Using seed respiration as a tool for calculating optimal soaking times for 'on-farm' seed priming of barley (*Hordeum vulgare*)

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Abstract

A low-cost technique named ‘on-farm’ seed priming is increasingly being recognised as an effective approach to maximise crop establishment. It consists of anaerobically soaking seeds in water before sowing resulting in rapid and uniform germination, and enhanced seedling vigour. The extent of these benefits depends on the duration soaking time. Current determination of optimal soaking time by germination assays and mini-plot trials is resource-intensive, as it is species/genotype-specific. This study aimed to determine the potential of seed respiration rate (an indicator of metabolic activity) and seed morphological changes during barley priming as predictors of the priming benefits and, thus, facilitate determination of optimal soaking times. A series of germination tests revealed that germination rate is mostly attributable to rapid hydration of embryo tissues as the highest gains occurred before the resumption of respiration. Germination uniformity, however, was not significantly improved until seed were primed for at least 8 h, i.e. after a first respiration burst was initiated. The maximum seedling vigour was attained when the priming was stopped just before the beginning of the differentiation of embryonic axes (20 h) after which vigour began to decrease (‘over-priming’). The onset of embryonic axes elongation was preceded by a second burst of respiration, which can be used as a marker for priming optimisation. Thus, monitoring of seed respiration provides a rapid and inexpensive alternative to the current practice. The method could be carried out by agricultural institutions to provide recommended optimal soaking times for the common barley varieties within a specific region.

Keywords: Germination; Imbibition; Seed morphology; Seed vigour; Seedling establishment; Agricultural sustainable practices.

1. INTRODUCTION

Seed germination involves an array of coupled morphological and respiratory changes that make up three distinct phases each of which are characterised by the dynamics of water uptake. Germination commences with 'imbibition' (phase I), a profuse uptake of water by the dry seed and a gradual increase of seed size, although this phase is associated with no or little metabolic activity (Bewley *et al.*, 2013). This is then followed by the onset of seed respiration as a result of the resumption of pre-germinative activity, primarily attributed to the activation of mitochondrial energy production, which has been associated with the resumption of phosphorylation to produce ATP (Botha *et al.*, 1992; Ma *et al.*, 2017). Subsequently, the 'lag' phase (or phase II) initiates involving an intense metabolic activity (including the transcription and translation of new genes) and a stabilisation of water uptake and respiration rate (Bove *et al.*, 2002). Lastly, active mobilisation of reserves to the growing embryo causes another profuse increase of seed respiration and demand for water uptake, leading to the emergence of the radicle through the seed coat, which marks the end of germination *sensu stricto* and the beginning of seedling growth ('post-germination' or phase III) (Bove *et al.*, 2002; Bewley *et al.*, 2013).

'On-farm' seed priming is a farmer-managed type of seed treatment that differs from industrial priming strategies as it simply consists of anaerobically soaking seeds in water for a number of hours prior to sowing (Harris, 2006). Seeds are subsequently surface-dried for 1-2 hours (to avoid clumping) and sown soon after. Once sown, seeds spend significant amounts of time absorbing water from the soil. However, by controlling the transition through the germination phases, i.e. allowing seeds to undergo the pre-germinative phases I and II but preventing the start of phase III, 'on-farm' primed seed retains the benefits of pre-germinative advancements and, concurrently, preserve desiccation tolerance (Harris, 2006; Bewley *et al.*, 2013). Subsequently, this can lead to quicker emergence and enhanced seedling vigour (and ultimately yield) when the primed seed is sown in the field as demonstrated for a range of crops (Carrillo-Reche *et al.*, 2018). Importantly, to fully exploit this method of seed priming, the safe limit (the maximum length of time that seeds can be soaked without

germination taking place before sowing) for each crop and cultivar first needs to be determined. However, the optimal duration for soaking seeds (in terms of yield benefits) is not necessarily the same as the safe limit, e.g. priming seeds to the safe limit could lead to seeds biochemically arrested at a very advanced stage in the transition from phase II to phase III (Salimi and Boelt, 2019). Therefore, as seed soaking times are specific to each crop species/genotype or even seed quality, the major obstacle for the determination of optimal 'on-farm' seed priming protocols is the large number of trials needed (Paparella *et al.*, 2015; Salimi and Boelt, 2019; Forti *et al.*, 2020).

