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RESEARCH PAPER

Quantifying the sensitivity of barley seed germination to oxygen, abscisic acid, and gibberellin using a population-based threshold model

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Abstract

Barley (*Hordeum vulgare* L.) seeds (grains) exhibit dormancy at maturity that is largely due to the presence of the glumellae (hulls) that reduce the availability of oxygen (O_2) to the embryo. In addition, abscisic acid (ABA) and gibberellins (GAS) interact with O_2 to regulate barley seed dormancy. A population-based threshold model was applied to quantify the sensitivities of seeds and excised embryos to O_2, ABA, and GA, and to their interactive effects. The median O_2 requirement for germination of dormant intact barley seeds was 400-fold greater than for excised embryos, indicating that the tissues enclosing the embryo markedly limit O_2 penetration. However, embryo O_2 thresholds decreased by another order of magnitude following after-ripening. Thus, increases in both permeability of the hull to O_2 and embryo sensitivity to O_2 contribute to the improvement in germination capacity during after-ripening. Both ABA and GA had relatively small effects on the sensitivity of germination to O_2, but ABA and GA thresholds varied over several orders of magnitude in response to O_2 availability, with sensitivity to ABA increasing and sensitivity to GA decreasing with hypoxia. Simple additive models of O_2–ABA and O_2–GA interactions required consideration of these O_2 effects on hormone sensitivity to account for actual germination patterns. These quantitative and interactive relationships among O_2, ABA, and GA sensitivities provide insight into how dormancy and germination are regulated by a combination of physical (O_2 diffusion through the hull) and physiological (ABA and GA sensitivities) factors.

Key words: Abscisic acid, barley, germination, gibberellin, *Hordeum vulgare* L., model, oxygen, sensitivity.

Introduction

Seeds (caryopses or grains) of cereals from temperate climates often exhibit primary dormancy at harvest that is most evident at warm temperatures (>15 °C) (Corbineau and Côme, 1980; Lenoir *et al*., 1983). Insufficient dormancy can result in pre-harvest sprouting in humid climates, while excessive dormancy can interfere with utilization of the grain for planting or malting (Benech-Arnold, 2004). The glumellae (lemma and palea) adhering to the caryopses, for example in barley (*Hordeum vulgare* L.) and oat (*Avena fatua* L.) grains, are primarily responsible for imposition of dormancy, as germination is much improved by their removal (Corbineau and Côme, 1980; Lenoir *et al*., 1986; Benech-Arnold *et al*., 1999). The dormancy present at harvest is lost during after-ripening (dry storage) as the temperature range for germination expands and germination occurs more rapidly and uniformly (Côme *et al*., 1984).

It has been proposed that the effect of the glumellae on dormancy is to reduce the availability of oxygen (O_2) to the embryo (Pollock and Kirsop, 1956; Lenoir *et al*., 1986; Lecat *et al*., 1992). Fresh intact barley grains germinated poorly even in 100% O_2, while removal of

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the glumellae or after-ripening allowed complete germination to as low as 10% or 3% O_2, respectively (Lenoir *et al*., 1986). However, the improvement in germination and lower O_2 requirement of naked grains following after-ripening suggests that factors in the embryo may also be involved. Abscisic acid (ABA) in particular plays a major role in regulating dormancy during seed development (Feurtado and Kermode, 2007), and the ABA content of barley embryos was greater in a more dormant variety than in a less dormant variety (Benech-Arnold *et al*., 1999). Similarly, embryonic ABA content decreased quickly after imbibition in embryos of non-dormant barley grains or in embryos of dormant grains imbibing at 20 °C (where germination occurred), but remained elevated in embryos of dormant grains imbibing at 30 °C (where germination was prevented) (Benech-Arnold *et al*., 2006). In addition, the sensitivity of germination to exogenous ABA was greater for dormant embryos than for non-dormant embryos, particularly at 30 °C (Benech-Arnold *et al*., 2006). Gibberellins (GAS) are required for seed germination and can stimulate the germination of dormant grains, while inhibition of GA biosynthesis during seed development increases dormancy (Lecat *et al*., 1992; Benech-Arnold *et al*., 1999). Provision of GA to dormant oat grains markedly reduced their sensitivity to low O_2 percentages and largely overcame the inhibitory effect of the glumellae (Lecat *et al*., 1992).

These studies with cereals and recent investigations with *Arabidopsis thaliana* and other species have confirmed that the balance of ABA and GA action plays a critical role in regulating seed germination and dormancy and that these hormones can have reciprocal effects on their respective biosynthesis and inactivation pathways (Gonai *et al*., 2004; Cadman *et al*., 2006; Finch-Savage and Leubner-Metzger, 2006; Seo *et al*., 2006; Finch-Savage *et al*., 2007; Oh *et al*., 2007). Hormone action is dependent on the combination of the hormone content (the net result of rates of synthesis and metabolism) and the sensitivity of the cells to the hormone (Bradford and Trewavas, 1994). Hormone sensitivity is generally measured by observing the response of the targeted phenotypic or physiological event to a range of hormone concentrations. In the case of seed germination, wide variances in hormone sensitivity have been observed among seeds in a single population (Ni and Bradford, 1992, 1993). Thus, it is necessary to characterize the distribution of hormone sensitivities among seeds and/or compare populations or treatments at a specific percentage in order to make valid comparisons. Population-based threshold models have proven to be a robust approach to characterize and quantify the germination and dormancy behaviour of seeds in response to a diverse range of physiological and environmental factors (Bradford, 1995; Finch-Savage and Leubner-Metzger, 2006; Allen *et al*., 2007). This model allows the sensitivity of germination to ABA, GA, and O_2 to be quantified based upon germination time courses conducted at a range of hormone concentrations or O_2 percentages (Ni and Bradford, 1992, 1993; Bradford *et al*., 2007). Parameters quantifying the overall speed, uniformity, and sensitivity of germination in relation to the regulatory factor(s) are derived from the model. In addition, interactions among these factors can also be modelled to test whether they are additive or exhibit other types of relationships (Ni and Bradford, 1993).

Here the population-based threshold model is applied to examine the effects of seed coverings, after-ripening, and temperature on the sensitivities of germination to O_2, ABA, and GA. The results allow a quantitative assessment of the effect of seed coverings on O_2 availability and of the effect of O_2 availability on hormone sensitivity and vice versa. The results support a proposed scenario for the interaction of these factors in primary dormancy of barley seeds and its alleviation by after-ripening.

Materials and methods

Plant material and germination at different O_2 percentages

Seeds of barley (*Hordeum vulgare* L. cv. Pewter) harvested in 2000 or 2002 were stored at –20 °C to maintain dormancy (Lenoir *et al*., 1986) or in the open air for 5 or 9.5 months at 25 °C for after-ripening (Corbineau and Côme, 1996). Germination experiments with dormant and non-dormant seeds and embryos were performed as described in Benech-Arnold *et al*. (2006) utilizing the apparatus and methods previously described to control O_2 in the surrounding atmosphere (Côme and Tissaoui, 1968). A seed was regarded as germinated when the radicle had pierced the seed covering structures or had reached 2–3 mm in length in isolated embryos. Germination counts were made daily for 7 d or 8 d. The results presented are the means of the germination percentages obtained in the two replicates.

