PHYSIOLOGICAL BASIS OF CYTOKININ INDUCED DROUGHT TOLERANCE IN WHEAT

(Triticum aestivum L.)

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PHYSIOLOGICAL BASIS OF CYTOKININ INDUCED DROUGHT TOLERANCE IN WHEAT (Triticum aestivum L.)

BY

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This is to certify that the thesis entitled “Physiological Basis of Cytokinin Induced Drought Tolerance in Wheat (Triticum aestivum L.)” submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi in partial fulfillment of MASTER OF SCIENCE in PLANT PHYSIOLOGY, embodies the results of bonafide research work carried out by Shivani Nagar; Roll No. 4906 under my guidance and supervision, and that no part of this thesis has been submitted for any other degree or diploma. This assistance and help availed during the course of investigation as well as source of information have been duly acknowledged by him.

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# CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>TITLE</th>
<th>PAGE NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>INTRODUCTION</td>
<td>1-3</td>
</tr>
<tr>
<td>2.</td>
<td>BACKGROUND</td>
<td>4-11</td>
</tr>
<tr>
<td>3.</td>
<td>MATERIALS AND METHODS</td>
<td>12-25</td>
</tr>
<tr>
<td>4.1</td>
<td>RESEARCH PAPER-I</td>
<td>26-52</td>
</tr>
<tr>
<td>4.2</td>
<td>RESEARCH PAPER-II</td>
<td>53-68</td>
</tr>
<tr>
<td>5.</td>
<td>DISCUSSION</td>
<td>69-73</td>
</tr>
<tr>
<td>6.</td>
<td>SUMMARY AND CONCLUSION</td>
<td>74-76</td>
</tr>
<tr>
<td>7.</td>
<td>ABSTRACT (English)</td>
<td>77</td>
</tr>
<tr>
<td>8.</td>
<td>ABSTRACT (Hindi)</td>
<td>78</td>
</tr>
<tr>
<td>9.</td>
<td>BIBLIOGRAPHY</td>
<td></td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Title</th>
<th>After page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1.1.</td>
<td>Effect of cytokinin (Benzyl amino purine; BAP (40 µM)) on relative water content (RWC; %) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.2.</td>
<td>Effect of cytokinin BAP (40 µM) on membrane stability index (MSI; %) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.3.</td>
<td>Effect of cytokinin BAP (40 µM) on chlorophyll-a content (mg g⁻¹ dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.4.</td>
<td>Effect of cytokinin BAP (40 µM) on chlorophyll-b content (mg g⁻¹ dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.5.</td>
<td>Effect of cytokinin BAP (40 µM) on total chlorophyll content (mg g⁻¹ dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.6.</td>
<td>Effect of cytokinin BAP (40 µM) on chlorophyll-a/chlorophyll-b ratio at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.7.</td>
<td>Effect of cytokinin BAP (40 µM) on carotenoid content (mg g⁻¹ dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.8.</td>
<td>Effect of cytokinin BAP (40 µM) on photosynthesis rate (µmoles CO₂ m⁻² s⁻¹) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.9.</td>
<td>Effect of cytokinin BAP (40 µM) on stomatal conductance (cm</td>
<td>52</td>
</tr>
<tr>
<td>4.1.10.</td>
<td>Effect of cytokinin BAP (40 µM) on photochemical efficiency (Fv/Fm) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.11.</td>
<td>Effect of cytokinin BAP (40 µM) on total sugar content (mg g(^{-1}) dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.12.</td>
<td>Effect of cytokinin BAP (40 µM) on total starch content (mg g(^{-1}) dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.13.</td>
<td>Effect of cytokinin BAP (40 µM) on total carbon content (mg g(^{-1}) dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Effect of cytokinin BAP (40 µM) on total biomass (gm plant(^{-1})) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>68</td>
</tr>
<tr>
<td>4.2.2.</td>
<td>Effect of cytokinin BAP (40 µM) on total leaf area (cm(^2) plant(^{-1})) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>68</td>
</tr>
<tr>
<td>4.2.3.</td>
<td>Effect of cytokinin BAP (40 µM) on nitrate reductase activity (µmoles g(^{-1}) dw h(^{-1})) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>68</td>
</tr>
<tr>
<td>4.2.4.</td>
<td>Effect of cytokinin BAP (40 µM) on glutamine synthetase activity (µmoles g(^{-1}) dw h(^{-1})) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>68</td>
</tr>
<tr>
<td>4.2.5.</td>
<td>Effect of cytokinin BAP (40 µM) on total soluble leaf protein (mg g(^{-1}) dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>68</td>
</tr>
<tr>
<td>4.2.6.</td>
<td>Effect of cytokinin BAP (40 µM) on total protease activity (µmoles g(^{-1}) dw h(^{-1})) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>68</td>
</tr>
<tr>
<td>4.2.7.</td>
<td>Effect of cytokinin BAP (40 µM) on total leaf nitrogen (%) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.8.</td>
<td>Effect of cytokinin BAP (40 µM) on total crude protein (%) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.9</td>
<td>Effect of cytokinin BAP (40 µM) on number of tillers at harvest at in two contrasting cultivar affected by water deficit stress in wheat</td>
<td></td>
</tr>
<tr>
<td>4.2.10</td>
<td>Effect of cytokinin BAP (40 µM) on grain yield per plant (gm) at harvest at in two contrasting cultivar affected by water deficit stress in wheat</td>
<td></td>
</tr>
<tr>
<td>4.2.11</td>
<td>Effect of cytokinin {Benzyl amino purine; BAP (40 µM)} on grain number per panicle at harvest at in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.12</td>
<td>Effect of cytokinin BAP (40 µM) on 1000 grain weight (gm) at harvest at in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.13</td>
<td>Effect of cytokinin BAP (40 µM) on harvest index at harvest at in two contrasting cultivar affected by water deficit stress in wheat</td>
<td></td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Title</th>
<th>After page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1.1.</td>
<td>Effect of cytokinin (Benzyl amino purine; BAP (40 µM)) on relative water content (RWC; %) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.2.</td>
<td>Effect of cytokinin BAP (40 µM) on membrane stability index (MSI; %) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.3.</td>
<td>Effect of cytokinin BAP (40 µM) on chlorophyll-a content (mg g⁻¹ dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.4.</td>
<td>Effect of cytokinin BAP (40 µM) on chlorophyll-b content (mg g⁻¹ dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.5.</td>
<td>Effect of cytokinin BAP (40 µM) on total chlorophyll content (mg g⁻¹ dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.6.</td>
<td>Effect of cytokinin BAP (40 µM) on chlorophyll-a/chlorophyll-b ratio at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.7.</td>
<td>Effect of cytokinin BAP (40 µM) on carotenoid content (mg g⁻¹ dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.8.</td>
<td>Effect of cytokinin BAP (40 µM) on photosynthesis rate (µmoles CO₂ m⁻² s⁻¹) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.9.</td>
<td>Effect of cytokinin BAP (40 µM) on stomatal conductance (cm s⁻¹) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>4.1.10.</td>
<td>Effect of cytokinin BAP (40 µM) on photochemical efficiency (Fv/Fm) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.1.11.</td>
<td>Effect of cytokinin BAP (40 µM) on total sugar content (mg g(^{-1})dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.1.12.</td>
<td>Effect of cytokinin BAP (40 µM) on total starch content (mg g(^{-1})dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.1.13.</td>
<td>Effect of cytokinin BAP (40 µM) on total carbon content (mg g(^{-1})dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.1</td>
<td>Effect of cytokinin BAP (40 µM) on total biomass (gm plant(^{-1})) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.2</td>
<td>Effect of cytokinin BAP (40 µM) on total leaf area (cm(^2) plant(^{-1})) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.3</td>
<td>Effect of cytokinin BAP (40 µM) on nitrate reductase activity (µmoles g(^{-1})dw h(^{-1})) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.4</td>
<td>Effect of cytokinin BAP (40 µM) on glutamine synthetase activity (µmoles g(^{-1})dw h(^{-1})) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.5</td>
<td>Effect of cytokinin BAP (40 µM) on total soluble leaf protein (mg g(^{-1})dw) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.6</td>
<td>Effect of cytokinin BAP (40 µM) on total protease activity (µmoles g(^{-1})dw h(^{-1})) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.7.</td>
<td>Effect of cytokinin BAP (40 µM) on total leaf nitrogen (%) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.8.</td>
<td>Effect of cytokinin BAP (40 µM) on total crude protein (%) at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.9</td>
<td>Effect of cytokinin BAP (40 µM) on number of tillers at harvest at in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.10</td>
<td>Effect of cytokinin BAP (40 µM) on grain yield per plant (gm) at harvest at in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.11</td>
<td>Effect of cytokinin {Benzyl amino purine; BAP (40 µM)} on grain number per panicle at harvest at in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.12</td>
<td>Effect of cytokinin BAP (40 µM) on 1000 grain weight (gm) at harvest at in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>4.2.13.</td>
<td>Effect of cytokinin BAP (40 µM) on harvest index at harvest at in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td></td>
</tr>
<tr>
<td>Plate No.</td>
<td>Title of plate</td>
<td>After pages</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>4.1.1</td>
<td>Effect of cytokinin (Benzyl amino purine; BAP (40 µM)) on RT-PCR expression analysis of Rubisco SSU gene at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.2</td>
<td>Effect of cytokinin (Benzyl amino purine; BAP (40 µM)) on RT-PCR expression analysis of COR- gene at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.3</td>
<td>Effect of cytokinin (Benzyl amino purine; BAP (40 µM)) on RT-PCR expression analysis of DHN- gene at three different growth stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
<tr>
<td>4.1.4</td>
<td>Effect of cytokinin (Benzyl amino purine; BAP (40 µM)) on morphology at anthesis stages in two contrasting cultivar affected by water deficit stress in wheat.</td>
<td>52</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important staple food crops grown over 200 mha in the range of environment throughout the world with an annual production likely to reach more than 650 million metric tons in 2009-10 (Yadav *et al.*, 2010). The production and productivity of wheat crop were quite low, when India became independent in 1947. The production of wheat was only 6.46 million tonnes and productivity was merely 663 kg per hectare during 1950-51, which was not sufficient to feed the Indian population. After the Green Revolution in the late 60’s the production of wheat has shown a huge increase and now India is the second largest producer of wheat in the world and produced a record 80.7 million tons during the year 2010 (Economic Survey of India, 2010) with 28.5 million ha area under wheat production and 2830 kg/ha productivity. Major wheat growing states in India are Uttar Pradesh, Punjab, Haryana, Rajasthan, Madhya Pradesh, Gujarat and Bihar.

Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for major crop plants by more than 50%. It includes stress condition like drought, salinity, extreme temperatures and heavy metals. All these abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity. Drought is the most serious environmental factor limiting the productivity of agricultural crops, with devastating economical and sociological impact. Estimates indicate that 25% of the world’s agricultural lands is now affected by water stress. Moreover, the faster-than-predicted change in global climate (Intergovernmental Panel on Climate Change, 2007) indicated that drought episodes will become more frequent because of the long-term effects of global warming. Emphasizing the urgent need to develop adaptive agricultural strategies for a changing environment. It can be said that it is one of the most devastating environmental stresses. In 2009, 19% of India’s land area experienced ‘moderate drought’; 10 % suffered ‘severe drought’. More than 246 districts in the country have been affected by deficient rainfall (Economic Survey of India, 2009). Drought stress remains an ever-growing problem that severely limits
crop production and causes important agricultural losses particularly in arid and semiarid areas (Boyer et al., 1982). The percentage of drought affected land areas more than doubled from the 1970s to the early 2000s in the world. Understanding how plants respond to drought and co-occurring stresses can play a major role in stabilizing crop performance under drought conditions and in the protection of natural vegetation. Although drought can strike at any time, depending on which stage of growth a plant experiences drought stress, it reacts quite differently to the stress. The plants are most prone to damage due to limited water during flowering time.

Plants can use a combination of different strategies to avoid or tolerate drought stress. In arid regions, for example, winter annuals combine a relatively short life cycle with a high growth rate during the wet season to avoid drought altogether. Other types of avoidance include closing of stomata to minimize water loss, adjusting source/sink allocation by increasing root growth, and decreasing canopy by reducing growth and shedding of older leaves. Accelerated leaf senescence and leaf abscission are associated with drought in nature as a means to decrease canopy size. In perennial plants, this strategy contributes to the survival of the plant and the completion of the plant life cycle under drought stress. In contrast, this strategy reduces the yields of annual crops, with concomitant economical loss to farmers. We hypothesized that it is possible to enhance the tolerance of plants to drought stress by delaying the drought-induced senescence of leaves during the drought episode. Our hypothesis is that senescence is due to a type of cell death program that could be activated in different plants during drought. Suppressing it could therefore enable plants to mount a vigorous acclimation response that would result in enhanced drought tolerance with reduced yield losses. Water availability limits wheat productivity in many parts of the world. Stress responses in wheat plant dependent on the developmental stage of the plant (Bray, 1997). Wheat yield is particularly susceptible to drought during two important growth periods, the first is between terminal spikelet (TS) stage (Tottman and Broad, 1987) and boot stage (BT), and the second between boot stage and anthesis (Zhang and Oweis, 1999).

Various physiological and biochemical effects of phytohormone and plant growth regulators (PGR’s) on plant systems have been documented. These include effects on ion uptake, membrane permeability, photosynthesis, mitochondrial respiration, etc. These are also important signal molecules for modulating plant
responses to various kinds of stresses. It is now clear that various PGR’s are involved in a number of stress responses that involve oxidative stress as a major factor including such varied stresses as pathogen infection, UV-irradiation, heat, salinity, osmotic and others. In addition, it is now becoming clear that PGR’s interacts both negatively and positively with other major signalling pathways including those regulated by jasmonic acid and ethylene.

Cytokinins (CKs) are known to regulate several aspects of plant growth and development, including the response of plants to abiotic stress (Rivero et al., 2007). CKs regulate stomatal behaviour (Hegele et al., 2008), the formation and protection of cellular structures (Chiappetta et al., 2006), and the induction and activation of protein synthesis (Chernyad, 2005). There important role as senescence-retarding hormones has been found and their exogenous application has been demonstrated to prevent the degradation of chlorophyll and photosynthetic protein.

Photosynthesis is among the primary processes affected by water stress induced change in stomatal behaviour, degradation of chlorophyll and carotenoid content and decrease in rubisco activity. The cytokinins played protective role, increasing the stability of the photosynthetic apparatus and maintain the rate of photosynthesis under water stress condition. Drought stress also has a negative effect on plant nitrogen concentrations, free aminoacids or soluble protein contents accompanied with a decline of activity of nitrate reductase and glutamine synthetase in many plant species, such as in winter wheat (Xu and Yu, 2005, 2006). CKs can enhance activity of enzymes of nitrogen metabolism. However, the precise physiological and molecular mechanism of cytokinin-regulated stress responses is not clear yet. Therefore, an experiment was conducted to study the effect of CKs on drought stress responses of wheat. Keeping the aforementioned background in mind, present study was undertaken with the objective “The effect of cytokinin on physiological/biochemical and molecular changes in wheat under water stress”.

2. BACKGROUND
Wheat is one of the premier cereal crops of worldwide importance which is grown under a wide range of climatic conditions. India is one of the major producers of wheat and it is maintaining its second position of wheat producing nations after China. Record breaking wheat harvest to the tone of 80.71 million tonnes was obtained during 2009-10 crop season from 28.0 million ha area. It is contributing about 30% to the food basket of the country. Water stress at stages before anthesis can reduce number of ear heads and number of kernels per ear (Dencic et al., 2000; Mary et al., 2001). While, water stress imposed during later stages might additionally cause a reduction in number of kernels/ears and kernel weight (Gupta et al., 2001). Zhang and Oweis (1999) reported that wheat crop was found to be more sensitive to water stress from stem elongation to heading and from heading to milking.

Water stress is a global problem, which is a severe threat for sustainable agriculture (Shao et al., 2005). The global water crisis seriously influences crop productivity particularly in most of the Asian countries where irrigated agriculture accounts for 90% of total diverted fresh water (Huaqi et al., 2002). Plant experiences water stress either when the water supply to the roots becomes difficult or when the transpiration rate becomes very high. Water stress tolerance is seen in almost all plant species but its extent varies from species to species (Chaitanya et al., 2003). With increase of population, deficit of water resources and degrading ecoenvironmental conditions on the globe, crop stress physiology has become one of the hot topics of plant biology (Chaves et al., 2003; Deng et al., 2004). About 60 percent of the globe belongs to arid and semiarid zone (Shao et al., 2005). And in developing countries 37% of the area is semiarid in which available moisture is the primary constrain to wheat production (Dhanda and Sethi, 2002).

