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Dormancy breakage and germination are tightly controlled by hypoxic submergence water on *Echinochloa crus-galli* seeds from an accession resistant to anaerobic germination

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Abstract

In wetlands, dormancy may be a key functional trait enabling seeds to avoid underwater germination, which could be lethal for seedling establishment. Our objectives were to find out (i) if shallow dormant (i.e. conditionally dormant) *Echinochloa crus-galli* seeds from an anaerobic germination resistant accession can break dormancy under hypoxic submergence and (ii) if underwater germination can be restored in scarified, non-dormant seeds. Shallow dormant *E. crus-galli* seeds perceived diurnally alternating temperatures (AT) and red light (R) pulses (i.e. dormancy-breaking cues) under hypoxic submergence; however, an inhibitory far-red light pulse given at the end of the 4-d inundation period demonstrated that most of the seeds (85%) were unable to break dormancy. Scarified *E. crus-galli* seeds, which did not express dormancy under drained conditions, were unable to germinate under hypoxic submergence, despite being exposed to dormancy-breaking cues (AT + R). Lastly, the temporal window for germination sensitivity to the inhibitory action of hypoxia, once dormancy-breaking signals have been applied, is progressively lost and bounded to approximately 18 h for half of the seed lot. These results highlight the importance of dormancy as a trait enabling *E. crus-galli* seeds to avoid underwater germination, a risky scenario for seedling emergence and establishment in this facultative hydrophyte.

Introduction

Wetland mudflats are clear examples of unpredictable environments where the risk of the unsuccessful establishment during flooding periods is high due to fluctuating oxygen levels (from normoxia to severe hypoxia) (Hook, 1984; Baskin et al., 2004; Winkel et al., 2013). Moreover, opportunistic cultivation of lowlands and flood-prone areas leave non-vegetated mudflats free to weed colonization and encroachment, once crops have been damaged by floods (Hall et al., 1992; Ghersa et al., 2007). In this scenario, as in others in which environmental conditions are highly dynamic, it is important to study the functional traits that enable seeds to coordinate germination timing with suitable conditions for ensuring seedling establishment (Long et al., 2015; Saatkamp et al., 2019). In the case of weeds, seed physiological traits are key to understand the timing of seedling emergence and seed bank formation and persistence at a site (Harper, 1977; Benech-Arnold et al., 2000; Batlla and Benech-Arnold, 2007), which ultimately define the assemblage of the weed community (Radosevich et al., 2007).

The soil–water interphase can be hypoxic under flooding (Barclay and Crawford, 1982; Sasidharan et al., 2017). Wetland plants, such as some *Echinochloa* species, are able to germinate under anaerobic conditions, although hypoxia can seriously damage seedlings or rapidly waste seed reserves (Kennedy et al., 1980; Chauhan and Johnson, 2011; Ismail et al., 2012; Estioko et al., 2014). Despite the fact that some aquatic species germinate underwater (Hook, 1984; Keeley, 1988; Bissels et al., 2005), a more common scenario for seed germination and seedling establishment of wetland plants is under moist drained conditions (Lenssen et al., 1998; Nishihiro et al., 2004; Carta, 2016). Dormancy, defined as an innate property of the seeds that restricts the environmental conditions in which they are able to germinate (Finch-Savage and Leubner-Metzger, 2006), does not change between binary states but among a continuum of transitional states (conditional dormant states) in where seeds express different degrees of dormancy in otherwise optimal conditions for a non-dormant seed to germinate (Baskin and

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Baskin, 2004). In this way, conditionally dormant seeds, which had their dormancy alleviated through after-ripening or stratification, may still require environmental signals like diurnally alternating temperatures and light to become non-dormant and get the capacity to germinate (Benech-Arnold et al., 2000; Finch-Savage and Leubner-Metzger, 2006). Seed dormancy is a key control point of the emergence process that might avoid lethal underwater germination in facultative wetland weeds (Mollard and Insausti, 2009, 2011; Peralta Ogorek et al., 2019); however, we still do not know the specific states in where hypoxic conditions could affect dormancy and seeds ability to germinate.

