

Evolution of aerenchyma formation in a maize breeding program

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Abstract

Approximately 28 million hectares of intermittently flooded land with agricultural potential in Brazil could be used for rice crop rotation to increase production. Understanding the flood tolerance mechanisms in ‘Saracura’ is of paramount importance for maize and other crops. Thus, we evaluated the expression levels of the superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase, and catalase antioxidant genes and cell wall loosening (XET) enzyme in maize roots under flooding as well as associated expression with the initiation of aerenchyma formation, which is the most important plant adaptation to flooding. We collected data on ‘Saracura’ at the beginning of the breeding program (cycle 1), a time considered more sensitive to flooding, and during the last selection cycle (cycle 18), a time considered more resistant to flooding. Maize plants were flooded for 12 and 24h, and roots were collected for anatomic (aerenchyma density in the root cortex) and gene expression analyses by qRT-PCR. Our results showed that there was a proportional graded increase in aerenchyma formed in the root cortex with increased flooding time that was most pronounced during selection cycle 18. Antioxidant enzyme gene expression levels at both time points were similar: high expression just after germination, decreased expression after 12h, and increased expression after 24h of flooding. Aerenchyma formation was detectable after 24h of flooding, which coincided with the highest antioxidant enzyme gene expression levels. These results indicate that the correlation between antioxidant enzyme activities and flooding can act as a molecular flag to improve flood tolerance in ‘Saracura’. Our results are important to understand the induction of aerenchyma formation and flooding tolerance in ‘Saracura’.

Keywords: Flooding; gene expression; antioxidant system enzymes; *Zea mays* L.; aerenchyma formation.

Abbreviations: CO₂_carbon dioxide; O₂_oxygen; ROS_reactive oxygen species; H₂O₂_hydrogen peroxide; CAT_catalase; GPX_guaiacol peroxidase; POD_ascorbate peroxidase; XET_xyloglucan endotransglucosylase; SOD_superoxide dismutase; CRD_completely randomized; LPC_lysophosphatidylcholine; PCR_polymerase chain reaction; CT_threshold cycle; PA_proportional area.

Introduction

Approximately 28 million intermittently flooded hectares in Brazil have agricultural potential (Kavalco et al., 2014). This environmental condition leads to restricted diffusion of atmospheric air, increased accumulation of carbon dioxide (CO₂), and decreased oxygen (O₂) in soil, known as hypoxia ([O₂] < 4 %) or anoxia, in extreme cases ([O₂] ~0%) (Muhlenbock et al., 2007; Pezeshki and DeLaune, 2012). Under oxygen-deficient conditions plant growth is drastically inhibited, photosynthetic rate is gradually decreased, and mitochondrial membrane integrity is lost, thereby blocking aerobic respiration. Thus, energy production is restricted to fermentation, with much lower energy yields (Fukao and Bailey-Serres, 2004; Sairam et al., 2008; Banti et al., 2013; Sayed and Gadallah, 2014). Moreover, roots grow only at the soil surface and cannot fully exploit soil volume as under normal oxygenation conditions. In addition, plants produce more reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂) and other free radicals (Sairam et al., 2008; Guo et al., 2011; Porto et al., 2013).

Plants subjected to hypoxia undergo various morphoanatomical changes to withstand the low oxygen concentrations (Gibbs et al., 2011). Two of the most well-

known adaptations are aerenchyma formation and adventitious root development (Bailey-Serres and Voesenek, 2008; Yu et al., 2015). These two adaptations allow plants to keep air inside their cells and survive under hypoxic conditions.

