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# Nitric oxide accelerates seed germination in warm-season grasses

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ORIGINAL ARTICLE

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**Abstract** The nitric oxide (NO) donor sodium nitroprusside (SNP) significantly promoted germination of switchgrass (*Panicum virgatum* L. cv Kanlow) in the light and in the dark at 25°C, across a broad range of concentrations. SNP also promoted seed germination in two other warm-season grasses. A chemical scavenger of NO inhibited germination and blocked SNP stimulation of seed germination. The phenolic (+)-catechin acted synergistically with SNP and nitrite in promoting seed germination. Acidified nitrite, an alternate NO donor also significantly stimulated seed germination. Interestingly, sodium cyanide, potassium ferricyanide and potassium ferrocyanide at 200 μM strongly enhanced seed germination as well, whereas potassium chloride was without effect. Ferrocyanide and cyanide stimulation of seed germination was blocked by an NO scavenger. Incubation of seeds with a fluorescent NO-specific probe provided evidence for NO production in germinating switchgrass seeds. Abscisic acid (ABA) at 10 μM depressed germination, inhibited root elongation and essentially abolished coleoptile emergence. SNP partially overcame ABA effects on radicle emergence but did not overcome the effects of ABA on coleoptile elongation. Light microscopy indicated extension of the radicle and coleoptiles in seeds maintained on water or on SNP after 2 days. In contrast, there was minimal growth of the radicle and coleoptile in ABA-treated seeds even after 3–4 days. These data indicate that seed germination of warm-season grasses is significantly influenced by NO signaling pathways and document that NO could be an endogenous trigger for release from dormancy in these species.

**Keywords** Microscopy · Nitric oxide · Seed dormancy · Seed germination · Switchgrass · Warm-season prairie grasses

**Abbreviations** *ABA*: Abscisic acid · *DAF-FM*: 4-amino-5-methylamino-2',7' difluorofluorescein · *NO*: Nitric oxide · *PTIO*: 2-Phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide · *SNP*: Sodium nitroprusside

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## Introduction

Seed dormancy and subsequent germination are physiologically adaptive processes that allow a species to optimize seedling establishment. Dormancy mechanisms prevent seed germination even under favorable conditions, and are present to various degrees in most angiosperm seeds (Bewley 1997; Koornneef et al. 2002). Dormancy could arise from physical barriers, physiological barriers or from a combination of the two. Structural barriers are present at the time of seed maturation, and are removed through an external environmental event, such as fire, cold, etc. Physiological barriers to germination most frequently reside in the embryo and require post-maturation changes to occur in order to be removed. These after-ripening processes enhance the ability of a seed to germinate (Shen et al. 1999; Veasey et al. 2004). Release from dormancy favors germination, whereas, the germination process is still affected by internal and external stimuli (Bewley 1997; Koornneef et al. 2002; Bethke et al. 2004b).

Both precocious germination and extended dormancy are detrimental for crops established from seed, where predictability of germination and seedling establishment are crucial for agronomic success. It is well known that plant hormones can impact seed dormancy and germination and have been extensively studied in dicots and monocots. For example, abscisic acid (ABA) generally inhibits germination (Gubler et al. 2005), whereas gibberellic acid promotes germination (Romagosa et al. 2001; Zentella et al. 2002; da Silva et al. 2004). More recently, it has become apparent that molecules, such as nitric oxide (NO) and reactive oxygen intermediates, can significantly impact plant growth and development (Neill et al. 2002; Lamattina et al. 2003; del Rio et al. 2004; Apel and Hirt 2004; Lamotte et al. 2005). Treatment with NO or reactive oxygen donors, such as sodium nitroprusside (SNP) or hydrogen peroxide respectively can enhance germination and/or break dormancy in seeds (Beligni and Lamattina 2000; Neill et al. 2002; Bethke et al. 2004b). These findings highlight the complex interplay of endogenous mechanisms and exogenous signals in controlling seed dormancy and germination. It is also known that different plant species frequently exhibit significant variations in their germination responses to exogenously imposed conditions (Bewley 1997; Koornneef et al. 2002). Knowledge gained from individual species can assist in developing optimized conditions for enhancing germination and also provide insights into the commonality of underlying molecular processes.

Warm-season ($C_4$) grass seeds consist of a caryopsis covered by two bracts, the lemma and the palea. The caryopsis contains the embryo, a starchy endosperm, the scutellum and the aleurone layer. Once the seed has imbibed water, radicle extension precedes coleoptile emergence. This process requires weakening of the endosperm cap, cell elongation of the coleorhiza and eventual emergence of the radicle, followed by the development of root hairs (Loch et al. 2004). Initial radicle extension occurs from cell elongation, and further development of the root requires cell division. Thus, seeds can exhibit radicle extension, and still not produce a viable seedling. As observed for other species, $C_4$-prairie grasses also exhibit seed dormancy and variable germination. Treatment with various chemicals can influence germination in these grasses (see review by Loch et al. 2004). The most commonly applied conditions to assay warm-season grass seed germination and predict establishment under field conditions are chilling for 2 weeks followed by germination on 0.2% nitrate (Loch et al. 2004; Handbook of the Association of Official Seed Analysts 2003; http://www.aosaseed.com). The use of nitrate suggests that NO could be a regulator of warm-season prairie grass seed germination and potentially a breaker of seed dormancy.

In this study we have evaluated the effect of two NO donors SNP and acidified nitrite, the NO scavenger—2-Phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), and the chemicals, nitrite, nitrate, cyanide, ferrocyanide, ferricyanide, ABA and (+)-catechin predominantly on the germination of switchgrass (*Panicum virgatum* L.). SNP, cyanide ferrocyanide and ferricyanide significantly enhanced germination. PTIO by itself inhibited germination and also blocked the initial and prolonged stimulation of germination observed in the presence of SNP, cyanide, and ferrocyanide. Interestingly, the phenolic, (+)-catechin alone had minimal effect on switchgrass seed germination rates, however, in combination with SNP or nitrite it acted synergistically to promote germination. Using a specific cellular probe for NO, it was possible to document NO production in germinating switchgrass seeds. ABA significantly reduced germination and essentially abolished coleoptile emergence. SNP could partially overcome ABA inhibition of radicle emergence, but did not mitigate ABA effects on coleoptile emergence.

