

# Reduced metabolic rate indicates declining viability in seed collections: an experimental proof-of-concept

Emma L. Dalziell<sup>1,2,3,\*</sup> and Sean Tomlinson<sup>1,2</sup>

<sup>1</sup>Department of Environment and Agriculture, Curtin University, Kent Street, Bentley, Western Australia 6102, Australia

<sup>2</sup>Kings Park & Botanic Garden, 2 Kattidj Close, Kings Park, Western Australia 6005, Australia

<sup>3</sup>School of Biological Sciences, University of Western Australia, 35 Stirling Highway, Crawley, Western Australia 6009, Australia

\*Corresponding author: Botanic Gardens and Parks Authority, Biodiversity Conservation Centre—Science Directorate, 2 Kattidj Close, Kings Park, 6005 Western Australia, Australia. Tel: +61 84803648. Email: emma.dalziell@dbca.wa.gov.au

There is increasing investment globally in seed storage facilities for a wide array of purposes, from food security to biodiversity conservation. Best practice when storing seeds in this manner is to periodically test collections for viability, such that declining viability can be used as a trigger for management actions. Typically, viability testing is time consuming and/or destructive, involving germination testing, cut-testing or a range of potential biochemical indicators. Given that respiration (i.e. metabolic activity) is the basic chemical reaction common to all forms of life, measuring metabolic rate should provide a less-destructive, simple and repeatable correlate of seed viability. We compared the viability of seed collections of known proportions of alive and dead seeds to their metabolic rates, calculated as CO<sub>2</sub> production ( $\dot{V}CO_2$ ) measured using flow-through respirometry. To maximize the activity of the seeds and our likelihood of measuring metabolic rates, we imbibed the seeds from 12 species of angiosperms, and measured them using an open system respirometer. Measuring metabolic rate in seeds from diverse evolutionary and ecological backgrounds required us to adopt an allometric approach to account for the effects of seed size upon metabolic rate. After doing so, however, we found significant linear relationships between the known viability of our seed collections and their metabolic rates, but these relationships were unique for each species measured. These data provide substantial support to the prospect that measuring metabolic rates can be used to estimate viability of seeds in storage, however, we advocate the development and adoption of more sensitive respirometry equipment, specifically engineered for this purpose in order to achieve truly non-destructive measurements.

**Key words:** Allometry, *ex situ* storage, genebank, orthodox storage behaviour, seed conservation, viability monitoring

**Editor:** Kevin Hultine

Received 19 July 2017; Revised 14 September 2017; Editorial Decision 14 September 2017; accepted 22 September 2017

**Cite as:** Dalziell EL, Tomlinson S (2017) Reduced metabolic rate indicates declining viability in seed collections: an experimental proof-of-concept. *Conserv Physiol* 5(1): cox058; doi:10.1093/conphys/cox058.

## Introduction

The ultimate goal of any seed genebank is that seeds be viable, germinable and usable when withdrawn from storage (Hay and Whitehouse, 2017). Seedbanks are recognized for

their value in food security (FAO, 2014), and are increasingly utilized as part of the conservation toolkit to protect threatened flora (Crawford *et al.*, 2007; Walters, 2015) and for use in degraded land restoration (Merritt and Dixon, 2011; Erickson *et al.*, 2016a). While several decades of

research have optimized the conditions under which many species of orthodox seeds can be stored in order to improve longevity (i.e. suitable reductions in temperature and humidity), seeds do not survive indefinitely, and all seed collections in a genebank will eventually age and decline in viability (Ellis and Roberts, 1980). Therefore, the implementation of effective monitoring is essential in order to detect changes in viability prior to precipitous decline (Walters, 2015), enabling genebank managers to make decisions regarding the utility of a particular collection, and whether re-collection of the species is required (Hay and Whitehouse, 2017).

Germination tests are the most commonly used method of determining whether or not seeds within a collection are germinable (FAO, 2014). Germination testing requires *a priori* knowledge of whether seeds are dormant, how dormancy may be overcome, and the optimal conditions (i.e. temperature, light and chemical stimulant) required to promote germination (Hay and Whitehouse, 2017). For many agriculturally important crop species, this information is available via the International Seed Testing Association (ISTA, 2017), along with a series of standard protocols. For non-crop species, particularly wild species, this information may be more limited and often requires significant investment, both in terms of time and the number of seeds required to fully understand dormancy and optimal germination conditions prior to a collection's successful lodgement in a genebank (Gosling, 2003). For example, some non-dormant seeds germinate quickly and germination testing may be completed in a matter of days, however, seeds that have complex dormancy mechanisms may require lengthy pre-treatment (e.g. stratification or after ripening), that take several weeks or months to complete (Gosling, 2003; Baskin and Baskin, 2014). Other methods of determining viability such as cut testing (ISTA, 2017), measuring the conductivity of electrolytes in leachates from seeds (Powell, 1986) or staining with chemicals such as tetrazolium chloride (ISTA, 2017) or fluorescein diacetate (e.g. Pritchard, 1985) can be time consuming, especially for species where optimized methodologies and protocols are not available. Furthermore, these techniques are completely destructive, and seeds are not useable after testing. In large stores of domesticated cultivars this may not be prohibitive, but conservation collections are often small, resulting from opportunistic collection that cannot be easily replicated. The implicit value of these unique collections means that their periodic destructive monitoring represents a very genuine cost and impact upon the efficacy of a conservation seedbank. Therefore, establishing non-destructive methods of determining seed viability, both sustainably and accurately from small collections containing dormant seeds, is imperative to the successful *ex situ* storage of all species.

There have been recent investigations into novel non-destructive methods and technologies to test seed viability and deterioration (Fu *et al.*, 2015). The basis of all these techniques, however, is the measurement of various products

of respiration by seeds (Kranter *et al.*, 2010; Mira *et al.*, 2010, 2016; Colville *et al.*, 2012), and assumes that the metabolic activity of seeds changes with increasing age and decreasing viability and quality. It also assumes that dormant seeds remain metabolically active (*sensu* Guppy and Withers, 1999), although to our knowledge, this remains largely unquantified. The measurement of metabolic rates has a long heritage (Lighton, 2008) and is most typically measured using respirometry (Frappell, 2006; Lighton, 2008) to record oxygen consumption ( $\dot{V}O_2$ ), carbon dioxide production ( $\dot{V}CO_2$ ) or both. Traditionally, the lower detection limit of most respirometers has obviated their use in measuring the metabolic rates of plant seeds, but technological advances have recently seen the publication of several studies linking whole seed or seed-lot respiration with seed quality (Zhao and Zhong, 2012; Bradford *et al.*, 2013; Patanè and Avola, 2013; Xin *et al.*, 2013; Bello and Bradford, 2016). Most notably, Patanè and Avola (2013) adapted the gas analyser of a LI-COR portable photosynthesis system to measure instantaneous  $\dot{V}CO_2$  in seeds of corn (*Zea mays*), cotton (*Gossypium hirsutum*) and sorghum (*Sorghum bicolor*).

