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Uranium-induced oxidative stress in *Arabidopsis thaliana:* influence of pH on uranium toxicity

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Woord vooraf

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Summary

Uranium (U) is a naturally and commonly occurring radioactive element and heavy metal. Due to anthropogenic activities, such as U mining and milling, large areas have been contaminated with U. Uranium has a complex chemistry and its behaviour, mobility and bioavailability in the soil is strongly dependent on the U speciation. One of the important factors controlling the speciation is the pH value. Toxicity of U in plants (e.g. *Arabidopsis thaliana*), is mainly investigated in lab experiments under ideal growth conditions (pH 5.5). Through this, the contribution of different U species, generated through pH changes, has not been taking into account. Therefore, when evaluating the environmental impact of U contamination, it is important to investigate this under different environmentally relevant setups that can influence U toxicity.

The main objective of this research is to analyse the **effects of environmentally relevant parameters on U toxicity**. The main focus is to investigate the influence of the pH on U toxicity at different levels of biological complexity (from the individual to the subcellular and molecular level) in order to study the mechanisms that play an important role in the U-induced stress responses in plants. Exposing plants to 25 µM U at different pH levels, ranging from 4.5 to 7.5 (chapter 4), resulted in a high uptake of U but only a limited fraction was transported to the leaves at low pH. At pH 7.5, less U was taken up by the roots, but it was more easily transported to the leaves. The differences in translocation can possibly be related to the differences in speciation. At low pH, the highly reactive uranyl ion (UO_2^{2+}) was predicted to be the dominant U species present. This ion will precipitate with e.g. phosphate moieties present in root cell membranes leading to its immobilization. At higher pH, carbonate species are prevalent. They are less reactive and can therefore more easily be transported to the leaves.

In addition to the large differences in U uptake and translocation, the biomass and the capacities of enzymes related to the antioxidative defence system were differentially affected at the different pH levels. To investigate the underlying mechanisms of U toxicity in more detail, **dose-dependent responses** were

studied at pH 4.5 and pH 7.5 (chapter 5-7). Some U responses could be observed that were present at both pH levels. An increased *MIR398b/c* expression, which is involved in the regulation of the expression of specific genes of the antioxidative defence system, was observed after U exposure at both pH levels. As such, the role of miRNA398b/c in the regulation of the U-induced oxidative stress responses was shown for the first time. In addition, U exposure induces the biosynthesis of ascorbate (AsA) in the leaves at both pH levels. Since an increased AsA content was also observed before in *Arabidopsis thaliana* plants exposed to U at pH 5.5, it seems that AsA plays an important role in the antioxidative defence mechanisms during U stress. In an attempt to elucidate the role of AsA in the U response, AsA deficient *vtc* mutants of *Arabidopsis thaliana* were exposed to 25 µM U (chapter 9). Since the miRNA398b/c response after U stress was more pronounced in the *vtc* mutants as compared to the wild-type plants, this further supports the hypothesis that AsA is a regulator of the U-induced stress responses.

Based on the **dose-response curves**, the EC50 values for growth reduction were calculated. The EC50 value for the **roots** at pH 4.5 was about 2.5 times lower than the value observed at pH 7.5. In addition, a number of changes in the antioxidative pathway, such as the activation of miRNA398b/c, were observed at a lower U concentration at pH 4.5 as compared to pH 7.5. Finally, the AsA redox balance could not be determined in roots exposed to the higher U concentrations used at pH 4.5, indicating that those roots were seriously damaged, and a significant decrease in the GSH redox balance was observed. At pH 7.5, the AsA redox state shifted to the oxidized state, but the GSH redox balance could be maintained. These results indicate that U causes more adverse effects at low pH in the roots. This can possibly indicate that U is more toxic at low pH. In addition, the differences can possibly be explained by the fact that for similar nominal U concentrations, U was more readily taken up at low pH, possibly leading to more adverse effects.

Although the U concentration in the **leaves** of U-exposed plants was low, a significant reduction in leaf fresh weight could be observed at both pH levels. Simlar as in the roots, the EC50 for leaf growth reduction at pH 4.5 was approximately two times lower than the EC50 value observed at pH 7.5. In

addition, the concentration at which miRNA398b/c were activated was lower at pH 4.5 as compared to pH 7.5. Although the U concentration in the leaves of plants exposed to U at pH 4.5 was lower than at pH 7.5, those results indicate that U caused more adverse effects at low pH. This supports the hypothesis that U is more toxic at low pH. This can be related to differences in U speciation or to the involvement of root-to-shoot signalling. Since the roots exposed to U at low pH are seriously stressed, this can lead to the activation of defence reactions in the leaves via still unknown root-to-shoot signalling molecules.

Since organisms are typically exposed to multiple stressors in the environment, the **effects of U in combination with Cu** were investigated (chapter 9). In the roots, the expression of *LOX1*, an enzyme involved in the production of reactive oxygen species, was induced 20 times after exposure to U and Cu as compared to the expression when U or Cu were applied as single stressors. Although more research is needed, this points towards an important role for LOX1 in the plant response when plants are exposed to a combination of U and Cu.

In conclusion, the large differences in U uptake and translocation can be related to the different U species present at pH 4.5 and pH 7.5. Uranium seems to be more toxic at low pH, which can be attributed to a higher uptake of U but also to the high UO_2^{2+} concentrations present in the medium at low pH, which is supposed to be the most toxic U species. Although at pH 7.5 the U concentration in the leaves was about 3 times higher than at pH 4.5, most effects were observed at low pH. This possibly indicates an important role for root-to-shoot signalling. The differences in U toxicity at different pH levels observed in this research stress the need to take site-specific characteristics into account when making a risk assessment for U-contaminated areas.

Samenvatting

Uranium (U) is een natuurlijk, veel voorkomend radioactief element en zwaar metaal. Door antropogene activiteiten zoals het ontginnen en verwerken van U werden grote gebieden gecontamineerd met U. Het gedrag, de mobiliteit en de biobeschikbaarheid van U in de bodem is sterk afhankelijk van de U speciatie die bepaald wordt door o.m. de zuurtegraad (pH). De toxiciteit van U op planten, waaronder *Arabidopsis thaliana*, werd tot nu toe voornamelijk onderzocht in labo-experimenten onder ideale groeicondities en dus enkel bij een pH van 5.5. De mogelijke bijdrage van de verschillende U species, gegenereerd door pH veranderingen, aan de toxiciteit wordt hierbij niet in rekening gebracht. Voor een correcte risicoanalyse van de impact van U verontreiniging op het milieu, is het daarom belangrijk om rekening te houden met de verschillende omgevingsparameters die de U toxiciteit kunnen beïnvloeden.

De belangrijkste doelstelling van dit onderzoek is om **de effecten van relevante omgevingsparameters op de U toxiciteit** te onderzoeken. De focus ligt hierbij op de invloed van de pH op de toxiciteit van U op verschillende niveaus van biologische complexiteit: van het individuele tot het subcellulaire en moleculaire niveau. Dit geeft de mogelijkheid om mechanismen die belangrijk zijn voor de U toxiciteit in planten te bestuderen. Wanneer planten werden blootgesteld aan 25 µM U bij verschillende pH niveaus, variërend van 4.5 tot 7.5 (hoofdstuk 4), werd bij lage pH veel U opgenomen door de wortels, maar hiervan werd een relatief kleine fractie getransporteerd naar de blaadjes. Bij pH 7.5 werd er minder door de wortels opgenomen maar in verhouding meer naar de blaadjes getransporteerd. Dit verschil in translocatie naar de blaadjes kan mogelijk verklaard worden door het verschil in U speciatie bij de verschillende pH niveaus. Zo is bij pH 4.5 voornamelijk het reactieve uranyl ion (UO_2^{2+}) aanwezig. Dit ion kan gemakkelijk binden aan vb. fosfaatgroepen die aanwezig zijn in de celmembranen waardoor het geïmmobiliseerd wordt. Bij pH 7.5 zijn voornamelijk U-carbonaten aanwezig. Deze zijn minder reactief en kunnen daardoor gemakkelijker naar de blaadjes getransporteerd worden.

Naast het grote verschil in U opname en translocatie werden ook verschillen gevonden op biomassa en op de capaciteiten van de enzymen betrokken in het antioxidatieve verdedigingssysteem. Om de onderliggende mechanismen van U toxiciteit meer in detail te bestuderen, werden de **dosis-afhankelijke responsen** bij pH 4.5 en pH 7.5 bestudeerd (hoofdstuk 5-7). Hierbij werden enkele U responsen geïdentificeerd die bij beide pH niveaus aanwezig waren. Zo lijkt miRNA398b/c, dat betrokken is bij de regulatie van de expressie van bepaalde genen van het antioxidatieve verdedigingssysteem, een belangrijke rol te spelen in de U-geïnduceerde stress reacties. Na U blootstelling werd namelijk een stijging van de *MIR398b/c* expressie waargenomen zowel in de blaadjes als in de wortels en dit voor beide pH niveaus. Uraniumblootstelling induceert ook de biosynthese van ascorbate (AsA) bij pH 4.5 en pH 7.5, wat ook al geobserveerd was indien planten blootgesteld werden aan U bij pH 5.5. Dit kan wijzen op een belangrijke rol voor dit metaboliet in de antioxidative verdedigingsmechanismen tijdens U stress. Om de rol van AsA verder te onderzoeken werden *Arabidopsis thaliana* planten met een verlaagd AsA gehalte (*vtc* mutanten) blootgesteld aan 25 µM U (hoofdstuk 9). Vermits de miRNA398b/c respons onder U stress meer uitgesproken was in de *vtc* mutanten dan in de wild-type planten, lijkt dit de hypothese te bevestigen dat AsA een regulator is van de U-geïnduceerde stress responsen.

Op basis van de **dosis-respons curves**, werd een EC50 waarde berekend voor de groeireductie. De EC50 waarde in de **wortels** bij pH 4.5 was ongeveer 2.5 keer lager dan deze bij pH 7.5. De veranderingen in de antioxidatieve verdedigingsmechanismen, waaronder de activatie van miRNA398b/c, gebeurde bij lagere U concentratie bij pH 4.5 in vergelijking met pH 7.5. Daarnaast kon de AsA redox balans niet bepaald worden in de wortels die blootgesteld werden aan de hogere U concentraties bij pH 4.5, wat erop wijst dat de wortels ernstig beschadigd waren. Ook werd in deze wortels een daling in de glutathion (GSH) redox balans waargenomen. In de wortels blootgesteld bij pH 7.5 verschoof de AsA redox balans naar de geoxideerde toestand, maar de GSH redox balans kon behouden blijven. Uit deze resultaten lijkt het dat U in de wortels meer toxisch is bij lage pH. Dit kan te wijten kan zijn aan een verschil in U speciatie maar kan mogelijk ook verklaard worden door het feit dat bij gelijke nominale U

concentraties, meer U opgenomen werd bij een lage pH, wat mogelijk kan leiden tot meer nadelige effecten.

In de **blaadjes** was de U concentratie laag maar werd toch een significante daling in het versgewicht waargenomen bij beide pH niveaus. Net als in de wortels was de EC50 waarde voor groeireductie in de blaadjes bij pH 4.5 ongeveer 2 keer lager dan deze bij pH 7.5. Ook was de concentratie waarbij miRNA398b/c geactiveerd werd bij pH 4.5 lager in vergelijking met pH 7.5, hoewel de U concentratie in de blaadjes bij pH 7.5 hoger is dan bij pH 4.5. Deze resultaten wijzen er weer op dat U meer toxische effecten veroorzaakt bij lage pH, wat mogelijk te wijten kan zijn aan een verschil in speciatie. Een andere mogelijke verklaring voor deze resultaten is de signalering van wortel naar blad. Omdat de U-blootgestelde wortels bij lage pH ernstig gestresseerd zijn, kan dit leiden tot de activatie van verdedigingsmechanismen in de blaadjes via tot nu toe onbekende moleculen.

Omdat organismen meestal blootgesteld worden aan meer dan één stressor, werd ook het **effect van U in combinatie met Cu** onderzocht (hoofdstuk 9). Zo werd in de wortels na blootstelling aan U+Cu de expressie van *LOX1*, een enzym betrokken in de productie van reactieve zuurstofspecies, 20 maal geïnduceerd ten opzichte van de expressie geobserveerd indien U en Cu apart toegediend werden. Hoewel meer onderzoek nodig is duidt dit op een belangrijke rol van LOX1 in de respons van planten na blootstelling aan U en Cu.

Als conclusie kunnen we stellen dat de verschillen in U opname en translocatie gerelateerd kunnen worden aan de verschillende U species die aanwezig zijn bij pH 4.5 en pH 7.5. Ook lijkt U meer toxisch bij lage pH, wat te wijten kan zijn aan een hogere opname van U, maar ook aan de hoge concentratie van UO_2^{2+} in het medium, sinds deze vorm van U wordt beschouwd als de meest toxische. Hoewel de U concentratie in de blaadjes bij pH 7.5 ongeveer 3 keer hoger was dan bij pH 4.5, werden de meeste effecten waargenomen bij lage pH. Dit kan wijzen op een belangrijke rol voor signalering van wortel naar blad. De verschillen in U toxiciteit bij verschillende pH niveaus die tijdens dit onderzoek geobserveerd werden, wijzen op het belang om rekening te houden met plaats-specifieke karakteristieken wanneer men een inschatting maakt van de nadelige effecten van U in de omgeving.

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Abbreviations

APX	ascorbate peroxidase
AsA	ascorbate
Ca	calcium
CAT	catalase
Cd	cadmium
CSD	copper/zinc superoxide dismutase
Cu	copper
DHA	dehydroascorbic acid
DTNB	dithiobis(2-nitro-benzoic acid)
DTT	dithiothreitol
DW	dry weight
ECx	effective concentration causing x per cent effect
ETR(II)	electron transport rate of photosystem II
F_0	minimum fluorescence in dark adapted leaves
Fe	iron
F_m	maximum fluorescence in dark adapted leaves
F'_m	maximum fluorescence in light adapted leaves
FSD	iron superoxide dismutase
F_v	variable fluorescence in dark adapted leaves
FW	fresh weight
GPX	guaiacol peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	oxidized glutathione
H	hydrogen
H_2O	water
H_2O_2	hydrogen peroxide
LOX	lipoxygenase
MDHA	monodehydroascorbate
Mg	magnesium
miRNA398	microRNA 398
Mn	manganese
MSD	manganese superoxide dismutase
NADPH	nicotinamideadeninedinucleotide
$^1O_2$	singlet oxygen
O_2	atmospheric oxygen
$O_2^{\bullet-}$	superoxide
$OH^{\cdot}$	hydroxyl radical
PAM	pulse amplitude modulated
Pb	lead
PCR	polymerase chain reaction
PCS	phytochelatin synthase
PSII	photosystem II
PUFA	polyunsaturated fatty acid
Px	peroxidases
qL	coefficient of photochemical quenching

RBOH	respiratory burst oxidase homologue
ROS	reactive oxygen species
SOD	superoxide dismutase
SPL7	SQUAMOSA promoter-binding protein-like 7
SPX	syringaldazine peroxidase
TBA-rc	thiobarbituric acid reactive compounds
U	uranium
UO_2^{2+}	free uranyl ion
Vtc	vitamin C
WT	wild-type
Y(II)	effective PSII quantum yield
Y(NO)	quantum yield of nonregulated energy dissipation
Y(NPQ)	quantum yield of regulated energy dissipation
Zn	zinc

Chapter 1

Introduction

1.1. Uranium

1.1.1. Uranium properties

Uranium (U) is a naturally and commonly occurring radioactive element and heavy metal, with an atomic weight of 238.0289 g mol^{-1}. As member of the actinide series, it has atomic number 92. Uranium has a silver-white colour and is the heaviest chemical element found in nature. In addition, it is more abundant than gold, silver or cadmium (WHO, 2001; Gavrilescu *et al.*, 2009). There are three natural isotopes of U: ^{234}U, ^{235}U and ^{238}U. Uranium-235 and ^{238}U are the head of two different decay chains, the U series and the actinium series, respectively (Bleise *et al.*, 2003; Gavrilescu *et al.*, 2009). All three U isotopes behave the same chemically because they all have 92 protons, but they have different radioactive properties (Table 1.1) (ATSDR, 1999; Bleise *et al.*, 2003). The most abundant naturally occurring U isotope is ^{238}U. It has the longest half-life and, consequently, the lowest specific activity (Bleise *et al.*, 2003).

Table 1.1: Properties of the three naturally occurring uranium isotopes (Bleise *et al.*, 2003).

Isotope	Abundance (%)	Half-life (year)	Specific activity (Bq g^{-1})
^{234}U	0.006	2.46×10^5	231×10^6
^{235}U	0.72	7.04×10^8	80011
^{238}U	99.3	4.47×10^9	12455

All naturally occurring U isotopes are weakly radioactive. They will disintegrate by emitting alpha particles, i.e. positively charged ions consisting of two protons and two neutrons (Scientific Committee on Health and Environmental Risks, 2010). Further disintegrations will occur by emitting alpha, beta or gamma radiation, which will lead to the formation of a stable lead isotope (ATSDR, 1999; Scientific Committee on Health and Environmental Risks, 2010). Due to their large size and charge, alpha particles have little penetrating power since they lose their energy fast by colliding with other molecules. However, they will

cause a number of ionizations over a short pathway because of their high LET-value (linear energy transfer). As a result, U mainly represents an internal radiation hazard (Ribera *et al.*, 1996; Bleise *et al.*, 2003). In addition, U is classified as a heavy metal since it will be more chemotoxic than radiotoxic, due to the long half-life of natural U (Ribera *et al.*, 1996). Its chemical toxicity mimics that of lead (Shahandeh *et al.*, 2001).

1.1.2. Natural occurrence of uranium

Uranium is a primordial metal which is widely distributed in nature. The average concentration of U is 3 mg kg^{-1} in the earth's crust. In continental waters, the concentration varies between 10^{-6} and 10^{-3} mg kg^{-1}, while the concentration in the sea is approximately 3.3 x 10^{-3} mg kg^{-1} (Garnier-Laplace *et al.*, 2001). However, local abundance is strongly dependent on the concentration of U in surrounding rocks and soil. The main natural sources of U are hydrothermal veins, sedimentary rocks and pyritic conglomerate beds. Uranium also occurs in different minerals. However, in most minerals the concentration of U is very low. In others (e.g. zircon) and rare earths, the level of U is considerably elevated, with activities up to 70 kBq kg^{-1} (Vandenhove, 2004). In addition, U is found in lignite, monazite sands, phosphate rock and phosphate fertilizers (Ribera *et al.*, 1996; Bleise *et al.*, 2003; Vandenhove, 2004). The main geographical locations for U are for example Australia, Canada, France, West Africa and Russia (Ribera *et al.*, 1996; WHO, 2001). Natural phenomenon's such as erosion, wind activity and volcanic eruptions can cause a redistribution of U in the environment. However, anthropogenic activities such as U mining and milling, phosphate mining and heavy metal mining have caused enormous damage to the environment by means of improper disposal of the radioactive waste material. Furthermore, nuclear weapon facilities, above ground nuclear testing, nuclear reactor operations and nuclear accidents contribute to the U contamination (Vandenhove, 2002; Vandenhove, 2004; Gavrilescu *et al.*, 2009).

1.1.3. Applications of uranium

Uranium has historically been used in the colouring of ceramics and glass, producing a fluorescent yellow or light green colour. Until the early 1980s U has also been used in the production of dental porcelains to obtain a natural colour

and fluorescence. Another important application of U is as fuel for nuclear power stations and for nuclear weapons. At present, depleted U is used for the shielding of gamma radiation, as counterbalance weights in boats and aircrafts and in military munitions and armour (ATSDR, 1999; WHO, 2001).

1.1.4. Uranium behaviour in water and soil

Uranium can adopt four valances resulting in the ions U^{3+} (III), U^{4+} (IV), UO_2^{+} (V) and UO_2^{2+} (VI) (Ribera *et al.*, 1996). Uranium +4 and +6 are the most important oxidation states of U (Gavrilescu *et al.*, 2009). The oxidized uranyl ion can form complexes with carbonate or sulphate ions which will lead to a soluble and easily transportable ion. Under reducing conditions, U occurs as the tetravalent ion and will bind to organic material which will lead to precipitation and immobilization. As such, U can be found in the soils as sorbed, complexed, precipitated and reduced forms, which will have an impact on U mobility (Sheppard *et al.*, 2005; Gavrilescu *et al.*, 2009).

The behaviour, mobility and bioavailability of U in the soil are strongly dependent on the speciation. A chemical species can be described as a specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure. The speciation describes the distribution of species in a system (Templeton *et al.*, 2000). Uranium can be present in a wide variety of species. Important factors controlling the speciation of U are for example pH, redox potential and the availability of inorganic or organic ligands (Bernhard, 2005). The pH dependent speciation of U has extensively been studied (Figure 1.1). In the soil and in most waters below a pH of 5, U is mainly present as the hexavalent species UO_2^{2+} (Ebbs *et al.*, 1998; Vandenhove, 2004). This is the most stable form of U (Shahandeh *et al.*, 2001; Vandenhove, 2004). Hydroxide (e.g. UO_2OH^{+}, $(UO_2)_2(OH)_2^{2+}$) and phosphate complexes (e.g. UO_2HPO_4, $UO_2(HPO_4)_2^{2-}$) form under neutral conditions, while at higher pH complexes are formed with natural ligands such as inorganic ions (e.g. carbonate (e.g. UO_2CO_3, $UO_2(CO_3)_2^{2-}$)) and natural organic matter (e.g. humic acids) (Ebbs *et al.*, 1998; Laurette *et al.*, 2012). In the presence of phosphate, U-phosphate complexes will form, which will reduce the level of the free uranyl ion and U hydroxides and will lead to immobilization. According to Ebbs *et al.* (1998) addition of phosphate to a U containing solution overcomes

the toxic effects of U, most likely due to complexation of U with phosphate (Ebbs *et al.*, 1998). Conversely, complexation to carbonate or citrate increases U solubility (Laurette *et al.*, 2012).

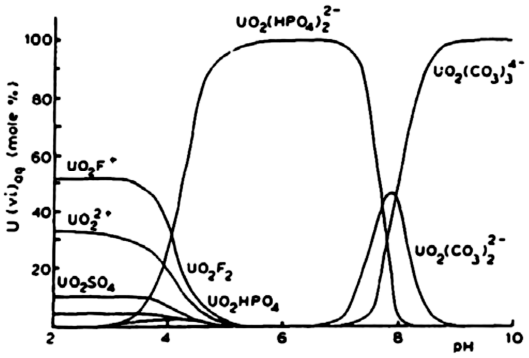

Figure 1.1: Distribution of some uranyl complexes versus pH for some typical ligand concentrations in groundwaters of the Wind River formation at 25°C. $P_{CO_2}=10^{-2.5}$ atm, F=0.3 mg l^{-1}, Cl=10 g l^{-1}, SO_4=100 mg l^{-1}, PO_4=0.1 mg l^{-1}, SiO_2=30 mg l^{-1} (Langmuir, 1978).

In surface soil and water, i.e. aerobic conditions, U is mainly present in the +6 oxidation state. In this oxidation state, the speciation is mainly controlled by pH, redox potential and the presence of complexing agents (Laurette *et al.*, 2012). In normal groundwaters, U is mainly present as carbonate species, although sulphate and phosphate complexes may also form. The carbonate complexes are highly mobile in most soils. Under acidic conditions, stable complexes will be formed with soil organic matter which will lead to retention and accumulation of U (WHO, 2001).

1.1.5. Effect of uranium on animals and humans

Although U has no known metabolic function in humans and animals, and as such is considered as a nonessential element, exposure to U is unavoidable since it is found everywhere in small amounts (WHO, 2001). The doses of external exposure with natural U are negligible. The alpha emitted is innocuous since it cannot reach the basal layer of the epidermis. However, the risk will increase during beta and gamma disintegrations (Ribera *et al.*, 1996). Uranium poses a risk by internal exposure of humans and animals. This can occur by inhalation of atmospheric particles, ingestion of food or water containing U or through wounds (Ribera *et al.*, 1996; ATSDR, 1999). Although natural U is only a weak radioactive material, it also has a chemotoxic potential (Bleise *et al.*, 2003).

Concerning radiological toxicity, only enriched U presents a problem (Ribera *et al.*, 1996). When breathing U dust, part of it is exhaled but it also partially stays in the lungs. Big particles are blown out or pushed to the throat and swallowed. Small particles stay in the lower part of the lungs and stay there or dissolve into the blood. As such, the more soluble compounds (e.g. uranyl tetrachloride, uranyl nitrate hexahydrate) are less toxic to the lungs, but they can be absorbed more easily from the lungs into the blood and be transported to distal organs (ATSDR, 1999). However, most of the U in the blood will leave the body via urine in a few days (approximately 90%). More insoluble U salts and oxides (e.g. U tetrafluoride, U dioxide) may be retained in the lungs for weeks up to years. In the lungs, they can decay into daughter products causing a radiation hazard. The daily ingestion of U is estimated to be 1-2 µg via food (e.g. bread, fruit, vegetables, meat, milk) and 1.5 µg via drinking water. People living near U mines, working at factories that process U or working with phosphate fertilizers have a chance of taking in more U than other people (ATSDR, 1999; Anke *et al.*, 2009). Uranium can enter the food chain via the soil-plant-animal pathway. In addition, the use of contaminated drinking water can also lead to a direct exposure of humans and animals (Vandenhove, 2004). By the oral exposure route, water-soluble compounds are more toxic because of the greater ease of absorption in the gastrointestinal tract (ATSDR, 1999). However, most (>98%) of the U introduced into the gastrointestinal tract is excreted via faeces. The amount of U that is absorbed will be transferred to the bone, the liver and the kidneys and accumulates there (Ribera *et al.*, 1996; Scientific Committee on Health and Environmental Risks, 2010). The kidneys are the most sensitive target of U, with toxic effects as cellular necrosis and atrophy in the tubular wall, resulting in a decreased reabsorption efficiency (ATSDR, 1999). Thus, U can affect the organs in the body where it is retained. The retention time of U in the body can be split into a biological and physical retention time. The first parameter depends on mechanisms and kinetics of transfer between organs, while the second one depends on the declining radioactivity of U. Due to its long half-life, the retention of U will mainly depend on the biological retention time. In addition, the long half-life of naturally occurring U also makes it more chemotoxic than radiotoxic. The chemical toxicity depends on the chemical combination. The soluble U compounds are highly toxic, while the non-soluble

compounds are less dangerous. The aqueous UO_2^{2+} ion causes the main U chemical toxicity. Since U can induce the production of reactive oxygen species (ROS), it can induce damage to biological molecules such as nucleic acids, proteins and lipids, leading to e.g. DNA damage, enzyme inactivation and membrane damage (Ribera *et al.*, 1996; Tasat *et al.*, 2007). If the DNA damage results in repair errors, this can lead to gene mutations or chromosomal aberrations. These effects may be manifested as cancer. However, intake of U at low concentrations usually ingested is not likely to cause cancer (ATSDR, 1999).

1.1.6. Effect of uranium on plants

Since U is found in all soils, it can be present in all plants. However, the concentration found in plants is strongly dependent on the soil type, plant species and radionuclide physicochemical form (Ribera *et al.*, 1996). In general, roots accumulate much more U than stems, leaves and seeds, resulting in low root-to-shoot transfer factors (Ribera *et al.*, 1996; Shahandeh *et al.*, 2001; Vandenhove, 2004). In addition, more U is found in leaves and stems than in the grains or fruits (Ribera *et al.*, 1996; Garnier-Laplace *et al.*, 2001).

Plants take up essential metals predominantly via channels and transporters in the root plasma membrane. Since metals can be toxic, the uptake and distribution of metals is regulated. However, sometimes absorption of toxic non-essential metals occurs because of the chemical similarity between essential and toxic metals. However, plant uptake mechanisms for U have not been studied well (Mortvedt, 1994), although it is suggested that many plant species take up U via calcium uptake mechanisms and incorporate it into their biomass (Shahandeh *et al.*, 2001; Markich *et al.*, 2002). In addition, it is generally observed that plants species differ in U accumulation (Shahandeh *et al.*, 2001). Ebbs *et al.* (2001) demonstrated that e.g. *Phaseolus acutifolius* and *Beta vulgaris* take up much more U then e.g. *Brassica rapa* or *Medicago sativa*. Duquène *et al.* (2006) also observed large differences in U uptake by different plants species.

In addition, U uptake by the roots and transfer to the shoots depends on chemical species available to the plant (Bernhard, 2005). A recent study of Laurette *et al.* (2012) shows that at low pH, when there was mainly the

presence of UO_2^{2+} and $UO_2(SO_4)$ in the medium, U is adsorbed onto the plant root surfaces and/or accumulated in the root but is not translocated to the shoots. As such, U precipitates with phosphate and calcium on the root epidermis. Inside the roots, U was mainly precipitated in the walls and membranes of parenchymal and vascular cells. Some of the U is not fixed and can be transported to the leaves via the apoplasm or symplasm. In sunflower roots, they also observed U precipitates on the membrane of endocytosis vesicles, indicating that U may enter the cell via endocytosis and translocate to the vascular cylinder by passing through plasmodesmata and passage cells. In addition, Misson *et al.* (2009) observed that the U taken up by the root cells was trapped within phosphate-rich granules inside subcellular structures such as vacuoles or the nucleus. This will lead to a decreased mobility of U inside the cells. During the transfer to the shoots, U can gradually precipitate with phosphate moieties present in in the root cell membranes or react with cellulose-, pectin- or glycoprotein-rich compounds of cell membranes and cell walls, leading to a low translocation efficiency. However, this can be a defence mechanism leading to neutralization of reactive U forms. When plants were exposed to U in the presence of phosphate, root uptake of uranium was moderate. Phosphate probably increases U adsorption on the roots' surface but inhibits its absorption. An efficient uptake and transfer was observed by exposing plants to U in a medium containing carbonate, at neutral pH, or citrate. Complexation with carbonate or citrate leads to the formation of neutral complexes, which are less reactive than the free uranyl ion, allowing transfer to the shoots. Since U in the leaves was mainly associated with cell walls and membranes, the U citrate and carbonate complexes may dissociate in the leaves, leading to precipitation with phosphate-rich cell walls and membranes (Laurette *et al.*, 2012).

As U will be more chemotoxic than radiotoxic, metal toxicity can arise from direct interaction of the metal with proteins, stimulated generation of ROS or displacement of essential cations from their binding sites (Sharma *et al.*, 2009). Since U is a redox-active metal, it can elicit ROS generation directly (Sharma and Dietz, 2009; Viehweger *et al.*, 2011). It is stated before that U toxicity is predominantly caused by UO_2^{2+} (Ribera *et al.*, 1996). It can replace calcium and magnesium, which can lead to structural changes in cell membranes, enzyme

inactivation and damage to RNA and DNA. Because UO_2^{2+} can also interact with phosphate moieties, DNA and membrane damage can also occur via this pathway (Vanhoudt *et al.*, 2008). However, also other U species including UO_2OH^+ and carbonated complexes can contribute to U toxicity (Zeman *et al.*, 2008).

Little information is available on U toxicity in terrestrial plants. In addition, the existing information on U toxicity is contradictory. Sheppard *et al.* (2005) summarized the available literature for chemical toxicity of U to non-human biota for different endpoints. For terrestrial plants, normal background levels of 0.5 - 5 mg kg^{-1} dry soil have been cited as toxic. In other studies, no toxicity effects have been reported at concentrations up to 1000 mg kg^{-1} dry soil (Sheppard *et al.*, 2005). Vandenhove *et al.* (2006) reported after 4 days exposure of *Phaseolus vulgaris* to 1000 µM U leaf chlorosis and roots started to turn yellow. In addition, they observed an increase in growth parameters after exposure to 1 or 10 µM U, alluding to a hormesis effect. A hormesis effect was also observed by Straczek *et al.* (2009) after exposure of hairy roots of carrots to 2.5 and 5 mg U l^{-1}. In *Arabidopsis thaliana* hormesis was observed by Misson *et al.* (2009) after exposure to 2 µM U. Vanhoudt *et al.* (2011a) also reported hormesis after exposure of *Arabidopsis thaliana* to 1 and 10 µM U during three days. A reason for these apparent contradictory results is, in addition to the different sensitivity of different plant species, the influence of U speciation on its bioavailability (Vandenhove, 2004). Therefore, it is important to take into account environmental parameters while examining toxic effects of U.

1.2. Copper

1.2.1. Copper properties and occurrence

Copper (Cu) is a naturally occurring reddish metal present in rock, soil, water and sediment. In the environment, it usually has a valence of +2. However, it can also exist in the +1 and +3 valence states (World Health Organization, 1998). The average Cu concentration in non-contaminated areas is ca. 50 mg kg^{-1} soil and less than 2 µg kg^{-1} in natural waters (Wojcik *et al.*, 2003; ATSDR, 2004). In contaminated areas, it can reach levels up to a hundred times higher (Yruela, 2009). Copper is an essential micronutrient being part of several

proteins involved in a variety of biological processes (López *et al.*, 2011). However, exposure to higher Cu concentrations results in toxic effects (Yruela, 2005; Nagajyoti *et al.*, 2010). Natural processes such as windblown dust, volcanic eruptions, forest fires or weathering of rocks can cause an enhanced level of Cu in the atmosphere and waterways. In addition, anthropogenic activities such as mining and smelting, agricultural activities or emission from diesel engines can increase the Cu concentration in the environment. Copper ores are mined, smelted and processed to produce commercial and industrial products. As such, it is widely used in alloys and for electrical wires, plumbing, air conditioning, automotive industry, bactericides and fungicides (World Health Organization, 1998; ATSDR, 2004).

1.2.2. Copper and uranium co-occurrence

In association with U deposits and wastes from U mines, a number of heavy metals may occur, leading to multiple stressors exposures. Those heavy metals can pose a risk if they migrate into the groundwater. As such, Cu is often associated with the waste of U mines (U.S. Environmental Protection Agency, 2008). Additionally, also Cu containing ores are known to have U-associated waste (U.S. Environmental Protection Agency, 2008). Due to Cu extraction and beneficiation processes, U (and other radioactive materials) may become concentrated, leading to concentrations above background levels (U.S. Environmental Protection Agency, 1999). The wastes produced during the milling processes are the principal source of health and environmental hazards, since they can leach out, resulting in increased soil, surface water or groundwater metal concentrations (U.S. Environmental Protection Agency, 1999; U.S. Environmental Protection Agency, 2008).

For example, in the early 1970s, low levels of U were discovered in a developing Cu mine in Arizona. Later in 1975, the U price had considerably increased, making U extraction economically feasible. As such, secondary U mines originated (U.S. Environmental Protection Agency, 1999).

1.2.3. Effect of copper on humans

Copper is essential for human health, although exposure to higher doses can lead to toxic effects. In humans, the main sources of Cu are food and drinking water. Dietary sources are for example organ meat (liver), oysters and seeds. Copper uptake through inhalation or dermal routes is negligible. Copper can rapidly enter the bloodstream and is distributed throughout the body after dietary intake. However, this Cu intake does not pose a health risk because Cu absorption, storage and excretion is regulated over a wide range of dietary exposure levels. Copper will leave the body via faeces and urine (ATSDR, 2004; López *et al.*, 2011). Acute effects of Cu toxicity are characterized by stomach problems causing nausea, vomiting and diarrhoea. High intakes of Cu can cause damage to the liver and kidneys and even death (ATSDR, 2004). Chronic Cu exposure can result in Wilson's disease, mainly caused by hepatic Cu accumulation. Clinical manifestations depend on Cu deposition in specific organs, mainly liver, brain and the cornea. Patients suffer from liver disease and/or neurological or psychiatric impairments frequently accompanied with kidney malfunction (López *et al.*, 2011). Therapeutic strategies consist of decreasing Cu absorption and/or increasing Cu excretion. Other examples of chronic Cu exposure are Indian Childhood Cirrhosis and Idiopathic Chronic Toxicosis, most likely caused by a combination of a genetic defect in Cu metabolism and high Cu intake. Besides its toxic effects, Cu is an essential metal for cell division. As such, health effects can also be related to Cu deficiency. Copper-deficient disorders are e.g. bone malformation, poor immune response and poor cardiovascular health. In addition, Cu deficiency is associated with a faster decline in cognitive ability in Alzheimer's disease (Matés *et al.*, 2010; López *et al.*, 2011).

1.2.4. Effect of copper on plants

The uptake of Cu by plants is dependent on plant species, developmental stage of the plant, the concentration of Cu and environmental factors such as pH. The free cupric ion (Cu^{2+}) is the most predominant form at pH < 6.5, while at higher pH levels, cupric carbonate and hydroxyl species are present (Riethmuller *et al.*, 2000). As such, the pH will influence Cu toxicity. However, different studies are

available that report both a decreased or increased toxicity with a decreasing pH (Riethmuller *et al.*, 2000).

Copper is taken up by the root cells via a conserved Cu transporter (CTR) family. In *Arabidopsis thaliana*, the CTR family is known as Cu transporter (COPT) (Puig *et al.*, 2007; Burkhead *et al.*, 2009). Copper mainly accumulates in the roots with only limited transfer to the shoots. However, little is known about Cu acquisition and transport into and within cells (Yruela, 2005).

Plants require Cu as essential micronutrient for normal growth and development. It is involved in many physiological processes such as photosynthesis, cell wall metabolism, protein trafficking machinery, oxidative stress responses and hormone signalling. In addition, Cu is a cofactor in many enzymes e.g. Cu/ZnSOD (Yruela, 2005; Nagajyoti *et al.*, 2010). However, at higher concentrations, Cu can become toxic. Therefore, plants have developed mechanisms to tightly control Cu uptake and distribution (Puig *et al.*, 2007). Symptoms of Cu toxicity are e.g. chlorosis, necrosis, reduced biomass and inhibition of shoot and root growth. Due to its redox properties, that make Cu an essential nutrient, Cu can catalyse the formation of hydroxyl radicals ($OH^{\cdot}$) directly via the Haber-Weiss and Fenton reactions (Figure 1.2) causing oxidative damage. This can lead to lipid peroxidation, decreased lipid content and changes in fatty acid composition of thylakoid membranes. When this leads to an altered structure and composition of the thylakoid membrane, the function and conformation of photosystems can be influenced. Photosynthesis can also be inhibited since Cu can substitute the central Mg ion of chlorophyll, resulting in an impairment of the correct function of the chlorophyll-complexes. In addition, Cu can modify the pigment and protein composition of photosynthetic membranes since Cu interferes with the biosynthesis of the photosynthetic machinery. The chlorophyll content can also be reduced due to an Cu-induced Fe deficiency, which can be explained by a competing ion-uptake of Fe and Cu (Yruela, 2009). Furthermore, Cu also directly interacts with proteins due to its affinity for thiol-, histidyl- and carboxyl-groups leading to an inhibition of enzyme activities or protein functions, an induction of deficiency of other essential ions and an impairment of cell transport processes. This, in turn, can alter the function of the

target protein and as such change the cell metabolism (Yruela, 2005; Sharma and Dietz, 2009; Yruela, 2009).

1.3. Oxidative stress

Exposure of plants to biotic or abiotic stresses can increase the concentration of reactive oxygen species (ROS), leading to a disturbance of the equilibrium between ROS production and scavenging in favour of the former. This state is called oxidative stress. ROS are produced during normal cell metabolism, e.g. during photosynthesis and respiration. They possibly have a role in the regulation of growth, development and defence pathways. However, under normal conditions, ROS production in cells is low, while under stress conditions (e.g. exposure to heavy metals) the production will be enhanced. Plants have evolved an antioxidative defence system, consisting of enzymes and metabolites, to scavenge ROS (Mittler, 2002; Karuppanapandian *et al.*, 2011; Sharma *et al.*, 2012).

1.3.1. Biochemical properties of ROS

Plants require atmospheric oxygen (O_2) for efficient production of energy, needed for their own developmental processes. However, the O_2 molecule is a free radical since it has two unpaired electrons. The two electrons have the same spin quantum number, preferring O_2 to accept electrons one at a time (Halliwell, 2006). ROS are partially reduced forms of O_2. They can be generated by a transfer of energy or electrons to O_2 (Mittler, 2002; Foyer *et al.*, 2003; Karuppanapandian *et al.*, 2011). By an energy input (e.g. the transfer of excitation energy of a chlorophyll triplet state to O_2) the spin restriction can be removed, giving the formation of singlet oxygen ($^1O_2$), which is no free radical. However, $^1O_2$ is highly destructive since it can transfer its excitation energy or it can react with biological molecules. It can oxidize proteins, polyunsaturated fatty acids and DNA and it can trigger cell death (Arora *et al.*, 2002; Halliwell, 2006). If one electron is supplied to O_2, the short-living superoxide radical ($O_2^{\bullet -}$) is formed. This radical is highly reactive in a hydrophobic environment such as the interior of membranes and it can oxidize specific amino acids and can cause lipid peroxidation. However, it cannot cross biomembranes and is easily dismutated to hydrogen peroxide (H_2O_2) (Dat *et al.*, 2000; Karuppanapandian *et al.*, 2011).

Addition of a second electron to O_2 gives rise to the peroxide radical, while the addition of four electrons produces water (Halliwell, 2006). In biology, the two-electron reduction product of O_2 is H_2O_2, which is no free radical since all its electrons are paired. It has a relatively long half-life and can diffuse some distance from its site of production (Dat *et al.*, 2000). The H_2O_2 is moderately reactive and is able to oxidize thiol groups which can lead to enzyme inactivation, while at high concentrations, it can orchestrate programmed cell death (Sharma *et al.*, 2012). Its toxicity can be enhanced in the presence of metal ions (e.g. Fe^{2+}, Cu^{+}) and $O_2^{\bullet-}$ through the Fenton or Haber-Weiss reactions (Figure 1.2) (Dat *et al.*, 2000; Mittler, 2002; Bhattacharjee, 2005).

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$	(Fenton reaction)
$Fe^{3+} + O_2^{\bullet-} \rightarrow O_2 + Fe^{2+}$	
$H_2O_2 + O_2^{\bullet-} \rightarrow OH^{\cdot} + OH^{-} + O_2$	(Haber-Weiss reaction)

Figure 1.2: Fenton and Haber-Weiss reactions.

The last species generated by these reactions is the hydroxyl radical ($OH^{\cdot}$). Since no enzymatic scavenging mechanisms are present to remove $OH^{\cdot}$, the reactions that lead to its generation should be controlled to avoid oxidative damage (Apel *et al.*, 2004). Hydroxyl radicals are highly reactive since they can react with all biological molecules. They can attack DNA and proteins and can initiate lipid peroxidation (Arora *et al.*, 2002). A consequence of lipid peroxidation is a decreased membrane fluidity which makes it easier for phospholipids to exchange between the two halves of the bilayer. This leads to an increased leakiness of the membrane, damaged membrane proteins and an inactivation of receptors, enzymes and ion channels (Halliwell, 2006).

Oxidation of organic substrates may proceed by two reactions: an addition or an abstraction reaction. In the addition reaction, an $OH^{\cdot}$ is added to an organic molecule which is further oxidized by Fe^{3+} and O_2 to form a stable oxidized product (Arora *et al.*, 2002). The abstraction reaction consists of three stages: initiation, progression and termination (Figure 1.3) (Bhattacharjee, 2005). In the initiation step, a hydrogen atom is abstracted from the organic substrate leading to the formation of water and an organic radical ($R^{\cdot}$). The organic compound has a single unpaired electron and can thus react with O_2 in its

ground state (propagation step). This can lead to the formation of a peroxy radical ($ROO^{\cdot}$) which in turn, can abstract hydrogen from another organic molecule leading to the formation of a second organic radical. This chain reaction is more damaging than any other reaction catalysed by ROS (Arora *et al.*, 2002). In the presence of Fe^{2+} (or other reduced metal ions), the lipid peroxides (ROOH) are unstable and can participate in a Fenton reaction. This leads to the formation of the reactive alkoxyl radical ($RO^{\cdot}$). The alkoxyl radical is as damaging as $OH^{\cdot}$. It is thus able to start a cascade of oxidation reactions, leading to an exponential increase of the propagation step. When two radicals of the previous reactions react with each other, this results in the formation of fatty acids or peroxide bridged dimers (termination step) (Arora *et al.*, 2002; Bhattacharjee, 2005).

$RH + OH^{\cdot} \rightarrow R^{\cdot} + H_2O$	Initiation step
$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$	Propagation step
$ROO^{\cdot} + RH \rightarrow R^{\cdot} + ROOH$	
$ROOH + Fe^{2+} \rightarrow OH^{-} + Fe^{3+} + RO^{\cdot}$	
$R^{\cdot} + R^{\cdot} \rightarrow R + R$	Termination step
$R^{\cdot} + ROO^{\cdot} \rightarrow ROOR$	
$ROO^{\cdot} + ROO^{\cdot} \rightarrow ROOR + O_2$	

Figure 1.3: The oxidation of organic substrates – stages of the abstraction reaction.

1.3.2. Sources of ROS

ROS are by-products of various metabolic processes, as an inevitable result of membrane-linked electron transport that leaks electrons onto O_2 (Bhattacharjee, 2005). However, during stress conditions, their production can be aggravated (Karuppanapandian *et al.*, 2011). Most cellular compartments can become a source of ROS (Figure 1.4). The chloroplasts are one of the most powerful sources of ROS in plants producing $O_2^{\bullet -}$. Under conditions limiting CO_2 fixation, ROS production will be enhanced (Foyer *et al.*, 1994; Sharma *et al.*, 2012). The major sources in the chloroplasts are the Mehler reaction, via electron leaking from reduced ferredoxin to O_2, and the photorespiratory cycle. If ROS generated

in the chloroplast accumulate, they can damage the photosynthetic apparatus. In addition, the production of $O_2^{\bullet-}$ can trigger a chain reaction leading to the production of more toxic radicals (Dat *et al.*, 2000; Bhattacharjee, 2005). Moreover, $^1O_2$ can be produced in the chloroplasts, affecting membrane proteins and lipids near the site of its production. The mitochondrial electron transport system generates ROS (including $O_2^{\bullet-}$, H_2O_2 and $OH^{\cdot}$) during respiration at several sites (Bhattacharjee, 2005). However, ROS production in mitochondria also takes place under normal conditions during the reduction of O_2 to water. Under stress conditions, an over-reduction of electron carriers of the electron transport system can occur, leading to an enhanced formation of ROS. However, the presence of the alternative oxidase (AOX) may help to reduce ROS production. AOX prevents electrons from reducing O_2 to $O_2^{\bullet-}$ and they reduce the overall level of O_2, the substrate of ROS production, by reducing it to H_2O (Mittler, 2002; Keunen *et al.*, 2011). The peroxisomes generate $O_2^{\bullet-}$ as a consequence of their normal metabolism (Quan *et al.*, 2008). In addition, they are also a major site of H_2O_2 production during the glycolate oxidase reaction, fatty acid β-oxidation, the enzymatic reaction of flavin oxidase and the dismutation of $O_2^{\bullet-}$ (Foyer and Noctor, 2003; Sharma *et al.*, 2012). In the endoplasmic reticulum, $O_2^{\bullet-}$ are generated during the detoxification reactions catalysed by cytochrome P450 (Bhattacharjee, 2005; Sharma *et al.*, 2012). Another potential source of ROS are the plasma membrane bound NADPH-dependent oxidases (NADPH oxidase). Plant NADPH oxidases (also called respiratory burst oxidase homologues (RBOH)) generate ROS in the apoplast during the oxidative burst. This is a common response of plant cells to environmental fluctuations (e.g. pathogens, metals, wounding, ultraviolet light and ozone), which lead to a rapid increase in ROS, primarily $O_2^{\bullet-}$ and H_2O_2 (Neill *et al.*, 2002; Bhattacharjee, 2005). Also cell-wall-bound peroxidases and amine oxidases play a role in the oxidative burst (Mittler, 2002). The H_2O_2 generated during this response can diffuse into the cell and activate plant defence mechanisms, depending on the steady-state level of the produced ROS. Also lipoxygenases (LOX) can be involved in ROS production. They catalyse the dioxygenation of polyunsaturated fatty acids (PUFA) such as linoleic acid and linolenic acid. When the hydroperoxyderivates of PUFAs degrade, they can

produce radicals that, in turn, will initiate lipid peroxidation. In addition, LOX can also mediate the formation of $^1O_2$ and $O_2^{\bullet -}$(Blokhina *et al.*, 2003).

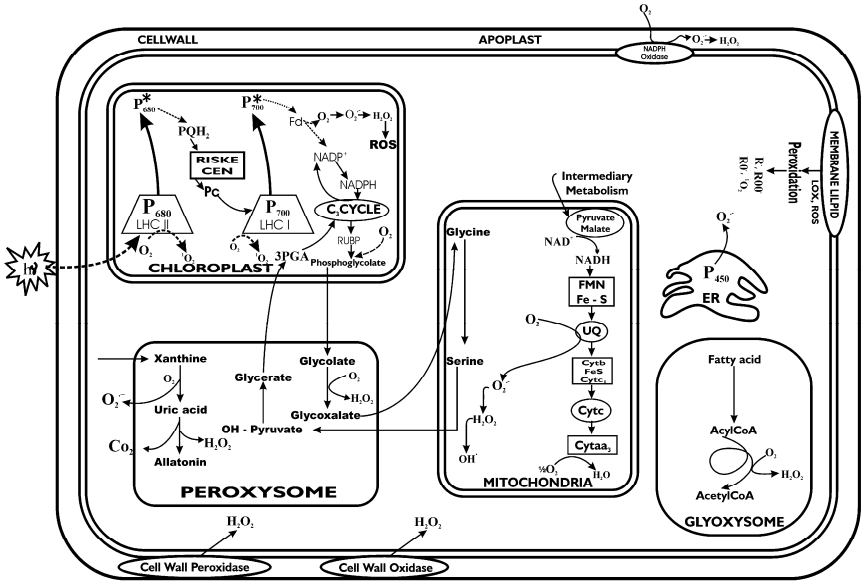

Figure 1.4: Sources of ROS in plant cell (Bhattacharjee, 2005).

