ROS - To Live and Let Die

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Karlsruhe, den 01. Juni 2015

Holger Michael Rolf Ludwig

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Abstract

Zusammenfassung

Biotischer und abiotischer Stress auf Pflanzen verursacht weltweit enorme Schäden in der Landwirtschaft. Ein verringertes Wachstum sowie die dadurch verminderte Produktivität führen zu erheblichen Ertragsminderungen und Ernteausfällen. Um diesen Effekt zu minimieren, sowie dem erhöhten Bedarf an Lebensmitteln in der Zukunft gerecht zu werden, ist es notwendig, verbesserte Bedingungen für Pflanzenwachstum zu schaffen. Um dieses Ziel zu erreichen, ist es von großer Bedeutung die pflanzenspezifischen Signalwege zur Wahrnehmung, Verarbeitung und Antwort auf solche Stresssituationen zu identifizieren, um dann Pflanzen optimal in der Abwehr unterstützen zu können. An diesen Signalwegen sind stets mehrere Signalmoleküle - z.B. Ca^{2+}-Ionen, Reaktive Sauerstoff Species (ROS) oder auch Phytohormone wie Jasmonsäure und Abscisinsäure - beteiligt, die dann, je nach Intensität des Stresses, zur spezifischen Anpassung und Abwehr führen.

Als Modellsystem für die Analysen wurden zwei unterschiedliche Zellkulturen aus Weinreben verwendet, die sich in ihrer Anpassung an Stress deutlich unterscheiden. *Vitis rupestris*, eine Wildrebe, stammend aus dem Süden und Westen Nordamerikas, repräsentiert eine Art, die normalerweise in freier Wildbahn an sonnigen, felsigen Standorten lebt. Daher ist sie an trockene und warme Lebensbedingungen angepasst. Im Gegensatz dazu ist *V. vinifera* cv. 'Pinot Noir' noir eine Weinrebe, die von der Europäischen Wildrebe *Vitis vinifera ssp. sylvestris* abstammt und durch Züchtung

erhalten wurde. Dieses Kultivar zeichnet sich durch ihre hochwertige Weinqualität aus, lässt sich jedoch nur in den kühleren Anbaugebieten, z.B. in Mitteleuropa, kultivieren - in wärmeren Anbauregionen ist sie wegen ihrer schlechten Anpassung an Wärme, Wassermangel und Trockenheit weniger geeignet.

In dieser Dissertation wurde die Beteiligung von ROS als Signalmolekül - sowohl in Intensität als auch im zeitlichen Verlauf - unter Stresssituationen untersucht. Als Stresssituation wurde ionischer als auch osmotischer Druck in Form von NaCl und Mannitol eingesetzt. Beide Arten zeigten als schnelle Antwort auf ionischen und osmotischen Stress eine erhöhte Produktion an ROS. Dieser Anstieg war signifikant, vorübergehend und konzentrationsabhängig. Aus diesem Verlauf schließt sich die Funktion eines Signalmoleküls. Die Bildung dieses ROS-Signals durch diese Stressarten konnte mittels der Hemmung des Enzyms NADP(H)-Oxidase, das die Reaktionskaskade von O_2 über $^1O_2$, H_2O_2 und $OH^{\cdot}$ zu H_2O initiiert, komplett unterdrückt werden. Darüber hinaus konnte gezeigt werden, dass sowohl durch ionischen als auch osmotischen Stress die Sterberate der Zellen enorm anstieg. Der Effekt von osmotischem Stress war in allen Fällen schwächer als der von ionischem Stress, da dieser zur ionischen Komponente auch osmotischen Stress darstellt. Anschließende Studien zeigten einen Zusammenhang von ROS, dem Zellzyklus sowie dem Zelltod. Diese Korrelation zeigte sich in einem Anstieg an ROS beim Übergang der Zellen aus der ruhenden Phase zur Zellteilung. Die Hemmung des Enzyms NADP(H)-Oxidase, also die Unterdrückung der ROS-Bildung, führte genauso wie die stress-induzierte ROS-Erhöhung zum Zelltod. Die Fusion beider Experimente, einer Doppelbehandlung aus Stress und Inhibitor führte zu einem teilweisen Heilungseffekt - einer Reduktion der Sterblichkeit.

Im weiteren Verlauf dieser Dissertation wurde die Beteiligung von Abscisinsäure (ABA) als Interaktionspartner von ROS an diesen zellulären Reaktionen untersucht. Die Behandlung mit ABA führte zu einer Zunahme an Zellvolumen und gleichzeitiger Reduktion des ROS-Niveaus in der Zelle. Die Sterblichkeit der Zellen wurde durch

ABA nur minimal beeinflusst, jedoch konnte die stress-bedingte Sterblichkeit auch teilweise - in ähnlichem Maße wie durch die Hemmung der NADP(H)-Oxidase - geheilt werden. Zusätzlich konnte gezeigt werden, dass durch die Behandlung mit ABA, ein Anthocyan gebildet wurde. Dies ist sehr spektakulär, da die Bildung solcher Sekundärmetabolite in einer pflanzlichen Stammzelle sehr ungewöhnlich ist und normalerweise in ausdifferenzierten Zellen stattfindet.

Abstract

Worldwide biotic and abiotic stresses cause tremendous damage in agriculture. Reduced growth and productivity of stressed plants lead to substantial losses of yield and crop failure. To minimise this effect - as well as to cover the rising demand of foods in the future - it is necessary to work out better conditions for plant growth. To reach this goal it is very important to understand the plant-specific signal pathways for perception, processing and reaction to such stress situations, so that the plant can be supported optimally. Several Signal molecules - such as Ca^{2+} ions, reactive oxygen species (ROS) as well as phytohormones like Jasmonate und Abscisic acid - are participating and will lead to specific adaptation or defence.

Cell suspension cultures of two different grapevine species, which differ clearly in their adaptation to stress, were used for these analyses. *Vitis rupestris*, a wild species of grapevine, native to Southern and Western parts of Northern America, represents a species which usually inhabits rocky, sunny locations. Thereon it is well-adapted to dry and warm life conditions. In contrast to this *V. vinifera* cv. 'Pinot Noir' is a grapevine species, which has been derived from the European wild species *Vitis vinifera ssp. sylvestris* by breeding. One of the most important qualities of this cultivar it its valuable wine, but can just be cultivated in cooler areas, such as Central Europe - in warmer

regions *V. vinifera* cv. 'Pinot Noir' is not cultivated, because of its poor adaptation to heat, water deficiency, and drought.

In this dissertation the participation of ROS as a signal molecules - in intensity as well as in time-dependent change - in stress situations have been investigated. Osmotic an ionic pressure has been used as stressor. Both species showed a fast reaction to the stressor by induced production of ROS. This increase was rapid, significant, transient and concentration dependent. This results a function as signal molecule. This ROS production could be completely suppressed by inhibiting the enzyme NADP(H) oxidase, which initiates the reaction cascade starting from O_2 via $^1O_2$, H_2O_2, $OH^{\cdot}$ to H_2O. Further it could be shown that ionic and osmotic stressors lead to increased mortality. This effect was more stringent in case of ionic stress, because of the fact that ionic stress also comprises osmotic components. Further studies revealed a correlation of ROS, cell cycle and cell death. This correlation showed a induction of ROS when cell transition into mitosis. The inhibition of the enzyme NADP(H) oxidase - the suppression of ROS formation - does also lead to cell dead. The fusion of both experiments, a double treatment of stress factor and inhibitor - lead to a partial healing effect - a reduction in mortality.

In further experiments the participation of abscisic acid as an interaction partner of ROS has been investigated in these cellular reactions. The treatment of ABA leads to an increase of cell volume and simultaneously to a reduction of ROS levels. The mortality was just influenced minimally, but the stress-induce mortality could also be healed - comparable to the inhibition of the NADP(H) oxidase. Further it could be shown that ABA-treated cell produce a secondary metebolite compound, in this case an anthocyanin. This is very spectacular, because of the fact that secondary metabolite compounds are usually not produced in undifferentiated stem cells. Secondary metabolite compounds are usually an indication for differentiated cells.

Contents

Abbreviations

abbreviation	description
ABA	Abscisic Acid
AOC	allene oxide cyclase
AOS	allene oxide synthase
CAMTA	camodulin binding transcription activators
CDK4, CDK6	cyclin-dependent kinase
Chk1	Checkpoint kinase 1
ddH2O	double distilled water
DNA	deoxyribonucleic acid
DPI	diphenyleneiodonium chloride
E2F	transcription factor family E2F
gpx1	glutathione peroxidase 1
GTPase Rac/Rop	RHO-like GTPase
H_2O_2	hydrogen peroxide
H_3O	hydronium ion
JA	Jasmonic Acid
JAZ/TIFY	jasmonate ZIM/tify-domain
KOH	potassium hydroxyde
LEA	late embryogenesis abundant proteins

abbreviation	description
LOX	lipoxygenase
MDA	malone dialdehyde
MEP/DOXP	Methylerythritolphosphat/ 1-Desoxy-D-xylulose-5-phosphat-Synthase
MI	mitotic index
MS	Murshige and Skoog
MYC1, MYC2h, MYC3	transcription factor family named for myelocytomatose
NaCl	sodium chloride
NADP(H)	nicotinamide adenine dinucleotide phosphate
$^1OH_2$	superoxide radicals
$OH^{\cdot}$	hydroxyl radical
OPDA	12-oxophytodienoic acid
OPR3	OPDA reductase 3
OPC	3-oxo-2(2'[Z]-pentenyl)cyclopentane-1-octanoic acid
PCV	packed cell volume
PP2Cs	protein phosphatases which fall under the category of type 2C; some members are negative regulators of ABA-induced responses.
RCAR/PYR/PYL	regulatory components of ABA receptor/pyrabactin resistance protein1/PYR-like proteins; a family of START domain proteins; demonstrated to inhibit clade A PP2Cs.
RboH	Respiratory burst oxidase
RNS	reactive nitrogen species
ROS	reactive oxygen species

abbreviation	description
SDS	sodium dodecyl sulphate
SOS, SOS1, SOS2, SOS3	Salt Overly Sensitive
SnRK	Sucrose non-fermenting1-related protein kinases
TBA	2- thiobarbituric acid
TPL	Groucho/Tup1-type corepressor TOPLESS
WEE1	protein kinase found in *Schizosaccharomyces pombe*
ZIM/tify	zinc-finger protein expressed in inflorescence meristem

1 Introduction

1.1 Why is research on plant stress necessary?

Biotic and abiotic stresses cause tremendous problems in agriculture. Reduced growth and productivity of stressed plants lead to substantial losses of yield and cause a shortage of food supplies. In the year 2014 the estimated loss of yield was about 25% of the whole food production on earth. The biggest loss was estimated for wheat (in China 40 million tons, in the US 50 million tons; just in 2014). As a result that the world population is increasing faster and faster, malnutrition is one of the major factors for human diseases and the risk of being affected is getting higher and higher. The increase in world population, which is just one reason for malnutrition, will lead to a situation that the agricultural production has to be enhanced by more than 50% over the next decades to cover the rising food demand for our human population. This problem is enhanced further by the fact, that the society demands more and more products of ecological cultivation, which leads to the necessity of new growing methods to keep and increase in yield.

Facing these facts it will not be easy to improve the state of malnutrition. Combined with the situation that agricultural areas are more and more irreversibly degraded this will lead to several additional problems. At first, water limitation is considered as one of the major restricting factors in many countries. Second, non-sustainable farming and soil erosion often destroy agricultural areas significantly, but also the creation of new industrial fields or economical and structural reorganisation leads to a reduction in agricultural usable

areas. Third, the growing impact of the global climate changes seriously affect crop growth. Fourth, the usage of crops has changed to keep the balance of carbon dioxide in the atmosphere, which is under suspicion influencing global warming. Therefore crops are increasingly used for expanding biofuel production.

All of this leads to a loss in agricultural area ore to a decrease in availability of food which results in increasing prices, which mostly affects people in poor countries. Considering the fact that the agricultural area will not increase tremendously, the demand for food supplies has to be covered almost exclusively by increased productivity. Therefore it is important to understand how growth and yield can be promoted, which situations represent stress for a plant, and which defence mechanisms plants do have.

1.2 What does stress mean for a plant?

During a life cycle organisms experience favorable and non-favorable conditions. In the latter case stress is imposed on organisms, which needs to be handled. In former times the concept of stress was often used imprecisely for a wide range of situations. Therefore many rough assembled terms have been proposed until the biomedical scientist Hans Seyle (Seyle, 1936) coined the term “general adaptation syndrome” as a biomedical concept in stress, which can be described by several steps. At first a detection and general alarm occures, followed by a multi-step cellular reaction by producing several different defence-related metabolites and finally initiating counteraction by the trial to eliminate the stressor.

But stress in plants is different from those in other organisms. Since plants use photosynthesis for energy generation they need a large surface. While living as an aquatic organism, that was no problem, but since the shore leave in the Ordovician (about 460 to 480 million years ago), plants were faced to big static problems and had to change to a sessile way of life because of this. This immobility results in the requirement of monitoring permanently the surrounding environment. Therefore plants need several

possibilities of adaptation and defence mechanisms to possible stressors. A further fact is that a situation which is stressful for one species does not necessarily be a stress factor for another. Moreover, even individuals or varieties in one species react different in the same stress situation.

However, it is possible to transfer the stress concept of Seyle (Seyle, 1936) to plant stress, as it has been worked out for seed stress (Kranner et al., 2010). In this example, the first "general alarm" phase results in post-translational modifications and stress signaling by the cross-talk of reactive oxygen species (ROS), reactive nitrogen species (RNS), and phytohormones (like Jasmonic Acid or Abscisic Acid) to challenge seeds to induce transcriptome modifications. The second phase, "adaptation", is reached when the resistance response has been successively assembled the and the final step, the so-called exhaustion phase, can occur resulting in seed death.

A widely accepted definition considers plant stress as any external factor that negatively affects the plant (Taiz and Zeiger, 2010). These stress factors are divided into two groups: biotic or abiotic stress. Biotic stress refers to biological factors such as insects, weeds, and pathogens. Whereas, abiotic stress includes stress situations like nutrient deficiency, salinity, drought, flooding, heat, cold and mechanical damage. All of these stressors are very different - and if a plant wants to survive - have to lead to a specific reaction by the plant. In order to this plants must have several signals pathways - including several signal molecules - to react to different kinds of stress. All these signal molecules do not act individually - they all interact in a small-meshed network.

1.3 Stress signalling in plants

1.3.1 Calcium as a second messenger

Calcium is the most famous ubiquitous second messenger in cells over all species. However, calcium is essential for life, but continuous high levels of calcium activate degadation pathways or apoptosis. Therefore the intracellular Ca^{2+} homeostasis has to be tightly regulated. This homeostasis is achieved by several different mechanisms like cell export or compartimentalization. The normal level of Ca^{2+} is about 100-200 nM, in organelles it is about 1-2 mM. Many Ca^{2+} channels have been detected by patch-clamp studies, which export Ca^{2+} out of the cell or into compartments like chloroplasts, mitochondria or the vacuole (White, 2000). When a Ca^{2+} channel opens, it transports approximately a million Ca^{2+} ions per second driven by the concentration-gradient between the apoplast and the cytoplasm (Clapham, 2007). It has been shown that Ca^{2+} signals are a result of Ca^{2+} release from internal storages (Peiter, 2011), which is then processed in a signal pathway as seen in biotic or abiotic stress situation (Hirschi, 2001).

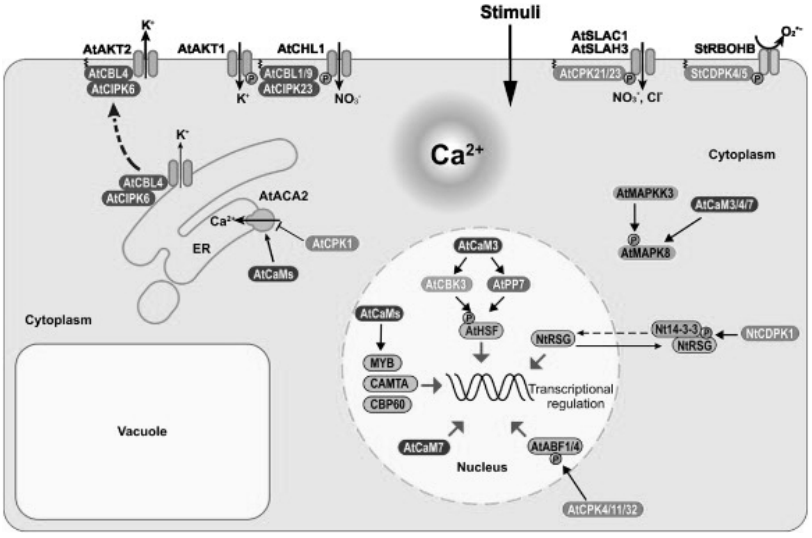

Figure 1.1: Calcium signaling in plants (Batistic and Kudla, 2012).

Such a change in Ca^{2+} levels is processed by adaptor proteins or Ca^{2+}-modulated proteins which function as sensors for the Ca^{2+} signals and regulate down-stream signalling events. Some of these down-stream signalling events are shown in Fig 1.1, all of them have been identified in plants like *Arabidopsis*, tomato or tobacco (Batistic and Kudla, 2012). Ca^{2+} signals are highly connected to signalling pathways involved in the response to nearly all developmental, hormonal, and stress situations. Whether a stress challenge will be met by successful adaptation depends on the timely and balanced activation of these cellular and molecular events (Ismail et al., 2014).