There is a shortage of commercial species adapted to flooded conditions due to low survival, except flooded rice (Silva, 2007). According to FAO (2013), maize (*Zea mays*) is the most produced cereal in Brazil and contributes to about 80% of the country's grain production along with soybeans. Maize can adapt to most climatic conditions due to its allogamous nature (Allard, 1960; Rowan and Barret, 2008). Researchers from the Brazilian Agricultural Research Corporation, EMBRAPA Maize and Sorghum, developed the maize BRS-4154 variety ‘Saracura’ after considering these genetic characteristics and planting crops in flooded areas immediately after rice is planted to better use the flooded land in Brazil (Parentoni et al., 1997). After several mass selection cycles under intermittent flooding conditions, this variety has proved to be one of the most suitable for cultivation in flooded areas (Alves et al., 2002). Further studies have shown significant genetic gains during selection

cycles in different experiments related to flooding (Souza et al., 2010; Souza et al., 2012). Increased catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (POD1) antioxidant enzyme activities have been observed in roots throughout the selection cycles for 'Saracura' (Pereira et al., 2010). These antioxidant enzymes specifically removed cellular H₂O₂ (Moller et al., 2007). Pereira et al. (2010) reported increased root density of aerenchyma in 'Saracura', suggesting a relationship between antioxidant enzyme activities and increased area of aerenchyma, which is an essential feature of flood tolerance. Thus, the aim of this study was to evaluate the gene expression levels of three antioxidant enzymes [ascorbate peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD)] and cell wall loosening enzyme [xyloglucan endotransglucosylase (XET)], of 'Saracura' seedlings roots during cycles 1 and 18 and correlate these changes to the induction of aerenchyma formation.

Results

Anatomical analysis

The anatomical results revealed significant differences in the proportion of root cortex area occupied by aerenchyma. About 4% of the root cortex was occupied by aerenchyma on cycle 1, without flooding, whereas it increased to almost 7% on cycle 18, demonstrating good breeding program efficiency. The proportion of area occupied by aerenchyma increased rapidly to about 12% on the cycles 1 and 18 after 12h of flooding (Table 1). The proportion of cortex occupied by aerenchyma reached 15% on the cycle 1 and about 20% in cycle 18 after 24h of flooding. The differences between flooding time and between cycles were significant.

Roots in cycle 1 presented a smaller proportion of cortex area occupied by aerenchyma than those in cycle 18 regardless of flooding time. These results indicate a gradual increase in the proportion of aerenchyma area during flooding (Table 1 and Fig. 1), and the cortex area occupied by aerenchyma was larger during cycle 18 even in unflooded plants. These results demonstrate that the flood tolerance trait is incorporated in the genome.

Expression of antioxidant genes and cell wall enzymes

XET and POD1 were highly expressed, whereas CAT and SOD were expressed at low levels just after germination of 'Saracura' roots during selection cycle 1 before flooding (Fig. 2). The expression levels of all genes decreased after 12h of hypoxia, but POD1 remained the highest expressed gene, followed by XET, and CAT. SOD had practically no expression during selection cycles 1 and 18 and after 3h of flooding (Fig. 2). The expression levels of all of these genes increased again after 24h of flooding and maintained the levels observed after germination (Fig. 2).

The gene expression results for 'Saracura' roots during selection cycle 18 followed the same pattern observed during cycle 1; high expression of POD1 and XET and lower expression of CAT and SOD. In general, the expression level of all genes increased, (Fig. 2), decreased after 12h of flooding (Fig. 2), and then increased again after 24h of flooding (Fig. 2), but did not reach the initial level. Although similar gene expression patterns were observed during cycles 1 and 18, the levels were always higher during cycle 18 than those during cycle 1, which may be related to the better

adaptability of this maize cultivar to flooding and formation of aerenchyma.

Discussion

The results of this study agree with those reported by Pereira et al. (2008), who observed a significant increase in the proportion of cortex area occupied by aerenchyma in 'Saracura' roots subjected to 2 months of hypoxic stress.

