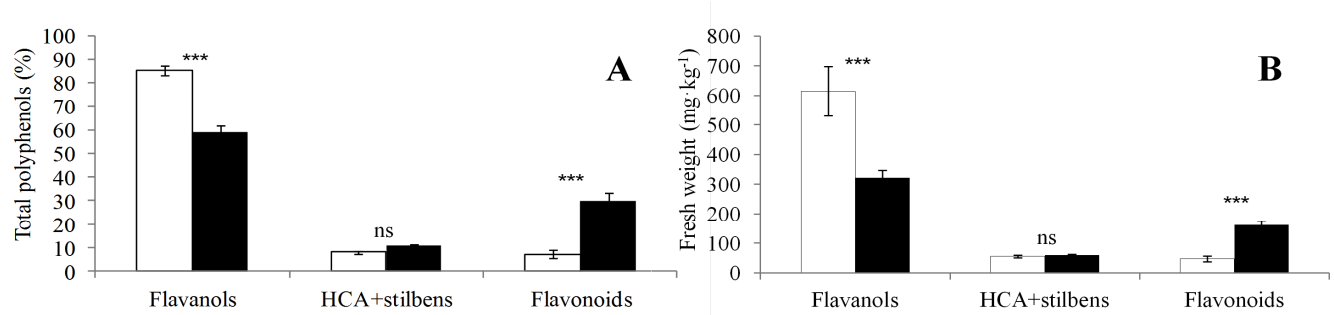

Figure 1. Mean values of representative plant phenolics of Chardonnay, expressed as a *(i)* percentage ratio of Σ polyphenols [A] and *(ii)* real concentrations [B] at the `verasion` stage [□] and before harvest [■]. Flavanols group is represented by sum of catechin and epicatechin; HCA+stilbenes by sum of gallic acid, caftaric acid, courtaric acid, fertaric acid, trans-rescveratrol, cis-resveratrol, total resveratrol, trans-piceatannol, trans-astringin and total piceatannol; and Flavonoids group is represented by the sum of Rutin, Quercetin-3-Glc, Quercitrin, Myricetin, Quercetin, Kaemferol and Isorhamnetin. Chemical analysis was performed by using HPLC system. Columns represent average value of 4 measuring dates for `verasion` and before harvest, during ripening season 2012. Error bars are showing standard error of the mean (n=24, SEM). ns, * , ** , *** – non-significant or significant at probability level 5%, 1% and 0.1%, respectively.

the most dominant compounds of the flavonoid group shown in our experiment were found to be quercetin 3-glucoside and rutin, with an significant increase from 16 to 97 $mg \cdot kg^{-1}$ and 21 to 30 $mg \cdot kg^{-1}$, respectively ($P<0.001$, Figure 1B).

Non-flavonoids compounds (HCA + stilbenes, Figure 1A, B), were showing the smallest, no significant changes throughout the whole ripening season (ca. 3% increase, $P>0.05$, from 55 to ca. 60 $mg \cdot kg^{-1}$). As shown by Singelton et al. (1978), HCA's are present mostly in the flesh of the grape berries. A major compound and representative member of the HCA's in this study, caftaric acid, did not show any significant change in concentration before harvest and at early ripening season ($P>0.05$, ca. 40 $mg \cdot kg^{-1}$), which is in accordance with previous results of our group (data not shown). In contrast to HCA present mostly in the berry flesh, majority of stilbenes has been reported to be found in the grape skin (Versari et al., 2001; Wang et al., 2002; Jaendet et al., 2002). In our study, visible increase in the concentration of the total resveratrol was observed between harvest and the onset of veraison (0.27 to 0.69 $mg \cdot kg^{-1}$), showing similar trend to Guifree (2013).

Changes in the content of phenolic compounds during the ripening season 2012 however, did not follow a clear, well defined trend (Fig. 2). Instead, the curves revealed big variations in the phenolic contents between different time points. These differences in concentrations could be explained by `date to date` fluctuations in critical environmental factors (reviewed by Teixeira et al., 2013). The temperature effect, as one of the most critical factor connected with the phenolic ripeness of the grape berries, was maximally attenuated by the sampling procedure used in this study (see materials and methods). With the increasing sunlight intensity however, there is a connected temperature effect. As reported by Berquist et al. (2001), the temperature increases linearly with the sun exposure and its optimum for berry growth is in the range of 25-30℃ (Hale and Buttrose, 1974). Many of the grapevine biochemical pathways are mutually light and temperature sensitive (Kliewer, 1970) but separating the effects both light and temperature remains technically impossible. Still the light regime reportedly plays a more crucial role during berry ripening compared to the other environmental factors (Dokoozlian and Kliewer, 1996) and thus has been named as a key factor influencing the synthesis of the phenolic compounds. Since the shading, caused by leaf area density and canopy architecture (Dokoozlian and Kliewer, 1995; Gladstone and Dokoozlian, 2003; Tarara et al., 2005) could influence the light reaching the clusters, usual agro- technical leaf removal treatments were applied prior to the measurements to minimize the effect of canopy shading (Bledsoe et al., 1988; Reynolds et al., 1995), Similarly, to avoid the effect of vineyard orientation (Grifoni et al., 2008) and/or microclimatic influences inside vineyard itself (Pereira et al., 2006), sampling setup was adapted according to the experiment's purpose (see materials and methods).

Average ambient light intensity showed decreasing trend throughout the whole season (Figure 2). Maximum level, at the beginning of the season (August, ca. 300 $W \cdot m^{-2}$), was followed by few minima during wet and cloudy days, when light intensity dropped to ca. 100 $W \cdot m^{-2}$, followed by reaching the maximum and decreasing slowly towards the 220, 160 and 100 $W \cdot m^{-2}$ (Fig. 2, 1DE) at the end of the season (October), respectively. The maximum average light intensity of 300 $W \cdot m^{-2}$ was proportional to a total sunshine of 12 hours per day in contrast to 100 $W \cdot m^{-2}$ which corresponded to 0.3 hours of total sunshine per day (data not shown). To investigate the connection between the ambient sunlight intensity and the concentration of the phenolic compounds throughout the ripening season, estimate of the phenolic concentration of wine and grapes (OD_{280}; Pirie and Mullins, 1976; Somers and Evans, 1977) have been plotted against the specific amount of solar radiation (see materials and methods, Figure 2). The same approach was implemented when using the real concentrations measured by HPLC analysis. The mutual dependence between the amount of solar radiation and

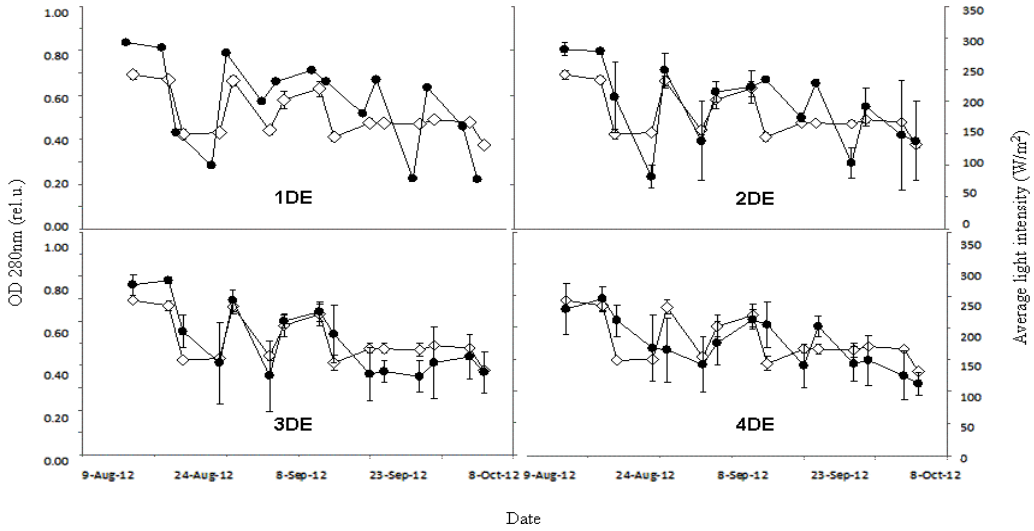

Figure 2. Time trend of OD 280 nm and average light intensity [W·m^{-2}] during ripening season. Average value of absorbance at 280 nm for Chardonnay [◊], measured by using spectrophotometer SPECOR 210 from the beginning of the season to harvest time. Average sunlight intensity [●] in W·m^{-2}, represented for A: one day before sampling-experiment date [1DE]; B: average value of sunlight intensities during two days before sampling-experiment date [2DE]; C: average value of sunlight intensities during three days before sampling-experiment date [3DE]; D: average value of sunlight intensity during four days before sampling-experiment date [4DE]. Error bars represents standard error of the mean (n=6, SEM).

relevant concentrations are summarized by the regression coefficients (R^2, Table 1). As expected, no or very low correlation was found between phenolics concentration and average sunlight intensity measured on the same day as berries were sampled (day of experiment – DE, Table 1). Due to the consistent, early morning (08:00 AM) sampling, when fluctuations of the light intensity were minimal, such changes in the average light could not have had a major influence on the actual concentrations of phenolics. Values of regression coefficients across the ripening season (Table 1) showed increasing trend up to 3DE, when they either reached a constant value or started to decrease.

Table 1. Regression coefficients (R^2) of average sunlight intensity and berry sample extracts.

	OD 280nm	Total polyphenols [mg/kg]	Flavanols [mg/kg]	Catechin [mg/kg]	Catechin +Epicatechin [mg/kg]	HCA +Stilbenes [mg/kg]
DE	0.36*	0.06^{ns}	0.14^{ns}	0.21^{ns}	0.15^{ns}	0.00^{ns}
1DE	0.59***	0.23^{ns}	0.31*	0.34*	0.37*	0.09^{ns}
2DE	0.49*	0.26*	0.28*	0.38*	0.34*	0.15^{ns}
3DE	0.67***	0.41**	0.47**	0.59***	0.53**	0.21^{ns}

Dependence of ambient sunlight intensity on optical density at 280nm [OD 280 nm], total polyphenols, flavanols, catechin, sum of catechin and epicatechin, HCA and stilbenes concentrations [mg·kg^{-1}] respectively. ns, * , ** , *** – non-significant or significant at probability level 5%, 1% and 0.1%, respectively.

No significant correlation (P>0.05) was found in the case of HCA and stilbenes, where values were in the range between 0.00 and 0.21. On the other hand, strong relationship was found between average light intensity 3DE and: OD 280 nm (R^2=0.71, P< 0.001); catechin (R^2 = 0.59, P<0.001), sum of catechin and epicatechin (R^2 = 0.53, P<0.001), flavanols (R^2 = 0.47, P<0.01) and total polyphenols (R^2 = 0.41, P<0.009). In agreement with our study, Keller and Torres-Martinez (2004) showed that flavonols are those phenolic compounds that are severely affected by UV light in both grapevine leaves and berries of Chardonnay variety, while the HCA's not. Also, Crippen and Morrison (1986) confirmed the effect of sun exposure on concentration of soluble phenols. They found significant differences between the sun-exposed and shaded grapes of Cabernet Sauvignon and an increasing trend in the percentage of phenols from verasion to harvest, which is in accordance with our experiment. The novelty of our study consists in the fact that we aim to provide a more realistic insight into the influence of the sunlight regime on the phenolic profiles in vineyards under field conditions, in a time dependent manner. Most studies in the past are based on experimental setups that do not occur in the field. Several studies reported the shielding approach, where in some case the UV light was either filtered (e.g. Spayd et al., 2002; Berli et al., 2008), or enhanced by using UV lamps (e.g. Petit et al., 2009). Surprisingly, relatively high concentrations of flavonols was also found in the absence of UV light (Keller and Torres-Martinez, 2004), suggesting their possible role not only in the protection against excess UV light, but also their role in other light dependent metabolic processes. The significance of our experimental setup consists in keeping the natural light conditions manipulation free. In this way we could only assume, that higher average light intensity comes with higher UV. Contrary to earlier studies (Cortell and Kennedy, 2006; Zhao et al., 2006) we dealt with a mixture of the berries that represents usual conditions in vineyards

management and harvest, i.e. collection of all berries from the cluster with no space for segregation of particular ones.

Undoubtedly for most of the modern wineries, the main aim is to produce wines of superior quality and thus increasing competitiveness on a national and/or international scale, but also to reduce input costs to a minimum. Our study of temporal influence of ambient sunlight intensity on the phenolic profiles of grapes offers a realistic insight into the crossplay between light regimes and a series of secondary metabolites that eventually result in phenolic ripeness. Better understanding of these dynamics will help wine production management and improve the existing strategies to grow higher quality wine grapes in an ever-changing environment.

In this work, we demonstrate the effects of the ambient sunlight on the temporal phenolic profiles of *Vitis vinifera* cv. Chardonnay. Significant correlation between the phenolics content and sunlight irradiance was found throughout the ripening phase, from the `verasion` to harvest. The main novelty of our study consists in the use of field conditions with non-manipulated light regime, therefore providing realistic, de facto trends for the light dependence of the phenolic ripeness. These, even one season, correlation models between light intensity, analytical description of the phenolics profile (HPLC) and spectrophotometric analysis, can contribute to a better understanding of the optimal phenolic ripeness, helping to increase the production of higher quality grapes and consequently better quality final products.

Acknowledgements

The authors are thankful to Prof. Jan Tříska and Dr. Naděžda Vrchotová for their helpful discussion and comments. This work was supported by project GA 522/09/1565 and FP7-SME-2010-1-262011-PREMIVM and forms a part of research supported by the Research Infrastructure project EPPN (FP7; Grant Agreement No. 284443). Participation of the first and third author was supported by the EfCOP-IPo project ENVIMET (CZ.1.07/2.3.00/20.0246) and is also an output of the grant number L01415 (Ministry of Education, Youth and Sports, Czech Republic).

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3.2.2. On-the-go to development of methodological and technological platform for non-invasive UV fluorescence and reflectance screening of phenolic compounds in grapevine berries

Prepared manuscript

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Abstract

In this study a method allowing rapid, non invasive and accurate estimation of phenolic content in berries of *Vitis vinifera* L. was designed. The method itself is based on the recording of *in vivo* UV induced fluorescence and/or reflectance from grape berry. As a first step, main phenolic compounds representing quality components of grapevine berry were identified; respective chemical standards were excited by their particular absorption maxima. Resulting emission spectra were analyzed and wavelengths suitable for the detection of these compounds were identified. *In vivo* fluorescence of grape berry however, slightly changed compared to the chemical standards, while emission peaks were centered at 400 nm. This approach has been tested during on-site field campaign carried out by using new CMOS radiometer instrument (prototype WinePen), in two independent seasons (2011 and 2012). As a reference, the same sets of measurements were carried out using hi-tech laboratory instrument. Phenolic profiles of white [Cahrdonnay (CH), Riesling (R)], and red [Pinot Noir (PN), Saint Laurent (SL)] cultivars were monitored from `verasion` till `harvest`, by using high pressure liquid chromatography (HPLC). Subsequently, changes in concentrations were correlated with optical signals. Our results showed high accuracy of the CMOS prototype along with the laboratory instrument. Moreover, we were able to identify specific phenolic compounds *in vivo* based on the UV induced fluorescence, both for white and red cultivars. In case of red cultivars however, better results were obtained in case of reflectance measurements. The key UV induced fluorescence and/or reflectance wavelengths/parameters have been summarized and are discussed.

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***Abstract*—In this study a method allowing rapid, non invasive and accurate estimation of phenolic content in berries of *Vitis vinifera* L. was designed. The method itself is based on the recording of *in vivo* UV induced fluorescence and/or reflectance from grape berry. As a first step, main phenolic compounds representing quality components of grapevine berry were identified; respective chemical standards were excited by their particula absorption maxima. Resulting emission spectra were analyzed and wavelengths suitable for the detection of these compounds were identified. *In vivo* fluorescence of grape berry however slightly changed compared to the chemical standards, while emission peaks were centered at 400 nm. This approach has been tested during on-site field campaign carried out by using new CMOS radiometer instrument (prototype WinePen), in two independent seasons (2011 and 2012). As a reference, the same sets of measurements were carried out using hi-tech laboratory instrument. Phenolic profiles of white [Cahrdonnay (CH), Riesling (R)], and red [Pinot Noir (PN), Saint Laurent (SL)] cultivars were monitored from `verasion` till `harvest`, by using high pressure liquid chromatography (HPLC). Subsequently, changes in concentrations were correlated with optical signals. Our results showed high accuracy of the CMOS prototype along with the laboratory instrument. Moreover, we were able to identify specific phenolic compounds *in vivo* based on the UV induced fluorescence, both for white and red cultivars. In case of red cultivars however, better results were obtained in case of reflectance measurements. The key UV induced fluorescence and/or reflectance wavelengths/parameters have been summarized and are discussed.**

***Key words*—grape berries, *in vivo*, phenolic compounds, reflectance, UV induced fluorescence, viticulture**

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Introduction

Grapevine (*Vitis vinifera* L.) has been produced in Czech Republic (mainly in South Moravia) over a century. In spite of the fact that the beginning of wine growing is associated with Roman colonizers, it is possible to date main expansion of vineyards back to Great Moravian Empire. Nowadays, wine is an integral component of the culture, a form of entertainment and undoubtedly part of the life style thanks to its charm difficult to define (Bisson et al., 2002). In August 2009, reform of the Common Market Organization for wine (CR $N^0$479/2008, 29-04-2008) was fully implemented. This reform aims at reducing 24 million ha/year surplus of basic low quality wine. This in fact endangers all EU winegrowing small and medium-sized enterprises. To survive, winegrowers have to increase crop value, reduce the production of basic wine and convert part of the production into premium quality. Such conversion however, requires implementation of precision agriculture technologies – a new and more effective field control methods.

