Response of intact seeds of wild rice (Oryza) species to dry heat treatment and dormancy-breaking chemicals

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Abstract

Seed dormancy is an important factor that limits effective and efficient conservation, evaluation, distribution and utilisation of wild Oryza species seeds. In this study, 15 accessions from the International Rice Genebank (IRG) representing different wild Oryza species were grown in a screen-house on the Zeigler Experiment Station at IRRI in the Philippines, harvested manually and dried at 15% RH and 15°C. Seeds were subjected to dry heat treatment and to treatments with: 0.001 M KNO₃, 0.0029 M gibberellic acid (GA₃), 1 M H₂O₂ and 0.1 M HNO₃. Initial assessment confirmed high levels of dormancy among the different wild Oryza species tested, with only three species having > 50% germination. The dormancy of intact wild Oryza species seeds was alleviated by dry heat treatment at 50°C for 14 days and sowing directly in KNO₃ or by pre-soaking heat-treated seeds in GA₃ or H₂O₂ for 18 hours before soaking in distilled H₂O for a further 18 hours prior to sowing. It is recommended for species with compact and thick seed coverings, that dry heat-treated seeds should be pre-soaked in HNO₃ for 18 hours before soaking in GA₃ or H₂O₂ for 18 hours prior to sowing for germination.

Keywords: dormancy-breaking chemicals, dry heat treatment, seed dormancy, wild Oryza species

Introduction

Rice is one of the world’s most important food crops. With more than 50% of the world’s population dependent on rice, there is an undisputed importance to conserve rice germplasm. The International Rice Genebank (IRG) of the T.T. Chang Genetic Resources Center (GRC), at the International Rice Research Institute (IRRI) in the Philippines, currently conserves more than 127,000 accessions of rice from around the world. This collection includes two cultivated species, Oryza sativa L. and O. glaberrima Steud., and
25 wild *Oryza* species (GRiSP, 2013). All accessions are stored following the international guidelines for genebank storage (FAO/IPGRI, 1994; FAO, 2014) and most are held *in trust* and distributed under the terms of the International Treaty on Plant Genetic Resources for Food and Agriculture.

The conservation of wild *Oryza* species is an important endeavour as it is an integral part of rice genetic resource management programmes. Wild *Oryza* species are a potential source of useful genes for tolerance to various stresses (Waheed et al., 2012). However, seed dormancy is still a major challenge in the effective utilisation and evaluation of wild *Oryza* species. Naturally, dormancy is an advantageous state for plant species since it allows the seeds to survive until favourable conditions for plant growth are available (Harlan, 1992). On the other hand, excessive seed dormancy is a problem for breeding, research and conservation. Wild *Oryza* species are known to have stronger seed dormancy than cultivated rice, but the level of dormancy varies between species (Naredo et al., 1998).

From previous studies, it is clear that seeds of different wild *Oryza* species respond differently to various dormancy-breaking treatments (Waheed et al., 2012). Common seed dormancy-breaking methods include germination at alternating temperatures, dry heat treatment (> 40°C), palea and lemma (hull) removal, and the use of dormancy-breaking chemicals. The most common method used for wild *Oryza* seeds, including at GRC, is removal of the seed hull (Naredo et al., 1998). However, hull removal is a tedious process and risks damaging the seeds which may reduce germination. Therefore, there is an urgent need to explore efficient and effective seed dormancy-breaking treatments or combinations of treatments that can promote germination in wild *Oryza* species seeds without the need to remove the hull. Some dormancy-breaking chemicals can increase the germination of seeds after partial after-ripening (Cohn et al., 1983). Dormancy-breaking treatments should be able to address not only the seed coat-imposed dormancy, but also the possible presence of embryo-imposed dormancy (Bewley and Black, 1994; Adkins et al., 2002). In this study the response of intact seeds (lemma, palea and glumes retained) of different wild *Oryza* species to dry heat treatment and dormancy-breaking chemicals were examined.

