DISCUSSION

Chlorophylls form one of the most prominent classes of plant pigments which play an essential role in light energy absorption during photosynthesis. The very process of Chl biosynthesis is also dependent upon light. Besides light the greening process, which includes biosynthesis and assembly of Chl, proteins, lipids and nucleic acids, is also dependent upon the environmental growth temperature. Both low and high temperatures have profound effect on chloroplast biogenesis. In Delhi and many parts of northern India, the temperature in winter falls to 3-10°C and in summer it rises to 40-45°C. In the present investigation the effect of chill- (7°C) and heat- (42°C) stress on Chl biosynthesis has been studied.

In order to elucidate the effect of temperature stress on Chl biosynthesis, concentrations of different intermediates of Chl biosynthetic pathway need to be quantified. In green plants, fluorescing compounds of Chl biosynthetic pathway are Proto IX, MPE, Pchlide, Chl(ide) a and Chl(ide) b. Coprogen III is one of the non-fluorescing (in the visible range) intermediates of the pathway. However, in certain mutants and under certain growth and experimental conditions Copro III, an oxidation product of Coprogen III, accumulates. While estimating the Coprogen oxidase, which converts Coprogen III to Protogen IX, Copro III accumulates due to non-enzymatic oxidation of Coprogen III to Copro III. Unlike Coprogen III, which is non-fluorescent, Copro III fluoresces at 622 nm in neutral and alkaline pH [Fig. 1]. Copro III also fluoresces substantially at 632 nm, the fluorescence peak of Proto IX [Fig. 2]. Similarly Proto IX also fluoresces at 622 nm, the peak of Copro III fluorescence. Therefore while estimating Copro III or Proto IX, appropriate corrections at 622 nm or 632 nm, were applied to take into account the fluorescence due to Proto IX or Copro III (Hukmani and Tripathy, 1992).

Sensitivity and resolution of room temperature fluorescence spectroscopy has been utilized as an analytical tool for the quantitative estimation of tetrapyrroles. It is often extremely difficult to separate minute
quantities of pigments by chromatography which sometimes results in either loss or destruction of pigments. Therefore, elimination of the need for separation and purification of various tetrapyrroles prior to quantitative analysis of pigments, which is a unique feature of the spectrofluorometric technique, was developed in the present investigation. As the present spectrofluorometric technique can estimate as low as one picomole concentration of pigments from their mixture, it offers significant advantage over the other available methods. Convenience, rapidity and accurate quantification of minute concentration of pigments and elimination of analytical uncertainties due to recovery losses caused by chromatography, are unique advantages of the present spectrofluorometric method of analysis of pigments from their mixture. Estimation of Copro III and Proto IX from their mixtures is accurate upto ± 5% [Table 2-3].

Illumination (30 µmol m⁻² S⁻¹) of 4-d old cucumber plants in chill- and heat-stress conditions resulted in inhibition of Chl biosynthesis by 90% and 60%, respectively [Fig. 6]. This signifies that Chl biosynthesis is more inhibited in chill-stress than in heat-stress conditions. Chl biosynthesis has been shown to be severely inhibited by chill-stress in maize seedlings (Hodgins and van Huystee, 1986; van Huystee and Hodgins, 1989).

In order to investigate the mechanism of Chl biosynthesis in chill- and heat-stressed plants, synthesis of ALA (the committed precursor of Chl) was monitored. The reduction of ALA synthesis in chill and heat-stressed plants demonstrates that inhibition of Chl biosynthesis is due to impairment of ALA biosynthesis. Hodgins and van Huystee, (1986) have also demonstrated inhibition of ALA biosynthesis in chill-stressed maize plants. In the present investigation, ALA biosynthesis in cucumber was inhibited almost to a similar extent both in chill- (81%) and heat-stress (70%) conditions [Fig. 7]. However as stated above, the inhibition of Chl biosynthesis under identical conditions in chill- and heat-stressed plants was 90% and 60%, respectively. To account for the discrepancy, between the inhibition of ALA and Chl biosynthesis in chill- and heat-stress conditions, synthesis of Chl biosynthetic
intermediates and activities of different enzymes involved in Chl biosynthesis was monitored.