## Materials and methods

### Plant materials and germination assays

*P. virgatum L.* cv Kanlow seeds were obtained from plants grown in the field at Mead, NE. Seeds were cold stratified at 4–8°C for at least 6 weeks before initiation of experiments. Seeds were placed in a tea-leaf strainer and surface sterilized in 5% commercial bleach for 15 min with stirring. Seeds were washed extensively with autoclaved distilled water (3 × 5 min), and placed on autoclaved glass petriplates containing two layers of Whatman No. 1 filter paper wet with 10 ml of the appropriate solution, excess liquid was discarded before plating with seeds. Compounds selected for germination assays have been previously shown to impact seed germination in other plants and/or used as a source for generating reactive nitrogen species, cyanide or a combination of these species (e.g.,; Loch et al. 2004; Bethke et al. 2005). The concentrations of KCl, cyanide, ferrocyanide and ferricyanide tested were based on the optimal concentration of SNP (200 μM) used for most of the germination assays.

All compounds including ABA, SNP, PTIO, catechin and other salts were freshly prepared as stock solutions in sterilized double distilled water for each experiment, and diluted to the appropriate concentrations prior to application to filter papers. The pH of a 200-μM SNP solution was approximately 6.5 (equivalent to distilled water). Measured pH values of solutions containing (+)-catechin alone or in combination with SNP or nitrite were between 4.8 and 4.9. In all experiments, except for the one described next, seeds were placed directly on filter paper wetted with the appropriate solutions.

Acidified nitrite was also used as another means of generating gaseous reactive nitrogen oxides (Wu et al. 2001). For seeds germinated in the presence of acidified nitrite, 10 ml of 100 mM HCl containing 10 mM sodium nitrite was poured into a small donor plastic

petridish (5-cm diameter), which was then placed in the center of the larger glass petriplates. Seeds were arranged on the periphery. For controls, the central donor dish contained 100 mM HCl or 10 mM nitrite.

Approximately 0.1–0.2 g of seed (75–150 seeds) was used with duplicate or triplicate plates for each treatment. Plates were placed in temperature-controlled incubators (Precision Scientific Co.) under continuous light (25 umol $m^{-2}$ $s^{-1}$) or covered tightly with aluminum foil (dark) at the indicated temperature. Seed germination, and when needed, coleoptile emergence was scored daily. Seeds were considered as germinated when the radicle had protruded through the seed coat. All germination experiments were repeated at least thrice and usually six times and the data were analyzed from this pooled set. Statistical analyses were performed using the statistical routines available in Microsoft Excel. Critical values of the *t*-distribution values 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 earlier.

### Direct detection of NO in seeds

Seeds were imbibed on filter papers wetted with the indicated solutions containing 2.5 μg/ml of 4-amino-5-methylamino-2',7' difluorofluorescein diacetate (DAF-FM), a specific cell permeable probe for NO (Kojima et al. 1999), which has been used as a marker for detecting NO in plants (Corpas et al. 2004). 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 10 seeds were viewed for each treatment and the experiment was repeated thrice. Representative images are shown.

### Microscopy

Seeds were fixed in 2.5% glutaraldehyde/4% formalin in 0.1 M phosphate buffer pH 7.0, (Ruzin1992) for 2–4 h, washed twice with phosphate buffer and processed through a graded alcohol series and stored in 70% ethanol. For light microscopy, tissue was dehydrated through 95% ethanol, embedded in JB-4 plastic, sectioned and stained with 0.1% aqueous Toluidine blue and observed using an Olympus BX 51 light microscope.

## Results

### SNP enhances switchgrass seed germination at 25°C in continuous light

Switchgrass seeds incubated at 25°C under continuous light show a burst in germination after 3 days on water, with a total germination of 56.7% occurring by 6 days after imbibition (Table 1). There were no effects of dark or changes in germination percentage even after 14 days (not shown). These studies indicated that optimal germination as scored on radicle emergence was complete by 4–5 days on water. The relative increase was approximately threefold from day 2 to day 3 (14.8% versus 44.6%). In contrast, seeds treated with a wide range of SNP concentrations showed significantly greater germination by day 2 and a 20–30% enhancement of seed germination over water controls by day 6. SNP caused continuing increases in germination until day 6. No further changes were seen after this time (not shown). SNP concentrations at 30, 200 and 500 μM significantly enhanced seed germination over time, although total percent germination on day 6 was lowest at 30-μM SNP and highest for 200-μM SNP (Table 1).

Coleoptile emergence is a requisite for seedling establishment, and SNP at 30- and 200 μM significantly

**Table 1** Dose–response of switchgrass seed germination at 25°C in continuous light to varying concentrations of SNP

| Treatment | Percent emergence | | | | | |
|---|---|---|---|---|---|---|
| | Days after imbibition | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 |
| *Radicle* | | | | | | |
| Water | 0 a | 14.8 b | 44.6 c | 52.3 d | 56.7 e | 56.7 e |
| 30-μM SNP | 0.7 a | 24.3 f | 45.2 c | 48.2 c | 59.8 e | 65.5 g |
| 200-μM SNP | 0 a | 20.9 f | 52.7 d | 57.8 e | 63.9 f | 71.2 h |
| 500-μM SNP | 0.8 a | 23.3 f | 46.7 c | 58.4 e | 68.9 h | 70.4 h |
| $LSD_{0.05}$ = 3.35 | | | | | | |
| *Coleoptile* | | | | | | |
| Water | 0 a | 0 a | 5.8 b | 36.7 c | 54.0 d | 68.7 e |
| 30-μM SNP | 0 a | 2.5 a | 14.7 f | 42.0 c | 63.0 g | 71.2 e |
| 200-μM SNP | 0a | 4.0 a | 18.2 f | 53.2 d | 73.2 e | 80.2 h |
| $LSD_{0.05}$ = 4.63 | | | | | | |

Values followed by different *letters* were significantly different at $P \leq 0.05$

