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Using hyperspectral imaging to determine germination of native Australian plant seeds

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ABSTRACT

We investigated the ability to accurately and non-destructively determine the germination of three native Australian tree species, *Acacia cowleana* Tate (Fabaceae), *Banksia prionotes* L.F. (Proteaceae), and *Corymbia calophylla* (Lindl.) K.D. Hill & L.A.S. Johnson (Myrtaceae) based on hyperspectral imaging data. While similar studies have been conducted on agricultural and horticultural seeds, we are unaware of any published studies involving reflectance-based assessments of the germination of tree seeds. Hyperspectral imaging data (110 narrow spectral bands from 423.6 nm to 878.9 nm) were acquired of individual seeds after 0, 1, 2, 5, 10, 20, 30, and 50 days of standardized rapid ageing. At each time point, seeds were subjected to hyperspectral imaging to obtain reflectance profiles from individual seeds. A standard germination test was performed, and we predicted that loss of germination was associated with a significant change in seed coat reflectance profiles. Forward linear discriminant analysis (LDA) was used to select the 10 spectral bands with the highest contribution to classifications of the three species. In all species, germination decreased from over 90% to below 20% in about 10–30 days of experimental ageing. P_{50} values (equal to 50% germination) for each species were 19.3 (*A. cowleana*), 7.0 (*B. prionotes*) and 22.9 (*C. calophylla*) days. Based on independent validation of classifications of hyperspectral imaging data, we found that germination of *Acacia* and *Corymbia* seeds could be classified with over 85% accuracy, while it was about 80% for *Banksia* seeds. The selected spectral bands in each LDA-based classification were located near known pigment peaks involved in photosynthesis and/or near spectral bands used in published indices to predict chlorophyll or nitrogen content in leaves. The results suggested that seed germination may be successfully classified (predicted) based on reflectance in narrow spectral bands associated with the primary metabolism function and performance of plants.

1. Introduction

Ex situ seed banks play an important role in accessing and propagating seeds for restoration of degraded and fragmented native habitats [1] and for the conservation of threatened species [2–5]. Accordingly, many botanic gardens and conservation agencies worldwide have established seed banks to address ongoing concerns about habitat destruction and in particular the loss of biodiversity. As well as providing seeds for use in conservation and restoration programs, seed banks provide unique opportunities for studies of the biology, ecology, and evolution of native species as well as opportunities for the commercialization of species with horticultural and agricultural potential. Given the value of these collections and in many cases the irreplaceable nature of the accessions held, it is imperative that seed banks are managed in ways that minimize age-related seed losses. Consequently, the maintenance of seed viability during long-term storage is a major focus of seed banks. However, the longevity of seed viability varies greatly among plant species and is significantly impacted by seed storage conditions [6]. Therefore, the success of long-term *ex situ* seed conservation is dependent on regular monitoring using standardized and reproducible approaches [7]. The International Seed Testing Association (http://seedtest.org/en/home.html) has as one of its important objectives to develop and test methods used to quantify seed germination.

Existing methods to test seed viability include X-ray analysis [8–10], tetrazolium staining [11–15], a cut test [16–18], in some cases the careful extraction and sterile culture of zygotic embryos under aseptic *in vitro* conditions [10,19], but germination tests

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[11,20,21] are probably the most widely used. Each method has advantages and disadvantages and consequently no single method provides a definitive means of accurately quantifying seed viability. For example, while relatively new and non destructive the X-ray assessment of seeds only indicates whether seeds are structurally intact (i.e. possess an endosperm/embryo), not whether the seeds are actually alive so while the seeds can be placed back into storage following assessment the use of this test on its own does not provide any certainty as to whether any structurally sound seeds are specifically alive [22]. Although the germination test is probably one of the most accurate ways to assess seed lots, it is labor intensive, time consuming (several days to many weeks to establish the level of germination), and it may dependent upon knowledge concerning the optimal germination conditions and dormancy breaking requirements of the species in question [22]. In addition, a significant concern from a seed bank management perspective is the fact that germination tests are "irreversible", so if seeds are viable and therefore germinate, the genetic information that they represent will be lost if not grown to maturity which may not possible in large seed banks. This is a particular concern with critically endangered species, as seed stocks may be exceptionally rare and in some cases impossible to source again. With currently available seed viability tests being largely irreversible, generally destructive, and sensitive to subjective data interpretation, there is a pressing need for development of non-destructive and quantitative methods to assess viability and germination of precious seed stocks.