**Materials and methods**

*Seed materials and dormancy-breaking chemicals*

Fifteen accessions from the International Rice Genebank (IRG) representing 15 wild *Oryza* species (table 1) were grown in the GRC screen-house at the Zeigler Experiment Station in 2016. Seeds were collected manually by shaking individual panicles in mesh bags. Seeds were then dried and stored in a drying room (15% RH, 15°C) until sufficient seeds were attained. Dry intact seeds were subjected to dry heat treatment and to dormancy-breaking chemicals as follows: (a) 0.001 M KNO$_3$, (b) 0.0029 M gibberellic acid (GA$_3$), (c) 1 M H$_2$O$_2$ and (d) 0.1 M HNO$_3$ (Sigma-Aldrich Co. LLC.).
Viability test and assessment of dormancy
The viability of all accessions used was determined by a tetrazolium test (ISTA, 2015) prior to the dormancy-breaking treatments. For each accession, 10 dehulled seeds were pre-conditioned by immersing in distilled water at 20°C for 24 hours. Seeds were cut longitudinally through the embryo and ¾ of the endosperm, and soaked in 1% aqueous 2,3,5-triphenyl tetrazolium solution in 60 mm-diameter × 15 mm-deep Petri dishes. Petri dishes were wrapped with aluminum foil to exclude light and placed at 30°C for two hours. Seeds were washed several times with distilled water after incubation to remove excess solution. Seeds were considered viable when at least 33% of the embryo tissue had stained.

Initial germination was determined by sowing two replicates of 25 intact seeds (awns cut off for species with long awns) in 94 mm-diameter × 16 mm-deep Petri dishes lined with two layers of Whatman no. 1 filter papers (90 mm-diameter) moistened with 7.5 ml distilled water. Seeds were placed over water for 24 hours prior to sowing for germination testing. Seeds were allowed to germinate at 30°C with 12 hours illumination per day for 28 days. Seeds with at least 5 mm shoots and roots were considered as germinated. Scoring of germination was done at 2, 3, 5, 7, 14, 21 and 28 DAS.

Dry heat treatment and sowing with KNO₃
Two groups of samples with 100 seeds each were prepared for each accession. The first group was stored at 50°C for 14 days while the second group was temporarily stored in the drying room (15% RH, 15°C). After 14 days, seed samples were taken out from storage and placed over water for 24 hours in desiccation boxes at room temperature (21°C). Seeds were sown (as four replicates of 25 seeds each) in 94 mm-diameter × 16 mm-deep Petri dishes lined with two layers of Whatman no.1 filter papers moistened with 7.5 ml distilled water or 0.001 M KNO₃. Seeds were placed to germinate at 40/30°C with 12 hours illumination during the warm phase, for 28 days. Scoring for germination was conducted at 4, 8, 12, 16, 20, 24 and 28 DAS.

Pre-soaking with dormancy-breaking chemicals
A separate batch of 16 samples with 100 seeds each for each accession was placed at 50°C for 14 days. Each sample was then subjected to one of 16 2-stage pre-soaking treatments. For the first pre-soaking treatment, the seeds for four of the samples were soaked in each of either 0.2 M HNO₃, 1 M H₂O₂, 0.0029 M GA₃ or distilled water for 18 hours. One of the four samples from each initial soaking treatment was then transferred to each of either 0.2 M HNO₃, 1 M H₂O₂, 0.0029 M GA₃ or distilled water for a further 18 hours. Seeds were rinsed with distilled water three times and blot-dried in paper towels on the laboratory bench between pre-soaking treatments and before sowing for germination. Seeds were sown for germination testing at 40/30°C, as before.

Data analysis
All analyses were carried out using GenStat for Windows, Version 17 (VSN International Ltd., Hemel Hempstead, UK). Generalised Linear Models (GLM) with a logit link function was fitted to the data, taking into account the binomial error distribution of seed
germination, and used to compare the germination response to the different dormancy-breaking treatments. Additionally, a Wald test statistic was used to determine the significance of the main and interaction effects among the different dormancy-breaking treatments and to identify which terms could be dropped from the full model which included all the terms (i.e. main effects and interactions).

Results

Seed germination and viability
The tetrazolium tests showed that the viability of the seed lots ranged between 80 and 100% (table 1). The seeds of most of the species showed 100% viability while five of the remaining species showed 90% viability and only the O. latifolia showed 80% viability. Seed germination on the other hand differed among the different species tested (table 1). The highest germination was observed for O. grandiglumis seeds with 78% germination, followed by O. glumaepatula and O. minuta seeds with 64 and 54%, respectively. Most of the species showed less than 50% germination (4 - 48%) while seeds of three species (O. barthii, O. malampuzhaensis and O. rhizomatis) showed no germination.