The ALA dehydratase (ALAD) is the first enzyme leading to porphyrin synthesis. Its activity was reduced by 24% and 45% in chill- and heat-stressed plants [Fig. 9]. ALAD is a heat-stable enzyme. However, when plants were grown at high temperature ALAD activity was inhibited more in heat-stressed plants than in chill-stressed plants. Thus higher inhibition of ALAD activity in heat-stress condition may be due to its reduced synthesis.

Similarly PBG deaminase (PBGD) activity was also reduced more in heat-stress than in chill-stress conditions [Fig. 10]. On the contrary, Proto IX synthesis from Urogen increased by 155% in heat-stress conditions and decreased by 65% in chill-stressed conditions [Fig. 11]. Stimulation of UDC activity in heat-stressed plants may be due to increased synthesis of this enzyme at an elevated temperature. Coprox activity was not affected and remained same as control in heat-stress conditions. However, in chill-stressed plants Coprox activity was partially reduced [Fig. 12]. Similarly Protox activity was not affected in heat-stress conditions, although there was a 60% decline in its activity in chill-stressed plants [Fig. 13]. Thus all the porphyrinogen oxidizing enzymes involved in Proto IX synthesis, were not affected in heat-stressed plants. Rather UDC activity was stimulated. This suggests that Proto IX biosynthesis is relatively insensitive to heat-stress as compared to that of chill-stress. Although ALAD and PBGD are partially inactivated in heat-stressed plants, they were probably well-compensated due to stimulation of UDC activity. Inhibition of all the enzymes involved in Proto IX synthesis in chill-stressed plants is may be partially due to their reduced synthesis.

Mg-chelatase catalyzes the insertion of magnesium into Proto IX and is the first enzyme of Mg-branch of metallo-porphyrin biosynthesis. It was inhibited in chill-stressed (60%) and heat-stressed (85%) plants [Fig. 14]. MPE cyclase, that catalyzes the conversion of MPE to Pchlide, was inhibited by 60% and 36% in chill- and heat-stressed plants, respectively [Fig. 21]. These two enzymes are involved in Pchlide biosynthesis. Inhibition or
stimulation of different enzymes involved in porphyrin biosynthesis in chill- or heat-stressed plants may be due to reduced or increased synthesis of the enzymes which could be verified by utilizing immuno precipitation techniques. The reduced or increased synthesis of the proteins could be proportional to the mRNA level, present in chill- or heat-stressed plants which could be monitored by running appropriate northern-blots. Post-translational modifications of proteins in chill- and heat-stressed conditions may contribute to reduced or increased activity of the enzymes. Further investigations are needed to ascertain the mechanism of inhibition or stimulation of enzymes in chill- and heat-stressed plants.

Pchlide synthesis was inhibited by 90% in chill-stress and 70% in heat-stress conditions, respectively [Fig. 8]. Severe inhibition of Pchlide biosynthesis in chill-stressed plants may be due to decline in Proto IX synthesis coupled with the inhibition of Mg-chelatase and MPE cyclase activities. Plants usually do not accumulate Proto IX as it is immediately metabolized to Pchlide. Therefore, actual amounts of Proto IX synthesized from the endogenous substrate could not be measured. The Protogen oxidizing enzymes are either stimulated or not inhibited in heat-stressed plants. Hence, synthesis of Proto IX in heat-stressed plants is likely to be higher than that of chill-stressed plants. Although heat-stressed plants had reduced activity of Mg-chelatase and MPE cyclase, they accumulated higher amounts of Pchlide than that of chill-stressed plants due to continued availability of its substrate, Proto IX.

