

Fluorescein Diacetate and Orchid Seed Viability in UK and Kenyan Species



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Summary

The vital stain fluorescein diacetate (FDA) was used to assess the viability of 36 orchid species from the UK and nine from Kenya. For UK orchid seeds, there was a linear relationship between the highest recorded values for asymbiotic germination and FDA viability, with a slope close to unity. Thus, for UK orchids, the FDA test provides a fast, dependable and accurate measure of viability. In contrast, the FDA test substantially underestimated the viability of Kenyan species tested, due to problems with stain permeability. Clearly, further research is needed before the FDA test can be adopted for seed viability testing throughout the *Orchidaceae*.

Introduction

A vital aspect of conserving seed germplasm is the ability to obtain an accurate measurement of seed quality. This is usually achieved through germination testing and/or the use of vital stains, particularly tetrazolium chloride, TZ (ISTA, 1999). However, routine testing of orchid seed quality is rather more complicated than for other groups of species. Firstly, the seeds are very small; for example, many terrestrial orchids have seeds around 0.1 to 2.0 mm in length (Arditti *et al.*, 1980; Healey *et al.*, 1980) and epiphytic species can weigh in the region of 1 mg (Stoutamire, 1983). This makes assessment of topographical staining by TZ difficult. Secondly, the physiology of germination is unique, often requiring mycorrhizal association in the natural environment. Nonetheless, germination is possible under laboratory conditions on defined and complex nutrient media (Olivia and Arditti, 1985; Pritchard, 1984; 1985a), although for terrestrial species, the process can take several months to complete (Olivia and Arditti, 1985; Rasmussen, 1995). As a consequence, the development of a fast and easy method for determining orchid seed viability is highly desirable.

Tetrazolium salt is the favoured vital stain for seed quality assessment for the majority of species, particularly those that are dormant or slow to germinate (ISTA, 1999). However, particular care is needed with the interpretation of results for seeds that have not been previously tested (Justice, 1972). The method has been specifically adapted for use on Western European orchid species to include a seed sterilisation step and a 1 d soak in sterile water (Van Waes and Deberg, 1986). It has also been used to examine the viability of stored orchid seeds (Shoushtari *et al.*, 1994). In both studies, a positive correlation was observed between TZ viability and germination. However, the assessment of TZ staining in some orchid seeds can be difficult; for example,

the brown embryos of several *Epipactis* spp. can obscure the developed stain and the multifaceted seed coats of *Spiranthes* spp. present permeability problems (F. G. Prendergast, Royal Botanic Gardens, Kew, pers. comm.).

Fluorescein diacetate (FDA) has also been used to assess plant cell viability, and its potential (comparative) superiority over TZ as a viability stain has been noted (Widholm, 1972; Sedgley and Harbard, 1993). FDA is a non-polar, non-fluorescent fatty ester of fluorescein and freely enters living cells where it is rapidly hydrolysed by esterase enzymes to fluorescein. As fluorescein is a polar molecule, its rate of escape out of cells containing an intact plasmalemma is slower than the rate of entry of FDA. Thus, for a period, fluorescein accumulates and fluorescence can be easily detected using UV-blue light (Rotman and Papermaster, 1966).

FDA was first used as an indicator of viability in mammalian cells (Rotman and Papermaster, 1966) and has since been used on bacteria and algae cells (Medzon and Brady, 1969), fungi (Soderstrom, 1977), plant culture cells (Widholm, 1972), pollen (Heslop-Harrison and Heslop-Harrison, 1970), pollen tubes (Yang, 1986), stomatal guard cells (Hall *et al.*, 1996), root cortical cells (Lacaris and Deacon, 1991), pine buds (Kuoksa and Hohtola, 1991) and tree roots (Noland and Mohammed, 1997). FDA has also been used on seeds, including green ash (Noland and Mohammed, 1997), jack pine and black and white spruce (Noland *et al.*, 2001) and orchids (Pritchard, 1985b; Pritchard and Prendergast, 1990; Batty *et al.*, 2001; Wood and Pritchard, 2003).