Bahrun et al. (2002) observed that individual plant respond to most stresses by changing their hormonal balance, frequently producing more ABA and less gibberellins. Hormones mediate the adaptation of plant growth and development to changing environmental conditions (Wolters and Jurgens, 2009) and extensive overlaps exist between drought-associated genes of plants in response to hormones including ABA, auxins, cytokinins (CKs), gibberellic acid, jasmonic acid and brassinosteroids (Huang et al., 2009 ). However, CKs and ABA have been clearly associated with senescence and drought responses.
CKs promote cell division and, acting both in synergy and antagonism with other plant hormones, influence a wide range of events during plant growth. The major portion of CKs are produced in meristematic regions in the root system and transported via xylem to the shoot. These CKs, along with the locally synthesized CKs, control development and senescence of the whole plant. CKs promote leaf expansion, accumulation of chlorophyll and conversion of etioplasts into chloroplasts, and delay leaf senescence. Information on how endogenous CKs are affected under stress is meagre. In the light of the important regulatory role played by CKs in modulating development, it seems feasible also to anticipate their involvement in response to adverse environmental conditions (Hare et al., 1997; Brault and Maldiney, 1999). Water stress inhibits synthesis and causes degradation of CKs, (Rivero et al., 2007, 2009). CKs may play important roles in regulating plant responses to water stress. Plant responses to CKs are often judged from their responses to exogenously applied CKs. However, on applying CKs exogenously it can increase content of endogenous CKs by their uptake and by promotion of CK biosynthesis (Hare et al., 1997; Kaminek et al., 1997 a, 1997 b).

CK content and activity was reduced in xylem exudates and/or leaves of stressed plants. The response is usually rapid and CK activity returns to a normal level after a release of stress. The explanation for the detection of reduced CK content under water stress is either a reduction in CK biosynthesis or enhanced degradation. Decreased content of CKs was found in alfalfa under drought (Goicoechea et al., 1995, 1997). This CK content decrease was accompanied with accelerated senescence. The effects were less pronounced in plants inoculated with *Glomus or Rhizobium* than in controls. The contents of zeatin, zeatin riboside, isopentenyladenine, and isopentenyladenosine in rice substantially decreased with the decrease in soil moisture but they only slightly increased after rewatering (Bano et al., 1993).

Under water stress CKs also known to regulate several aspects of plant growth and development, and plant response to abiotic stress (Haberer and Kieber, 2002; Rivero et al., 2007, 2009). CKs regulate stomatal behaviour (Reeves and Emery, 2007; Haisel et al., 2008; Hegele et al., 2008), the formation and protection of cellular structures (Chiappetta et al., 2006), and the induction and activation of protein synthesis (Selivankina et al., 2004; Chernyadev, 2005). CKs maintain stomata open and thus increase stomatal conductance (Blackman and Davies, 1985). On, the basis of above background we can hypothesis the role of cytokinin under water stress.
Research paper-I

Cytokinin induced changes of photosynthesis during water deficit stress in wheat (*Triticum aestivum* L.)

Photosynthesis is a chemical process that converts carbon dioxide into organic compounds, especially sugars, using the energy from sunlight (Bryant *et al.*, 2006). Water availability mostly affects growth of leaves and roots, photosynthesis and dry matter accumulation (Blum, 1996). One of the initial responses of plants to water stress is the decrease of leaf elongation rate and reduction in plant total leaf area. Cytokinin can induce water stress tolerance by delaying senescence, increasing chloroplast stability and by increasing cell membrane integrity.

Relative water content (RWC) of leaves is an important character, which is directly related to soil water content and plant water status (Sarker *et al.*, 1999). RWC has been reported as an important indicator of water stress in leaves (Merah, 2001). Stress exposed plants immediately lower down RWC of their leaves, the decrease in leaf water potential and osmotic potential is also reported (Grover *et al.*, 2004). Sairam and Saxena (2000) found decrease in the membrane stability index of leaves under water stress and ageing. Water stress also severely decreases photosynthesis and other related traits.

Chlorophyll a and b are the most important pigments active in the photosynthetic process. In photosynthesis, antenna pigments in leaf chloroplasts absorb solar radiation, total chlorophylls, Chl-a and Chl-b, are the most essential of these pigments, and are thus virtually necessary for the oxygenic conversion of light energy to the stored chemical energy that is the source of energy for the biosphere. From a physiological perspective, leaf Chl content (for example, how it varies both between and within species) is therefore a parameter of significant interest in its own right. The accessory pigment, carotenoids also have a very important role in photosynthesis. Biosynthesis of carotenoids in plants is a genetic characteristic, but environmental conditions also play a significant role (Bojovic and Stojanovic, 2005). Leaf chlorophyll content directly related with photosynthesis and due to decrease in chlorophyll there is decrease in photosynthesis rate.

Richmond and Lang (1957) initially reported that exogenous cytokinins could delay senescence in detached leaves. Although plant senescence is a complex
syndrome of biochemical and physiological changes, the antisenescent effect of cytokinins has generally been attributed, in whole or in part, to retarding chlorophyll loss, inhibiting protein degradation, preventing effluent carbohydrate translocation, and mobilizing metabolites. Kraus (1995) discovered that exogenous cytokinins could retard senescence in intact bean (*Phaseolus vulgaris*) leaves. CKs accelerate the regeneration and the de novo formation of chloroplasts by regulating membrane formation and the synthesis of components of the electron transport system (Chernyadev, 2000; Pospisilova *et al.*, 2000). In transgenic plants overexpressing IPT gene transcriptional activation of several genes coding for enzymes involved in chlorophyll biosynthesis was enhanced e.g. porphobilinogen deaminase and protochlorophyllide deoxidoreductase under water stress (Reviro *et al.*, 2007).

Photosynthesis, which is the basic process influencing crop productivity, is inhibited by water stress (Chaves and Oliveira, 2004). The major effect of water deficiency is the closure of stomata that leads to reduced transpiration rate and a lowered internal concentration of CO₂, which in turn inhibits the Calvin-cycle even at moderate water deficits (Horton *et al.*, 1996). The reduction in photosynthesis as a result of water stress can be attributed to both stomatal and non-stomatal limitations (Graan and Boyer, 1990; Shangguan *et al.*, 1999). Stomatal control of water loss has been identified as an early event in plant response to water stress under field conditions leading to limitation of carbon uptake by the leaves (Chaves, 1991; Cornic, 2000). The foliar photosynthetic rate of higher plants is known to decrease concomitant with the decrease in relative water content (RWC) (Lawlor and Cornic, 2002). Stomatal limitation was generally accepted to be the main determinant of reduced photosynthesis under water stress (Cornic, 2000). Stomata close in response either to a decline in leaf turgor and/or water potential, or to a low-humidity atmosphere (Maroco *et al.*, 1997). When the photosynthetic rate drops below certain threshold level leaf senescence is initiated. Reduction in photosynthesis level also affect carbon metabolism of plants. Rubisco activity was down regulated in the water stress due to tight binding with inhibitors under water stress condition (Farooq *et al.*, 2009). Decreased supply of CO₂ to Rubisco under both mild and severe water deficit is primarily responsible for the decrease in CO₂ fixation in *Hordeum vulgare* (Lal *et al.*, 1996).
CKs have been shown to stimulate stomatal opening (Dodd, 2003). High concentrations of CKs can supersede the effects of ABA on stomata (Davies and Zhang, 1991), and many of the ABA-mediated processes induced by drought (closing of stomata, leaf abscission, etc.) and helps in drought tolerance (Dodd, 2003). Zeatin is an active form of CKs it also influence the activity of the stomatal apparatus (Jones et al., 1998). CKs played a protective role, by increasing the stability of the photosynthetic apparatus (Chernyad, 2005). The content of Rubisco in wheat plants exposed to water deficiency was reduced less significantly than the activity of the enzyme. Preliminary treatment of plant seeds with a preparation with cytokinin activity, reduced the dehydration-induced inhibition of enzymatic activity. Similar, results were also observed by Parry (2002) that cytokinin is known to stimulate the expression of photosynthetic enzymes like Rubisco and, more generally, the development of functional chloroplasts.

Sugars produced during photosynthesis are the substrates of carbon and energy metabolism and are used in the biosynthesis of polysaccharides like starch and cellulose in plants thus these are required for plant growth and development (Dodd, 2003). Water stress leads to major alterations in carbohydrate metabolism (Hare et al., 1998). Sugars play a significant role during plant growth and development under abiotic stresses by regulating carbohydrate metabolism. Induction of a large number of stress responsive genes by glucose has also been reported, indicating the role of sugars in environmental responses (Price et al., 2004).

Late embryogenesis abundant (LEA) proteins have also been implicated in water deficit stress (Xu et al., 1996; Goyal et al., 2005). LEA proteins have been classified into five major groups based on amino acid sequences (Bake et al. 1988; Dure et al., 1989). These proteins are part of evolutionarily conserved group of hydrophilic proteins termed “hydrophilins” involved in various adaptive responses to hyperosmotic conditions (Garay-Arroyo et al., 2000). The majority of LEA proteins display a preponderance of hydrophilic and charged amino acid residues. Expression of LEA genes, which often appears to be abscisic acid-dependent, was detected not only in seeds but also in vegetative tissues with water deficit associated with drought, salt, and cold stresses (Ingram and Bartels, 1996; Thomashow, 1998; Cuming, 1999; Grelet et al., 2005). Both the pattern of expression and the structural features of LEA proteins suggest a general protective role in desiccation tolerance (Ingram and Bartels,
Xu et al., (1996) transformed HVA1 gene encoding group 3 LEA protein from barley (Hordeum vulgare L.) to rice and the tolerance to water deficit and salt stress of the transgenic rice was improved under the greenhouse conditions.

