Effect of supplementary ultraviolet-B radiation on young wheat seedlings

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Effect of supplementary UV-B radiation (1 mW m^{-2}) on photosynthesis, flavonoid content and anatomical changes was studied in young wheat leaves. It was observed that supplementary UV-B radiation did not affect PS II activity, assayed as water to phenylenediamine. However, PS I activity, assayed as reduced dichlorophenol indophenol to methyl viologen, showed an increase. UV-B treatment resulted in qualitative and quantitative changes in UV-B absorbing phenolic compounds. Flavonol (Kaempferol) and caumarin showed quantitative increase due to supplementary UV-B exposure to the wheat leaves. Synthesis of cinnamic acid was observed only after 4 days of UV-B treatment. UV-B treatment resulted in significant anatomical changes in the leaves. There was a massive increase in the cutin synthesis. Epidermal cells were largely destroyed, while hypodermal cells were seen to replace the epidermal cells.

HIGH fluence rate of short wavelength (<320 nm) reaching the earth is increasing due to ongoing depletion of stratospheric ozone layer[1]. Plants use sunlight for photosynthesis and as a consequence are exposed to the ultraviolet radiation. The enhanced levels of UV-B radiation induce damage to various plant processes, particularly resulting in lowered photosynthesis and inhibited growth[2]. A large number of plants exhibit UV-B response, but the photoreceptor responsible for important biochemical parameters such as photosynthesis remains to be conclusively identified[3–6]. The damages due to UV-B radiation can be classified into two categories: damage to DNA (it can cause heritable mutations) and damage to physiological processes such as photosynthesis and proteins, subsequently resulting in decline in plant productivity[7].

To protect itself against enhanced UV radiation, plants have developed protective responses which may prevent damage to the various physiological processes. UV-B-absorbing phenolic compounds (flavonoids) are thought to protect photosynthetic tissue by acting as screening pigments[8,9]. Besides, anatomical changes may occur to reduce the level of UV-B reaching the susceptible site(s).

In this study, we report the effect of UV-B radiation on photosynthesis (PS II activity), qualitative and quantitative changes in UV-B absorbing compounds (flavonoids and other phenolic compounds) and anatomical changes that were observed in wheat leaves in response to UV-B treatment.

Wheat (*Triticum aestivum* L. cv. HD 2380) seeds from Indian Agricultural Research Institute, New Delhi, were grown in 10 cm × 10 cm plastic pots containing vermiculite, and bottom-irrigated routinely using 1/2 strength Hoagland's solution. Plants were grown in a growth chamber illuminated with incandescent bulbs and fluorescent tubes for 12 h photoperiod having a photon flux density (PFD) of 200 µmol $m^{-2}s^{-1}$, measured with a radiometer (model LI-189, Licor, USA). The day/night temperature was maintained at 25 ± 2°C.

Pots containing 4-day-old wheat seedlings were transferred to another growth chamber with identical PFD, photoperiod and temperature described under growth conditions and were supplemented with UV-B radiation. The source of UV-B was a Vilbour-Lourmat (France) T-6 M source (1 × 6 W fluorescent tube with a permanent filter) with a peak at 312 nm (Figure 1). The UV-B radiation was measured using a UV-B radiometer (with a sensor for 312 nm) from the same manufacturer (Vilbour-Lourmat). The intensity of UV-B irradiation was measured at base, middle and top of the seedlings by placing the sensor of the UV-B radiometer in close contact with the leaves in the middle of the pot. The average intensity of UV-B radiation was 1 mW m^{-2}. Care was taken to maintain the average level of UV-B irradiance at 1 mW m^{-2} during the treatment while plants were growing by changing the distance between source and seedlings and placing the bandage cloth in between.

Isolation of chloroplast from control and treated wheat seedlings and PS II activity (assayed as H_2O to phenylenediamine; O_2 evolution) and PS I activity (assayed as $DCIPH_2$ to MV; O_2 uptake) were assayed according to Sharma and Singhal[10].

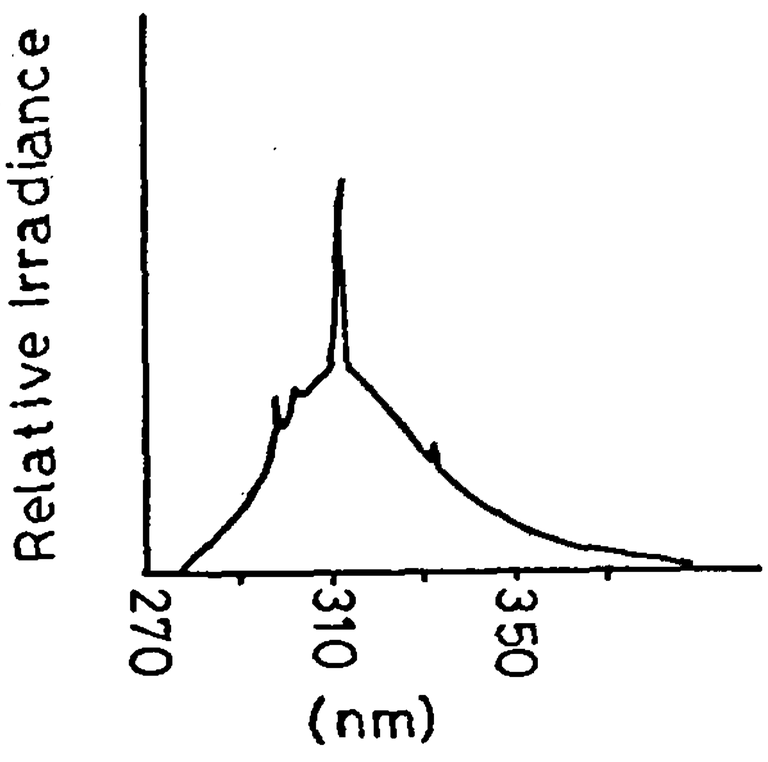

Figure 1. Spectral curve of UV-B source. Filter life time unlimited. Source intensity 6 mW cm^{-2}.

