Results & Discussions
Carotenoids in photosynthesizing organisms act as accessory pigments in light absorption. Carotenoids also act as antioxidants and provide protection against photooxidative stress to the pigment protein complexes (Niyogi 2000). They have been shown to be indispensable structural components in the assembly of pigment protein complexes (Trebst and Depka 1997). Considering their significance, various strategies have been employed to study the impact of carotenoid depletion on the development and maintenance of photosynthetic apparatus, especially the thylakoid membranes. Norflurazon, an experimental herbicide that inhibits carotenoid biosynthesis is being used for carotenoid depletion studies (Mannan and Bose 1988, Karapetyan 1993). It inhibits the enzyme phytoene desaturase, a key enzyme in the carotenoid biosynthesis. Norflurazon is also known to nonspecifically inhibit other desaturases, at low molar treatments, however, the primary effect of norflurazon is phytoene desaturase (Britton et al 1989, St John 1976).

Chloroplasts, the photosynthesizing plastids in higher plants are derived from the proplastids of the meristematic tissues under natural growth conditions. This development is light-dependent and in the absence of light the proplastids and chloroplasts develop into the achlorophyllous etioplasts. However, upon illumination these etioplasts develop into chloroplasts.

1. Effect of norflurazon on wheat leaves growing under light/dark cycle

1.1 Results

The proplastids have a very rudimentary membrane system lacking pigment protein complexes and the typical membrane organization of the thylakoids from mature chloroplasts. This maturation of thylakoid membrane is a complex process and depend the availability of various components like the pigments and
proteins. Therefore the absence of any of these components like carotenoids affects the photosynthetic process. The effect of carotenoid biosynthesis inhibition through norflurazon treatment on the development of thylakoids has been studied.

Two day old dark grown wheat seedlings were treated with different doses of norflurazon and illuminated (40 \(\mu\)M/m\(^2\)/sec) under light/dark cycle (14/10 h). Initial screening with a series of concentrations above 25 \(\mu\)M of norflurazon was found to be necrotic and lethal to the seedlings. Therefore a lower concentration of 25 \(\mu\)M was selected to examine the effect of norflurazon on carotenoid contents and thylakoid development. Accumulation patterns of the pigments were analysed in the primary leaves after the norflurazon treatment along with the polypeptides profiles of the thylakoid membranes from the norflurazon affected regions of the primary leaves. The effect of norflurazon on pigment protein complexes in thylakoids were characterised by taking a room temperature fluorescence emission spectrum between 650-800 nm after exciting the chlorophylls at 440 nm.

1.1A. Effect of norflurazon on carotenoids and chlorophylls in leaves: No change was observed till the second day in the pigment contents of the primary leaves of treated seedlings. However, from third day onwards the carotenoid levels started declining from the basal growing region of the leaves, reflective of norflurazon action. There was nearly 30% decrease in the carotenoid levels on the third and forth days. The loss in carotenoids reached to 50% by fifth and sixth days (Figure 1). When carotenoid levels were compared at the different regions of the primary leaves, the bottom halves of the leaves from the treated seedlings showed almost 60-70% decrease on third day reaching to more than 90% by the
Figure 1: Carotenoid contents from norflurazon treated leaves.
Results & Discussions

sixth day of treatment. On the other hand, the upper half of the leaves did not show appreciable changes in its carotenoid content till the fifth day.

In case of chlorophylls, their levels started declining from fourth day. The maximum loss of around 40% was observed on sixth day. It may be mentioned that the loss was mainly confined to the lower half of the leaf (Figure 2). On eighth day almost the entire leaf except its tip got bleached (Figure 3). In the young secondary leaves, developed after the herbicide treatment, the lamina was getting completely bleached with little of pigments.

1.1B. Thylakoid protein profiles in norflurazon treated leaves: Norflurazon treatment was shown to induce changes in the assembly of pigment protein complexes in thylakoids of several plants (Bolychvestseva et al 1993, Tonkyn et al 1992). These changes were found to be dependent on the light intensities and were most likely due to photooxidation. To know the effect of norflurazon on the thylakoid protein accumulation under the bleaching conditions the thylakoid polypeptides were analyzed through SDS-PAGE (12% acrylamide), from different areas of the six days illuminated norflurazon treated wheat leaves (Figure 4). The samples were analyzed on equal chlorophyll basis. We observed that there were differences between the tip and middle of the leaves in their polypeptide composition (Figure 4). These differences were prominent among the polypeptides of 30 kDa, and at low molecular peptides of around 14 kDa. Since, alterations in this molecular range of polypeptides are indicative of changes in LHCII and PSII, the loss of carotenoids and chlorophylls in this region may have caused a loss of these membrane proteins.

1.1C. Fluorescence emission spectrum from the thylakoids: In the present study, the room temperature absorption spectrum from the thylakoids of
Figure 2: Chl contents in norflurazon treated wheat leaves.
Figure 3: 8 days illuminated norflurazon treated wheat plants.
Figure 4: Thylakoid protein profiles from 6 day illuminated norflurazon treated wheat leaves. Lane 1: Control leaf tip region. 2: Control middle portion. 3: Treated leaf tips. 4: Treated middle region.
norflurazon treated wheat leaves showed a change in its intensity without major shifts in the peaks. Norflurazon is also known to inhibit the photosynthetic electron transport by binding to the $Q_B$ binding site of PSII leading to a rise in the fluorescence emission at this site (Tischer and Strotmann 1977). We therefore measured the room temperature fluorescence emission spectrum in the range of 650 to 800 nm, after exciting at 440 nm where chl absorbs, in the dark incubated thylakoid membranes isolated from the middle portion of 6 days illuminated norflurazon treated wheat leaves (Figure 5). The emission spectrum excited at 440 nm showed an emission peak at 683 nm (F683) with a hump at longer wavelength. The main absorption peak F683 originates from the PSII core and the longer wavelength peak from LCHII. The treated sample showed a similar fluorescence with a change in the intensity of about 21% at F683. Since the measurement was made on equal chl basis, this may reflect a change in the fluorescence yield of PSII.