Several studies have demonstrated the potential of reflectance-based spectroscopy methods in studies of plant seeds, including detection of internal infestations by weevils (*Bruchus pisorum*) in dry field peas (*Pisum sativum*) [23,24], classification of near isogenic maize lines (*Zea mays*) [25], ageing of cabbage seeds [26], classification of near isogenic maize lines (*Z. mays*) [27], differentiation between black walnut (*Juglans nigra*) shell and pulp [28], sorting of lettuce (*Lactuca sativa*) seeds [29], and viability of horticultural seeds [26,30,31]. These spectroscopy studies are based on the fundamental assumption that reflectance data acquired from the seed coat provides indicative information about the quality/germination of the given seed. The research objective is therefore to identify portions of the wavelength spectrum, in which seeds show a significant and measurable change in certain parts of the examined reflectance spectrum and associated that change in reflectance with certain traits, such as, germination (yes/no). A wide range of classification methods have been used as part of using reflectance data to characterize seeds and food products; these classification methods include support vector machine (SVM) [32], variogram analysis [33,34], partial least square analysis [35], and linear discriminant analysis (LDA) [36]. LDA is based on discriminant functions, which are linear combinations of features (in this case reflectance values in spectral bands) and with one function for each target class. For each observation, a discriminant score is calculated and the observation is assigned to the class for which the discriminant function generates the highest discriminant score.

In this study, we used hyperspectral imaging data to determine the germination of seeds from three native Australian tree species [*Acacia cowleana* Tate (Fabaceae), *Banksia prionotes* L.F. (Proteaceae), and *Corymbia calophylla* (Lindl.) K.D. Hill & L.A.S. Johnson (Myrtaceae)]. These species represent the Australian flora with many different species held in seed banks across Australia as well as internationally including a number of critically endangered taxa [6,20,37,38]. Seeds were exposed to standardized rapid ageing conditions (60 °C and 60% eRH) [6,20], and at each assessment point were subjected to germination testing and hyperspectral imaging. We hypothesized that there would be detectable difference in seed coat reflectance between germinating and non-germinating seeds, and that changes in reflectance profiles would be most pronounced in spectral bands near known pigment peaks involved in photosynthesis and/or near spectral bands used in published indices to predict chlorophyll or nitrogen content in leaves. The potential benefits of developing accurate machine vision systems to automate non-destructive monitoring of seed germination are discussed in the context of management of seed banks, botanic gardens, and implementation of vegetation restoration programs.