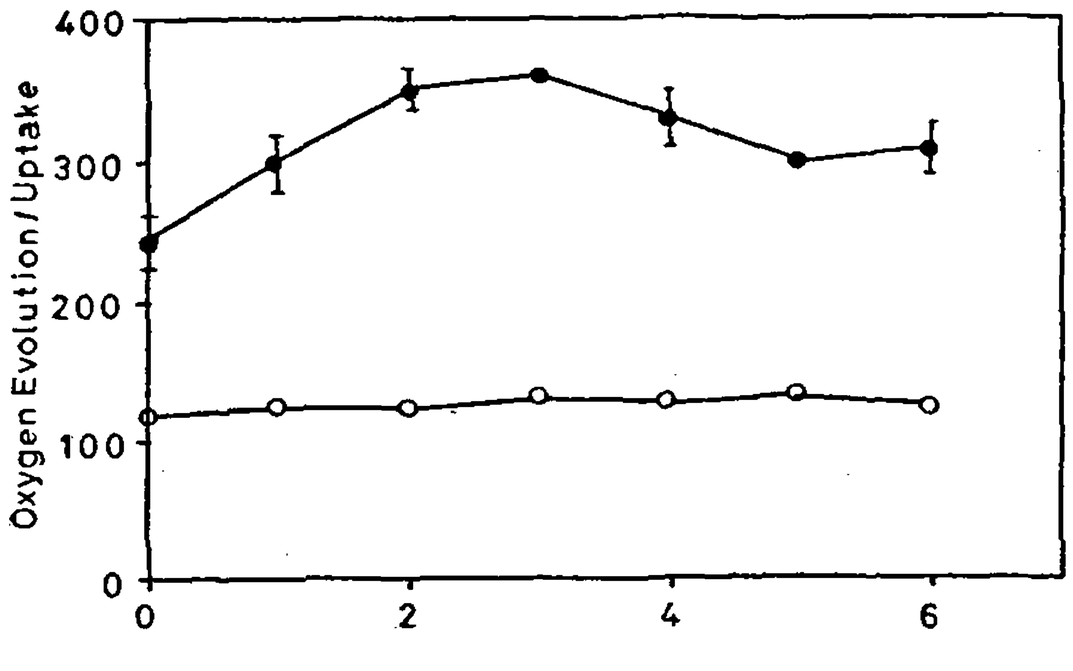

Figure 2. Effect of UV-B radiation (1 mW m^{-2}) on PS II (–O–) assayed as H_2O to phenylenediamine (oxygen evolution) and PS I activity (–●–) assayed as reduced dichlorophenol indophenol to methyl viologen (oxygen uptake). The PPFD during assays was 1200 µmol m^{-2} s^{-1}. Rates are expressed as µmol O_2 evolved or consumed/mg chlorophyll/hour. Each point represents a mean value of 4 experiments. Standard error (±8) for PS II is not visible on the scale.