It is assumed that the perception of signals that break seed dormancy and promote germination is essential to avoid underwater germination in wetland species (Thompson and Grime, 1983; Pons and Schroder, 1986; Ekstam and Forseby, 1999; Mollard and Insausti, 2011; Peralta Ogorek et al., 2019). It was shown that *Echinochloa crus-galli* seeds can continuously monitor environmental cues under hypoxic floodwaters, thereby perceiving dormancy-breaking signals when submerged (Peralta Ogorek et al., 2019). Seed dormancy breaking occurs due to the coordinated action of several signalling pathways, jointly acting both on embryos and the structures surrounding them (Finch-Savage and Leubner-Metzger, 2006). Moreover, the perception of environmental cues involves highly regulated processes such as phytochrome conversions (Chen et al., 2004). Despite the research in controlled atmospheres performed in recent years (Benech-Arnold et al., 2006; Bradford et al., 2007, 2008; Hoang et al., 2013), the information about dormancy and germination inhibition by anaerobiosis is still fragmentary. Furthermore, it is still not known if hypoxic submergence acts on one or more control points or pathways, for example, inhibiting either the embryo growth potential or seed coat weakening.

Scarification treatments have been used to break coat-imposed dormancy in graminoids and produce readily germinating seeds (Rosbakh et al., 2019). These treatments not only weaken covers but also improve O_2 diffusion to the embryo in those seeds where coats impose dormancy by chemically limiting oxygen supply to the embryo [reviewed in Corbineau and Côme (1995) and Rodríguez et al. (2015)]. Thus, scarification can be used to test if seeds, in which mechanical or gas diffusion restrictions to germination have been removed, are still able to germinate under hypoxic conditions.

The weed *E. crus-galli* has anaerobic germination (AG) sensitive accessions able to germinate under anoxic atmospheres, and AG resistant accessions that are not able to germinate in anaerobiosis (Kennedy et al., 1980; Fukao et al., 2003). For this study, we selected an AG resistant accession, as our objective was to find out if *E. crus-galli* seeds can break dormancy under hypoxic submergence and if underwater germination can be restored in seeds that do not express seed dormancy. The questions that guided this research were (i) Are *E. crus-galli* seeds able to break dormancy and germinate under hypoxic submergence? (ii) Are scarified *E. crus-galli* seeds able to germinate under hypoxic submergence? and (iii) How long is the temporal window in where *E. crus-galli* seed germination can be inhibited by hypoxic submergence?

Material and methods

Freshly harvested mature dispersal units of *E. crus-galli* (L.) P. Beauv. var. *crus-galli*, hereafter referred to as seeds, were collected from wild plants growing on a pond located in the cropping region of Buenos Aires province, Argentina (35°33′15″S, 58°58′08″W). The agricultural wetland is seasonally flooded during winter and early spring, and therefore, floods may occur during the early growing season of *E. crus-galli*. Mean annual precipitation in the area is 911 mm with large interannual variation, with mean monthly temperature ranging from 23°C in January (summer) to 9.1°C in July (winter) (De Fina, 1992). Seeds were harvested at the time of their natural dispersal and were stored in a chamber (dry seeds, 20°C in darkness, 30–60% RH) until the experiments commenced. Dry conditions during storage ensured the partial attenuation of primary dormancy through the after-ripening process. For this reason, shallow dormant (i.e. conditionally dormant) 15-months after-ripened seeds, with requirements of diurnally alternating temperatures and exposure to high red to far-red light pulses to break dormancy and germinate, were used in the experiments.