1.3.3. ROS signalling

An enhanced production of ROS has in the past been categorized under 'oxidative stress', which is a negative term. However, when ROS are present at low concentrations, they can act as secondary messengers for the activation of stress responses and defence pathways (Foyer and Noctor, 2003; Foyer *et al.*, 2005). ROS signalling is a dynamic process that can occur between different organelles within one cell or over long distances between cells (Mittler *et al.*, 2011). The responses it can induce, include stomatal closure, lignin biosynthesis, enzyme activation, gene expression modification and programmed cell death (Neill *et al.*, 2002; Sharma *et al.*, 2012). Since plant cells can sense ROS via ROS receptors, redox-sensitive transcription factors or via direct inhibition of phosphatases, the ROS signals can be translated into an appropriate cellular response (Mittler *et al.*, 2004; Sharma *et al.*, 2012). Hydrogen peroxide is a good candidate for intercompartmental signalling since it has the ability to cross biological membranes and has a relative long lifetime (Blokhina *et al.*, 2010). As such, the movement of H_2O_2 is facilitated by water channels that serve as conduits for trans-membrane H_2O_2 transport. H_2O_2 can activate several

mitogen-activated protein kinase (MAPK) cascades that play a central role in mediating cellular responses to stress. H_2O_2 can also oxidize cysteine residues of protein phosphatases that can alter the activity of transcription factors (Figure 1.5). This in turn, will affect gene expression and can possibly activate the ROS-scavenging pathways (Apel and Hirt, 2004; Mittler *et al.*, 2004).

In addition to H_2O_2, the oxygenated products of lipid degradation, i.e. oxylipins, can act as second messenger. They serve as inter- and intracellular signalling compounds involved in abiotic and biotic stress responses. As such, jasmonates and other oxylipins have been associated with plant defence responses since they can induce accumulation of secondary metabolites such as H_2O_2 (Mithöfer *et al.*, 2004; Quan *et al.*, 2008). Finally, a change in the ratios of the reduced and oxidized forms of ascorbate (AsA) and glutathione (GSH) may also be involved in the regulation of gene expression (Noctor *et al.*, 1998).

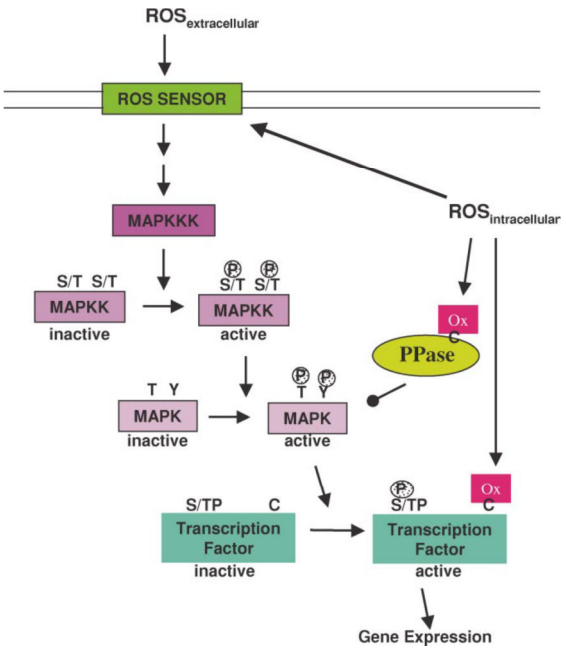

Figure 1.5: Schematic depiction of cellular ROS sensing and signalling mechanisms (Apel and Hirt, 2004). ROS sensors can sense extracellular and intracellular ROS. Intracellular ROS can influence the ROS-induced mitogen-activated protein kinase (MAPK) signalling pathways which regulate gene expression by altering transcription factor activity through phosphorylation of serine and threonine residues. ROS can also oxidize cysteine residues of protein phosphatases (PP) or downstream transcription factors, which will affect gene expression.

1.3.4. Scavenging mechanisms

Exposure of plants to biotic or abiotic stresses can disturb the metabolic balance, resulting in an increased concentration of ROS. Antioxidant defence mechanisms catalyse reactions involved in removing ROS, preventing the ROS from exceeding toxic thresholds (Noctor and Foyer, 1998; Sharma and Dietz, 2009). An antioxidant is capable of quenching ROS without itself undergoing conversion to a destructive radical (Noctor and Foyer, 1998). The major scavenging enzymes are superoxide dismutase (SOD), AsA peroxidase (APX) and catalase (CAT). The balance between the activities of those enzymes is important to prevent the formation of $OH^{\cdot}$. However, ROS can also be detoxified non-enzymatically via AsA, GSH, tocopherols and carotenoids. The localization of different ROS scavenging mechanisms is depicted in figure 1.6.

Superoxide dismutase acts as the first line of defence against ROS. It removes $O_2^{\bullet-}$ by catalysing its dismutation to H_2O_2 and O_2 (1) (Halliwell, 2006).

$$2\ O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

Depending on the co-factor used, different SODs can be distinguished. SODs containing manganese at the active-site (MnSOD) are mainly localized in the mitochondrial matrix. Copper and zinc SOD (CuZnSOD) are found in the chloroplast, the cytosol and possibly in the mitochondria, while iron-containing SODs (FeSOD) are mainly found in the chloroplast but have also been reported in cytosol, mitochondria and peroxisomes (Arora *et al.*, 2002; Halliwell, 2006; Sharma *et al.*, 2012).

Catalases are responsible for the removal of excess H_2O_2 producing water and O_2 (2).

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2 \qquad (2)$$

However, they are not involved in the removal of H_2O_2 generated by the dismutation of $O_2^{\bullet-}$ by SOD, since there is little CAT in mitochondria and chloroplasts, where much $O_2^{\bullet-}$ is generated (Halliwell, 2006). Catalases are mainly present in peroxisomes and glyoxysomes (Dat *et al.*, 2000). During

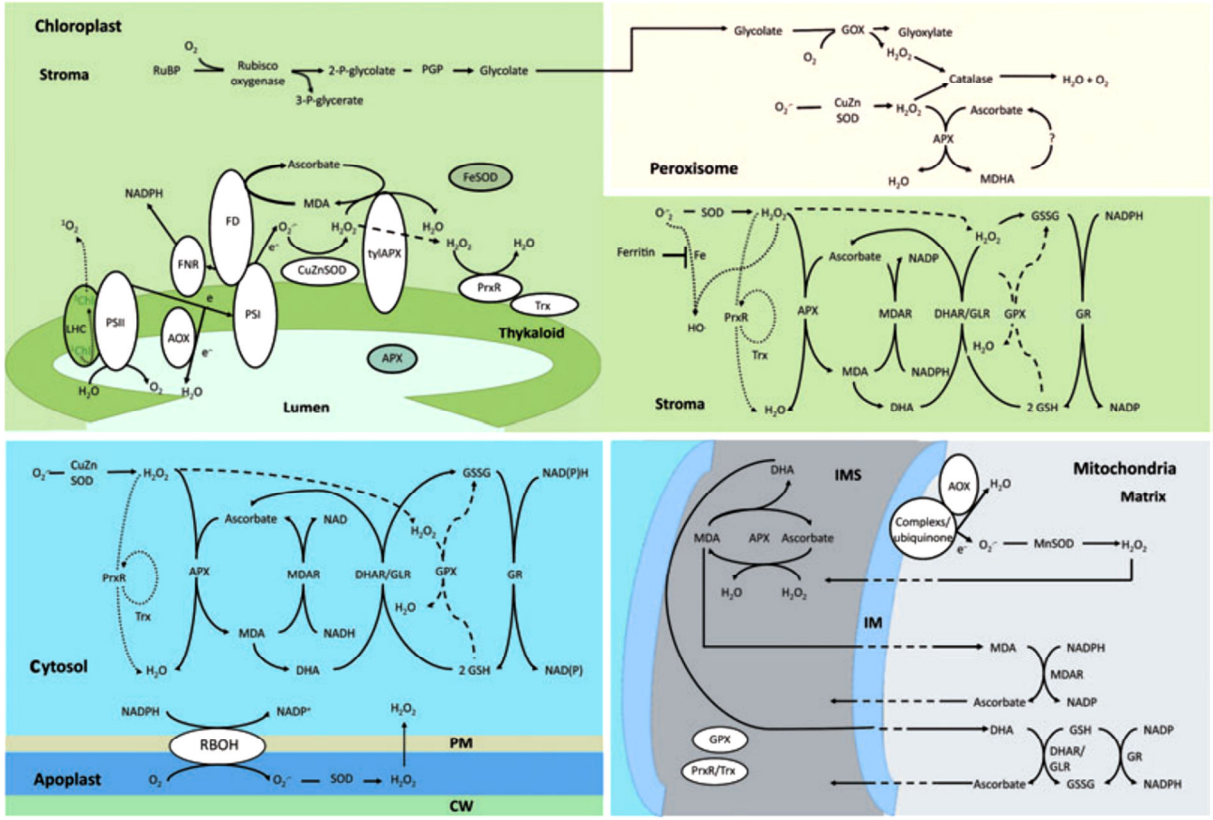

Figure 1.6: Localization of reactive oxygen species generation and scavenging pathways in plant cells (Miller *et al.*, 2010). Membrane-bound enzymes are depicted in white, glutathione peroxidase pathways are indicated by dashed lines and peroxiredoxin pathways are indicated by dotted lines in the stroma and cytosol. AOX: alternative oxidase; APX: ascorbate peroxidase; CAT: catalase; Chl: Chlorophyll; CuZnSOD: copper/zinc superoxide dismutase; CW: cell wall; DHA: dehydroascorbate; DHAR: DHA reductase; FD: ferredoxin; GR: glutathione reductase; GOX: glycolate oxidase; GPX: glutathione peroxidase; GSH: reduced glutathione; GSSG: oxidized glutathione; IM: inner membrane; IMS: IM space; LHC: light harvesting complex; MDA: monodehydroascorbate; MDAR: MDA reductase; PGP: phosphoglycolate phosphatase; PM: plasma membrane; PrxR: peroxiredoxin; PSI: photosystem I; PSII: photosystem II; RBOH: respiratory burst oxidase homolog; RuBP: ribulose-1,5-bisphosphate; Rubisco: RuBP carboxylase oxygenase; Trx: thioredoxin.

stress, peroxisomes can proliferate, which might help in scavenging H_2O_2 that can diffuse from the cytosol (Karuppanapandian *et al.*, 2011). Catalases have a high catalytic rate but low affinity for H_2O_2, since the reaction requires the access of two H_2O_2 molecules at the active site (Noctor and Foyer, 1998). As such, CAT might be responsible for the removal of excess H_2O_2 during stress. There are three main isoforms of catalase: CAT1, CAT2 and CAT3 which are differentially expressed. CAT1 is mainly present in vascular tissue. CAT2 is mainly found in leaves and is responsible for the removal of H_2O_2 during photorespiration. CAT3 is highly abundant in seeds and young seedlings (Dat *et al.*, 2000).

Peroxidases are haem-containing glycoproteins that remove H_2O_2 by using it to oxidize a cosubstrate (Halliwell, 2006). In addition to ROS scavenging, they play a role in lignification, cross-linking of cell wall structure proteins and defence against pathogens (Quan *et al.*, 2008). Ascorbate peroxidase is mainly present in the chloroplast and cytosol, but its presence in mitochondria, peroxisomes and apoplast has also been reported (Arora *et al.*, 2002; Mittler, 2002). Ascorbate peroxidase has a higher affinity for H_2O_2 than CAT and is therefore involved in the fine modulation of ROS for signalling (Mittler, 2002). It uses two molecules of AsA as a hydrogen donor to dismutate H_2O_2 to H_2O with a concomitant generation of two molecules of monodehydroascorbate (MDHA) (Karuppanapandian *et al.*, 2011; Sharma *et al.*, 2012). In the chloroplast, APX and SOD are present as soluble and membrane-bound enzymes. Thus $O_2^{\bullet-}$ generated at the membrane surface can be trapped and converted to H_2O_2, which can immediately be scavenged by APX (Arora *et al.*, 2002; Karuppanapandian *et al.*, 2011). Glutathione peroxidases remove H_2O_2 by coupling its reduction to water with the oxidation of reduced GSH to oxidized glutathione (GSSG), which consists of two GSH molecules linked by a disulphide bridge. GSSG can be converted back to GSH by glutathione reductase (GR) (Halliwell, 2006). GR is located in chloroplasts, mitochondria and cytosol (Arora *et al.*, 2002). However, in plants, the main role of glutathione peroxidase is lignin biosynthesis, degradation of indole-3-acetic acid and resistance to pathogens (Asada, 1992). Another peroxidase is guaiacol peroxidase (GPX). They are a group of non-donor specific peroxidases in plant cells, for which guaiacol is common donor (Quan *et al.*, 2008). They preferably oxidize aromatic

electron donors to scavenge H_2O_2 in the cytosol, vacuole, cell wall and the extracellular space. In addition, they have a role in the biosynthesis of lignin (Karuppanapandian *et al.*, 2011; Sharma *et al.*, 2012).

Besides enzymes, there are also some low mass antioxidants important in ROS scavenging, i.e. AsA, GSH, tocopherols and carotenoids (Blokhina *et al.*, 2003). Ascorbate is the most important ROS detoxifying compound in plants, since it has a great ability to donate electrons. The final step of the AsA synthesis occurs in the inner mitochondrial membrane. From here, it will be transported to the chloroplast, cytosol, vacuole and apoplastic space of leaf cells (Foyer *et al.*, 1994; Blokhina *et al.*, 2003; Bielen *et al.*, 2013). Ascorbate reduces H_2O_2 to H_2O via the APX reaction. In addition, it can react directly with $O_2^{\bullet -}$, OH^- and $^1O_2$. However, a high ratio of reduced to oxidized AsA is essential for ROS scavenging (Apel and Hirt, 2004). Ascorbate also acts as a cofactor of violaxantin de-epoxidase to sustain the dissipation of excess excitation energy. Moreover, AsA is a secondary antioxidant as it is involved in the regeneration of tocopherol from tocopheroxyl radicals, providing membrane protection. Finally, AsA also has a number of non-antioxidant functions. As such, it is involved in the regulation of cell division, cell cycle progression from G1 to the S phase and in cell elongation (Blokhina *et al.*, 2003). Glutathione is a tripeptide synthesised in the chloroplast and cytosol. It is found in virtually all cell compartments such as the endoplasmatic reticulum, vacuole, chloroplast, mitochondria and cytosol. It scavenges H_2O_2 and reacts non-enzymatically with other ROS. In addition, GSH is a potent detoxifier of xenobiotics, it can serve as a precursor of phytochelatins, it can participate in the regulation of the cell cycle and it plays a role in sulphur metabolism (Blokhina *et al.*, 2003; Jozefczak *et al.*, 2012). It maintains a redox balance together with its oxidized form which is important for the fine-tuning of the cellular redox environment. However, the central role of GSH is the regeneration of AsA via the AsA-GSH cycle (Blokhina *et al.*, 2003; Karuppanapandian *et al.*, 2011). Tocopherols are lipophilic antioxidants found in all plant parts as component of biological membranes. They are involved in scavenging $^1O_2$ and other ROS and they protect membranes from lipid peroxidation by scavenging lipid peroxy radicals (Halliwell, 2006; Sharma *et al.*, 2012). In addition, they protect the structure and function of photosystem II by reacting with O_2 in chloroplasts (Karuppanapandian *et al.*, 2011; Sharma *et al.*,

2012). Finally, carotenoids quench the triplet excited states of chlorophyll and as such prevent the formation of $^1O_2$ (Triantaphylidès *et al.*, 2009).

The AsA-GSH cycle is important in H_2O_2 scavenging (Figure 1.7). It takes place in chloroplasts, cytosol, mitochondria, apoplast and peroxisomes (Mittler, 2002). In this cycle, two molecules of AsA are used to reduce H_2O_2 to water. This leads to the generation of two molecules MDHA. If MDHA is not reduced to AsA by MDHA reductase, it will disproportionate spontaneously to dehydroascorbate (DHA). Subsequently, GSH is used as reductant to reduce DHA to AsA. This reaction is catalysed by DHA reductase. During this reaction, the reduction to AsA is coupled to the oxidation of GSH, by which GSSG is generated. GR will re-reduce GSSG to GSH by using NADPH as reducing power.

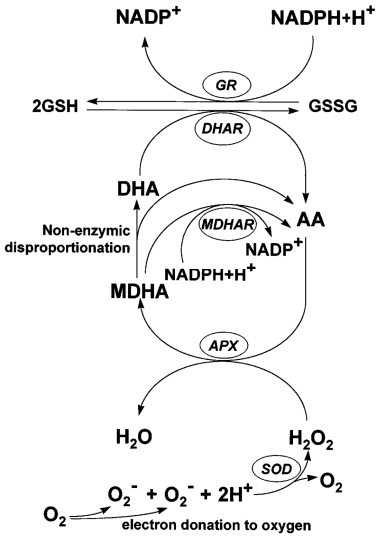

Figure 1.7: ascorbate-glutathione cycle (Noctor and Foyer, 1998). AA: reduced ascorbate; APX: ascorbate peroxidase; DHA: dehydroascorbate; DHAR: DHA reductase; GR: glutathione reductase; GSH: reduced glutathione; GSSG: oxidized glutathione; MDHA: monodehydroascorbate; MDHAR: MDHA reductase; $NADP^+$: nicotinamide adenine dinucleotide phosphate; NADPH: reduced nicotinamide adenine dinucleotide phosphate; SOD: superoxide dismutase.

1.3.5. Metal-induced oxidative stress

It has been demonstrated that exposing plants to heavy metals leads to the production of ROS and induces the antioxidative defence mechanisms. As such, a significant increase in the H_2O_2 concentration in *Arabidopsis thaliana* roots was observed after exposure to 2 µM Cu (Opdenakker *et al.*, 2012) or 5 or 10 µM cadmium (Cd) (Cuypers *et al.*, 2011; Smeets *et al.*, 2013). This increase is possibly due to NADPH oxidases localised in the plasma membrane, since the expression levels of *RBOHC/D* (2 µM Cu), *RBOHC/E* (5 µM Cd) or *RBOHD* (10 µM

Cd) significantly increased. The observation that Cd increased the expression of NADPH oxidases was also observed by Remans *et al.* (2010). Vanhoudt *et al.* (2011b) observed a significant increase in *RBOHD* expression after exposure of *Arabidopsis thaliana* plants to 100 µM U during 3 days, indicating that also exposure to U induces an oxidative stress response.

In addition to an increased production of ROS, an increased lipid peroxidation was found after exposure to Cd, Cu and U (Smeets *et al.*, 2009; Cuypers *et al.*, 2011; Vanhoudt *et al.*, 2011; Opdenakker *et al.*, 2012; Smeets *et al.*, 2013) which can result from the formation of $OH^{\cdot}$ or from the oxygenation of fatty acids via LOX. As such, an increase in *LOX1* or *LOX2* expression was observed after exposure to Cd, Cu and U. Remans *et al.* (2012) also observed a significant increase in *LOX1* and *LOX2* after exposure to an excess zinc (Zn). The changes in *LOX* gene expression may also be involved in oxylipin signalling (Vanhoudt *et al.*, 2011; Opdenakker *et al.*, 2012)

As a response to the disturbed oxidative balance, organisms alter their antioxidative defence system in order to reach a new cellular equilibrium (Smeets *et al.*, 2013). After exposure to Cd or Cu, an upregulation of different ROS scavenging enzymes was observed, both at protein and transcription level (Cuypers *et al.*, 2011). Also under U stress, an increased capacity of different enzymes was observed, indicating an increased defence against ROS (Vanhoudt *et al.*, 2011; Vanhoudt *et al.*, 2011b). However, depending on the stressor, the cellular and physiological response was different, which can be caused by the activation and interplay of signalling networks (Smeets *et al.*, 2013). As such, it has been demonstrated that exposure to Cd or Cu induces the MAPK signalling pathway (Opdenakker *et al.*, 2012; Smeets *et al.*, 2013). This information is not yet available for U-exposed plants.

In conclusion, it is known that heavy metal exposure causes oxidative stress in plants. However, studies under controlled lab conditions generally take not into account differences in environmental parameters. Since U speciation is strongly dependent on the pH, it is important to investigate the impact of U contamination under different environmentally relevant conditions.

Scope and objectives

Due to anthropogenic activities such as uranium (U) mining and milling and the phosphate industry, large areas have been contaminated with U. The bioavailability of U not only depends on its concentration but is also strongly dependent on environmental factors such as pH value, redox potential, ionic strength, availability of inorganic and organic ligands and possibly also the presence of other co-contaminants. It has been demonstrated before that U can cause oxidative stress in *Arabidopsis thaliana* plants when they are exposed at pH 5.5, the ideal pH for growing this plant (Vanhoudt *et al.*, 2008; 2011; 2011b). However, when evaluating the environmental impact of U contamination, it is important to investigate this under different environmentally relevant setups.

The main objective of this research was to analyse the influence of different environmentally relevant setups on the toxicity of U. The main focus was to investigate the effect of the pH on U toxicity, in order to unravel the mechanisms by which plants respond to U exposure at different pH levels. Since oxidative stress seems to be an important regulator of heavy metal stress responses, the role of reactive oxygen species production and scavenging under U stress was further elucidated. Effects were analysed at morphological, physiological, biochemical and molecular level in *Arabidopsis thaliana* plants. To achieve this objective, the effects of the pH on U uptake and oxidative stress responses were investigated by exposing plants to one U concentration at different pH levels (chapter 4). Subsequently, the dose-dependent effects were studied more profoundly at pH 4.5 (chapter 5 (roots) and chapter 6 (leaves)) and at pH 7.5 (chapter 7), while the dose-dependent effects at pH 5.5 were analysed before by Vanhoudt *et al.* (2011; 2011b). In addition, the effect of U on the photosynthetic efficiency of *Arabidopsis thaliana* leaves was investigated, both at pH 4.5 and pH 7.5 (chapter 8).

Since organisms are generally always exposed to multiple stressors and since one stressor can influence the toxicity of another stressor, it is important to investigate the toxic effects of U under multiple stressor conditions. Therefore, in the last part of this work, the effects of U in a multiple stressor setup were investigated by exposing *Arabidopsis thaliana* plants to U in combination with copper (Cu) (chapter 9). The importance of the production of reactive oxygen species and the antioxidative defence systems in the plant were analysed at protein and molecular level. In addition the importance of ascorbate (AsA) in the oxidative stress response during U stress was investigated by using AsA deficient *vtc* mutants of *Arabidopsis thaliana* (*vtc1* and *vtc2*) (chapter 9).

Materials and methods

3.1. Plant culture and treatment

Arabidopsis thaliana wild-type (WT), *vtc1-1* and *vtc2-1* mutant seeds (Columbia ecotype) were surface sterilized and incubated in the dark for three days at 4°C on moist filter paper to synchronize germination. The *vtc1* mutant has a defect in the GDP-D-mannose pyrophosphorylase enzyme and less than 30% of the leaf AsA of WT plants. The *vtc2* mutant has lower AsA levels than the *vtc1* mutant. They are defective in the GDP-L-galactose phosphorylase enzyme (Olmos *et al.*, 2006). Seeds were sown on plugs from 1.5 ml polyethylene centrifuge tubes filled with 0.6% agar in Hoagland solution with low phosphate content (Vanhoudt *et al.*, 2008). The plugs were positioned in a PVC cover capable of holding 36 plugs. Next, the cover was placed on a container filled with 1.35 l of a modified Hoagland solution with a pH of 5.5 (1 mM KNO_3, 0.3 mM $Ca(NO_3)_2$, 0.2 mM $MgSO_4$, 0.1 mM $NH_4H_2PO_4$, 1.62 µM $FeSO_4$, 0.78 µM EDTA, 4.6 µM H_3BO_3, 0.9 µM $MnCl_2$, 32 nM $CuSO_4$, 55.6 nM H_2MoO_4, 76.5 nM $ZnSO_4$). Plants were grown in a growth chamber (Microclima 1000E, Snijders Scientific B.V.) under a 14 h photoperiod (photosynthetic photon flux density of 150 µmol m^{-2} s^{-1} at the leaf level, supplied by Sylvania BriteGro F36WT8/2084 and F36WT8/2023), with day/night temperatures of 22°C/18°C and 65% relative humidity. After 18 days preculture, the pH of the nutrient solution was adjusted with NaOH or HCl to different pH levels ranging from 4.5 to 7.5. To retain the pH at a constant level, 500 µM MES (2-(N-morpholino)ethanesulfonic acid) and 500 µM TRIS (tris(hydroxymethyl)-aminomethane) were added. Plants were exposed to 0, 6.25, 12.5, 25, 50, 75 or 100 µM uranium (U). In a multi-pollution setup (chapter 9), plants were exposed to 25 µM U, 2.5 µM copper (Cu) or 12.5 µM U + 1.25 µM Cu at pH 7.5. Uranium was added as $UO_2(NO_3)_2.6H_2O$ (SPI chemicals, USA) from a 100 mM stock solution to the Hoagland nutrient solution. Copper was added as $CuSO_4$ from a 10 mM stock solution. Since roots can exudate organic acids or anions (Akhtar *et al.*, 2009), the pH of the nutrient solution was adjusted twice a day. During the exposure

time, a modified Hoagland solution was used with 0.025 mM $NH_4H_2PO_4$ (Vanhoudt *et al.*, 2008). After 3 days of exposure, plants were harvested. Leaf and root fresh weight was determined and samples were snap frozen in liquid nitrogen and stored at -80°C. Leaf and root growth was determined as [(fresh $weight_{day\ 21}$ - fresh $weight_{day\ 18}$)/(fresh $weight_{control\ plants\ day\ 21}$ - fresh $weight_{control\ plants\ day\ 18}$)]*100.

3.2. Metal analysis

Leaf and root samples were taken for the determination of U and Cu accumulation. Roots were washed twice for 10 min in 1 mM $Pb(NO_3)_2$ and once for 10 min with distilled water to exchange surface-bound U or Cu. Afterwards, root and shoot samples were dried for at least one week at 70°C. The oven-dried samples were calcinated in a muffle furnace at 550°C. After cooling down to room temperature, the plant material was digested in 1 M HCl. The U-238 and Cu concentrations in these samples were determined by using a quadrupole inductively coupled plasma - mass spectrometer (ICP-MS) (XSeries II, Thermo Scientific, Bremen, Germany) equipped with a PFA-ST Nebulizer (Elemental Scientific, Omaha, Nebraska, USA) and a peltier cooled (2°C) cyclonic quartz spray chamber for sample introduction. Calibration curves were established using U and Cu standard solutions (0 to 10 µg l^{-1}) prepared from a single element stock solution (SPEX Industries Inc., Edison, NJ, USA). The instrumental detection limit for U was 2 ng l^{-1}, while this was 50 ng l^{-1} for Cu. Typical precision for samples with U or Cu concentrations well above the limit of detection was below 5% (relative standard deviation, 10 replicates). Based on U or Cu concentrations in roots and shoots, root-to-shoot transfer factors were defined as concentration in the shoot divided by the concentration in the root.

3.3. Pigment concentration

For the determination of chlorophyll a, chlorophyll b and carotenoids, one fresh leaf rosette (approximately 65 mg) was extracted overnight at 4°C in 1 ml dimethylformamide. After 1 min centrifugation at 20000 x *g*, the absorbance of the supernatant was measured at 664 nm, 647 nm and 480 nm. Pigment concentrations were calculated according to Wellburn *et al.* (1994). Since the results in chapter 8 derive from leaf samples harvested during two separate

experiments (dose-dependent effects at pH 4.5 and dose-dependent effects at pH 7.5), data presented are data normalized against the pigment concentration of leaves grown at pH 5.5, which were taken as reference group in both experiments.

Anthocyanins were determined according to Porter *et al.* (2009). Approximately 100 mg leaves were grounded in 1 ml ice-cold ethanol supplemented with 1% (v/v) HCl. After centrifugation for 5 min at 16000 x *g* at 4°C, a back extraction in chloroform was carried out, according to Vanderauwera *et al.* (2005). Therefore, 600 µl supernatant was diluted in 600 µl deionized water and 120 µl chloroform. After a second centrifugation step at 16000 x *g* for 5 min at 4°C, absorbance of the upper phase was determined at 535 nm. Since the results in chapter 8 derive from leaf samples harvested during two separate experiments (dose-dependent effects at pH 4.5 and dose-dependent effects at pH 7.5), data presented are data normalized against the anthocyanin concentration of leaves grown at pH 5.5, which were taken as reference group in both experiments.

3.4. Chlorophyll *a* fluorescence measurements

Chlorophyll *a* fluorescence was measured in the youngest full grown leaf (normally fourth youngest leave), dark adapted for at least 30 min. Measurements were made with the Dual-PAM-100 chlorophyll fluorescence measuring system (Walz, Effeltrich, Germany). Different parameters were analysed based on the induction curve (Heinz Walz GmbH, 2009). The minimum (F_0) and maximum fluorescence (F_m) were determined. F_0 can be determined after dark adaptation so that all photosystem II (PSII) reaction centres are open and maximal photochemical quenching is observed (Heinz Walz GmbH, 2009). F_m is the maximal fluorescence, assessed after a saturation pulse (SP). After 40 s of delay, actinic light was switched on. Briefly hereafter, a second saturation pulse is given, which was followed by further saturation pulses applied at regular intervals to determine F'_m (Heinz Walz GmbH, 2009). Based on these values, different parameters were calculated. For the calculations of Y(II), Y(NO) and Y(NPQ), the Dual-PAM-100 software uses formulas that contain the parameter F'_0. According to Klughammer and Schreiber (2008), reliable determination of F'_0 is problematic. Therefore, they proposed new equations for

the complementary quantum yields in terms of fluorescence yield parameters that can be readily determined by the SP method. Those formulas are used in the present study to calculate the parameters of Y(II), Y(NO) and Y(NPQ) (Klughammer and Schreiber, 2008). The effective PSII quantum yield (Y(II)) is a measure of how much of the absorbed quanta are converted into fixed energy by the PSII reaction centres. The quantum yield of regulated energy dissipation (Y(NPQ)) is a measure of non-photochemical fluorescence quenching, reflecting down-regulation of PSII as a protective mechanism against excess light intensity. The excess energy is dissipated in a regulated way via the xanthophyll cycle (Foyer *et al.*, 2000). The quantum yield of non-regulated energy dissipation (Y(NO)) is another measure of non-photochemical fluorescence quenching. However, a high Y(NO) value indicates that both photochemical energy conversion and protective regulatory mechanisms are inefficient. The ETR(II) parameter is a relative measure of the rate of electron transport (Heinz Walz GmbH, 2009). Finally, the coefficient of photochemical quenching (qL) is a measure of the fraction of open PSII reaction centres based on the lake model of PSII antenna pigment organization (Heinz Walz GmbH, 2009). In chapter 8, experimental curves were fitted with mathematical functions for the different parameters to quantify the dynamic responses of the photosynthetic processes (D'Haese *et al.*, 2004). Since the results in chapter 8 derive from leaf samples harvested during two separate experiments (dose-dependent effects at pH 4.5 and dose-dependent effects at pH 7.5), data presented are data normalized against the photosynthetic parameters of leaves grown at pH 5.5, which were taken as reference group in both experiments.

3.5. Determination of lipid peroxidation

The thiobarbituric acid reactive compounds (TBA-rc) were used as a measure for membrane damage. Approximately 120 mg of shoots or 90 mg of roots were homogenized in 1 ml 0.1% trichloroacetic acid (TCA) using an ice-cold mortar and pestle. After centrifugation at 20000 x *g* for 10 min, 250 µl supernatant was diluted with 1 ml TBA/TCA solution (0.5 % TBA in 20 % TCA). The mixture was 30 min incubated at 95°C and quickly cooled down in an ice bath. After another centrifugation step of 10 min at 20000 x *g*, the absorbance of the supernatant was determined spectrophotometrically at 532 nm and corrected for the non-

specific absorbance at 600 nm (Dhindsa *et al.*, 1981). The content of TBA-rc was calculated according to the law of Lambert-Beer (ε = 155 mM^{-1} cm^{-1}) taking into account the fresh weight and the dilutions made.

In chapter 9, samples were disrupted under frozen conditions using steel beads (diameter 3 mm) and the Retsch Mixer Mill MM400. After 1 ml 0.1% TCA was added, the samples were centrifuged and 400 µl supernatant was diluted with 1 ml TBA/TCA solution (0.5 % TBA in 20 % TCA). Hereafter, measurements were performed as described above.

3.6. Metabolite measurements

Oxidized and reduced forms of ascorbate (AsA) and glutathione (GSH) were measured spectrophotometrically using a plate-reader assay according to the method described in Queval and Noctor (2007). Approximately 120 mg of shoot or 80 mg of root samples were ground in liquid nitrogen and then extracted in HCl (Table 3.1). After 15 min centrifugation (20000 x *g*, 4°C), the pH of 300 µl supernatant was adjusted to 4-5 with 30 µl 0.2 M NaH_2PO_4 (pH 5.6) and ca. 230 µl 0.2 M NaOH. For the measurement of total AsA levels (reduced AsA + dehydroascorbic acid (DHA)), 100 µl supernatant was incubated with 25 mM dithiothreitol (DTT) in a 120 mM NaH_2PO_4 (pH 7.5) buffer during 15 min at 20°C to convert DHA to AsA. Hereafter, the pH of the incubated supernatant was adjusted to pH 5.5, the optimal pH for ascorbate oxidase (AO). Total and reduced AsA levels were measured in 100 µl 200 mM NaH_2PO_4 (pH 5.6) buffer, 55 µl H_2O and 40 µl extract. After the absorbance at 265 nm was recorded, 5 µl AO (40 U ml^{-1}) was added and the decrease in A_{265} was monitored. Stable values were taken 5 min after AO addition.

Glutathione measurements relied on the GR-dependent reduction of 5,5-dithiobis(2-nitro-benzoic acid) (DTNB), monitored at 412 nm. Without pre-treatment, total GSH (reduced GSH + oxidized GSH (GSSG)) was measured. When samples were incubated with 2-vinylpyridine, a specific measurement of GSSG was achieved by blocking any free reduced GSH. The reactions for total GSH were performed in 100 µl 200 mM NaH_2PO_4 – 10 mM EDTA (pH 7.5) buffer, 60 µl dH_2O, 10 µl 10 mM NADPH, 10 µl 12 mM DTNB, 10 µl sample and 10 µl GR. The increase in A_{412} was monitored. Standards were run concurrently. GSSG

was measured by the same principle, except that 20 µl sample and 50 µl H_2O were used.

Table 3.1: Extraction volume of HCl.

Chapter	Plant organ	Volume HCl
Chapter 4	Roots	600 µl
	Leaves	700 µl
Chapter 5 and 6	Roots	600 µl
	Leaves	800 µl
Chapter 7	Roots	600 µl
	Leaves	800 µl
Chapter 9	Roots	400 µl
	Leaves	600 µl

3.7. Analysis of enzyme capacities

Approximately 100 mg frozen leaf or root tissue was homogenized in 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM DTT and 4% insoluble polyvinylpyrrolidone (PVP) using a mortar and pestle. The homogenate was squeezed through a nylon mesh and centrifuged at 20000 x *g* and 4°C for 10 min. The enzyme capacities were determined spectrophotometrically in the supernatant at 25°C. For the enzyme measurements of chapter 9, the root and shoot samples were disrupted under frozen conditions using two stainless steel beads (diameter 3 mm) in the Retsch Mixer Mill MM400 during 3.5 min at 30 Herz after a spatula tip of PVP was added. Hereafter, 1.5 ml extraction buffer was added and the samples were vortexed and centrifuged at 20000 x *g* at 4°C during 10 minutes. After 0.5 ml of the supernatant was diluted in 0.5 ml extraction buffer, the enzyme capacities were determined. For the determination of the ascorbate peroxidase (APX) capacity, an extraction buffer was used which contained 10 mM sodium-AsA since APX loses stability in the absence of AsA, which will lead to a declined activity of the enzyme (Dabrowska *et al.*, 2007).

Guaiacol peroxidase and syringaldazine peroxidase (GPX, SPX, EC 1.11.1.7) capacities were measured at 436 nm and 530 nm according to Bergmeyer *et al.*

(1974) and Imberty *et al.* (1984), respectively. Analysis of superoxide dismutase (SOD, EC 1.15.1.1) capacity was based on the inhibition of cytochrome c at 550 nm according to McCord and Fridovich (1969). Ascorbate peroxidase (APX, EC 1.11.1.11) capacity was measured at 298 nm following the method of Gerbling *et al.* (1984). Analysis of the capacities of catalase (CAT, EC 1.11.1.6) were performed as described by Bergmeyer *et al.* (1974). Analysis of glutathione reductase (GR, EC 1.6.4.2) capacity was based on the reduction of GSSG (340 nm), using NADPH as described by Bergmeyer *et al.* (1974).

3.8. Gene expression analysis

Frozen root or leaf tissue (approximately 80 mg) was disrupted in 2 ml microcentrifuge tubes under frozen conditions using steel beads (diameter 3 mm) and the Retsch Mixer Mill MM400. RNA was extracted using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer's instructions. The RNA quantity and integrity were determined spectrophotometrically at 230, 260 and 280 nm (Nanodrop, Isogen Life Science) and via gel electrophoresis (Bioanalyzer, Agilent Technologies), respectively. Before cDNA synthesis RNA samples were incubated in gDNA wipeout buffer at 42 °C for 2 min to remove contaminating genomic DNA. First strand cDNA synthesis was primed with a combination of oligo(dT)-primers and random hexamers according to the manufacturer's instructions using QuantiTect Reverse Transcription Kit (Qiagen). Equal amounts of starting material were used (800 ng for chapter 5 and 6; 1 µg for chapter 7, 8 and 9). Quantitative PCR was performed with the ABI Prism 7500 (Applied Biosystems), using SYBR Green chemistry. PCR amplifications were performed at universal cycling conditions (10 min 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C) in a total volume of 10 µl, containing 2.5 µl cDNA sample, 5 µl Fast SYBR Green Master Mix (Applied Biosystems), 0.3 µM forward primer, 0.3 µM reverse primer and 1.9 µl RNase-free H_2O. Primers used for gene expression analyses are given in table 3.2. The amplification efficiencies of the primers were calculated according to Wong and Medrano (2005) by making a 4-fold serial dilution over 4 dilution points of a mixed sample and were accepted when they were greater than 80%. Multiple reference genes were used for root and shoot normalization (Table 3.3). The expression stability of the reference genes was evaluated by geNorm (version 3.5) (Vandesompele *et al.*, 2002).

Gene expression data were calculated relative to the control treatment following the $2^{-\Delta\Delta Ct}$ method (Livak *et al.*, 2001), normalized to a normalization factor based on the expression level of multiple reference genes.

Table 3.2: Reference genes used for the different experiments.

Experiment	Chapter	Reference genes leaves	Reference genes roots
Uranium exposure pH 4.5	5 and 6	*At2g28390* *At5g08290* *At5g15710* *At4g05320* *At4g26410* *At5g25760* *At5g55840* *At4g34270*	*At2g28390* *At5g08290* *At5g15710* *At4g05320* *At4g26410* *At5g25760* *At5g55840* *At4g34270*
Uranium exposure pH 7.5	7	*At5g55840* *At5g25760* *At4g26410* *At4g34270*	*At2g28390* *At5g08290* *At5g15710* *At4g05320* *At4g26410* *At5g25760* *At5g55840* *At4g34270*
Uranium and copper + vtc mutants	9	*At4g34270* *At2g28390* *At5g08290* *At5g15710*	*At5g55840* *At4g34270* *At2g28390* *At5g15710* *At5g08290* *At4g05320*

Table 3.3: Sequences of forward and reverse primers used in gene expression analysis.

Locus	Gene	Forward primer	Reverse primer
Reference genes			
At2g28390	*SAND family protein*	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC
At4g26410	*expressed*	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC
At4g34270	*TIP41-like*	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA
At5g08290	*Mitosis protein YSL8*	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT
At5g15710	*F-box protein*	TTTCGGCTGAGAGGTTCGAGT	GATTCCAAGACGTAAAGCAGATCAA
At5g25760	*UBC*	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC
At5g55840	*PPR gene*	AAGACAGTGAAGGTGCAACCTTACT	AGTTTTTGAGTTGTATTTGTCAGAGAAAG
At4g05320	*UBQ10*	GGCCTTGTATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAAACATAGT
Other genes			
At1g07890	*APX1*	TGCCACAAGGATAGGTCTGG	CCTTCCTTCTCTCCGCTCAA
At1g20630	*CAT1*	AAGTGCTTCATCGGGAAGGA	CTTCAACAAAACGCTTCACGA
At4g35090	*CAT2*	AACTCCTCCATGACCGTTGGA	TCCGTTCCCTGTCGAAATTG
At1g20620	*CAT3*	TCTCCAACAACATCTCTTCCCTCA	GTGAAATTAGCAACCTTCTCGATCA
At1g08830	*CSD1*	TCCATGCAGACCCTGATGAC	CCTGGAGACCAATGATGCC
At2g28190	*CSD2*	GAGCCTTTGTGGTTCACGAG	CACACCACATGCCAATCTCC
At5g18100	*CSD3*	GTTGTTGTGCATGCGGATCC	CACATCCAACTCTCGAGCCTG
At4g25100	*FSD1*	CTCCCAATGCTGTGAATCCC	TGGTCTTCGGTTCTGGAAGTC
At5g51100	*FSD2*	TTGGAAAGGTTCAAGTCGGCT	CATTTGCAACGTCAAGTCTATTCG
At5g23310	*FSD3*	AACGGGAATCCTTTACCCGA	TGTCTCCACCACCAGGTTGC
At3g24170	*GR1*	CTCAAGTGTGGAGCAACCAAAG	ATGCGTCTGGTCACACTGC
At3g54660	*GR2*	GCCCAGATGGATGGAACAGAT	TAGGGTTGGAGAATGTTGGCG
At4g23100	*GSH1*	CCCTGGTGAACTGCCTTCA	CATCAGCACCTCTCATCTCCA
At5g27380	*GSH2*	GGACTCGTCGTTGGTGACAA	TCTGGGAATGCAGTTGGTAGC

Table 3.3 (continued)

Locus	Gene	Forward primer	Reverse primer
At1g55020	*LOX1*	TTGGCTAAGGCTTTTGTCGG	GTGGCAATCACAAACGGTTC
At3g45140	*LOX2*	TTTGCTCGCCAGACACTTG	GGGATCACCATAAACGGCC
At3g10920	*MSD1*	ATGTTTGGGAGCACGCCTAC	AACCTCGCTTGCATATTTCCA
At5g44070	*PCS1*	TGGTGTTGAATGCTCTTTCTATCG	GGTTCGCAGCAATCCAACAT
At5g14545	*pri-miRNA398b*	AGTAATCAACGGCTGTAATGACGCTAC	TGACCTGAGAACACATGAAAACGAGAG
At5g14565	*pri-miRNA398c*	TCGAAACTCAAACTGTAACAGTCC	ATTTGGTAAATGAATAGAAGCCACGGGCCACG
At5g51060	*RBOHC*	TCACCAGAGACTGGCACAATAAA	GATGCTCGACCTGAATGCTC
At5g47910	*RBOHD*	TATGCATCGGAGAGGCTGCT	TAGAGACAACACGTTCCCGGG
At1g64060	*RBOHF*	GGTGTCATGAACGAAGTTGCA	AATGAGAGCAGAACGAGCATCA
At5g18830	*SPL7*	GAGCTGGAGGGCTATATCCG	GGAAGAGGCTCGATGACTGT

3.9. Uranium speciation

Uranium speciation in Hoagland nutrient solution was calculated in function of the pH using the speciation software React (Geochemist's Workbench® version 8.0). The Thermo_Minteq database was used. This database is the thermodynamic database from Visual MINTEQ release 2.40 (Bethke *et al.*, 2010). The temperature was fixed to 25°C. Redox simulations were enabled, while precipitation of solids was disabled.

3.10. Statistical analysis

Uranium, pH and U x pH interaction effects were determined by two-way ANOVA using SAS 9.1 software (SAS Institute Inc., Cary, NC, USA) (chapter 4). The analysis of the uranium effects in chapter 5 and 6 was done by one-way ANOVA using SAS 9.1 software (SAS Institute Inc., Cary, NC, USA). For the statistical analysis of chapter 7, 8 and 9 the freeware software package GNU R (version 2.15.0) (R Foundation for Statistical Computing, Vienna, Austria) was used. Uranium effects in chapter 7 were analysed with one-way ANOVA. To analyse the photosynthetic parameters for U, pH and U x pH interaction effects (chapter 8), two-way ANOVA was used. In chapter 9, treatment effects within the genotypes were determined by one-way ANOVA. Differences within the same treatment for the different genotypes were analysed by two-way ANOVA.

Statistical differences in group means were determined after Tukey adjustment for multiple comparisons. Normal distribution of the data was tested using Shapiro-Wilk (SAS and R) and the Kolmogorov-Smirnov test (only SAS). Logarithmic or square root transformations were applied where necessary to obtain normal distribution of the data. If the assumption of normality was not fulfilled, a non-parametric Wilcoxon rank sum test was carried out. Homoscedasticity was evaluated by a plot of group residues in SAS. To evaluate the homoscedasticity in R, the Bartlett's test was used.

Effects of pH on uranium uptake and oxidative stress responses induced in *Arabidopsis thaliana*

Eline Saenen, Nele Horemans, Nathalie Vanhoudt, Hildegarde Vandenhove, Geert Biermans, May Van Hees, Jean Wannijn, Jaco Vangronsveld, Ann Cuypers (2013). Effects of pH on uranium uptake and oxidative stress responses induced in *Arabidopsis thaliana*. Environmental toxicology and chemistry 32(9), 2125-2133.

Abstract

Uranium (U) causes oxidative stress in *Arabidopsis thaliana* plants grown at pH 5.5. However, U speciation and as such its toxicity strongly depends on environmental parameters, e.g. pH. It is unknown how different U species determine U uptake and translocation within plants and how they might affect the oxidative defence mechanisms of these plants. The present study aimed to analyse U uptake and oxidative stress-related responses in *Arabidopsis thaliana* (Columbia ecotype) under contrasted U chemical speciation conditions. Eighteen-day-old seedlings were exposed for 3 days to 25 µM U in a nutrient solution of which the pH was adjusted to 4.5, 5.5, 6.5 or 7.5. Results indicate that there is a different rate in U uptake and translocation at the different pH levels, with high uptake and low translocation at low pH and lower uptake but higher translocation at high pH. After U exposure, an increased glutathione reductase activity and total glutathione concentration were observed in U-exposed roots, pointing towards an important role for glutathione in the root defence system against U either by chelation or antioxidative defence mechanisms. In leaves, antioxidative defence mechanisms were activated upon U exposure, indicated by an increased superoxide dismutase and catalase activity. As it seems that U toxicity is influenced by the pH, it is important to consider site-specific characteristics when making U risk assessments.

4.1. Introduction

Uranium (U) is a naturally occurring radionuclide and heavy metal, with a greater risk of chemical toxicity than radiological toxicity because of its low specific activity (Sheppard *et al.*, 2005). Uranium-238 has a specific activity of 1.25 x 10^4 Bq g^{-1} U due to its large physical half-life of 4.47 x 10^9 years (Sheppard *et al.*, 2005). It is naturally present in most groundwaters and surface soils with an average concentration of 3 mg kg^{-1} dry soil (Bleise *et al.*, 2003). Anthropogenic activities such as U mining and milling, metal mining and smelting and the phosphate industry have caused enhanced U levels in the environment in many countries (Vandenhove, 2002).

Chemical toxicity of U is dependent on U speciation (Franklin *et al.*, 2000). The speciation describes the chemical state of elements in solutions. U can be present in a wide variety of chemical species, which can be divided into 3 predominant species: uranyl cation (UO_2^{2+}), uranyl hydroxides (e.g. UO_2OH^+, $(UO_2)_3(OH)_7^-$) and uranyl carbonates (e.g. UO_2CO_3, $UO_2(CO_3)_2^-$) (Ebbs *et al.*, 1998). Important factors controlling the speciation are for example pH value, redox potential, ionic strength and availability of inorganic and organic ligands (Bernhard, 2005). The pH-dependent speciation of U has been extensively studied. According to Ebbs *et al.* (1998), UO_2^{2+} is the predominant U species in soils under acidic conditions. Hydroxide complexes and phosphate complexes are generally formed under more neutral conditions, while carbonate complexes are majorly present under alkaline conditions. As Nagao *et al.* (2002) observed that the pH of pore waters varied between 4.9 and 7.9, it is important to evaluate the environmental impact of U under different ecologically relevant conditions. It is stated before that U toxicity is predominantly caused by UO_2^{2+} (Ribera *et al.*, 1996; Vandenhove *et al.*, 2006). It can replace Ca^{2+} and Mg^{2+}, which can lead to structural changes in cell membranes, enzyme inactivation and damage to RNA and DNA. Because UO_2^{2+} can also interact with phosphate moieties, DNA and membrane damage can also occur via this pathway (Vanhoudt *et al.*, 2008). However, also other U species including UO_2OH^+ and carbonated complexes can contribute to U toxicity (Zeman *et al.*, 2008).

Exposure of plants to environmental stress conditions (e.g. heavy metals) can lead to oxidative stress (Smeets *et al.*, 2008; Cuypers *et al.*, 2011). Oxidative stress is the disturbance of the cellular redox status, caused by an inhibition of the antioxidative defence system (enzymes and metabolites) and/or increased production of reactive oxygen species (ROS) (Remans *et al.*, 2010). ROS include the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1O_2$). They are generated in plant cells during normal metabolic processes at low rate. However, under stress conditions, the production of ROS can considerably rise (Arora *et al.*, 2002; Mittler, 2002). A dual role has been ascribed to the presence of ROS. They are toxic by-products of aerobic metabolism that can lead to the oxidative destruction of cells. However, ROS are also key regulators of growth, development and defence pathways (Mittler *et al.*, 2004). To regulate the amount of ROS, plants possess an antioxidative defence system. Superoxide dismutase (SOD) acts as the first line of defence against ROS and dismutate $O_2^{\bullet -}$ to H_2O_2. Catalase (CAT) and peroxidases (Px) subsequently detoxify H_2O_2. The ascorbate (AsA)-glutathione (GSH) pathway also plays an important role in the antioxidative mechanism in which metabolites and enzymes act together to detoxify H_2O_2 (Apel and Hirt, 2004).

