

Research review

Physiological and cellular aspects of phytotoxicity tolerance in plants: the role of membrane transporters and implications for crop breeding for waterlogging tolerance

Author for correspondence:
Sergey Shabala
Tel: +613 62267539
Email: sergey.shabala@utas.edu.au

Sergey Shabala
School of Agricultural Science, University of Tasmania, Hobart, Tas. 7001, Australia

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Summary

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Waterlogging affects large areas of agricultural land, resulting in severe economic penalties because of massive losses in crop production. Traditionally, plant breeding for waterlogging tolerance has been based on the field assessment of a range of agronomic and morphological characteristics. This review argues for a need to move towards more physiologically based approaches by targeting specific cellular mechanisms underlying key components of waterlogging tolerance in plants. Also, while the main focus of researchers was predominantly on plant anoxia tolerance, less attention was given to plant tolerance to phytotoxins under waterlogged conditions. This paper reviews the production of major elemental and organic phytotoxins in waterlogged soils and describes their adverse effects on plant performance. The critical role of plasma membrane transporters in plant tolerance to secondary metabolite toxicity is highlighted, and ionic mechanisms mediating the this tolerance are discussed. A causal link between the secondary metabolite-induced disturbances to cell ionic homeostasis and programmed cell death is discussed, and a new ethylene-independent pathway for aerenchyma formation is put forward. It is concluded that plant breeding for waterlogging tolerance may significantly benefit from targeting mechanisms of tolerance to phytotoxins.

Breeding for waterlogging tolerance

Waterlogging affects *c.* 10% of the global land area (Setter & Waters, 2003) and is one of the most important constraints imposed on agricultural crop production. The yield penalty resulting from waterlogging may vary between 15% and 80%, depending on the species, soil type and duration of the stress (reviewed by Zhou, 2010). In the USA alone,

16% of soils are affected by waterlogging, and economic penalties for crop production are estimated to be the second largest after drought (Zhou, 2010). Not surprisingly, developing waterlogging tolerant varieties has long been a top priority for plant breeders.

The agronomic definition of waterlogging tolerance is the maintenance of relatively high grain yields under waterlogging relative to non-waterlogged conditions (Setter &

Waters, 2003). Accordingly, numerous phenotype-based indices have been used both in genetic studies (Parelle *et al.*, 2010) and in breeding programs (Zhou, 2010). As a result, nearly all published papers reporting quantitative trait loci (QTL) associated with waterlogging stress tolerance deal with agronomical and morphological characteristics (Table 1). While it is often argued that agronomic characteristics represent the combined genetic and environmental effects on plant growth, such a generic approach is insufficient, if one is searching for specific gene(s) conferring waterlogging tolerance. For example, 34 different QTL controlling waterlogging tolerance in maize were detected based on analysis of agronomical traits such as plant height, root length, and root and shoot DW; these QTL were mapped to eight different chromosomes (Qiu *et al.*, 2007). Given this breadth, many undesirable or unwanted genes will be inevitably transferred during the breeding process while attempting to transfer 'good' genes from these multiple loci. The problem may be resolved only if more specific mechanisms conferring waterlogging tolerance are targeted.

Over the recent years, an impressive amount of information has been accumulated on the molecular, biochemical,

physiological, morphological, anatomical and metabolic responses to waterlogging stress in plants. Surprisingly, only a very limited number of these characteristics have been used in QTL mapping. The most likely explanation is that most breeders still prefer to conduct their screening in the field, and the majority of techniques used in studies on mechanisms of waterlogging tolerance are not easily (if at all) applicable under the field conditions.

Among the targeted traits, aerenchyma formation and development of lateral roots are most directly related to a plant's superior ability to adapt to anoxic conditions (Jackson & Armstrong, 1999; Evans, 2003; Pang *et al.*, 2004; Colmer & Voesenek, 2009) and, thus, have been used in QTL mapping (Huang *et al.*, 1994; Mano *et al.*, 2007; Mano & Omori, 2008, 2009). However, it appears that even such a relatively straightforward trait as aerenchyma formation is conferred by at least four QTL mapped to three different chromosomes (Mano *et al.*, 2007); these QTL explained < 50% of plant's phenotypic variation under waterlogged conditions. Hence, there are repeated calls for the shift from mostly field-based screening techniques towards more physiologically oriented assays for specific mechanisms conferring waterlogging tolerance (Khabaz-Saberi *et al.*, 2006).