Our primary aim was to (i) determine whether flow-through respirometry can be used to measure  $\dot{V}CO_2$  in seeds from a range of orthodox, wild and crop species and infer the viability of seeds within a seed lot. Given that mass is a pervasive influence on metabolic rates, however, and allometric effects are evident on the metabolic rates of seeds (Garwood and Lighton, 1990), we also took this opportunity to (ii) compare our data to the only previously published allometry between mass and metabolic rate of plant seeds in order to further explore the appropriate scaling relationship for angiosperm seeds.

## Materials and methods

### Species selection, seed collection and quality assessment

We investigated the relationship between the metabolic rates and viability of 12 species representing 10 families from a range of ecosystems and evolutionary contexts, and with a broad diversity of seed traits (Table 1). Commercially available lots of tomato (*Solanum lycopersicum*, Mr Fothergill's Heirloom Garden, Amish Paste), corn (*Zea mays* Mr Fothergill's Sweet Corn, terrific F1), onion (*Allium cepa* Mr Fothergill's Hunter River Brown) and climbing beans (*Phaseolus vulgaris* Mr Fothergill's climbing bean, Vitalis) were purchased in hermetically sealed, laminated foil bags in Perth, Western Australia in 2014, and were used as representatives of commonly available crop species. Drupes of blackberry (*Rubus anglocandicans*) were collected in 2013 from wild populations near Pemberton, Western Australia, where it is considered an invasive weed. Blackberry seeds were cleaned from the fleshy drupe, then washed and air-dried (*ca.* 25°C) prior to transport. Seeds of *Acacia ancistrocarpa*,

**Table 1:** Characteristics of the 12 species used in this study.

Family (aceae)	Species	Preferred climate or habitat at collection location	Mono/dicotyledenous	Life form	Dormancy	Dormancy alleviated	Embryo type	1000 seed dry weight (g)
Domesticated								
Amaryllid-	<i>Allium cepa</i> L.	Temperate	Mono	Bulbous perennial	ND	N/A	L (st)	4.02
Fab-	<i>Phaseolus vulgaris</i> L.	Temperate	Di	Herbaceous annual	ND	N/A	Be	172.67
Solan-	<i>Solanum lycopersicum</i> Mill.	Temperate	Di	Vine/shrub	ND	N/A	L (cu)	2.38
Po-	<i>Zea mays</i> L.	Temperate	Mono	Grass	ND	N/A	Lat	136.80
Introduced								
Ros-	<i>Rubus anglocandicans</i> A.Newton	Temperate south-west of Western Australia	Di	Scrambling shrub	PD	No	S	3.43
Wild								
Fab-	<i>Acacia ancistrocarpa</i> Maiden & Blakely	Semi-arid/arid Pilbara region of Western Australia	Di	Shrub or tree	PY	Yes (HW)	In	29.02
Sapind-	<i>Atalaya hemiglauca</i> (F. Muell.) Benth.	Semi-arid/arid Pilbara region of Western Australia	Di	Tree	ND	N/A	F	69.99
Prote-	<i>Banksia attenuata</i> R. Br.	Mediterranean Swan Coastal Plain region of Western Australia	Di	Tree	ND	N/A	In	87.72
Myrt-	<i>Eucalyptus gamophylla</i> F. Muell	Semi-arid/arid Pilbara region of Western Australia	Di	Tree	ND	N/A	F	1.81
Prote-	<i>Grevillea wickhamii</i> Meisn.	Semi-arid/arid Pilbara region of Western Australia	Di	Shrub or tree	ND	N/A	In	24.17
Nymph-	<i>Nymphaea lukei</i> S.W.L. Jacobs & Hellq.	Wetlands in the wet-dry tropics (savannah) of northern Western Australia	Basal	Herbaceous macrophyte	PD	No	Br	8.98
Surian-	<i>Stylobasium spathulatum</i> Desf.	Semi-arid/arid Pilbara region of Western Australia	Di	Shrub	PY	Yes (HW)	L (cu)	277.39

Dormancy: ND = non-dormant, PD = physiological dormancy, PY = physical dormancy as per Baskin and Baskin (2004). Physical dormancy was alleviated in seeds of *Acacia ancistrocarpa* and *Stylobasium spathulatum* by immersing seeds in hot water (95°C) for 2 min (HW). Embryo type: Be = bent, Br = broad, In = investing, F = folded, L (cu) = linear (curved), L (st) = linear (straight), Lat = lateral, S = spathulate as per Martin (1946)

*Eucalyptus gamophylla*, *Grevillea wickhamii* and *Stylobasium spathulatum* were collected from wild populations in the Pilbara region of Western Australia in 2012. Seeds of *Atalaya hemiglauca* were purchased from a commercial supplier (Arid Land Seeds) in 2009 and were collected from wild populations near Newman, Western Australia. Seeds of *Banksia attenuata* were purchased from a commercial supplier (Tranen Revegetation Systems) in 2012

and were collected from wild populations in the Swan Coastal Plain region of Western Australia. Seeds of the waterlily *Nymphaea lukei* were collected from wild populations in the Kimberley region of Western Australia in June 2013. All seeds collected from the wild were collected at the point of natural dehiscence and were fully mature. All seeds, including those purchased from commercial suppliers, were stored in open laminated foil bags in a Controlled

Environment facility (CE: 15% RH, 15°C) at Kings Park and Botanic Garden, Western Australia prior to experimental work. For convenience of handling, and to reduce the likelihood of microbial contamination during respirometry and germination trials, seeds of *A. hemiglauca* and *S. spathulatum* were removed from their covering structures. In order to ensure 100% seed fill prior to respirometry trials, all seeds used were x-rayed (Faxitron Specimen Radiography System MX-20 Cabinet, AZ, USA), and seeds showing absence of or damage to the embryo and endosperm were removed. In order to determine seed dry weight, three replicate samples of between 5 and 50 seeds (depending on seed size) were dried in an oven at 65°C for 4 days before being weighed (Mettler-Toledo PB405-S Greifensee, Switzerland).

