

Review

# Waterlogging Stress Physiology in Barley

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**Abstract:** Barley (*Hordeum vulgare* L.) is the most susceptible cereal species to excess moisture stress. Waterlogging-induced hypoxia causes major morphological, physiological, and metabolic changes, some of which are regulated by the action of plant growth regulators and signal molecules including nitric oxide. Recent studies have evidenced the participation of phytochemicals in attenuating hypoxic stress during conditions of excessive moisture through their ability to scavenge nitric oxide and influence the synthesis and response of growth regulators. This review will highlight major cellular changes linked to plant responses to waterlogging stress with emphasis on phytochemicals.

**Keywords:** waterlogging; barley; hypoxic stress; nitric oxide; reactive oxygen species; phytochemicals

## 1. Introduction

In global cereal production, barley is the fourth most important following wheat, rice, and maize. In 2019, barley production totaled 159 million tonnes over 50 million hectares (M ha), giving a yield of over three tonnes per hectare (t/ha) and led by the Russian Federation, France, and Germany, with Canada ranking fourth [1]. Barley is a versatile cereal grain with varieties that have high protein and low starch grown for animal feed, as well as varieties with high starch and low protein used for malting [2]. Strict requirements on malting quality make it difficult for farmers worldwide to grow malting varieties that meet all those parameters. In 2019, the top worldwide exporters of malt were France (\$474 M), Belgium (\$426 M), Germany (\$376 M), Australia (\$343 M), and Canada (\$339 M) [3]. It is assumed that barley was first used as human food but evolved into a feed, malting, and brewing grain, in part due to the rise in prominence of wheat and rice. However, recent research into barley's low glycemic index, low cholesterol, and high antioxidant and vitamin contents [4,5] is slowly increasing the demand for the crop to be grown for human consumption as a health-forward product.

Barley production is often limited by unsuitable landscapes and the presence of diverse conditions of abiotic stress, including excess moisture. From the years 2006 to 2016, 65% of global crop loss due to abiotic stresses was caused by excess moisture [1]. An estimated 10% of total arable land is hindered by waterlogging [6]. In Canada, the Prairie Provinces (Alberta, Manitoba and Saskatchewan), where barley is primarily produced, are the most affected. The combination of factors, such as the flat topography, the impact of land management practices, the large accumulation of snowfall, and early spring rainfall among others, participate in making the prairies susceptible to flooding and excess moisture [7]. In Saskatchewan, heavy precipitation caused 49% of crop losses from 2006–2015 [8]. In Manitoba, excess moisture was the leading cause of crop loss at 38% from 1966–2015. Data from 2016–2017 showed that it remained to be the leading cause, yet at a much higher value of 71% [9]. Because of this, the need for different solutions in enhancing barley production and imparting excess moisture tolerance persist, especially in maintaining Canada's economic position when it comes to barley.



**Citation:** De Castro, J.; Hill, R.D.; Stasolla, C.; Badea, A. Waterlogging Stress Physiology in Barley. *Agronomy* **2022**, *12*, 780. <https://doi.org/10.3390/agronomy12040780>

Academic Editor: Jerzy Henryk Czembor

Received: 18 February 2022

Accepted: 21 March 2022

Published: 24 March 2022

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With the advent of climate change, precipitation will also continue to be severe in the coming years [10]. An increase in both intensity and frequency is expected, along with unpredictable storm patterns. Simulation studies that use climate models predict large increases in flooding in the areas of Southeast Asia, India, and East Africa [11]. In Canada, Eastern Manitoba, Western and Southern Ontario, and the Atlantic provinces are expected to be affected the most [12]. To prepare for the worsening probabilities of excess moisture stress in the future, extra attention must be directed to understanding the physiological and molecular mechanisms contributing to hypoxia tolerance in barley, as its demand continues to grow.

Organized into five main sections: barley susceptibility to waterlogging; hypoxic stress; nitric oxide; reactive oxygen species; and phytooglobins (Pgbs). This review provides an overview of waterlogging stress in barley, highlighting the major cellular changes with an emphasis on Pgbs.

## 2. Barley Susceptibility to Waterlogging

In an agronomic perspective, tolerance to waterlogging is perceived as the capacity of the plant to maintain grain yield despite the conditions [13] and compared to other cereal crops, barley (*Hordeum vulgare* L.) is the most susceptible to waterlogging stress [14]. A comparative study on wheat and barley under natural waterlogging resulted in 19–82% yield reduction in wheat, and 51–84% in barley [15]. The yield trait that waterlogging affects in barley is also different from wheat: a greater reduction in the number of spikes per plant is more prominent in barley, while wheat tends to show a greater reduction in the number of fertile florets per spike [16]. The growth stage of the crop is also crucial in determining susceptibility to waterlogging for barley, The range between leaf emergence and the booting has been identified as the most vulnerable [17]. In barley, excess moisture also causes a significant reduction (up to 50%) in chlorophyll a + b content and this decline was alleviated when phytoglobin (Pgb) was upregulated [18]. Another common symptom observed in waterlogged barley is a reduction in stomatal conductance. After a week of early-waterlogging, stomatal conductance was reduced to 38% of its control and even more so to 27% of control after a week of late-waterlogging [19]. Despite being very susceptible to excess moisture, when compared to other cereals, barley has a demonstrated capacity to recover. Most gas exchange parameters recover after a week following the removal of the stress [14,15]. Improving this characteristic is the focus of several current breeding programs.