Currently, optimal 'on-farm' seed priming times have been determined for a range of crops by testing different seed soaking times (usually on moist filter paper) followed by sowing in mini-plot trials at research stations (e.g., Harris *et al*., 1999; Rashid *et al.*, 2004, 2006; Virk et *al.*, 2006). However, this process is resource-intensive and only provides retrospective information about its effectiveness. Moreover, information on the soaking times from these trials are limited to the specific crop variety and trial conditions; published or recommended soaking times, therefore, tend to be conservative and are likely to compromise any yield benefits that would have been gained from utilising 'on-farm seed priming. Thus, farmers performing 'on-farm' seed priming have used conservative soaking times, for simplicity commonly "overnight", despite this most likely being far from the optimum (Harris, 2006). Consequently, there is a need for the development of cost-effective methods that facilitate rapid determination of optimal soaking times for 'on-farm' seed priming.

Increases in respiration at the end of phase II are associated with the initiation of starch metabolism and have been used to predict seedling vigour of different species and cultivars (Patanè *et al.*, 2006; Patanè and Avola, 2013; Wang *et al.*, 2016). Therefore, detecting indicators of seed metabolic changes (as the flux of either O_2 uptake or CO_2 release) during seed soaking could provide a useful marker for the optimisation of 'on-farm' seed priming. Using barley as a model crop, this study aimed to determine: a) whether seed morphology and/or seed respiration changes can be used to detect metabolic changes that occur during 'on-farm' seed priming; and b) whether changes in morphology and/or respiration are associated with optimal soaking times and, thus, can be used as a marker for optimising the duration of 'on-farm' seed priming.

2. MATERIAL AND METHODS

2.1. Plant material and priming treatments

Barley (*Hordeum vulgare* L.) cultivars Concerto (Limagrain, Rothwell, UK) and RGT Planet (RAGT Seeds, Ickleton, UK) were chosen as they represent a benchmark variety for spring barley in the UK and a modern elite cultivar respectively. However, these cultivars are more correctly representative of genotype x environment x management differences as genotype represents only one factor in seed batch comparisons. The priming treatments applied in all experiments consisted of seeds soaked in distilled water (1:6 (w/v)) in 100 mL plastic pots, at 20 °C in the dark. After treatment, seeds were allowed to air-dry on paper towel for an hour (unless specified otherwise). In all cases, non-primed dry seeds were used as controls.

2.2. 'On-farm' seed priming soaking times and germination

Soaking times and moisture content determination

Samples of 150 seeds were soaked for either 4, 8, 12, 16, 20, 24 or 28 h (28h was established as the upper limit as it was when the coleorhiza tip became visible for some seeds) in triplicate for each soaking time. Three samples of unsoaked seeds (5 g each aprox.) were oven dried at 103 °C for 17 h to determine initial moisture content (Mci) (ISTA, 2015). The soaked samples were weighed before and after each soaking time to determine final moisture content (Mc), which was calculated as:

$$Mc = \frac{Wi \ * Mci + \Delta W}{Wf}$$

where *Wi* and *Wf* are seed weight before and after drying respectively, and ΔW is the difference between *Wf* and *Wi*.