The premium quality depends mainly on the composition of grapevine metabolites. Grape berry can contain over thousand different chemical constituents making it very complex solution (Ali et al., 2010) however, main diversities are mainly influenced by the presence of phenolic compounds (reviewed by e.g. Teixeira et al., 2013; Kuhn et al., 2014). The overall function of plant phenolics has been greatly reviewed by e.g. Bennet and Wallsgrove (1994) and, even though these components are present in grapevines in relatively small quantities, are presumed to play crucial role in sensory properties (Bakker and Clarke, 2012). Moreover, plant phenolics nowadays achieve attention for their health benefit impact thanks to antioxidant activity (Guilford and Pezzuto, 2011). In fact, antioxidant activity of grapes is positively correlated with concentration and/or composition of phenolic compounds. From the composition perspective, phenolics are so to say anthocyanins, flavonols, flavan-3-ols, hydroxybenzonates etc. (Orak, 2007). With the demand for

phenolic rich high-quality production (Kennedy et al., 2006), precise on site detection of phenolic compounds directly in grapevine berry becomes a main bottleneck facing disadvantages of traditional destructive laboratory methods (Harbertson and Spayed, 2006). These time-consuming and costly analyses are recently replaced by fast and accurate, non invasive optical methods allowing *in vivo* field measurements.

During ripening, grape berry posses dramatic changes in pigment composition, which affects both the absorption and emission of the incident light (reflectance, fluorescence). Near infrared (NIR) and visible spectroscopy is ruled by content, composition and localization of pigments and thus, can be used to predict grape skin and/or inner properties (Cao et al., 2010; Rustioni et al., 2014). The absorption characteristics of pigments show their typical bands (Buschmann et al., 2012), which could serve as a tool for *in vivo* characterization of grapevine berries (Lamb et al., 2004; Hall et al., 2011; Rustioni et al., 2013 a,b). Decrease of chlorophyll (Chl) content during ripening of grape berries has been monitored by recording chlorophyll fluorescence (Chl-F), excited by visible radiation (Kolb et al., 2006; Lenk et al., 2007). Here, authors proved decrease of Chl-F signal in parallel to increasing sugar content, a part of the ripening process in grape berries under natural conditions. Compared to Chl-F excited by visible light, UV excitation can emit blue fluorescence, green fluorescence, red fluorescence, and far-red fluorescence (Buschmann et al., 2000; Meyer et al., 2003). A dual UV-excitation method has been proven as technique for screening phenolic compounds in leaves (Bilger et al., 1997; Ounis et al., 2001; Cerovic et al., 2002) and subsequently, have served as a platform for development of handheld instrument Dualex (Force-A, Orsay, France) (Goulas et al., 2004; Cerovic et al., 2012). This instrument allows assessment of polyphenolic compounds in plant from the measurement of UV absorbance of the leaf epidermis by double excitation of Chl-F. In fact, it takes advantage of a feedback loop that equalizes fluorescence level induced by a reference red light to the UV-light-induced fluorescence level (Goulas et al., 2004).

Applicability of this technique of several excitation wavebands have also been proven to follow fruit phenolic maturation. Here, anthocyanins (Anth) were selected as a main markers of ripening. By using this method, Anth were detected in e.g. olives (Agati et al., 2005) and apples (Hagen et al., 2006). Subsequently, this technique has been successfully extended for Anth detection at either single (Agati et al., 2007) or the whole grape bunches (Cerovic et al., 2008). In these studies authors proved that the higher the Anth concentration in berry skin, the lower the Chl-F signals is. Development and implementation of portable LED-based sensor Multiplex (Force-A, Orsay, France) allowed large `in field` measurements, which proved applicability of this technique for high area and/or number of clusters per plot (Ghozlen et al., 2010; Bramley et al., 2011; Tuccio et al., 2011). In fact, this device uses Chl-F (or re-emitted light) as well as the screening effect of polyphenols on Chl-F.

The role of UV absorbing phenolic compounds in screening of harmful UV light is well known phenomena (e.g. Agati and Tattini, 2010). Apart from the above mentioned excellent methods, there is also possibility for their direct detection. With the regards to the brightness under UV ilumination, imaging technique have been proven to detect some flavonoids and carotenoids in flowers (Fukuta et al., 2005; Nakayama et al., 2006). This approach however, gives only poor information about quantitative characteristics of these compounds. Appart from the imaging method, phenolic compounds absorbing in UV are excellent fluorophores (reviewed by e.g. Garcia-Plazaola et al., 2015; Talamond et al., 2015) and thus, their fluorescence can be directly excited by UV light. Either with or without the impact of environmental stresses of both biotic and abiotic origin, UV-excited phenolics such as e.g. phenolic acids (Hutzler et al., 1998; Lichtenthaler and Schweiger, 1998; Buschmann et al., 2000; Chaerle et al., 2007), flavonoids (Agati et al., 2002; Meyer et al., 2003), or stilbenes (Hillis and Ishikura, 1968; Poutaraud et al., 2007, 2010; Bellow et al., 2012; Šebela et al., 2014) were detected *in vivo* in plant leaves. Although there are structural differences between leaf and fruit tissue, most of the essential optical properties are similar both for leaves and grapes. In fact, the main significant difference here having potential effect on the optical properties of fruit tissue can be intercellular spaces, since their size vary with the ripening in several fruits, e.g. apples (Pieczywek and Zdunek, 2012). However, despite the potential of UV excitation to direct estimation of phenolic compounds concentration, there is a lack of *in vivo* studies available. The UV excitation method has been tested in e.g. strawberries (Yoshioka et al., 2013), where the strong UV-excited fluorescence signals were markedly correlated with the levels of those fluorescence compounds as evaluated by high-performance liquid chromatography (HPLC). To the best of our knowledge however, effectiveness of this technique has not been attempted in direct estimation of particular phenolic compounds concentration in grapevine berries. Thus, the aims of our study were: *(i)* to identify phenolic compounds which are important for grape maturity evaluation and to identify their spectral and fluorescence properties, *(ii)* to systematically monitor optical properties, main grape maturity components and changes of phenolic compounds concentrations throughout the ripening season and *(iii)* to validate the optical properties of particular compounds through the classical chemical analysis.

Materials and methods

Plant material

Three white cultivars [cv. `Pinot Blanc` (PB), cv. `Riesling` (R) and cv. `Chardonnay` (CH)], and four red cultivars [cv. `Cabernet Sauvignon` (CS), cv. `Malverina` (Mal), cv. `Saint Laurent` (SL) and cv. `Pinot Noir` (PN)] were selected and investigated throughout this study. Since the study itself was divided into two parts, specifics of the cultivars used in *(1) Laboratory study* and *(2) Field campaign* are detailed in each sub division bellow. As per standard recommendation, plants received approx. same treatments

during all growing seasons implemented in this study. Briefly, treatment against Oidium *Uncinula necator* (Kumulus WG, Falcon 460EC, Discus) in the early spring was followed by treatment against Peronospora *Plasmopara viticola* (Pergado, Rhidomyl Gold and Folpan 50 WP). During the early ripening stage, the treatment against *Botrytis cinerea* was applied (Mythos 30 SC and Theldor 500 SC). The detailed plant protection treatments are presented in Table 1.

Table 1. Plant protection treatments

Date	Substance	Amount*
05/ (2010, 2011, 2012)	Kumulus WG *(80% sulfur)*	0.30
05/ (2010, 2011, 2012)	Falcon 460 EC *(Tebuconazolu, triamidenol, spiroxamine)*	0.40
	Discus *(kresoxim-methyl)*	0.20
05/ (2010, 2011, 2012)	Pergado *(mandipropamid, folpet)*	1.25
	Ridomyl Gold *(metalaxyl-M, folpet)*	2.00
	Folpan 50 WP *(Folpet)*	2.50
06/ (2010, 2011, 2012)	Mythos 30 SC *(pyrimethanil)*	2.00
	Theldor 500 SC *(fenhexamid)*	0.75

*amount is expressed in kg/ha

Site and soil characteristics

Grape berries were sampled from two nearby located vineyards during this study. Both vineyards have similar characteristics - thanks to soil and climatic conditions being one of the best in Czech Republic. Vineyards are situated in corn production type, subtype barley. The area itself is characterized as warm, dry sub-region, district warm and dry with mild winters. According to agro-climatic conditions, area is warm (only once or two times per 10 year with the temperature below -20ºC; the long-term average temperature is 9ºC; the average annual rainfall is 516.6 mm). Mostly southeast winds (bringing much rain) are streamlined by Pálava hills. The local climate is influenced by Pavlov hills (Pavlovské vrchy), which produces relatively large rain shadow. Rain therefore comes from the northwest, in relatively small quantities. The land of both vineyards is mostly steep, slightly sloped to the southeast. Soil is loam-sandy containing 20-24% clay particles. Soils itself are light and porous. Humus horizont is 0.4-0.6 m deep.

(1) Laboratory study

Laboratory study was carried out during summer 2010. It can be divided into four sub steps, i.e. the aims were *(i)* to identify main phenolic compounds which are important for grapevine maturity, *(ii)* to identify the absorption maxima of these phenolic compounds, and *(iii)* to investigate if these compounds are fluorescing. As the last `summary` step, we tried *(iv)* to identify wavelengths suitable for *in vivo* detection of phenolic compounds.

Plant material

Grape berries cultivated at experimental vineyard (Department of Viticulture and Oenology, Lednice, Czech Republic, 48°47'24.16"N; 16°47'53.61"E) were used. Namely, SL was used to identify the main phenolic compounds of interest. Also, white cultivar (PB) and two red cultivars (Mal and CS) were sampled and analyzed to know their particular phenolic concentrations right before harvest. Sampling procedure (just for one sampling date) for selected cultivars was done the same way as prescribed in *(2)* Field campaign section.

Extraction of polyphenols

The extraction was done according to Kumšta et al., (2012). Briefly, whole grape berries (i. e. including skin, flesh and seeds) from both red and white grapes were smashed in a solution containing 90% methanol (MeOH) and 1mM potassium metabisulfite ($K_2H_2O_5$) in 1:1 ratio (mg berry/ml MeOH+ $K_2H_2O_5$), and left in the dark at room temperature for 2 weeks. Following extraction, homogenate was centrifuged at 15.000 rpm for 10 min. Supernatants were than filtered through 0.45 µm Millipore filters (Merck Millipore, Prague, Czech Republic) and stored at -20°C until the analysis.

HPLC analysis

HPLC analysis was performed by using Digital High-Pressure LC-1A system with SCL-10Avp (Schimadzu, Japan) system controller. Polymer IEX H form 10 mm packing, 250x8mm + 10x8 mm column (Watrex, Czech Republic) was used for the separation. Using column thermostat with manual spraying valve Rheodyne CTO-10Acvp (Shimadzu, Japan), the column was kept in 60ºC during analysis to shorten the analysis time and to facilitate the separation. Isocratic elution was used with 2mM H_2SO_4 composition of mobile phase. The applied flow rate, provided by LC-10 Advp pump, was 0.75 ml/min.; the injected sample volume was 20µl. The different phenolic compounds were identified based on their retention times and/or UV-Vis spectra, and by comparison with the pure standards. The reagents constituting the mobile phase were ordered as follows: methanol from Chromservis (Czech Republic), acetonitrile from Chem-Lab NV (Belgium) and perchloric acid from Acros Organics (Czech Republic). All these reagents were HPLC pure.

Optical measurements

HPLC pure standards purchased from PhytoPlan (Heidelberg, Germany) were used to measure their optical properties (absorbance, fluorescence). Absorption maxima of main selected phenolic compounds were measured by using spectrophotometer SPECORD 210 Plus BU (Analytic Jena AG, Germany). Fluorescence emission spectra of these compounds were measured with Fluormax-P (Jobin Yvon, Horiba Scientific, Japan).

(2) Field campaign

Field campaign was conducted during two seasons (2011 and 2012), at two nearby located sites. During 2011 season in vineyard Winberg Winery Ltd. in Mikulov, Czech Republic (N 48°47'50.67" E 16°40'22.97"), during 2012 season in experimental vineyard (Department of Viticulture

and Oenology, Lednice, Czech Republic, 48°47'24.16"N; 16°47'53.61"E).

Sampling procedure

Monitoring of the gape berry ripening at several timings prior to harvest is a current practice in wine industry worldwide. Thus, berry samples were taken and monitored at several time points, similarly during both seasons. Briefly, the time course of the berry ripening has been selected according to Coombe (1992), i.e. from `verasion` (the term corresponding to early ripening season) to `harvest` (the term corresponding to maturity of grapes). The samples itself were taken at pre-defined time intervals, alternatively each second and fifth day, in the period between August and October (2011 and 2012), respectively. In each date, samples were collected in the vineyard at approx. same time (08:00 AM). Immediately after collection, samples were transported to the laboratory and undergone *[(a) Indoor measurements]*. To prevent drying of the samples, each cluster was placed into a plastic bag and transported at controlled temperature of 15°C. To compare the differences, the same set of optical measurements were done *[(b) On site measurements]*, too.

Plant material

Namely, two white (Chardonnay, Riesling) and two red cultivars (Saint Laurent, Pinot Noir) were used in this field campaign.

Extraction of polyphenols

Two independent extraction methods were selected for 2011 and 2012 seasons, respectively. The extraction protocol for *2011 season* was carried out according to Jensen et al., (2007), only with small modifications. Briefly, whole berries (i.e. including skin, flash and seeds) both for red and white grapes were powdered in mortar and then extracted with an aqueous solution of methanol (50 % methanol) + 0.1 mM HCl at a 1:1 w/v (grape fresh weight/ volume of aqueous methanol HCl) ratio. After homogenization, the extract was kept for 10 minutes in 40 °C, and then centrifuged for 20 minutes at 15.000 rpm. The extraction protocol for *2012 season* was carried out according to Kumšta et al., (2012), the same extraction method prescribed in *(1)* Laboratory study. In both cases (2011 and 2012 seasons), supernatants were also filtered through 0.45 µm Millipore filters (Merck Millipore, Prague, Czech Republic) and stored at -20°C until the analysis.

HPLC analysis

Plant phenolics in both seasons were analyzed using two different HPLC systems. In *2011 season*, the analysis of extracts was performed by Smartline HPLC system (Knauer Instruments, Berlin, Germany). In *2012 season*, the same approach was performed by Agilent 1200 Infinity Series Instrument (Agilent Technologies, California, USA). In both cases the same column [Alltech Alltima C18 3µm particle sized reversed phase column (150 x 3 mm)] was used for the separation and identification of main phenolic compounds. The mobile phase employed was as follows: 50 % acetonitrile, 15 mM $HClO_4$, 10 % methanol as solvent `A` and 15 mM $HClO_4$ as solvent `B`, with a flow rate of 0.5 ml min^{-1}; while the gradient applied was as follows: 0min, 2 % `A`, 20min, 26 % `A`, 30min, 45% `A`, 35min, 70% `A`, 37min, 100% `A`, 39 min 0% `A`, 50 min, 2% `A`. The different phenolic compounds were also identified based on their retention times and/or UV-Vis spectra, and by comparison with the pure standards as in 2010.

Absorption maxima

Concentration of total phenolics was obtained by recording absorbance at 280nm (OD_{280}; Pirie and Mullins, 1976; Somers and Evans, 1977), by using spectrophotometer SPECORD 210 Plus BU (Analytic Jena AG, Germany).

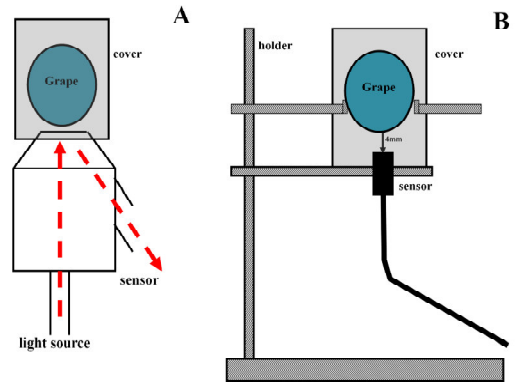
Optical measurements

Since the changes of phenolic composition during ripening can affect both the absorption and emission of the incident light, reflectance and fluorescence emission spectra were measured throughout the whole experiment. Also, to prove applicability of the presented technique for assessment phenolic compounds *in vivo* in field conditions, both *(a)* `Indoor` and *(b)* `On site` measurements were conducted.

(a) Indoor measurements

Reflectance measurements in laboratory conditions were done by using optical spectrometer (Optical Spectrometer SM 9000, PSI, Czech Republic). Reflectance spectra were measured in the range from UV/VIS to NIR (400-800 nm, with the integration time 300 ms), using deutherium and halogen lamp; reflectance itself was measured under angle 45º (Fig. 1A). To prevent inaccuracy caused by influence of incident light, samples were covered during measurements. Reflectivity of each single berry was measured 20 times in the different position. Subsequently, selected vegetation index (Vis) was calculated from raw reflectance spectra: ` anthocyanin reflectance index` (ARI; Gitelson et al., 2001). Briefly, [ARI = $(\rho^{-1}_{green}-\rho^{-1}_{red\ edge})$], where: `$\rho^{-1}_{green}$` is average reflectance in λ_{green} band (540-560 nm) and `$\rho^{-1}_{red\ edge}$` is average reflectance in $\lambda_{red\ edge}$ band (690-710 nm).