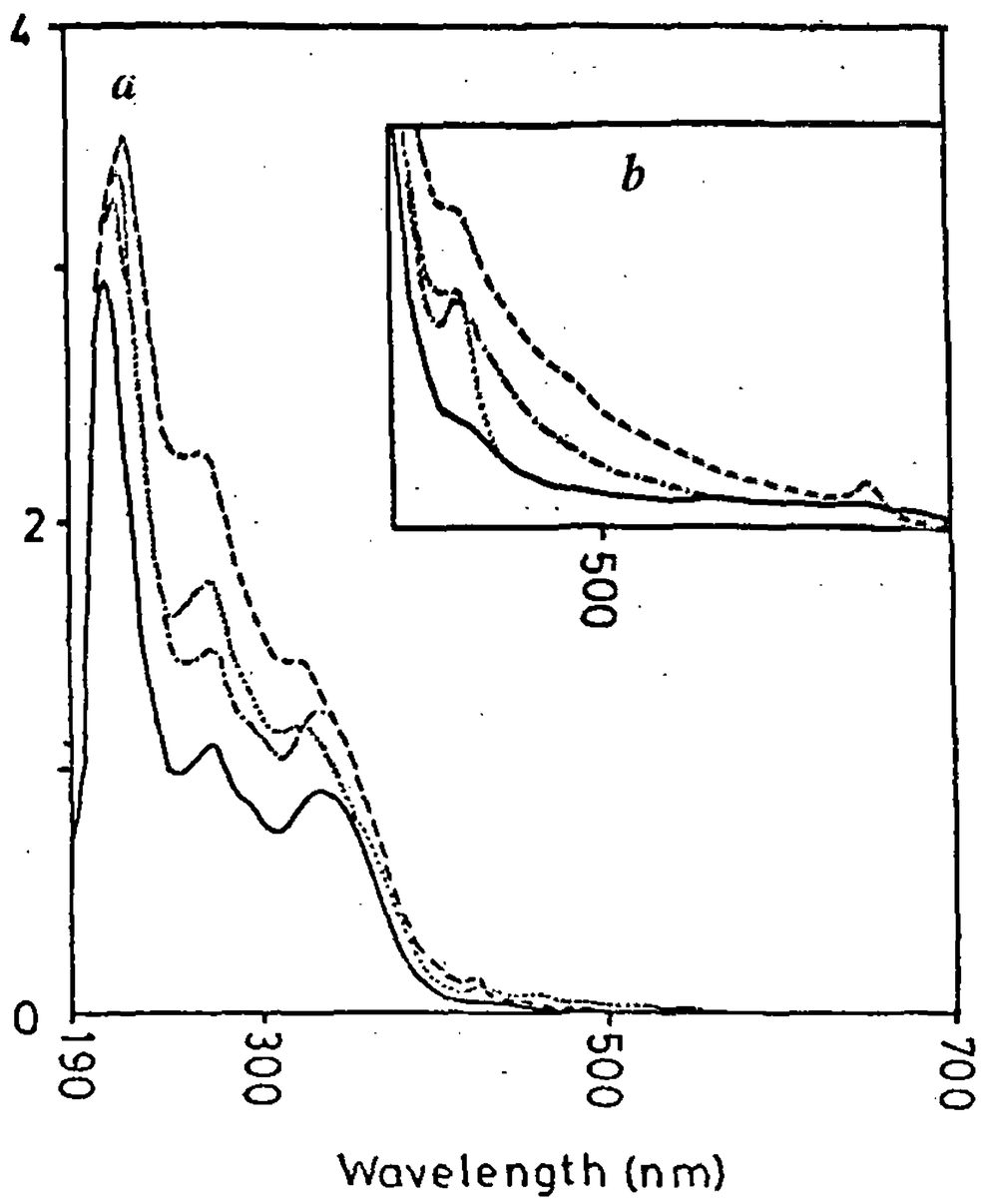

Figure 3. Effect of supplementary UV-B radiation on quantitative change in flavonoid contents in wheat seedlings. *a*, Spectral scan at 1:5 dilution; *b*, Spectral scan at 1:3 dilution. Control, i.e. 0 day UV-B treatment (------), 3 day UV-B treatment (.....), 5 day UV-B treatment (–.–.–.–.–.–.) and 7 day UV-B treatment (———).

Fresh leaves (2 g) were excised and homogenized in 20 ml of 80% methanol with 1% HCl (final volume) in a mortar. The homogenate was incubated for 2 h at room temperature in the dark and centrifuged at 1600 *g* for 20 min. The supernatant was collected for absorption spectra, paper chromatography and HPLC (Spectraphysics).

Spectral scan of UV-B absorbing compound was carried out with diluted extract using spectrophotometer (Shimadzu UV240).

For paper and HPLC analysis, 5 ml of each sample was fully dried by flushing N_2 and re-dissolved in 200 µl of methanol. 20 µl of sample was loaded on a Whatman filter paper no. 1 and resolved using *n*-butanol:acetic acid:water (6:1:2) at room temperature and observed under UV photoilluminator.

Identification and separation of flavonoids was carried out using HPLC (Spectraphysics) with a C18 reverse-phase column (ET 250/4 Nucleosil 100-5C_{18} ODS) Spectraphysics SP 8800 ternary HPLC pump with SP 4270 integrator and Spectra 100 variable wavelength detector. The 100 µl of re-dissolved sample was diluted in 1 ml methanol and 25 µl of this was injected into the HPLC column. The separation was carried out using an isocratic system (0.5% phosphoric acid and methanol in 1:1 ratio) over 20 min with a 1.0 ml/min flow rate at 280 nm and 418 nm at room temperature.

Wheat leaves were fixed in a solution containing 50% alcohol, 10% acetic acid and 10% formic acid. The fixed material was passed through a series of 90, 70, 50, 30 and 100% of alcohol for 2 h to overnight duration. This was followed by a series (50, 70, 85, 95 and 100%) of tertiary butyl alcohol (TBA) for 2 h duration followed by TBA and paraffin oil (1:1) for 2 h. The plant material was then fixed in paraffin wax (60°C) blocks and cut into sections using microtome (Sipcon SP1110). Sections were stained with safranine and fast green and mounted in the DPX. The anatomical observations were recorded by light microscope (Leica) with photographic attachment (Leica).

It was observed that plants treated with supplementary UV-B radiation did not result in significant changes in the PS II activity, assayed as H_2O to phenylenediamine (PD), compared to the plants grown without supplementary UV-B radiation (0 h). However, PS I activity assayed as dichlorophenol indophenol ($DCIPH_2$) to methyl viologen (MV), showed a significant increase in the activity in UV-B supplemented plants compared to non-supplemented plants (Figure 2).