### Dormancy break and imbibition

Four species used in this study were dormant at the time of collection (Table 1). Seeds of *A. ancistrocarpa* and *S. spathulatum* possessed physical (PY) dormancy, which was alleviated by immersion in hot water (95°C) for 2 min (Erickson *et al.*, 2016b, 2016c) the day prior to respirometry trials. In order to determine whether the assessment of seed viability via respirometry was possible in dormant seeds, *R. anglocandicans* and *N. lukei* were physiologically dormant at the time of experimentation.

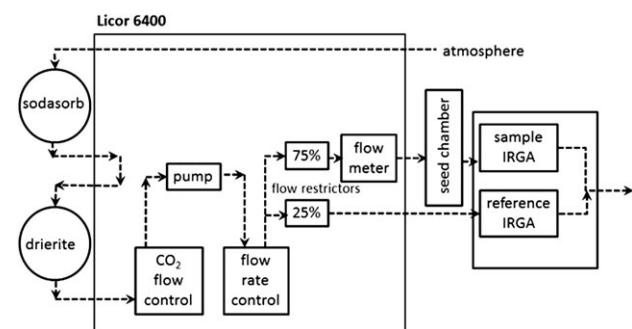
Our preliminary experiments conducted on dry seeds of corn and climbing beans showed limited  $\dot{V}CO_2$ , or  $\dot{V}CO_2$  below the detection limits of our system, which is consistent with published evidence of a significant increase in aerobic metabolic activity in seeds that have been hydrated (Walters *et al.*, 2005; Bewley *et al.*, 2013). Therefore, we conducted a water-uptake experiment on seeds of all species in order to discern the approximate time taken to reach stage two of imbibition, where respiration slows prior to germination (Bewley *et al.*, 2013). The initial weight of dry seeds was determined by weighing three replicates of 20 dry seeds. Seeds were then placed into 90 mm Petri dishes lined with glass filter paper and irrigated with 12 mL of water, and blotted dry and reweighed at 0.5, 2, 4, 6, 8, 24, 48, 72 and 96 h. Seeds were considered to have reached stage two of imbibition when water uptake plateaued after an initial rapid increase, which ranged in time between species from 8 (e.g. *S. lycopersicum*) to 24 h (e.g. *Z. mays*) (data not shown). Prior to respirometry measurements, seeds were imbibed to the pre-determined imbibition time.

### Respirometry trials

Thirty minutes prior to the respirometry measurements, half of the imbibed seeds were killed using a conventional microwave (830 W for 5 min) and marked with acrylic paint. For the larger seeded species *A. ancistrocarpa*, *A. hemiglauca*, *B. attenuata*, *P. vulgaris*, *S. spathulatum* and *Z. mays*, pools of 100 seeds, both alive and dead, were established, while two pools of 200 seeds each were established for *G. wickhamii*. For the remaining smaller-seeded species, pools consisted of at least 2 g of seed each. Respirometry trials were

conducted using a randomized, repeated measures design; whereby blends of seeds with differing proportions of living and dead seed (0, 25, 50, 75 and 100%) were made from the two larger pools of living and dead seed. Each blend of percentage alive (i.e. 0, 25, 50, 75 and 100%) was replicated six times. For larger seeds, the same number of seeds was used per species for every respirometry trial (*A. ancistrocarpa*, *A. hemiglauca*, *B. attenuata*, *P. vulgaris* and *Z. mays*  $n = 8$ ), while smaller seeded species' seed lots were made up to ~0.5 g. Pools of seeds were kept in closed Petri dishes containing moistened filter paper while respirometry measurements were not being made.

A LI-COR 6400XT portable photosynthesis system with an infra-red CO<sub>2</sub> gas analyser (LI-COR Inc. Lincoln, NE, USA) was adapted for use as a flow-through respirometry system (Fig. 1), similar to that described by Withers (2001). Incurrent air was passed through the LI-COR's soda lime (predominantly calcium hydroxide, LI-COR, Inc. Lincoln, NE, USA) and Drierite (anhydrous calcium sulphate, W.A. Hammond, Xenia OH, USA) columns to remove CO<sub>2</sub> and H<sub>2</sub>O vapour, respectively. Using the 'Insect RD' configuration on the LI-COR and a custom built plastic chamber (ranging in size from 1 to 10 cm<sup>3</sup> depending on the size of the seeds), the airflow from the LI-COR was passed through the chamber containing the experimental seed lot at a rate of 60–100 mol s<sup>-1</sup>. The excurrent air was then analysed with the LI-COR's infra-red gas analyser (IRGA) for CO<sub>2</sub> (μmol mol<sup>-1</sup> of air). Baseline readings of background CO<sub>2</sub> were run for 20 min before and after each 20-min trial period. The IRGA was calibrated at the end of the respirometry trial using 1325 ppm CO<sub>2</sub> calibration gas (BOC, Australia). All measurements were made at room temperature of ~24.5°C. The resulting F<sub>E</sub>CO<sub>2</sub> was used to calculate metabolic rate following Withers (2001) using a custom-written Visual Basic program (P. Withers, pers. contribution).



**Figure 1:** Schematic of the LI-COR 6400 XT unit modified to enable flow-through respirometry of seeds. Modification involves the placement of a metabolic chamber (a syringe barrel ranging in volume from 1 to 10 cm<sup>3</sup>) between the flow metre and the sample IRGA, external to the analysis head, which would usually incorporate a leaf photosynthesis chamber, and a shortening of all external hoses to decrease washout rate. No internal modifications were made. Dashed arrows indicate direction of airflow.

## Post-respirometry germination and viability assessment

After completion of respirometry trials, both living and dead non-dormant seeds were plated on to sterile, solidified water agar (0.7%) contained within 90 mm Petri dishes. Dishes were wrapped in plastic film to prevent water loss, and placed in an incubator at 25°C (Erickson *et al.*, 2016a) under a daily photoperiod of 30  $\mu\text{mol m}^{-2}\text{s}^{-1}$ , 400–700 nm cool white fluorescent light. Seeds of *R. anglocandicans* were plated on agar containing 2.89 mM gibberellic acid ( $\text{GA}_3$ ) and incubated at 25°C, while seeds of *N. lukei* were placed in 10 mL plastic tubes containing 10 mL of sterile deionised water and incubated at 35°C (Dalziell, 2016). Seeds were scored for germination; defined as radical emergence  $\geq$  2 mm, every 2–3 days for a total period of 42 days. Seeds that did not germinate were subject to a cut-test, and in some cases, a tetrazolium test, where uniform bright red/pink staining of the embryo confirmed viability (ISTA, 2017).