Figure 1. Scheme of reflectance and fluorescence measurements



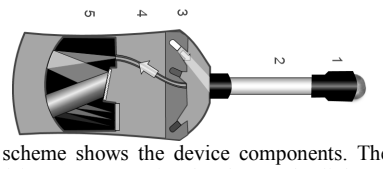
Schematic diagram for reflectance (panel A) and fluorescence (panel B) measurements. Both instruments capable to measure leaf reflectance and/or fluorescence were slightly modified to allow grape berry measurements. Red arrows indicate the direction of light incident to berry sample and/or reflected light (panel A). Black lining (panel B) shows the holder system. Here, grape berry is fixed at the distance 4 mm from the sensor. Movable holder can be set to the diameter of the berry sample. In both cases, samples and sensors were covered during measurements to avoid incoming light during measurements.

Fluorescence measurements Fluorescence emission spectra in laboratory conditions were measured with Fluormax-P (Jobin Yvon, Horiba Scientific, Japan), by using external adaptor connected with the instrument by fiber optic bundle. Briefly, instrument contained xenon lamp supplying a wild range of excitation light. This excitation light was focused to the entrance slit of the excitation monochromator by an elliptical mirror. On the excitation side, the slits were adjusted to 1nm to control the band pass (range) of light incident to the sample. The slits of emission monochromator were also adjusted to 1nm, to determine the intensity of the emitted fluorescence recorded by the detector. Increment of the emission spectra was adjusted to 2nm. Based on the results from independent experiment (signal dependence upon the distance from the sensor, data not shown), sample was fixed by the holder at approx. 4 mm distance from the sensor (Fig. 1B). To prevent inaccuracy caused by incident light, both sample and sensor were also covered during all measurement.

(b) **On-site measurements**

All field spectral measurements were performed using prototype of handheld instrument `WinePen` (PSI, Brno, Czech Republic). `WinePen` (Fig. 2) is portable spectrophotometer equipped with internal light sources capable of measuring both reflectance and fluorescence in the spectral range 325 – 780 nm. Measuring head can hold berry sample up to 20 mm in the diameter. The following light sources are used: incandescent xenon lamp (emission from 380 to 780 nm) for reflectance measurements, blue LED maximum at about 435 nm for chlorophyll fluorescence, and 2 LEDs with the maxima at 280 nm and 320 nm provide light excitation. Signal detection is achieved by compact polychromator integrated with a reflection grating and CMOS linear image sensor (Hamamatsu, Japan) with the spectral response half width of 9 nm and wavelength reproducibility +/- 0.5 nm. For calibration of reflectance measurements two Zenith standards (SphereOptics, Uhldingen, Germany) with 5 and 20% reflectance were used.

Figure 2. Scheme of the prototype of hanheld instrument (PSI, Czech Republic)



The principle scheme shows the device components. The grape berry sample is fixed in open measuring head (1). The light sources (3) are illuminating the sample (1) through the optical glass rod (2). The radiometer (5) is than detecting the radiation (reflectance or fluorescence) coming from the sample through a light fiber (4).

Data analysis

ChromGate V3.1.6. software (Knauer Instruments, Berlin, Germany) and Open Lab CDS ChemStation software (Agilent Technologies, California, USA) were used to integrate chromatographic peaks, to create standard calibration curves and to calculate real concentrations of selected phenolic compounds. Regression analysis, significance of the correlation coefficients was tested by regression analysis, ANOVA; standard errors were calculated by MS Excel.

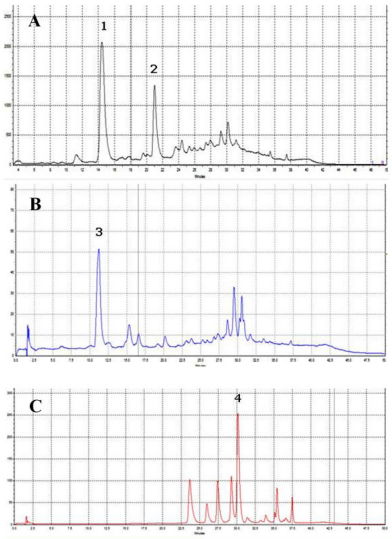
Results and Discussion

As prescribed above, study itself comprises two independent parts. At first part, we tried to identify the main phenolic compounds of interest and their optical properties during (1) Laboratory study. Subsequently, all the optical parameters identified during this laboratory study were tested and verified during (2) Field campaign.

1. Laboratory study

(i) to identify main phenolic compounds Main phenolic compounds important for maturity evaluation of grapevine berries were identified. As shown in Fig. 1, typical HPLC chromatograms of analyzed grape extract (SL) exhibit several peaks at different retention times and/or wavelengths. While detected at 280 nm, two main compounds from the group of flavan-3-ols (catechin, epicatechin, Fig. 1A) were identified. When detected at channel 315 nm, main representative of hydroxycinnamic acids + stilbenes (caftaric acid, Fig. 1B; + resveratrol) and/or flavonols (quercetin); and at channel 520 nm main representative of Anth (Malvidin-3glucoside, Fig. 1C) was identified, too. The main identified phenolic compounds in our study were similar to those mentioned by Teixeira et al. (2013). Since from the literature there are similarities for both red and white grapevine cultivars (Teixeira et al., 2013), the typical chromatograms and compounds identified are presented just for red cultivar (SL, Fig. 3). While comparing red and white cultivars, the only exception appears in the case of Anth (Fig. 3C), which differs red cultivars from white ones.

Figure 3. HPLC chromatogram of analyzed grape berry extract

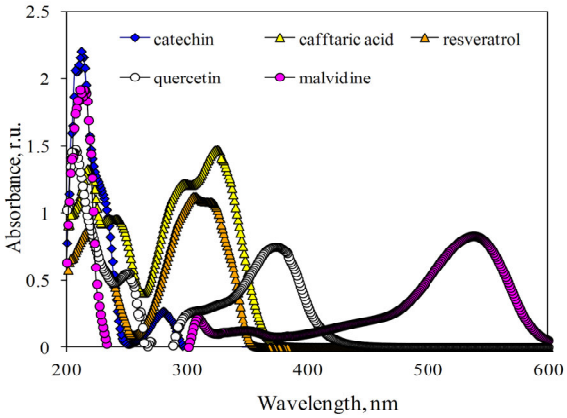


The typical chromatograms of analyzed extract (Saint Laurent) depicting selected phenolic compounds with their retention times, recorded at wavelengths (280 nm panel A, 315 nm panel B and 520 nm panel C). Numbers indicate detected compounds: 1-catechin, 2-epicatechin, 3-caftaric acid, and 4-mlavidin-3-glucoside. Also, if recorded at wavelengths 315 nm, representatives of stilbenes (resveratrol and quercetin, panel B) can be detected.

While comparing our study with the literature, also the effect of extraction procedure can largely influence the final determination of phenolic compounds in grapevine berries (Gambuti et al., 2009). Thus, two verified methods were used (Nicoletti et al., 2008; Kumšta et al., 2012; see materials and methods), respectively. Such selection of two independent extraction methods could overcome possible negative effect of (e.g. solvent concentration, contact time etc.) on the resulting concentrations of phenolic compounds in extract. Knowing the concentrations of pure standards analyzed by HPLC (catechin, epicatechin, caftaric acid, resveratrol, quercetin and malvidin-3-glucoside), real concentrations of these compounds in grape berries were calculated. Several studies have shown that real concentrations of these compounds can fluctuate throughout the ripening season, depending mainly on the environmental stimuli (e.g. Teixeira et al., 2013). To quote some example, Šebela et al., (section *3.2.1.*) have shown dependence of ambient sunlight intensity on the phenolic profiles of cv. `Chardonnay`. Also, Addams (2006) reported changes in concentrations of these phenolic compounds during ripening, which is in accordance to our study.

(ii) to identify the absorption maxima of these phenolic compounds As the second step, optical properties of main groups of phenolic compounds were investigated. The typical absorption spectra of selected compounds are shown in Fig. 4.

Figure 4. Absorption spectra of selected phenolic compounds

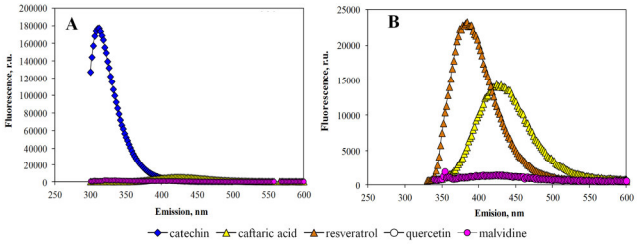


Absorption spectra of selected phenolic compounds (catechin, cafftaric acid, resveratrol, quercetin and Malvidin-3-glucoside) measured as a methanolic solution.

While measuring absorbance of pure chemical standards, typical spectra can be seen for each main representative. In the case of all phenolic compounds, absorption maxima are visible in the UV range (200-220 nm). As the main representative of flavan-3-ols: catechin (Fig. 4, blue diamonds) showed absorption spectra with two main bands (at ca. 210 and 280 nm). Caftaric acid (Fig. 4, yellow triangles), the main compound of hydroxycinnamic acids, expressed several absorption maxima bands, while the most important were identified as either 220 nm or the wavelengths in interval 300-320 nm. Resveratrol, the main compound of stilbenes, showed similar absorption spectra to those of caftaric acid (Fig. 4, orange triangles). Here, the main bands were also identified as belonging to the interval 300-320 nm. Absorption spectrum of quercetin, the main compound of flavonols, exhibit several main absorption bands, while the most important were identified as belonging to the interval 360-370 nm (Fig.4, white circles). The compound which is present only in red grapevine cultivars (Malvidin-3-glucoside, the main representative of Anth group) showed main absorption peak at ca 530 nm (Fig. 4, violet circles). Although phenolic compounds have usually more than one absorption peak, findings similar to our study are also presented by e.g. He et al. (2010) and Zhang et al. (2013), respectively.

(iii) to investigate if these compounds are fluorescing As a subsequent step, fluorescence emission spectra were recorded. With regards to the absorption maxima of each selected phenolic compound provided above, such emission spectra were recorded by using specific excitation wavelengths. Simplified, each standard (diluted in methanol) was excited by the wavelengths of its absorption maxima and resulting emission spectra were detected.

Figure 5. Fluorescence emission spectra of selected phenolic compounds



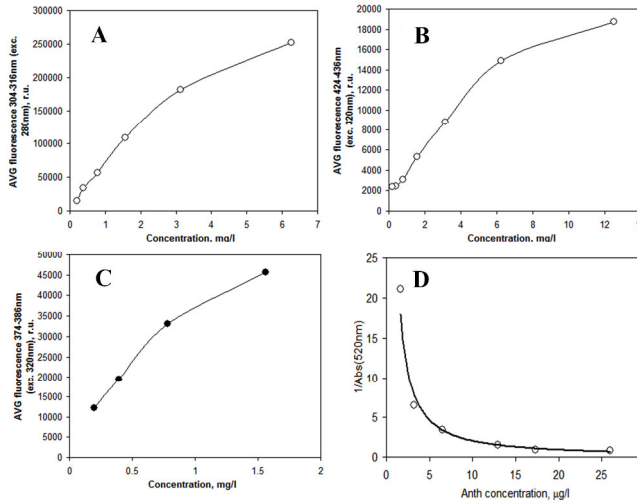
Fluorescence emission spectra of selected phenolic compounds (catechin, caftaric acid, resveratrol, quercetin and malvidin) under excitation 280 nm (Panel A) and 320 nm (Panel B), respectively.

When excited by wavelengths in the range 205-220 nm (maximum absorption of all presented phenolic compounds), no emission was detected, possibly due to re-absorption of emitted light in a longer wavelengths. With the longer wavelengths (230-260 nm), emission spectra were not detected for all phenolic compounds of interest, except catechin. Increase in intensity of this emission for catechin was visible throughout the increasing wavelengths up to 280nm, when it reached its maximum (Fig. 5A). This emission was characterized by peak centered at 310 nm. While exciting in longer wavelengths (280-300 nm), decrease in intensity of this catechin peak was visible however, two compounds (caftaric acid and resveratrol) were characterized by slow increase in intensity in their emission (300-310 nm). The maximum intensity of these peaks was reached while excited by 320 nm. Under this excitation, emission peaks centered at 420 and 380 nm were found, respectively. When excited in longer wavelengths (420-530 nm), visible decrease in the intensity of these peaks appeared in both cases. Other compounds (quercetin and malvidin-3-glucoside) did possess little or no fluorescence under these excitation wavelengths presented above. Thus, the main phenolic compounds which can be directly excited by UV light and detected at specific wavelengths were identified as follows: *(a)* catechin [exc. 280

nm/em. 310 nm], *(b)* caftaric acid [exc. 320 nm/em. 420 nm] and *(c)* resveratrol [exc. 320 nm/em. 380 nm], respectively.

With regards to the selected excitation and/or emission maxima provided above, one can note that there should be evidence for the dependence of signal intensity upon the real concentration of these selected phenolic compounds. Thus, the influence of the real concentration of particular compound upon the resulting fluorescence signal was investigated, too. By exciting the methanolic solution at certain concentrations, emission signal gradient can be seen in Fig. 6A, B, C.

Figure 6. Signal emitted by UV induced fluorescence and its dependence upon real concentration.



Fluorescence signal dependence upon concetration (mg/l) of selected phenolic compounds: catechin (panel A), caftaric acid (panel B), and resveratrol (panel C); and dependence of relative absorbance [1/Abs (520 nm)] upon the concentration (µg/l) of Anthocyanins (Malvidin-3-glucoside, panel C).

In all inspected compounds (catechin, caftaric acid and resveratrol), fluorescence signal increased with the increase of concentration. The highest signal (in relative units of emitted fluorescence) was observed in the case of catechin (exc. 280 nm/ AVG fluorescence em. 304-316; Fig. 5A). Here, the signal at concentration ca 2mg/l was higher than signal resulting while excited by 320nm in the case of: caftaric acid (ca 25 times, Fig. 5B) and resveratrol (ca 3 times, Fig. 5C).

Since there is little or no fluorescence in the case of qurcetin and/or Malvidin-3-glucoside, the concentration gradient and its dependence upon the absorption at main absorption maxima of these particular compounds were inspected, too. The absorbance of Malvidin-3-glucoside was found to be proportional to its concentration. With its increasing there is increasing absorbance as well and thus, relative absorbance (1/Abs 520 nm) decreases (Fig. 5D). The same phenomena was however not observed in the case of quercetin, where the absorbance around 360-370 nm was not proportional to its concentration (data not shown).

(iv) to identify wavelengths suitable for in vivo detection of phenolic compounds The wavelength suitable for detection particular compounds of interest were identified however, each grapevine berry contains complex mixture of these compounds. This in fact can influence resulting optical signal in the way of re-absorption of the emitted light from one fluorophore molecule by another one. As shown in table 2, concentrations of particular phenolic compounds varied across all, one white (PB) and two red (Mal, CS) cultivars.

Table 2. Real concentrations of particular phenolic compounds

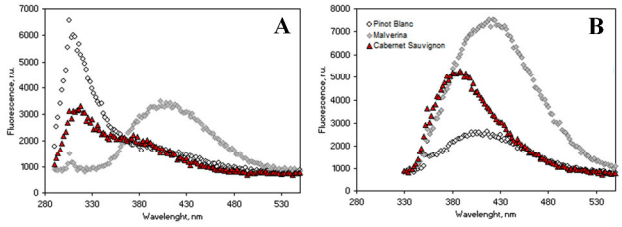
	Pinot Blanc	Malverina	Cabernet Sauvignon
catechin*	3.43	1.58	3.28
caftaric acid*	2.76	14.86	1.125
resveratrol*	0.005	0.08	0.365
quercetin*	0.03	0.035	0.06
Malvidin-3 -Glucoside*	0	1.85	234.1

* Concentration is expressed in mg/l

The highest concentration of catechin was detected in white cultivar PB, while other two red cultivars expressed visibly higher amount of caftaric acid in case of Mal and/or malvidin in case of CS. Compared to CS, only trace amount of resveratrol and double the amount of quercetin was found in the case of PB and Mal, respectively. As presented in the literature, concentrations of various phenolic compounds can vary either upon the stage of development, environmental and viticultural factors (e.g. Haselgrove et al., 2000), or can also differ with cultivars. Differences in phenolic (Ky et al., 2014) as well as Anth profiles (Pomar et al., 2005) across several cultivars supports our observations in this study.

Knowing the real concentrations, mixtures representing three selected cultivars (PB, Mal and CS) were prepared and their optical properties were investigated, too. Since the main excitation wavelengths were already identified, the mixtures were excited by 280nm (Fig. 7A) and 320 nm (Fig. 7B), respectively.