1.2. Discussion

Norflurazon, a substituted pyridazinone, treatment has differential effect on thylakoids at different stages of development and leaves at different stages of growth as seen in the secondary leaves. Previous studies with norflurazon have shown that leaves at different stages of development respond differently to its treatment (Tonkyn et al. 1992). Our results with wheat are in agreement with these reported observations.

We illuminated norflurazon treated growing wheat seedlings to study the effect of norflurazon on both the developing and developed thylakoids. We found that the chl and carotenoid accumulation after the herbicide treatment is drastically reduced in the growing regions of the primary leaf and in the entire young secondary leaves. The differential accumulation of pigments in the primary
and secondary leaves may be due to a difference in the rate of carotenoid synthesis and in the stability of the photosynthetic apparatus in young and mature tissues. It is established that biosynthesis inhibitors mainly cause chlorosis only in developing tissues, whereas inhibitors of photosynthetic electron transport cause bleaching in the mature and developed tissues (Moreland 1980). It has been reported that carotenoids are essential for the assembly and photoprotection of PSII (Trebst and Depka 1997, Telfer et al. 1994). Higher rates of carotenoid biosynthesis might be required during the development of thylakoids in growing tissues to protect the chls from photooxidation. In mature tissues however, the carotenoids may be needed as photoprotectants of chls or as structural components only when the pigment protein complexes were turned over, and thus leading to very little impact on the mature tissue, in normal conditions as our observation revealed.

The lag between the norflurazon treatment and onset of bleaching in the primary leaves was perhaps reflective of the cumulative nature of the herbicide effect, where a threshold concentration of herbicide may be needed to inhibit the carotenoid biosynthesis. Together with the growth rate of the leaves, it may lead to a gradient effect of bleaching resulting in the mature tip portions escaping the carotenoid inhibition and developing into normal chloroplasts while developing chloroplasts in the middle and bottom levels getting effected and bleached.

In addition to the morphological changes in the leaves and in their pigment levels, norflurazon treatment also caused changes in the membrane protein accumulation in thylakoids. In the bleached regions of the wheat leaves SDS-PAGE analysis has shown differences in the protein profiles in the range of 30 kDa and below. The polypeptides in this range of molecular weight are generally associated with the pigment protein complexes of PSII and LHCII and other
antenna complexes and carotenoid inhibition through norflurazon treatment may have caused a change in these pigment protein complexes.

It is well known that carotenoid inhibition leads to depletion of PSII and LHCII in thylakoids in wheat, barley and several other plants (Dahlin 1988, Bolchvetseva et al. 1993). The reaction centers of PSI and PSII have β-carotene and the assembly of D1 protein into PSII is found to be dependent on the β-carotene (Trebst and Depka 1997). Also PSII assembly has been shown to be more sensitive to the reduced levels of carotenoids than PSI (Markgraff and Oelmuller 1991). As constitutive components of LHCII carotenoids are seen spanning the lipid bilayer and connect various components of the complex on the both the surfaces in its crystal structure (Kuhulbrandt et al 1994). Recently it has been hypothesized that carotenoid biosynthesis has a role in photoprotection (Niyogi 2000) through their dependence on reduced quinone produced from PSII and other non-photochemical sources. In addition to this carotenoid deficiency due to norflurazon treatment appears also to impair protein import into chloroplasts (Dahlin and Timko 1997, Paulsen 1999). Due to norflurazon treatment some of these factors might have been affected leading to a changes in the assembly of the thylakoid components.

Norflurazon is known to inhibit the photosynthetic electron transport by competitively binding to the \( Q_B \) site of PSII similar to DCMU binding (Tischer and Strotmann 1977). To ascertain whether electron transport is inhibited in our samples we took a room temperature fluorescence emission spectrum of the thylakoid membranes from 6 day illuminated norflurazon treated wheat leaves after exciting the chls at 440 nm. The yield of fluorescence emission at 683 (F683) at room temperature, which is ascribed to PSII, has decreased by about 21% (Figure 5) in the treated samples. This indicates that the inhibition of
Figure 5: Room temperature fluorescence emission spectrum of thylakoid membranes isolated from 6 day illuminated norflurazon treated wheat leaves.

(----- Control, ------ Treated)
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electron transport by norflurazon in PSII may not be the reason for this decline. Though there are reports indicating the number of PSII reaction centers increasing in wheat treated with a pyridazinone, SAN9785 (Mannan and Bose 1985), our study indicates that the decrease in the fluorescence yield after norflurazon treatment may be due to a decrease in the number of PSII reaction centers in thylakoid membranes. However, further studies are needed to precisely know the nature of PSII decline.

Thus the present study shows that 25 μM of norflurazon caused a basipetal bleaching of primary leaves and extensive arrest of pigment accumulation in the secondary leaves. Norflurazon is possibly affecting PSII and LHCII as shown in the changes of protein profiles (Figure 4) at around 30 kDa and also a decrease in the yield of fluorescence emitting from PSII.

2. Effect of norflurazon treatment on wheat etioplasts

Etioplasts differ markedly from chloroplasts in their pigment composition and membrane organization. Etiolated plants have only carotenoids as the pigments in their plastids. The other pigments, chls are inhibited at Pchlide level since the enzyme that converts Pchlide to chlide is a photoenzyme. Membranes in etioplasts lack the typical thyalkoid organization of chloroplasts. They have a crystalline prolamellar bodies surrounded by the membranous prothylakoids. The carotenoids and and some lipids are aggregated in the form of plastoglobuli. These inner membranes have some of the polypeptides and their complexes associated with mature thyalkoids (Balakrishna et al 1999).

