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ORIGINAL ARTICLE

Reactive oxygen species, ABA and nitric oxide interactions on the germination of warm-season C_4-grasses

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Abstract Hydrogen peroxide (H_2O_2) as a source of reactive oxygen species (ROS) significantly stimulated germination of switchgrass (*Panicum virgatum* L.) seeds with an optimal concentration of 20 mM at both 25 and 35°C. For non-dormant switchgrass seeds exhibiting different levels of germination, treatment with H_2O_2 resulted in rapid germination (<3 days) of all germinable seeds as compared to seeds placed on water. Exposure to 20 mM H_2O_2 elicited simultaneous growth of the root and shoot system, resulting in more uniform seedling development. Seeds of big bluestem (*Andropogon gerardii* Vitman) and indiangrass [*Sorghastrum nutans* (L.) Nash] also responded positively to H_2O_2 treatment, indicating the universality of the effect of H_2O_2 on seed germination in warm-season prairie grasses. For switchgrass seeds, abscisic acid (ABA) and the NADPH-oxidase inhibitor, diphenyleneiodonium (DPI) at 20 μM retarded germination (radicle emergence), stunted root growth and partially inhibited NADPH-oxidase activity in seeds. H_2O_2 reversed the inhibitory effects of DPI and ABA on germination and coleoptile elongation, but did not overcome DPI inhibition of root elongation. Treatment with H_2O_2 appeared to enhance endogenous production of nitric oxide, and a scavenger of nitric oxide abolished the peroxide-responsive stimulation of switchgrass seed germination. The activities and levels of several proteins changed earlier in seeds imbibed on H_2O_2 as compared to seeds maintained on water or on ABA. These data demonstrate that seed germination of warm-season grasses is significantly responsive to oxidative conditions and highlights the complex interplay between seed redox status, ABA, ROS and NO in this system.

Keywords ABA · Diphenyleneiodonium · Hydrogen peroxide · Nitric oxide · Reactive oxygen species · Seed germination · Switchgrass · Warm-season C_4 grasses

Abbreviations

AscPx	Ascorbate peroxidase
DPI	Diphenyleneiodonium
GPx	Guaiacol peroxidase
DAF-FM	4-Amino-5-methylamino-2',7' difluorofluorescein
H_2DCFDA	2',7'-Dichlorodihydrofluorescein diacetate
MAT	Methionine-adenosyl transferase
NBT	Nitroblue tetrazolium
NO	Nitric oxide
PTIO	2-Phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide
Px	Hydrogen peroxide
Rboh	Respiratory burst oxidases
ROS	Reactive oxygen species

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Introduction

Exogenously supplied donors of NO significantly enhanced germination and overcame residual dormancy in three

warm-season C_4 grass seeds (Sarath et al. 2006b). In a response similar to barley (*Hordeum vulgare* L.) and Arabidopsis seeds (Bethke et al. 2004, 2005) cyanide-releasing compounds also stimulated germination of switchgrass (*Panicum virgatum* L.) seeds. 2-Phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), a chemical scavenger of NO, blocked both the NO and cyanide-responsive stimulation of grass seed germination, indicating that adequate levels of endogenous NO were required for germination (Sarath et al. 2006b). For switchgrass seeds, application of abscisic acid (ABA) significantly retarded radicle elongation and essentially abolished coleoptile growth. Simultaneous imbibition with external donors of NO failed to overcome the inhibitory effects of ABA, suggesting that ABA and NO targeted different parts of the germination machinery (Sarath et al. 2006b).

An important link between ABA and NO action in plants are reactive oxygen species (ROS) (Neill 2005; Bright et al. 2006) and plants appear to have redundancy in signaling mechanisms for these molecules (Kwak et al. 2003; Apel and Hirt 2004; Shi et al. 2005; Zago et al. 2006). Therefore, cross-talk between different elicitation pathways could result in identical biological outputs for different stimuli. This is especially true for complex events such as plant growth processes (Zhang et al. 2005; Torres and Dangl 2005) where the same signaling molecule elicits differential responses in different tissues.

Whereas the cellular interactions between these small regulatory molecules are not fully known, exogenously supplied sources of ROS, such as hydrogen peroxide (H_2O_2) can impact a wide variety of plant responses, including seed germination and breaking of dormancy (Ogawa and Iwabuchi 2001; Clerkx et al. 2004; Gechev and Hille 2005). Plants have evolved several mechanisms that control seed dormancy and germination (Koornneef et al. 2002). For example, for physiological barriers to embryo germination to be removed post-maturation changes are frequently involved. These after-ripening processes enhance the ability of a seed to germinate (Heggie and Halliday 2005; Gubler et al. 2005). Although release from dormancy favors germination, the germination process is still affected by internal and external events (Koornneef et al. 2002; Bethke et al. 2004).

The roles and interactions of plant hormones in imposing seed dormancy and promoting seed germination have been extensively studied in monocots and dicots (for example, Loch et al. 2004; Chiwocha et al. 2005; Gubler et al. 2005). Studies from a number of systems reviewed by Gubler et al. (2005) indicate that ABA levels in the seed are of overriding importance in the control of dormancy. As a seed exits from dormancy, ABA levels decrease, with concomitant increases in other hormones such as GAs which promote germination (also see Seo and Koshiba 2002; Heggie and Halliday 2005). Plant hormones catalyze their cellular actions through receptors. Subsequent interactions between the receptors and their cognate proteins then drive signaling cascades resulting in biological outputs. Additionally, second messengers such as calcium, NO, ROS and G-proteins are intrinsically linked to most hormone responsive cellular processes (Lovegrove and Hooley 2000; Zentella et al. 2002; Chen et al. 2004; Higuchi et al. 2004; Verslues and Zhu 2005; Chiwocha et al. 2005; Okamoto et al. 2006; Pandey et al. 2006).

In this study we evaluated the response of warm-season grass seed germination to exogenously supplied H_2O_2 (source of ROS), ABA and their intersection with endogenous NO. H_2O_2 significantly enhanced germination at two different temperatures and elicited a simultaneous development of the seedling root and shoot system. ABA and the NADPH-oxidase inhibitor, diphenyleneiodonium (DPI), significantly inhibited seed germination. DPI in addition strongly retarded root growth and seeds imbibed on DPI had lowered levels of NADPH-oxidase activities. H_2O_2 substantially reversed the effects of ABA and DPI on seed germination and seedling growth. A scavenger of NO partially inhibited germination of switchgrass seeds and abolished the stimulation in seed germination observed upon peroxide treatment. We also assessed aleurone activation status through analyses of hydrolytic enzymes and protein markers known to accompany seedling growth. Levels of α-amylase and guaiacol peroxidase (GPx) increased sooner and more dramatically in seeds imbibed on peroxide. Likewise, protein levels for ascorbate peroxidase (AscPx) and methionine-adenosyl transferase (MAT) increased earlier in seeds imbibed in the presence of 20 mM H_2O_2. Levels of all of these proteins were significantly reduced by ABA. Our results indicate that exogenous one-off hydrogen peroxide strongly stimulates seed germination in switchgrass and appears to overcome endogenous/exogenous inhibition modulated by ABA. Both H_2O_2 and ABA appear to intersect with NO production and NADPH-oxidases during the germination process in switchgrass.

