TEMPLATE APPLICATON FORM (based on NWO Open Competition Domain Science – KLEIN-1)

Part A – Applicant

A.1 Applicant

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Part B – Scientific proposal

B.1 BASIC DETAILS

B.1.1 Title

Sink nor swim: Potato (*Solanum Tuberosum*) root survival during waterlogging and hypoxia

B.1.2 Abstract

Flooding and waterlogging conditions are detrimental to tuber development in *Solanum tuberosum* and change nutritional composition. As extreme weather events are becoming increasingly common, waterlogging stress affecting potato production poses a threat to food security. Currently, the below-ground architecture under waterlogging in potato is not well-characterized in mature plants. The research proposed here aims to better understand how potato roots are affected by waterlogging, and to explore different pathways that will help improve potato production under increasingly wet conditions. First, we will develop a novel set-up in order to understand to which extend hypoxia contributes to the damage done by waterlogging stress in potatoes. Next, we will develop an molecular oxygen-sensor that will elucidate the role of hypoxia in different tissues under waterlogging conditions. Finally, we will investigate the gene expression and alternative splicing activity during waterlogging and hypoxia treatments in root tissue. Building forth on what is known about model root systems such as that of model species *Arabidopsis thaliana*, we will translate and extend the existing body of knowledge regarding potato roots under waterlogged conditions. This will provide a solid foundation of knowledge for future improvements of this stress-sensitive and economically important staple crop.

B.1.3 Layman's summary

Unlike animals, plants do not have the ability to run away from predators or other threats. Instead, they have had to find a way to survive a broad range of harmful conditions. These can include extreme temperatures, flooding, drought, lack of light or diseases. If during a flooding event only the roots become submerged, we speak of waterlogging. Waterlogging limits oxygen supply to the roots, causing the to stop growing and eventually die. A concentration of less than 21% of oxygen is called hypoxia. Hypoxia is one of the main reasons root tissue cannot survive under waterlogging conditions, but is also an important signal for the plant that it is in trouble. In this NWO proposal, we will investigate and look to improve the resistance of potato plants under waterlogging and root hypoxia. Potato plants are very important because they are a staple that constitute and important part of many people's diet.

First, we will develop a new method to expose potato roots to hypoxia and compare potato varieties of differing resistance to waterlogging. By separating the stress that hypoxia gives from the stress that waterlogging as a whole gives, we can learn

NWO-ENW v190703

more about the problems that the potato roots experience. This will aid us finding processes that actually help potato roots survive.

Next, we will develop a molecular oxygen sensor that will show which parts of the roots become hypoxic first, how long it takes for the roots to become hypoxic, and finally how hypoxic they finally get. Because the creation of this sensor may be a lengthy process, we will develop two sensors alongside each other as a back-up.

Last but not least we will investigate how gene expression is affected by waterlogging and hypoxia. In order to do so, we will take samples from potato plants at different moments during waterlogging treatment and during the subsequent recovery. The data will be analysed for changes in amount of gene transcripts, but also the amount of change in these transcripts by so-called 'alternative splicing'. This is common process that can heavily influence the production of protein because it creates different versions (isoforms) of gene transcripts.

Together, the research proposed here will greatly help the future development of more flooding-resistant potatoes. This will be much needed in our rapidly changing climate that causes more and more extreme weather.

B.1.4 Keywords

Potato, *Solanum tuberosum*, waterlogging, hypoxia

B.2 SCIENTIFIC PROPOSAL

Relevance

The total global food demand is expected to increase with up to 56% between 2010 and 2050 (van Dijk et al., 2021). Currently, 1.3 billion people on our planet depend on potatoes as a staple food (Devaux et al., 2021). At the same time, a warmer climate will intensify very wet and very dry weather and climate events and seasons, with implications for flooding. In its latest report, the IPCC stated that at merely 1.5°C global warming, heavy precipitation and associated flooding are projected to intensify and be more frequent in most regions in Africa and Asia, North America and Europe (IPCC 2021).This area overlaps with the currently most important potato growth zones. The highest concentration of potato production is found in the temperate zone of the northern hemisphere where the crop is grown in summer (Devaux et al., 2021). Flooding and waterlogging conditions are detrimental to tuber development and change its nutritional composition (Ekanayake, 1994; Orsák et al., 2020, 2021). Thus, waterlogging stress affecting potato production increasingly poses a threat to food security. Currently, the below-ground architecture under waterlogging in potato is not well-characterized in mature plants. Moreover, waterlogging has been relatively understudied compared to whole-plant submergence even though flooding is a growing field. The research proposed here aims to better understand how potato roots are affected by waterlogging, and to explore different pathways that will help improve potato production under increasingly wet conditions.

The terminology used to describe oxygen concentrations has been debated in the past. To prevent confusion, this proposal will use the terms normoxia (20.95% O_2), hypoxia (any concentration lower than normoxia) and anoxia (the total absence of O_2) as advised by Sasidharan et al., 2017.

Waterlogging and hypoxia stress in plants

As plants are sessile, they require rapid and versatile strategies to adapt to changes in their surrounding environment. This translates into high plasticity in growth and development. One abiotic stress is flooding. Flooding can range from waterlogging, during which only the below-ground organs are exposed to flooded soils, or complete submergence. Waterlogging or submergence impose changes in oxygen availability, CO_2, light, ethylene, mineral nutrients and reactive oxygen species (ROS) (Voesenek & Bailey-Serres, 2015). In contrast to complete submergence, waterlogging does not block gas exchange in the plant's foliage and respiration of the laminar tissue is not directly hampered. However, it is still detrimental to roots and can lead to local and systemic (i.e. shoot-root communication) changes in response to flood water surrounding the roots (Perata et al., 2011). As a result of the low diffusion rate of oxygen in water, the level of oxygen in the plant root zone decreases to seemingly anoxic levels (Vashisht et al., 2011). For this reason, hypoxia treatment is an often-used proxy for flooding stress. In addition to reducing oxygen levels in the rhizosphere, waterlogging also acidifies the soil, cools the soil down and affects accessibility of nutrients such as nitrogen, sulphur, zinc, iron, manganese, phosphorous and potassium (Araki et al., 2012; Fukao et al., 2019). Yet, plant growth or even survival under waterlogging strongly depends on molecular responses that enhance the tolerance of hypoxia (Shiono et al., 2008; Voesenek & Bailey-Serres, 2015).