Monocots are ideal experimental systems to study the development of chloroplast via etioplasts. They have larger leaves under etiolation and produce more plastids per cell under these conditions (Tevini 1977). Wheat has been
extensively used as a model system for understanding etiolation and photomorphogenesis of chloroplasts from etioplasts (Axelsson et al 1982, Dahlin 1988). Plastids in monocots are known to be at different stages of development along the leaf blade, and hence are differentially susceptible to various treatments in a basipetal manner. Therefore the experiments in the present study were performed on the middle part of the leaf lamina, of 8 day old dark grown norflurazon treated seedlings, to avoid complications arising out of this differential susceptibility. To avoid excessive oxidative damage norflurazon induced carotenoid inhibition levels were kept at 50% during etiolation of leaves.

2.1 Results
The effect of 50% inhibition of carotenoid biosynthesis on the etioplast membrane organization and its polypeptide composition has been studied by TEM and SDS-PAGE. Changes in the lipid-protein interactions in the etioplast inner membranes have been estimated with fluorescence emission studies from the ANS that is known bind to these membranes. Moreover, the antioxidant function of carotenoids in etioplasts was determined in terms of TABRS formation

2.1A. Effect of norflurazon on carotenoid levels of etiolated wheat leaves: The levels of carotenoids decreased with increasing concentration of norflurazone. It is clearly visible that the 2 μM of norflurazon was able to inhibit 50% of carotenoids in the middle of the leaf (Figure 6). The middle region of the lamina was paler in color when compared to a white basal region and normal yellow tips indicating the gradient effect of the herbicide.

2.1B. Effect of norflurazone on structure of etioplasts: Carotenoids are known to reside in the plastoglobuli of etioplasts (Goodwin 1977). These structures are located in the stroma of the plastids and often in the prolamellar
Fig. 6: Carotenoid inhibition by different norflurazon doses in 8 day old etiolated wheat leaves
bodies (PLBs). The etioplasts from dark grown wheat leaves treated with higher doses of norflurazon have been reported to have changes in the plastoglobuli structure (Axelsson et al 1982). However, there is no information on the effect of norflurazon at its lower concentration. Therefore in the present work we examined the effect of norflurazon at 2 µM on the wheat etioplasts through electron microscopy. It was found that at these treatments the gross structure of etioplast's inner membrane arrangement was affected. The results are shown in the figure 7C and D. The etioplasts from treated leaves have smaller and less prominent PLBs with fewer PTs surrounding them. Also the number of plastoglobuli has increased in the treated plastids, indicating the accumulation of intermediates of carotenoids into them.

2.1C. SDS-PAGE analysis of membrane proteins: Carotenoid deficiency due to norflurazon treatment was suggested to have impaired protein import into isolated chloroplasts (Paulsen 1999). Since the major protein component of PLB, the enzyme POR, is a nuclear encoded product (Rienbothe et al 1995) we were interested to see whether there was any change in the protein accumulation into the etioplast inner membranes due to norflurazon treatment. We performed a SDS PAGE of the etioplast inner membranes isolated from the treated and control plants (Figure 8). Interestingly a new band of size approximately 75 kDa was observed in the etioplast isolated from norflurazon treated seedlings. An increase in the intensity of a 45 kDa band was also observed in the treated samples. However the prominent band seen at approximately 40 kDa, in control was completely missing from the treated plastids. This missing polypeptide is likely to correspond to the POR (Ikeuchi and murakami 1982) in the control, is missing in the treated samples. However, this needs to be confirmed.
Figure 7: Transmission electron micrographs of etioplasts from 8 day old dark grown wheat leaves. Panel A and B control, panel C and D treated (magnification 16000x).
Figure 8: Polypeptide profiles from norflurazon treated etioplast inner membranes. M- Marker, C- Control and T- Treated.
2.1D. Effect of norflurazon on the lipid protein interactions in etioplast inner membranes: It is well known that ANS binds to both the membrane proteins and to the lipid phase, preferentially in the area of the lipid’s polar head (Minkov 1997). Therefore ANS is likely to provide the information on the lipid protein interactions.

The determination of the number of binding sites for the ligand ANS on the membranes and the dissociation constant of the binding reflect changes in the membrane structure. The fluorescence emission spectrum of ANS bound to the inner membranes of the etioplasts from the norflurazon treated leaves was measured between 400-700 nm by exciting at 390 nm, showed an increase fluorescence yield (Figure 9) when compared to the control.

A graphical analysis of the fluorescence intensity at the emission maximum 480, by plotting its reciprocal values Vs the membrane protein concentration gave the maximal fluorescence ($F_{\text{max}}$) attainable for bound ANS. The maximal fluorescence ($F_{\text{max}}$) at 480 nm has decreased by about 50% in the norflurazon treated membranes suggesting a decrease in the lipid protein interactions in the membranes.

The ANS bound to unit proteins were calculated by analyzing $F_{\text{max}}$ followed by construction of a Scatchard plot by plotting ANS bound Vs bound/free. This plot gives the number of binding sites for ANS and dissociation constant of binding ($K_d$) (Figure 10). The number of binding sites for ANS per unit protein has increased by six times in the carotenoid deficient membranes. However the dissociation constant ($K_d$) of binding is slightly lesser in the treated membranes when compared to the control (Table 1).
Figure 9: Fluorescence emission spectrum of etioplast inner membrane bound ANS. (------ Control, -------- Treated).
Figure 10: Scatchard plots of etioplast inner membranes from ANS binding assay.
Table 1: Data from ANS binding assays of etiolated inner membranes isolated from norflurazon treated etiolated 8 day old wheat leaves.