2. Methods

2.1. Plant seeds and data collection

Seeds from *A. cowleana* Tate, *B. prionotes* L.F., and *C. calophylla* (Lindl.) K.D. Hill & L.A.S. Johnson were included in this study and were sourced from either Kings Park collections (*A. cowleana*) or a commercial seed supplier (*B. prionotes* and *C. calophylla*) in March 2014 (Fig. 1a–c). According to Kodym et al. [9], seed fill was initially assessed on one lot of 100 seeds using an MX-20 digital X-ray radiography system (Faxitron, Tucson, AZ, USA) and determined to be >95% across all three species. The bradysporous species *B. prionotes* and *C. calophylla* are non dormant at maturity so required no further treatments to promote germination. However the seeds of *A. cowleana* are physically dormant when released so prior to seed ageing and germination assessment all *A. cowleana* seeds were placed into hot water (~90 °C) for 1 min to render these water permeable. Regarding *Acacia* seeds (Fig. 1a), only reflectance profiles from the actual seed (not the yellow aril) were included. The exclusion of reflectance profiles from the arils were excluded through simple radiometric filtering (segmentation) of hyperspectral images. This radiometric filtering also excluded reflectance profiles from the white background. For each species, we initially established eight subsamples of 25 seeds, and all 24 subsamples (three species × eight subsamples) were experimentally aged according to published methods [6,20]. In short, seed lots were first sealed in small nylon bags and initially stored in a polycarbonate electrical box (28 × 28 × 14 cm; NHP Fibox, Australia) above a non-saturated solution of LiCl (370 g L^{-1}; anhydrous, Sigma®, Australia), generating a relative humidity (RH) of 47% at 20 °C [39]. Seeds were stored under these conditions for two weeks then a subsample of seeds were transferred to a rapid ageing box (polycarbonate electrical box as previously described) above a non saturated solution of LiCl (300 g L^{-1}) generating 60% eRH and placed inside an electronic oven set (Contherm Thermotec 2000 oven, Contherm Scientific Ltd., Wellington, NZ) set to 60 °C. After 0, 1, 2, 5, 10, 20, 30, and 50 days of ageing under these conditions a subsample of seeds were removed (one nylon bag of 25 seeds) and immediately subjected to hyperspectral imaging and germination assessment. Thus, we were able to directly associate reflectance data from each seed with its germination status (yes = 1 or no = 0).

2.2. Reflectance data acquisition

A push-broom hyperspectral camera (PIKA II, Resonon Inc., Bozeman, MT, USA) was mounted 40 cm above the seeds, and hyperspectral images were acquired with the spatial resolution of 50 pixels per mm^2 under artificial lighting (two 15 W and 12 V light bulbs mounted on either side of the lens). The main specifications of the hyperspectral camera were: Firewire interface (IEEE 1394b), 12 bit digital output, 240 spectral bands from 392 to 889 nm (spectral resolution = 2.1 nm) (spectral) by 640 pixels (spatial). The objective lens had a 35 mm focal length (maximum aperture of F1.4) with a 7° field of view, and it was optimized for the near-infrared and visible near-infrared spectra. During

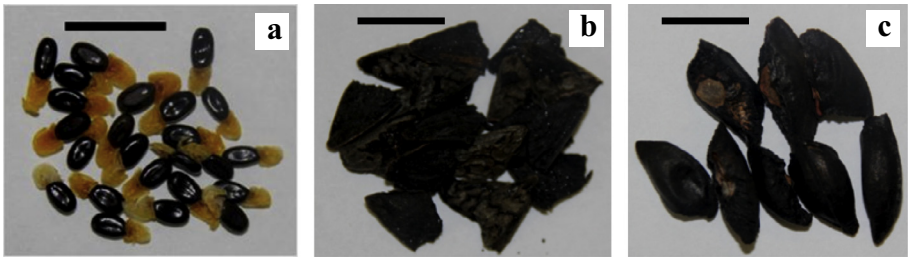

Fig. 1. Images of the native Australian seed species included in the study: *Acacia cowleana* Tate (Fabaceae) (a), *Banksia prionotes* L.F. (Proteaceae) (b), and *Corymbia calophylla* (Lindl.) K.D. Hill & L.A.S. Johnson (Myrtaceae) (c). Bar = 1 cm.

hyperspectral image acquisition in the lab, RH was between 30% and 40% and temperature 19–22 °C. A piece of white Teflon (K-Mac Plastics, MI, USA) was used for white calibration. Reflectance value was referred to relative reflectance and compared to that obtained from white Teflon. Colored plastic cards (green, yellow, and red) were imaged at all hyperspectral imaging events, and average reflectance profiles from these cards were used to confirm high consistency of hyperspectral image acquisition conditions (-less than 2% variance in individual spectral bands).