Phototransformation of Pchlide to Chlide is mediated by the enzyme protochlorophyllide oxidoreductase (POR). POR activity measured as disappearance of Pchlide after exposure of etiolated plants to cool-white fluorescent light (30 \( \mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \)), increased in heat-stress conditions by 46% over that of control [Fig. 15-16]. Increase in the activity of POR in heat-stressed conditions may be due to increased synthesis of the enzyme and/or conversion of non-phototransformable Pchlide to transformable form. POR activity is not affected in chill-stressed plants. This is contrary to the previous report (van Huystee and Hodgins, 1989) of inhibition of phototransformation
of Pchlide to Chlide in chill-stressed conditions. This discrepancy may be due to different methodology followed by van Huystee and Hodgins, 1989. They had used exogenous substrate ALA to accumulate Pchlide and then phototransform Pchlide to Chlide by exposing plants to light. However, it is known that certain amounts of Pchlide synthesized from exogenous substrate ALA, are non-phototransformable (Chakraborty and Tripathy, 1992).

Taking into account of degree of inhibition of Pchlide biosynthesis in chill- and heat-stress conditions and stimulation of POR activity resulting in increased phototransformation (in heat-stress conditions) of Pchlide to Chlide and subsequent esterification with phytol to Chl, the calculated percent inhibition of Chl biosynthesis in chill- (92%) and heat-stressed (60%) plants matches well with the inhibition of the actual amount of Chl measured in chill- (90%) and heat-stress (60%) conditions (Fig. 6).

To study if temperature has similar effect on Chl biosynthetic enzymes in green plants as compared to etiolated plants, temperature induced changes of a few selected enzymes in greening cucumber cotyledons were investigated. The effect of chill- and heat-stress on Chl biosynthetic enzymes appear to be different in etiolated and green plants, i.e. in heat-stress conditions PBGD was inhibited in etiolated plants but increased in green plants [Fig. 18]. Similarly in heat-stress conditions, Protox was not affected in etiolated plants but inhibited in green plants [Fig. 19]. Mg-chelatase activity was inhibited in heat-stressed etiolated plants whereas it was stimulated in heat-stressed green plants [Fig. 20]. Significance of the differential behaviour of temperature on Chl biosynthetic enzymes in etiolated and green plants, is not understood and needs further investigation.

In order to compare the effects of growth temperature on Chl biosynthesis in dicot and monocot plants, the activities of a few enzymes of Chl biosynthetic pathway were monitored in wheat plants and compared with that of cucumber plants under similar temperature stress conditions.

In etiolated wheat plants, the PBGD activity was inhibited by 13% in chill- and 42% in heat-stress conditions [Fig. 22]. This is similar to the pattern of inhibition of PBGD reaction, observed in chill- and heat-stressed cucumber.
plants. PBGD was affected more in heat-stressed plants than in chill-stressed plants in both wheat and cucumber. Further the net Pchlide content was measured in wheat plants in both chill- and heat-stress conditions. Pchlide synthesis was reduced by 80% in chill-stressed while in heat-stress plants the reduction was only 60% [Fig. 23]. In control and chill-stressed wheat plants, phototransformation was almost same i.e. 57%. Phototransformation in heat-stress conditions was substantially higher (86%) [Fig. 24, 25] and was possibly due to induction of POR synthesis at elevated temperature. Similar pattern of POR activity was observed in cucumber plants. These results suggest that temperature has similar effect on Chl biosynthetic enzymes in cucumber (dicot) and wheat (monocot).