**Table 2** Effect of SNP on germination of two native prairie grasses. Surface-sterilized seeds were placed on filter papers wet with 200-μM SNP or water. Data are the means of two experiments

| Species | Percent emergence | | | | | | |
|---|---|---|---|---|---|---|---|
| | Days after imbibition | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| *Big bluestem* | | | | | | | |
| Water | 2.0 a | 3.0 a | 7.2 b | 15.1 cd | 26.2 e | 30.1 e | 30.1 e |
| SNP | 9.7 b | 13.9 c | 18.5 d | 32.3 f | 44.2 g | 46.6 g | 46.6 g $LSD_{0.05}$ = 3.80 |
| *Indiangrass* | | | | | | | |
| Water | 0.0 a | 4.7 b | 9.6 c | 16.3 d | 21.1 e | 21.1 e | 21.1 e |
| SNP | 0.0 a | 1.8 a | 5.3 b | 25.1 f | 39.1 g | 39.1 g | 39.1 g $LSD_{0.05}$ = 3.04 |

Values followed by different *letters* were significantly different at $P \leq 0.05$

enhanced coleoptile growth (Table 1). By 3 days post-imbibition there was a threefold increase in germinated seeds with elongating coleoptiles when maintained on SNP. The number of germinated seeds with coleoptiles increased with time. In seeds maintained on water, approximately 69% of the germinated seeds showed emergent coleoptiles by day 6. SNP at 30 μM did not significantly enhance the number of germinated seeds with coleoptiles, but did affect the rate of coleoptile emergence during the early stages of germination. In contrast, seeds maintained on 200-μM SNP exhibited both a significant enhancement in the rate of coleoptile growth as well as the total number of germinated seeds containing coleoptiles (Table 1). These results indicated that SNP significantly enhanced germination in the light at 25°C, and that SNP at 200 μM had the greatest effect. Thus, 200-μM SNP was used in all subsequent experiments.

## SNP promotes germination in other warm-season prairie grasses

Seed germination of big bluestem and Indiangrass was promoted by 200-μM SNP (Table 2). For big bluestem, effects of SNP on seed germination were significantly different on each day as compared with the water controls. Final germination was almost 17% greater when seeds were imbibed on SNP as compared with water (Table 2). For Indiangrass, SNP appeared to have a delayed effect and germination on water was significantly greater at day 3 (Table 3). However, by day 4, germination on SNP was significantly greater than that observed for seeds maintained on water, and a maximal germination of 39.1 % was observed by day 5. In contrast, germination on water was 21.1 % after 5 days post-imbibition (Table 2).

## The NO scavenger PTIO inhibits switchgrass seed germination and blocks SNP stimulation of this process

PTIO is a potent and specific scavenger of NO (Goldstein et al. 2003) and has been used to block NO-responsive events in plants (Lamattina et al. 2003; Bethke et al. 2004b). Incubation of switchgrass seeds with 200-μM PTIO in the light at 25°C significantly inhibited germination as compared with water controls at 2 days post-imbibition (12% versus 20% for water) (Fig. 1). With time, the negative effect of PTIO decreased, and germination after 3 days post-imbibition were statistically similar to seeds maintained on water. Rewetting the filter paper with a fresh PTIO solution each day and/or keeping the plates in the dark did not enhance the extent of inhibition, nor prevent the loss on inhibitory activity by day 3.

PTIO significantly reduced germination of switchgrass seeds in the presence of SNP, 2 days post-imbibition. The relative suppression of seed germination by PTIO was observed at 3 days post-imbibition with seeds maintained on solutions containing both SNP and PTIO as compared to seeds maintained on SNP alone (Fig. 1).

**Table 3** Acidified nitrite, and nitrite + catechin, stimulate seed germination at 25°C in the light. Acidified nitrite and acid controls were placed in a central donor dish within a larger petriplate containing seeds. Data are the means of two experiments with triplicate plates

| Treatment | Percent radicle emergence | | |
|---|---|---|---|
| | Days after imbibition | | |
| | 2 | 3 | 4 |
| Water | 23.9 a | 48.7 b | 60.9 c |
| 100-mM HCl (Donor dish) | 25.3 a | 43.8 d | 59.3 c |
| 10-mM Nitrite (Donor dish) | 24.1 a | 49.2 b | 60.2 c |
| 10-mM Nitrite + 100-mM HCl (Donor dish) | 36.6 e | 67.6 f | 73.2 g |
| 200-μM Nitrite | 25.3 a | 47.9 b | 60.1 c |
| 200 μM Nitrate | 23.9 a | 44.0 b | 58.1 c |
| 200 μM Nitrite + 300 μM Catechin | 38.2 e | 55.3 h | 63.2 c |
| $LSD_{0.05}$ = 2.81 | | | |

Values followed by different *letters* were significantly different at $P \leq 0.05$

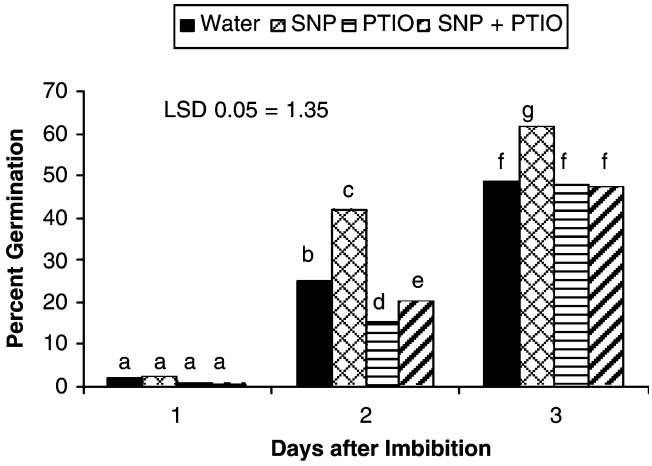


**Fig. 1** PTIO inhibits seed germination and negates SNP-induced stimulation. Seeds were germinated on solutions containing water, PTIO, PTIO + SNP and SNP. Germination was scored daily for 3 days. Data were pooled from two different experiments and analyzed. Each experiment was conducted with duplicate plates containing 120–160 seeds each. *Bars* with different *letters* were significantly different at $P \leq 0.05$