Total crop losses due to waterlogging have historically averaged around \$74 billion USD [20]. In Australia, \$300 million AUD make up the total crop losses due to waterlogging, with its wheat and barley as the two most affected [21]. In two of Canada's provinces, Manitoba and Saskatchewan, excess moisture insurance claims were on par with those of drought and heat from 1966–2005 [22]. However, from 2005 to 2015, excess moisture claims surpassed drought claims by almost 30% [22]. With excess moisture as the new lead cause of yield loss in barley and the projected 30% increase in precipitation by 2030 [23], the need for new tolerance strategies is clearly becoming more urgent. Currently, most work that targets an improvement of waterlogging tolerance relies on quantitative trait loci (QTLs) [24]. Those identified in barley so far have involvements in root aerenchyma formation [25], root membrane potential [26], and reactive oxygen species (ROS) formation [27], yet these traits are not necessarily the primary genes responsible for the response to waterlogging [28]. Proteomic analyses conducted by Luan et al. [29] have also identified pyruvate decarboxylase (PDC), 1-aminocyclopropane-1-carboxylic acid-oxidase (ACO), and glutathione S-transferase (GST) as candidate genes after observing upregulation in a waterlogging-tolerant genotype. Based on genome-wide analysis of gene expression, Borrego-Benjumea et al. [30] also identified the GST, indicating that its activity under waterlogging could be an important mechanism in the overall barley resistance to waterlogging stress. The main waterlogging stress responses and metabolic adaptive traits for waterlogging tolerance in plants were recently reviewed by Tong et al. [31].

### 3. Hypoxic Stress

#### 3.1. Waterlogging and Oxygen Deprivation

Plants exposed to excess moisture can experience diverse forms of oxygen deprivation conditions, which are referred to as hypoxia or anoxia. Hypoxia occurs when the partial pressure of O<sub>2</sub> limits the adenosine 5'-triphosphate (ATP) production in mitochondria [32]. Anoxia, on the other hand, occurs when ATP production via oxidative phosphorylation is negligible compared to the output of glycolysis and fermentation [32]. Hypoxia tends to occur during waterlogging, which is when plant roots are fully submerged in water while the shoot is above the water level. In contrast, anoxia's definition is more specific: true anoxia is only achievable if there is no capacity to perform photosynthesis and respiration. Therefore, the environment would need to be totally devoid of oxygen and sunlight which rarely occurs in nature [33]. For instance, research work that imposes anoxic conditions would not only require darkness, but also the substitution of atmosphere with an inert gas [34]. Waterlogging-induced hypoxia occurs when the pores or air pockets in poor-draining soil become saturated with water, leading to the slow movement of dissolved O<sub>2</sub> into the roots [35], since O<sub>2</sub> diffuses through water 10<sup>4</sup>-fold slower than in air [36]. Hypoxic conditions can further develop if the oxygen level is further lowered by the aerobic activity of the soil microflora competing with the root for oxygen [37]. Waterlogging is often dependent on soil type and characteristics, with poor drainage enhancing the stress, but it can also occur because of cultivation practices, such as a rice-wheat rotation that leads to periodic soil compaction [30]. The negative effect of waterlogging in plants relies on several factors, such as the depth and duration of flooding, sensitivity of the crop and genotype, plant developmental stage, and temperature [30].

#### 3.2. Effects of Waterlogging on Plants

The development and function of both shoot and root tissue are compromised during hypoxia, since the limited availability of O<sub>2</sub> interferes with metabolic pathways [38]. A switch from aerobic respiration to fermentation contributes to the production of ATP in the roots, albeit at much lower levels than under normoxic conditions [38]. Accumulation of the products of fermentation, such as lactate and ethanol, can also contribute to root injury and death [36], although the disruption of metabolic processes because of ATP limitation are the major causes of cell and tissue damage. For example, the lack of ATP disrupts phosphorylation of aquaporins—proteins that facilitate water transport—in root cells, thus lowering the total soil-to-leaf hydraulic conductance that results in wilting, which is observed in waterlogged plants, including Arabidopsis, wheat, and maize [38]. In addition to having lowered access to O<sub>2</sub>, hypoxic roots exhibit symptoms of nutrient deficiency and senescence [39]. The access of roots to sugars from the shoot is also impeded as ATP is often required for active transport of sugars from the phloem to sink tissues [36]. Hypoxia also damages the root apical meristem (RAM) as reported in maize seedlings subjected to the stress, resulting in growth arrest and cell death [40].