The oxygen-time threshold model

The oxygen-time threshold model has been described in detail by Bradford *et al*. (2007) based upon similar threshold models describing seed germination responses to temperature, water potential, and plant hormones (Ni and Bradford, 1992, 1993; Bradford, 1995; Allen *et al*., 2007). Briefly, the model can be defined by the equation:

$$\theta_{Ox} = [Ox - Ox_b(g)]t_g \quad (1)$$

where θ_{Ox} is the oxygen-time constant, Ox is the O_2 percentage in the air surrounding the seed, $Ox_b(g)$ is the base or threshold value of Ox just allowing germination of percentage g of the viable seed population, and t_g is the time to germination of percentage g. θ_{Ox} is a constant, so as the difference between Ox and $Ox_b(g)$ decreases (i.e. the O_2 percentage approaches the threshold limit for a particular seed fraction), t_g increases proportionately, and vice versa. In the case of O_2 effects on germination, the germination response is proportional to the logarithm of the O_2 percentage, so the actual model used is:

$$\theta_{Ox} = [\log Ox - \log Ox_b(g)]t_g \quad (2)$$

A probit regression method was used to estimate the median and standard deviation of the normal distribution of O_2 thresholds

within the seed population (Bradford, 1990; Bradford *et al*., 2007). The fit of the model was optimized by testing different values of θ_{Ox} until the R^2 (coefficient of determination) of the regression was maximal. The median [$Ox_b(50)$] and standard deviation (σ_{Oxb}) of log $Ox_b(g)$ values define this distribution, which, along with θ_{Ox}, can generate predicted germination time courses at any value of log Ox. Graphs and predicted time courses were generated using CoPlot software (www.cohort.com).

ABA- and GA-time threshold models

A similar approach was used to model the germination responses to ABA and GA, as has been described previously (Ni and Bradford, 1992, 1993). Germination responses to these hormones are also logarithmic, so the models can be defined as:

$$\theta_{ABA} = [\log ABA - \log ABA_b(g)]t_g \quad (4)$$

and

$$\theta_{GA} = [\log GA - \log GA_b(g)]t_g \quad (5)$$

where ABA and GA indicate the concentration of ABA or GA, respectively, in the imbibition solution (molar) and $ABA_b(g)$ and $GA_b(g)$ represent the sensitivity thresholds for ABA and GA of germination fraction g. As for O_2, the time constants (θ_{ABA}, θ_{GA}), median thresholds [$ABA_b(50)$, $GA_b(50)$], and standard deviations of the threshold distributions (σ_{ABAb}, σ_{GAb}) can be used to compare hormonal sensitivities and to predict germination time courses at any ABA or GA concentration.

Models of O_2–ABA and O_2–GA interactions

Once the effects of O_2 concentrations on ABA and GA responses and vice versa were determined, a simple additive model was used to investigate interactions between these factors. As described in Ni and Bradford (1993), factors such as reduced O_2 or increased ABA delay germination relative to the rate at which it would occur in 21% O_2 and no added ABA. Thus, the time to germination in a combination of reduced O_2 and added ABA [$t_g(Ox, ABA)$] can be represented as:

$$t_g(Ox, ABA) = t_g(0) + d_{OX} + d_{ABA} \quad (6)$$

where $t_g(0)$ is the time to germination in water and 21% O_2, and d_{Ox} and d_{ABA} refer to the delays in germination due to O_2 and ABA, respectively. Since the time to germination in either reduced O_2 [$t_g(Ox)$] or ABA [$t_g(ABA)$] alone is equal to $t_g(0)+d_{Ox}$ or $t_g(0)+d_{ABA}$, respectively, then $d_{Ox}=t_g(Ox)–t_g(0)$ and $d_{ABA}=t_g(ABA)–t_g(0)$. Thus, the time to germination when both factors are present is equal to

$$t_g(Ox, ABA) = t_g(0) + \left[t_g(Ox) - t_g(0)\right] + \left[t_g(ABA) - t_g(0)\right] \quad (7)$$

or

$$t_g(Ox, ABA) = t_g(Ox) + t_g(ABA) - t_g(0) \quad (8)$$

Thus, interactions were modelled by adding the predicted times to germination at the specific O_2 percentage and ABA concentration when the other factor was not present and subtracting the predicted time to germination in water at 21% O_2. However, since O_2 percentage strongly affected ABA sensitivity, the model parameters for the appropriate O_2 level were used in calculating $t_g(ABA)$. Using the same reasoning, the advancement of germination due to the addition of GA (a_{GA}) would reduce the time to germination, so that the combined effect of O_2 and GA on time to germination would be $t_g(0)+d_{Ox}–a_{GA}$. As $a_{GA}=t_g(0)–t_g(GA)$, the following equations describe the simple additive interaction between O_2 percentage and GA concentration:

$$t_g(Ox, GA) = t_g(0) + \left[t_g(Ox) - t_g(0)\right] - \left[t_g(0) - t_g(GA)\right] \quad (9)$$

or

$$t_g(Ox, GA) = t_g(Ox) + t_g(GA) - t_g(0) \quad (10)$$

Results

Germination sensitivity to O_2 in relation to dormancy and after-ripening

Dormant intact seeds harvested in 2000 germinated <50% in water at 20 °C even in 21% O_2, and lower O_2 percentages reduced germination to zero at about 1% O_2 (Fig. 1A). By contrast, embryos excised from dormant seeds germinated rapidly and completely under those conditions, and about 50% of the seeds completed germination even in 0.5% O_2 (Fig. 1B). The O_2-time model fits these germination time courses well, with R^2 values of 0.954 for seeds and 0.948 for embryos (Table 1). The model predicted that 36.3% O_2 would be required for 50% germination of intact dormant seeds, illustrating their high O_2 requirement and low sensitivity to O_2. Dormant embryos, on the other hand, were predicted to require only 0.123% O_2 to achieve 50% germination (Table 1). Seeds also exhibited greater variation in O_2 thresholds, having a σ_{Oxb} value of 1.01 (10.2%) versus only 0.752 (5.65%) for embryos (Table 1). The time courses predicted by the O_2-time model matched well to the actual time courses for both seeds and embryos (Fig. 1A, B).

Germination rates and percentages improved following after-ripening for both seeds and embryos (Fig. 1C–F). Five months of after-ripening allowed almost complete germination of seeds in 21% O_2, but germination remained highly sensitive to O_2 percentage (Fig. 1C), with an $Ox_b(50)$ value of 2.87% (Table 1). After an additional 4.5 months of after-ripening, $Ox_b(50)$ had decreased to only 0.30% O_2 and germination was relatively insensitive to O_2 concentrations above 5% (Fig. 1E, Table 1). The threshold O_2 percentage for germination of seeds decreased approximately 10-fold for every 5 months of after-ripening (Fig. 2). Similar changes occurred in germination of excised embryos following after-ripening (Fig. 1D, F), and $Ox_b(50)$ decreased to only 0.013% after 9.5 months (Table 1). The total change in O_2 sensitivity was less for embryos, however, being about 10-fold lower after 9.5 months than at harvest, compared with 100-fold less for seeds (Fig. 2). The O_2-time constant (θ_{Ox}) values were not consistently altered by after-ripening or embryo excision, but the standard deviation in