Respiration measurements

Immediately after soaking, the concentration of CO_2 released by the seeds was measured with an EGM-4 CO_2 infrared gas analyser (PP Systems, Amesbury, Massachusetts, USA). Briefly, 100 mL plastic pots were hermetically closed with a lid connected to the infrared analyser through inlet and

outlet tubing, in order to create a closed system to monitor the flux of [CO_2]. The net CO_2 flux was calculated as the increment within 1 min (average of three sequential readings representing one replicate) prior to allowing CO_2 to accumulate within the tubing system for 15 s (modified from Patanè et al. (2006)). Seed respiration rates (SRR), expressed as μmol CO_2 s^{-1} g^{-1} seed DW (dry weight), for each soaking time were calculated as follows:

$$SRR = \left(\frac{\Delta CO_2}{\Delta t}\right) x \left(\frac{V}{RT}\right)$$

where $\Delta CO_2/\Delta t$ (μmol CO_2 s^{-1}) is the change in CO_2 concentration over the measurement time; V (m^3) is the total volume of the system (volume of priming pot, tubing and gas analyser); R (kPa m^3 mol^{-1} K^{-1}) is the ideal gas constant, and T (K) is the temperature in the incubator.

Histological observations

To examine the morphology changes over time, seeds were transversally sectioned with a razor blade after each soaking time. Seed embryo structures were observed under a stereomicroscope (magnification x 9, Leica GZ6) and photographed using a digital camera (Nikon Coolpix 950).

Germination test

One hundred seeds per soaking time were placed over four sheets of paper towel covered with another two sheets previously moistened with 30 mL of sterile distilled water and incubated for 72 h in plastic containers (304 x 216 x 55mm) with lids at 20 ºC in darkness. Seeds were considered to have germinated when the radicle length was greater than 2 mm. In order to accurately determine germination dynamics, counts were made every 2 h from the start of germination until cumulative germination was above 75 %. Each soaking time and germination assay were carried out three times.

Desiccation tolerance test

To simulate a delay before "sowing", the same soaking times were repeated (as in Soaking times and moisture content determination) and seeds allowed to air-dry to original moisture on paper towel

for 30 days at nearly ambient temperature (20 °C) in the dark, and then a germination test carried out as described above.

2.3. 'On-farm' seed priming soaking times and seedling vigour

Based on the principles of a cold test (Hampton and TeKrony, 1995), a modified cold test was carried out to assess seedling vigour. Soaking times of 16, 20 and 24 h were selected (based on the germination test results) for this test, together with an un-soaked treatemtn as a positive control. Seeds were sown in vermiculite in three seed tray inserts (60 cells per tray). All treatments were equally present in each trait and their position was randomised within each tray (replicate). Trays were watered to reach 80 % saturation, covered with aluminium foil to avoid evaporation, and kept at 10 °C in the dark. This setup provided high water availability, good aeration of the substrate and low temperature to minimise any potential head-start related to seed water content. After seven days, the trays were uncovered and moved to a growth chamber at 20 °C, 12 h photoperiod and 70 % relative humidity for 5 days. Each tray was watered with 75 mL of distilled water every other day and emergence recorded daily. After 5 days, seedlings were removed from the inserts and categorised as either healthy (viable enough to turn into a healthy plant), or abnormal, e.g. damaged, or deformed or decayed as a result of infection (Supplementary Fig. S1 for illustration of abnormality criteria). All healthy seedlings per replicate were dried at 110 °C for 17 h to obtain dry weights. The experiment was repeated three times.

2.4. Data analysis

Indices for time to 50 % germination (G_{50}), time to 50 % emergence (E_{50}), uniformity (U), calculated as the time interval between 25% and 75% of seeds to germinate/emerge, the percentage of total germinated seeds (%TG), and the percentage of healthy emerged seedlings (%TE) were calculated using the 'Germinator' tool (Joosen *et al.*, 2010). Effect of cultivar (Cv), soaking time (Tr) and their interaction on germination variables were assessed by analysis of variance (ANOVA) and emergence variables by linear mixed-effects model (LMM), with experiment repetitions as a random term, in R version 3.3.0 (R Development Core Team, 2016). Assumption of normality and