It has already been demonstrated that U can cause oxidative stress in *Arabidopsis thaliana* plants grown at pH 5.5 (Vanhoudt *et al.*, 2008). However, it is unknown how the different U species present at different pH levels determine U uptake and translocation within plants and as such affect the antioxidative defence mechanisms of these plants. The aim of the present study was to analyse the biological effects of U induced in *Arabidopsis thaliana* under different ecologically relevant conditions (i.e. different pH levels). *Arabidopsis thaliana* seedlings were exposed to 0 µM U or 25 µM U at different pH levels ranging from 4.5 to 7.5. The effects of the pH on the antioxidative defence system were determined under control conditions. In addition, the influence of the pH on U speciation and U uptake and translocation was investigated, while the phytotoxicity of U under contrasted U chemical speciation conditions (through pH change) was also analysed. *Arabidopsis thaliana* was used since it is a model organism for flowering plants to study cellular and molecular processes in plants. Furthermore, this plant is easy to grow, a validated hydroponic setup is available

(Smeets *et al.*, 2008), it has a short life cycle and can produce a considerable amount of offspring. In addition, its entire genome has been sequenced and annotated (Poole, 2007).

4.2. Results

4.2.1. Uranium speciation

It was already shown that pH is a factor that influences U speciation. However the speciation distribution of U in Hoagland solutions with different pH levels was not reported before. U speciation in the different Hoagland solutions with low phosphate content was modelled using the Geochemist's Workbench® modelling software. Table 4.1 shows the U species distribution at the used pH levels. At pH 4.5, UO_2^{2+} was the major species (57.28%) present. When the pH increased to 5.5, a hydroxide ($(UO_2)_3(OH)_5^+$) and phosphate (UO_2HPO_4 (aq)) complex were calculated to be mainly present in the medium. By further increasing the pH, the majority of U consisted of $(UO_2)_2CO_3(OH)_3^-$ species.

Table 4.1: Uranium speciation calculations. Calculations were made with the Geochemist's Workbench® modelling software using the thermo_Minteq database at different pH levels in the Hoagland nutrient solution. Individual species are shown if their abundance is above 1% of the total U. aq = aqueous.

	pH 4.5	**pH 5.5**	**pH 6.5**	**pH 7.5**
UO_2^{2+}	57.28	7.34		
UO_2HPO_4 (aq)	26.54	30.12	5.75	
UO_2OH^+	8.58	11.00	1.98	
UO_2SO_4 (aq)	3.50			
$(UO_2)_2(OH)_2^{2+}$	3.09	5.08		
$(UO_2)_3(OH)_5^+$		34.45	20.22	
$UO_2PO_4^-$		4.16	7.94	1.24
$(UO_2)_4(OH)_7^+$		2.96	3.13	
$UO_2(OH)_2$ (aq)				
$(UO_2)_2CO_3(OH)_3^-$		1.75	56.81	93.14
UO_2CO_3 (aq)		1.65	2.98	1.20
$UO_2(CO_3)_2^{2-}$				2.72
Others	1.00	1.48	1.18	1.70

4.2.2. Uranium uptake and translocation

To investigate the effect of the pH on U uptake and translocation into the plants, the U concentration in *Arabidopsis thaliana* roots and shoots was determined after 3 days exposure to 25 µM U at different pH levels (Figure 4.1). In the roots (Figure 4.1A), the highest U concentration was found in plants exposed to pH 4.5. The U concentration in the roots was more than 200 times higher than in the leaves, indicating a small root-to-shoot transfer. However, the transfer at pH 7.5 was 10 times higher than at pH 4.5 (transfer factor 5.10^{-3} (pH 7.5) and 5.10^{-4} (pH 4.5)). This led to a shoot U concentration at pH 7.5, which was at least twice as high as compared to the other pH levels (Figure 4.1B).

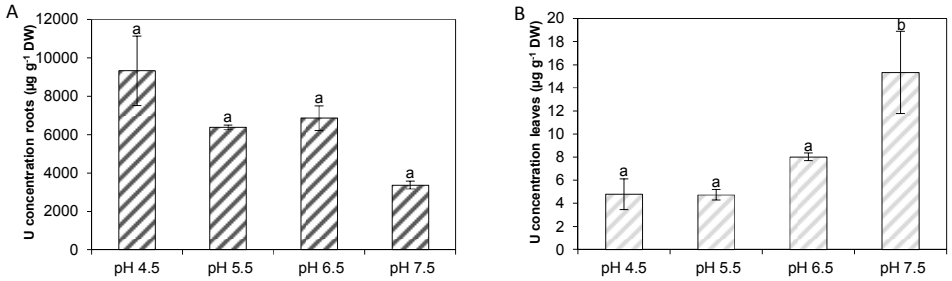

Figure 4.1: Uranium concentration (µg g^{-1} DW) in *Arabidopsis thaliana* roots (A) and leaves (B), treated with 25 µM U during 3 days at different pH levels. Statistical analyses were done separately for leaves and roots. Each point represents the mean ± S.E. of at least 4 replicates. Data points with different letters are significantly different (p<0.05).

4.2.3. Growth responses

There was no effect of the pH on the biomass of control plants (without U) (Figure 4.2). Whereas a significant decrease of shoot biomass was observed in U-exposed plants at pH 4.5 and 5.5 as compared to the control plants, no differences were detected at higher pH. In the U-exposed plants, there was a decreased root and shoot biomass at pH 4.5 as compared to higher pH ranges. On the other hand, leaf biomass increased in the plants exposed to U at pH 6.5 and pH 7.5 as compared to pH 5.5.

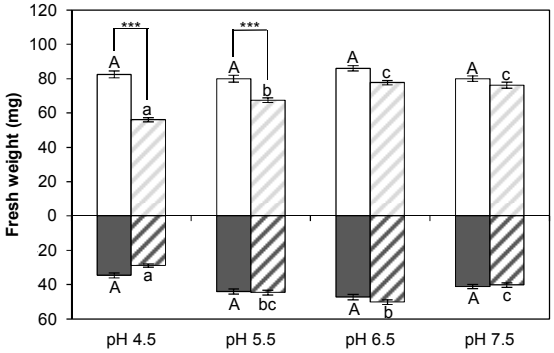

Figure 4.2: Fresh weight (mg/plant) of *Arabidopsis thaliana* leaves (upper part of the graph) and roots (lower part of the graph) of the non-exposed plants (solid bars) and plants exposed to 25 µM U (shaded bars) at different pH levels (pH 4.5, 5.5, 6.5, 7.5). Values represent the mean ± S.E. of at least 100 biological replicates. Statistical analyses were done separately for leaves and roots. Significant differences ($p<0.05$) between the different pH conditions are given in capital letters (control plants) and small letters (U-exposed plants). Significant differences between control plants and U-exposed plants at the same pH are indicated with *** ($p<0.001$). Since the data represented are the merged results from 2 separate experiments, only the significant differences present in both experiments are shown.

In control plants, an increased percentage dry weight (expressed as % of fresh weight) of roots at pH 4.5 was observed in comparison to the plants exposed to the higher pH levels (Table 4.2). The same result was observed in both roots and leaves when plants were exposed to U. Comparing control to U-exposed plants, a significant increase in leaf percentage dry weight was observed only at the lowest pH (Table 4.2).

Table 4.2: Percentage dry weight of *Arabidopsis thaliana* leaves and roots[a].

	ROOTS		**LEAVES**	
	Control	**25 µM U**	**Control**	**25 µM U**
pH 4.5	14.4 ± 5.1^{A}	10.5 ± 3.3^{a}	12.5 ± 1.6^{A}	$15.9 \pm 1.5^{a*}$
pH 5.5	5.1 ± 0.7^{B}	6.3 ± 1.1^{b}	11.0 ± 0.9^{A}	11.7 ± 1.0^{b}
pH 6.5	4.7 ± 0.4^{B}	4.6 ± 0.4^{b}	10.7 ± 0.7^{A}	11.1 ± 0.7^{b}
pH 7.5	4.0 ± 0.1^{B}	4.2 ± 0.2^{b}	10.5 ± 0.6^{A}	11.2 ± 0.5^{b}

[a]Percentage dry weight is expressed as % of fresh weight of the control plants and plants exposed to 25 µM U at different pH levels (pH 4.5, 5.5, 6.5, 7.5). Values represent the mean ± S.E. of at least 4 biological replicates. Statistical analyses were done separately for leaves and roots. Significant differences ($p<0.05$) between the different pH conditions are given in capital letters (control plants) and small letters (U-exposed plants). Significant differences between control plants and U-exposed plants at the same pH are indicated with * ($p<0.05$).

4.2.4. Photosynthesis

The photosynthetic efficiency of *Arabidopsis thaliana* leaves was determined by measuring chlorophyll *a* fluorescence in dark-adapted leaves. By analysing the results from high to low pH, a decreasing trend in Y(NPQ) was observed (Table 4.3). This means that less energy was quenched non-photochemically at low pH in both control and U-exposed plants. The increased effective photosystem II (PSII) quantum yield [Y(II)] indicated that there are more electrons effectively used for photosynthesis at low pH under both conditions. This was in combination with a higher electron transport rate [ETR(II)] that also occurred at low pH. In comparison with the control plants at similar pH, U had no significant effect on the different photosynthetic parameters measured.

Table 4.3: Saturation points of the different parameters of photosynthesis. Saturation points are points at 341 s. Data points are averages ± S.E. of 4 replicates. Different capital letters indicate differences in control plants (p<0.05). Different small letters indicate differences in U-exposed plants (p<0.05).

		Y(NPQ)	**Y(II)**	**Y(NO)**	**ETR(II)**
Control	pH 4.5	7.25 ± 0.22^{A}	69.45 ± 0.53^{A}	23.30 ± 0.35^{A}	43.75 ± 0.35^{A}
	pH 5.5	9.45 ± 1.02^{A}	65.40 ± 0.89^{B}	25.15 ± 0.24^{AB}	41.23 ± 0.56^{A}
	pH 6.5	11.20 ± 0.39^{AB}	62.38 ± 0.26^{C}	26.43 ± 0.21^{B}	39.30 ± 0.18^{B}
	pH 7.5	17.40 ± 1.83^{B}	58.13 ± 0.90^{C}	24.53 ± 0.73^{A}	36.58 ± 0.57^{B}
25 µM U	pH 4.5	6.88 ± 0.24^{a}	69.13 ± 0.64^{a}	24.00 ± 0.41^{a}	43.58 ± 0.42^{a}
	pH 5.5	10.23 ± 1.31^{ab}	64.73 ± 0.55^{b}	25.08 ± 0.37^{ab}	40.78 ± 0.98^{b}
	pH 6.5	10.05 ± 0.63^{ab}	63.65 ± 0.52^{bc}	26.33 ± 0.56^{ab}	40.08 ± 0.33^{bc}
	pH 7.5	13.93 ± 2.55^{b}	59.78 ± 2.74^{c}	26.35 ± 0.78^{b}	39.23 ± 1.73^{c}

4.2.5. Lipid peroxidation

Lipid peroxidation was analysed by measuring the thiobarbituric acid reactive compounds (TBA-rc) (Figure 4.3). Under control conditions, pH did affect lipid peroxidation neither in roots (Figure 4.3A), nor in leaves (Figure 4.3B). A significant increase in the TBA-rc was found after U exposure at low pH in *Arabidopsis thaliana* leaves both as compared to the control plants and to the U-exposed plants at higher pH levels. In the roots, there was an increasing trend in the TBA-rc after U exposure in comparison to the control plants, although not significant.

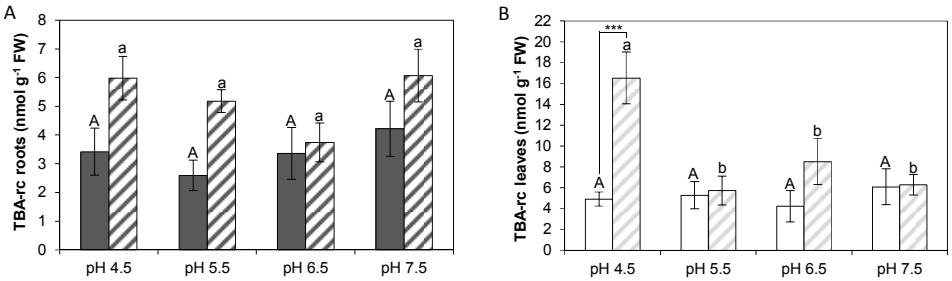

Figure 4.3: Level of lipid peroxidation, based on the amount of thiobarbituric acid reactive compounds (TBA-rc), in *Arabidopsis thaliana* roots (A) and leaves (B) of control plants (solid bars) and plants exposed to 25 µM U (shaded bars) at different pH levels (pH 4.5, 5.5, 6.5, 7.5). Values represent the mean ± S.E. of at least 3 biological replicates. Statistical analyses were done separately for leaves and roots. Significant differences (p<0.05) between the different pH conditions are given in capital letters (control plants) and small letters (U-exposed plants). Significant differences between control plants and U-exposed plants at the same pH are indicated with *** (p<0.001).

4.2.6. Antioxidative metabolites

To evaluate the importance of the AsA-GSH pathway under U stress, the AsA and GSH concentrations were determined (Table 4.4). In the roots, a significant increase in the concentration of total and reduced AsA was observed under control conditions at pH 4.5 in comparison to higher pH ranges. In control conditions, pH had no significant effect on DHA content. A significant decrease in total AsA concentration was seen in U-exposed plants with increasing pH. Nevertheless, the decreases observed in reduced AsA and DHA were not significant. In comparison with control plants, a significant increase in total AsA was observed at pH 5.5 and pH 6.5 after U exposure. At pH 6.5, this increase was due to an increase in reduced AsA, while at pH 5.5, there was a small increase both in reduced AsA and DHA. At pH 4.5, a significant decrease in reduced AsA and a significant increase in DHA was observed after U exposure.

For GSH, plant growth at different pH levels had no effect on total GSH, reduced GSH and GSSG concentrations, neither under control conditions nor in U-exposed roots. However comparing control and U-exposed plants at similar pH, a significant increase in total and reduced GSH was observed after U exposure starting at pH 5.5 and higher.

Table 4.4: Ascorbate and glutathione concentrations (nmol g^{-1} FW) in leaves and roots of *Arabidopsis thaliana*. Concentrations are given for control plants and plants exposed during 3 days to 25 µM U at different pH levels (4.5, 5.5, 6.5, 7.5). Each point represents the mean of at least 4 biological replicates ± S.E. Statistical analyses were done separately for leaves and roots. Different capital letters indicate differences in control plants (p<0.05). Different small letters indicated differences in U-exposed plants (p<0.05). Differences between control plants and U-exposed plants at the same pH are indicated with ▮ or ▮ for increased or decreased concentration respectively (p<0.05). AsA = reduced ascorbate, DHA = dehydroascorbate, % red AsA = reduced AsA / total AsA; GSH = reduced glutathione, GSSG = oxidized glutathione, % red GSH = reduced GSH / total GSH.

		Control				25 µM U			
		pH 4.5	**pH 5.5**	**pH 6.5**	**pH 7.5**	**pH 4.5**	**pH 5.5**	**pH 6.5**	**pH 7.5**
ROOTS	AsA+DHA	1176 ± 90^{A}	671 ± 24^{B}	660 ± 31^{B}	698 ± 26^{B}	1079 ± 99^{a}	944 ± 46^{ab}	936 ± 40^{ab}	708 ± 42^{b}
	AsA	879 ± 112^{A}	434 ± 26^{B}	436 ± 35^{B}	455 ± 32^{B}	536 ± 105^{a}	540 ± 52^{ab}	664 ± 38^{b}	426 ± 17^{a}
	DHA	298 ± 48^{A}	221 ± 12^{A}	225 ± 20^{A}	234 ± 30^{A}	509 ± 83^{a}	377 ± 49^{ab}	292 ± 25^{b}	292 ± 64^{ab}
	%red AsA	73 ± 5^{A}	67 ± 2^{A}	66 ± 4^{A}	70 ± 2^{A}	50 ± 11^{a}	60 ± 5^{a}	68 ± 3^{a}	60 ± 7^{a}
	GSH+GSSG	152 ± 7^{A}	119 ± 6^{A}	119 ± 4^{A}	128 ± 4^{A}	182 ± 13^{a}	190 ± 10^{a}	210 ± 19^{a}	173 ± 10^{a}
	GSH	147 ± 8^{A}	116 ± 6^{A}	117 ± 5^{A}	125 ± 5^{A}	176 ± 14^{a}	185 ± 10^{a}	205 ± 18^{a}	169 ± 10^{a}
	GSSG	2.5 ± 0.7^{A}	1.7 ± 0.4^{A}	1.3 ± 0.3^{A}	1.5 ± 0.4^{A}	3.1 ± 0.5^{a}	2.3 ± 0.2^{a}	2.6 ± 0.6^{a}	2.2 ± 0.3^{a}
	% red GSH	97 ± 1^{A}	97 ± 0.8^{A}	98 ± 0.5^{A}	98 ± 0.6^{A}	96 ± 0.8^{a}	98 ± 0.2^{a}	98 ± 0.4^{a}	97 ± 0.3^{a}
LEAVES	AsA+DHA	4585 ± 29^{AB}	3841 ± 174^{A}	5091 ± 58^{B}	5339 ± 57^{B}	6623 ± 277^{a}	6030 ± 200^{a}	5635 ± 305^{a}	5582 ± 440^{a}
	AsA	4227 ± 121^{AB}	3564 ± 194^{A}	4802 ± 195^{B}	4577 ± 60^{B}	6376 ± 23^{a}	5754 ± 245^{ab}	5164 ± 191^{b}	4983 ± 281^{b}
	DHA	358 ±137^{A}	335 ±79^{A}	581 ±36^{A}	762 ±43^{A}	550 ±132^{a}	406 ±32^{a}	471 ±144^{a}	599 ±168^{a}
	%red AsA	93 ± 3^{A}	93 ± 2^{A}	94 ± 4^{A}	87 ± 2^{A}	100 ± 4^{a}	93 ± 1^{a}	92 ± 2^{a}	90 ± 2^{a}
	GSH+GSSG	289 ± 13^{A}	298 ± 14^{AB}	394 ± 27^{BC}	449 ± 31^{C}	317 ± 14^{a}	320 ± 7^{a}	361 ± 12^{a}	352 ± 19^{a}
	GSH	283 ± 14^{A}	286 ± 13^{AB}	378 ± 26^{BC}	435 ± 31^{C}	301 ± 13^{a}	306 ± 6^{a}	346 ± 12^{a}	335 ± 18^{a}
	GSSG	3.9 ± 0.8^{A}	5.8 ± 0.3^{AB}	8.2 ± 0.7^{B}	6.8 ± 0.9^{AB}	8.0 ± 1.0^{a}	7.1 ± 0.8^{a}	7.0 ± 0.1^{a}	8.5 ± 1.0^{a}
	% red GSH	97 ± 0.4^{A}	96 ± 0.2^{A}	96 ± 0.2^{A}	97 ± 0.5^{A}	95 ± 0.6^{a*}	96 ± 0.4^{a}	95 ± 0.2^{a}	95 ± 0.5^{a}

In the leaves, total AsA concentrations increased under control conditions at pH 6.5 and 7.5 as compared to plants grown at pH 5.5. This could be attributed to an increase in the concentration of reduced AsA. Nevertheless, an increasing trend in DHA concentrations with increasing pH was also observed, but generally the redox state was maintained at the different pH levels. While under control conditions, the reduced AsA concentration increased at pH 6.5 and 7.5, a reduction in total and reduced AsA content was observed when plants were exposed to U. Comparing control plants and U-exposed plants grown under similar pH conditions, a significant increase in total and reduced AsA was found after U exposure at pH 4.5 and pH 5.5. However, no significant differences were observed at pH 6.5 and pH 7.5.

For GSH, a significant increase in total GSH concentration was observed in control plants at pH 7.5 as compared to pH 5.5, mainly due to an elevation of the reduced GSH concentration. Uranium exposure did not affect the total GSH concentrations at the different pHs. However, when comparing U-exposed plants to control plants grown at similar pH, a significant decrease in total and reduced GSH was observed at pH 7.5 after U exposure. At low pH, GSSG significantly increased after U exposure. At higher pH, there were no differences between control and U-exposed plants for GSSG.

4.2.7. Enzyme capacities

Enzymes of the antioxidative defence system were analysed to evaluate the importance of the cellular redox balance in *Arabidopsis thaliana* plants exposed to U at different pH levels (Figure 4.4). In the roots, SOD (Figure 4.4A) and guaiacol peroxidase (GPX) (Figure 4.4B) capacities were higher at pH 4.5 than at the other pHs under control conditions. The same effect was observed for U-exposed plants for the capacities of glutathione reductase (GR) (Figure 4.4C) and GPX. When comparing non-exposed to U-exposed plants at similar pH, an increased capacity of the ROS scavenging enzymes GR and GPX was only observed at the lowest pH. Whereas an increasing trend in the SOD capacity was shown at pH 6.5 and 7.5 after U exposure, a general decrease was observed in CAT (Figure 4.4D) capacity regardless of the external pH.

In the leaves, there were almost no differences in enzyme capacities under control conditions when comparing plants grown under different pH regimes. After U exposure, a decrease was observed for SOD capacity with increasing pH; a trend that was also observed for CAT capacity at pH 5.5 and 7.5 as compared to U-exposed plants grown at pH 4.5. When comparing non-exposed versus U-exposed plants grown at similar pH, an increasing trend in CAT capacity at all pH levels was observed. The SOD capacity significantly increased at pH 4.5 and 5.5, whereas GR capacity decreased at the lowest pH.

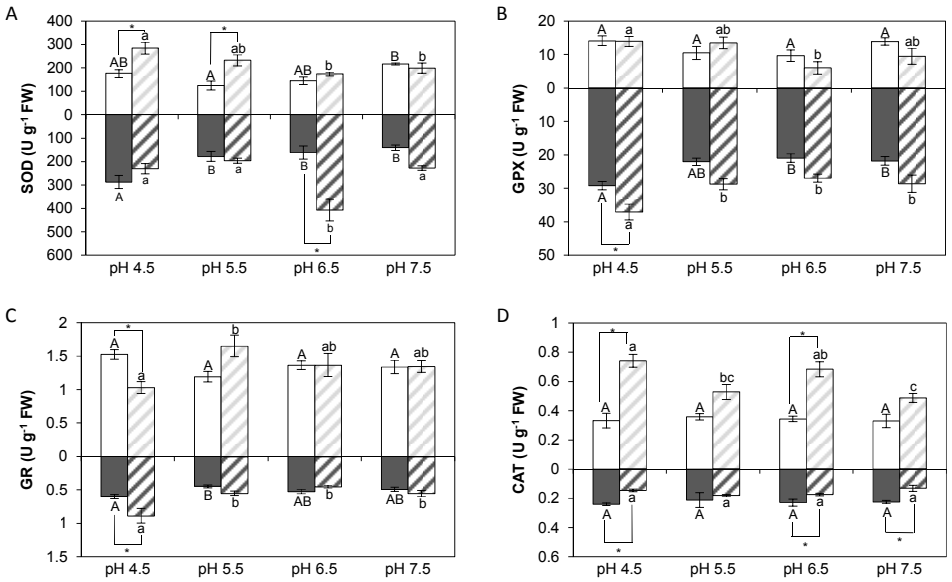

Figure 4.4: Enzyme capacities (units (U) g^{-1} FW) of superoxide dismutase (A, SOD), guaiacol peroxidase (B, GPX), glutathione reductase (C, GR) and catalase (D, CAT) for *Arabidopsis thaliana* leaves (upper part) and roots (lower part) of the non-exposed plants (solid bars) and plants exposed to 25 µM U (shaded bars) at different pH levels (pH 4.5, 5.5, 6.5, 7.5). Values represent the mean ± S.E. of at least 5 biological replicates. Statistical analyses were done separately for leaves and roots. Different capital letters indicate significant differences in the control plants (p<0.05). Different small letters indicate significant differences between the U-exposed plants (p<0.05). Differences between control plants and U-exposed plants at the same pH are indicated with * (p< 0.05).

4.3. Discussion

The speciation of U is strongly influenced by environmental factors, such as the pH level. This can have its impact on U uptake and translocation (Ebbs *et al.*, 1998). So far, most research on *Arabidopsis thaliana* was carried out under standard conditions with a growth medium of pH 5.0 – 5.7 (Vanhoudt *et al.*, 2008; Misson *et al.*, 2009; Horemans *et al.*, 2011; Vanhoudt *et al.*, 2011b).

However, pH of pore water varies naturally. Nagao *et al.* (2002) reported pH values of pore water ranging from 4.9 to 7.9. Therefore, it is necessary to evaluate the impact of U under different ecologically relevant conditions. The present study aimed to analyse the effects of the pH on the antioxidative defence system under control conditions (without U). Also the influence of the pH on U speciation and U uptake was investigated. Finally, the phytotoxicity of U under contrasted U chemical speciation conditions (through pH change) was analysed. For these purposes, *Arabidopsis thaliana* plants were exposed to 25 µM U during 3 days at different pH levels.

By analysing biomass parameters under control conditions (without U), no effect of the pH was observed on root and shoot fresh weight of *Arabidopsis thaliana* plants. However, a significant increase in root dry weight was observed at pH 4.5. This indicates that growing *Arabidopsis* plants at low pH caused a disturbed water balance in the roots and plants started to wilt. To evaluate whether these effects of the pH are also observed at biochemical level, different parameters of the antioxidative defence system, i.e. AsA and GSH concentrations and enzyme capacities, were measured. In *Arabidopsis thaliana* roots at pH 4.5, there was a significant increase in total AsA, attributed to an increase in reduced AsA, but a steady-state situation for DHA (Table 4.4). These results indicate that by increasing the amount of total AsA, *Arabidopsis thaliana* roots increase the capacity to detoxify H_2O_2. Increased synthesis of AsA under stress conditions was observed before. Gupta *et al.* (1999) found a significant increase in total AsA concentrations after exposure of *Phaseolus vulgaris* to Cu. In addition, enzymes of the antioxidative defence system were activated at low pH in the roots. This again indicates that *Arabidopsis thaliana* roots are sensitive to low pH, which was also reflected by the increased dry weight of those roots. The disturbed water balance at low pH can affect shoot biomass. However, this effect was not yet observed after 3 days. Besides the water balance, photosynthesis is a pivotal process in biomass production, one of the key parameters in the food web of an ecosystem (Snel *et al.*, 2000). It is an essential process of plant life, which begins with the absorption of light by chlorophyll as energy for photosynthesis (Snel and Dassen, 2000). Non-photochemical quenching decreased significantly at pH 4.5 as compared to pH 7.5. This could indicate that at low pH, the photosystem is damaged and plants are no longer able to quench

the excess energy in a regulated way [Y(NO)] or alternatively, that the plant uses more excitation energy for photosynthesis [Y(II)]. At pH 4.5, an increase in Y(II) was observed, indicating that more of the absorbed quanta are effectively used for photosynthesis. This observation was confirmed by measuring the ETR(II). When ETR(II) increases, more electrons can be accepted from PSII by the electron transport chain. This, in turn, will increase the amount of photons accepted by PSII, reflected by an increased Y(II). These results indicate that under mild stress conditions (e.g. low pH treatment) plants can increase their photosynthetic efficiency.

To investigate the effect of pH on U speciation, theoretical speciation calculation using the Geochemist's Workbench® modelling software were made. Results indicated that mainly UO_2^{2+} species were present at pH 4.5. In contrast to Ebbs *et al.* (1998) who found in his medium at pH 5.5 approximately 30 % UO_2^{2+} and 65% U-hydroxyl complexes, in our modified Hoagland solution, $(UO_2)_3(OH)_5^+$ and UO_2HPO_4 species were present at pH 5.5 and only 7.48% UO_2^{2+} was found. At pH 6.5 and 7.5, $(UO_2)_2CO_3(OH)_3^-$was the dominant species. These results are partially in agreement with Ebbs *et al.* (1998), who predicted that carbonate species would be mainly present at a pH higher than 7. To investigate the effect of the pH on U uptake and translocation, the U concentration in *Arabidopsis thaliana* roots and shoots was evaluated. Uranium mainly accumulates in the roots with limited transfer to the shoots. A small root-to-shoot transfer of U is in agreement with previous studies (Vandenhove *et al.*, 2006; Vanhoudt *et al.*, 2008). However, large differences in translocation to the shoots among the different pH levels were observed here. Although at pH 7.5 the concentration of U in the roots was approximately 3 times lower than at pH 4.5, the translocation at pH 7.5 was 10 times higher than at pH 4.5. The differences in U translocation to the shoots can be explained by the differences in U speciation at the different pH levels as suggested by Laurette *et al.* (2012). They suggest that U transfer through plants is mainly regulated by its precipitation with phosphate residues. According to the Geochemist's Workbench® modelling, U is at low pH mainly present in a highly reactive chemical form (UO_2^{2+}). This species can immediately precipitate with phosphate moieties present in the root cell membranes or react with cellulose-, pectin- or glycoprotein-rich compounds in the cell wall, which leads to its immobilization.

Laurette *et al.* (2012) suggest that at higher pH, when U is mainly present as a complexed (e.g. as U carbonate) and hence less reactive form, it is more mobile and can be transferred to plant shoots more easily. In agreement with this theory the lowest root-to-shoot transfer was found here at pH 4.5. However, our results are in strong contrast with those reported by Ebbs *et al.* (1998). They studied U uptake in peas grown at different pH levels and found the highest shoot U concentration at pH 5.0 when U was mainly present as UO_2^{2+} and found the highest root U concentrations at pH 6.0 and 8.0 (Ebbs *et al.*, 1998).

It was demonstrated before that exposure to U induces physiological and morphological effects in *Arabidopsis thaliana* plants at pH 5.5 (Vanhoudt *et al.*, 2011a). In the present study, the phytotoxicity of U under contrasted U chemical speciation conditions was determined by exposing plants to 25 µM U at different pH levels. It is stated before that toxicity of U would be predominantly caused by UO_2^{2+} (Ribera *et al.*, 1996). However, also other U species such as UO_2OH^+ and carbonated complexes possibly contribute to U toxicity (Zeman *et al.*, 2008). Root and shoot biomass of U-exposed plants at pH 4.5 decreased significantly as compared to pH 5.5. Since no significant differences were found under control conditions (without U), this decrease in fresh weight can probably be attributed to a difference in U-speciation. An increased leaf and root dry weight at pH 4.5 in U-exposed plants as compared to higher pH ranges indicate that those plants are water stressed. An increased leaf and root dry weight after U exposure was observed before by Vanhoudt *et al.* (2011a). In contrast, Ebbs *et al.* (1998) found an increase in leaf dry weight of U-exposed peas with increasing pH, but root dry weight at pH 5.0 was significantly higher than at pH 6.0 or 8.0.

Exposure of plants to heavy metals can lead to an inhibition of the antioxidative defence system and/or an increase production of ROS (Remans *et al.*, 2010). The increased ROS production can cause lipid peroxidation in plants cells resulting in membrane damage. Comparing control and U-exposed plants within one pH level, a significant increase in lipid peroxidation was only observed in the leaves at pH 4.5, indicating an affected membrane integrity and functionality, regardless of the low U concentration that was found in those leaves compared to pH 7.5. It seems that at low pH, plants are already suffering stress from the

low pH, which will be further enhanced after exposure to U, so plants will no longer be able to maintain their normal cell metabolism.

While oxidative stress responses for U at pH 5.5 (Vanhoudt *et al.*, 2008; Vanhoudt *et al.*, 2011; Vanhoudt *et al.*, 2011b) and for other heavy metals (Cuypers *et al.*, 2002; Smeets *et al.*, 2005) are well investigated, it is not known how U exposure at different pH levels will affect the antioxidative defence pathways. By analysing effects both at protein and metabolite level within one pH level, it seems that the antioxidative defence mechanisms were activated after U exposure. In roots at pH 4.5, the total AsA concentration remained stable after U exposure. However, a decreased reduced AsA concentration combined with a higher DHA level indicated that the redox balance shifted towards a more oxidized form, although not significant. Since DHA accumulation is considered as a negative event for cell metabolism (Drazkiewicz *et al.*, 2003), this can indicate that those roots are stressed. As only reduced AsA is capable of donating electrons, which makes it the main ROS-detoxifying compound in aqueous phase (Blokhina *et al.*, 2003), the shift towards the more oxidized form indicates that there is a decreased capacity to detoxify ROS. A decrease in percentage reduced AsA was observed before in *Arabidopsis thaliana* roots exposed to U at pH 5.5 (Vanhoudt *et al.*, 2011b). On the other hand, the GSH redox balance could be maintained and GR capacity increased. GR is important in the recycling of GSSG to GSH. Glutathione in its turn is a precursor of phytochelatins, metal binding peptides that are important in detoxification of toxic heavy metals (Hirata *et al.*, 2005). Total GSH concentrations significantly increased in the roots after U exposure at pH 5.5 or higher, as compared to the controls. Although the induction of phytochelatin synthesis to detoxify U in plants has not been shown before, the increased GR capacity at low pH and the increased reduced GSH concentrations can possibly indicate an increased capacity to produce phytochelatins. However, no increased phytochelatin concentration could be measured after U exposure (results not shown). Another mechanism to reduce the free cellular U concentration can be to limit the entry of this toxic element. Since peroxidases play an important role in cell wall lignification, the increased GPX capacity after U exposure can be a defence reaction to limit the entry of U into the roots (Ederli L, 2004).

In the leaves, an increased SOD capacity was observed after U exposure at pH 4.5 and 5.5, while CAT capacity increased at all pHs. The increased SOD and CAT capacity, together with the increase in total and reduced AsA levels at pH 4.5 and 5.5 after U exposure, indicates that the antioxidative defence mechanisms in the leaves are activated. This indicates that U disrupts the cellular redox balance. However, plants are still able to respond to U stress by increasing their antioxidative capacity. An activation of the antioxidative defence mechanism after U exposure was observed before in *Arabidopsis thaliana* leaves at pH 5.5 (Vanhoudt *et al.*, 2011).

In conclusion, it seems that the U exposure at different pH levels affect U uptake and translocation in the plants, with high uptake and low translocation at low pH and lower uptake but higher translocation at high pH. After U exposure, the antioxidative defence mechanisms in the leaves were activated. In the roots however, plants try to avoid the toxic effects of U by reducing the free cellular U on one hand by limiting the entry of U, on the other hand by increasing the capacity to produce phytochelatins and hence complexing U. The fact that a physicochemical variable such as the pH seems to influence U toxicity in *Arabidopsis thaliana* indicates that it is important to consider site-specific characteristics, including the pH, when making U risk assessments.

Dose-dependent effects induced by uranium at pH 4.5 in *Arabidopsis thaliana* roots

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Abstract

Oxidative stress responses after uranium (U) exposure have been investigated before in *Arabidopsis thaliana* plants at pH 5.5, the ideal pH for growing plants in a hydroponic setup. However, U speciation, and as such its toxicity, is strongly dependent on the pH. Therefore, the aim of this study is to investigate the stress responses induced in roots of *Arabidopsis thaliana* plants exposed to different U concentrations at pH 4.5. While exposure to low U concentrations resulted in a hormesis effect, a significant decrease in root fresh weight was observed after exposure to 50 µM U or higher. Results indicate that U is extremely toxic at low pH since no intact RNA could be extracted in the roots exposed to 75 and 100 µM U. In addition, the ascorbate redox balance was completely disturbed at higher U concentrations indicating that the roots are seriously damaged. Concerning the antioxidative defence system, it seems that miRNA398b/c is involved in the regulation of the SOD response after U exposure. As such, a significant increase in *MIR398b/c* expression was observed, accompanied by a decreased *CSD1/2* expression. While the involvement of miRNA398b/c was already reported before under Cu or Cd stress, this is the first time that it is reported for U.

5.1. Introduction

Environmental uranium (U) contamination is widespread due to anthropogenic activities such as U mining and milling, the phosphate industry and coal mining (Vandenhove, 2004). Uranium-238 is a naturally occurring radionuclide and heavy metal with a greater risk for chemical toxicity than radiological toxicity. This is due to the very long decay half-life of U (4.47×10^9 years) giving it a low specific activity of 1.25×10^4 Bq g^{-1} U [2, 3]. The bioavailability of U is strongly dependent on the physicochemical form of the element, which in turn depends on environmental parameters such as the pH level (Bernhard, 2005). The free uranyl ion (UO_2^{2+}) is mainly present at more acid pH conditions. At neutral pH, a number of aqueous hydroxide complexes (e.g. UO_2OH^+, $(UO_2)_3(OH)_7^-$) are formed while under alkaline conditions, the carbonate complexes dominate (eg. UO_2CO_3, $UO_2(CO_3)_2^-$)(Ebbs *et al.*, 1998). Uranium accumulation and distribution has been reported by several authors (Ebbs *et al.*, 1998; Laroche, 2005; Tomé *et al.*, 2009; Straczek *et al.*, 2010; Laurette *et al.*, 2012). However, little information on U toxicity at the cellular level is available for plants. Toxicity of U would be predominantly caused by UO_2^{2+} (Ribera *et al.*, 1996). It can replace Ca^{2+} and Mg^{2+}, what can lead to structural changes in cell membranes, enzyme inactivation and damage to RNA and DNA. Because UO_2^{2+} can also interact with phosphate moieties, DNA and membrane damage can also occur via this pathway. However, also other U species including UO_2OH^+ and carbonated complexes can contribute to U toxicity (Zeman *et al.*, 2008).

Exposure of plants to environmental stress conditions (e.g. heavy metals) can lead to oxidative stress (Cuypers *et al.*, 2001; Smeets *et al.*, 2008; Cuypers *et al.*, 2011). During oxidative stress, there is an imbalance between reactive oxygen species (ROS) production and ROS scavenging (Sorg, 2004). A dual role has been ascribed to the presence of ROS. On one side, they are toxic by-products of aerobic metabolism that can lead to the oxidative destruction of cells. On the other, ROS are key regulators of growth, development and defence pathways (Arora *et al.*, 2002; Mittler *et al.*, 2004). Enzymatic sources of ROS are NADPH oxidases and lipoxygenases (LOX). Plant NADPH oxidases, also called respiratory burst oxidase homologues (RBOHs), catalyse the formation of superoxides ($O_2^{\bullet -}$). LOX catalyse the dioxygenation of polyunsaturated fatty

acids, producing hydroperoxy fatty acids (Remans *et al.*, 2010). Vanhoudt *et al.* (2011) already demonstrated that LOX can be an important source of ROS during U-induced oxidative stress in *Arabidopsis thaliana*. To scavenge ROS, plants have evolved different mechanisms that are responsible for maintaining a low baseline of ROS. Those mechanisms include enzymes such as superoxide dismutases (SOD), catalases (CAT) and peroxidases (Px) and antioxidants such as ascorbate (AsA) and glutathione (GSH) (Mittler *et al.*, 2004). SOD constitutes the first line of defence and removes $O_2^{\bullet-}$ by catalysing its dismutation to hydrogen peroxide (H_2O_2). Catalases and Px subsequently detoxify H_2O_2. Furthermore, the AsA-GSH pathway plays an important role in the antioxidative defence mechanism in which metabolites and enzymes act together to detoxify H_2O_2 (Apel and Hirt, 2004; Foyer *et al.*, 2005).

Most research on toxicity of U in *Arabidopsis thaliana* plants was carried out under standard conditions with a growth medium of pH 5.0 – 5.7 (Vanhoudt *et al.*, 2008; Misson *et al.*, 2009; Horemans *et al.*, 2011; Vanhoudt *et al.*, 2011b). As such, it has already been demonstrated that U can cause oxidative stress in *Arabidopsis thaliana* plants exposed to U at pH 5.5 (Vanhoudt *et al.*, 2011; Vanhoudt *et al.*, 2011b). However, pH of soil pore water varies naturally. Nagao *et al.* (2002) reported pH values of pore water ranging from 4.9 to 7.9. When aiming to assess the impact of radioactive contamination on the environment, the effects of U contamination on the vegetation have to be investigated under different ecologically relevant conditions. In chapter 4, we investigated U uptake and related stress responses at four different pH levels. We observed high U uptake and low translocation at pH 4.5 and lower uptake but higher translocation at pH 7.5. Concerning the antioxidative responses, the defence mechanisms were activated in the leaves with an increased SOD and CAT capacity. In the roots plants try to avoid the toxic effects of U by an increased guaiacol peroxidase (GPX) capacity. This possibly leads to an enhanced cell wall lignification to limit the entry of U into the roots. Since only one U concentration was used in chapter 4, the present study aims to perform a more profound investigation of U-induced stress responses in the roots at pH 4.5, including the concentration dependency of the effects. For this purpose, *Arabidopsis thaliana* seedlings were exposed to different U concentrations ranging from 0 to 100 µM U at pH 4.5. We investigated effects at biochemical and molecular level.

5.2. Results

5.2.1. Uranium uptake and growth responses

The U concentration was determined in roots of 18-day-old *Arabidopsis thaliana* plants exposed to different U concentrations during 3 days at pH 4.5. The U concentration increased significantly with increasing U concentration added to the nutrient solution (Table 5.1).

Table 5.1: Uranium concentration (µg g^{-1} DW) in roots of *Arabidopsis thaliana* plants, exposed to different U concentrations during 3 days at pH 4.5. Each point represents the mean ± S.E. of at least 4 biological replicates. Data points with different letters are significantly different (p<0.05).

	U concentration (µg g^{-1} DW)
0 µM U	4.1 ± 0.8[a]
6.25 µM U	2289 ± 95[b]
12.5 µM U	4021 ± 275[c]
25 µM U	6784 ± 399[d]
50 µM U	39836 ± 5762[e]
75 µM U	68918 ± 3731[e]
100 µM U	76090 ± 10935[e]

After exposure of *Arabidopsis thaliana* plants to 50, 75 or 100 µM U, a significant decrease in root fresh weight was observed compared to control plants (Figure 5.1). However, root fresh weight significantly increased after exposure to 6.25 or 12.5 µM U, alluding to a hormesis effect present at these concentrations.

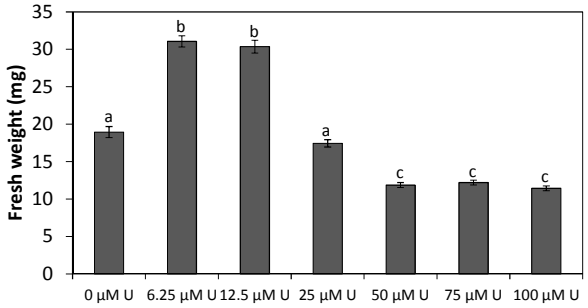

Figure 5.1: Fresh weight (mg/plant) of roots of *Arabidopsis thaliana* plants exposed to different U concentrations for 3 days at pH 4.5. Values represent the mean ± S.E. of at least 100 biological replicates. Data points with different letters are significantly different (p<0.05).

The percentage dry weight (expressed as % of fresh weight) was analysed by drying the roots for one week at 70°C. An increasing trend in dry weight was observed after U exposure, with a significant increase after exposure to 75 µM U as compared to the control roots (Table 5.2).

Table 5.2: Percentage dry weight of *Arabidopsis thaliana* roots exposed to different U concentrations for 3 days at pH 4.5. Values represent the mean ± S.E. of at least 4 biological replicates. Data points with different letters are significantly different ($p<0.05$).

	Percentage dry weight (expressed as % fresh weight)
0 µM U	5.00 ± 0.12^{ab}
6.25 µM U	4.85 ± 0.24^{a}
12.5 µM U	5.49 ± 0.43^{ab}
25 µM U	5.13 ± 0.61^{ab}
50 µM U	5.82 ± 0.44^{ab}
75 µM U	9.43 ± 0.80^{c}
100 µM U	7.34 ± 0.58^{bc}

For the reduction in relative root growth as compared to the control roots (= 100% growth), a dose-response curve was modelled using the Cedergreen-Ritz-Streibig model (Cedergreen *et al.*, 2005; Ritz *et al.*, 2005) in the statistical software package R (R Foundation for Statistical Computing, Vienna, Austria) since this model fitted out data the best. This model provides a log-logistic model for describing hormesis (Cedergreen *et al.*, 2005). The curve fitting enabled to calculate effective concentration (EC) levels together with the corresponding standard error. The ECx is the concentration that causes x per cent effect. The EC10, EC30 and EC50 for root growth reduction after 3 days exposure to U were 23.90 ± 1.06, 25.75 ± 1.20 and 28.14 ± 1.59 µM U, respectively.

5.2.2. Antioxidative metabolites

Ascorbate and GSH are both key components of the AsA-GSH cycle, essential to normal cell functioning in plant cells. The concentrations of both metabolites were determined spectrophotometrically in *Arabidopsis thaliana* roots after exposure to different U concentrations at pH 4.5 (Table 5.3). Despite several attempts to measure AsA concentrations in roots exposed to 50, 75 or 100 µM U, we were unable to measure those. The interference of U with the AsA

Table 5.3: Ascorbate and glutathione concentrations (nmol g^{-1} FW) in *Arabidopsis thaliana* roots exposed to different U concentrations for 3 days at pH 4.5. Values represent the mean ± S.E. of at least 3 biological replicates. Data points with different letters are significantly different ($p<0.05$). *n.d. = not detectable. AsA = reduced ascorbate, DHA = dehydroascorbate, Total AsA = AsA + DHA, % red AsA = reduced AsA/total AsA, GSH = reduced glutathione, GSSG = oxidized glutathione, Total GSH = GSH + GSSG, % red GSH = reduced GSH/total GSH.

	0 µM U	**6.25 µM U**	**12.5 µM U**	**25 µM U**	**50 µM U**	**75 µM U**	**100 µM U**
Total AsA	675 ± 76^{a}	628 ± 70^{a}	546 ± 90^{a}	693 ± 107^{a}	n.d.*	n.d.*	n.d.*
AsA	319 ± 61^{a}	394 ± 53^{a}	405 ± 78^{a}	362 ± 86^{a}	n.d.*	n.d.*	n.d.*
DHA	355 ± 50^{a}	291 ± 68^{a}	196 ± 12^{a}	331 ± 81^{a}	n.d.*	n.d.*	n.d.*
% red AsA	46 ± 7^{a}	66 ± 11^{a}	72 ± 3^{a}	53 ± 8^{a}	n.d.*	n.d.*	n.d.*
Total GSH	191 ± 21^{a}	129 ± 3^{bc}	135 ± 4^{bc}	170 ± 14^{ab}	126 ± 4^{bc}	121 ± 7^{c}	118 ± 7^{c}
GSH	183 ± 19^{a}	126 ± 3^{bc}	131 ± 4^{bc}	165 ± 14^{ab}	115 ± 4^{c}	110 ± 5^{c}	103 ± 5^{c}
GSSG	4.4 ± 0.8^{acd}	1.5 ± 0.2^{b}	1.8 ± 0.1^{ab}	2.6 ± 0.3^{bc}	5.2 ± 0.4^{cde}	5.5 ± 0.9^{de}	7.6 ± 0.9^{e}
% red GSH	96 ± 1^{a}	98 ± 0.3^{a}	97 ± 0.1^{a}	97 ± 0.2^{a}	92 ± 1^{b}	91 ± 1^{b}	87 ± 1^{c}

measurement was tested by spiking a control sample with U. However, it seems that U did not interfere with the measurement.

After exposure to 6.25, 12.5 or 25 µM U, the levels of total AsA remained unaffected. For reduced AsA an increasing trend was observed up to 12.5 µM U. The opposite result was found for DHA, where a decreasing trend was found up to 12.5 µM U. This is also reflected in the % reduced AsA, where roots exposed to 0 and 25 µM U have a lower % reduced AsA as compared to 6.25 or 12.5 µM U (not significant).

For GSH, a significant decrease in the concentration of total and reduced GSH was found after U exposure, except for 25 µM U. An increasing trend in GSSG was observed with increasing U concentrations, with a significant increase after exposure to 100 µM U as compared to the control. A corresponding significant decrease in % reduced GSH was also present at higher U concentrations.

5.2.3. Enzyme capacities

Enzyme capacities of some relevant enzymes of the antioxidative defence system were determined at protein level to evaluate the importance of the cellular redox balance in *Arabidopsis thaliana* roots exposed to U (Figure 5.4). An increasing trend was observed in the capacities of SOD, glutathione reductase (GR) and guaiacol peroxidase (GPX). The ascorbate peroxidase (APX) capacity increased up to 50 µM U, while CAT capacity showed a decreasing trend with a significant decrease after exposure to 75 µM U as compared to the control.