Aerenchyma begins forming after 24h of hypoxia, and the proportion of cortex occupied by aerenchyma reaches 12% and then gradually increases to 50% after 4 days of flooding (Dantas et al., 2001; Lopes et al., 2005). According to these studies, aerenchyma forms in 'Saracura' seedlings during the first hours of hypoxia, reaching 12% of the cortex area 24h after the stress starts. Thereafter, aerenchyma forms progressively to include 50% of the total cortex area after 4 days and 98% after 7 days of flooding (Souza et al., 2012). Pereira et al. (2008) reported a progressively increasing proportion of aerenchyma in the root cortex after the 'Saracura' maize genetic selection cycle 18. Aerenchyma allows oxygen to reach internal root tissues (Bouranis et al., 2006; Voesenek and Bailey-Serres, 2015), which is crucial to maintain aerobic metabolism in roots in hypoxic or anoxic environments (Insausti et al., 2001; Gunawardena, 2008). This may be the most important strategy adopted by the 'Saracura', as it enables the plant to grow and survive for much longer periods during flooding, compared to other cultivars from the Brazilian germplasm. Previous studies (Dantas et al., 2001; Alves et al., 2002; Lopes et al., 2005; Pereira et al., 2008; Souza et al., 2009; Pereira et al., 2010; Souza et al., 2012) confirmed the superior flood tolerance of the 'Saracura' cultivar compared to that of the BR 107 cultivar, which is consistent with the increased proportion of cortex occupied by aerenchyma found in this study.

In another study conducted by Pereira et al. (2010), CAT and GPX activities decreased during the last few selection cycles, whereas POD1 activity increased. This reduction in antioxidant activity is related to an imbalance in H₂O₂ degradation, which promoted the increase in the amount of aerenchyma formed during the final cycles. CAT gene expression was upregulated during cycle 1 after 12h of flooding and POD1 was upregulated after 24 h of flooding. SOD was not expressed during either cycle after 12 and 24h of flooding, whereas XET gene expression increased during cycle 18 after 12 and 24h of flooding (Fig. 2).

The enhanced gene expression observed during cycle 18 suggests that 'Saracura' roots are more responsive to the oxygen deficit and that more genes related to oxidative stress are expressed. In fact, these two stressors are closely related, as the second one is a consequence of the first (Gunawardena et al., 2001; Evans, 2004; Deuner et al., 2008; Zanandrea, 2010; Steffens, 2014; Kamal and Komatsu, 2015b). Many researchers have associated XET, POD1, CAT, and SOD enzyme activities with increased flood tolerance in 'Saracura' (Dantas et al., 2001; Lopes et al., 2005; Pereira et al., 2010; Porto et al., 2013). Tolerance to anaerobic stress can be improved by increasing antioxidant enzyme activities (Mehlhorn et al., 1996; Biermelt et al., 1998; Jiang and Huang, 2001; Lin et al., 2007; Chiang et al., 2014). In the present study, the increased flood tolerance of the 'Saracura' occurred along with increased expression of antioxidant enzyme genes during the two selection cycles. Hypoxia induces cellulase and XET activities, leading to disorders of cell wall components and formation of aerenchyma (Brett

Table 1. Proportion of aerenchyma in the total cortex area (PA) of ‘Saracura’ maize roots and growth during selection cycles 1 and 18 at different flooding times.

Cycle 1	PA (%) C1	Cycle 18	PA (%) C18
0 h	4.36 A a	0 h	6.9 B a
12 h	11.57 A b	12 h	12.03 B b
24 h	15.75 A b	24 h	19.55 B c

Uppercase letters represent differences between cycles and lowercase letters represent differences between different flooding times within the same cycle.

