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Aged Switchgrass Seed Lot's Response to Dormancy-breaking Chemicals

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ABSTRACT

Aged switchgrass seed lots can display various levels of dormancy. Understanding the causes for this dormancy will provide better insight into seed physiology, and potentially lead to treatments that reduce variability in seed germination assays. The effects of sodium nitroprusside, potassium ferrocyanide and hydrogen peroxide on the germination of eight aged switchgrass (*Panicum virgatum* L.) seed lots, seven of which were produced in the same year at a single experiment station, were examined. Seed germination demonstrated a significant seed lots × days and treatments × days effect. However, responses of individual seed lots to specific chemicals varied considerably. Genetically related seed lots did not display similar responses to the treatments, while switchgrass derived from a different germplasm exhibited a more similar response. Coleoptile emergence was significantly improved by chemical treatments and showed a significant seed lots × treatment interaction. Together, these results indicate (1) chemicals releasing reactive nitrogen species or peroxide can overcome residual dormancy and stimulate seed germination and coleoptile emergence in diverse switchgrass seed lots, and (2) multiple mechanisms, to some extent under genetic control, appear to direct switchgrass seed germination.

INTRODUCTION

Seed dormancy can be caused by several sources and can act to prevent precocious germination of seeds under unfavorable environmental conditions (Bewley, 1997). In seeds such as switchgrass, dormancy mechanisms are frequently present in the embryo and can prevent subsequent germination, leading to poor stand establishment (Shen et al., 2001; Schmer et al., 2006). Although uniform seed germination may not directly correlate with stand establishment due to environmental stress, it will contribute to good stands under favorable conditions. Most often, freshly harvested switchgrass seeds have primary dormancy. Primary dormancy resides principally in the embryo, and requires both an after-ripening period as well as cold-stratification to permit optimal germination. Dormancy that persists in after-ripened, or aged, seeds is called secondary, or latent, dormancy, and can have multiple physiological causes. Various environmental and chemical treatments can be used to break secondary dormancy (Loch et al., 2004). Switchgrass seeds which have undergone secondary dormancy-breaking treatments, but still exhibit poor germination possess residual dormancy (Sarath et al., 2006). Residual dormancy in switchgrass seeds is responsive to chemicals that alter the endogenous levels

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of nitric oxide (NO) and/or reactive oxygen species (ROS) (Sarath et al., 2006; 2007a). In some cases, switchgrass seeds after exiting from after-ripening or following priming and drying enter deep dormancy (Shen et al., 2001; Loch et al., 2004). Deeply dormant seeds generally do not respond to secondary dormancy-breaking treatments. If cold-stratified seeds are dried, they undergo "dormancy reversion" and exhibit poor germination; however, extended cold stratification prevented this reversion, although some seeds still demonstrated symptoms of residual dormancy (Shen et al., 2001).

Interactions between plant hormones and seed dormancy have been thoroughly studied in many plant species (Koornneef et al., 2002; Loch et al., 2004). Abscisic acid (ABA) is a key regulator of dormancy in seeds (Gubler et al., 2005). Decrease in ABA levels combined with increases in other important hormones and signaling molecules such as gibberellic acid (GA), NO, and calcium will result in germination (Zentella et al., 2002; da Silva et al., 2004; Heggie and Halliday, 2005; Lefebvre et al., 2006; Sarath et al., 2006). The cellular perception of ABA requires both NO and ROS (Neill et al., 2002; Bright et al., 2006; Hancock et al., 2006). Ogawa and Iwabuchi (2001) and Bethke et al. (2006) demonstrated external sources of NO, cyanide and ROS (for example: hydrogen peroxide will break dormancy in several dicots). Although the overall process of seed dormancy and germination is complex and requires several shifts in metabolism (Chiwocha et al., 2005; Fait et al., 2006), external seed treatments can be used to effectively stimulate seed germination.

Warm-season grass seed germination is generally assayed after chilling on 0.2% potassium nitrate (KNO_3) moistened media for two weeks followed by germination (Loch et al., 2004; AOSA, 2003). Previously, studies using one seed lot ('Kanlow', lot #2302) indicated that a significant number of viable seeds would not germinate under normal testing conditions (0.2% KNO_3; AOSA, 2003), but showed significant enhancement in germination when treated with sodium nitroprusside (SNP), potassium ferrocyanide (ferrocyanide) and hydrogen peroxide (peroxide) (Sarath et al., 2006; Sarath et al., 2007a). The universality of this response and influence of genetic background across switchgrass seed lots has not been evaluated.

