Table 3. Effect of desiccation on seed germination of *P. pinnata*

Period (days)	Moisture content (%)	Seed viability: Germination (%)	Seed viability: TTC staining pattern	Vigour index*	Days for final count
Control	14.32	100.0 ± 2.21	100	2698	15
2	8.32	100.0 ± 2.17	100	2584	18
7	6.95	95.7 ± 1.89	95	2549	20
15	4.97	93.2 ± 2.21	92	2288	22
20	3.67	90.0 ± 2.10	90	2223	28
28	3.39	90.0 ± 2.21	90	2200	30

*Vigour index = Germination % × (root length + shoot length).

Table 4. Effect of chilling on seed viability of *P. pinnata*

Storage temperature	Storage duration (months)	Germination (%)
Control (fresh seeds; mc 14.3%)	–	100
Desiccated seeds (mc 6.0%)	–	95
+4°C	3	90
–20°C	3	90

mc = moisture content.

the subsequent days. Seed viability of 90–95% was maintained at all the desiccation levels. The seeds took about 28 days to achieve lowest moisture content of 3.39% (critical moisture), at which viability was maintained up to 90%. The vigour index followed a similar trend (Table 3). The patchy and less TTC staining pattern appears to correlate with progressive decline in seed viability and seed deterioration indicated by loss in seedling vigour with the advancement of desiccation, reflecting the efficacy of this technique in assessment of seed viability and quality of *Pongamia* seeds (Table 2). This result is in conformity with earlier observations[12], suggesting that the seed deterioration is associated with the loss of vigour. On desiccation, the germination period was extended to 22–30 days compared to 15 days in the case of control, indicating induction of dormancy as the seeds progressed to maturity under desiccation. The dried seeds maintained 80–100% viability when exposed to low temperature (4°C and –20°C) for different durations (Table 4). No significant adverse effect of desiccation and chilling on viability was observed (90% germinability) even at 6.0% moisture and at –20°C, confirming the orthodox nature of *Pongamia* seeds (Table 4).

Higher seed moisture content (40–60%), large seed size and weight have often been associated with recalcitrant storage behaviour of seeds (weight of 1000 seeds exceeding 500 g)[13]. However, *Pongamia*, despite having tropical/subtropical habitat and large and heavy seeds with moisture content of about 14% on fresh-weight basis, is not sensitive to desiccation and chilling. Therefore it can be stored normally with drying of seeds to 5–10% moisture level under low temperature conditions according to the objectives.

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Secondary emissions from spectrofluorimeters

Heisenberg's uncertainty principle states that when a wave of frequency λ is generated in a finite space, it is accompanied by its overtones (2λ, 3λ, 4λ, etc.) and undertones ($\lambda/2$, $\lambda/3$, $\lambda/4$, etc.). According to eigenfunctions, the intensity of these harmonics dies out exponentially. As a result, only the first or second harmonics of several-fold lower intensity are of significance[1,2]. A description of the harmonics obtained from a two-channel digital real-time oscilloscope (Model Tektronix TDS 220) shows that when a wave of 2555 Hz is generated, it is accompanied by frequencies of 5110 and 7665 Hz, which represent the first and the second overtones respectively. The ratio of their intensities is 100 : 1.73 : 0.27 (Figure 1). Thus the uncertainty in generating monochromatic light cannot be solved by improving instrumentation.

Presence of harmonics in a wave being universal, we have investigated whether the overtones and undertones generated at the excitation window of spectrofluorimeters are perceivable. If so, additional Rayleigh scattering and fluorescence emission, depending on the spectral property of the test sample, will be observed. In a spectrofluorimeter where the excitation and emission wavelengths are the same, Rayleigh scattering is obtained. These observations could be made if the samples are scanned between the entire spectral zone of the instrument, i.e. 200–800 nm when a xenon lamp source is used.