In shoot tissue, chloroplasts are damaged through the deterioration of the membranes and grana lamellae, causing chlorosis and eventually tissue senescence [30]. Hypoxia also increases the production of ROS, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide (O<sub>2</sub><sup>-</sup>), which can cause extensive damage to the photosynthetic machinery, leading to decreased photosynthetic rate and stomatal conductance [19,41]. These effects contribute to chlorosis-reduced growth and ultimately decreased seed production [42]. It is therefore apparent that waterlogging-induced hypoxic stress not only compromises the function of the root, the initial organ perceiving the stress, but also above-ground organs.

#### 3.3. Plant Response to Waterlogging

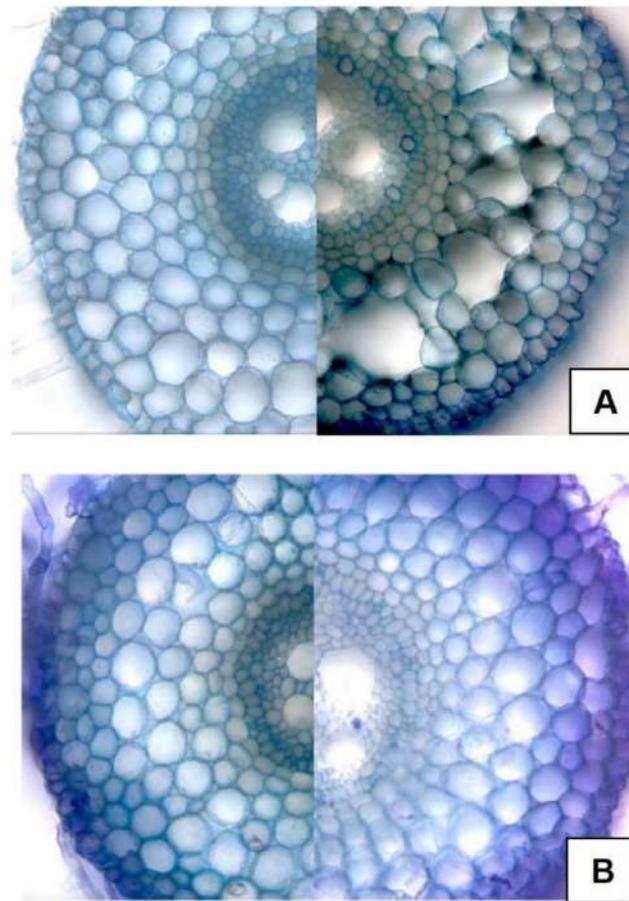
To cope with waterlogging stress, plants have evolved effective mechanisms that allow survival in O<sub>2</sub>-deprived environments. They undergo metabolic changes to accommodate the energy deficit following the stress, as well as anatomical changes to grant access to more O<sub>2</sub>. Some of these mechanisms are avoidance-based, whereby tissues undergo

morphological changes, such as selective cell death during aerenchyma formation or shoot elongation away from flooded soil, to gain more access to O<sub>2</sub> [43]. Other mechanisms are tolerance-based, which ensure longer-term survival in prolonged waterlogged conditions by involving alternative pathways to return to an energy status typical of the new hypoxic environment [36].

#### 3.4. Structural Changes

As a crop that requires a flooded environment for its cultivation, rice has been one of the preferred systems to study hypoxia. During submergence, rice coleoptiles and internodes increase elongation to maintain the above-water access to O<sub>2</sub> [44]. This avoidance strategy was also documented in the petioles of the wetland species *Rumex palustris* [45]. Other aquatic species have cell walls reinforced with suberin and lignin around the root tip to further conserve and distribute O<sub>2</sub> near the meristematic zone and prevent radial loss out into the soil pores [46]. Without this adaptation, the low redox potential of the hypoxic environment would have otherwise been a strong sink for O<sub>2</sub>, redirecting the minute amount of O<sub>2</sub> available away from the meristematic zone of the root [35]. Other cereal species not adapted to flooded conditions, such as wheat and barley, do not tend to elongate during waterlogging, but rather undergo structural modifications in the root system to favor O<sub>2</sub> acquisition. This includes one of the most documented and most studied avoidance mechanisms against hypoxia, which is the morphological adaptation of forming aerenchyma (Figure 1). These large intracellular spaces within the root (and most of the time, shoot tissue as well) allow low-resistance pathways for gas exchange from shoot to root [47]. Aerenchyma can form through cell separation (schizogenous formation) or the controlled death of cells to produce these air spaces (lysigenous formation) [38]. Like the adaptations mentioned previously, this mechanism is not present in all species. For instance, aerenchyma were found to occupy an area of 20–22% in wheat roots and 13–19% in barley roots, but other crop species, such as rapeseed and field pea, are devoid of aerenchyma [19].