## Statistical analysis

The effect of seed dry mass on absolute  $\dot{V}\text{CO}_2$  ( $\mu\text{L h}^{-1}\text{seed}^{-1}$ ) was interrogated by applying a non-linear regression to the 100% alive blends using the *nls* procedure in the R statistical environment (R Core Team, 2017). The allometric relationship fitted was a power function:

$$\dot{V}\text{CO}_2 = a \times M^b$$

where,  $a$  is a constant (intercept),  $M$  is the dry mass of the seed in grams and  $b$  is the allometric scaling exponent. In order to compare our measurements of allometrically corrected  $\dot{V}\text{CO}_2$  to data previously published by Garwood and Lighton (1990),  $\dot{V}\text{CO}_2$  measurements were converted to  $\dot{V}\text{O}_2$  by assuming a respiratory exchange ratio (RER) of 1, i.e. a 1:1 exchange of  $\text{O}_2$  consumed to  $\text{CO}_2$  produced.

In order to quantify the relationship between seed viability and metabolic rate,  $\dot{V}\text{CO}_2$  for individual species was allometrically corrected using the scaling factor resolved above to  $\mu\text{L}(\text{CO}_2)\text{g}^{-1.09}\text{h}^{-1}$ . We used a model-comparison approach where two global linear regression models were fitted between experimental viability blends and the allometrically corrected  $\dot{V}\text{CO}_2$  using the *nls* protocol. The first of these models fitted both a slope and intercept, while the second assumed that  $\dot{V}\text{CO}_2$  of a completely dead seed lot (where viability = 0%) should be zero. We selected the most appropriate of these two candidate models on the basis of the Akaike Information Criterion (AIC) (Burnham and Anderson, 2002) using the R package *AICcmodavg* (Mazerolle, 2016). To separate unique relationships between viability and  $\dot{V}\text{CO}_2$  for individual species, the parameters of the most appropriate models were grouped by species (Ritz and Streibig, 2008), and the grouped model was compared to the global model, again using AIC. The assumption

underpinning this technique is that a model that explains statistically greater proportions of the variability in the data indicates effects of the factor by which the data were grouped, in this case interspecific differences. Data are presented as means  $\pm$  1 standard error.

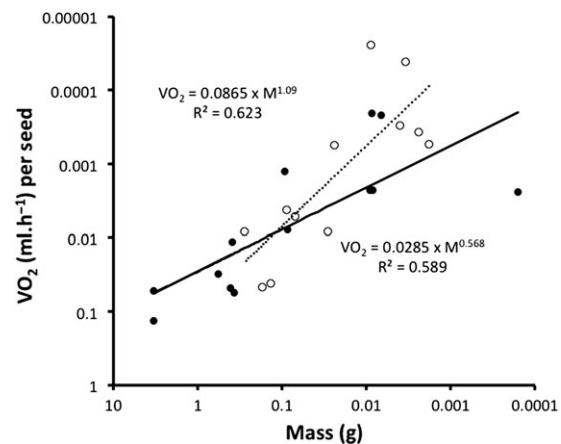
## Results

### Effects of seed mass on $\dot{V}\text{CO}_2$

In seed batches of 100% viability,  $\dot{V}\text{CO}_2$  increased with increasing dry mass (log likelihood =  $-129.04$ ,  $\text{df} = 3$ ), with an allometric scaling exponent of 1.09. However, there was a significant amount of variation in the relationship [residual sum of squares (RSS) = 151.9]. For example, high metabolic rates were measured seeds of *E. gamophylla* ( $532.6 \pm 93.6 \mu\text{L g}^{-1.09}\text{h}^{-1}$ ), *A. ancistrocarpa* ( $396.1 \pm 34.0 \mu\text{L g}^{-1.09}\text{h}^{-1}$ ), *Z. mays* ( $367.5 \pm 21.6 \mu\text{L g}^{-1.09}\text{h}^{-1}$ ) and *P. vulgaris* ( $320.20 \pm 25.0 \mu\text{L g}^{-1.09}\text{h}^{-1}$ ), compared to *S. spathulatum* ( $34.0 \pm 4.4 \mu\text{L g}^{-1.09}\text{h}^{-1}$ ), *G. wickhamii* ( $32.5 \pm 2.3 \mu\text{L g}^{-1.09}\text{h}^{-1}$ ), *R. anglocandicans* ( $20.5 \pm 1.6 \mu\text{L g}^{-1.09}\text{h}^{-1}$ ) and *N. lukei* ( $4.2 \pm 1.0 \mu\text{L g}^{-1.09}\text{h}^{-1}$ ). When converted to  $\dot{V}\text{O}_2$  and compared to Garwood and Lighton's (1990) previous allometry of metabolic rate in angiosperm seeds (Fig. 2), our data are well within a similar range of values, although a high level of variability is present in both relationships.

### Effects of seed viability on $\dot{V}\text{CO}_2$

Across the 12 species tested, there was a significant positive relationship between increasing seed viability and allometrically corrected  $\dot{V}\text{CO}_2$  (log likelihood = 268.6,  $\text{df} = 2$ ; RSS = 4.23). The regression model assuming  $\dot{V}\text{CO}_2$  of zero in dead seed batches ( $K = 2$ ,  $\text{AICc} = -533.1$ ,  $\Delta\text{AICc} = 0.00$ , log-likelihood = 268.6) better explained the patterns in the data



**Figure 2:** The allometric relationship between seed dry mass and oxygen consumption ( $\dot{V}\text{O}_2$ ).  $\text{CO}_2$  consumption data from the present study were converted to  $\dot{V}\text{O}_2$  by assuming an RER of 1 (open circles, dashed line) and plotted against data presented by Garwood and Lighton (1990) (closed circles, solid line).

than the model that fitted an intercept other than zero ( $K = 3$ ,  $AICc = 531.3$ ,  $\Delta AICc = 1.86$ ,  $\log\text{-likelihood} = 268.7$ ). The best fitting model resulted when the slope of the zero-intercept global model was grouped by species ( $K = 2$ ,  $AICc = -533.1$ ,  $\Delta AICc = 429.1$ ,  $\log\text{-likelihood} = 268.6$ ). This model was also more appropriate than the unique models fitted to each individual species ( $K = 13$ ,  $AICc = 1025.3$ ,  $\Delta AICc = 0.00$ ,  $\log\text{-likelihood} = 526.2$ ).