Experiment 1

A first experiment was carried out to test if *E. crus-galli* seeds were able to break dormancy under hypoxic floodwaters. Groups of 30 seeds each were submerged under 8 cm N_2-flushed distilled water [dissolved oxygen (DO) levels between 1 and 1.5 mg l^{-1}] in airtight flasks. Seeds were subjected during 4 d (i.e. enough time to break dormancy) to diurnally alternating temperatures 20/30°C (9/15 h d^{-1}) and daily red light (R) pulses for 30 min each (R/FR = 4.03, irradiance = 3.8 µmol m^{-2} s^{-1}, λ_{max} = 610 nm, FR = far-red light), provided by two fluorescent Philips TL 40 W/15 tubes covered in red acetate. The R light treatment was performed during the high-temperature phase. After each irradiation period, the flasks were covered with opaque black polyethylene. At the end of the fourth day (i.e. hypoxic submergence conditions), seeds were removed from flasks and placed in drained conditions on Whatman No. 3 filter paper soaked in distilled water in transparent crystal polystyrene boxes (6 × 7×1 cm). The groups of 30 seeds coming from flasks were randomly split between two light treatments and four temperature regimes amounting to eight conditions to assess germination ($n = 4$ for each combination). The light treatments were (i) constant darkness and (ii) an only 1 h saturating FR light pulse in recently drained seeds and then seeds transferred to constant darkness. The FR light pulses had R/FR = 0.012, irradiance = 0.5 µmol m^{-2} s^{-1} provided by an Osram incandescent quartz bulb of 150 W filtered with 10 cm of water and Schott RG9 filters (Schott, Mainz, Germany) of 2 mm width, λ_{max} = 760 nm. The rationale for the FR pulse treatment was to reverse phytochrome Pfr back to inactive Pr form to reveal if seeds were able to break dormancy under hypoxic submergence (i.e. similar germination percentages both under constant darkness and after the FR light pulse). Seeds were also exposed to either of the following temperature regimes: a diurnally alternating temperature regime (20/30°C [9/15 h d^{-1}]) or constant temperature regimes of 30, 25 or 20°C. Germination was monitored after 10 days. DO levels were monitored with an LT Lutron DO-5510 (Lutron Electronic Enterprise, Taipei, Taiwan).

Experiment 2

The second experiment was conducted to test if scarified *E. crus-galli* seeds, which did not express dormancy under drained conditions, were able to germinate under hypoxic floodwaters. For this experiment, part of the seed lot was scarified for 3 min in H_2SO_4 1N and then thoroughly rinsed in water for 5 min.

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Scarified and non-scarified seeds (i.e. whole seeds) were subjected to the following treatments: (i) hypoxic submergence: seeds submerged in airtight flasks under 8 cm N_2-flushed distilled water (DO levels between 1 and 1.5 mg l^{-1}), (ii) normoxic submergence: seeds in open flasks submerged under 8 cm distilled water where DO range between 7.0 and 8.4 mg l^{-1} with water being partially replaced daily and (iii) seeds under drained conditions and placed on Whatman No. 3 filter paper soaked in distilled water in transparent crystal polystyrene boxes as above-mentioned. These treatments were combined with the following procedures: (i) seeds exposed to diurnally alternating temperatures 20/30°C (9/15 h d^{-1}) plus daily red light pulses (i.e. exposure to dormancy-breaking signals) and (ii) control: seeds incubated at constant 25°C and darkness (i.e. no dormancy-breaking signals). The flasks and germination boxes exposed to dormancy-breaking signals received daily light pulses during the first 5 days of incubation applying the same protocols as in Experiment 1. Flasks and boxes were covered with opaque black polyethylene, except during irradiation periods. Water replacement in normoxic flasks was carefully done under safe dim green light. Each treatment consisted of groups of 30 seeds ($n = 4$). Germination percentages were monitored after 10 days as in Experiment 1.