Absorption spectra of 80% methanolic extract with 1% HCl is shown in Figure 3 *a*. It was observed that plants treated with UV-B radiation showed an increase in the absorption peaks at 210–220 (caumarin). UV-B treated plants showed a slight shift in the absorption peak of caumarin from 210 to 220 nm, 270–280 nm (Kaempferol) and 320–330 nm (flavone). Absorption spectra of concentrated extract also showed slight changes in the absorption at 418 and 660 nm (anthocyanin) in the visual region (Figure 3 *b*, Table 1).

UV-B-absorbing compounds showed qualitative changes in response to UV-B radiation which were

Table 1. Effect of supplementary UV-B radiation (1 Mw m^{-2}) in combination with visual radiation (60 µmol m^{-2} s^{-1}) at 25°C on flavonoids and its derivatives in wheat seedlings. Comparative data of spectrophotometric analysis and paper chromatogram seen under visual and UV-B transilluminator before and after NH_3 fuming. According to Swain[11] the colour of spot under visible and UV light with and without NH_3 treatment of the chromatogram indicate type of flavonoid

Spectrophotometric data		Paper chromatogram					
			Spot colour				
					+NH_3 fumes		
Lamda max	Compound	*Rf* × 100	Visible	UV	Visible	UV	Compound
270	Flavonol (Kaempferol)	84	Brownish yellow	Brownish yellow	Brownish yellow	Brownish yellow	Flavonol
210	Caumarin	79	Colourless	Blue	Colourless	Blue	Caumarin
		59	Colourless	Lightblue arrowshape	Colourless	Light blue arrowshape	Cinnamic acid
418	Anthocyanin	56	Brownish yellow	Brownish yellow	Brownish yellow	Brownish yellow	Anthocyanin
320–330	Flavone	34	Colourless	Purplish brown	Yellow	Yellow	Flavone
655–660	Anthocyanin	31	Brown	Purplish brown	Bright yellow	Bright yellow	Anthocyanin

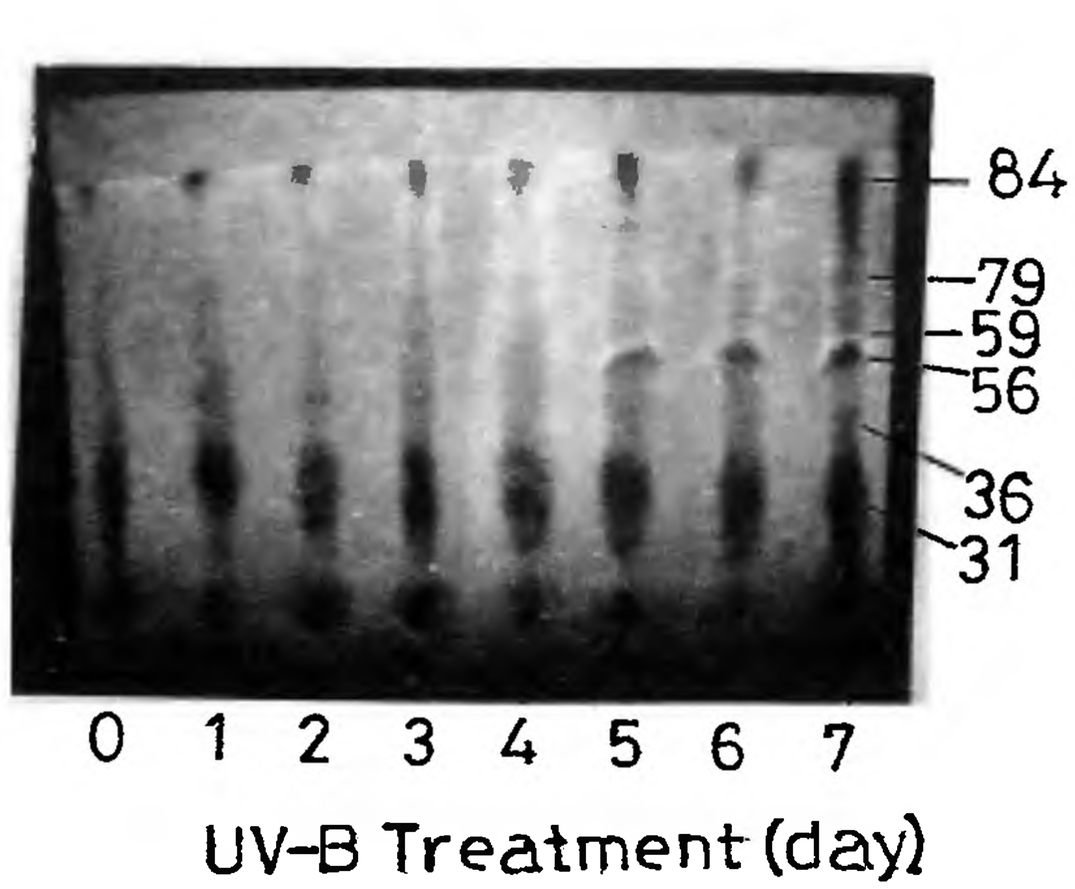

Figure 4. Effect of supplementary UV-B radiation for 0 to 7 day (left to right in the figure) on flavonoid content in wheat seedlings. The *Rf* value and colour composition under visual light with and without NH_3 fumes and nature of compounds are given in Table 1.

determined by paper chromatography. All major compounds visualized in extract from UV-B-irradiated plants were seen in control except cinnamic acid which appeared after day 4 of UV-B treatment (Figure 4). The possible group of flavonoids were determined on the basis of *Rf* value, and colour characteristics under visual and UV transilluminator (with and without treatment with NH_3 fumes) and their absorption spectra according to Swain[11] (Table 1).