2.3. Germination assessment and probit analysis

Immediately after hyperspectral imaging at each sampling time, the subsamples of seeds were transferred to Petri dishes with semi-solidified water agar (0.7% w/v) for germination testing [22]. Each seed was sequentially placed onto the dish and individually labelled with a permanent marker (outside of dish). Petri dishes were sealed with plastic film, wrapped in aluminium foil (to exclude light) and incubated in a 15 °C growth chamber (Contherm Biosyn 6000CP Growth Chamber, Contherm Scientific Ltd., Wellington, NZ) and checked weekly for germination. Presence of emerged radicle to 2 mm was considered indication of germination success. For determination of the predicted time (days) required for germination to decline to 50% (P_{50}) probit analysis was performed in Genstat version 10.0 (VSN International Ltd., UK). Seeds were aged at 60 °C rather than 45 °C as is more common in seed ageing experiments [6,20]. P_{50} values were calculated using the original data (i.e. storage at 60 °C) then adjusted to 45 °C by multiplying 60 °C P_{50} values by 8.44 as described in previous studies [6,20].

2.4. Hyperspectral imaging data analysis

A customized software script in Spectronon (Resonon Inc., Bozeman, MT, USA) was used to convert all hyperspectral imaging files into txt-format. The first 15 and last five spectral bands were omitted from each hyperspectral data file, as these were considered to be associated with stochastic noise. Consequently, only 220 spectral bands from 423.6 nm to 878.9 nm were included in the analysis. In addition, we conducted 1×2 spectral binning (decreased the spectral resolution from 2.1 to 4.2 nm), which resulted in 110 spectral bands being included in the analysis. Spectral binning was deployed, as it has been shown to increase classification accuracy [23,40]. Similar previously published studies [24,40], a radiometric filter was applied to exclude background, so that a pixel was only included, if the reflectance value of *Acacia* and *Banksia* seed coat at 660 nm (R660) met the following criterion:

$$0.050 < R660 < 0.250$$

The radiometric selection criterion for pixels of *Corymbia* seed coat was:

$$0.035 < R660 < 0.250$$

Pixels with R660 reflectance values outside the stipulated ranges were excluded before average reflectance profiles were generated for each seed. We obtained 200 average reflectance profiles from each species (25 seeds $\times$ 8 time points), and relationships between results from the germination tests and hyperspectral imaging data were tested using LDA classification [36]. To select an optimized subset of the 10 "best" spectral bands, we conducted a forward stepwise LDA based on all average reflectance profiles for each of the three seeds species, and only these 10 spectral bands were used. Although not presented in this study, we conducted additional classification analyses with both more (15, 20, and 25) or fewer (3, 5, and 8) spectral bands, and negligible classification accuracy was gained by including more than 10 spectral bands, and significant classification accuracy was lost by reducing the number of spectral bands. Thus, using the "best" 10 spectral bands was considered an optimum for this particular application. Classification accuracies of each LDA was based on independent validation, as the original 200 average reflectance profiles and germination test results from each seed species were randomly divided (using a random number function) into 80% training data and 20% independent validation data, This random division of data into training and validation data was repeated five times, and we calculated the average classification accuracy from the five randomized divisions of each data set.

3. Results

3.1. Germination of seeds

The effect of experimental ageing showed that within 0–10 days, germination of *Acacia* and *Corymbia* seeds was above 90%, but both species of seeds showed a considerable decrease in germination (%) after 10–20 or 20–30 days of experimental ageing, respectively (Fig. 2). From the onset of ageing, there was an exponential decline in germination of *Banksia* seeds, and none of the seeds germinated after 30 days of experimental ageing. Germination for all three species was below 10% after 30 days of ageing, with *B. prionotes* seeds showing the steepest decline in germination with a P_{50} of only 7.0 days (60 °C). For comparison, the P_{50} values for *A. cowleana* and *C. calophylla* seeds were 19.3 and 22.9 days respectively. Adjusted P_{50} values (45 °C) for all three species were 163.3, 59.1 and 193.3 days for *C. calophylla*, *A. cowleana* and *B. prionotes* respectively. With 25 seeds and eight sampling events (a total of 200 seeds of each species), we obtained the following numbers of germinating and non-germinating seeds: (1) *Acacia* (135 germinated and 65 non-germinated), (2) *Banksia* (86 germinated and 114 non-germinated), and (3) *Corymbia* (141 germinated and 59 non-germinated).