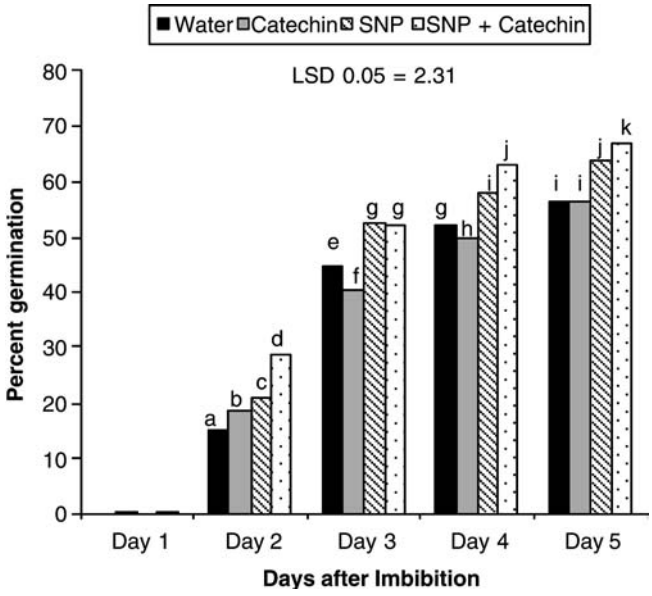


**Fig. 2** Catechin acts synergistically with SNP to promote switchgrass seed germination. Seeds were germinated at 25°C under continuous light on filter paper wet with water, 200-μM SNP, 300-μM catechin or 300-μM catechin + 200-μM SNP. Germination as determined by radicle emergence was recorded daily for 5 days. Data are the means of two triplicate experiments. *Bars* with different *letters* were significantly different at $P \leq 0.05$

## (+)-Catechin affects seed germination in the presence of SNP

SNP is a NO donor and phenolics, such as (+)-catechin are known to play a role in enhancing NO formation in the apoplast of isolated aleurone cells (Bethke et al. 2004a). Initial experiments indicated that concentrations of (+)-catechin below 200 μM had no effect and concentrations greater than 500 μM showed some inhibition of germination. A (+)-catechin concentration of 300 μM was selected for further studies. At 25°C in continuous light, (+)-catechin at 300 μM did not significantly affect seed germination as compared with water (Fig. 2), but acted synergistically with SNP to significantly enhance germination by day 2 (14.7% for water versus 28.7 for SNP + catechin). Germination of switchgrass cv Kanlow seeds on SNP and (+)-catechin were significantly different on days 3 through 5 as compared with germination on water or (+)-catechin alone (Fig. 2). When compared with the treatment with SNP, seeds imbibed on (+)-catechin and SNP together, exhibited greater germination on all days except for day 3. As observed earlier (see Table 1), SNP treatment by itself, significantly stimulated germination as compared with water controls on days 2 through 5 (Fig. 2).

## Acidified nitrite, an alternate source of NO, stimulates switchgrass seed germination

Acidified nitrate is known to generate NO as well as other gaseous nitrogen oxides (Carlsson et al. 2001; Wu et al. 2001). The effects of this alternate source of NO was evaluated along with nitrite, nitrate and nitrite + catechin on switchgrass seed germination (Table 3). As anticipated, treatment with gaseous vapors produced by acidified nitrite significantly stimulated switchgrass seed germination (73.2% versus 60.9% for water controls). The stimulatory effect of acidified nitrite was more pronounced than that of all the other treatments across all days (Table 3). Seeds imbibed on 200 μM + 300 μM catechin showed enhanced germination with respect to controls on day 2 and 3. However, total germination on day 4 was statistically similar to water controls (Table 3). Seeds incubated in the presence of vapors produced from 100 mM HCl or 10 mM nitrite (contained in a central donor dish), or imbibed directly on 200 μM $NaNO_2$, 200 μM $KNO_3$, and 200 μM KCl germinated with essentially identical rates as those observed on water alone (Table 3).

## Ferrocyanide and ferricyanide also stimulate switchgrass seed germination

Interestingly, potassium ferricyanide and ferrocyanide also significantly stimulated germination (Table 4). This stimulation was particularly dramatic at 2 days post-imbibition, where both compounds elicited over a 200% increase in germinated seeds as compared with water controls (42.3 and 45.6% versus 19.9% for seeds imbibed on ferricyanide, ferrocyanide and water, respectively) (Table 4). Ferrocyanide treatment induced the greatest stimulation of seed germination after 4 days, 77.6% versus 60.8% for seeds maintained on water.

## Ferrocyanide and cyanide stimulation of switchgrass seed germination is blocked by PTIO

Ferrocyanide stimulation of germination prompted us to study the effects of cyanide on switchgrass seeds and to test if PTIO could reverse the stimulatory effects of these chemicals. As shown in Fig. 3, cyanide by itself had a modest stimulatory effect on seed germination especially after 2 days as compared with seeds maintained on water or on ferrocyanide (30.6% versus 27.7% and 40.7% for seeds on cyanide, water or ferrocyanide, respectively). PTIO abolished the stimulatory effects of both cyanide and ferrocyanide at day 2, and germination percentages were statistically similar to those observed for seeds maintained on PTIO alone (21.3% versus 24.1 and 21.7% for seeds on PTIO, ferrocyanide + PTIO and cyanide + PTIO, respectively). After 3 and 4 days the strong stimulatory effects of ferrocyanide were markedly evident (Fig. 3). Interestingly, PTIO appeared to depress ferrocyanide- or cyanide- induced stimulation of switchgrass seed germination even after 4 days (62.3% and 61.6 versus 71.8 and 67.0% for seeds maintained on PTIO + ferrocyanide, PTIO + cyanide and ferrocyanide and cyanide alone) (Fig. 3).