Figure 7. Fluorescence emission spectra of representative mixtures



Fluorescence emission spectra of mixtures prepared from standards of representative phenolic compounds and reflecting real concentrations of white cultivar (Pinot Blanc) and two red cultivars (Malverina and Cabernet Sauvignon). Panel A represents emission spectra under excitation 280 nm, while Panel B represents emission spectra under excitation 320 nm, respectively.

Under the excitation of 280nm (Fig. 6A), the highest peak centered at 310 nm was observed in PB. As identified above, this emission peak is originating from catechin and thus, reflecting its concentration (Table 2). CS is also showing

this peak originated from catechin, which can be also supported by its concentration in the mixture (Table 2). As seen in this table, caftaric acid is dominating phenolic compound in the case of Mal. This in fact is reflected in both emission spectra (Fig. 6A, B). The lower concentration of caftaric acid in the case of PB however, is also causing emission peak centered at around 420-430 nm, while in the case of CS with the lowest caftaric acid concentration this peak is shifted towards 390 nm, possibly due to the influence of catechin. Nonetheless, for both standards measured separately and the mixtures of standards, typical excitation and/or emission spectra were identified and verified. Similar observation was found in the case of the grape extracts (data not shown).

2. Field campaign

The subsequent field campaign was designed to evaluate whether the findings observed during laboratory studies can be implemented and further expanded to direct measurements of the phenolic compounds concentrations *in vivo*, on the whole grape berry level.

(i) HPLC analysis of grape phenolics and evidence for correlation with the optical parameters

Despite other changes, grapevine ripening is characterized by softening and coloring of the berry. Overall, one of the main visible change between `verasion` and harvest is increase in berry size. Most of the solutes accumulated in the grape berry before `verasion` (Kennedy et al., 2000, 2001) remain also at harvest however, their concentrations are significantly reduced due to this increase in berry volume. The same phenomena can be supported by our experimental evidence, while the concentrations of phenolic compounds expressed in mg/kg were changed according to the literature (e.g. Pirrie and Mullins, 1977; Kennedy et al., 2000, 2002; Adams, 2006). In fact, overall trend was characterized as a decrease of total phenolics in white and slight increase of total phenolics in red cultivars. Such differences can be prescribed by anthocyanin production, being the most obvious compounds of importance in red cultivars (reviewed by Flamini et al., 2013). The changes of particular phenolics throughout the ripening season are however, above the scope of this study and thus were used just for correlation libraries presented in *(iv) Correlation library for chemical and optical measurements.*

(ii) In vivo fluorescence of grape berry

The optical properties of main compounds obtained during *1. Laboratory studies* were used to investigate applicability of this technique for *in vivo* detection of phenolic compounds directly in the grapevine berry. The potential problems here, compared to the extract/pure standard, can be caused by penetration of fluorescence signal. One of the main factors which can influence this signal penetration is undoubtedly morphology of grapes. It has been reported by several authors that there is a structural distribution of these compounds inside the grape berry (reviewed by Teixeira et al., 2013), which can vary throughout ripening. The most important stage in this study is technological ripeness and thus, all statements refer to this stage. Catechin is very abundant compound presented mainly in grape seeds however, in lower concentrations can be found both in flesh and skin. Contrary, flesh of the grape berry is mostly occupied by caftaric acid which is however, presented in the skin and seeds. Majority of resveratrol, quercetin and Malvidin-3-glucoside is presented in the skin of the berry. Whereas resveratrol and quercetin can be found in flesh and seeds, the presence of malvidin in flesh occurs only in teinturier cultivars (Adams, 2006; Conde et al., 2007). These cultivars whose flesh and juice is red in color were however not inspected in our study.

For all cultivars inspected in this field campaign, emission spectrum was not influenced by detecting emission spectra recorded *in-vivo* (data not shown). For both red and white cultivars, two main emission peaks were identified under excitation of 280 nm; one centered at around 310 nm and the other one located at around 380 nm. While excited by 320 nm, the same peak at longer wavelengths (380 nm) was observed in all cultivars, too. In fact, the excitation energy as well as fluorescence emission can undergo multiple scattering and re-absorption processes before it is sensed by the detector. To overcome these negative effects, several ways how to identify exact localization of particular compounds are prescribed in literature. It can be done by means of i.e. measuring absorption spectra, or transmittance and epifluorescence microscopy of the tissue of interest (Agati et al., 2005, 2007). As discussed by authors, partial overlapping can appear in the case of Chl fluorescence which is however, not our case. Here we are presenting method based on two excitation (280 and 320 nm) wavelengths, which both are out of the absorption maxima of chlorophyll a as well as chlorophyll b, respectively. The high energy UV light which is used to excite particular phenolic compounds here is thus using effectively for direct excitation in case of compounds of interest located throughout grape berry, mostly in the skin (Teixeira et al., 2013). The emission spectrum measured *in vivo* is not affected compared to the measurements done on extract/solutions of standards (Fig. 6.) and thus, these wavelengths were selected and further investigated.

(iii) Comparison of in vivo fluorescence signal from field and indoor measurements

Since the method which we are trying to develop would aim to serve as a platform for non invasive high-throughput screening based tool for phenolic compounds detection directly in the vineyard, both on site and indoor experiments were conducted. Also, since on site measurements would require fast and accurate repeatable readings, portable fluorometer `WinePen` (see material and methods) was developed. The presented approach however, has to be verified and newly developed instrument has to be tested throughout both fluorescence and reflectance measurements. As shown in table 3, both laboratory and the field readings were showing significant relationship between both high-tech instrument and portable fluorometer.

Table 3. Regression coefficient (R^2) of laboratory and on site measurements

Fluorescence[1]	**^{UV}280/400**	**^{UV}320/400**	**^{B}435/683**	**^{B}435/735**
White cultivars	0.34 ***	0.39 ***	0.86 ***	0.82 ***
Red cultivars	0.40 ***	0.70 ***	0.64 ***	0.47 ***
Both red and white	0.42 ***	0.69 ***	0.93 ***	0.75 ***

Reflectance[2]	**R_{550}**	**R_{700}**
White cultivars	0.89 ***	0.86 ***
Red cultivars	0.93 ***	0.91 ***
Both red and white	0.93 ***	0.89 ***

Comparison of *in vivo* measurements from laboratory (FluorMax) and field (prototype WinePen) experiment, both for fluorescence and reflectance readings. Regression coefficients (R^2) are presented for *(a)* UV induced fluorescence (excitation 280 and 320 nm) detected at 400 nm (^{UV}280/400; ^{UV}320/400), *(b)* for Chl-F excited by blue light (435 nm), and detected at either 683 or 735 nm (^{B}435/683; ^{B}435/735), and (c) the values in selected wavelengths of reflectance at 550 and 700 nm (R_{550} and R_{700}). [ns, *, **, ***]- non-significant or significant at probability level 5%, 1% or 0.1%, respectively. Regression coefficients were calculated separately for *(i)* `white cultivars` (chardonnay and riesling), *(ii)* `red cultivars` (pinot noir and saint laurent), and (iii) `both red and white cultivars` (with no segregation of red and white cultivars). [(1)] n=1056; [(2)] n= 720 berries.

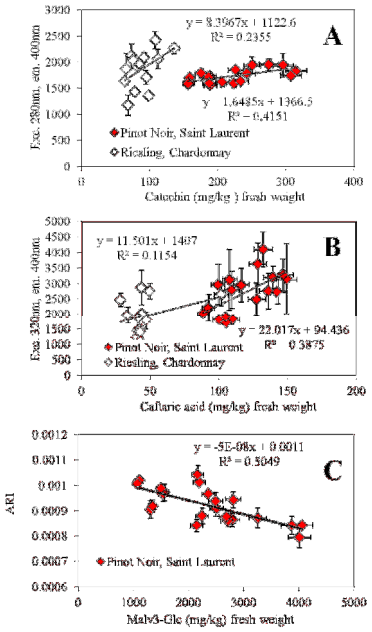
The highest values of R^2 were found in case of reflectance measurements, while there were just a slight differences when comparing red and white cultivars. Since the reflected light from either leaf or fruit surface have been shown to be similar (Buschmann, 2007), our selected wavelengths are referenced to the main wavelengths for anthocyanin reflectance index (ARI; Gitelson et al., 2001) calculation. In case of white cultivars, UV induced fluorescence [(^{UV}280/400) and (^{UV}320/400)] detected at 400 nm was showing lower R^2 (lower accuracy in comparison of both instruments), compared to those of Chl-F readings [(^{B}435/683); (^{B}435/735)]. Nonetheless, Chl-F expressed higher accuracy compared to red cultivars. It can be prescribed by the fact, that the excitation light in white cultivars is not penetrated (Buschmann, 2007) compared to red cultivars, where it is hindered by Anth (Agati et al., 2005). Also, the higher accuracy of UV induced fluorescence can be influenced either by the presence of Anth, or by particular concentrations of phenolic compounds differing throughout cultivars (Teixeira et al., 2013). However, while measuring both red and white cultivars, either UV induced or Chl-F appeared to be effective in both cases. For both white and red cultivars thus, high significant relationship between hi-tech laboratory measurements and those obtained on-site by prototype measurements demonstrate usability and applicability of our approach.

(iv) Correlation library for chemical and optical measurements

Since both fluorescence and reflectance spectra were measured *in vivo* from berry surface, spectral properties were mostly determined by berry skin. Although there are some structural differences between leaf and berry tissue (Jackson 2008), both techniques are commonly used for leaves as well as fruits.

Fluorescence Regression coefficients for fluorescence readings and chemical analysis are presented *(i)* right before harvest in technological ripeness of berries (Fig. 8A, B), and *(ii)* for the whole time duration from `verasion` till harvest (table 4). As shown in Fig. 8, higher R^2 values were obtained in the case of red cultivars (Pinot Noir, Saint Laurent) compared to white (Riesling, Chardonnay). Also, more accurate results (R^2=0.23 for white and R^2=0.41 for red cultivars) were obtained in case of catechin, compared to the caftaric acid estimation (0.11 and 0.38, respectively).

Figure 8. Correlation between optical parameters and HPLC analysis, harvest stage



Correlation of *in vivo* measurements and chemical analysis is presented for ripened grapes. Y axis represents the specific excitation/emission selected for catechin (A), caftaric acid (B) and anthocyanin reflectance index [ARI = $(R550)^{-1}$-$(R700)^{-1}$] for Malvidin-3-glucoside (Malv-3-Glc; C). X axis depicts the amount of individual compounds determined through HPLC (mg/kg fresh weight). Coefficients of determination R^2 together with the equation of the regression line are presented for *(i)* white (Riesling and Chardonnay) and *(ii)* red cultivars (Pinot Noir and Saint Laurent), respectively. Each data point represents average of 6 berries ± SEM.

Compared to the harvest stage (Fig. 8), several changes were visible both for white and red cultivars, while the data from whole ripening stage were analyzed together. Being probably the most contrasting one, caftaric acid expressed non-signifficant correlations between optical measurements and chemical analysis. Although caftaric acid can be found in grapevine skin, it is abundantly present in the flesh and seeds as well (Teixeira et al., 2013). In the harvest stage, the morphological changes in the skin allows light to penetrate deeper inside the grape, which can explain higher R^2 in this stage compared to the lower value in the case of whole season (Fig. 8). With the period between `verasion` and harvest, catechin concentration (dominantly presented in skin and seed) has decreasing trend. In white cultivars, such decrease can explain higher R^2 across the season (Table 4),

compared to observation right before harvest (Fig. 8), where the highest concentration is not reached. For red cultivars however, more complex changes appear during ripening. One of the most crucial, anthocyanin accumulations in the skin is affecting optical properties the most. While there are different levels of anthocyanin synthesis throughout the ripening (Ryan and Revilla, 2003), the signal posses significant changes, which can explain lower R^2 compared to the phase right before harvest (Fig.).

Table 4. Regression coefficients for optical measurements and chemical analysis, whole ripening season

		OD 280nm	Catechin [mg/kg]	Total polyphenols [mg/kg]	Caftaric acid [mg/kg]	°RS
White	$^{UV}280/400$	0.27 **	0.38 **	0.38 **	0.02 ^{ns}	0.06 ^{ns}
	$^{UV}320/400$	0.11 ^{ns}	0.23 **	0.23 **	0.02 ^{ns}	0.02 ^{ns}
	$^{UV}280/400^{norm}$	0.47 **	0.69 **	0.69 **	0.03 ^{ns}	0.42 **
	$^{UV}320/400^{norm}$	0.33 **	0.59 **	0.59 **	0.03 ^{ns}	0.45 **
Red	$^{UV}280/400$	0.01 ^{ns}	0.17 *	0.17 *	0.10 ^{ns}	0.04 ^{ns}
	$^{UV}320/400$	0.00 ^{ns}	0.09 ^{ns}	0.07 ^{ns}	0.00 ^{ns}	0.11 ^{ns}
	$^{UV}280/400^{norm}$	0.15 *	0.21 *	0.21 *	0.08 ^{ns}	0.18 **
	$^{UV}320/400^{norm}$	0.03 ^{ns}	0.21 *	0.18 *	0.05 ^{ns}	0.05 ^{ns}

Regression coefficients (R^2) of *in vivo* fluorescence measurements and chemical analysis are presented for white (Riesling and Chardonnay) and red (Pinot Noir and Saint Laurent) cultivars, respectively. The real concentrations obtained from HPLC analysis (catechin, caftaric acid and total polyphenols) are expressed in mg/kg fresh weight, while optical density at 280 nm (OD 280 nm) and sugar content (°RS) in respective units. UV induced fluorescence excited by 280 and 320 nm ($^{UV}280$ and $^{UV}320$) was detected at 400 nm ($^{UV}280/400$ and $^{UV}320/400$), respectively. For both white and red cultivars, UV excited fluorescence was also normalized by chlorophyll fluorescence (^{norm}): ratio of fluorescence excited by blue light at 435 nm ($^{B}435$), detected at 683 and 735 nm, respectively [($^{B}435/683$)/($^{B}435/735$)]. R^2 represents regression coefficient of optical measurements and chemical analysis obtained during ripening season of both 2011 and 2012 seasons. [ns, *, **, ***]- non-significant or significant at probability level 5%, 1% or 0.1%, respectively.

Not only phenolic compounds synthesis, but also changes in other pigments are being crucial for the light penetration into/outside the grapevine berry. During ripening, decrease of chlorophyll concentration is well known phenomena. Such chlorophyll effect was maximally attenuated by normalizing of UV induced fluorescence by Chl-F. As shown in table 4, R^2 significantly improved both for white and red cultivars. In the case of red cultivars however, the light penetrating into the berry is affected also by anthocyanin accumulation in the berry skin (Buschmann, 2007).

Although similar approaches have been already attempted, methodological differences along with the overall uniqueness of our technique bring up advantage of our study compared to the literature. Majority of studies reported applicability of Chl-F to direct estimation of Anth concentration. Moreover, the Anth concentrations in these studies were mostly determined spectroscopically from fruit skin discs (Agati et al., 2005, 2007; Hagen et al., 2006; Cerovic et al., 2008), with just two reports related to the whole grape berry extracts (Ghozlen et al., 2010; Bramley et al., 2011). Such approach brings advantage in case of compounds dominantly presented in the skin (Anth) however, could potential fail in application to the real practice. Evidence of Chl-F to follow Anth content obtained through HPLC analysis from grape skin extracts (Tuccio et al., 2011; Agati et al., 2013) is the most similar approach to our study however, do not deal with the direct detection of other compounds than Anth. In the case of phenolic compounds presented also in the flesh and seeds (Teixeira et al., 2013), the extracts done on the whole grape basis can give more accurate information about overall concentrations and thus, present more realistic insight. As shown in Fig. 7 and table 4, the regression coefficients R^2 are influenced by this methodological approach however, still offers a unique way how to detect phenolic compounds in more realistic way *in vivo*, with high accuracy.

Reflectance techniques have been broadly applied and effectively used in remote sensing as well as in contact measurements of anthocyanin content in leaves, including grapevine (Gitelson et al., 2001, 2009; Steele et al., 2009). The raw spectra measured in our study (325-790 nm) are mostly affected by the absorption of photosynthetic pigments (Knipling 1970), affecting reflected light from berry surface. Despite photosynthetic pigments, also other factors can play crucial role in resulting reflectance from berry surface. The wax layer, size of the cells, as well as the intercellular space has significant effect in scattering of NIR leaf reflectance (Buschmann et al., 2012). However, the main attribute connected with ripening of red cultivars in our study was accumulation of anthocyanins. As shown in Fig. 7C, ARI (Gitelson et al., 2001) was potent for direct estimation of anthocyanin content of grape berries right before harvest. For calculation of this Vis, the main spectral feature of anthocyanin absorption in vivo as a peak around 550 nm was selected. With berry ripening, the strong decrease in reflectance in this green region is not caused by the chl decrease, but due to the anthocyanin accumulation observed also in the surface of e.g. olives (Agati et al., 2005) or grapes (Rustioni et al., 2014). Several studies have proven reflectance at 700 nm and its close relationship to 550 nm being effective for chlorophyll estimation in anthocyanin-free (e.g. Gitelson and Merzlyak, 1994) or anth-containing leaves (e.g. Gitelson et al., 2001). Also in the case of fruits, the close correlation of these two wavelengths was found in the case of anthocyanin-free fruits (Merzlyak et al., 2003). Navrátil and Buschmann (2016) have investigated changes of reflectance spectra of grapevine berries affected by the content of pigments (chlorophylls and anthocyanins). Contrary to our study, authors also ascertain applicability of chlorophyll related indices to estimate compounds of interest extracted from berry skin, which in fact gives disadvantage compared to real practice results presented in our study.