Another commonly employed strategy to cope with hypoxia involves the formation of adventitious roots (ARs) that re-route water and nutrient uptake away from the site of the stress [29]. They exhibit negative geotropism and develop horizontally from the stem tissue, granting them more access to O<sub>2</sub> and eventually replacing the damaged primary root system. A positive correlation exists between formation of ARs and aerenchyma and tolerance to waterlogging, as demonstrated in maize [48], cucumber [49], and barley [29]. In barley, aerenchyma formation also occurs in ARs to further facilitate the movement of oxygen [29].



**Figure 1.** Light micrographs of cross sections of adventitious roots of barley waterlogging tolerant genotype Deder 2 (A) and waterlogging sensitive genotype Naso Nijo (B); no waterlogging treatment (left half) and after 3 days of waterlogging treatment (right half). Images taken from De Castro [50].

### 3.5. Physiological Changes

The anatomical adaptations occurring during hypoxia-induced waterlogging are the results of changes in cell physiology triggered by O<sub>2</sub> deprivation. During anaerobic conditions, plant cells rely on fermentation pathways, producing ethanol and lactic acid, to produce ATP, since regular mitochondrial respiration is impaired in O<sub>2</sub> limiting environments [44]. An increase in starch and other soluble sugars also occurs in waterlogged plants and in the root tissue; this increase in starch is quickly utilized to maintain proper root functioning [51]. With the rest of the plant, the starch fuels the transition to anaerobic fermentation, causing the increase in the activity of fermentation enzymes, such as alcohol dehydrogenase (ADH) and PDC, often used as biochemical markers in hypoxic studies [29,45]. Other markers used in hypoxic stress tolerance studies include lactate dehydrogenase (LDH), and ethylene-response transcription factors (ERFs) [52].

Under hypoxic stress, nitric oxide (NO) is generated through the reduction of nitrite [53]. While acting as an important signal molecule in many developmental and stress-related processes [54], as described in the next section, NO can cause severe cellular damage by favoring the production of ROS. Reactive oxygen species induce oxidative stress when present in excess [55], contributing to cell and tissue damage, especially in photosynthetic tissues [56].

Alterations in NO levels in hypoxic tissues also contribute to changes in hormone synthesis and response. For example, the levels of jasmonic acid (JA), indole-3-acetic acid (IAA), and ethylene are found to increase in hypoxic tissue (reviewed in [57]). In rice, besides inducing the expression of *SNORKEL* and *ERF-VII* which are involved in

internode elongation responses [45], hypoxia induced by waterlogging or submergence increases the expression of ACO and 1-aminocyclopropane-1-carboxylic acid-synthase (ACS), which participate in ethylene biosynthesis (reviewed in [58]). Ethylene is required for the formation of lysigenous aerenchyma in barley [53], as well as other species including rice (reviewed by [39]), wheat [59], and maize [60]. The ethylene-induced formation of aerenchyma is mediated by ROS signaling culminating in programmed cell death (PCD) in specific root domains [38]. Jasmonic acid can also alter NO homeostasis, which modulates the activity of several stress-related enzymes including GST, an attenuator of oxidative damage [61], as well as the expression of the pathogenesis-related protein TaBWPR-1.2 in root tissue [62].

An increase in gibberellic acid (GA) was also observed in hypoxic tissues; in waterlogged rice this increase promotes the internode elongation [52]. A similar response was observed in *R. palustris* where it induces petiole elongation [52]. When it comes to physiological changes in the shoot, abscisic acid (ABA) regulates systemic responses as demonstrated by the use of ABA-insensitive mutants [63]. A rise in ABA following waterlogging contributes to the closing of the stomata to reduce transpiratory processes minimizing oxygen escape [41].

The generation and development of ARs is mainly mediated by the interaction between auxin and ethylene [51]. This has been observed in maize, soybean, cucumber, tomato, and barley exposed to hypoxic stress (reviewed in [64]). Adventitious root formation allows the tolerant plants to obtain O<sub>2</sub> from the atmosphere as, in most cases, they form above the water level. It has been proposed that because of the positive correlation between tolerance and AR formation, the presence of ARs represents a phenotypic indicator for hypoxic tolerance [29].