Materials and methods

Plant materials and germination assays

Panicum virgatum L. cv Kanlow seeds were obtained from plants grown in the field at Mead, NE. Seeds were surface sterilized and assayed for germination. For biochemical analyses 0.20 g of seeds were used in duplicate. Plates were sealed with parafilm and placed in temperature-controlled incubators. Conditions for seed sterilization and germination were as described earlier (Sarath et al. 2006b), except experiments were conducted at 35°C unless noted otherwise.

Seeds were considered as germinated when the radicle had protruded through the seed coat. All germination experiments were repeated thrice and data were analyzed from this pooled set. Statistical analyses for analysis of variance (ANOVA) were performed using the statistical routines available in Microsoft Excel. Critical values of the *t*-distribution were obtained from published tables (Steel and Torrie 1982). Seeds of big bluestem (*Andropogon gerardii* Vitman) and indiangrass [*Sorghastrum nutans* (L.) Nash] were surface-sterilized using bleach and germinated and counted as described above. Root and coleoptile lengths when measured were performed 6 days post-imbibition using a dissecting microscope and a plastic scale with mm markings. Root and coleoptile lengths were measured from the tip to the seed and rounded to the nearest mm.

Any direct degradation of ABA by peroxide was monitored by incubating ABA in a solution of H_2O_2 for a period of 4 days in the incubator used for seed germination assays. Triplicate aliquots corresponding to approximately 30 ng ABA were removed daily, dried and analyzed as its silyl esters using *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane using manufacturer recommended protocols (Pierce Chemical Company; Rockford, IL). Gas chromatography-mass spectrometry analyses were performed as described by Sarath et al. (2006a). We did not observe a loss of ABA (inferred from peak heights) even when the ratio of ABA to peroxide was increased from 1:1,000 (conditions of assay) to 1:1,00,000 (data not shown). Based on these observations it is unlikely that peroxide contributed to a direct chemical degradation of ABA within the conditions of our assay.

Confocal microscopy

Seeds were imbibed on filter papers wetted with the indicated solutions containing 2.5 μg ml^{-1} of 4-amino-5-methylamino-2',7' difluorofluorescein diacetate (DAF-FM) a specific cell permeable probe for NO (for example, Sarath et al. 2006b). Seeds from different experimental groups were examined and imaged with an Olympus confocal laser scanning microscope (FluView500, Olympus, USA). The confocal settings including laser power were maintained the same during the period of imaging. Several seeds were imaged from each treatment, and fluorescence in the green channel (fluorescein) and in the red channel (Cy 3; autofluorescence) and the merged images were obtained. At least ten seeds were viewed for each treatment and the experiment repeated thrice. Representative images are shown.

Seed extraction and enzyme assays

Germinating seeds were harvested 2 days after imbibition, frozen on dry-ice and stored at −20°C until analyzed. This time point was chosen to detect changes, if any, in enzymes and proteins known to accompany seed germination and seedling development. We anticipated obtaining data that would indicate relative aleurone activity in switchgrass seeds as a response to treatments.

Seeds (0.2 g) were ground to a fine powder with liquid nitrogen in a mortar and pestle, weighed and transferred to 2 ml tubes and kept on ice. One milliliter of cold (4°C) 10 mM Na-phosphate buffer, pH 6.0 was added to each tube and the contents were mixed and sonicated using a microtip for 15 s (Branson Digital Sonifier 250D, VWR Corp). Homogenates were clarified by centrifugation at 13,400 rpm for 15 min at 4°C in a refrigerated microcentrifuge (MicroGPR, Thermo Electron Corp). Clarified extracts were used for all subsequent analyses. Initial experiments with desalted or non-desalted extracts did not show any differences in enzyme activities; and centrifuged crude homogenates were used in all subsequent analyses. Soluble protein was quantitated by the BCA assay (Pierce Chemical Co) using bovine serum albumin as a standard. Non-specific peroxidases were assayed at pH 6.0 in phosphate buffer essentially according to Córdoba-Pedregosa et al. (2003), and GPx activity was calculated using an extinction coefficient of 26.6 mM^{-1} cm^{-1}. Alpha-amylase was assayed using the Megazyme kit (Megazyme International Ltd., Ireland) except the protocol was adapted as follows: aliquots of seed homogenates were diluted with an equal amount of 2× assay buffer (Megazyme kit) and "activated" by incubation at 37°C for 30 min. Five microliter aliquots of "activated" extracts were assayed in triplicate in a total volume of 20 μl that contained 5 μl of amylase substrate, 5 μl of 4× assay buffer and 5 μl of water for a total time of 5 min. Reactions were stopped by adding 230 μl of 1× stop solution (Megazyme kit) and the absorbance measured at 410 nm. Enzyme activity was calculated using an extinction coefficient of 17.4 mM^{-1} cm^{-1}. Absorbance measurements were performed using a Spectramax Plus plate reader (Molecular Devices, Sunnyvale, CA, USA). NADPH-oxidases were assayed essentially according to Van Gestelen et al. (1997) using nitroblue tetrazolium (NBT) and NADPH. Crude seed homogenates (0.2 ml) were precipitated with acetone (9:1 acetone:homogenate) at −20°C for 15 min. Precipitated proteins were recovered by centrifugation at 14,000×*g* for 10 min at 4°C. Protein pellets were resuspended in buffer (50 mM Tris–Cl, 0.1 mM $MgCl_2$, 0.25 M Suc, 0.1% Triton-X-100, pH 8.0) and used to assay for NADPH-oxidases. Oxidase activities were calculated from the difference in NBT reduction using an extinction coefficient of 12.8 mM^{-1} cm^{-1} in the absence or presence of 50 U ml^{-1} superoxide dismutase (bovine erythrocytes, Sigma-Aldrich, St. Louis, MO, USA).

All assays were routinely done in triplicate and the germination experiments repeated at least thrice.