The following reagents were obtained from Sigma; N-acetyltryptophan amide (NATA), acrylamide, 1-anilino 8-naphthelene sulphonic acid (ANS, a nonspecific hydrophobic fluorophore for proteins), bovine serum albumin (BSA, fraction III), DNA (calf thymus, Type I), ethedium bromide (EtBr, fluorescence interchelator for DNA), fluorescein isothiocyanate I-Celite mixed isomers (FITC), rhodamine B isothiocyanate-Celite mixed isomers (RITC), NADH and KI. UDP-galactose 4-epimerase (epimerase) was purified from *Kluyveromuces fragilis*[3]. Distilled water was purified through high performance reverse osmosis purification system (Arium 61315, Sartorius).

Epimerase (100 µg in 250 mM Na-carbonate, pH 9.2) was treated with 0.5 mg FITC or RITC for 30 min at 25°C in the dark. Unreacted reagent was removed from the fluorophore-conjugate by passing through a Sephadex G-50 spin column[4]. Binding of BSA (1 mg/ml) with ANS (100 µM) was allowed in 50 mM Na-phosphate, pH 7.5. Quantum yield being low, the unbound reagent was not separated from the protein conjugate[5]. A Hitachi F 4500 spectrofluorimeter was used with excitation and emission wavelength ranging between 240–600 and 240–800 nm respectively. Excitation and emission slit widths were of variable combinations of 2.5, 5 and 10 nm. Sampling interval and scan speed were 5 nm (for both ex. and em.) and 12,000 nm/min respectively.

Fluorescence quenching data were analysed using the Stern–Volmer or Lehrer equation[6]. No corrections for the inner filter effect were made because of low absorbance of the fluorophores at the excitation or emission wavelengths. Absorption profile of the microscope glass slide used as UV-filter was generated by an Analytical Jena Specord 200 recording spectrophotometer in the range 190–1100 nm. A sharp drop of absorbance from >2.000 (190–310 nm) to 0.001 (360–1100 nm) was observed. Absorbance between 310 and 360 nm at an interval of 10 nm was: 2.214, 1.010, 0.399, 0.144, 0.027 and 0.001. In the text, normal fluorescence has been referred to those emissions for which a fluorophore is widely referred in the literature.

In this analysis, one group of fluorophores has been selected, e.g. NATA, NADH and BSA–ANS complex, where normal excitation wavelengths are relatively short so that the first overtone is likely to be observed within the emission zone of the instrument (Group A). Conversely, these fluorophores may provide normal emission when excited at the first overtone. The second group consists of fluorophores where the normal excitation wavelengths are relatively long and therefore, the first undertone is likely to be observed. Epimerase–FITC, epimerase–RITC and DNA–EtBr complexes belong to this group (Group B). Emission scans of one from each group have been presented in Figure 2 *a* and *b*. Spectral parameters of the fluorophores have been summarized in Table 1. Emissions from aromatic residues of FITC and RITC–epimerase conjugates after near-UV excitation have not been included in Table 1.

Features of Table 1 are: observance of Rayleigh scattering at the excitation wavelength together with scattering at the first overtone or undertone as applicable. Intensity of the secondary scatterings was low, as they are reduced exponentially. For overtones, relatively lower intensity was due to scattering being inversely proportional to the fourth power of the wavelength of irradiation[7]. These data also show fluorescence at unaltered emission maxima when excited not only at normal wavelength, but also at the first overtone (Figure 2 *a*) or undertone (Figure 2 *b*). It may be noted that in Figure 2, depending on the wavelength of excitation, slit widths and emission scales were adjusted to obtain near normalized profiles.