Furthermore Ca^{2+} also plays a crucial role in salinity tolerance involving different signalling elements. Upon salinity several calmodulin binding transcription activators are upregulated like CAMTA1 to 6 (Yang and Poovaiah, 2002). The Salt Overly Sensitive (SOS) signaling pathway that comprises the SOS proteins is activated through phosphorylation. SOS3/SOS2 complex phosphorylates SOS1 which leads to the export of Na^{+} out of the cell (Zhu, 2002). Additionally, Ca^{2+} also acts directly without the need for a protein adaptor. In the year 2006 the salt stress-responsive gene 1 (AtNIG1), the first known calcium-binding transcription factor, which is involved in salt-stressed plant, was identified (Kim and Kim, 2006). Further Ca^{2+} ions interact also with H_2O_2, ABA and JA in the activation of the potassium outward channels causing stomatal closure (Allen et al., 2000). Beside Ca^{2+} also the Apoplatic Alkalinisation plays an important role for the stress adaptive reaction of plant.

1.3.2 Apoplastic Alkalinisation

The apoplastic alkalinisation is another fast reaction of the cell, which is involved in the stress adaption and defence process. Alkalinisation - a rise in pH - is a result of a change in H_30^{+} ion concentration. The pH scale is a model calculating the concentration of H_30^{+} in a negativ logarithmic manner. In cells this pH value is monitored and regulated by proton transport mechanisms in the plasmalemma and by buffering in the cytosol (Kurkdjian and Guern, 1989). This regulation is very important since proton activity

acts either directly or by cross-talk with Ca^{2+} or phytohormones. E.g. cytoplasmic alkalinisation transmits Jasmonate and Abscisic Acid signalling during stomatal closure (Suhita et al., 2004). Moreover, several studies revealed that plant cells respond on an elicitor induced stress by a rapid and transient increase of apolastic pH. E.g. tomato cells elicited by chitin or bacterial flagellin (Felix et al., 1993, 1999), root hair cells of *Sinapis alba* elicited by fusicoccin (Bertl and Felle, 1985) and *Vitis* cells elicited by the bacterial elicitor Harpin (Qiao et al., 2010). Additionally apoplastic alkalinisation is also involved in plant responses such as salinity, auxin, and gravity (Gao et al., 2004; Fasano et al., 2001; Kurkdjian and Guern, 1989). And - at last - until now there are many more contexts in which apoplastic alkalinisation is discussed as a signal, e.g. drought, light, and microbial stress (Felle, 2001), in which also Reactive Oxygen Species seem to be involved.

1.3.3 Production of ROS

Reactive Oxygen Species (ROS) are continuously produced in plant compartments like mitochondria, chloroplasts or peroxisomes as a by-product of metabolic processes like photosynthesis or respiration. The accumulation of ROS causes oxidative damage to DNA, proteins, carbohydrates and lipids. However, they also could function as signaling molecules regulating responses of development and various aspects of stress. Therefore ROS represent a good example to illustrate the importance of stress signatures (Mittler et al., 2010).

This group of chemically reactive molecules is produced by partial reduction of oxygen (Fig 1.2). In this pathway, the so called primary ROS - singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$) - are produced from ground state dioxygen (O_2) by a stepwise transfer of electrons through the enzymes NADP(H) oxidase (from O_2 to $^1O_2$); superoxide dismutase (from $^1O_2$ to H_2O_2), and by interaction with Fe^{2+} ions (from H_2O_2 to $OH^{\cdot}$) (Krieger-Liszkay, 2005; Halliwell, 2006). This sequence of reactions is initiated by the NADP(H) oxidase, which is located in the plasma membrane,

and thus plays a central role for stress signalling (Torres and Dangl, 2005). These primary ROS are mainly responsible for lipid peroxidation and underlie the so called oxidative burst (Gill and Tuteja, 2010).

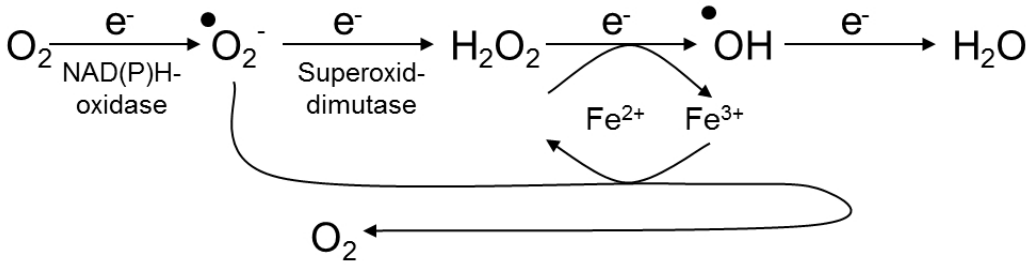

Figure 1.2: The pathway of univalent reduction of O_2 (Liszkay et al., 2004).

This oxidative burst is initiated by singlet oxygen and leads to peroxidation of membrane lipids. The peroxidation seems to be stoichiometric, i.e. one ROS will produce just one oxidised lipid product rather than triggering a chain reaction (Triantaphylides et al., 2008; Triantaphylides and Havaux, 2009). To prevent the cell from damage, singlet oxygen is oxidised by superoxide dismutase to hydrogen peroxide. Hydrogen peroxide, a lower reactive species is very instable. In presence of Fe^{2+} ions hydrogen peroxide is converted to hydroxyl radicals, which are very dangerous to the cell, because they are much more reactive. Hydroxyl radicals abstract hydrogen from fatty acids, which in turn oxidises additional lipids in a chain reaction - resulting in the accumulation of malone dialdehyde (MDA). This peroxidation leads to degradation and conformational changes of membrane lipids, which results in an extension of the bilayer as well as an increase of its fluidity and permeability (Wong-Ekkabut et al., 2007). Therefore, the abundance of MDA as end product of lipid peroxidation is a good indicator for the amount of produced ROS and thus the amplitude of oxidative stress.

It is likely, that ROS are involved in stress processing and adaptation simultaneously and as well are utilized for the regulation of normal growth. ROS participate in numerous stress responses, act as second messengers of Abscisic acid (Kwak et al., 2003), for instance, by activating Ca^{2+} channels during stomatal closure (Allen et al., 2000) while

drought stress. Singlet oxygen as primary ROS is not only an important stress signal, but participates in the activation of the small GTPaseRac/Rop proteins by auxin and thus represents an early transducer in auxin signaling (Wu et al., 2011). Modulation of the oxidative stress level in the fungus *Asperillus nidulans* activates production of mycotoxins and induces conidiation (Zheng et al., 2015). For example in salt stress, increased H_2O_2 levels lead to the activation of the glutathione peroxidase 1 promoter (gpx1) in tobacco (Avsian-Kretchmer et al., 2004). Furthermore, it could be shown, that anionic glutamate dehydrogenases, the enzyme for proline synthesis, is induced by salt-stress stimulated ROS formation (Skopelitis et al., 2006). These examples illustrate that sub lethal levels of stress and the resulting stress signals can also be used for promoting cell growth and cell proliferation by activation of ion channels, defence genes and stomatal closure (Allen et al., 2000). However, cell growth and cell proliferation is not just controlled by ROS - control of cell growth and cell proliferation is also done by phytohormones.

1.3.4 The role of phytohormones

Phytohormones are molecules, which coordinate plant life by biochemical activity. Until now many phytohormones like auxin, abscisic acid, jasmonatic acid, gibberellin, ethylene or cytokinins have been identified. These phytohormones have been studied very well in the last century, because of their important functions in the plant. Furthermore there are many other molecules, which also belong to this group of active molecules, like brassinosteroids, salicylic acid and nitric oxide. These are just the most famous phytohormones - all of them are involved in stress adaption, stress defence, plant growth and development. Among those plant hormones, jasmonic acid and abscisic acid play a central role in plant adaptation to stress.

Jasmonatic Acid and its derivatives

Jasmonates (JA) - Jasmonic Acid and its related molecules - are members of the oxylipin family, which are formed by oxidation of polyunsaturated fatty acids. Jasmonate is derived from α-linolenic acid via the octadecanoid pathway (Wasternack, 2002). Several key enzymes, including, lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC), which are located in the chloroplasts, catalyze the biosynthetic pathway from α-linolenic acid to 12-oxophytodienoic acid (OPDA). OPDA reductase 3, located in the peroxisome, reduces OPDA to OPC, which in turn is converted to Jasmonate via several steps of beta-oxidation (Browse, 2009; Wasternack, 2002).

The molecule which could be isolated and identified first was methyl jasmonate (Demole et al., 1962) and got accepted as a phytohormone, when a role in plant stress adaptation became clearer. JA fulfils the function as the master switch in plant responses to abiotic and biotic stress situations. Drought, salt stress, mechanical wounding as well as pathogen infection and insect attack (Wasternack, 2007) are among these stress situations. JA regulates gene expression of plant defence proteins (jasmonate-induced proteins). E.g. enzymes of phytoalexin synthesis (e.g. resveratrol in grapevine) are upregulated and genes for housekeeping proteins such as Rubisco are down regulated (Wasternack, 2007). Moreover, Jasmonates regulate development and growth such as fruit ripening, seed germination, root growth, leaf abscission and senescence.

However, in response to environmental stress or developmental signals that stimulate the biosynthesis of JAs, the elevated level of jasmonate isoleucin promotes the interaction of JAZ/TIFY repressors by binding to Coi1, an F-Box protein. This complex is part of the SCF-komplex which funcions as a E3 ubiquitin ligase which results in an polyubiquitination of JAZ/TIFY proteins. This followes the degradation of JAZ/TIFY proteins via the 26S proteasome, which results in release of MYC transcription factors (MYC1, MYC2h, MYC3) (Wager and Browse, 2012). Activation of MYC2 induces transcription of early JA-responsive genes including the JAZ/TIFY genes themselves.

(Chung et al., 2009). Moreover, it also acts as a transcriptional repressor mediated by the interaction to the Groucho/Tup1-type corepressor TOPLESS (TPL), which thereon regulate JA responses negativly (Pauwels et al., 2010). These interactions of JA show a strong correlation to stress, in contrast to this Abscisic Acid ist more involved in the regulation of growth.

Abscisic Acid

Abscisic Acid (ABA) has been identified by two research groups in the 1960s, which studied different effects in plants. On the one hand it was observed that ABA is involved in the abscission of leaves, on the other hand it could be shown that it supports dormancy. ABA belongs to the group of sesquiterpenes and is synthesized in plastids by two pathways - the Mevalonat pathway and the MEP/DOXP pathway (Methylerythritolphosphat/1-Desoxy-D-xylulose-5-phosphat-Synthase). The basic molecule is the isopentenyl diphosphate structure xanthoxal and is formed by oxidative cleavage of the tetraterpenoid structure of neoxanthin. Xanthoxal is transported to the cytoplasm, where it is converted to ABA (Taiz and Zeiger, 2010).

The function of ABA is very versatile. ABA regulates numerous developmental processes like dormancy, inhibition of root growth, inhibition of shoot growth and regulation of germination and seed development. Furthermore, ABA leads to the synthesis of storage reserves and late embryogenesis abundant (LEA) proteins during the induction of seed dormancy (Xiong and Zhu, 2003). Additionally ABA plays a central role in the adaptive response to several stress situations like drought, salt and pathogen infection. Reducing the transpirational water loss while salt or drought stress ABA is involved in stomatal closure. This closure is a multi-step reaction in which Ca^{2+} channels in guard cells are activated (Allen et al., 2000) which results in a turgor reduction. This leads to effusion of water and the stomata is closing. But there are more well-known functions of ABA in plants. ABA also induces the production of proteins to protect membranes (Hasegawa

et al., 2000; Zhu, 2002) or the accumulation of anthocyanins (Jackson, 2008). The role of ABA is antagonistic to other plant hormones like Auxin, Ethylene or JA (Taiz and Zeiger, 2010).

ABA perception and signalling have been extensively studied by biochemical, molecular and genetic approaches (Cutler et al., 2010; Hasegawa et al., 2000; Xiong and Zhu, 2003; Zhu, 2002). In 2009 the pyrabactin resistance 1 receptor was identified, which contributes a lot of understanding in ABA signalling (Ma et al., 2009; Park et al., 2009). This receptor belongs to the family of soluble ABA-binding proteins, which undergoes a conformal change after ABA binding (Nishimura et al., 2009). In the absence of stress factors, the ABA signal is switched off by the clade A protein phosphatases, that negatively regulate downstream kinases. After salt- or droughtstress inducing, the receptor is activated by binding ABA, a conformational change is induced in the receptor family PYR/PYL/RCAR, which subsequently binds and inhibits the protein phosphatase type PP2Cs, which results in the activation of the sucrose non-fermenting1-related protein kinases (SnRK2.2, SnRK2.3, and SnRK2.6). These kinases phosphorylate transcription factors, or activate ion channels or NADP(H) oxidases (Umezawa et al., 2010; Hubbard et al., 2010).

Until now many downstream targets of ABA signalling, like transcription factors and second messengers, have been identified (Cutler et al., 2010; Hasegawa et al., 2000; Allen et al., 2000) - many act in stress adaptation, defence as well as cell development and cell proliferation.

1.4 Stress signals are involved in cell proliferation

Cell proliferation, cell division and cell growth are nearby related to each other. All these comprise developmental steps in the plants, which are effected by abiotic stress factors. Cell division is the best regulated mechanism in the cell. In prokaryotic cells, cell division is just regulated by environmental factors and not by cell-internal

checkpoints. In eukaryotic cells this is completely different. The eukaryotic cell cycle comprises mainly four phases. (I.) The M-phase (mitosis), in which the cell is dividing (condensing, sorting and separating the chromosomes and the cytokinesis); (II.) the G_1-phase, which is a gap phase; (III.) the S-phase, in which the DNA is replicated and (IV.) the G_2-phase, which is a second gap phase. As a reason, that in most organisms eukaryotic cells work together and have to sense and to keep responsible for each other, so that there is no unstructured cell division, the transition of these phases are strongly monitored and regulated by so-called checkpoint kinases (Campbell and Reece, 2009).

In mammals the transcriptional repressors Rb (retinoblastoma), p107 and p130, which bind E2F transcription factors (inactivation by binding activator E2F transcription factor E2F1-3) stop G_1-to-S transition. Once the decision to progress comes cyclin D level rises and forms a complex with checkpoint kinases (CDK4 and CDK6). This complex phosphorylates E2F transcription factor complexes. As a result these E2F transcription factor complexes are degraded and the transcriptional activation of downstream targets to promote the G_1-to-S transition are activated (Bertoli et al., 2013). A second checkpoint is between G_2 and M-phase. The mechanism is similar to the checkpoint in G_1-to-S transition. Activation of Chk1 holds the cell in the G_2 phase until it is ready to enter the mitotic phase. This delay leads to DNA repair immediately, or in case of irreversible DNA damage, to programmed cell death (Meuth, 2010). In plants such proteins can also be found, e.g. in *Arabidopsis* the protein WEE1 inhibits Chk1 by phosphorylating which results in a delay of M-phase entry (Cools et al., 2011). Also stress induced signal molecules like Ca^{2+}, ROS or phytohormones are interacting within the cell cycle control kinases (Verbon et al., 2012). Especially the role of ROS is increasingly focussed.

Then singlet oxygen - produced by the NADP(H) oxidase RboH - seems to play a dual role, as a factor transducing the auxin-triggered activation of phospholipase D through Rac/Rop proteins (Wu et al., 2011) and, simultaneously, acts as a central signal in stress adaptation and defence. Moreover, the role of singlet oxygen is more

complex than expected until now, e.g. the modulation of the oxidative stress level in the fungus *A. nidulans* activates production of mycotoxins and induces conidiation (Zheng et al., 2015). Moreover, a leak in ROS will not only arrest proliferation, but channel the arrested cells for cell death. Further it is suggested, that, similar to animal cells, non-differentiated plant cells will die, if they are not allowed to divide.

To summarise - stress adaptation binds energy - resources that otherwise would be used for growth and development. These resources have to be repartitioned, and this will impinge on fitness during the interaction of the stressed plant with non-stressed competitors (Stamp, 2003). Therefore, stress adaptation has to be regulated and tuned with normal development, which means that stress-signalling must somewhere interact and overlap with those signals that govern normal growth and development.

1.5 Why do we use grapevine as a model in plant biology?

Every model organism in biology - whether in botany, zoology or microbiology - has been chosen because of its own qualities. As a plant most famous are *Arabidopsis thaliana*, *Nicotiana tabacum* and *Oriza sativa*. E.g. *Arabidopsis thaliana* has been chosen, because of its easy growth conditions, fast generation cycle and small genome size. Based on this qualities, many genetic approaches have been developed for *Arabidopsis thaliana*, which improved the status as an experimental model. Also *Arabidopsis thaliana* has been studied extensively as a model to analyse stress pathways (Ma et al., 2006), including several genomic and proteomic analyses (Jiang, 2007; Zhu, 2000), but the economical and agricultural value of *Arabidopsis thaliana* is not important. *Nicotiana tabacum*, as well a very famous model plant for botanists, can be easily cultivated and modified genetically. Therefore *Nicotiana tabacum* is commonly used to study pattern formation

and plant cell interaction by the use of cell suspension cultures. The use of *Oriza sativa* or *Vitis vinifera* is mainly motivated by zhe importance for human life.

Grapevine is the economically most important fruit species worldwide. It has been domesticated about 7000 years ago and is correlated to human civilisation in nearly all parts of the European and Asian continent. Therefore grapevine has reached important traditional, religious, and economic values. In the year 2013, about 7.2 million hectares agricultural area have been cultivated with a yield of 77 million tons worldwide. This represents about 10% of all yields of fruits (FAOSTAT data, 2010. http://faostat.fao.org). Because of its major importance for mankind, grapevine has been intensively studied for functional genetics and is the third plant, after *Arabidopsis* and rice, for which a genome project had been completed. Moreover, grapevine as a model plant is increasingly focussed of applied plant research, especially for stress adaptation and stress defence research. The reason for this intensive research for stress adaptation and stress response of grapevine is becuase grapevine is a plant, which is effected very easily by plant pathogens. The amount of pesticides applied to cultivated grapevine areas is higher than compared to any other crop plant. The estimated amount of pesticides, which is used in viticulture (3.5 % of the cultivated area in Germany), is about 15 % of the total pesticide usage (in Germany) (http://www.dlr.rlp.de).