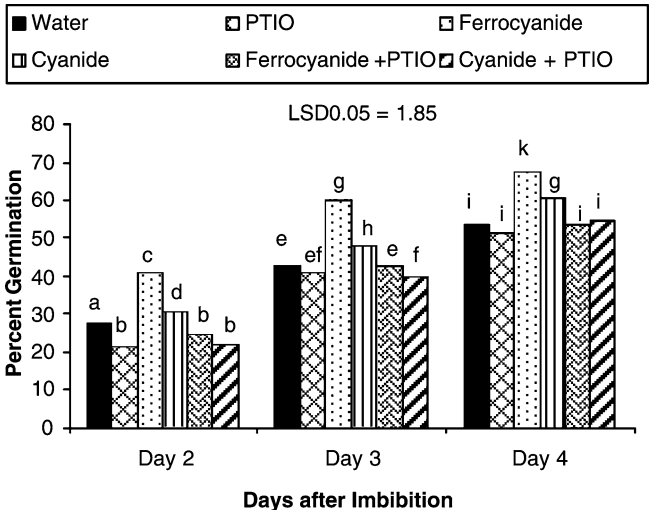


**Fig. 3** PTIO blocks ferrocyanide- and cyanide- induced stimulation of switchgrass seed germination. Seeds were germinated at 25°C on filter paper wet with water, 200 μM PTIO, 200 μM potassium ferrocyanide, 200-μM sodium cyanide, 200-μM PTIO + 200-μM ferrocyanide or 200-μM PTIO + 200-μM cyanide. Plates were sealed with parafilm and covered with aluminum foil. At 2 days post-imbibition, the foil was removed and seed germination was scored. Plates were thereafter maintained in the light. 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 $P \leq 0.05$

## Switchgrass seeds produce NO during seed germination

The strong response of switchgrass seed germination to exogenous NO suggested that these seeds could produce NO during the germination process. To document endogenous NO production, seeds were imbibed on water, 200 μM PTIO, 200 μM SNP, 200 μM nitrite + 300 μM catechin or 200 μM ferrocyanide in the absence or presence of the NO-specific fluor, DAF-FM diacetate for 2 days in the dark. Seeds were then imaged by confocal microscopy. Results from this experiment are shown in Fig. 4. NO signal (green fluorescence) detectable by the specific interaction between the dye and NO was clearly observed in all seeds imbibed in the presence of DAF-FM (white arrows in panel c for + DAF-FM treatments). As expected, seeds maintained on water, SNP and catechin + nitrite showed high specific fluorescence in seeds that were germinated or about to germinate (Fig. 4). However, the extent of the signal and the number of seeds with fluorescence was diminished in the presence of PTIO. Interestingly, for seeds maintained on ferrocyanide + DAF-FM, fluorescence was observed in seeds about to germinate, but was generally undetectable in seeds that had germinated (where the radicle had broken through the seed coat). Green fluorescence was not observed for seeds imbibed in the absence of DAF-FM. This experiment was repeated at least thrice, and several seeds were observed for each treatment. A representative image for each treatment is shown. Results were essentially identical for each trial.

**Table 4** Potassium ferrocyanide and ferricyanide significantly enhance switchgrass seed germination. Data are the means of three separate experiments using duplicate plates

| Treatment | Percent radicle emergence | | |
|---|---|---|---|
| | Days after imbibition | | |
| | 2 | 3 | 4 |
| Water | 19.9 a | 47.8 b | 60.8 c |
| 200-μM KCl | 22.4 a | 44.1 b | 57.9 c |
| 200-μM Ferricyanide | 42.3 d | 55.4 e | 68.2 f |
| 200-μM Ferrocyanide | 45.6 bd | 68.3 f | 77.6 g |
| $LSD_{0.05}$ = 2.63 | | | |

Values followed by different *letters* were significantly different at $P \leq 0.05$

## ABA inhibits switchgrass seed germination and blocks coleoptile emergence

ABA at 10 μM significantly depressed radicle emergence in Kanlow seeds, although approximately 30% of the seeds exhibited some signs of germination. In many of these seeds initial radicle elongation had occurred, but further radicle growth was not observed. Co-imbibing seeds with SNP at 200 μM and ABA enhanced radicle emergence after 4 days as compared to seeds imbibed on ABA alone (Fig. 5a). At all time points, seeds placed on water or SNP alone exhibited significantly greater germination. SNP treatment resulted in greatest stimulation of seed germination (Fig. 5a).