Comparison of data in Table 1 showed that the first overtone and undertone were observed within ± 1.7 nm, except for Group B fluorophores where the first overtone appeared at <+10 nm. The wider deviation was an artifact as emissions

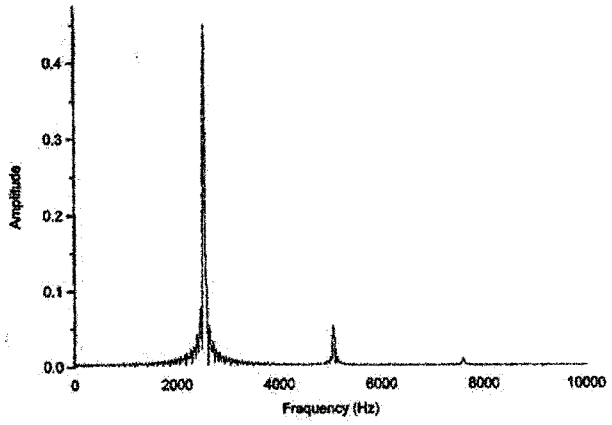

Figure 1. Presence of first and second overtones of frequencies 5110 and 7665 Hz while generating a wave of 2555 Hz using an oscilloscope.

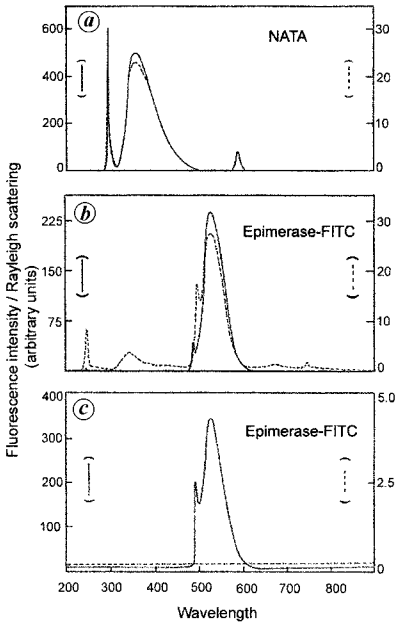

Figure 2. *a*, Emission from NATA. (—), ex: 295 nm, ex/em slit widths: 5/5 nm; (····), ex: 590 nm, 10/10 nm. *b*, Emission from epimerase–FITC. (····), ex: 245 nm, 3/3 nm; (—), ex: 490 nm, 3/3 nm. *c*, Emission from epimerase–FITC after excitation at 245 nm (····) and 490 nm (—) having a glass filter between the light source and the sample.

Table 1. Spectral characters of fluorophores excited at different wavelengths

		Normal emission					Additional emission		
			Fluorescence					Fluorescence	
Fluorophore	Ex (nm)	Rayleigh scattering (nm)	$\lambda_{max, em}$ (nm)	Intensity (slit width ex, em in nm)	Over-tone	Under-tone	Rayleigh scattering (nm)	$\lambda_{max, em}$ (nm)	Intensity (slit width ex, em in nm)
Group A									
NATA	295	295.6 ± 0.4	353.0 ± 1.4	939.0 ± 11 (5/10)	√		589.7 ± 0.7	NA[a]	NA
	590	589.6 ± 0.4	NA	NA		√	296.3 ± 0.3	353.0 ± 3.2	5.34 ± 0.08 (5/10)
NADH	340	341.1 ± 0.7	455.5 ± 0.9	1140 ± 26 (10/5)	√		679.0 ± 1.8	NA	NA
	680	680.4 ± 0.6	NA	NA		√	340.2 ± 1.2	456.0 ± 3.8	28.2 ± 1.1 (10/5)
ANS–BSA	375	376.4 ± 0.5	469.5 ± 0.8	3972 ± 11 (5/5)	√		749.8 ± 1.2	NA	NA
	750	751.2 ± 0.6	NA	NA		√	375.4 ± 1	467.6 ± 1.4	294.9 ± 0.2 (5/5)
Group B									
Epimerase–FITC	490	491.5 ± 0.1	521.7 ± 0.1	235.9 ± 1.1 (3, 3)		√	246.2 ± 0.2	351.0 ± 1.2	0.28 ± 0.01 (3/3)
	245	249.6 ± 0.2	344.3 ± 0.3	4.73 ± 0.07 (3, 3)	√		499.2 ± 0.2	521.9 ± 0.7	26.9 ± 0.6 (3/3)
Epimerase–RITC	570	571.6 ± 0.4	654.7 ± 1.3	46.6 ± 3.2 (10, 10)		√	286.7 ± 0.1	342.9 ± 0.5	199.1 ± 14.6 (10/10)
	285	289.4 ± 0.2	343.3 ± 0.1	2190 ± 257 (10, 10)	√		580.3 ± 0.3	657.3 ± 0.5	449.6 ± 29.9 (10/10)
DNA–EtBr	545	547.4 ± 0	586 ± 0.6	99.3 ± 0.3 (10/10)		√	274.1 ± 0.1	342.0 ± 0.2	3.6 ± 0.1 (10/10)
	273	278.3 ± 0.3	340.6 ± 0.2	52.5 ± 0.5 (10/10)	√		557.7 ± 0.3	583.6 ± 0.2	302.3 ± 1.5 (10/10)