Furthermore to its huge part in social life grapes also contain health-promoting contents. In former times wine has been used in precise amounts for dietary purpose or to treat chronic diseases. This effect is related to the ability of grapevine to accumulate specific phytoalexins, e.g. resveratrol, at different developmental stages, which is very interesting because of its anti-inflammatory impact. These remarkable characteristics - along with the availability of the *Vitis* genome - raise grapevine to an excellent model to stress related plant adaptation and defence.

1.6 Aim of this study

The way how plants sense stress and find their way to defend and adapt is of great interest of plant biologists. In the last decades many stress elicitiors could be identified, which leads to an improved knowledge in the interaction of different organisms. Furthermore, intensive research projects generated a lot of information about plants signalling in defence and adaption mechanisms in short and long-term response. This response is performed by the plant by either small simple molecules (e.g. Ca^{2+}, ROS) or complex structures (e.g. JA, ABA), which have to be synthesised in specific pathways.

However, for some hormones, it is not clear, whether their increase is adaptive or simply a manifestation of stress damage, e.g. it has been shown, that JA is induced by osmotic stress, but is also increased in salt tolerant plants (Pedranzani et al., 2003). JA is just one example (and JA research lasts for more than 50 years), in which there is no total clearity about the function in plants. This situation is made worse by the amount of signal molecules we identify and by the fact that there is an interplay between all these signal compounds. Further it has been shown that many of these signal molecules can be connected to different kinds of stress, e.g. Ca^{2+} shifts are a result of mechanical, cold, and pathogenic stress. The difference in these signals seems to be a time-dependent process - the so-called signature - not the appearance of a signal molecule. ROS - small radical molecules - are further very important signal molecules. ROS are just studied in the last decade after it was obviously clear that they seem to play a bigger role in the plant cell than it has been expected.

Initially ROS had been supposed to be toxic as chemical radicals, which lead to oxidation of DNA, proteins or lipids, which harms the cell. But meanwhile this opinion has changed and several results show that ROS are involved in a small-meshed network of signal molecules in the plant to adapt or defend against stress situation. One of these biological functions of ROS is the activation of phospholipase D through Rac/Rop proteins (Wu et al., 2011) or an activation of mycotoxin production and thereon

promotion of conidiation (Zheng et al., 2015). Further it has been shown that ROS homeostasis is involved in stomatal closure (Allen et al., 2000) and also is changed due to light and heat stress (Suzuki et al., 2013). Based on these findings it was clear that ROS do have a singal character in stress adaptation and defence, but there are several question which are unsolved until now:

1. Which role do ROS really play in plant cells?
2. How is the signal signature of ROS?
3. What happens, if ROS homeostasis is changed?
4. Do ROS concern influence development and proliferation?
5. Do ROS interact with other signal compounds?
6. Does ABA influence ROS production?
7. Does ABA influence ROS-mediated cell development?

2 Materials & Methods

2.1 Materials

A list of all chemicals and their producers is shown below. Buffers and solutions were solved in demineralized water using a Milipore device (ddH_2O) and autoclaved for 20 min and 121° C temperature at 1 bar overpressure. Heat sensitive chemicals were filter sterilized using a 0.22 μm grid size PVDF filter.

2.1.1 Used chemicals

Table 2.1: content of MS-medium

Chemical	Amount	Company
MS salts	4.3 g/l	Duchefa, Haarlem, The Netherlands
Sucrose	30 g/l	Carl Roth, Karlsruhe, Germany
Potassiumdihydrogenphosphat	0.2 g/l	Merck, Darmstadt, Germany
(myo)-Inositol	0.1 g/l	Sigma-Aldrich, Steinheim, Germany
Thiamine	1 mg/l	Carl Roth, Karlsruhe, Germany
2,4-Dichlorophenoxyeacetic acid	0.2 mg/l	Sigma-Aldrich, Steinheim, Germany
pH adjusted by KOH to 5.8		Carl Roth, Karlsruhe, Germany
Agar danish	8 g/l	Carl Roth, Karlsruhe, Germany

Table 2.2: Used chemicals for cell treatment

Chemical	Company
Absisic acid	Sigma-Aldrich, Steinheim, Germany
Diphenyleneiodonium Chloride	Sigma-Aldrich, Steinheim, Germany
Mannitol	Carl Roth, Karlsruhe, Germany
Sodium Cloride	Carl Roth, Karlsruhe, Germany
Ethanol 100%	Carl Roth, Karlsruhe, Germany
Dimethylsulfoxide	Carl Roth, Karlsruhe, Germany

Table 2.3: Used chemicals for determination of cell viability

Chemical	Amount	Company
2.5% w/v Evansblue	25 mg/ml	Carl Roth, Karlsruhe, Germany
Hoechst 33258	1 mg/ml	Sigma-Aldrich, Steinheim, Germany

Table 2.4: Used chemicals for MDA quantification

Chemical	Amount	Company
sodium phosphate buffer pH 7.4	10 mM	Merck, Darmstadt, Germany
sodium dodecyl sulfate	8.1% (w/v)	Carl Roth, Karlsruhe, Germany
acetic acid (pH 3.5)	20% (w/v)	Merck, Darmstadt, Germany
2-thiobarbituric acid	0.8% (w/v)	Sigma-Aldrich, Steinheim, Germany
ddH_2O		

Table 2.5: Used chemicals for HPLC analysis

Chemical	Amount	Company
acetonitrile		Merck, Darmstadt, Germany
methanol		Merck, Darmstadt, Germany
formic		Merck, Darmstadt, Germany
ddH_2O		

2.1.2 Used materials

Table 2.6: Used materials

Material	Company
sterile filter 0,22 μm	Carl Roth, Karlsruhe, Germany
StrataX 33 μm Polymeric Reversed filter column	Phenomenex, Aschaffenburg, Germany
Synergie HYDRO-RP column	Phenomenex, Aschaffenburg, Germany
falcons - cellstar® Tubes	Greiner Bio-One GmbH, Frickenhausen, Germany
metal beads	Carl Roth, Karlsruhe, Germany
Eppendorf tubes	Diagonal GmbH, Münster, Germany

2.1.3 Used equipment

Table 2.7: Used equipment part 1

Equipment	Type	Company
laminar flow bench	Heraeus Hera guard	Thermo Scientific, Langenselbold, Germany
orbital shaker	KS 260 basic	IKA Werke GmbH, Stauffen, Germany
millipore device	SG millipore device	SG GmbH, Barsbüttel, Germany
autoclave	Systec VE-95	Systec, Wettenberg, Germany
thermoblock	Thermomix 5436	Eppendorf, Hamburg, Germany
centrifuge	Heraeus Pico17	Thermo Scientific, Langenselbold, Germany
centrifuge	Hermle Z232K	M&S Laborgeräte, Wiesloch, Germany
photometer	Uvicon 930	Kontron, Rossdorf, Germany
microscope	Axio Imager Z1 Apotome	Carl Zeiss AG, Jena, Germany
tissue lyzer	Tissue Lyzer	Qiagen, Hilden, Germany
ice mashine	Scotsman AF 80 AS-E	Scotsman Ice Systems, Milan, Italy
vacuum pump	CVC2	Vacuubrand, Wertheim, Germany
ultrasonic device	UP100H	Hielscher Ultrasonics GmbH, Teltow, Germany
HPLC	Agilent 1200	Agilent Technologies, Santa Clara CA, USA
glassware		Schott, Mainz, Germany
pipettes		Eppendorf, Hamburg, Germany

2.1.4 Used software

Table 2.8: Used software

Software	Company
AxioVision Rel. 4.8 Software	Carl Zeiss AG, Jena Germany
Microsoft Office Excel	Microsoft Cooperation, Redmond, USA
Matlab	Mathworks, Ismaning, Germany
Vector-NTI	Invitrogen, Darmstadt, Germany

2.2 Methods

2.2.1 Cultivation & Treatment

Cultivation of cell lines

Suspension cell cultures of *V. rupestris* and *V. vinifera* cv. 'Pinot Noir' were used in all experiments. They were cultivated in liquid MS-medium containing 4.3 g/l Murashige and Skoog salts, 30 g/l sucrose, 200 mg/l KH_2PO_4, 100 mg/l inositol, 1 mg/l thiamine and 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), pH was set to 5.8 by KOH (Tab. 2.1). Cells were subcultured weekly when they reached stationary phase by inoculating 5 ml into 30 ml of fresh medium in 100 ml Erlenmeyer flasks. The cell suspensions were incubated at 26 °C in the dark on an orbital shaker (Tab. 2.7) at 150 rpm. As a back-up, calli were subcultured monthly on the same medium solidified with 0.8% (w/v) Danish agar. Both cell lines *V. rupestris* and *V. vinifera* cv. 'Pinot Noir' were generated from leaves as described in Seibicke (Seibicke, 2002).

Treatment of cell lines

To induce cellular responses, the suspention cell cultures were treated with aequous solutions of either sodium chloride (NaCl) or mannitol. Experimental concentrations were 100 μM and 200 μM for sodium cloride, 170 μM and 340 μM for mannitol. Furthermore cells were treated with diphenyleneiodonium chloride (DPI) with concentrations of 0.2 nM, 2 nM and 20 nM. DPI was dissolved in dimethylsulphoxide (DMSO). The treatment with abscisic acid was done with concentrations between 1 and 100 μM, which was dissolved in Ethanol. Negative controls contained the corresponding concentrations of solvent without the active ingredient (Tab. 2.2).

2.2.2 Groth-related mesuremnts

Quantitative analysis of mortality

To determine, how many cells were killed due to the applications, mortality was measured using an assay based on the membrane-impermeable dye Evans Blue (Gaff and Okong'O-Ogola, 1971). 500 μl of the treated cell suspension culture were drained in a custom-made staining chamber using a sieve with a mesh-size of 70 μm, and then transferred into 1 ml of 2.5% Evans Blue (w/v) in MS medium (Tab. 2.3). After incubation for 5 min, the dye was drained and cells were washed three times for 5 min with fresh MS medium. Living cells did not take up the dye, whereas dead cells could be detected rapidly by a strong blue staining. The mortality was determined as relative proportion of stained cells out of a sample of 300 cells scored for each data point in three independent measurements by using differential interference contrast microscopy (Tab. 2.7).

Quantitative analysis of mitotic index

To investigate the proliferation status of treated cells, the mitotic index (MI) was measured as described in Maisch & Nick ((Maisch and Nick, 2007)) by using the

nuclear dye Hoehchst 33258 (2'-(4-hydroxyphenyl)5-(4-methyl-1-piperazinyl)-2,5' -bi(1H-benzimidazole)-tri-hydrochloride) (Tab. 2.3). 1 ml of the treated cell suspension culture was transfered to a new reaction vessel and 1 μl of 1 mg/ml Hoechst 33258 was added. The MI was determined as relative proportion of mitotic cells - cells in prophase, metaphase, anaphase, and telophase - from a sample of 500 cells scored for each data point in three independent measurements. The MI samples were viewed by fluorencence microscopy (Tab. 2.7) using the filter set 49 (excitation at 365 nm, beamsplitter at 395 nm, and emission at 445 nm).

Quantitative analysis of packed cell volume

To monitor culture growth after treatment, the packed cell volume (PCV) was recorded as described in Kühn et al. (Kühn et al., 2013). The increment in PCV was used as measure of the growth response. Each data point represents the mean from three independent duplicate measurements collected 5d after subcultivation. The non treated sample was set to 100 %. In each independent experiment duplicates were measured and the total experiment was repeated twice, so that in total 6 values were collected.

2.2.3 Detection of oxidative burst

Quantitative analysis of malon dialdehyde

The lipid peroxidation level in plant tissues was determined by measuring the malon dialdehyde (MDA) content via a 2-thiobarbituric acid (TBA) reaction (Hodgson and Raison, 1991). To test the response of MDA content to different stress situations, stationary cells (at day 7 after subcultivation) were treated with different concentrations of salt (NaCl), mannitol or DPI (diphenyleneiodonium chloride), respectively. Samples were collected at specific time points up to three hours after onset of the treatment. Samples were centrifuged at 8,000 g for 5 min at room temperature and the supernatant was removed and discarded. Further the sediments were shock-frozen in liquid nitrogen and homogenized with 5 mm steel beads by using a tissue lyser (Tab. 2.7). 1 ml of

10 mM sodium phosphate buffer (pH 7.4) was added to each sample, homogenized and briefly spun down. 200 μl of the supernatant were added to a reaction mixture containing 100 μl of 8.1% (w/v) sodium dodecyl sulfate (SDS), 750 μl of 20% (w/v) acetic acid (pH 3.5), 750 μl of 0.8% (w/v) aqueous TBA, and 200 μl of Milli-Q water (Tab. 2.4). An identical reaction mixture in which the 200 μl of supernatant were substituted by an equal volume of buffer was simultaneously set up as blank. Both reaction mixtures were then incubated at 98 °C for 1 h. After cooling down to room temperature, the mixtures were centrifuged for 5 min. Absorbance at 535 nm was measured and corrected for nonspecific absorbance at 600 nm. The level of lipid peroxidation was expressed as micromoles of MDA as derived from the difference in absorbance at 535 and 600 nm using an extinction coefficient of 155 * mM^{-1} * cm^{-1}. In each independent experiment triplicates were measured and the total experiment was repeated twice, so that in total 9 values were collected.

2.2.4 Anthocyanin determination

extraction of anthocyanins

The cell suspension cultere of *V. vinifera* cv. 'Pinot Noir' was treated with 100 μM absisic acid while subcultivation. On day 4 after subcultivation the colour in the treated cell suspension culture changed to a light red and got stronger and stronger until day 7. To isolate the anthocyanin-like compounds 30 ml of a 7 day old cell suspention culture was acidified by formic acid and lysed by an ultrasonic device (Tab. 2.7) using a frequency of 30 kHz at 100W for 4 min. The lysat was centrifuged for 10 min at 10,000 g, the pellet was discarded and the supernatant was kept for further extraction. A Roti 100 filter column was equilibrated twice with 5 ml methanol. To accelerate the flow through a vaccum pump with an underperssure of 400 kPa was set up. After equilibration the collectet supernatant was added to the column. After binding of the red coloured compounds the column was washed twice with 5 ml ddH_2O. For eluatation 5 ml of a mixture of 95% methanol acidified by 5% formic acid was added. The eluated fraction

was vaccum distilled to a final volume of 1 ml (Tab. 2.7). Further as a comparison the same extract method was used for a bottle of a single-variety Spätburgunder red wine (Blankenhornsberger Spätburgunder Rotwein, Spätlese trocken, Staatsweingut Freiburg).

detection of antocyanins

To investigate the isolated anthocyanin-like compounds the methanolic extracts of the ABA-treated cell suspension cultere of *V. vinifera* cv. 'Pinot Noir' were analyzed using a HPLC (Tab. 2.7). 10 μl of each extract was used for the HPLC analysis with a Phenomenex Synergie Hydro RP column and a flow rate of 0.8 ml/min. The solvents acetonitrile (A), formic acid (B) and ddH_2O (C) (Tab. 2.5) were used with changing solvent gradients over time that was applied to the samples (Tab. 2.9).

Table 2.9: timetable of solvent mixture while HPLC

time	A	B	C
0 min	5.8%	10%	84.2%
15 min	17.1%	10%	72.9%
30 min	26.5%	10%	63.5%
35 min	26.5%	10%	63.5%
41 min	5.8%	10%	84.2%

3 Results

3.1 Mitotic index correlates with level of MDA

To get insight into modulations of the oxidative status during the culture cycle, the abundance of malon dialdehyde (MDA) was followed over time in suspension cell cultures of the grapevine species *V. rupestris* and *V. vinifera* cv. 'Pinot Noir'. Since MDA is produced by lipid peroxidation, quantifications of MDA report on the oxidative status of the cell. In parallel, the mitotic index as the percentage of mitotic cells was determined to monitor the physiological status of the cell culture under control conditions. For

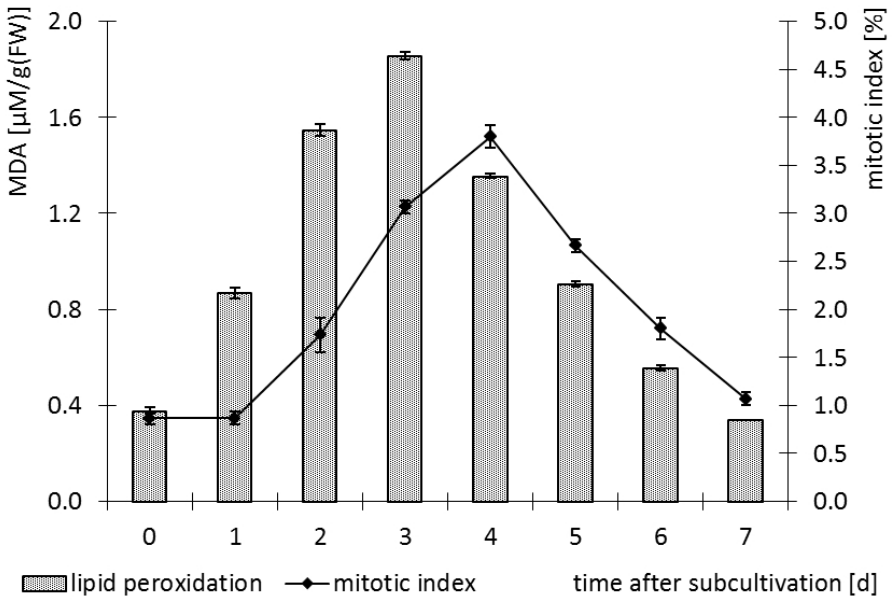

Figure 3.1: Correlation between mitotic index and MDA content in a suspension cell culture of *V. rupestris* during the culture cycle of one week duration. All values represent means and standard errors from three independent measurements for each time point.