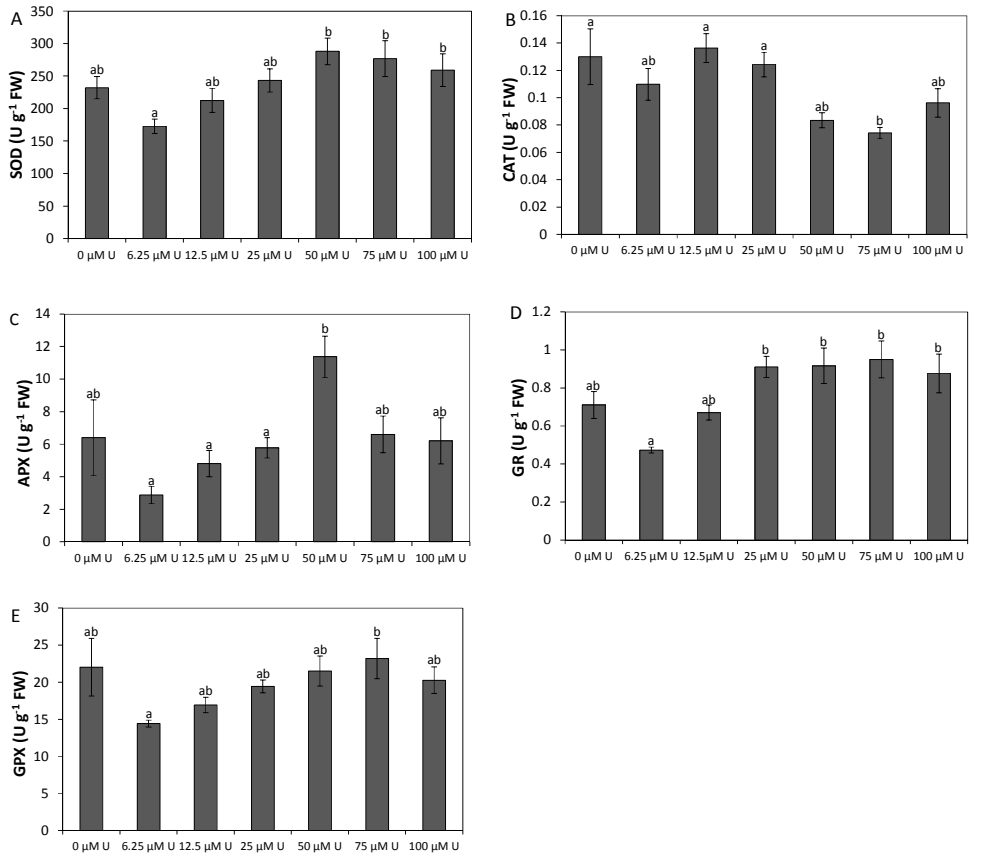

Figure 5.4: Enzyme capacities (units (U) g^{-1} FW) of superoxide dismutase (A, SOD), catalase (B, CAT), ascorbate peroxidase (C, APX), glutathione reductase (D, GR) and guaiacol peroxidase (E, GPX) of *Arabidopsis thaliana* roots exposed to different U concentrations during 3 days at pH 4.5. Values represent the mean ± S.E. of at least 4 biological replicates. Data points with different letters are significantly different (p<0.05).

5.2.4. Gene expression analysis

To evaluate the importance of oxidative stress related responses in *Arabidopsis thaliana* roots exposed to U, transcript levels of different ROS-producing and antioxidative enzymes were analysed using quantitative real-time PCR. Unless several attempts, no intact RNA could be extracted from roots exposed to the highest two U concentrations (75 and 100 µM). Therefore, the effects at molecular level of U exposure could not be determined in those roots.

First, gene expression of several ROS-producing enzymes was analysed (Table 5.4). Based on the results of Vanhoudt *et al.* (2011; 2011b), different NADPH

Table 5.4: Relative expression levels of the genes involved in ROS production and scavenging in roots of *Arabidopsis thaliana* plants after exposure to different U concentrations at pH 4.5. Gene expression is expressed relative to the control roots, which was set to 1. Values represent the mean ± S.E. of at least 3 biological replicates. Significant differences compared to the control plants are indicated with $p<0.01$ $p<0.05$ or $p<0.05$ $p<0.01$ for down- or up-regulation, respectively.

	Gene	Subcellular localization	6.25 µM U	12.5 µM U	25 µM U	50 µM U
Pro-oxidative marker genes	*RBOHC*	Cytoplasm	0.38 ± 0.12	1.17 ± 0.19	0.21 ± 0.07	0.09 ± 0.02
	RBOHD	Cytoplasm	0.31 ± 0.04	0.35 ± 0.04	0.16 ± 0.05	0.37 ± 0.12
	RBOHF	Cytoplasm	0.52 ± 0.16	0.41 ± 0.10	0.10 ± 0.04	0.27 ± 0.06
	LOX1	Cytoplasm	0.44 ± 0.12	1.09 ± 0.08	0.83 ± 0.33	0.83 ± 0.28
Anti-oxidative defence marker genes	*CSD1*	Cytoplasm	0.38 ± 0.07	0.39 ± 0.06	0.06 ± 0.02	0.11 ± 0.02
	CSD2	Plastid	0.32 ± 0.08	0.48 ± 0.11	0.04 ± 0.02	0.12 ± 0.01
	CSD3	Peroxisome	0.44 ± 0.08	0.48 ± 0.08	0.27 ± 0.05	1.13 ± 0.20
	FSD1	Plastid	0.89 ± 0.08	3.50 ± 0.95	5.29 ± 1.68	11.27 ± 3.60
	FSD2	Plastid	0.07 ± 0.01	0.14 ± 0.03	0.10 ± 0.01	0.37 ± 0.10
	FSD3	Plastid	0.27 ± 0.01	0.37 ± 0.05	0.25 ± 0.03	0.87 ± 0.23
	MSD1	Mitochondrion	0.57 ± 0.12	1.19 ± 0.29	0.51 ± 0.17	0.43 ± 0.08
Gene expression regulating genes	*MIR398b*		1.73 ± 0.28	2.55 ± 0.50	2.93 ± 0.40	5.55 ± 1.50
	MIR398c		2.59 ± 0.20	2.26 ± 0.75	4.01 ± 0.79	9.84 ± 2.55
Anti-oxidative defence marker genes	*CAT1*	Peroxisome	0.40 ± 0.08	0.41 ± 0.06	0.13 ± 0.01	1.03 ± 0.16
	CAT2	Peroxisome	1.20 ± 0.43	0.88 ± 0.13	0.15 ± 0.06	0.10 ± 0.02
	CAT3	Peroxisome	0.58 ± 0.06	0.29 ± 0.04	0.55 ± 0.09	0.49 ± 0.12
Genes involved in AsA-GSH cycle	*APX1*	Cytoplasm	0.80 ± 0.20	1.14 ± 0.30	0.43 ± 0.04	1.01 ± 0.09
	GR1	Cytoplasm	0.53 ± 0.16	0.64 ± 0.13	0.29 ± 0.02	0.49 ± 0.14
	GR2	Plastid	1.47 ± 0.14	1.08 ± 0.19	0.46 ± 0.05	0.58 ± 0.14
Genes involved in GSH and PCs synthesis	*GSH1*	Plastid	0.86 ± 0.22	0.93 ± 0.15	0.35 ± 0.02	0.42 ± 0.09
	GSH2	Cytoplasm	1.08 ± 0.26	1.23 ± 0.18	0.48 ± 0.04	0.69 ± 0.20
	PCS1	Cytoplasm	1.11 ± 0.33	1.30 ± 0.21	0.49 ± 0.08	0.84 ± 0.24

oxidases located in the plasma membrane were analysed on transcriptional level (*RBOHC*, *RBOHD* and *RBOHF*). A decreased expression was observed for *RBOHD* and *RBOHF* after exposure to all U concentrations. Similarly, *RBOHC* expression significantly decreased after U exposure, except after exposure to 12.5 µM U. For *LOX1* expression, no significant differences were detected.

Secondly, transcript levels of different isoforms of SOD were investigated (Table 5.4). A significant decrease in *CSD1* (cytoplasmic copper (Cu)/zinc (Zn) SOD) transcript levels was noticed after exposure to all U concentrations. *CSD2* (plastidic Cu/Zn SOD) expression significantly decreased after exposure to 25 and 50 µM U, while the expression of *CSD3* (peroxisomal Cu/Zn SOD) only decreased after exposure to 25 µM U. A significant increase in the transcript levels of *FSD1* (plastidic iron (Fe) SOD) was observed after exposure to 25 or 50 µM U. In contrast, the *FSD2* (plastidic FeSOD) and *FSD3* (plastidic FeSOD) expression decreased significantly after U exposure. No significant differences were observed in *MSD1* (mitochondrial manganese SOD) expression. CuZnSOD transcript levels are known to be negatively regulated by miRNA398b/c (Sunkar *et al.*, 2006). Exposure of *Arabidopsis thaliana* plants to U resulted in an increased *MIR398b/c* expression, with a significant increase after 50 µM U.

Subsequently, gene expression of H_2O_2 scavenging enzymes was examined (Table 5.4). The expression of *CAT1* (peroxisomal CAT) decreased after exposure to 6.25, 12.5 or 25 µM U, while the expression remained at control level after exposure to 50 µM U. In addition, a decreasing trend in *CAT2* (peroxisomal CAT) expression was detected with increasing U concentration, while a significant decrease in *CAT3* (peroxisomal CAT) expression was only observed at 12.5 µM U. APX is another enzyme important in H_2O_2 scavenging. Additionally, it has a crucial role in the AsA-GSH cycle. However, no changes were detected in the expression levels of *APX1*. The expression of *GR1* (cytoplasmic GR) and *GR2* (plastidic GR), also important in the AsA-GSH cycle, did not change significantly after U exposure.

Finally, genes involved in the GSH and phytochelatin production were analysed (Table 5.4). *GSH1* codes for γ-glutamylcysteine synthetase, while *GSH2* codes for GSH synthetase (Cairns *et al.*, 2006). Concerning phytochelatin production, only phytochelatin synthase 1 (*PCS1*) (Vatamaniuk *et al.*, 1999) was measured

since the available primers for *PCS2* had a low efficiency. No significant differences were observed in the transcript levels of *GSH1/2* or *PCS1* after U exposure.

5.3. Discussion

The aim of this study was to investigate the dose-dependent stress responses induced by U at low pH and hence at high UO_2^{2+} concentration. Therefore, 18-day-old *Arabidopsis thaliana* plants were exposed to a U concentration range from 0 to 100 µM U during 3 days at pH 4.5.

Roots are exposed to U via direct contact with the nutrient solution. As such a dose-dependent increase in the U concentration in the roots was observed. This increased U content was accompanied by a significant increase in root fresh weight after exposure to 6.25 µM U or 12.5 µM U as compared to the control roots (Figure 5.1). The increased root fresh weight at low U concentrations alludes to a transient hormesis effect as was observed before by several authors. Vanhoudt *et al.* (2011a) reported an increased root fresh weight of *Arabidopsis thaliana* seedlings after exposure to 1 µM or 10 µM U for 3 days at pH 5.5. A transient hormesis effect was also observed by Straczek *et al.* (2009) for hairy roots of carrots exposed to 2.5 and 5 mg U l^{-1}. As reported by Vanhoudt *et al.* (2011a), this increase in fresh weight is probably not attributable to a higher nitrate concentration, since U is added to the plants as uranyl nitrate. The extra nitrate added to the plants is negligible compared to the nitrate present in the Hoagland medium. Viehweger *et al.* (2008) proposed a secretion of phenolic compounds from cell cultures of *Brassica napus* after U contact. At low concentration, phenolic compounds can stimulate root elongation (Wang, 1991). In contrast to the lower U concentrations, root fresh weight decreased significantly after exposure to 50, 75 or 100 µM U (Figure 5.1). A decreased fresh weight of *Arabidopsis thaliana* roots was also observed by Vanhoudt *et al.* (2011a) after exposure to 100 µM U at pH 5.5. The decreased fresh weight, together with the increased dry weight observed after exposure to 75 and 100 µM U, is an indication for a disturbed water balance, which in turn indicates that plants start to wilt. Similar results were reported by Vanhoudt *et al.* (2011a) where the relative water content in *Arabidopsis* thaliana roots

exposed to 10 and 100 µM U at pH 5.5 was reduced, indicating that those plants might be water stressed.

The EC values that caused 10, 30 or 50 % root growth reduction were calculated using the Cedergreen-Ritz-Streibig model (Cedergreen *et al.*, 2005; Ritz and Streibig, 2005). The EC50 value (28.14 ± 1.58 µM U) is close to the EC50 value for root fresh weight reduction reported by Vanhoudt *et al.* (2011a) for *Arabidopsis thaliana* roots exposed to U at pH 5.5 (26.0 ± 17.3 µM U). Since plants in the present study are exposed to concentrations well above the EC50 value of 28.14 µM U, deleterious effects are expected. Toxicity of U was evidenced by a decreased fresh weight, an increased dry weight and a reduction in the percentage reduced GSH after exposure to 50, 75 and 100 µM U. In addition, AsA concentrations could not be determined in those roots, indicating that the roots are seriously damaged. Another consequence of prolonged metal exposure for plant cells can be DNA/RNA degradation (Lin *et al.*, 2012). Since no intact RNA could be extracted from roots exposed to 75 or 100 µM U, those plants are probably suffering severe stress. Additionally, the absence of intact RNA also indicates that there will no longer be *de novo* protein synthesis. Misson *et al.* (2009) reported an affected cell viability after exposure to 50 or 500 µM U, which can possibly explain the absence of intact RNA in the roots exposed to 75 and 100 µM U.

Gene expression levels of some plasma membrane bound NADPH oxidases were analysed in roots exposed to 0, 6.25, 12.5, 25 or 50 µM U (Table 5.4), since they are an important source of ROS production. In contrast to the results of Vanhoudt *et al.* (2011b), who observed a significant increase in *RBOHD* expression after exposure to 100 µM U at pH 5.5, a decreased *RBOHC*/*D*/*F* expression was observed after U exposure at pH 4.5. This indicates that the NADPH-mediated oxidative burst probably is not an important ROS-generating pathway in *Arabidopsis thaliana* roots under U stress at pH 4.5. The decreased RBOH expression is also in contrast with the expression observed after Cd exposure, where the RBOHs are an important source of ROS (Remans *et al.*, 2010; Cuypers *et al.*, 2011). Another source of ROS production in plants is LOX. They catalyse the dioxygenation of polyunsaturated fatty acids, producing hydroperoxy fatty acids (Porta *et al.*, 2002). However, *LOX1* transcripts were not

significantly affected in *Arabidopsis thaliana* roots after U exposure at pH 4.5. These results are again in contrast with the results of Vanhoudt *et al.* (2011b), where an increased *LOX1* expression was found after exposure to 100 µM U during 3 days at pH 5.5. Also after Cd or Cu exposure, an increased *LOX1* expression was observed before (Remans *et al.*, 2010). Comparing the different experimental set-ups, it is clear that the other studies were performed in *Arabidopsis* seedlings grown on hydroponics with an elevated pH, above pH 5. Since it seems that growing *Arabidopsis* plants at low pH causes already some stress (chapter 4), it is possible that there is already an elevated *RBOH* and *LOX* expression under control conditions, which can possibly explain no further significant increase in expression after U exposure.

Being a $O_2^{\bullet-}$ detoxifying system, SOD constitutes the first line of defence against ROS (Apel and Hirt, 2004). An increasing trend in SOD capacity was observed after U exposure (Figure 5.4), indicating an increased detoxification of $O_2^{\bullet-}$. Correspondingly, the *FSD1* expression significantly increased after U exposure (Table 5.4). However, a decrease in *CSD1* and *CSD2* expression was observed. The CSD transcripts are known to be negatively regulated by miRNA398b/c. The general notion is that under stress conditions, *MIR398b/c* is down-regulated to ensure an increased defence by CuZnSOD (Sunkar *et al.*, 2006). However, under U stress the *MIR398b/c* expression is induced. This led to a decrease in *CSD1* and *CSD2* expression, which is probably compensated by an increased *FSD1* expression. In accordance with our results, an induction of *MIR398b/c* expression was also observed in plants exposed to toxic but sublethal Cd concentrations (Cuypers *et al.*, 2011). Yamasaki *et al.* (2007) proposed that miRNA398 is involved in the regulation of Cu homeostasis. This will lead to a shut off of the *CSD1* and *CSD2* expression when Cu is limiting, while the Fe-requiring *FSD1* transcripts will be up-regulated. Consequently, *CSD1* and *CSD2* transcripts will not accumulate, leading to a decreased biosynthesis of the Cu requiring CuZnSODs when Cu is below a critical threshold. As such, Cu remains available and can be transported to plastocyanin, which is essential in photosynthesis in higher plants. Thus Cu will be saved for the most essential functions during limited Cu supply (Abdel-Ghany *et al.*, 2008).

Ascorbate peroxidase is an important scavenger of H_2O_2. More specific, APX is involved in the fine regulation of the H_2O_2 content (Mittler, 2002). It uses AsA as an electron donor to scavenge H_2O_2. During this reaction, AsA will be oxidized to DHA. Ascorbate on itself can also directly scavenge different ROS (Karuppanapandian *et al.*, 2011). At the lowest U concentration (6.25 µM U), a small decrease in APX capacity was observed (Figure 5.4), while after exposure to 50 µM U, the APX capacity increased. This could indicate an enhanced H_2O_2 detoxification. Unfortunately, the corresponding AsA concentrations at the higher U concentrations could not be determined. Together with the fact that no intact RNA could be extracted at the higher U concentrations, this can indicate that the roots are seriously damaged under these conditions. In addition, APX is probably no longer able to scavenge H_2O_2 as reflected by a decreased APX capacity at 75 and 100 µM U as compared to 50 µM U (Figure 5.4).

Like AsA, GSH is also an important metabolite occurring in virtually all cellular components. In combination with its oxidized form, it maintains redox equilibrium in the cellular compartments (Karuppanapandian *et al.*, 2011). Glutathione scavenges H_2O_2 and reacts non-enzymatically with other ROS. However, another important role of GSH is its ability to regenerate AsA from DHA via the AsA-GSH cycle (Arora *et al.*, 2002). Finally, GSH is a precursor of phytochelatins, heavy metal-binding peptides. The total glutathione concentration decreased significantly after exposure to 50, 75 and 100 µM U (Table 5.3). These results are in agreement with Vanhoudt *et al.* (2011b), where a significant reduction in the total GSH concentration was observed after exposure to 100 µM U at pH 5.5. The decrease in total GSH concentration can be related to a decreasing trend in the expression levels of *GSH1* and *GSH2* after U exposure (Table 5.4). *GSH1* and *GSH2* code for γ-glutamylcysteine synthetase and GSH synthetase respectively, two enzymes essential in GSH biosynthesis (Noctor *et al.*, 2011; Jozefczak *et al.*, 2012). A reduction in the transcript levels of *GSH1* and *GSH2* possibly indicate a reduced synthesis of GSH. In addition to a decreased GSH content, an increasing trend in the GR capacity was observed. GR normally reduces GSSG to GSH to keep GSH in its reduced state. In the present study, however, a significant decrease in reduced GSH and a significant increase in GSSG was observed after exposure to 50 µM and 100 µM U,

respectively. This led to a significant reduction in the % reduced GSH, indicating that roots of *Arabidopsis thaliana* plants exposed to U at pH 4.5 are stressed.

In conclusion, this study aimed to analyse the dose-dependent effects induced by U in *Arabidopsis thaliana* roots at low pH. Results have shown that U exposure caused deleterious effects at higher concentrations. As such, the AsA concentrations could not be determined and no intact RNA could be extracted indicating that the roots are seriously damaged. In addition, a significant decrease in root fresh weight, an increased percentage dry weight and a significant reduction in the percentage reduced GSH were observed at higher U concentrations, indicating that the roots are suffering from stress. Concerning the antioxidative defence mechanisms, SOD constitutes the first line of defence. It seems that miRNA398b/c is involved in the regulation of the SOD response after U exposure. This was already demonstrated before for other heavy metals (Gielen *et al.*, 2012). However, this is the first time that the involvement of miRNA398b/c is reported for U. Finally, the increased enzyme capacity of APX and GR could possibly indicate an important role for the AsA-GSH cycle in U-induced stress responses. However, further research is needed to determine the importance of AsA and GSH under U stress.

Dose-dependent effects induced by uranium at pH 4.5 in *Arabidopsis thaliana* leaves

Eline Saenen, Nele Horemans, Nathalie Vanhoudt, Hildegarde Vandenhove, Geert Biermans, May Van Hees, Jean Wannijn, Jaco Vangronsveld, Ann Cuypers (2013). Dose-dependent effects induced by uranium at pH 4.5 in *Arabidopsis thaliana* leaves. *In preparation for submission to Journal of Plant Physiology.*

Abstract

Anthropogenic activities have led to a widespread uranium (U) contamination in many countries. Uranium-induced stress responses in plants have already been investigated at pH 5.5, the optimal pH for hydroponically grown plants. However, since the speciation of U, and hence its toxicity, is strongly dependent on environmental factors such as the pH, it is important to investigate the effects of U at different environmentally relevant pH levels. In addition, although the U concentration in the leaves is low, it has been demonstrated that toxic effects in the leaves were already visible after 1 day exposure at pH 5.5, although only when exposed to relatively high (100 µM) U concentrations. Therefore, the present study aimed to analyse the dose-dependent effects induced by U at pH 4.5 in leaves of *Arabidopsis thaliana* plants. Results indicate that miRNA398b/c is involved in the regulation of the SOD response in the leaves. As such, an increased *MIR398b*/c expression was observed leading to a decreased transcript level of *CSD1*/*2*. In addition, a decreased expression of *CAT2* accompanied by an induction of *CAT3* expression, a decreased CAT capacity and an increased lipid peroxidation, possibly indicate that U induces early senescence in *Arabidopsis thaliana* leaves.

6.1. Introduction

Uranium (U) contamination has occurred in many countries due to anthropogenic processes such as U mining and milling, metal mining and smelting and the phosphate industry (Vandenhove, 2002). Uranium-238 is a naturally occurring radionuclide and heavy metal with a greater risk for chemical toxicity than radiological toxicity, because of the very long decay half-life of U (4.47×10^9 years) giving it a low specific activity of 1.25×10^4 Bq g^{-1} (Sheppard *et al.*, 2005). Typical concentrations of U in the soil range from 0.3 – 11.7 mg kg^{-1} soil, while concentrations in surface- or ground-waters vary between 3×10^{-2} and 2.1 µg l^{-1} (Bleise *et al.*, 2003). However, in some minerals (e.g. zircon) and rare earths, the concentration may be elevated up to 800 mg kg^{-1} (Vandenhove, 2002). The distribution, mobility and biological availability of U not only depends on its concentration, but is strongly dependent on the physicochemical form of the element, which in turn depends on environmental parameters such as the pH level (Bernhard, 2005). Since the pH level of pore waters can vary strongly (Nagao *et al.*, 2002), it is important to investigate the environmental impact of U under different ecological relevant conditions. Previously, we calculated the pH-dependent speciation of U in Hoagland nutrient solution (chapter 4). The free uranyl ion (UO_2^{2+}) was the major species (57.28%) present at pH 4.5. At pH 5.5, a hydroxide ($(UO_2)_3(OH)_5^+$) and phosphate (UO_2HPO_4 (aq)) complex were calculated to be mainly present in the medium. By further increasing the pH, the majority of U consisted of $(UO_2)_2CO_3(OH)_3^-$ species (chapter 4). U accumulation and distribution in plants has been reported by several authors (Ebbs *et al.*, 1998; Laroche, 2005; Tomé *et al.*, 2009; Straczek *et al.*, 2010; Laurette *et al.*, 2012). However, little information on U toxicity at the cellular level is available. Toxicity of U would be predominantly caused by UO_2^{2+} (Ribera *et al.*, 1996). The UO_2^{2+} can bind to phosphate groups of the cell membranes, leading to structural changes in the cell membranes. In addition, it will compete with Ca^{2+} and Mg^{2+} for binding sites, e.g. as cofactor in different enzymes, which can lead to enzyme inactivation and damage to RNA and DNA. However, also other U species including UO_2OH^+ and carbonated complexes can contribute to U toxicity (Zeman *et al.*, 2008).

It has been demonstrated that U and other heavy metals can induce oxidative stress related responses in plants (Vandenhove *et al.*, 2006; Smeets *et al.*, 2008; Vanhoudt *et al.*, 2008; Cuypers *et al.*, 2011; Vanhoudt *et al.*, 2011, chapter 4). Under normal conditions, ROS are produced as second messengers in many processes associated with plant growth and development (Foyer and Noctor, 2005). However, under stress conditions, the production of ROS can be enhanced or the ROS scavenging mechanisms can be impaired, a state that is called oxidative stress (Foyer and Noctor, 2005). Plant NADPH oxidase, also called respiratory burst oxidase homologues (RBOHs), have been identified in plants as a source of ROS by transferring electrons from cytoplasmatic NADPH to molecular oxygen (O_2) to form superoxide ($O_2^{\bullet-}$) (Mittler, 2002; Karuppanapandian *et al.*, 2011). The peroxidation of membrane lipids by lipoxygenases (LOX) is another possible source of ROS (Karuppanapandian *et al.*, 2011). To control the ROS production, plants have evolved an antioxidative defence system consisting of enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (Px), and antioxidants such as ascorbate (AsA) and glutathione (GSH) (Mittler *et al.*, 2004). SOD acts as the first line of defence against ROS by dismutating $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2). Catalases and Px subsequently detoxify H_2O_2 (Apel and Hirt, 2004). Concerning the non-enzymatic antioxidative systems, AsA can directly scavenge $O_2^{\bullet-}$, hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) and can reduce H_2O_2 to H_2O via ascorbate peroxidase (APX). Glutathione can also react non-enzymatically with ROS. However, the central role for GSH is its ability to regenerate AsA via the AsA-GSH cycle (Karuppanapandian *et al.*, 2011).

Vanhoudt *et al.* (2011; 2011b) showed before that U exposure can affect the transcript levels of several antioxidative and ROS-producing genes in *Arabidopsis thaliana* at pH 5.5. As such, an increased ROS production was evidenced by an increased *RBOHD* and *LOX1* expression in *Arabidopsis thaliana* roots accompanied with an increased SOD and APX capacity to scavenge the ROS after exposure to 100 µM U at pH 5.5 (Vanhoudt *et al.*, 2011b). In addition, in the leaves responses were already visible after 1 day U exposure, although the U concentrations were negligible at that time. This indicates a possible role for root-to-shoot signalling in the oxidative stress responses after U exposure (Vanhoudt *et al.*, 2011). However, the latter studies have been carried out at pH

5.5 which is the optimal pH of the Hoagland solution normally used to grow plants hydroponically. Since U speciation and hence its toxicity is strongly dependent on the pH and since the pH of pore water can vary (Lovley *et al.*, 1992; Nagao *et al.*, 2002), it is important to study the effects of U under different environmental relevant conditions. In chapter 4, we showed that the U uptake, translocation and the induced stress responses are strongly influenced by the pH. However, as in that study only one U concentration (25 µM) was applied, a more profound research is needed to further unravel the oxidative stress responses after U exposure. The present study will further unravel U-induced oxidative stress responses in *Arabidopsis thaliana* leaves at low pH. For this purpose, 18-day-old *Arabidopsis thaliana* plants were exposed to different U concentrations ranging from 0 to 100 µM U at pH 4.5 during 3 days.

6.2. Results

6.2.1. Uranium uptake and growth responses

Arabidopsis thaliana plants were exposed for 3 days to different U concentrations at pH 4.5. The U concentration in the leaves increased with increasing U concentration added to the Hoagland nutrient solution (Table 6.1).

Table 6.1: Uranium concentration (µg g^{-1} DW) in leaves of *Arabidopsis thaliana* plants, exposed to different U concentrations during 3 days at pH 4.5. Each point represents the mean ± S.E. of at least 4 biological replicates. Data points with different letters are significantly different (p<0.05).

	U concentration (µg g^{-1} DW)
0 µM U	n.d.
6.25 µM U	0.90 ± 0.13^{a}
12.5 µM U	1.49 ± 0.40^{ab}
25 µM U	2.68 ± 0.26^{bc}
50 µM U	5.00 ± 0.75^{cd}
75 µM U	8.44 ± 2.59^{de}
100 µM U	18.17 ± 3.91^{e}

Leaf fresh weight significantly decreased when plants were exposed to 25, 50, 75 or 100 µM U (Figure 6.1). For the reduction in leaf growth (growth of control plants = 100%), a dose-response curve was modelled using the Cedergreen-Ritz-Streibig model (Cedergreen *et al.*, 2005; Ritz and Streibig, 2005) in the

statistical software package R (R Foundation for Statistical Computing, Vienna, Austria) since this model was the best-fit model for our data. The model provides a log-logistic model for describing hormesis (Cedergreen *et al.*, 2005). The curve fitting enabled to calculate effective concentration (EC) levels together with the corresponding standard error. The ECx is the concentration that causes x per cent effect. However, in order to obtain a good fit, the growth results of 25 µM U were not taken into account. The EC10, EC30 and EC50 for leaf growth reduction after 3 days exposure to U were 14.84 ± 6.69, 21.14 ± 4.22 and 27.13 ± 5.20 µM U, respectively.

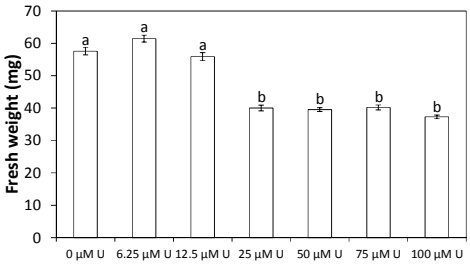

Figure 6.1: Fresh weight of leaves of *Arabidopsis thaliana* plants exposed to different U concentrations during 3 days at pH 4.5. Values represent the mean ± S.E. of at least 100 biological replicates. Data points with different letters are significantly different (p<0.05).

The percentage dry weight (expressed as % of fresh weight) was analysed by drying the leaves during 1 week at 70°C. A significant increase in leaf percentage dry weight was observed when plants were exposed to 25, 50, 75 or 100 µM U (Table 6.2).

Table 6.2: Percentage dry weight of *Arabidopsis thaliana* leaves exposed to different U concentrations during 3 days at pH 4.5. Values represent the mean ± S.E. of at least 4 biological replicates. Data points with different letters are significantly different (p<0.05).

	Percentage dry weight (expressed as % fresh weight)
0 µM U	12.96 ± 0.23^{a}
6.25 µM U	11.55 ± 0.26^{a}
12.5 µM U	12.52 ± 0.34^{a}
25 µM U	16.90 ± 0.96^{b}
50 µM U	17.25 ± 0.58^{b}
75 µM U	17.31 ± 0.64^{b}
100 µM U	17.28 ± 0.57^{b}

6.2.2. Lipid peroxidation

As a measure for the level of lipid peroxidation in *Arabidopsis thaliana* leaves, the amount of thiobarbituric acid reactive compounds (TBA-rc) was determined (Figure 6.2). A significant dose-dependent increase in lipid peroxidation was observed following exposure to 25 µM U or higher, as compared to the control leaves.

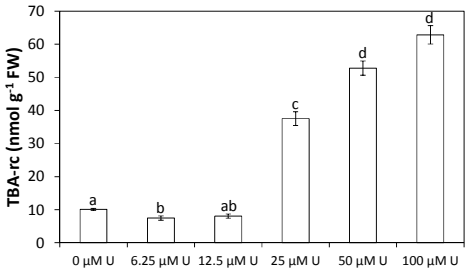

Figure 6.2: Level of lipid peroxidation, based on the amount of thiobarbituric acid reactive compounds (TBA-rc), in *Arabidopsis thaliana* leaves exposed to different U concentrations during 3 days at pH 4.5. Values represent the mean ± S.E. of at least 3 biological replicates. Data points with different letters are significantly different (p<0.05).

6.2.3. Antioxidative metabolites

To evaluate the importance of the AsA-GSH cycle in the response to U stress, the AsA and GSH concentrations were determined spectrophotometrically (Table 6.3). A significant increase in the total AsA concentration was observed when leaves were exposed to 25, 50, 75 or 100 µM U as compared to the control leaves. This increase is mainly due to a significant increase in reduced AsA while only a small non-significant increase in DHA was observed. In contrast to AsA, total GSH concentrations decreased significantly after U exposure to 100 µM U as compared to the control leaves, with a corresponding significant decrease in reduced GSH concentration. No significant changes in GSSG were observed. When analysing the percentage of reduced antioxidants, no significant differences for neither AsA nor GSH were observed.

Table 6.3: Ascorbate and glutathione concentrations (nmol g^{-1} FW) in *Arabidopsis thaliana* leaves exposed to different U concentrations during 3 days at pH 4.5. Values are mean values ± S.E. of at least 4 biological replicates. Data points with different letters are significantly different (p<0.05). AsA = reduced ascorbate, DHA = dehydroascorbate, Total AsA = AsA + DHA, % red AsA = reduced AsA/total AsA, GSH = reduced glutathione, GSSG = oxidized glutathione, Total GSH = GSH + GSSG, % red GSH = reduced GSH/total GSH.

	0 µM U	**6.25 µM U**	**12.5 µM U**	**25 µM U**	**50 µM U**	**75 µM U**	**100 µM U**
Total AsA	5433 ± 394^{a}	5717 ± 211^{a}	5595 ± 207^{a}	7311 ± 356^{b}	8395 ± 364^{b}	8243 ± 314^{b}	7192 ± 323^{b}
Reduced AsA	4973 ± 326^{a}	5137 ± 192^{a}	5166 ± 239^{a}	6755 ± 340^{b}	7850 ± 349^{b}	7741 ± 290^{b}	6675 ± 259^{b}
DHA	460 ± 98^{a}	580 ± 105^{a}	429 ± 45^{a}	556 ± 132^{a}	544 ± 57^{a}	502 ± 97^{a}	612 ± 54^{a}
% red AsA	92 ± 1^{a}	90 ± 2^{a}	92 ± 1^{a}	92 ± 2^{a}	94 ± 1^{a}	94 ± 1^{a}	93 ± 1^{a}
Total GSH	369 ± 19^{ab}	391 ± 8^{a}	361 ± 6^{ab}	343 ± 16^{ab}	315 ± 11^{bc}	324 ± 11^{bc}	273 ± 10^{c}
Reduced GSH	351 ± 18^{ab}	356 ± 15^{a}	345 ± 7^{ab}	327 ± 17^{ab}	298 ± 11^{bc}	308 ± 9^{ac}	257 ± 10^{c}
GSSG	9.3 ± 1.0^{a}	9.9 ± 2.2^{a}	7.6 ± 0.6^{a}	7.7 ± 1.0^{a}	8.4 ± 0.6^{a}	8.0 ± 1.2^{a}	8.0 ± 0.5^{a}
%red GSH	95 ± 1.0^{a}	96 ± 0.4^{a}	96 ± 0.2^{a}	95 ± 0.5^{a}	95 ± 0.4^{a}	95 ± 1.0^{a}	94 ± 0.4^{a}

6.2.4. Enzyme capacities

To investigate the influence of U contamination on protein level, enzyme capacities of the antioxidative defence system (SOD, CAT, APX, glutathione reductase (GR), guaiacol peroxidase (GPX) and syringaldazine peroxidase (SPX)) were examined in *Arabidopsis thaliana* leaves. A decreasing trend in APX and CAT capacity was observed after exposure to 75 and 100 µM U, while an increasing trend was detected in SOD capacity. However, none of these results showed significant differences (results not shown).

6.2.5. Gene expression analysis

Some important ROS-producing and –scavenging enzymes were analysed at transcriptional level using quantitative real-time PCR to evaluate their importance during U stress.

First, several plasma membrane bound NADPH oxidases (RBOH) and lipoxygenases (LOX) were analysed since they play an important role in ROS-production. An increased *RBOHC* expression was observed after U exposure, although not significant (Table 6.4). In contrast, the expression of *RBOHD* and *RBOHF* decreased after U exposure. The *LOX1* and *LOX2* expression increased after exposure to 6.25 or 12.5 µM U, although not significantly. By further increasing the U concentration, *LOX1* expression decreased, while *LOX2* expression decreased only after 25 or 100 µM U (not significant).

In order to study the antioxidative defence system, different isozymes of SOD were analysed. For the expression of the iron (Fe) SODs (*FSD1*, *FSD2* and *FSD3*), a significant decrease in the transcript levels of *FSD1* (100 µM U) and *FSD2* (all U concentrations) was observed. *FSD3* expression levels did not change significantly. A significant decrease in the expression of *CSD1* (cytoplasmic copper (Cu)/zinc (Zn) SOD) was observed after exposure to 12.5 µM U or higher, while the *CSD2* (plastidic Cu/Zn SOD) expression decreased after exposure to 25 µM U or higher (Table 6.4). No clear pattern was noticed in *CSD3* (peroxisomal Cu/Zn SOD) transcript levels. SOD transcript levels are known to be negatively regulated by miRNA398b/c (Ding *et al.*, 2009).

Table 6.4: Relative expression levels of the genes involved in ROS production and scavenging in leaves of *Arabidopsis thaliana* plants after exposure to different U concentrations at pH 4.5. Gene expression is expressed relative as compared to the control, which was set to 1. Values represent the mean ± S.E. of at least 3 biological replicates. Significant differences compared to the control plants are indicated with $p<0.01$ $p<0.05$ or $p<0.05$ $p<0.01$ for down- or up-regulation, respectively.

	Gene	Subcellular localization	6.25 µM U	12.5 µM U	25 µM U	50 µM U	100 µM U
Pro-oxidative marker genes	*RBOHC*	Cytoplasm	0.88 ± 0.49	1.77 ± 0.97	2.59 ± 1.52	1.01 ± 0.62	2.33 ± 0.13
	RBOHD	Cytoplasm	0.29 ± 0.13	0.30 ± 0.11	0.14 ± 0.02	0.25 ± 0.08	0.20 ± 0.08
	RBOHF	Cytoplasm	0.53 ± 0.08	0.17 ± 0.01	0.06 ± 0.01	0.31 ± 0.12	0.27 ± 0.14
	LOX1	Cytoplasm	1.84 ± 1.03	2.78 ± 0.80	0.51 ± 0.07	0.70 ± 0.22	0.34 ± 0.13
	LOX2	Plastid	1.23 ± 0.51	1.56 ± 0.33	0.39 ± 0.11	1.71 ± 0.70	0.77 ± 0.40
Anti-oxidative defence marker genes	*CSD1*	Cytoplasm	0.48 ± 0.09	0.23 ± 0.04	0.17 ± 0.05	0.18 ± 0.06	0.07 ± 0.03
	CSD2	Plastid	0.57 ± 0.12	0.45 ± 0.19	0.04 ± 0.01	0.10 ± 0.03	0.11 ± 0.06
	CSD3	Peroxisome	0.46 ± 0.14	0.37 ± 0.10	0.68 ± 0.14	1.87 ± 0.58	0.56 ± 0.20
	FSD1	Plastid	0.62 ± 0.12	1.32 ± 0.52	0.51 ± 0.11	0.74 ± 0.20	0.38 ± 0.22
	FSD2	Plastid	0.23 ± 0.09	0.14 ± 0.05	0.07 ± 0.01	0.18 ± 0.05	0.07 ± 0.02
	FSD3	Plastid	0.73 ± 0.20	0.64 ± 0.20	0.47 ± 0.03	0.69 ± 0.22	0.28 ± 0.12
	MSD1	Mitochondrion	0.96 ± 0.33	1.26 ± 0.40	0.63 ± 0.06	0.98 ± 0.25	0.72 ± 0.28
Gene expression regulating genes	*miRNA398b*		1.28 ± 0.31	0.62 ± 0.05	2.01 ± 0.54	2.90 ± 0.62	0.83 ± 0.20
	miRNA398c		0.75 ± 0.24	1.16 ± 0.41	1.81 ± 0.31	2.70 ± 0.51	1.38 ± 0.13
Anti-oxidative defence marker genes	*CAT1*	Peroxisome	0.88 ± 0.23	1.30 ± 0.27	0.27 ± 0.16	0.50 ± 0.14	0.40 ± 0.15
	CAT2	Peroxisome	0.80 ± 0.34	0.89 ± 0.22	0.16 ± 0.08	0.12 ± 0.04	0.04 ± 0.02
	CAT3	Peroxisome	2.40 ± 0.53	1.67 ± 0.28	3.11 ± 0.40	1.94 ± 0.67	1.06 ± 0.31
Genes involved in AsA-GSH cycle	*APX1*	Cytoplasm	1.01 ± 0.26	0.80 ± 0.10	0.81 ± 0.10	1.17 ± 0.26	0.43 ± 0.07
	GR1	Cytoplasm	0.72 ± 0.23	0.81 ± 0.19	0.57 ± 0.15	0.48 ± 0.01	0.30 ± 0.08
	GR2	Plastid	0.74 ± 0.18	0.75 ± 0.16	0.41 ± 0.04	0.61 ± 0.21	0.30 ± 0.03
Genes involved in GSH and PCs biosynthesis	*GSH1*	Plastid	0.93 ± 0.26	0.56 ± 0.12	0.40 ± 0.07	0.36 ± 0.07	0.23 ± 0.04
	GSH2	Cytoplasm	0.61 ± 0.20	0.41 ± 0.13	0.54 ± 0.16	0.64 ± 0.16	0.35 ± 0.06
	PCS1	Cytoplasm	0.50 ± 0.14	0.42 ± 0.11	0.44 ± 0.13	0.44 ± 0.11	0.24 ± 0.03

Exposure of *Arabidopsis thaliana* to U resulted in an increased *MIR398b/c* expression in the leaves after exposure to 50 µM U.

Subsequently, gene expression of H_2O_2 scavenging enzymes was examined. A decreased expression of *CAT1* (peroxisomal CAT) and a significant decrease in *CAT2* (peroxisomal CAT) transcript levels were observed after exposure to 25, 50 and 100 µM U as compared to the control (Table 6.4). The expression of *CAT3* (peroxisomal CAT) increased after U exposure, with a significant increase at 25 µM U. APX is another enzyme important enzyme in H_2O_2 scavenging. In addition, it also plays an important role in the AsA-GSH cycle. However, no differences in *APX1* expression were detected in the leaves. Another enzyme important in the AsA-GSH cycle is GR. *GR1* and *GR2* expression levels did not change significantly after U exposure. However, a decreasing trend was noticed in *GR1* expression.

The possible production of phytochelatins under U stress was evaluated by measuring the transcript levels of *GSH1*, *GSH2* and *PCS1*. *GSH1* codes for γ-glutamylcysteine synthetase, while *GSH2* codes for GSH synthetase (Cairns *et al.*, 2006). Concerning phytochelatin production, only phytochelatin synthase 1 (PCS1) (Vatamaniuk *et al.*, 1999) was measured. No significant differences in those transcript levels could be observed after U exposure as compared to the control.

6.3. Discussion

It is known that U speciation, and as such its toxicity, strongly depends on the pH level of the external medium. Therefore, it is important to investigate the environmental impact of U contamination under different environmentally relevant conditions. In chapter 4, we demonstrated that exposing *Arabidopsis thaliana* plants to U at low pH resulted in a high uptake but low translocation of U, which could be related to the high presence of UO_2^{2+}. At higher pH, the main U species present were carbonyl species which resulted in a low uptake but higher translocation (chapter 4). Since only one U concentration was used in the latter study, the present study aimed to perform a more profound investigation of U induced stress responses in *Arabidopsis thaliana* leaves at a low pH level and hence at high UO_2^{2+} concentrations. Therefore, 18-day-old *Arabidopsis*

thaliana plants were exposed during 3 days to a U concentration range from 0 to 100 µM U at pH 4.5.

After 3 days, the *Arabidopsis thaliana* plants showed a dose-dependent accumulation of U in the leaves (Table 6.1). Although U concentrations were very low in the leaves of U-exposed plants, effects were already observed after exposure to 25 µM U. This is in agreement with the results of Vanhoudt *et al.* (2011) who observed stress responses in *Arabidopsis thaliana* leaves after 1 day U exposure to 100 µM U, when the U concentrations in the leaves were negligible. They suggest that the oxidative stress was probably generated via root-to-shoot signalling. In the present study, the increased U content was accompanied with a significant decrease in leaf growth (Figure 6.1) and a significant increase in leaf percentage dry weight (Table 6.2) after exposure to 25 µM U or higher. A decreased leaf fresh weight for *Arabidopsis thaliana* plants after U exposure was also shown by several authors (Misson *et al.*, 2009; Vanhoudt *et al.*, 2011). *Arabidopsis thaliana* seedlings exposed to 100 µM U during 3 days at pH 5.5 showed a significant reduced leaf fresh weight (Vanhoudt *et al.*, 2011a). Misson *et al.* (2009) reported a 25 % and 38.5 % reduction in *Arabidopsis* leaf biomass after exposure to 50 and 500 µM U at pH 5.7, respectively. The significant increase in leaf percentage dry weight possibly indicates that U exposure caused a disturbed water balance and plants start to wilt. Similar results were reported by Vanhoudt *et al.* (2011a), who observed an increased dry weight of *Arabidopsis thaliana* leaves exposed to 100 µM U at pH 5.5. An increased dry weight after exposure to 25 µM U at pH 4.5 was also reported in chapter 4, indicating that those plants might be water stressed.

Based on the leaf growth reduction, the EC10, EC30 and EC50 could be calculated. The EC50 value from the present experiment is remarkably lower than the EC50 value observed before by Horemans *et al.* (2011). They reported an EC50 value of 66 µM U for leaves of *Arabidopsis thaliana* plants exposed to U during 3 days at pH 5.5. The discrepancy in EC50 value can possibly be related to the different pH at which plants were exposed to U. Since the EC50 value we found is about 2.3 times lower than the value reported by Horemans *et al.* (2011), this can indicate that U induces more toxic effects at low pH. Similar results were reported before in chapter 4, where a decreased fresh weight, an

increased dry weight, an increased lipid peroxidation and an activation of antioxidative enzymes were mainly observed at pH 4.5.

Generally, under stress conditions, the generation of toxic ROS species is increased (Arora *et al.*, 2002). An important source of ROS production are the plasma membrane bound NADPH oxidases. They catalyse the formation of $O_2^{\bullet-}$ (Apel and Hirt, 2004). Gene expression of some important NADPH oxidases was analysed. However, there was no induction in *RBOHC/D/F* expression after U exposure. This suggests that under U stress at pH 4.5 the NADPH-mediated oxidative burst is not an important ROS-generating pathway in leaves of *Arabidopsis thaliana* plants, as was also observed in the roots of those plants (chapter 5). Other sources of ROS in plants are the lipoxygenases (LOX). They catalyse the dioxygenation of polyunsaturated fatty acids, producing hydroperoxy fatty acids (Porta and Rocha-Sosa, 2002). However, LOX transcript levels were not significantly affected in *Arabidopsis thaliana* leaves under U stress. These results are partially in agreement with the results of Vanhoudt *et al.* (2011) who reported no involvement of *LOX1* but an increasing trend in *LOX2* expression after exposure of *Arabidopsis thaliana* to U at pH 5.5 during 3 days. Although no differences in LOX transcripts were observed in the present study, U exposure resulted in a significant increase in the amount of TBA-rc in the leaves. This possibly indicates that the observed lipid peroxidation is ROS generated. Since U is a redox-active metal, it can enhance the production of $OH^{\cdot}$ and further stimulate lipid peroxidation (Cuypers *et al.*, 2011). The produced oxidized polyunsaturated fatty acids can act as precursors for signalling molecules like jasmonic acid and other oxylipins that enable plants to adequately respond to stress (Sharma and Dietz, 2009).

To counteract the toxicity of ROS, plants possess an antioxidative defence system composed of ROS scavenging enzymes (e.g. SOD, CAT, APX) and metabolites (AsA and GSH) (Dat *et al.*, 2000). SOD constitutes the first line of defence against ROS by removing $O_2^{\bullet-}$. SODs are present at different subcellular locations. Depending on the metal co-factor that is used, different isoforms of SOD can be distinguished (Alscher *et al.*, 2001). The total SOD capacity was not significantly affected by the U treatment. This can indicate that either the production of $O_2^{\bullet-}$ did not increase to significant levels, or that the activity was

sufficient to tackle the $O_2^{\bullet-}$ produced (Srivastava *et al.*, 2010). By analysing the gene expression of the different isoforms of SOD, there was a significant decrease in *CSD1* and *CSD2* expression after exposure to 12.5 and 25 µM U respectively, or higher U concentrations. It was already demonstrated that miRNA398b/c is involved in the regulation of the SOD response after exposure to U in *Arabidopsis thaliana* roots (chapter 5). It seems that the miRNA398b/c also plays a role in the leaf responses after U exposure since the *MIR398b/c* expression was up-regulated, with a significant induction after exposure to 50 µM U. This can explain the inhibition of *CSD1/2* expression, as it is known that miRNA398 negatively regulates the *CSD1/2* expression at post-transcriptional level (Sunkar *et al.*, 2006). In contrast to the roots, where the decreased *CSD1*/2 expression was compensated by an increased expression of *FSD1*, no compensation by *FSD1* was observed in the leaves. Vanhoudt *et al.* (2011a) reported a decreasing trend in the leaf Fe concentrations after exposure of *Arabidopsis thaliana* plants to U, with a significant decrease after exposure to 100 µM U. A decreased Fe concentration can be an explanation for the lack of *FSD1* compensation in the leaves, since under Fe-limiting conditions the expression of FeSOD can decline. However, to confirm this hypothesis, Fe concentrations in the leaves of *Arabidopsis thaliana* plants of the present study should be determined.

Besides ROS scavenging enzymes, also metabolites are important in the defence mechanisms against oxidative stress. Ascorbate is the primary antioxidant in plants that counteracts ROS (Noctor and Foyer, 1998). The increase in total AsA and reduced AsA levels under U stress suggests that U induces *de novo* synthesis of AsA. This, in turn, could indicate the importance of this compound in the antioxidant metabolism after U exposure. An increase in the total AsA concentration after heavy metal exposure has been reported before in different plant species. Vanhoudt *et al.* (2011) noticed a significant increase in total AsA and reduced AsA in *Arabidopsis thaliana* leaves after exposure to 10 and 100 µM U. Márquez-García *et al.* (2012) observed an increase in total AsA and reduced AsA after exposure of *E. andevalensis* to different Cd concentrations. Another important antioxidant is GSH. It protects thiol groups on enzymes, is an important molecule in the regeneration of AsA and reacts directly with singlet oxygen and $OH^{\cdot}$ (Arora *et al.*, 2002). After U exposure, a decreasing trend in

total GSH and reduced GSH concentrations was found, with a significant decrease after exposure to 100 µM U. This decrease can possibly be related to the decreasing trend in *GSH1* and the small decrease in *GSH2* expression after U exposure. *GSH1* and *GSH2* code for γ-glutamylcysteine synthetase and GSH synthetase respectively, two enzymes essential in GSH biosynthesis (Noctor *et al.*, 2011; Jozefczak *et al.*, 2012). A decrease in GSH concentration after heavy metal exposure was noticed before for *E. andevalensis* after Cd exposure (Márquez-García *et al.*, 2012). However, Vanhoudt *et al.* (2011) found a significant increase in total GSH and reduced GSH in *Arabidopsis thaliana* leaves after exposure to 10 µM U during 3 days at pH 5.5. This increase was only transient since no significant difference in total GSH was observed after exposure to 100 µM. Besides its antioxidative properties, GSH is a precursor of phytochelatins, which are crucial in controlling free cellular heavy metal concentrations (Noctor and Foyer, 1998). In contrast to other heavy metals, induction of phytochelatins has not been reported under U stress before. Here, based on gene expression results, we also do not find evidence for active phytochelatin biosynthesis since no increase in *PCS1* transcript levels was observed after U exposure. Although this could indicate that phytochelatins are probably not synthesised after U exposure at pH 4.5, more research is needed to investigate their role under U stress.