homoscedasticity of variances were checked by QQ-plots and residuals against fitted value plots respectively. When these assumptions were not met, data was transformed. G_{50} data from germination test were square-root transformed and continuous proportional data, i.e., percentage of germination (%TG) and percentage of healthy emerged seedlings (%TE) were arcsine transformed to approximate normality. Post-hoc Fisher's LSD tests were performed to separate significant differences at *P* values < 0.05 with *predictmeans* package (Luo *et al.*, 2014). *P* values were adjusted to avoid Type I errors (false positives) using the Benjamini–Hochberg correction (Waite and Campbell, 2006). Means for significant main effects are presented based on the highest order of factorial combination that was significant in the ANOVA or LMM.

In order to investigate the relative contribution of initial moisture content and advancement of germination to speed of germination at each germination phase, moisture content (Mc) and cumulative CO_2 (ΣCO_2) at the moment of sowing were used as predictors of G_{50}. Data from both cultivars were pooled for this test. The relative importance of predictor variables and their bootstrapped 95 % confidence intervals were calculated with the *relaimpo* package (Grömping, 2006) in R. Absence of collinearity between the two variables was verified by variance inflation factor.

3. RESULTS

3.1. Changes in seed morphology and respiration during 'on-farm' seed priming

Barley seeds showed clear morphological differences indicative of the transition from one germination phase to another (

Fig. **1**). After the first 4 h of imbibition, the wetting of the embryonic tissues was visually evident. This was reflected in moisture content as almost half of the total water absorbed occurred within the first 4 h of soaking, which is characteristic of the phase I "imbibition" stage (Fig. 2a). From 4 h to 20 h, no major morphological changes were observed, although the overall seed size increased gradually concurrent with a progressive increase in moisture content. Typically, both differentiation and expansion of the embryonic axis began at 24 h, accompanied by seed coat loosening and wetting of the endosperm. At 28 h, emergence of the coleorhiza tip through the micropylar was visually distinguishable for most of the seeds. Soaking times beyond 28 h did not result in further visual morphological development of the seed and only marginal increments in moisture content.

The initiation of respiration about 4 h after imbibition marked the primary activation of germinative metabolism (Fig. 2b). The onset of respiration was followed by a steep rise in respiration until about 16 h where the rate of respiration became constant. This plateau, characteristic of the phase II "lag" stage, was punctuated by a second release of CO_2 after 20 h of soaking, which corresponds with the major morphological changes at 24 h (

Fig. **1**). This burst of respiration had declined by 28 h, and soaking times beyond this did not result in further increases of water content or seed respiration which typically marks the onset of phase III.

Respiration curves for both cultivars showed a similar triphasic-like shape with some disparity in the initiation in respiration (Fig. 2b), i.e., the onset of cultivar-specific respiration. For RGT Planet, this occurred within the first 4 h of soaking, whereas for Concerto this happened after 4 h. Cumulatively, although RGT Planet had earlier metabolism, both cultivars had released similar amounts of CO_2 by the end of the experiment (Fig. 2c). This cumulative respiration was later used as a proxy of seed germination advancement (ΣCO_2).

3.2. Effect of different soaking times on germination parameters

Germination tests were carried out to determine the most promising soaking times for each cultivar. There was a significant interaction between cultivar and soaking time ($P < 0.001$) in time to 50 % emergence. Longer soaking times reduced the time to 50 % germination, although the residual increment after each soaking interval decreased progressively to a minimum between 24 and 28 h (Table 1). For both cultivars, the most effective durations were ≥ 16 h. In terms of uniformity of germination, soaking time but not cultivar had a significant effect ($P < 0.001$). Soaking times greater than 4 h significantly improved uniformity, with 16 h being the most effective duration for both cultivars (Table 2). However, regarding total percentage of germination, there was no soaking time effect ($P = 0.13$) but cultivar effect ($P < 0.001$) with Concerto having a higher percentage than RGT Planet. Overall, soaking times exerted very similar effects on germination parameters of both cultivars, thus, based on these results, soaking times of 16 h, 20 h and 24 h were selected for the subsequent seedling vigour tests. Although 28 h soaking time achieved similar values to those of the selected soaking times, it was considered excessively long as the coleorhiza tip was visible in some seeds, indicative of 'over-priming' (liable to loss of vigour, desiccation and damage during sowing).