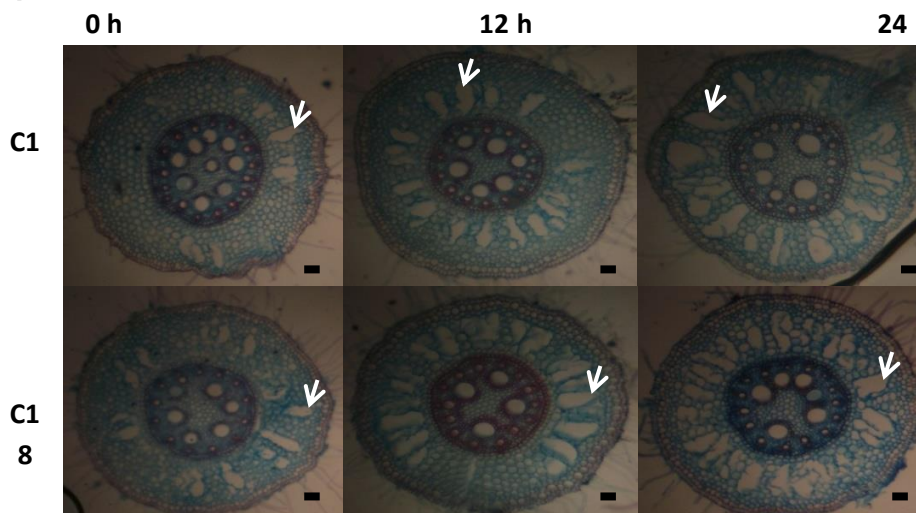


Fig 1. Aerenchyma formation in ‘Saracura’ root during selection cycles 1 and 18 after flooding the substratum. Bars = 100 μ m. Arrows show aerenchyma.

And Waldron, 1990; Vitorino et al., 2001; Manzur et al., 2014). Porto et al. (2013) demonstrated that expression of the poligalacturonase gene, which is a cell wall loosening enzyme in *Saracura coleoptile* that appears just after germination, is high, decreases after 4.5 days of hypoxia, and then increases on day 6. The XET gene allows for the growth and development of seedlings immediately after seed germination through a cell wall loosening effect (Asada, 1999; Fries et al., 2007; Porto et al., 2013). This may explain the high XET expression after germination and the subsequent decrease after 12h of flooding in this study.

The aerenchyma that forms in maize roots under hypoxic conditions has a lysigenous origin, high cellulase activity, and is induced by apoptosis (Dantas et al., 2001; Gunawardena et al., 2001a; Gunawardena et al., 2001b; Gadjev et al., 2008; Petrov et al., 2015). The activity of the cell wall loosening enzyme is closely linked to formation of aerenchyma (Saab and Sachs, 1996), which is usually found during the early stages of flooding in the maize coleoptile (Dantas et al., 2001). The high antioxidant enzyme activities that are observed immediately after germination are due to neutralization of the free radicals formed during germination and plant growth (Asada, 1999; Fries et al., 2007; Porto et al., 2013; Balakhnina et al., 2015). Dismutation of O_2^- is catalyzed by SOD. CAT, POD1, and other peroxidases reduce H_2O_2 to H_2O . Thus, these enzymes constitute the frontline defense against ROS (Léon et al., 2002; Xi et al., 2010; Chiang et al., 2014).

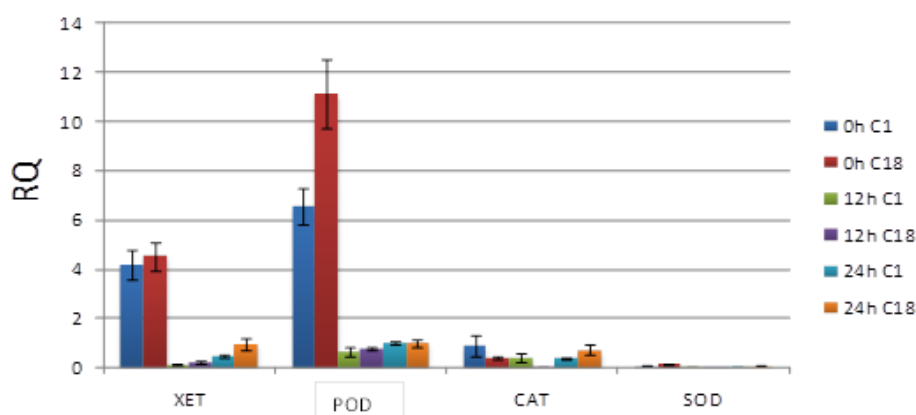
Kamal (2015a) found that the POD1 protein was downregulated in soybean cotyledons under flooding stress, which is the opposite of what was found in this study. This contrary result can be explained since the upregulation of POD1 was detoxifying and may explain the ability of ‘Saracura’ to survive longer under intermittent flooding. Another study that supported these results was reported by

Chiang (2014a), who created flood-tolerant rice plants by transforming them with the eggplant POD1 gene fused with a constitutive promoter.