Finally, it seems that U induced early senescence in *Arabidopsis thaliana* leaves exposed to U during 3 days at pH 4.5. As such, a significant decrease in *CAT2* expression was found after exposure to 25 µM U or higher. This was in agreement with the results of Vanhoudt *et al.* (2011) where the *CAT2* expression significantly decreased after 3 days exposure of *Arabidopsis thaliana* leaves to 10 or 100 µM U at pH 5.5. Zimmerman *et al.* (2006) demonstrated that *CAT2* down-regulation is an initial step in producing elevated H_2O_2 levels, which would lead to the induction of *CAT3* expression. An increased *CAT3* expression was indeed observed after U exposure as it was also found before under Cd stress (Cuypers *et al.*, 2011). This could be a regulatory mechanism during senescence. The decreases in *CAT1* and *CAT2* expression are accompanied by a small decrease in total CAT enzyme activity (not significant). A decreased CAT activity in early senescence has also been reported by Dhindsa *et al.* (1981) and by Kukavica and Jovanovic (2004). The enhanced H_2O_2 level can play a role in

lipid peroxidation. As such, a significant and concentration-dependent increase in lipid peroxidation was observed after exposure to 25 µM U or higher. Increased levels of TBA-rc indicating lipid peroxidation have been described before during senescence (Dhindsa *et al.*, 1981; Prochazkova *et al.*, 2001; Zimmermann *et al.*, 2005). The increased lipid peroxidation can affect membrane integrity and functionality, which can cause leakage of nutrients from the cell. An increased potassium leakage possibly due to enhanced membrane damage after U exposure was also suggested by Vanhoudt *et al.* (2011). In addition to a decreased *CAT* expression and increased lipid peroxidation, Jiménez *et al.* (1998) found a significant decrease in the GR activity at the 11th day of senescence in *Pisum sativum*. Although we did not observe a decrease in GR activity, the expression levels of *GR1* and *GR2* decreased (not significant), indicating that U exposure at low pH can induce early senescence in *Arabidopsis thaliana* leaves.

In conclusion, U induces adverse effects in leaves of *Arabidopsis thaliana* plants after 3 days exposure at pH 4.5, although the U concentrations in the leaves were low. In accordance with the roots, miRNA398b/c seems to be involved in the regulation of the SOD response in the leaves, although the decreased *CSD* expression was not compensated by an up-regulation of *FSD1* as was found in the roots. This can possibly be explained by a decreased iron content in the leaves, which was observed before in *Arabidopsis thaliana* plants exposed to U. However, nutrient profiles should be determined to confirm this hypothesis. Finally, it seems that U can induce early senescence in *Arabidopsis thaliana* leaves since different characteristics of early senescence were observed in the leaves of *Arabidopsis thaliana* plants exposed during 3 days to U at pH 4.5.

Dose-dependent effects induced in *Arabidopsis thaliana* after uranium exposure at pH 7.5

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Abstract

To evaluate the environmental impact of uranium (U) contamination, it is important to unravel the mechanisms by which plants respond to U stress. Since U speciation strongly depends on the environmental pH and since U uptake and hence its toxicity is influenced by U speciation, it is important to investigate the effects of U at different ecologically relevant pH levels. The present study aimed to investigate dose-dependent effects of U at pH 7.5. Therefore, *Arabidopsis thaliana* plants were exposed during 3 days to a U concentration range at pH 7.5. Results indicate that U causes stress in *Arabidopsis thaliana* plants leading to a dose-dependent decrease in leaf and root fresh weight and an increased dry weight. In addition, in the roots a significant decrease in reduced ascorbate (AsA) and an increase in dehydroascorbate was found, indicating that the roots are stressed after exposure to U. The increased capacities of ascorbate peroxidase and glutathione reductase in the roots after U exposure possibly indicate an important role for the AsA-glutathione (GSH) cycle in the scavenging of hydrogen peroxide during U-induced stress. The leaves were able to counteract the oxidative stress by upregulating the AsA and GSH biosynthesis. In accordance with the roots, this can indicate that the AsA-GSH cycle plays an important role in the antioxidative defence systems in *Arabidopsis thaliana* leaves exposed to U at pH 7.5. In addition, small inductions of enzymes of the antioxidative defence system were observed at lower U concentrations. However, at higher U concentrations a reduction in enzyme activities and gene expression levels was observed. As such, it seemed that the antioxidative defence system of the leaves collapses at higher U concentrations.

7.1. Introduction

Uranium (U) is a naturally occurring radionuclide and heavy metal, with an average concentration of 3 mg kg^{-1} in the earth's crust (Bleise *et al.*, 2003). However, large areas have been contaminated with U due to activities such as U mining and milling, metal mining and smelting and the phosphate industry (Vandenhove, 2002). Uranium causes both a radiation dose and chemical toxicity. However, the chemical toxicity will be of greater concern than its radiotoxicity due to the large physical half-life of 4.47 x 10^9 years, giving U-238 a low specific activity of 1.25 x 10^4 Bq g^{-1} U (Sheppard *et al.*, 2005).

The mobility and bioavailability of U is dependent on the physicochemical form of U. Uranium can be present in a wide variety of chemical species including uranyl cation (UO_2^{2+}), uranyl hydroxides (e.g. UO_2OH^+,$(UO_2)_3(OH)_5^+$), uranyl phosphate (e.g. UO_2HPO_4, $UO_2(HPO_4)_2^{2-}$) and uranyl carbonates (e.g. UO_2CO_3, $(UO_2)_2CO_3(OH)_3^-$) (Ebbs *et al.*, 1998). Important factors controlling the speciation are for example pH value, redox potential, ionic strength and availability of inorganic and organic ligands (Bernhard, 2005). In chapter 4, the speciation of U in the Hoagland solution (nutrient solution of *Arabidopsis thaliana* plants) was calculated. At pH 4.5, UO_2^{2+} was mainly present. At pH 5.5, U phosphate (UO_2HPO_4) and $(UO_2)_3(OH)_5^+$ were present, while at pH 6.5 and 7.5 $(UO_2)_2CO_3(OH)_3^-$ was the dominant species. The difference in speciation resulted in remarkable differences in U uptake and translocation in the plant, with high uptake and low translocation at pH 4.5 and lower uptake but higher translocation at pH 7.5. U accumulation and distribution in plants has been reported by several authors (Ebbs *et al.*, 1998; Laroche, 2005; Straczek *et al.*, 2010; Laurette *et al.*, 2012). However, little information on U toxicity at the cellular level is available. Toxicity of U would be predominantly caused by UO_2^{2+} (Ribera *et al.*, 1996; Vandenhove *et al.*, 2006). The UO_2^{2+} can bind to phosphate groups of the cell membranes, leading to structural changes in the cell membranes. In addition, it will compete with Ca^{2+} and Mg^{2+} for binding sites, e.g. as cofactor in different enzymes, which can lead to enzyme inactivation and damage to RNA and DNA. However, also other U species including UO_2OH^+ and carbonated complexes can contribute to U toxicity (Zeman *et al.*, 2008).

It was already demonstrated that U and other heavy metals can induce oxidative stress related responses in plants (Smeets *et al.*, 2008; Vanhoudt *et al.*, 2008; Cuypers *et al.*, 2011; Vanhoudt *et al.*, 2011; chapter 4; chapter 5; chapter 6). During oxidative stress, an imbalance between the rate of reactive oxygen species (ROS) production and their degradation will occur (Sorg, 2004). There are different sources of ROS, some of which are involved in normal cell metabolism, such as photosynthesis and respiration (Mittler, 2002). Under stress conditions, ROS production may be enhanced, possibly leading to an abnormal metabolism, loss of physiological functions and death (Sorg, 2004). However, the ROS produced can also act as signals for the activation of stress responses and defence pathways (Mittler, 2002). An important source of ROS under stress are NADPH oxidases (respiratory burst oxidase homologs, RBOH). They transfer electrons from cytoplasmatic NADPH to molecular oxygen (O_2) to form superoxide ($O_2^{\bullet -}$) (Mittler, 2002; Sagi *et al.*, 2006). The peroxidation of membrane lipids by lipoxygenases (LOX) is another possible source of ROS production in plants (Karuppanapandian *et al.*, 2011). To regulate the intracellular concentration of ROS, plant cells evolved an antioxidative defence system consisting of enzymes such as superoxide dismutase (SOD), peroxidases (Px) and catalase (CAT), and antioxidants such as ascorbate (AsA) and glutathione (GSH) (Mittler *et al.*, 2004). SOD removes $O_2^{\bullet -}$ by catalysing its dismutation to hydrogen peroxide (H_2O_2) and O_2. The intracellular level of H_2O_2 is regulated by CAT and Px (Halliwell, 2006). Furthermore, the AsA-GSH pathway plays an important role in the antioxidative mechanism in which metabolites and enzymes act together to detoxify H_2O_2 (Apel and Hirt, 2004; Foyer and Noctor, 2005).

It has already been demonstrated that U can induce oxidative stress related responses in *Arabidopsis thaliana* (Vanhoudt *et al.*, 2008; Vanhoudt *et al.*, 2011; Vanhoudt *et al.*, 2011b). However, these studies have been carried out at pH 5.5, the optimal pH for hydroponically grown plants. Since the environmental pH can vary naturally (Lovley and Phillips, 1992; Nagao *et al.*, 2002), and since U speciation and as such its uptake and toxicity are strongly dependent on the pH, it is important to investigate the effects of U under different ecologically relevant conditions. In chapter 4, we already demonstrated that U responses are strongly dependent on the pH. However, since only one U concentration (25 µM U) over a

broad pH range was used in that study, a more profound investigation is needed to further unravel the oxidative stress responses after U exposure. In the present study, we aimed to set up a dose-response curve of U at pH 7.5. In addition, we focus on the U-induced oxidative stress responses in *Arabidopsis thaliana* plants grown in an environment at pH 7.5. For this purpose, 18-day-old *Arabidopsis thaliana* plants were exposed to different U concentrations ranging from 0 to 100 µM U at pH 7.5 during 3 days.

7.2. Results

7.2.1. Uranium uptake

Exposure of *Arabidopsis thaliana* plants to U during 3 days resulted in a concentration-dependent increase of the U concentration in the roots and shoots (Figure 7.1). However, the U concentration in the roots is about 300 times higher than in the shoots, indicating a small root-to-shoot transfer of U.

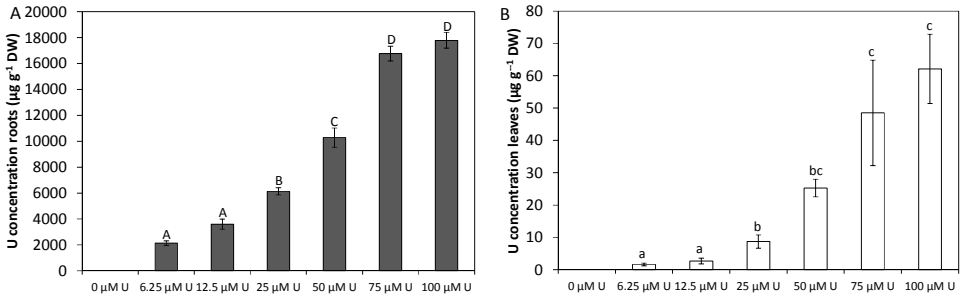


Figure 7.1: Uranium concentration (µg g^{-1} DW) in *Arabidopsis thaliana* roots (A) and shoots (B) exposed to different U concentrations during 3 days at pH 7.5. Statistical analyses were done separately for leaves and roots. Each point represents the mean ± S.E. of at least 4 biological replicates. Data points with different letters are significantly different (p<0.05).

7.2.2. Growth responses

After U exposure, a significant decrease in root and shoot fresh weight was observed (Figure 7.2). This decrease was already significant after exposure to the lowest U concentration tested (6.25 µM).

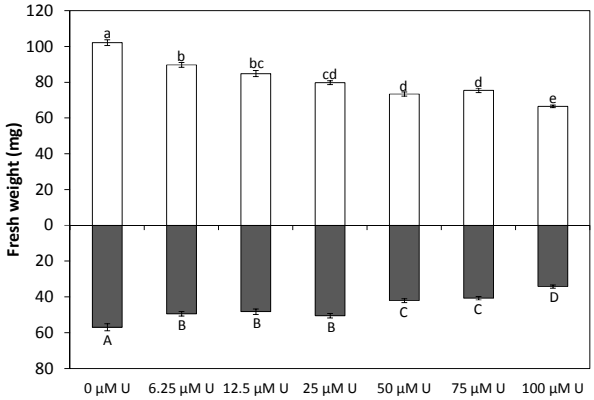


Figure 7.2: Fresh weight (mg per plant) of *Arabidopsis thaliana* roots (grey bars) and shoots (white bars) of *Arabidopsis thaliana* plants exposed to different U concentrations during 3 days at pH 7.5. Statistical analyses were done separately for leaves and roots. Values represent the mean ± S.E. of at least 100 biological replicates. Data points with different letters are significantly different (p<0.05).

For the growth reduction in roots and shoots (growth of control root and shoot = 100%), a dose response curve was modelled using the four-parameter Weibull function (Seber *et al.*, 1989; Ritz, 2010) in the statistical software package R (version 2.15.0) (R Foundation for Statistical Computing, Vienna, Austria) since this model seems to be the best-fit model for our data. The curve fitting enabled to calculate effective concentration (EC) levels together with a corresponding standard error. The ECx is the concentration that causes x per cent effect. For roots exposed during 3 days to U, the EC10, EC30 and EC50 values for root growth reduction were 2.84 ± 1.80, 22.67± 6.43 and 70.24 ± 10.48 µM U, respectively. For leaf growth reduction 1.08 ± 0.30, 13.55 ± 1.43 and 53.74 ± 3.51 µM U were calculated to be the EC10, EC30 and EC50, respectively.

The percentage dry weight of the roots (expressed as % of fresh weight) significantly increased after exposure to 25 µM U or higher. Also in the shoots, an increasing trend in the percentage dry weight was observed, with a significant increase after exposure to 100 µM U (Table 7.1).

Table 7.1: Percentage dry weight (expressed as % fresh weight) of *Arabidopsis thaliana* roots and leaves exposed to different U concentrations during 3 days at pH 7.5. Statistical analyses were done separately for leaves and roots. Values represent the mean ± S.E. of at least 4 biological replicates. Data points with different letters are significantly different (p<0.05).

	ROOTS	**LEAVES**
0 µM U	5.20 ± 0.07^{A}	10.32 ± 0.44^{a}
6.25 µM U	5.73 ± 0.28^{AB}	10.50 ± 0.51^{a}
12.5 µM U	5.43 ± 0.55^{A}	10.58 ± 0.11^{a}
25 µM U	8.42 ± 0.70^{CD}	11.18 ± 0.73^{ab}
50 µM U	7.43 ± 0.24^{BC}	11.31 ± 0.22^{ab}
75 µM U	9.26 ± 0.50^{CD}	12.04 ± 0.34^{ab}
100 µM U	9.51 ± 0.33^{D}	13.02 ± 0.53^{b}

7.2.3. Lipid peroxidation

The amount of thiobarbituric acid reactive compounds (TBA-rc) was determined as a measure of lipid peroxidation in leaves of U-exposed *Arabidopsis thaliana* plants. A significant increase in lipid peroxidation was observed after exposure to 100 µM U as compared to the control leaves (Figure 7.3).

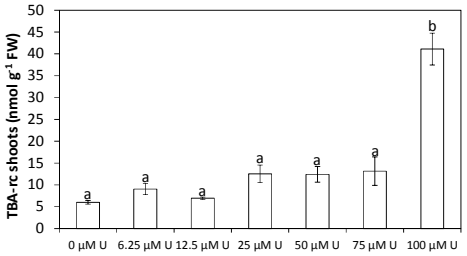


Figure 7.3: Level of lipid peroxidation, based on the amount of thiobarbituric acid reactive compounds (TBA-rc) in *Arabidopsis thaliana* leaves exposed to different U concentrations during 3 days at pH 7.5. Values represent the mean ± S.E. of at least 4 biological replicates. Data points with different letters are significantly different (p<0.05).

7.2.4. Antioxidative metabolites

To analyse the importance of the AsA-GSH cycle under U stress, the concentrations of both metabolites were determined spectrophotometrically. In the roots, U exposure did not affect total AsA concentrations (Table 7.2). However, a significant decrease in reduced AsA was observed after exposure to

Table 7.2: Ascorbate and glutathione concentrations (nmol g^{-1} FW) in leaves and roots of *Arabidopsis thaliana* plants exposed to different U concentrations at pH 7.5 during 3 days. Each point represents the mean of at least 4 biological replicates ± S.E. Statistical analysis were done separately for leaves and roots. Different capital letters indicate significant differences in the roots (p<0.05). Different small letters indicate significant differences in the leaves (p<0.05). AsA = reduced ascorbate, DHA = dehydroascorbate, Total AsA = AsA + DHA, % red AsA = reduced AsA/total AsA, GSH = reduced glutathione, GSSG = oxidized glutathione, Total GSH = GSH + GSSG, % red GSH = reduced GSH/total GSH.

		0 µM U	**6.25 µM U**	**12.5 µM U**	**25 µM U**	**50 µM U**	**75 µM U**	**100 µM U**
ROOTS	Total AsA	587 ± 67^{A}	657 ± 87^{A}	803 ± 51^{A}	603 ± 25^{A}	782 ± 33^{A}	688 ± 23^{A}	680 ± 78^{A}
	AsA	512 ± 48^{AB}	575 ± 92^{A}	678 ± 55^{A}	477 ± 48^{AB}	530 ± 45^{A}	230 ± 31^{BC}	242 ± 38^{C}
	DHA	75 ± 26^{A}	82 ± 20^{A}	126 ± 17^{A}	125 ± 43^{A}	252 ± 44^{AB}	368 ± 17^{B}	439 ± 85^{B}
	% red AsA	97 ± 6^{A}	99 ± 7^{A}	84 ± 2^{A}	86 ± 9^{A}	68 ± 5^{AB}	47 ± 3^{BC}	38 ± 8^{C}
	Total GSH	120 ± 8^{AB}	115 ± 8^{AB}	137 ± 6^{A}	110 ± 3^{B}	139 ± 5^{A}	120 ± 4^{AB}	121 ± 4^{AB}
	GSH	117 ± 7^{AB}	113 ± 8^{AB}	133 ± 6^{A}	107 ± 3^{B}	133 ± 5^{A}	115 ± 4^{AB}	115 ± 4^{AB}
	GSSG	1.3 ± 0.4^{A}	1.3 ± 0.4^{A}	2.1 ± 0.2^{AB}	1.3 ± 0.4^{A}	2.9 ± 0.3^{B}	2.4 ± 0.2^{AB}	3.0 ± 0.3^{B}
	% red GSH	98 ± 1^{A}	98 ± 1^{A}	97 ± 0.7^{AB}	98 ± 0.7^{A}	96 ± 0.3^{AB}	96 ± 0.4^{AB}	95 ± 0.4^{B}
LEAVES	Total AsA	2917 ± 271^{a}	3752 ± 272^{ab}	5041 ± 311^{b}	4246 ± 414^{ab}	5029 ± 265^{b}	4714 ± 180^{b}	4940 ± 363^{b}
	AsA	2676 ± 277^{a}	3476 ± 261^{ab}	4498 ± 286^{b}	4008 ± 461^{ab}	4858 ± 236^{b}	4570 ± 189^{b}	4962 ± 346^{b}
	DHA	241 ± 35^{a}	276 ± 55^{a}	543 ± 153^{a}	238 ± 93^{a}	170 ± 31^{a}	145 ± 16^{a}	248 ± 34^{a}
	% red AsA	92 ± 1^{a}	93 ± 1^{ab}	89 ± 3^{a}	94 ± 3^{a}	97 ± 1^{bc}	97 ± 1^{c}	95 ± 1^{ac}
	Total GSH	235 ± 13^{a}	279 ± 18^{ac}	324 ± 41^{bc}	322 ± 29^{bc}	333 ± 20^{c}	256 ± 15^{ab}	329 ± 60^{ac}
	GSH	219 ± 14^{a}	257 ± 17^{ac}	304 ± 38^{c}	301 ± 28^{bc}	305 ± 22^{c}	234 ± 12^{ab}	297 ± 56^{ac}
	GSSG	8.0 ± 1.4^{a}	11 ± 2.9^{a}	9.8 ± 1.9^{a}	11 ± 0.9^{a}	14 ± 1.5^{a}	11 ± 2.1^{a}	16 ± 2.1^{a}
	% red GSH	93 ± 1^{a}	92 ± 2^{a}	94 ± 1^{a}	93 ± 1^{a}	91 ± 1^{a}	92 ± 1^{a}	90 ± 1^{a}

75 and 100 µM U as compared to the control roots, which was accompanied by a significant increase in DHA. Correspondingly, the percentage reduced AsA decreased with a significant decrease after exposure to 75 and 100 µM U. For GSH, no significant differences were observed in total and reduced GSH concentrations as compared to the control roots. However, a significant increase in GSSG was observed after exposure to 50 and 100 µM U, with a corresponding significant decrease in percentage reduced GSH at 100 µM U.

In contrast to the roots, a significant increase in the total AsA concentration was observed in the leaves after exposure to 12.5 µM U or higher (Table 7.2). This increase corresponds to a significant increase in reduced AsA with no significant changes in DHA. This led to an increasing trend in percentage reduced AsA after U exposure. For GSH, an increasing trend in total and reduced GSH was observed, with a significant increase after 12.5, 25 or 50 µM U. However, the total and reduced GSH concentrations after exposure to 75 and 100 µM U were not significantly different as compared to the control. No significant differences were observed in the GSSG concentration or in the percentage reduced GSH (Table 7.2).

7.2.5. Enzyme capacities

Capacities of several enzymes related to the antioxidative defence system were analysed at protein level to investigate their importance under U stress (Figure 7.4 and Figure 7.5). In the roots, a significant increase in APX capacity was observed after exposure to 25 µM U or higher as compared to the control plants (Figure 7.4A). Also, an increasing trend in the capacity of glutathione reductase (GR) was observed after U exposure, with a significant increase after exposure to 100 µM U (Figure 7.4B). No significant differences were observed in the capacities of CAT, guaiacol peroxidase (GPX), syringaldazine peroxidase (SPX) and SOD (Supplementary Table S7.1).

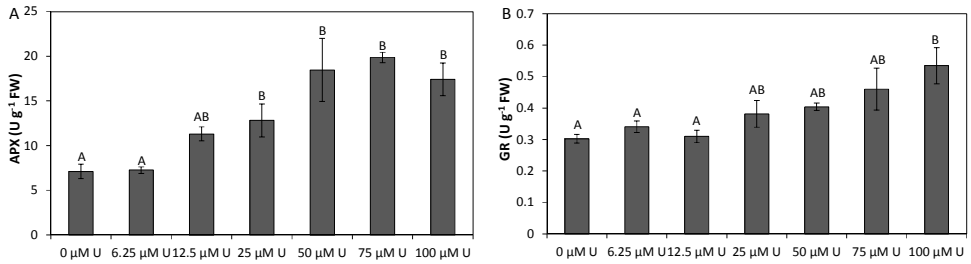


Figure 7.4: Enzyme capacities (units (U) g^{-1} FW) of ascorbate peroxidase (A, APX) and glutathione reductase (B, GR) in *Arabidopsis thaliana* roots exposed to different U concentrations during 3 days at pH 7.5. Values represent the mean ± S.E. of at least 4 biological replicates. Data points with different letters are significantly different (p<0.05).

In the leaves, no significant differences were observed in enzyme capacities after U exposure. However, for CAT, GR, GPX and SPX, an increasing trend in the enzyme capacities was observed up to 25 µM U, after which the activities declined again (Figure 7.5). This trend was not observed in the SOD and APX capacities (Supplementary Table S7.1).

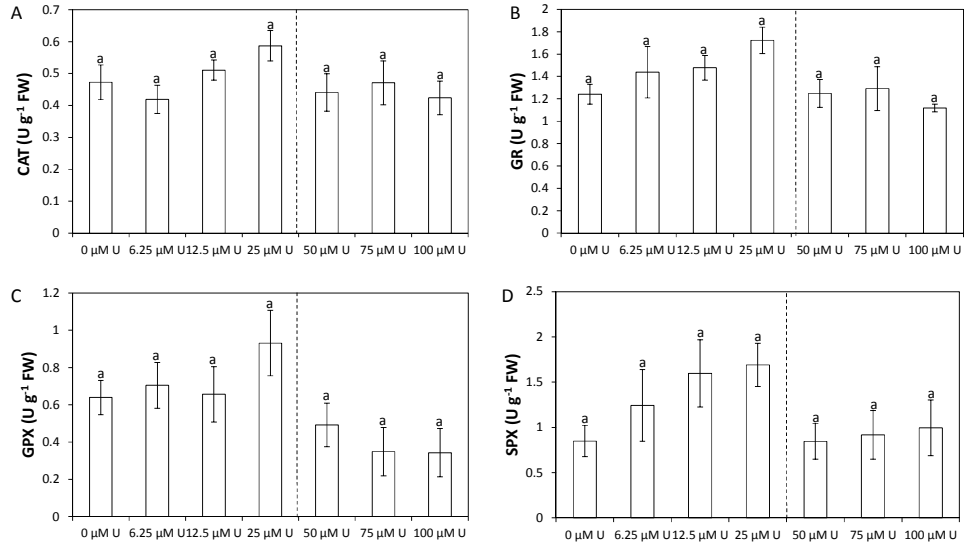


Figure 7.5: Enzyme capacities (units (U) g^{-1} FW) of catalase (A, CAT), glutathione reductase (B, GR), guaiacol peroxidase (C, GPX) and syringaldazine peroxidase (D, SPX) in *Arabidopsis thaliana* leaves exposed to different U concentrations during 3 days at pH 7.5. Values represent the mean ± S.E. of at least 4 biological replicates. The vertical line indicates the transition from the increasing trend in enzyme capacities to the decreased capacity.

7.2.6. Gene expression analysis

To evaluate the importance of oxidative stress related responses in *Arabidopsis thaliana* plants exposed to U, some important ROS-producing and -scavenging enzymes were analysed at transcriptional level using quantitative real-time PCR.

First, gene expression of ROS-producing enzymes was analysed in the roots. The plasma membrane bound NADPH oxidases (*RBOHC*/*D*/*F)* are induced in different biotic and abiotic stress conditions as an important source of $O_2^{\bullet-}$ production (Apel and Hirt, 2004). In the roots, a significant decrease in the expression levels of *RBOHC* was observed after exposure to 100 µM U as compared to the control roots (Table 7.3). Expression levels of *RBOHD*/F remained stable for all treatments. Another source of ROS production are the lipoxygenases. They catalyse the dioxygenation of polyunsaturated fatty acids, producing hydroperoxy fatty acids (Remans *et al.*, 2010). A significant increase in *LOX1* expression was observed in the roots after exposure to 75 µM U.

Concerning gene expression levels of different isoforms of SOD in the roots, the expression level of *CSD2* decreased significantly as compared to the control roots after exposure to 75 and 100 µM U (Table 7.3), while a decreasing trend for *CSD1* was observed. After exposure to 25 µM U or higher, the *FSD1* expression increased significantly. It is known that the CSD transcript levels are post-transcriptionally regulated by miRNA398b/c (Zhu *et al.*, 2011). A significant increase in the transcript levels of *MIR398b*/*c* was observed after exposure to 100 µM U.

Furthermore, gene expression of enzymes important in H_2O_2 scavenging and enzymes related to the AsA-GSH cycle were analysed. In the roots, an increased *CAT1* expression was observed after exposure to 100 µM U. No differences were observed in *APX1* expression. *GR1* and *GR2* expression decreased significantly after exposure to all U concentrations (*GR1*) and 100 µM U (*GR2*). Finally, enzymes involved in GSH and phytochelatin production were analysed at transcriptional level. However, no clear pattern was observed for the *GSH1*, *GSH2* and *PCS1* expression (Table 7.3).

Table 7.3: Relative gene expression levels in *Arabidopsis thaliana* roots of the genes involved in ROS production and scavenging after exposure to different U concentrations at pH 7.5. Gene expression is expressed relative as compared to the control, which was set to 1. Values represent the mean ± S.E. of at least 3 biological replicates. Significant differences compared to the control plants are indicated with $p<0.01$ $p<0.05$ for down-regulated and $p<0.01$ $p<0.05$ for up-regulated genes.

	Gene	ROOTS					
		6.25 µM U	**12.5 µM U**	**25 µM U**	**50 µM U**	**75 µM U**	**100 µM U**
Pro-oxidative marker genes	*RBOHC*	0.87 ± 0.14	0.85 ± 0.14	1.08 ± 0.13	0.81 ± 0.05	0.80 ± 0.18	0.23 ± 0.03
	RBOHD	1.62 ± 0.30	1.13 ± 0.34	1.24 ± 0.13	1.20 ± 0.13	1.39 ± 0.14	1.07 ± 0.14
	RBOHF	1.05 ± 0.08	1.24 ± 0.30	1.12 ± 0.17	1.07 ± 0.23	1.48 ± 0.22	0.98 ± 0.14
	LOX1	0.64 ± 0.20	0.62 ± 0.07	0.72 ± 0.07	0.90 ± 0.09	1.92 ± 0.17	1.53 ± 0.22
Anti-oxidative defence marker genes	*CSD1*	1.22 ± 0.11	1.22 ± 0.26	0.91 ± 0.10	0.99 ± 0.08	0.91 ± 0.19	0.61 ± 0.07
	CSD2	0.99 ± 0.14	0.85 ± 0.15	0.64 ± 0.05	0.64 ± 0.04	0.49 ± 0.07	0.36 ± 0.03
	CSD3	0.43 ± 0.06	0.62 ± 0.03	0.60 ± 0.05	0.49 ± 0.03	0.85 ± 0.14	0.49 ± 0.09
	FSD1	1.93 ± 0.02	1.57 ± 0.11	5.21 ± 1.71	5.10 ± 0.23	6.51 ± 13.03	13.03 ± 0.97
	FSD2	0.49 ± 0.09	0.56 ± 0.07	0.62 ± 0.06	0.67 ± 0.03	0.78 ± 0.11	0.61 ± 0.10
	FSD3	1.23 ± 0.12	0.95 ± 0.16	1.13 ± 0.09	1.38 ± 0.36	1.58 ± 0.37	1.62 ± 0.21
	MSD1	2.11 ± 0.14	2.07 ± 0.43	1.71 ± 0.33	1.78 ± 0.23	2.23 ± 0.50	2.40 ± 0.18
Gene expression regulating genes	*miRNA398b*	1.74 ± 0.17	1.00 ± 0.29	1.74 ± 0.25	1.59 ± 0.12	2.59 ± 0.52	3.65 ± 1.04
	miRNA398c	1.27 ± 0.22	1.10 ± 0.37	1.89 ± 0.22	1.66 ± 0.16	2.72 ± 0.55	5.29 ± 1.50
Anti-oxidative defence marker genes	*CAT1*	0.90 ± 0.12	1.02 ± 0.18	1.07 ± 0.17	1.06 ± 0.14	0.79 ± 0.24	1.90 ± 0.19
	CAT2	1.52 ± 0.18	1.01 ± 0.16	0.95 ± 0.16	1.12 ± 0.14	0.50 ± 0.10	0.62 ± 0.05
	CAT3	0.84 ± 0.19	0.73 ± 0.19	1.27 ± 0.38	0.69 ± 0.12	0.77 ± 0.09	0.91 ± 0.25
Genes involved in AsA-GSH cycle	*APX1*	0.93 ± 0.08	1.18 ± 0.18	1.20 ± 0.15	1.23 ± 0.17	1.28 ± 0.13	0.98 ± 0.08
	GR1	0.53 ± 0.06	0.42 ± 0.03	0.37 ± 0.06	0.36 ± 0.05	0.48 ± 0.07	0.30 ± 0.01
	GR2	0.76 ± 0.10	0.79 ± 0.10	0.99 ± 0.16	0.76 ± 0.13	0.78 ± 0.11	0.52 ± 0.06
Genes involved in GSH and PCs biosynthesis	*GSH1*	0.73 ± 0.05	0.55 ± 0.04	0.67 ± 0.15	0.59 ± 0.09	0.65 ± 0.08	0.52 ± 0.09
	GSH2	0.94 ± 0.05	0.73 ± 0.10	0.95 ± 0.16	0.88 ± 0.12	1.24 ± 0.04	0.88 ± 0.15
	PCS1	1.04 ± 0.18	0.83 ± 0.06	0.84 ± 0.08	1.24 ± 0.15	1.20 ± 0.08	1.23 ± 0.21

Table 7.4: Relative gene expression levels in *Arabidopsis thaliana* leaves of the genes involved in ROS production and scavenging after exposure to different U concentrations at pH 7.5. Gene expression is expressed relative as compared to the control, which was set to 1. Values represent the mean ± S.E. of at least 3 biological replicates. Significant differences compared to the control plants are indicated with $p<0.01$ $p<0.05$ for down-regulated and $p<0.01$ $p<0.05$ for up-regulated genes.

	Gene	LEAVES					
		6.25 µM U	**12.5 µM U**	**25 µM U**	**50 µM U**	**75 µM U**	**100 µM U**
Pro-oxidative marker genes	*RBOHC*	2.44 ± 1.41	2.16 ± 0.57	1.04 ± 0.18	1.11 ± 0.46	0.23 ± 0.05	0.54 ± 0.07
	RBOHD	0.79 ± 0.03	1.65 ± 0.34	0.98 ± 0.23	0.91 ± 0.34	0.92 ± 0.20	1.01 ± 0.12
	RBOHF	1.34 ± 0.17	1.32 ± 0.19	0.73 ± 0.20	0.56 ± 0.08	0.51 ± 0.13	0.41 ± 0.05
	LOX1	0.81 ± 0.16	0.92 ± 0.23	0.44 ± 0.14	0.64 ± 0.15	0.63 ± 0.10	0.55 ± 0.09
	LOX2	0.73 ± 0.15	0.90 ± 0.19	0.66 ± 0.22	1.37 ± 0.30	1.88 ± 0.35	1.82 ± 0.10
Anti-oxidative defence marker genes	*CSD1*	0.48 ± 0.12	0.64 ± 0.09	0.36 ± 0.08	0.23 ± 0.05	0.11 ± 0.01	0.05 ± 0.01
	CSD2	0.38 ± 0.10	0.43 ± 0.011	0.12 ± 0.02	0.09 ± 0.01	0.04 ± 0.00	0.01 ± 0.00
	CSD3	0.52 ± 0.07	0.70 ± 0.14	1.07 ± 0.27	0.86 ± 0.20	0.81 ± 0.11	0.34 ± 0.05
	FSD1	1.03 ± 0.20	1.43 ± 0.23	2.59 ± 0.73	1.46 ± 0.12	0.45 ± 0.08	0.28 ± 0.09
	FSD2	0.68 ± 0.12	0.83 ± 0.26	0.77 ± 0.27	0.59 ± 0.10	0.42 ± 0.04	0.18 ± 0.04
	FSD3	0.79 ± 0.04	1.05 ± 0.26	0.47 ± 0.11	0.72 ± 0.15	0.47 ± 0.05	0.37 ± 0.08
	MSD1	0.78 ± 0.08	0.91 ± 0.13	0.75 ± 0.19	0.70 ± 0.15	0.73 ± 0.18	0.46 ± 0.09
Gene expression regulating genes	*miRNA398b*	1.04 ± 0.19	1.49 ± 0.22	0.92 ± 0.05	1.65 ± 0.39	2.38 ± 0.40	2.72 ± 0.47
	miRNA398c	1.04 ± 0.18	1.46 ± 0.21	0.74 ± 0.09	1.80 ± 0.36	2.84 ± 0.44	2.79 ± 0.53
Anti-oxidative defence marker genes	*CAT1*	0.85 ± 0.13	0.84 ± 0.26	1.33 ± 0.51	0.65 ± 0.47	0.68 ± 0.24	0.50 ± 0.16
	CAT2	0.91 ± 0.18	0.42 ± 0.14	1.03 ± 0.38	0.58 ± 0.08	0.34 ± 0.08	0.16 ± 0.05
	CAT3	0.67 ± 0.10	1.25 ± 0.24	2.17 ± 0.50	1.98 ± 0.53	1.07 ± 0.08	0.78 ± 0.27
Genes involved in AsA-GSH cycle	*APX1*	0.98 ± 0.04	1.06 ± 0.08	0.77 ± 0.21	0.59 ± 0.13	0.61 ± 0.12	0.41 ± 0.02
	GR1	0.92 ± 0.05	0.67 ± 0.03	0.54 ± 0.09	0.48 ± 0.07	0.49 ± 0.02	0.46 ± 0.03
	GR2	0.75 ± 0.09	0.76 ± 0.08	0.58 ± 0.08	0.53 ± 0.11	0.43 ± 0.08	0.29 ± 0.03
Genes involved in GSH and PCs biosynthesis	*GSH1*	0.89 ± 0.06	1.04 ± 0.09	1.17 ± 0.19	0.88 ± 0.12	0.57 ± 0.07	0.46 ± 0.03
	GSH2	1.23 ± 0.09	1.33 ± 0.12	1.29 ± 0.13	1.53 ± 0.17	1.65 ± 0.24	1.80 ± 0.25
	PCS1	1.52 ± 0.26	1.73 ± 0.30	1.28 ± 0.29	0.89 ± 0.19	1.17 ± 0.24	1.08 ± 0.10

In the leaves, no significant differences were observed in the ROS-producing enzymes (*RBOHC*/*D*/*F* and *LOX1*/*2*) as compared to the control (Table 7.4). Concerning the ROS-scavenging mechanisms, a significant decrease in *CSD1*/2 transcript levels was observed after exposure to 25 µM U or higher. As mentioned before, the transcript levels of CSDs are down-regulated by microRNA398b/c. As such, a significant increase in *MIR398b*/c expression was observed after exposure to 75 and 100 µM U. A transient increase in *FSD1* expression was observed up to 25 µM U, after which the expression decreased again, with a significant decrease after exposure to 100 µM U. A significant decrease was also observed in *FSD2* expression after exposure to 100 µM U.

In the expression of H_2O_2 scavenging enzymes, a significant decrease in the transcript levels of *CAT2* and *APX1* was observed after exposure to 100 µM U. The expression of *CAT3* showed a transient increase up to 50 µM U after which the expression declined again. The *GR1* and *GR2* expression in the leaves were significantly decreased after exposure to 12.5 µM U or higher. Finally, a significant decrease in the expression of *GSH1* was observed after exposure to 75 and 100 µM U, while an increasing trend for the *GSH2* expression was observed, with a significant increase after exposure to 100 µM U. No differences were observed in *PCS1* expression.

7.3. Discussion

The influence of U on the oxidative defence system of plants has mostly been investigated at a pH range of 5.0 – 5.7 (Vanhoudt *et al.*, 2008; Misson *et al.*, 2009; Horemans *et al.*, 2011; Vanhoudt *et al.*, 2011b) as this is the optimal pH range for hydroponic growth of plants. However, since chemical speciation of U, and hence its toxicity, is strongly dependent on the pH, it is important to investigate the environmental impact of U contamination under different ecologically relevant pH values. While the dose-dependent effects at pH 4.5 were described in chapter 5 and 6, the present study aimed to set up a dose-response curve of U at pH 7.5. In addition, the oxidative stress responses after U exposure at pH 7.5 were investigated. Therefore, 18-day-old *Arabidopsis thaliana* plants were exposed to different U concentrations ranging from 0 to 100 µM U during 3 days at pH 7.5.

Roots are in direct contact with the nutrient solution, which resulted in a dose-dependent increase in the U concentration of the roots. This increase was accompanied by a significant reduction in root fresh weight, which was already observed after exposure to the lowest U concentration applied (6.25 µM) (Figure 7.2). In addition, an increased percentage dry weight was observed after exposure to 25 µM U or higher, indicating a disturbed water balance as was observed before by Vanhoudt *et al.* (2011a) for roots exposed to U at pH 5.5. For root fresh weight reduction, the EC10, EC30 and EC50 were calculated using the four-parameter Weibull function (Seber and Wild, 1989; Ritz, 2010). For U-exposed roots at pH 7.5, the EC50 (70.24 ± 10.48 µM U) was remarkably higher than the value observed for root fresh weight reduction at pH 4.5 (28.14 ± 1.59 µM U) in chapter 5. This can possibly be related to the fact that at acidic pH, U is more easily taken up by the roots which will lead to a higher U content in the roots and as such to a faster decrease in root growth (chapter 5). In addition, the differences in speciation can possibly contribute to the discrepancy in EC50 values. At pH 4.5, there was mainly the presence of UO_2^{2+}, while at pH 7.5 the carbonate species were mainly present. Since UO_2^{2+} is suggested to be the most toxic U species, this can possibly explain the fact that more toxic effects were observed at low pH.

In the leaves, a dose-dependent increase of U was also observed. However, the U concentration in the leaves is about 300 times lower than in the roots, indicating a low root-to-shoot transfer factor ranging between 3.5×10^{-3} and 7.7×10^{-4}, depending on the U concentrations added. A small root-to-shoot transfer of U has been reported before (Vandenhove *et al.*, 2006; Vanhoudt *et al.*, 2011a; chapter 4). The increased U content in the leaves led to a significant decrease in leaf fresh weight (Figure 7.2). Based on the leaf fresh weight reduction, the EC10, EC30 and EC50 were calculated using the four-parameter Weibull function (Seber and Wild, 1989; Ritz, 2010). The leaf EC50 (53.74 ± 3.51 µM U) is comparable to the EC50 observed before by Horemans *et al.* (2011) for leaf fresh weight reduction after U exposure at pH 5.5. They observed an EC50 value of 66 µM U. However, the EC50 for reduction in leaf fresh weight observed before after U exposure at pH 4.5 was remarkably lower (27.13 ± 5.20 µM U) (chapter 6). Since the U translocation at pH 7.5 is higher than at pH 4.5, resulting in a higher leaf U content at pH 7.5 following exposure to the same U

concentration, this possibly indicates that U is more toxic in the leaves after exposure to U at acidic pH. However, the large differences in U uptake by the roots, with high uptake at pH 4.5 and lower uptake at pH 7.5, can possibly also contribute to the different EC50 value due to root-to-shoot signalling.

After exposure to 100 µM U, a significant increase in leaf percentage dry weight was observed, indicating that U causes a disturbed water balance and leaves start to wilt at higher U concentrations. This was in agreement with previous studies. As such, we reported in chapter 6 an increased dry weight in *Arabidopsis thaliana* leaves after exposure to 25 µM U or higher at pH 4.5. In addition, Vanhoudt *et al.* (2011a) also reported a significant increase in leaf dry weight after exposure of *Arabidopsis thaliana* plants to U at pH 5.5, indicating those plants may be water stressed.

Generally, under stress conditions the generation of toxic ROS species is increased (Arora *et al.*, 2002). Since U is a redox-active metal it can directly induce ROS formation non-enzymatically through Fenton and Haber-Weiss reactions (Halliwell, 2006; Viehweger *et al.*, 2011). In addition, the membrane bound NADPH oxidases are an important source of ROS. They catalyse the formation of $O_2^{\bullet -}$ (Apel and Hirt, 2004). Gene expression of some important NADPH oxidases was analysed. However, no induction of *RBOHC/D/F* was observed in the roots. This indicates that the NADPH-mediated oxidative burst is probably not involved in the ROS production in the roots after U exposure at pH 7.5. These results are partially in agreement with the results of Vanhoudt *et al.* (2011b). As such, they observed a significant induction of the *RBOHD* expression after exposure to 100 µM U at pH 5.5, but no increased expression of *RBOHC* and *RBOHF*. In addition, the NADPH-mediated oxidative burst was also not involved in the roots of *Arabidopsis thaliana* plants exposed to U at pH 4.5 (chapter 5). Another source of ROS are LOX. These enzymes catalyse the dioxygenation of polyunsaturated fatty acids, producing hydroperoxy fatty acids (Porta and Rocha-Sosa, 2002). A transient dose-dependent induction of *LOX1* was observed in roots after U exposure, with a significant induction after exposure to 75 µM U (Table 7.3). This indicates that LOX1 can be an important source of ROS in the roots after U exposure at pH 7.5. In contrast, no induction of *LOX1* was observed when plants were exposed to different concentrations of

U at pH 4.5 (chapter 5). Since LOX are involved in the initiation of the synthesis of oxylipins, which serve as inter- and intracellular signalling compounds involved in abiotic and biotic stress responses (Mithöfer *et al.*, 2004), the increased *LOX1* expression observed in the present study could also induce an enhanced production of precursors for signalling molecules (Porta and Rocha-Sosa, 2002). However, this was not a specific response for plants exposed to U at pH 7.5 since an increased expression of LOX was suggested before by Vanhoudt *et al.* (2011) in roots exposed to U at pH 5.5. In the leaves of *Arabidopsis thaliana* plants exposed to U at pH 7.5, it seems that the NADPH oxidases and LOX are not involved in the U-induced stress responses since no significant differences were observed in their gene expression levels.

To counteract the toxicity of the U-induced oxidative stress, plants possess an antioxidative defence system composed of ROS-scavenging enzymes (e.g. SOD, CAT, APX) and antioxidative metabolites (AsA and GSH) (Dat *et al.*, 2000). Concerning ROS-scavenging enzymes, SOD constitutes the first line of defence by dismutating $O_2^{\bullet-}$ to H_2O_2. SODs are present at different subcellular locations. Depending on the metal co-factor that is used, different isoforms of SOD can be distinguished (Alscher *et al.*, 2001). At enzymatic level, no induction of the SOD capacity in the roots was observed. However, at transcriptional level, a shift in the expression of the different SOD isoforms was noticed. As such, a significant reduction in *CSD2* expression was observed after exposure to 75 and 100 µM U. Since the miRNA398b/c targets *CSD1* and *CSD*2 (Sunkar *et al.*, 2006), the decreased expression of *CSD2* can be related to the induction of *MIR398b*/c after exposure to 75 and 100 µM U, as was shown before under U stress at low pH (chapter 5). Since the CSDs are indispensable, the loss of CSD2 can be compensated by an increased *FSD1* expression, which is observed after exposure to 25 µM U or higher. Yamasaki *et al.* (2007) proposed that miRNA398 is involved in the regulation of Cu homeostasis. Under conditions where Cu is limited, the expression of the Cu-requiring CSDs will be downregulated. Although the Cu content in the roots of our plants was not decreased (results not shown), Vanhoudt *et al.* (2011a) showed before that the Cu content significantly decreased in leaves exposed to 100 µM U at pH 5.5. We also observed a decreasing trend in the Cu content in the leaves (results not shown). As such, by decreasing the expression of the CSDs in the roots, more free Cu

will be available in the roots which can be transported to the plastocyanins, which is essential in photosynthesis in higher plants. Thus Cu will be saved for the most essential functions during limited Cu availability (Abdel-Ghany and Pilon, 2008).

The significant increase in APX and GR capacities in the roots observed after U exposure possibly indicates an important role for the AsA-GSH cycle in the scavenging of H_2O_2 under U stress. APX has a high affinity for H_2O_2 and is able to tightly control the H_2O_2 concentrations, rendering it the ideal candidate to control the H_2O_2 levels for signalling (Cuypers *et al.*, 2011). The significant induction of APX after exposure to 25 µM U or higher indicates an increased detoxification of H_2O_2. Since APX needs AsA as a reductant and since no increased biosynthesis of AsA was found after U exposure (Table 7.2), the increased APX activity was accompanied by a significant reduction in reduced AsA and a significant increase in DHA. GSH is used as reducing substrate to reduce DHA to AsA by DHA reductase. During this reaction, GSH will be oxidized to GSSG, which in turn will be re-reduced to GSH. This reaction is catalysed by GR (Noctor and Foyer, 1998). The increased GR capacity observed after U exposure indicates that the roots try to keep GSH in its reduced state to ensure DHA reduction to AsA. However, the increased GR activity is not sufficient to maintain AsA in its reduced state, since only 38% reduced AsA was present after exposure to 100 µM U. Since DHA accumulation is considered as a negative event for cell metabolism (Drazkiewicz *et al.*, 2003), this indicates that the roots exposed to 50 µM U or higher at pH 7.5 are seriously stressed.