The proportional contribution of moisture content (expressed as the moisture content at sowing) and germination advancement (expressed as accumulated CO_2 at the moment of sowing) to time to 50

% germination was resolved through linear regression for each phase (Table 3). At imbibition, 97 % of the total variability was explained by the model and showed that reductions in time to 50 % germination can be largely ascribed to the moisture content rather than cumulative CO_2 (90 % vs. 7 %) (Fig. 3). However, this situation was reversed during the lag phase as cumulative CO_2 contributed 1.5-fold more than moisture content to the total explained variance (87 %).

3.3. Vigour: optimization of soaking times and desiccation tolerance

In order to assess the effect of the soaking times, a cold test was designed to offset initial water content at sowing so that potential changes in seedling biomass would be attributable to greater vigour rather than initial water content. No differences in emergence of healthy seedlings were found at the cultivar ($P = 0.12$) or treatment level ($P = 0.80$), or their interaction ($P = 0.73$) indicating that seed viability remained unaffected under prolonged exposure to soaking and high moisture (Supplementary Table 1). Similarly, no significant differences for time to 50 % emergence were found among soaking times and control ($P = 0.49$); therefore, the experimental design was effective for counteracting the effect of initial moisture content (Supplementary Table 1).

In contrast, both cultivar and treatment effects significantly affected seedling biomass ($P < 0.001$ and $P < 0.01$ respectively) but not the interaction ($P = 0.09$), indicating that the effect of soaking time was similar in both cultivars (Fig. 4). Soaking for 20 h produced the highest amount of seedling biomass of all soaking times and was significantly higher than seeds soaked for 16 h ($P < 0.01$) and 24 h ($P < 0.05$). Based on these results, 20 h was considered the optimum soaking time for both cultivars.

Analysis of variance for the effect of desiccation on time to 50 % emergence showed significant differences for cultivar and soaking time ($P < 0.001$) but not for the interaction ($P = 0.94$). The seeds of RGT Planet were more affected than Concerto by the 30-day desiccation period (Table 4). For both cultivars, seeds soaked for 24 and 28 h needed significantly longer to attain 50 % of germination compared with the rest of the soaking times. Soaking for 8 h yielded the shortest time to 50 % emergence and 28 h soaking the longest time. Differences in total germination were due to cultivar

effect ($P < 0.001$), where again RGT Planet was more sensitive to desiccation. No significant differences among soaking times ($P = 0.27$) or the interaction ($P = 0.40$) were found (Table 4). Comparison of time to 50 % germination and total germination of (unsoaked) control treatments relative to the corresponding control showed a negative effect in germination performance that was attributable to storage conditions (i.e. 30 d at 20 °C). These effects were most apparent for RGT Planet with +26.4 and -4.5 % change in time to 50 % germination and total germination respectively; whilst the effect for Concerto was negligible, +1.8 and -0.7 % respectively.

4. DISCUSSION

4.1. Seed respiration as a tool for detecting the activation of metabolic processes during 'on-farm' seed priming

The present work has shown that monitoring of CO_2 flux patterns is a reliable tool for detecting key germination events during barley 'on-farm' seed priming. As under regular germination conditions, barley respiration during priming describes a triphasic curve where the transition from one germination phase to another is marked by a burst of seed respiration, providing useful information on the timing of metabolic changes that occur during the course of priming. The highest seedling vigour for both cultivars was attained in seeds primed for 20 h, which morphologically, corresponds with stopping the priming process just before the differentiation of embryo tissues into coleoptile and coleorhiza; and before the second burst of CO_2 flux. Therefore, both seed morphology and CO_2 flux patterns can be used as a marker for 'on-farm' priming optimisation.