Plant roots are hypoxic to some extend even under well-drained conditions (Vashisht et al., 2011). Root growth in anoxic waterlogged soils depends on the distance oxygen can travel to sustain the respiratory demands of the root apex as it grows further away from the shoot base and the source of the oxygen (Armstrong, 1980). Three important morphological strategies used by plant roots to cope with flooding are (i) the prevention of radial oxygen loss (ROL), (ii) growth of new adventitious roots and (iii) formation of aerenchyma. (i) The passive diffusion of oxygen present in the roots to the rhizosphere is termed radial oxygen loss (ROL). Many wetland-adapted plants like rice have been shown to increase suberin biosynthesis in the roots, creating a barrier to prevent ROL. (ii) New adventitious roots may be formed from the stem in order to restore root functioning (Yamauchi et al., 2018). These can compensate, at least partially, for the growth inhibition or even death of roots during waterlogging. New adventitious root formation under waterlogging conditions has been found in two relatives of potato, *Solanum lycopersicum* (tomato) (Vidoz et al., 2016) and *Solanum dulcamara* (Dawood et al., 2014; Zhang et al., 2015). Although potato roots are also known to grow adventitious roots, this adaptive trait under waterlogging is not yet shown in potato. (iii) Aerenchyma is a spongy tissue that creates air channels in tissues such as roots, which allows rapid exchange of gases between the shoot and the root. The formation of aerenchyma through induced cell-death provides an internal route for low resistance gas-phase diffusion of

oxygen from above-ground tissues to the root system (Yamauchi et al., 2018). Aerenchyma have been proven to be present in or inducible under hypoxic conditions in a range of plant species such *as Zea mays, Oryza sativa, Pisum sativum, Triticum aestivum* and *Arabidopsis thaliana* (Loreti & Perata, 2020). It has been hypothesized that root thickening in potato is a sign of its ability to form aerenchyma (Munoz-Arboleda et al., 2006) yet no studies have directly evidenced the presence of this tissue in potato. There is great diversity among oxygen homeostasis between plant species, which makes it crucial to conduct this research directly in potato. Using existing literature we will be able to corroborate similarities with model species such as *A. thaliana* while discovering what the differences exist of. *S. tuberosum* has a more complex below-ground system than other plant species commonly studied in this field. Furthermore, apart from being an economically important crop, it can serve as a model for other closely related commercial crops such as tomato and other *solanum* species.

Molecular mechanisms of hypoxia

To make the physiological changes described above possible under waterlogging conditions, oxygen and the lack there-of needs to be sensed by the roots. Oxygen levels are sensed by a class of proteins that belong to group VII of the Ethylene Response Factors (ERF-VIIs). ERF-VIIs activate a range of anaerobic genes by binding to their Hypoxia-Responsive Promotor Element (HRPE) (Gasch et al., 2016). In *Arabidopsis*, five ERF-VII genes (*HRE1*, *HRE2*, *RAP2.2*, *RAP2.3*, *RAP2.12*) are recognized as key regulators for flooding and hypoxia tolerance (Fukao et al., 2019). Several ERF-VIIs homologs are found in potato, such as *stRAP2.12, stHRE1, stHRE2a* and *stHRE2b* (Shi et al., 2021). Of these, *stRAP2.12* expression has been shown to be upregulated in the shoot under hypoxia, but not in the root (Hartman et al., 2020). *StHRE1, stHRE2a,* and *stHRE2b* are affected by hypoxia at the transcriptional level, although the hypoxic responsiveness is not identical (Licausi et al., 2011). Interestingly, reduced oxygen levels induced StHRE1 in the leaves but repressed it in tubers and roots. Instead, StHRE2a and StHRE2b were ubiquitously induced by exposure to low oxygen concentrations. Therefore, these genes are induced or repressed by hypoxia in a tissue-specific manner (Licausi et al., 2011).

The ERF-VIIs are transcription factors characterized by a cysteine residue at their N-terminal (Giuntoli & Perata, 2018). ERF-VIIs are constitutively degraded under normoxic conditions as the cysteine residue is oxidized. This oxidization targets the ERF-VIIs for degradation by the proteasome. Under hypoxia, however, ERF-VIIs are not degraded and can be instead translocated to the nucleus.

It is important to note that besides hypoxia due to external causes such as flooding (acute hypoxia), natural variation of oxygen concentrations throughout plant tissues and organs such as fruits, tubers, seeds and meristems has also been reported (Weits et al., 2021). This chronic hypoxia has been shown to be crucial for potato tuber development (Licausi et al., 2011), and in lateral root primordia and shoot apical meristem, hypoxic conditions are required for meristem activity (Shukla et al., 2019; Weits et al., 2019).