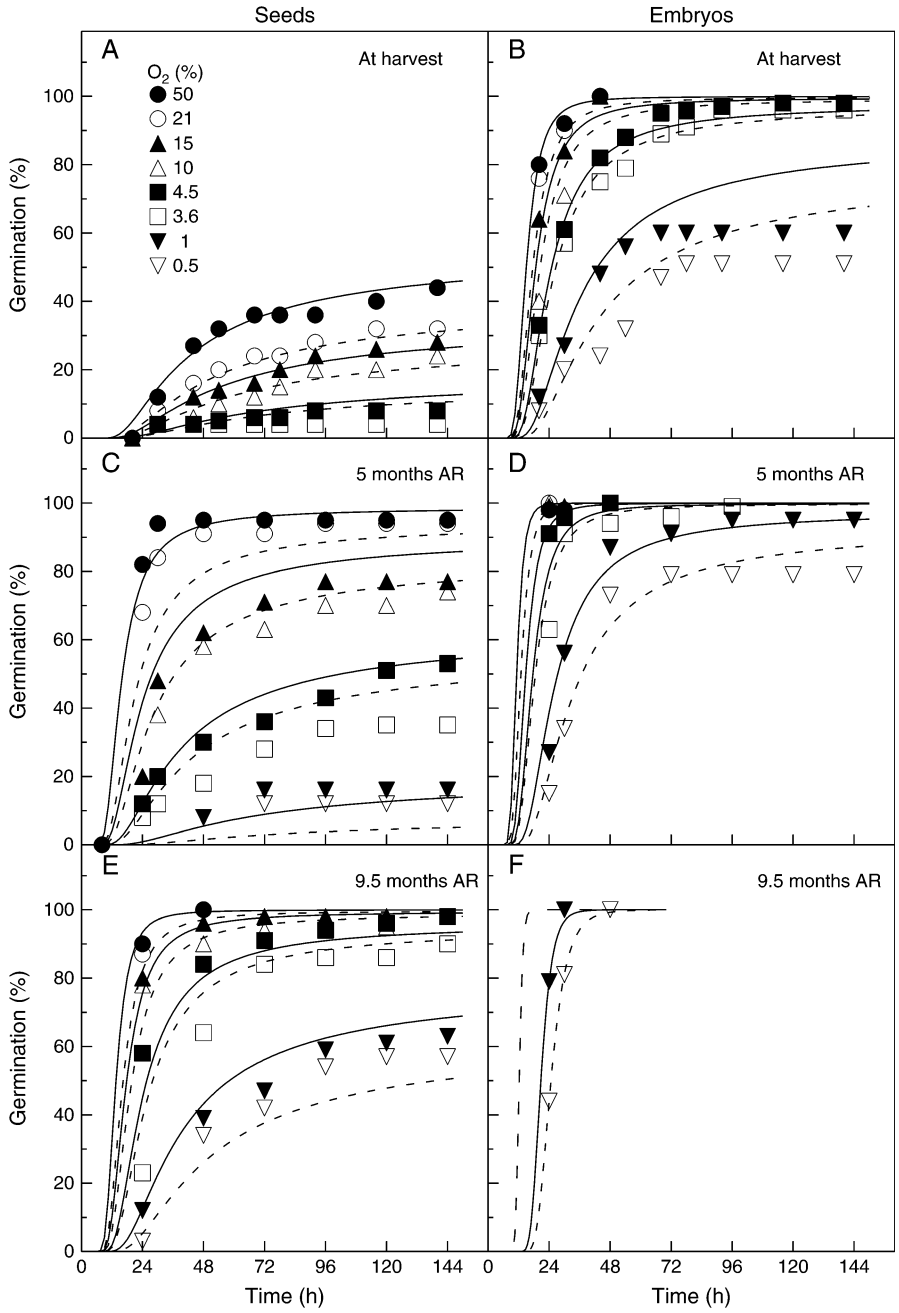

Fig. 1. Germination time courses of barley seeds (caryopses) (A, C, E) and embryos (B, D, F) at 20 °C in a range of O_2 percentages at harvest (A, B) and after 5 months (C, D) and 9.5 months (E, F) of after-ripening (AR). Symbols show the actual data at the indicated O_2 percentages, and continuous and dashed lines (corresponding to filled and open symbols) show the time courses predicted by the O_2 threshold model using the parameter values in Table 1. Within each panel, predicted time courses are generated by varying only the O_2 percentage in the model using the appropriate O_2 sensitivity parameters for that condition. Embryos after 9.5 months of after-ripening (F) germinated 99–100% in <24 h at O_2 percentages >1%. A dashed curve illustrates the predicted time course in 21% O_2. Seeds from the 2000 harvest were used.

Ox_b values (σ_{Oxb}) decreased during after-ripening in both seeds and embryos (Table 1). Thus, the major effect of after-ripening in improving germination of both seeds and embryos was the reduction in the minimum O_2 threshold (Fig. 2), with less variation in thresholds among seeds also contributing to the more uniform germination following after-ripening.

Sensitivity of germination to ABA and O_2

The separate and combined effects of O_2 and ABA on the germination of dormant barley seeds was tested at 20 °C and 30 °C. As barley seed dormancy is expressed at higher temperatures, few intact seeds germinated at 30 °C under any conditions. Thus, results were analysed for seeds and embryos at 20 °C and for embryos at 30 °C. In

Table 1. *Oxygen response parameters of germination of dormant barley seeds and embryos at 20 °C in relation to after-ripening*

Seeds or embryos	After-ripening period (months)	log $Ox_b(50)$ (log % O_2)	$Ox_b(50)$ (% O_2)	σ_{Oxb} (log % O_2)	θ_{Ox} (log % O_2 h)	R^2
Seeds	0	1.56	36.3	1.01	36	0.954
	5	0.457	2.87	0.546	20	0.884
	9.5	−0.517	0.304	0.641	30	0.944
Embryos	0	−0.908	0.123	0.752	39	0.948
	5	−1.170	0.068	0.501	32	0.962
	9.5	−1.88	0.013	0.315	39	0.999

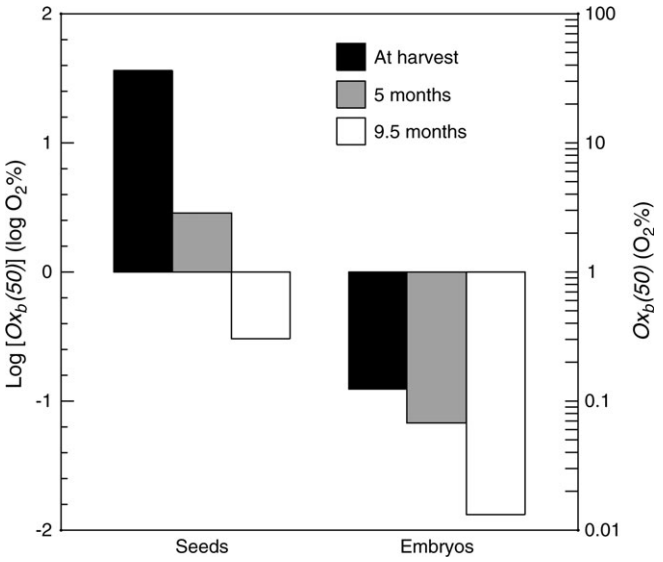

Fig. 2. Values of $Ox_b(50)$ for barley seeds and embryos at harvest and after 5 months and 9.5 months of after-ripening.

the absence of ABA, germination at 20 °C was somewhat greater in seeds harvested in 2002 as compared with those described above that were harvested in 2000, with intact seeds germinating about 70% in 21% O_2 and exhibiting an $Ox_b(50)$ value of 15.7% O_2 (Fig. 3A, B, Table 2). Dormant embryos germinated much better and had an $Ox_b(50)$ value of 4.80% (Fig. 3C–E, Table 2). High temperature reduced and delayed germination of embryos, primarily due to a 3-fold increase in the time constant, while the median O_2 threshold increased only slightly to 6.25% (Fig. 3F–H, Table 2). Thus, the major effect of embryo excision was to lower the O_2 threshold for germination, while supra-optimal temperature mainly delayed germination without markedly affecting O_2 sensitivity.