Table 1. Initial germination (without any dormancy-breaking treatment) and viability of the Oryza species seed lots used for the dormancy-breaking experiments.

| Species                | IRGC accession number | Global Information System Link | Germination (%) | Viability (%) |
|------------------------|-----------------------|--------------------------------|-----------------|--------------|
| O. alta Swallen        | 105143                | https://doi.org/10.18730/4K46= | 46              | 90           |
| O. australiensis Domin | 86533                 | https://doi.org/10.18730/460T9 | 30              | 90           |
| O. barthii A. Chev     | 105507                | https://doi.org/10.18730/4KC1V | 0               | 100          |
| O. eichingeri Peter    | 81803                 | https://doi.org/10.18730/423Y9 | 28              | 90           |
| O. glumaepatula Steud. | 88806                 | https://doi.org/10.18730/48775 | 64              | 100          |
| O. grandiglumis (Döll) Prodoehl | 105156 | https://doi.org/10.18730/4K4H9 | 78              | 100          |
| O. latifolia Desv.     | 100885                | https://doi.org/10.18730/4GZHN | 48              | 80           |
| O. malampuzhaensis Krish. et Chand. | 100957 | https://doi.org/10.18730/4H1JC | 0               | 100          |
| O. meridionalis N.Q. Ng | 104086               | https://doi.org/10.18730/4JBX= | 4               | 100          |
| O. minuta J. Presl. & C. Presl. | 82048 | https://doi.org/10.18730/42BK* | 54              | 90           |
| O. nivara Sharma et Shastry | 89166 | https://doi.org/10.18730/48JF* | 18              | 100          |
| O. officinalis Wall     | 106382                | https://doi.org/10.18730/4M3D= | 14              | 100          |
| O. punctata Kotschy ex. Steud. | 101429 | https://doi.org/10.18730/4H840 | 24              | 90           |
| O. rhizomatis D.A. Vaughan | 105660              | https://doi.org/10.18730/4KDQ7 | 0               | 100          |
| O. rufipogon Griff.     | 93116                 | https://doi.org/10.18730/4BHBS | 40              | 100          |

1 International Rice Genebank Collection.
2 Based on the tetrazolium test conducted for the initial assessment of seed viability.
Dry heat treatment and sowing with KNO₃
Seed germination varied among species and in response to the different treatments (figure 1). In the control treatment (no heat treatment and sown in H₂O), the highest germination was recorded for *O. grandiglumis* (98%), while no germination was observed for *O. rhizomatis*. The Wald tests showed that among the species tested, only *O. eichingeri* and *O. alta* showed significant interaction effects between the dry heat and KNO₃ treatments (*P* = 0.024 and 0.003 for *O. eichingeri* and *O. alta*, respectively). In *O. echingeri*, both dry-heat treatment (*P* < 0.001) and sowing in KNO₃ (*P* < 0.001) showed significant effects. Dry heat treatment increased germination in seed lots sown in distilled water and in KNO₃ from 35 to 74% and from 85 to 90%, respectively. In contrast, sowing in KNO₃ increased germination in the non-heat-treated and heat-treated seeds from 35 to 85% and from 74 to 90%, respectively. In the case of *O. alta*, significant main effects were only observed with KNO₃ (*P* < 0.001) increasing germination in the non-heat-treated and heat-treated seed lots from 58 to 86% and from 67 to 69%, respectively. All the remaining species only showed significance in one of the main effects. Thus for these species, the interaction effects was dropped from the model.

Using the simplified model, five species showed significant main effects with both dry heat treatment and KNO₃ including *O. glumaepatula* (*P* = 0.006 for both), *O. malampuzhaensis* (*P* < 0.001 and 0.009, respectively), *O. punctata* (*P* = 0.006 and 0.002, respectively), *O. meridionalis* (*P* < 0.001 for both) and *O. officinalis* (*P* < 0.001 for both). Dry heat treatment increased the germination of seeds sown in distilled water and KNO₃ from 77 to 86% and 86 to 96%, respectively, in *O. glumaepatula*; from 8 to 19% and 12 to 35%, respectively, in *O. malampuzhaensis*; from 35 to 44% and 46 to 64%, respectively, in *O. punctata*; from 10 to 37% and 77 to 95%, respectively, in *O. meridionalis*; and from 20 to 40% and 33 to 76%, respectively, in *O. officinalis*. In contrast, treatment with KNO₃ increased germination in both the non-heat-treated and heat-treated seeds from 77 to 86% and 86 to 96%, respectively, in *O. glumaepatula*; from 8 to 12% and 19 to 35%, respectively, in *O. malampuzhaensis*; from 35 to 46% and 44 to 64%, respectively, in *O. punctata*; 10 to 77% and 37 to 95%, respectively, in *O. meridionalis*; and 20 to 33% and 40 to 76%, respectively, in *O. officinalis*.