Root development of *S. tuberosum*

Root morphological and anatomical traits determine root growth and functioning in anoxic waterlogged soils (Yamauchi et al., 2018). The potato root system is notoriously inefficient compared to other crops, due to weak and shallow soil penetration and its poor ability to extract water and minerals (Joshi et al., 2016). Potatoes are also highly susceptible to flooding and waterlogging stress. There is however, a plethora of different potato cultivars, a number of which have been shown to more tolerant to submergence than others. Cultivars Festien and Avarna, for example, are relatively tolerant to waterlogging, whereas Seresta, Ambition, and Atlantic are relatively sensitive (Hartman et al., 2020; Munoz-Arboleda et al., 2006).

Potato roots are classified as adventitious roots (AR), as opposed to primary roots that derive from an embryonic radicle. AR develop post-embryonically from cells neighbouring vascular tissues in shoots, stems, tubers, or leaves (Bellini et al., 2014). After a sprout has emerged from the mother or seed tuber, lateral roots called 'basal roots' develop from the junction between tuber and sprout (Figure 1). Posterior, hooked rhizomes known as stolons develop from the stem of the sprout (Figure 1). Their onset is crucial for potato yield, as tubers develop from the swollen part of stolons (Struik, 2007). Therefore, the research proposed here will focus on the development of the potato root system leading up to the formation of tubers.
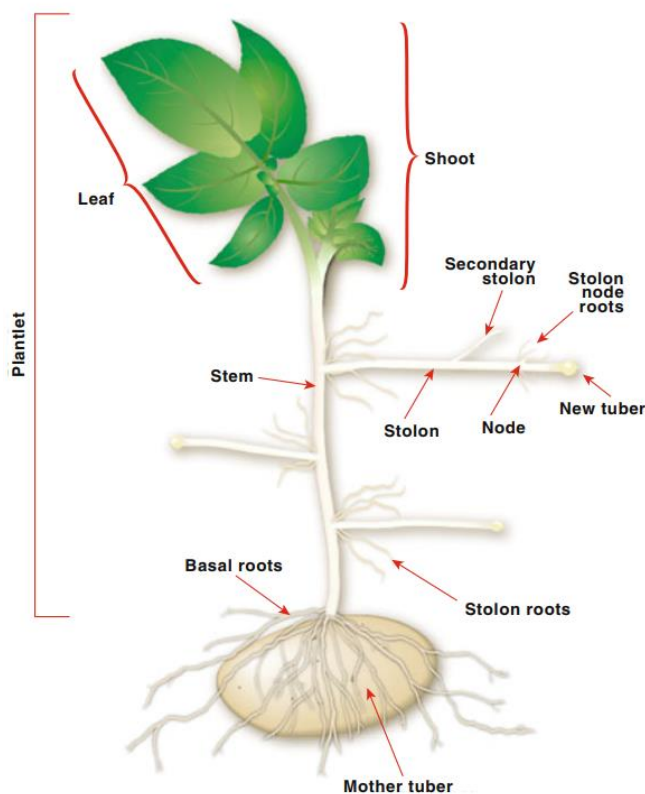
Figure 1 Schematic representation of *S. tuberosum* showing different potato organs. (Adapted from Wishart et al., 2013).

Potato tuber yield and quality reduction under waterlogging stress is determined by the time, duration, developmental stage and severity of the stress, as well as the genotype (Orsák et al., 2020, 2021). This is not surprising, considering that final tuber yield has been shown to be negatively correlated with basal root length and positively although weakly with total root weight (Wishart et al., 2013). Thus, a plant that has a root system that reaches deeper rather than wider will have a higher yield than a plant with a shallow root system due to waterlogging damage.

Objectives

A lot of fundamental research uncovering molecular mechanisms involved in responses to waterlogging has been performed in species such as *Arabidopsis thaliana*. This has led to an extensive body of knowledge regarding plant physiology and molecular pathways activated under waterlogging conditions in a small number of species. However, in agriculturally and economically important crops including *S. tuberosum* (potato), this is a barely explored field. Therefore, this project will aim to increase our current understanding of the physiological and molecular pathways occurring in waterlogged potatoes. Building forth on what is known about model root systems such as that of model species *A. thaliana*, we will translate and extend the existing body of knowledge regarding potato roots under waterlogged conditions. This will provide a solid foundation of knowledge for future improvements of this stress-sensitive and economically important staple crop.

Hypoxia is used as a proxy for flooding and waterlogging. Waterlogging is a compound stress, however, not solely consisting of hypoxia. Therefore, the first objective of this study will be to assess to what extend hypoxia contributes to flooding stress in potato. This will include developing an experimental set-up that can be used to assess potato root development under normal and hypoxic conditions. Secondly, we will quantify oxygen levels in different root tissues under hypoxic conditions. For this purpose, we will develop a molecular oxygen sensor. This biosensor will allow to visualize oxygen levels with high spatial and temporal resolution in vivo. Next, we will investigate how hypoxic and waterlogging soil conditions affect the oxygen concentrations in the potato roots. The third and final objective of the proposed project will consist of corroborating the findings in *A. thaliana* with those in potato. A transcriptome and alternative splicing analysis will allow for the detection of transcriptional and post-transcriptional changes over time under hypoxic conditions. Using a small number of well-chosen

checkpoints, we will be able to model to what extend discoveries done in *A. thaliana* can be translated to and manipulated in *S. tuberosum*. Therefore, we will be able to confirm which relevant biological and cellular processes especially regarding roots understood in *A. thaliana* are conserved in potato and could be used for the development of novel waterlogging-tolerant potato varieties.

WP1: Potato roots under waterlogging and hypoxic conditions

The goal of the first work package will be to understand to which extend hypoxia contributes to the damage done by waterlogging stress in potatoes. For this package we will use one variety that is regarded in literature as typical resistant and one that is regarded typical sensitive to waterlogging stress. First, we will investigate the physiological changes occurring under waterlogging stress, focussing on the roots and their environment. Next, we will identify early physiological responses to waterlogging stress. Then, we will develop a novel experimental set-up studying hypoxia in potato roots. Finally, we will characterise potato development and architecture under root hypoxia.