Increasing ABA concentrations consistently delayed and reduced germination of seeds and embryos (Fig. 3). At 20 °C, ABA concentrations (up to 10 μM) had relatively little effect on the O_2 response parameters of seeds (Table 2, Fig. 4). ABA had a greater impact on O_2 sensitivity of embryos, with essentially log-linear increases in log $Ox_b(50)$ with increasing ABA concentrations (Fig. 4). The slope of this increase was greater at 30 °C than at 20 °C. In addition, reduced O_2 percentages increased the sensitivity of germination to ABA. For seeds and embryos at both 20 °C and 30 °C, the inhibitory effect of a given ABA concentration on germination was much greater as the O_2 concentration decreased (Figs 3, 4). The median ABA threshold for seeds imbibing at 20 °C decreased by 0.44 log units for every 1% decrease in O_2; the relationship was less steep for embryos, being 0.15 log units/% O_2 at 20 °C and 0.17 log units/% O_2 at 30 °C (Fig. 4B). Effects of O_2 percentage on other model parameters were smaller and less consistent (Table 3), suggesting that the major effect of reduced O_2 on responses to ABA is to increase the median threshold ABA sensitivity.

The ABA sensitivity model provides additional indirect information about the effective internal concentrations of ABA in seeds and embryos. As the response to ABA is logarithmic, it is not possible to plot a predicted curve for 0 μM ABA using the model. For seeds and embryos imbibing at 20 °C, the time courses for 0 μM and 1 μM ABA are essentially identical, so only the predicted curve for 1 μM is shown. Since this concentration had no effect on germination, while 10 μM did have an effect, the action threshold for ABA (i.e. the combination of ABA uptake, content, and sensitivity) is equivalent to ~1 μM exogenous ABA. For embryos at 30 °C, however, 1 μM ABA resulted in a noticeable reduction in germination (Fig. 3F–H). Using the model, different values for ABA concentration were tested to identify the highest one that would match the time course with no exogenous ABA. The predicted time courses in Fig. 3F, G, and H for 0 ABA are plotted using values of –6.3, –6.3, and –6.8 for log ABA concentrations (0.50, 0.50, and 0.16 μM) at 21, 15, and 10% O_2, respectively, which match well to the actual data. Thus, the effective minimum exogenous ABA response threshold (i.e. the result of the combination of ABA uptake, content, and sensitivity) was 2- to 7-fold lower in embryos imbibing at 30 °C compared with embryos imbibing at 20 °C.

In general, the ABA threshold model using the parameter values for each O_2 percentage (Table 3) resulted in good agreement between the predicted and actual germination time courses (Fig. 3). However, there are some notable exceptions, particularly for embryos at

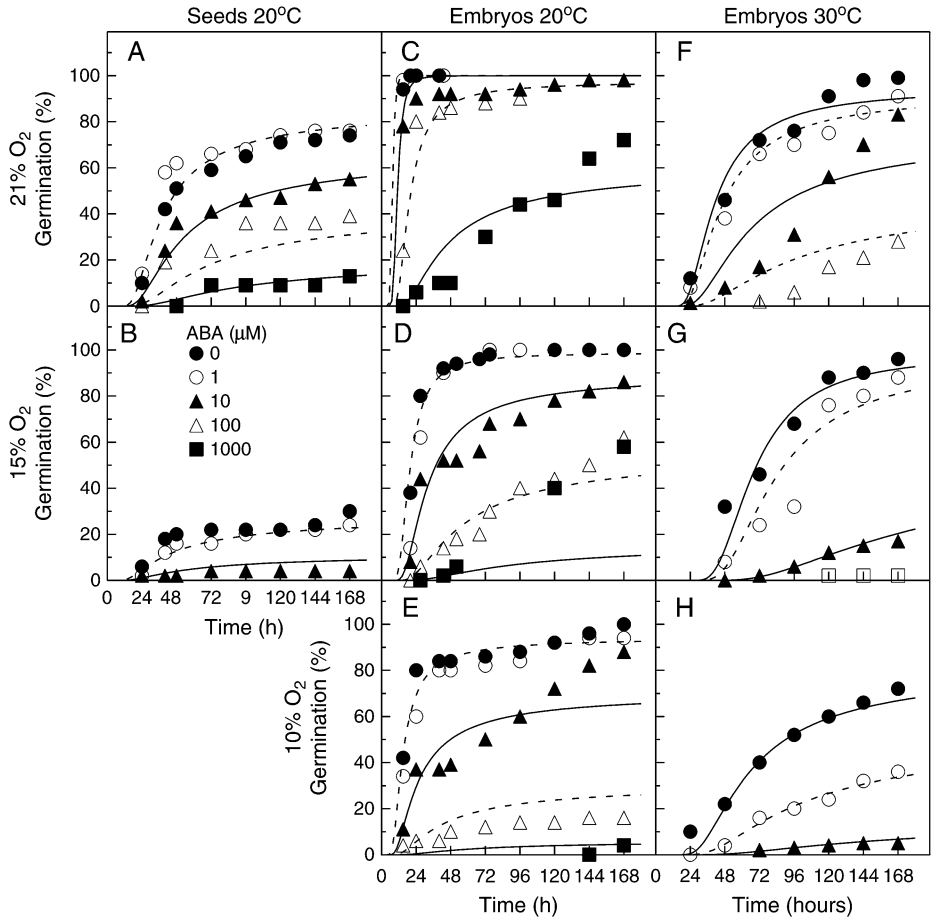

Fig. 3. Germination time courses of dormant barley seeds and embryos which imbibed at 20 °C or 30 °C in a range of ABA concentrations and O_2 percentages. The symbols represent the actual data, and the curves are predicted by the ABA threshold model for each ABA concentration (parameters in Table 3). For seeds and embryos which imbibed at 20 °C, predicted time courses are not shown for 0 μM ABA, as the data are essentially identical for 0 μM and 1 μM ABA. For embryos which imbibed at 30 °C, the 0 μM ABA curves are plotted using values of 0.50, 0.50, and 0.16 μM (log *ABA*=–6.3, –6.3, and –6.8) for 21, 15, and 10% O_2, respectively, since the logarithmic response precludes use of 0 μM in the equation. These values estimate the minimum median ABA response threshold at each O_2 concentration. Seeds from the 2002 harvest were used.