## 4. Nitric Oxide

### 4.1. Roles in Plant Development

Nitric oxide is a gaseous, water and lipid-soluble metabolite that has signaling functions in plants [65]. It is involved in pathways that pertain to development, including those that regulate seed germination and dormancy, flowering, and root development [66], but also in homeostatic pathways such as transpiration management and tissue senescence [67]. Higher NO concentrations (40–80 ppm) have been reported to impede the growth of tomato, lettuce, and pea, while lower concentrations stimulated their growth [68,69]. Nitric oxide acts as a signal molecule involved in the ABA driven stomatal closure by regulating K<sup>+</sup> and Cl<sup>-</sup> channels [70]. As a radical, NO's reactivity makes it unstable around O<sub>2</sub> and ROS, and readily available to form reactive NO species (RNOS) that cause damage via oxidative reactions [71]. Because of these properties, extensive information on NO physiology relates to cell death.

### 4.2. Nitric Oxide Synthesis

The main enzymatic pathway proposed in the synthesis of NO during hypoxia has nitrate reductase (NR) as its main catalyst. During root hypoxia, NR itself is upregulated and it uses NAD(P)H to reduce NO<sub>2</sub><sup>-</sup> into NO [72]. Other heme proteins that synthesize NO during hypoxia include cytochrome c oxidase and the bc<sub>1</sub> complex, both of which also reduce NO<sub>2</sub><sup>-</sup> and belong to the mitochondrial electron transport chain [73]. However, some heme proteins retain an oxygenated state for extended periods of time and have a very high affinity for O<sub>2</sub>, such as hemoglobins (in plants, termed phytohemoglobins [74]), and therefore cannot reduce NO<sub>2</sub><sup>-</sup>. Instead, they operate the reverse reaction of scavenging NO to produce NO<sub>2</sub> or NO<sub>2</sub><sup>-</sup> [75].

### 4.3. Roles of Nitric Oxide in Biotic and Abiotic Stress

In biotic stresses, NO involvement in the hypersensitive response (HR) has been well documented, with NO being a messenger during the necrotic response around the site of infection following pathogen attack [65]. Soybean inoculated with *Pseudomonas syringae*

accumulated NO, as well as exhibited high expression of defense-related genes such as phenylalanine ammonia-lyase (PAL). In potatoes treated with NO donors, there was an accumulation of the phytoalexin rishitin, an antimicrobial terpenoid [65]. The increased NO production soon after infection and the results of its exogenous application suggest that NO acts upstream in defense signaling against biotic stress.

Abiotic stresses such as hypoxia, drought, ultraviolet radiation and ozone exposure induce plant responses that are also found to be moderated by NO and one such example is its signaling role in stomatal closure [65]. In *Tradescantia* spp. and *Vicia faba*, NO has been confirmed to be downstream of ABA in the signal transduction for stomatal closure [76]. Relative to normoxic conditions, NO is produced at the onset of hypoxia, as observed in maize and alfalfa cells [56]. The same authors suggested the rise in NO to be sufficient enough to contribute to the death program of the cortical cells and formation of aerenchyma [57].

Despite its roles in signal transductions during stress responses, the accumulation of NO could also lead to the formation of toxic reactive NO species (RNOS), such as peroxynitrite ( $\text{ONOO}^-$ ) and dinitrogen trioxide ( $\text{N}_2\text{O}_3$ ), which would lead to nitrosative stress. Damages resulting from nitrosative stress include single strand breaks in DNA, inhibition of repair mechanisms, and interference with mitochondrial respiration [45,47].

## 5. Reactive Oxygen Species

### 5.1. Deleterious Effects of Reactive Oxygen Species

Reactive oxygen species (ROS), such as  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$ , are produced due to homeostatic processes but also in response to stresses [77]. In peroxisomes and mitochondria, they are synthesized in various electron transport reactions and by membrane-bound NADPH oxidase, with  $\text{O}_2^-$  reacting with superoxide dismutase (SOD) to synthesize the more stable  $\text{H}_2\text{O}_2$  [78]. In chloroplasts, the non-radical singlet oxygen ( $^1\text{O}_2$ ) is a product of photodynamic reactions within the photosystem II [79]. Overproduction of ROS has been reported from plant exposure to pathogen infection [80], drought [81], pollutants [82], and waterlogging [83] stresses. As strong oxidants, they damage plant cells by causing oxidative damage on lipids, proteins, and nucleic acids [58]. A well-studied effect is the PCD-mediated aerenchyma formation induced by ROS as a mechanism to cope with hypoxic stress [84]. Depending on the response, plants can limit the amount of oxidative damage through the activation of antioxidant enzymatic reactions and production of antioxidants such as glutathione, ascorbic acid, and polyphenols [51].

In hypoxic maize roots, the rise in ROS is governed by the rapid increase in ethylene driven by an overproduction of NO [40]. The cell death caused by this rise in ROS was observed primarily in the quiescent center (QC) of the RAM, preventing root growth [40]. Therefore, a system that scavenges NO as one of the earlier players in the stress response would be an important factor in avoiding further ROS-oxidative damage and excess PCD.