<table>
<thead>
<tr>
<th></th>
<th>$n$ (μM/mg protein)</th>
<th>$K_d$ (μM)</th>
<th>$F_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.32</td>
<td>11.53</td>
<td>152</td>
</tr>
<tr>
<td>Treated</td>
<td>7.42</td>
<td>10.15</td>
<td>99</td>
</tr>
</tbody>
</table>

$n$: number of binding sites, $K_d$: dissociation constant of ANS binding, $F_{max}$: maximum fluorescence intensity attainable
2.1E. Oxidative damage of etioplast inner membranes: The presence of lipids with highly unsaturated fatty acids in the inner membranes of plastids likely makes them susceptible to the peroxidative damage. Since carotenoids function as antioxidants in photosynthetic systems we were interested to know the relationship between carotenoid inhibition by norflurazon and peroxidative damage in membranes of etioplasts. Around 120% increase in TBARS formation was observed in herbicide treated membranes when compared to the control (Table 2). This observation suggests that the membranes of etioplasts are undergoing oxidative stress even in darkness, when carotenoids are inhibited. Thus these results show a close link between the carotenoid levels and oxidative stress in etioplasts. This observation is in conformity with ANS fluorescence analysis that shows a decrease in lipid protein interactions thereby perhaps inducing peroxidative damage.

2.2 Discussion

Etioplasts accumulate large quantities of Pchlide, which is a precursor of chl. This Pchlide is suggested to act as a photosensitizer during early stages of greening leading to photooxidative damage (Thomas 1997). Perhaps to obviate this possibility etioplasts have carotenoids which are likely to serve as quenchers of any free radicals that might be generated by photosensitization of Pchlide. Since etioplasts lack pigment binding proteins in their inner membranes carotenoids are localized either in their envelope membrane or in plastoglobuli (Goodwin 1977). Therefore it was interesting to see whether carotenoids have any structural role other than the photoprotection in etioplasts and treated wheat seedlings particularly with sublethal doses of norflurazon to inhibit carotenoid biosynthesis.
Table 2: Oxidative stress induced in the etioplast inner membranes of norflurazon treated dark grown wheat leaves.

<table>
<thead>
<tr>
<th></th>
<th>TBARS (µM/mg protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Treated</td>
<td>58 ± 5</td>
</tr>
</tbody>
</table>

*Values are mean of three independent experiments ± SE.
Our results described have shown that carotenoid inhibition by norflurazon is concentration dependent. It is important to note that norflurazon has also a gradient effect on the etiolated wheat leaves. Etioplasts in monocot leaves are found to be at different stages of development, depending on their position in the leaf lamina (Tevini 1971). Our observations indicate that norflurazon might have a cumulative effect on the etioplasts of basal and middle regions of the leaf resulting in a paler color in the treated leaves compared to their yellow tips and the control leaves.

Carotenoid depletion through norflurazon has caused a change in the size of PLBs in etioplasts as observed in the electron micrographs in the carotenoid depleted etioplasts. It is important to note that these changes in PLBs occurred at the lower concentrations of norflurazon. It is surprising that Axelsson et al (1982) did not observe any major alterations in the PLB structure in wheat even at higher concentrations of norflurazon at 100 \( \mu \text{M} \). This difference cannot be easily explained except for the fact that it may be due to varietal difference or mode of application of the herbicide or both. The increase in the number of plastoglobuli could be due to the accumulation of intermediates of carotenoid biosynthesis like phytoene (Dahlin and Ryberg 1986).

The reasons for changes observed in the PLBs in carotenoid depleted etioplasts are not properly understood. However, the major polypeptide constituent of PLBs is the nuclear encoded enzyme POR a 40 kDa protein. Reinbothe et al (1995) have reported that the accumulation of POR in the plastids is dependent upon the Pchlide levels. On reducing the levels of Pchlide by ALA treatment or by illumination, POR is not transported into the etioplasts. Other reports suggested that norflurazon treatment has affected the protein uptake by the isolated chloroplasts (Paulsen 1999). But it is not known if this
change in protein uptake is due to carotenoid inhibition or by some other action of norflurazon or whether this inhibition has similar effect on POR transport into etioplasts. We observed in norflurazon treated etioplast inner membranes a band in the range of 40 kDa, which may correspond to POR, is missing from the SDS-PAGE profile. This observation is significant. However, to get an insight at the molecular level on the role played by carotenoids in POR levels of plastids further studies are needed.

As mentioned earlier ANS binds to both the membrane proteins and to the lipid phase of the membrane, preferentially in the area of the lipid polar heads (Minkov 1997). Carotenoids particularly those involved in the xanthophyll cycle are hypothesized to stabilize lipid bilayers by directly getting inserted in to the membrane matrix in plastid membranes (Gruszecki 1999). Since etioplasts have similar xanthophyll composition, the deletion of carotenoids due to norflurazon may cause some perturbations in the hydrophobic matrix of the membranes. ANS is also shown to bind to the thylakoid membranes and its proteins (Andley et al 1981, Andley and Singhal 1983, Prasad et al 1977, Minkov 1997, Denev and Minkov 1997). Therefore any perturbations in the structure and function of thylakoid membranes are likely to reflect on the fluorescence property on the ANS. The increase in the number of binding sites and changes in the fluorescence intensity in the emission spectrum of the ANS bound to the etioplast inner membranes from norflurazon treated samples suggests that the carotenoid inhibition is leading to changes in the lipid protein interactions in the membrane.

The presence of lipids with highly unsaturated fatty acids in the plastid inner membranes makes them susceptible to the peroxidative damage. Peroxidation of the membrane lipids is an indication of oxidative stress in biological systems. Since carotenoids have antioxidant function it was interesting
to see whether there is any oxidative stress in the norflurazon treated etioplast in which carotenoid levels were depleted. It may be mentioned that the oxidative damage measured in terms of TBARS enhanced by 120% in the treated membranes. This enhancement might be due to non-availability of sufficient amounts of carotenoids. Our observation suggests that the etioplast membranes are undergoing oxidative stress even in darkness, when carotenoids are depleted. This is surprising, since most of the reports attributed the oxidative stress in plastids to their role of photosynthesis and the perturbations involved in this process (Asada 1999).