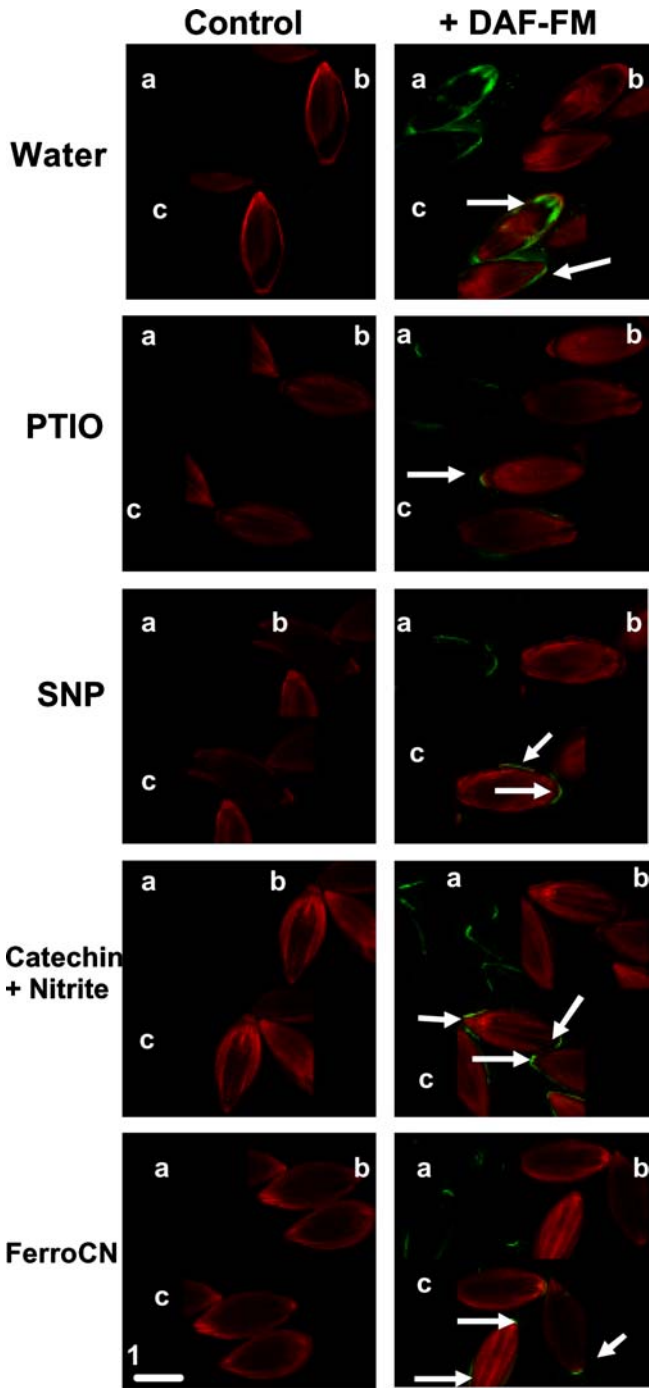


**Fig. 4** Switchgrass seeds produce endogenous NO during germination. Seeds were germinated in the dark on filter paper wet with water, 200-μM SNP (SNP), 200-μM potassium ferrocyanide (ferrocyanide), or 200-μM nitrite + 300-μM (+)-catechin (catechin + nitrite) in the absence or presence (+DAF-FM) of 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. Panel **a** is the image of the *green fluorescence*; Panel **b** is the image of the *red* (auto) *fluorescence*; Panel c is the *merged image* of fluorescence signal shown in Panels **a** and **b** and indicates any *green fluorescence* specifically attributable to the reaction between DAF-FM and NO, and is indicated by *white arrows*

Although a small positive effect of SNP co-treatment in partially overcoming ABA inhibition was observed for radicle extension, a similar effect on coleoptile growth was not observed. ABA both in the presence or absence of SNP greatly diminished coleoptile growth as compared with seeds maintained on water or on SNP alone (Fig. 5b). As observed earlier (Table 1), SNP enhanced the number of germinated seeds with emerged coleoptiles as compared with water controls.

## Light microscopy reveals changes in seeds maintained on SNP and ABA

In order to obtain some insights occurring at the tissue level, seeds kept on water, 10-μM ABA or 200-μM SNP were collected after 1, 2 and 3 days post-imbibition, fixed, embedded in plastic and sectioned. Sections were observed after staining with 0.1% Toluidine blue (Fig. 6). For seeds kept in water, there were no

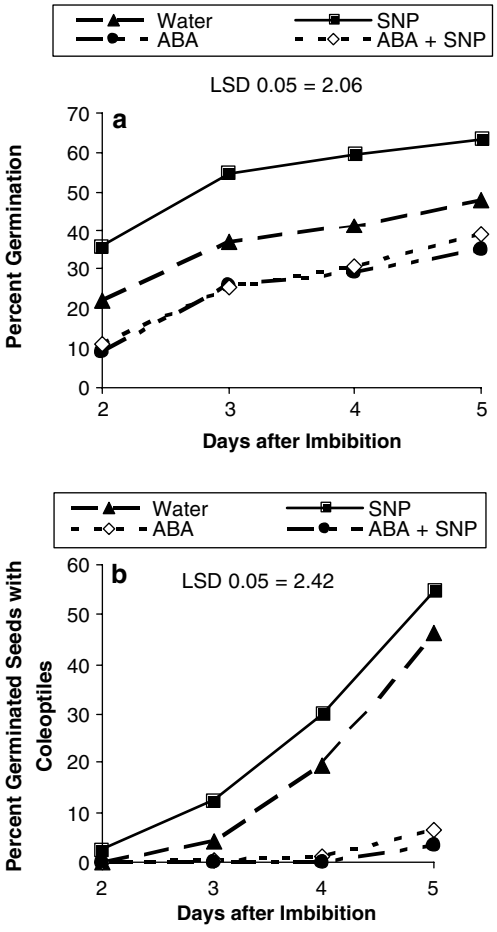


**Fig. 5** ABA significantly retards switchgrass germination and essentially abolishes coleoptile growth. Seeds were germinated in the light at 25°C on filter paper wet with water, 200-μM SNP, 10-μM ABA or 200-μM SNP + 10-μM ABA. Germination was determined by radicle emergence (a). Coleoptile growth was also scored as a percent of germinated seeds (b). Data are the means of duplicate plates from two independent experiments

apparent changes after 1 day of imbibition (Fig. 6a). After 2 days, extension of the coleorhiza through the endosperm cap was evident (Fig. 6b). By day 3, radicle elongation had occurred and the nascent root had emerged from the seed (Fig. 6c). For seeds maintained on ABA (Fig. 6d–f), there was minimal extension of the coleorhiza even after 3 days post-imbibition and negligible changes at the plumule end of the embryo. After 3 days, some elongation of the coleorhiza had occurred (Fig. 6f). In marked contrast, by day 1 post-imbibition for seeds maintained in SNP, there was some indication for coleorhizal elongation and the beginnings of the rupture of the endosperm cap (Fig. 6g). By day 2, the coleorhiza had emerged from the confines of the seed, and elongation of the radicle and coleoptile were evident (Fig. 6h). After 3–4 days post-imbibition on 200-μM SNP significant growth of the radicle and coleoptile were evident (Fig. 6i). These data were consistent with the germination data presented previously.

## Discussion

Data presented in this study for the first time that NO is a potential regulator of seed germination in warm-season $C_4$-grasses, such as switchgrass, big bluestem and Indiangrass. Seeds imbibed in SNP or in the presence of acidified nitrite, both known donors of NO (Beligni and Lamattina 2000; Wu et al. 2001; Bethke et al. 2005), elicited a significant enhancement in seed germination as compared with water controls. NO can break seed dormancy in Arabidopsis and barley (*Hordeum vulgare* L.) (Bethke et al. 2004b; Libourel et al. 2005), suggesting that NO is an endogenous regulator of seed germination in these two species. Data presented in this paper confirm and extend these findings to the warm-season $C_4$ grasses.