### 5.2. Reactive Oxygen Species in Signaling and Stress Response

Reactive oxygen species have long been hypothesized to have a signaling function during homeostatic physiological processes including plant growth [85], PCD [86], and especially in response to environmental stimuli [59]. In response to stress and other stimuli, a burst of ROS is produced, and this is often followed by the activation of the antioxidant system [58]. It is this interplay between ROS production and their scavenging by the antioxidant system that regulates cellular ROS homeostasis modulating plant responses to stress conditions [60]. In particular,  $\text{H}_2\text{O}_2$  has been linked to waterlogging stress signaling; it promotes aerenchyma formation in rice [87] and adventitious root formation in cucumber [48]. Suppression of RBOH in rice roots decreases ROS accumulation required to trigger aerenchyma formation during waterlogging stress [88].

As previously mentioned, ROS are synthesized typically as by-products by enzymes active in homeostatic processes including photorespiration [89]. The enzymes responsible for ROS synthesis are a family of NADPH oxidases comprising respiratory burst oxidase

homologs (RBOHs) [90]. Environmental stimuli, such as drought, salinity, cold temperatures, wounding, and excess moisture [48], increase ROS through induced RBOH [62]. In *Arabidopsis*, two of these oxidases, AtrbohD and AtrbohF, work together to generate ROS responsible for the induction of hypoxia markers ADH, LDH, and PDC [59]. Other RBOHs are reported to mediate the establishment of crucial symbiotic nodules in *Medicago*, as well as having functions in the hypersensitive response against pathogens [91]. Because of their involvement in signal transduction, RBOHs are also commonly utilized as a marker for ROS production [48].

Reactive oxygen species and ethylene control of aerenchyma formation is also a heavily studied response following excess moisture induced hypoxia: H<sub>2</sub>O<sub>2</sub> and ethylene together regulate the development of aerenchyma in deepwater rice, as well as the hypoxic response of *Arabidopsis* [63]. In maize roots, O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> levels increased due to sulfate starvation in cells that underwent PCD to form aerenchyma [38]. Given that NO is required to destabilize the ERFs that sense fluctuations in O<sub>2</sub>, it is the crosstalk between ROS and NO that is then believed to be the basis for anaerobic metabolism and stress acclimation [63].

## 6. Phytooglobins

Plant hemoglobins (Hgbs) are hemoproteins that were first discovered in soybean after finding high concentrations of the protein within the plant's root nodules [92]. Like their counterparts in animals, they bind O<sub>2</sub> as well as other ligands including NO, carbon monoxide (CO), and hydrogen sulfide (HS) [93]. The symbiotic leghemoglobins (LegHgbs), as they were later referred to, are involved in the regulation and provision of O<sub>2</sub> for N<sub>2</sub>-fixing bacteria to use [67]. Eventually, two other types of Hgbs were discovered: nonsymbiotic Hgbs, recently and appropriately renamed as Pgbs [74], which are subdivided into class 1, found in both monocots and dicots, and class 2, which is exclusive to dicots [94] as well as the truncated Pgbs (class 3), termed as such due to its different protein structure [95]. Class 1 Pgbs have the strongest affinity for O<sub>2</sub> [66], with class 3 having the least [96].

### 6.1. Classes of Phytooglobins

Leghemoglobins are like animal myoglobins in their relation to oxygen. They are expressed in root nodules of leguminous plants that established symbiotic relationships with N<sub>2</sub>-fixing soil bacteria such as *Rhizobium* [97] and function to buffer oxygen concentrations in the nodules [98]. Their pentacoordinate structure permits ligand binding with O<sub>2</sub> as the most common ligand with which it reacts [43]. The mobility of its distal histidine sidechain allows O<sub>2</sub> to not only bind rapidly to it, but also have O<sub>2</sub> be readily released [99].

Given the high affinity of Pgbs for O<sub>2</sub>, it has been speculated that Pgbs are not involved in the transport of O<sub>2</sub> (reviewed by [45]). Rice Pgb1's O<sub>2</sub> affinity, for instance, was calculated to be 78 times stronger than soybean LegHgb a [100], showing that once O<sub>2</sub> binds with Pgb, it is stabilized and unlikely to be released. Instead, class 1 Pgbs scavenge NO and participate in the redox balance during the waterlogging-induced hypoxic stress (reviewed in [43]) and other abiotic stresses. Class 1 Pgbs also have roles in different plant organs at varying stages of development. Rice Pgbs were found expressed in the cytoplasm of differentiating tissues including the scutellum, root cap, and tracheary elements [63]. However, increased levels of transcripts and protein were better detected when plants were stressed. Focus on class 1 Pgb and its involvement in hypoxic responses will be discussed in later sections.