Only the dry heat treatment resulted in significant main effects on the germination of *O. minuta* (*P* < 0.001), *O. australiensis* (*P* < 0.001), *O. nivara* (*P* < 0.001) and *O. rufipogon* (*P* < 0.001) seeds. Dry heat treatment increased the germination of seeds sown in distilled water and KNO₃ from 71-72 to 99% in *O. minuta*; from 34-36 to 52-56% in *O. australiensis*; from 25-29 to 67-71% in *O. nivara*; and from 68-76 to 87-92% in *O. rufipogon*. In contrast, only the KNO₃ treatment was had a significant effect in *O. latifolia* (*P* < 0.001) which increased the germination in the non-heat-treated and heat-treated seed lots from 67 to 78 and 88%, respectively.

There were no significant effects of either dry heat treatment or sowing with KNO₃ on the germination of *O. grandiglumis* seeds (*P* = 0.25 and 1.0, respectively) where there was 96-98% germination in all treatments, and in *O. barthii* (*P* = 0.703 and 0.269, respectively) and *O. rhizomatis* (*P* = 1.0 and 1.0, respectively) where germination was between 0 and 3% across treatments.
Figure 1. Germination of intact wild Oryza species seeds with and without dry heat treatment (50°C for 14 days) and sowing in H2O or KNO3.
Pre-soaking with dormancy-breaking chemicals

**O. alta**
For the first soaking treatment, significant main effects were only observed with pre-soaking in HNO$_3$ ($P = 0.01$). Germination increased from 72 (control) to 81-87% when soaked successively in H$_2$O, GA$_3$, or HNO$_3$ (figure 2A). Additionally, significant interaction effects were also observed when seeds were soaked again in HNO$_3$ where germination increased to 75%. On the other hand, significant main effects were observed with soaking in H$_2$O$_2$ ($P = 0.027$) or HNO$_3$ ($P = 0.002$) for the second soaking period. In the case of H$_2$O$_2$, germination increased to 85-93% when initially soaked in H$_2$O, GA$_3$, H$_2$O$_2$ or HNO$_3$. Soaking seeds in HNO$_3$ following H$_2$O and H$_2$O$_2$ increased germination to 90-91%. Significant interaction effects were also observed with seeds initially soaked in GA$_3$ or HNO$_3$, which increased germination to 75% for both treatments.

**O. australiensis**
Pre-soaking seeds in H$_2$O$_2$ for the first soaking period showed significant main effects ($P = 0.014$) increasing germination from 54 (control) to 69-74% when seeds were subsequently soaked in H$_2$O or GA$_3$ (figure 2B). In contrast, germination decreased to 52 and 37% when seeds were soaked in H$_2$O$_2$ or HNO$_3$, respectively, after the initial soaking in H$_2$O$_2$. Significant main effects were also found with pre-soaking in HNO$_3$ ($P < 0.001$) for the first soaking period. While germination increased to 83 and 65% with soaking in H$_2$O or GA$_3$, respectively, following pre-soaking in HNO$_3$, germination decreased to 48 and 12% when seeds were soaked in H$_2$O$_2$ or HNO$_3$, respectively. On the other hand, significant main effects with soaking seeds in HNO$_3$ ($P < 0.001$) during the second soaking decreased germination after soaking in H$_2$O, GA$_3$, H$_2$O$_2$ or HNO$_3$. For these respective treatments, germination decreased to 28, 29, 37 and 12%, respectively. Furthermore, significant interaction effects were also observed with HNO$_3$ and GA$_3$ treatments ($P = 0.003$), HNO$_3$ and H$_2$O$_2$ ($P = 0.004$) and HNO$_3$ ($P < 0.001$) alone for the consecutive soaking periods.

**O. barthii**
Low germination (< 20%) was observed in general across treatments while no germination was observed in the control treatment. There were no significant main and interaction effects observed overall across treatments. However, when the seeds were pre-soaked in HNO$_3$ followed by soaking in H$_2$O$_2$, germination increased from 0 (control) to 83% (figure 2C).