Unlike regular germination, the continuation of phase III beyond its initiation is impeded during 'on-farm' seed priming, and longer soaking times do not result in further development of the coleorhiza tip nor a sharp increment of water uptake. Due to the hypoxic conditions within the seed, the energy demands for early barley seed development are mostly provided through oxygen-independent metabolic pathways, e.g. glycolysis and alcohol fermentation (Østergaard *et al.*, 2004; Zhang *et al.*, 2004). However, in late phase II, the further development of the embryo requires oxygen-dependent cycles such as tricarboxylic acid (TCA) that are more efficient for active

mobilization of storage reserves and cannot be fulfilled by anaerobic respiration alone (He *et al.*, 2015; Ma *et al.*, 2017). When exogenous O_2 is available, a profuse second burst of CO_2 flux is followed by the appearance of the coleorhiza tip and more water uptake (Bewley *et al.*, 2013; Ma *et al.*, 2017). However, this second burst declines soon after and is not followed by an increase of water uptake under the hypoxic conditions imposed by 'on-farm' seed priming. Although respiration remains active possibly through fermentation, further root development is impeded as a mechanism to avoid anoxia (Borisjuk *et al.*, 2007; Ma *et al.*, 2016).

Sectioning and observation of seed morphology seems useful for detecting the beginning of phase III, which corresponds with the elongation of the coleoptile and coleorhiza tissues in the embryo, but not for other metabolic processes. As observed for other cereal seeds, although an enlargement of the seed size throughout soaking could be seen by eye, actual changes in seed structures are minimal even under the microscope until phase III (An and Lin, 2011; He *et al.*, 2015).

Cultivars showed distinct seed vigour from one another, although this was not only due to genotype differences but also to differential seed quality (as manifested by the notable deterioration of RGT Planet germination performance after a storage period under unfavourable conditions). However, both cultivars performed similarly with an optimal soaking time of 20 h, suggesting that seed vigour and/or seed quality have minor influence in soaking times. Although it is tempting to generalise that 20 h is the optimal soaking time for barley, it is still to be elucidated the extent to what extent seed vigour and/or seed quality components can influence priming soaking times. Seed phenotypical characteristics (e.g. seed coat, grain composition and size), ageing and the make-up of the maternal tissues are known to alter the germination process and, by extension, likely to affect seed priming soaking times (Finch-Savage and Bassel, 2016; Salimi and Boelt, 2019).

4.2. Mechanistic of the priming benefits: Timing and contribution of its drivers

In order to better leverage 'on-farm' seed priming, it is critical to understand the timing and contribution of the two main drivers for rapid germination: 1) a hydrated seed, and 2) being developmentally more advanced than dry seeds at the moment of sowing. The rapid germination of

304 ‘on-farm’ primed seeds can be mainly ascribed to the rapid hydration of internal tissues rather than to
305 the germination advancement gained during the soaking time. In this study, few hours of soaking (~4
306 h) were sufficient to dramatically reduce the time for germination relative to dry barley seeds (35 %
307 out of the 53 % average total gain), after which residual gains from longer soaking times were
308 gradually ascribable to developmental advancement. Longer soaking times (≥8 h) are needed to
309 significantly enhance uniformity of barley germination, after which no further improvements in
310 uniformity are attained. This suggests the occurrence of metabolic changes at early lag phase which
311 completion ensures that all barley seeds have reached, by way of checkpoint, a common stage in the
312 germination programme.