1.1 Targeted screen of potato cultivars

The large number of different cultivars used in agriculture today present an equally large spectrum of ability to deal with environmental stress such as waterlogging (Pradel et al., 2019). As data on a number of cultivars considered waterlogging resistant or waterlogging sensitive is readily available, a thorough literature search will be able to render a selection of each category (resistant/sensitive). Then, three cultivars of each category will be subjected to a relatively simple waterlogging experiment to assess their response. Promising candidates might be Festien, Avarna (Hartman et al., 2020) for tolerant cultivars and Seresta, Ambition (Hartman et al., 2020), and Atlantic (Munoz-Arboleda et al., 2006) for sensitive varieties. A list with possible backups will be compiled to refer to in case the selected varieties proof impossible to obtain or cultivate, or if challenges arise during the experimental phase. Based on this targeted waterlogging screen, one cultivar of each category will be used for the remainder of WP1. They will serve as a model by representing each end of the waterlogging-response spectrum. For reproducibility and experimental set-up purposes, we will use *in vitro* propagated potato plants rather than seed-potato grown plants.

Plants will be three weeks old at the time of experiments. These plants are still plastic and not as sensitive to stresses as very young plants. At this age, potatoes are maturing plants that are not yet flowering nor producing tubers. This means that the plants have not yet entered the tuberization stage and will therefore be mature enough while not too old. As the plant enter the flowering and tuberization stage, significant hormonal shifts and developmental changes will dilute crucial information about waterlogging and hypoxia.

1.2 Characterise potato development and root architecture under waterlogging conditions

Our second objective will be to characterise root physiology during waterlogging stress compared to control conditions. In this work package, extensive phenotyping will be carried out in order to obtain a physiological understanding of waterlogging stress in potato roots. This will aid in discovering which physiological parameters are reliable indicators of waterlogging stress in potato in WP1.2 and will be used in the following work packages as indicators. Priority traits for easy assessment indicators of waterlogging tolerance might be less reduction of leaf size (Hartman et al., 2020) and a decline of stomatal conductance (Khan et al., 2020). However, very few indicators regarding root physiology or root function are known. We will compare waterlogged plants of both selected varieties with controls in order understand what coping strategies give the resistant variety its relative resilience and what traits make that the sensitive variety does not cope well. During this assessment, special attention will be paid to three adaptive traits found in other plants, that show potential for potatoes. These will be the prevention of radial oxygen loss (ROL), growth of adventitious roots and formation of aerenchyma.

The next step will be to determine a number of parameters to be measured. Candidate parameters are timing of stolon formation, stolon shape, stolon length, root hair count, and number of branches for root development, and stomatal conductance for adaptation. These parameters are often used in to assess root health under abiotic stress conditions, but little is known about them in potato under waterlogging conditions and subsequent recovery.

Furthermore, we propose to use a model for further exploration of the collected the data. Recently, a new model was developed to assess potato root development by Zhao et al. This 3D visual model of the tuber-root system was constructed and verified by experiments. We will use this model (Zhao et al., 2020) to map changes in root architecture under waterlogging conditions. The use of this model will aid the reproducibility of our experiments but, more importantly, it will document changes in growth in an unbiased manner and give a complete overview. This way, also changes that might lead to novel insights and will otherwise fail to be observed, will be recorded. So far, this model has been based on Fujin, Zaodabai, and Helanshiwu, cultivars commonly grown in Northeast China. By integrating phenotyping data of our selected cultivar in this unbiased model, we will obtain an improved understanding of physiological changes during waterlogging stress in potato roots. Reliable physiological indicators and possible adaptive traits will not only be identified, but also recorded in a

standardized manner. These indicators can both be applied in the field and will aid rapid progress in WP1.3 and WP2.

Finally, the absolute reduction of oxygen levels over time in the soil and the rhizosphere during waterlogging experiments will be measured using a microsensor. These state-of-the-art probes can obtain results with high spatial and/or temporal resolution via non-destructive infiltration. The smallest type of O2 microsensors currently developed have a tip diameter of ~3 μm (Pedersen et al., 2020). This makes it more than suitable for our measurements. Different measurements will be taken over a to be determined time period during the treatment and subsequent recovery fase, at several soil depths, and at several distances to the roots. The information thus obtained about O_2 concentrations in the soil will be utilised in WP1.3 in order to accurately mimic hypoxic conditions as they may be found in waterlogged soils.

1.3 Develop novel experimental set-up for hypoxia

A novel experimental set-up will be developed that will allow real-time monitoring of both root development and oxygen concentrations present in the root. This experimental set-up will isolate hypoxia from the compound stress that waterlogging is. Such a set-up will replicate physiological relevant growth conditions that will allow studying a natural change of oxygen concentrations as occur during waterlogging. The most important aspect of this set-up is the oxygen concentration surrounding the roots. Plant roots are hypoxic even under well-drained conditions but rapidly become virtually anoxic under flooding conditions (Vashisht et al., 2011). Therefore, empirical obtained data in WP1.1 will be used to mimic accurate soil oxygen concentration, both for well-drained and for waterlogged conditions.