Table 2. *Oxygen response parameters of germination of dormant barley seeds and embryos at 20 °C and 30 °C in relation to ABA concentrations*

Seeds or embryos and temperature	ABA (μM)	log $Ox_b(50)$ (log % O_2)	$Ox_b(50)$ (% O_2)	σ_{Oxb} (log % O_2)	θ_{Ox} (log % O_2 h)	R^2
Seeds, 20 °C	0	1.20	15.7	0.161	6.4	0.938
	1	1.21	16.4	0.110	4.5	0.965
	10	1.27	18.7	0.097	5.5	0.962
Embryos, 20 °C	0	0.681	4.80	0.239	5.5	0.849
	1	0.794	6.22	0.191	3.9	0.911
	10	0.850	7.08	0.280	5.9	0.769
	100	1.08	12.0	0.224	5.8	0.726
	1000	1.16	14.5	0.224	15.4	0.823
Embryos, 30 °C	0	0.796	6.25	0.363	18.0	0.842
	1	0.960	9.11	0.274	17.8	0.876
	10	1.22	16.6	0.172	13.3	0.790

higher ABA concentrations (Fig. 3C–F). In these cases, the model predicted a sigmoidal or asymptotic pattern, while actual germination percentages increased essentially linearly. Therefore an attempt was made to model explicitly the interaction of reduced O_2 percentages combined with exogenous ABA applications using the approach developed by Ni and Bradford (1993) for modelling interactions of ABA and reduced water

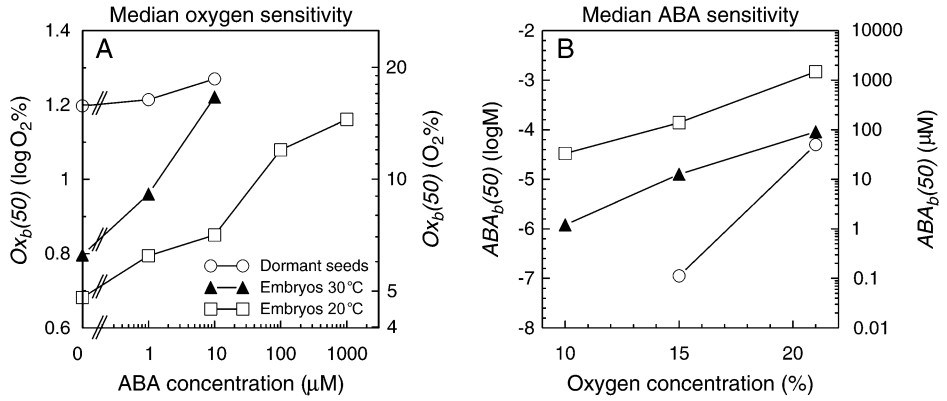

Fig. 4. Median threshold sensitivities to O_2 and ABA of germination of dormant barley seeds and embryos. Values of $Ox_b(50)$ as a function of ABA concentration (A) and of $ABA_b(50)$ as a function of O_2 percentage (B) are shown for seeds (grains) which imbibed at 20 °C and for excised embryos which imbibed at both 20 °C and 30 °C. No data are shown for ABA concentrations or O_2 percentages where germination was insufficient to allow modelling of the responses.

Table 3. *ABA response parameters of germination of dormant barley seeds and embryos at 20 °C and 30 °C in relation to oxygen concentrations*

Seeds or embryos and temperature	Oxygen (%)	log $ABA_b(50)$ (log M)	$ABA_b(50)$ (μM)	σ_{ABAb} (log M)	θ_{ABA} (log M h)	R^2
Seeds, 20 °C	21	−4.30	50	−1.59	−71	0.940
	15	−6.96	0.11	−1.63	−43	0.891
Embryos, 20 °C	21	−2.83	1470	−0.579	−24	0.905
	15	−3.86	137	−0.892	−43	0.943
	10	−4.48	33	−0.959	−25	0.921
Embryos, 30 °C	21	−4.04	91	−1.30	−100	0.866
	15	−4.90	13	−0.586	−98	0.942
	10	−5.92	1.2	−0.931	−79	0.987

potential in inhibiting germination (see Materials and methods for details). Since ABA had relatively less effect on O_2 response parameters (Table 2), the O_2 response parameters for 0 μM ABA were used and only O_2 concentration was changed in that component of the combined model. Since O_2 level markedly altered the ABA response parameters (Table 3), the parameters estimated for 15% O_2 were used in combination with the ABA concentration. The O_2 and ABA response functions were then combined as described in equation 8 to generate the predicted time courses for combinations of reduced O_2 and increasing ABA concentrations. Overall, this approach accounted fairly well for the combined effects of reduced O_2 and exogenous ABA, except at 100 μM ABA and 10% O_2, where the model overestimated actual germination (Fig. 5). However, if the ABA response parameters for 10% O_2 were used instead of those for 15% O_2, the match was improved somewhat at this ABA concentration (Fig. 5, dashed curve), but not at lower ones, where predictions underestimated actual germination (not shown). The combined model also did not predict the continuing increases in germination percentage that occurred at longer incubation times in a number of cases.

Sensitivity of germination to GA and O_2

Dormant cereal seeds can be stimulated to germinate by GA, and this effect is attenuated by reduced O_2 (Lecat *et al.*, 1992; Corbineau and Côme, 1996). Therefore the interaction of GA and O_2 effects on germination of dormant barley seeds were modelled. Consistent with the results presented above for after-ripening (Figs 1, 2), modelling the O_2 response in the absence of GA resulted in $Ox_b(50)$ values for non-dormant seeds that were about 100-fold lower than for dormant seeds (Fig. 6A, B, Table 4). High concentrations of GA caused $Ox_b(50)$ values to decrease by about 50% and θ_{Ox} values to increase somewhat, although not to the value for non-dormant seeds (Table 4). When the GA sensitivity of dormant seeds was modelled at different O_2 percentages, large effects on GA sensitivity were evident (Fig. 6C–E, Table 5). The median threshold for GA response increased by almost six orders of magnitude between 21% and 10.4% O_2. That is, in 21% O_2 the estimated $GA_b(50)$ for dormant seeds was 2.7 nM; in 15.5% and 10.4% O_2, this value increased to 12 μM and 1.7 mM, respectively (Table 5). This indicates that as O_2 percentages decreased, hypoxia increasingly became the primary limitation to

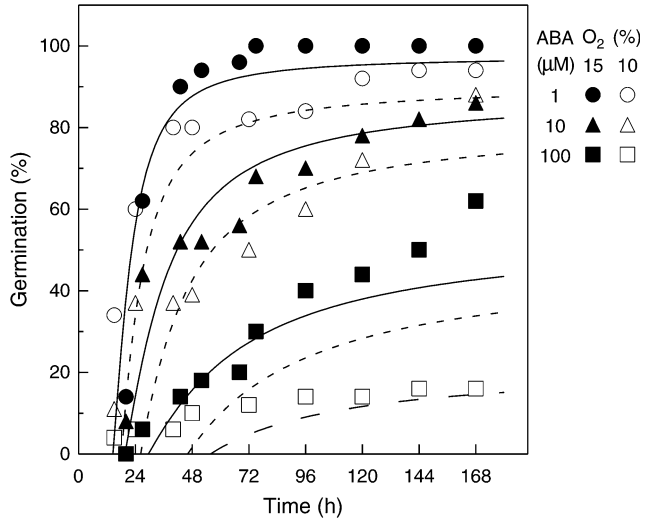

Fig. 5. Germination time courses illustrating the interaction of hypoxia and ABA on germination of dormant barley embryos at 20 °C. The symbols are the data for germination under the indicated combination of O_2 and ABA levels. The curves (continuous and dotted) are the time courses predicted from the threshold model by combining the responses to O_2 and to ABA in an additive manner (equation 8). This was done by assuming that ABA did not affect the response to O_2, but that hypoxia increased the sensitivity of germination to ABA according to the parameters for 15% O_2 (Table 3). For 100 μM ABA and 10% O_2, an additional predicted curve (dashed) is shown where the ABA response parameters for 10% O_2 were used instead.

germination, and even high concentrations of GA could not compensate for the O_2 requirement.