It also appears that waterlogging tolerance in plants is highly dependent on the environment, and the most waterlogging tolerant varieties in one trial may end up being intolerant in other trials (Setter *et al.*, 2009). Obviously, factors other than the ability of a plant to provide adequate oxygen supply or retention in the root play an important role in overall waterlogging tolerance. These factors include elemental and secondary metabolite toxicities that occur in waterlogged soils (Drew & Lynch, 1980; Armstrong *et al.*, 1996; Armstrong & Armstrong, 1999, 2001, 2005).

Waterlogging and production of phytotoxins

Excess water causes a sharp decrease in soil redox potential, resulting in very significant changes to the soil elemental profile. As soon as the free oxygen is depleted, nitrate is used by soil microorganisms as an alternative electron acceptor in respiration. Manganese (Mn) oxides are the next electron acceptors, followed by iron and sulphate. This results in a dramatic build up in the amount of soluble iron (Fe^{2+}) and Mn^{2+} in the soil solution, often to above toxic levels (Marschner, 1991). It should be also kept in mind that the solubility of potentially toxic metal cations is strongly dependent on soil pH, and the toxicity of some of these elements may increase even further in some soil types (such as Mn^{2+} in acid soils; Khabaz-Saberi *et al.*, 2010). It should be noted in this context that waterlogging usually results in a drift towards neutrality, thus causing changes in nutrient solubility. As waterlogging stress progresses, a further decrease in redox potential results in the reduction of sulphate to hydrogen sulphide. Finally, methane formation,

Table 1 Traits targeted during quantitative trait loci (QTL) experiments aimed at identifying waterlogging-tolerance related loci in different species

Traits targeted	Reference
Survival rate	Parelle <i>et al.</i> (2010)
Germination ability	Parelle <i>et al.</i> (2010)
Seedling emergence	Parelle <i>et al.</i> (2010)
Degree of leaf injury	Parelle <i>et al.</i> (2010); Zhou (2010)
Leaf senescence	Parelle <i>et al.</i> (2010); Zhou (2010)
Leaf chlorosis	Li <i>et al.</i> (2008)
Epinasty	Parelle <i>et al.</i> (2007)
Hypertrophied lenticels	Parelle <i>et al.</i> (2007)
Total dry weight	Li <i>et al.</i> (2008); Qiu <i>et al.</i> (2007)
Shoot dry weight	Qiu <i>et al.</i> (2007)
Shoot growth rate	Parelle <i>et al.</i> (2010)
Plant height	VanToai <i>et al.</i> (2001)
Root dry weight	Yeboah <i>et al.</i> (2008); Qiu <i>et al.</i> (2007)
Seminal root length	Parelle <i>et al.</i> (2010)
Seminal root growth rate	Parelle <i>et al.</i> (2010)
Lateral root number	Parelle <i>et al.</i> (2010)
Lateral root length	Parelle <i>et al.</i> (2010)
Adventitious root formation	Yeboah <i>et al.</i> (2008); Reyna <i>et al.</i> (2003)
Aerenchyma formation	Mano <i>et al.</i> (2007), Mano & Omori (2008, 2009)
Total grain yield	Parelle <i>et al.</i> (2010)
Kernel weight	Xue <i>et al.</i> (2010)
Number of grains per spike	Xue <i>et al.</i> (2010)
Number of spikes per plant	Xue <i>et al.</i> (2010)
Spike length	Xue <i>et al.</i> (2010)

from the reduction of carbon dioxide and certain organic acids, is initiated at redox potentials of *c.* -200 mV (at pH 7; Drew & Lynch, 1980).

Impeded gas exchange during soil waterlogging also leads to high partial pressure of CO_2 (pCO_2) in the root zone, with some serious consequences for root growth and metabolism (e.g. Boru *et al.*, 2003; Greenway *et al.*, 2006). High root zone pCO_2 will lead to high concentrations of HCO_3^- in the cytoplasm, with implications for metabolic regulatory systems. Rice and other wetland plants can tolerate high pCO_2 but nonwetland species suffer rapid damage (reviewed by Greenway *et al.*, 2006). Also, in waterlogged soils, the main form of plant-available nitrogen (N) is NH_4^+ (Kirk, 2004), and plant adaptations to NH_4^+ vs NO_3^- nutrition may be important under prolonged waterlogging. Some wetland species are particularly efficient at absorbing NO_3^- , possibly to scavenge NO_3^- formed in the rhizosphere by nitrifiers depending on O_2 released from roots (Kirk & Kronzucker, 2005).