V. rupestris the MI started at a low level (0.5 %) at the day of subcultivation (day 0), increases continuously, reaching a maximum at day 4 (4 %) and subsequently decreased in almost linear until day 7 when the stationary phase of the culture cycle had been reached (0.4 %). The MDA content started at a low level (0.4 μM/g(FW)) at the day of subcultivation, increasing continuously until it reached a maximum at day 3 (1.8 μM/g(FW)), and then decreased continuously until day 7 (0.3 μM/g(FW)). Data is shown in Fig. 3.1. The data for *V. vinifera* cv. 'Pinot Noir' are similar and are shown in the Appendix (Chap. 5.1 on page XXXIII).

3.2 MDA is induced by ionic and osmotic stress

To verify whether MDA monitors the oxidative status of a cell culture, stress treatments that are known to induce oxidative burst were administered. The stress-related formation of reactive oxygen species is expected to lead to an increase of MDA by lipid peroxidation. To avoid that the results were distorted by the mitosis-associated MDA level, this experiment was conducted in stationary cells, where MDA content had returned to low levels.

To induce stress, suspension cultures of *V. rupestris* and *V. vinifera* cv. 'Pinot Noir' were treated with salt (0, 100 and 200 mM NaCl). Since salinity stress comprises both osmotic as well as ionic components, a treatment with mannitol (0, 170 and 340 mM) was further investigated, that just will cause equivalent osmotic, but not ionic stress. The concentration of 170 mM of mannitol was chosen because it is osmotically equivalent to 100 mM NaCl, whereas 340 mM mannitol causes the same osmotic potential as 200 mM NaCl.

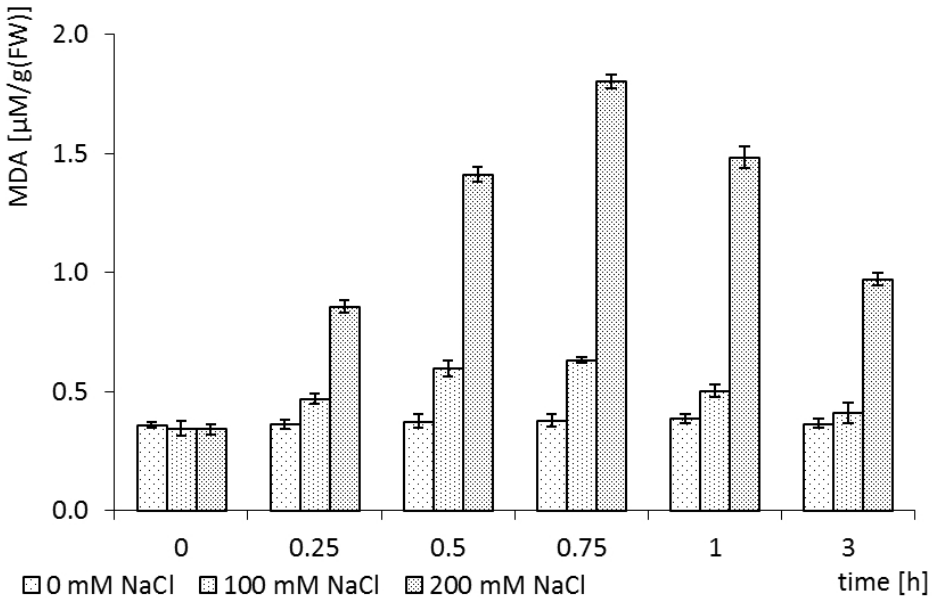

Figure 3.2: Time course of stress-dependent MDA formation in a 7 day old suspension cell culture of *V. rupestris* after treatment with 100 mM and 200 mM NaCl over three hours. Values represent means and standard errors from three independent triplicate measurements.

For *V. rupestris* the treatment with NaCl leads to an increase of MDA within 45 min to a maximum of 1.8 μM/g(FW) and decreases fast until it reaches its normal level after 24 hours. Mannitol also leads to a fast increase after 45 min to a maximum of 1.2 μM/g(FW) and decreases to its normal level after 3 hours (compare Fig. 3.2, Fig. 3.3). In fact, both stressors showed a rapid increase of MDA levels. This response was rapid (onset within 0.25 h), transient (peak at 0.75 h), and concentration dependent.

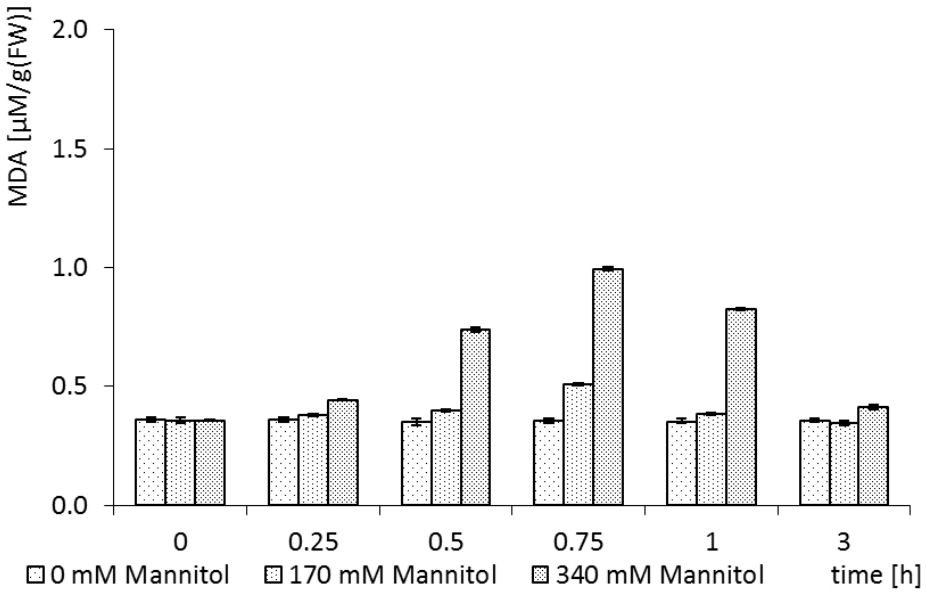

Figure 3.3: Time course of stress-dependent MDA formation in a 7 day old suspension cell culture of *V. rupestris* after treatment of 170 mM and 340 mM mannitol over three hours. Values represent means and standard errors from three independent triplicate measurements.

3.3 Inhibition of the NADP(H) oxidase can suppress salt-induced MDA formation

Salinity signaling in plants involves an oxidative burst produced by a membrane-bound enzyme complex, which contains the enzyme NADP(H) oxidase. NADP(H) oxidase generates superoxide by transferring electrons from intracellular NADP(H) across the membrane, coupling these to molecular oxygen and thus producing the superoxide anion, a reactive free radical. This enzyme can be blocked by diphenylene iodonium (DPI). If the induction of MDA by salinity is caused by the NADP(H) oxidase, inhibition of this enzyme by DPI should also inhibit the accumulation of MDA.

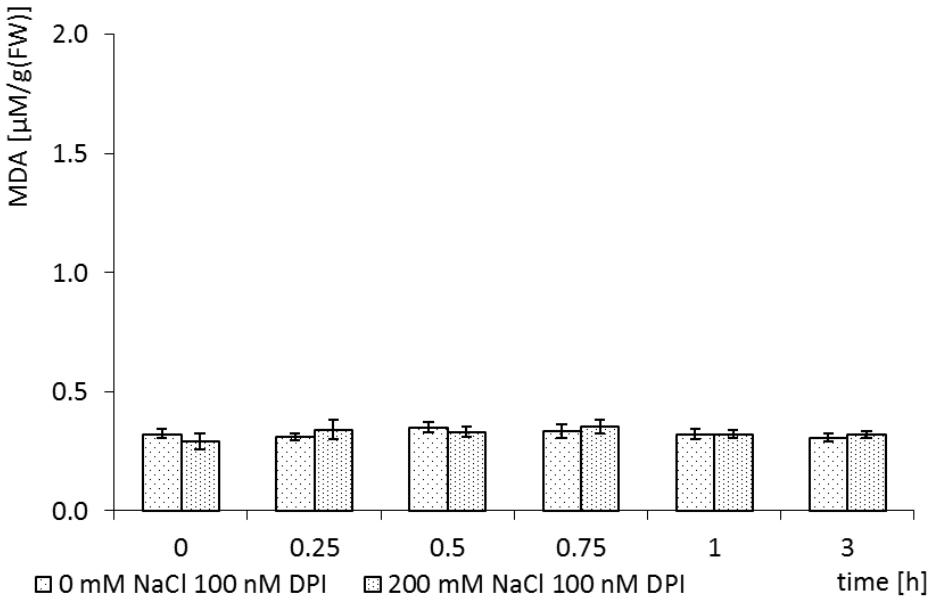

Figure 3.4: ROS dependent MDA formation in a 7 day old suspension cell culture of *V. rupestris* after induced salt stress with 200 mM NaCl and blocking NADP(H) oxidase by 100 nM DPI for 5 min prior to addition of salt. Values represent means and standard errors from three independent triplicate measurements.

In fact, a pretreatment of a *V. rupestris* suspension cultures (Fig. 3.4 & Fig. 3.5) with 100 nM DPI for 5 min prior to addition of 200 mM NaCl can completely suppress the salt-induced accumulation of MDA. A control experiment, where 100 nM DPI was administered in the absence of salt revealed that the low residual level of MDA in control cells was not altered.

The same experiment was also performed with a cell suspension culture of *V. vinifera* cv. 'Pinot Noir'. The salt-induced MDA formation could also be suppressed in *V. vinifera* cv. 'Pinot Noir'. The data is shown in the Appendix (Chap. 5.3: Fig. 5.4 & Fig. 5.5 on page XXXV).

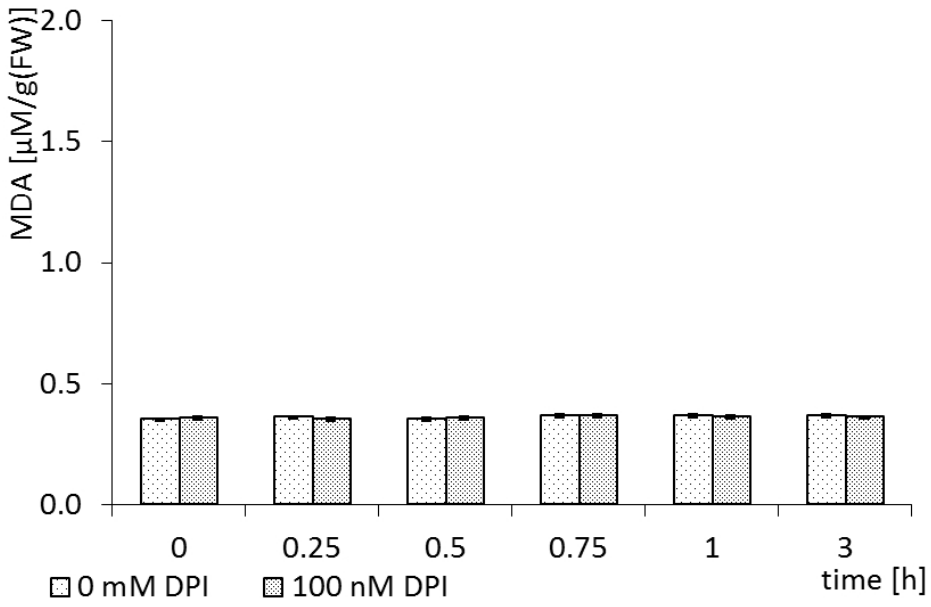

Figure 3.5: ROS dependent MDA formation in a 7 day old suspension cell culture of *V. rupestris* after blocking NADP(H) oxidase by 100 nM DPI. Values represent means and standard errors from three independent triplicate measurements.

3.4 Inhibition of the NADP(H) oxidase can trigger cell death

To monitor the physiological response to stress-induced oxidative burst, the decrease in packed cell volume (PCV) as indicator for culture growth was measured. A dose-response curve of cell growth (monitored as the increment of PCV, ΔPCV) for different concentrations of NaCl (0-200 mM) and mannitol (0-680 mM) measured at day 5 after subcultivation (at the decline of cycling) was performed for the suspension cell culture of *V. rupestris*.

Fig 3.6 shows a dose-dependent inhibition. The dose-response curve was shifted to higher values for mannitol if compared to NaCl. This shift was in the range of almost one order of magnitude. 1.7 times higher molar concentrations of mannitol are required

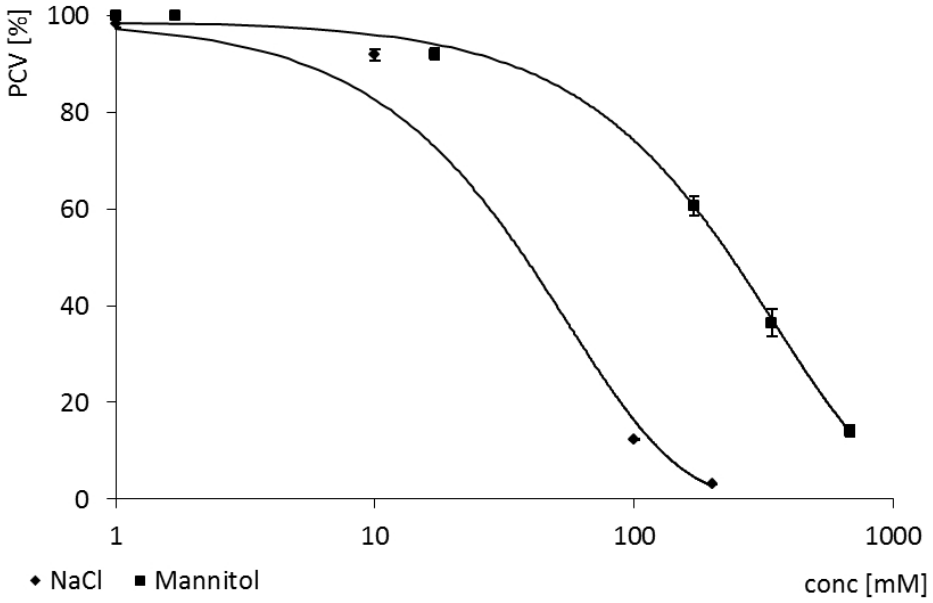

Figure 3.6: Sustained effect of a *V. rupestris* cell suspension culture after NaCl or mannitol treatment. An increase in packed cell volume (ΔPCV) in presence of different concentrations of NaCl or mannitol relative to the values obtained for untreated controls on day 5 after subcultivation. Values represent means and standard errors from three independent duplicate measurements.

- compared to NaCl - to obtain equivalent osmotic potentials. Similar results could be shown by the same treatment of a suspension culture of *V. vinifera* cv. 'Pinot Noir' (see Chap. 5.4: Fig 5.6 on page XXXVII).

In the next step, the progression of mortality in the suspension cultures were determined for NaCl (0, 100 and 200 mM) and osmotically equivalent concentrations of mannitol (0, 170 and 340 mM). Mortality increased depending on increasing concentration and time. For *V. rupestris* the highest salt concentration (200 mM NaCl) leads to total mortality within the first 3 days, for the lower concentration (100 mM NaCl), mortality increased as well during this time span, but reached a saturation at around 20 %, which means that 80 % of the cells had successfully adapted (Fig 3.7). Again, mannitol stress was less stringent. Even for the highest concentration (340 mM mannitol, osmotically equivalent to 200 mM NaCl), after 3 days, around 70 % cells were alive and resisted cell death much longer (Fig 3.8). The 50 % mortality values were reached at day 5, which was

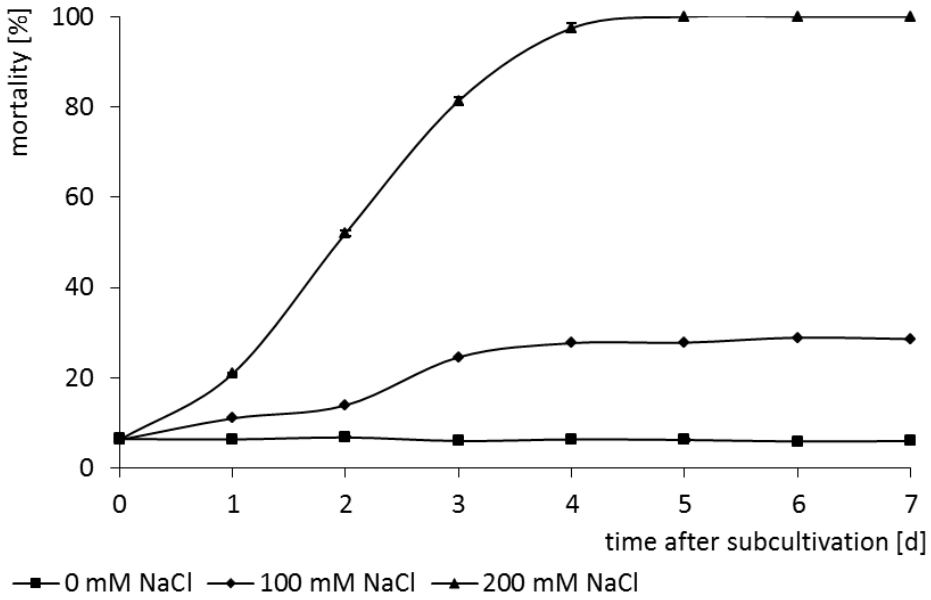

Figure 3.7: Time courses of the mortality of a *V. rupestris* cell suspension culture for different concentrations of NaCl. Values represent means and standard errors from three independent measurements.

3 days later than for the equivalent concentration of salt. For *V. vinifera* cv. 'Pinot Noir' the results were very similar (data shown in Chap 5.4: Fig 5.7 & Fig 5.8 on page XXXVII), but all treatments caused more severe stress as compared to *V. rupestris*.