**O. eichingeri**
Significant main effects were observed with pre-soaking seeds in GA$_3$ ($P = 0.023$) or HNO$_3$ ($P = 0.04$) for the first soaking period. In the case of GA$_3$, germination increased from 82 (control) to 83-93% when seeds were pre-soaked in GA$_3$ prior to soaking in distilled H$_2$O, GA$_3$, H$_2$O$_2$ or HNO$_3$; pre-soaking in HNO$_3$ prior to H$_2$O, GA$_3$ or H$_2$O$_2$ increased germination to 91-93% (figure 2D). Significant interaction effects were also observed between GA$_3$ and HNO$_3$ ($P = 0.034$), and in GA$_3$ ($P = 0.025$) or HNO$_3$ ($P = 0.005$) alone for the consecutive soaking periods.
Figure 2. Germination of intact dry heat-treated seeds of *O. alta* (A), *O. australiensis* (B), *O. barthii* (C) and *O. eichingeri* (D) following pre-soaking in dormancy-breaking chemicals.
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**O. glumaepatula**
Overall, significant effects were found only with pre-soaking seeds in HNO₃ ($P < 0.001$) during the second soaking period. Pre-soaking in H₂O, GA₃, H₂O₂ and HNO₃ prior to soaking in HNO₃ decreased germination from 89 (control) to 62, 55, 57 and 44%, respectively (figure 3A).

**O. grandiglumis**
Significant main effects were observed in pre-soaking the seeds in HNO₃ ($P < 0.001$) for the second soaking period. Germination decreased from 93 (control) to 32, 34, 37 and 16% when soaked initially in H₂O, GA₃, H₂O₂ or HNO₃, respectively (figure 3B). Additionally, significant interaction effects were also observed in pre-soaking seeds successively in GA₃ ($P = 0.009$) for the two soaking periods which increased germination to 95%.

**O. latifolia**
Pre-soaking in GA₃ ($P = 0.025$) or HNO₃ ($P = 0.015$) prior to soaking in other chemicals showed significant main effects on seed germination. In the case of GA₃, germination increased from 88 (control) to 95-97% when seeds were successively soaked in H₂O or GA₃ (figure 3C). While no significant effect on germination was observed with successive soaking with H₂O₂, germination was decreased to 75% when successively soaked in HNO₃, where significant interaction effects were also detected ($P = 0.031$). In the case of HNO₃, germination increased to 90-99% with successive soaking in H₂O, GA₃ or H₂O₂. Significant interaction effects were found when seeds were soaked in HNO₃ for both the first and second soaking ($P < 0.001$). However, for this treatment, germination decreased to 15%. Furthermore, significant main effects were found with soaking seeds in GA₃ ($P = 0.047$), H₂O₂ ($P = 0.02$) and HNO₃ ($P = 0.044$) for the second soaking period. In the case of GA₃, germination increased to 95-99% when seeds were pre-soaked in H₂O, GA₃, H₂O₂ or HNO₃. For H₂O₂, while no change was observed with initial soaking in GA₃, germination decreased to 75% when seeds were first soaked in H₂O. In contrast, germination was increased to 90-92% when seeds were initially soaked in H₂O₂ or HNO₃. In the case of HNO₃, germination decreased to 77% when seeds were soaked initially in H₂O. Significant interaction effects were also observed when seeds were initially soaked in GA₃ ($P = 0.031$), H₂O₂ ($P < 0.001$) or HNO₃ ($P < 0.001$) where germination decreased to 75, 36 and 15%, respectively.

**O. malampuzhaensis**
There were significant main effects of pre-soaking in GA₃ ($P < 0.001$), H₂O₂ ($P = 0.046$) and HNO₃ ($P < 0.001$). In the case of GA₃, germination increased from 9 (control) to 39, 40 and 82% when the seeds were placed in H₂O, H₂O₂ or HNO₃ for the second soaking period, respectively (figure 3D). Significant main and interaction effects were observed when seeds were soaked again in GA₃ ($P = 0.001$) for the second soaking period where germination increased to 38% relative to the control. Initial soaking with H₂O₂ increased germination to 19, 13 and 71% when successively soaked in H₂O, H₂O₂ or HNO₃, respectively. Significant main and interaction effects were detected when seeds were successively soaked in GA₃ ($P = 0.047$) where germination increased to 28%. In
Figure 3. Germination of intact dry heat-treated seeds of *O. glumaepatula* (A), *O. grandiglumis* (B), *O. latifolia* (C) and *O. malampuzhaensis* (D) following pre-soaking in dormancy-breaking chemicals.
the case of HNO$_3$, germination increased to 66, 86 and 84% when soaked successively in H$_2$O, GA$_3$ or HNO$_3$, respectively. Significant main and interaction effects were observed when seeds were again placed in HNO$_3$ (P < 0.001) for the second soaking period where germination increased to 70%. Significant main effects were also observed with soaking seeds in GA$_3$ (P < 0.001) or HNO$_3$ (P < 0.001) after pre-soaking with other chemicals. In the case of GA$_3$, germination increased to 32, 38, 28 and 86% when initially soaked in H$_2$O, GA$_3$, H$_2$O$_2$ or HNO$_3$, respectively. In the case of HNO$_3$, germination increased to 65, 82, 71 and 70% when seeds were initially soaked in H$_2$O, GA$_3$, H$_2$O$_2$ or HNO$_3$, respectively.