In rice, iron (Fe) toxicity is reported over a wide range of Fe^{2+} concentrations in the soil solution, ranging from 1000 mg l^{-1} to only 10 mg l^{-1} in soils with poor nutrient status (e.g. in poorly-drained sandy soils; Kirk, 2004). In such cases the toxicity may result from the inability to exclude Fe^{2+} , and plant traits for internal tolerance or detoxification of Fe^{2+} will be important (Dufey *et al.*, 2009). As a result of Fe^{2+} oxidation in the rhizosphere, and plant cation–anion intake imbalance under NH_4^+ nutrition, the rhizosphere pH in flooded soils can be significantly lowered. This difference may range from 0.5 pH units in clay soils to as much as 1 to 2 pH units in poorly buffered soils (Kirk, 2004). Thus, the concentration of HCO_3^- (which is the dominant anion in most flooded soil solutions) may be significantly lowered, in which case any Fe^{2+} entering the root will be accompanied by other anions such as Cl^- or SO_4^{2-} rather than HCO_3^- . If Fe^{2+} enters with HCO_3^- , the acidity generated by the oxidation of Fe^{2+} is neutralized by the conversion of HCO_3^- to CO_2 ; however, if it enters roots with a nonvolatile anion, Fe^{2+} oxidation will produce the equivalent amount of free H^+ in the plant, causing cytosolic acidification and impairing cell metabolism.

Can plants do something about it? Oxidation of the rhizosphere by the increased radial oxygen loss (ROL) is considered to be essential for the detoxification of these elemental phytotoxins (Armstrong & Armstrong, 2005). However, while it is beneficial for the apical region of the exploring adventitious root to release oxygen, the continuing release from the maturing parts might result in the shrinkage of the oxidized rhizosphere, owing to a build-up of microorganisms under the influence of the exudates and oxygen. Therefore, it is beneficial for some ROL barrier to form. Indeed, roots of many wetland species contain a barrier to ROL in the basal zones (Jackson & Armstrong, 1999; Colmer, 2003), while the losses of O_2 from the basal

zones of adventitious roots of wheat and barley can be substantial (McDonald *et al.*, 2001; Garthwaite *et al.*, 2003; Pang *et al.*, 2006). Accordingly, it was suggested that enhancing a barrier to diminish ROL in roots should be a priority in breeding programs aimed at improving cereal tolerance to waterlogging (reviewed in Colmer *et al.*, 2005). Thus, it appears that many physiologists and breeders consider increased ROL as a *negative* trait, and while attempting to increase the ability of a plant to retain oxygen in the root by minimising ROL, breeders will inevitably reduce its ability to detoxify phytotoxins, unless the ROL barrier is confined strictly to mature zone, and lateral roots are dense enough to provide an extensive oxidized zone for protection.

In addition to inorganic phytotoxins such as Fe^{2+} , Mn^{2+} or H_2S , a significant accumulation of organic substances (e.g. ethanol, acetaldehyde and various short-chain fatty acids and phenolics), also occurs in waterlogged soils. These are secondary metabolites, produced as a result of anaerobic metabolism in both plants (Lynch, 1977; Armstrong & Armstrong, 1999) and rhizosphere microorganisms (Drew & Lynch, 1980). The type and amount of secondary metabolites produced depends upon the fermentative character of the microflora, the type and amount of organic materials added, the duration of waterlogging, and on the prevailing soil conditions. Ethylene can accumulate to phytotoxic concentrations in wet soils, and its production appears to be the results of microbial action (Drew & Lynch, 1980; Setter & Waters, 2003). Numerous volatile (short-chain) fatty acids and phenolics also accumulate in soils during prolonged waterlogging (Drew & Lynch, 1980; Armstrong & Armstrong, 1999). As discussed below, each of these substances may significantly affect plant physiological performance, both at the cellular and the whole-plant level, calling for the need to target plant tolerance to soil phytotoxins in breeding programs (Mano *et al.*, 2007).

Phytotoxins and plant responses

Elemental toxicity

Iron toxicity was repeatedly named as a primary limitation to rice cropping in flooded areas around the globe (Borges *et al.*, 2001; Shimizu *et al.*, 2005). High concentrations of both Mn^{2+} and Fe^{2+} in waterlogged soils were also named as major constraints for growing sensitive wheat cultivars in waterlogging-prone areas of Western Australia and north-eastern Victoria wheat belts (Khabaz-Saberi *et al.*, 2010), with two- to ten-fold increase in the shoot Mn and Fe concentrations reported (Khabaz-Saberi *et al.*, 2006). Importantly, genotypes showing superior Mn and Fe tolerance under drained conditions tolerated waterlogging much better, outperforming intolerant genotypes by 30–50% (Khabaz-Saberi & Rengel, 2010).