As for O_2–ABA interactions, interactions between O_2 and GA were also directly modelled. As was the case for ABA, GA concentration had less effect on O_2 sensitivity (Table 4) than O_2 percentage had on GA sensitivity (Table 5). Thus, the model parameters for O_2 sensitivity at 0 μM GA were used in the interaction model to account for the effect of reduced O_2, and parameters for GA sensitivity at different O_2 percentages were used to account for the effects of GA. However, this approach did not result in predicted germination time courses that matched well to the actual data (not shown). In particular, germination at 10% O_2 was underestimated at all GA concentrations. However, the interaction model can be applied in a reverse sense, i.e. to test different values for the GA sensitivity parameters that would result in better matches to the actual data. Modifying only the GA sensitivity parameters resulted in quite good matches to the experimental data for GA concentration effects at 10.4% and 15.5% O_2 (Fig. 7). In the predicted curves shown, σ_{GAb} was maintained constant at 2.5 log M, and only θ_{GA} and $GA_b(50)$ values were modified. θ_{GA} values were varied within the range found for the GA model (between 65 and 140 log M h; Table 5), while log $GA_b(50)$ values from –4 to –7 were required to obtain good fits to the data (Fig. 7). In the case of 1000 μM GA and 15.5% O_2, this still underestimated final germination percentages, but this could be accounted for by simultaneous modification of the O_2 sensitivity parameters (Fig. 7, dashed curve). These modelling exercises, while not rigorous tests, suggest that seeds have the physiological capacity to modify their sensitivity thresholds to O_2 and GA in response to the prevailing levels of these factors. It is somewhat arbitrary, therefore, to define specific response parameters under these conditions, but the model provides insight into the types of sensitivity changes that appear to be required to account for the observed germination patterns.

Discussion

The population-based threshold model has been applied to quantify seed germination responses to many environmental and physiological factors (Bradford, 1995; Finch-Savage *et al*., 2005; Finch-Savage and Leubner-Metzger, 2006; Allen *et al*., 2007). It is able to quantify the sensitivity of germination responses to those factors using only three parameters that address the overall speed of germination (factor-time constant), the median sensitivity to the factor (median base threshold), and the variation in sensitivity among seeds in the population (standard deviation of base thresholds). Diverse work with this approach has led to the recognition that the values of the parameters of this model are not constant, but rather change in response to physiological and environmental inputs. For example, changes in base water potential thresholds can account for inhibition of germination at supra-optimal temperatures (Alvarado and Bradford, 2002) or by ABA (Ni and Bradford, 1992; Alvarado and Bradford, 2005) and for promotion of germination due to dry after-ripening (Christensen *et al*., 1996; Alvarado and Bradford, 2005; Bair *et al*., 2006), moist chilling (Batlla and Benech-Arnold, 2004), fluctuating temperatures (Huarte and Benech-Arnold, 2005), and GA (Ni and Bradford, 1993; Fennimore and Foley, 1998). Application of the model allows quantification of the sensitivity of the seed population to factors that regulate germination and, in some cases, insight into the potential physiological mechanisms that regulate germination and dormancy (Finch-Savage and Leubner-Metzger, 2006; Allen *et al*., 2007). Dormancy and germination of barley seeds are known to be sensitive to temperature, after-ripening, O_2 availability, GA, and ABA (Lenoir *et al*., 1986; Lecat *et al*., 1992; Corbineau and Côme, 1996; Benech-Arnold *et al*., 2006). Thus, application of the population-based threshold model to analyse post-harvest dormancy of barley seeds may provide useful insights into how these diverse factors interact to regulate germination.

At harvest, intact barley grains exhibit dormancy and germinate poorly. Much of this restriction is attributable to the presence of the glumellae or hulls, as their removal or excision of the embryos greatly improves germination

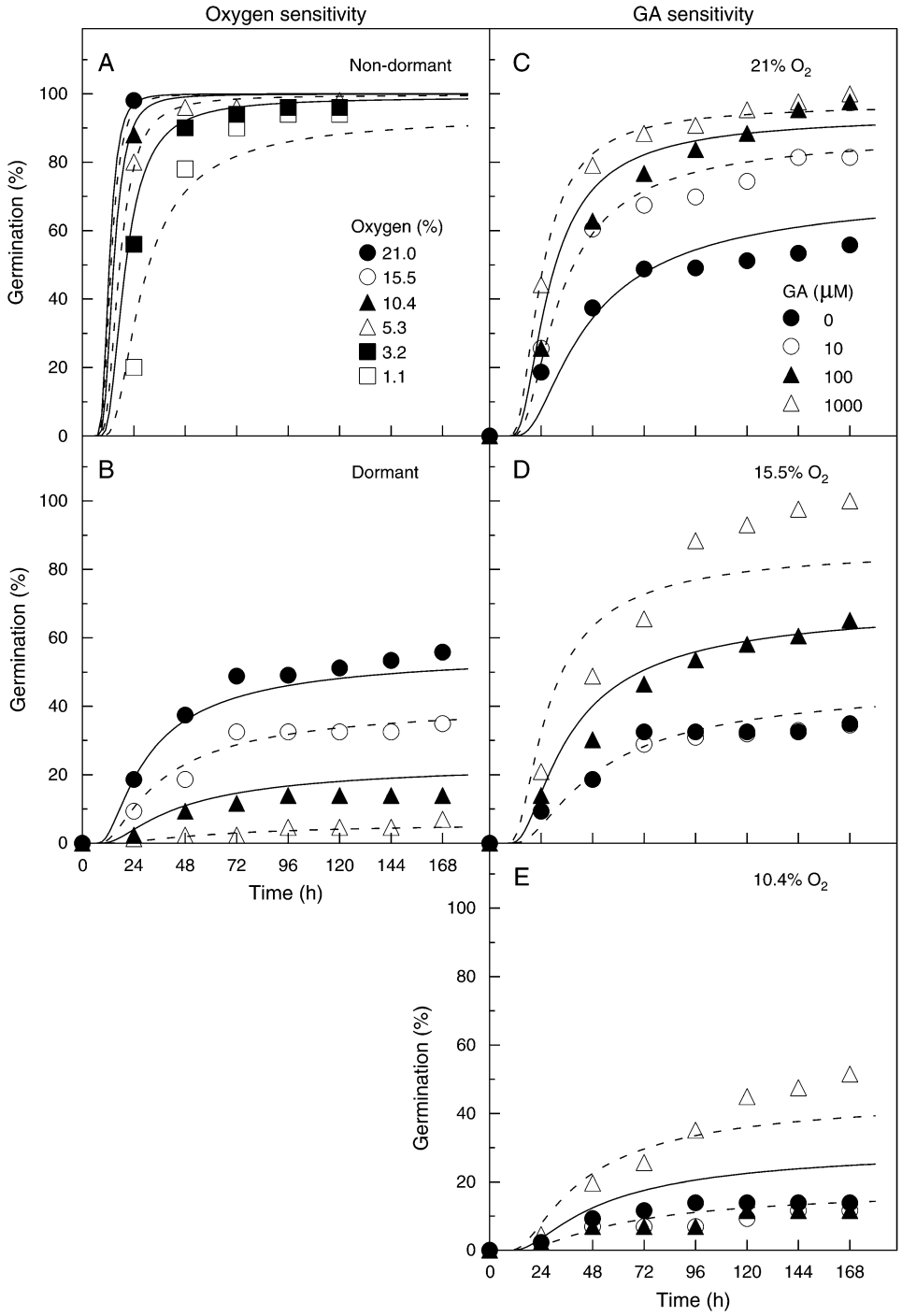

Fig. 6. Germination time courses of non-dormant and dormant barley seeds which imbibed at 20 °C in a range of GA concentrations and O_2 percentages. Time courses for non-dormant (A) and dormant (B) seeds which imbibed without added GA illustrate the large reduction in O_2 threshold and improvement in germination that accompany loss of dormancy. Addition of GA can partially substitute for after-ripening and promote germination of dormant seeds when the O_2 percentage is high (C), but is less effective as O_2 levels are reduced (D, E). The symbols represent the actual data, and the curves are predicted by the O_2 threshold model for each O_2 percentage for non-dormant and dormant seeds (panels A, B; parameters in Table 4 for 0 μM GA) or by the GA threshold model at each O_2 concentration (panels C–E; parameters in Table 5).