To understand the mechanistic detail of the impairment of Chl biosynthetic machinery in chill- and heat-stressed plants and study the adaptive responses of plants grown at low and high temperatures, plastids isolated from 7°C-, 25°C- and 42°C-grown plants were incubated with ALA in dark at different temperatures and the net syntheses of various tetrapyrroles were monitored. The optimum temperature for the synthesis of MPE and Pchlide was 35°C in control [Fig. 26]. In the chill-stressed plants the optimum temperature for MPE and Pchlide synthesis was observed as 30°C, i.e. there was a 5°C shift towards lower temperature [Fig. 27]. In heat-stressed plants, the temperature for optimum Pchlide biosynthesis was having broader profile and slightly shifted towards the higher temperature [Fig. 28A]. Enzymes involved in Pchlide biosynthesis are membrane-bound. Therefore, shift in optimum temperature in chill- and heat-stressed plants may be due to changes in the composition of membrane lipids. It has been reported that the fatty acid composition of membrane lipids play a major role in the low temperature acclimation of plants and cyanobacteria. Chilling-sensitive plants are known to contain high levels of saturated phosphatidylglycerol (PG) in their thylakoid membranes whereas chilling-resistant species have the majority of their PG in unsaturated form (Murata, 1983). Several mutants of Arabidopsis with defects in chloroplast membrane polyunsaturation were found to be chlorotic and deficient in chloroplast membranes when grown at
low temperatures. The fab1 mutant of *Arabidopsis* which contains increased levels of saturated fatty acids, was dramatically affected by low temperature treatment. There was almost complete loss of the photosynthetic function and associated destruction of chloroplasts within the leaf cells whereas wild-type plants maintained quantum efficiency of PSII at approximately 0.7 for at least 35 d at 2°C, this parameter declined rapidly in the mutant after 7 d and reached a value of less than 0.1 after 28 d at 2°C. These results provide a good demonstration of the importance of chloroplast membrane unsaturation to the proper growth and developments of plants at low temperature (Wu et al., 1997). It has also been reported that alterations in fatty-acid unsaturation of glycerolipids in thylakoid membranes can be achieved by changing the growth temperatures of photosynthetic organisms. Pearcy (1978) and Raison et al., (1982) observed that increases in growth temperature increase the level of saturated fatty acids in membrane lipids and enhance the heat stability of photosynthesis and suggested that saturation of fatty acids increases heat stability. Thus shift in optimum temperatures of MPE and Pchlide biosynthesis in chill- and heat-stressed plants may be due to desaturation or saturation of membrane lipids in low and high temperatures, respectively.

Proto IX synthesis continued at a substantial rate in both chill- and heat-stress conditions and the optimum temperature under experimental condition was 42°C. Continued synthesis of Proto IX may be due to stimulation of UDC or non-inhibition of Coprox and Protox at higher temperatures. Inhibition of Mg-chelatase and MPE cyclase at higher temperatures would also contribute to the accumulation of Proto IX.

To probe further chloroplasts isolated from 7°C-, 25°C- and 42°C-grown plants, were heated at different temperatures and syntheses of different tetrapyrroles were monitored at 25°C. Heating of plastids isolated from plants grown at control and chill-stressed conditions, resulted in the inhibition of MPE and Pchlide biosynthesis and accumulation of Proto IX. Maximum synthesis of Proto IX was observed when chloroplasts were heated at 50°C. At that point MPE and Pchlide synthesis was completely abolished.
As shown in [Fig. 29-31] the increase in Proto IX synthesis cannot be explained in terms of non-conversion of ProtoIX to MP(E) and Pchlide, as the total amount of MP(E) and Pchlide if added together, accounts only for 30-35% of Proto IX accumulated at 50°C. These results demonstrate that accumulation of Proto IX in heated chloroplasts is due to continued Proto IX synthesis at high temperatures. Proto IX synthesizing enzymes at high temperatures are comparatively more active than Mg-chelatase and MPE cyclase, responsible for Pchlide synthesis. A broader peak of Proto IX synthesis i.e. relatively higher synthesis of Proto IX at 55°C in heat-stressed plants, may be due to acclimation of heat-stressed plants containing Protox, bound to envelope membranes of the plastids (Matringe et al., 1992a).