The microscopic nature of orchid seeds, such that embryos frequently contain less than 100 cells, means that whole orchid embryos may be especially suited to viability testing by FDA. Previous studies have been limited to just a few species, and there is, therefore, no evidence that the test can be applied with validity across the family. Here, we extend orchid FDA studies to the seeds of nine Kenyan and 36 UK species, including *Cypripedium calceolus* L. which is considered to be critically endangered in the wild (IUCN/SSC Orchid Specialist Group, 1996). In addition, we also consider the effects of seed pre-sterilisation on FDA analysis. Orchid seeds are routinely sterilised prior to asymbiotic germination *in vitro*, thus maintaining sterility (Olivia and Arditti, 1985; Pritchard, 1985b; Pritchard *et al.*, 1999; Rasmussen, 1995), but also stimulating germination in some species (Stoutamire, 1963; Van Waes, 1984; Purves and Hadley, 1976; Arditti, 1982). Although, earlier orchid – FDA studies involved seed sterilisation prior to FDA addition (Pritchard, 1985b), the effects of sterilisation time on test results were not assessed.

Materials and Methods

1. Seed Pre-Treatments

Seeds from dehisced capsules were stored, after equilibrium to c. 15% relative humidity (RH), 15°C, at either 5°C (*Dactylophiza fuchsii*) or -20°C (UK and Kenyan bank collections; Table 26.1)), in hermetically sealed screw-cap aluminium vials.

Initially, dry seeds of *Dactylophiza fuchsii* (Druce) Soó were re-hydrated over distilled water for up to 48 h at 20°C in sealed containers and then FDA stain development followed. Based on the results, all following experiments used a 3 h re-hydration period prior to analysis.

To determine the effect of sterilisation on FDA staining, *D. fuchsii* seeds were re-hydrated for 3 h and then surface sterilised for 20 min in filter-paper packets (Pritchard *et al.*, 1999) using 10% commercial bleach solution, followed by three washes in sterile distilled water.

2. FDA Analysis

Following re-hydration over distilled water, seeds were sandwiched between two microscope slides in a drop of water immediately prior to FDA analysis (Pritchard, 1985b). The slides were then gently rotated in opposite directions to rupture the membranous testa and to isolate the embryo. This was attempted on all species investigated.

FDA was prepared at 0.5% (w/v) in absolute acetone (Widholm, 1972) and mixed, on a slide, 1:1 (v/v) with either isolated embryos (UK species) or intact seeds (Kenyan species) in distilled water (for explanation, see Results). Stain development in *D. fuchsii* was assessed after periods up to 24 h, the optimum being 6 h, and this period was used in all other tests.

Staining was viewed under UV light using a Nikon Diaphot inverted microscope (Nikon Instruments, Surrey, UK) with an epi-fluorescent attachment TMD-EF. Only embryos exhibiting fluorescence over their entirety, when viewed at $\times 100$ magnification, were considered to be positively stained (Figure 26.1A). In all cases, two assessments were made on > 100 isolated embryos. Still photographs were taken using Kodak Ektachrome 64T film (100 ASA); the exposure time was typically 20 s. Background fluorescence was determined by comparing the staining intensity of a 2.5 mm area immediately surrounding the isolated embryo, to that of the embryo itself. If the surrounding fluorescence was greater than that of the embryo, a score for background fluorescence was recorded, enabling the percentage of embryos exhibiting background fluorescence to be determined.