capacity. The restriction of germination by the hull has been attributed largely to its ability to reduce the availability of O_2 to the embryo (Lenoir *et al.*, 1986). This was supported here by using an O_2-time threshold model (Bradford *et al.*, 2007) to quantify the base O_2 thresholds for germination of intact dormant barley seeds and excised embryos. For intact highly dormant seeds, an $Ox_b(50)$ value of 36% O_2 (above ambient levels) was

Table 4. *Oxygen response parameters of barley seed germination at 20 °C in relation to GA concentrations*
Non-dormant seeds were assessed only in water.

Seed dormancy	GA (μM)	log $Ox_b(50)$ (log % O_2)	$Ox_b(50)$ (% O_2)	σ_{Oxb} (log % O_2)	θ_{Ox} (log % O_2 h)	R^2
Dormant	0	1.26	18.4	0.352	8.9	0.953
	10	1.18	15.1	0.256	7.7	0.866
	100	0.99	9.8	0.258	11.2	0.851
	1000	0.92	8.2	0.233	10.0	0.889
Non-dormant	0	−0.829	0.15	0.546	27	0.876

Table 5. *GA response parameters of dormant barley seed germination at 20 °C in relation to O_2 concentrations*

Oxygen (%)	log $GA_b(50)$ (log M)	$GA_b(50)$ (μM)	σ_{GAb} (log M)	θ_{GA} (log M h)	R^2
21.0	−8.57	0.00268	2.85	140	0.908
15.5	−4.93	11.8	1.70	65	0.882
10.4	−2.76	1720	2.51	80	0.880

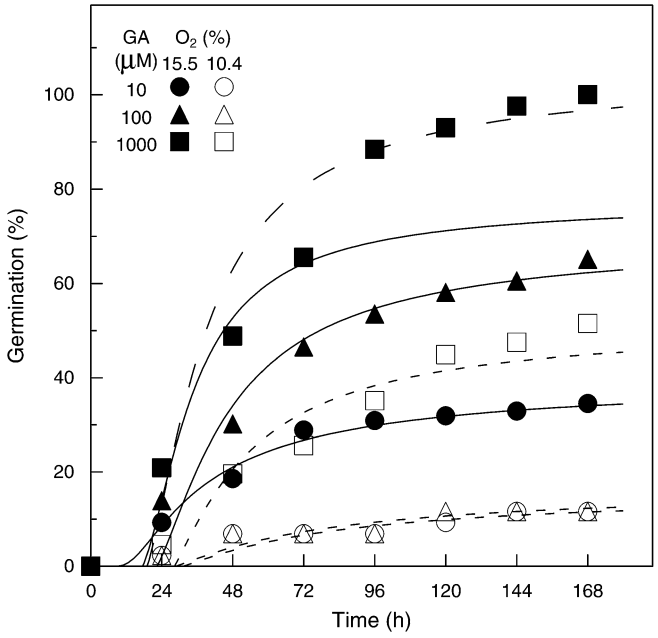

Fig. 7. Germination time courses illustrating the interaction of hypoxia and GA on germination of dormant barley seeds at 20 °C. The symbols are the data for germination under the indicated combinations of O_2 and GA levels. The curves are the time courses predicted from the threshold model by combining the responses to O_2 and to GA in an additive manner (equation 10). It was assumed that GA did not affect the response to O_2, so the parameters for O_2 response of dormant seeds in 0 μM GA (Table 4) were used with the appropriate O_2 percentage. However, using the values for the GA sensitivity predicted at each O_2 percentage (Table 5) did not result in good predictions of the experimental data (not shown). Thus, the values for the parameters in the GA component of the model were adjusted until better matches were obtained. A value of 2.5 log M was used for σ_{GAb} in all cases, and θ_{GA} (log M h) and $GA_b(50)$ (log M) values for the curves shown (continuous and dotted) for each combination were: 10 μM GA and 15% (65, –5.4) or 10% O_2 (65 and –5.0); 100 μM GA and 15% (140, –6.8) or 10% O_2 (65 and –4.0); 1000 μM GA and 15% (140, –7.0) or 10% O_2 (140 and –5.7). An additional curve (dashed line) is given for 1000 μM GA and 15% O_2 using the values above for GA sensitivity but changing the O_2 sensitivity parameters to θ_{Ox}=11 log % O_2 h, $Ox_b(50)$ = 1.06 log % O_2, and σ_{Oxb}=0.25 log % O_2.

estimated to be required to achieve 50% germination, and a maximum germination of only 67% would be predicted even in 100% O_2. The corresponding $Ox_b(50)$ value for excised embryos was only 0.12%, a reduction of 2.6 orders of magnitude (400-fold) (Fig. 2, Table 1). However, $Ox_b(50)$ for excised embryos decreased by almost another order of magnitude to 0.013% O_2 following 9.5 months of after-ripening, indicating that while physical O_2 limitation is a primary restraint on germination of dormant barley grains, physiological dormancy was also present in the embryos at harvest. In intact grains, after-ripening reduced $Ox_b(50)$ >100-fold to 0.3% O_2. The difference in log $Ox_b(50)$ between intact grains and excised embryos was only 1.36 log units following after-ripening for 9.5 months, compared with 2.64 log units at harvest (Fig. 2, Table 1). The resistance to O_2 penetration attributable to the hull therefore declined by over 10-fold during after-ripening, while the O_2 sensitivity threshold of the embryos also decreased by a similar amount. In the case of the hull, one can imagine that oxidation of phenolic compounds or other constituents could utilize O_2 and reduce its penetration to the embryo, and that as these materials are exhausted over time, the effectiveness of this barrier might decrease. In fact, Lenoir *et al.* (1986) demonstrated that after-ripening was associated with a 12-h delay in O_2 fixation by the glumellae, probably long enough to allow initiation of germination. Alternatively, structural changes leading to an increased porosity of the glumellae (Lenoir *et al.*, 1983) could increase the physical permeability of the tissues to the diffusion of O_2. The basis for the change in embryo sensitivity to O_2 is less clear, but could be related to the presence of respiratory inhibitors or other factors that might contribute to the quiescence of the embryo during maturation. These might be oxidized or chemically degraded during after-ripening (Oracz *et al.*, 2007), allowing respiration to be more efficient at low O_2 levels following imbibition.

Dormancy might also delay or prevent the repair or activation of mitochondria following imbibition.