The objectives of this study were to evaluate the variations in germination of aged switchgrass seed lots obtained from the same year of harvest, in response to three chemical treatments, SNP, ferrocyanide and peroxide, and to indirectly evaluate the physiological status of seed lots with or without genetic similarities. The long-term goal of this research is to discover biochemical and molecular events that control switchgrass seed dormancy and germination.

MATERIALS AND METHODS

Plant materials

Seven seed lots were from isolated polycross nurseries of experimental switchgrass strains developed by the USDA-ARS and University of Nebraska (ARS-NE) cooperative grass breeding project. These seed lots were produced at the University of Nebraska Agricultural Research and Development Center located near Mead, NE in 2004. Seed lots were stored at room temperature, 22

± 2 °C during the course of the experiment. One seed lot, #2302, was 'Kanlow' Foundation seed, which was obtained from the USDA NRCS Plant Materials Center, Manhattan, KS, in 2004. Seeds from lot #2302 have been used in our previous studies and served as a positive control (Sarath et al., 2006; 2007a). Germination percentages for aged, cold-stratified (on water at 4–8 °C for 7 d, then blotted dry) and stored chilled seeds from these lots varied from 51 to 92%, when assayed on water utilizing the protocol described by Sarath et al., 2007a. The two experimental strains #2369 and #2372 were developed from the cultivar 'Pathfinder' by three or four cycles of selection for forage yield and in vitro dry-matter digestibility. Two of the experimental seed lots, #2370 and #2373, have the cultivar 'Trailblazer' genetic background. Seed lots #2368 and #2371 are based on a population created by selecting plants from cultivars 'Pathfinder', 'Blackwell', and two earlier ARS-NE synthetic populations of similar maturity. Seed lot #2374 was from an experimental strain developed by two generations of breeding for improved forage yield and in-vitro dry-matter digestibility from the cultivar 'Cave-in-Rock'. 'Kanlow' is a tetraploid, lowland cultivar while the experimental strains are all octaploid, upland ecotypes.

Germination assays (All weight)

Seeds (0.6 g) were surface sterilized in 5% (v/v) commercial bleach and washed three times for 5 min (15 min total) with sterile distilled water. Approximately 0.15 g of surface sterilized seeds were placed in each of the four replicate petriplates for a given seed lot. The number of seeds per 0.6 g and numbers of seeds on an individual plate varied within each seed lot, (ranged from 200 to 285 seeds per 0.6 g). Assays were conducted utilizing the method described in Sarath et al., 2006; 2007a. Each 9 cm petri plate contained two layers of Whatman No. 1 filter paper (Fisher Scientific) moistened with 10 ml of the appropriate solution (water, 200 μM SNP, 200 μM ferrocyanide or 20 mM peroxide) Solid potassium ferrocyanide (SNP) (Sigma-Aldrich, St. Louis, MO) required to make 10 mL of a 1 mM solution were weighed, transferred into a plastic test tube and dissolved in water. This stock solution was diluted to obtain the 200 μM working solution. The peroxide, solution was obtained by adding 23 μL of 30% hydrogen peroxide solution to 10 mL of water to obtain a 20 mM solution. The chemical concentrations utilized were based on earlier studies (Sarath et al., 2006; 2007a) that demonstrated a positive impact to seed germination in the cultivar 'Kanlow' (seed lot #2302; this seed lot was used as a control in our current studies). All chemicals were prepared fresh with sterile distilled water for each experiment. Any excess liquid was discarded prior to plating seeds. Seeds were arranged to avoid overlap with each other and the plates were sealed with Parafilm strips. Petri plates were arranged in a single layer in a temperature-controlled incubator maintained at 35 °C with continuous light provided by fluorescent bulbs (approximately 20 μmoles photons m^{-2} s^{-1} at shelf height). Seeds were considered germinated when the radicle protruded from the seed coat. Coleoptiles were scored as emerged when the coleoptiles became visible.The radicles were at least 2–3 mm in length, and the coleoptiles at least 3 mm in length. Coleoptiles visible within seeds, but which

had not broken through the seed coat were not counted. Germination, as determined by radicle protrusion from the seed coat was scored every 2 d over a period of 6 d. Coleoptile emergence was scored after 4 and 6 d respectively.