The slopes of the species-specific relationships between viability and  $\dot{V}CO_2$  generally demonstrated positive linear relationships between increasing seed viability and  $\dot{V}CO_2$ , but with substantially different slopes (Fig. 3). For seeds of *A. ancistrocarpa*, *B. attenuata*, *G. wickhamii*, *P. vulgaris*, *R. anglocandicans*, *S. spathulatum* and *Z. mays*, seed viability was a good predictor of  $\dot{V}CO_2$  (Fig. 3). For other species such as *A. hemiglauca*, *N. lukei* and *S. lycopersicum*, viability explained less of the variance in  $\dot{V}CO_2$ .

### Germination testing

Living seeds of most species germinated to 100% within 28 days, except *B. attenuata* (98%), *G. wickhamii* (84%), *N. lukei* (77.5%) and *R. anglocandicans* (4.2%). Cut testing confirmed the viability of the non-germinated seeds of *B. attenuata*, *G. wickhamii* and *N. lukei*, while a combination of cut testing and tetrazolium staining confirmed viability in seeds of *R. anglocandicans*. No germination was recorded for any of the dead seed batches.

### Discussion

The accurate assessment of seed viability is essential for the ongoing success of *ex situ* seed storage. Traditional methods of assessing viability are often time consuming (e.g. germination), destructive (e.g. cut-testing) or inconclusive (e.g. tetrazolium staining) in their result. These difficulties are particularly evident when working with the heterogeneity of dormancy and germination patterns associated with seeds of wild species. Our data are strongly suggestive that measuring metabolic rates ( $\dot{V}CO_2$ ) via flow-through respirometry is indicative enough of seed viability to provide a quicker, less destructive viability assessment method. The novelty of applying this approach to plant seeds has brought some considerations to light that need to be addressed in order for this technique to gain broad applicability.

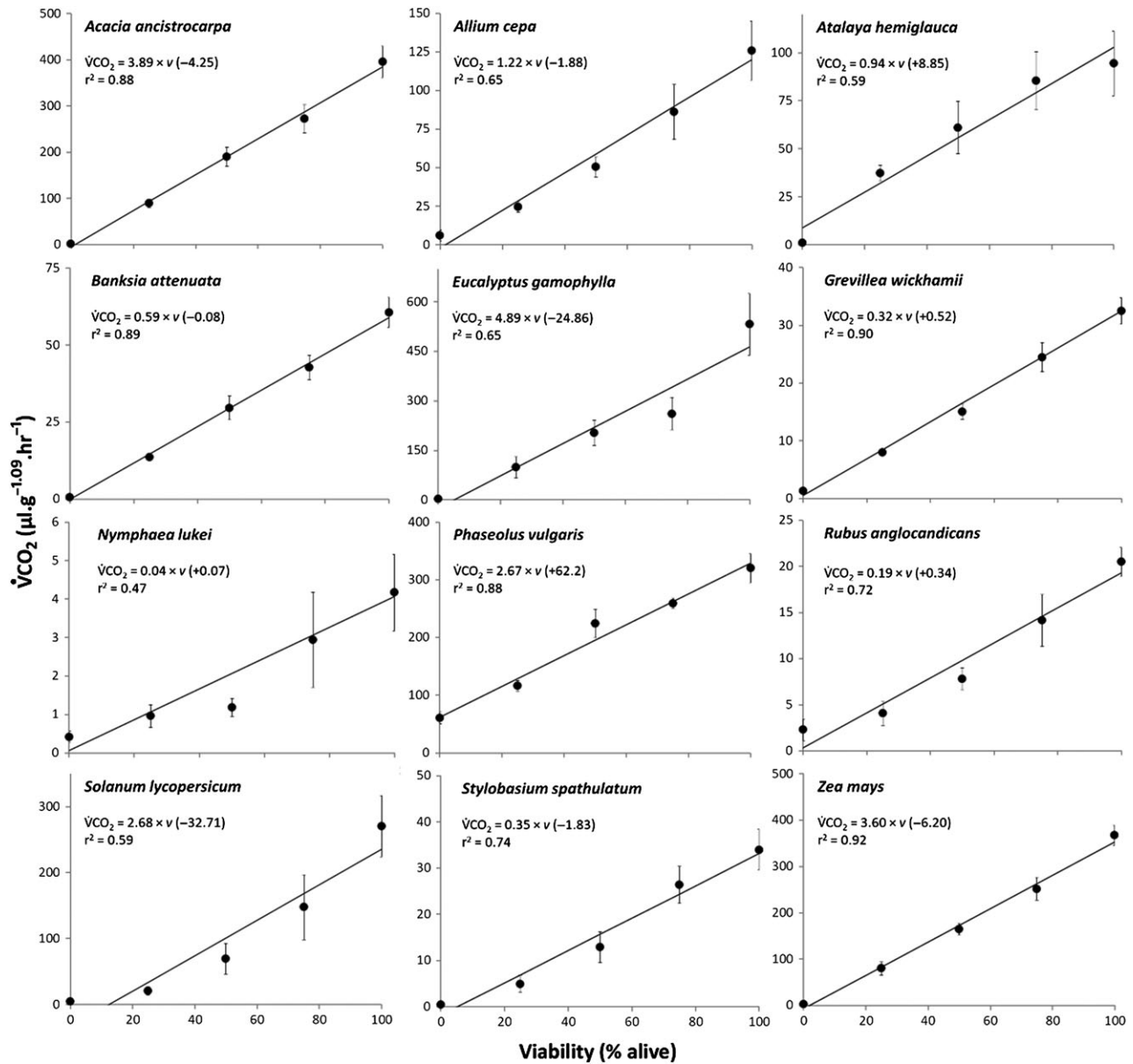
### Allometric effects on $\dot{V}CO_2$

Although our overarching aim was to investigate relationships between seed viability and  $\dot{V}CO_2$ , our first challenge was to account for the effects of interspecific mass differences on  $\dot{V}CO_2$ , as with all comparative studies of metabolic rate (White *et al.*, 2007). The positive relationship in which  $\dot{V}CO_2$  increased proportionally with seed mass (Fig. 2) is consistent with the wider literature investigating the effect that mass has metabolic rate in animals (Kleiber, 1932) and

plants (e.g. see Reich *et al.* (2006), Mori *et al.* (2010) and Price *et al.* (2010)) and the scaling exponent of 1.09 is within the range discussed for universal scaling laws between mass and metabolic rate (White *et al.*, 2007). Although this value is not consistent with either of the major theories of metabolic scaling (West *et al.*, 1999; Brown *et al.*, 2004), we remain dubious of this, at least until a larger body of data can be subject to appropriate phylogenetically corrected analysis (Feder, 1987; Garland and Adolph, 1994). Furthermore, our data are relatively consistent with those presented by Garwood and Lighton (1990), although we have assumed a RER (i.e. the ratio between  $CO_2$  produced and  $O_2$  consumed during respiration) of 1 in order to convert between  $\dot{V}CO_2$  and  $\dot{V}O_2$ . This would be consistent for respiration of entirely carbohydrate substrates that are the predominant fuel in pre-germinative metabolism (Withers, 1992; Bewley *et al.*, 2013). Our data become less congruent with those of Garwood and Lighton (1990) if RER is reduced by the introduction of a greater proportion of lipids. Given that there is no evidence for the most appropriate RER in angiosperm seeds, this assumption remains untested.