Abscisic acid is a major regulator of seed dormancy, both during seed development and for the maintenance of dormancy following imbibition (Finch-Savage and Leubner-Metzger, 2006; Feurtado and Kermode, 2007). Benech-Arnold *et al.* (2006) found that there was a strong interaction between ABA and O_2 in regulating germination of dormant barley embryos. Reduced O_2 resulted in maintenance of higher ABA contents following imbibition, and also increased the sensitivity of germination to applied ABA. In that study, only the final germination percentages were compared under different conditions of ABA concentration and O_2 percentage. While increases in ABA sensitivity due to hypoxia were evident, it was not possible to quantify them clearly. Here, the complete germination time courses have been utilized for those same experiments (and additional data not reported there) and the population-based threshold model applied to quantify the interactions of ABA and O_2 in controlling germination of dormant barley seeds and embryos. For intact dormant seeds, $Ox_b(50)$ values were high (16–19% O_2) and were relatively insensitive to the presence of ABA (Fig. 4A). Limitation of O_2 availability to the embryo is apparently the primary regulator of germination, and ABA, while further inhibiting germination, had little additional effect on O_2 sensitivity *per se*. Dormant embryos, however, were much less sensitive to O_2 limitation, with $Ox_b(50)$ values of 5–9% in water or low ABA concentrations, which then approximately doubled at higher ABA concentrations (Fig. 4A). High ABA concentrations and high temperature could essentially replace the effect of the hull on O_2 sensitivity; the combination of 10 μM ABA and 30 °C imbibition resulted in a median O_2 threshold equal to that for intact seeds imbibing at 20 °C in water (Table 3). At 20 °C, an ABA concentration >1 mM would be required to completely replace the effect of the hull. Part of the difference in the effect of exogenous ABA on O_2 sensitivity is likely to be due to the 2-fold greater ABA content of barley embryos imbibed at 30 as compared with 20 °C (Benech-Arnold *et al.*, 2006). With a higher endogenous ABA content at 30 °C, it would be expected that exogenous ABA would have a greater effect. However, the doubling of ABA content can account for only a small fraction of the change in dose response to ABA, unless ABA is compartmentalized in cells and bulk ABA content does not reflect effective endogenous concentrations at its sites of action.

A more significant effect may be due to changes in ABA sensitivity at reduced O_2 percentages. As noted by Benech-Arnold *et al.* (2006), ABA sensitivity increased by several orders of magnitude as O_2 percentages decreased. For example, reducing O_2 from 21% to 10% increased the sensitivity of germination to ABA by almost 500-fold for seeds or 45- to 75-fold for embryos (Fig. 4). The importance of this change in ABA sensitivity was supported by direct modelling of the interaction of reduced O_2 and increased ABA on germination of embryos at 20 °C. In the combined model, the parameters for O_2 sensitivity were left unchanged (i.e. only the values determined for germination in 0 μM ABA were used), but the ABA response parameters had to be adjusted to account for the effect of hypoxia on ABA sensitivity in order to give even an approximate match to the experimental data (Fig. 5). Better matches to the data could be obtained by modifying both the ABA and O_2 response functions (results not shown; see, for example, Fig. 7), suggesting that the assumption of independent additive effects, even taking into account the changes in ABA sensitivity, is too simple to completely account for their interaction. Based on prior results indicating that seeds can shift their sensitivities to water potential, hormones, or O_2 after extended incubation under inhibitory conditions (Ni and Bradford, 1992; Dahal and Bradford, 1994; Bradford *et al.*, 2007), it is expected that this is occurring here as well. It is likely to require a continuous adjustment of response sensitivity parameters over time to fully match the actual germination responses when such changes in sensitivity are occurring.

While ABA is a critical hormone for inducing and maintaining dormancy, most seeds also require GA action to complete germination (Finch-Savage and Leubner-Metzger, 2006; Feurtado and Kermode, 2007; Hilhorst, 2007). Thus, GA biosynthesis and sensitivity may also be involved in the germination patterns observed here. Germination of dormant barley seeds could be promoted by GA_3, but relatively high concentrations were required, particularly as O_2 percentage decreased (Fig. 6). As for ABA, there were changes in O_2 sensitivity with increasing GA concentrations, but the changes were relatively small compared with those seen with after-ripening, for example. On the other hand, GA sensitivity of intact dormant seeds was remarkably responsive to O_2 availability, varying by almost six orders of magnitude between 21% and 10% O_2 (Table 5). At low O_2 levels, seeds became essentially unresponsive to GA. As for ABA–O_2 interactions, accounting for the effects of hypoxia on GA sensitivity was essential in modelling the interactive effects of GA and O_2 on germination of dormant barley seeds (Fig. 7).

It is worth noting that a wide range of sensitivity thresholds is present among seeds in the population for O_2, ABA, and GA, as reflected in σ_{Oxb}, σ_{ABAb}, and σ_{GAb} values of 0.1–0.3, 0.6–1.6, and 1.7–2.8 log units, respectively. As two standard deviations on each side of the mean encompass 96% (from 2% to 98%) of the population, these large σ values indicate the wide variance in dormancy/germination states present among seeds even of this relatively uniform crop species. This variation tended to decrease during after-ripening and is

an important factor contributing to the changes in germination rates and percentages that occur during release from dormancy or in response to other factors. Given these wide variances and shifting means of overlapping sensitivity distributions to multiple factors, it is easy to conceive how the complex patterns of germination exhibited under various combinations of factors can be generated. Simple additive models must adjust sensitivity distributions in response to multiple factors in order to account for the observed germination patterns (e.g. Figs 5, 7). Feedback loops among response regulators are present and need to be considered for a comprehensive description of dormancy induction, maintenance, and release (Feurtado and Kermode, 2007).

Together, these results suggest a scenario for the potential interactions among O_2, ABA, and GA amounts and sensitivities in barley seed dormancy and after-ripening. At seed maturity and shedding there is some embryo dormancy present, but the major factor maintaining dormancy is the limitation of O_2 penetration to the embryo by covering tissues. This directly prevents germination and also maintains higher ABA content and particularly greater ABA sensitivity, which together further restrict germination potential, likely via maintenance of a high water potential threshold for germination by reducing the growth potential of the embryo (Nonogaki *et al*., 2007). In addition, in *Arabidopsis* seeds it has been shown that ABA down-regulates genes in the GA biosynthetic pathway (Seo *et al*., 2006), so higher ABA content and sensitivity may also decrease GA synthesis. Low O_2 availability in the intact seed would also tend to reduce the sensitivity to any GA that is present. During after-ripening, the permeability of the hull to O_2 increases, reducing the restraint on germination by hypoxia and increasing sensitivity to GA, while the sensitivity to ABA decreases (Benech-Arnold *et al*., 2006), both of these contributing to increasing embryo growth potential (Nonogaki *et al*., 2007). In fully after-ripened seeds, even though there is still a considerable barrier to O_2 diffusion through the hull, the shift in O_2 thresholds to very low levels and the consequent reduction in ABA sensitivity and increase in GA sensitivity would combine to allow germination of intact seeds. Thus, O_2 availability to the embryo, regulated by the hull, would have additional effects on the synthesis of, and sensitivity to, ABA and GA, altering their balance of potential action to either promote or retard germination (Finch-Savage and Leubner-Metzger, 2006). The repressive effects of high temperature can also be accounted for by this scenario, as ABA contents (Gonai *et al*., 2004; Benech-Arnold *et al*., 2006) and water potential thresholds (Alvarado and Bradford, 2002) are higher in seeds which imbibe at supra-optimal temperatures.

As our knowledge of the molecular events underlying dormancy and germination phenomena expand (Cadman *et al*., 2006; Bradford and Nonogaki, 2007; Finch-Savage *et al*., 2007), modelling approaches such as the one applied here to quantify phenotypic responses and to account for the population nature of seed biology will be important tools for integrating across the levels of biological organization to link changes in gene expression and protein action to germination and emergence in agricultural and natural environments.

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