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QUANTIFICATION OF XANTHOPHYLL CYCLE PIGMENTS IN *HEVEA BRASILIENSIS*

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The present study was aimed at optimizing a method using HPLC for qualitative and quantitative assessment of xanthophyll cycle pigments in young plants of *Hevea brasiliensis*. Successful separation of xanthophyll cycle pigments and other photosynthetic pigments was achieved by this method. In the current modified method co-elution of lutein and zeaxanthin was rectified and effective separation of these pigments was obtained. Diurnal variation in xanthophyll pigment content was very much evident according to the change in light intensity, from 08.30 to 16.30 hr. Diurnal changes in xanthophyll cycle pigment pool size and de-epoxidation state were apparently evident in sun exposed young *Hevea* plants. De-epoxidation rate was high at midday (12.30 hr) indicating increased energy dissipation during high light condition. The result suggests that the increased production of zeaxanthin and antheraxanthin with progressive increase in light intensities protects the photosystems from excess light energy and avoid photoinhibitory damage during sunny days in young plants of *H. brasiliensis*.

Keywords: Energy dissipation, *Hevea brasiliensis*, HPLC, Photoprotection, Xanthophyll pigments

INTRODUCTION

During the process of photosynthesis plants often absorb higher amount of light energy than what is required for driving photosynthesis. Photoinhibition of photosynthetic apparatus occurs unless the excess energy is dissipated safely. The excess energy is dissipated as fluorescence and as heat by chlorophyll and xanthophyll cycle pigments in photosystems (Gilmore, 1997; Niyogi *et al.*, 1998; Munné-Bosch and Alegre, 2000). In this cycle the excess excitation energy induces the formation of zeaxanthin through de-epoxidation of violaxanthin *via* an intermediate antheraxanthin. Both these pigments scavenge the excess excitation energy present in the reaction centers of the light harvesting complexes and dissipate it as heat. Under low light condition this reaction is reversed and violaxanthin is formed (Eskling *et al.*, 1997). This phenomenon of diurnal cycle involving interconversion of xanthophylls operates in plants.

The photoprotective role of xanthophylls has been very well established in other plant species (Demmig-Adams and Adams, 1996; Niyogi *et al.*, 1998; Goss and Jakob, 2010; Pompelli *et al.*, 2010; Golovko *et al.*, 2012). Other carotenoids such as lutein also take part in non-photochemical quenching of chlorophyll fluorescence to some extent (Casper-Lindly and Björkman, 1998). Carotenoid pigments have been

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reported to have important role in quenching excitation energy (Nilkens *et al.*, 2010; Li *et al.*, 2009; Vaz and Sharma, 2011) and are essential components of the light harvesting complexes playing a crucial role in combating oxidative stress (Lu *et al.*, 2001).

The magnitude of changes in the level of photosynthetic and accessory pigments in photosynthetic apparatus particularly carotenoids are of paramount importance in stress responses and tolerance in plants. The aim of the present study was to optimize a method for estimating xanthophyll cycle pigments qualitatively and quantitatively and the fluctuations of the same in *H. brasiliensis* at different time of the day varying with light intensities.

MATERIALS AND METHODS

Plant material

The study was conducted in young plants of *H. brasiliensis*, clone RRII 414 grown in polybags at the experimental farm of Rubber Research Institute of India, Kottayam. Routine cultural practices as per the package of practices recommendations were carried out to maintain these plants. After attaining 6-7 months growth these plants were kept in diffused light under laboratory conditions for two days. Further these plants were transferred to direct sunlight and exposed through the day/night cycle for five days. Leaf samples for the pigment analysis were collected from the sun exposed top whorl at 8:30, 10:30, 12:30, 14:30 and 16:30 hrs of the day.