NO can be produced enzymatically or non-enzymatically in plants (del Rio et al. 2004; Bethke et al. 2004a; Delledonne 2005; Planchet et al. 2005). In our system, the endogenous source(s) of NO are not currently known, but could arise from both enzymatic and apoplastic routes. For example, nitrite is efficiently reduced in an acidic environment, such as the apoplast to produce NO, and phenolic compounds, such as catechin can accelerate this process (Bethke et al. 2004a). The results indicate that there is a synergistic effect of SNP and catechin (Fig. 3) and micromolar levels of nitrite and catechin (Table 3) on switchgrass seed germination. SNP decays to yield cyanide, NO and nitrite (Bethke et al. 2005) and would be expected to generate sufficient nitrite that can act as a feed-forward mechanism for the synthesis of additional NO in the presence of catechin in an acidic environment, such as the apoplast (Bethke et al. 2004a). Solutions containing 300 μM (+)-catechin were acidic (~pH 4.8), thus, SNP or nitrite in the presence of catechin would be expected to stimulate switchgrass seed germination through enhanced production of exogenous NO.

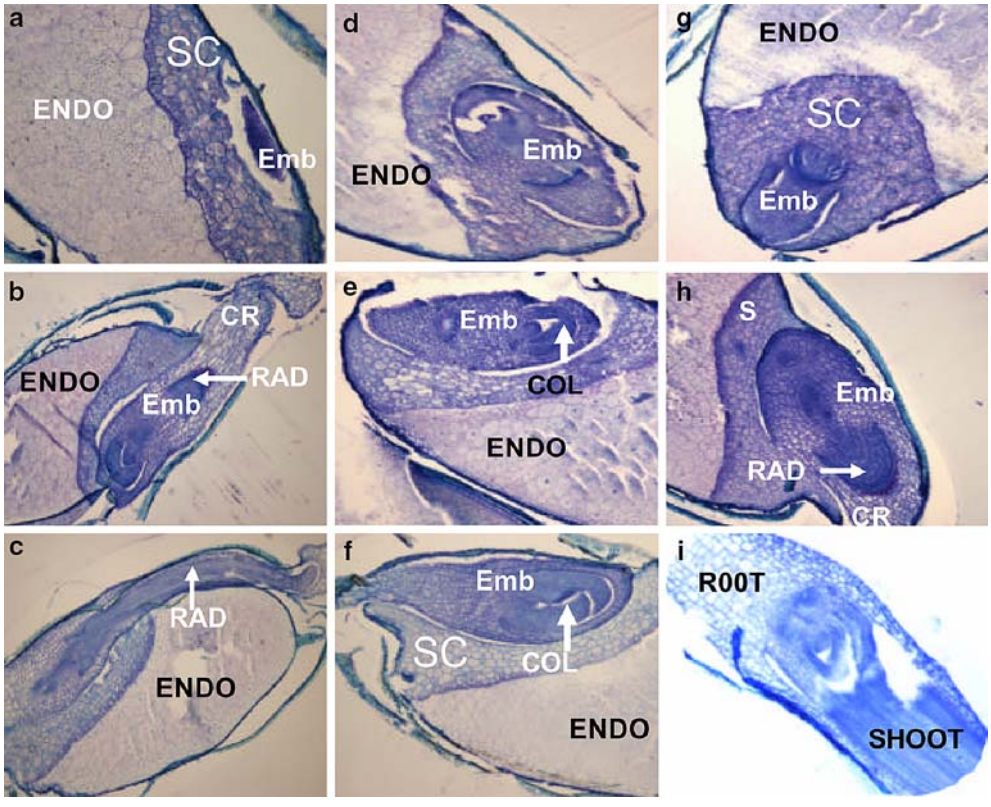


**Fig. 6** Sections of germinating switchgrass seeds observed by light microscopy. Seeds from different treatments were fixed, embedded in plastic and 5-μm sections stained with 0.1% Toluidine blue and observed using an Olympus BX 51 light microscope. a–c Sections of seeds imbibed on water; d–f On 200-μM SNP; g–i On 10-μM ABA for 1, 2 and 3 days, respectively. COL coleoptile; CR coleorhiza; Emb embryo; ENDO endosperm; RAD radicle; SC scutellum

NO is also known to enhance de-etiolation and promote greening in young seedlings (Beligni and Lamattina 2000; Delledonne 2005). For switchgrass seeds, SNP treatment significantly enhanced the number of germinated seeds with elongated coleoptiles at 25°C. These results imply that NO has a strong positive effect on seedling establishment, by stimulating germination, root and shoot growth. Seed size positively affected germination and adventitious root emergence in switchgrass (Smart and Moser 1999), but did not impact plant growth after 6–8 weeks following germination. It is possible that larger seeds might generate greater amounts of NO potentially through interactions between seed-associated phenolics and nitrite in the soil thereby stimulating germination and early root growth, processes known to be influenced by NO (Lamattina et al. 2003; Correa-Aragunde et al. 2004). Subsequent seedling establishment will be dependent on environmental conditions, principally the availability of water, and would be independent of the germination process. SNP treatment stimulated seed germination in big bluestem and Indiangrass as well, suggesting a universal role for NO during germination in warm-season prairie grasses. The data show a strong link between NO, germination and coleoptile elongation, especially at 25°C, which is consistent with soil surface temperatures expected during mid spring in the upper Midwest of the continental US. It is possible that exogenously produced NO due to soil warming in early spring combined with endogenous NO production occurring upon seed imbibition could stimulate germination and/or break dormancy in several prairie grasses.