3. Seed Germination

Seeds were surface sterilised, as previously described. Seeds of UK and Kenyan species were then sown onto solid (1% agar) Norstog (Norstog, 1973) or Phytamax (Sigma-Aldrich Co. Ltd., Poole, UK) nutrient media, respectively, in sterile 9 cm-diameter Petri dishes. All media components were of analytical grade and sterilised by autoclaving at 121°C for 20 min. Sowing operations were performed in a laminar flow cabinet. For UK species, sealed dishes containing seeds were enclosed in two layers of aluminium foil (to exclude light) and incubated at $21 \pm 2^\circ\text{C}$. Kenyan species were incubated at $25 \pm 1^\circ\text{C}$ in sealed dishes under light (12 h photoperiod). For UK species, germination was assessed every 4 weeks for up to a year, under at least $\times 25$ magnification. Germination of Kenyan species was recorded every week, over a four month period, also at $\times 25$ magnification. Seeds were considered to have germinated when the embryo had enlarged sufficiently to emerge from the testa, i.e., forming a spherical protocorm. Germination is presented as the percentage of the total number of full seeds sown per treatment, based on two replicates of c. 100 seeds.

4. Statistical Analysis

To compare the slopes of linear regressions of germination and FDA results (Figure 26.3) with a null line of slope one, the following *t*-test was used (Sokal and Rohlf, 1995):

$$t_s = b - H/SE_b$$

where *H* is the slope of the null line, *b* is the slope of the regression line and SE_b is the standard error of *b*, and t_s was tested against $t_{0.05 [n - 2]}$.

Results and Discussion

The vast majority of microscopic seed maintained as *ex situ* collections within the Millennium Seed Bank are represented by the *Orchidaceae*, amounting to > 50 species. Although the geographical coverage of these collections is wide, most come from the UK and Kenya (see Table 26.1).

When stained with FDA, viable cells within orchid embryos were characterised by yellow fluorescence emitted from the cell cytoplasm (brightness in Figure 26.1A). As previously observed (Pritchard, 1985b), non-viable embryos (Figure 26.1B) exhibited no or little staining, indicating that the esterase enzymes responsible for the conversion of FDA to fluorescein are only active within the cytoplasm of viable cells (Rotman and Papermaster, 1966).

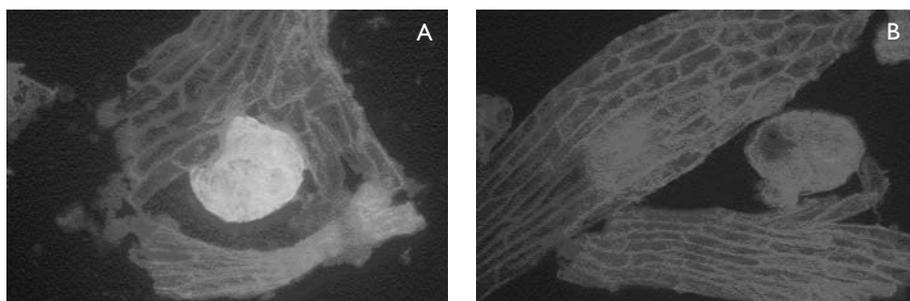


Figure 26.1 Comparison between fluorescence after FDA staining of *Dactylorhiza fuchsii* seed embryos when viable (A) and inviable (B). Ruptured seed testas are clearly visible. Photographic exposure was 20 s, microscope magnification was $\times 100$ and embryos were c. 150 μm long.