Although the relationship between seed mass and  $\dot{V}CO_2$  is largely consistent with expectations, there is a substantial amount of unexplained variation within the relationship ( $R^2 = 0.62$ , Fig. 2). For example, seeds of *E. gamophylla*, *A. ancistrocarpa*, *Z. mays* and *P. vulgaris* show a high  $\dot{V}CO_2$  relative to their mass, while *N. lukei* and *R. anglocandicans* show a low  $\dot{V}CO_2$  relative to their mass. All four highly active species germinated to 100% within 4 days in post-respirometry germination testing, whereas the two low-activity species reached 77.5 and 4.2% germination, and were slower to germinate (rate data not shown), suggesting both dormancy and seed vigour play a role in overall metabolic rate (Bradford *et al.*, 2013; Bello and Bradford, 2016). However, there is currently an incomplete understanding of the relationship between seed dormancy and whole seed respiration. Previous studies have shown that dormant and non-dormant bitterbrush seeds (*Purshia tridentata*) produce the same amount of  $CO_2$  (Booth and Sowa, 2001), while experiments with red rice (*Oryza sativa*) showed almost four times less  $O_2$  consumption on a per seed basis in dormant seeds compared with non-dormant seeds (Footitt and Cohen, 1995), and dormant tomato seeds (*S. lycopersicum*, GA-deficient mutant line) consumed significantly less  $O_2$  than non-dormant seeds (ABA-deficient mutant line) (Bello and Bradford, 2016).

Allometric studies within broad groups of animals have offered insights into many organisms' physiological ecology (McKechnie and Wolf *et al.*, 2004; Chown *et al.*, 2007; White *et al.*, 2007), and divergence from the expected patterns have raised questions concerning the phylogenetic and ecological correlates of this deviation (Withers *et al.*, 2006). The species tested here represent a wide range of phylogenetic heritage and ecotypes, along with adaptive variation unique to plant seeds, such as seed dormancy type, embryo type and plant life form (Table 1). The breadth of variability



**Figure 3:** The linear relationship between seed viability and  $\dot{V}CO_2$  in seeds of 12 angiosperm species. Linear regressions were fitted to raw  $\dot{V}CO_2$  data, with means  $\pm$  standard errors presented. The equations describe the relationship between viability and metabolic rate for each species. Intercepts in parentheses denote the less parsimonious viability function, where the metabolic rate of dead seeds was not presumed to be zero.

in these traits far exceeds that of most groups studied so far, and the 34 species represented by including our data with those reported by Garwood and Lighton (1990) reflect only 0.009% of the estimated global plant diversity (RBG Kew, 2016). This suggests to us that the investigation of phylogenetic and ecological correlates of metabolic rate in plant seeds is an intriguingly open and potentially lucrative field of study, which may offer unique insights for seed biology, in

terms of relationships between metabolism and seed dormancy, and seed storage and seed viability.

### Viability effects on $\dot{V}CO_2$

We found a consistent, significant linear relationship between seed viability and  $\dot{V}CO_2$ , which is consistent with the linear relationship between  $O_2$  influx and individual seed

viability presented by Xin *et al.* (2013). By measuring batches of imbibed seed from 12 agriculturally important and native Australian species, the significant linear relationship that we found between seed viability and metabolic rate (Fig. 3) indicates that, with appropriate replication and construction of calibration curves, measurements of metabolic rate can be used to infer seed viability in many other species. The significant interspecific variation in this relationship, however, suggests there to be equally powerful patterns of phylogeny and ecotype in the relationship between seed viability and  $\dot{V}CO_2$  as there are between seed mass and  $\dot{V}CO_2$ .

For seeds of *A. ancistrocarpa*, *B. attenuata*, *G. wickhamii*, *P. vulgaris* and *Z. mays*, seed viability explained the majority of the relationship with  $\dot{V}CO_2$  (i.e.  $r^2 > 0.80$ ), compared to seeds of *A. hemiglauca*, *N. lukei* and *S. lycopersicum* ( $r^2 < 0.6$ ) which were more variable. The *Nymphaea* are an ancient member of the ANA-grade basal angiosperms, and there is currently a limited understanding of their seed dormancy (Dalziell, 2016). Therefore, it is entirely likely that variation in dormancy depth between individual seeds in each experimental blend contributed substantial uncontrolled variation to our data. The *Solanum* seeds that we sourced, however, were from a commercial supplier of agricultural cultivars. Seeds such as these are typically bred for very low levels of interspecific variability and predictably high levels of germinability. Likewise, the *Atalaya* seeds, although sourced from the wild are non-dormant (Cook *et al.*, 2008). As such, the potential source of variance to their metabolic rates remains elusive, and indicates our naivety concerning the factors that contribute to patterns of metabolic rates, even in the species with which we are most familiar.

Whilst the measurement of seed metabolism as we have described here can be used to infer viability in a batch of seeds, there are a few considerations to note when using this technique. For example, seeds of *N. lukei* produced only  $4.2 \pm 1.0 \mu\text{L g}^{-1.09} \text{h}^{-1}$  of  $CO_2$ , which is close to the lower detection limit of the IRGA used to measure  $CO_2$ . As such, the number of small seeds required to obtain an identifiable signal may not be sustainable for small seed collections. Furthermore, this is greatly amplified if seed dormancy results in substantial metabolic depression (Guppy and Withers, 1999). Care must also be taken to ensure seeds are appropriately hydrated during the measurement periods, as drying may reduce overall metabolic activity (Garwood and Lighton, 1990; Walters *et al.*, 2005; Bewley *et al.*, 2013).