Pigment extraction

Leaf samples were collected and immersed immediately in liquid nitrogen. Photosynthetic pigments were estimated from fully expanded and physiologically mature leaves. Leaf discs (5 mm) were taken using a sharp, punch and homogenised in a cold mortar with ice cold acetone containing 0.1 per cent BHT (w/v). The homogenate was centrifuged for 10 minutes at 4 °C for 5000 rpm and supernatant was collected. The pellets were re-extracted with minimum amount of acetone and the supernatant was pooled. The extracts were analyzed on the same day to reduce loss of pigments during analysis. All analytical procedures were carried out under dim light.

HPLC analysis

Identification and separation of pigments were done on a reverse phase column, Waters Spherisorb ODS -5 µm column (250 x 4.6 mm) in HPLC. Samples were injected with a Rheodyne 7010 injector with a 20 µL loop at 30 °C for 60 minutes. The mobile phases were pumped with a Waters 600 high pressure pump at a flow rate of 0.5 mL per minute. The methods developed by De Las Rivas *et al.* (1989), Thayer and Björkman (1990) and Janik *et al.* (2008) were used for pigment analysis. Among these Thayer and Björkman (1990) method was found suitable but there was no clear cut separation of lutein (L) and zeaxanthin (Z). Thus, the pigment analysis was carried out using modified steps for the present study. The solvent system consisted of acetonitrile:methanol:water (solvent A, 84:14:2) and methanol:ethyl acetate (solvent B, 68:32). The column was equilibrated with mobile phase prior to injecting each sample. The gradient used was 0 to 20.5 minutes 100 per cent solvent A, 20.5 to 25.5 minutes decreasing to zero per cent solvent A, 25.5 to 40 minutes 100 per cent solvent B, 40.0 to 40.1 minutes increasing to 100 per cent solvent A and finally from 40 to 60 minutes 100 per cent solvent A. Peaks were detected at 450 nm with waters 996 PDA detector and integrated using Empower software. Peaks were identified using standard methods.

Standards of xanthophylls lutein, zeaxanthin, chlorophyll *a*, chlorophyll *b* and β-carotene from M/s. Sigma Aldrich were used for the study. Pigments were identified by comparing their absorption spectra and retention time with standards. Standard curves for quantification of pigment were made by plotting concentration against absorbance responses *i.e.* peak area (Almela *et al.*, 1990). The analysis was done in triplicates. Lutein, zeaxanthin, β-carotene, chlorophyll *a* and *b* were quantified according to their respective standards. The response factor of lutein standard was used for quantifying the pigments *viz.* neoxanthin, violaxanthin and antheraxanthin. The concentration of pigment was determined from the standard curve and expressed as μg cm^{-2}. The level of de-epoxidation and epoxidation state of xanthophyll cycle pigments were calculated as (Z+0.5A)/(V+A+Z) and (V+0.5A)/(V+A+Z), respectively.

RESULTS AND DISCUSSION

Various xanthophyll pigments of the extract was separated out on ODS column and evaluated under a range of mobile phase conditions. Acetonitrile, methanol and ethyl acetate were the primary mobile phases and modified according to the selectivity of pigments with appropriate modifiers. In this case, water was used as the modifier (Tee and Lim, 1992; Craft, 2001). Presence of water influences the separation of polar pigments, whereas non polar pigments are relatively insensitive. Therefore, water was included in the solvent A. Column temperature regulation is essential for a reproducible elution profile of all the major carotenoids. Hence, a temperature profile ranging from 25 to 50 °C was tried and