HPLC analaysis of methenolic extract at 280 nm showed qualitative changes in the flavonoids. UV-B exposure resulted in an appearance of two new peaks (peak no. 3 and 7; cinnamic acid and anthocyanin respectively) compared to the control. Peaks 1 and 2 (kaempferol and caumarin respectively) showed increase due to the supplementary UV-B exposure. HPLC analysis using visual range (418 nm) also showed significant changes in the anthocyanin (Figure 5 *b*). The nature of new peaks observed in Figure 5 *b* could not be determined.

The leaf section showed a massive increase in the deposition of cuticle wax in the UV-B treated leaves compared to control (Figure 6 *a–d*). Epidermis was damaged to a greater extent (Figure 6 *b* and *c*). Mesophyll cells were converted into hypodermal cells (Figure 6 *c*) to replace the damaged epidermal cells (Figure 6 *c* and *d*).

Our study shows that supplementary UV-B radiation did not cause significant changes in the PS II activity but the PS I activity showed a significant increase. Qualitative and quantitative changes in the flavonoids and other phenolic (phenyl propane) derivatives using analytical techniques such as spectrophotometry and chromatography were also observed.

Response of photosynthetic apparatus to UV-B radiation has been studied by several workers[9,12–16]. A few reports have suggested photosystem II as the UV-B-sensitive component[14,15], but the action spectrum of UV-B does not suggest a specific target molecule[5,6,13].

Jie He *et al.*[15] observed decrease in PS II activity measured as *Fv/Fm* ratio in rice and pea leaves. However, they have not measured photosynthetic electron