Statistical Analyses

The experiment consisted of four blocks with one replicate of four treatments for each of the eight seed lots per block. Each block was replicated four times on different days. Within each block (four treatments × eight seed lots) 32 trays were randomly assigned to a single shelf within one growth chamber. All experiments were performed in a single chamber. The effects of the four treatments (water, 200 μM SNP, 200 μM ferrocyanide or 20 mM peroxide) on germination of the eight seed lots were analyzed with day (two, four, or six) and coleoptile emergence with day (four and six; only day six data is reported) as a repeated measure using PROC Mixed in the SAS system (Littell et al., 1998). Germination or coleoptile emergence were the dependent variables for these analyses.

RESULTS

Seed lot showed a dominant influence on the outcome of the experiment as indicated by the significant differences between the seed lots and their responses to the chemical treatments. Analysis of variance for seed germination indicated significance ($P < 0.0001$) of the main effects of seed lots, treatments, and days; and the interactions of seed lots × days and treatments × days. All other effects were not significant.

Seed lots with shared genetic composition displayed different germination responses. For example, lots 2369 and 2372 which were derived from the cultivar 'Pathfinder' exhibited similar levels of germination; whereas lots 2368 and 2371, derived from synthetic population showed more marked differences in total germination over time. After 2 d, the mean germination across treatments for lot 2368 was ~22% as compared to ~50% for the genetically related lot 2371 (Fig. 1). A highly significant interaction between treatments and days was observed for this dataset (Table 1). The peroxide treatment elicited the highest germination across seed lots after 2 d and 4 d of treatment. After 6 d no significant effects on germination were observed (Table 1).

Germination of individual seed lots to applied treatments are shown in Fig. 2 to highlight underlying physiological differences (and variation) in germination observed for these lots. Chemical treatments increased seed germination as compared to the water treatment for all seed lots, except lot 2374. Based on germination responses to applied chemicals, seed lots with similar genetic backgrounds exhibited different responses to the treatments (Fig. 2), and could be broadly categorized into three groups. In the first group were seed lots 2302, 2369, 2373, and 2371. These seed lots had elevated germination after 2 d of peroxide treatment; for example, 2302 seeds displayed over 200% stimulation in germination as compared to water treatments. Although with time, germination stimulated by ferrocyanide approached levels seen with peroxide treatment for these seed lots. In these four seed lots SNP had a smaller but still significant promotive effect on seed germination (Fig. 2).

Seed lots, 2370 and 2372 fell into a second category, characterized by stronger

FIGURE 1. Switchgrass mean germination (radicle emergence) response of eight seed lots to three treatments (200 µM SNP, 200 µM ferrocyanide or 20 mM peroxide) and water evaluated at 2 (white bars), 4 (striped bars), and 6 (black bars) days after treatment for the seed lot × day interaction. Single, double and triple lines under the seed lots on the X-axis identify seed lots that share similar genetic backgrounds (see materials and methods).