The gene expression patterns observed in this study during cycles 1 and 18 can be explained based on these relationships. SOD was active during germination, as it generates H_2O_2 that needs to be eliminated by peroxidases and catalases, justifying the high expression of their genes, particularly POD1, soon thereafter. SOD enzyme expression does not change during flooding (Fries et al., 2007; Chiang et al., 2014b). Several authors have shown that POD1 activity increases at the detriment of CAT (Dantas et al., 2001; Lopes et al., 2005; Pereira et al., 2010) during flooding. CAT is specialized to remove high concentrations of toxic cellular peroxides (Mittler, 2002), suggesting that there some ROS may not be removed by POD1 (Madhusudhan et al., 2003). Greater formation of aerenchyma in maize roots during cycle 18 was detected after 12 h of flooding (Table 2), when the levels of antioxidant enzyme gene expression were lower, compared to the initial period (Fig. 2). This low expression may have led to a decrease in enzyme synthesis and an accumulation of cellular ROS. Accumulating ROS lead to lipid peroxidation and cell death (Cakmak and Horst, 1991; Mittler et al., 2004; Zanandrea et al., 2010; Baxter et al., 2013), which can generate lysigenous aerenchyma. The accumulation of ROS is a molecular flag in response to various stimuli, including hypoxia after 12–24 h of flooding (Neill et al., 2002). Antioxidant gene expression is activated, and there is a direct relationship between enzyme synthesis and expression level. The increased antioxidant and cell wall loosening enzyme activities we observed (Fig. 2) prevented cell collapse by scavenging free radicals. Nevertheless, these enzymes increased flooding tolerance and subsequent survival of ‘Saracura’ during hypoxia. The significant increase in the proportion of aerenchyma in the root cortex

Table 2. Polymerase chain reaction primers with NCBI and GenBank identification.

Gene	Orientation	Sequence 5' -----3'	NCBI/GenBank	Amplicon (bp)
Ascorbate Peroxidase (POD1)	Forward	GCTGAGTGACCCTGTCTTCC	FJ797426.1	102
	Reverse	AGTTCGGAGAGCCTTGAGGTG		102
Catalase (CAT1)	Forward	CCAAACTATCTGATGCTTCC	X60135.1	63
	Reverse	ATCATGGTGGTTATTGTGGT		63
Xyloglucan Endotransglycosylase (XET)	Forward	TCTACCTGTCGTCGCAGAACTC	NM_001155512.1	98
	Reverse	CCGGTTGCCAGGAACT		98
Superoxide Dismutase (SOD1)	Forward	GCATAGCCGAGGCAACCAT	AB093581.1	106
	Reverse	AACTGAATTTGCGCCAGTCAA		106
Ubiquitin	Forward	AAGGCCAAGATCCAGGACAA	XM_008657649.1	69
	Reverse	TTGCTTTCCAGCGAAGATGA		69
Alcohol Dehydrogenase (ADH)	Forward	AGGACGCTGAGTTAAGACC	XM_008650471	104
	Reverse	CACATTTGGCAGATCAGTGC		104

**Fig 2.** Ascorbate peroxidase (POD1), xyloglucan endotransglycosylase (XET), catalase (CAT), guaiacol peroxidase (GPX), and superoxide dismutase (SOD) gene expression in ‘Saracura’ roots during selection cycles 1 and 18. Time: 0, 12, and 24 h of hypoxia. RQ, relative quantity

during cycle 18 was absent during cycle 1 but started to increase after 12 h of flooding. This behavior was attributed to the increased formation of ROS in the region.