*O. meridionalis*
Pre-soaking in GA$_3$ (P < 0.001) or H$_2$O$_2$ (P < 0.001) prior to soaking in the other chemical treatments showed significant main effects on seed germination. In the case of GA$_3$, successive soaking in H$_2$O, GA$_3$, H$_2$O$_2$ or HNO$_3$ increased germination from 11 (control) to 39, 40, 40 and 66%, respectively (figure 4A). Pre-soaking in H$_2$O$_2$ increased germination to 42, 61, and 38% in seeds soaked in H$_2$O, GA$_3$, or H$_2$O$_2$, respectively. Additionally, significant interaction effects were also observed when seeds were soaked in HNO$_3$ again for the final soaking which increased germination to 94%. In the case of HNO$_3$, seed germination increased to 75, 96 and 84% when soaked in H$_2$O, GA$_3$, or H$_2$O$_2$, respectively. Significant interaction effects were also observed when seeds were soaked again in HNO$_3$ for the final soaking which increased germination to 54%. On the other hand, significant main effects were also detected in soaking seeds in HNO$_3$ (P < 0.001) for the second soaking. Germination increased to 35, 66, 94 and 54% when seeds were initially soaked in H$_2$O, GA$_3$, H$_2$O$_2$ or HNO$_3$, respectively.

*O. minuta*
Significant main effects were observed with soaking seeds in H$_2$O$_2$ (P = 0.047) or HNO$_3$ (P = 0.021) for the second soaking period. No significant interaction effects were detected among the other soaking treatments. While there were no effects when seeds were pre-soaked in GA$_3$ before soaking in H$_2$O$_2$, pre-soaking in H$_2$O$_2$ and HNO$_3$ prior to H$_2$O$_2$ decreased germination from 98 (control) to 91, 86 and 75%, respectively (figure 4B). Additionally, pre-soaking seeds in H$_2$O, GA$_3$, H$_2$O$_2$ and HNO$_3$, prior to soaking in HNO$_3$ decreased germination to 89, 79, 85 and 76%, respectively.

*O. nivara*
Significant main effects were observed in treatments involving HNO$_3$, either before (P = 0.005) or after (P = 0.015) pre-soaking with other chemicals. In the case of initially soaking in HNO$_3$, germination increased from 77 (control) to 92-99% when soaked in H$_2$O, GA$_3$ and H$_2$O$_2$ (figure 4C). In contrast, soaking in HNO$_3$ after pre-soaking in H$_2$O, GA$_3$ and H$_2$O$_2$ decreased germination to 61, 54 and 57%, respectively. Additionally, there were significant interaction effects with consecutive soaking in HNO$_3$ (P < 0.001) where germination decreased to 32%.
Figure 4. Germination of intact dry heat-treated seeds of *O. meridionalis* (A), *O. minuta* (B), *O. nivara* (C) and *O. officinalis* (D) following pre-soaking in dormancy-breaking chemicals.
O. officinalis
Significant main effects were observed with pre-soaking seeds in GA\(_3\) \((P = 0.008)\), H\(_2\)O\(_2\) \((P = 0.036)\) and HNO\(_3\) \((P < 0.001)\) prior to soaking in other chemical treatments. In the case of GA\(_3\), germination increased from 26 (control) to 43-46% when soaked in H\(_2\)O, GA\(_3\) or H\(_2\)O\(_2\) (figure 4D). Significant interaction effects were also observed with soaking in HNO\(_3\) \((P < 0.001)\) which increased germination to 73%. Pre-soaking in H\(_2\)O increased germination to 40 and 32% when seeds were successively soaked in H\(_2\)O or H\(_2\)O\(_2\), respectively. Significant interaction effects were also observed with soaking in GA\(_3\) \((P < 0.001)\) where germination decreased to 24%. On the other hand germination increased to 79% with successive soaking in HNO\(_3\) \((P = 0.029)\). In the case of HNO\(_3\), germination increased to 82-88% when seeds were successively soaked in H\(_2\)O or H\(_2\)O\(_2\).