Table 26.1 List of Kenyan and UK species used in the germination and FDA tests

Kenyan Species	UK Species (continued)
<i>Angraecopsis breviloba</i> Summerh.	<i>Epipactis helleborine</i> (L.) Crantz
<i>Chamaeangis odoratissima</i> Schltr.	<i>Epipactis leptochila</i> Godfrey
<i>Chamaeangis sarcophylla</i> Schltr.	<i>Epipactis phyllanthes</i> G.E.Sm.
<i>Cribbia brachyceras</i> (Summerh.) K.Senghas	<i>Epipactis purpurata</i> Sm.
<i>Diaphanthe lorifolia</i> Summerh.	<i>Goodyera repens</i> (L.) R.Br.
<i>Habenaria malacophylla</i> Rchb.f.	<i>Gymnadenia conopsea</i> (L.) R.Br.
<i>Liparis bowkeri</i> Harv.	<i>Hammarbya paludosa</i> Kuntze
<i>Polystachya bennettiana</i> Rchb.f.	<i>Herminium monorchis</i> R.Br.
<i>Polystachya cultriformis</i> Lindl. ex Spreng.	<i>Himantoglossum hircinum</i> Spreng.
UK Species	<i>Listera ovata</i> (L.) R.Br.
<i>Aceras anthropophorum</i> f. <i>purpurata</i> M.Balayer	<i>Neottia nidus-avis</i> (L.) L.C.Rich.
<i>Anacamptis pyramidalis</i> (L.) L.C.Rich.	<i>Ophrys apifera</i> Huds.
<i>Cephalanthera damasonium</i> Druce	<i>Ophrys fuciflora</i> (F.W.Schmidt) Moench
<i>Cephalanthera longifolia</i> Fritsch	<i>Ophrys sphegodes</i> Mill.
<i>Coeloglossum viride</i> Hartm.	(Orchis) <i>Anacamptis laxiflora</i> (Lam.) R.M. Bateman, A.M. Pridgeon & M.W. Chase
<i>Corallorhiza trifida</i> Chatel.	<i>Orchis mascula</i> L.
<i>Cypripedium calceolus</i> L.	<i>Orchis militaris</i> L.
<i>Dactylorhiza fuchsii</i> (Druce) Soó	(Orchis) <i>Anacamptis morio</i> (L.) R.M. Bateman, A.M. Pridgeon & M.W. Chase
<i>Dactylorhiza incarnata</i> (L.) Soó	<i>Orchis ustulata</i> L.
<i>Dactylorhiza lapponica</i> (Laest. ex Hartm.) Soó	<i>Platanthera bifolia</i> (L.) L.C.Rich.
<i>Dactylorhiza maculata</i> (L.) Soó	<i>Platanthera chlorantha</i> Custer ex Rchb.f.
<i>Dactylorhiza praetermissa</i> (Druce) Soó	<i>Pseudorchis albida</i> (L.) A. & D.Löve
<i>Dactylorhiza purpurella</i> (T. & T.A. Stephenson) Soó	<i>Spiranthes spiralis</i> (L.) Chevall.

1. Seed Re-Hydration, Embryo Isolation and Stain Development

The importance of seed rehydration before stain application was assessed. For *D. fuchsii* seeds dried to 4% moisture content, < 30% of embryos tested positive when FDA solution was added directly to the dry seeds (data not shown). This problem of poor staining of embryos in whole seeds could be due to numerous reasons. Firstly, the seeds could be sensitive to rapid imbibition. If this were the case, cold temperatures in particular would exaggerate the effect (Tilden and West, 1985). However, seeds of *D. fuchsii* and *Dendrobium anosmum* Lindl. have been shown to be relatively unaffected by the temperature of the rehydration solution (Pritchard *et al.*, 1999).

A second reason might be the poor permeation of the stain through the testa and into the embryo. If this were the case, then the isolation of embryos would be essential for effective stain development. For all UK species tested, embryo isolation was possible, resulting in good stain development in viable material (Figures 26.1A and 26.3). However, for all the Kenyan species, it was not possible to rupture the seed testa prior to FDA analysis by rolling the seeds between glass slides. Consequently, stain development was poor, and in comparison with *in vitro* germination, staining substantially underestimated seed viability (Figure 26.3). Alternative methods to breach the testa, such as mechanical vibration (Uchida and Yamamoto, 2002), may need to be employed on such material.

Finally, there may be the need for the seeds to reach a critical moisture content for the instigation of metabolism (see Vertucci and Farrant, 1995), and thus stain conversion. Figure 26.2 shows that stain development in *D. fuchsii* seeds was improved by the use of a 3 h rehydration period prior to FDA analysis.