To test, whether the salt-induced mortality is linked to the oxidative burst induced by salt treatment, the activity of the NADP(H) oxidase was blocked using 20 nM DPI. A control experiment using incubation with only the inhibitor (in the absence of salt stress) produced a surprising result: the mortality of *V. rupestris* increased rapidly, already within 3 hours after addition of the inhibitor, and after 3 days most cells had undergone cell death (Fig 3.9). This time course was very similar to that observed for the treatment with 200 mM NaCl. Also *V. vinifera* cv. 'Pinot Noir' treated with DPI showed this rapid and fast increasing mortality, also more stringent than compared with *V. rupestris*. Data is shown in Chap. 5.4: Fig 5.9 on page XXXVII.

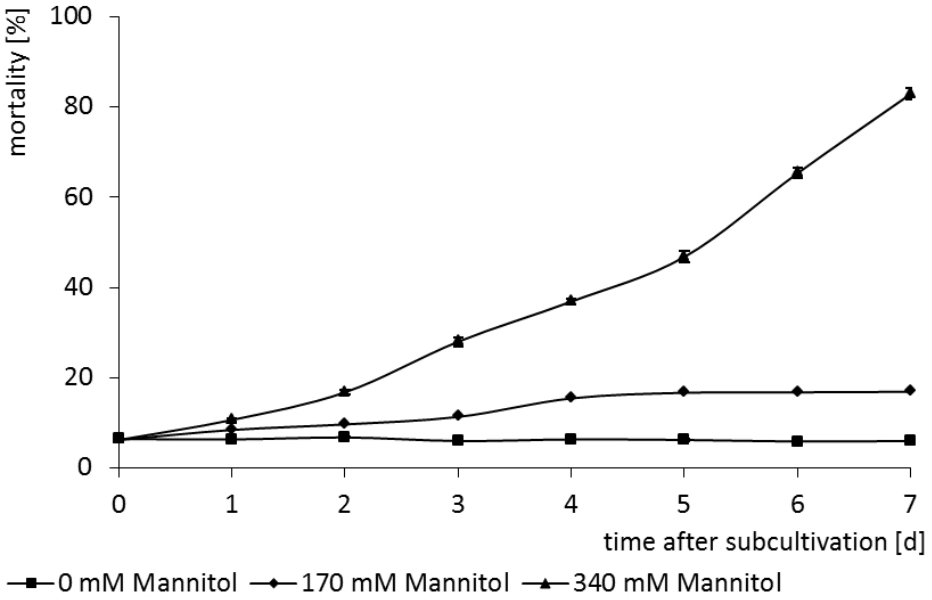

Figure 3.8: Time courses of the mortality of a *V. rupestris* cell suspension culture for different concentrations of mannitol. Values represent means and standard errors from three independent measurements.

To clarify, if this similar behaviour of the two treatments is caused by the same cellular mechanisms, a co-treatment of 200 mM NaCl and 20 nM DPI was further tested. The mortality was increasing slower and weaker and reached saturation at about 25 % after one day for *V. rupestris*. For *V. vinifera* cv. 'Pinot Noir' this saturation could also be observed, but it was much higher at about 50 %. The data is shown in Chap 5.4: Fig 5.10 on page XXXVII.

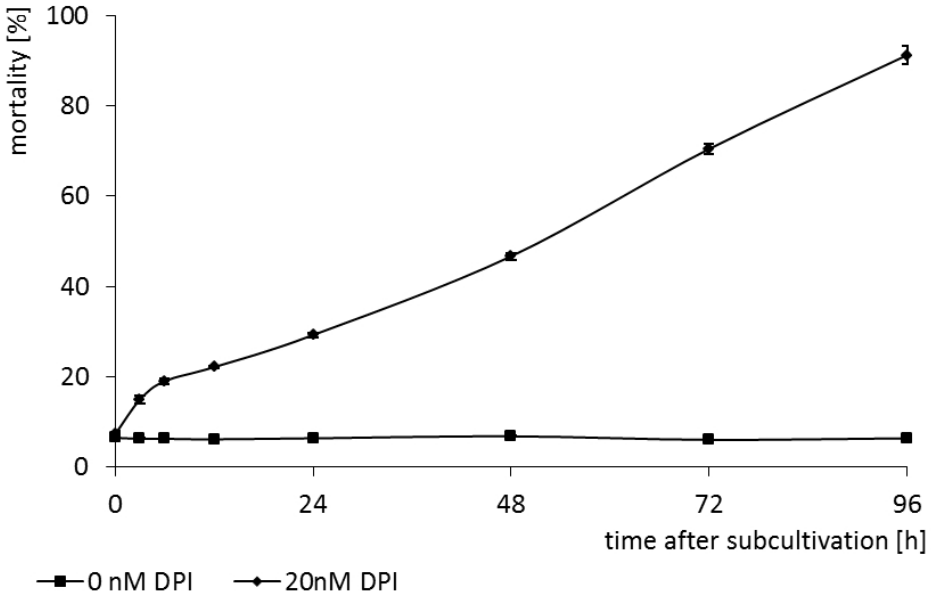

Figure 3.9: Time courses of the mortality of a *V. rupestris* cell suspension culture in response to a treatment with 20 nM DPI. Values represent means and standard errors from three independent measurements.

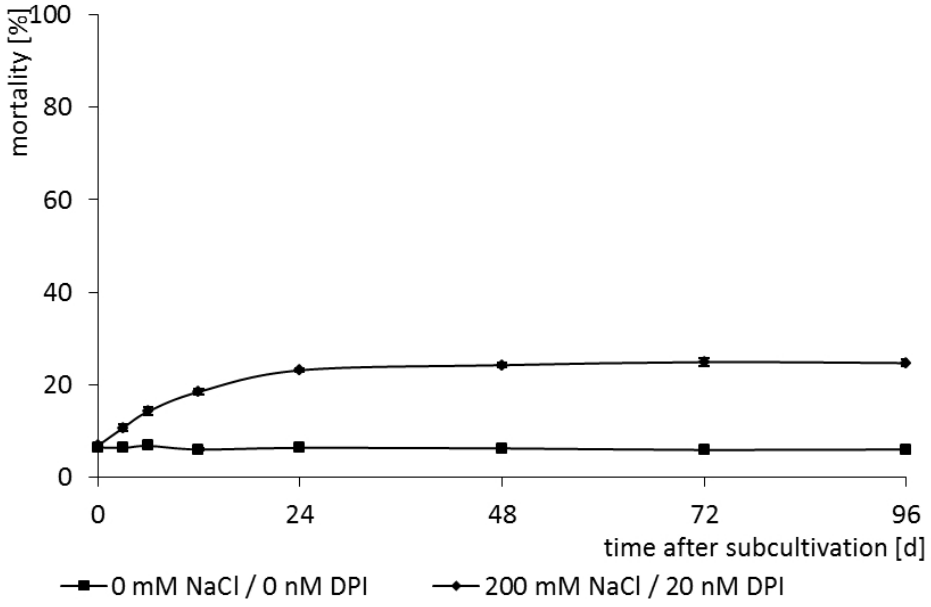

Figure 3.10: Time courses of the mortality of a *V. rupestris* cell suspension culture in response to a co-treatment with 20 nM DPI and 200 mM NaCl. Values represent means and standard errors from three independent measurements.

3.5 Abscisic acid triggers growth, but also cell death

To get more insight into the complex of stress, cell cycle and cell death, cell suspenstion cultures of *V. rupestris* and *V. vinifera* cv. 'Pinot Noir' were treated with the stress-related plant hormone abscisic acid (ABA). Therefore, the mortality was determined after a treatment with 100 μM ABA. In *V. rupestris* there was no change in mortality (Fig 3.11) compared to an untreated sample. At any time the mortality was about 6 %. In case of *V. vinifera* cv. 'Pinot Noir' the mortality increased slightly, but significantly, from 6 % to 8 % after day 3 (Fig 3.12).

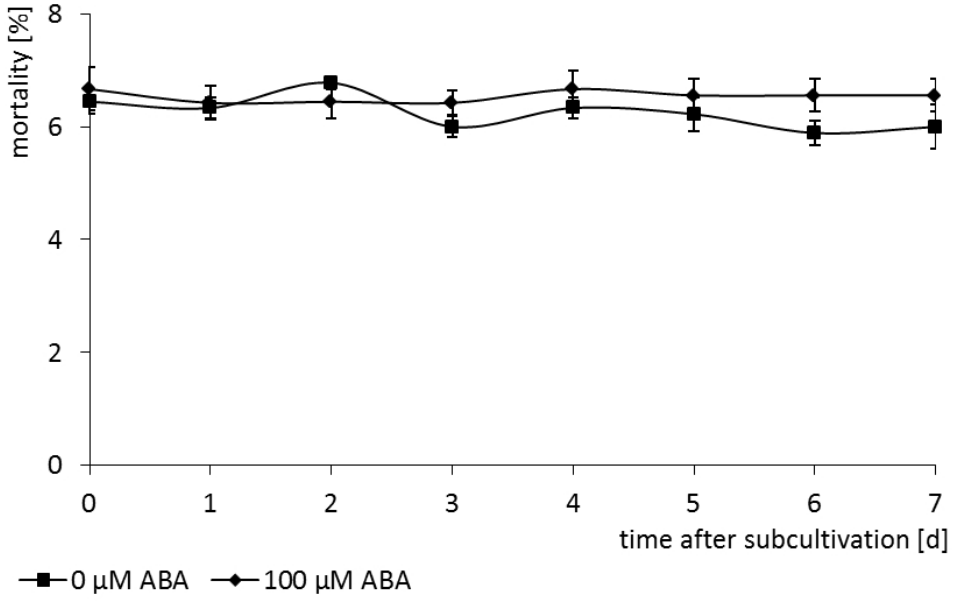

Figure 3.11: Time courses of the mortality of a cell suspension culture of *V. rupestris* in response to a treatment with 100 μM ABA. Values represent means and standard errors from three independent measurements.

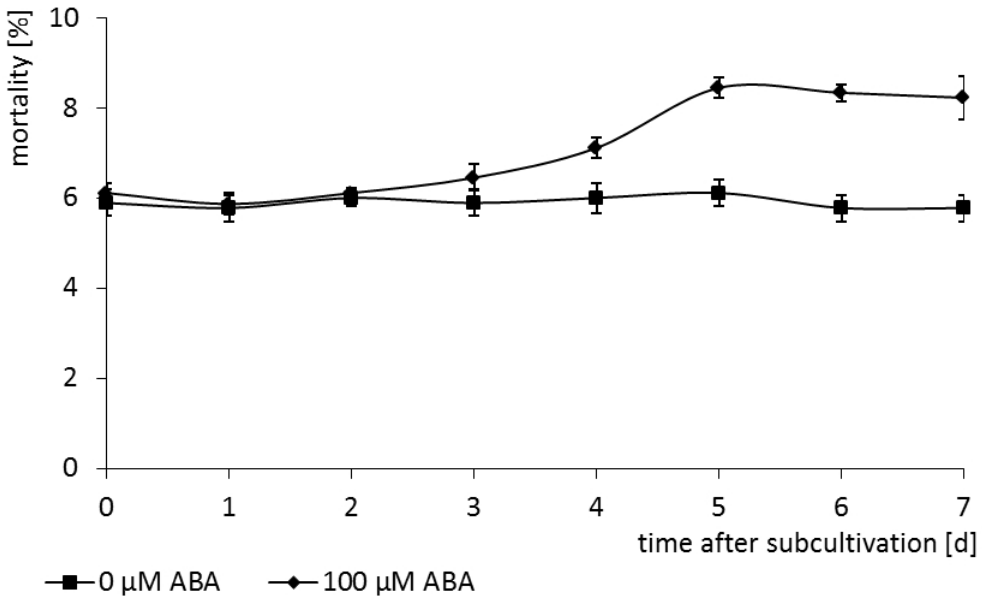

Figure 3.12: Time courses of the mortality of a cell suspension culture of *V. vinifera* cv. 'Pinot Noir' in response to a treatment with 100 μM ABA. Values represent means and standard errors from three independent measurements.

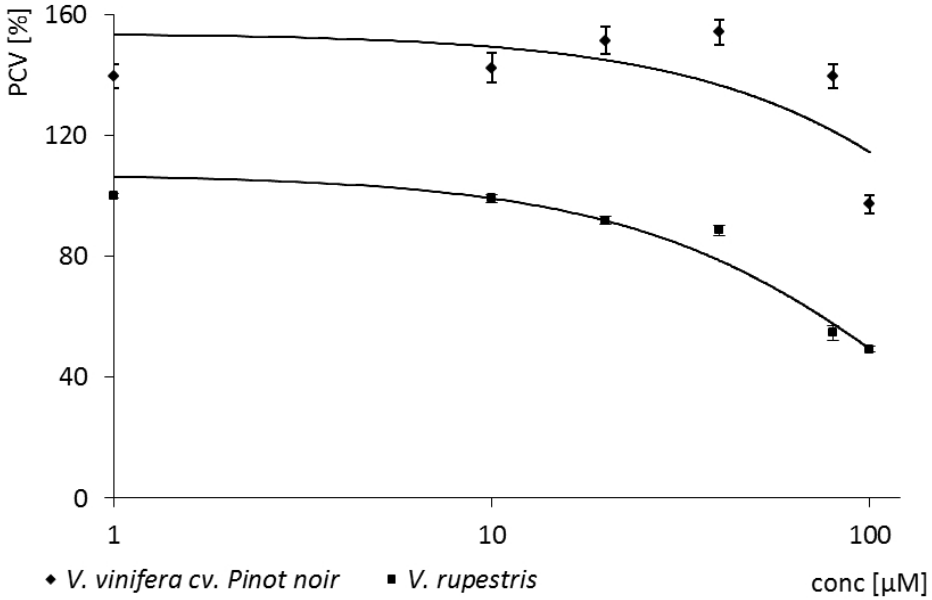

Figure 3.13: Change in packed cell volume (ΔPCV) of *V. rupestris* and *V. vinifera* cv. 'Pinot Noir' in presence of different ABA concentrations relative to the values obtained for untreated controls on day 5 after subcultivation. Values represent means and standard errors from three independent duplicate measurements.

In addition also the increase of packed cell volume in presence of different concentrations of abscisic acid (1 to 100 μM) for both cell suspension cultures was measured. Also in this experiment *V. vinifera* cv. 'Pinot Noir' showed a different behaviour compared to *V. rupestris*. The PCV of *V. rupestris* demonstrated a fast decrease with a reduction to 50 % at about 40 μM. *V. vinifera* cv. 'Pinot Noir' did not show such an effect. Even more, *V. vinifera* cv. 'Pinot Noir' pointed an increase of about 50 % in PCV, which is nullified at about 100 μM (Fig 3.13).

3.6 Abscisic acid leads to a reduced groundlevel concentration of MDA in stationary cell phase

In the next step, cell suspension cultures of *V. rupestris* and *V. vinifera* cv. 'Pinot Noir' were tested for MDA formation after treatment with ABA, to see whether the change in mortality and growth is correlated with the status of oxidative burst.

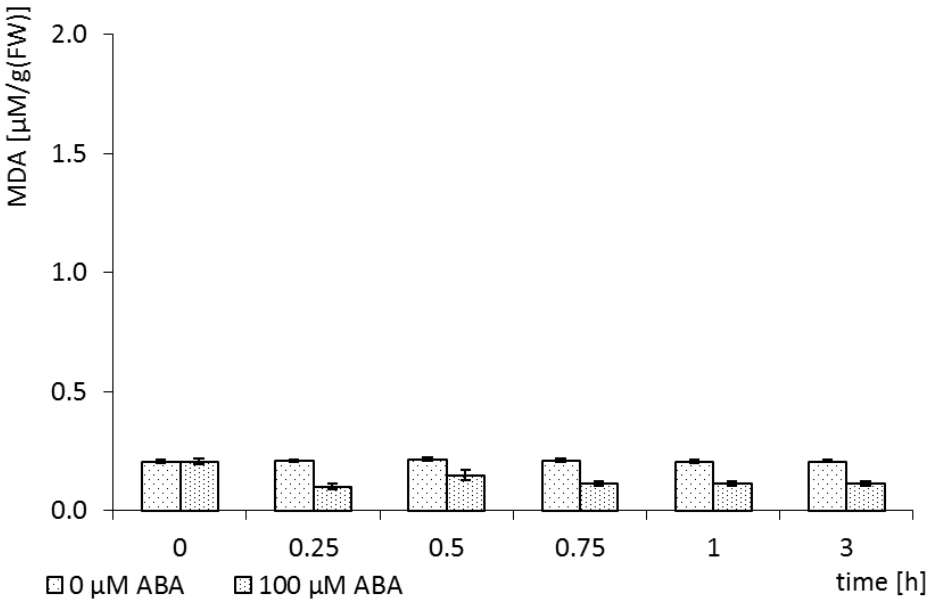

Figure 3.14: Time courses of MDA formation of a *V. vinifera* cv. 'Pinot Noir' cell suspension culture after addition of 100 μM ABA. Values represent means and standard errors from three independent triplicate measurements.

For *V. vinifera* cv. 'Pinot Noir' the MDA level in stationary cell phase is reduced by the addition of 100 μM ABA to 50 % within 15 min (Fig 3.14). For *V. rupestris* this decrease in MDA amount could not be observed (Fig 3.15).