Concerning ROS-scavenging in the leaves, the AsA-GSH pathway possibly plays an important role. In contrast to the roots, a significant increase in the total AsA and GSH concentration after U exposure was observed (Table 7.2), which possibly indicates an enhanced detoxification of H_2O_2. An increase in the antioxidative metabolites after heavy metal exposure has been observed before in different plant species. Vanhoudt *et al.* (2011) observed a significant increase in total and reduced AsA and in total and reduced GSH in *Arabidopsis thaliana* leaves after exposure to 10 µM U during 3 days at pH 5.5. However, in the present study, the increase in total GSH was only transient, with a significant increase after exposure to 12.5-50 µM U but no significant differences after

exposure to 75 and 100 µM U. This can be related to the decreased GSH biosynthesis as suggested by a decreased expression of *GSH1* that was observed at the two highest U concentrations (Table 7.4), since this is the rate-limiting step in GSH production. In addition, transient increases in several enzyme capacities and gene expression levels are observed. As such, a small (not significant) induction was observed in the CAT, GR, GPX and SPX capacity up to 25 µM U (Figure 7.5). At higher U concentrations, the enzyme activities declined again. Also at gene expression level, the *CAT1*, *CAT3* and *FSD1* expressions are slightly upregulated, up to 50 µM U after which their expression declined (Table 7.4). This biphasic response can possibly indicate that at lower U concentrations, leaves are able to defend themselves against U-induced oxidative stress by upregulating the antioxidative defence mechanisms (i.e. increasing trend in enzyme activities and increased biosynthesis of AsA and GSH), while at higher U concentrations the leaves can no longer cope with the U-induced stress and the defence mechanisms collapse.

Similar to the roots, miRNA398b/c is involved in the regulation of the *CSD1*/2 expression in the leaves. A significant increase in *MIR398b/c* expression was observed after exposure to 75 and 100 µM U, accompanied by a decreased *CSD1/2* expression in leaves of plants exposed to 50 µM U or higher. As such, the miRNA398b/c response seems to be a general U response, since similar results were observed in the leaves of plants exposed to U at pH 4.5 (chapter 6). In contrast to the roots, this decrease in *CSD1*/2 was not compensated by an increased *FSD1* expression at higher U concentrations (Table 7.4), as was also observed in the leaves exposed to U at pH 4.5 (chapter 6). Vanhoudt *et al.* (2011a) reported before that U exposure at pH 5.5 disturbed the nutrient uptake and distribution of several nutrients in *Arabidopsis* seedlings. As such, they reported a decreased Fe concentration in *Arabidopsis thaliana* leaves after exposure to 100 µM U during 3 days. In addition, the Fe content also decreased in our leaves after U exposure (results not shown). This decreased Fe content can explain the lack of compensation by *FSD1* at higher U concentrations, since under Fe limiting conditions the *FSD1* expression can decline. The decreased expression of both *CSD1/2* and *FSD1* can lead to a decreased capacity to scavenge $O_2^{\bullet -}$, which in turn can lead to oxidative stress.

In conclusion, U exposure at high pH resulted in a significant reduction in leaf and root fresh weight, which already was observed after exposure to the lowest U concentration used (6.25 µM). However, EC50 values for root and leaf growth reduction were higher at pH 7.5 as compared to pH 4.5, possibly indicating that U causes more toxic effects at low pH. In addition, stress at pH 7.5 was evidenced by a significant reduction in the AsA redox state in the roots after exposure to 75 and 100 µM U. However, the increased APX and GR capacities point towards an important role for the AsA-GSH cycle in the U-induced stress responses in the roots. In contrast to the roots, the leaves seemed capable to defend themselves against the oxidative stress by increasing the AsA and GSH biosynthesis and by upregulating some enzymes of the antioxidative defence system. However, at higher concentrations, the antioxidative defence system collapsed also in the leaves with a reduction in enzyme capacities and a decreased gene expression of several enzymes. In addition, the decreased expression of *CSD1/2* was not compensated by an increased *FSD1* expression at higher U concentrations. This can indicate a decreased capacity to scavenge $O_2^{\bullet -}$, which in turn can lead to oxidative stress.

Supplementary data

Supplementary Table S7.1: Enzyme capacities (units g^{-1} FW) of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX) and syringaldazine peroxidase (SPX) of *Arabidopsis thaliana* roots and SOD and ascorbate peroxidase (APX) capacities of *Arabidopsis thaliana* leaves exposed to different U concentrations during 3 days at pH 7.5. Values represent the mean ± S.E. of at least 4 biological replicates. Data points with different letters are significantly different ($p<0.05$).

		0 µM U	6.25 µM U	12.5 µM U	25 µM U	50 µM U	75 µM U	100 µM U
ROOTS	SOD	124 ± 14^{A}	130 ± 19^{A}	135 ± 25^{A}	123 ± 23^{A}	99 ± 12^{A}	154 ± 26^{A}	128 ± 21^{A}
	CAT (x10^{-3})	45 ± 8^{A}	66 ± 10^{A}	57 ± 11^{A}	81 ± 9^{A}	54 ± 8^{A}	77 ± 12^{A}	60 ± 4^{A}
	GPX	8.9 ± 0.9^{A}	12.58 ± 1.3^{A}	12.7 ± 1.6^{A}	14.7 ±1.2^{A}	13.1 ± 1.5^{A}	15.9 ± 1.7^{A}	17.0 ± 3.2^{A}
	SPX	0.57 ± 0.21^{A}	1.33 ± 0.38^{A}	0.78 ± 0.19^{A}	1.81 ± 0.36^{A}	1.07 ± 0.15^{A}	1.24 ± 0.44^{A}	1.12 ± 0.47^{A}
LEAVES	SOD	134 ± 7^{a}	159 ± 12^{a}	154 ± 25^{a}	159 ± 12^{a}	153 ± 17^{a}	171 ± 23^{a}	169 ± 12^{a}
	APX	11.4 ± 1.6^{a}	15.3 ± 2.8^{a}	16.7 ±3.1^{a}	13.8 ± 2.3^{a}	10.5 ± 1.4^{a}	16.8 ± 2.1^{a}	14.2 ± 3.2^{a}

Uranium exposure at low pH increases the photosynthetic efficiency in *Arabidopsis thaliana*

Eline Saenen, Nele Horemans, Nathalie Vanhoudt, Hildegarde Vandenhove, Geert Biermans, May Van Hees, Jean Wannijn, Jaco Vangronsveld, Ann Cuypers (2013). Uranium exposure at low pH increases the photosynthetic efficiency in *Arabidopsis thaliana*. *In preparation for submission to Plant Physiology and Biochemistry.*

Abstract

It is known that metals such as Cd, Cu and Zn strongly interfere with the functioning of the chloroplast, while this information for U is scant. Since the inhibition of photosynthesis can affect the physiological state of a plant, it is important to investigate the impact of U contamination on the photosynthetic machinery at different pH levels. Eighteen-day-old *Arabidopsis thaliana* plants were exposed to different U concentrations (0 – 100 µM U) during 3 days at pH 4.5 or pH 7.5. At pH 7.5, U did not influence photosynthesis. However, at pH 4.5, it seems that an optimization of the photosynthetic processes takes place, since more of the absorbed quanta are effectively used for photosynthesis, leading to a decreased fluorescence quenching. In addition, an increased electron transport rate was observed. Since the enhanced photosynthesis at low pH was accompanied by a decreased growth of the plants, it is conceivable that the produced energy will be used for defence reactions of the plants that are activated upon U exposure.

8.1. Introduction

Nuclear energy production encompasses a variety of activities such as mining and milling and waste management. These processes release radioactive substances such as uranium (U) into the environment which will lead to a more widespread distribution of U. Although U is a radionuclide, the chemical toxicity will be of greater concern than its radiotoxicity since U has a long half-life of 4.47 x 10^9 years, giving it a low specific activity of 1.24 x 10^4 Bq g^{-1} U (Sheppard *et al.*, 2005).

Uranium speciation, and as such its chemical toxicity, is strongly dependent on environmental factors. Important factors controlling the speciation are for example pH value, redox potential, ionic strength and availability of inorganic and organic ligands (Bernhard, 2005). In chapter 4, we calculated the U speciation in a Hoagland solution. We observed at pH 4.5 mainly the presence of the free uranyl ion (UO_2^{2+}). At pH 5.5, U phosphate (UO_2HPO_4) and U hydroxyl species ($(UO_2)_3(OH)_5^+$) were dominating, while at pH 6.5 and 7.5 mainly U carbonate species ($(UO_2)_2CO_3(OH)_3^-$) were present (chapter 4). Exposure of *Arabidopsis thaliana* plants to U at those different pH levels resulted in differences in U uptake by the roots and translocation to the shoots, with high uptake and low translocation at pH 4.5 and low uptake but higher translocation at pH 7.5.

It is known that heavy metals such as cadmium (Cd), copper (Cu) and zinc (Zn) can interfere with the functioning of chloroplasts (Maksymiec, 1997; Miao *et al.*, 2005; Filek *et al.*, 2010). However, the effects of U on photosynthesis are largely unknown. Since the inhibition of photosynthesis can affect a plant's physiological state (Juneau *et al.*, 1999), it is important to investigate the impact of U contamination on the photosynthetic machinery. In chapter 4, we analysed the photosynthetic parameters of plants exposed to 25 µM U at different pH levels, but no significant differences between U exposed and control plants were observed at the different pH levels. However, only the saturation points of the photosynthetic parameters were analysed. More information can be obtained by fitting the experimental curves with mathematical functions (i.e. nonlinear regression analysis) to quantify the dynamic responses of the

photosynthetic processes (D'Haese *et al.*, 2004). Therefore, the present study gives a more detailed analysis of the photosynthetic parameters. In addition, a more profound investigation of the effect of U on the photosynthesis was carried out by using different U concentrations ranging from 0-100 µM at two different pH levels (pH 4.5 and pH 7.5).

Photosynthesis was analysed by chlorophyll *a* fluorescence since it is considered as the most effective way to analyse the influence of stressors on photosynthesis *in vivo* (Schreiber *et al.*, 1987). Therefore, the initial fluorescence in a dark adapted leaf (F_0) and the maximum fluorescence after a saturating light pulse in a dark adapted leaf (F_m) were measured. At regular time intervals, saturation pulses were applied, leading to the determination of the maximum fluorescence in a light adapted leaf (F'_m) in function of the time (Kooten *et al.*, 1990). Based on those parameters, other parameters can be calculated that give a more detailed insight into the photosynthetic process under stress conditions. The effective photosystem II (PSII) quantum yield (Y(II)), the relative measure of the rate of electron transport (ETR(II)) and the fraction of open reaction centres according to the lake model of PSII antenna pigment organization (qL) are indicators for the capacity of photochemical processes (Linger *et al.*, 2005). A reaction centre is considered open when the primary quinone acceptor of PSII is oxidized and as such is able to accept an electron, i.e. photoreduction (Baker, 2008). By non-photochemical quenching, the plants dissipate the excess of absorbed energy (Allen *et al.*, 2008). The parameters for regulated energy dissipation via the xanthophyll cycle (Y(NPQ)) and the non-regulated energy dissipation (Y(NO)) are a measure of non-photochemical fluorescence quenching. To investigate the effects of U on the photosynthesis of *Arabidopsis thaliana* plants, plants were exposed to different U concentrations at pH 4.5 or pH 7.5. We addressed the following questions: a) Does U negatively influence photosynthesis? b) Is the effect different for U exposure at different pH levels?

8.2. Results

8.2.1. Uranium concentration

After 3 days exposure of *Arabidopsis thaliana* plants to different U concentrations at pH 4.5 or pH 7.5, the U content in the leaves was determined (Table 8.1). Under both pH conditions, an increased U content in the leaves was found with increasing U concentration added to the nutrient solution. By comparing the two pH levels within one U concentration, the U concentration in the leaves at pH 7.5 is higher than in the leaves exposed to U at pH 4.5. For example, after exposure to 100 µM U, about 3.4 times more U was present in the leaves at pH 7.5 compared to pH 4.5. Since U uptake by the roots was approximately 4 times higher at pH 4.5 as compared to pH 7.5, the differences in translocation resulted in a root-to-shoot transfer factor that is approximately 15 times higher at pH 7.5 after exposure to 100 µM U as compared to pH 4.5.

Table 8.1: U concentration (µg g^{-1} DW) and transfer factors of *Arabidopsis thaliana* leaves exposed to different U concentrations during 3 days at pH 4.5 or pH 7.5. U concentration values are mean values ± S.E. of at least 4 biological replicates. Different small letters indicate significant differences between different U treatments at pH 4.5 (p<0.05). Different capital letters indicate significant differences between different U treatments at pH 7.5 (p<0.05). Differences within one U concentration for the different pH treatments are indicated with *(p<0.05).

	pH 4.5	**Transfer factor**	**pH 7.5**	**Transfer factor**
0 µM U	n.d.	n.d.	n.d.	n.d.
6.25 µM U	0.90 ± 0.13^{a}	0.00039	1.64 ± 0.43^{A}	0.00077
12.5 µM U	1.49 ± 0.40^{ab}	0.00037	2.70 ± 0.87^{A}	0.00075
25 µM U	*2.68 ± 0.26^{bc}	0.0004	8.73 ± 2.06^{B}	0.0014
50 µM U	*5.00 ± 0.75^{cd}	0.00013	25.26 ± 2.72^{BC}	0.0025
75 µM U	*8.44 ± 2.59^{de}	0.00012	48.53 ± 16.30^{C}	0.0029
100 µM U	*18.17 ± 3.91^{e}	0.00024	62.14 ± 10.71^{C}	0.0035

8.2.2. Growth responses

Exposure to U resulted in a significant decrease in the relative leaf growth under both pH conditions (growth control plants = 100%) (Table 8.2). Comparing both pH levels within one U concentration, the relative growth at pH 4.5 was significantly lower after exposure to 25 µM U or higher as compared to the growth observed at pH 7.5.

Table 8.2: Growth responses of *Arabidopsis thaliana* leaves exposed to different U concentrations during 3 days at pH 4.5 or pH 7.5. Relative growth is expressed as % growth compared to the control plants (100 % growth). The leaf growth of control plants is indicated between brackets. Values are mean values ± S.E. of at least 50 biological replicates. Different small letters indicate significant differences between different U treatments at pH 4.5 (p<0.05). Different capital letters indicate significant differences between different U treatments at pH 7.5 (p<0.05). Differences within one U concentration for the different pH treatments are indicated with *(p<0.05).

	Relative leaf growth	
	pH 4.5	**pH 7.5**
0 µM U	100 ± 5.24^{a} (31.46 mg)	100 ± 2.62^{A} (58.77 mg)
6.25 µM U	*101.75 ± 4.37^{a}	78.8 ± 2.29^{B}
12.5 µM U	*97.15 ± 5.28^{a}	70.48 ± 2.88^{BC}
25 µM U	*30.46 ± 3.79^{b}	61.88 ± 1.86^{CD}
50 µM U	*37.52 ± 2.83^{b}	51.1 ± 2.07^{E}
75 µM U	*35.72 ± 2.53^{b}	54.43 ± 1.81^{DE}
100 µM U	*28.13 ± 2.89^{b}	39.4 ± 1.30^{F}

8.2.3. Pigment concentrations

Chlorophyll a, chlorophyll b and carotenoid concentrations (Figure 8.1) were measured and calculated according to Wellburn *et al.* (1994). After U exposure at pH 4.5, a transient increase in chlorophyll a, chlorophyll b and carotenoids was detected. After U exposure at pH 7.5, no significant differences in chlorophyll content were observed. However, a steady increase in the pigment concentrations was detected. By comparing the pigment concentrations within one U concentration, it seems that at pH 4.5 the chlorophyll a and b content was slightly higher than in the plants exposed to U at pH 7.5. For the carotenoids, this effect was only visible after exposure to 0 and 50 µM U.

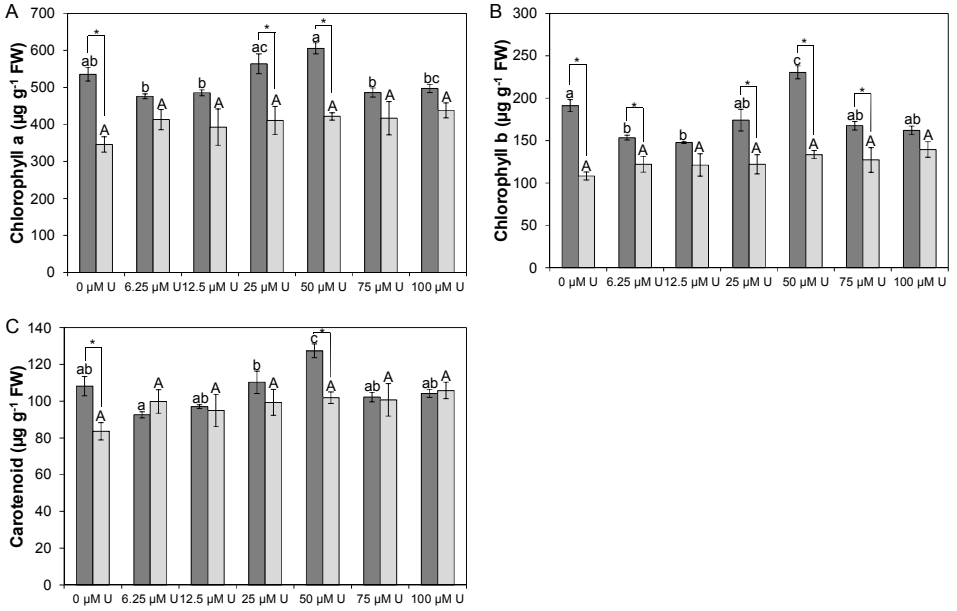


Figure 8.1: Chlorophyll a, chlorophyll b and carotenoid concentrations (µg g^{-1} FW) of *Arabidopsis thaliana* plants exposed to different U concentrations at pH 4.5 (dark grey) or pH 7.5 (light grey). Each data point represents the mean ± S.E. of at least 3 biological replicates. Different small letters indicate significant differences between different U treatments at pH 4.5 (p<0.05). Different capital letters indicate significant differences between different U treatments at pH 7.5 (p<0.05). Differences within one U concentration for the different pH treatments are indicated with *(p<0.05).

Concerning the anthocyanin content (Figure 8.2), an increasing trend in anthocyanins was found after U exposure at both pH levels. No differences were detected by comparing both pH levels within one U concentration.

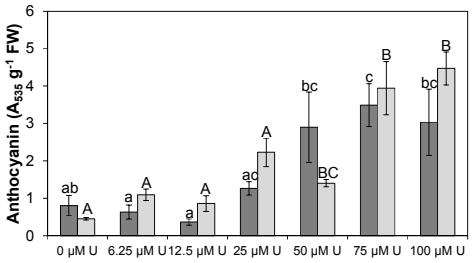


Figure 8.2: Anthocyanin concentrations (A_{535} g^{-1} FW) of *Arabidopsis thaliana* plants exposed to different U concentrations at pH 4.5 (dark grey) or pH 7.5 (light grey). Each data point represents the mean ± S.E. of at least 3 biological replicates. Different small letters indicate significant differences between different U treatments at pH 4.5 (p<0.05). Different capital letters indicate significant differences between different U treatments at pH 7.5 (p<0.05).

8.2.4. Photosynthetic parameters

Chlorophyll *a* fluorescence of the youngest full grown leaf (normally fourth youngest leave), dark adapted during at least 30 min, was measured using a Dual-PAM-100 chlorophyll fluorescence measuring system. The minimum (F_0) and maximum (F_m, F'_m) fluorescence were determined. From the F_0, F_m and F'_m values different parameters can be calculated that give a measure of the photosynthetic efficiency of PSII. One of those values is $(F_m-F_0)/F_m$ (F_v/F_m with $F_v=F_m-F_0$), a relative measure of the maximum quantum efficiency. Exposure to 25 µM U at pH 4.5 caused a significant increase in F_v/F_m as compared to the control (Figure 8.3). Uranium exposure at pH 7.5 did not affect F_v/F_m. By comparing the two pH levels within one U concentration, a significant increase in F_v/F_m was observed at pH 7.5 compared to pH 4.5 at almost all exposure conditions.

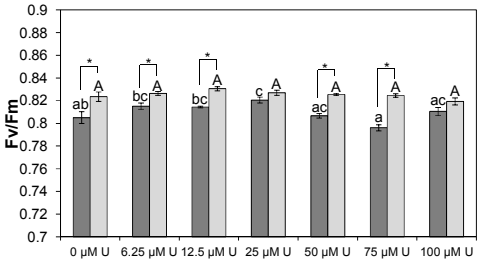


Figure 8.3: Maximum quantum efficiency (F_v/F_m, with $F_v=F_m-F_0$) of *Arabidopsis thaliana* plants exposed to U at pH 4.5 (dark grey) or at pH 7.5 (light grey). Each data point represents the mean value ± S.E. of 4 biological replicates. Different small letters indicate significant differences between different U treatments at pH 4.5 ($p<0.05$). Different capital letters indicate significant differences between different U treatments at pH 7.5 ($p<0.05$). Differences within one U concentration for the different pH treatments are indicated with *($p<0.05$).

Since F_v/F_m should not be used as a rigorous quantitative value of the quantum yield of PSII (Blankenship, 2002), other parameters are taken into account to assess the effect of U on photosynthesis. As such, the amount of open reaction centres during photosynthesis was estimated (qL) (Supplementary Figure S8.1). To estimate the rate at which the reaction centres open, a curve of qL was modelled using nonlinear regression analysis in the freeware software package GNU R (version 3.0.1). An $alpha_{qL}$ parameter could be determined, accounting for the slope of the qL curves (Figure 8.4A). At low pH, a small increase (not significant) in $alpha_{qL}$ was detected at higher U concentrations. Also by

comparing the saturation points (i.e. last point of the curve) at different U concentrations (Figure 8.4B), a small increase was found at low pH level. In contrast, at pH 7.5, a small decrease in $alpha_{qL}$ was observed after exposure to 50 µM U. Comparing both pH levels within one U concentration, no difference in the rate of the opening of the reaction centres could be detected under control conditions, since there were no differences in $alpha_{qL}$. However, after U exposure, significant increases in $alpha_{qL}$ and in saturation points were observed at pH 4.5 as compared to pH 7.5.

As for qL, a curve was modelled for ETR(II) and subsequently, an $alpha_{ETR(II)}$ parameter could be defined, accounting for the slope of the curve . By analysing the ETR(II) (Supplementary Figure S8.2) an increasing trend in the $alpha_{ETR(II)}$ could be observed after U exposure at pH 4.5 (Figure 8.4C). In addition, a small increase in the ETR(II) saturation points was observed after exposure to 25 µM U or higher at low pH (Figure 8.4D). At pH 7.5, no increases in $alpha_{ETR(II)}$ or saturation points were detected. By comparing both pH levels within one U concentration, a significant increase in $alpha_{ETR(II)}$ and saturation points were observed after exposure to 25 µM U or higher at low pH as compared to pH 7.5.

Also for the quantum yield of photochemical energy conversion (Y(II)) a curve was modelled with a resulting $alpha_{Y(II)}$ parameter. The Y(II) gives an indication of the fraction of the absorbed energy that is effectively used for photosynthesis (Supplementary Figure S8.3). After U exposure at pH 4.5, the Y(II) increased more rapidly after U exposure as indicated by an increased $alpha_{Y(II)}$ (Figure 8.4E). In addition, an increasing trend in the Y(II) was observed at the saturation point (Figure 8.4F). At pH 7.5, no differences were observed in the Y(II) parameters as compared to the control plants. When comparing both pH levels at one U concentration, no differences were observed under control conditions. However, a significant increase in $alpha_{Y(II)}$ was found after exposure to 6.25 µM U or higher, while the saturation points increased after exposure to 25 µM U or higher at pH 4.5 as compared to pH 7.5

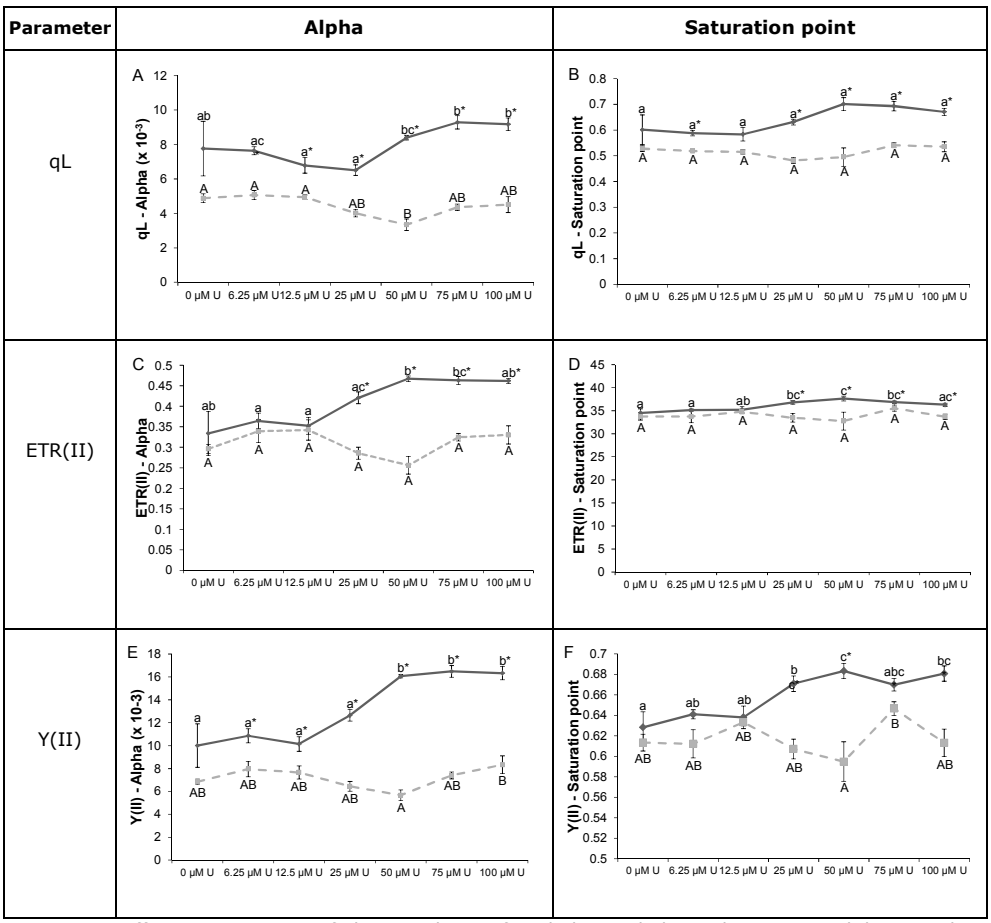


Figure 8.4: Different parameters of photosynthesis of *Arabidopsis thaliana* plants exposed during 3 days to different U concentrations at pH 4.5 (solid line) or pH 7.5 (dashed line). Alpha is the slope of the initial part of the curve. Saturation points are the points at the end of the curve (340s). Peak height is the maximum value of the curve. A+B: Coefficient of photochemical quenching as estimation of the amount of open reaction centres. C+D: Electron transport rate of photosystem II. E+F: Effective quantum yield. G+H: Quantum yield of regulated energy dissipation. I+J: Quantum yield of non-regulated energy dissipation. Values presented are mean ± S.E. of at least 3 biological replicates. Error bars not shown are smaller than symbol size. Data points with different letters are significantly different (p<0.05). Differences between the two pH levels within the same U concentration are indicated with * (p<0.05)

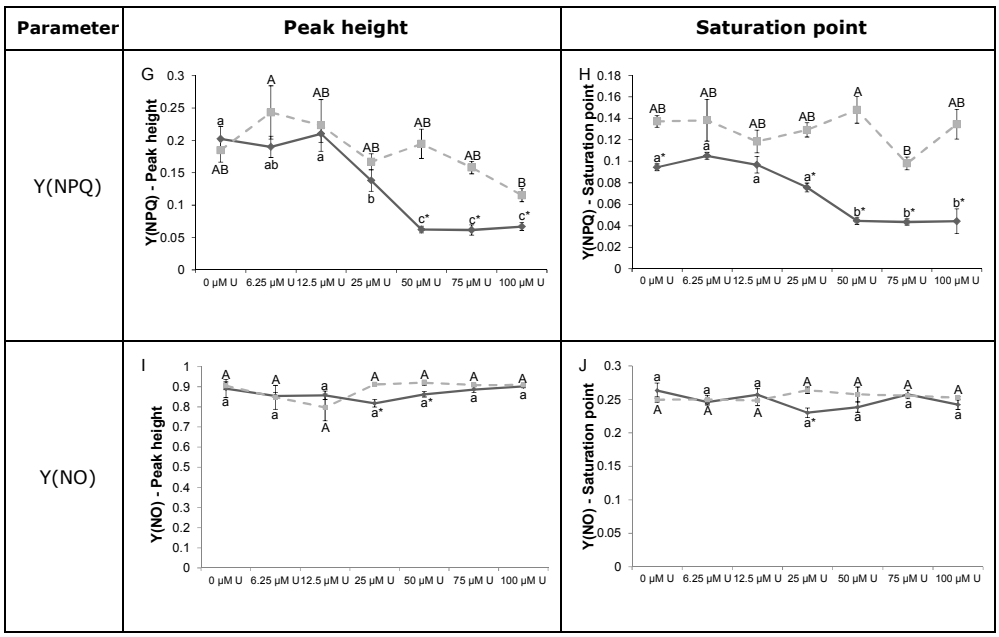


Figure 8.4 (continued)

Finally, two mechanisms for energy dissipation were evaluated, namely Y(NPQ) and Y(NO). Y(NPQ) corresponds to the fraction of energy dissipated in form of heat via a regulated and photoprotective mechanism, i.e. xanthophyll cycle (Supplementary Figure S8.4). Y(NO) reflects the fraction of energy that is passively dissipated in form of heat and fluorescence, mainly due to closed PSII reaction centres (Supplementary Figure S8.5). For these parameters, the peak height and the saturation points were analysed. At low pH, a significant decrease in the peak height of Y(NPQ) was observed after exposure to 25 µM U or higher (Figure 8.4G). This decrease was also detected at the saturation point (Figure 8.4H). At pH 7.5, a small decrease in peak height was found after exposure to 75 and 100 µM U (not significant). Concerning the saturation points, no significant differences were observed. By analysing both pH levels within one U concentration, the peak height and the saturation points were significantly lower at pH 4.5 as compared to pH 7.5 after exposure to 50 µM U or higher.

No differences were found for the different U concentrations at both pH levels in Y(NO) (figure 8.4I+J). However, a significant increase in peak height and saturation point was observed at pH 7.5 as compared to pH 4.5 after exposure to 25 or 50 µM U.

8.3. Discussion

Chemical toxicity of U is strongly dependent on environmental factors such as the pH. In chapter 4, we investigated the effect of pH on U uptake and translocation in *Arabidopsis thaliana* plants and observed a high uptake but low translocation at pH 4.5 and a low uptake but higher translocation at pH 7.5. In addition, differences in leaf fresh weight were observed depending on the pH level at which plants were exposed to U (25 µM). Since photosynthesis is the first step in biomass production (Snel and Dassen, 2000) and since the inhibition of photosynthesis can affect a plant's physiological state (Juneau and Popovic, 1999), it is important to investigate the impact of U contamination on the photosynthetic machinery. It has been demonstrated that heavy metals such as Cd, Cu and Zn can interfere with the functioning of chloroplasts (Maksymiec, 1997; Miao *et al.*, 2005; Filek *et al.*, 2010). However, the effects of U on photosynthesis are still largely unknown. In chapter 4, no effect of U on the photosynthesis was observed. However, in that study only saturation points of the photosynthetic parameters were analysed. Moreover, only one U concentration (25 µM) was used. Therefore, the present study provides a more detailed analysis of the photosynthetic parameters by carrying out nonlinear regression analyses on the full induction curve and is as such not only looking at the final outcome. In addition, this is the first study to carry out a profound investigation of the effect of U exposure on photosynthesis at different pH levels. Photosynthetic responses were measured in *Arabidopsis thaliana* plants exposed to different U concentrations during 3 days at pH 4.5 or pH 7.5.

After exposure to U, the U concentrations in the leaves increased with increasing U concentration added to the nutrient solution (Table 8.1). Under both pH conditions, the root-to-shoot transfer factors were small, indicating that most U is retained in the roots (Table 8.1), as was reported before by several authors for different plants species (Vandenhove *et al.*, 2006; Vanhoudt *et al.*, 2008; chapter 4; chapter 7). However, after exposure to 100 µM U at pH 7.5, the U concentration in the leaves is about 3.4 times higher than at pH 4.5. A possible explanation for the differences in root-to-shoot transfer for U is given by Laurette *et al.* (2012). They suggested that when U is present in a highly reactive form it immediately precipitates with phosphate moieties, leading to

immobilization of U. However, when U is complexed, it will be less reactive and can thus more easily be transported to the shoots driven by sap flow, through xylem vessels (Laurette *et al.*, 2012). In chapter 4, we observed that in the Hoagland nutrient solution the highly reactive UO_2^{2+} is mainly present at low pH. At higher pH, U forms complexes with carbonate groups which probably decrease the reactivity of U, leading to more mobile species. According to the suggestion of Laurette *et al.* (2012), this explains the observed differences in root-to-shoot transfer factors between low and higher pH levels. Those results are in agreement with the results of chapter 4, where we detected a higher transfer of U from roots to shoots in *Arabidopsis thaliana* plants at pH 7.5 as compared to pH 4.5 after exposure to 25 µM U.

The increased U concentration in the leaves was accompanied by a significant reduction in the growth of the leaves of *Arabidopsis thaliana* plants at both pH levels (Table 8.2). After exposure to 100 µM U, the relative growth at pH 4.5 was significantly more reduced than at pH 7.5, although the U concentration at low pH was approximately 3.5 times lower than at pH 7.5. This could possibly indicate that U exposure at low pH causes more adverse effects than exposure at higher pH. Since photosynthesis is a pivotal process in biomass production, the differences in growth reduction might be explained by a different effect of U on the photosynthesis when plants are exposed to U at different pH levels. Photosynthesis is an essential process of plant life, which begins with the absorption of light by chlorophyll as energy (Snel and Dassen, 2000). It has been stated before that one of the injury factors of heavy metal exposure is a decreased chlorophyll content (Ernst, 2000). However, we did not found a decreased chlorophyll a or chlorophyll b content (Figure 8.1). In fact, we observed a significant increase in chlorophyll b concentration after exposure to 50 µM U at pH 4.5, while a steady increase could be detected in the pigment concentration at pH 7.5. Those results are in contrast with the results of several authors (Aery *et al.*, 1997; Shtangeeva *et al.*, 2006). As such, Shtangeeva *et al.* (2006) found a decreased chlorophyll content in plantains exposed to 25 mg kg^{-1} U in soils. A decrease in chlorophyll a and b was also observed by Aery *et al.* (1997). They noticed a 25 % reduction in total chlorophyll content after exposure of *Triticum aestivum* to 10 mg kg^{-1} U. Also exposure to other heavy metals generally induced a decrease in the chlorophyll content. Baek *et al.*

(2012) observed a decreased chlorophyll content in *Arabidopsis thaliana* after exposure to Cu, Mn, Pb and Zn. Therefore, further investigations are needed to explain the fact that the chlorophyll content did not change in U-exposed *Arabidopsis thaliana* plants.

Heavy metal exposure can also affect the anthocyanin and carotenoid synthesis. Both pigments are known to be involved in protecting the plant from stress (Baek *et al.*, 2012). Anthocyanins are photoprotective since they are able to screen the visible radiation and to scavenge ROS (Zhang *et al.*, 2012). Accordingly, a significant increase in anthocyanins was observed after U exposure at both pH levels, indicating anthocyanins possibly also have a photoprotective role under U stress. Carotenoids directly protect the photosystem by scavenging triplet chlorophyll and singlet oxygen (via β-carotene) and lowering the formation of triplet chlorophyll by quenching excited single state of chlorophyll via the xanthophyll cycle (Choudhury *et al.*, 2001). Previous studies reported increased (Drakiewicz *et al.*, 2005), decreased (Ekmekci *et al.*, 2008) or no changes (Mishra *et al.*, 2006) in carotenoid concentrations following exposure of plants to Cd. In the present study, an increased carotenoid concentration was only observed after exposure to 50 µM U at the lowest pH, while at pH 7.5 a steady increase was detected. This could indicate an increased capacity to protect the photosystem against photo-oxidation.

To investigate the effect of U exposure at different pH levels on the photosynthetic efficiency, chlorophyll *a* fluorescence in dark-adapted leaves was measured using a Dual-PAM-100 chlorophyll measuring system. After a saturation pulse, the actinic light was switched on and the evolution of F'_m was followed in function of time. From the F_0, F_m and F'_m values, different parameters could be calculated that give an impression of the photosynthetic efficiency. F_v/F_m values are a measure for the maximum photochemical efficiency often reported to decrease in severe stress conditions (Baker, 2008). In the present study, significant differences were found at low pH with a transient increase in F_v/F_m. By comparing both pH levels within one U concentration, a significant reduction in F_v/F_m was detected at low pH as compared to pH 7.5. This effect was present at almost all tested conditions.

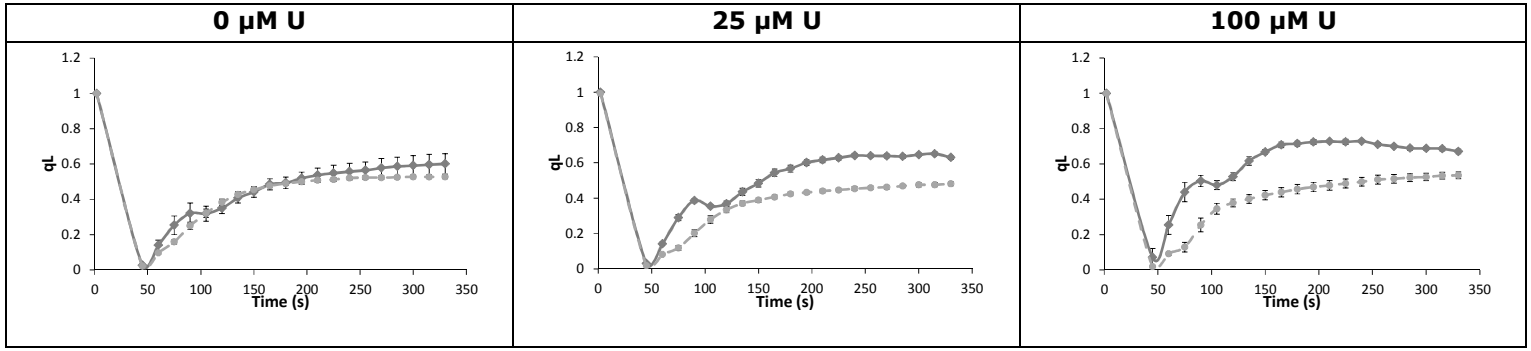
However, the average values fall in the range of 0.79-0.84, which is considered as the F_v/F_m range of normal conditions for vascular plants (Maxwell *et al.*, 2000). As such, no biological relevant differences were observed in the F_v/F_m values in the U-exposed plants at different pH levels, which indicates no strong photo-inhibition. However, Blankenship *et al.* (2002) stated that the F_v/F_m value should not be used as a rigorous quantitative value of the quantum yield of PSII, since it requires some assumptions that are not necessarily correct for all situations. For example, it is assumed that the fluorescence of both F_0 and F_m are emitted from a homogeneous system where all the excited states of the chlorophylls are the same (Baker, 2008). Therefore, it is important to take into account other parameters to investigate the influence on photosynthetic efficiency. The qL parameter estimates the fraction of open PSII centres based on the lake model (Kramer *et al.*, 2004). Although no significant differences were observed under control conditions, it seems that after U exposure the reaction centres open more quickly (reflected by a higher $alpha_{qL}$) at pH 4.5 as compared to the plants at the same U concentration at pH 7.5 (Figure 8.4A). Also at the saturation level, more reaction centres are open at pH 4.5 as compared to pH 7.5 (Figure 8.4B). A reaction centre is open when the primary quinone acceptor of PSII, i.e. plastoquinone A, is able to accept an electron from pheophytin. When plastoquinone A has accepted an electron, the reaction centre is closed until the electron is transferred to plastoquinone B, which in turn transfers the electron further down the electron transport chain. Hence, the faster opening of the reaction centres can possibly be explained by the fact that the ETR(II) is more efficient at low pH after exposure to 50 µM U or higher as compared to the control conditions and also as compared to pH 7.5 as reflected in the increased $alpha_{ETR(II)}$ (Figure 8.4C). As such, after U exposure at low pH levels, electrons are being transferred from the plastoquinones to the ETR(II) more easily, which will lead to an increased openness of the reaction centres. Also at saturation level, the ETR(II) was significantly higher at pH 4.5 after exposure to 25-100 µM U as compared to their counterparts at high pH or as compared to the control situation (Figure 8.4D). This indicates that after U exposure at low pH, more electrons will flow through the electron transport chain. However, the differences in ETR(II) should be taken with care, since for the calculations of ETR(II) it is assumed that 84% of incident photosynthetically

active photon flux density is absorbed by the leaves. The presence of non-photosynthetic pigments, such as anthocyanins, which are shown here to change in U-exposed plants, can influence this parameter, possibly leading to errors in the calculation of ETR(II) (Baker, 2008).

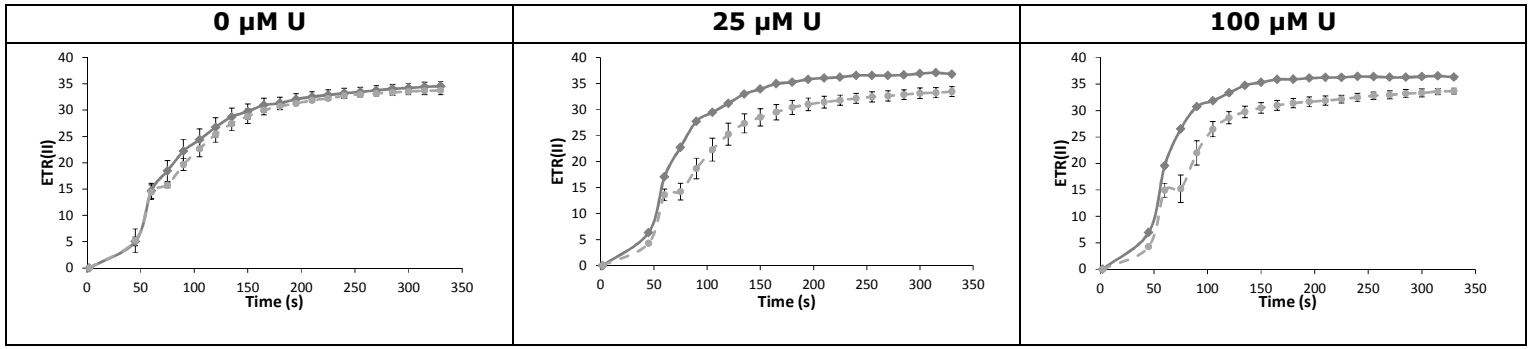
The increased ETR(II) at low pH was accompanied by a significant increase in Y(II) after exposure to 50-100 µM U. This indicates that more of the absorbed quanta are converted into chemically fixed energy at the PSII reaction centres. Since more energy will effectively be used for photosynthesis, this also implicates that less of the absorbed energy should be quenched. The quenching mechanisms encompass two different strategies, namely Y(NPQ) (i.e. the regulated quenching of energy via the xanthophyll cycle) or Y(NO) (i.e. the non-regulated heat dissipation). While no significant differences were found in Y(NO), a significant decrease in Y(NPQ) at pH 4.5 at higher U concentrations was detected, indicating that less energy will be non-photochemically quenched at the regulated way via the xanthophyll cycle. This effect is also observed at pH 7.5, however only after exposure to 100 µM U.

In conclusion, our results indicate that U does not have a negative effect on the photosynthetic processes after 3 days exposure. While almost no differences were observed at pH 7.5, an optimization of the photosynthesis took place when plants were exposed to U at low pH. The differences between the two pH levels can possibly be attributed to the fact that at high pH, more U was transferred to the shoots than at low pH (Table 8.1) and as such direct adverse effects of U on the photosynthesis are more likely at pH 7.5 than at 4.5. However, since the more efficient photosystem at low pH was accompanied by a decreased growth of the plants, it is more conceivable that due to the high U concentration present in the roots when plants are exposed to U at pH 4.5, the plants need a higher sink for sugars that are needed during defence responses. To increase the synthesis of sugars and adenosine triphosphate (ATP) that can be used for detoxification, the photosynthetic processes are upregulated. This was also suggested before by Linger *et al.* (2005) for Cd-exposed *Cannabis sativa*. As such, the energy produced during photosynthesis will not be used for growth but for defence reactions against U-induced oxidative stress.

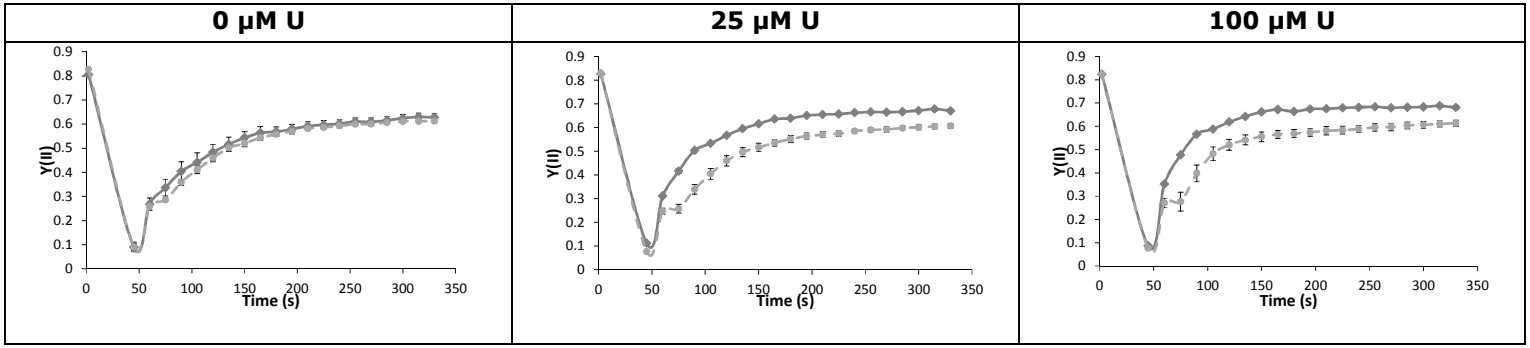
Supplementary data



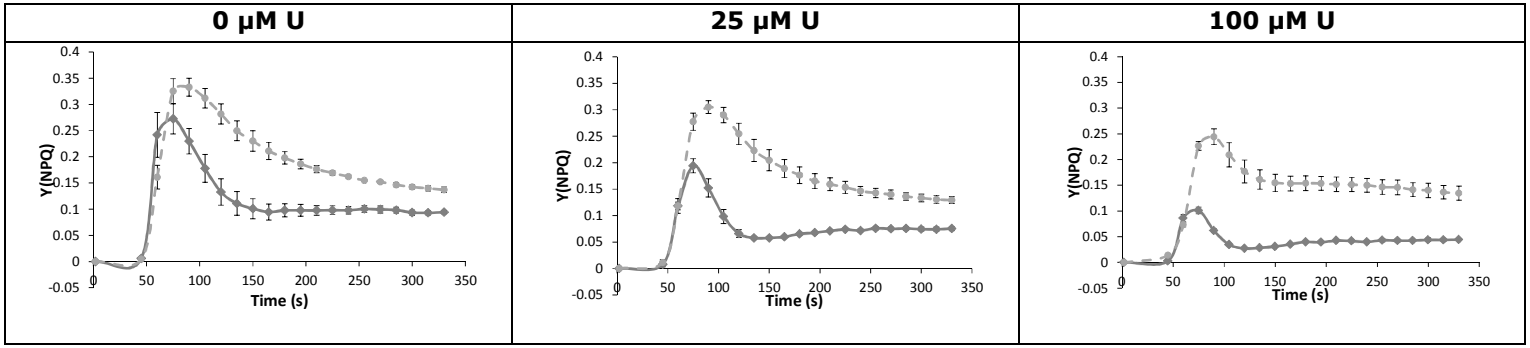
Supplementary Figure S8.1: Coefficient of photochemical quenching as amount of open reaction centres (qL) for *Arabidopsis thaliana* plants exposed to U at pH 4.5 (dark grey) or at pH 7.5 (light grey). Values represented are mean values ± S.E. of at least 3 biological replicates. Error bars not shown are smaller than symbol size.



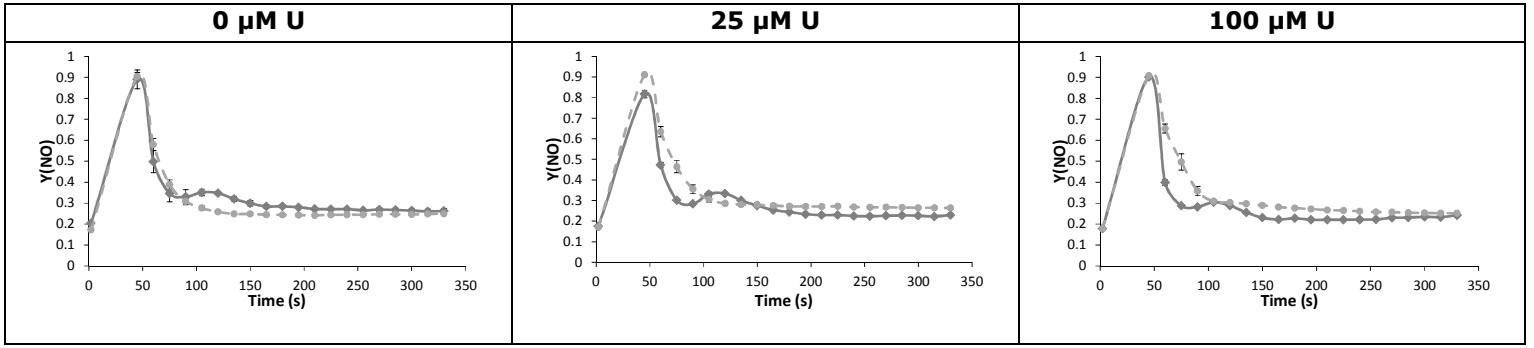
Supplementary Figure S8.2: Electron transport rate of photosystem II (ETR(II)) for *Arabidopsis thaliana* plants exposed to U at pH 4.5 (dark grey) or at pH 7.5 (light grey). Values represented are mean values ± S.E. of at least 3 biological replicates. Error bars not shown are smaller than symbol size.