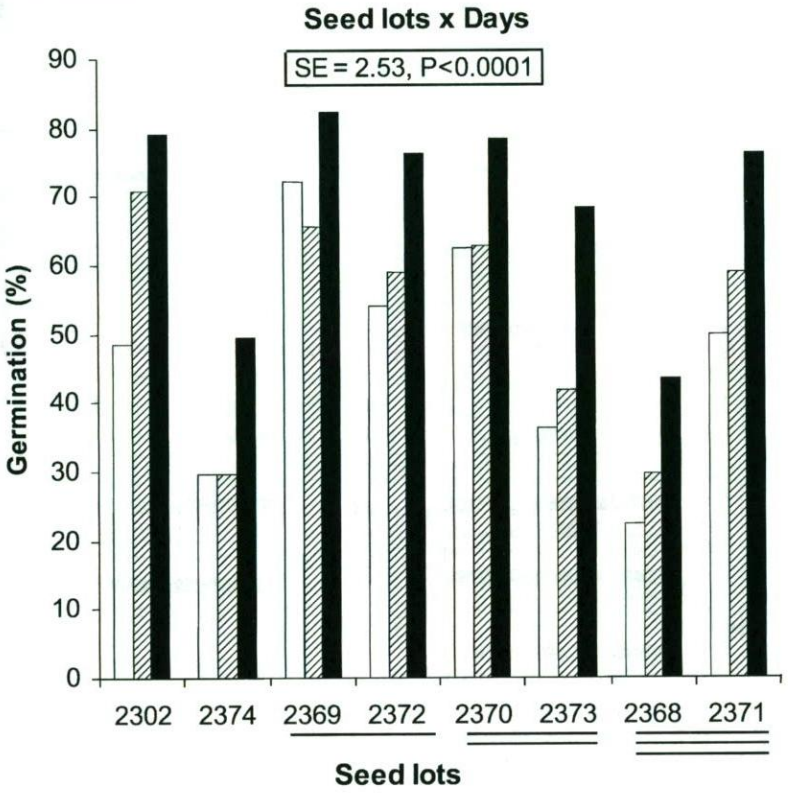
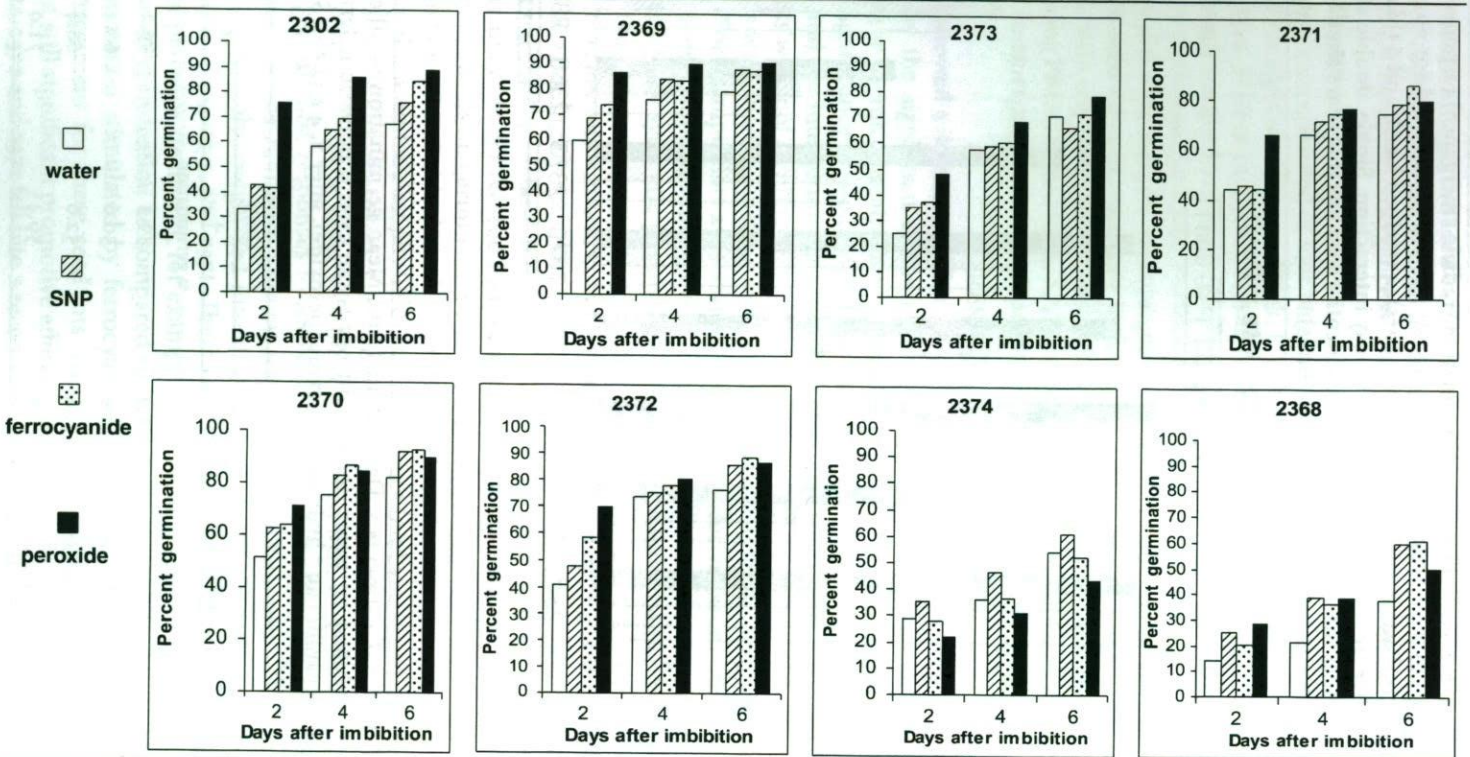


TABLE 1. Treatments × Days interactions. Mean germination of all eight seed lots imbibed on water, sodium nitroprusside (SNP), potassium ferrocyanide (ferrocyanide) and hydrogen peroxide (peroxide), after 2, 4 and 6 d. S.E = 2.27, P < 0.0001