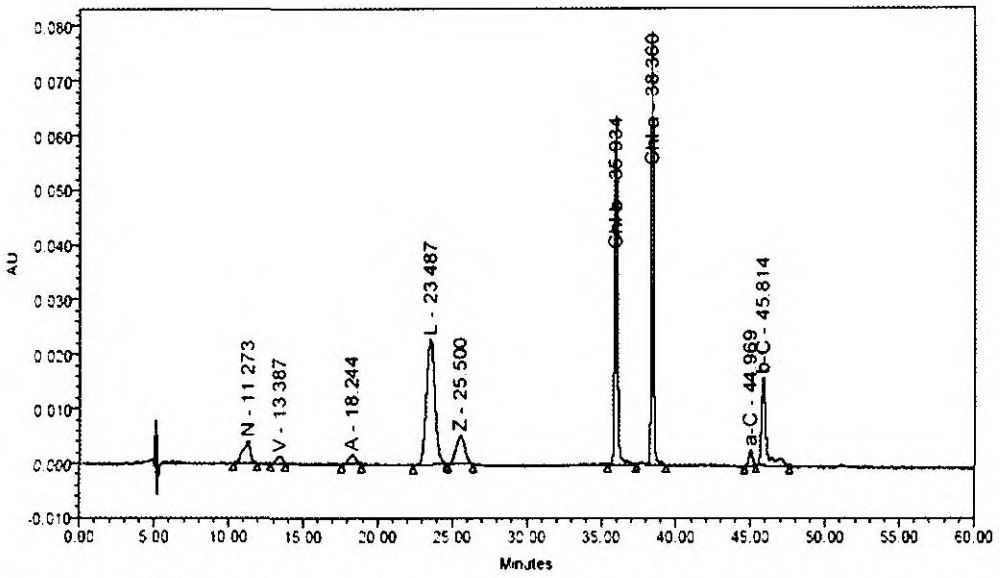

Fig. 1.The chromatographic profile of photosynthetic pigments after exposure to sunlight. Pigments *viz.*, neoxanthin (N), violaxanthin (V), antheraxanthin (A), lutein (L), zeaxanthin (Z), chlorophyll *b* (Chl.*b*), chlorophyll *a* (Chl.*a*), α- carotene (a-C), and β- carotene (b-C) are indicated

Table 1. **Composition of photosynthetic pigments (μgcm^{-2}) in *H. brasiliensis* leaves at different times of the day**

Pigments	8:30 hr.	10.30 hr.	12.30 hr.	14.30 hr.	16.30 hr.	CD (P< 0.05)
N	0.534	0.705	1.125	1.131	0.986	0.063
V	1.140	0.317	0.210	0.644	0.824	0.050
A	-	0.371	0.320	0.384	0.342	0.049
L	3.135	3.798	6.249	6.190	5.416	0.130
Z	-	0.584	0.899	0.501	0.071	0.049
α carotene	0.126	0.356	2.900	2.199	1.988	0.030
β carotene	2.078	3.252	4.811	5.370	4.488	0.440
Chl *a/b*	2.607	2.628	2.390	2.450	2.171	0.130

finally 30 °C was chosen in combination with the mobile phase.

A gradient system comprising of *acetonitrile:methanol:water* (84:14:2) and ethyl acetate:methanol (68:32) was adopted with ODS column for pigment separation. A problem related to co-elution of lutein and zeaxanthin was rectified in this method and found suitable for effective separation of these pigments (Fig. 1).

Initial identification of individual pigment was based on chromatographic profile and elution behavior *i.e.*, order of elution and their visible absorption spectra, as the wavelength of maximum absorption and spectral structures are characteristics of each chromophore. The quantitative distribution of xanthophyll pigments, carotenes and chlorophyll *a/b* are given in Table 1. The order of elution of xanthophylls was based on order of decreasing polarity. It has been reported that polar compounds elutes first and non polar later (Tee and Lim, 1992). As expected, on the reverse column, neoxanthin (N) was eluted first followed by violaxanthin (V), antheraxanthin (A), luetin (L) and zeaxanthin (Z). Separation of lutein and zeaxanthin was very prominent in this study. Chlorophylls *a* and *b* and carotenes (α and β carotenes) were eluted after the solvent change. The chromatogram profile of chlorophylls, xanthophylls and other carotenes are shown in Figure 1.