NA, Not applicable; √, Overtone or undertone as applicable; n = 2–4.
Group A and Group B fluorophores have been classified based on their wavelength of excitation between 250–400 nm and 500–600 nm.

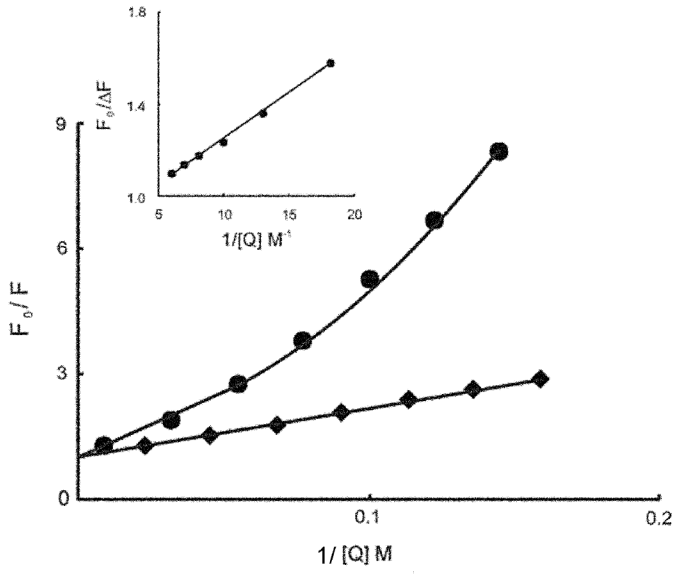

Figure 3. Stern–Volmer plot from secondary emission. NATA was excited at 560 nm and emission at 340 nm was measured in the presence of quenchers like acrylamide (♦) or KI (•) at the concentrations indicated. (Inset) Leher plot of the zone that showed nonlinearity in the Stern–Volmer plot. F and F_0 indicate emission intensities in the presence and absence of quenchers, where $\Delta F = F_0 - F$.

from these fluorophores were too close to their excitation wavelength. As a result, Rayleigh scattering from these overtones partly superimposed with fluorescence, leading to its apparent red shift. Table 1 also shows that variations of emission maxima were ± 2.5 nm when excited either at normal wavelength or first harmonics, e.g. in the case of NATA, excitation at 295 and 590 nm yielded scattering from the first harmonics at 589.7 ± 0.7 and 296.3 ± 0.3 nm respectively. Corresponding emission maxima between 300 and 500 nm were 353 ± 1.4 and 353 ± 3.2 respectively (n = 4). Low emissions from the harmonics were consistent with their low intensity. Exact quantification was difficult as variable slit widths were often used to get accurate signals.