Conclusions

According to our results, UV induced fluorescence and reflectance have been found to be potent in direct estimation of phenolic compounds in grape berries. This work has also demonstrated the potential of the prototype of handheld

instrument `WinePen` for rapid and accurate on-site measurements. Such optical instrument along with the novelty of our presented method appear to be well suited as tool for integration in precise grapevine management, since it can solve the problem of carefully defining phenolic maturation, an important advance in superior quality grapevine production.

Acknowledgements

This work was supported by project GA 522/09/1565 and FP7-SME-2010-1-262011-PREMIVM and forms a part of research supported by the Research Infrastructure project EPPN (FP7; Grant Agreement No. 284443). Participation of the first and third author was supported by the EfCOP-IPo project ENVIMET (CZ.1.07/2.3.00/20.0246) and is also an output of the grant number L01415 (Ministry of Education, Youth and Sports, Czech Republic).

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3.2.3. Temporal chlorophyll fluorescence signals to track changes in optical properties of maturing rice panicles exposed to high night temperature

Field Crops Research, 2015

David Šebela, Cherryl Quinones, Julie Olejníčková, Krishna SV Jagadish

Abstract

High night temperature (HNT) significantly influences rice grain filling dynamics. A novel phenotyping approach using chlorophyll fluorescence was employed to track changes in the optical properties of maturing rice panicles exposed to control and HNT. Two contrasting rice genotypes, Gharib (HNT sensitive) and N22 (highly tolerant), were exposed to control (23 ˚C) and HNT (29 ˚C), from panicle initiation till maturity. Changes in the optical properties of rice panicles throughout maturity were evaluated under field conditions by measuring *(i)* effective quantum yield of photosystem II efficiency (Φ_{II}), *(ii)* steady-state chlorophyll fluorescence (F_S) and *(iii)* ratio of emitted chlorophyll fluorescence at 690 and 735 nm, under excitation at 435 nm (F_{690}/F_{735}). Numerous vegetative indices (Vis) were correlated with fluorescence measurements to prove the accuracy of the phenotyping method. Φ_{II} was selected as the most potent fluorescence parameter *(i)* to track changing optical properties of maturing rice panicles under both control and HNT and *(ii)* to estimate the elusive change point initiating rice panicle senescence. Detection of Φ_{II} change point allows for larger genetic diversity scans under field conditions and for identifying novel donors for increasing rice yields and incorporating resilient strategies to reduce impact of HNT stress on grain-filling.

Field Crops Research 177 (2015) 75–85

Contents lists available at ScienceDirect

Field Crops Research

journal homepage: www.elsevier.com/locate/fcr

Temporal chlorophyll fluorescence signals to track changes in optical properties of maturing rice panicles exposed to high night temperature

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ARTICLE INFO

Article history:
Received 11 December 2014
Received in revised form 26 February 2015
Accepted 26 February 2015

Keywords:
Chlorophyll fluorescence (Chl-F)
Grain filling
High night temperature (HNT)
Maturing panicle
Reflectance
Rice (*Oryza sativa*)

ABSTRACT

High night temperature (HNT) significantly influences rice grain filling dynamics. A novel phenotyping approach using chlorophyll fluorescence was employed to track changes in the optical properties of maturing rice panicles exposed to control and HNT. Two contrasting rice genotypes, Gharib (HNT sensitive) and N22 (highly tolerant), were exposed to control (23 °C) and HNT (29 °C), from panicle initiation till maturity. Changes in the optical properties of rice panicles throughout maturity were evaluated under field conditions by measuring (i) effective quantum yield of photosystem II efficiency (Φ_{II}), (ii) steady-state chlorophyll fluorescence (F_S) and (iii) ratio of emitted chlorophyll fluorescence at 690 and 735 nm, under excitation at 435 nm (F_{690}/F_{735}). Numerous vegetative indices (Vis) were correlated with fluorescence measurements to prove the accuracy of the phenotyping method. Φ_{II} was selected as the most potent fluorescence parameter (i) to track changing optical properties of maturing rice panicles under both control and HNT and (ii) to estimate the elusive change point initiating rice panicle senescence. Detection of Φ_{II} change point allows for larger genetic diversity scans under field conditions and for identifying novel donors for increasing rice yields and incorporating resilient strategies to reduce impact of HNT stress on grain-filling.

1. Introduction

Flowering in rice is a critical developmental stage, when seed numbers are determined. Flowering, along with the seed-filling phase, is considered highly sensitive to temperatures above critical thresholds (Shah et al., 2011). One major component accompanying climate change is the rapid increase in minimum night temperature compared with maximum day temperature at the global (Vose et al., 2005), country (Zhou et al., 2004; Welch et al., 2010; Rao et al., 2014) and farm level (Peng et al., 2004). A significant negative impact of high night temperature (HNT) stress (Jagadish et al., 2014) on rice yield has been confirmed by controlled-environment studies (Kanno and Makino, 2010; Mohammed and Tarpley, 2009, 2010, 2011; Mohammed et al., 2013) and recently from field experiments (Shi et al., 2013). Interestingly, under field conditions, the major cause of reduced yield under HNT was due to the overall reduction in biomass and non-structural carbohydrate (NSC) content, particularly in the panicles (Shi et al., 2013). In addition, the authors documented reduced grain width and 1000 grain weight, which could potentially be a result of reduced active grain filling duration (Kim et al., 2011), that is not captured by the routine protocols established to determine grain maturity.

Anthesis or flowering of rice (reviewed by Yoshida and Nagato, 2011) generally begins upon panicle emergence or on the following day and is consequently considered synonymous with heading, depending on the genotype under investigation. Rice follows a top-down flowering pattern, with the entire panicle flowering lasting for about 4 to 7 days (Moldenhauer and Slaton, 2005). Each spikelet on a rice panicle initiates its grain filling process 4 to 5 days past flowering, with the initiation of starch granule formation (Fitzgerald and Resurreccion, 2009). Superior spikelets are defined as those that are generally located on the apical primary branches

Abbreviations: Cc, total carotenoids; Chl-*a*, chlorophyll *a*; Chl-*b*, chlorophyll *b*; Chl-F, chlorophyll fluorescence; CP, fitted change point; DAF, days after flowering; DS, dry season; Φ_{II}, effective quantum yield of photosystem II efficiency-QY; F_S, steady-state chlorophyll fluorescence level; HNT, high night temperature; NSC, non-structural carbohydrate; PRI, photochemical reflectance index; PSII, photosystem II; Vis, vegetative indices; WS, wet season.

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http://dx.doi.org/10.1016/j.fcr.2015.02.025
0378-4290/

of a rice panicle, they flower first and produce larger and heavier seeds grains, while inferior spikelets are located on the proximal secondary branches, flower late and are either sterile or produce poorly filled grains (Yang and Zhang, 2010). The grain filling and maturation stage, occurring after anthesis, are characterized by grain growth and increases in weight, and are concomitant with the translocation of stored assimilates in the culms or from current photosynthesis (Fu et al., 2011; Yang and Zhang, 2010). The translocated sugars, which is sucrose in the case of rice, are converted to starch in the grain with the help of invertases, starch synthase, sucrose synthase, adenosine diphosphate-glucose pyrophosphorylase and starch branching enzyme and other enzymes involved in sugar metabolism (Yang and Zhang, 2006, 2010). All these changes are reflected in the progressive change in grain color as grain filling continued. A feature of rice panicle ripening is the change in color as a consequence of chlorophyll disappearance, when the yellowish coloration, due to other unknown pigments, becomes perceptible.

The presence of functional chlorophyll, a major photosynthetic pigment, showed that the photochemical efficiency of photosystem II (PS II) in fruit is similar to that in leaves (Carrara et al., 2001), since fruits from numerous species develop as a green photosynthetic tissue (Gillaspy et al., 1993). Rice panicles contribute to photosynthesis as well (Imaizumi et al., 1997). On the basis of chlorophyll, the photosynthetic capacity of a spikelet was found to be similar, while the estimated gross amount of photosynthetically assimilated carbon in the panicle is 30% of that in a flag leaf (Imaizumi et al., 1990). In addition, it was highlighted that the transpiration rate of the rice panicle reached maximum at heading, when the panicle color is similar to the leaf and decreased thereafter with age as grain filling progressed (Ishihara et al., 1990), providing evidence of carbon entry facilitating assimilation in the rice floral tissue. In addition, it has been highlighted, that rice genotypes characterised with rapid leaf senescence also exhibit rapid panicle senescence (Seo et al., 1981). In principle, photosynthesis suffers a substantial decrease during the period of color change, in which chlorophyll or chloroplast function is lost (Bean et al., 1963). These color changes can be followed by measurements of different optical signals. Chlorophyll fluorescence (Chl-F) provides a fast and non-destructive assessment of the loss of chloroplast function, and has been effectively used to follow senescence and/or ripening in a range of harvested plant tissues and organs (Adams et al., 1990; Armstrong et al., 1997). Since Chl-F is induced by direct excitation of chlorophyll molecules of PSII by light and their immediate relaxation, its characteristic is altered by functionally poor or rich PSII. During ripening, the level of Chl-F emission can be affected by two major changes—(i) a decrease in chlorophyll content or (ii) a loss of photosynthetic competence per unit chlorophyll. A decline in Chl-F has been reported to follow a decrease in chlorophyll content in papaya fruit (Bron et al., 2004), and was reflected in the loss of chloroplast function with advancing maturation, for example, in apples (Song et al., 1997).

Extending rice grain filling period has been a target of rice breeding programs during the past couple of decades. The underlying benefit is to delay panicle senescence, which would allow sufficient time for assimilates to fill the reproductive units efficiently, a novel route to increase stagnating rice yields. The grain filling duration in rice under temperate conditions such as Japan is much longer, whereas a significantly shorter duration under tropical conditions such as the Philippines is considered as a key bottleneck for further yield enhancement. Progress has not been made in this direction due to the lack of a standardized phenotyping protocol to establish the right approach to ascertain and track progress during grain filling. To ensure that a larger diversity of rice accessions is explored to address this highlighted bottleneck, a high-throughput phenotyping approach is needed. To date, there are no reports related to the use of Chl-F for the detection of rice panicle maturity, even though previous findings showed that Chl-F measurements in conjunction with other conventional methods appear to be a useful tool to follow ripening in chlorophyll-containing fruits, and thus potentially rice panicles. Hence, the objectives of our studies were (i) to investigate temporal changes in Chl-F and supportive reflectance signals of rice panicles exposed to control and HNT during ripening, (ii) to unravel the association between known vegetative tissue reflectance indices (Vis) and those obtained from panicle fluorescence and reflectance measurements, and (iii) to identify the best Chl-F parameter that can detect the elusive point trigerring panicle senescence and its rate of advancement under HNT.

2. Materials and methods

Field and greenhouse experiments were conducted at the International Rice Research Institute (IRRI), Los Baños, Philippines (14°11′N, 121°15′E). Three independent experiments were carried out during the wet season (WS) of 2013 and dry season (DS) of 2014.

2.1. Plant material

Based on phenotypic data obtained from a larger genotypically diverse set of 36 rice genotypes in response to HNT stress (Zhang et al., 2013), followed by a comprehensive physiological and molecular characterization (Shi et al., 2013), N22 (HNT tolerant) and Gharib (a susceptible genotype), were selected for this study. Seed dormancy was broken by exposure to 50 °C for 3 days, followed by pre-germination and sowing in seeding trays. Fourteen-day-old seedlings were transplanted on July 11 during the 2013 WS and on January 5 during the 2014 DS, at a spacing of 0.2 × 0.2 m, with four seedlings per hill. During the 2013 WS, phosphorus (15 kg P ha^{-1} as single superphosphate), potassium (20 kg K ha^{-1} as KCl) and zinc (2.5 kg Zn ha^{-1} as zinc sulfate) were incorporated one day before transplanting. Nitrogen fertilizer in the form of urea was applied in four splits (30 kg ha^{-1} as basal, 20 kg ha^{-1} at mid-tillering, 20 kg ha^{-1} at panicle initiation and 30 kg ha^{-1} just before heading). The amount of P, K and Zn fertilizers was doubled in the 2014 dry season as per the standard recommendations, while the 150 kg N was applied in four splits with a proportional increase in each of the splits. Manual weeding was employed to maintain weed-free plots. Need-based chemical spraying was undertaken to control whorl maggots (*Hyddrelia philippina* Ferino) during the early vegetative stage and yellow stem borers (*Scirpophaga incertulas*) at the flowering stage.

2.2. HNT tents and stress treatment

Six field-based temperature-controlled tents were used to study the impact of HNT on the optical properties of rice panicles during the grain filling and ripening stages. For details on the facility and the operation of the tents, readers are directed to Shi et al. (2013). A photographic view and details of the facility are also provided as Supplementary data Fig. S1. In brief, the tents were designed to impose HNT with high accuracy—by ensuring they were leak-proof and provided with sufficient air flow to avoid buildup of CO_2 and relative humidity. Plants were exposed to HNT (29 °C [actual = 28.6 ± 0.1 °C during 2013 WS and 28.6 ± 0.5 °C during 2014 DS]) and 23 °C [23 ± 0.1 °C during 2013 WS and 22.9 ± 0.1 °C during 2014 DS] as control temperature. Each tent of size 18 m^2 was considered as an independent replicate, two (in 2013) and three (in 2014) each for control and HNT treatments. The tents were covered during nights (1800–0600) to impose HNT stress and completely opened during the day (0600–1800) to avoid other confounding factors, such as reduced light, etc.; and to obtain results that would

be more applicable to real-world conditions (Supplementary data Fig. S1).

2.3. Chl-F and reflectance measurements

For better ecophysiological information and to know more about the potential intrinsic capacity of photosynthesizing tissue, it is usually recommended to measure light-response curves (Rascher et al., 2000) however, dark adaptation of plant tissue before measuring light response curves is essential. Also, under field conditions, accurate recording of light-response curves often appears to be technically impossible, since in the strict sense the steady-state conditions in each light are obligatory (Serodio et al., 2006). Therefore, to avoid inaccuracy in interpreted data, all field measurements were carried out during clear days, with approximately the same ambient light intensity, which does not affect individual observations (Buschmann, 1994). Thus for further analysis, fluorescence parameters which allows fast and accurate measurements under field conditions and without the need for dark adaptation (Roháček, 2002; Maxwell and Johnson, 2000) of the sample: (1) Φ_{II} (Genty et al., 1989) and (2) F_S (Krause and Weis, 1991) were selected.

The Chl-F measurements of rice panicles were performed by the portable fluorometer FluorPen (FluorPen FP 100 with detection area of 0.385 cm^2, Photon System Instruments, Ltd., Brno, Czech Republic; Supplementary data Fig. S2, for detailed information readers are directed to www.psi.cz). Saturating light (intensity approx. 3,000 μmol (photons) m^{-2} s^{-1}) and measuring light (intensity approx. 0.09 μmol (photons) m^{-2} per pulse) were applied to measure the effective quantum yield of PSII in light-adapted state (Φ_{II}; Genty et al., 1989) and steady-state fluorescence yield in light-adapted state (F_S; Krause and Weis, 1991). In addition, the ratio of emitted fluorescence excited by 435 nm and detected at 690 and 735 nm (F_{690}/F_{735}; Buschmann and Schrey, 1981; Buschmann et al., 1994; Buschmann, 2007) was measured by a prototype of handheld instrument based on platform of SpectraPen SP 100, WinePen (WinePen, detection area of 0.636 cm^2, Photon System Instruments, Ltd., Brno, Czech Republic, Supplementary data Fig. S2, for detailed informations readers are directed to www.psi.cz). Small detection area of both instruments helped discriminate false readings caused by panicle heterogenity. With regard to diurnal variations of Chl-F (e.g. Cerovic et al., 1996; Zhang and Rongfu, 1999; Zarco-Tejada et al., 2000; Ji-Yong et al., 2004; Ač et al., 2009; Panda, 2011), all measurements were made in the morning (0800 to 1100) to avoid confounding of results with afternoon photosynthetic depression.

Panicle reflectance measurements were also performed by using WinePen (Photon System Instruments, Ltd., Brno, Czech Republic, Supplementary data Fig. S2, for detailed informations readers are directed to www.psi.cz), a portable spectrophotometer equipped with internal light sources capable of measuring reflectance in the spectral range 325–780 nm. For reflectance, an incandescent xenon lamp was used. Signal detection was achieved by a compact polychromator integrated with a reflection grating and CMOS linear image sensor with the spectral response half width of 9 nm and wavelength reproducibility of ±0.5 nm. For reflectance measurement calibration, two Zenith standards were used. Based on the literature review, several Vis were used in connection with rice leaves/panicles:

(i) PRI [photochemical reflectance index (Gamon et al., 1992; Chen et al., 2006)],
(ii) mSR_{705} (Sims and Gamon, 2002; Chen et al., 2006),
(iii) mND_{705} (Sims and Gamon, 2002; Chen et al., 2006),
(iv) R_{470}/R_{570},
(v) R_{520}/R_{675},
(vi) R_{570}/R_{675} (Kobayashi et al., 2001); With the reflectance parameters related to steady-state fluorescence level:
(vii) R_{686}/R_{630} (Ač et al., 2009); Or with the estimation of chlorophyll concentration in leaves:
(viii) R_{750}/R_{700} (Lichtenthaler et al., 1996) and
(ix) $Log(R_{750}/R_{700})$ (Buschmann and Nagel, 1993) were calculated and used as a reference for fluorescence measurements.