In contrast to Arabidopsis and barley seeds (Bethke et al. 2004b), switchgrass seeds displayed incomplete dormancy. In the experiments reported here, there was approximately 50–60% germination for seeds plated on water. However, under conditions favoring NO-production, such as treatment with SNP or acidified nitrite, two events occurred: (1) stimulation of the rates of germination and (2) significant enhancement in the total percent of seeds that germinated. These data indicated that germinable switchgrass seeds respond positively to NO, and that there is residual dormancy in cold-stratified switchgrass seeds, which can be broken by NO. Results from the PTIO treatments (Fig. 1) buttress the role for NO in warm-season grass seed germination. First, PTIO initially blocked the increase in germination rates elicited by SNP and second, PTIO reinforced residual dormancy observed in these seeds. These data are consistent with the earlier observations with stratified Arabidopsis seeds (Bethke et al. 2004a, b). For these seeds, treatment with cPTIO (a PTIO analog), had minimal effect on germination, indicating that non-dormant (i.e., germinable) seeds are unaffected by NO scavengers and that they can produce adequate amounts of NO required for germination. Data from confocal microscopy (Fig. 4) of seeds imbibed with the NO-specific fluor DAF-FM supports this contention for switchgrass as well. Recently, it has been shown that sorghum seeds produce NO during germination (Simontacchi et al. 2004). Fluorescent signal attributable to endogenous NO was observed for switchgrass seeds imbibed in water as well as other treatments, specifically in seeds that had just germinated or were about to germinate. We interpret these results to show that NO is produced by switchgrass seeds during germination, and that dormant seeds require exposure to higher levels of NO (such as treatments with SNP etc.) that then leads to germination. An evaluation of other chemicals that generate $CN^-$ or reactive nitrogen oxides in situ or in vitro also revealed a significant stimulation of switchgrass seed germination. In a related earlier study on Arabidopsis (Bethke et al. 2005), cyanide, ferricyanide and ferrocyanide strongly stimulated seeds germination, and these responses were blocked by the NO-scavenger cPTIO. The authors performed a detailed analysis of these responses, and have shown that $CN^-$ apparently affects germination processes upstream of NO, but requires NO production for eliciting germination (Bethke et al. 2005). They also determined that the first volatile breakdown product of SNP is a cyanide. For switchgrass seeds, PTIO blocked the enhanced germination observed by treatment with cyanide-releasing agents (Table 4, Fig. 3). These results are consistent with the observations with SNP, and lend further support for the postulated roles for cyanide and NO in seed germination and dormancy processes (Bethke et al. 2005). However in contrast to findings with Arabidopsis, nitrite or nitrate alone did not significantly stimulate switchgrass seed germination (Table 3), and cyanide was less effective as compared with ferrocyanide (Fig. 3, Table 4). Despite the differences in germination behavior among the various species, the data strongly support a role for NO as an important signal for seed germination. That switchgrass seeds imbibed in the presence of ferrocyanide and the NO-detector DAF-FM exhibited fluorescence (Fig. 4) would lend additional support for this hypothesis. Useful future experiments would be to determine endogenous levels of cyanide or cyanide-related compounds during warm-season grass germination, and to evaluate the time course of appearance of $CN^-$ relative to NO for switchgrass seeds imbibed in water.

Among potential cellular targets for $CN^-$or NO would be hemoproteins, such as cytochromes, hemoglobins, heme-containing reductases and heme-containing oxidases (e.g., Kundu et al. 2003; Igamberdiev et al. 2004). NO, in addition can *S*-nitrosylate plant proteins (Lindemayr et al. 2005). However, specific protein targets for NO have yet to be unequivocally identified in plants (Delledonne 2005). One intriguing possibility is that cyanide directly stimulates a NO-producing reaction during seed germination and NO targets proteins critical for cell elongation (radicle emergence) and cell growth (coleoptile extension). NO is known to regulate ion channels in plant cells by increasing intracellular $Ca^{2+}$, which impacts protein phosphorylation cascades (Sokolovski et al. 2005). Furthermore, in tobacco leaves,

NO formation requires mitochondrial electron flow and the production of nitrite by nitrate reductase (Planchet et al. 2005). Conceivably similar interactions could be occurring in germinating switchgrass seeds. Given the range of plant processes controlled or impacted by NO (Delledonne 2005), it is possible that there may be specific cellular targets for NO unique to each plant organ or cell type.

That ABA depresses seed germination in switchgrass is not unexpected, since it is known that seed dormancy and germination in switchgrass will respond positively to exogenously supplied gibberellic acid (Zarnstorff et al. 1994; Loch et al. 2004), and SNP (this study), two compounds known to affect pathways that are inhibited by ABA in other systems (Bethke et al. 2004b). However, the use of ABA allowed us to gain insights into the germination process of switchgrass seeds. At the anatomical level, SNP treatment appeared to stimulate embryo growth by day 1 as compared with water controls. Subsequent germination resulted in the elongation of the coleorhiza and the radicle, followed by the emergence of the coleoptile. ABA treatment appeared to significantly retard coleorhizal elongation and essentially shut-down shoot growth. There is strong evidence that ABA, hydrogen peroxide and NO share cross-signaling pathways in guard cells (Desikan et al. 2004), and NO is part of the ABA response. In contrast, ABA and NO are antagonistic for seed germination (Bethke et al. 2004a; Gubler et al. 2005; this study) with coleoptile elongation being more affected by ABA even in the presence of optimal levels of SNP (Fig. 5b). Contrasting effects of ABA and NO have also been observed during leaf senescence and accumulation of ammonia in rice leaves (Hung and Kao 2003, 2005). ABA inhibition of seed germination in many species is well established, and it is possible that ABA interferes with processes downstream of initial NO action for both radicle emergence and coleoptile growth. Results from this study and the work of Bethke et al. (2004a, 2005) would suggest that PTIO and ABA are probably working in different routes. PTIO appears to be directly related to scavenging of NO, whereas ABA apparently affects other growth processes during seed germination.

In conclusion, the data show for the first time that NO is produced during switchgrass seed germination, and that NO could be an endogenous regulator of seed germination in several warm-season prairie grasses. Specifically, for switchgrass, exogenous sources of NO significantly stimulated germination. In addition, it has been demonstrated that the response of warm-season grass seeds to cyanide and cyanide-releasing compounds is similar to those reported for Arabidopsis. We anticipate that our studies can assist in developing new methods to study dormancy and germination in these species.

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