3.2. Seed coat reflectance of germinated and non-germinated seeds

Average reflectance profiles acquired after 5 (*Banksia*) (Fig. 3b), 20 (*Acacia*) (Fig. 3a), and 20–30 (*Corymbia*) (Fig. 3c) days of

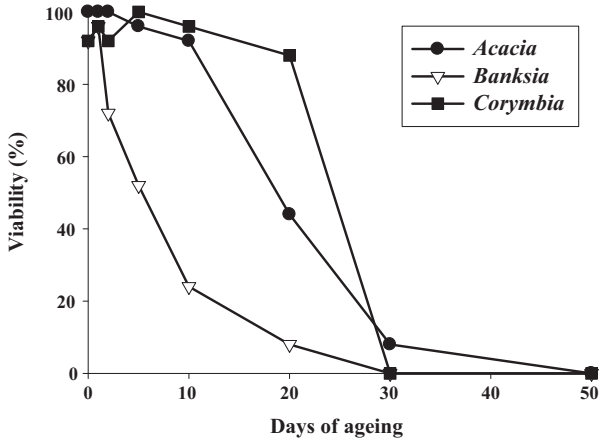

Fig. 2. Germination (%) of subsamples of 25 seeds of each species at eight time points during exposure to standardized rapid ageing conditions.

standardized rapid ageing were used to illustrate the difference in reflectance between germinating and non-germinating seeds on days with similar numbers of seeds in both categories. Average reflectance profiles from the seed coat of the three species showed similar pattern with relative reflectance values commencing around 0.10 in spectral bands near 400 nm and gradually increasing reflectance in spectral bands near 900 nm. Loss of germination caused a decrease in reflectance in *Banksia* and *Corymbia* seeds, while it caused an increase in reflectance in *Acacia* seeds. We wish to highlight that it was virtually impossible to distinguish germinating and non-germinating seeds on the basis of visual inspection. This statement is confirmed by the fact that reflectance values across the visual part of the spectrum were very similar.

Based on independent validation of LDA classifications, we found that germination of *Acacia* and *Corymbia* seeds could be classified with over 85% accuracy, while it was about 80% for *Banksia* seeds (Table 1). Regarding *Acacia* and *Banksia* seeds, we obtained similar classification accuracies of germinating and non-germinating seeds, but for *Corymbia* the classification accuracy associated with non-germinating seeds was considerably higher than for germinating seeds. We examined the relationships between days of ageing and classification accuracies, and it was revealed that the classification accuracy of (Fig. 4): (1) *Corymbia* seeds was above 80% at all time points, (2) the classification accuracy of *Acacia* seeds was above 90% in the beginning and end of the study period but was below 65% around the time point with 50% germination, and (3) *Banksia* seeds was below 80% during the time period with a marked decline in germination but around 90% in the beginning and end of the study period. In other words, seeds sampled during the gradual decrease in germination, in the transition from germination to non-germination, were generally classified with lower accuracy.