Under water stress cytokinin can delayed leaf senescence as it directly affect photosynthetic parameters, e.g., chlorophyll and photosynthetic protein synthesis and degradation, chloroplast composition and ultrastructure, electron transport, and enzyme activities (Synkova et al., 1997). Cytokinin is known to stimulate the expression of photosynthetic enzymes like Rubisco and, more generally, the development of functional chloroplasts. One obvious approach for manipulating senescence is by engineer plants that will overproduce cytokinins. Stress-induced leaf senescence could be delayed in transgenic plants expressing isopentenyltransferase (IPT), an enzyme that catalyzes the rate-limiting step in CK synthesis under the control of SARK, a maturation and stress-inducible promoter in tobacco (Rivero et al., 2007).

Research paper-II

Effect of cytokinin on nitrogen metabolism and yield attributes under moisture deficit stress condition in wheat (Triticum aestivum L.)

Nitrogen is one of the most important mineral nutrients for plants and is taken up by the root system predominantly in inorganic forms (NH$_4^+$ and NO$_3^-$). Twenty percent (20%) of the N fertilizer is consumed for wheat production (Behrens, et al., 2004) as it is one of the most important cereals in the world. Plants assimilate nitrogen as a source for growth, biomass production and development. It is a constituent of numerous important compounds, including proteins, nucleic acids, chlorophyll, and several plant hormones. Wheat contains more protein than the other cereals. Water stress reduces nitrogen use efficiency of plants.

Nitrate assimilation is primary pathway by which reduced nitrogen is accumulated in plants it requires eight electrons to reduce NO$_3^-$ to NH$_4^+$. Nitrate assimilation involves a consecutive action of two enzymes: Nitrate reductase (NR), a cytosolic substrate inducible enzyme that reduces nitrate to nitrite using NADPH as electron donor, and nitrite reductase, a plastidic enzyme that reduces nitrite to ammonium. It is highest energy-consuming reaction of metabolism after CO$_2$ assimilation. That is why decreases in Rubisco activity and photosynthesis of plants as a response to drought are in agreement with the lower foliar N
concentrations (Llorens et al., 2003). NR regulates the major rate limiting stage in the reduction of nitrate to ammonium, which is then incorporated in amino acids (Hopkins et al., 1995).

Nitrogen concentration of plants varies with soil N status (Blankenau et al., 2000) and soil moisture (Pande and Becker, 2003). Drought results in a decrease in leaf N concentration (Sinclair et al., 2000; Xu and Zhou, 2005), and drought stress and N limitation significantly reduce net photosynthetic rate and Rubisco activity, although drought alone does not affect Rubisco activity (Heitholt et al., 1991). Glutamine synthetase (GS) converts inorganic form NH$_4^+$ to glutamine which later on converted to glutamate (first primary amino acid) by glutamate synthase enzyme. GS activity in maize was reduced under water stress condition. It results in decrease in leaf N and protein content (Baki et al. 2000) with increases in GS activity, protein content was also found to be enhanced in the cytosol leaves of the black cumin plants as compared to the control plant with cytokinin treatment (Shah S.H., 2011).

Drought stress has a negative effect on plant N concentrations, free aminoacids or soluble protein contents accompanied with a decline of NR and GS activity in many plant species, such as winter wheat (Xu and Yu, 2006), and in other plants (Xu and Zhou, 2005) correlation was observed between decrease in extractable foliar NR activity and CO$_2$ assimilation, which indicates a co-regulation of C and N metabolisms in higher plants (Xu and Zhou, 2005).

Total protease activity is induced by abiotic stresses such as drought. Enhanced protease activity might occur for proteolysis of proteins released as a result of membrane damage. Proteolysis may serve to release amino acids for synthesis of stress induced/responsive proteins (Hameed et al., 2009). Effect of cytokinins like N6-benzyladenine (BA) and N1-(2-chloro-4-pyridyl)-N2-phenylurea (CPPU) was studied on protease activity of one week and in 1-month-old plants of rose cv. Motrea showed lower protease activity than that in the control in response both to CPPU and BA (Yakimova et al., 2000). Cytokinin treatment resulted in an increase in NR activity and thus elevated the useable form of nitrogen (ammonia) to produce a larger pool of amino acids/amides, in addition to stimulating their incorporation to polypeptide synthesis (by activating transcription and/or translation) and optimizing protein formation so overall increase in protein content in plants leaves. Leaves of plants treated with cytokinin not only photosynthesized at a faster rate, but also possessed an extended period of metabolic activity, because of the delayed senescence
caused by the cytokinin (Blackman et al., 1985). As a result, plants accumulated a large quantity of metabolites, which can be seen from increase in their dry matter in black cumin (Shah, 2011).

Duggan and Fowler (2006) found that although genotype X environment interactions were important contributors to variation in the yield determination factors, genotype and position of kernel in the spike had the major influence on kernel weight. Large differences in kernels/spikelet and kernel weight indicated that these two variables were responsible for yield adjustments to stress during spikelet and kernel development phase. Grain yield generally depends on spike length, number of effective tillers and 1000-kernel weight. Under drought conditions production of new photosynthetic products become limited and remobilization of reserves towards grain also reduce during grain filling (Gaspar et al., 2005). Production of shrivelled kernels due to hastened maturity due to the shortage of moistures it leads to decrease in 1000-kernel weight.

Baser et al. (2004) studied the effect of water stress on the yield and yield components of winter wheat and found a decrease of about 40% in yield under water stressed conditions as compared to control. Weight of grains/spike was reported by many researchers as the most closely linked variable related to grain yield per unit area and was often used in selecting high yielding wheat strains (Kumbhar et al., 1983). And 1000-grain weight had also been shown as the main yield component accounting for 20% of variation in wheat grain yield (Collaku, 1989). Less decrease in wheat yield under water stress is usually observed in water stress tolerant cultivars as compared to sensitive ones (Gaspar et al., 2005).

### 3. MATERIALS AND METHODS

Present investigations were undertaken in pot culture facility at Division of Plant Physiology, Indian Agricultural Research Institute, New Delhi (latitude of 28°N and longitude of 77°E, and about 250 m above mean sea level) in rabi season during 2010-2011.
3.1 Plant material

Two wheat varieties were selected, C-306 (drought tolerant) and PBW 343 (drought sensitive), as suggested by breeders and procured from Division of Genetics, Indian Agricultural Research Institute, New Delhi.

Sowing was done in 30 cm earthen pots with clay loam soil and farmyard manure in 3:1 ratio during winter season and supplied with 60, 60 and 60 kg ha\(^{-1}\) of N, P, K, respectively, in the form of urea, single super phosphate and muriate of potash at the time of sowing. Remaining 60 kg N ha\(^{-1}\) was given after 25 days of sowing.

3.2 Experimental Treatment

Plants were subjected to water stress after 55 days after sowing for providing water deficit stress condition during reproductive stage by control irrigation. Cytokinin treatment was given by spraying benzyl amino purine (BAP; 40 µM) concentration at 40 DAS and 60DAS. Following four different treatments were maintained;

- Control- Normal irrigation condition
- Control + BAP - Normal irrigation with BAP treatment
- Waters stress- Withholding irrigation
- Waters stress + BAP- Withholding irrigation with BAP treatment

3.3 Plant Sampling

The plants were sampled and observations were taken for growth, physiological, biochemical parameters and molecular study at pre-anthesis (67 DAS); anthesis (77 DAS) and post-anthesis (87 DAS) of the wheat plants described above. Three replications with five pots per replication were taken for each variety. Upper most fully expanded flag leaf was used for recording observation.

3.4 Chemicals, kits and other materials

All the chemicals used in this study were of either analytical grade (AR) or molecular biology grade. The chemicals were procured from Sigma Chemicals, Qualigens, Sisco Research Laboratory (SRL), Merck, Stratagene, and Qiagen. In the work related to molecular biology, the kits used for RNA extraction was RNeasy® Plant Minikit (Qiagen), RT-PCR kit from ABM (Applied Biological Materials) and Protoscript (New England Biolabs).

3.5 Growth Parameters

Following growth parameters were measured at different growth stages from samples collected from ambient and elevated CO\(_2\) plots.

3.5.1 Leaf area
The leaves were separated from the stem and cleaned thoroughly. All the green leaves from each replication (three plants per replication) were taken. Leaf area of flag leaf, main shoot leaves and total plant leaf area was measured using a standard leaf area meter (Model LiCOR 3100) and was expressed as cm$^2$ plant$^{-1}$.