313 It follows from the above discussion that simply soaking for several hours, e.g. 8 h as equivalent
314 to the “overnight” practice proposed for most tropical crops (Harris, 2006), is enough to obtain
315 significant germinative benefits from planting hydrated seeds. However, if primed seeds are sown in
316 soil at field capacity, this rapid hydration effect compared to dry seeds may be limited, although the
317 benefits of being developmentally advanced still remain. In an agricultural context yield benefits
318 associated with sowing hydrated seeds will vary depending on local soil moisture, with the most
319 beneficial associated with sowing ‘on-farm’ primed seeds in water-stressed soils (Carrillo-Reche *et*
320 *al.*, 2018). Imbibition is primarily a passive process and is a driver for the resumption of metabolic
321 activity (reflected by the increase in respiration), so the priming duration must be long enough to
322 ensure that germination processes are sufficiently advanced to enable pre-germinative benefits once
323 the seed is sown. Since the actual timing for these events will vary depending on cultivar, seed quality
324 and priming conditions (e.g. temperature), focusing on the germination advancement stages rather
325 than a particular soaking time seems to be the best strategy for the optimisation and standardisation of
326 ‘on-farm’ seed priming in order to maximise seed vigour.

327 Seedling vigour is the most important seed quality trait as the post-germination pre-emergence
328 seedling growth phase is considered the most vulnerable stage and, thereby, the usefulness of seed
329 priming (Finch-Savage and Bassel, 2016). When the advantage of partial hydration is kept out of the
330 equation, enhanced seedling vigour is evident when the priming process is stopped just before the

beginning of the differentiation of embryo tissues into coleoptile and coleorhiza, but not before or after, highlighting the specificity of optimal priming protocols. At this stage, most of the pre-germinative metabolism has already taken place, i.e. mitochondrial multiplication, gene transcription, synthesis of amino acids and new proteins, but is still prior to the induction of post-germinative metabolism, i.e. cell division and expansion, which ensures that root emergence only occurs after sowing (He *et al.*, 2015; Wojtyla *et al.*, 2016; Ma *et al.*, 2017). Furthermore, there is increasing evidence that the activation of cellular repair is the key process enhancing seed vigour following seed priming, so that it is likely that this optimal soaking time corresponds with the maximum DNA repair and antioxidant response to recover from prior oxidative damage (Sharma and Maheshwari, 2015; Wojtyla *et al.*, 2016; Forti *et al.*, 2020). However, these invigorating effects are not arrested when seeds are dehydrated to their original moisture content and then allowed to 're-germinate'. Dehydration, unfavourable storage conditions, and re-hydration lead to extensive oxidative damage that may revoke the seed repair attained during the priming process (El-Maarouf-Bouteau *et al.*, 2013; Waterworth *et al.*, 2019).

The onset of embryonic axes differentiation can be understood as the milestone marking the transition from seed to seedling and, although technically falls within the 'safe limits' (as no germination is externally visible even when let air-dry), must be prevented. The declines in seed/seedling performance in both desiccation and vigour tests at and after this milestone are clear signs of excessively long priming duration ('over-priming'). The probable reason for this phenomenon is the loss of desiccation capacity. Type I such as late embryogenesis abundant proteins (which are involved in preventing membrane disintegration and protecting mitochondrial enzymes under dehydration) progressively deplete after imbibition, and thus compromise desiccation tolerance (Grelet *et al.*, 2005; Yang *et al.*, 2007; An and Lin, 2011). In addition, it is possible that toxic fermentation products accumulate in excess in response to the prolonged hypoxic conditions during 'on-farm' seed priming conditions contributing to a gradual loss of vigour (Benvenuti and Macchia, 1995).

4.3. Implications and practical considerations of 'on-farm' seed priming

In practice, farmers using 'on-farm' seed priming need to be able to distinguish between 'optimal' and 'safe' soaking times. When conditions allow seeds to be sown within a few hours after priming, optimising soaking times to produce maximal moisture content and advancement benefits would be the best strategy. Air humidity and a long drying period after priming may impair the optimal soaking times by, for example, promoting the proliferation of fungal damage. Thus, when there is a risk of delayed sowing (e.g. due to heavy rain, or having to passively dry seeds overnight after priming), shorter soaking times can ensure that germination does not occur before planting. Current safe recommendations for 'on-farm' seed priming of barley is for "overnight" priming (~8 h) (Harris, 2006).