Several breeding programs were carried out to increase rice production under Fe toxicity conditions using traditional methods (Dufey *et al.*, 2009 and references within). However, resistance to Fe toxicity appears to be a rather complex trait, both genetically and physiologically. Several resistance mechanisms have been suggested (Dufey *et al.*, 2009) including Fe²⁺ oxidation in the rhizosphere, exclusion of Fe²⁺ from root uptake, storage of the excessive Fe²⁺ in the apoplast and vacuole, and detoxification of Fe-induced reactive oxygen species (ROS) by antioxidant enzymes. None of these mechanisms were directly targeted in breeding programs. Instead, a large number of QTL have been reported for easily measurable traits such as leaf bronzing index, shoot and root dry weight, tiller number, plant height and root length, and Fe accumulation in the shoot (Wan *et al.*, 2003; Shimizu *et al.*, 2005; Dufey *et al.*, 2009; all for rice). Given the fact that at least 24 putative QTL on six chromosomes have been discovered (Dufey *et al.*, 2009), genetic manipulation of these traits is not an easy task.

Mechanisms of plant tolerance to Mn toxicity remain largely unknown (Wang *et al.*, 2002), although great genotypic variation in Mn toxicity tolerance was reported for some species such as rice. Again, multiple QTL appear to govern this trait (for soybean see Kassem *et al.*, 2004); these were mapped by assessing leaf chlorosis and root necrosis rather than targeting specific physiological mechanism at the cellular level. It should be added that, to the best of my knowledge, none of the attempts of QTL mapping for either Mn or Fe toxicity tolerance has considered a potential interaction between the elemental toxicity for these nutrients and hypoxia.

Phenolics and volatiles

Adverse physiological effects of secondary metabolites of plants have been a subject of extensive investigation over the past decades. Allelopathic properties have been attributed to the large number of phenolic compounds (see the Supporting Information, Table S1), as well as to all known volatile (short-chain) fatty acids (Table S1). These metabolites were reported to affect a broad range of physiological processes related to all aspects of plant growth and metabolism (Table S2). Given such a broad spectrum of effects of secondary metabolites, it remains to be discovered which of them originate from the direct interaction of phytotoxins with some cellular structures.

Plant phenolics are a major chemical group implicated in allelopathy (Harper & Lynch, 1982; Siqueira *et al.*, 1991; Booker *et al.*, 1992). Phenolic compounds in plants are second only to carbohydrates in abundance. They display a great structural heterogeneity, ranging from derivatives of simple phenols to complex polymeric structures (Bertin *et al.*, 2003). The total concentration of phenolics typically varies between 100 and 500 mg kg⁻¹ dry matter (Glass,

1973; Wu *et al.*, 2001), with the free acid concentrations in the root rhizosphere estimated to be between 50 µM and 100 µM in nonflooded soils (Glass, 1974; Kuiters, 1990).

Both phenolic and volatile fatty acids are most active in the undissociated form (Drew & Lynch, 1980). As the pH of many waterlogged soils tends towards neutrality, most of these would be fully dissociated and largely ineffective. However, the pH of the rhizosphere may often be much lower than the bulk soil (Armstrong & Armstrong, 2001; Kirk, 2004). Under these conditions, concentrations of organic acids *c.* 1 mM (or even less) would then become injurious (Drew & Lynch, 1980; Wu *et al.*, 2001; Armstrong & Armstrong, 2001). Another important point is that under natural conditions, plants are likely to be exposed to cocktails of phytotoxins, not just to a single component. Indeed it has been shown that the cocktails of dilute volatile acids can be harmful, even at pH 6, when the undissociated molarity of each acid has previously been shown to be innocuous (Armstrong & Armstrong, 1999). Importantly, the above phytotoxic effects are observed both under hypoxic and normoxic conditions. Finally, very significant pH gradients exist along the root surface, with the elongation zone being much more acidic than the mature root zone (Shabala *et al.*, 1997). This pattern appears to be a trade-off between the requirement of a plant to acidify the cell wall to drive the expansion growth, and an increased risk of phytotoxicity. It should be also mentioned that it is the root apex and, specifically, the distal elongation zone, that appears to be most sensitive to elemental toxicity to ions such as aluminum (Al³⁺; Delhaize & Ryan, 1995), copper (Cu²⁺; Madejon *et al.*, 2009) and sodium (Na⁺; Chen *et al.*, 2005). It is logical to also add secondary metabolite toxicity to this list.