Spectrofluorimeters are operated between 200 and 800 or 900 nm, and excitation below 240 nm is frequently interfered by absorption from solvents or impurities from reagents. Thus, scattering from the second overtone could only be demon-

strated between 720 and 795 nm while exciting between 240 and 265 nm. Fluorophores having emission between 720 and 795 nm being rare, the second overtone is difficult to demonstrate. For similar reasons, the effect of the first undertone could be demonstrated only for those fluorophores that absorb effectively between 480 and 750 nm. Thus, it remains virtually impossible to demonstrate the effect of the first overtone and undertone of excitation together in the spectral zone covered by spectrofluorimeters. The harmonic intensities being low, secondary emissions could only be detected from sensitive fluorophores under favourable instrumental conditions like large slit width and high concentration of fluorophores. In this study, Rayleigh scattering has been considered all along with fluorescence data because scattering indicates the presence of radiation of that wavelength.

Fluorescence quenchers were applied to study the nature of emission from excitation harmonics. NATA was excited at 560 nm (first overtone of 280 nm) and the effect of KI (static quencher) and acrylamide (dynamic quencher) in the emission zone 300–400 nm was studied. Corresponding Stern–Volmer and Lehrer plots (where deviation from linearity was observed in the Stern–Volmer plot) were constructed. Linear dependencies of quenching parameters with inverse of quencher concentrations demonstrated that the short wavelength emission was indeed fluorescence by nature (Figure 3).

When a glass slide is placed between the excitation monochromator and the sample, UV-radiation between 200 and 340 nm is largely absorbed offering a convenient way to filter majority of the undertones. Epimerase–FITC was scanned between 220 and 800 nm after excitation at 245 and 490 nm in the presence of the filter. The former was devoid of all scatterings and emissions, while the latter showed scattering at 490 nm as well as emission between 520 and 600 nm, but no scattering or emission related to excitation at 295 nm (Figure 2 *c*). For other fluorophores, the glass filter had similar effects without exception. This confirmed that the harmonics were generated at the light source and not related to the samples.

Spectrofluorimeter users should be cautious about the stability of test samples, particularly with undertones, irrespective of their detection. As samples are rarely scanned below their excitation wavelengths or above normal emission zones, incidents related to harmonic waves remain unnoticed. There are several examples of damage of biomolecules or initiation of chemical reactions related to UV-irradiation, e.g. formation of thymine dimers in DNA and RNA resulting in the inhibition of replication, and dissociation of disulphide bonds affecting tertiary structures leading to denaturation of proteins. Decarboxylation, deamidation and breaking of ring structures are also common in UV-absorbing biomolecules, e.g. amino acids (tyrosine, tryptophan, phenylalanine, cysteine), nitrogen-containing rings (pyridine, imidazole, pyrimidine, cytosine, thymine, uracil), six-membered carbon rings, double, triple and quadruple rings and other cofactors, vitamins, and steroids (NADH, quinines, vitamin A and K, phterins, flavins and porphyrins)[8–10]. In the extreme or vacuum UV, i.e. below 190 nm, the damaging effect however remains insignificant as water and air absorb radiation in that zone[11].

Considering the usual practice of excitation of fluorophores between 280 and 600 nm, the first overtone extends from the visible to infrared zone, where the energy of radiation is low. Nevertheless, many photochemical reactions are induced by radiation at that wavelength, e.g. photolysis, elimination reaction, reduction, oxidation, *cis-trans* isomerization, rearrangement, cyclization, intramolecular cyclo-addition, etc.[12,13]. The overtone in particular may interfere with fluorescence resonance energy transfer (FRET) analysis, where the combination of acceptor–donor covers an extended zone of emission. FRET between tryptophan (ex: 280 nm, em: 300–400 nm) and NADH (ex: 340 nm, em: 400–600 nm) or dansyl chloride (ex: 335 nm, em: 400–600 nm) is likely to be interfered by the first overtone at 560 nm.

The additional Rayleigh scattering and fluorescence do not yield additional information over the normal spectrum, but explain several distinct emissions in analyses such as three-dimensional excitation–emission matrix contour plots (ex: 240–600 nm, em: 240–800 nm, using Hitachi F-4500 spectrofluorimeter). These profiles compulsorily include scatterings from the first overtone and undertone and also fluorescence emerging from the undertone[14,15]. However, the instrument manufactures do not provide a clear description of the origin of these signals[16].

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