It is important that farmers can obtain information on optimal soaking times for their own seeds and specific 'on-farm' priming conditions. From the methods proposed in this study for determining optimal soaking times, sectioning for microscopic observation of seed morphological changes is the simplest option. Having identified embryo axis differentiation as the marker for "over-priming", this method could be performed by farmers with a razor blade and a magnifying glass. However, the reproducibility of this within the farm context would be a challenge, and specific training for the identification of these subtle embryo differences would be required. The second method of monitoring seed respiration as a marker is a non-invasive technique and allows the accurate identification of both the initiation of phase II (which can be used for recommendation of safe limits) and the initiation of phase III (for recommendation of optimal soaking time). Although this method is not designed to be carried out by farmers, it could be performed by agricultural institutions for providing recommendations of general practices for common varieties within their region produced under comparable growing conditions. Both methods represent a much more rapid and cost-effective alternative to the current optimisation approach through a series of germination assays and mini-plot trials and, therefore, could facilitate the widescale adoption of 'on-farm' seed priming.

5. CONCLUSIONS

This study emphasises the importance of the two drivers of 'on-farm' seed "priming" benefits: moisture content and advanced germination at the moment of sowing. In an agricultural context, the former largely determines the time to germination but its magnitude will vary depending on soil moisture. However, the extent of the benefits from germination advancement will depend on the moment of stopping the priming process and, thereby, the importance of optimising the soaking times in order to exploit the full benefits from this technology. Therefore, it is proposed that to achieve maximum seedling performance priming is stopped prior to the differentiation of the embryonic axis and/or the second burst of respiration. This optimal timing can be deduced from morphological observation of the embryonic axis or CO_2 flux patterns for each cultivar and priming conditions. These methods could easily be implemented for determining the optimal soaking times of other cultivars of barley. Extrapolation of these methods to other crops seems feasible although further testing would be required as seed respiration and germination rates can vary greatly depending on crop-specific characteristics of the seed, e.g. starch seeds versus oil seeds.

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Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

The datasets generated during the current study are available in the Stirling Online Repository for Research Data repository, [the link for data sharing can be produced on acceptance of the manuscript].

Supplementary data

The following is Supplementary data to this article:

Supplementary Fig. 1.

Supplementary Table 1.

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Fig. 1. Structural morphology of barley seeds at the end of each soaking time. Transversal embryo observation by stereomicroscopy. From left to right, red arrows show wetting of the germ, wetting of the endosperm, expansion of the coleorhiza, expansion of the coleoptile and emergence of the radicle tip.

Fig. 2. The effects of 'on-farm' seed priming on, (a) seed moisture content, (b) seed respiration rate (SRR) and (c) cumulative SRR at specific intervals for Concerto (open circles) and RGT Planet (closed triangles) barley seeds. Vertical bars show ± SE (only if the SE is greater than the symbol size).

Fig. 3. Percentage of variance explained by moisture content (Mc) and Cumulative CO_2 (ΣCO_2) to time to 50 % germination during phase I "imbibition" and phase II "lag". Vertical bars show 95% bootstrap confidence intervals.

Fig. 4. Average dry weight of seedlings at the end of the cold test. Linear mixed-effects model *P* values are for factor cultivar (Cv) and soaking time (Tr). Bars with different letters differ significantly according to LSD test ($P < 0.05$). $LSD_{Cv} = 0.02$; $LSD_{Tr} = 0.02$. Vertical bars show the mean + SE.

Fig. S1. Evaluation criteria for seedling abnormalities. a) damaged seedling missing side roots, b) seedling with a deformed etiolated shoot, c) decayed seedling presenting a fungal infection around the seed coat; d) un-germinated seed due to a primary infection around the germ; and e) non-viable seed.