Treatments	Days		
	2	4	6
	% germination		
Water	37.2	49.3	69.2
SNP	45.9	50.6	68.5
Ferrocyanide	45.9	52.6	68.4
Peroxide	58.7	56.4	71.0

FIGURE 2. Mean germination response of eight switchgrass seed lots to three treatments (200 μM SNP, 200 μM ferrocyanide or 20 mM peroxide) and water evaluated at 2, 4, and 6 days after treatment. Although this 3-way interaction was not significant (P = 0.2491), the figure is shown to demonstrate physiological differences in seed lot response to water, SNP, ferrocyanide, and peroxide.

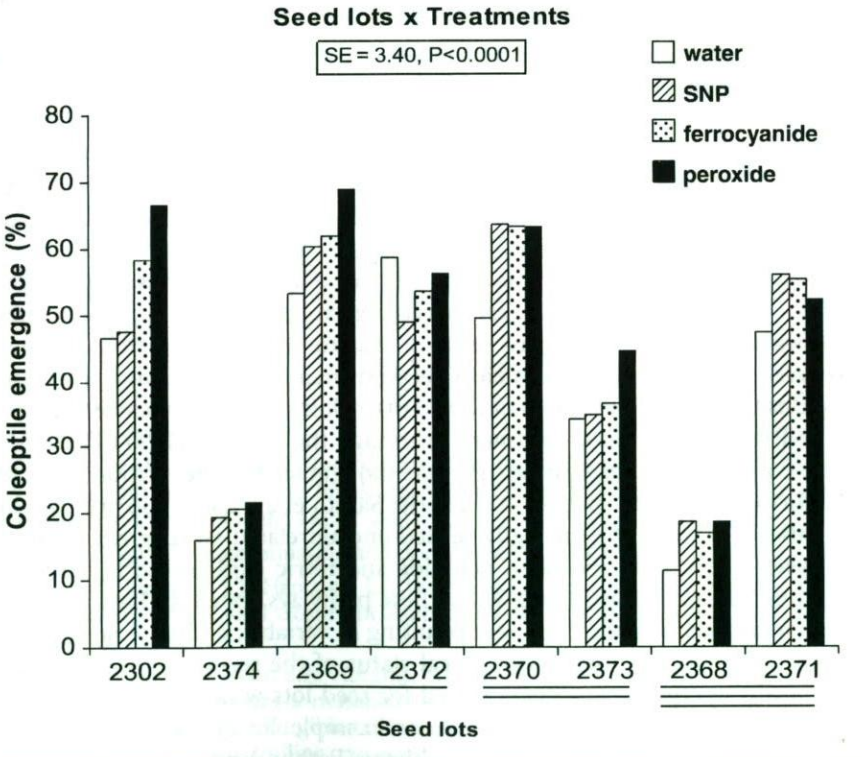


responses to SNP and ferrocyanide treatment as compared to peroxide treatment. All three compounds significantly stimulated germination in these two seed lots as compared to water treatment (Fig. 2). Although peroxide caused the maximal change in germination levels after 2 d, by 6 d, germination levels were highest in seeds maintained on SNP and ferrocyanide.

The last group consisted of two seed lots, 2374 and 2368. Both of these lots exhibited lower overall germination on water (Fig. 2) and displayed a strong positive response to treatment with SNP. For lot 2374, peroxide depressed germination after 6 d relative to the other treatments. For lot 2368, all three chemicals stimulated germination as compared to the water treatment, but the levels were greater in seeds maintained on SNP and ferrocyanide (Fig. 2)

Statistical analyses of coleoptile emergence revealed a significant response to coleoptile seed lots and days, and the interaction of seed lots × treatments ($P < 0.0001$). All other effects were not significant. For seed lots × treatments (Fig. 3), there were significant differences both in total coleoptile emergence

FIGURE 3. Coleoptile emergence of eight switchgrass seed lots to three treatments (200 μM SNP, 200 μM ferrocyanide or 20 mM peroxide) and water, taken 4 and 6 d after treatment. Single, double and triple lines under the seed lots on the X-axis identify seed lots that share similar genetic backgrounds. Data are the combined means of percent coleoptile emergence for each seed lot after 4 and 6 d.



as well as differences in the response of individual seed lots to treatments. Genetically related seed lots exhibited similar (2370 and 2373), or different (2368 and 2371) coleoptile emergence in response to the treatments (Fig. 3). Conceivably, coleoptile emergence might have continued after 6 d, but was not evaluated in the current experiment. Overall, seeds treated with peroxide exhibited the greatest coleoptile emergence percent for six of eight seed lots. Ferrocyanide significantly enhanced coleoptile emergence relative to water treatment in seven of the eight lots analyzed, and SNP was effective in five of eight seed lots. Interestingly, in genetically related seed lots 2369 and 2372, as compared to water treatments, chemical treatments significantly enhanced coleoptile emergence in lot 2369, but not in 2372 (Fig. 3).