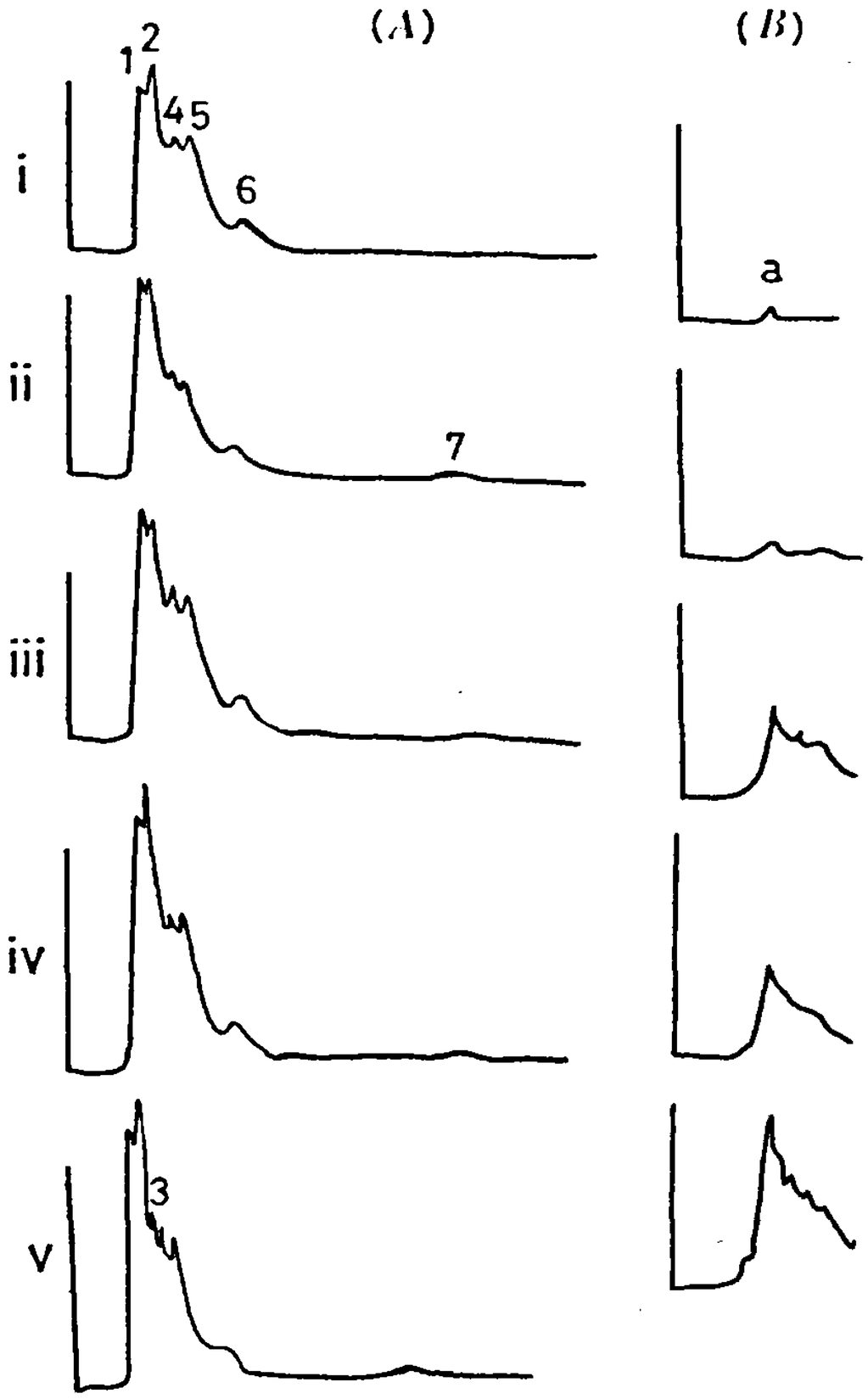

Figure 5. HPLC analysis showing qualitative and quantitative changes in wheat seedlings under supplementary UV-B exposure for 0 (i), 2 (ii) 3, (iii) 5 (iv) and 7 (v) days. *A*, HPLC analysis at 280 nm. 1, Kaempferol; 2, Caumarin; 3, Cinnamic acid; 4 and 5, Flavone; 6 and 7, Anthocyanin; *B*, HPLC analysis at 418 nm. *a*, anthocyanin. Other peaks seen are not identified.

transport. Middleton and Teramura[9] showed that the reduction in photosynthesis, under UV-B radiation, was primarily associated with a stomatal limitation rather than the damage to PS II.

In our study we have not observed any damage to PS II activity (Figure 2). This may be due to low UV-B fluence rate (1 mW) for a short duration (up to 6 h only). Also, the effect of supplementary visual radiation (200 µmol m^{-2} s^{-1}) during the UV-B exposure, may have resulted in preventing the damage due to PS II. There have been reports that UV-B-mediated effects are dampened by the background intensities of photosynthetic active radiation[17–20].

The increase in the PS I activity may be owing to micro-environmental changes in the thylakoid membrane (lipids/protein) due to oxidative damage[21], making reduced DCIP more assessable to the site of action (electron donation).

In this study we observed quantitative and qualitative changes in the UV-B absorbing flavonoids in response to supplementary UV-B radiation (Figures 3–5). It has been suggested that flavonoids and phenyl derivatives are involved in the protection against UV-B exposure in higher plants[8,12,15,22,23]. Flavonoids and anthocyanins absorb UV-B radiation and these generally accumulate in the epidermis. Here they could keep UV-B radiation from reaching photosynthetic tissues and other sensitive sites (protein, DNA, etc.).

Lois[8] observed that irradiance of *Arabidopsis* with UV-B light resulted in accumulation of flavonoids in the aerial parts of the plants. He correlated this increase with the protection (less damage to plant growth) against UV-B damage.

Lois and Buchanan, working with *Arabidopsis* mutant deficient in flavonoids accumulation, found that mutant displayed a dramatic increase in the sensitivity (reduced plant growth) to UV-B radiation compared with wild type, suggesting a protection role for flavonoids against UV-B radiation.

Li *et al.*[16], working with *Arabidopsis* mutant with defects in the synthesis of flavonoids, found them more sensitive (less dry weight) to UV-B than the wild type when grown under UV-B radiation.

Jie He *et al.*[15], working with rice and pea plants, found that rice cultivar showed less decrease in *Fv/Fm* ratio as compared to pea. They correlated this to the higher levels of UV-B absorbing compounds in rice as compared to pea. This indicates a role of flavonoids in the protection against UV-B in higher plants.

In this study we also observed a slight shift in the absorption peak of caumarin (210–220 nm; Figure 3 *a*). This shift in lambda maximum for caumarin may be due to its glycosylation[23].

Our study shows massive increase in the cuticle layer in the leaves in response to UV-B radiation (Figure 6 *a–d*). Earlier studies have shown that UV-B radiation causes epidermal deformation[12], changes in cuticular wax composition[24]. These changes may result in preventing harmful UV-B radiation to reach sensitive site. Hahlbroack and Scheel[25] and Robberecht and Caldwell[26] reported that cutin deposition along with flavonoid synthesis in upper epidermis could block transmittance of 95–99% of incoming UV-B radiation.

The increase in the cuticle layer under UV-B exposure may prevent the penetration of the UV-B radiation by reflecting large amounts of it. Development of hypodermal cells from mesophyll cells (Figure 6 *c* and *d*) may replace the damaged epidermis. Also the development of hypodermal cell may prevent the UV-B radiation from reaching the more sensitive sites by providing layer(s) of cells which accumulate large amounts of