Class 2 Pgbs, studied extensively from *A. thaliana* and *L. esculentum*, are interestingly closer to symbiotic LegHgbs in structure and O<sub>2</sub> binding characteristics, despite not having a symbiotic function, as well as having a lower O<sub>2</sub>-affinity relative to class 1 Pgbs [101]. Transgenic *Arabidopsis* overexpressing class 2 Pgb showed an increase in fatty acid content in seed tissue, leading to the assumption that it has roles in delivering O<sub>2</sub> to developing tissues [102]. Although it shares a NO-scavenging function with class 1 Pgbs, a greater expression of class 2 Pgbs was observed following cytokinin and low temperature treatments [103], making it unlikely to have primary roles in hypoxic stress tolerance.

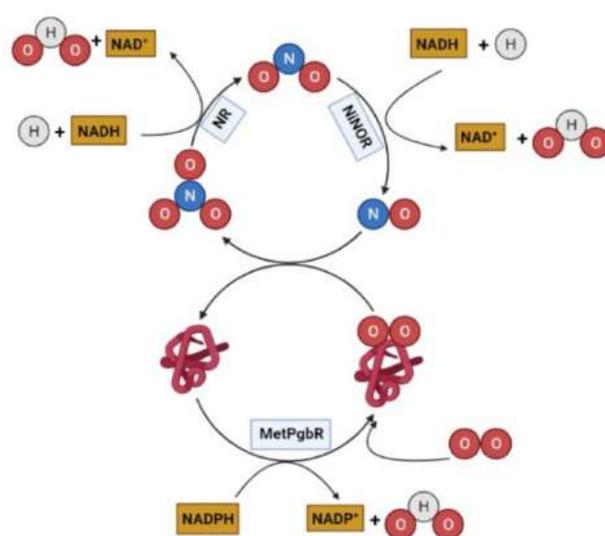
Class 3 or the truncated Pgb (TruncPgbs) are the least studied with still unclear functions in plant development. There has been more attention paid to their roles in bacterial and unicellular eukaryotes [68]. In the algae *Chlamydomonas eugametos*, TruncPgbs are induced during active photosynthesis in the thylakoid membranes and in cyanobacterium *Nostoc commune*, at the cytoplasmic side of the cell membrane during anaerobic conditions [65].

### 6.2. Class 1 Phytoglobin: Structure and Roles in Nitric Oxide Scavenging

The discovery of Pgb in non-leguminous plant species that do not form mutualistic relationships with  $N_2$ -fixing bacteria [94], including barley and other monocot species [104], broadened the physiological functions of Pgb. The hexacoordinate structure of class 1 Pgb is what allows it to have a very strong affinity for  $O_2$  compared to the pentacoordinate LegHgb and class 2 Pgb; therefore allowing it to function even during low  $O_2$  availability [66]. In hypoxia-tolerant genotypes, a notable decrease in NO concentrations was reported when the gene *Pgb1* is upregulated (reviewed in [45]). The strong affinity for  $O_2$  and its antagonistic relationship with NO is what contributes to Pgb's NO-scavenging function, thus permitting the PCD-derived adaptations without the uncontrolled damage from excess NO and ROS.

Class 1 Pgb in cereals are expressed in aleurone and embryo tissue during germination [69]. In roots, they are expressed during abiotic stresses, such as nutrient deprivation [105], drought [70], and hypoxia [69], but almost undetectable in normal conditions. Dordas et al. [67] have hypothesized that, since it is unlikely for them to function as  $O_2$  transporters, class 1 Pgb would instead have regulatory functions on NO levels that increase during hypoxic stress. This was confirmed by hypoxic alfalfa roots down-regulating *Pgb1* which exhibited NO levels 2.5-fold higher than that of Pgb-overexpressing roots [56].

The scavenging properties of Pgb have been documented in the Pgb/NO cycle elaborated in transgenic maize and alfalfa root culture [71] (Figure 2). Class 1 Pgb's strong affinity for  $O_2$  allows it to retain its oxygenated form (OxyPgb), which is the form responsible for scavenging NO [57]. In the cytoplasm where OxyPgb and NAD(P)H levels are high, OxyPgb oxidizes NO to produce nitrate ( $NO_3^-$ ) and ferric Pgb (MetPgb). MetPgb is then reduced by MetPgb reductase with NAD(P)H. The high affinity of Pgb for  $O_2$  causes an almost immediate oxygenation back to its OxyPgb form, and the cycle repeats [67].



**Figure 2.** Pgb1-NO cycle (Adapted from Gupta et al. [66]); Diagram generated using BioRender.com; NO, nitric oxide; NR, nitrate reductase; NiNOR, NO reductase; MetPgbR, metphytoglobin reductase.