Although the exact source of oxidative stress in dark grown carotenoid deficient plastids is not understood, recent reports indicating that carotenoid biosynthesis is linked to a chlororespiratory pathway in plastids through a terminal oxidase which is hypothesized to have a role in the detoxification, of 
$H_2O_2$ through a NADPH-palstoquinone oxidoreductase complex (Carol and Kuntz 2001). This complex is reported to have a prominent role in etioplasts of barley (Guera et al 2000). Perhaps, a similar chain is operational in wheat etioplasts as well, which is effected by inhibition of carotenoid biosynthesis. This along with the changes in the hydrophobiosis of the membranes as observed in the changes of ANS fluorescence characteristics may be inducing an oxidative damage in dark grown plastids.

Our observations clearly shown that 50% carotenoid inhibition through norflurazon treatment in etiolated wheat leaves has caused prominent changes in the protein profiles and lipid protein interactions in the inner membranes of etioplast. The important observation is the likely affect of norflurazon, perhaps through carotenoid inhibition on the transport of POR a nuclear encoded protein into the etioplasts. This is reflected as a change in the significant 40 kDa band
which might correspond to enzyme POR. It appears that carotenoids might be directly linked with the transportation of nuclear encoded proteins like POR into the plastids. The observations on changes in the membrane hydrophobicity and peroxidative damage in the dark grown membranes are very important observations that might highlight the importance of carotenoids in the normal etioplast development.

3. Norflurazon action on greening of etioplasts
Upon illumination the etioplasts are transformed into chloroplasts. During this process the Pchlides accumulated in etioplasts are converted into chl. The pigment binding apoproteins, certain of them present in the etioplasts, and others synthesized upon illumination, bind to the pigments carotenoids and chls and start assembling into the membranes in a stepwise manner. The lipids associated with PLBs and PTs of the etioplasts phase get reorganized into thyalkoids of chloroplasts. These reorganized lipids are stabilized into a bilayer through their interactions with the pigment protein complexes. All these chain of events dependent on the availability of several components and are regulated by several factors like light and age of the leaves etc.

3.1. Results
8 day old etiolated wheat seedlings, in which carotenoid biosynthesis was 50% inhibited were illuminated under continuous white light to study the effect of carotenoid inhibition on the development of chloroplasts from etioplasts. To avoid photooxidative bleaching in the presence of norflurazon, the illumination was kept at a low intensity of 5 μM/m²/sec. As mentioned earlier wheat leaves show a gradient of carotenoid inhibition under the norflurazon treatment. We have taken the middle portion of the lamina (where the carotenoid levels are 50%
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inhibited during etiolation) and studied further the process of greening and associated structural changes during the plastid morphogenesis.

We reported the accumulation patterns of the pigments and proteins were studied during the illumination of norflurazon treated etiolated seedlings. We also analysed the changes due to norflurazon treatment in membrane organization of thylakoids at macrolevel and microlevel in the plastids after the illumination, using the electron microscopy and native gel assays. Further protein assembly into thylakoids and their interactions with lipids during different intervals of illumination have been analysed by SDS-PAGE and fluorimetric analysis of membrane bound ANS respectively. The apoprotein levels of the PSI reaction centers and manganese satbilising protein of PSII oxygen evolving complex in the norflurazon treated thyalakoids have been screened through immunoblotting. To know the oxidative stress levels during illumination of the norflurazon treated seedlings we assayed for the oxidative damage of thylakoid membranes and the specific activities SOD and catalase.

3.1A. Pigment and protein accumulation patterns during the greening process: Under low light intensity the treated plants looked pale green at the middle regions with complete bleaching at the basal regions and the tips in yellow color indicating the differential effect of the herbicide on the leaf (Figure 11). We observed that the 50% inhibition of carotenoid biosynthesis during etiolation has got a marginal effect on accumulation of chls till 4 h of greening. At this stage the treated leaves have only 60% of the amounts of chl while the carotenoid levels remain unaffected (Table 3). However, the chl levels were the same in both the control and the treated, during later stages of greening (8 h and 12 h). In case of carotenoid levels treatment of norflurazon reduced to 50% till 4 h. From 8 h onwards the levels of carotenoids were almost same in treated and
Figure 11: 12 h illuminated, 8 day old dark grown norflurazon treated wheat seedlings. N- treated, C-control.
Table 3: Amounts of pigments and proteins from norflurazon treated greening wheat leaves.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control*</th>
<th>Treated*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Chl</td>
<td>Car</td>
</tr>
<tr>
<td>4</td>
<td>200±13</td>
<td>180±15</td>
</tr>
<tr>
<td>8</td>
<td>440±22</td>
<td>170±12</td>
</tr>
<tr>
<td>12</td>
<td>535±66</td>
<td>180±16</td>
</tr>
</tbody>
</table>

*Values (for pigments µg/g f. wt. and for proteins mg/g f.wt) are means of three independent experiments ± SE.
control. Thus norflurazon inhibition of carotenoids after 8 h of illumination has become ineffective. There were no significant changes in the total protein contents between the treated and control leaves during greening (Table 3).

3.1B. Electron micrographic study of greening etioplasts: Carotenoid inhibition through norflurazon treatment has lead to the absence of grana in the weak red light illuminated wheat and barley chloroplasts (Axelson et al 1982, Bolychevetseva et al 1993). Since a recovery of pigments was observed during greening, in the norflurazon treated leaves where carotenoid inhibition was 50% under etiolation, we were interested to see whether this recovery has any impact on thylakoid membrane organization.