O. punctata
There were significant main effects of pre-soaking seeds in HNO\(_3\) \((P < 0.001)\) which increased germination from 52 (control) to 78 and 89% when soaked successively in H\(_2\)O or H\(_2\)O\(_2\), respectively (figure 5A). There were also significant interaction effects when seeds were soaked in GA\(_3\) \((P < 0.001)\) or HNO\(_3\) \((P < 0.001)\) following pre-soaking in HNO\(_3\) where germination decreased to 50 and 34%, respectively. Additionally, significant main effects were also observed with soaking in H\(_2\)O\(_2\) either before \((P = 0.022)\) or after \((P < 0.001)\) soaking with other chemical treatments. For seeds pre-soaked in H\(_2\)O\(_2\), germination increased to 68-75% when soaked in H\(_2\)O, GA\(_3\) or HNO\(_3\). There were also significant interaction effects when seeds were in H\(_2\)O\(_2\) \((P = 0.032)\) for the second soaking which increased germination to 70%. On the other hand, soaking in H\(_2\)O following pre-soaking in H\(_2\)O or HNO\(_3\) increased germination to 75 and 89%. Significant interaction effects were also observed when seeds were pre-soaked in GA\(_3\) \((P = 0.005)\) prior to final soaking in H\(_2\)O\(_2\) where germination increased to 61%.

O. rhizomatis
Significant main effects were observed with pre-soaking either for the first or second soaking period in GA\(_3\) \((P = 0.004\) and 0.016, respectively), H\(_2\)O\(_2\) \((P = 0.34\) and 0.001, respectively) or HNO\(_3\) \((P < 0.001)\). While no germination was observed with the control treatment, germination of seeds initially soaked in GA\(_3\) increased to 2% when soaked successively in H\(_2\)O or H\(_2\)O\(_2\), and to 14% when soaked successively in HNO\(_3\) (figure 5B). In the case of pre-soaking in H\(_2\)O\(_2\), germination increased to 2 and 7% when seeds were successively soaked in H\(_2\)O and HNO\(_3\), respectively. Pre-soaking with HNO\(_3\) increased germination to 4, 17, 21 and 10% when seeds were successively soaked in H\(_2\)O, GA\(_3\), H\(_2\)O\(_2\) and HNO\(_3\), respectively.
**O. rufipogon**

There were significant main effects of pre-soaking in H$_2$O$_2$ ($P = 0.044$) prior to soaking in H$_2$O, HNO$_3$, GA$_3$ or H$_2$O$_2$. Germination decreased from 94 (control) to 85-87% with successive soaking in H$_2$O or HNO$_3$ (figure 5C). In contrast, an increase to 95-97% was observed with successive soaking in GA$_3$ or H$_2$O$_2$. Additionally, there were significant interaction effects with successive soaking in H$_2$O$_2$ and GA$_3$ ($P = 0.017$; 97% germination) and HNO$_3$ for the two soaking periods ($P < 0.001$; 50% germination).

![Figure 5](image_url)

**Figure 5.** Germination of intact dry heat-treated seeds of *O. punctata* (A), *O. rhizomatis* (B) and *O. rufipogon* (C) following pre-soaking in dormancy-breaking chemicals.
Discussion

In a seed genebank, where regular germination testing is performed to monitor seed viability, seed dormancy may result in misleading viability data which may lead to wrong management decisions. Furthermore, accurate measurement of seed viability is important since the genebank needs to ensure distribution of viable seeds. Additionally, poor germination during multiplication or regeneration will exhaust seed stocks.

Dormancy levels among the wild *Oryza* species tested varied, as expected (Naredo *et al*., 1998; Waheed *et al*., 2012). It is also worth noting that the initial germination results (table 1) were generally lower than the results from the control treatment of the first experiment (dry heat treatment and sowing in KNO$_3$; figure 1) where the seeds were treated in the same way as in the initial germination tests. This difference could be due to some dormancy loss that might have occurred during the 14-day storage period while waiting for seeds to come out of the dry heat treatment.