2.4. Methodology

Considering that there are limited studies reporting usage of reflectance measurements and none using Chl-F measurements on rice panicles even under controlled conditions, we have investigated methodology tests to overcome possible confounding factors. Four possible scenarios were investigated on five randomly selected rice panicles in both the greenhouse and field conditions: (A) 10 continuous reflectance measurements in the field (5 N22 + 5 Gharib) recorded at the same place of the panicle to quantify the error caused by the instrument itself (fluorescence measurements were omitted due to effect of repeated saturating pulse application on fluorescence signal); (B) 10 independent and repeated reflectacance measurements in the field (5 N22 + 5 Gharib) recorded at the same place of the panicle with repeated removal and clasping of the instrument to estimate the error caused by the user (fluorescence measurements were omitted, too); (C) 10 independent fluorescence and reflectance measurements under field conditions (5 N22 + 5 Gharib), recorded at different places throughout the panicle to estimate the error caused by panicle heterogenity; and (D) 10 fluorescence and 5 reflectance measurements made under greenhouse conditions (genotype PSBRc4), on alternative days from flowering to maturity, in the top and bottom portions of the panicles, to quantify temporal and spatial error throughout rice panicle itself, during maturation. Methodology involving scenarios A, B and C was carried out in field experiments (see section 2.6) and scenario D in the greenhouse experiment (see section 2.5) below.

2.5. Greenhouse experiment

A greenhouse experiment was carried out from 27 January to 20 February 2014. Five uniform main tiller panicles at the time of heading (genotype PSBRc4) were selected and tagged following the procedure detailed in Shi et al. (2013), on which fluorescence and reflectance measurements were than recorded. On each panicle, 10 fluorescence and 5 reflectance spectra were measured in the top and/or bottom portion of the panicle. Measurements on alternative days were always aimed for completion between 0800 and 1100, going from 100% flowering till maturity. This experiment was used to test the methodology, to explore and fine-tune the protocol and to establish a standardized phenotyping approach for measuring chlorophyll flourescence and calculating reflectance indices on a maturing rice panicle.

2.6. Field experiments

Two HNT experiments were carried out during WS 2013 and DS 2014. Readings during the WS were obtained from 3rd to 24th September 2013 and during the DS from 1st to 21st March 2014. Fluorescence and reflectance signals were measured on clear days under ambient conditions by using the standardized approach established under greenhouse and field conditions. Measurements were done on alternate days in both years from 0800 to 1100. Measurements were shifted to afternoons in some cases or even cancelled because of continuous rainfall during 2013 WS, but that was not the case during 2014 DS, and data were collected on every alternate day. Two independent tents each during WS 2013 and three during DS 2014 were used for each temperature treatments

of 23 °C (control) and 29 °C (HNT), respectively. To maintain uniformity across all target panicles, five uniform main tiller panicles at the time of heading of each genotype (N22 and Gharib) were selected and tagged in each selected tent following the procedure detailed in Shi et al. (2013). On each panicle, 10 fluorescence and five reflectance measurements were recorded: (a) randomly throughout the whole panicle in 2013 (in total, 100 fluorescence and 50 reflectance measurements on every measurement day); and to separate errorneous readings caused by top-down flowering pattern in rice (b) separately in the top and bottom portion of the panicle in 2014 (in total, 150 fluorescence and 75 reflectance measurements in each portion of the panicle, on every measurement day).

2.7. Chlorophyll and carotenoid estimation of lemma and palea

Experimental setup, which was focused on obtaining highly accurate results, did not allow to quantify chlorophylls and carotenoids concentrations at multiple points during grain filling period. To ascertain the photosynthetic pigment concentration at flowering stage and to ensure that the starting point of concentrations is equal for all treatments, five panicles from control and HNT tents were sampled for genotypes Gharib and N22, in the 2014 dry season. Before further analysis, panicles were stored at −80 °C. To quantify the heterogeneity of pigment composition between control and HNT, respectively, samples of both control and HNT and spikelets at different positions along rice panicles, that is the top and bottom portions of the panicles, were selected for following analysis. By using forceps, lemma and palea were separated from each panicle for further investigation. In total, two samples of lemma and palea for the category "top" and "bottom" from all five selected panicles, resulting in five independent replicates, were homogenized and extracted in 96% acetone. To hydrate homogenized freeze-dried lemma and palea samples, approx. 1.2 mg of panicle dried tissue was splashed and ground with 100 μl of distilled water. Homogenate with 10 ml of 96% ethanol was than incubated at room temperature overnight in test tubes wrapped in aluminum foil. Chlorophyll *a* (Chl-*a*), total chlorophylls (Chl-*a* + *b*) and total carotenoids (Cc) were estimated from the extract. Absorption spectra of each extract at pre-defined wavelengths (470 nm, 652.4 nm and 665.2 nm) were measured by using a spectrophotometer (Shimadzu UV-1800, Japan). Chl-*a*, Chl-*a* + *b* and Cc concentrations were than calculated according to Lichtenthaler (1987), and pigment content was expressed in mg g^{-1} fresh weight.

2.8. Statistical analysis

A fitted change point (CP) model with unknown CP was applied on effective quantum yield of PS II (Φ_{II}) by using SAS software (SAS ver. 9.3, SAS Institute Inc., Cary, NC, USA), in which the slope of the line before and after CP is characterized by β_1 (slope before CP) and $\beta_1 + \beta_2$ (slope after CP). Standard error for the slope after CP [SE($\beta_1 + \beta_2$)] was calculated using the formula $\sqrt{\mathrm{SE}(\beta_1)^2 + \mathrm{SE}(\beta_2)^2 + 2\mathrm{COV}(\beta_1, \beta_2)}$, where $\mathrm{COV}(\beta_1, \beta_2) = \rho(\beta_1, \beta_2)\,\mathrm{SE}(\beta_1)\,\mathrm{SE}(\beta_2)$ is model-generated output. To prove the significance of CP in a piece wise regression, a (1) reduced model with combined data and a (2) full model with separate parameter estimates for each treatment were fitted. Models (1) and (2) were than compared using Sum of Squares Reduction Test. The (i) contrasting rice genotypes (Gharib and N22), (ii) treatments (control and HNT) and (iii) different places throughout the panicle (top and bottom portions of the panicles) comparisons were done by *t*-test. The photosynthetic pigment content (Chl-*a*, Chl-*a* + *b*, Cc) comparisons across treatments (control and HNT) and different portions (top and bottom) on the panicle were made by using Genstat (GenStat 14th Edition, Rothamsted Experimental Station, Harpenden, UK). Regression analysis of Chl-F and Vis were determined using Microsoft Excel.

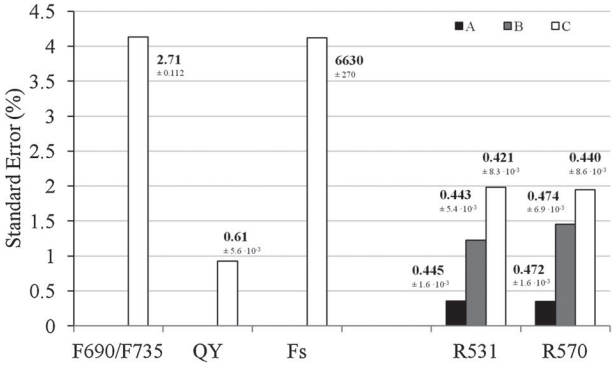

Fig. 1. Fine-tuning the phenotyping protocol to estimate optical properties of a maturing rice panicle. SE [%, $n = 10$] was calculated using Abs[(SE − Avg)/Avg × 100]. Three different field scenarios are represented by columns: (A) same place; (B) same place with repeated removal and clasping of the instrument and (C) different places throughout the panicle (see Section 2). Parameters for which SE (%) was calculated: (1) ratio of emitted fluorescence excited by 435 nm and detected at 690 and 735 nm (F_{690}/F_{735}); (2) effective quantum yield of photosystem II efficiency (QY-Φ_{II}); (3) *steady-state Chl-F (Fs)*; and (4) wavelengths used to calculate photochemical reflectance index [PRI = $(R_{531} - R_{570})/(R_{531} + R_{570})$; i.e. value of reflectance at 531 nm and 570 nm, respectively]. Numbers beside the bars represent mean ± SE.

3. Results

3.1. Methodology

To overcome possible confounding factors, that could challenge the novel approach presented to measure the optical properties of maturing rice panicles, four possible scenarios were investigated (see Section 2 for details). To evaluate possible inaccuracy of the method, the percentage difference of SE of the mean was calculated. Fig. 1 represents the percentage SE of four possible scenarios, which can potentially confound obtained results. The values of reflectance at 531 nm and 570 nm showed a very small difference with measurements made at the same place on the panicle (scenario A; Fig. 1), while the same was comparatively larger in scenarios B and C (Fig. 1, columns B and C). The highest percentage SE for fluorescence measurements was recorded in scenario C for the ratio of fluorescence excited by 435 nm and emitted at 690 and 735 nm (F_{690}/F_{735}) and steady-state Chl-F (Fig. 1, column C). The value of SE for wavelengths for PRI calculation (531 nm and 570 nm) varied around 2% under scenario C (Fig. 1, column C) and scenario d (Supplementary data Fig. S3B), but recorded a lower percentage SE across scenarios a and b (Fig. 1, columns A and B). Compared to other fluorescence parameters, a different trend was found in the case of effective quantum yield of PS II efficiency (Φ_{II}, Fig. 1, Supplementary data Fig. S3A), for which the highest value (ca 3.5%) was associated with scenario D, followed by a decrease in scenario C (ca 1%). With measurements made across the panicle (Fig. 1, column C) and in the top and bottom portions of the panicle (Supplementary data Fig. S3A), which resemble the actual measurement procedure followed, effective quantum yield (Φ_{II}) was identified as the most stable parameter with the least error. Standard errors (%) of the whole fluorescence spectrum under excitation (435 nm) and reflected spectrum, from which Vis with known physiological interpretation were calculated, are presented in Supplementary data Fig. S4A and B. As shown in Supplementary data Fig. S3A and B, the percentage SE of measured parameters throughout panicle maturity phase has a slightly increasing trend, with the values for the top portion of the panicle expressing higher standard error.

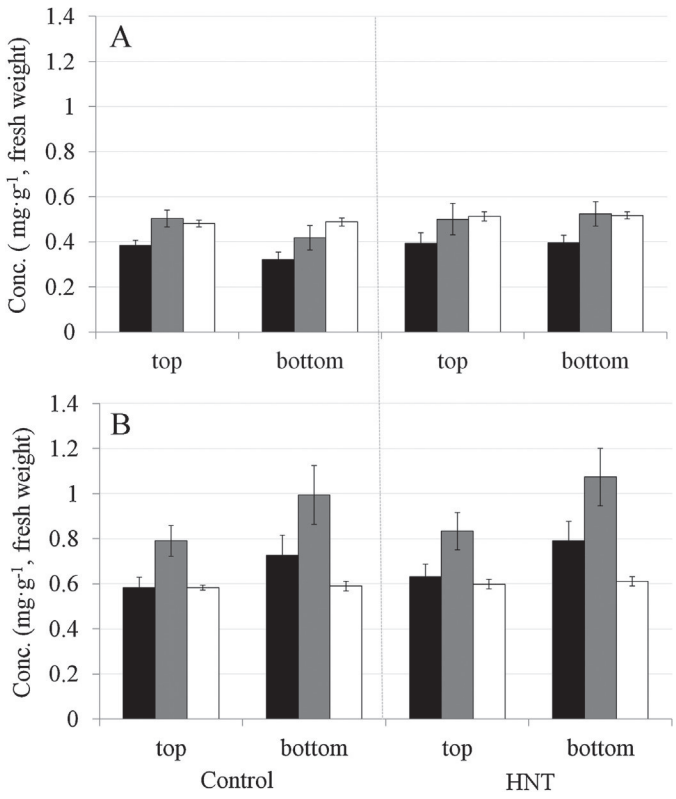

Fig. 2. Concentration of photosynthetic pigments in lemma and palea of N22 (a) and Gharib (b) panicles at flowering stage. Mean values of chlorophyll *a* (Chl-*a*, black columns), total chlorophylls (Chl-*a* + *b*, gray columns) and total carotenoids (Cc, white columns) for control and HNT treatments, "top" and "bottom" portions of the panicle, respectively, are expressed in mg g^{-1} per fresh weight ± SE.

3.2. Pigment content of rice lemma and palea

Estimating senescence in maturing rice panicles through Chl-F would require ensuring, that the starting point of the measurements did not discriminate genotypes either across the treatments (control versus HNT) or in the position along the panicle (top versus bottom portion of the panicle) and that the consequent differences in panicle senescence were recorded only during subsequent grain filling. To evaluate photosynthetic pigment concentration at the flowering stage, Chl-*a*, Chl-*a* + *b* and Cc were recorded. As shown in Fig. 2, the HNT treatment applied from panicle initiation has no significant effect on Chl-*a*, Chl-*a* + *b* and Cc concentration in lemma and palea across treatments ($p > 0.11$) and portions of the panicle ($p > 0.43$).

3.3. Chlorophyll fluorescence

Uniform concentrations of Chl-*a* at the flowering stage (0 DAF), with non-significant difference between treatments, on top and bottom portions of the panicles (Fig. 2), ensured identical conditions at the start of Chl-F measurements. Effective quantum yield of PS II efficiency (Φ_{II}, Figs. 3 and 4), steady-state fluorescence level (F_S, Supporting informations Fig. S5A and B) and ratio of emitted fluorescence excited by 435 nm and detected at 690 and 735 nm (F_{690}/F_{735}; F_{690}, Supplementary data Fig. S6A and B) have been used to follow the changes in optical properties of Gharib and N22 panicles during maturation, directly under field conditions. Also, to explore the impact of HNT on the changes in optical properties during maturation, results are presented as control and HNT (Figs. 3 and 4; Supplementary data Fig. S5A and B; Fig. S6A and B). Based on the above, the best-fitting fluorescence parameter (Φ_{II}) was selected for further analysis.

Because of a top-down flowering pattern in rice, optical measurements and chemical analysis (at the flowering stage, Fig. 2) were carried out in the top and bottom portion of the panicle. Also, to estimate the phenotypic response to HNT within a panicle, independent analyses for top and bottom portions of the panicles, for both control and HNT conditions, are presented (Fig. 3). Both genotypes followed a similar pattern of Φ_{II} in the top and bottom portion of the panicle exposed to control treatment (Fig. 3A–D), with Φ_{II} remaining constant after flowering until the genotype-dependent CP was reached (Fig. 3A–D; open symbols). In contrast, rice panicles exposed to HNT treatment (Fig. 3E–H; closed symbols) recorded a decrease in Φ_{II} parameter immediately after the flowering stage.

To evaluate the trend of Φ_{II} parameter numerically, the shape of the CP curve was characterized by the values derived from CP analysis (time [in days] to reach CP; slope before and after CP, Table 1). The time to reach CP under control conditions in Gharib panicles occurred 13–14 DAF in 2013 or with the top portion of the panicle in 2014 and decreased by up to 4 days ($p < 0.01$) under HNT. The CP in the bottom portions of Gharib panicles was unchanged between control and HNT (10 DAF, Table 1, Fig. 3C and G). The shortened duration to reach the CP under HNT in Gharib panicles was also seen with data averaged across both years. The CP of N22 panicles exposed to HNT and control treatment occurred at 12–15 DAF during both seasons. However, panicles of N22 exposed to HNT took a longer period to reach CP in 2013 (+1 day, $p < 0.01$), and in the case of the bottom portions of panicles during the 2014 season (+ 2 days, $p < 0.01$), compared with their respective controls, and the trend was similar with the combined analysis (Table 1).

Slope before and after CP is characterized by β_1 and $(\beta_1 + \beta_2)$ values, respectively (Table 1). The positive and negative values reflect the increasing and decreasing trend of Φ_{II}, respectively. Negative values of $(\beta_1 + \beta_2)$ characterized decreasing slope after CP in both inspected rice genotypes. Across both genotypes, the effective quantum yield under control conditions was consistently stable till CP, after which there was a sharp decline, while HNT induced a declining trend starting immediately after flowering (Fig. 3E–H; and Table 1). Although the start and end points of Φ_{II} were about the same, the declining trend across the control and HNT was strikingly different as mentioned above. A greater decline in the slope was observed after CP across all conditions and both genotypes, with a faster rate of decline recorded under control conditions than with HNT. Within the panicle, the CP in the bottom portion of the panicle under control conditions was lower by 2 to 3 days ($p < 0.01$) than the CP in the top portion in both genotypes. This difference disappeared with HNT exposure, with the CP becoming almost the same across the entire panicle.