For switchgrass seeds, published colorimetric methods for determining tissue peroxide levels (Park et al. 2004; Cheeseman 2006) were unsuccessful primarily due to a high background arising from extractable components that interfered with these assays. Attempts to clean extracts by passage through normal or C-18 reverse phase matrices conditioned with phosphate buffer containing 5 mM KCN (Cheeseman 2006) were also unsuccessful. Indirect estimation of total ROS produced in switchgrass seeds using the fluorescent dye DFCDA, either after uptake by seeds or after base-catalyzed removal of acetate esters were inconsistent. A problem noticed during these studies was significant quenching of fluorescence by even trace levels of free peroxide/and or extractables present in switchgrass seeds. We therefore did not ascertain the actual levels of peroxide present in switchgrass seeds during germination or in response to the different chemical treatments. However, the bulk of our results presented below would suggest that endogenous levels of ROS are important during switchgrass seed germination.

Immunoblotting

For immunoblotting, aliquots containing approximately 20 µg protein were separated by SDS-PAGE using 12% gels (Laemmli 1970). Duplicate gels were stained with Coomassie blue to confirm equal loading (data not shown). Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus in 10 mM CAPS-NaOH, pH 11.0 containing 8% (v/v) methanol. Membranes were probed with antibodies raised to plant proteins and antigen-antibody complexes were detected by chemiluminescence (Xiang et al. 2002). Sources of antigens were as follows: AscPx (soybean root nodules, rabbit; Dalton et al. 1993), MAT [peptide antibody based on C-terminal of *Arabidopsis thaliana* MAT-3 protein, *accession number*: gi 15450421, sequence (C)DPDFTWEVVKPLKWDKPQA-AMIDE, rabbit], and PPDK (recombinant maize leaf, rabbit, generous gift from Dr Chris Chastain, Morehead State University, MN). All other chemicals were reagent grade or better and obtained from commercial sources. Initial experiments did not reveal any apparent changes in the level of PPDK protein between the experimental treatments 2 days post-imbibition, and these antibodies were used as a control to verify equal transfer of proteins separated by gel electrophoresis.

Results

ROS strongly stimulates switchgrass seed germination

Switchgrass seeds incubated at 35°C on water exhibited about 50% germination after 2 days, and were essentially fully germinated after 4 days (73.4%) (Fig. 1a). Imbibing seeds on various concentrations of H_2O_2 induced a pronounced stimulation in germination with an apparent concentration threshold of about 10 mM. Both 10 and 20 mM peroxide treatment elicited a significant increase in seed germination at all time points tested as compared to water controls (Fig. 1a). In contrast to seeds maintained on water, seeds imbibed on 20 mM peroxide exhibited almost 88% of full germination after only 2 days of imbibition (74.1% on day 2 vs 84.3% on day 6). Treatment with H_2O_2 concentrations

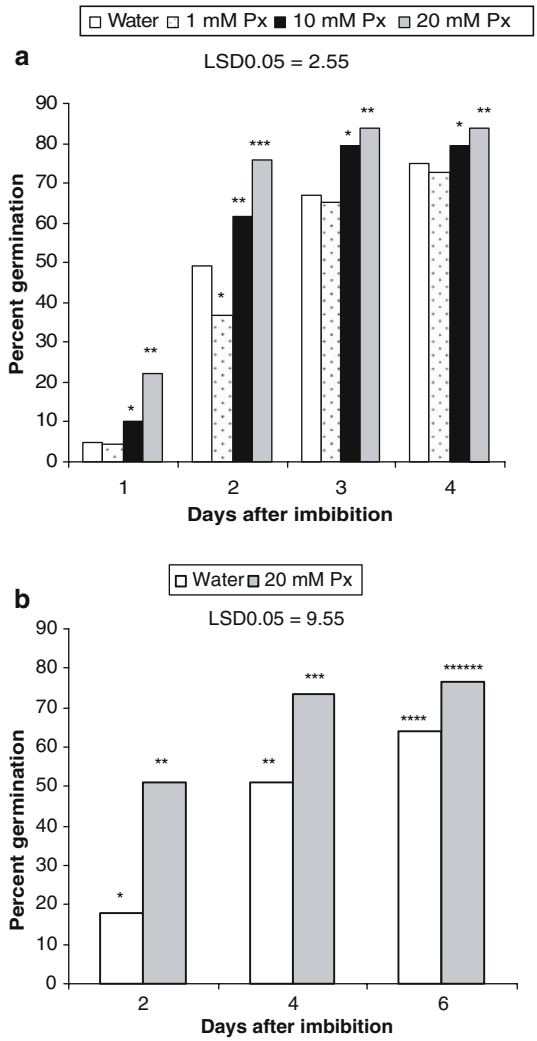

Fig. 1 Reactive oxygen species strongly promotes switchgrass seed germination. Seeds were germinated at 35°C (**a**) or at 25°C (**b**) under continuous light on filter paper wet with water, or the indicated concentrations of hydrogen peroxide (Px). Germination as determined by radicle emergence was recorded every 2 days as indicated. Data were pooled from three different experiments and analyzed. Each experiment was conducted with duplicate plates. In panel **a,** *bars* with different numbers of *asterisks* were significantly different at $P < 0.05$ as compared to water controls. In panel **b,** *bars* with different numbers of *asterisks* were significantly different at $P < 0.05$. $LSD_{0.05}$ are indicated for each experiment

of up to 60 mM did not produce a significant negative effect on switchgrass seed germination (not shown). Imbibing seeds on 1 mM H_2O_2 showed an initial inhibition of germination after 2 days, but was otherwise similar to seeds maintained on water (Fig. 1a).

Peroxide treatment (20 mM) at 25°C resulted in a significant enhancement in switchgrass seed germination after 2 days as compared to water controls (Fig. 1b). Germination after 2 days of imbibition on peroxide was equal to germination on water after 4 days, 50.1% vs 49.6% respectively. Percent germination in seeds imbibed on 20 mM peroxide at 25°C continued to be significantly greater across all days as compared to water controls (Fig. 1b). At both temperatures tested, 20 mM H_2O_2 resulted in a near simultaneous development of the coleoptile along with the root.

ROS accelerates seed germination in other warm-season grasses

We evaluated the responses of seeds from two other warm-season grasses, big bluestem (*A. gerardii* Vitman.) and indiangrass [*S. nutans* (L.) Nash] to peroxide treatment. Data for each species was analyzed separately and are shown in Table 1. Seed germination in both species was significantly stimulated by H_2O_2 at 35°C. However the concentrations of H_2O_2 required to elicit maximal response were different for the two species. For big bluestem, all concentrations of peroxide tested significantly accelerated germination as compared to seeds maintained on water. For seeds maintained on 20 mM or 40 mM H_2O_2, the percent germination 2 days post-imbibition on was essentially equal to the percent germination for seeds maintained on water for 7 days (46.1 and 45.7% vs 47.4% respectively). At 60 mM H_2O_2 germination after 2 days was greater than twofold as compared to water controls (62.6% vs 27.3% respectively). Highest germination was observed for big bluestem seeds maintained on 60 mM H_2O_2 for 7 days.