2. Background Fluorescence and Seed Sterilisation

Both seed hydration time and stain development time influenced the interpretation of the FDA test. Immediately after the application of the stain to *D. fuchsii* embryos, there was a consistently high degree of background fluorescence surrounding c. 40% of embryos (Figure 26.2). Moreover, pre-sterilisation approximately doubled the level of background fluorescence (Figure 26.2), making determinations of stained embryos particularly difficult. Background fluorescence did diminish over a period of c. 6 h (Figure 26.2), and this response was independent of seed pre-hydration time. Presumably this extra-cellular fluorescein lost its fluorescence properties, perhaps by being converted to a non-fluorescent chemical in the slide solute.

Embryos isolated from both sterilised and non-sterilised seeds tended to retain their staining over the same (6 h) time period. However, whilst *in vitro* germination of sterilised seed was $90 \pm 6\%$, FDA staining was only $72 \pm 12\%$. By comparison, $88 \pm 6\%$ of embryos from non-sterilised seed stained with FDA. Even though a one-way ANOVA revealed no significant ($P > 0.05$) effect of

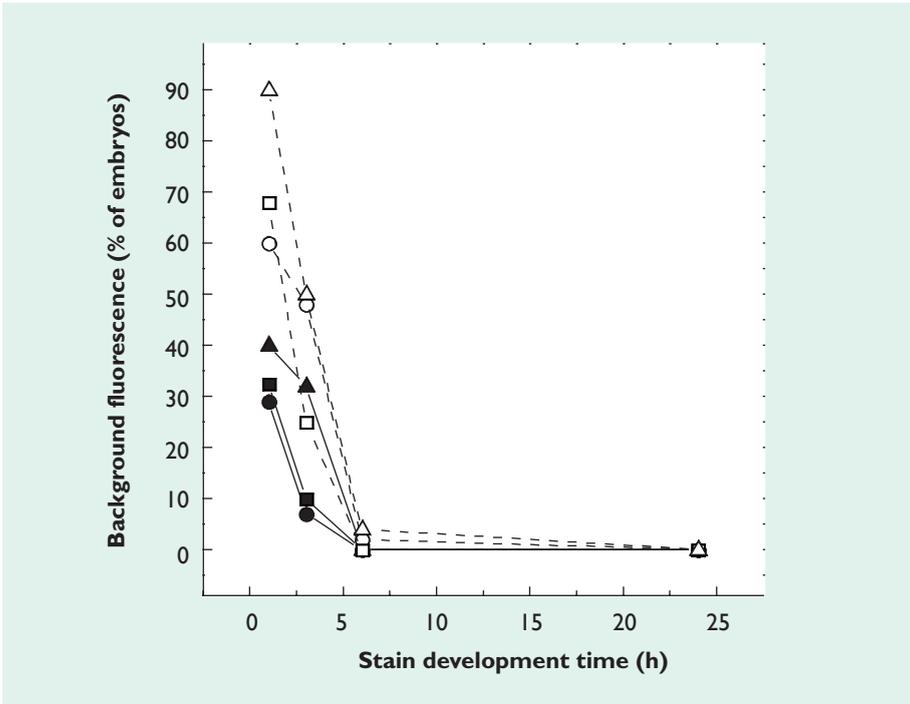


Figure 26.2 The effect of stain development and re-hydration time on the percentage of *Dactylophiza fuchsii* seed embryos surrounded by background fluorescence. Results are presented for sterilised (open symbols) and non-sterilised seed embryos (closed symbols) in combination with re-hydration times of (●) 0 h, (■) 1 h and (▲) 3 h.

sterilisation on the level of embryo staining, this lower value might indicate a modest level of stress to the embryo as a result of the sterilisation procedure. For example, the chlorine solution may have weakened the plasmalemma of the embryo cells, making them leak fluorescein, thereby contributing to the extra background fluorescence (Figure 26.2). Similar detrimental effects of sterilisation have been observed in other membrane systems, including erythrocytes (Zandovik *et al.*, 2000), reverse-osmosis cellulose-acetate membranes (Han and Bhattacharyya, 1991) and polysulfone dialyzers (Kaplan *et al.*, 1995, Kunas *et al.*, 1996; Scott *et al.*, 1999). It is worth noting however, that germination of orchid seeds is generally not adversely affected by pre-sterilisation (Figure 26.3; Pritchard, 1985b; Rasmussen, 1995).