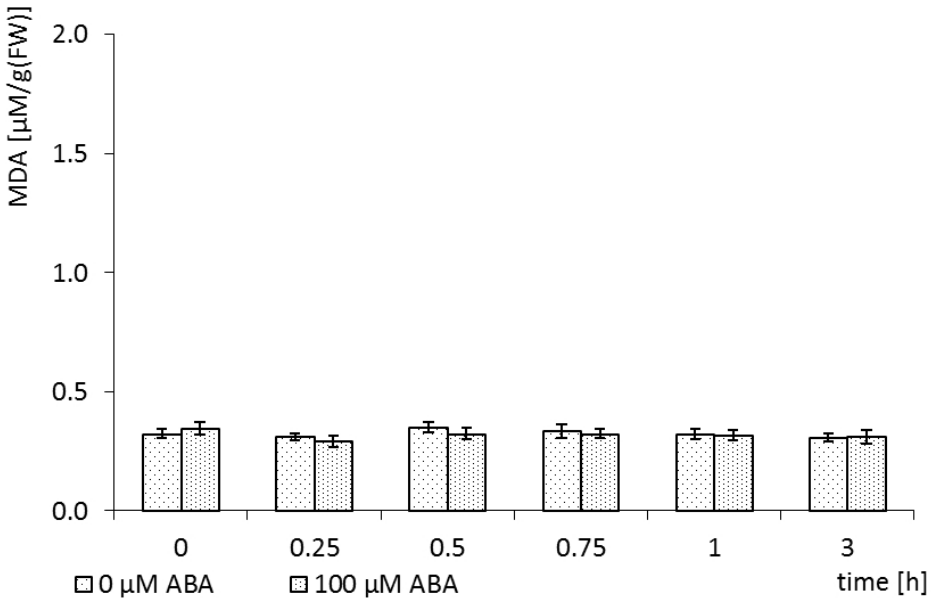

Figure 3.15: Time courses of MDA formation of a *V. rupestris* cell suspension culture after treatment of 100 μ ABA. Values represent means and standard errors from three independent triplicate measurements.

3.7 Abscisic acid quenchs the salt-triggered cell death

Furthermore, the mortality of *V. vinifera* cv. 'Pinot Noir' was determined for a double treatment with ABA and NaCl. This experiment was also performed for *V. rupestris*. In case of *V. vinifera* cv. 'Pinot Noir' the salt-induced cell death, which reached 100 % on day 3 could be quenched tremendiously to a survival rate of 80 % by the addition of ABA (Fig 3.16). The treatment of *V. rupestris* did not show such a big effect as compared to *V. vinifera* cv. 'Pinot Noir', but also showed a reduction in mortality to a survival rate of about 50 % compared to the single treatment of NaCl (Fig 3.17).

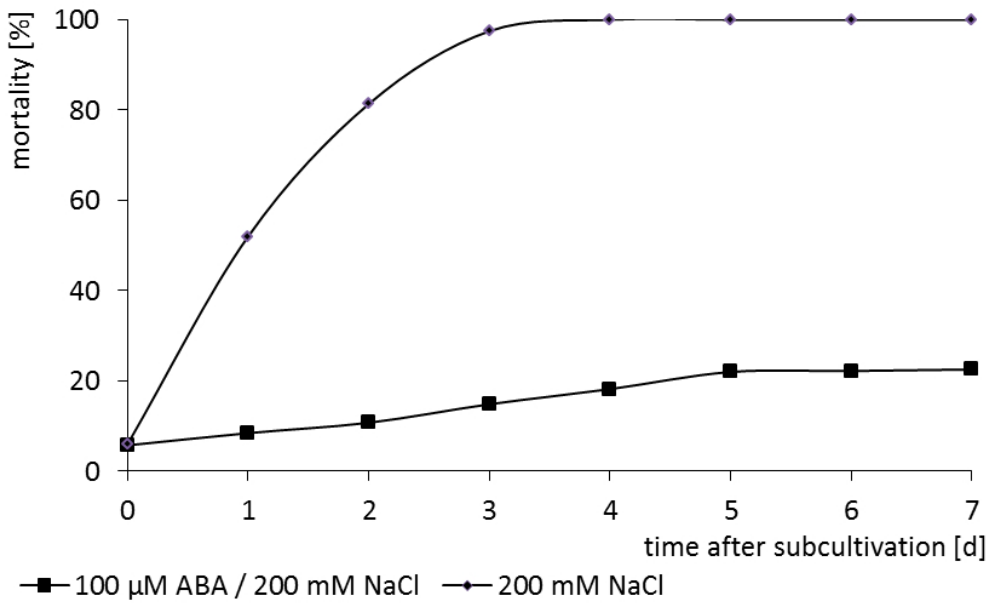

Figure 3.16: Time courses of the mortality of a *V. vinifera* cv. 'Pinot Noir' cell suspension culture after addition of a co-treatment with 200 mM NaCl and 100 μ ABA. Values represent means and standard errors from three independent measurements.

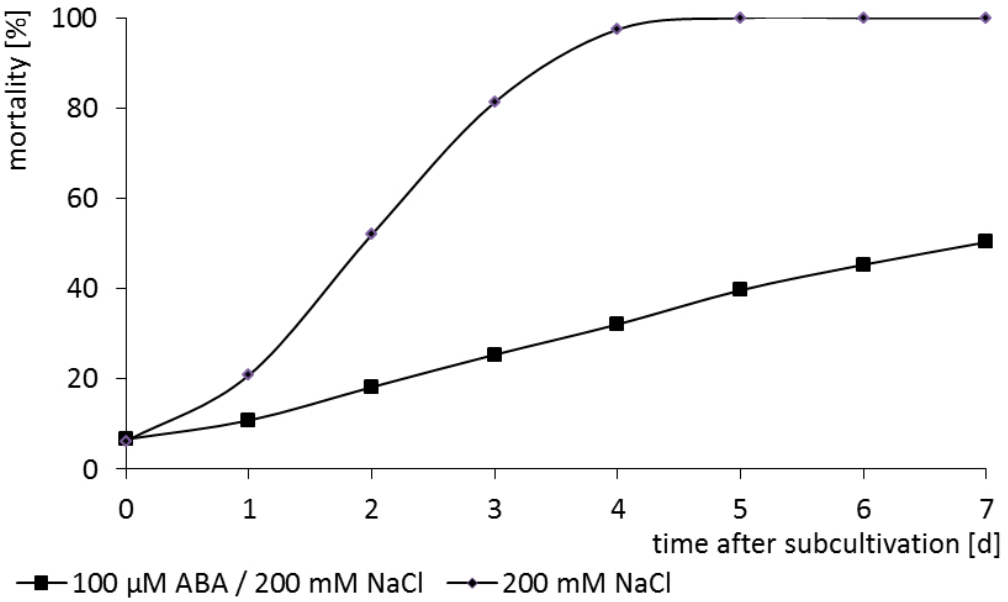

Figure 3.17: Time courses of the mortality of a *V. rupestris* cell suspension culture after a addition of a co-treatment with 200 mM NaCl and 100 μ ABA. Values represent means and standard errors from three independent measurements.

3.8 Abscisic acid leads to antocyanine production

To investigate the cause for the change in colour of a *V. vinifera* cv. 'Pinot Noir' cell suspension culture (A) after a treatment of 100 μM abscisic acid, the intracellular compounds were isolated and separated by a HPLC. Further a total absorbance spectrum was recorded by a photodiode. To compare, also an extract of a red wine (a single-variety Spätburgunder, B) was performed and analysed by a HPLC. The recorded spectra are shown in Fig 3.18.

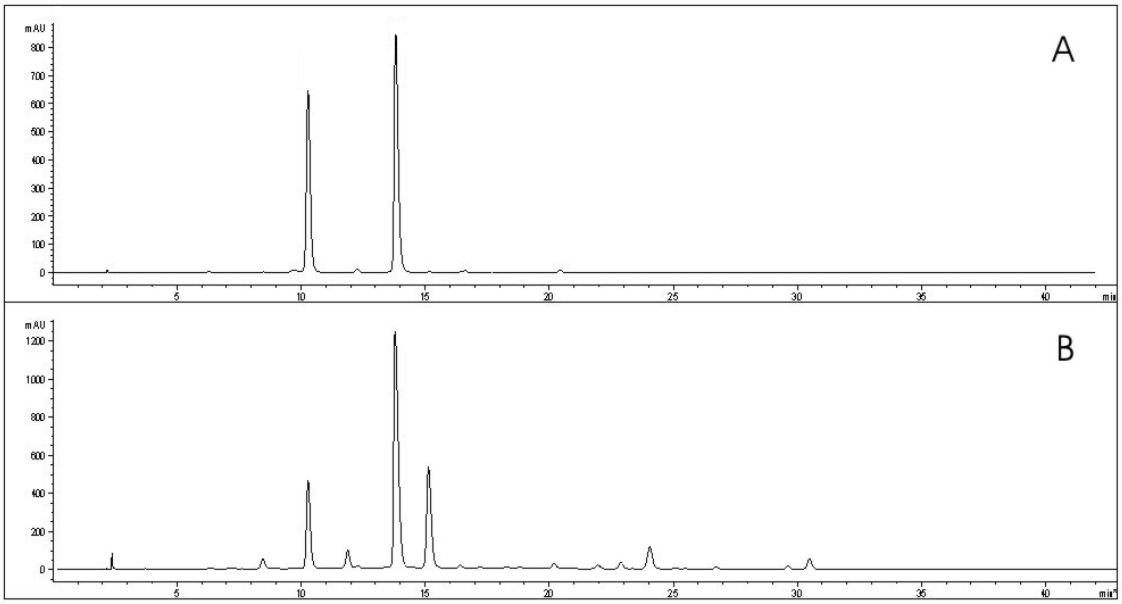

Figure 3.18: Example spectra for the HPLC analysis of a cell extracts of a suspension culture of *V. vinifera* cv. 'Pinot Noir' after treatment with ABA and an authentic Spätburgunder red wine. Retention times are shown on the x-axis, intensity in mAu on the y-axis.

The pattern for the anthocyanins computed by HPLC for both extract were quite different, but the tho main peaks at the retention times od 10.2 min and 13.8 min are similar. This fact suggests that the produced anthocianidic compounds are the same as found in red wine. To get further the two peaks (A, retention time 10.2 min; B, retention time 13.8 min), which appeared in the HPLC analysis of the cell suspension culture of

V. vinifera cv. 'Pinot Noir' were analysed more exactly by checking the total spectra (Fig 3.19). The totals spectra of both peaks (HPLC analysis of a cell suspention culture) a similar with a main peak at 518 nm.

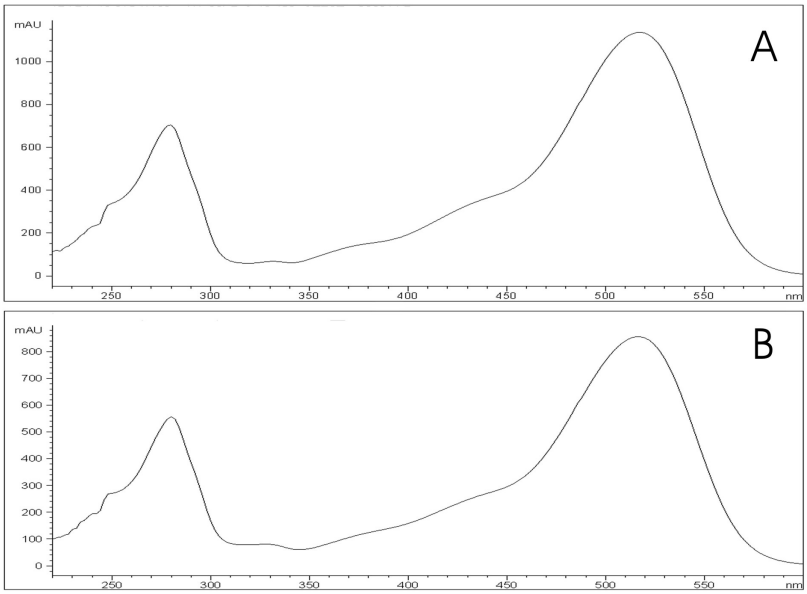

Figure 3.19: Total absorbance spectra for the HPLC analysis of a cell extracts of a suspension culture of *V. vinifera* cv. 'Pinot Noir' at the retention times of 10.2 min (A) and 13.8 min (B)

To compare, if these two peaks, which were found in HPLC analysis of a cell suspension culture are also found in authentic Spätburgunder red wine the total spectra of both peaks (A, overlay of spectra at retention time 10.2 min; B, overlay of spectra at retention time 13.8 min) were overlayed in the range from 350 nm to 600 nm, including the main peak at 518 nm (Fig 3.20). The overlay of the peaks at correlated retention times show the same absorbance spectra, so that we strongly suggest, that the compounds in a cell suspension culture after ABA treatment are the same than in red wine.

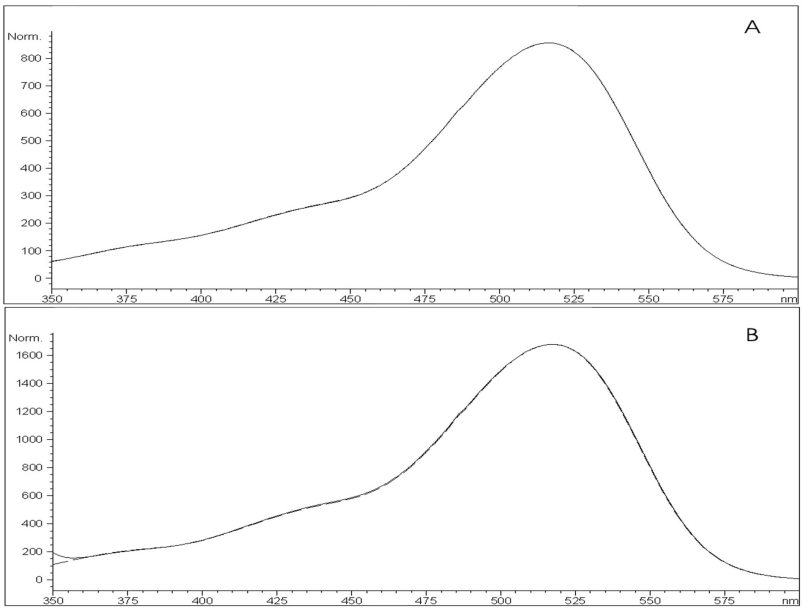

Figure 3.20: Overlay of of absorbance spectra for the HPLC analysis of a cell extracts of a suspension culture of *V. vinifera* cv. 'Pinot Noir' at the retention times of 10.2 (A) min and 13.8 min (B)

To conclude, it is not complete evidence which specific anthocyanin it is. Compared to the literature (Berente, 2004), we suggest it could be a cyanidin- or a peonidin which do have their absorption maximum at 518 nm. But to be sure and to tighten the suggestion the isolated compuds shoud be analysed by NMR and compared with comercial references. Further it is possible to check if the two produced compunds by the ABA-treated cell suspension culture are the anthocyanin and its correlated aglycone (anthocyanin without an attached glycosid).

4 Discussion

4.1 MDA indicates the activity of RboH

Over many years, reactive oxygen species (ROS) have been merely understood as undesired by-product of oxidative metabolism (Bereiter-Hahn, 2014). However, more recently, ROS have emerged as important stress signal (Mittler et al., 2010), and deliberate release of ROS was found to stimulate efficient adaptation. The plasma-membrane located RboH NADP(H) oxidases (for respiratory burst) have been recognised as a central tool for deliberate ROS release in plants (Marino et al., 2013). This group of enzymes can generate superoxide by partial reduction of oxygen, and is activated by different challenges, including ionic and osmotic stress. However, a certain ground level of superoxide is also required for normal growth and development, for instance to drive auxin-dependent signalling mediated by the activity of small G-proteins of the Rac/Rop family (Wu et al., 2011).

In this work, malone dialdehyde (MDA) has been used as readout for the activity of RboH-generated oxidative burst. It has been shown that for *V. rupestris* as well as *V. vinifera* cv. 'Pinot Noir', (i) salinity stress and equivalent osmotic stress administered through mannitol cause a fast and drastic increase in MDA content (Fig. 3.2, 3.3, 5.2, 5.3), (ii) this increase of MDA content can be completely inhibited by pre-incubation with diphenylene iodonium chloride (DPI), an inhibitor of RboH (Fig. 3.4, 5.4), (iii) as to be expected for a reporter of a signalling events, the increase of MDA content remains transient, (iv) the increase of MDA content depends on the strength of the stressor,

with higher concentrations of NaCl or mannitol yielding a higher amplitude of MDA induction. These four criteria support that the increase of MDA is in fact indicating the activity of RboH.

Diphenylene iodonium chloride (DPI) blocks the activity of NADP(H)-dependent flavoproteins, and can also inhibit a specific group of membrane-bound nitric oxide synthases (Stuehr et al., 1991) in mammalian cells. Nitric oxide has also been found to participate in a broad spectrum of stress responses by a cross-talk between Ca^{2+}, H_2O_2 and plant hormone signalling (Mur Luis et al., 2013), which leads to the question, whether DPI might also affect MDA levels by blocking NADP(H)-dependent nitric oxide synthase (NOS). However, although a membrane-located putative NOS activity has been published for tobacco roots (Stöhr et al., 2001), so far, despite intensive search, no molecular homologue of the mammalian NADP(H)-dependent NOS could be identified in plants, and the majority of the NO in plant cells derives from the cytosolic nitrate reductase (Mur Luis et al., 2013). Even, if one would assume the existence of a NADP(H)-dependent NOS as target of DPI, this would be expected to cause an increase of MDA and not the observed decrease of MDA, since NO can scavenge ROS by its ability to neutralize Fenton-type oxidative damage. On the base of this argumentation, MDA is utilised as a reporter for modulations in RboH activity.

4.2 Transient activation of RboH is required to sustain cell proliferation

After it has been shown, that MDA can be used as a readout for the activity of RboH in the response to osmotic stresses, the next step is asking, whether modulations of MDA can be detected during normal development. Most suspension cultures that have been developed to a sufficient degree of homogeneity, follow a characteristic developmental sequence (Opatrny et al., 2014), where an initial proliferation phase (characterized by an increase in mitotic index - in the current cell system at day 4 after subcultivation) is

followed by a phase of cell expansion, where the mitotic index decreases. The transition from proliferation to this stationary phase is regulated by auxin (Campanoni and Nick, 2005). A characteristic time course of mitotic activity is preceded by a parallel time course in the level of MDA (Fig. 3.1, 5.1). The time interval between the transient increase of MDA and the transient increase in mitotic index is one day. The amplitude of this MDA peak is comparable to that induced by 200 mM NaCl (compare Fig. 3.1, 5.1 and Fig. 3.2, 3.3, 5.2, 5.3).