Since many variables such as environmental conditions and management practices, including different levels of fertilizer application etc., can influence rice panicle maturation and/or changes in optical properties during ripening, a more robust phenotyping approach is needed. Hence, changes in fluorescence signals during the ripening of rice panicles under different conditions such as DS and WS, and measurements taken throughout the panicle and top or bottom portions of the panicle, are combined and summarized in Fig. 4. Control treatments showed a similar trend with both WS and DS and across genotypes, with a genetic variation of 2 days (Gharib—11 DAF and N22–13 DAF) in reaching the CP under control conditions (Fig. 4A and B; Table 1). The impact of HNT treatment was characterized by the shortening of duration to reach CP by 2 days in Gharib (Fig. 4C) compared with the control conditions. The trend in Φ_{II} as seen with independent seasons, genotypes or different portions of the panicle (Fig. 3) or a combined analysis of

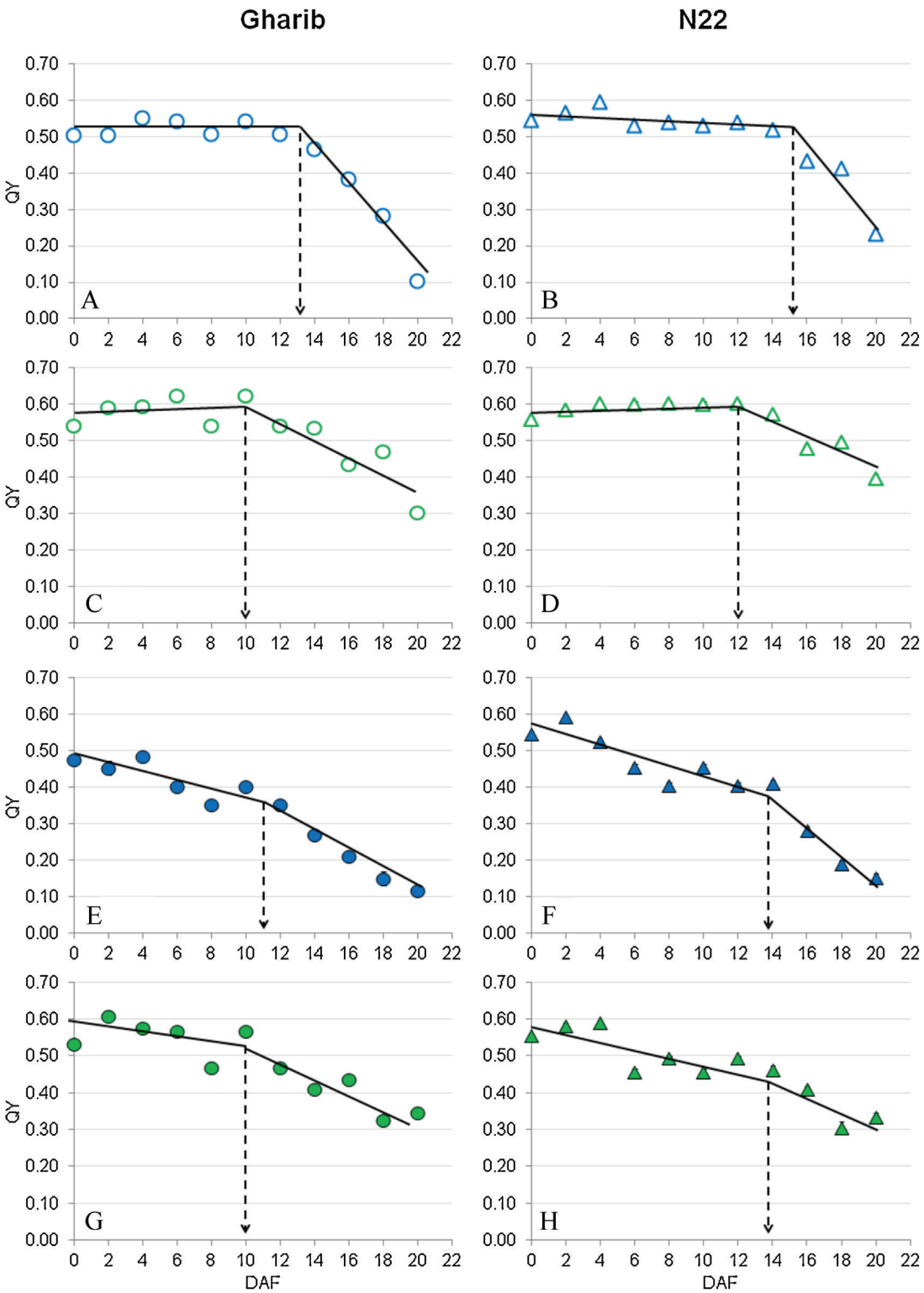

Fig. 3. Time trend of effective quantum yield of PSII (QY). Respective values for 2014 season, genotypes Gharib and N22, in days after flowering (DAF), for two different positions within panicles. Blue symbols represent top and green symbols bottom portions of the panicles for control (23 ± 0.1 °C; open symbols, panels a, b, c, d) and HNT (28.6 ± 0.1 °C; closed symbols, panels e, f, g, h), respectively. Each data point represents mean value of fluorescence measurements (n = 150, see Section 2) ± SE. The interpolating line represents regression optimized by SAS software, where the change point (CP) is highlighted by a broken line. (i) Rice genotypes ($p < 0.01$), (ii) the treatments ($p < 0.01$) and (iii) top and bottom portions of the panicles ($p < 0.01$) differed significantly. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

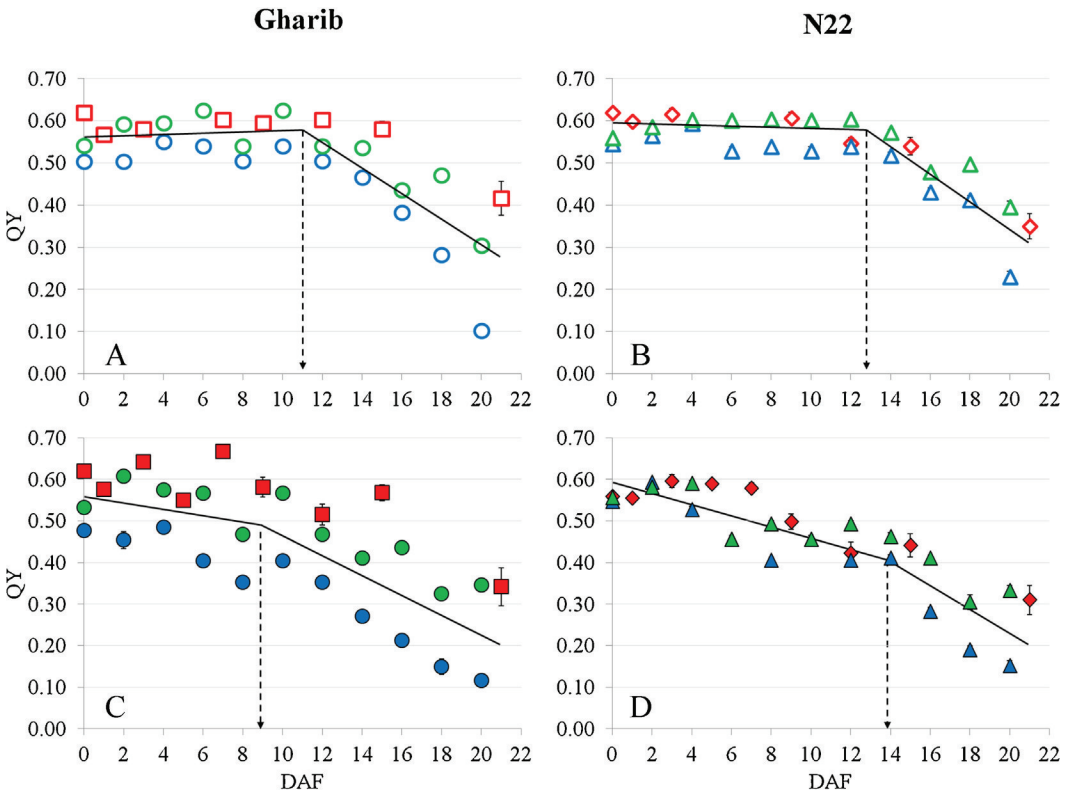

Fig. 4. Time trend of effective quantum yield of PSII (QY). Respective values for 2013 and 2014, genotypes Gharib and N22, in days after flowering (DAF) for control (panels a and b) and HNT (panels c and d) treatments, respectively. Red squares—(panels a and c) and red diamonds (panels b and d) represent average values of 2013 season for genotypes Gharib and N22, respectively. Blue and green open and closed symbols (circles for Gharib and triangles for N22) represent average values with respect to position in the panicle; open symbols for control and closed symbols for HNT treatment; blue represents top and green bottom part of the panicles, respectively. Each data point represents mean value of fluorescence measurements ($n = 100$ for 2013 and $n = 150$ for 2014; see Section 2) ± SE. The interpolating line represents regression optimized by SAS software, where the change point (CP) is highlighted by a broken line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

all three cases (Fig. 4) indicated the robustness of the parameter in estimating maturing panicle senescence under control and HNT conditions [across environments (two seasons), temperature (control and HNT), developmental stages (from flowering to maturity), genotypes (Gharib and N22) and different position of the panicle (top and bottom portion of the panicle)].

3.4. Reflectance measurements

To strengthen the panicle fluorescence hypothesis and to support fluorescence measurements, additional reflectance measurements in visible light (400–800 nm) were carried out on the same panicle used for fluorescence measurements. With the

Table 1
Individual observations of change point (CP) analysis for 2013 and 2014, top^{T} and bottom^{B} portions of the panicles. Duration to reach CP (in days after flowering) and the slopes before (β_1) and after CP ($\beta_1 + \beta_2$) are presented for genotypes Gharib and N22 ± standard errors (SE). + and − signs indicate increasing and decreasing trend of the slope.

	CP	β_1	SE	$\beta_1 + \beta_2$	SE
2013					
Gharib _control	14	+0.00176	*0.00617*	−0.03254	*0.00798*
Gharib _HNT	10	+0.00006	*0.00595*	−0.02193	*0.00715*
N22_control	14	−0.00425	*0.00374*	−0.03165	*0.00925*
N22_HNT	15	−0.01040	*0.00369*	−0.02190	*0.00925*
2014					
Gharib_control^{T}	13	+0.00061	*0.00264*	−0.05949	*0.00625*
Gharib_control^{B}	10	+0.00421	*0.00551*	−0.02689	*0.00729*
Gharib _HNT^{T}	11	−0.01070	*0.00335*	−0.02970	*0.00442*
Gharib_HNT^{B}	10	−0.00643	*0.00729*	−0.01853	*0.00551*
2014					
N22_control^{T}	15	−0.00290	*0.00271*	−0.04860	*0.01240*
N22 _control^{B}	12	+0.00372	*0.00406*	−0.02458	*0.00537*
N22_HNT^{T}	14	−0.01390	*0.00332*	−0.03900	*0.00785*
N22 _HNT^{B}	14	−0.00900	*0.00262*	−0.02276	*0.01201*
Combined					
Gharib_control	11	+0.00176	*0.00617*	−0.03254	*0.00798*
Gharib_HNT	9	−0.00613	*0.00742*	−0.02283	*0.00648*
N22_control	13	−0.00155	*0.00278*	−0.03285	*0.00662*
N22_HNT	14	−0.01180	*0.00277*	−0.02750	*0.00661*

Table 2
Linear regression coefficients of fluorescence and reflectance measurements. Relationship presented represents all three genotypes (PSBRc4, Gharib and N22), both seasons (wet and dry season), experimental treatments (control and HNT), across greenhouse and field conditions. *** and ** represent significance at probability level 0.1% and 1%, respectively. NS = non-signficant, $n = 135$. The '+' and '−' signs indicate the positive and negative association between the fluorescence and reflectance parameters.

Reflectance	Fluorescence		
	F_S (Krause and Weis, 1991)	Φ_{II} (Genty et al., 1989)	F_{690}/F_{735} (Buschmann, 2007)
PRI (Gamon et al., 1992)	+0.47***	+0.71***	+0.37***
mND_{705} (Sims and Gamon, 2002; Chen et al., 2006)	+0.35***	+0.62***	−0.29**
mSR_{705} (Sims and Gamon, 2002; Chen et al., 2006)	+0.30**	+0.54***	0.24NS
R_{470}/R_{570} (Kobayashi et al., 2001)	−0.06***	−0.003***	+0.16***
R_{520}/R_{675} (Kobayashi et al., 2001)	+0.54***	+0.70***	−0.52***
R_{570}/R_{675} (Kobayashi et al., 2001)	+0.54***	+0.58***	−0.52***
R_{686}/R_{630} (Ač et al., 2009)	−0.44***	−0.52***	+0.43***
R_{750}/R_{700} (Lichtenthaler et al., 1996)	+0.41***	+0.49***	−0.29***
$Log(R_{750}/R_{550})$ (Buschmann and Nagel, 1993)	−0.36***	−0.50***	+0.30***

progression of panicle maturity, panicle reflectance tended to decrease in the green region (500–570 nm) and increase in the blue and red region (400–510 nm; 610–700 nm), where chlorophyll and carotenoids absorb strongly. Particular Vis with known physiological interpretation were than calculated to follow the changes in chlorophyll and carotenoids during maturation (Table 2). Among others, photochemical reflectance index (PRI; Gamon et al., 1992) was tested as the best-fitting Vis to Φ_{II} fluorescence measurements. The PRI value expressed almost the same patterns as those of fluorescence measurements (Supplementary data Fig. S7A and B; Table 2), while it gradually decreased as maturation progressed. To prove the accuracy and reproducibility of the experiment, the significance and strength of mutual dependence was tested by means of linear regression analysis. From Table 2, regression analysis indicated that PRI and Φ_{II} decreased significantly ($p < 0.001$) with the progress in panicle maturity. This strong positive correlation was characterized by coefficient of determination $R^2 = 0.71$ for PRI and Φ_{II}.

4. Discussion

In addition to flag leaves, chlorophyll-containing rice panicles have the potential to photosynthesize, and thus allow investigation of photosynthetic apparatus indirectly by measuring Chl-F (Papageorgiou and Govindjee, 2004; Baker, 2008). As reported by Imaizumi et al. (1990), on a chlorophyll basis, the assimilation rate was found to be similar, while the estimated amount of photosynthetically assimilated CO_2 in the whole panicle reached 30% of the flag leaves. In addition, 20–30% of the carbohydrates accumulated in the grain are a result of direct CO_2 assimilation of the panicle after its emergence (Ishihara et al., 1990). Thus, during panicle maturation, disappearance of chlorophyll and/or loss of chloroplast function results in lower photosynthetic activity, which can be captured through changes in fluorescence signals (Maxwell and Johnson, 2000; Roháček, 2002; Papageorgiou and Govindjee, 2004). Such chlorophyll disappearance from the lemma and palea, which directly influence the fluorescence signal preceeding senescence is, however, not uniform on a temporal basis, and can be either accelerated or slowed during the grain filling stage influenced by the phenotype (Fujita et al., 1984) and/or prevailing environmental conditions (Liang et al., 2011; Chen et al., 2012). To date, there are no reports on evaluating changes in the Chl-F signal of rice panicles during maturation to estimate the break point initiating panicle senescence and a decline in predicted grain filling in rice panicles. Chl-F is a widely used, fast and accurate method to investigate photosynthetic alterations (reviewed by Papageorgiou and Govindjee, 2004; Baker, 2008), and has been recently used to reveal the physiological status of (i) senescing plant leaves (Adams et al., 1990) or (ii) during the ripening period of certain fruits, for example apples (Armstrong et al., 1997), tomatoes (Carrara et al., 2001), or papaya (Bron et al., 2004). As generally agreed, a change in Chl-F signal in senesencing fruit is connected to either a loss of chloroplast function (Bean et al., 1963) or a decrease in chlorophyll content (Bron et al., 2004). The morphological architecture of the plant material, however, is also one of the key components that determine light capture, amount of emitted Chl-F and/or photosynthetic efficiency in the plant canopy, in a single leaf or in organs such as fruits/spikes/panicles (Parveaud et al., 2008). Thus, potential application of Chl-F measurements and the use of appropriate instruments should be tested to address these limitations based on the objective of the study. As presented in Fig. 1 and Supplementary data Fig. S3, by considering four possible scenarios which can confound the measurements, we have demonstrated that the SE of the instrumentation used in this study was found to be negligible and hence appropriate for testing the target hypothesis.

Among other Chl-F parameters, maximum quantum yield of PS II (F_V/F_M, Kitajima and Butler, 1975) is closely related to the activity of photosynthetic plant tissue and has been broadly applied to study plant-stress response (e.g., Nedbal and Whitmarsh, 2004; Cséfalvay et al., 2009; Šebela et al., 2014), as well as to investigate the ripening period of certain fruits mentioned above. However, application of this parameter to field studies is limited, since it requires species-dependent, appropriate dark adaptation (Roháček, 2002). As an additional tool, the use of effective quantum yield of PSII (Φ_{II}; Genty et al., 1989), steady-state chlorophyll fluorescence level (F_S; Krause and Weis, 1991) and ratio of fluorescence excited by 435 nm and detected at 690 and 735 nm, respectively (F_{690}/F_{735}, Buschmann and Schrey, 1981; Buschmann et al., 1994; Buschmann, 2007), allow field application, since they do not require dark adaptation of the sample, and thus facilitated the use of these parameters directly in our field experiments under ambient conditions. Numerous studies presented experimental evidence for a close relationship between maximum and effective quantum yield of PS II across different plant species (e.g. Genty et al., 1989; Schreiber et al., 1995). On the contrary, a similar strength in the relationship with steady-state (F_S; Krause and Weis, 1991) and (F_{690}/F_{735}) fluorescence measurements is missing (Flexas et al., 2000; Ounis et al., 2001). Moreover, the sensitivity of F_S and F_{690}/F_{735} to water-limited conditions (Cerovic et al., 1996; Flexas et al., 2000) is a limiting factor to study rice panicle senescence, for which the reducing water content during ripening would confound the results (Hay and Spanswick, 2006). Thus, effective quantum yield of PS II (Φ_{II}), which is considered to be a potent indicator of plant response to environmental stress (Rascher et al., 2000; Braun and Neuner, 2005), to follow leaf senescence (Falqueto et al., 2009) and/or fruit ripening (Meyerhoff and Pfundel, 2008), under in situ light conditions in the field, was found to be the most potent indicator of maturing rice panicles (Figs. 1, 3, 4) with the lowest SE (Supplementary data Fig. S3).