Experiment 3

In the third experiment, we followed an adaptation of the photomorphogenic test of escape time of a plant morphological response from R–FR reversibility (Schäfer and Nagy, 2006) to determine the kinetics of the inhibition of germination imposed by the hypoxic submergence after a saturating red light pulse under an alternating temperature regime (i.e. exposure to dormancy-breaking signals). Seeds were placed on Whatman No. 3 filter paper, soaked in distilled water in drained transparent crystal polystyrene boxes at a 20/30°C (9/15 h d^{-1}) regime for 4 d. Then, seeds were exposed to a 2 h red light pulse with the same light sources as in the first experiment, to trigger dormancy breaking through the phytochrome signalling pathway. Afterwards, groups of seeds ($n = 4$, 30 seeds each) were, at different times, either exposed to a saturating FR light pulse or submerged in airtight flasks under 8 cm N_2-flushed distilled water (DO levels between 1 and 1.5 mg l^{-1}) leaving intervals of 0, 3, 6, 9 12, 15 18 or 24 h between the end of the red light pulse and the start of the inhibitory condition (FR pulse or hypoxic submergence). The FR light pulse followed the protocol of Experiment 1. The diurnally alternating temperature treatment was maintained along with the incubation period with the initial red light pulse given at the end of the fourth 20°C phase. Germination was monitored after 10 days.

Statistical analysis

Seed germination was analysed using germination percentages. Dead seeds were subtracted from the total number of seeds per box or flask to calculate germination percentages. For each replicate, dead or alive seeds were sorted out by gauging their hardness with histology tweezers (Herron et al., 2000). Also, routine tetrazolium chloride tests (1% solution of 2,3,5-triphenyl tetrazolium chloride (w/v) for 24 h in the dark at 25°C) were performed to determine if submerged-induced hypoxia treatments changed seed viability. Germination percentages were analysed with parametric *n*-way ANOVAs after rank transformation of data to satisfy ANOVA assumptions. ANOVAs were followed by *post hoc* Tukey tests with $\alpha = 0.05$. All statistical analyses were conducted using STATISTICA version 10 (StatSoft Inc., Tulsa, OK, USA). Results are presented as non-transformed means of four replicates ± standard error (SE).

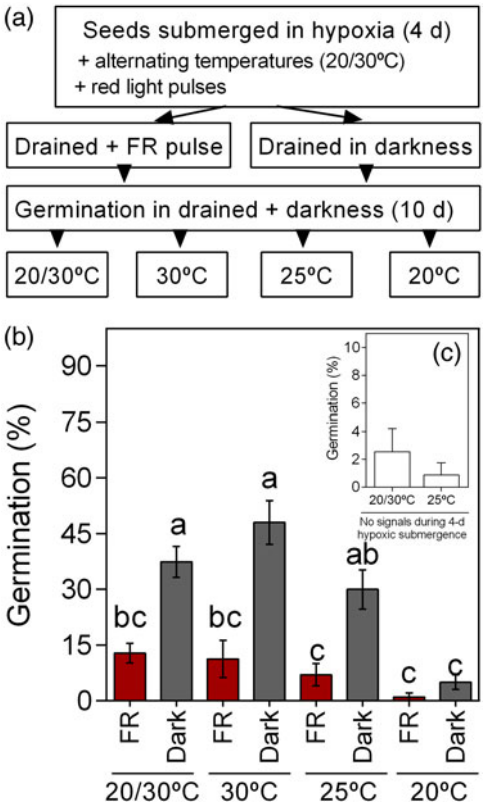

Fig. 1. Dormancy breaking in *E. crus-galli* seeds submerged under hypoxic water (dissolved oxygen < 1.5 mg l^{-1}). (a) Scheme of treatments application where shallow dormant seeds were submerged and exposed to diurnally alternating temperatures (20/30°C, 9/15 h) and daily red light pulses for 4 d. Then, seeds were drained on wet filter paper and subjected to either constant darkness (Dark) or a final far-red light pulse, and then transferred to darkness (FR). After being drained, the groups of seeds were incubated at the given temperature regimes. (b) Germination percentages. (c) Inset: Germination percentages of seeds which were not exposed to light and temperature signals either during the 4-d hypoxic submergence period or after being transferred to drained conditions in darkness at 20/30 or 25°C. Data are presented as means ± SE ($n = 4$). Bars not sharing the same letter are significantly different ($P < 0.05$).