Ethanol and acetaldehyde

A lack of oxygen under waterlogged conditions shifts the energy metabolism from aerobic to the anaerobic mode (Gibbs & Greenway, 2003). Both alcoholic and lactic acid fermentation play an important role in anaerobic stress tolerance by maintaining lower redox potential and allowing the continuation of glycolysis via pyruvate consumption and recycling NADPH to NADP (Drew, 1997; Sairam *et al.*, 2009). However, anaerobic respiration in roots and other submerged organs may also lead to the accumulation of acetaldehyde and ethanol, causing cell death in roots and injury to shoots during waterlogging (Drew & Lynch, 1980; Sairam *et al.*, 2009). Importantly, inhibition of growth induced by exogenously applied ethanol in coleoptiles was strikingly different between rice and oat (Kato-Noguchi, 2002), with a nearly sevenfold higher sensitivity of oat compared with rice. Given the fact that rice is one of the few facultative waterlogging tolerant species, this poses the question as to the extent to which this trait is conferred by the superior tolerance of rice to ethanol.

The precise mechanisms mediating the above tolerance remain elusive. It is believed that self-poisoning by ethanol is not the main cause of injury to anoxia sensitive plants, and a possible role of acetaldehyde on the ethanol-induced injuries has been suggested (Perata & Alpi, 1991, 1993). It is assumed that the *in vivo* conversion rate of ethanol to acetaldehyde is lower in tolerant species (Kato-Noguchi, 2002), but the molecular targets of acetaldehyde remain unexplored.

Ethylene

Another potential toxic metabolite found in waterlogged soils is ethylene. Ethylene concentrations in agricultural soils are assumed to be between 1 and 10 $\mu\text{l l}^{-1}$, and these concentrations have been shown to cause a marked reduction in the extension of roots of several species, even under aerated solutions (Drew & Lynch, 1980). However, the inhibitory role of ethylene is unlikely to be related to its toxicity *per se*, but rather is caused by the disturbance to signal transduction pathways. Ethylene has been implicated in control of root hairs (Petruzzelli *et al.*, 2003) and aerenchyma (Evans, 2003) formation, K^+ starvation responses (Jung *et al.*, 2009), and oxidative stress signalling (Laohavisit & Davies, 2007; Jung *et al.*, 2009). It is assumed that ethylene functions through interaction with other hormones or signalling pathways (reviewed by Laohavisit & Davies, 2007). This issue is considered in more detail in other papers in this volume and so is not discussed here.

Membrane transporters and secondary metabolite phytotoxicity

Secondary metabolites and ion transport activity

Despite an impressive bulk of phenomenological observations, the specific mode of action of secondary metabolites remains elusive. Among possible targets, root plasma membrane transporters are the most suitable candidates. First, these transporters are located at the root–rhizosphere interface so may directly interact with secondary metabolites accumulated in waterlogged soils. Second, numerous papers report dramatic alterations in membrane transport activity caused by phytotoxins; 40–95% reduction in both phosphorus (P) and potassium (K) uptake was reported in barley roots after application of submillimolar concentrations of cinnamic and benzoic acid derivatives (Glass, 1973, 1974). These effects were observed within minutes (Glass, 1974) and were reversible (Glass, 1973), suggesting a direct effect upon the cell membrane. Lower amounts of organic acids have been also shown to reduce the uptake of other nutrients such as magnesium (Mg), calcium (Ca), and N in a range of plant species (Booker *et al.*, 1992; Armstrong & Armstrong, 2001). Finally, uptake of phytotoxins *per se* is apparently mediated by membrane transporters (e.g. for rats endothelial

cell line; Kido *et al.*, 2000). The considerable differences in tolerance to exogenously applied phenolics in different species (Glass, 1973), makes membrane transporters an attractive target for genetic manipulation in breeding programs.

Ionic basis of tolerance to phytotoxins

It has long been suggested that non-specific changes to membrane permeability are the main reasons for the observed phytotoxic effects of secondary metabolites (Glass & Dunlop, 1974). These conclusions were derived mostly from the reported ability of phenolic acids to cause membrane depolarization (Glass, 1973; Yu & Matsui, 1997). Indeed, the chemical nature of these compounds implicates their interactions with cellular membranes, an interaction that leads to phospholipid oxidation and a subsequent change in membrane integrity (Erlejman *et al.*, 2004; Campos *et al.*, 2009). However, several lines of evidence argue against this over-simplified model.