For indiangrass, imbibition on 20 mM H_2O_2 caused the most rapid germination and by day 2 the percent germination was already significantly greater than those observed for seeds maintained on water for 7 days (59.3% vs 46.1% respectively) (Table 1). With increasing concentrations of peroxide, seed germination rates declined, and at 60 mM H_2O_2, germination was essentially similar to water controls (Table 1). Indiangrass seeds imbibed on 40 mM H_2O_2 exhibited an intermediate level of germination as compared to seeds maintained on 20 or 60 mM H_2O_2.

Table 1 Exogenous hydrogen peroxide (H_2O_2) accelerates germination of two other native prairie grasses

Species	Percent radicle emergence		
	Days after imbibition		
	2	4	7
Big bluestem			
Water	27.3 a	40.3 b	47.4 b
20 mM H_2O_2	46.1 b	62.3 c	74.2 d
40 mM H_2O_2	45.7 b	70.4 cd	72.9 d
60 mM H_2O_2	62.6 cd	77.5 d	80.1 d
$LSD_{0.05}$ = 8.42			
Indiangrass			
Water	27.2 a	42.5 b	46.1 bc
20 mM H_2O_2	59.3 d	71.1 e	73.6 ef
40 mM H_2O_2	45.3 bc	64.4 d	66.9 de
60 mM H_2O_2	29.6 a	44.1 b	51.0 bc
$LSD_{0.05}$ = 6.41			

Data are the means of three experiments with duplicate plates. Germination data were analyzed separately for each species across all days, and values followed by a different letter were significantly different at $P < 0.05$ within each species. $LSD_{0.05}$ values are indicated for each dataset

Inhibitory effects of ABA and DPI on switchgrass seed germination are reversed by ROS

Earlier studies had shown that ABA significantly retarded switchgrass seed germination and essentially abolished coleoptile elongation at 25°C. External compounds that generated NO did not appreciably reverse the inhibitory effects of ABA (Sarath et al. 2006b), suggesting that ABA was affecting processes both upstream and downstream of NO action. To more fully understand these relationships, switchgrass seed germination and early seedling growth were monitored in the presence or absence of ABA, H_2O_2 and an inhibitor of NADPH oxidases, DPI.

Germination data as scored by radicle emergence and coleoptile emergence were analyzed independently of each other and are shown in Table 2. Treatment with 20 mM H_2O_2 significantly stimulated switchgrass seed germination 2 days post-imbibition as compared to water controls (56.2% vs 31.6% respectively). Both 20 μM DPI and 20 μM ABA significantly retarded radicle emergence after 2 days as compared to seeds maintained on water or on H_2O_2 (Table 2). Over the next 4 days about 66% of seeds maintained on water had germinated, as compared to 83.5% germination for seeds maintained on H_2O_2 and 44.3 and 51.7% for seeds maintained on DPI and ABA respectively. In marked contrast, imbibing switchgrass seeds on filter paper wet with solutions containing either DPI or ABA and H_2O_2 resulted in a significant reversal of the inhibitory effects observed in the presence of these compounds alone. Co-treatment with H_2O_2 + DPI resulted in germination percentages which were essentially similar to seeds that were maintained on H_2O_2 alone. For seeds imbibed on solutions

Table 2 Negative effects of DPI and ABA on switchgrass seed germination are reversed by H_2O_2. Seeds were germinated in the light at 35°C and radicle and coleoptile emergence score 2 and 6 days after imbibition

Treatment	Percent emergence	
	Days after imbibition	
	2	6
Radicle		
Water	31.6 a	65.7 d
20 mM H_2O_2	56.2 b	83.5 e
20 μM DPI	19.1 c	44.3 f
20 μM ABA	17.9 c	51.7 b
20 μM DPI + 20 mM H_2O_2	63.2 d	78.8 e
20 μM ABA + 20 mM H_2O_2	54.9 b	71.2 g
$LSD_{0.05}$ = 5.69		
Coleoptile (percent of germinated seeds)		
Water	15.8 a	79.3 f
20 mM H_2O_2	45.2 b	86.1 g
20 μM DPI	17.9 a	84.4 gh
20 μM ABA	0.0 c	41.2 e
20 μM DPI + 20 mM H_2O_2	6.7 d	81.3 fh
20 μM ABA + 20 mM H_2O_2	38.4 e	69.6 i
$LSD_{0.05}$ = 3.14		

Data are the means of three different experiments containing duplicate or triplicate plates. Radicle and coleoptile emergence data were analyzed independent of each other and across all days. Values followed by different letters are significantly different at $P < 0.05$ for each parameter analyzed. $LSD_{0.05}$ values are indicated for each dataset

containing ABA + H_2O_2, germination on day 2 was almost threefold greater than for seeds imbibed on ABA alone (54.9% vs 19.1% respectively). After 6 days, total germination was significantly greater than that observed for seeds maintained on water or on ABA alone (71.2% vs 65.7% and 51.7% respectively) but less than for seeds imbibed on H_2O_2 alone (Table 2). Although radicle emergence was used as a measure of seed germination, seedling development also requires coleoptile and root growth. Coleoptile emergence was scored for germinated seeds, and results are shown in Table 2. For seeds maintained on water about 16% of the germinated seeds had visible coleoptiles after day 2 and this percentage increased to nearly 80% after day 6, as compared to 45.2% of seeds containing visible coleoptiles in seeds maintained on H_2O_2 after day 2 and 86% after day 6. DPI did not appear to have an inhibitory effect on coleoptile emergence in contrast to its inhibitory effect on germination (Table 2), and after day 6 approximately 81% of germinated seeds had a shoot system. ABA strongly retarded coleoptile development as well as radicle emergence, and after 6 days approximately 41% of germinated seeds maintained on ABA had functional shoots (Table 2). For seeds imbibed on solutions containing ABA or DPI + H_2O_2, there was a marked enhancement in both germination as well as coleoptile emergence. In the case of DPI, co-treatment with H_2O_2 did not stimulate coleoptile emergence after 2 days, but after 6 days coleoptile emergence was similar to water controls and seeds maintained on H_2O_2 alone (Table 2). For seeds imbibed on solutions with ABA and H_2O_2, the reversal of inhibition was even more striking. After 2 days, the percentage of germinated seeds with coleoptiles in the ABA +H_2O_2 treatment was approximately twofold greater as compared to water controls (38.4% vs 15.8% respectively). After 6 days, this number had increased to almost 70% for seeds imbibed on ABA + H_2O_2 as compared to 41% for seeds maintained on ABA alone.