The electron micrographs of the treated plastids after 12 h of illumination have shown that the shape of the plastids is altered. They are elongated when compared to more oval shaped control plastids (Figure 12). Plastids of the control chloroplasts have shown a typical thylakoid structure with stacked granal regions and unstacked stromal regions. In some, as expected the PLBs surrounded by the stacked thylakoids are seen. This pattern, however, was missing in the treated samples where the membranes have relatively fewer granal stackings. It was also observed that even in the areas where there is stacking, the numbers of membranes per stacking were fewer when compared to the control thylakoids. This was similar to the earlier observations where plants have depleted carotenoid levels all along and were illuminated under dim red light which implies that the recovery from initial carotenoid inhibition did not lead to reorganization into normal thylakoids in the norflurazon treated plastids.

3.1C. Green gel assay of thylakoid membranes: In norflurazon treated and weak light grown plants it has been reported that the assembly of pigment protein
Figure 12: Transmission electron micrograph of Chloroplasts from 8 day old dark grown wheat leaves after 12 h of illumination. Panel A and B control, panel C and D treated (Magnification 16000x).
complexes in the thylakoids has been affected due to carotenoid inhibition (Dahlin 1988, Bolychvetseva et al 1993). It is well known that the presence of certain types of pigment protein complexes, like LHCIIIs, facilitates membrane stacking into grana in thyalkoid membranes (Garab and Mustardy 1999, Simidjiev et al 2000). Since there was a significant change in the membrane stacking in the norflurazon treated chloroplasts we wondered if there is a change in their pigment protein complex composition which might be leading to changes in the thylakoid stackings. To test this possibility fractionation of thylakoid membranes from 12 h illuminated seedlings was performed under nondenaturing conditions through LDS-PAGE. Figure 13 shows that there were differences between the control and treated thylakoids in the composition of their pigment protein complexes.

The CP-I complex that comprises of PSI has reduced drastically in the treated samples. Similarly the intensity of a band corresponding to CP-II that is supposed to have derived from LHCII was also much reduced in the treated thylakoids. The amount of free pigments has also decreased in the norflurazon treated samples. When these nondenaturing gels were stained with Coomassie blue we observed differences in the intensity of the protein bands in the corresponding positions. Thus these results imply that carotenoid biosynthesis inhibition during early stages of greening affected the assembly of pigment protein complexes into the thylakoids.

3.1D. Thyalkoid membrane bound ANS fluorimetric analysis: Pigments and their binding proteins get integrated into thylakoids during the development of these membranes. Since there are several polypeptides in thylakoids whose assembly into it is not directly dependent on the composition of pigments, knowing the over all changes in the membranes would give us a better
Figure 13: Non denaturing green gels from thylakoids of 12 h illuminated, 8 day old dark grown wheat seedlings. Panel A: Green gel. Panel B: Coomassie blue stained green gel. Lane 1 - control, Lane 2 - treated. CP I and II - Chlorophyll binding protein I and II.
understanding. ANS that binds to hydrophobic domains of the proteins and the lipid membranes, has been used as a probe to study the structural changes in the biological membranes (Andley et al. 1981, Andley and Singhal 1983, Prasad et al. 1977, Minkov 1997, Denev and Minkov 1997). Therefore ANS was used as a fluorescent probe to understand the changes in membrane structure during greening after norflurazon treatment.

Fluorescence emission spectra between 400-700 nm of ANS bound to the norflurazon treated thylakoids have shown a decrease in the fluorescence intensity at both time intervals (4 h and 12 h) of greening (Figure 14). The dissociation constant for ANS binding has not appreciably changed in the membranes of norflurazon treated samples. On the other hand the number of binding sites have decreased several folds in these membranes when compared to the control (Table 4).

We graphically analysed through Scatchard plots fluorescence intensities obtained through ANS binding to the membranes to estimate the number of binding sites on them (Figure 15). When we compared the number of binding sites among the treated samples at different time intervals we notices they were going up as illumination progressed from 4 h to 12 h, where as in control their number is decreasing with illumination. The maximal ANS fluorescence ($F_{max}$) obtainable at 480 nm in the treated samples has shown a massive raise when compared to the control at 4 h of illumination. These values are very close to those generally associated with lipid depleted membranes (Andley et al. 1981). As the illumination progresses to 12 h the $F_{max}$ has decreased considerably but still higher than that of the controls. In case of control membranes the $F_{max}$ declined with illumination time (Table 4).
Figure 14: Fluorescence emission spectrum of thylakoid membrane bound ANS. Panel A thylakoids from 4 h illuminated plants. Panel B thylakoids from 12 h illuminated plants. (Control, Treated).
Table 4: Data from ANS binding assays of thylakoid membranes isolated from norflurazon treated etiolated and greening wheat leaves.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>( n ) (( \mu \text{M/mg Protein} ))</th>
<th>( K_d(\mu \text{M}) )</th>
<th>( F_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td>4</td>
<td>67.55</td>
<td>1.23</td>
<td>57.89</td>
</tr>
<tr>
<td>12</td>
<td>32.36</td>
<td>5.77</td>
<td>53.42</td>
</tr>
</tbody>
</table>

\( n \): number of binding sites, \( K_d \): dissociation constant of ANS binding, \( F_{\text{max}} \): maximum fluorescence intensity attainable.
Figure 15a: Scatchard plot of 4 h illuminated norflurazon treated wheat leaves from ANS binding assay
Figure 15b: Scatchard plot of thylakoids from 12h illuminated norflurazon treated wheat leaves for ANS binding assay.
3.1E. SDS-PAGE analysis of thylakoid polypeptides: To see whether there is a correlation between the number of ANS binding sites reflected on the protein profiles of the membranes we performed SDS-PAGE analysis of thylakoids isolated after different illumination intervals was performed. Figure 16 showed both the control and the treated samples have shown a similar trend in the assembly of proteins into the membranes at different time intervals there are differences in the intensities of some polypeptide bands between the treated and the control samples. The prominent of these differences are being in the range of 20–45 kDa. The polypeptides of this molecular range are generally associated with the pigment-protein complexes in the thylakoid membranes whose assembly in to the membrane has been reported to be dependent on the illumination (Balakrishna et al 1999).