- Once within the tissues, phenolics are rapidly conjugated with glucose or other molecules, and their toxicity is reduced (Glass, 1973). This argues for an apoplastic rather than a symplastic mode of interaction with membrane transporters.
- Very rapid (within 1 min) changes in net K^+ and Ca^{2+} fluxes were measured from barley roots in response to the application of physiological concentrations of various phenolic and monocarboxylic acids (Pang *et al.*, 2007), while nonspecific changes in membrane permeability operate at a much slower time-scales (Jackson & St John, 1980; Jackson & Taylor, 1970).
- While the K^+ leak gradually increased with time, the Ca^{2+} efflux was short-lived and returned to control values within 10–15 min after treatment (Pang *et al.*, 2007). This strongly suggests that the fluxes of these two ions were mediated by two different transport systems and could not be attributed to a general change in membrane permeability.
- Secondary metabolite-induced K^+ loss from roots was prevented by known blockers of K^+ -permeable ion channels (Pang *et al.*, 2007), ruling out nonspecific changes in membrane permeability.

The possibility of direct regulation of ion transporter activity by phenolics and other phytotoxins is also supported by the data from animal literature. Phenolic compounds inhibited Kv1.3 channels activity in human T lymphocytes (Teisseyre *et al.*, 2009) and prevented ATP-induced activation of voltage-gated Ca^{2+} channels in PC12 cells (Guo *et al.*, 2010). Two pore domain, mechano-sensitive K^+ TREK channels were stimulated by a range of polyunsaturated fatty acids (Lesage *et al.*, 2000). L-type Cav1.2 calcium channels in rat ventricular myocytes were strongly inhibited by Mn^{2+} (Yang *et al.*, 2006), and phenolic compounds inhibited the activity of plasma membrane Ca^{2+} -ATPase in cardiovascular and smooth muscle tissues

(Roufogalis *et al.*, 1999). Several molecular targets were also reported for acetaldehyde. The so-called transient receptor potential (TRP) ion channels were shown to be activated by acetaldehyde in both human and mouse tissues (Bang *et al.*, 2007). Acetaldehyde also increased the open probability of the RyR Ca^{2+} -release channel in the sarcoplasmic reticulum (Oba & Maeno, 2004).

Surprisingly, no direct studies have ever been attempted on plant systems to investigate the possibility of direct regulation of ion channel activity by any of the secondary metabolites mentioned; direct patch-clamp experiments are urgently needed to address this issue.

Secondary metabolites transport across the plasma membrane

It is traditionally believed that most phenolic acids cross the cell membrane in an undissociated form via passive diffusion (Jackson & St John, 1980). Once inside the cell, permeated phenolic acids dissociate and acidify the cytosol (Ehness *et al.*, 1997). This acidification is expected to activate the plasma membrane H^+ -ATPase (Felle, 1996), increasing H^+ extrusion and causing plasma membrane hyperpolarization. Indeed, a 40% higher ATP hydrolytic activity compared with the control is reported in plasma membrane vesicles from barley roots treated with 200 μM of 2-hydroxybenzoic acid for 30 min (Pang *et al.*, 2007). However, other reports showed that ATPase is deactivated in response to secondary metabolites (Erdei *et al.*, 1994),

and a pronounced plasma membrane depolarization has been reported elsewhere (Glass, 1973; Yu & Matsui, 1997). This questions the validity of the passive diffusion model.

The recent cloning and functional characterization of the monocarboxylic acid transporter (MCT) family suggests that uptake of both monocarboxylic acids and benzoic acid occur via a H^+ -coupled cotransport mechanism in animal systems (Kido *et al.*, 2000; Tamai *et al.*, 1999) and > 10 isoforms of MCT have been identified in animals so far (reviewed in Pang & Shabala, 2010). It is also reported that MCT transporter may mediate plasma membrane transport of a wide range of phenolic acids and I suggest that a similar scenario is also applicable to plant tissues (Fig. 1).

KOR and NSCC potassium-permeable channels as possible targets

Net K^+ efflux induced by physiological concentrations of hydroxybenzoic and acetic acids was strongly inhibited in barley roots by TEA^+ , a known blocker of voltage-gated Shaker type K^+ channels (Pang *et al.*, 2007). Along with the observed membrane depolarization, it implies that depolarization-activated outward rectifying K^+ channels are downstream targets for secondary metabolites. Assuming that phenolic uptake is mediated by MCT transporters (Fig. 1), an H^+ -coupled movement of undissociated phenolic acid across the plasma membrane will result in a net H^+ influx, as well as causing a substantial membrane depolarization. Indeed, both these effects were observed in direct experiments (Pang *et al.*,