Using the enzyme system oxyrase, oxygen can be removed form liquids and semi-solids such as agar. Watering plants with an oxyrase solution will most accurately mimic field growing conditions while assuring oxygen depletion (Figure 2A). The use of oxyrase has been successful for creating a stable anaerobic condition for plants grown both in hydroponics (Guangchen et al., 2021; Nishimura et al., 2020) and agar (Lokdarshi et al., 2016; Sorenson & Bailey-Serres, 2014) but to our knowledge not yet for soil-grown plants. Nevertheless, this method can be easily implemented into standard plant-growing facilities and is relatively low in costs. If oxyrase does not prove to be a reliable method for quickly and consistently creating anoxic soil conditions, we will be able to resort to stagnant (semi-solid) agarose or hydroponics (Figure 2B). With the appropriate controls these will also provide the desired experimental conditions, but are more labour-intensive and expensive. Alternatively, the air surrounding the shoot of the plant might be separated from the soil using a physical barrier (Figure 2C). The resulting compartment in which the roots grow can then be filled with oxygen-depleted air consisting of humidified 100% N2 gas. This method is similar to the use of air-tight closed glass desiccators in Hartman et al. (2019). The physiological parameters determined during work package 1.1 will then provide easy and rapid insight in the stress response of the potato plant.
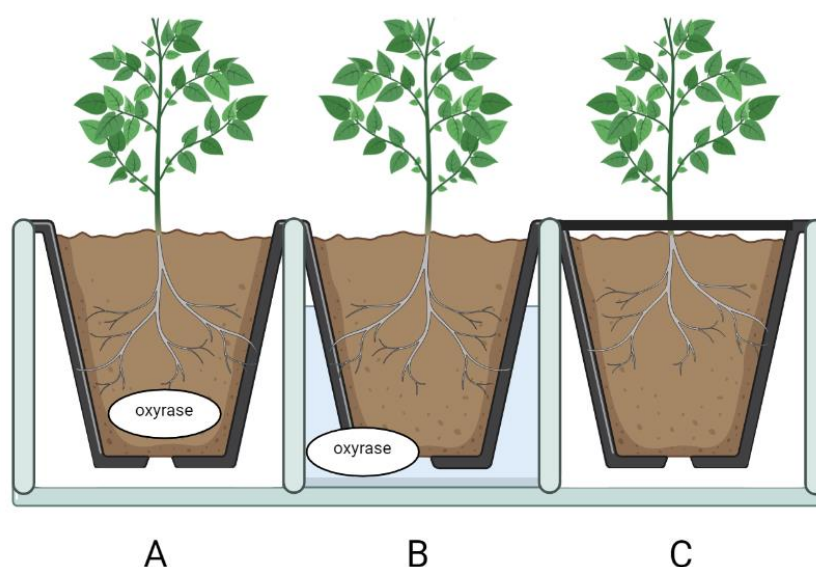
Figure 2 Potential experimental set-up for hypoxia treatment to potato roots, with A) watering with an oxyrase solution, B) an oxyrase solution/oxyrase-containing medium, or C) using an air-tight barrier and filling the compartment with oxygen-depleted air (humidified 100% N2 gas).

1.4 Characterise the effect of root hypoxia

The final step of WP1 will be to assess the effect of isolated hypoxia on the growth of potato roots. Using the robust indicators from WP1.2 and the novel experimental set-up from WP1.3, we will be able to assess the effect of acute hypoxia to potato roots. We will monitor the growth and development of root tips to see how potato roots cope under these conditions. Moreover, we will be able to tell if hypoxia is a signal for the plant root to adapt to waterlogging conditions by comparing changes in root architecture with waterlogged plants. Moreover, we will measure ethylene and NO to detect if the response to hypoxia is follows similar patterns to that of Arabidopsis. Arabidopsis reacts to whole-plant anoxia by immediately elevating ethylene levels (Sasidharan et al., 2018). Sometime after submergence, a NO burst will take place (Sasidharan et al., 2018). Our measurements will be able to determine whether findings in Arabidopsis correspond with potato. This will show to what extend we can see Arabidopsis as a reliable model under waterlogging and hypoxia conditions. During these experiments, we will also monitor the oxygen levels in the soil with a probe to see if the plant transports measurable amounts of oxygen to the soil through its roots. Beside the physiology of the plant itself, special attention will be paid to possible increases of ethylene and its precursors such as ACC.

WP2: Quantify oxygen under hypoxic conditions in different root tissues and stolons

Absolute oxygen concentration changes in the rhizosphere and root tissue will be established under waterlogging and hypoxic conditions. Root conditions are usually hypoxic compared to normoxic conditions, but the roots tend to be supplied with oxygen by the shoots through the vasculature. This work package will first investigate how hypoxic potato roots are under typical well-drained conditions by quantifying oxygen concentrations in vivo. For this, we will make use of a biosensor to be developed in WP2.1. This biosensor will allow to visualize oxygen levels with high spatial and temporal resolution in vivo. Next, we will investigate how hypoxic and waterlogging soil conditions affect the oxygen concentrations in the potato roots. We will pay special attention to differences between tissues. Moreover, we will be looking to answer the question whether the shoot can supply roots with oxygen as observed in Arabidopsis.

2.1 Development of an oxygen-sensor for potato tissue

Although clark probes - i.e. O2 microsensors -, like the one that will be made use of during WP1.2 are suitable for measurements in plant tissues, they have several shortcomings that make them not the preferred method used for our research. Instead, we will develop an endogenously encoded biosensor. Unlike the usage of clark probes, this is a non-invasive method that will allow a high special and temporal resolution. Therefore, in order to be able to accurately quantify oxygen levels in potato tissue, we will develop a new molecular fluorescence sensor based on state-of-the-art oxygens sensors recently being created for *A. thaliana*. We will transform our cultivar with this oxygen sensor, and initially test it in young plantlets. The choice of biosensor should take into account its pH sensitivity, specificity to O2 and possible side effects on plant physiology (Weits, 2021).