Root and coleoptile lengths are affected by the chemical treatments

Seeds with visible roots and coleoptiles obtained from experiments shown in Table 2, were used to evaluate coleoptile and root growth as influenced by the imbibition conditions. Seeds maintained on water exhibited the longest primary roots, which were significantly different than those for the other treatments (Fig. 2a). Imbibing switchgrass seeds on H_2O_2 caused a moderate inhibition in primary root elongation. ABA induced a more profound change in root elongation relative to root lengths for seeds imbibed on water. DPI essentially abolished root growth in the time frame of the experiment (approximately 12% of water controls). We did observe new lateral growth in seeds treated with DPI after about 5–6 days, but these roots were too small to measure.

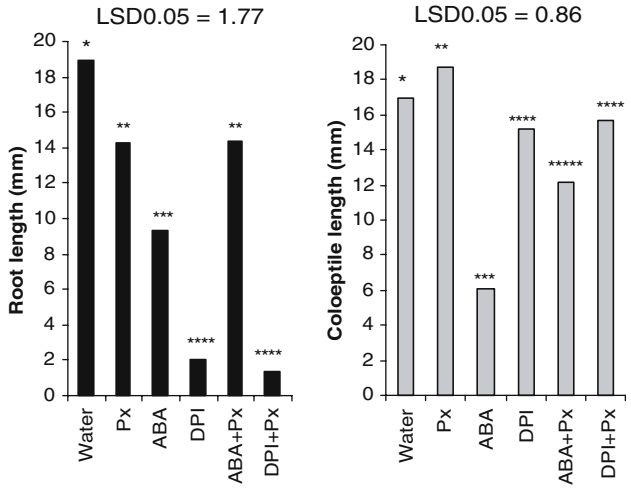

Fig. 2 Root (**a**) and coleoptile (**b**) lengths in germinated switchgrass seeds are strongly influenced by ABA and DPI. Root and coleoptile lengths were measured 6 days after imbibition in 20 germinated seedlings chosen at random from triplicate plates (at least six seedlings from each plate). The data were analyzed from this pooled set using the statistical routines available in Microsoft Excel. $LSD_{0.05}$ is indicated in each panel. *Bars* with different numbers of *asterisks* were significantly different at $P < 0.05$

Primary root growth inhibition induced by ABA was reversed by co-treatment with H_2O_2, however peroxide was without effect when supplied along with DPI (Fig. 2a).

Switchgrass seeds imbibed on 20 mM H_2O_2 had longer coleoptiles as compared to water controls (Fig. 2b). ABA treatment induced a drastic reduction in coleoptile length (approximately 32% of seeds imbibed on H_2O_2 alone). DPI treatment resulted in a smaller, but significant reduction in coleoptile length. For seeds germinated with ABA + H_2O_2, coleoptile lengths were twofold greater as compared to seeds germinated on ABA alone (12.3 mm vs 6.1 mm). Coleoptile lengths were reduced for seeds imbibed on DPI alone or in combination with H_2O_2 as compared to seeds maintained on water or on H_2O_2 by itself (15.1 and 15.7 mm vs 17.0 and 18.7 mm respectively) (Fig. 2b).

The NO scavenger PTIO inhibits ROS-induced stimulation of switchgrass seed germination

Previously it was shown that germination of warm-season grass seeds was stimulated by exogenous sources capable of generating NO, and this stimulation was blocked by the NO scavenger, PTIO (Sarath et al. 2006b). Since peroxide induced a marked stimulation of switchgrass seed germination, we studied the interaction between the NO scavenger, PTIO and H_2O_2 on seed germination. This experiment was conducted at 25°C.

Imbibing switchgrass seeds on 20 mM H_2O_2 significantly stimulated germination relative to all the other treatments over the 6 days of the experiment at 25°C (Fig. 3). After 6 days approximately 82% of all seeds maintained on peroxide had germinated, as compared to 62% for seeds imbibed on water. PTIO by itself significantly inhibited germination after 2 days as compared to water controls (23.7% vs 33.6% for PTIO and water respectively) and was then without apparent effect (Fig. 3). However, when seeds were imbibed on filter paper wet with both PTIO and H_2O_2, there was an inhibition in seed germination relative to the germination of seeds maintained on H_2O_2 by itself; although the percent germination was significantly enhanced over water and PTIO treatments across all 6 days of the experiment (Fig. 3). After 2 days, PTIO in the presence of peroxide had reduced germination by 18% as compared to seeds maintained on peroxide alone. This inhibition of switchgrass seed germination by PTIO in the presence of H_2O_2 was significant even after 6 days (Fig. 3).

NO is produced in seeds imbibed on H_2O_2

The results obtained with PTIO and H_2O_2 (Fig. 3), and the reversal by H_2O_2 of ABA-induced inhibition of switchgrass seed germination indicated that NO was a potential common denominator in these reactions. Previous studies had shown that endogenous NO is produced by germinating switchgrass seeds (Sarath et al. 2006b).

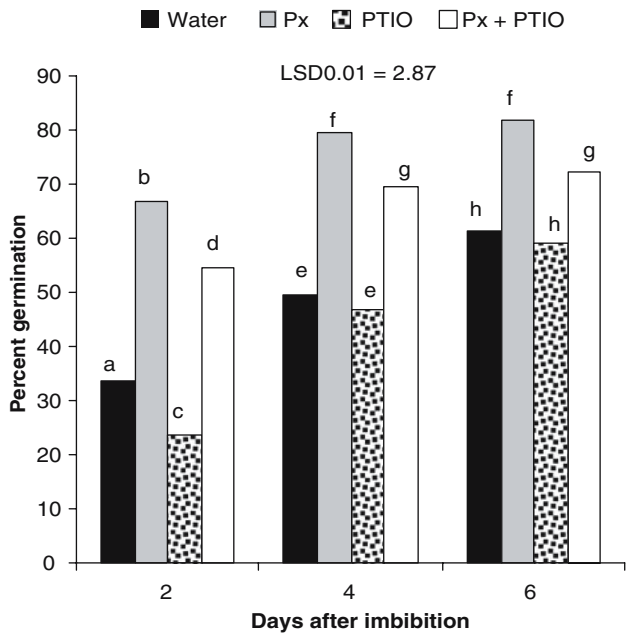

Fig. 3 2-Phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide blocks ROS induced stimulation of switchgrass seed germination. Seeds were germinated at 25°C on filter paper wet with water, 200 µM PTIO, 20 mM Px or 200 µM PTIO + 20 mM Px. Germination was determined by radicle emergence. Data are the means of duplicate plates from three independent experiments. *Bars* with different *letters* were significantly different at $P < 0.01$

As expected, signal due to endogenous NO was detected in germinating seeds across all treatments (Fig. 4a–d), although the relative signal appeared to be weaker in seeds imbibed on ABA alone (Fig. 4b). Fluorescent signal intensity was enhanced in seeds imbibed on ABA and H_2O_2 together (Fig. 4d). Hand sections of seeds indicated that the fluorescent signal was largely present in the aleurone layer (Fig. 4e). Green fluorescence was not observed for seeds imbibed in the absence of DAF-FM, however strong autofluorescence was seen at the chalazal end of the seeds (yellow color, arrow, Fig. 4d). Experiments to detect fluorescence were repeated at least thrice as were hand sectioning of germinating seeds. A representative image for each treatment is shown. Results were essentially identical for each trial.