This observation would be consistent with a model, where a transient activation of RboH would be necessary to initiate cell proliferation. This model would be congruent with findings from other organisms: It already has been shown that superoxide is required for the transition from G_0-to-G_1 in mammalian cells (Menon and Goswami, 2007), and in fungi, mycotoxins can regulate oxidative stress which in turn induces conidiation (Zheng et al., 2015). For plants, the link between ROS and the cell cycle is ambiguous. On the one hand, there is evidence that ROS are needed for the G_0-to-G_1 transition, probably through activation of CDKA1 (Feher et al., 2008). On the other hand, artificial induction of ROS by menadione (2-methyl-1,4-naphthoquinone) disturbs DNA replication, followed by cell-cycle arrest at low concentrations of menadione, and cell death at high concentrations (Reichheld et al., 1999). However, this artificial compound may not act through generation of superoxide, but by formation of non-conventional radical species (McCormick et al., 2000).

To clarify the link between the oxidative burst reported by MDA and subsequent activation of cell proliferation, RboH was inhibited by DPI at the time of subcultivation (when a new wave of proliferation was triggered by addition of the artificial auxin 2,4-D). The block of RboH also blocked proliferation completely. Instead, cells progressively initiated cell death at a time course matching that of MDA accumulation under control conditions (compare Fig. Fig. 3.9, 5.9 with Fig. 3.1, 5.1). This observation shows that the activity of RboH is necessary to initiate or sustain the cell cycle.

The next experiment (Fig. 3.10, 5.10), in which the effect of DPI was tried to mitigate by additionally administering a salinity stress treatment (200 mM NaCl) was found to induce a level of MDA equivalent to that observed during proliferation (2 μM/g(FW)). In fact, this salinity stress (which by itself would cause high mortality, see (Fig. 3.7, 5.7), was able to rescue the DPI treated cells from cell death. The resulting mortality (saturated at around 20% for *V. rupestris*, repectivly 40% for *V. vinifera* cv. 'Pinot Noir') was comparable to that produced by mild salinity stress (100 mM NaCl, Fig. 3.7, 5.7).

In other words: a mild oxidative burst (generated by RboH) is necessary and sufficient to sustain cell proliferation. Both, the absence of this burst (by DPI) as well as excessive amplitudes of this burst (high salinity or strong hyperosmotic stress) will block proliferation and cause cell death. These findings are consistent with the proposition, that a homeostasis of ROS is required for the plant cell cycle (Livanos et al., 2012). By the way, the existence of this homeostasis argues against a viewpoint, where ROS are merely interpreted as manifestation of cellular damage. A molecule which is actively kept in a state of equilibrium dependent on cellular development is more likely involved in signalling rather than in damage. In fact, the observation that the plant cell cycle depends on redox homeostasis is discussed with respect to maintenance of a functional microtubular cytoskeleton (Livanos et al., 2014). A second potential target might be the mitogen-activated protein kinase cascade (MAPK), which is modulated by different phytohormonal pathways in a ROS-dependent manner (Xia et al., 2015).

The arrest of the cell cycle is followed in many organisms by a developmental switch that will result in programmed cell death. This mechanism might safeguard genetic integrity in multicellular organisms by eliminating cells, where due to DNA damage the progression of the cell cycle is deviant. For instance, the mammalian protein p53 can sense DNA damage and then initiate arrest of the G_0-to-G_1 transition, which will activate apoptotic cell death. Plant cells lack homologues of this protein (Yoshiyama et al., 2009), but nevertheless are able to initiate programmed cell death, for instance in the context of

defence (Ohno et al., 2011). Although the underlying molecular mechanisms have not been fully elucidated, the general link between arrested cell cycle and programmed cell death seems to be valid for plants as well, similar to other organisms (Gilchrist, 1998).

4.3 Abscisic acid - a stress-related co-operator with ROS

Abscisic acid, a plant hormone, which was discovered in the 1960s and since this has been intensively studied is correlated to stress situations such as salt, drought, and heat stress. The most famous function of ABA is that as a regulator of stomatal closure, which is also crucial for the plant's response to such stress situations. In this mechanism several other signal compounds like ROS and Ca^{2+} are involved (Allen et al., 2000). Until now more than 100 genes and several secondary messengers, also including ROS, have been identified as downstream targets of early ABA signalling events (Cutler et al., 2010; Suhita et al., 2004). E.g. an accumulation of ABA in systemic leaves was found to be transient and dependent on ROS production by RboHD (Suzuki et al., 2013). This result suggests that ROS accumulation or ROS waves (Mittler and Blumwald, 2015) may be required for the accumulation and dergradation of ABA (Galvez-Valdivieso et al., 2009). This change in ABA concentration in turn is required for activation of gene expression. Since this interaction of ABA and ROS, it is clear that ABA interacts in the cell proliferation as a regulatory element to release RboH-dependent ROS.

A characteristic time course of MDA amout has shown a decrease after treatment with ABA (Fig. 3.13). This reduction appears fast and persisting. Further the time course of the mortality (Fig. 3.12) shows an increase. These results - combined with the findings of Lizkay and Schopfer (Liszkay and Schopfer, 2006), who showed that RboH-dependent ROS can trigger cell wall loosening during cell extension growth - tighten the model, where a transient activation of RboH is necessary to initiate cell proliferation. To clarify the link between ABA and the salt-stress-dependent increase in mortality a co-treatment

was performed. In fact, ABA was able to rescue the salt-stressed cells from cell death (Fig. 3.14). Also this observation shows that a specific amout of ROS - or in detail a specific activity of RboH is necessary to initiate or sustain the cell cycle.

4.4 Abscisic acid as a trigger for proliferation and development

ABA signalling is also correlated to the activity and inactivity of small G-proteins of the Rac/Rop family. In *Arabidopsis thaliana* the ROP6/AtRac1 protein inhibited ABA-mediated stomatal closure (Lemichez et al., 2001). A loss of function mutant of ROP10 in *Arabidopsis thaliana* showed a hypersensitivity to ABA in root elongation response, stomatal closure and enhanced seed dormancy, which is usually regulated by ABA. A promoter analysis of the ROP10 promoter showed its down-regulated activity in root tips by ABA (Zheng et al., 2002). These results suggest a mutual inhibition between ROP10 signaling and ABA signaling. The ROP10-mediated pathway negatively regulates ABA signaling, while ROP10 transcripts are down-regulated by ABA (Kadomura-Ishikawa et al., 2015). Since Rac/Rop proteins interact in auxin-dependent signaling, it is clear that ABA is also included in the network of cell proliferation.

ABA also might have a function upstream of ROS. Such an effect of ROS on ABA could be observed after the treatment of *Arabidopsis thaliana* seedlings with gamma irradiation. Seedlings, which were treated with gamma irradiation showed higher contents of hydrogen peroxide and correlated antioxidant enzymes - further endogenous ABA production was induced and growth was stimulated (Qi et al., 2015). The upstream function of ABA on ROS could be determinex by an experiment with ABA-deficient mutants which do not produce ROS after gamma irradiation. Furthermore, the reduction of ROS showed no obvious effects on the ABA induction (Qi et al., 2015).

Further ABA and ROS lead to fruit ripening in the fruit skin of grapevine berries. ABA, the phytohormone which is correlated to dormancy in seeds, is accumulated in fruit skins of grapevine berries and leads to fruit ripening. In this case the function of ABA seems also to be upstream. Also ROS and ethylen are involved in fruit ripening. ROS are accumulated after the developmental-related induction of ABA-related genes (Pilati et al., 2014). Also ethylen-related genes are activated later than ABA. Fruit ripening is a very late phase in cell development and differentiation. It is correlated with fruit colouring, which is based on the accumulation of anthocyanins in grapevine berries. This colouring could also be observed in a cell suspension culture of *V. vinifera* cv. 'Pinot Noir'. Extracts of a cell suspension culture 7 days after subcultivation showed an accumulation of anthocyanins (Fig. 3.18, 3.19, 3.20). This result is very unexpected, because of the fact that a cell suspension culture contains stem cells and undifferentiated cells usually do not produce secondary metabolic compounds such as anthocyanins. But - more in detail - if cells do not divide because of an enhanced dormancy effect, triggered by ABA, they might differentiate. This differentiation or developmentel process in the cell also might be a reason for a increased mortality as observed after ABA treatment (Fig. 3.12).

4.5 Eustress versus distress: auxin and RboH negotiate on growth versus stress adaptation?

The dual role of singlet oxygen as a stress signal and as a regulator of cellular development has been investigated and arrived at the conclusion that a certain level of this signal is essential for normal development. If the physiological range for this factor is either exceeded (salinity or mannitol stress) or not sustained (DPI), this will impair cell proliferation and result in cell death as abortive outcome of an arrested cell cycle. In other words, if singlet oxygen represents a stress signal, this means that a moderate level of stress is beneficial, which is congruent with the eustress concept proposed by Seyle

(Seyle, 1936). Eustress should not be perceived as fixed state, but comprises a certain amplitude of fluctuations in external (or internal) factors, that can be compensated by adaptive changes. Only, when this range is persistently exceeded, the organism will not be able to restore homeostasis and enter a state of exhaustion culminating in progressive damage, so called distress. This General Adaptation Syndrome was seminal for animal stress physiology, but can also been transfered successfully to plants (Kranner et al., 2010).

When singlet oxygen generated by RboH can signal an eustress situation in the context of cell proliferation, it must interact at one point with the regulatory circuit governing the cell cycle. In our cell culture system, cell division is under control of auxin, which will activate cell division through a G-protein dependent pathway (Campanoni and Nick, 2005). Early auxin-triggered signalling provides to our knowledge the earliest target, where singlet oxygen might interfere with cell cycle regulation: superoxide anions transduce the auxin-triggered activation of phospholipase D (PLD) through Rac/Rop GTPases (Wu et al., 2011). The activation of proliferative auxin signalling will therefore lead to an increased demand of singlet oxygen in and close to the plasma membrane, which is provided by the stimulated activity of RboH. Inhibition of RboH by DPI will therefore shut down this pathway culminating in cell cycle arrest and subsequent cell death. Overstimulation of RboH by high salinity stress will cause DNA damage, which will also arrest the cell cycle by a system analogous (not homologous!) to mammalian p53. Partial activation of DPI-inhibited RboH by salinity stress will restore a physiological level of ROS (eustress) sufficient to sustain auxin signalling without leading the cellular redox homeostasis to a distress situation. In the result, two evils can recombine into a beneficial situation. The antagonism between auxin signalling and oxidative distress is also supported by recent results (Chang et al., 2015) in the same cell line, where cell death is induced by the bacterial elicitor Harpin can be quelled by simultaneous application of auxin, and where the auxin effect involves DPI-dependent ROS as well as the activity of phospholipase D.

4.6 Outlook

Future research will be dedicated to understand the link between oxidative distress and cell cycle progression by inducing or reducing ROS to specific points in time. Further the characteristic and the mechanisms of ensuing cell death response after ROS inducing has to be identiefied. Also the interplay of ABA and RboH-dependent ROS production has to be further investigated by correlations to mortality, mitotic index, cell cycle control and cell death. Also it is interesting which molecules do also interact with ROS in these or other cellular mechanisms.

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5 Appendix

5.1 Mitotic index correlates with level of MDA

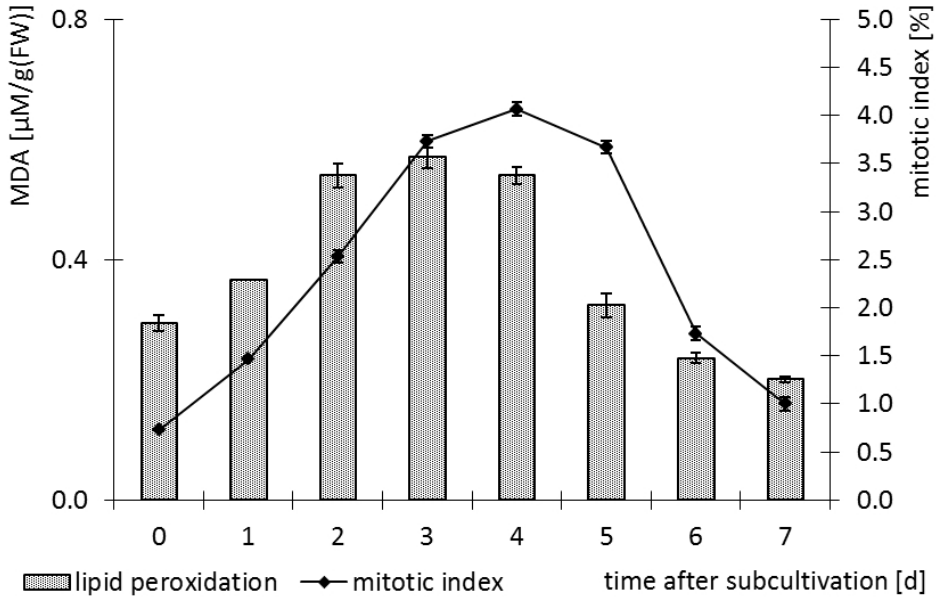

Figure 5.1: Correlation between mitotic index and MDA content in a suspension cell culture of *V. vinifera* cv. 'Pinot Noir' during the culture cycle of one week duration. MDA values represent means and standard errors from three independent triplicate measurements. Mitotic indices represent means and standard errors from three independent measurements for each time point.

The MI of *V. vinifera* cv. 'Pinot Noir' tstarted at a low level (0.5 %) at the day of subcultivation (day 0), increasing continuously, reached a maximum period from day 3 till day 5 (4 %) and subsequently decreased in a continuous manner until day 7 when the stationary phase of the culture cycle had been reached (1 %). The MDA content started

at a low level (0.3 μM/g(FW)) at the day of subcultivation, increasing continuously until it reached a maximum period from day 2 till day 4 (0.6 μM/g(FW), and then decreased continuously until day 7 (0.2 μM/g(FW)). Data is shown in Fig. 5.1.

5.2 MDA is induced by ionic and osmotic stress

The treatment with NaCl of a cell suspension culture of *V. vinifera* cv. 'Pinot Noir' lead to an increase of MDA within 45 minutes to a maximum of 0.6 μM/g(FW) and decreased fast to its normal level after 3 hours. Mannitol also lead to a fast increase after 45 minutes to a maximum of 0.5 μM/g(FW) and decreased to its normal level after 3 hours (compare Fig. 5.2, Fig. 5.3).

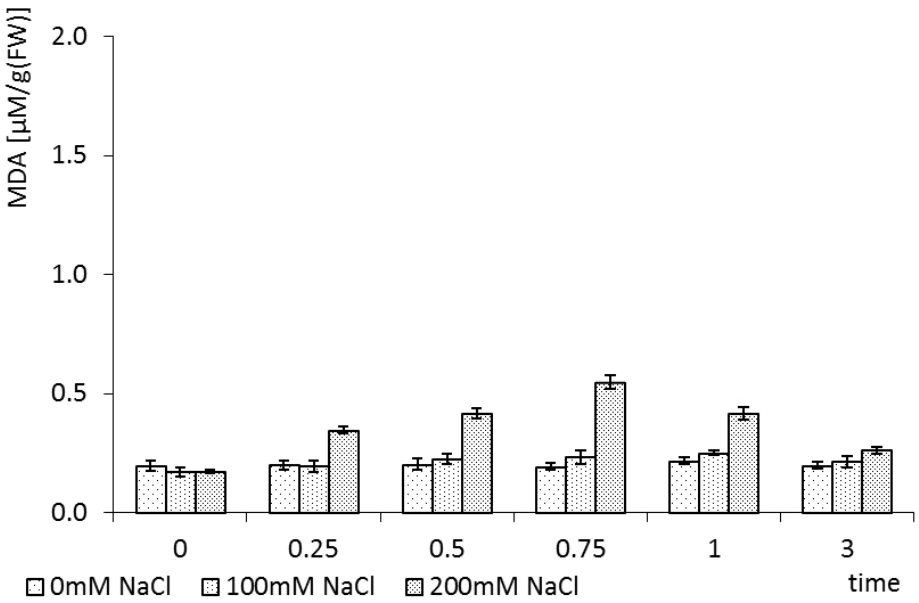

Figure 5.2: Time course of stress-dependent MDA formation in a 7 day old suspension cell culture of *V. vinifera* cv. 'Pinot Noir' after treatment of 100 mM and 200 mM NaClin term of three hours. Values represent means and standard errors from three independent triplicate measurements.

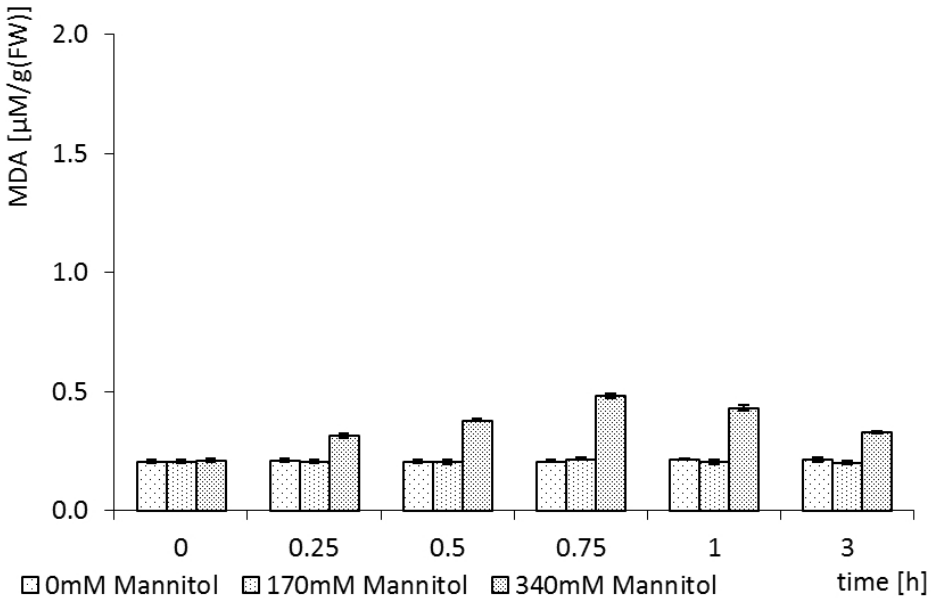

Figure 5.3: Time course of stress-dependent MDA formation in a 7 day old suspension cell culture of *V. vinifera* cv. 'Pinot Noir' after treatment of 170 mM and 340 mM mannitol in term of three hours. Values represent means and standard errors from three independent triplicate measurements.