Since none of the Chl-F studies have focused on rice panicles during maturation, a literature review and characteristics of the spectra of reflected light from rice panicles were used as a reference in our analysis. As mentioned in Section 1, the raw spectral response of healthy rice panicles in the flowering stage is similar to that of the green leaf in the visible and near infrared (NIR) regions (Liu et al., 2010). To represent more clearly the reflectance response of maturing panicles, selected Vis with known physiological interpretation were extracted from captured reflectance spectra (Table 2). The above is based on a strong link between plant pigment concentrations and certain Vis across the species, tissue structures and vegetative stages (Sims and Gamon, 2002). In our study, Chl-F and chlorophyll-related Vis were selected and investigated.

For statistical significance with directly measured Chl-F parameters, several Vis were tested in grassland species (Ač et al., 2009). At the leaf level, PRI and R_{686}/R_{630} were used for tracking the spatial variation of the Chl-F parameters (F_S and Φ_{II}). However, the R_{686}/R_{630} index designed to track changes in F_S and identified as a useful indicator of early-stress plant response (Zarco-Tejada et al., 2000) failed during this study, since gradually limiting chlorophyll pigment content affected the response (Buschmann and Nagel, 1993; Lichtenthaler et al., 1996). Compared with those on leaves, studies connecting with spectral properties of rice panicles are limited. A number of Vis have been tested in rice panicles from the milky ripening stage to harvest (Chen et al., 2006) and have been identified as indicators of rice panicle blast (Kobayashi et al., 2001), or as a potent parameter for leaf blast detection (Kobayashi et al., 2003). Kobayashi et al. (2001) suggested Vis for rice blast detection, which followed a decrease in the absorbtion of photosynthetic pigments and changes in the internal structure of rice panicles during senescence. As panicle ripening advanced, NIR rather than visible reflectance was a more reliable indicator of panicle infection at the maturity stage, which does not allow tracking changes in optical properties of rice panicles by means of visible reflectance measurements, used in this study. As reported by Chen et al. (2006), Vis were correlated with the pigment content of rice panicles and flag leaves. It was suggested that PRI (Gamon et al., 1992), mND_{705} (Sims and Gamon, 2002) and mSR_{705} (Sims and Gamon, 2002) could be used to estimate and apply as spectral parameters in rice maturation monitoring, since they were significantly related to chlorophyll and carotenoid/chlorophyll ratio. Moreover, as we demonstrate in this study, the mentioned Vis were significantly correlated with fluorescence parameter (Φ_{II}) during rice panicle maturation (Table 2), suggesting potential application of Chl-F in rice panicle research.

By using Φ_{II} as a most reliable indicator, the loss of chlorophyll content or photosynthetic activity was estimated throughout panicle maturity among rice genotypes contrasting for their response to HNT. As suggested by Kim et al. (2011), early termination of grain filling in temperate rice under high temperature results from the loss of sink activity owing to the earlier panicle senescence, and not from the lack of assimilate supply due to leaf senescence, which extends the usage of Φ_{II} in connection with panicle sensecence and grain filling duration. Interestingly, a distinct CP was estimated under control conditions, but, with HNT response, a gradual decline throughout panicle maturity starting at the end of flowering was observed (Figs. 3 and 4). A clear genotypic variation even within the two gentoypes tested was seen with the fitted CP, indicating the potential of extending the introduced phenotyping technique to larger and more diverse rice panels in an attempt to identify donors with the CP occurring at a later point, to extend the predicted active grain filling duration under non-stress conditions. In addition, genotype N22, known for its reproductive-stage heat tolerance (Shi et al., 2013; Coast et al., 2014), had minimal change in CP under HNT compared with the sensitive Gharib, supporting earlier studies that have quantified their contrasting response to heat stress. The above provides evidence for the effectiveness of the technique to study maturing rice panicles even under stress conditions. Further, to reduce the negative effect of HNT on predicted grain filling, genotypes with the potential to retain their effective quantum yield of PS II between 0.5 and 0.6 post-flowering could provide avenues to overcome HNT-induced grain yield and quality losses caused by early initiation of panicle senescence. Although a number of other physiological processes such as overall biomass, non-structual carbohydrate reserves and their allocations, and efficiency of starch-converting enzymes could play a role in the overall grain filling process, particularly under HNT (Shi et al., 2013), the technique presented allows tracking the inherent potential of the sink to remain active and receptive to the incoming assimilates for efficient, predicted grain filling duration. In summary, effective quantum yield of PS II (Φ_{II}) with supportive PRI measurements was found to be the best Chl-F indicator, which can be used as a high-throughput means to precisely estimate the dynamics encountered with a maturing rice panicle. Our next step is to use these key parameters and carry out similar experiment on large rice core collections in an attempt to quantify genetic diversity for this trait and to set up a high-throughput platform based on the successful standardization of the protocol to measure signals from maturing rice panicles with high accuracy. The novel approach presented, with appropriate crop specific adjustments can potentially be extended to other cereals such as wheat, to overcome bottlenecks with predicted grain filling duration both under non-stress and stress condition.

Acknowledgments

The Federal Ministry for Economic Cooperation and Development, Germany, is thanked for supporting the first author's visit and stay at IRRI. Participation of the first and third author was also supported by the EfCOP-IPo project ENVIMET (CZ.1.07/2.3.00/20.0246), by the MEYS of CR within the National Sustainability Program I (NPU I), grant number LO1415, and by the EU (FP7) EPPN project (284443).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fcr.2015.02.025.

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4. CONCLUSIONS

In this thesis, methodological and technological platform for noninvasive monitoring of phenolic compounds in leaves and berries have been investigated.

The first part of this study has been designed as extension of existing laboratory study (Cséfalvay et al., 2009). For this purpose, chlorophyll-a fluorescence imaging technique (Nedbal et al., 2000) have been used. It has been shown that F_V/F_M parameter is effective to discriminate symptomatic and asymptomatic leaf tissue infected by fungal infection *Plasmopara viticola*. Tříska et al. (2012) have firstly identified highly fluorescing phenolic compound synthesized in grapevine leaves under *P. viticola* attack. As shown in this thesis, this compound (2,4,6-trihydroxyphenanthrene-2-*O*-glucoside) can be detected *in vivo*, by means of UV induced fluorescence measurements (Šebela et al., 2014). Moreover, Olejníčková et al. (chapter *3.1.2.* in this thesis) found close connection between the concentration of this compund and concentration of *trans*-resveratrol. In both cases also, particular concentrations were well correlated with the value of F_V/F_M parameter. Combinations of these optical signals with the other method developed in our laboratory (Bellasio et al., 2012; appendixes I.) could potentially be explored and serve as an effective, noninvasive tool for either *(i)* early detection of *P. viticola* infection in field, *(ii)* direct estimation of concentrations of phenolic compounds synthesized by grapevine under *P. viticola* attack, or *(iii)* 3D visualisation of *P. viticola* spread throughout the leaf tissue.

The second part of this study dealt with possible detection of phenolic compounds in fruits of grapevine and other important field crop-rice. In accordance to the literature, it has been proven that grapevine phenolics are synthesized under UV light and thus, serve as protective compounds (Šebela et al., chapter *3.2.1.*). In this study we have demonstrated the effect of ambient sunlight on the temporal phenolic profiles in model grapevine cultivar. The novelty of this approach consisted in the use of field conditions, which in fact provides realistic insight with non-manipulated doses of UV light throughout ripening season. Systematic mapping of this grapevine phenolic profiles throughout ripening and their direct correlation with optical signals allowed us to develop UV fluorescence based method (Šebela et al., chapter *3.2.2.*). Our results showed high accuracy of the CMOS prototype designed for this purpose, along with the laboratory instrument. By using this method we were able to identify specific phenolic compounds in grapevine berries *in vivo*. Other approach has been tested in the case of rice (Šebela et al., 2015 chapter *3.2.3.*; Šebela et al., 2015 apendixes III). Here, measurements of

panicle Chl-F have found to be potent to track changes of optical properties of maturing rice panicles and to estimate elusive point initiating panicle senescence. Here we assume, that this signal can be also used as potent indicator of phenolic compounds synthesized in rice during ripening, eventhough direct correlation Chl-F and specific phenolic concentrations are missing.

Both for leaves and berries, optical methods suitable for detection phenolic compounds *in vivo* are presented. This complex study bringed up several approcahes which could improve, along with the traditional wet chemistry, usability of phenolic compounds having undoubtedly high potential in modern medicine thanks to their undisputable health benefit.

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http://en.citizendium.org/wiki/Carotenoid
http://biology.westfield.ma.edu/biol129labs/sites/default/files/Figure%204.gif
http://www.uic.edu/classes/bios/bios100/lectures/ps01.htm

6. APENDIXES

Apendixes contains title pages of publications, which where not included in this thesis.

Apendix I

Bellasio C, Olejníčková J, Tesař R, Šebela D, Nedbal L (2012) Computer reconstruction of plant growth and chlorophyll fluorescence emission in three spatial dimensions. Sensors 12: 1052-1071.

Apendix II

Šebela D, Olejníčková J, Župčanová A, Sotolář R (2012) Response of grapevine leaves to *Plasmopara viticola* infection by means of measurement of reflectance and fluorescence signals. Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis 8: 229-238.

Apendix III

Šebela D, Quinones C, Olejníčková J, Jagadish KSV (2015) High-night temperature induced acceleated maturation of rice panicles can be detected by chlorophyll fluorescence. Global Change: A Complex Challenge, Conference Proceedings 1, 134-137.

Sensors **2012**, *12*, 1052-1071; doi:10.3390/s120101052

sensors

ISSN 1424-8220

www.mdpi.com/journal/sensors

Article

Computer Reconstruction of Plant Growth and Chlorophyll Fluorescence Emission in Three Spatial Dimensions

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Received: 4 January 2012; in revised form: 13 January 2012 / Accepted: 16 January 2012 / Published: 18 January 2012

Abstract: Plant leaves grow and change their orientation as well their emission of chlorophyll fluorescence in time. All these dynamic plant properties can be semi-automatically monitored by a 3D imaging system that generates plant models by the method of coded light illumination, fluorescence imaging and computer 3D reconstruction. Here, we describe the essentials of the method, as well as the system hardware. We show that the technique can reconstruct, with a high fidelity, the leaf size, the leaf angle and the plant height. The method fails with wilted plants when leaves overlap obscuring their true area. This effect, naturally, also interferes when the method is applied to measure plant growth under water stress. The method is, however, very potent in capturing the plant dynamics under mild stress and without stress. The 3D reconstruction is also highly effective in correcting geometrical factors that distort measurements of chlorophyll fluorescence emission of naturally positioned plant leaves.

ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume LX 27 Number 8, 2012

RESPONSE OF GRAPEVINE LEAVES TO *PLASMOPARA VITICOLA* INFECTION BY MEANS OF MEASUREMENT OF REFLECTANCE AND FLUORESCENCE SIGNALS

D. Šebela, J. Olejníčková, A. Župčanová, R. Sotolář

Received: September 13, 2012

Abstract

ŠEBELA, D., OLEJNÍČKOVÁ, J., ŽUPČANOVÁ, A., SOTOLÁŘ, R.: *Response of grapevine leaves to Plasmopara viticola infection by means of measurement of reflectance and fluorescence signals.* Acta univ. agric. et silvic. Mendel. Brun., 2012, LX, No. 8, pp. 229–238

Response of grapevine leaf tissue naturally infected by *Plasmopara viticola* in field was measured by means of chlorophyll fluorescence and reflectance signals. Three susceptible grapevine varieties (Cabernet Sauvignon, Pinot Blanc and Pinot Gris) were used in this study. Since the infection impairs photosynthetic activity, distribution of F_V/F_M parameter (maximum quantum yield of Photosystem II) over the leaf was effective to discriminate healthy and naturally infected leaf tissue. F_V/F_M was reduced ~ 25% in all infected leaf parts. Infected leaf spots expressed significantly altered chlorophyll fluorescence induction kinetics expressing much slower electron transport rate both on donor and acceptor site of PSII. Vegetation reflectance indices followed the variations in pigment content after the fungal infection. R750/R700 ($R^2 = 0.877$) and CRI (carotenoid reflectance index; $R^2 = 0.735$) were the most potent to follow changes in chlorophylls and carotenoids contents, respectively. Infected leaf tissue exhibited decrease in chlorophyll a (~50%) as well as carotenoids (~70%). We conclude that combination of chlorophyll fluorescence and reflectance measurements can be used as an effective non-invasive tool for an early detection of *Plasmopara viticola* in field as well as for estimation of the level of infection.

Plasmopara viticola, downy mildew, grapevine, leaf tissue, susceptible varieties, chlorophyll fluorescence imaging, reflectance

Abbreviations

Car..... carotenoids
CRI..... carotenoid reflectance index
CCD ..... charge coupled device
Chl..... chlorophyll
Chl-F.... chlorophyll fluorescence
F_0..... minimum chlorophyll fluorescence yield in dark – adapted state
F_M..... maximum chlorophyll fluorescence yield in dark – adapted state
F_P..... peak chlorophyll fluorescence yield measured when the actinic light is switched on, it is measured after ca 1s of actinic light
F_S..... steady state chlorophyll fluorescence yield in light – adapted state
Fd/2 = (FP – FS)/2
LED..... light emitting diode
PSII ..... photosystem II
VI..... reflectance vegetation index

Grape vine (*Vitis vinifera* L. subsp. vinifera Hegi, 1753) has many potential pests, which may cause decreases, or in the most serious cases complete yield destruction if no chemicals are applied. One of the major diseases of the grapevine is downy mildew [*Plasmopara viticola* (Berk. & Curtis ex de Bary) Berlese & de Toni, 1888]. *Plasmopara viticola* is the casual agent of grape downy mildew (e.g. Kortekamp and Zyprian, 2003). It is an obligate biotrophic oomycete that grows in the intercellular spaces

High night temperature-induced accelerated maturation of rice panicles can be detected by chlorophyll fluorescence

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ABSTRACT

Rice panicle maturation is considered to be highly sensitive to environmental conditions. Since one of the factors accompanying global climate change is increases in minimum night temperatures more pronounced than those in maximum day temperatures, the effect of high night temperature (HNT) on rice panicle maturation was investigated. Two rice genotypes with contrasting HNT responses, N22 (highly tolerant) and Gharib (susceptible), were exposed to control temperatures (ca 23°C) and HNTs (ca 29°C) from flowering until maturity. Loss of photosynthetic activity and/or pigments during rice panicle maturation were evaluated temporally by measuring (i) effective quantum yield of photosystem II efficiency (Φ_{II}), and (ii) steady-state chlorophyll fluorescence level (F_S). To prove the accuracy of the new approach presented in this study, several vegetative indices were calculated from reflectance measurements and correlated with fluorescence parameters. It has been observed that Φ_{II} tracks the accelerated maturation of rice panicles exposed to HNT better than does F_S. Employing a newly identified chlorophyll fluorescence-based parameter could potentially enable larger genetic diversity scans and identification of novel genotypes with longer panicle maturation periods so as to increase rice yields directly under field conditions.

INTRODUCTION

Rice (*Oryza sativa* L.) production worldwide is affected by many environmental factors. As one of the main impacts of global climate change, minimum night temperatures are increasing at a much faster pace than are maximum day temperatures at a global as well as a farm level. Rice panicle maturation, including flowering and the subsequent grain-filling phase, is considered to be highly sensitive to temperature above a critical threshold. Negative effects on rice yield from high night temperatures (HNT) have been proven in controlled-environment studies and more recently by a field experiment (Shi et al. 2013), where reduced yields could potentially be a result of a shortened active grain-filling period. In addition, significantly shorter grain-filling periods under tropical conditions are in contrast to much longer periods under temperate conditions, and they are thus considered to be a key bottleneck for further yield enhancement. The main benefit of breeding programs would thus be in delaying panicle maturation, providing sufficient time for assimilates to fill grains efficiently. However, no progress or even attempt has been made in this direction due to the lack of a standardized phenotyping tool to ascertain and track grain-filling senescence over time.

Rice panicle maturation is accompanied by a change in colour as a consequence of disappearing chlorophyll. Given that based on chlorophyll spikelets' photosynthetic capacity has been found to be similar to that of flag leaves (Imaizumi et al. 1990), such disappearance and/or loss of photosynthetic capacity per unit chlorophyll can be temporally tracked by chlorophyll fluorescence (Chl-F). However, no reports had previoulsy been

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