3.5.2 Plant Biomass

The plants were harvested at various growth stages and separated into stems and leaves then dried in an oven at 80 ºC for four hours and then at 60 ºC till constant dry weight was recorded. Dry weights were recorded and expressed as g plant$^{-1}$.

3.5.3 Harvest Index

Plants were collected at the time of the harvest. Grains were separated and weighed (economic yield). The whole plant dry weight was measured as biological yield. The harvest index was calculated as the ratio of the economic yield to biological yield and was expressed as percentage (Gardner et al., 1985).

\[
HI = \frac{\text{Economic yield}}{\text{Biological Yield}} \times 100
\]

3.5.4 Yield components

Following yield components were recorded at the time of final harvest; number of tillers, number of grains per ear, grain yield per plant, 1000-grain weight (g).

3.6 Physiological observations

3.6.1 Relative water Content

Leaf relative water content (RWC) was estimated by recording the turgid weight of 0.5 g fresh leaf samples by keeping in water for 4 h, followed by drying in hot air oven till constant weight was achieved (Weatherley, 1950).

\[
\text{RWC} = \frac{[(\text{Fresh wt.} - \text{Dry wt.}) / (\text{Turgid wt.} - \text{Dry wt.})] \times 100}{(\text{Turgid wt.} - \text{Dry wt.})}
\]

3.6.2 Membrane stability index

Membrane stability index (MSI) was estimated as per Sairam et al. (1997). For estimation of membrane stability index 100 mg leaf material, in two sets, was taken in test tubes containing 10 ml of double distilled water. One set was heated at 40 ºC for 30 min in a metabolic water bath, and the electrical conductivity of the solution was recorded on a conductivity bridge ($C_1$). Second set was boiled at 100 ºC on a boiling water bath for 10 min, and its conductivity was measured on a conductivity bridge ($C_2$). Membrane stability index was calculated as:
3.6.3 Total chlorophyll and carotenoid estimation

The procedure for estimation of chlorophyll content in plants is based on the absorption of light by chlorophyll extracts prepared by incubating the leaf tissues in DMSO (Dimethyl sulfoxide). DMSO renders plasmalemma permeable thereby, causing the leaching of the pigments (Hiscox and Israelstam ,1979). The absorbance of the known volume of solution containing known quantity of leaf tissue at two respective wavelengths (663 and 645 nm) was determined for chlorophyll content and at 470 nm for total carotenoid contents. Chlorophyll content was estimated using the formula given by Arnon (1949). Fifty milligram fresh leaf samples (small discs) were added to the test tubes containing 10 ml DMSO. Tubes were kept in dark for 4 hrs at 65 °C. The absorbance was recorded at 663 and 645 nm using DMSO as blank and was expressed as mg g⁻¹ dw.

Chlorophyll ‘a’ = \((12.7 \times OD_{663} - 2.69 \times OD_{645}) \times \frac{V}{1000 \times w}\)

Chlorophyll ‘b’ = \((22.9 \times OD_{645} - 4.68 \times OD_{663}) \times \frac{V}{1000 \times w}\)

Total chlorophyll = \((20.2 \times OD_{645} + 8.02 \times OD_{663}) \times \frac{V}{1000 \times w}\)

Total carotenoids = \((1000 \times OD_{470}) - (3.29 \times \text{Chl a}) - (104 \times \text{Chl b}) / 198\)

Where,

\(OD_{663}\) = Absorbance values at 663 nm
\(OD_{645}\) = Absorbance values at 645 nm
\(OD_{470}\) = Absorbance values at 470 nm
\(W\) = Weight of the sample in mg
\(V\) = Volume of the solvent used (ml)

Chl ‘a’ = \((12.21 \times OD_{663}) - (2.81 \times OD_{645})\)
Chl ‘b’ = \((20.13 \times OD_{645}) - (5.03 \times OD_{663})\)

3.6.4 Net photosynthetic rate

Rate of photosynthesis was measured on flag leaves using portable Infrared Gas Analyzer (IRGA LI-6400 Model). The rate of photosynthesis was measured by operating the IRGA in the closed mode. The photosynthetic rate was determined at different growth stages in the upper most fully expanded flag leaf between 10 am and 11.30 am by providing artificial light source of light intensity 1000 CO₂ µmol m⁻² s⁻¹. The net photosynthetic rate was expressed as µmoles CO₂ m⁻² s⁻¹.

3.6.5 Stomatal conductance

The stomatal conductance was also measured using IRGA (LI-COR 6400). The stomatal conductance was expressed as cm s⁻¹.
3.6.6 Photochemical Efficiency

The photochemical efficiency was measured using IRGA (LI-COR 6400). The photochemical efficiency was expressed as Fv/Fm ratio.

3.7 Biochemical estimation

3.7.1 Total Soluble Sugars

3.7.1.1 Preparation of reagents

(i) Sulphuric acid (95%): To 95.9 ml of sulphuric acid 4.1 ml of distilled water is added to make volume upto 100 ml.

(ii) Anthrone reagent: Two hundred mg anthrone was dissolved in 100 ml of ice cold 95 per cent sulphuric acid. Fresh reagent was prepared each time.

Weighed amount of leaf samples were plunged in 95% ethanol and preserved. There were three replications for each determination.

3.7.1.2 Extraction of sugars

For sugar analysis, the sample was boiled and the supernatant decanted into a beaker. The extraction was repeated four times (three times with 20 ml of 80% (v/v) ethanol in water and finally with 20 ml of distillated water) by boiling the sample for 4-5 min and decanting the supernatant (McCready et al., 1950). The combined sugar extract was made up with distillated water in a 100 ml volumetric flask.

3.7.1.3 Clarification of the sugar extract

For clarification, 50 ml aliquot of the above sugar extract was evaporated in a water bath, taking care not to let the liquid dry out completely. Subsequently, the sample was treated with 1 ml saturated solution of lead acetate to precipitate the colloidal substances. It was then filtered into a 50 ml volumetric flask and made up to the volume. An aliquot of this solution was used for determining the total sugar by anthrone reagent method.

3.7.1.4 Determination of Total sugars

One ml of sugar sample was taken and to this 4 ml solution of anthrone regent was added. The mixture is heated on a boiling water bath for 8 min followed by cooling. The optical density of green to dark green colour was read at 630 nm in UV-visible spectrophotometer (model Specord Bio-200, AnalytikJena, Germany). A blank and two freshly prepared glucose standards were also included with each set of samples.

3.7.2 Estimation of starch content
Starch content was also determined by Anthrone method (McCready et al., 1950). The dry residue left after sugar extraction was powdered and 50 mg of it was hydrolyzed by boiling with 10 ml of 1N HCl for 30 minutes in a glycerine bath at 112-115 °C. After cooling, the samples were transferred into a 100 ml volumetric flask. The residue was repeatedly washed with distilled water until a negative test (iodine test) was obtained. The extract was collected and made to 100 ml. An aliquot of (0.5 ml) the above extract was made to 2.5 ml with distilled water. This was then mixed thoroughly with 10 ml of freshly prepared anthrone reagent (100 mg anthrone was dissolved in 100 ml chilled concentrated H_2SO_4), in a cold water bath. Subsequently, the tubes containing this mixture were kept in a boiling water bath for 15 minutes and then rapidly cooled in running tap water. Absorbance was measured at 620 nm. A reference standard curve was prepared using glucose in the range of 25-300 µg. Starch content was calculated by multiplying the glucose values (1OD = 600 µg/g dw with 0.9.

3.7.3 Estimation of total carbon

Estimation of carbon content was done by wet digestion following the modified Walkley-Black method (1934). The dried sample was oxidized with a mixture of potassium dichromate and concentrated sulphuric acid using the heat of dilution of acid. The unused potassium dichromate was estimated by back titration with ferrous ammonium sulphate.

3.7.3.1 Reagents

Standard potassium dichromate solution (1N): 49.0 g of AR grade potassium dichromate (K_2Cr_2O_7) (dried at 105 °C for 2 hours) was dissolved in distilled water and diluted to 1000 ml in a volumetric flask.

Ferrous ammonium sulphate solution (0.5N): 196.1 g of AR grade ferrous ammonium sulphate (FeSO_4 (NH_4)_2SO_4.6H_2O) was dissolved in about 800ml of distilled water. 200 ml of concentrated sulphuric acid was added to it and solution was allowed to cool. The solution was diluted to 1000 ml in a volumetric flask. Titrating against 10 ml 1N K_2Cr_2O_7 solution standardized the reagent. Ferrous ammonium sulphate was preferred to ferrous sulphate because of the more stable nature of the former.