Materials and Methods

Plants and experimental design

We used the caryopsis of the BRS 4154 maize cultivar, which is also called ‘Saracura’. The selection program has been conducted by Embrapa maize and sorghum since 1986. Selection was carried out in trays in poorly drained alluvial soil. Flooding was initiated about 30 days after planting by intermittently filling the pan every 2 days to a 20 cm water depth and allowing it to drain naturally. Mass selection was conducted after the stress was applied and only the best plants were used for cycles 1–18 (Parentoni et al. 1998). Caryopses were grown for these two cycles in plastic trays filled with vermiculite and were divided horizontally. The caryopses were arranged on each side to avoid possible environmental interference. The trays were kept in a growth chamber at $26 \pm 2^\circ\text{C}$ and a 12h photoperiod for 4 days. To avoid possible hypoxia during germination, perforations were made in the tray bottoms, and the holes were sealed with Durepox® to induce hypoxia. Water was added above the substrate level until a 1 cm blade formed and remained for different periods (12 and 24h). Plants that were not watered were used as a control.

Detecting the formation of aerenchyma

Primary roots were collected from three seedlings per replicate for the anatomical analysis and were fixed in formalin, acetic acid, and 70% ethanol for 72h and then stored in 70% ethanol until data collection (Kraus and Arduini, 1997). Two cm cuts were made into the pilifera root zone for the evaluation. The pilifera root fragments were cut into cross sections using a microtome, clarified with 5% sodium hypochlorite for 10 min, rehydrated for 10 min, stained with “astrablau” (7.5:2.5, safranin solution: astrablau), and assembled on slides with 50% glycerin (Kraus and Arduini, 1997). Photographs of the sections were obtained with an optical microscope coupled to a digital camera, and total cortex area, area of the aerenchyma, and the proportion of the cortex area occupied by aerenchyma were measured with the UTHSCSA ImageTool analysis software. Three replicates were taken to calculate the mean for each anatomical characteristic. The proportion of area in the cortex occupied by the aerenchyma was calculated by dividing total aerenchyma area by total cortex area.

RNA extraction

RNA was extracted from roots using TriReagent (Sigma, St. Louis, MO, USA), according to the manufacturer’s instructions with some modifications.

The samples were stored at -20°C . RNA integrity was assessed spectroscopically, and quality was evaluated by agarose gel electrophoresis. DNase Free was applied from the DNase Turbo Free kit (Ambiom, Austin, TX, USA). RNA was quantified using Nanodrop® Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA), and cDNA was synthesized using the High Capacity cDNA Reverse Transcription cDNA Kit (Applied Biosystems, Foster City, CA, USA).

The primers were designed using the Primer Express 3 program (Applied Biosystems) (Table 2), and cDNA quality by PCR amplification using oligonucleotides corresponding to endogenous ubiquitin and alcohol dehydrogenase (Livak and Schmittgen, 2001; Scgikdberg et al., 2009). Gene expression was quantified using the ABI PRISM 7500 Real-Time PCR (Applied Biosystems) and the SYBR Green detection system. Relative gene expression was determined by the comparative CT method after diluting the samples 1:10. The expression analysis was processed in triplicates. After amplification, data were analyzed using 7500 Fast Software ver. 2.1 (Applied Biosystems). Each sample was normalized with the mean expression value of the endogenous genes and the equation $\Delta\text{CT} = \text{CT} (\text{target gene}) - \text{CT} (\text{endogenous})$. Relative quantification values were obtained using $-2^{\Delta\Delta\text{CT}}$ and calibrated with the equation $\Delta\Delta\text{CT} = \Delta\text{CT} (\text{sample}) - \Delta\text{CT} (\text{calibrated sample})$. Expression of all genes in a 12 h flood treatment sample was analyzed as a calibrator. All qPCR analyses followed the MIQE guidelines for qPCR analysis (Bustin et al., 2009).

Statistical analysis

The experimental design was completely randomized and each tray was considered a repeat. Three replicate measurements were taken for each anatomical characteristic. Tukey's test was used to make comparisons. A p -value < 0.05 was considered significant. Sisvar software (Ferreira, 2011) was used to conduct the analysis.

Conclusion

We conclude that antioxidant enzyme gene expression was correlated with formation of aerenchyma and acted as a molecular flag to activate the flood-tolerance mechanism in 'Saracura'. These results are for understanding the mechanisms of flood tolerance in maize. These results could be extrapolated to other cultivars adapted to micro-environments or other flood-tolerant crop species.

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