Results

Experiment 1

The germination of drained *E. crus-galli* seeds was negligible when the requirements of dormancy-breaking signals had not been fulfilled during the 4-d hypoxic submergence period (Fig 1 insert). *E. crus-galli* seeds submerged in hypoxic water for 4 days perceived diurnally alternating temperatures and red light pulses and were later able to germinate during the drained phase, under constant darkness at most of the tested temperatures (Fig. 1). Noticeably, there was an unexpected significant temperature effect ($P < 0.002$, interaction temperature × treatment $P > 0.05$) as low germination percentages were recorded at 20°C after the drained period. By contrast, under other temperature regimes (continuous 25 and 30°C, or alternating 20/30°C), a major fraction of germinable seeds (60%) was inhibited to germinate by a final FR light pulse despite having had enough

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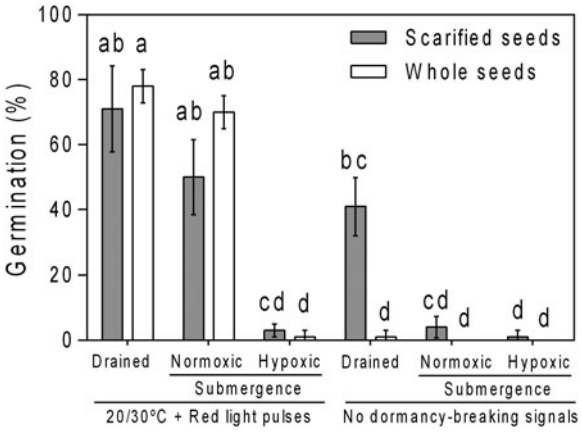

Fig. 2. Germination of scarified *E. crus-galli* seeds submerged under hypoxic water. Shallow dormant seeds either H_2SO_4 scarified or whole were incubated submerged under hypoxic (dissolved oxygen < 1.5 mg l^{-1}) or normoxic (dissolved oxygen > 7 mg l^{-1}) distilled water or imbibed on wet drained filter paper and exposed to dormancy-breaking signals [alternating temperature regime (20/30°C, 9/15 h) + red light pulses] or without signals (no dormancy-breaking signals). Germination percentages are presented as means ± SE ($n = 4$). Bars not sharing the same letter are significantly different ($P < 0.05$).

time (i.e. 4 d) to fully perceive and transduce environmental cues (daily red light pulses, diurnally alternating temperatures) and, therefore, break seed dormancy under hypoxic submergence ($P < 0.001$; Fig. 1).

Experiment 2

Seed scarification, submergence treatments and the exposure to dormancy-breaking cues (diurnally alternating temperatures, daily red light pulses) significantly interacted to determine *E. crus-galli* germination percentages ($P = 0.002$; Fig. 2). Scarified seeds that did not receive the dormancy-breaking signals were able to germinate when kept under constant temperature (25°C) and darkness, only under drained imbibed conditions but not under submergence, either in normoxic or hypoxic water (Fig. 2). On the other hand, seeds that received the dormancy-breaking signals showed high germination (50–70%) under normoxia (drained or normoxic submergence) regardless of whether seeds were scarified or not. By contrast, hypoxic submergence blocked germination in both scarified and whole seeds even when dormancy-breaking requirements were applied (Fig. 2).