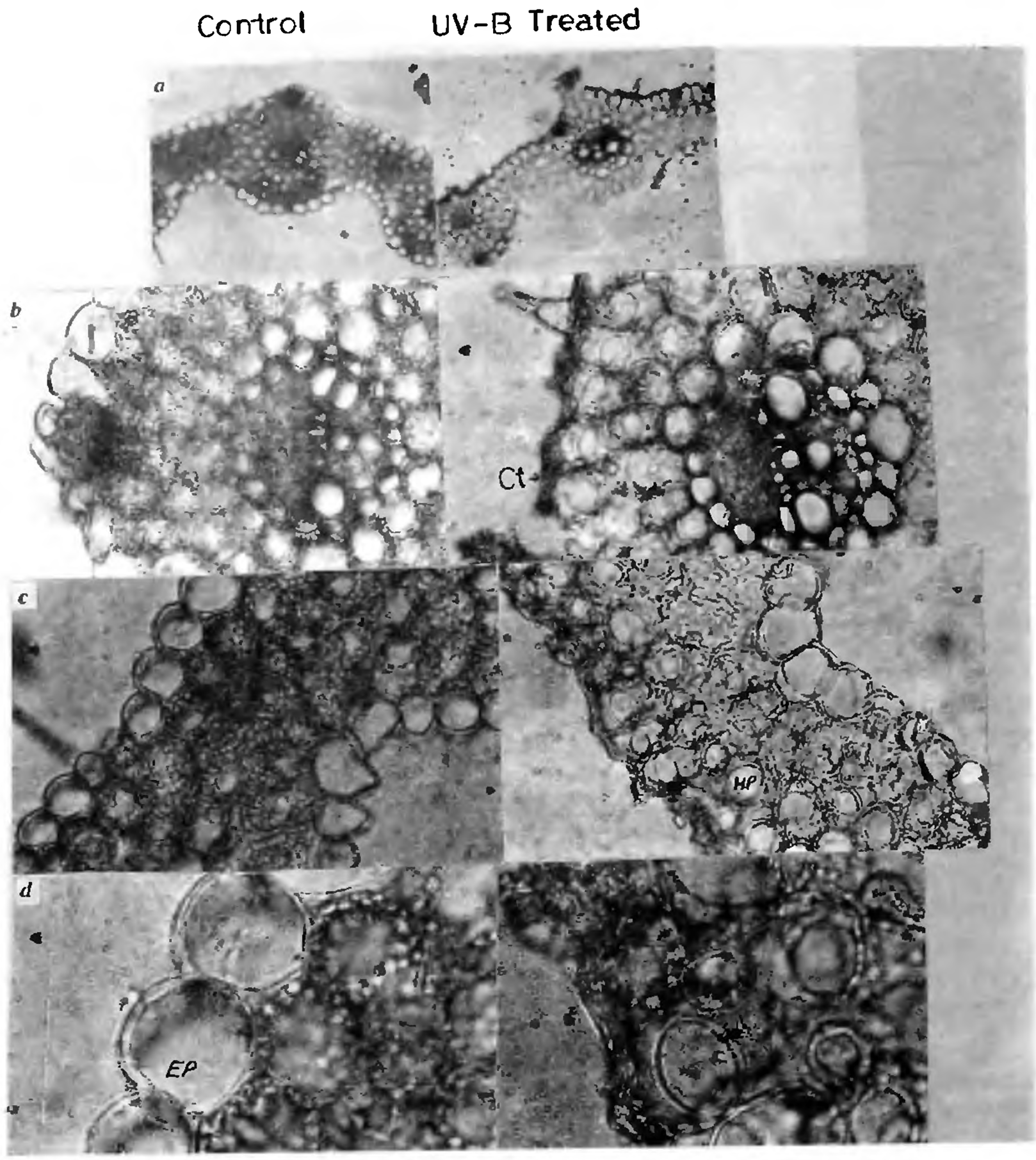

Figure 6. Effect of supplementary UV-B radiation on leaf anatomy. *a*, TS of mid rib portion of control and UV-B treated leaf respectively (10×); *b*, TS of mid rib portion of control and UV-B treated leaf respectively (40×). Ct, cuticle; *c*, TS of control and UV-B treated leaf (non-midrib region) respectively (40×). HP, hypodermal cell; *d*, TS of control and UV-B treated leaf respectively (100× oil). EP, Epidermal cell.

phenolic compounds (flavonoids). This may further help in absorbing the UV-B radiation, thus protecting more sensitive sites, such as DNA and enzymes, from UV-B radiation.

1. Rozma, J., Lenssen, G. M., Arp, W. J. and Van De Staaij, W. M., in *Ecological Responses to Environmental Stresses* (eds Rozema, J. and Verklei, J. A. C.), Kluwer Academic, The Netherlands, 1991, pp. 220–331.
2. Kelly, G. J., *Trans. Menzies Found.*, 1989, **15**, 187–190.
3. Rudiger, W. and Lopez-Figueroa, F., *Photochem. Photobiol.*, 1992, **55**, 949–954.
4. Short, T. W. and Briggs, W. R., *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1994, **45**, 143–171.
5. Iseki, M. and Wada, S., *Plant Cell Physiol.*, 1995, **36**, 1033–1040.
6. Coohil, T. P., *Photochem. Photobiol.*, 1989, **4**, 1353–1358.
7. Stapleton, A. E., *The Plant Cell*, 1992, **4**, 1353–1358.
8. Lois, R., *Planta*, 1994, **194**, 498–503.
9. Middleton, E. M. and Teramura, T. H., *Plant Physiol.*, 1993, **103**, 741–752.
10. Sharma, P. K. and Singhal, G. S., *J. Photochem. Photobiol.*, 1992, **13**, 83–94.
11. Swain, T., in *Chemistry and Biochemistry of Plant Pigments* (ed. Goodwin, T. W.), Academic Press, London, 1976, pp. 425–461.
12. Tevini, M., Braun, J. and Fiesser, G., *Photochem. Photobiol.*, 1991, **53**, 329–333.
13. Renger, G., Voelker, M., Eckert, H. J., Fromme, R., Hohmveit, S. and Graeber, P., *Photochem. Photobiol.*, 1989, **49**, 97–105.
14. Kulandaivelu, G. and Noorudeen, A. M., *Physiol. Plant.*, 1983, **58**, 389–394.