Supplementary Figure S8.3: Effective quantum yield of photosystem II (Y(II)) for *Arabidopsis thaliana* plants exposed to U at pH 4.5 (dark grey) or at pH 7.5 (light grey). Values represented are mean values ± S.E. of at least 3 biological replicates. Error bars not shown are smaller than symbol size.



Supplementary Figure S8.4: Quantum yield of regulated energy dissipation(Y(NPQ)) for *Arabidopsis thaliana* plants exposed to U at pH 4.5 (dark grey) or at pH 7.5 (light grey). Values represented are mean values ± S.E. of at least 3 biological replicates. Error bars not shown are smaller than symbol size.



Supplementary Figure S8.5: Quantum yield of nonregulated energy dissipation (Y(NO)) for *Arabidopsis thaliana* plants exposed to U at pH 4.5 (dark grey) or at pH 7.5 (light grey). Values represented are mean values ± S.E. of at least 3 biological replicates. Error bars not shown are smaller than symbol size.

Biological effects and oxidative stress responses in *Arabidopsis thaliana* following exposure to uranium and copper: the role of ascorbate

Eline Saenen, Nele Horemans, Nathalie Vanhoudt, Hildegarde Vandenhove, May Van Hees, Jean Wannijn, Robin Nauts, Jaco Vangronsveld, Ann Cuypers (2013). Biological effects and oxidative stress responses in *Arabidopsis thaliana* following exposure to uranium and copper: the role of ascorbate. *In preparation.*

Abstract

Since organisms are almost always exposed to multiple stressors, it is important to investigate the toxicity effects in plants in a multiple stressor context to provide a more realistic estimate of environmental risks. Therefore, we evaluated the toxicity of U and Cu individually and in combination in *Arabidopsis thaliana* plants. In addition, we start to elucidate the role of ascorbate (AsA) in the U-induced stress responses by using AsA deficient vitamin C (*vtc*) mutants of *Arabidopsis thaliana*. In a multipollution setup, it seems that U influences the Cu uptake by the roots, while Cu inhibited U translocation to the shoots. In addition, a 20-fold increase in the expression of *LOX1* was observed in the wild-type roots when they are exposed to both U and Cu as compared to the single stressor conditions, indicating an important role for *LOX1* in the ROS production when plants were exposed to a combination of U and Cu. An induction in the transcript levels of *MIR398b/c* was observed both in leaves and roots under U stress, while under Cu stress or in a multipollution setup, the expression did not change (roots) or even decreased (leaves). Concerning the role of AsA, it seems that in the *vtc* mutants, the U-induced stress responses were more pronounced as compared to the wild-type plants, indicating that AsA plays an important role in the stress response after U exposure.

9.1. Introduction

Due to anthropogenic activities such as metal mining and milling, the presence of metals in the environment has been largely increased. As such, a number of heavy metals are associated with deposits and waste from uranium (U) mines, e.g. copper (Cu). In addition, also Cu-containing ores are known to have U-associated waste (U.S. Environmental Protection Agency, 2008). Both U and Cu are naturally occurring heavy metals. Typical concentrations of U in the soil range from 0.3 – 11.7 mg kg^{-1}, while the concentration in surface or groundwaters varies between 3 x 10^{-2} and 2.1 µg L^{-1} (Bleise *et al.*, 2003). Copper occurs in an average concentration of 50 mg kg^{-1} soil and less than 2 µg kg^{-1} in natural waters. In contaminated areas the concentration of both components can considerably increase (Vandenhove, 2004; Yruela, 2009).

It has been demonstrated that both U and Cu can cause adverse effects in *Arabidopsis thaliana* plants. Although Cu is an essential micronutrient required for growth and development, at higher concentrations it can become toxic leading to chlorosis, necrosis, reduced biomass and inhibition of shoot and root growth (Yruela, 2005; Yruela, 2009; Cuypers *et al.*, 2011). Uranium is a non-essential nutrient leading to growth reduction and a disturbed nutrient profile (Vanhoudt *et al.*, 2011; Vanhoudt *et al.*, 2011a; Vanhoudt *et al.*, 2011b; chapter 4 - chapter 7). In addition, it is known that both U and Cu can induce oxidative stress (Yruela, 2005; Vanhoudt *et al.*, 2008; Cuypers *et al.*, 2011; Vanhoudt *et al.*, 2011; Vanhoudt *et al.*, 2011b). During oxidative stress, an imbalance between the rate of reactive oxygen species (ROS) production and their degradation will occur (Sorg, 2004). The oxidative burst is a common response of plant cells to environmental fluctuations, leading to a rapid increase in the superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) concentrations. The ROS produced during the oxidative burst play an important role in the plant's defence system against pathogens (Schützendübel *et al.*, 2002). The oxidative burst is mediated by NADPH oxidases, also called respiratory burst oxidase homologues (RBOHs), by transferring electrons from cytoplasmatic NADPH to O_2 to form $O_2^{\bullet-}$ (Bhattacharjee, 2005; Sagi and Fluhr, 2006). In addition, lipoxygenases (LOX) catalyse the dioxygenation of polyunsaturated fatty acids, which can lead to the initiation of lipid peroxidation. The oxygenated products of lipid peroxidation (i.e.

oxylipins) serve as inter- and intracellular signalling molecules involved in abiotic and biotic stress responses (Blokhina *et al.*, 2003; Mithöfer *et al.*, 2004). To protect plant cells from oxidative damage but to allow signalling from ROS, plants have evolved an antioxidative defence system consisting of enzymes and metabolites (Mittler *et al.*, 2004). Superoxide dismutase (SOD) acts as the first line of defence by dismutating $O_2^{\bullet-}$ to H_2O_2. Catalase (CAT) and peroxidases (Px) subsequently detoxify H_2O_2. Also the ascorbate (AsA)-glutathione (GSH) cycle is important in the antioxidative defence in which metabolites and enzymes act together to detoxify H_2O_2 (Foyer and Noctor, 2005; Halliwell, 2006).

Effects in biota are generally studied under single stressor conditions. However, organisms are typically exposed to multiple stressors. The effects observed under a multiple stressor situation can deviate from the individually induced effects (Charles *et al.*, 2006). To provide a more realistic estimate of environmental risks, U- and Cu-induced effects need to be studied in a multiple stressor condition. Therefore, the present study aimed to evaluate the toxicity of U and Cu individually and in combination in *Arabidopsis thaliana* plants at pH 7.5. In addition, the importance of AsA under the different stress conditions was investigated by using AsA deficient vitamin C (*vtc*) mutants of *Arabidopsis thaliana* (*vtc1* and *vtc2*). The *vtc1* mutant has a defect in the GDP-D-mannose pyrophosphorylase enzyme and less than 30% of the leaf AsA of wild-type (WT) plants. The *vtc2* mutant has lower AsA levels than the *vtc1* mutant. They are defective in the GDP-L-galactose phosphorylase enzyme (Olmos *et al.*, 2006).

9.2. Results

9.2.1. Metal uptake and translocation

The concentrations of U and Cu administered to the plants were the derived EC30 values for plant growth reduction of wild-type plants at pH 7.5 based on the single dose-response curves (data not shown), resulting in 25 µM U and 2.5 µM Cu. In the multiple stressor setup, both stressors were added at half their EC30 value (12.5 µM U + 1.25 µM Cu).

Exposure to Cu under single stressor conditions resulted in a significant increase in Cu the concentrations in both roots and shoots as compared to the control plants (Table 9.1, Table 9.2). However, there was a limited transfer of Cu to the shoots, resulting in a low Cu concentration in the shoots. Uranium was also easily taken up by the roots under single stressor conditions, but was mainly retained in the roots as the concentration in the shoots was low. Uranium uptake by the roots was unaffected by the presence of both U and Cu in the mixed exposure conditions. However, the translocation of U to the shoots was almost completely inhibited, resulting in a transfer factor that was approximately 4 times lower than when U was applied as a single stressor. In addition, U seems to influence Cu uptake by the roots, since in the multipollution setup, where plants were exposed to 1.25 µM Cu, the uptake of Cu by the roots was less than half as compared to the uptake observed in a single stressor setup (Table 9.1). Uranium did not influence the root-to-shoot translocation of Cu.

Comparing the different genotypes within the same exposure condition, the leaf Cu concentration in the *vtc1* mutant was significantly decreased when plants were exposed to U, Cu and U+Cu as compared to the WT plants exposed to the same condition. The Cu concentration in the roots of *vtc2* plants exposed to U was significantly decreased as compared to the WT plants exposed to U.

Table 9.1: U and Cu concentrations (µg g^{-1} DW) in *Arabidopsis thaliana* roots exposed to 25 µM U, 2.5 µM Cu or 12.5 µM U + 1.25 µM Cu during 3 days at pH 7.5. Values are mean ± S.E. of at least 3 biological replicates. Data points with different letters within a genotype are significantly different (p<0.05). Within each treatment, differences between the genotypes as compared to the WT plants are underlined (p<0.05). n.d.: non detectable.

		ROOTS			
		Control	**25 µM U**	**2.5 µM Cu**	**U + Cu**
U	WT	n.d.	13080 ± 653^{a}	n.d.	5582 ± 275^{b}
	vtc1	n.d.	10433 ± 1043^{A}	n.d.	5374 ± 127^{B}
	vtc2	n.d.	13002 ± 792$^{a'}$	n.d.	6789 ± 588$^{b'}$
Cu	WT	10.34 ± 0.89^{a}	7.47 ± 2.49^{b}	285.90 ± 19.47^{c}	85.02 ± 3.07^{d}
	vtc1	9.17 ± 1.09^{A}	5.65 ± 0.32^{B}	265.28 ± 39.39^{C}	78.96 ± 0.52^{D}
	vtc2	10.40 ± 0.40$^{a'}$	4.73 ± 0.41$^{a'}$	319.36 ± 13.51$^{b'}$	112.63 ± 6.85$^{c'}$

Table 9.2: U and Cu concentrations (µg g^{-1} DW) in *Arabidopsis thaliana* leaves exposed to 25 µM U, 2.5 µM Cu or 12.5 µM U + 1.25 µM Cu during 3 days at pH 7.5. Values are mean ± S.E. of at least 3 biological replicates. Data points with different letters within a genotype are significantly different (p<0.05). Within each treatment, differences between the genotypes as compared to the WT plants are underlined (p<0.05). n.d.:non detectable.

		LEAVES			
		Control	**25 µM U**	**2.5 µM Cu**	**U + Cu**
U	WT	n.d.	3.62 ± 0.27^{a}	n.d.	0.42 ± 0.10^{a}
	vtc1	n.d.	2.34 ± 0.51^{A}	n.d.	0.21 ± 0.01^{A}
	vtc2	n.d.	4.24 ± 1.40$^{a'}$	n.d.	0.53 ±0.08$^{a'}$
Cu	WT	6.42 ± 0.22^{a}	4.75 ± 0.33^{a}	17.16 ± 1.16^{b}	10.37 ± 0.61^{c}
	vtc1	4.92 ± 0.29^{A}	3.30 ± 0.2^{A}	10.79 ± 0.37^{B}	7.68 ± 0.28^{C}
	vtc2	6.57 ± 0.17$^{a'}$	5.28 ± 0.33$^{b'}$	16.18 ± 1.36$^{c'}$	9.14 ± 0.39$^{d'}$

9.2.2. Growth responses

In the WT plants, a significant decrease in root (Figure 9.1A) and shoot growth (Figure 9.1B) was detected when plants were exposed to 2.5 µM Cu, while no significant effect was observed in the U-exposed plants. In a multipollution setup, no differences in root and shoot growth were observed in the WT plants as compared to the control plants.

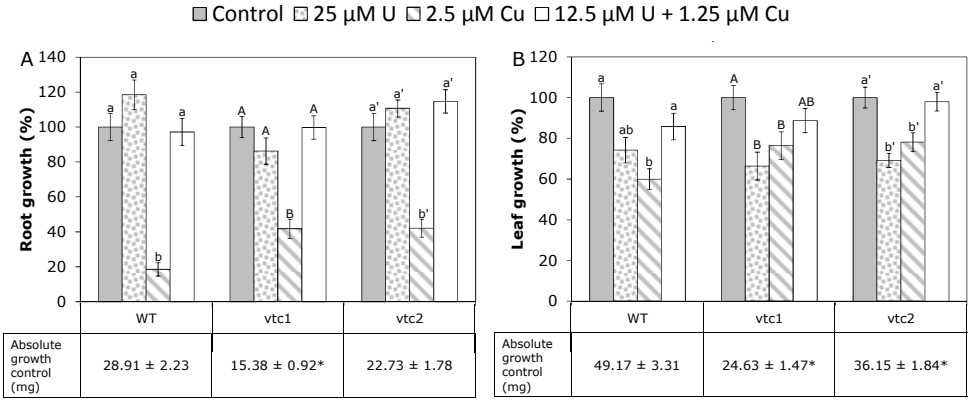


Figure 9.1: Root (A) and leaf (B) growth relative to the non-exposed plants (= 100%) of WT, *vtc1* and *vtc2 Arabidopsis thaliana* plants exposed to 25 µM U, 2.5 µM Cu or 12.5 µM U + 1.25 µM Cu during 3 days. Values are mean ± S.E. of 24 biological replicates. Statistical analyses were done separately for leaves and roots. Data points with different letters are significantly different (p<0.05).

In the *vtc* mutants, a significant decrease in the absolute growth of the roots (*vtc1*) and leaves (*vtc1* and *vtc2*) was detected under control conditions as compared to WT plants. As for WT plants, Cu significantly decreased root and

shoot growth in the *vtc* mutants as compared to their respective controls, but no significant differences in growth were found in the multipollution setup. In contrast to WT plants, a significant decrease in leaf growth was observed when *vtc* mutants were exposed to U as compared to the control.

In the roots, the significant decreased growth after Cu exposure was accompanied by a significant increase in root percentage dry weight in all genotypes (Table 9.3). A significant increase was also observed in the leaves of U-exposed *vtc1* plants as compared to their controls. By comparing the different genotypes within one treatment, a significant increase in leaf and root percentage dry weight was found in the *vtc1* mutants after exposure to U and in the multipollution setup as compared to both the WT and *vtc2* plants.

Table 9.3: Percentage dry weight (expressed as % of fresh weight) of WT, *vtc1* and *vtc2 Arabidopsis thaliana* plants exposed to 25 µM U, 2.5 µM Cu or 12.5 µM U + 1.25 µM Cu for 3 days. Values represent the mean ± S.E. of at least 3 biological replicates. Statistical analyses were done separately for leaves and roots. Data points with different letters within a genotype are significantly different (p<0.05). Differences between the genotypes as compared to the WT plants within the same treatment are underlined (p<0.05).

		Control	**25 µM U**	**2.5 µM Cu**	**U+Cu**
ROOTS	WT	4.81 ± 0.87^{a}	4.99 ± 0.04^{a}	7.13 ± 0.23^{b}	4.54 ± 0.15^{a}
	vtc1	5.43 ± 0.04^{A}	$\underline{6.15 \pm 0.10^{A}}$	7.61 ± 0.11^{B}	$\underline{5.30 \pm 0.07^{A}}$
	vtc2	$4.78 \pm 0.02^{a'}$	$4.88 \pm 0.12^{a'}$	$7.20 \pm 0.09^{b'}$	$4.50 \pm 0.16^{a'}$
LEAVES	WT	9.56 ± 0.10^{a}	10.63 ± 0.30^{a}	10.52 ± 0.20^{a}	10.16 ± 0.1^{a}
	vtc1	10.62 ± 0.30^{A}	$\underline{11.86 \pm 0.45^{B}}$	11.70 ± 0.32^{AB}	$\underline{11.72 \pm 0.21^{AB}}$
	vtc2	$9.83 \pm 0.22^{a'}$	$10.73 \pm 0.12^{a'}$	$10.35 \pm 0.18^{a'}$	$10.32 \pm 0.07^{a'}$

9.2.3 Enzyme capacities

Enzyme capacities of several enzymes of the antioxidative defence system were analysed at protein level to evaluate the importance of the cellular redox balance in WT plants*, vtc1* and *vtc2* mutants after exposure to U, Cu or to a combination of both. In the WT roots, a significant decrease in syringaldazine peroxidase (SPX) capacity (Figure 9.2B) was observed after exposure to U. After Cu exposure, the capacities of SOD (Figure 9.2A) and guaiacol peroxidase (Figure 9.2C) increased while the GPX capacity also increased in the multipollution setup. Those effects were also found in the *vtc* mutants, were the

SPX capacity significantly decreased after U exposure in the *vtc1* and *vtc2* mutants. The GPX and SOD capacities significantly increased after Cu exposure in the *vtc1* mutant, whereas a small non-significant increase was detected in the *vtc2* mutant. No significant differences were observed in the capacities of CAT, APX and GR as compared to the control plants (results not shown).

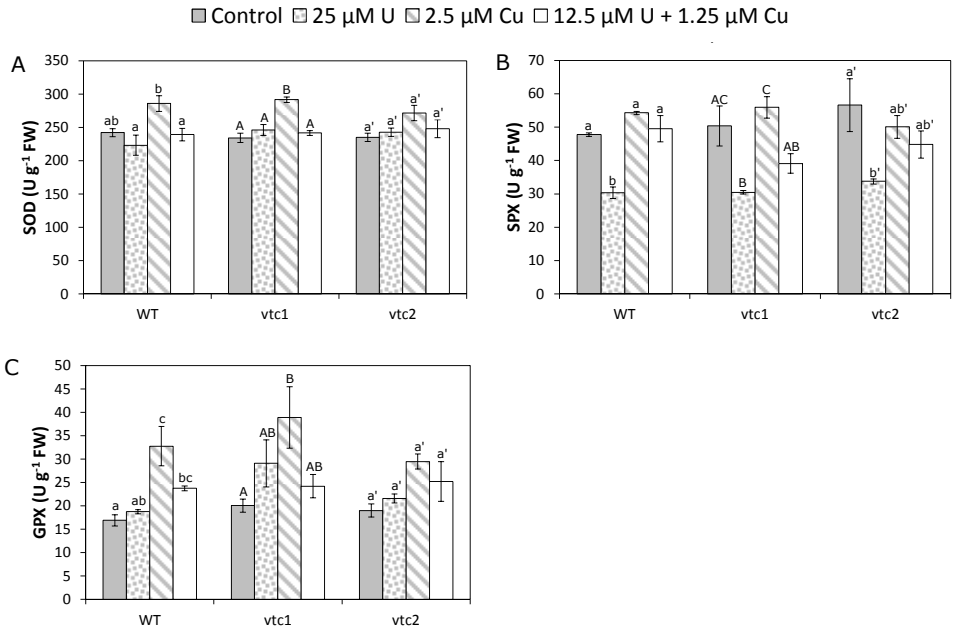


Figure 9.2: Enzyme capacities (units (U) g^{-1} FW) of superoxide dismutase (A, SOD), syringaldazine peroxidase (B, SPX) and guaiacol peroxidase (C, GPX) of roots of WT, *vtc1* and *vtc2 Arabidopsis thaliana* plants exposed to 25 µM U, 2.5 µM Cu or 12.5 µM U + 1.25 µM Cu during 3 days. Values represent the mean ± S.E. of at least 3 biological replicates. Data points with different letters are significantly different (p<0.05).

In the leaves of WT plants, the SOD capacity (Figure 9.3A) significantly decreased after exposure to U, Cu and U+Cu, while the APX capacity decreased only after Cu exposure (Figure 9.3B). The decrease in APX capacity after Cu exposure was also found in both the *vtc* mutants. In addition, a significant increase in GPX capacity was observed after U exposure in the *vtc1* mutant. By comparing the different genotypes within one treatment, a significant increase in the SOD capacity was detected in the *vtc1* leaves after exposure to U or Cu as compared to the WT plants, while the APX capacity increased after exposure to U+Cu. In addition, a significant increase in GPX capacity was found in the *vtc1* mutant under control conditions and after exposure to U and U+Cu as compared to the WT leaves. In the *vtc2* mutant, a significant decrease in SOD capacity was

observed under control conditions as compared to the WT leaves, while the APX capacity increased after exposure to U+Cu as compared to the WT leaves. In the enzyme capacities of CAT, GR and SPX no significant differences were detected as compared to the control plants.

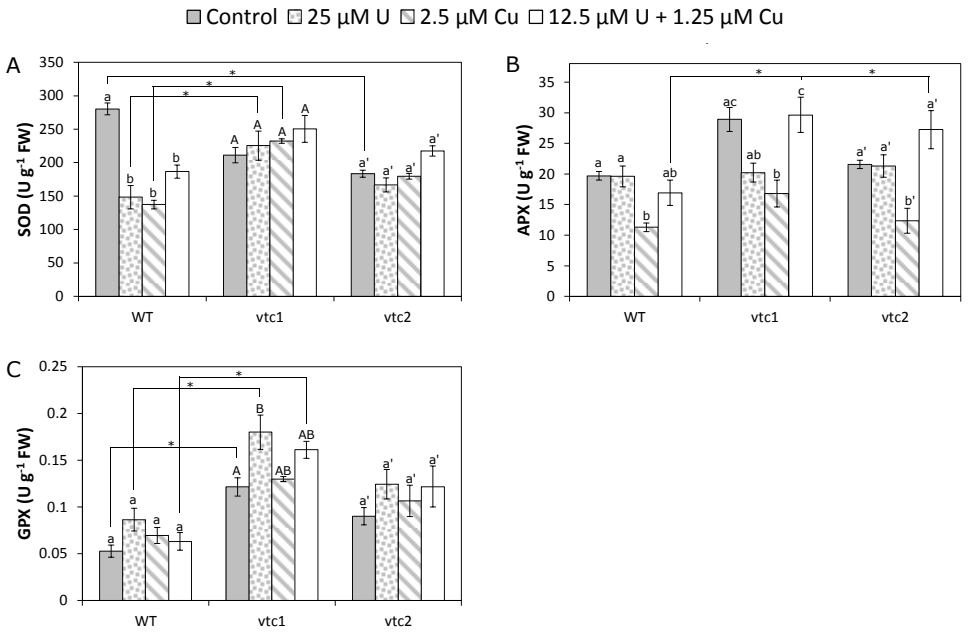


Figure 9.3: Enzyme capacities (units (U) g^{-1} FW) of superoxide dismutase (A, SOD), ascorbate peroxidase (B, APX) and guaiacol peroxidase (C, GPX) of leaves of WT, *vtc1* and *vtc2 Arabidopsis thaliana* plants exposed to 25 µM U, 2.5 µM Cu or 12.5 µM U + 1.25 µM Cu during 3 days. Values represent the mean ± S.E. of at least 3 biological replicates. Data points with different letters are significantly different (p<0.05). Differences between the genotypes as compared to the WT plants within the same treatment are indicated with * (p<0.05).

9.2.4. Antioxidative metabolites

Ascorbate and GSH are both key components of the AsA-GSH cycle, essential to normal cell functioning in plant cells. The concentrations of both low molecular weight antioxidants were determined spectrophotometrically in *Arabidopsis thaliana* plants. In the roots, no significant differences were found in the total or reduced GSH concentrations as compared to the control roots for the three genotypes (Supplementary Table S9.1). However, a significant increase in GSSG (Figure 9.4A) was observed in the WT plants in the multipollution setup, which resulted in a significant decrease in the percentage reduced GSH (Figure 9.4B). In the *vtc1* mutant, a significant increase in GSSG was detected after Cu

exposure with a corresponding decrease in percentage reduced GSH. No significant differences were observed in the *vtc2* roots.

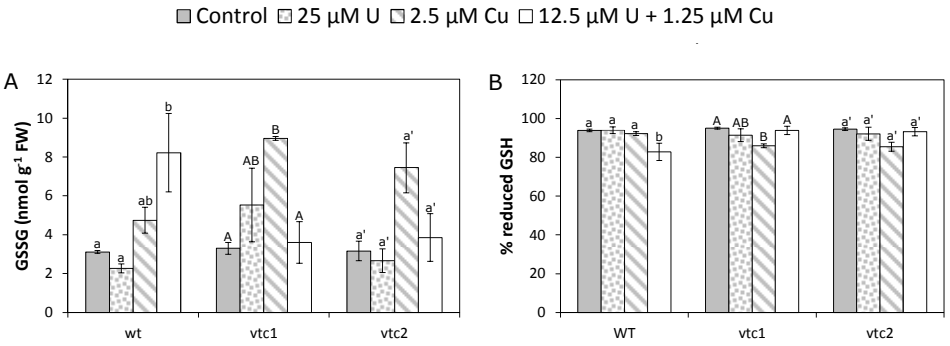


Figure 9.4: Oxidized glutathione (A, GSSG) concentration (nmol g^{-1} FW) and the % reduced GSH (B) in roots of WT, *vtc1* and *vtc2 Arabidopsis thaliana* plants exposed to 25 µM U, 2.5 µM Cu or 12.5 µM U + 1.25 µM Cu. Values are mean ± S.E. of at least 3 biological replicates. Data points with different letters are significantly different (p<0.05).

In the leaves, no significant differences were found in the AsA or GSH concentrations in WT, *vtc1* or *vtc2* plants under the different exposure conditions (Supplementary Table S9.1). By comparing the different genotypes, a significant decrease in the total and reduced AsA concentration was observed in the *vtc1* and *vtc2* mutants as compared to the WT plants under all conditions, as was expected (Figure 9.5A). While no differences in the percentage reduced AsA were detected under control conditions, a significant decrease was observed in the *vtc1* plants after exposure to U, Cu and U+Cu as compared to the WT plants (Figure 9.5B). A significant reduction in the percentage reduced AsA was also found in the *vtc2* plants as compared to the WT plants after exposure to U+Cu. In addition, an increased total GSH concentration (Figure 9.5C) was found in the *vtc1* mutant in all exposure conditions as compared to the WT plants.

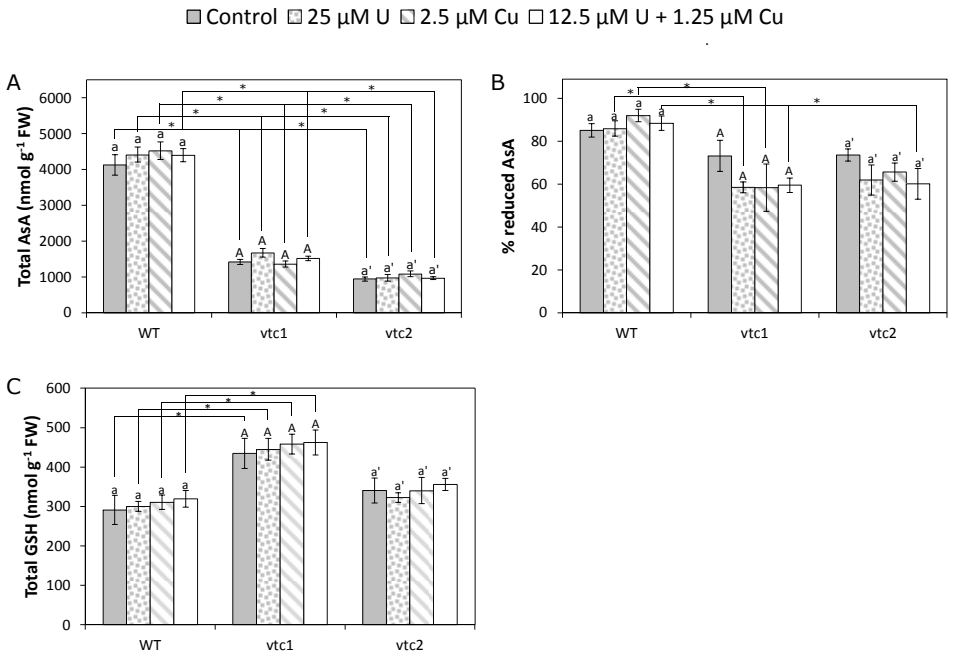


Figure 9.5: Total ascorbate (A) concentration (nmol g^{-1} FW), % reduced ascorbate (B) and total glutathione (C) concentration (nmol g^{-1} FW) in leaves of WT, *vtc1* and *vtc2 Arabidopsis thaliana* plants exposed to 25 µM U, 2.5 µM Cu or 12.5 µM U + 1.25 µM Cu. Values are mean ± S.E. of at least 3 biological replicates. Data points with different letters are significantly different (p<0.05). Differences between the genotypes as compared to the WT plants within the same treatment are indicated with * (p<0.05).

9.2.5. Gene expression analysis

Some important ROS-producing and -scavenging enzymes were analysed at transcriptional level using quantitative real-time PCR to evaluate their importance during heavy metal stress (Supplementary Table 9.2).

First, several plasma membrane bound NADPH oxidases (RBOH) and lipoxygenases (LOX) were analysed since they play a role in ROS production in different abiotic stress conditions. In the WT roots, a significant increase in the expression of *LOX1*, *RBOHD* and *RBOHF* was detected after U or Cu exposure (Figure 9.6B, C, D). When the roots were exposed to both U and Cu, the transcript levels of *RBOHD*/*F* significantly increased as compared to the other conditions. In addition, a 20-fold increase in the gene expression of *LOX1* was observed as compared to the WT roots exposed to U or Cu separately. In the *vtc* mutants, no increases in *LOX1* or *RBOHD*/F expression were detected after exposure to U or Cu as compared to the control roots, while the expression of

RBOHC increased in the *vtc1* roots after exposure to Cu. In the roots of the *vtc2* plants, a significant increase was observed in the transcript levels of *RBOHC* after exposure to U. In the multipollution setup the expression levels of *LOX1* and *RBOHC* significantly increased in the *vtc1* and *vtc2* roots as compared to their control plants. Comparing the different *Arabidopsis* genotypes within the same treatment, a significant increase in the transcript levels of *LOX1* (*vtc2*), *RBOHD* and *RBOHF* (*vtc1* and *vtc2*) was found under control conditions as compared to the WT roots.

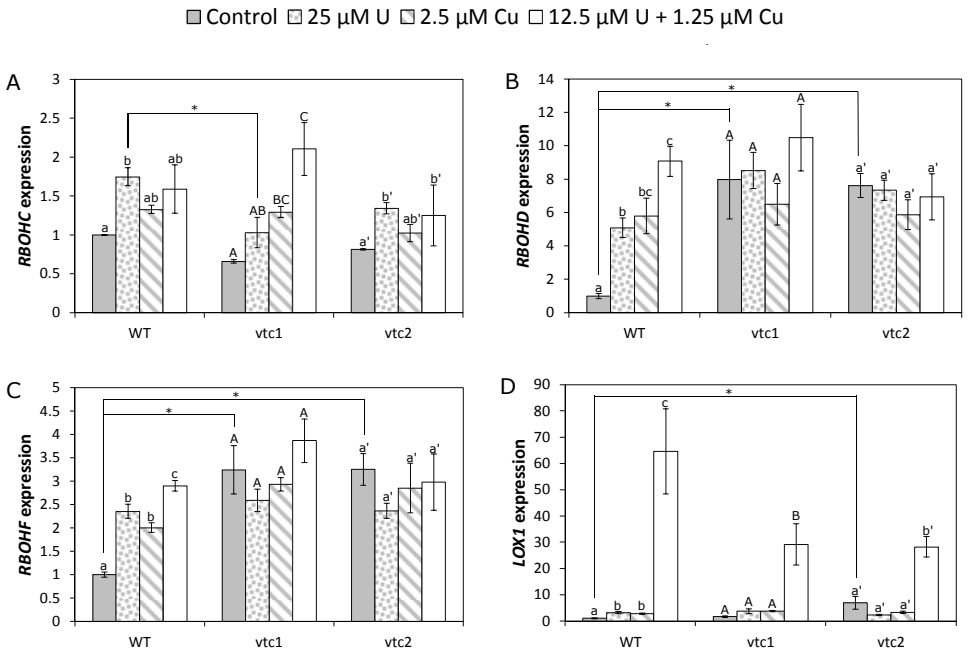


Figure 9.6: Relative expression levels (expression of control WT plants = 1) of the ROS-producing enzymes RBOHC (A), RBOHD (B), RBOHF (C) and LOX1 (D) in roots of WT, *vtc1* and *vtc2 Arabidopsis thaliana* plants exposed to 25 µM U, 2.5 µM Cu or 12.5 µM U + 1.25 µM Cu. Values are mean ± S.E. of at least 3 biological replicates. Data points with different letters are significantly different (p<0.05). Differences between the genotypes as compared to the WT plants are indicated with * (p<0.05).

Secondly, transcript levels of different isoforms of SOD, i.e. $O_2^{\bullet-}$ scavenging enzymes, were analysed. In WT roots, a significant increase in the expression of the *FSD1 (*plastidic iron (Fe) SOD) (Figure 9.7E) and *MIR398b (*Figure 9.7A) was detected after U exposure (Figure 9.7).

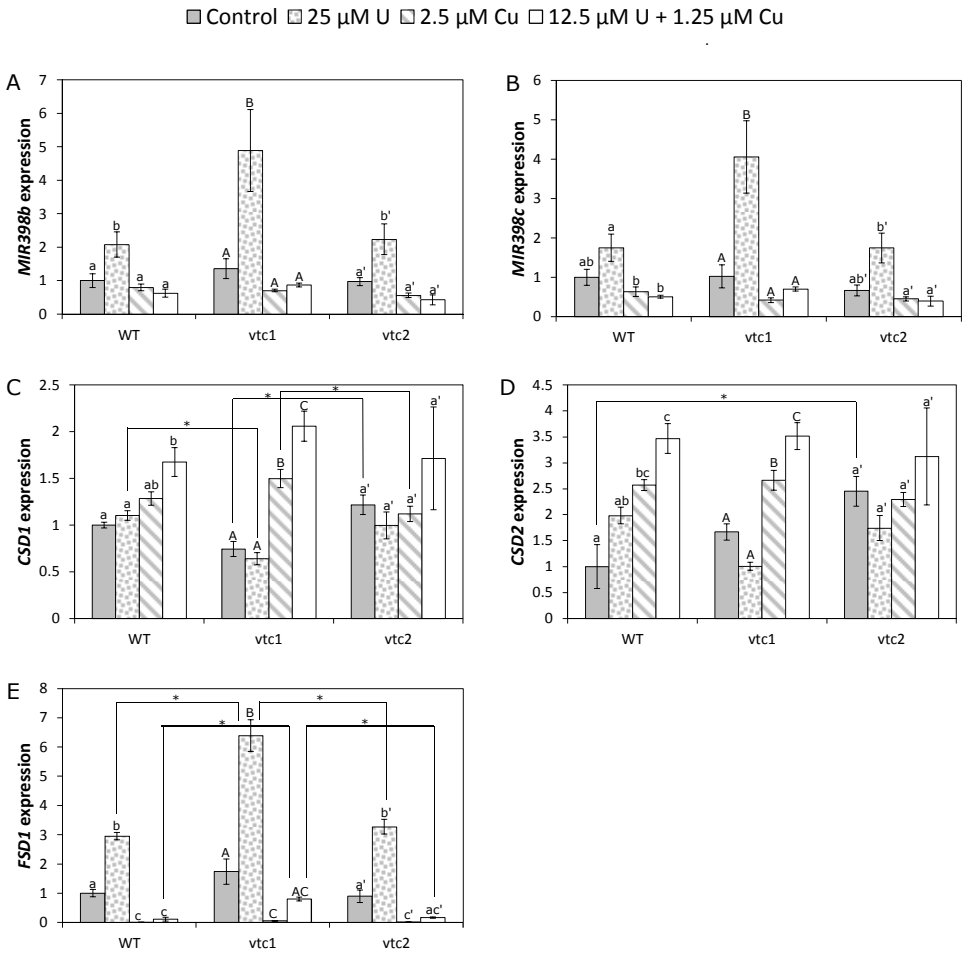


Figure 9.7: Relative expression levels (expression of control WT plants = 1) of miRNA398b (A), miRNA398c (B) and different isozymes of superoxide dismutase (C, D and E) in roots of WT, *vtc1* and *vtc2 Arabidopsis thaliana* plants exposed to 25 µM U, 2.5 µM Cu or 12.5 µM U + 1.25 µM Cu. Values are mean ± S.E. of at least 3 biological replicates. Data points with different letters are significantly different ($p<0.05$). Differences between the genotypes as compared to the WT plants are indicated with * ($p<0.05$).

The expression of the transcription factor SQUAMOSA promoter-binding protein-like 7 (SPL7) was analysed since it is involved in the regulation of the miRNA398b/c and FSD1 expression (Pilon *et al.*, 2011). No differences in the transcript levels of *SPL7* were found after U exposure (Supplementary Table S9.2). After Cu exposure, a significant increase was observed in *CSD2* (plastidic Cu/zinc (Zn) SOD) expression (Figure 9.7D), while the *FSD1* and *SPL7* expression decreased. Exposing plants to both U and Cu resulted in a significant increase in *CSD1* (cytoplasmic Cu/Zn SOD) (Figure 9.7C) and *CSD2* transcript

levels as compared to the control roots, while a decrease in *FSD1* and *SPL7* gene expression was found (Supplementary Table S9.2). The responses observed in the *vtc* mutants were comparable to those in the WT plants. In addition, the expression of *FSD1* increased significantly after exposure to U or U+Cu in the *vtc1* mutant as compared to the WT roots.

Finally, the expression of H_2O_2 scavenging enzymes and enzymes related to the AsA-GSH cycle were analysed (Supplementary Table S9.2). In the WT roots, a significant increase in *CAT1 (*peroxisomal CAT) and *CAT3* (peroxisomal CAT) transcript levels were observed after exposure to U+Cu, while the expression of *APX1* (cytoplasmatic APX) significantly decreased after exposure to Cu (Supplementary Table S9.2). In the roots of the *vtc1* mutant, a significant decrease in the expression of *CAT1* was observed after exposure to U.

In the leaves of WT plants, no significant increases in the transcript levels of genes coding for ROS-producing enzymes were found after exposure to U, Cu or in a multipollution setup as compared to the control plants. In the leaves of the *vtc* mutants, a significant increase was observed after U or Cu exposure in the expression levels of *RBOHD* (*vtc1*) and *RBOHF* (*vtc2*) as compared to the control plants (Supplementary Table S9.3).

Concerning the $O_2^{\bullet -}$ scavenging mechanisms in the leaves of WT plants, a increased *FSD1* expression (Figure 9.8E) was detected after U exposure, although not significant. In addition, a decrease in the transcript levels of *CSD1* (Figure 9.8C) and *CSD2* (Figure 9.8D) was observed. After Cu exposure or in a multipollution setup, the *MIR398b*/c (Figure 9.8A,B) and *FSD1* expression significantly decreased while the *CSD1* expression increased as compared to the control. In the *vtc* mutants, the U and Cu effects are comparable as those described for WT leaves. However, in a multipollution setup, the expression or *MIR398b*/c in the leaves of the *vtc* mutants was not significantly different from the control plants.

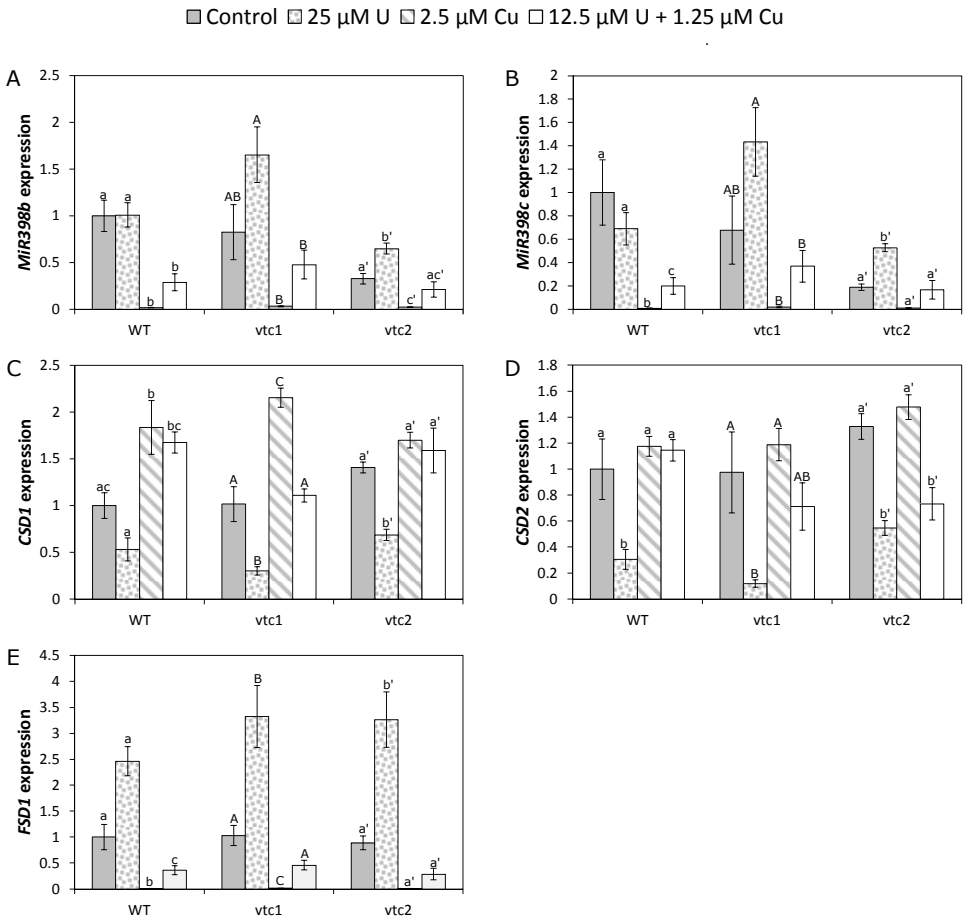


Figure 9.8: Relative expression levels of miRNA398b (A), miRNA398c (B) and different isozymes of superoxide dismutase (C, D and E) in leaves of WT, *vtc1* and *vtc2 Arabidopsis thaliana* plants exposed to 25 µM U, 2.5 µM Cu or 12.5 µM U + 1.25 µM Cu. Values are mean ± S.E. of at least 3 biological replicates. Data points with different letters are significantly different (p<0.05).

By analysing the transcript levels of the H_2O_2 scavenging enzymes (Supplementary Table S9.3), a significant increase in the expression of *CAT1* was found in the WT leaves after exposure to U+Cu, while the *APX1* and *CAT3* expression significantly decreased after Cu exposure. In the *vtc1* mutants, a significant increase in the transcript levels of *CAT2* and *CAT3* was observed after exposure to U (*CAT2/3*) and U+Cu (*CAT2*). Finally, in the *vtc2* mutants, the expression of *CAT1* and *APX1* significantly increased after exposure to U, while a significant decrease was observed in the expression of *CAT3* after exposure to Cu.

9.3. Discussion

It has been demonstrated before that U and Cu cause adverse effects in *Arabidopsis thaliana* plants under single stressor conditions (Cuypers *et al.*, 2011; Vanhoudt *et al.*, 2011; Vanhoudt *et al.*, 2011b; Opdenakker *et al.*, 2012). However, since organisms are exposed to multiple stressors and the effects observed under a multiple stressor situation can deviate from the individually induced effects, it is important to investigate the toxic effects of U and Cu in a multiple stressor setup (Charles *et al.*, 2006). In addition, since it seems that AsA plays an important role in U-induced stress responses (chapters 5-7), its function was further elucidated using the *vtc1* and *vtc2* mutants of *Arabidopsis thaliana* under multiple stressor conditions. Ascorbate-deficient mutants have less than 30 % of the leaf AsA of WT plants present (Olmos *et al.*, 2006), which was confirmed by our measurements (Figure 9.5). Eighteen-day-old WT, *vtc1* and *vtc2 Arabidopsis thaliana* plants were exposed during 3 days to U, Cu or a combination of both.

Under control conditions, a significant reduction in the growth of the roots of the *vtc1* and of the leaves of both *vtc1* and *vtc2* was observed as compared to the WT plants (Figure 9.1). A decreased growth in the *vtc* mutants has been observed before (Veljovic-Jovanovic *et al.*, 2001; Olmos *et al.*, 2006; Colville *et al.*, 2008). Veljovic-Jovanovic *et al.* (2001) and Olmos *et al.* (2006) suggested that both the *vtc1* and *vtc2* mutants have the same number of cells as compared to the WT plants. However, they prevented cell expansion early in development by an increased cross linking of cell wall components. Since the oxidized product of AsA (DHA) prevents cross linking of cell wall proteins and polysaccharides by reacting with lysine and arginine side residues present in cell walls which is leading to looser cell walls, low AsA content can lead to an increased cross linking of cell wall components, resulting in a more stiffened cell wall (Veljovic-Jovanovic *et al.*, 2001; Olmos *et al.*, 2006).

When *Arabidopsis thaliana* plants were exposed to U at pH 7.5, this resulted in a high U uptake by the roots and low translocation to the leaves in all three genotypes (Table 9.1 and Table 9.2), as was indicated before (Vanhoudt *et al.*, 2011a; chapter 4; chapter 7). In addition, when plants were exposed to Cu, this resulted in a high Cu uptake by the roots but low transfer to the shoots as was

noticed before by Smeets *et al.* (2009). The U concentration in the roots was unaffected by the presence of both U and Cu since about half as much U was found when plants were exposed to 12.5 µM U as compared to plants exposed to 25 µM U. However, the translocation of U to the leaves was seriously inhibited when both metals were present in the medium. The latter indicate that Cu interferes with the U translocation. In addition, in a multipollution setup the Cu uptake by the roots was less a half the uptake as when plants were exposed to 2.5 µM Cu. As such, it seems that U interferes with Cu uptake by the roots. These results might be specific for U and Cu as the results are not according to the results of Vanhoudt *et al.* (2010) in which a combination of U and Cd was investigated. In that study, a significant increase in the U root and leaf content was reported after exposure to 10 µM U + 5 µM Cd as compared to the U content when plants were exposed to 10 µM U as a single stressor. On the other hand, it seems that the effects on metal uptake and translocation are strongly dependent on the ratio of metals used. As such, no differences in U or Cd uptake and translocation were observed when plants were exposed during 24 h to 12.5 µM U and 2.5 µM Cd, (Hendrix S., personal communication). In addition, after exposure to 33 µM U and 10 µM Cd, no difference in U and Cd uptake were found but the translocation of Cd from the roots to the shoots was inhibited (Horemans N. personal communication). Therefore, to confirm the effects observed in the present study, additional experiment with different U and Cu concentrations and ratios are needed.

The increased Cu content in roots and leaves of *Arabidopsis thaliana* WT plants resulted in a significant decrease in root and shoot growth (Figure 9.1). This is in accordance with the results of Opdenakker *et al.* (2012). They observed a significant decrease in root and leaf fresh weight after exposure to 2 µM Cu during 24h. Cu induced the same decrease in growth in the *vtc* mutants as compared to the WT plants. Uranium did not significantly affect the growth of the WT plants. This was in contrast to the expectations, since the concentration of U (25 µM U) was calculated to be the EC30 for plant growth reduction. In addition, a significant decrease in root and leaf fresh weight was observed after exposure to 25 µM U at pH 7.5 (chapter 7). In the *vtc* mutants, a significant decrease in leaf growth was observed after exposure to U, which was not

observed in the WT plants. This can possibly indicate that due to the low AsA content, those plants are more sensitive to U-induced stress.

Gene expression of some ROS-producing enzymes was analysed to investigate their importance in the U and Cu-induced oxidative stress responses. In the roots of WT plants exposed to U or Cu under single stressor conditions, a significant increase in *LOX1*, *RBOHC*, *RBOHD* and *RBOHF* expression was observed (Figure 9.5). The NADPH oxidases generate ROS in the apoplast resulting in an oxidative burst, a response of plant cells to biotic and abiotic stress (Bhattacharjee, 2005). Lipoxygenases can also lead to the production of ROS such as singlet oxygen and $O_2^{\bullet -}$. In addition, they initiate the synthesis of oxylipins, which serve as inter- and intracellular signalling compounds involved in abiotic and biotic stress responses (Mithöfer *et al.*, 2004). As such, the increased *RBOHC/D*/F and *LOX1* expressions in the roots indicate that the oxidative burst plays a role under both U and Cu-induced oxidative stress. For U these results are in agreement with Vanhoudt *et al.* (2011b), who reported an increased *RBOHD* and *LOX1* expression in *Arabidopsis thaliana* roots exposed to 100 µM U at pH 5.5. An upregulation of the transcript levels of *RBOHD* and *LOX1* was also observed by Smeets *et al.* (2013) after exposure of *Arabidopsis thaliana* roots to 2 µM Cu during 24 h. In contrast, no induction of the expression of *RBOHC/D/*F was found in *Arabidopsis thaliana* roots exposed to 25 µM U at pH 4.5 or 7.5, although an increased *LOX1* expression was observed after exposure to 75 µM U at pH 7.5 (chapter 5, chapter 7). Although exposure to U or Cu probably induces an increased ROS production in the roots and Cu significantly reduced the root fresh weight of the plants, it seems that the roots can cope well with the U and Cu-induced stress when the metals are applied under single stressor conditions during 3 days. This hypothesis is supported by the lack of significant differences in the GSH redox state and almost no differences in the enzymatic antioxidative defence mechanisms at protein level in the exposed WT roots as compared to the controls.

In a multipollution setup, plants were exposed to a combination of both U and Cu. In the roots of WT plants, this resulted in a 60-fold increase of *LOX1* expression and a significant increase in *RBOHD*/*F* expression as compared to the control roots. As compared to the plants exposed to U and Cu under single

stressor conditions, a 20-fold increase in the transcript levels of *LOX1* was found. Since the observed effect is stronger than expected, this can possibly result from a synergistic effect between U and Cu. These are the first results showing possible synergistic reactions when plants are exposed to a combination of U and Cu. However, more research is needed to further characterize the interactions between U and Cu. The increased expression of *LOX1* detected in the multipollution setup indicates a significant increase in the metal-induced ROS production when plants are exposed to both U and Cu. In addition, an increase in lipid peroxidation can be expected, resulting in membrane damage but also in more precursors of signalling molecules. As the increased ROS production was accompanied by a significant increase in the GSSG concentration in the WT roots and a significant reduction in percentage reduced GSH, this indicates that the cellular redox state in the roots was affected. As such, LOX1 seems to play an important role in the induction of oxidative stress in the WT roots after exposure to U and Cu in a multipollution setup.