Despite potato being a tetraploid plant, it has been shown that stable gene expression can be achieved using commonly used techniques such as agrobacterium-mediated transformation or CRISPR-Cas9 (Bakhsh, 2020). We expect oxygen levels to be in the range of < 1% if present at all under anoxia treatment. Therefore, our oxygen sensor will require a very high sensitivity. The effect of the expression of significant amounts of oxygen-binding biosensors should be considered, especially since these may compete with biological processes that require oxygen, or lower the overall oxygen availability in the tissue even when it is already low (Weits, 2021).

A number of sensors have been developed so far, each with different advantages and disadvantages (Weits et al., 2021). Ideally, our sensor will sense relative or actual oxygen concentrations rather than hypoxia responsiveness. Therefore, we propose the adaptation of the novel sensor developed by Iacopino et al. (2019) (Figure 3). The reason we wish to select two candidates for optic sensors in root systems, is that the creation of these sensors is a complicated and long pipeline that would greatly benefit from having a ready-to-use backup. The development of two sensors will cost far less time and material than developing a second sensor after the development of the first has stagnated at a late phase. The sensor developed by Iacopino et al. is a synthetic sensor that drives gene expression in an oxygen-dependent fashion in plants. It is bioengineered from a mammalian oxygen sensor mechanism. In animals, Hypoxia-Inducible Factor (HIF) regulates the expression of oxygen-response genes. HIF interacts with the VON HIPPEL-LINDAU TUMOUR SUPPRESSOR (VHL) in an oxygen-dependent manner. This interaction is the basis of this bioengineered oxygen sensor, and has been shown to be sensitive enough to detect oxygen levels from normoxia to anoxia in *A. thaliana* mesophyll protoplasts.
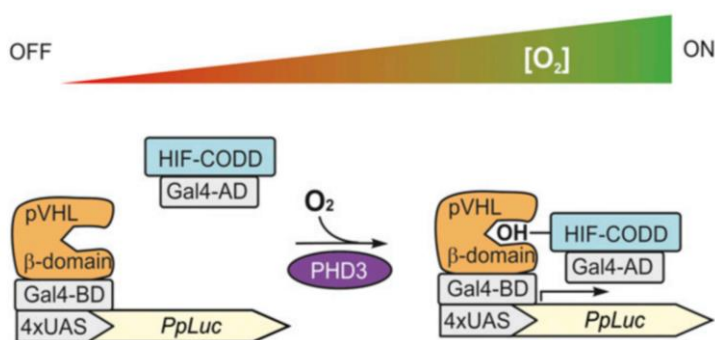

Figure 3 Schematic overview of the proposed oxygen sensor to be adapted to potato roots. Adapted from (Iacopino et al., 2019).

After inserting each sensor in our cultivar of choice, we will test both sensors using young plantlets grown on agar medium with oxyrase and the appropriate controls to see if our systems works *in vitro* and *in vivo*. Should the suggested experimental set-up not allow for the use of this preferred sensor, we will resort to a set of GUS-reporters that report the expression of hypoxia-induced PCO1 and PCO2 (Weits et al., 2014). The effect of PCOs on the molecular response to hypoxia involves RAP2.12 and is independent of its DNA binding properties (Weits et al., 2014). PCO1 and PCO2 are induced by RAP2.12, thereby generating a feedback loop that enables homoeostatic control of the anaerobic response (Weits et al., 2014). This GUS system reports the response of the plant to hypoxia rather than the absence of oxygen. Therefore, despite this being an indirect reporter, it will still be able to serve as an indication of hypoxia despite being likely less sensitive.

2.2 Quantify oxygen levels in-vivo under hypoxia conditions and waterlogging conditions

The final step of this work package will be measuring the oxygen levels of different root tissues and stolons with the developed biosensor. Some tissues are naturally hypoxic, such as the root apical meristem (RAM) and the lateral root primordia (LRP), making for a great natural control of our sensor. Besides the quantification of reduction in cellular O_2 levels, we will also perform a time series experiment. This will aid to determine how long it takes for different tissues to experience hypoxic stress after the hypoxia or waterlogging treatment has been started. Tissues of special interest will be root tips, young root tissue, LR primordia, stolons, and adventitious roots. Leaves will also be investigated, as waterlogging affects stomatal conductance.

## WP3: Hypoxia and the ethylene pathway in waterlogged potato

In the third and final work package, we will look in more detail at the different varieties used for a pre-screen in WP1. During this work package, mRNA samples will be collected in a time series experiment. This data will be used for two analyses. First, we will investigate the gene expression levels. Secondly, we will run a separate analysis for alternative splicing. During both analyses, special attention will be paid to genes known to be involved in hypoxia or flooding in other plant species while also leaving room for novel discoveries. Special attention will be paid to the potential of ethylene (C_2H_4) as a signalling molecule.

3.1 Time series experiment for transcriptome analysis

During a large-scale time series experiment, root tissue will be collected, poly-A purified, and RNA-sequenced in order for the transcriptomics analyses. These samples will later be analysed both for expression levels and alternative splicing events in WP3.2 and WP3.3, respectively. As these harvests will be destructive, they cannot be combined with the phenotyping screen in WP1. Moreover, for this experiment a limited number of harvest moments will be selected during the treatment phase and recovery phase. We will investigate plants under both waterlogging and hypoxia conditions using the experimental set-ups developed in WP1, in order to better understand the role of hypoxia in waterlogging. The harvest moments will be selected using a qPCR screen over a large number of time points. For each harvest moment selected, one variety will yield three samples (i.e. control, root hypoxia, and waterlogging) with sufficient replicates each per time point.