In general, the wild *Oryza* species included in this study responded differently to dry heat treatment and sowing or pre-soaking in dormancy-breaking chemicals. Dry heat treatment showed a positive effect on germination overall, although the level of the response varied. Similarly, Veasey *et al*. (2004) observed different patterns of dormancy loss with after-ripening among cultivated and wild rice species. However, heat treatment was not effective in removing dormancy in some of the wild *Oryza* species included in this study, especially *O. punctata*, *O. latifolia* and *O. grandiglumis* where germination of dry heat-treated seeds did not vary from the control, and with *O. barthii* and *O. rhizomatis* where no germination was observed at all.

In general, sowing seeds in KNO$_3$ had positive effects on germination (figure 1). Soaking in KNO$_3$ was also found to be very effective in removing dormancy in cultivated rice (Seshu and Dadlani, 1991). Significant improvements in germination of non-heat-treated dormant seeds by sowing in KNO$_3$ were observed in eight of the wild *Oryza* species, notably in *O. meridionalis* where the largest increase in germination was observed relative to the control (from 10 to 77%). Bellairs *et al*. (2015) similarly reported that HNO$_3$ could overcome some of the dormancy in *O. meridionalis* seeds, although not in *O. rufipogon*. While significant effects by combining dry heat and KNO$_3$ treatments were only observed in two species (*O. eichingeri* and *O. alta*), maximum germination was achieved by sowing heat-treated seeds in KNO$_3$ in all the species except *O. alta* where germination decreased and *O. rhizomatis* where no germination was observed. Heat treatment (25, 35 or 60°C) for 28 or 60 days also overcame dormancy in *O. meridionalis* and *O. rufipogon* (Bellairs *et al*., 2015).

Dormancy in rice seeds is attributed to factors in the palea and lemma and/or seed coat (Gu *et al*., 2005; Bellairs *et al*., 2015). While the effect of heat treatment was positive overall, germination of some species was still low (<50%) and most species did not reach full germination. Dormancy persisted in seeds of *O. australiensis*, *O. barthii*, *O. malampuzhaensis*, *O. meridionalis*, *O. officinalis*, *O. punctata* and *O. rhizomatis* after subjecting to dry heat treatment and pre-soaking in distilled H$_2$O. However, germination improved with pre-soaking in chemicals or combinations of chemicals. For these species, highest germination was achieved mostly with soaking in HNO$_3$ either before or after
soaking in H₂O, GA₃ or H₂O₂. Chemical scarification by soaking in acids was also effective in alleviating seed dormancy in sorghum (Shanmugavalli et al., 2007), Zaleya petandra (L.) C. Jeffrey (Munawar et al., 2015) and Ornithopus pinnatus (Mill.) Druce (Lopes et al., 2015). The action of H₂O₂ is similar to that of HNO₃ such that it also disintegrates the seed covering and makes it more permeable. Additionally, H₂O₂ may also help in promoting germination by oxidising inhibitors that may be present in the seed coat (Ogawa and Iwabuchi, 2001). On the other hand, the combination of soaking in HNO₃ followed by soaking in GA₃ may have promoted germination by weakening the seed coverings due to acid scarification. Significant interaction effects suggest that the timing of soaking of seeds in a particular dormancy-breaking chemical needs to be considered especially in the case of HNO₃ where majority of the species showed decrease in germination when soaked in other chemicals first before in HNO₃ or in HNO₃ throughout the 2-stage soaking.

Results in the current study suggest that the dormancy of intact wild Oryza species seeds can be safely alleviated by dry heat treatment at 50°C and sowing directly in KNO₃ or by pre-soaking heat-treated seeds in GA₃ or H₂O₂ for 18 hours before soaking in distilled H₂O for a further 18 hours prior to sowing. On the other hand, for species with a compact and thick seed covering, it is recommended that the heat-treated seeds should be pre-soaked in HNO₃ for the first 18 hours before soaking in GA₃ or H₂O₂ for 18 hours prior to sowing for germination. Otherwise, further testing is required to determine the response of intact seeds to varying chemical concentrations and soaking durations, especially in species where the current treatments were ineffective, e.g. O. rhizomatis, as well as the response of other accessions within each species to dry heat treatment and different dormancy-breaking chemicals.

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