Experiment 3

In a follow-up experiment, we studied the kinetics of escape time of drained imbibed seeds subjected to diurnally alternating temperatures in darkness (4 d) followed by a saturating pulse of red light. Then, we assessed the effect on the germination of different intervals (h) between the red light pulse and two inhibitory treatments: (i) a final saturating FR light pulse to revert phytochromes to the inactive form or (ii) hypoxic submergence. Seeds progressively escaped from the control of phytochrome or the inhibition of hypoxic submergence on dormancy and germination, yet following different kinetics, as there was a delay time between loss of FR reversibility and that of hypoxic submergence inhibition (interaction treatment × time: $P < 0.001$; Fig. 3). The higher germination percentage of the seed lot under optimal drained conditions and exposed to the signals that break dormancy was 80%

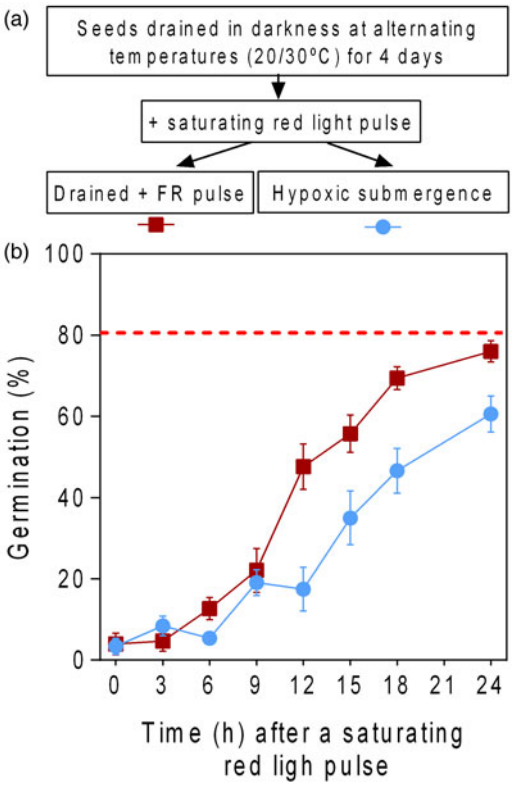

Fig. 3. Kinetics of the escape time to the control of seed dormancy and germination by phytochromes or inhibiting hypoxic submergence. (a) Scheme of treatments application where shallow dormant seeds were exposed to diurnally alternating temperatures (20/30°C, 9/15 h) for 4 d on wet filter paper. At the end of the fourth day, seeds were exposed to a saturating red light pulse and then transferred, at different times, to a final saturating far-red (FR) light pulse (squares) or submerged under hypoxic water (circles). Seeds were exposed to a 20/30°C regime during the experiment. (b) Germination percentages are presented as means ± SE ($n = 4$). The dashed line indicates seed lot germination percentages under optimal drained conditions. Bars not sharing the same letter are significantly different ($P < 0.05$).

(i.e. germinable seeds). It took approximately 12 h for 50% of the seeds to escape from the reversal of phytochrome driven by a saturating FR light pulse (30% of the seed lot was still dormant; Fig. 3); at that time, only 20% of the seeds germinated under hypoxic submergence (i.e. 60% of the germinable seeds were still inhibited by hypoxia; Fig. 3). After 24 h, none of the germinable seeds was further inhibited by FR light pulses, while the germination of 20% of the germinable seeds was still blocked by hypoxic submergence (Fig. 3).

Discussion

This research reveals some previously unknown control points of hypoxic conditions inhibiting underwater seed germination of an *E. crus-galli* accession resistant to anaerobic germination. It is known that germination is inhibited in many wetland species under stagnant floodwaters (Hook, 1984; Baskin et al., 1998; Mollard and Insausti, 2011). Nevertheless, the functional mechanisms by which submersion underwater may lead to such an inhibition are still not clear. It was previously demonstrated that a major fraction of *E. crus-galli* seeds is able to track dormancy-breaking cues even under hypoxic submergence (Peralta Ogorek et al., 2019); however, downward physiological processes that lead to dormancy breakage and germination may be blocked by

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hypoxic floodwaters. This contribution highlights that seed dormancy, for a facultative wetland weed such as *E. crus-galli*, is a key functional trait enabling seeds to avoid underwater germination under hypoxic floodwaters, which in case it happens, would be lethal for the emerging seedlings.

