Short communication

Alleviation of dormancy by reactive oxygen species in *Bidens pilosa* L. seeds

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Abstract

Bidens pilosa L. is a weedy species in the Asteraceae producing dimorphic one-seeded fruits, with longer black seeds centrally situated in the capitulum, and shorter dormant brown peripheral seeds. While the outer seeds germinate readily after seed shedding, the shorter seeds possess various dormancy mechanisms, including requirements for after-ripening and red light. Here we show that applications of reactive oxygen species (ROS)-generating reagents can remove dormancy in the short seeds. In *B. pilosa*, reagents that generate hydroxyl radicals ($^{\cdot}OH$) and superoxide ($O_2^{\cdot-}$) partially replaced the requirement for after-ripening, while $O_2^{\cdot-}$ generation replaced the requirement for red light. Hence, ROS appear to be implicated in the alleviation of dormancy in the seeds of *B. pilosa*.

1. Introduction

Orthodox seeds enter a resting state at the end of development, which continues until external conditions create an environment that allows the seed to germinate (Finch-Savage and Leubner-Metzger, 2006; Kozlowski and Gunn, 1972). Such a seed is quiescent, but not necessarily dormant, which may be defined as the failure of an imbibed, viable seed to germinate under seemingly favorable conditions (Bewley, 1997). The block to germination may be either physical (e.g. a hard testa) or physiological (Finch-Savage and Leubner-Metzger, 2006; Finkelstein et al., 2008). Seed dormancy and subsequent germination are generally agreed to involve the modulation of the concentrations of, and sensitivity to, abscisic acid (ABA) and gibberellins (GA). ABA is involved in the induction of dormancy, while GA acts in the promotion of germination (Finch-Savage and Leubner-Metzger, 2006; Finkelstein et al., 2008). A recent model proposes that a heterodimeric protein complex exists that promotes germination, and that the abundance of one monomer is influenced by ABA and the

other by GA (Penfield and King, 2009). This complex also regulates GA and ABA metabolism in seeds, and this creates feedback necessary to maintain the dormant and germinating states. One component of a dimeric complex has been proposed to be a phytochrome interaction factor, which may explain why for the seeds of some species under the canopy of trees, far-red (FR) light represses seed germination (Piskurewicz et al., 2009).

ROS also appear to be involved in dormancy alleviation. Many studies over decades have shown that exogenous application of ROS and pro-oxidants can break dormancy in a variety of seeds (for review see El-Maarouf Bouteau and Bailly, 2008). Several recently published studies provide intriguing examples indicating how ROS may participate with hormones in the regulation of normal seed development. For example, in sunflower seeds after-ripening involves the progressive accumulation of ROS in the embryonic axes (Oracz et al., 2007). These ROS cause targeted changes in protein carbonylation patterns and gene expression, apparently leading to a loss of dormancy. Oracz et al. (2009) later showed that ethylene can also overcome the requirement for after-ripening in sunflower seeds, and ROS can also activate ERF1, a component of the ethylene signaling pathways. Further evidence for ROS in the control of dormancy come from the observation that

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Arabidopsis lines with reduced transcript levels of NADPH oxidase (a membrane-bound enzyme complex involved in extracellular $O_2^{\cdot-}$ production) displayed reduced protein carbonylation and failed to after-ripen (Müller et al., 2009a). ROS are also important in endosperm weakening and radicle elongation. For example, in cress, electron paramagnetic resonance spectroscopy showed that polysaccharides are oxidized *in vivo* by the developmentally regulated action of apoplastic ·OH in radicles and endosperm caps (Müller et al., 2009b). The production of ·OH was inhibited by ABA, and this inhibition could be reversed by GAs. Although ROS produced at excess levels are clearly deleterious, it has been suggested that an "oxidative window" exists in which the generation of strictly regulated concentrations of ROS is required for germination (Bailly et al., 2008). The main aim of the present study was to investigate the potential of exogenous ROS such as hydrogen peroxide (H_2O_2), $O_2^{\cdot-}$ and ·OH, to break dormancy in a weedy species. Here, the ability to force germination has potential applications in weed control, and could also be useful in the context of seed-banking and conservation.