While imbibing seeds prior to making measurements of metabolism obviously negates the ability of seeds to be returned to storage, the technique we have presented here is much less destructive than traditional cut-testing and tetrazolium staining. Furthermore, we were able to make accurate measurements of seed viability, even from dormant seeds, in a matter of hours (i.e. 20 min for each replicate), compared to the number of weeks or months sometimes required for germination testing. For larger-seeded species (i.e. 7 out of

12 species) this was also achieved using the same number of seeds routinely used for germination testing (i.e. the number of living seeds ranged from 100 to 200 seeds). While we did require more of the smaller seeds than is used for an average germination test, further refinements in this technology could improve the detection limits of the system, and may also offer a truly non-destructive measurement of seed viability.

## Future developments in seed metabolic ecology

The measurement of metabolic rates has a long heritage, and there is a diversity of approaches to it, each with their own strengths and limitations (Lighton, 2008). Traditionally, closed-system respirometry would be used where the metabolic rates are low in comparison to background variation (Vleck, 1987; Lighton, 2008) and this approach has been used in previous studies measuring  $\dot{V}O_2$  of plant seeds (Garwood and Lighton, 1990; Bradford *et al.*, 2013; Bello and Bradford, 2016). There are several advantages to measuring seed  $\dot{V}CO_2$  in a flow-through system, as we have done here, however. As air is allowed to pass through the metabolic chamber during the measurement period, neither hypoxia nor hypercapnia become limiting (as long as appropriate flow rate is maintained). For this reason, there is no impact of measurement technique on the resulting metabolic rates, nor on post-measurement germination. Secondly, simply imbibing seeds, rather than requiring them to germinate, significantly reduces the amount of time required to investigate viability or vigour, which is especially important for slow-germinating species. Nevertheless, handling time with the LI-COR system that we used here is high, because only single seed batches can be measured at any one time, and the development of a more specifically engineered respirometry system, capable of measuring multiple seed batches at once, would be a substantial step forward. Finally, the establishment of an appropriate RER for seed metabolic rates is clearly critical in understanding many aspects of seed metabolic ecology. Nevertheless, given that the measurement of metabolic rate provides an alternative technique for the investigation of seed viability, and may be a useful addition to the viability monitoring 'toolkit' for seedbank managers, these future developments are sure to be fruitful avenues of investigation.

## Acknowledgements

Thanks to David Merritt and Philip Withers for their ongoing collaboration and support of the seed metabolism project, and additionally to Phil Withers for access to the custom-written Visual Basic software used for metabolic analyses; Wolfgang Lewandrowski for his assistance with the LI-CORs; Todd Erickson and Jason Stevens for initial conversations about the experiment; and Todd Erickson (Botanic Gardens and Parks Authority/University of Western Australia) and Paul Yeoh and John Scott (CSIRO) for access to seeds used in this study. The authors also thank Dr Kevin Hultine and two anonymous



reviewers for valuable comments that improved a previous version of this article.

## Funding

This work was supported by an Australian Research Council (ARC) Linkage Grant [LP160100381] and the Botanic Gardens and Parks Authority. During the initial stages of this research, ELD was supported by an Australian Postgraduate Award and a University of Western Australia PhD completion scholarship. During the period of data collection ST was supported by Australian Research Council Linkage Grant [LP110200304] and an Australian Department of Human Services Newstart Unemployment Allowance. Subsequently salary for ST was provided through the Australian Research Council Grant IC150100041.

## References

- Baskin CC, Baskin JM (2014). *Seeds. Ecology, Biogeography, and Evolution of Dormancy and Germination*, Ed 2. Academic Press, San Diego (CA, USA), London (UK), Waltham (MA, USA).
- Baskin JM, Baskin CC (2004) A classification system for seed dormancy. *Seed Sci Res* 14:1–16.
- Bello P, Bradford KJ (2016) Single-seed oxygen consumption measurements and population-based threshold models link respiration and germination rates under diverse conditions. *Seed Sci Res* 26:199–221.
- Bewley JD, Bradford KJ, Hilhorst HWM, Nonogaki H (2013) *Seeds. Physiology of Development, Germination and Dormancy*, Ed 3. Springer Science & Business Media, New York, Heidelberg, Dordrecht, London.
- Booth DT, Sowa S (2001) Respiration in dormant and non-dormant bitterbrush seeds. *J Arid Environ* 48:35–39.
- Bradford KJ, Bello P, Fu J-C, Barros M (2013) Single-seed respiration: a new method to assess seed quality. *Seed Sci Technol* 41:420–438.
- Brown JH, Gillooly JF, Allen AP, Savage VM, West GB (2004) Toward a metabolic theory of ecology. *Ecology* 85:1771–1789.
- Burnham KP, Anderson DR (2002) *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*, Ed 2. Springer Science & Business Media, NY, USA.
- Chown SL, Marais E, Terblanche JS, Klok CJ, Lighton JB, Blackburn TM (2007) Scaling of insect metabolic rate is inconsistent with the nutrient supply network model. *Funct Ecol* 21:282–290.
- Colville L, Bradley EL, Lloyd AS, Pritchard HW, Castle L, Kranner I (2012) Volatile fingerprints of seeds of four species indicate the involvement of alcoholic fermentation, lipid peroxidation and maillard reactions in seed deterioration during ageing and desiccation stress. *J Exp Bot* 63:6519–6530.
- Cook A, Turner S, Baskin J, Baskin C, Steadman K, Dixon K (2008) Occurrence of physical dormancy in seeds of Australian Sapindaceae: a survey of 14 species in nine genera. *Ann Bot* 101:1349–1362.
- Crawford AD, Steadman KJ, Plummer JA, Cochrane A, Probert RJ (2007) Analysis of seed-bank data confirms suitability of international seed-storage standards for the Australian flora. *Aust J Bot* 55:18–29.
- Dalziel EL (2016) Seed biology and *ex situ* storage behaviour of Australian *Nymphaea* (water lilies): implications for conservation. PhD thesis. University of Western Australia, Perth, WA.
- Ellis R, Roberts E (1980) Improved equations for the prediction of seed longevity. *Ann Bot* 45:13–30.
- Erickson TE, Barrett RL, Merritt DJ, Dixon KW (2016a) *Pilbara Seed Atlas and Field Guide. Plant Restoration in Australia's Arid Northwest*. CSIRO Publishing, Victoria, Australia.
- Erickson TE, Barrett RL, Symons DR, Turner SR, Merritt DJ (2016b) An Atlas to the plants and seeds of the Pilbara Region. In Erickson TE, Barrett RL, Merritt DJ, Dixon KW (eds). *Pilbara Seed Atlas and Field Guide: Plant Restoration in Australia's Arid Northwest*. CSIRO Publishing, Victoria, Australia. 43–251.
- Erickson TE, Merritt DJ, Turner SR (2016c) Overcoming physical seed dormancy in priority native species for use in arid-zone restoration programs. *Aust J Bot* 64:401–416.
- FAO (2014). *Genebank Standards for Plant Genetic Resources for Food and Agriculture*. Rev Ed., FAO Rome.
- Feder ME (1987) The analysis of physiological diversity: the prospects for pattern documentation and general questions in ecological physiology. In Feder ME, Bennett AF, Burggren WW, Huey RB (eds). *New Directions in Ecological Physiology*. Cambridge University Press, Cambridge, UK. 38–75.
- Footitt S, Cohn MA (1995) Seed dormancy in red rice (*Oryza sativa*). IX. Embryo fructose-2,6-bisphosphate during dormancy breaking and subsequent germination. *Plant Physiol* 107:1365–1370.
- Frappell P (2006) Respirometry, the gold standard. *Physiologist* 49:12.
- Fu YB, Ahmed Z, Diederichsen A (2015) Towards a better monitoring of seed ageing under *ex situ* seed conservation. *Conserv Physiol* 3: cov026. doi:10.1093/conphys/cov026.
- Garland TJ, Adolph SC (1994) Why not to do two-species comparative studies: limitations on inferring adaptation. *Physiol Zool* 67:797–828.
- Garwood NC, Lighton JB (1990) Physiological ecology of seed respiration in some tropical species. *New Phytol* 115:549–558.
- Gosling PG (2003) Viability testing. In Smith R,D, Dickie JB, Lington SH, Pritchard HW, Probert RJ (eds). *Seed Conservation: Turning Science into Practice*. Great Britain: Royal Botanic Gardens, Kew. 445–506.
- Guppy M, Withers P (1999) Metabolic depression in animals: physiological perspective and biochemical generalizations. *Biol Rev* 74:1–40.
- Hay FR, Whitehouse KJ (2017) Rethinking the approach to viability monitoring in seed genebanks. *Conserv Physiol* 5:cox009. doi:10.1093/conphys/cox009.
- ISTA (2017). *International Rules for Seed Testing*. International Seed Testing Association. Bassersdorf, Switzerland.