15. Jie He, Huang, Lin-Ke, Chow, W. S., Whitecross, M. I. and Anderson, J. M., *Aust. J. Plant Physiol.*, 1993, **20**, 129–142.
16. Li, J., Ou-Lee, T. M., Raba, R., Amundson, R. G. and Last, R. L., *The Plant Cell*, 1993, **5**, 171–179.
17. Teramura, A. H. and Sullivan, J. H., *Photosynth. Res.*, 1994, **39**, 463–473.
18. Teramura, A. H., Biggs, R. H. and Kossuth, S., *Plant Physiol.*, 1980, **65**, 483–488.
19. Reid, D. M., Beallard, F. D. and Pharis, R. P., in *Plant Physiol: A Treatise, Growth and Development* (ed. Bidwell, R. G. S.), Academic Press, San Diego, 1991, pp. 65–181.
20. Jansen, M. A. K., Gava, V., Greenberg, B. M., Mattoo, A. K. and Edelman, M., *The Plant J.*, 1996, **5**, 693–699.
21. Hu, M. L. and Tappel, A. L., *Photochem. Photobiol.*, 1992, **56**, 357–363.
22. Beggs, C. and Wellman, E., *Photochem. Photobiol.*, 1985, **41**, 481–486.
23. Okamura, N., Haraguchi, H., Hashimoto, K. and Yagi, A., *Phytochemistry*, 1994, **5**, 1463–1466.
24. Tevini, M. and Steinmuller, D., *J. Plant Physiol.*, 1987, **131**, 111–121.
25. Hahlbrook, K. and Scheel, D., *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1989, **40**, 347–369.
26. Robberecht, R. and Caldwell, M. M., *Oecologia*, 1978, **32**, 277–287.

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Temporal patterns of visitation among avian frugivores at fruiting strangler figs in a tropical evergreen forest in the Western Ghats, Southern India

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Frugivory observations were conducted at fruiting strangler figs in Karian Shola National Park in the Anaimalai hills from January to March 1993. A total of 123 hours of fruit use by frugivores was observed at fruiting trees of four species of *Ficus*. Frugivore activity was seen throughout the day with a major peak in visitation between 7.00 AM and 9.30 AM and a minor one at 2.30 PM. The common frugivore species exhibited preferences for different species of fruiting strangler figs, occurring in larger numbers at these trees. Of the seven common species of avian frugivores, only two pairs exhibited similar temporal patterns of visitation to the fruiting strangler figs, the Small Green Barbet–Crimsonthroated Barbet pair at *F. drupacea* and bulbul spp.–Golden Oriole pair at *F. microcarpa*. The pressures of competition, past or contemporary may be the reason behind the difference in the temporal pattern of visitation. However, it is unlikely that active competition is the cause since very few inter-specific aggressive interactions were noted. What is unclear is the similar temporal pattern of visitation in the case of the bulbul spp.–Golden Oriole pair at *F. microcarpa*, their preferred tree and the barbet pair at *F. drupacea*, not their preferred tree.

FRUITING fig trees, especially those that are bird-dispersed, attract avian frugivores in large numbers[1]. This has led to a number of studies on frugivore composition and their interactions at this superabundant fruit resource[2–6]. Visitations to the fruiting trees may depend on physiological needs of the frugivores and the local factors peculiar to each fruiting tree. Time spent by a frugivore at a fruiting tree would depend on its nutritional needs, diet and activity budgets. The presence of predators and the interactions among the frugivores could also affect visitation rates[7,8]. Kantak[7] surmised that the different visitation patterns of the common frugivores could be due to interference competition. This paper presents the results from a study of temporal partitioning of the fruit resource among avian frugivores visiting fruiting strangler figs in a tropical evergreen forest in the southern Western Ghats, India. The null hypothesis of no differences in the temporal patterns of visitation was tested against the alternative hypothesis of temporal partitioning of the fruit resource by the common frugivores.

The study was conducted from 21 January to 31 March 1993, at Karian Shola National Park in the Indira Gandhi Wildlife Sanctuary, Pollachi district, Tamil Nadu, India. The Karian Shola National Park (10°27'N, 76°51'E; altitude c. 765 m) is spread over 506 ha of tropical evergreen forest. It is contiguous with similar forest across the Kerala–Tamil Nadu border in the Parambikulam Wildlife Sanctuary. It is classified as the west coast tropical evergreen forest with the characteristic tree species being *Hopea parviflora* and *Messua ferrea*[9]. The national park is surrounded by moist deciduous forests and teak plantations. Most of the precipitation in this region occurs during the south-west monsoon (from June to August) but the effect of the north-east monsoon (during November and December) is also felt. This area received 1778.2 mm of rain in 1992 and 27.8 mm during the three-month study period.

A total of 123 hours of frugivory observations was conducted at three trees of *Ficus drupacea* (67 h), one tree of *F. microcarpa* (40 h) and two trees of *F. amplissima* (16 h). Observations were conducted between 7.00 AM and 5.00 PM. The bird species and their numbers were noted during every alternate 5-minute period.

The trees chosen for observations had to satisfy certain criteria. They had to have large synchronously ripening fruit crops; usually bird-dispersed fruiting characteristics in the old world tropics[1]. The trees had to be relatively shorter and located in more open areas. In