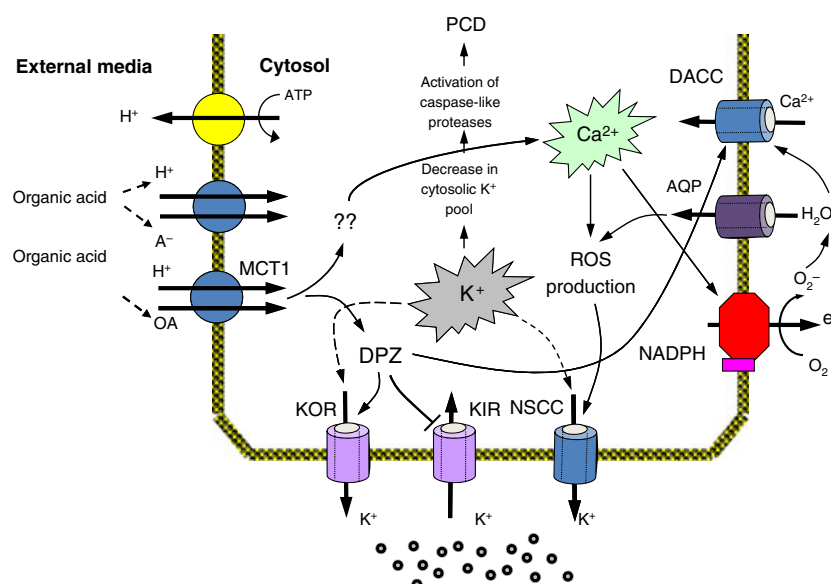


Fig. 1 Signal transduction pathways mediating secondary metabolite-induced programmed cell death in plant roots. Plasma membrane depolarization resulting from the movement of undissociated organic acid, and prolonged increase in the net calcium (Ca^{2+}) uptake leading to dramatic rise in cytosolic reactive oxygen species (ROS) levels, are responsible for the reduction in cytosolic potassium (K^+) pool. This reduction activates caspase-like proteases and results in a programmed cell death (PCD). MCT, monocarboxylic acid transporter; KOR, depolarization-activated outward-rectifying K^+ channel; KIR, hyperpolarization-activated inward-rectifying K^+ channel; NSCC, nonselective cation channel; AQP, aquaporin; DACC, depolarization-activated Ca^{2+} channel; DPZ, depolarization.

2007). Such a depolarization will significantly affect intracellular K^+ homeostasis by reducing K^+ uptake via inward-rectifying K^+ channels (KIR) and enhancing K^+ efflux via depolarization-activated outward-rectifying K^+ channels (KOR; Fig. 1). This may explain the rapid shift towards net K^+ efflux after phenolics application (Pang *et al.*, 2007).

This K^+ loss from barley roots was also prevented by gadolinium (Gd^{3+}) and lanthanum (La^{3+}), two known nonselective cation channels (NSCC) blockers (Pang *et al.*, 2007; Pang & Shabala, 2010). Given the fact that NSCC show only weak voltage dependence (Demidchik *et al.*, 2002), the depolarization-induced K^+ efflux through NSCC is highly unlikely. The plausible scenario to consider would be NSCC activation by ROS (Fig. 1). Arguments in support of this are given in the following sections.

Secondary metabolites and programmed cell death

Waterlogging and oxidative stress

Generation of ROS is characteristic for hypoxia and especially for reoxygenation. Hydrogen peroxide (H_2O_2) and superoxide are both produced in a number of cellular reactions (Blokina *et al.*, 2003). Both H_2O_2 and superoxide are highly reactive and may cause substantial damage to lipids, proteins, carbohydrates and nucleic acids. The formation of ROS is controlled by a large number of antioxidants (Noctor & Foyer, 1998). Nonetheless, although many phenolic compounds are considered to have antioxidant properties (Sairam *et al.*, 2009), experiments on overexpression of antioxidant production do not always result in an increase in antioxidative defence (Blokina *et al.*, 2003), and a waterlogging-induced decline in the activity of antioxidant enzymes has been reported (Sairam *et al.*, 2009). Obviously, the causal relationship between the antioxidant activity and waterlogging is not as straightforward as was initially believed.