The diurnal variation in pigment profile is shown in Figure 2. The xanthophyll pigment profile showed significant difference in the diurnal course of observations. Lutein was the most abundant pigment among xanthophylls throughout the day time. It was found that the content of pigments N, L, Z and α-carotene registered a steady increase from morning hours to midday, thereafter the content of these pigments showed a marked decline (Table 1). The concomitant increase of xanthophylls and carotenes with the increase of light intensity is attributed to the involvement of these pigments in dissipation of excess energy. It was reported that progressive development of oxidative stress leads to an increase in β -carotene content (Alscher and Cumming, 1990). This was observed in the case of leaves of young *H. brasiliensis* also. The magnitude of variation in xanthophyll cycle pigment is a clear indicator to analyze the role of xanthophyll cycle dependendent excess energy dissipation in response to high solar light condition (Table 2). Similar pattern of changes of pigments have been reported in plants from different families (Thayer and Björkman, 1990). They observed that V+A+Z

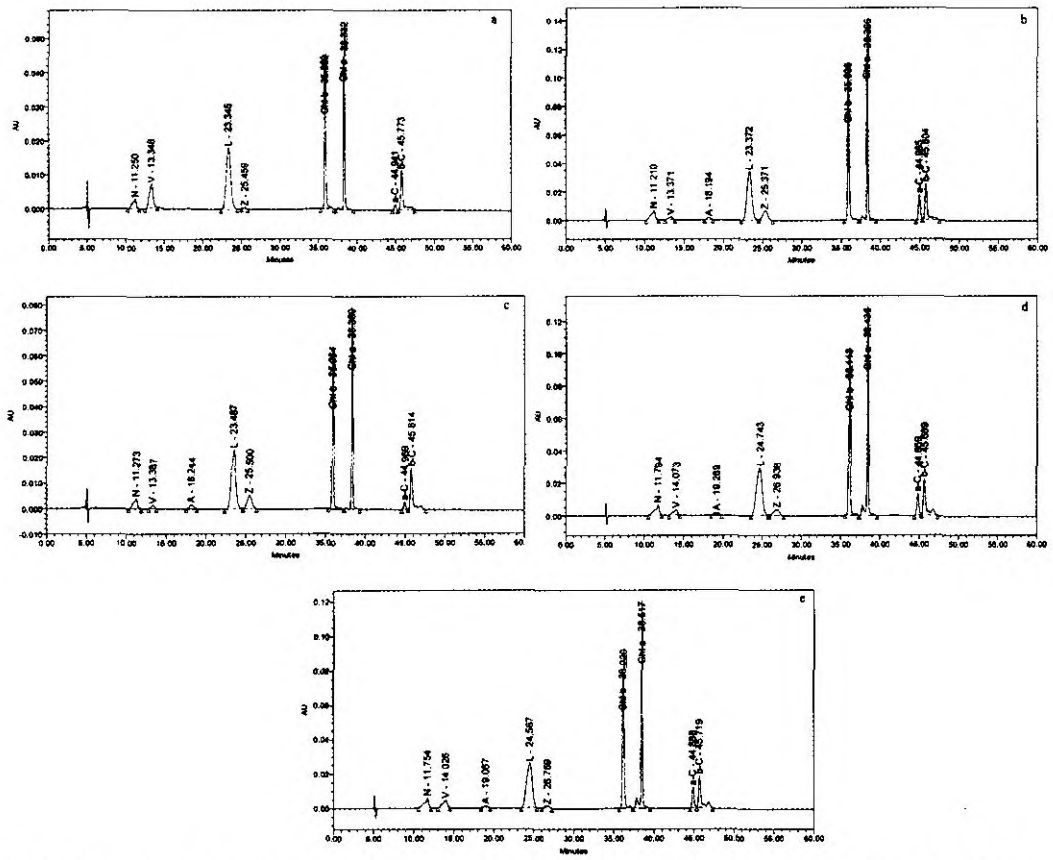

Fig. 2. Chromatographic profiles showing the variation in pigment compositions (a) 8:30 hrs (b) 10:30 hrs (c) 12:30 hrs (d) 14:30 hrs and (e) 16:30 hrs

pool size was determined by the factors like light intensity and its distribution during the day.