Diphenylamine indicator: 0.5 g diphenylamine [(C_6H_5)_2NH_4] was dissolved in a mixture of 20 ml distilled water and 100 ml concentrated H_2SO_4 (specific gravity 1.84 and concentration > 96%) and was stored in a coloured bottle.
Orthophosphoric acid (85% v/v); 85 ml of orthophosphoric acid was taken in measuring cylinder and then volume was made up to 100 ml by adding distilled water.

3.7.3.2. Estimation of carbon

0.05 g of dried powdered sample was taken in a 500 ml conical flask to which 10 ml of 1N K₂Cr₂O₇ was added. The flask was gently swirled to disperse the sample in the reagent and was then placed on asbestos sheet. 20 ml of concentrated H₂SO₄ was rapidly added to it followed by immediate swirling of the flask. First gently until the sample and the reagent were mixed and then vigorously. The flask was swirled 2 or 3 more times and allowed to stand for 30 min. This was followed by addition of 200 ml of distilled water and 10 ml of orthophosphoric acid. After adding 1 ml of diphenylamine indicator the contents were titrated with ferrous ammonium sulphate solution till the colour changed from blue violet to green. A blank titration was also carried out (without sample). The carbon content was calculated using the following formula and was expressed in percentage.

\[ C\% = \frac{10 (x-y) \times 0.003 \times 100}{X \times A} \]

Where A is the weight of the sample, X is the volume (ml) of ferrous ammonium sulphate solution required for blank titration; y is the volume (ml) of ferrous ammonium sulphate needed for titration of the sample. The final carbon percentage was obtained by multiplying this carbon percent with a constant 1.3 as only 77 percent recovery was presumed from the oxidation of samples in this procedure.

3.7.4 Nitrate reductase activity (NR; EC 1.6.6.1)

Nitrate reductase activity was assayed following the method of Klepper et al., (1971) as modified by Nair and Abrol (1973). Fully expanded flag leaves were harvested and immediately preserved in ice. Sampling was done at 10:30 a.m on a bright and sunny day. The leaves were cleaned thoroughly and cut into small pieces of 2-3 cm each after the removal of the midrib. 0.3 gram of the cut leaf sample was taken in triplicate in ice cold infiltration medium containing: 3 ml of 0.1M Phosphate buffer (pH 7.5), 3 ml of 0.4 M potassium nitrate and 0.1 ml isopropanol. A blank was run simultaneously without any leaf tissue. The solution was infiltrated into the leaf tissues under vacuum for five minutes or till the tissues sank to the bottom of the tubes. All operations were performed on ice. These were then incubated at 33 °C for 30 minutes in the water bath. The reaction was stopped by placing the tubes in the water bath at 70 °C for 3-4 min. Known volume of aliquot (0.2 ml) was taken in the fresh test tube and one ml each of
sulphanilamide (1%) in 1 N HCl and NEDD solution [N-(1-Naphthyl) ethylene diamine dihydrochloride solution (0.02%) was added and colour was allowed to develop for 20 minutes. The final volume was made to 6 ml with distilled water. The absorbance was recorded at 540 nm using spectrophotometer (SPECORD 200). Zero was set using the blank without aliquot. The NR activity was calculated as the amount of nitrite formed from the standard curve of nitrite (1 OD= 152.46 nmoles nitrite). The NR activity was expressed as µmoles NO₂ formed g⁻¹ dw h⁻¹.

3.7.4.1 Reagents:

i. Phosphate buffer (1M K₂HPO₄ stock: prepared by dissolving 17.418 g of dipotassium hydrogen phosphate in 100ml. 1M KH₂PO₄ stock: prepared by dissolving 13.61g in 100ml. Both solutions were diluted 10 times to get 0.1 M solutions. 80ml of 0.1 M K₂HPO₄ and 20ml of 0.1M KH₂PO₄ mixed and pH set to 7.5.

ii. 0.4 M KNO₃: 20.216g KNO₃ dissolved in 500ml distilled water.

iii. 1% Sulphanilamide: 1g of sulphanilamide dissolved in 100 ml of 1N HCl

iv. 0.02% NEDD: 20 mg of NEDD dissolved in 100 ml distilled water

3.7.5 Activity of glutamine synthetase (GS; EC 6.3.1.2)

Glutamine synthetase activity was assayed following the method of Mohanty and Fletcher (1980).

3.7.5.1 Extraction of protein

Flag leaf samples were collected in ice in the field as mentioned above for NR activity. After cleaning thoroughly the leaf samples were weighed (1g) and enzyme protein was extracted in the 6 ml ice cold extraction buffer. The extraction step was performed using chilled pestle and mortar in a tray filled with ice. Extract was centrifuged at 10,000 rpm for 20 min in a centrifuge (SIGMA 3K30). The supernatant was subsequently used for the assay of enzyme activity.

3.7.5.2 Assay of enzyme activity

The reaction mixture consisted of (0.1 M Tris 1.25 ml, 10 µM hydroxyl amine 0.6 ml, 100 µM MgCl₂ 0.3 ml,10 µM ATP 0.2 ml , enzyme extract 50 µl ) except sodium glutamate, which is the substrate for the enzyme were added in the assay tubes along with 0.05 ml enzyme extract. All the procedures were done on ice. The tubes were incubated in water bath at 33 °C for 5 min. to let the solutions attain the desired temperature and then 0.6 ml of sodium glutamate (250 µM) was added to each of the tube. Blank contained all the reagents except sodium glutamate. After 15 min. of incubation reaction was stopped by adding 0.3 ml FeCl₃ TCA. The brown precipitate was
formed. The volume was made to 6 ml with distilled water and centrifuged at 16,000 rpm for 10 min. to remove the precipitate. The absorbance was recorded at 540 nm. The GS activity was calculated from the standard curve of γ-glutamyl hydroxymate as the amount of ferric γ-glutamyl hydroxymate formed (1 OD=3.72 µmoles). The GS activity was expressed µmoles γ-glutamyl hydroxymate formed g⁻¹ dw h⁻¹.

3.7.5.3 Reagents

Extraction buffer (pH 7.8)

i. 1M Tris stock: 12.11g of Tris was dissolved in 50ml distilled water. pH was set to 7.8 and the volume made to 100ml with distilled water.

ii. 0.5 M EDTA: 20.23g of EDTA was dissolved in 60ml distilled water and pH set to 8 and the volume made to 100ml.

iii. 1mM DTT: 7.72g of dithriothretol was dissolved in 50ml of distilled water.

iv. 300mM MgCl₂: 0.075g of magnesium chloride (hepta hydrate) was dissolved in 100ml of distilled water.

v. 1X Protease inhibitor mix: 0.01ml.

Assay Buffer (pH 7.5)

i. 0.1M Tris: 1.211g Tris dissolved was in 100ml distilled water.

ii. 10 µM hydroxyl amine hydrochloride: 0.278g of hydroxyl amine hydrochloride was dissolved in 50ml distilled water.

iii. 100 µM MgCl₂: 3.05g of MgCl₂ dissolved in 50ml distilled water.

iv. 10µM ATP: 0.2308g of ATP was dissolved in 10ml distilled water.

v. 250µM sodium glutamate: 2.35g of sodium glutamate was dissolved in 40ml distilled water.

3.7.6 Determination of total reduced nitrogen

Total N in tissues was measured using N₂ analyser (Gerhardt, Turbotherm digestion unit and Gerhardt Vapodest distillation unit).

**REAGENTS USED**

i. NaOH 40%: 400 g NaOH was dissolved in distilled water and volume was made up to 1 lit.

ii. Boric acid indicator solution: 40 g of pure H₃BO₃ was dissolved in one litre distilled water.

iii. Mixed indicator: Dissolved 0.07 g methyl red with 0.1 g bromocresol green in 100 ml of 95% ethanol (alcohol).

iv. Concentrated Sulphuric acid.
v. Catalyst mixture of potassium sulphate and copper sulphate in the ratio of 10:1.

100 g of finely powdered homogenous sample was weighed and put in a clear and dry digestion tube. To this 5.5 g of K\textsubscript{2}SO\textsubscript{4}: CuSO\textsubscript{4} mixture was added and then 10 ml H\textsubscript{2}SO\textsubscript{4} was also added. The sample was digested at 600 °C for 15 min and then at 450 °C for 1 h. When the samples were colorless tubes were taken out from the digestion unit.