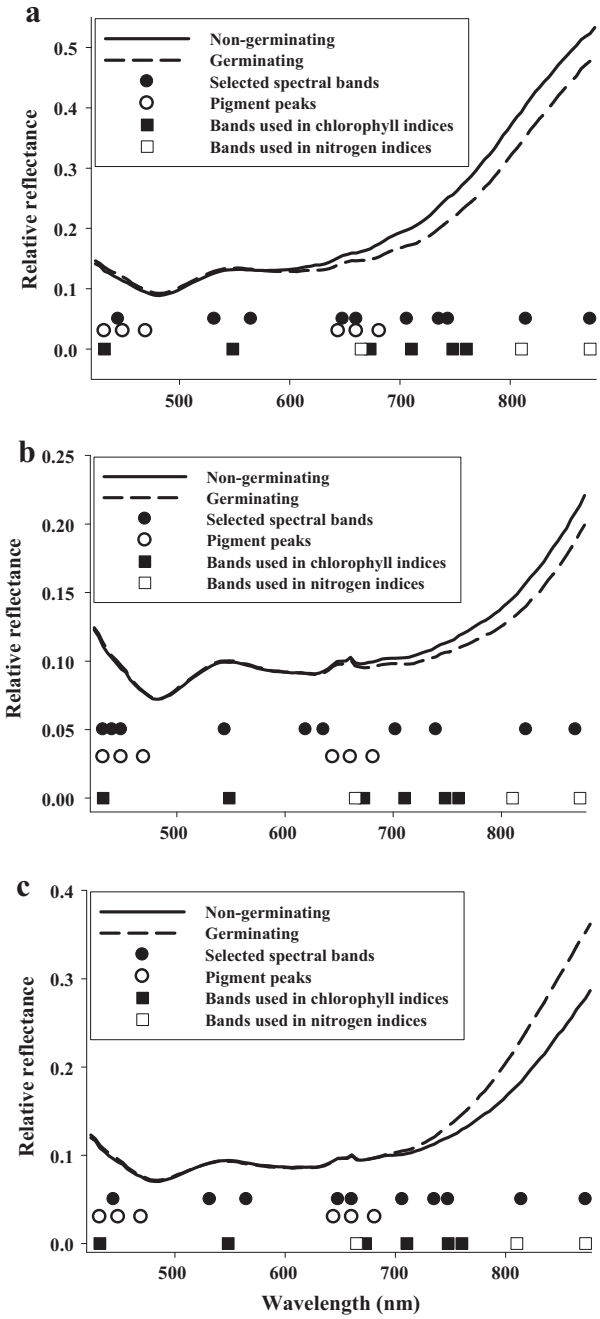

Fig. 3. Average reflectance profiles from germinating and non-germinating seeds of each species: *Acacia cowleana* Tate (Fabaceae) (a), *Banksia prionotes* L.F. (Proteaceae) (b), and *Corymbia calophylla* (Lindl.) K.D. Hill & L.A.S. Johnson (Myrtaceae) (c). Filled dots denote the wavelengths of the 10 spectral bands selected in each classification, open dots denote wavelengths of pigment peaks, and squares denote wavelengths of spectral bands used in chlorophyll and nitrogen indices.

4. Discussion

Despite growing interest in use of reflectance based methods to determine the germination of horticulture and agriculture seeds, we are unaware of any published studies involving the assessment of native seeds for conservation or vegetation restoration purposes. We demonstrated that the germination of seeds can be accurately classified (predicted) on the basis of commonly used classification methods, such as, LDA. Thus, it may be possible to replace time-consuming and destructive germination tests with non-destructive reflectance based technologies as part of improved management of seed banks. The germination of *Acacia* and *Banksia* seeds decreased from above 90% to below 20% in about 10 days of experimental ageing. The decline in germination of *Corymbia* seeds was less pronounced from over 90% to about 10% in 20 days.

Numerous studies have demonstrated successful use of reflectance-based spectroscopy as part of studies into seed germination. Shetty et al. [31] used near-infrared spectroscopy to classify viable/non-viable cabbage (*Brassica oleracea*) and radish (*Raphanus sativus*) seeds of different sizes with classification accuracies exceeding 90%. Similarly, Ahn et al. [29] used a combination of hyperspectral imaging and fluorescence lighting to discriminate between viable and non-viable Brassica seeds with over 90%

Table 1
Classification accuracy (%) with linear discriminant analysis (LDA).

Acacia cowleana			*Banksia prionotes*			*Corymbia calophylla*		
Overall	Viable	Non-viable	Overall	Viable	Non-viable	Overall	Viable	Non-viable
86.37	86.44	86.29	79.38	78.79	80.00	89.35	86.24	92.47

Overall classification accuracy is the average of five classifications of independent validation data.