The model chosen for the present study was *Bidens pilosa* L., a weedy species in the Asteraceae (Forsyth and Brown, 1982). The plant produces dimorphic one-seeded fruits (referred to here as seeds for convenience). Longer black seeds are centrally situated in the capitulum, and are more numerous than the shorter, dormant, brown peripheral seeds. While the outer seeds germinate readily after imbibition, the shorter seeds possess more than one physiological dormancy mechanism. Dormancy may be partially overcome by storing the seeds for 14 days after collection (after-ripening) and then typically display *c*. 20% total germination (TG). For higher TG, the shorter seeds require red light in what appears to be a classical phytochrome-mediated dormancy-breaking mechanism (Amaral-Baroli and Takaki, 2001; Forsyth and Brown, 1982). Two sets of experiments were carried out. Firstly, it was determined whether ROS can replace the after-ripening requirement for moderate TG, and secondly, whether ROS can replace the requirement for red light for a more complete TG.

2. Materials and methods

2.1. Plant material and chemicals

Seeds were collected close to the Life Sciences campus of the University of KwaZulu-Natal in Pietermaritzburg, South Africa. Seeds were sorted according to length, placed in brown paper bags inside a glass jar enclosed in aluminium foil, and stored in the dark at 20 °C until required. The short, dormant seeds were used for experimental purposes. Total germination was scored based on radicle protrusion. To generate $O_2^{\cdot-}$ radicals, methyl viologen dichloride hydrate (MV, Aldrich, St Louis) was used. While MV is often used to generate $O_2^{\cdot-}$ radicals in green tissues, effective generation of $O_2^{\cdot-}$ radicals in non-green tissues also occurs (Slooten et al., 1995). To generate ·OH radicals, three concentrations of Fenton reagent were prepared, for convenience referred to by their H_2O_2 concentrations, containing 1% H_2O_2 and 3 mg mL^{-1} FeSO$_4$, 3% H_2O_2 and 10 mg mL^{-1} FeSO$_4$, and 10% H_2O_2 and 30 mg mL^{-1} FeSO$_4$.

2.2. Alleviation of the requirement for after-ripening

Seeds were collected, stored for 1 day, and then subjected to treatments as outlined in Table 1. In the first set of experiments the three controls used were first, seeds germinated immediately with no other treatment applied and second, seeds after-ripened for 14 days at 20 °C, and then germinated ("conventional dormancy-breaking treatment"), and third, seeds soaked for 6 h at 10 °C in distilled water, and then placed on moist filter paper as for the first control. All treatments comprised three independent replicates of 50 seeds, and all treatments were carried out in the dark, except for scoring TG which was carried out under a green safe light (0.3 µmol m^{-2} s^{-1}). Treatment with MV solution was carried out by placing seeds in darkness at 10 °C for 3 h on a single layer of Whatman No. 1 filter paper in plastic Petri dishes (as recommend by Oracz et al., 2007). Treatment with H_2O_2 and Fenton reagent was done in the same way but for 6 h at room temperature. Seeds were rinsed three times in distilled H_2O to remove ROS-generating reagents, rapidly transferred to new Petri dishes that contained moistened filter paper and were wrapped in aluminium foil. Dishes were placed in an incubator at 25 ± 1 °C, and TG monitored daily for 5 days.

2.3. Alleviation of the requirement for red light

Seeds were after-ripened for 14 days at ambient temperature in the dark. The three controls were first, seeds placed on moist filter paper in the dark, second, seeds placed on moist filter paper and then exposed to red light (1.6 µmol m^{-2} s^{-1}) for 15 min (dishes were open). For the third control, seeds were soaked for 6 h at 10 °C in distilled water, and then placed on moist filter paper as for the first control. After-ripened seeds were treated with ROS-generating reagents as above, and TG monitored daily for 5 days.

2.4. Statistical analysis

Percentage germination data after 5 days were arcsine transformed and subjected to one-way ANOVA in combination with post-hoc comparison of means using the LSD test (SPSS 15 for Windows).

Table 1
Concentration and duration of treatment of *B. pilosa* seeds with ROS-generating compounds.

	Concentrations	Duration of treatment
H_2O_2	1, 3, 10%	6 h
Fenton reagent (% refers to H_2O_2 concentration)	1, 3, 10%	6 h
Methyl viologen	0.1 mM, 0.01 mM, 0.001 mM	3 h