3.1F. Western blotting against PSI RC and MSP antibodies: From the native gel analysis we observed that the pigment protein complexes corresponding to the PSI and LHCII are declining in the norflurazon treated thylakoid membranes. To know whether the apparent decline of PSI is due to absence of the apoproteins concerned we carried out immunoblotting against a specific antibody of the PSI reaction center. It was observed that there was no difference between the control and treated samples in their PSI RC polypeptide content at any stage of greening (Figure 17). It is clear that norflurazon is affecting pigment binding to the apoproteins.

The 32 kDa manganese stabilizing protein (MSP) of PSII oxygen evolving complex is known to be present in the etiolated membranes perhaps as an apoform. Upon illumination and subsequent assembly of PSII it gets associated with PSII. We used antibodies against the manganese stabilizing protein to check the status of intact PSII in the norflurazon treated membranes during greening.
Figure 16: Polypeptide profile of thylakoids from norflurazon treated 8 day old etiolated wheat leaves, isolated after different illumination periods. Lane 1, 3 and 5 represents thylakoids isolated from the control leaves after 4, 8 and 12 h of illumination respectively. Lanes 2, 4 and 6 represents treated thylakoids from similar conditions. M - protein marker
Figure 17: Western blotting against PSI (Panel A) and MSP (Panel B) antibodies. Lanes 1, 3 and 5 represent control thylakoids after 4, 8 and 12 h after illuminations respectively. Lanes 2, 4 and 6 represent thylakoids from norflurazon treated leaves after similar conditions.
Results & Discussions

There were no changes in the levels of MSP between control and treated at any stage of greening (Figure 17) indicating that the PSII may be compositionally the same.

3.1G. Assays for lipid peroxidation and antioxidative enzymes: Due to redox reactions of photosynthesis chloroplasts are prone to the frequent oxidative attacks (Asada 1999). Carotenoids because of their proximity to the chlorophylls in different pigment protein complexes, effectively quench free radicals that are inevitably generated under physiological stresses (Telfer et al 1994). Apart from carotenoids enzymes like SOD and catalase and several non-enzymatic molecules also participate in the detoxification of free radicals (Niyogi 2000). Etioplast inner membranes were found to undergo oxidative damage when carotenoids levels are depleted we assayed for lipid peroxidation trend in the treated and control thylakoids during greening at different illumination intervals. It is being observed that at 4 h of illumination there has been a marginal raise in the lipid peroxidation associated TBARS produced, when compared to the control. However, there was no difference between the control and treated samples in TBARS at later stages of the greening (Table 5).

Since there were no significant differences in terms of oxidative damage, it was possible that the free radicals generated by norflurazon treatment might have been efficiently scavenged by other enzymatic scavengers. Therefore the response of SOD and catalase over the treatment was examined. In this case also there were no appreciable changes in the specific activities of these enzymes at any stage of greening (Tables 6 and 7).
Table 5: Oxidative stress induced in the thylakoid membranes of norflurazon treated greening wheat leaves.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>TBARS (µM/mg protein)*</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>75 ± 4</td>
<td>87 ± 3</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>57 ± 7</td>
<td>57 ± 4</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>69 ± 5</td>
<td>67 ± 6</td>
<td></td>
</tr>
</tbody>
</table>

*Values are mean of three independent experiments ± SE.
Table 6: Specific activities of the enzyme catalase from greening norflurazon treated wheat leaf extracts.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Specific activity* (units/mg protein)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>14.5±1.4</td>
<td>15.0±0.9</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25.9±1.6</td>
<td>25.5±1.1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>26.0±1.9</td>
<td>20.0±1.5</td>
<td></td>
</tr>
</tbody>
</table>

*Values are means of three independent experiments ± SE.
Table 7: Specific activities of the enzyme superoxide dismutase from norflurazon treated greening wheat leaf extracts.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Specific activity* (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>4</td>
<td>24.0±0.8</td>
</tr>
<tr>
<td>8</td>
<td>31.0±1.1</td>
</tr>
<tr>
<td>12</td>
<td>24.0±1.3</td>
</tr>
</tbody>
</table>

*Values are means of three independent experiments ± SE.
3.2. DISCUSSIONS

As already mentioned in the previous section, norflurazon treated plants when grown under normal light intensities undergo photooxidative stress and get bleached. Thus the importance of carotenoids in the development and assembly of thylakoid membranes under natural conditions, by inhibiting their biosynthesis could not be assessed. However, various strategies have been used to overcome the photooxidative stress caused by carotenoid depletion and a common one being to grow the plants under intermittent flash illumination or under continuous red light (Axelsson et al 1982, Dahlin 1988, Bolychvetseva et al 1993). But to maintain more natural conditions we used a different approach while minimizing oxidative stress under carotenoid inhibition. The effective carotenoid inhibiton has been kept at its 150 at the beginning of greening levels and plants were illuminated under continuous low light intensities of 5 μM/m²/sec.

Illumination for 4 h of eight day old norflurazon treated etiolated seedlings resulted in 40% of inhibition in chl levels. However, these levels were restored to the normal levels by illuminating for 8 h and beyond. Almost similar effect was also observed in the case of carotenoid biosynthesis. This may be due to variable sensitivity of the enzyme phytoene desaturase to the norflurazon at different stages of plant growth. There are reports suggesting a raise in the amounts of carotenoids in greened plastids when compared to the etiolated (Young 1993). However, at the low light intensities this may not hold true, as indicated by the control plants where the carotenoid amounts remain the same both under etiolated and greening. Hence there could be some other mechanism operating which is influencing carotenoid biosynthesis leading to a recovery.