Onset of hydrolytic activities and increase in specific proteins is hastened by ROS

Our data indicated a significant promotive effect of ROS on grass seed germination and the ability of peroxide to reverse ABA-induced inhibition of germination. Since endogenous NO was produced in seeds maintained on

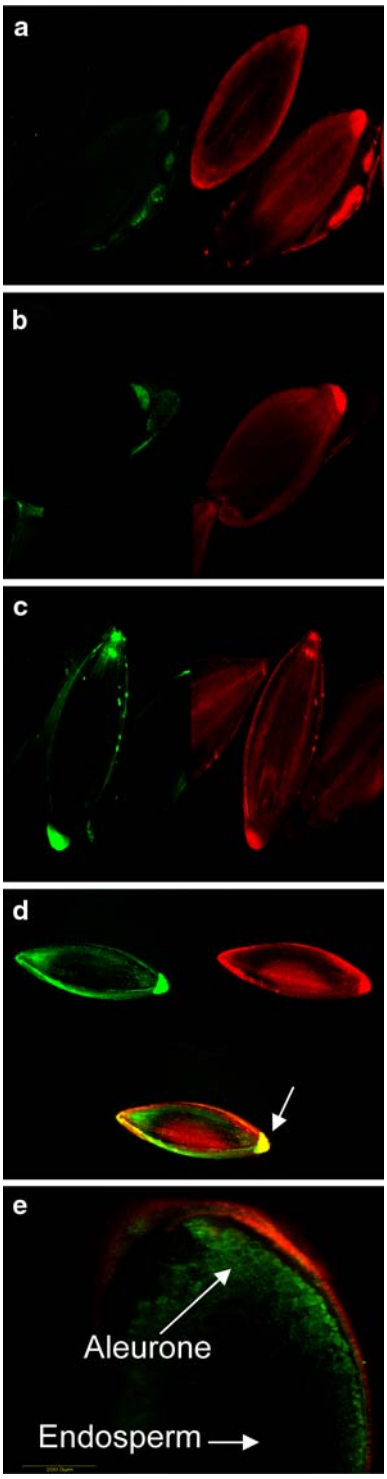

Fig. 4 Switchgrass seeds produce endogenous NO in the presence of ABA and hydrogen peroxide. Seeds were germinated in the dark on filter paper wet with water (**a**), 20 μM ABA (**b**), 20 mM H_2O_2 (**c**), or 20 μM ABA + 20 mM H_2O_2 (**d**) and hand-sectioned seed imbibed on water (**e**) in the presence of the cell-permeable form of the NO-specific fluor (DAF-FM diacetate) for 2 days in the dark at 25°C. Seeds were then viewed using an inverted confocal laser scanning microscope (FluView500, Olympus, USA). Representative images of single seeds are shown. For panels **a**, **b** and **c**, the *green image* includes signal from DAF and that due to autofluorescence. In panel **d** the merged image indicates *green fluorescence* specifically attributable to the reaction between DAF-FM and NO. The *white arrow points* to a zone of high autofluorescence at the chalazal end of the seed (*yellow* in the merged image). In panel **e** the endosperm (no fluorescence) and the aleurone (*green fluorescence*) are shown with *arrows*. Seeds imbibed on solutions in the absence of DAF-FM only exhibited *red fluorescence* and are not shown

ABA (Sarath et al. 2006b) with or without H_2O_2 (Fig. 4), we explored some biochemical changes occurring in switchgrass seeds 2 days post-imbibition to obtain an estimate of aleurone activation and seedling metabolism affected by the treatments. We did not attempt to dissect embryos and aleurone layers from these small seeds and the data represent proteins present in the aleurone, embryo and endosperm combined.

Guaiacol peroxidase activity was present in dry seeds and increased several-fold with time in all treatments. Seeds imbibed on water displayed an approximately sixfold increase in peroxidase activity. ABA treatment reduced GPx activity approximately 30% as compared to water controls. Seeds imbibed on peroxide or peroxide + ABA displayed an approximately eightfold greater activation in GPx levels as compared to seeds at the start of imbibition (Fig. 5a).

Trace levels of α-amylase present in dry seeds increased gradually in seeds maintained on water, with an approximately threefold increase over 2 days (0.05 nmol min^{-1} mg^{-1} protein vs 0.14 nmol min^{-1} mg^{-1} protein). For seeds imbibed on ABA, there was a more modest increase in α-amylase activity relative to dry seeds; this value was approximately 50% less compared to seeds imbibed on water alone (0.07 nmol min^{-1} mg^{-1} protein vs 0.14 nmol min^{-1} mg^{-1} protein, respectively). In the presence of 20 mM H_2O_2, the increase in α-amylase was more dramatic. By 2 days post-imbibition activity level were fivefold greater than that observed in seeds at the start of imbibition (day 0), and approximately 71% greater than for water controls. In seeds maintained on ABA + H_2O_2, levels of α-amylase were greater than in seeds maintained on ABA alone, but less than those imbibed on peroxide alone (Fig. 5b).

After 2 days post-imbibition, levels of MAT and AscPx protein were greater in extracts from seeds maintained on peroxide as compared to seeds imbibed on water. ABA abolished increases in the levels of these two proteins. For seed maintained on ABA and peroxide, the levels of AscPx and MAT were similar to those observed for seeds maintained on peroxide alone (Fig. 5c). As a blotting control, we used antibodies to PPDK. There was no apparent difference in the amount of signal for this protein between the experimental treatments (Fig. 5c) indicating approximate equal transfer in the different lanes. Protein loads were also verified by staining duplicate gels with Coomassie blue (data not shown).