- Kleiber M (1932) Body size and animal metabolism. *Hilgardia* 6:315–353.
- Kranner I, Kastberger G, Hartbauer M, Pritchard HW (2010) Noninvasive diagnosis of seed viability using infrared thermography. *Proc Natl Acad Sci USA* 107:3912–3917.
- Lighton JRB (2008) *Measuring Metabolic Rates: A Manual for Scientists*. Oxford University Press, New York.
- Martin AC (1946) The comparative internal morphology of seeds. *Am Midl Nat* 36:513–660.
- Mazerolle MJ (2016). AICcmodavg: model selection and multimodel inference based on (Q)AIC(c). R package version 2.1-0.
- McKechnie AE, Wolf BO (2004) The allometry of avian basal metabolic rate: good predictions need good data. *Physiol Biochem Zool* 77: 502–521.
- Merritt DJ, Dixon KW (2011) Restoration seed banks—a matter of scale. *Science* 332:424–425.
- Mira S, Gonzalez-Benito ME, Hill LM, Walters C (2010) Characterization of volatile production during storage of lettuce (*Lactuca sativa*) seed. *J Exp Bot* 61:3915–3924.
- Mira S, Hill LM, González-Benito ME, Ibáñez MA, Walters C (2016) Volatile emission in dry seeds as a way to probe chemical reactions during initial asymptomatic deterioration. *J Exp Bot* 67: 1783–1793.
- Mori SKY, Ishida A, Prokushkin SG, Masyagina OV, Hagihara A, Rafiqul Hoque ATM, Suwa R, Osawa A, Nishizono T, Ueda T, et al (2010) Mixed-power scaling of whole-plant respiration from seedlings to giant trees. *Proc Natl Acad Sci USA* 107:1447–1451.
- Patanè C, Avola G (2013) A seed respiration-based index of cold-sensitivity during imbibition in four macrothermal species. *Acta Physiol Plant* 35:911–918.
- Powell AA (1986) Cell membranes and seed leachate conductivity in relation to the quality of seed for sowing. *Seed Technol* 10:81–100.
- Price CA, Gilooly JF, Allen AP, Weitz JS, Niklas KJ (2010) The metabolic theory of ecology: prospects and challenges for plant biology. *New Phytol* 133:696–710.
- Pritchard HW (1985) Determination of orchid seed viability using fluorescein diacetate. *Plant, Cell & Environment* 8:727–730.
- R Core Team R (2017) *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- RBG Kew (2016). The State of the World's Plants Report-2016, Royal Botanic Gardens, Kew.
- Reich PB, Tjoelker MG, Machado J-L, Oleksyn J (2006) Universal scaling of respiratory metabolism, size and nitrogen in plants. *Nature* 439: 457–461.
- Ritz C, Streibig JC (2008) *Nonlinear Regression With R*. Springer Science & Business Media, New York, USA.
- Vleck D (1987) Measurement of O<sub>2</sub> consumption, CO<sub>2</sub> production, and water vapor production in a closed system. *J Appl Physiol* 62: 2103–2106.
- Walters C (2015) Genebanking seeds from natural populations. *Nat Area J* 35:98–105.
- Walters C, Hill LM, Wheeler LJ (2005) Dying while dry: kinetics and mechanisms of deterioration in desiccated organisms. *Integr and Comp Biol* 45:751.
- West GB, Brown JH, Enquist BJ (1999) The fourth dimension of life: fractal geometry and allometric scaling of organisms. *Science* 284: 1677–1679.
- White CR, Cassey P, Blackburn TM (2007) Allometric exponents do not support a universal metabolic allometry. *Ecology* 88:315–323.
- Withers P, Cooper C, Larcombe A (2006) Environmental correlates of physiological variables in marsupials. *Physiol Biochem Zool* 79: 437–453.
- Withers PC (1992) *Comparative Animal Physiology*. Brooks/Cole Thomson Learning, CA, USA.
- Withers PC (2001) Design, calibration and calculation for flow-through respirometry systems. *Aust J Zool* 49:445–461.
- Xin X, Wan Y, Wang W, Yin G, McLamore ES, Lu X (2013) A real-time, non-invasive, micro-optrode technique for detecting seed viability by using oxygen influx. *Sci Rep* 3:1–6.
- Zhao GW, Zhong TL (2012) Improving the assessment method of seed vigor in *Cunninghamia lanceolata* and *Pinus massoniana* based on oxygen sensing technology. *J For Res* 23:95–101.