3.2 Gene expression analysis of RNA-sequencing data

Next, we will analyse the obtained read data for increases and decreases in expression for all genes. This is unbiased way of looking at transcriptome data is with reason a widely used method. RNA-sequencing can provide high flexibility, sensitivity and accuracy to gene expression measurements. During this analysis, we will look out for genes known to be involved in hypoxia and waterlogging responses in other plants species and *S. tuberosum*. These include RAP2.12, ETR2, and PGB1. Previously, it was found that the expression of RAP2.12 does not increase under root hypoxia in *S. tuberosum* (Hartman et al., 2020) (Figure 4). In the same study, ethylene signalling gene ETR2 was found to be upregulated in the tolerant variety under hypoxia (Figure 4). Finally, NO-scavenging phytoglobin PGB1 activity was found in the roots of waterlogging-tolerant variety Festien, but not in waterlogging-sensitive variety Seresta (Hartman et al., 2020), indicating a possible benefit under waterlogging conditions (Figure 4). It must be notes though, that in the experimental used for this publication, plants were treated with whole-plant hypoxia. Given that the response to hypoxia is tissue-specific and the fact that during whole-plant hypoxia all above-ground tissue is affected as well, we expect to gain valuable novel insights with our set-up.
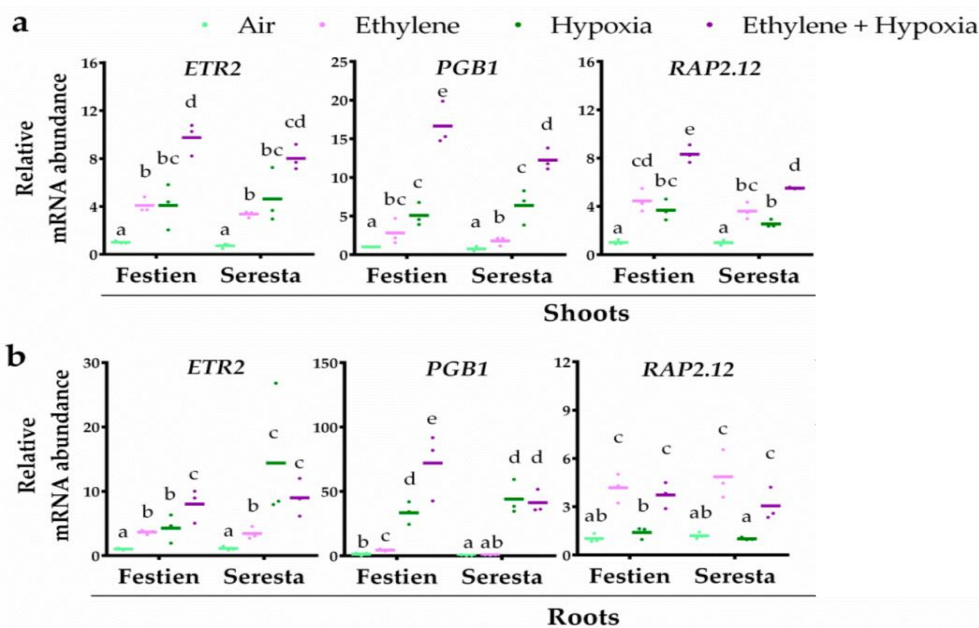
Figure 4 Gene expression in waterlogging-sensitive variety Festien en waterlogging-tolerant variety Seresta under different treatments in shoot (**a**) and root (**b**) tissue. Whole plants were treated with four hours of pre-treatment with air (light green) or ethylene (pink), followed by four hours of hypoxia (green and purple, respectively). Marker genes are orthologues of the Arabidopsis genes: ethylene signalling gene ETR2, NO-scavenging phytoglobin PGB1, ERFVII transcription factor RAP2.12 and hypoxia adaptive gene PDC1. Adapted from Hartman et al. (2020).
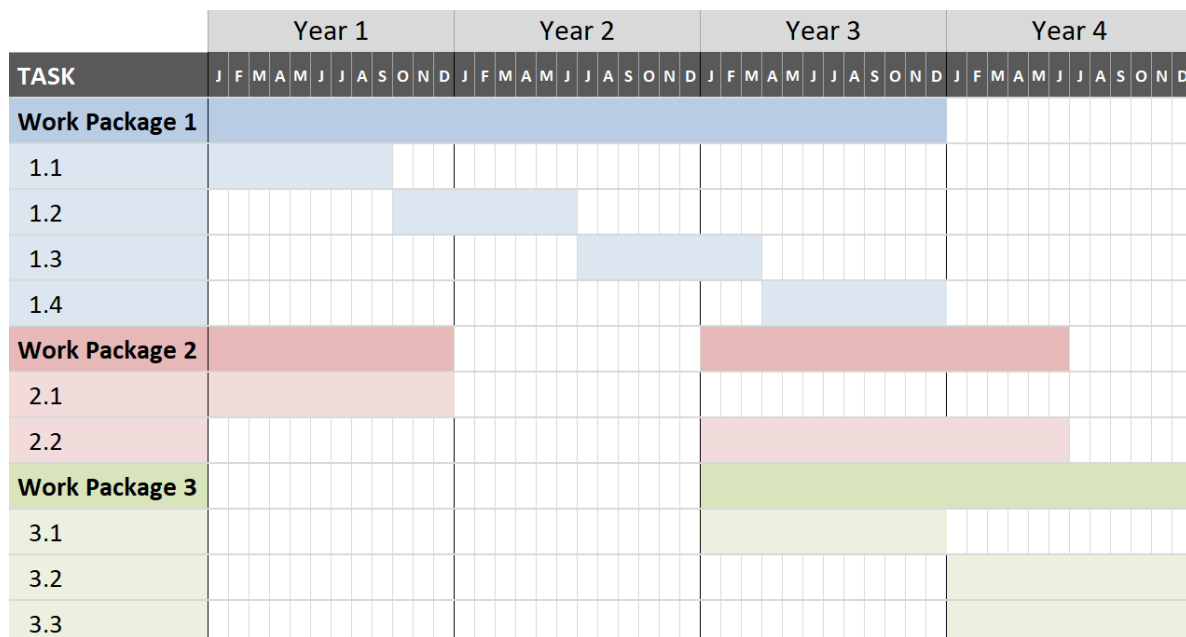
3.3 Alternative splicing analysis of RNA-sequencing data