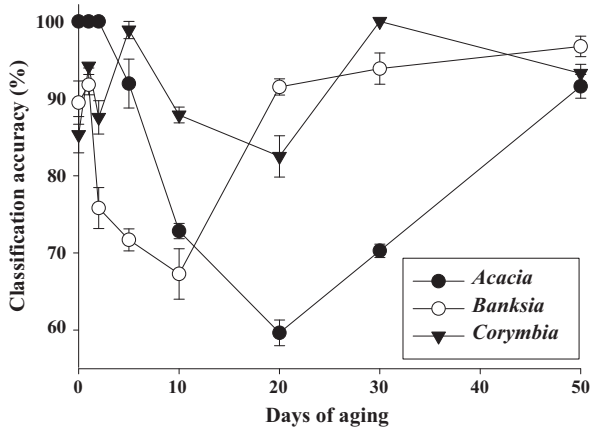

Fig. 4. Overall classification (%) (average classification accuracy of both germinating and non-germinating seeds) at each time point of exposure to standardized rapid ageing conditions.

accuracy. Ahn et al. [29] used the Fourier transform near-infrared reflectance technology technique to classify viable/non-viable water melon [*Citrullus lanatus* (Thunb.)] seeds and obtained classification accuracies also exceeding 90%. Finally, Esteve et al. [41] used near-infrared reflectance technology to detect heat and frost damage to corn (*Z. mays* L.) and to differentiate viable and non-viable corn and soybean (*Glycine max* L.) seeds. The authors concluded that only heat damage could be accurately predicted.

The exact associations between seed coat reflectance and primary seed metabolism are not known, so it is not possible to provide much more than speculations about the importance of certain changes in seed coat reflectance. However, some important insight may be gained by using knowledge gathered from reflectance studies of plant leaves. For instance, it is known that important plant pigments have maximum peaks at particular wavelengths [42,43]: chlorophyll a (430, 662, and 680 nm, chlorophyll b (448, 642 nm), and carotenoids (448, 471 nm). In addition, there is a wealth of simple two spectral band indices used to estimate chlorophyll content in leaves, including (R = reflectance): R430/R680 [44], R672/R550 [45], R710/R760 [46], R750/R550 [47]. Finally, there is a large body of research into use of reflectance-based methods to quantify nitrogen content in leaves, and these have recently been reviewed [48]. An important study analyzed the correlation between reflectance in spectral bands between R447–R1752 nm and leaf N accumulation in rice and wheat [49]. The authors found that leaf N accumulation was strongly correlated with reflectance at R660, R810, and R870 nm. We plotted the highlighted spectral bands into Fig. 3, and it is seen that the selected spectral bands in each LDA-based classification were located near known pigment peaks involved in photosynthesis and/or near spectral bands used in published indices to predict chlorophyll or nitrogen content in leaves. Thus, it appears that seed germination may be successfully classified based on reflectance in narrow spectral bands associated with the primary metabolism function and performance of plants.

Although this study has demonstrated proof of concept in the potential utilization of machine-vision systems for managing *ex situ* seed resources several questions remain. Seeds in this study were aged under artificial conditions (60 °C and 60% eRH) so the seed coat changes detected using hyperspectral analysis may not be indicative of changes that occur to seeds when stored under standard seed bank conditions. Across the plant kingdom, seeds vary greatly in colors, color patterns, shapes, and sizes. In addition, they have species-specific responses to environmental conditions. Different classification algorithms (combinations of spectral bands and discriminant coefficients) were therefore used for each of the three seed species, and successful use of this technology among other native plant species will require development of species-specific classification algorithms. Availability of machine-vision systems to automate non-destructive assessments of seed germination may greatly improve the management of seed banks in future once further more detailed assessments of the technology are undertaken. Indeed, it may also be possible that such machine-vision systems can be used in advanced research into seed dormancy and other studies of seeds and their responses to environmental conditions and thus provide fresh insights into the underlying seed biology and physiology.

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