### 6.3. Class 1 Phytoglobin and Stress Response

Instances of class 1 Pgb expression following biotic stresses have been reported in some species, such as *Lotus japonicus* and cotton following fungal infection [44]. Alteration of Pgb expression even enhanced defense mechanisms, as in the case of Pgb1-overexpression in *Arabidopsis* conferring tolerance to *Verticillium dahliae* and *Pseudomonas syringae* [106]. The role of Pgb1 has been associated more with abiotic stress responses, and especially hypoxia where Pgb1 exercises a protective role [42,107]. In hypoxic maize cultures overexpression of the barley Pgb, maintained a high energy status needed to cope with the stress [108]. When the same gene was overexpressed in alfalfa root cultures subjected to oxygen deprivation, root growth persisted. This was in contrast to root suppressing Pgb1, which exhibited a 30–70% decline in growth [56]. The protective role of Pgb1 was also demonstrated in whole plant systems: a strong positive correlation was observed between the expression of the maize Pgb1, *ZmPgb1*, and the ability to tolerate waterlogging stress [48]. In the same study, transgenic hypoxic lines over-expressing *ZmPgb1* retained a high photosynthetic rate and this effect was linked to a reduced accumulation of the ROS. In contrast, transgenic lines downregulating *ZmPgb1* had higher leaf injury, decreased photosynthetic rate, and increased ROS production [48]. The reduction in ROS and thus the reduction in oxidative damage in leaves, can therefore be attributed to the NO scavenging properties of Pgb upstream of ROS production. The deleterious effect of ROS during conditions of stress is most apparent at the root tip harboring the meristematic cells. In water stressed *Arabidopsis* roots, ROS accumulates within the (QC)—the undifferentiated organizing center of the RAM that maintains root growth and functioning, triggering the death program. These effects were mitigated by an elevation in Pgb1 and exacerbated by its suppression [70]. A similar effect was also documented in hypoxic maize root tips [74].

Other types of abiotic stress modulated by Pgb through ROS are cadmium toxicity [109] and iron deficiency [110]. Transgenic *Arabidopsis* plants over-expressing the tobacco Pgb1 (*NtHb1*) exhibited diminished ROS levels and better growth under cadmium toxicity [109].

### 6.4. Nonsymbiotic Class 1 Phytoglobin in Barley

Taylor et al. [104] were the first to characterize the barley Pgb1 and demonstrated its expression within the aleurone layers, and its induction at the onset of hypoxia. Barley class 1 Pgb has an O<sub>2</sub> affinity two orders of magnitude higher (2–3 nM) than that of cytochrome c oxidase (COX), which is another enzyme that converts NO<sub>2</sub><sup>−</sup> to NO [53]. It is also mobile and soluble, unlike COX which is membrane-bound, thus making Pgb a more efficient factor in the prevention of NO toxicity.

One of the earliest studies demonstrating the effect of the barley Pgb during excess moisture employed transgenic alfalfa root cultures. The hypoxic inhibition of root growth was attenuated by the over-expression of Pgb1, while augmented in roots suppressing the same gene. Suppression of the barley Pgb1 also resulted in the degradation of cortical cells, an observation that the authors interpreted as the formation of aerenchyma [52]. These effects were mediated by the ability of Pgb1 to scavenge NO.

Class 1 phytoglobin is also expressed in the seed's aleurone layer and was hypothesized to have major influences in germination [69]. Germination requires a high amount of energy and, especially upon imbibition, O<sub>2</sub> is consumed, thus creating an hypoxic environment. Zafari et al. [111] observed decreased germination rates in Pgb-knockdown barley lines compared to controls, while an enhanced germination rate and longer roots were observed in Pgb-overexpressing lines. These effects were ascribed to the ability of the barley Pgb1 to scavenge NO, which suppresses the activity of key enzymes essential for the maintenance of the redox balance.

## 7. Conclusions

Barley is the fourth most important cereal crop following wheat, rice, and maize, yet it is the most sensitive to waterlogging stress. Thus, the development of barley with improved tolerance to waterlogging stress is of high importance for breeding programs around

the world. One key factor modulating the plant response to stress, and ultimately tolerance to excess moisture, is NO. Modulation of this signal molecule has been shown to be critical for determining how plants cope with the stress. Therefore, the regulation of cellular NO homeostasis by Pgbs is a valid strategy to regulate how plants cope with the stress. Future directions involve the assessment of Pgb expression's potential as a marker for waterlogging tolerance in barley.

**Author Contributions:** Designed the manuscript, C.S. and A.B.; prepared the first draft, J.D.C.; reviewed and edited the manuscript, R.D.H., C.S. and A.B. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by the Manitoba Crop Alliance and the Canadian Agricultural Partnership Ag Action Manitoba—Research & Innovation (CAP Ag Action MB) (MWBGA 1956); and Agriculture and Agri-Food Canada and the National Barley Cluster led by the Barley Council of Canada through the CAP (J-002017).

**Acknowledgments:** The authors would like to thank Agriculture and Agri-Food Canada, CAP Ag Action MB, Manitoba Crop Alliance, The National Barley Cluster, The University of Manitoba, and the anonymous reviewers for their contribution.

**Conflicts of Interest:** The authors declare no conflict of interest.

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