Several workers have reported the light induced assembly of LHCII into thylakoids and formation of grana depends on carotenoids (Mullet 1983, Dahlin...
1988, Bolychevetseva et al 1993). Recently LHCII is shown to be responsible for the membrane stacking into grana (Garab and Mustardy 1999, Simedjeive et al 2000). EM studies into the ultrastructure of wheat plastids, developing from carotenoid depleted etioplasts demonstrated that grana are missing from their thylakoid membranes (Figure 12). Since the LHCII level is significantly reduced in the treated wheat thylakoids as shown in the form of reduction of CP2 band in the non-denaturing gel (Figure 13). This might indicate that LHCII are responsible for the missing of grana.

It is important to note that the carotenoid and chl levels are almost same in the control and treated after 12 h of greening. Several reports suggested that assembly of LHCII in thylakoids and grana formation is carotenoid dependent. However, carotenoids may be critically important in the initial stages of LHCII formation because the normal level of carotenoids at 12 h of greening could not lead to the formation of LHCII in our system. The reduced level of chl might also be causing an effect on LHCII. It has been shown in barley seedlings low rate of chl biosynthesis affects LHCII (Rodermel & Bogard 1985).

The MSP, an important component of PSII, levels were not altered in the greening of norflurazon treated membranes as indicated by immunoblotting (Figure 17B). However, the deficiency of carotenoids are required for the formation of PSII (Bolychevtseva et al 1995, Humbcek et al 1989, Markgraf and Oelmuller 1991). Also it was reported carotenoids are essential for the incorporation of D1 protein into the PSII reaction centers (Trebst and Depka 1997). But our observation is indicating that the PSII reaction centers in the carotenoid treated thylakoids are stable. It can be possible that the 50% carotenoid levels were sufficient for this stability.
The Western blotting against the PSI reaction center shows that there was no difference between the control and treated samples at protein level. However, the native PAGE shows no band corresponding to the pigment-protein complex at this level indicating either the pigments are not binding to the proteins or are loosely bound and due to the detergent treatment are getting detached. The latter case is probable, since the free pigment should have increased in the treated samples, which is not the case. Though, there are reports in *Scenedesmus obliques* was unable to form PSI antenna when treated with norflurazone (Romer *et al* 1991) the cause for this apparent lack of pigment bound PSI needs to be explored.

This paradox where the amount of pigments being normal and still the changes in the pigment protein complexes vis-à-vis PSI and LHCII may imply that the pigments are probably getting bound to certain other apoproteins like CP43 and 47 or other proteins like ELIPS or PsbS protein of PSII, which act as intermediate carriers, under stressed conditions (*Montane and Kloppstech* 2000, Niyogi 2001).

*ANS* has extensively been used to study the changes in plastid membranes and several other parameters in membrane integrity (*Andley et al* 1981, *Andley and Singhal* 1983, *Prasad et al* 1977, Minkov 1997, Denev and Minkov 1997). Fluorescence emission maximum analysis of membrane bound *ANS* during greening is significant as it indicates a decrease in the number of binding sites during greening in the treated samples when compared to the control. This seems reasonable when we take other observations that there are fewer pigment protein complexes in the treated membranes as indicated by changes in the stacking thylakoids and the native gel analysis. However, a decrease in the number of binding sites is not reflected in maximal fluorescence
(F_{max}) at 480 nm, which is increasing with greening in norflurazon treated membranes when compared to the control. ANS also binds to the lipids. During greening from etioplasts the lipids that are normally nonbilayer forming, like DGDG, gets stabilized by the presence of pigment protein complexes into a stable bilayers. Since we observed there are changes in the pigment protein complexes in the norfluorazon treated thylakoid membranes there could be changes at micro levels of the bilayer leading to perturbations which might be reflecting as changes in the F_{max}.

Our observation that there are virtually no changes in the lipid peroxidation levels or in the enzyme defense systems studied indicate that plants could recover from the oxidative stress observed in the initial stages of greening when the carotenoid levels are lower than the normal. There are many other membrane bound antioxidative systems both enzymatic and nonenzymatic whose activities we did not look into in the present study (Asada 1999). These may be participating in the repair of the membrane damage, which is seen recovering in the latter stages of greening when the carotenoid levels are recovering. Even when the lipid peroxidation levels are above normal there were no changes in activities of the two important enzymatic defense systems, SOD & CAT. Hence, the oxidative stress observed here in the membranes might be a free radical mediated rather than superoxide mediated. This assumption is supported by the presumed role of carotenoids in quenching 1O_2 and 3Chl formed in the reaction centers (Telfer et al 1994). However, further experiments are needed in order to establish the role 1O_2 or 3Chl in the present case.

Greening of etioplasts under continuous low intensity of illumination has probably for the first time suggested that the anti-oxidative and structural roles can be separated. Carotenoid biosynthesis inhibition at 50% levels during
etiolation and its impact on greening under low intensity continuous illumination has been a novel approach in studying the greening of etioplasts. It signifies the importance of carotenoids in the assembly of thylakoid membranes without the intervention of photooxidative stress. An important observation is the likely effect of carotenoid biosynthesis inhibition at the initial stages of development of various pigment protein complexes. We have shown that there are normal levels of apoproteins of PSI and perhaps PSII in the thylakoids developed from norflurazon treated etioplasts. However, from TEM studies and native gel assay it may be concluded that LHCII levels are affected which is likely reflected as loss of thylakoid membrane stackings into grana. Thus pigment binding to the apoproteins has a major effect on the lipid protein interactions as highlighted by changes in the ANS fluorescence characteristics. Significantly the oxidative damage as represented by lipid peroxidation levels during the initial stages of greening has gone down when the carotenoid levels started recovering implying a direct link between the carotenoid levels and oxidative stress during greening. This oxidative stress may be freeradical mediated since the enzymes SOD and catalase generally associated with superoxide involved stree responses are at normal levels during the intial stages where lipid peroxidation is observed.