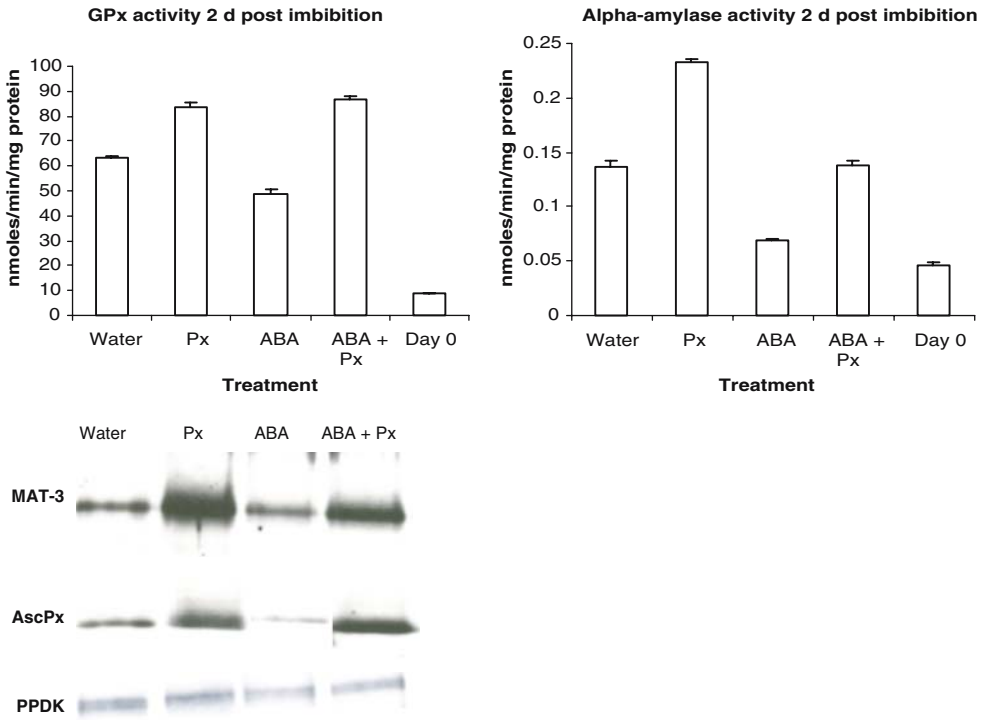

Fig. 5 Enzyme activity of guaiacol peroxidase, GPx, (**a**), alpha-amylase (**b**) and immunoblot of proteins (**c**) extracted from switchgrass seeds 2 days post-imbibition. Seeds from duplicate plates (0.20 g/plate) were ground in 20 mM phosphate buffer, pH 6.0 and clarified by centrifugation. Clarified homogenates were quantitated for total protein. Enzyme assays were performed in triplicate and *bars* show s.e.m for the assays. For immunoblotting, 20 µg aliquots obtained from seeds imbibed on water, H_2O_2 (Px), ABA, and ABA + H_2O_2 (ABA + Px) were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes probed with antibodies to the following proteins: *MAT*, methionine-adenosyl transferase, *AscPx*, ascorbate peroxidase and *PPDK*, pyruvate, *ortho*phosphate dikinase. PPDK was used as a control to establish approximately equal transfer of proteins separated by gel electrophoresis

NADPH-oxidases are inhibited by DPI

Among the potential sources of endogenous ROS in seeds are NADPH-oxidases, which utilize molecular oxygen and NADPH to produce superoxide anions, which can then participate in the formation of other ROS (Torres and Dangl 2005). After 2 days of imbibition on different solutions, activities of putative NADPH-oxidases were enhanced by treatment with H_2O_2 (Px; ~55%) and decreased in seeds imbibed on DPI (~30%) relative to seeds maintained on water (Fig. 6). Seeds imbibed in the presence of ABA possessed NADPH-oxidase activities similar to water controls. Addition of H_2O_2 to ABA and DPI enhanced NADPH-oxidase activities relative to imbibition of seeds on ABA or DPI alone, but were not as high as that observed for seeds imbibed on H_2O_2 by itself.

Discussion

Our data (Fig. 1; Tables 1and 2) show that treatment with H_2O_2 as an exogenous source of ROS significantly promotes seed germination in several warm-season C_4-grasses such as switchgrass, big bluestem and indiangrass. Treating seeds with H_2O_2 has been shown to promote germination in several other species, including zinnia (*Zinnia elegans* L.)

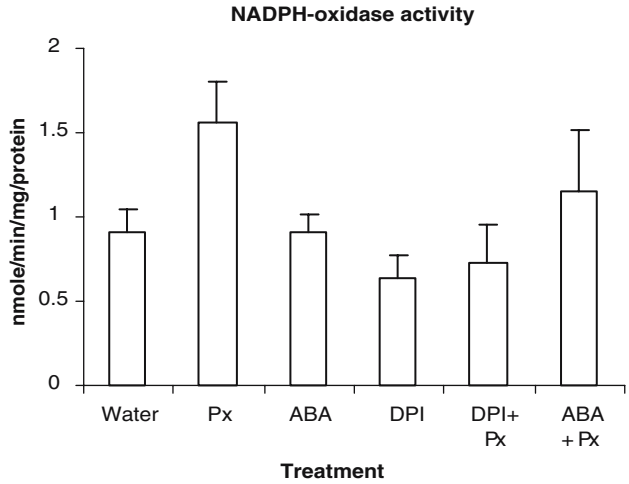

Fig. 6 Activity of NADPH-oxidases in switchgrass seeds in response to imbibition on different chemicals. Extracts were prepared from seeds from duplicate plates for each treatment, and used to assay for enzyme activities. Enzyme assays were performed in triplicate and *bars* show s.e.m for the assays

(Ogawa and Iwabuchi 2001), rice (*Oryza sativa* L.) (Naredo et al. 1998), wheat (*Triticum aesitvum* L.) (Wahid et al. 2007) and barley (*H. vulgare* L.) (Wang et al. 1998). What has not been explored in warm-season grasses is the relationship between H_2O_2, NO and ABA during seed germination. For switchgrass seeds, ABA inhibited germination and exogenous NO was unable to overcome ABA effects on seed germination, suggesting that NO responsive cascades could be effectively blocked by ABA (Sarath et al. 2006b). Negative effects of ABA were more pronounced on coleoptile growth as compared to radicle emergence, underscoring the differential hormonal responses of the young seedling tissues. In contrast, imbibition on peroxide resulted in the simultaneous development of the seedling shoot and root system (Table 2), and imbibition of switchgrass seeds on ABA + H_2O_2 resulted in a significant enhancement in shoot and root growth as compared to seeds maintained on ABA alone. These data demonstrated that for switchgrass seeds, addition of H_2O_2 could largely overcome the negative effects of ABA, possibly by swamping ABA-dependent signaling, as has been suggested for barley (Wang et al. 1998).

Conceivably, exogenous peroxide could act through several different routes in significantly promoting switchgrass seed germination. These are: (1) through interactions with existing ROS mediated GA/ABA/NO dependent signaling cascades (Beligni et al. 2002; Kwak et al. 2003; Malolepsza and Rozalska 2005; Bright et al. 2006; Pandey et al. 2006); (2) by oxidative destruction of germination inhibitors (Ogawa and Iwabuchi 2001) and enhanced oxygenation of tissues (for example, Wang et al. 1998); or (3) by a combination of ABA dependent and ABA-independent mechanisms.