DISCUSSION

Switchgrass seed lots responded differentially to applied chemicals, suggesting different endogenous mechanisms influence germination in this species. Seven of the eight seed lots were produced the same year at the same experiment farm in field isolations and some with similar genetic background. These results indicated that dormancy is likely related to genetic background and micro-environmental effects during seed production, harvesting and processing. Lot 2371 exhibited significantly greater germination as compared to lot 2368 across all treatments and across all days; suggesting traits that control these complex plant processes can be manipulated in switchgrass.

Externally supplied peroxide and NO are known to stimulate seed germination in other species such as arabidopsis (*Arabidopsis thaliana* L.; Bethke et al., 2006), zinnia (*Zinnia elegans* L.; Ogawa and Iwabuchi, 2001), wheat (*Triticum aestivum* L.; Wahid et al., 2007) and barley (*Hordeum vulgare* L.; Wang et al., 1998); suggesting that ROS and NO-reactive pathways are important during the germination process in plants. Studies with 'Kanlow' lot 2302 indicated both ROS and NO are involved in stimulating switchgrass germination and overcoming residual dormancy (Sarath et al., 2007b). Interestingly, this study demonstrated not all switchgrass seed lots showed a uniform response to peroxide. These results also suggest possible germination-specific biochemical pathways in specific switchgrass seed lots that are inhibited by peroxide. Alternatively, some seeds lots could have deep dormancy mechanisms that are unresponsive to external sources of ROS, but exhibit a stronger positive response to SNP and ferrocyanide treatment. Although the exact mode(s) of action of these compounds are not known, they are thought to elicit their actions through the formation of NO (Bethke et al., 2006; Sarath et al., 2006; 2007b). The differential responses of genetically related and unrelated switchgrass seed lots assayed in this study indicated peroxide and nitric oxide could be targeting different pro-germinative pathways. These pathways appear to have a genetic and an environmental component resulting in variable, and as of now, an unpredictable effect on the physiological status of the seeds. Germination and coleoptile emergence responses varied for seed lots with similarities in their genetic background (Fig. 2; Fig. 3). As an example, lot 2371 and lot 2368 were both derived from a combination of cultivars (Pathfinder and Blackwell among

other populations), and displayed the greatest difference in the responses to the treatments. However, both of these seed lots showed a stronger response to SNP compared to peroxide indicating that their shared genetic origin could result in similar physiological responses, but other factors were impacting overall germination and coleoptile emergence. As discussed earlier, seed dormancy and germination are complex processes controlled by genetics and the environment. Our results document that seed lots with significant dormancy, for example, 2374 and 2368 also displayed a tendency for lowered coleoptile emergence. It is plausible that for such seed lots, stimulating germination may not in itself stimulate coleoptile development indicating that coleoptile emergence required additional physiological events. It is as yet unclear if such a situation arises from developmental arrests in the embryo or from incomplete loss of dormancy in these seed lots. In both scenarios, these data have some practical implications, suggesting that scoring both germination and coleoptile emergence should yield better overall information about the ability of a given seed lot to have good field establishment. Utilizing switchgrass seed lots with different, but well characterized physiological responses should prove useful to understand the complexity of the germination and dormancy process in this species.

A lack of significance for a three-way interaction (seed lots × treatments × days) could reside in the large variations in levels of germination observed in these seed lots (see Fig. 2). Such variations might mask real differences in seed quality and physiology arising from genetics or gene × environment interactions. A greater understanding of both biochemical and molecular mechanisms controlling switchgrass dormancy could provide means to obtain cultivars with optimal seed qualities in this important biofuel species. The discovery of new germination stimulating and/or dormancy breaking chemicals could lead to the development of new rapid germination assays for native warm-season grasses, and potentially to novel means to obtain reliable seed germination and establishment under field conditions.

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