In the roots of the *vtc* mutants, a significant induction in the transcript levels of *LOX1* (*vtc2*), *RBOHD* and *RBOHF* (*vtc1* and *vtc2*) was detected under control conditions as compared to the WT roots (Figure 9.6). This might indicate an increased production of ROS under control conditions in the roots of the *vtc* mutants. As for the WT roots, a significant induction of *LOX1* was observed in the *vtc1* and *vtc2* roots when U and Cu were applied simultaneously. While in the WT roots a 20-fold induction was observed as compared to the single stressor conditions, the induction in the *vtc* mutants was only 7.5 times. However, more research is needed to investigate this large discrepancy in ROS production and the role of AsA herein.

In contrast to the roots, it seems that the oxidative burst is not an important source of ROS in the leaves of WT *Arabidopsis thaliana* plants since no induction in the transcript levels of the NADPH oxidases or LOX was found in all tested conditions (Supplementary Table S9.3). These results are in agreement with Vanhoudt *et al.* (2011) where no induction of *RBOHC/D/F* or *LOX1/2* expression was observed in *Arabidopsis thaliana* leaves after 3 days exposure to U at pH 5.5. In addition, no increase in the transcript levels of the *RBOHC/D/*F and *LOX1/*2 was observed in the leaves of *Arabidopsis thaliana* plants exposed to U

at pH 7.5 (chapter 7). This possibly indicates that the oxidative stress in the leaves is generated via root-to-shoot signalling since U and Cu are almost completely retained in the roots.

To regulate the amount of ROS present in the cells, plants have evolved an antioxidative defence system consisting of enzymes and metabolites. By evaluating several isoforms of SOD in WT plants, the changes in *FSD* and *CSD* gene expression in roots (Figure 9.7) and shoots (Figure 9.8) can be related to the expression of *MIR398b/c*. As such, the *CSD* levels are inversely proportional to the level of *MIR398b/c*, since miRNA398b/c will target the mRNA of CSD1/2 (Zhu *et al.*, 2011), whereas the expression levels of *FSD1* are proportional to the expression of *MIR389b/c*. In addition, SPL7 is essential for the expression of miRNA398b/c, while it is also involved in the regulation of the *FSD1* expression (Yamasaki *et al.*, 2009). SPL7 is a homologue of the Cu response regulator 1, the transcription factor in *Chlamydomonas reinhardtii* that is required for the Cu deficiency response (Kropat *et al.*, 2005). As such, an increased expression of *SPL7* is expected under Cu-limiting conditions, which can lead to an increased expression of *MIR398b/*c, while in Cu excess, a decreased *SPL7* expression is expected to lead to a decreased *MIR398b*/c expression. In the present study, when Cu was applied as a single stressor, possibly leading to an excess Cu, the transcript levels of *SPL7* showed a decreasing trend in the roots, as expected. However, the concomitant decrease in the expression of *MIR398b/c* after Cu exposure was not observed in the roots. In contrast, in the leaves no difference in the transcript levels of *SPL7* was detected after Cu exposure, but the *MIR398b/*c and the *FSD1* expression decreased. In addition, in both roots and shoots the expression levels of *CSD*2 increased under Cu exposure as was suggested by several authors (Burkhead *et al.*, 2009; Ding and Zhu, 2009). As such, the CSDs can detoxify superoxide which can be generated under heavy metal stress. In addition, the CSDs may serve as a Cu sink under conditions where Cu content is higher than the normal level (Ding and Zhu, 2009). After U exposure, a significant induction of *MIR398b* was observed in the roots which normally induce a decrease in *CSD1*/2 expression. However, the decreased expression of *CSD1/2* was not observed in the present study. These results are in agreement with the results obtained in the dose-response curve at pH 7.5 (chapter 7) since a decreased expression of *CSD1/2* was only observed after

exposure to 75 or 100 µM U. In WT leaves, no induction of *MIR398b/*c was detected after U exposure, while a decreased expression level of *CSD2* and an increased expression of *FSD1* were observed. In the mixed exposure condition, the miRNA398b/c response is comparable to the response that was observed when Cu was applied as a single stressor both in roots and shoots.

In the *vtc1* mutant, it seems that the U-induced responses are more pronounced in both roots and shoots as compared to the WT plants. As such, a marked increase in the expression of *MIR398b*/c and a significant upregulation of the transcript levels of *FSD1* was detected in roots (Figure 9.7) and shoots (Figure 9.8).

In conclusion, it seems that in the roots, U and Cu behave synergistic concerning ROS production, since a 20-fold increase in *LOX1* expression was found in the multipollution setup as compared to the single stressor conditions. Additional experiments, e.g. with different U and Cu concentrations and ratios, are needed to further elucidate the interactions between U and Cu. In addition, it is shown here for the first time that AsA probably plays an important role in the U-induced stress responses since the miRNA398b/c responses after U exposure of both roots and shoots were more pronounced in the *vtc1* mutant as compared to the WT plants.

Supplementary data

Supplementary Table S9.1: Ascorbate and glutathione concentrations (nmol g^{-1} FW) in roots and leaves of WT, *vtc1* and *vtc2 Arabidopsis thaliana* plants exposed to U, Cu or U+Cu at pH 7.5 during 3 days. Each point represents the mean of at least 4 biological replicates ± S.E. Statistical analysis were done separately for leaves and roots. Data points with different letters are significantly different (p<0.05). Differences between the genotypes as compared to the WT plants within the same treatment are underlined (p<0.05). AsA = reduced ascorbate, DHA = dehydroascorbate, Total AsA = AsA + DHA, % red AsA = reduced AsA/total AsA, GSH = reduced glutathione, GSSG = oxidized glutathione, Total GSH = GSH + GSSG, % red GSH = reduced GSH/total GSH. n.d.: non detectable.

			Total AsA	AsA	DHA	% red AsA	Total GSH	GSH	GSSG	% red GSH
ROOTS	WT	Control	n.d.	n.d.	n.d.	n.d.	102.64 ± 8.79^{ab}	94.18 ± 9.75^{ab}	3.11 ± 0.08^{a}	93.90 ± 0.65^{a}
		U	n.d.	n.d.	n.d.	n.d.	104.81 ± 3.59^{ab}	98.61 ± 4.43^{ab}	2.27 ± 0.23^{a}	93.99 ± 1.74^{a}
		Cu	n.d.	n.d.	n.d.	n.d.	121.08 ± 3.62^{a}	111.58 ± 3.18^{a}	4.75 ± 0.66^{ab}	92.20 ± 1.00^{a}
		U+Cu	n.d.	n.d.	n.d.	n.d.	97.08 ± 5.76^{b}	80.64 ± 7.26^{b}	8.22 ± 2.02^{b}	82.77 ± 4.40^{b}
	VTC1	Control	n.d.	n.d.	n.d.	n.d.	132.78 ± 7.80^{A}	126.18 ± 7.64^{A}	3.30 ± 0.30^{A}	95.00 ± 0.51^{A}
		U	n.d.	n.d.	n.d.	n.d.	135.70 ± 8.44^{A}	124.64 ± 11.43^{A}	5.53 ± 1.90A^{B}	93.38 ± 3.30^{AB}
		Cu	n.d.	n.d.	n.d.	n.d.	119.41 ± 2.13^{A}	102.64 ± 2.27^{A}	8.96 ± 0.09^{B}	85.95 ± 0.97^{B}
		U+Cu	n.d.	n.d.	n.d.	n.d.	121.26 ± 5.68^{A}	114.06 ± 7.39^{A}	3.60 ± 1.07^{A}	93.83 ± 2.12^{A}
	VTC2	Control	n.d.	n.d.	n.d.	n.d.	112.64 ± 7.84$^{a'}$	103.18 ± 8.58$^{a'}$	3.16 ± 0.50$^{a'}$	94.51 ± 0.64$^{a'}$
		U	n.d.	n.d.	n.d.	n.d.	114.86 ± 4.32$^{a'}$	105.88 ± 6.07$^{a'}$	2.66 ± 0.61$^{a'}$	92.11 ± 3.42$^{a'}$
		Cu	n.d.	n.d.	n.d.	n.d.	102.07 ± 5.60$^{a'}$	87.18 ± 5.50$^{a'}$	7.45 ± 1.28$^{a'}$	85.41 ± 2.28$^{a'}$
		U+Cu	n.d.	n.d.	n.d.	n.d.	113.35 ± 5.08$^{a'}$	105.64 ± 5.33$^{a'}$	3.86 ± 1.22$^{a'}$	92.21 ± 2.16$^{a'}$

Supplementary Table S9.1 (continued)

			Total AsA	AsA	DHA	% red AsA	Total GSH	GSH	GSSG	% red GSH
LEAVES	WT	Control	412.79 ± 28.55^{a}	351.33 ± 28.38^{a}	61.46 ± 14.50^{a}	85.07 ± 3.09^{a}	291.12 ± 36.99^{a}	260.46 ± 40.29^{a}	15.33 ± 2.31^{a}	88.46 ± 2.80^{a}
		U	441.05 ± 20.97^{a}	364.44 ± 6.56^{a}	64.37 ± 18.40^{a}	85.91 ± 3.59^{a}	300.30 ± 12.63^{a}	258.57 ± 8.35^{a}	17.49 ± 5.50^{a}	88.42 ± 3.38^{a}
		Cu	452.22 ± 24.75^{a}	417.80 ± 34.52^{a}	34.42 ± 12.52^{a}	92.05 ± 2.87^{a}	311.11 ± 18.14^{a}	281.50 ± 7.97^{a}	4.73 ± 1.96^{a}	96.80 ± 1.03^{a}
		U+Cu	439.49 ± 18.35^{a}	387.65 ± 13.88^{a}	16.51 ± 0.62^{a}	88.45 ± 3.37^{a}	319.36 ± 2.80^{a}	278.85 ± 21.38^{a}	15.76 ± 3.13^{a}	90.03 ± 1.89^{a}
	VTC1	Control	142.15 ± 7.54^{A}	104.73 ± 14.22^{A}	37.42 ± 10.96^{A}	73.18 ± 7.31^{A}	434.45 ± 38.09^{A}	361.94 ± 50.31^{A}	13.73 ± 2.00^{A}	93.43 ± 0.90^{A}
		U	167.63 ± 11.98^{A}	98.31 ± 9.43^{A}	69.32 ± 5.30^{A}	58.46 ± 2.58^{A}	445.08 ± 27.63^{A}	383.44 ± 7.36^{A}	30.82 ± 10.59^{A}	86.85 ± 3.86^{A}
		Cu	136.48 ± 8.47^{A}	80.28 ± 16.45^{A}	56.20 ± 13.94^{A}	58.36 ± 11.01^{A}	458.21 ± 25.44^{A}	429.28 ± 27.61^{A}	12.11 ± 0.41^{A}	94.78 ± 0.41^{A}
		U+Cu	151.92 ± 6.62^{A}	89.90 ± 4.02^{A}	62.02 ± 7.32^{A}	59.49 ± 3.37^{A}	462.49 ± 31.79^{A}	390.87 ± 27.70^{A}	21.21 ± 5.63^{A}	85.08 ± 4.96^{A}
	VTC2	Control	94.59 ± 5.69$^{a'}$	61.45 ± 11.09$^{a'}$	25.13 ± 1.78$^{a'}$	73.55 ± 2.89$^{a'}$	340.9 ± 31.56$^{a'}$	321.40 ± 27.13$^{a'}$	9.64 ± 2.32$^{a'}$	94.58 ± 0.92$^{a'}$
		U	97.90 ± 9.23$^{a'}$	60.40 ± 8.37$^{a'}$	37.50 ± 7.39$^{a'}$	61.89 ± 7.01$^{a'}$	322.60 ± 12.48$^{a'}$	292.78 ± 9.85$^{a'}$	14.91 ± 3.14$^{a'}$	90.86 ± 1.76$^{a'}$
		Cu	108.81 ± 7.98$^{a'}$	67.86 ± 3.95$^{a'}$	33.68 ± 4.72$^{a'}$	65.59 ± 4.28$^{a'}$	340.33 ± 33.09$^{a'}$	308.90 ± 25.68$^{a'}$	6.11 ± 0.57$^{a'}$	69.0 ± 30.65$^{a'}$
		U+Cu	97.02 ± 4.18$^{a'}$	59.09 ± 8.73$^{a'}$	37.93 ± 5.20$^{a'}$	60.14 ± 7.18$^{a'}$	355.85 ± 15.29$^{a'}$	327.02 ± 12.28$^{a'}$	14.41 ± 2.51$^{a'}$	91.98 ± 1.17$^{a'}$

Supplementary Table S9.2: Relative gene expression levels in the roots of WT, *vtc1* and *vtc2 Arabidopsis thaliana* plants of the genes involved in ROS scavenging after exposure to U, Cu or U+Cu during 3 days at pH 7.5. Gene expression is expressed relative as compared to the control of WT plants, which was set to 1. Values represent the mean ± S.E. of at least 3 biological replicates. Data points with different letters are significantly different ($p<0.05$). Significant differences compared to the WT roots within the same treatment are underlined ($p<0.05$).

			CAT1	***CAT2***	***CAT3***	***FSD2***	***FSD3***	***MSD1***	***APX1***	***GR1***	***SPL7***
ROOTS	WT	Control	1.00 ± 0.10^{a}	1.00 ± 0.02^{ab}	1.00 ± 0.08^{a}	1.00 ± 0.11^{a}	1.00 ± 0.20^{a}	1.00 ± 0.10^{a}	1.00 ± 0.11^{a}	1.00 ± 0.04^{ab}	1.00 ± 0.18^{a}
		U	0.94 ± 0.01^{a}	1.30 ± 0.21^{a}	0.83 ± 0.06^{a}	0.96 ± 0.13^{a}	1.38 ± 0.14^{a}	0.99 ± 0.10^{a}	1.44 ± 0.31^{a}	1.22 ± 0.13^{a}	1.04 ± 0.26^{a}
		Cu	0.95 ± 0.10^{a}	0.53 ± 0.07^{b}	1.08 ± 0.03^{a}	0.79 ± 0.07^{a}	1.10 ± 0.09^{a}	0.79 ± 0.09^{ab}	0.44 ± 0.03^{b}	0.70 ± 0.08^{b}	0.51 ± 0.16^{a}
		U+Cu	3.23 ± 0.35^{b}	1.41 ± 0.14^{a}	2.84 ± 0.92^{b}	0.68 ± 0.05^{a}	0.97 ± 0.08^{a}	0.61 ± 0.07^{b}	0.87 ± 0.05^{a}	0.84 ± 0.16^{ab}	0.49 ± 0.05^{a}
	vtc1	Control	$\underline{2.24 \pm 0.49}^{A}$	0.96 ± 0.12^{A}	1.19 ± 0.19^{AB}	0.79 ± 0.11^{A}	0.99 ± 0.14^{A}	0.91 ± 0.03^{AB}	0.89 ± 0.06^{A}	0.87 ± 0.09^{A}	1.11 ± 0.26^{A}
		U	1.08 ± 0.12^{B}	0.87 ± 0.08^{A}	0.69 ± 0.11^{A}	0.65 ± 0.02^{A}	0.81 ± 0.03^{A}	0.86 ± 0.05^{AB}	1.24 ± 0.20^{A}	1.08 ± 0.12^{A}	0.99 ± 0.14^{A}
		Cu	1.76 ± 0.07^{AB}	0.87 ± 0.11^{A}	1.46 ± 0.15^{B}	1.03 ± 0.05^{A}	1.22 ± 0.09^{A}	1.11 ± 0.07^{A}	0.72 ± 0.05^{A}	1.11 ± 0.12^{A}	0.98 ± 0.01^{A}
		U+Cu	2.01 ± 0.23^{AB}	1.19 ± 0.09^{A}	2.08 ± 0.58^{B}	0.77 ± 0.12^{A}	0.83 ± 0.11^{A}	0.73 ± 0.04^{B}	1.02 ± 0.16^{A}	0.85 ± 0.05^{A}	0.54 ± 0.04^{B}
	vtc2	Control	$1.51 \pm 0.29^{ab'}$	$1.11 \pm 0.15^{a'}$	$1.07 \pm 0.16^{ab'}$	$0.75 \pm 0.11^{ab'}$	$0.96 \pm 0.12^{a'}$	$0.80 \pm 0.10^{ab'}$	$0.94 \pm 0.08^{a'}$	$0.76 \pm 0.05^{a'}$	$0.83 \pm 0.13^{a'}$
		U	$0.96 \pm 0.05^{a'}$	$0.99 \pm 0.09^{a'}$	$0.77 \pm 0.05^{a'}$	$0.78 \pm 0.10^{ab'}$	$1.00 \pm 0.14^{a'}$	$0.90 \pm 0.05^{a'}$	$1.01 \pm 0.05^{a'}$	$0.83 \pm 0.06^{a'}$	$0.68 \pm 0.06^{ab'}$
		Cu	$1.21 \pm 0.14^{ab'}$	$0.77 \pm 0.10^{a'}$	$2.12 \pm 0.32^{b'}$	$1.22 \pm 0.14^{a'}$	$1.37 \pm 0.23^{a'}$	$1.03 \pm 0.04^{a'}$	$0.64 \pm 0.07^{a'}$	$0.83 \pm 0.05^{a'}$	$0.82 \pm 0.11^{a'}$
		U+Cu	$2.22 \pm 0.53^{b'}$	$1.13 \pm 0.24^{a'}$	$2.15 \pm 0.46^{b'}$	$0.61 \pm 0.09^{b'}$	$0.74 \pm 0.19^{a'}$	$0.47 \pm 0.09^{b'}$	$0.66 \pm 0.23^{a'}$	$0.61 \pm 0.22^{a'}$	$0.23 \pm 0.10^{b'}$

Supplementary Table S9.3: Relative gene expression levels in the leaves of WT, *vtc1* and *vtc2 Arabidopsis thaliana* plants of the genes involved in ROS production and scavenging after exposure to U, Cu or U+Cu during 3 days at pH 7.5. Gene expression is expressed relative as compared to the control of WT plants, which was set to 1. Values represent the mean ± S.E. of at least 3 biological replicates. Data points with different letters are significantly different (p<0.05). Significant differences compared to the WT leaves within the same treatment are underlined (p<0.05).

			RBOHC	***RBOHD***	***RBOHF***	***LOX1***	***LOX2***	***CAT1***	***CAT2***	***CAT3***
LEAVES	WT	Control	1.00 ± 0.25^{a}	1.00 ± 0.16^{ab}	1.00 ± 0.14^{ab}	1.00 ± 0.08^{ab}	1.00 ± 0.21^{a}	1.00 ± 0.09^{ac}	1.00 ± 0.07^{a}	1.00 ± 0.08^{ac}
		U	2.59 ± 0.93^{a}	1.29 ± 0.22^{a}	1.28 ± 0.11^{a}	0.64 ± 0.13^{a}	0.69 ± 0.21^{a}	1.54 ± 0.12^{bc}	0.66 ± 0.08^{a}	0.94 ± 0.13^{a}
		Cu	0.74 ± 0.12^{a}	1.16 ± 0.11^{ab}	1.34 ± 0.15^{a}	1.40 ± 0.12^{b}	0.87 ± 0.10^{a}	0.58 ± 0.12^{a}	0.73 ± 0.08^{a}	0.33 ± 0.07^{b}
		U+Cu	1.68 ± 1.09^{a}	0.54 ± 0.07^{b}	0.66 ± 0.08^{b}	1.06 ± 0.13^{ab}	0.97 ± 0.19^{a}	2.25 ± 0.41^{b}	0.70 ± 0.08^{a}	1.53 ± 0.20^{c}
	vtc1	Control	2.47 ± 0.72^{a}	0.96 ± 0.11^{a}	1.52 ± 0.11^{ac}	1.25 ± 0.41^{a}	0.60 ± 0.15^{a}	1.36 ± 0.29^{a}	0.46 ± 0.06^{a}	1.00 ± 0.12^{a}
		U	7.51 ± 2.09^{a}	2.33 ± 0.16^{b}	1.83 ± 0.21^{ab}	1.59 ± 0.17^{a}	1.39 ± 0.34^{a}	1.87 ± 0.26^{a}	0.96 ± 0.25^{b}	2.89 ± 0.07^{b}
		Cu	1.55 ± 0.26^{a}	1.89 ± 0.13^{b}	2.11 ± 0.14^{b}	1.22 ± 0.15^{a}	1.03 ± 0.18^{a}	1.31 ± 0.16^{a}	0.50 ± 0.09^{a}	0.82 ± 0.19^{a}
		U+Cu	3.25 ± 1.00^{a}	0.74 ± 0.09^{a}	1.08 ± 0.03^{c}	1.82 ± 0.42^{a}	1.17 ± 0.22^{a}	2.02 ± 0.35^{a}	0.49 ± 0.05^{a}	1.88 ± 0.14^{c}
	vtc2	Control	1.98 ± 0.55^{a}	1.37 ± 0.25^{a}	0.88 ± 0.04^{a}	0.91 ± 0.08^{a}	0.84 ± 0.12^{a}	1.70 ± 0.28^{a}	0.74 ± 0.08^{ab}	1.38 ± 0.20^{a}
		U	1.81 ± 0.50^{a}	2.17 ± 0.59^{a}	1.74 ± 0.36^{b}	1.50 ± 0.15^{b}	0.95 ± 0.19^{a}	2.66 ± 0.18^{b}	1.06 ± 0.19^{a}	1.61 ± 0.16^{a}
		Cu	1.50 ± 0.53^{a}	2.23 ± 0.28^{a}	1.67 ± 0.15^{b}	1.21 ± 0.03^{ab}	1.03 ± 0.13^{a}	0.93 ± 0.06^{a}	0.43 ± 0.03^{b}	0.58 ± 0.05^{b}
		U+Cu	4.60 ± 1.73^{a}	0.88 ± 0.19^{a}	0.94 ± 0.04^{a}	1.67 ± 0.15^{b}	0.27 ± 0.22^{a}	1.40 ± 0.30^{a}	0.45 ± 0.10^{b}	0.96 ± 0.05^{ab}

Supplementary Table S9.3 (continued)

			FSD2	***FSD3***	***MSD1***	***GR1***	***SPL7***
LEAVES	WT	Control	1.00 ± 0.11[a]	1.00 ± 0.11[a]	1.00 ± 0.06[a]	1.00 ± 0.14[a]	1.00 ± 0.16[a]
		U	1.36 ± 0.29[a]	0.9 ± 0.16[a]	1.23 ± 0.14[a]	0.91 ± 0.09[a]	0.66 ± 0.05[a]
		Cu	1.43 ± 0.21[a]	1.19 ± 0.11[a]	1.18 ± 0.14[a]	0.91 ± 0.05[a]	0.97 ± 0.12[a]
		U+Cu	1.30 ± 0.19[a]	0.94 ± 0.15[a]	1.06 ± 0.09[a]	0.85 ± 0.06[a]	0.95 ± 0.10[a]
	vtc1	Control	0.93 ± 0.13[a]	0.87 ± 0.11[a]	0.97 ± 0.05[a]	1.05 ± 0.10[a]	1.11 ± 0.21[a]
		U	0.89 ± 0.09[a]	0.95 ± 0.12[a]	0.98 ± 0.18[a]	0.98 ± 0.09[a]	1.13 ± 0.11[a]
		Cu	0.95 ± 0.07[a]	1.38 ± 0.27[a]	1.11 ± 0.09[a]	0.87 ± 0.14[a]	1.04 ± 0.07[a]
		U+Cu	1.08 ± 0.10[a]	1.15 ± 0.18[a]	1.12 ± 0.08[a]	1.24 ± 0.32[a]	1.19 ± 0.21[a]
	vtc2	Control	1.15 ± 0.14[a]	1.02 ± 0.13[a]	0.87 ± 0.06[a]	0.68 ± 0.12[a]	0.70 ± 0.15[a]
		U	1.09 ± 0.05[a]	0.95 ± 0.04[a]	0.83 ± 0.07[a]	0.76 ± 0.03[a]	0.80 ± 0.02[a]
		Cu	1.14 ± 0.16[a]	1.06 ± 0.08[a]	1.11 ± 0.04[a]	0.82 ± 0.06[a]	0.68 ± 0.05[a]
		U+Cu	0.89 ± 0.10[a]	0.86 ± 0.15[a]	1.12 ± 0.07[a]	0.83 ± 0.11[a]	1.23 ± 0.22[a]

Chapter 10

General discussion, conclusions and perspectives

10.1. Introduction

The main objective of this research concerns the effects of environmentally relevant parameters on U toxicity with the main focus on the influence of the pH. Therefore, hydroponically grown *Arabidopsis thaliana* plants were exposed to U at different pH levels during 3 days. Effects were analysed at different levels of biological complexity: at the level of the individual plants (macroscopic and physiological), at subcellular (biochemical) and at molecular level in order to study the mechanisms that play an important role in the U-induced stress responses in plants. Figure 10.1 gives a schematic overview of the effects that were observed in *Arabidopsis thaliana* roots and leaves after exposure to U at pH 4.5 and pH 7.5.

The second aim of this research is to investigate the effects of U in a multiple stressor setup. It was chosen to set up binary experiments with U and Cu as Cu is a possible co-contaminant in deposits and waste from U mines. In addition, Cu containing ores are also known to include U-associated waste. Finally, the importance of ascorbate (AsA) in the antioxidative stress responses during U exposure was investigated by using AsA deficient vitamin C (*vtc*) mutants of *Arabidopsis thaliana*.

Since the present research was carried out in a hydroponic setup, a direct comparison with the *in situ* or soil situations cannot be made. However, the hydroponic setup was preferred as experimental setup since it has many advantages as compared to soil systems. As such, more controlled experiments could be done by tightly controlling the concentrations of nutrients in the solution and the pH of the solution. *Arabidopsis thaliana* was used since it is a model organism for the study of cellular and molecular processes in plants. Furthermore, this plant is easy to grow, a validated hydroponic setup is available (Smeets *et al.*, 2008), it has a short life cycle and can produce a considerable

amount of offspring. Last but not least, its entire genome has been sequenced and annotated (Poole, 2007) enabling mechanistic studies at a transcriptional level and a huge amount of mutants and transgenic lines are available.

10.2. Toxic effects of U in Arabidopsis thaliana plants

After exposing *Arabidopsis thaliana* seedlings to toxic concentrations of U, a number of defence responses were observed both at molecular and physiological level: (1) the induction of lipoxygenases (LOX) probably inducing an oxidative burst as well as root-to-shoot signalling molecules, (2) the role of miRNA398b/c in regulating the expression of superoxide dismutases (SOD), (3) ascorbate (AsA) as an important antioxidant in U-induced stress responses, (4) the induction of an earlier senescence and (5) a higher photosynthetic efficiency. Although not all of these responses were as pronounced in the different conditions (pH, combination with Cu) or plant organs tested (leaves vs. roots), together they seem to characterise the U toxicity in plants.

Exposing plants to U at pH 4.5 or pH 7.5 resulted in a dose-dependent increase in the U concentration in the roots (Table 5.1 and Figure 7.1). At pH 7.5, this increased U content was possibly accompanied by an enhanced production of reactive oxygen species (ROS), as a significant induction of the transcript levels of *LOX1* was observed after exposure to 75 µM U (Table 7.3 and Figure 10.1). In addition, the increased *LOX1* expression can indicate an enhanced lipid peroxidation, leading to the production of precursors for signalling molecules such as oxylipins, as has been reported previously by Vanhoudt *et al.* (2011b).

Concerning ROS-scavenging, U exposure induced an increased expression of *MIR398b/c* both at pH 4.5 and pH 7.5 (Table 5.4, Table 6.4, Table 7.3, Table 7.4 and summarized in Figure 10.1). It is known that miRNA398b/c negatively affects the expression of *CSD1* (cytoplasmic copper (Cu)/zinc (Zn) SOD) and *CSD2* (plastidic Cu/Zn SOD) (Zhu *et al.*, 2011). As expected, an inhibition of the *CSD1/2* expression levels was detected after U exposure (Table 5.4, Table 6.4, Table 7.3 and Table 7.4). To counteract the decreased expression of *CSD1/2*, the *FSD1* expression was upregulated in the roots (Table 5.4 and Table 7.3). In contrast, in the leaves the decreased expression of the CSDs was not accompanied by an increased expression of *FSD1* at both pH levels (Table 6.4

and Table 7.4). A decreased Fe content was observed in the leaves after U exposure at pH 7.5, possibly indicating that U interferes with the Fe translocation in the plants, as was observed before by Vanhoudt *et al.* (2011a) in plants exposed to U at pH 5.5. Since Fe is a cofactor of the FeSOD enzymes, a deficiency of Fe can lead to a decreased *FSD1* expression.

In addition, U exposure seems to induce the biosynthesis of AsA in the leaves at both pH levels. Ascorbate is a metabolite involved in the scavenging of ROS. The increased AsA content in the present study may indicate the importance of AsA in the antioxidative defence mechanisms in the leaves during U stress. In an attempt to elucidate the role of AsA in the U response, AsA deficient *vtc* mutants of *Arabidopsis thaliana* (*vtc1* and *vtc2*) were exposed to 25 µM U. Results showed that the U-induced miRNA398b/c responses were more pronounced in the leaves of *vtc1* plants as compared to the WT plants. This further supports the assumption of AsA as a regulator of U-induced stress responses. However, additional research is needed to further elucidate the role of AsA under U stress and how it influences the *MIR398b/c* expression.

Another general response after U exposure is the induction of early senescence in *Arabidopsis thaliana* leaves. At both pH levels, a decreased expression of *CAT2*, accompanied by an increased *CAT3* expression and an increased lipid peroxidation were observed. In addition, although no decrease of GR activity was detected, the expression levels of *GR1* and *GR2* were markedly decreased, indicating the induction of early senescence in U-exposed leaves (Dhindsa *et al.*, 1981; Jiménez *et al.*, 1998; Zimmermann and Zentgraf, 2005).

Finally, when plants were exposed to different U concentrations at pH 4.5, photosynthesis seemed to be strongly influenced. In contrast to what is seen for most metal-stressed plants, an increased electron transport rate of photosystem II (PSII) was observed, possibly leading to an increased fraction of open reaction centres (Figure 8.4). In addition, more of the absorbed quanta were used for photosynthesis, leading to a decreased non-photochemical quenching of the energy. This suggests that at low pH, the plant is able to optimize its photosynthesis as a response to the U-induced stress. Since the enhanced photosynthesis was accompanied by a decreased growth of the plants, this could indicate that the energy produced during photosynthesis is not used for growth

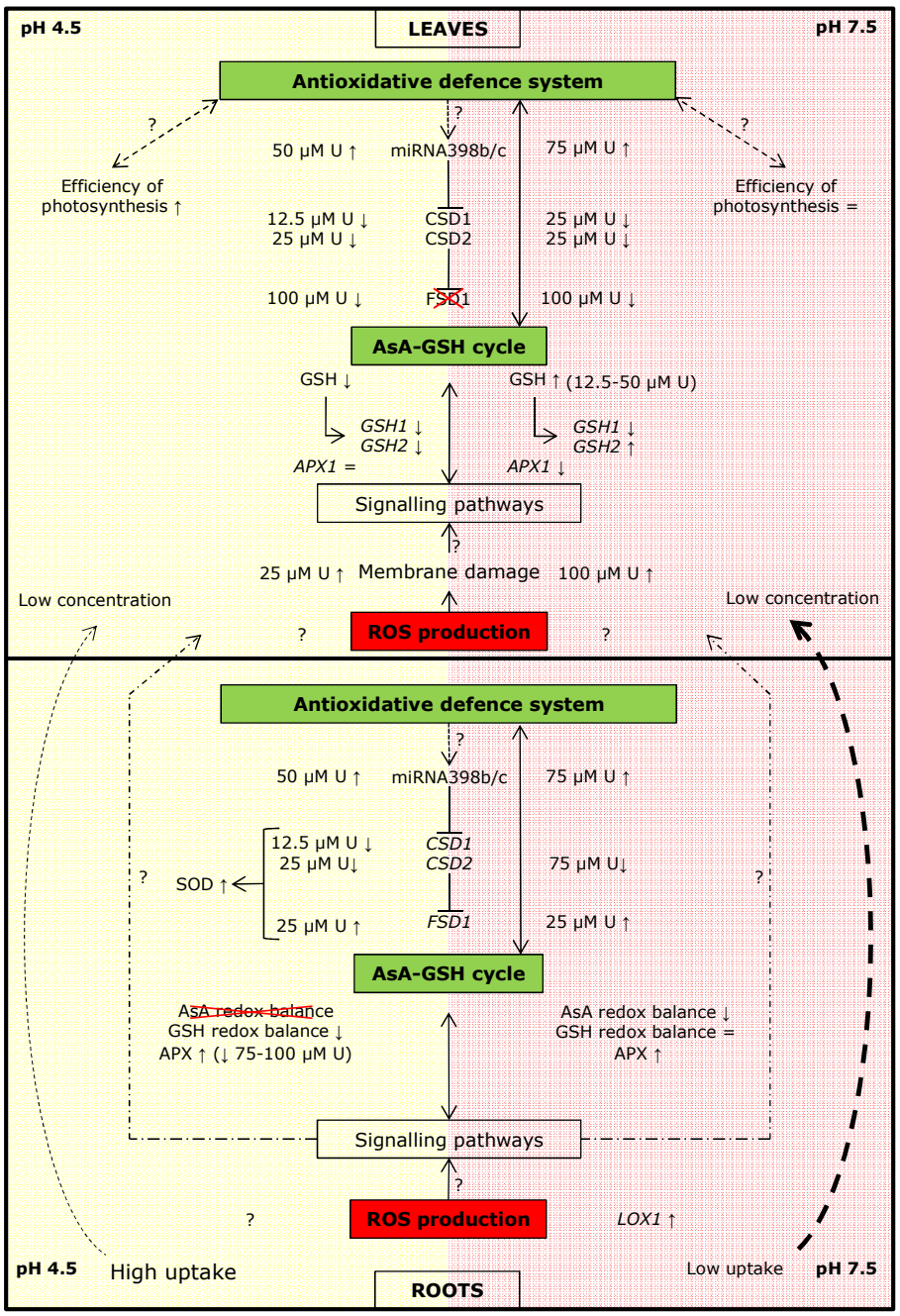


Figure 10.1: General scheme of the U-induced stress responses at pH 4.5 and pH 7.5 in *Arabidopsis thaliana* roots and leaves. APX: ascorbate peroxidase; AsA: ascorbate CSD: copper/zinc superoxide dismutase; FSD: iron superoxidedismutase; GSH: glutathione; miRNA398: microRNA398; ROS: reactive oxygen species.

but for defence responses against U-induced oxidative stress. As an increased efficiency in photosynthesis was not reported before during heavy metal stress, this might be a U-specific response.

10.3. Influence of the pH on U toxicity in Arabidopsis thaliana roots

Exposing roots of *Arabidopsis thaliana* seedlings to 25 µM U at different ecologically relevant pH levels, resulted in a three-fold higher uptake at pH 4.5 as compared to pH 7.5 (Figure 4.1A). The increased U content at low pH was accompanied by a significant decrease in root fresh weight (Figure 4.2) and an increased root dry weight (Table 4.2), indicating that those roots have a disturbed water balance. In addition, the AsA redox balance shifted towards a more oxidized form (Table 4.4) indicating that the roots exposed to U at low pH are stressed. These effects were not detected in the roots of *Arabidopsis thaliana* plants exposed to 25 µM U at pH 7.5. Due to the large differences observed between pH 4.5 and pH 7.5 after exposure to 25 µM U, with most effects being observed at low pH, the U-induced effects were analysed in more detail at those two pH levels by investigating the dose-dependent effects of U.

When plants were exposed to different U concentrations, ranging from 0 to 100 µM U at pH 4.5 or pH 7.5, a dose-dependent increase of the U concentration in the roots was observed at both pH levels (Table 5.1 and Figure 7.1). This increase was accompanied by a decreased root fresh weight (Figure 5.1 and Figure 7.2) and increased percentage dry weight (Table 5.1 and Table 7.1) at both pH levels. However, while at pH 4.5 a transient hormesis effect was observed at low U concentrations, this was not noticed at pH 7.5. Based on the root growth reductions found in the dose-response curves, an EC50 value for growth reduction was determined. The EC50 value for the inhibition of root growth at pH 4.5 (28.14 ± 1.59 µM U) is approximately 2.5 times lower than the EC50 value that was observed at pH 7.5 (70.24 ± 10.48 µM U). This may indicate that U is more toxic to plants at low pH. In addition, the differences in the EC50 value can be explained by the fact that for similar nominal U concentrations U was more readily taken up by the roots at low pH, possibly leading to more adverse effects.

In the roots of seedlings exposed to U at pH 7.5, an increased production of precursors for signalling molecules such as oxylipins can be present, since a significant induction of the transcript levels of *LOX1* was detected after exposure to 75 µM U (Table 7.3 and Figure 10.1). This agrees with the results of Vanhoudt *et al.* (2011b) who observed an increased *LOX1* expression in roots of *Arabidopsis thaliana* plants exposed to U at pH 5.5. In contrast, no induction of the NADPH oxidases or LOX was observed in the roots exposed to U at pH 4.5 (Table 5.4). This difference might be explained as a pH effect rather than an effect of U, since control plants of *Arabidopsis thaliana* grown in the absence of U but at low pH already showed some stress (chapter 4). This can lead to an elevated *RBOH* and *LOX* expression under control conditions. The latter might explain that there is no further increase in expression after U exposure. However, to confirm this hypothesis, transcript levels of ROS producing enzymes in plants grown at different pH levels should be investigated.

To allow ROS for signalling, but to limit oxidative damage, cells possess an antioxidative defence system comprising ROS-scavenging enzymes and metabolites (Arora *et al.*, 2002). SOD constitutes the first line of defence by dismutating superoxide ($O_2^{\bullet -}$). An increasing trend in the enzymatic SOD activity was detected at low pH, while this was not observed in the roots exposed to U at pH 7.5. At molecular level the observed responses concerning CSD, FSD and miRNA398b/c were similar, as described in §10.2. However, the concentrations at which those effects were detected are different (Figure 10.1). The increased expression in *MIR398b/c* was already observed at low pH after exposure to 50 µM U (Table 5.4), whereas at pH 7.5 a significant increase was found after exposure to 75 µM U (Table 7.3). In addition, the inhibition of *CSD1/2* occurred at pH 4.5 at lower concentrations than at pH 7.5.

Another important ROS-scavenging system is the AsA-GSH cycle. While the AsA redox balance shifted to the oxidized state at pH 7.5 (Table 7.2), the AsA levels in the roots of plants exposed to 50-100 µM U at low pH could not be determined, indicating that the roots were totally damaged. In addition, at low pH a reduced GSH content was observed accompanied by a significant decrease of the GSH redox balance. At pH 7.5, on the other hand, the GSH redox state could be maintained (Figure 10.1).

In conclusion, our results indicate that in the roots U causes more adverse effects at low pH. On one side, this can be linked to the higher U concentrations present in those roots. However, since it was stated before that the free uranyl ion (UO_2^{2+}) is the most toxic U species, the differences in toxicity can possibly also be linked to the fact that at pH 4.5 57.28% of U was present as UO_2^{2+} whereas at pH 7.5 less than 1% of this species was present (Table 4.1).

10.4. Influence of the pH on U toxicity in Arabidopsis thaliana leaves

When *Arabidopsis thaliana* plants were exposed to 25 µM U at different pH levels, low U concentrations were observed in the leaves, indicating a low root-to-shoot transfer of U, as was expected. However, large differences were observed between the different pH levels. As such, the highest U concentration in the leaves was found at pH 7.5, indicating that at high pH U can more easily be transported to the leaves, while at pH 4.5 transfer to the shoots is limited (Figure 4.1B). This can possibly be explained by the fact that at high pH, U-carbonate complexes are prevalent whereas at low pH, the dominant U species was predicted to be UO_2^{2+}. This free uranyl ion is a highly reactive ion which can rapidly precipitate with phosphate moieties present in the root cell membranes or react with cellulose-, pectin- or glycoprotein-rich compounds in the cell walls. These processes can lead to the immobilization of U at the root level, resulting in a lower root-to-shoot transfer at low pH. Uranium-carbonate complexes, on the other hand, are more mobile and can be transferred to plant shoots more easily (Laurette *et al.*, 2012).

The dose-dependent effects in the leaves of *Arabidopsis thaliana* plants were investigated to obtain a more detailed picture of the U-induced stress responses. After U exposure, a significant reduction in leaf fresh weight (Figure 6.1 and Figure 7.2) and an increased percentage dry weight (Table 6.2 and Table 7.1) was observed at both pH levels. This indicates that U caused a disturbed water balance and that plants start to wilt. Although the U concentration in the leaves at pH 4.5 was approximately 3 times lower than at pH 7.5, the EC50 value calculated for leaf growth reduction at pH 4.5 (27.13 ± 5.20 µM U) was approximately 2 times lower than the EC50 value observed at pH 7.5 (53.74 ±

3.51 µM U). This suggests that U is more toxic to the leaves when plants were exposed to U at low pH. The suggestions that the leaves are more stressed at low pH (and hence with lower U concentration in the leaves) is supported by the analysis of lipid peroxidation as a measure for membrane damage. As such, a significant increase in the TBA-reactive compounds was already observed after exposing *Arabidopsis thaliana* seedlings to 25 µM U at pH 4.5, while a significant increase at pH 7.5 was only observed after exposure to 100 µM U (Figure 10.1). The differences in concentration at which effects are observed, can possibly be attributed to the different species present at both pH levels. Another possible explanation might be the occurrence of root-to-shoot signalling. Since the roots exposed to U at low pH are seriously stressed, this can lead to the activation of defence reactions in the leaves via still unknown signals from the roots to the shoots.

The effects of U on photosynthesis were investigated since inhibition of photosynthesis can affect the physiological state of a plant. While at pH 4.5 an optimization of the photosynthetic processes occurred (§10.2), no effects of U on photosynthesis were observed at pH 7.5. A possible explanation for this discrepancy can be that at pH 7.5 more U was present in the leaves as compared to pH 4.5 and, as such, was leading to direct toxic effects. However more conceivable, it is possible that due to the high U concentration present in the roots of plants exposed to U at pH 4.5, a higher sink for sugars needed during plant defence responses in the roots or an altered nutrient profile in both roots and shoots contribute to the occurrence of root-to-shoot signalling, which in turn can lead to an optimization of the photosynthesis.

In the leaves of U-exposed plants at both pH levels, the investigated NADPH oxidases and LOX are not involved in the ROS production under U stress since no significant inductions in the expression levels of the NADPH oxidases or LOX genes were observed after U exposure (Figure 10.1). Therefore, the mechanisms of ROS production in the leaves of U-exposed *Arabidopsis thaliana* plants should be further investigated. Concerning the ROS-scavenging mechanisms, no significant differences were found in the enzyme capacities at both pH levels. At molecular level, it seems that U induces the miRNA398b/c response in the leaves at both pH levels (Table 6.4 and Table 7.4). Although the induction of

miRNA398b/c seems to be a general U response, the concentration at which this induction occurred differed between the two pH levels. At pH 4.5, the induction of *MIR398b*/c occurred already at 50 µM U while at pH 7.5 a significant induction was only observed from 75 µM U onwards. Like in the roots, it appears that U causes more effects at low pH. Since root-to-shoot signalling might be involved in the leaf responses, further investigations are needed to elucidate the role of root-to-shoot signalling and to investigate which molecules contribute to the signalling.

10.5. Uranium effects in a multipollution setup

Since organisms are typically exposed to multiple stressors, it is important to investigate the effects of U in multiple stressor conditions in order to provide a more realistic estimation of the environmental risks. To investigate the influence of U in a multipollution setup, *Arabidopsis thaliana* plants were exposed to U or Cu or a combination of both elements. A summary of the observed data is presented in table 10.1. Being an essential micronutrient, Cu was easily taken up by the roots, while its transfer to the shoots was limited. However, it seems that U interferes with the Cu uptake since in a multipollution setup, the Cu concentrations in the roots markedly decreased (Table 9.2). Although Cu did not affect U uptake by the roots, it interferes with the U translocation from roots to shoots. When plants were exposed to both U and Cu, the translocation of U to the shoots was strongly inhibited, resulting in a root-to-shoot transfer factor that was approximately 4 times lower than when U was applied as a single stressor.

Table 10.1: Scheme of the U, Cu and U+Cu induced stress responses in *Arabidopsis thaliana* roots and leaves.

ROOTS	U	Cu	U+Cu
Fresh weight	=	↓	=
GSSG	=	=	↑
RbohD	↑	↑	↑↑
RbohF	↑	↑	↑↑
LOX1	↑	↑	↑↑↑
MIR398b	↑	=	=
CSD1	=	=	↑
CSD2	=	↑	↑↑
FSD1	↑	↓	↓

LEAVES	U	Cu	U+Cu
Fresh weight	=	↓	=
APX1	=	↓	=
MIR398b	=	↓↓	↓↓
MIR398c	=	↓↓↓	↓
CSD1	=	↑	=
CSD2	↓	=	=
FSD1	=	↓↓	↓

In the roots, the ROS production was enhanced after exposure to U or Cu, illustrated by increased expressions of *RBOHD/F* and *LOX1* (Figure 9.5). However, in a multipollution setup, the expressions of *RBOHD, RBOHF* and *LOX1* were even more increased, with a 20-fold increase in *LOX1* expression as compared to the expression levels under single stressor conditions. This suggests an important role for LOX1 in the ROS production when plants are exposed to both U and Cu. However, more research is needed to characterize the interaction between U and Cu. While in the roots an enhanced ROS production was observed, no increased expression of the RBOHs or LOX was observed in the leaves.

As expected from previous research, the miRNA398b/c response observed in U-exposed roots is in contrast to what was found in Cu exposed roots (Figure 9.7), although the observed results are not always significantly different. In a multipollution setup, the responses concerning the regulation of SOD transcript levels followed the response found under Cu stress. As such, it seems that under Cu excess, the expression of *MIR398b/c* is downregulated.

Like in the roots, the *MIR398b*/c response observed in the leaves after exposure to U+Cu was similar to the response found under Cu stress. However, the responses concerning the AsA metabolism (APX capacity and *APX1* expression) under the mixed stressor condition tends to follow the U response. As such, no decrease in the *APX1* transcript levels and in the APX capacity was observed under U stress and in a multipollution setup. This again seems to indicate an important role for AsA in the antioxidative defence responses in *Arabidopsis thaliana* plants after U exposure, although more research is needed.

In conclusion, in a multipollution setup the observed ROS production was higher than expected, which might result from a synergistic effect between U and Cu. However, more research is needed to further elucidate the interactions between U and Cu by carrying out additional experiments with different U and Cu concentrations and ratios. In addition, the response regarding miRNA398b/c was opposite under Cu stress as compared to U stress, as was expected from previous research. Finally, it appears that AsA plays an important role in the antioxidative defence responses in *Arabidopsis thaliana* plants after U exposure,

as was also suggested in the studies concerning the dose-dependent effects of U at pH 4.5 and pH 7.5.

10.6. Conclusions

The aim of this study was first to investigate the influence of different environmental parameters on U toxicity, with the main focus on the pH. Next, the influence of a secondary stressor on the U-induced effects was analysed.

Exposing plants to U at pH 4.5 seems to be more toxic than exposing plants at pH 7.5. In the roots, this was reflected in a lower EC50 value, no intact RNA and a disturbed AsA and GSH balance in plants exposed to higher U concentrations at low pH. The differences in effects can on one side be linked to the presence of UO_2^{2+}, which was mainly present at low pH. However, since U is more easily taken up by the roots at low pH, this will possibly also contribute to the differences in toxicity.

Also in the leaves, effects were observed at low pH at lower nominal U concentrations as compared to pH 7.5, although the U concentration in the leaves at pH 4.5 was about 3 times lower than at pH 7.5. As in the roots, this can possibly be linked to the presence of UO_2^{2+} at low pH. In addition, this can indicate the importance of root-to-shoot signalling for the induction of the oxidative defence mechanisms.

In conclusion, it seems that pH strongly influences U toxicity, with more toxic effects observed at low pH. The differences in U toxicity at different pH levels observed in this research stress the need to take site-specific characteristics into account when making a risk assessment for U-contaminated areas.

10.7. Perspectives

The present research elucidated some general U-induced stress responses in *Arabidopsis thaliana* plants. To get a more profound insight in the U-induced stress response mechanisms and to identify possible biomarkers to better evaluate the environmental impact of U, the underlying mechanisms of the stress responses should be further investigated. A genome wide expression analysis can be used to identify some important mechanisms under U stress. In

addition, since root-to-shoot signalling seems to be involved in the U-induced stress responses, the role and induction of specific signal transduction pathways like jasmonates or other signalling molecules under U stress should be studied. Since it seems that AsA plays an important role in the U-induced stress responses, the role of the AsA-GSH cycle in U-induced stress responses should be further investigated by using AsA or GSH mutants and double AsA-GSH mutants of *Arabidopsis thaliana* plants. Knowledge on U uptake and translocation mechanisms can also contribute to a better understanding of U toxicity. In addition, the U speciation and the subcellular localization of U inside the plants should be examined to identify which species are contributing to the toxicity. Differences in the subcellular localisation might help to explain the differences in the root-to-shoot transfer of U.

Since the pH and other environmental parameters can strongly influence U speciation and toxicity, those parameter should be taken into account to prevent over- or underestimations of the environmental risk. To further study the influence of pH and other cations on U toxicity, a biotic ligand model (BLM) could be developed, *i.e.* a model to predict and evaluate the bioavailability and toxicity of metals. This model includes the complexation of the free metal ion with abiotic ligands and the competition with other cations for binding with the site of toxic action on the organism (Niyogi *et al.*, 2004). However, developing a BLM for *Arabidopsis thaliana* is rather challenging, since for different concentrations of at least 5 cations, dose-response curves have to be set up and analysed in function of different U species present in the medium. However, research in this direction will lead to a better evaluation of the environmental risks of U contamination.

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