100 ml conical flask containing 4% solution of boric acid (10 ml) was taken. To these one or two drops of indicator solution was added which gave the pink colour this solution was used to trap the ammonia liberated during distillation. For distilling the sample the digestion tube was placed in distillation unit and 50 ml water and 70 ml NaOH (40%) was added. After steam distillation, colour of the indicator dye changed to green as (NH\textsubscript{4})\textsubscript{2} SO\textsubscript{4} is trapped by boric acid. This was titrated against 0.01 N HCl until the pink colour reappeared. Each digestion set had two control tubes. N per cent in the tissues and the N content per plant was calculated using the formula

\[
\text{Weight of sample} = 0.5 \text{ g}
\]

\[
\text{Normality of HCl} = 0.1 \text{ N HCl}
\]

\[
\text{Titration value (T.V.)} = \text{Sample titration value} - \text{Blank titration value}
\]

\[
N = \frac{\text{T.V. x Normality of HCl x 1.4}}{\text{wt. of oven dry sample (g)}}
\]

3.7.7 Total Protein content

The N% estimated in the leaf samples from different treatments were multiplied by factor 6.25 to get the total (crude) protein content.

3.7.8 Estimation of total soluble proteins

3.7.8.1 Extraction of protein

The flag leaf samples were harvested. These were immediately wiped thoroughly and plunged in liquid nitrogen. The leaf tissue (100 mg) was powdered in liquid nitrogen and added to prewarmed extraction buffer (1: 5). These were centrifuged at 16,000 rpm for 10 min. The supernatant was subsequently used for the assay of protein content.

3.7.8.2 Estimation of protein

\[
\text{Total protease activity}
\]
3.7.9.1 Extraction of protease

1.0 gm of leaf tissue was taken and homogenizes the tissue sample. 10 ml of extraction buffer was added into the homogenate. Crude homogenate was then passed through 4 layers of muslin cloth. Centrifuge the filtrate at 25,000 g for 20 min at 4 °C. Take out the supernatant and use for the enzyme assay. To the 1.0 ml of the enzyme extract and added 1.0 ml of 1 % casein in 0.2 M diphosphate buffer (pH=7) and 1.0 ml of 0.2 M of phosphate buffer (pH=7). Incubate the solution at 40 °C for 90 min. Stop the reaction after the end of incubation period by adding 1.0 ml of 20 % TCA. Keep the reaction vials in refrigerator for 30 min. Centrifuge the reaction mixture at 3000 g for 15 min. Take out the supernatant and use for the amino acid analysis. A control was also be run along with sample.

3.7.9.1 Estimation of protease activity

0.5 ml of supernatant was used from the reaction mixture. Add 0.5 ml of 55% glycerol, 0.5 ml of ninhydrin solution. Make the volume to 6.0 ml by adding double distilled water. Boil for 20 min. Record the spectrophotometer reading at 570 nm. To calculate amount of amino acid formed due to protease activity, a standard curve was prepared by using 0.1 to 1.0 µM of glycine. The protease activity expressed as µM of amino acid produced per hour per gram per dry weight. (Nieri el al., 1998)

Reagents

(A) Extraction Medium

2.72 gm of potassium Phosphate (Mr. 136.1) in 400 ml of DDW (0.05 M)
280 µL mercaptoethanol
5.24 gm of EDTA
Final volume made to 500 ml

(B) Assay Mixture

1.0 gm casein in 100 ml of diphosphate buffer (pH = 7)
0.2 ml of phosphate buffer (pH=7)
1.0 ml of 20% TCA

(C) Reaction Mixture

55 ml of 55 % glycerol in 45 ml of DDW
1.5 gm of Ninhydrin in 0.5 M of citrate buffer

(D) Citrate buffer

1.05 gm of citric acid in 100 ml DDW
1.47 gm of sodium citrate in 100 ml DDW (0.5 M)
13.7 ml of citric acid and 36.3 ml of sodium citrate (pH = 5.6)
(E) Diphosphate buffer

2.72 gm of monobasic in 100 ml DDW
3.48 gm of dibasic in 100 ml DDW
39 ml of monobasic in 100 ml DDW and 67 ml of dibasic in 100 ml DDW

(pH=7)

3.8 Molecular study

3.8.1 Gene expression study for selected enzymes/proteins by RT-PCR.

Nucleotide sequences for candidate genes were obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/) was used to identify the homologs of candidate genes. For RT-PCR expression analysis and cloning of cDNAs, the following oligonucleotide primers were designed manually, and oligo quality (to avoid primer dimer, self dimer, etc.), GC % and Tm were analyzed by using Oligoanalyzer tool (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/, IntegratedDNA Technologies, Coralville, IA 52241, USA).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Length (bases)</th>
<th>GC %</th>
<th>Tm (ºC)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubisco SSU F</td>
<td>ACCCTTCCAGGGTCTCAAGT</td>
<td>20</td>
<td>55</td>
<td>53</td>
<td>320</td>
</tr>
<tr>
<td>Rubisco SSU R</td>
<td>TTGTCCAGTAGGACCCATCG</td>
<td>20</td>
<td>50</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>TADHN F</td>
<td>CATCGATGAGAAGTGGTAGGT</td>
<td>21</td>
<td>52</td>
<td>55</td>
<td>450</td>
</tr>
<tr>
<td>TADHN R</td>
<td>TTGTCCATGATCTTGGCCCAGT</td>
<td>21</td>
<td>48</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>WCOR F</td>
<td>CCCGCGCCGCCACAAGAA</td>
<td>17</td>
<td>71</td>
<td>53.5</td>
<td>490</td>
</tr>
<tr>
<td>WCOR R</td>
<td>GCCCAGCAAAATAAAACCACAAATA</td>
<td>21</td>
<td>43</td>
<td>53.5</td>
<td></td>
</tr>
</tbody>
</table>

*Oligo concentration 1.0 μM, Na+ concentration 50 mM.

3.8.1.1 Isolation of Total RNA

Total RNA was extracted from leaf tissues of salinity treated and control plants using RNA easy kit (Qiagen Inc., Chatsworth CA 91311, USA, Cat No: 749040) according to the manufacturer’s instruction. 100mg sample was taken for isolating the
RNA. The sample was powdered using liquid nitrogen in a pestle and mortar. The powdered samples were immediately soaked with 450 µl lysis buffer (RLT buffer) (containing 1 % β-mercaptoethanol), Vortexed for 10 sec and incubated at 56 °C for 3 min in water bath. Clear lysate was then passed through spin column and centrifuged at 8000g for 2 min in a bench top centrifuge at room temperature. The flow through was transferred to a new eppendorf tube and half volume of (225 µl) chilled ethanol (absolute) was added to clear lysate and mixed by pippeting. It was then passed through another spin column (pink column) and centrifuged for 1 min at room temperature at 8000 g. Flow through was discarded and 700 µl of RW1 buffer was added to the same column and centrifuged for 1 min. The same was repeated and centrifuged for 2 min. Finally RNA was eluted with 54 µl sterile RNAase free water by centrifuging at 8000 g for 1 min and was collected in an eppendorf. This was used as a template for reverse transcription polymerase chain reaction (RT-PCR).

3.8.1.1.1 Agarose Gel Electrophoresis

Material and Reagents

1. Agarose
2. 5X TBE Buffer (pH 8.0)
   Tris base - 54 g
   Boric acid - 27.5 g
   EDTA 0.5 M (pH 8.0) -20 ml
   Final volume 1000 ml with distilled water.

Gel loading dye (6X)

10 mM Tris-HCl (pH 7.6)
0.03 % bromophenol blue
0.03 % xylene cyanol FF
60 % glycerol
60 mM EDTA

Ethidium bromide stock solution: 10 mg per ml in water

3.8.1.1.2 Procedure:
Agarose gel (0.8 %) was prepared by heat dissolution of 0.40 g of agarose in 50 ml 1 X TBE buffer. Ethidium bromide from stock solution (10 mg ml⁻¹) was added to a final concentration of 0.5 mg ml⁻¹ of gel. Gel was allowed to cool to almost 50-55 ºC before pouring to the gel plate. Once the gel was ready, it was placed in the electrophoresis tank after removing the comb and tape. The electrophoresis tank was filled with 1X TBE buffer till the gel was completely submerged. The total RNA samples were mixed with appropriate volume of 6X loading dye before loading. The samples were loaded and run at 5 V/cm, with the help of an electric supply. After one hour of electrophoresis, the agarose gel was viewed using gel documentation system.