5.3 Inhibition of the NADP(H) oxidase can suppress salt-induced MDA formation

The pretreatment of a *V. vinifera* cv. 'Pinot Noir' suspension culture (Fig. 5.4 & Fig. 5.5) with 100 nM DPI for 5 minutes prior to addition of 200 mM NaCl can completely suppress the salt-induced accumulation of MDA. The control experiment, where 100 nM DPI was administered in the absence of salt, was also performed and revealed that the low residual level of MDA in control cells was not altered.

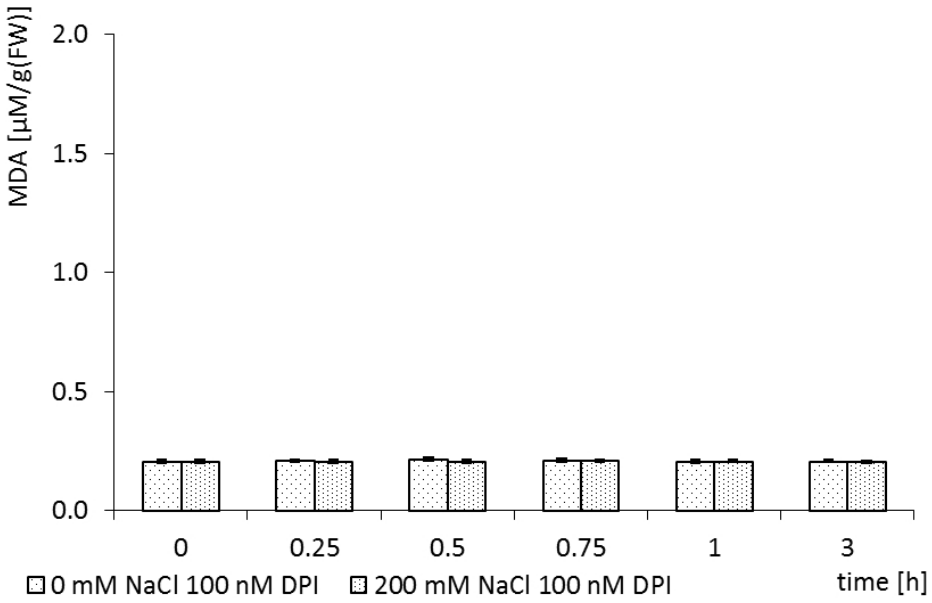

Figure 5.4: ROS dependent MDA formation in a 7 day old suspension cell culture of *V. vinifera* cv. 'Pinot Noir' after induced salt stress with 200 mM NaCl and blocking NADP(H) oxidase by 100 nM DPI for 5 minutes prior to addition of salt. Values represent means and standard errors from three independent triplicate measurements.

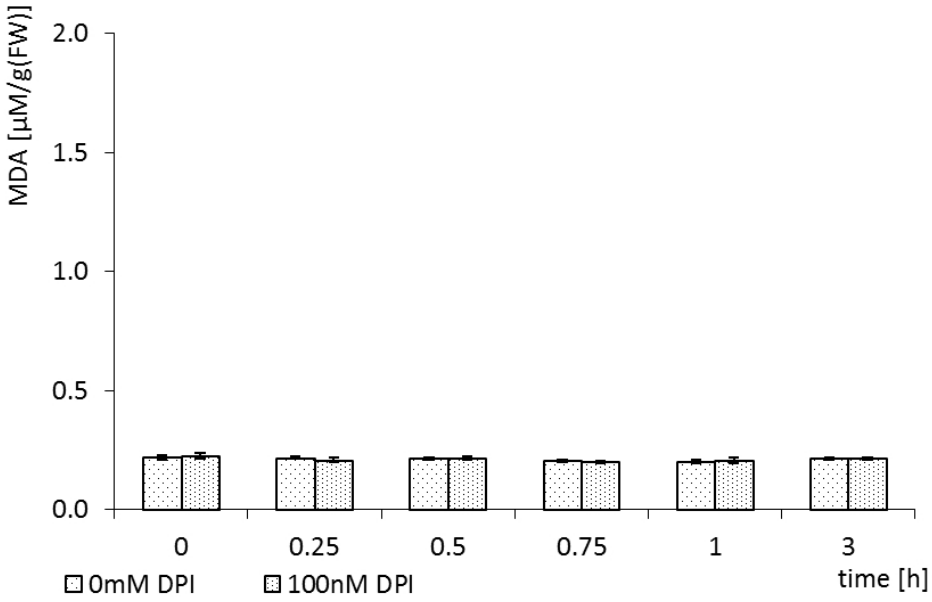

Figure 5.5: ROS dependent MDA formation in a 7 day old suspension cell culture of *V. vinifera* cv. 'Pinot Noir' after blocking NADP(H) oxidase by 100 nM DPI. Values represent means and standard errors from three independent triplicate measurements.

5.4 Inhibition of the NADP(H) oxidase can trigger cell death

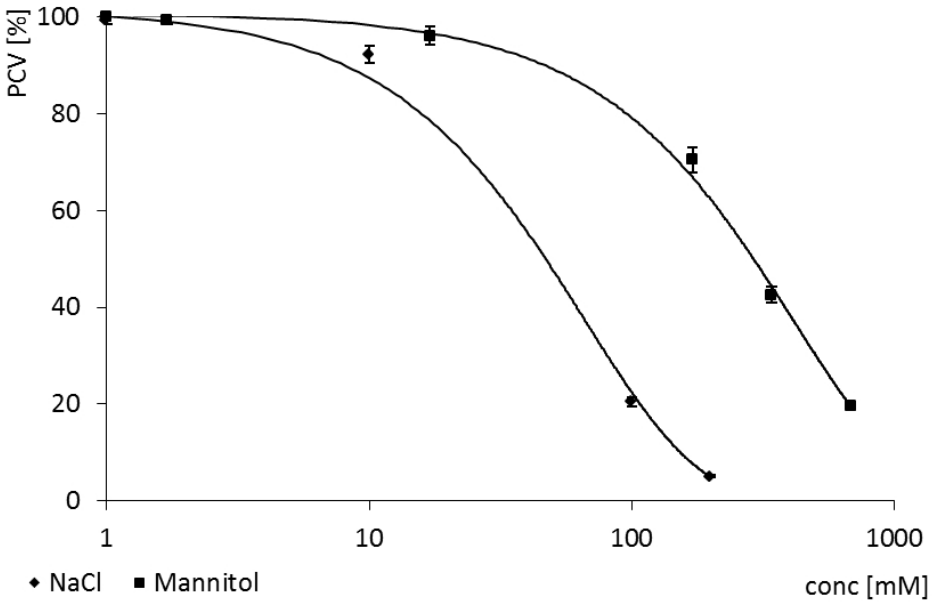

Figure 5.6: Sustained effect of a cell suspension culture of *V. vinifera* cv. 'Pinot Noir' after NaCl or mannitol treatment. An Increase in packed cell volume (ΔPCV) over different concentrations of NaCl or mannitol, respectively relative to the values obtained for untreated controls. Values were measured at day 5 after subcultivation.

The decrease in packed cell volume (PCV) as indicator for culture growth was measured for a cell suspension culture of *V. vinifera* cv. 'Pinot Noir' to monitor the physiological response to stress-induced oxidative burst. A dose-response curve of cell growth (monitored as the increment of PCV, ΔPCV) using different concentrations of NaCl (0-200 mM) and mannitol (0-680 mM) measured at day 5 after subcultivation was performed. Fig 5.6 shows a dose-dependent inhibition. The dose-response curve was shifted to higher values for mannitol if compared to NaCl as it could be shown for *V. rupestris*.

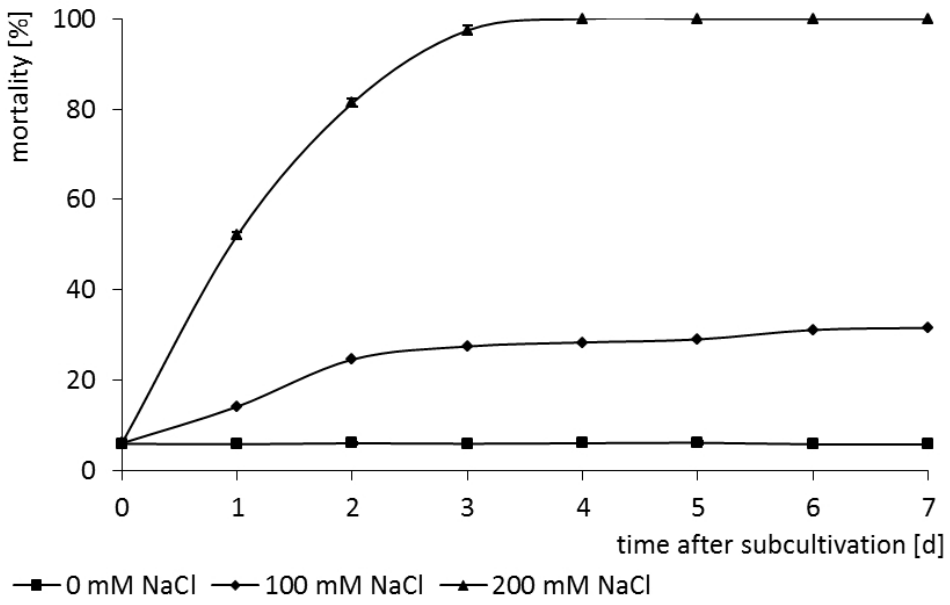

Figure 5.7: Time courses of the mortality of a cell suspension culture of *V. vinifera* cv. 'Pinot Noir' for different concentrations of NaCl. Values represent means and standard errors from three independent duplicates.

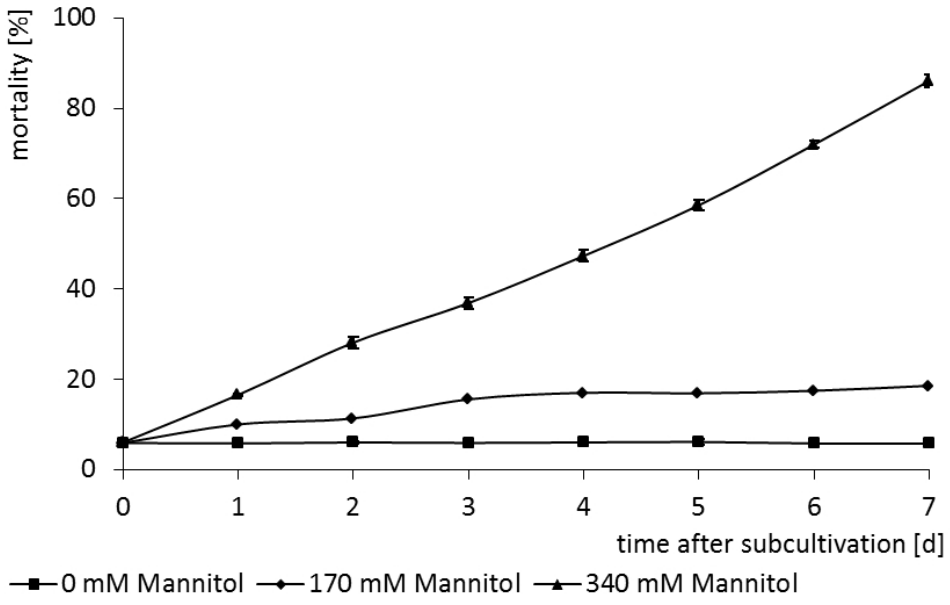

Figure 5.8: Time courses of the mortality of a cell suspension culture of *V. vinifera* cv. 'Pinot Noir' for different concentrations of mannitol. Values represent means and standard errors from three independent duplicates.

Further, the mortality of *V. vinifera* cv. 'Pinot Noir' was determined for NaCl (0, 100 and 200 mM) and osmotically equivalent concentrations of mannitol (0, 170 and 340 mM). Mortality increased depending on concentration and time. For *V. vinifera* cv. 'Pinot Noir' the results were very similar to *V. rupestris* and are shown in Fig 5.7 & Fig 5.8), but all treatments caused more stingent stress as compared to *V. rupestris*.

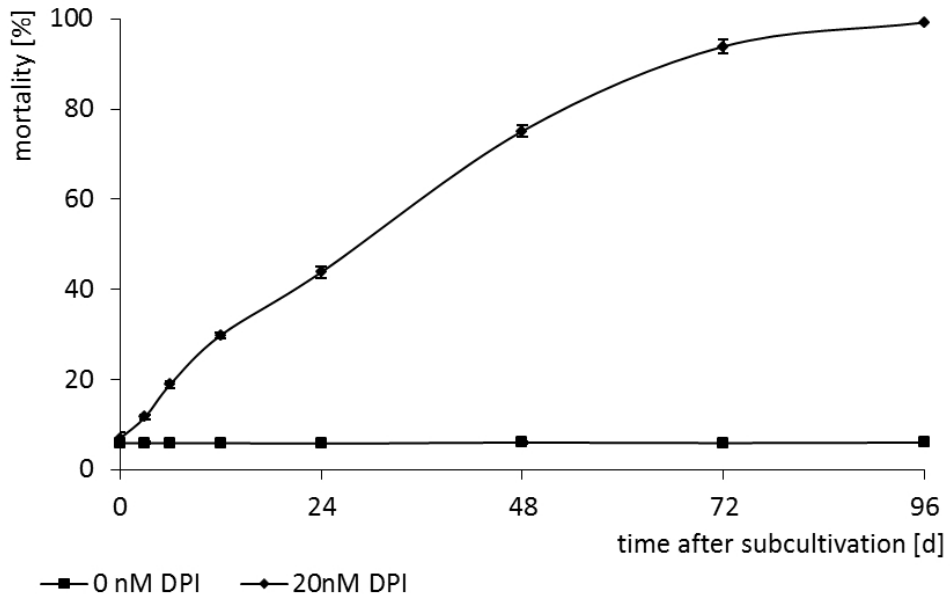

Figure 5.9: Time courses of the mortality of a cell suspension culture of *V. vinifera* cv. 'Pinot Noir' in response to a treatment with 20 nM DPI. Values represent means and standard errors from three independent duplicates

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A mortality experiment with a DPI treatment (inhibitor of the NADP(H) oxidase) was also performed for a cell suspension culture of *V. vinifera* cv. 'Pinot Noir'. This control experiment using incubation with the inhibitor alone, in the absence of salt stress, produced the same surprising result - the mortality increased rapidly, already within 3 hours after addition of the inhibitor, and after 3 days most cells had undergone cell death (Fig 5.9). This time course was very similar to that observed for the treatment with

200 mM NaCl. The co-treatment of 200 mM NaCl and 20 nM DPI was further tested and revealed the same result as seen for *V. rupestris* - a slower and weaker increase in mortality with a saturation at about 50% (Fig 5.10).

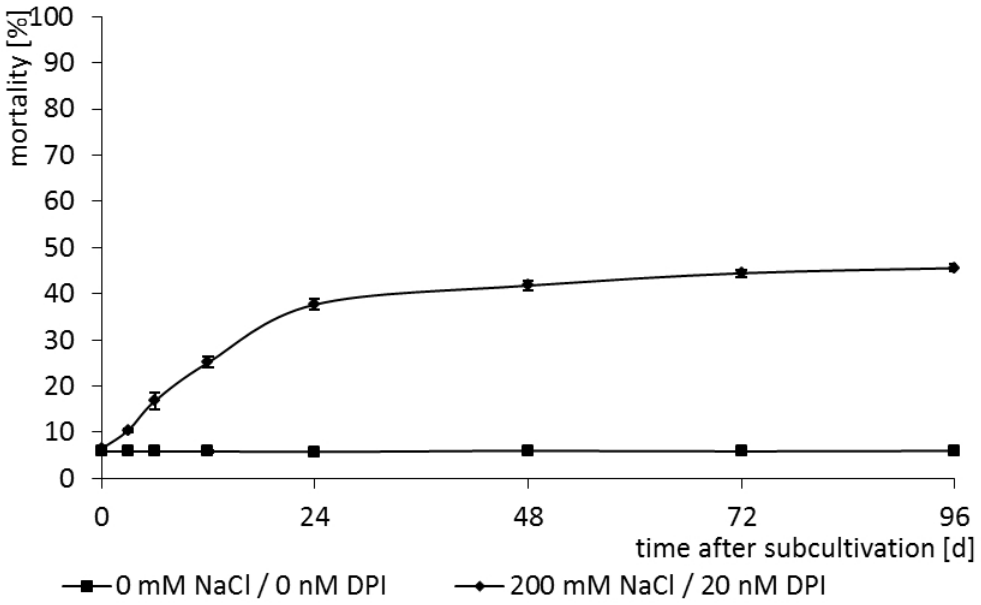

Figure 5.10: Time courses of the mortality of a cell suspension culture of *V. vinifera* cv. 'Pinot Noir' in response to a co-treatment with 20 nM DPIand 200 mM NaCl. Values represent means and standard errors from three independent duplicates.

Publications

Schneider, N., Ludwig. H., Nick, P. (2015). Suppression of tubulin detyrosination by parthenolide recruits the plant-specific kinesin KCH to cortical microtubules. Journal Experimental Botany 66, 2001-2011.

Ludwig, H., Nick, P. (2015). Divide or Die - Suppression of Stress Signalling Causes Cell Death in Grapevine. Unpublished manuscript.