Alternative splicing is a co-transcriptional modification of mRNA that can enhance transcriptome plasticity and proteome diversity. Whereas in constitutive splicing exons are retained and introns are removed from the pre-mRNA, sometimes there is a deviation during this process where an alternative mRNA isoform is created. This allows one gene to code for a number of mRNA isoforms. In some cases these mRNA isoforms code for slightly altered proteins, whereas in other cases it causes early termination of translation and thus inhibition of gene expression. For the analysis of the earlier obtained RNA-sequencing data, we will make use of the R package ASpli. ASpli is an integrative pipeline for R to analyse alternative splicing using RNA-sequencing data (Mancini et al., 2021). This package integrates both annotated and novel events. Due to this, its flexibility, high reliability, and relative ease of use make it a state-of-the-art option for the analysis of alternative splicing.

Alternative splicing has been shown to be an important regulator of many biological processes in plants, including but not limited to the circadian clock (Romanowski et al., 2020), response to temperature changes (John et al., 2021), and plant-microbe interactions (Rigo et al., 2019). Alternative splicing has also been show to play a role in waterlogging or flooding responses in several plant species, including sunflowers (Lee et al., 2021), maize (Lone et al., 2016), and *A. thaliana* (Veen et al., 2016). However, no findings have been published regarding the potential role alternative splicing plays in *S. tuberosum* under waterlogging conditions. We argue that this has been wrongly overlooked, as cold stress induces exon skipping in this species (Bournay et al., 1996). The fact that changes in alternative splicing are important under this condition suggests the same may very well be the case under waterlogging conditions, as waterlogging often goes together with a drop in temperature and these conditions together negatively affect tuber development in potatoes (Papathanasiou et al., 1999).

The same sequenced data used for the expression analysis can be used for this and are therefore this approach will be relatively low effort while high gain. The great advantage is that the elaborate experimenting and sample collections will not need to be repeated for this analysis and that changes in expression levels can be compared to changes in alternative splicing. We will keep an eye on potentially resistance- indicating candidate genes from literature, but it has to emphasized that this is a novel approach and that there is little literature that will help us predict possible genes of interest. However, we have ample experience with the ASpli package and possible collaborators should there be need for more expertise.

Time schedule

All four steps of Work Package 1 are expected to take nine months each (Figure 5). Work Package 2 can be started simultaneously with Work Package 1, as the oxygen sensor will need to be inserted into the potato variety of choice and tested in vitro. The second part of Work Package 2 will be carried out once a robust work flow for the hypoxia and waterlogging experiments have been established. This division of Work Package 2 also allows for some cushioning schedule-wise, as the new oxygen sensor will need to be thoroughly tested and this Work Package relatively high-risk is (see risk assessment). Work Package 3 can also be started once a robust work flow has been established for the experimental set-up. The second and third steps in this work package can be carried out simultaneously as soon as the RNA-sequencing data is obtained. It is advisable to do so, as the initial processing of the data will serve both steps and it will be insightful to do a cross-comparison of the results (e.g. genes of interest affected by either gene expression or alternative splicing).

TASK	Year 1	Year 2	Year 3	Year 4
Work Package 1				
1.1				
1.2				
1.3				
1.4				
Work Package 2				
2.1				
2.2				
Work Package 3				
3.1				
3.2				
3.3				

Figure 5 Gantt chart showing time schedule of the proposed research.

Risk assessment

We acknowledge that a small number of the endeavours suggested in this proposal contain a higher risk of failure than the rest. Therefore, we would like to address some key risks of the proposed research.

WP2.1: It is high risk to use a novel sensor, but it is exactly the novelty of the sensor that will make it interesting. This has not been attempted before and the step from *A. thaliana* to potato is considerable, but not insurmountable. We have experience in growing potatoes and access to the expertise of an oxygen-sensor expert. The risk is reduced by the development two sensors that have a slightly different approach and the use of a back-up system with a GUS-sensor. Moreover, once developed these sensors in potato could be used for a plethora of other research directions, such as those investigating potato tuber development, which to also require low oxygen concentrations.

WP3.3: To our knowledge, alternative splicing has not yet been shown to play a crucial role in flooding, waterlogging or hypoxia responses in any plant species. As the data is already collected, the costs will be relatively low, despite this step being very much into an unexplored area.

Scientific (a) and societal (b) impact

a) Each of the work packages of this research proposal will not only yield answers to the questions we ask, but also a tangible product for future research. First, the novel hypoxia set-up can be utilized for the study of root hypoxia in potato and, importantly, other plant species. This also goes for the root model that we will develop. Secondly, the oxygen biosensor may be used to answer many other standing questions in potato, including but not limited to questions regarding chronic hypoxia, hypoxia in other organs, and hypoxia in combinations with other environmental changes. Thirdly, the data collected in the third work package may inspire new research directions and can be used for different analysis, including cross-species meta-analyses.

b) Ultimately, this research will provide important stepping stones for the improvement of *S. tuberosum* as we face an increasingly changing and hostile climate.

Scientific (a) and societal (b) impact

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