Dormancy may allow hydrophyte seeds to avoid high water tables and germinate in shallow water or recently drained conditions (Baskin et al., 2004; Mollard et al., 2007; Peralta Ogorek et al., 2019). Moreover, low atmospheric oxygen tensions are able to control both seed dormancy and germination processes in grass seeds (Corbineau and Côme, 1995; Rodríguez et al., 2015). While *E. crus-galli* seeds from rice field accessions seem not to be controlled by oxygen availability as they germinate under anaerobic atmospheres (Kennedy et al., 1980; Chauhan and Johnson, 2011; Ismail et al., 2012), other accessions are notoriously sensitive to hypoxic conditions (Ismail et al., 2012; Peralta Ogorek et al., 2019). In this research, we found that the control of hypoxic floodwaters on shallow dormant *E. crus-galli* seed dormancy is positioned after dormancy-breaking cues are perceived, indicating that downward signalling or response pathways are the target for hypoxic submergence inhibition of seed germination.

Covering structures (i.e. glumellae plus pericarp and seed coat) impose mechanical restrictions as well as limiting oxygen diffusion into imbibed grass embryos, so germination generally improves after scarification (Lenoir et al., 1983; Corbineau and Côme, 1995; Benech-Arnold et al., 2006; Barrero et al., 2009). It was initially hypothesized that by damaging seed covers and then, alleviating previously stated restrictions to embryo growth potential, would turn seeds insensitive to the inhibition by hypoxic submergence. Scarified *E. crus-galli* seeds did not express dormancy under optimal drained conditions and germinated even in the absence of dormancy-breaking signals (diurnally alternating temperatures, red light pulses), which supports that seed coats play a role in controlling dormancy under normoxia (Corbineau and Côme, 1995; Rodríguez et al., 2015). On the other hand, in scarified seeds, germination was still blocked by hypoxic submergence. So, it would be possible to speculate that low oxygen for the embryo might regulate its abscisic acid (ABA) sensitivity as seen in barley grains, a mesophyte species whose seed dormancy expression is mainly driven by oxygen resistances of covering hulls (Hoang et al., 2013), and highpoints the embryo sensitivity to hypoxia in *E. crus-galli* seeds from an accession resistant to anaerobic germination.

The rate of escape of seed germination from R–FR reversibility followed kinetics similar to that previously reported in seeds of different plant species (Mancinelli, 1994; Schäfer and Nagy, 2006). Escape from the inhibition of hypoxic submergence was slower relative to the escape from photoreversibility, indicating that hypoxia can still block *E. crus-galli* seed dormancy breakage or germination even after Pfr action has been completed and phytochrome signalling towards dormancy breaking is initiated. This result also illustrates that the temporal window for hypoxic submergence inhibition of seed dormancy breaking is as short as 18 h for half of the seed lot (Fig. 3), time in which, according to the literature, the expression of most of the enzymes associated to cell wall modifications are expected to be induced in germinating seeds (Barrero et al., 2009; Bewley et al., 2013). These results pave the way to look for the putative sites for hypoxic submergence inhibition during dormancy breaking and map them into the pathways that lead to seed germination.

Conclusions

Seed dormancy controlled by hypoxia can be regarded as a novel functional trait for wetland species complementary to the well-known 'escape' and 'quiescence' strategies developed by adult plants facing submergence (Bailey-Serres and Voesenek, 2008). So, seed dormancy regulation by hypoxia – in addition to the environmental cues breaking dormancy (e.g. diurnally alternating temperatures) – can minimize the risks of lethal underwater germination in AG resistant seeds and can optimize seedling recruitment by ensuring seed germination when floodwaters subside, and normoxia is regained. In this scenario, dormancy breakage inhibition might help *E. crus-galli* keep a rich seed bank in flooded soils and, therefore, contribute to the persistence of this noxious weed in cultivated lowlands.

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Conflicts of interest. The authors declare no conflicts of interest.

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