A critical analysis of xanthophyll cycle pigments alone showed a higher level of violaxanthin and definite absence of antheraxanthin and zeaxanthin at 8:30 hrs. Transfer of polybag plants from the diffused light condition to sunlight lead to the reduction of violaxanthin and concomitant accumulation of antheraxanthin and zeaxanthin. The profile indicated that conversion of violaxanthin to zeaxanthin increases slowly and reaches maximum at midday. Similarly, the pool size of V+A+Z was significantly higher at midday than morning hours. The results indicate the significance of changes in xanthophylls in light adaptation process of *H. brasiliensis*. Photosynthetic apparatus of *Picea abies* showed acclimatization within two days of transfer to high light through an increase in both photochemical (q_p) and non photochemical (q_N) quenching processes (Špunda *et al.*, 1993). Photoprotection of the photosynthetic machinery and adaptation under natural conditions through xanthophyll pigments was observed in

Table 2. **Changes in sum of A+Z, xanthophyll cycle pigment pool (V+A+Z), the ratio of Z in xanthophyll cycle pigments (Z/V+A+Z) and epoxidation state (EPS) of xanthophyll cycle pigments in *H. brasiliensis* leaves at different time interval. Pigment content expressed as (μg cm^{-2})**

Time	A+Z	Xanthophyll cycle pigment pool size	Z/ V+A+Z	EPS
8:30 hr.	-	1.140	-	-
10:30 hr.	0.955	1.274	0.458	0.39
12:30 hr.	1.219	1.508	0.596	0.30
14:30 hr.	0.885	1.529	0.328	0.55
16:30 hr.	0.413	1.236	0.057	0.80
CD (P < 0.05)	0.020	0.100	0.005	0.007

Plantago media plants (Golovko *et al.*, 2012). Similarly, in the present study, *H. brasiliensis* plants transferred to open sunlight from laboratory condition seem to be adapted to high light condition through better dissipation of excess energy by xanthophyll cycle (Fig. 3 and Table 2). Higher level of zeaxanthin at midday indicates an increased distribution of absorbed light for energy dissipation as heat under higher solar light intensities (Table 2). The central role of zeaxanthin in thermal dissipation has been demonstrated and confirmed using *npq1* mutant of *Chalmydomonas reinhardii* and *Arabidopsis thaliana* deficient in violaxanthin de-epoxidase enzyme (VPE) activity (Niyogi *et al.*, 1998). The typical diurnal conversion

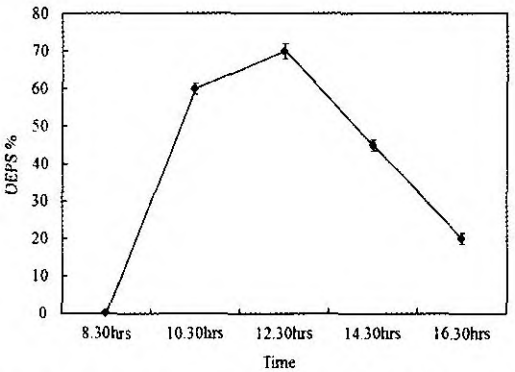

Fig. 3. Changes in de-epoxidation state (DEPS) of xanthophyll cycle pigment in *H. brasiliensis* leaf (significant P< 0.05 level)

of V to A and Z and its reversion back under low light intensity and role in dissipation of excess energy at photosytems are apparently evident in the present study.

In summary, the analytical method standardized in the present study enabled a good elution and quantification of photosynthetic pigments from *H. brasiliensis* leaves. The xanthophyll pigment pool size varies depending on the light intensity at a particular time of the day, with the highest level recorded at midday under high light intensity. This shows the adaptation of plants to excess light energy during day time through interconversion of xanthophyll cycle pigments. It is evident that the light dependent process of xanthophyll cycle pigments protects photosynthetic machinery against photoinhibition. Thus, under natural conditions plants require a better light management system like xanthophyll cycle pigments which switch over between efficient light harvesting mechanism in light limited condition and efficient photoprotection under high light condition.

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