Oxidative stress and aerenchyma formation

As commented earlier, aerenchyma formation is considered to be one of the most (if not the most) crucial adaptive traits for waterlogging tolerance. Two mechanisms of aerenchyma formation have been described: schizogeny, in which development results in cell separation, and lysogeny, in which cells die to create a gas space (Evans, 2003). Ethylene seems to be central to aerenchyma formation, regardless of whether produced endogenously or applied exogenously (Drew *et al.*, 1981; Jackson & Armstrong, 1999). However, the underlying mechanisms are far from being fully understood. Ethylene is reported to potentiate ROS production, thereby leading to cell death in tomato suspension cells (Moeder *et al.*, 2002), and the possibility of ethylene acting

upstream of an NADPH oxidase has been suggested (Laohavisit & Davies, 2007). Also, exogenously applied ethylene induced cell death in rice epidermis (Steffens & Sauter, 2005). Together, this evidence suggests a strong causal relationship between ethylene production and programmed cell death under waterlogged conditions.

Ethylene-independent aerenchyma formation

A critical role for ionic homeostasis in the apoptotic process has been endorsed for mammalian cells (Bortner & Cidlowski, 2007; Lang *et al.*, 2007), where a decrease in cytosolic K^+ pool was shown to be crucial in triggering key events in the apoptotic cascade, including caspase and endonuclease activation (Hughes & Cidlowski, 1999; Lang *et al.*, 2007). Although no caspase orthologs have been identified in plant genomes so far, eight distinct caspase-like enzymes have been reported in plants (Bonneau *et al.*, 2008). A model linking a decrease in the cytosolic K^+ pool and salinity-induced PCD in plant cells has recently been put forward (Shabala *et al.*, 2007; Shabala, 2009), and the central role of depolarization-activated outward-rectifying K^+ -permeable GORK channels in PCD in plants has been demonstrated (Demidchik *et al.*, 2010). Here, this model is extended to encompass a disturbance to ionic homeostasis caused by phytotoxins.

H^+ -coupled movement of undissociated phenolic acid across the plasma membrane and the resulting membrane depolarisation will significantly reduce cytosolic K^+ pool by reducing K^+ uptake via inward-rectifying KIR channels and enhancing K^+ efflux via depolarization-activated outward-rectifying KOR channels (Fig. 1). Root exposure to phenolics is also accompanied by a prolonged increase in the net Ca^{2+} uptake (Pang *et al.*, 2007). Depolarization-activated Ca^{2+} channels (DACC) are the most likely candidates to mediate this process, given the observed rapid depolarization of the plasma membrane (Pang *et al.*, 2007). Increases in the cytosolic free Ca^{2+} pool will lead to a dramatic rise in ROS levels resulting from $[Ca^{2+}]_{cyt}$ activation of NADPH oxidase via positive feedback mechanisms (Lecourieux *et al.*, 2002). This will cause an additional K^+ efflux via ROS-activated NSCC channels (Demidchik *et al.*, 2003, 2010), further decreasing cytosolic K^+ pool and causing the activation of caspase-like proteases that lead to PCD (Fig. 1). Thus, it is suggested that the existing ethylene-based models of aerenchyma formation by lysogeny (Evans, 2003) are complemented by the ethylene-independent signalling pathway – the disturbance to K^+ homeostasis, as described above.

Conclusions and prospects

No in-depth studies have ever been undertaken in plant systems to reveal the identity of the major ion transport systems mediating plant adaptive responses to phytotoxins

under waterlogging conditions. The model described here (Fig. 1) offers a possibility to validate some components of the stress signalling pathways in direct patch-clamp and molecular experiments. In addition to confirming the role of proposed transport systems, a number of additional components of the signalling network need to be properly integrated into the model. As such, the possibility of a NADPH-independent increase in the cytosolic Ca^{2+} pool by volatile fatty acids and phenols should be investigated. Also, the relationship between an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ and ROS accumulation in the cytosol remains elusive. In this context, a possible role for aquaporins should also be considered (Fig. 1). Rather than being simple water pores, aquaporins were recently shown to be permeable to a wide range of uncharged molecules, including hydrogen peroxide (Maurel *et al.*, 2009), and a possibility of H_2O_2 entering the cell through aquaporins (Fig. 1) may be envisaged.

Despite the calls to target plant's tolerance to soil phytotoxins while addressing the issue of plant waterlogging tolerance (Mano *et al.*, 2007; Setter *et al.*, 2009), this idea has not been properly implemented in the breeding programs. Meanwhile, the reported observations that genotypes with superior tolerance to elemental (Khabaz-Saberi & Rengel, 2010) and organic (Pang *et al.*, 2007) phytotoxins also possess better waterlogging tolerance suggest that targeting this (essentially unexplored) trait opens new and exciting prospects of utilizing available germplasm pool in breeding plants for waterlogging tolerance.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Secondary metabolites with reported allelopathic activity in plants

Table S2 Physiological processes affected by secondary metabolites produced in waterlogged soils

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