The known effects of NO, ABA and gibberellic acid on switchgrass seed germination (Zarnstorff et al. 1994; Loch et al. 2004; Sarath et al. 2006b) and processes such as after-ripening and cold-stratification that impact endogenous ABA levels in other species and prime seeds for germination (Loch et al. 2004; Gubler et al. 2005), would strongly favor a hormonal basis for controlling seed dormancy and germination in switchgrass. These results would suggest that non-ABA-related germination inhibitors are unlikely to be present in switchgrass, as has been suggested for Zinnia (Ogawa and Iwabuchi 2001). Furthermore, the switchgrass seeds used for our current experiments were after-ripened and extensively cold-stratified, and would be expected to be suitably primed for germination. In fact germination on water approached nearly 70% (for example, Fig. 1). In the absence of a significant direct degradation of ABA by H_2O_2 in-vitro, especially in the first 48 h, the ability of H_2O_2 to overcome ABA inhibition suggests that peroxide is acting on ABA-responsive mechanisms and potentially on other signaling pathways. Although our current data do not discriminate between these pathways, future experiments using dissected embryos and aleurone tissues could provide detailed insights into switchgrass seed signaling cascades.

The strong positive germination response to H_2O_2 indicated the potential for ROS involvement during switchgrass seed germination. Interestingly, DPI an inhibitor of NADPH-oxidases (Foreman et al. 2003) inhibited switchgrass seed germination and root growth (Table 2), but not coleoptile growth (Fig. 2a, b). This compound is known to block root development in Arabidopsis (Foreman et al. 2003) through inhibition of a specific NADPH-oxidase (AtRboh-C).

Simultaneous application of H_2O_2 overcame the inhibition of germination by DPI, but not its effect on root elongation, indicating the potential involvement of switchgrass Rbohs during root elongation in switchgrass as well. These data also provided further proof that H_2O_2 is probably not acting on an endogenous chemical inhibitor of seed germination, but more likely through hormone-responsive processes. Since DPI can inhibit flavin-containing enzymes (Foreman et al. 2003), it is possible that proteins other than NADPH-oxidases are also targets for DPI inhibition. However, our data on NADPH-oxidase activity and endogenous ROS levels suggests a role for oxidative signaling during switchgrass seed germination and highlights the fact that exogenous peroxide is able to override parts of the pathway influenced by ABA and DPI potentially acting through oxidative signaling arising from NADPH-oxidases.

Peroxide treatment markedly enhanced the levels of GPx, α-amylase activities and MAT and AscPx proteins during early stages of germination of switchgrass seeds. Increase in hydrolytic activities accompanies seed germination, and increases in α-amylase is a classical indicator of aleurone activation in grass seeds (for example, Loch et al. 2004; Gubler et al. 2005) MAT is required for generating *s*-adenosyl methionine, a key metabolite required for growth processes and for the formation of ethylene. In Arabidopsis, methionine synthase and MAT protein and activity are enhanced during seed germination (Gallardo et al. 2002). That MAT protein is upregulated in switchgrass seeds treated with H_2O_2 is also in concordance with the scheme presented by Zago et al. (2006) for tobacco (*Nicotiana tabacum* L). In tobacco, both NO and H_2O_2 apparently act on a combination of signaling pathways including ethylene synthesis via s-adenosylmethionine (Zago et al. 2006). For AscPx, peroxide treatment caused an earlier appearance of this protein as compared to the water controls. Interestingly, ABA treatment abolished the appearance of AscPx in concert with its inhibition of coleoptile development. Ascorbate metabolism and its importance to redox regulation in plant tissues is well established (Smirnoff et al. 2001). Levels of AscPx are high in metabolically active

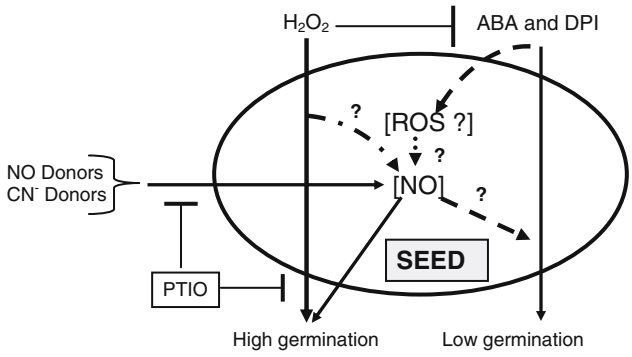

Fig. 7 A model integrating the observed effects of ABA, DPI, hydrogen peroxide (ROS) and nitric oxide during germination of switchgrass seeds. The rationale for the model is explained in Sect. "Discussion". Rboh, ROS generating NADPH-oxidases; ABA by itself is able to block pro-germinative cascades. Finally, external peroxide is able to overcome both DPI and ABA induced arrest of warm-season grass seed germination

tissues, and increase during seed germination (Tommasi et al. 2001). Our results indicated that MAT and AscPx proteins appear to be markers for seedling development and seedling vigor in switchgrass.

The relative inhibition by PTIO of H_2O_2-stimulated germination (Fig. 3) was similar in trend to that observed for switchgrass seeds imbibed on PTIO and NO donors (Sarath et al. 2006b) suggesting that residual dormancy in after-ripened switchgrass seeds is indeed more directly responsive to NO rather than other stimuli, as has been shown for Arabidopsis and barley (Bethke et al. 2004).

Based on our data presented previously (Sarath et al. 2006b) and in this manuscript, we propose the following model integrating information obtained with warm-season grass seeds (Fig. 7). In this model, exogenous ABA and DPI block germination potentially through the involvement of ROS and NO (broken arrows with question marks). Both ABA and DPI have been shown to interact with ROS, Rbohs and NO in other plants (Foreman et al. 2003; Torrey and Dangl 2005; Bright et al. 2006; Zago et al. 2006) and in switchgrass (this study).

Application of mM levels of external H_2O_2 stimulated seed germination and overcame the inhibitory effects of ABA and DPI. Similarly, external sources of NO or CN^- promoted germination in switchgrass (Sarath et al. 2006b). That PTIO blocked both H_2O_2 and NO/CN donor-induced increases in germination (Sarath et al. 2006b; Fig. 3) suggests that NO could be a common denominator (broken arrow and question mark in Fig. 7). How a switchgrass seed senses NO levels and how much NO is needed for seed germination is unknown. However, our data so far suggests that cellular NO level is likely to be a key indicator of germinability of switchgrass seeds.

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