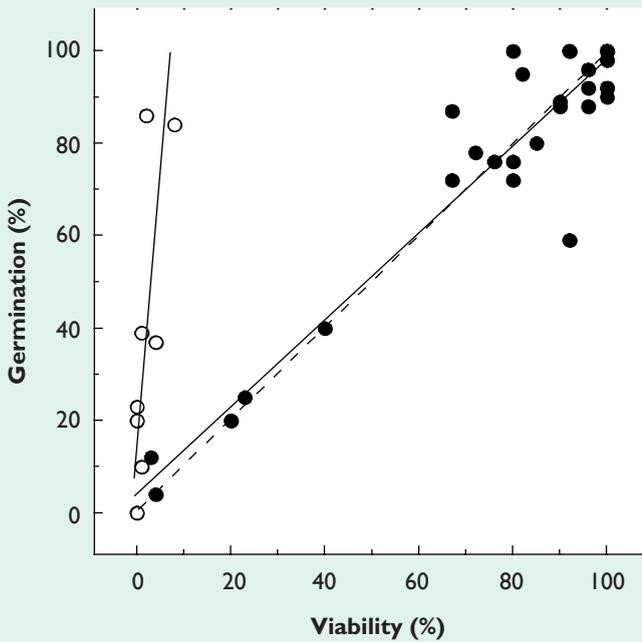


Figure 26.3 A comparison between seed germination and viability for 36 UK (●) and 9 Kenyan (○) orchid species. The equations of the fitted lines are $y = 5.76 + 0.929x$ and $y = 20.8 + 8.23x$, respectively. All tests were based on two replicates of at least 100 individual measurements. Error bars are omitted for clarity but in all cases the standard error of the mean was less than 12%. Results are based on the best germination performance for each species. The dashed line is a null line of slope = 1.

3. FDA versus Germination: Inter-Specific Variability

To determine the accuracy of the viability assessment obtained by FDA staining, a comparison was made with germination capacity in 36 UK and nine Kenyan terrestrial species (Table 26.1). For UK species, there was a highly significant correlation ($R^2 = 0.81$; $P < 0.001$) between seed germination and embryo FDA viability, with a slope that was not significantly different ($t = 0.004$, $d.f. = 34$; $P > 0.05$) from a null line (of slope = 1) (Figure 26.3). There was also a significant correlation ($R^2 = 0.46$; $P = 0.028$) between seed germination and embryo viability for the Kenyan species; however, the regression line slope was 8.23 and significantly ($t = 11.12$, $d.f. = 7$; $P < 0.001$) different from the null line. Thus FDA analysis significantly underestimated seed viability in the Kenyan species. As noted above, it was not possible to roll the embryos of Kenyan species out of the seed testa, unlike UK species.

Examination of seeds under a light microscope ($\times 100$ magnification) showed that the testa of the Kenyan orchid seeds investigated here was considerably thicker than that of all UK species and appeared to consist of several cellular layers. As a consequence, the passage of FDA to the embryo was likely hindered by the testa, resulting in lower staining results compared to those of *in vitro* germination. More detailed studies on embryo morphology/anatomy in Kenyan orchids seems to be warranted.

Conclusion

The results indicate that the FDA test can be a simple, rapid and accurate means of estimating orchid seed viability in more than 70% of UK species. However, FDA stained seeds are unable to subsequently germinate on growth media (data not shown). Thus we concur with the views of Noland and Mohammed (1997) and Batty *et al.*, (2001), that FDA analysis and routine germination studies should be used in parallel, employing separate seed samples for each test. The FDA test has not yet proved successful with Kenyan material, probably as a result of the physical properties of the seed testa. It is not clear how representative Kenyan orchids are of tropical, and in particular, dryland species. Clearly, further research is needed to assess the usefulness of the FDA test on a wider range of tropical orchids.

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