3.8.1.2 RT-PCR expression analysis of target genes

Reverse transcriptase-polymerase chain reaction (RT-PCR) mixture using Qiagen One Step RT PCR Kit with gene specific forward and reverse primers was prepared as per the protocol given below:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity for 50 µl of reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease free water</td>
<td>21.0 µl</td>
</tr>
<tr>
<td>5X RT buffer</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>(TrisCl, KCl, (NH₄)₂SO₄, 13.5 mM MgCl₂, DTT; pH 8.7 at 20 ºC)</td>
<td></td>
</tr>
<tr>
<td>dNTP Mix (10 mM each)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Forward + Reverse primers (10 µM)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>RNA template (1 µg)</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>RT Enzyme mix</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>50.0 µl</td>
</tr>
</tbody>
</table>

The above reaction mixture was prepared in 0.2 ml PCR tubes and Reactions were conducted using QB 96 Thermal cycler (Quanta biotech, England), under the following conditions. Number of cycles was standardized as 25-27 cycles by conducting semi quantitative RT-PCR, amplified products were not visible in susceptible cultivars when the number of cycles was less than 25.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcription</td>
<td>30 min 50 ºC</td>
</tr>
<tr>
<td>Initial PCR activation step</td>
<td>15 min 95 ºC</td>
</tr>
<tr>
<td>3-step cycling</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>1 min 94 ºC</td>
</tr>
<tr>
<td>Annealing</td>
<td>1 min 57 ºC</td>
</tr>
<tr>
<td>Extension</td>
<td>2 min 72 ºC</td>
</tr>
<tr>
<td>Final extension</td>
<td>10 min 72 ºC</td>
</tr>
</tbody>
</table>
Linear amplification for semi-quantitative RT-PCR was obtained with 27 cycles. To check the amplification, an aliquot of 5 μl of the reaction mixture was run on a 0.8 % (w/v) agarose gel stained with 0.5 μg ml⁻¹ ethidium bromide. 1 kb DNA ladder was included as a marker for size comparison of the amplified products. 1X TAE buffer was used to prepare the gel as well as for running buffer and electrophoresis was carried out at 5 V/cm (Sambrook et al., 1989). 6X loading dye were used to load the samples. The stained DNA products were photographed using Gel Documentation system.

3.9 Statistical analyses

The data was analysed statistically using 3 factorial CRD (Biochemical analysis and growth parameters) and 2 factorial CRD (Yield attributes) design and CD at 5% and ANOVA was calculated. The analysis was done using OPSTAT programme available online on CCS, HISAR web site.

4.1 RESEARCH PAPER-I

Cytokinin induced changes of photosynthesis during water deficit stress in wheat  
(Triticum aestivum L.)

Division of Plant Physiology  
Indian Agricultural Research Institute, New Delhi-110 012

4.1.1 Abstract

Water stress is one of the major limiting factors in wheat production which is more pronounced at reproductive stage. Experiments were carried out to study the effect of cytokinin (6-Benzyl amino purine; 40 μM) on photosynthesis and related traits under two different water regimes in two contrasting wheat cultivars differing in their tolerance to drought at pre-anthesis, anthesis and post-anthesis stages of wheat development. Decrease in RWC and MSI were observed under water stress condition but application of cytokinin enhanced the RWC and MSI in both the conditions. There was reduction in chlorophyll and carotenoid content under water stress condition whereas application of cytokinin helped in maintaining the stability of chlorophyll
and carotenoid in both conditions. Maximum photosynthesis and related components were found at anthesis stage. Application of cytokinin significantly improved the rate of photosynthesis and its components in both the cultivars and water regimes. Wheat cultivar C-306 performed better under water stress condition compared to PBW-343. Highest level of Rubisco SSU expression was observed at anthesis stage and expression of desiccation tolerance genes was more under water stress condition and at later developmental stages.

**Key words:** Water stress, Cytokinin, Photosynthesis, Rubisco.

### 4.1.2. Introduction

Abiotic stresses such as drought, salinity and heat together with the growing world population and per capita food consumption threaten stable global food availability. Among the abiotic factors water availability is the most important. Drought stress remains an ever-growing problem that severely limits crop production worldwide and causes important agricultural losses particularly in arid and semiarid areas (Boyer et al., 1982). The percentage of drought affected land areas more than doubled from the 1970s to the early 2000s in the world (Feller and Fisher, 2007). Climate models have indicated that drought episodes will become more frequent because of the long-term effects of global warming. Drought stress tolerance is seen in almost all plants but its extent varies from species to species and even within species (Jaleel et al., 2007). Water deficit stress is global issues to ensure survival of agricultural crops and sustainable food production (Sankar et al., 2007).

Understanding of physiological mechanisms that enable plants to adapt to water deficit and maintain growth and productivity during stress period could help in screening and selection of tolerant genotypes and using this trait in breeding programs (Zaharieva et al., 2001). Among the physiological processes, photosynthesis is the basic determinant of plant growth and productivity and the ability to maintain the rate of carbon assimilation under environmental stress is fundamental importance to plant production (Lawlor, 1995). Photosynthesis is the primary source of dry matter production and grain yield in crop plants. The improvements of leaf photosynthesis have occurred with the advance of breeding high-yielding cultivars (Jiang et al., 2002). Therefore, increasing single-leaf photosynthesis could be the main way to substantially enhance grain yield. However, because of the close correlation between
leaf area during maturation and grain yield in wheat, early senescing which seriously restricts the potential for heterotic vigour remains a significant physiological problem (Jiao et al., 2003). Wheat genotypes vary in the timing of senescence initiation and also in the subsequent rate of leaf senescence. The quest of the causes of differences in leaf photosynthetic rate among interspecies and/or intraspecies of crops may be one of the important strategies of crop engineering (Jiang et al., 2002). So delaying leaf senescence has become an agronomically desirable trait (Quirino et al., 2000; Subhan and Murthy, 2001). Flag leaf photosynthesis in wheat contributes about 30-50% of the assimilates for grain filling (Sylvester-Bradley et al., 1990) and initiation of grain filling coincides with the onset of senescence, therefore, photosynthesis of the flag leaf is the most important basis of the formation of grain yield, and the onset and rate of senescence are important factors for determining grain yield.

Grain growth in wheat depends on carbon from three sources: current assimilation, remobilization of pre-anthesis assimilates stored in the stem and other plant parts, and re-translocation of assimilates stored temporarily in the stem after anthesis (Kobata et al., 1992). Post-anthesis drought reduces carbon assimilation and hence the availability of current assimilates for grain filling, but probably does not affect the translocation of carbon to the grain (Wardlaw, 1967). Approximately 70 to 90% of the final grain yield is derived from photosynthates made during the grain filling periods under favourable conditions (Austin et al., 1977; Bidinger et al., 1977).

The effects of drought on leaf photosynthesis are well documented (e.g. Kaiser, 1987; Chaves, 1991). Sharkey and Seemann (1989) concluded that reductions in whole leaf photosynthesis caused by mild drought stress are primarily the results of stomatal closure and that there is no indication of damage to chloroplast reactions (Cornic, 2000). At more severe drought stress, photosynthesis continues to decrease, while the ratio of intercellular/ambient CO₂ concentration increases significantly to values similar to those obtained in well watered plants (Rekika et al., 1998). Thus, the decrease in photosynthesis could result from non-stomatal factors affecting photosynthetic capacity. There exist genotypic variations in the effect of drought stress on stomatal conductance and net photosynthetic rate (Johnson et al., 1987; Matin et al., 1989).
Research on biochemical changes that occur during leaf senescence has focused on loss of photosynthetic pigments, degradation of protein, and re-absorption of mineral nutrients. The drastic decline in activities of PS II, PS I and whole chain electron transport has also been reported in several senescing systems (Subhan and Murthy, 2001), indicating that the photochemical activity inhibits photosynthesis during leaf senescence (Harding et al., 1990). Most of these studies were conducted under controlled conditions; however, only a few reports are related to senescence of field grown plants (He et al., 2003). Additionally, the changing direction of Chl a/b ratio is still controversial during natural leaf senescence and therefore is worth to note further.

Association between wheat grain yield and photosynthesis has been reported (Loomis and Amthor, 1999; Reynolds et al., 2000). Water stress causes reduction in the rates of photosynthesis and transpiration in many crop species (Guo Li et al., 2000; Condon et al., 2002). The reduction in photosynthesis can be attributed to stomatal and non-stomatal factors (Ahmadi, 1998; Del Blanco et al., 2000). The importance of stomatal closure in regulating photosynthesis under water stress condition can be recognized by the findings showing parallel reduction in photosynthesis and stomatal conductance (Del Blanco et al., 2000; Koc et al., 2003). Although stomatal closure limits CO₂ flux to the site of its fixation in chloroplast, it could contribute to the maintenance of leaf turgor and thus improve drought tolerance of plant by limiting water transpirational rate. Frederick et al. (1989) reported that cultivars with greater stability in yield performance over a range of water conditions showed greater drought-induced increase in stomatal resistance.

Genotypic variation for photosynthesis rate, stomatal and non-stomatal parameters under water stress conditions have been reported (Koc et al., 2003; Stiller et al., 2005). This variation indicates potential for genetic advancement through selection programs. The extent to which these physiological parameters can contribute to grain production as well as drought resistance in wheat cultivars has not been well investigated. The present work was conducted to investigate leaf gas exchange parameters and water status as well as the relative importance of stomatal factors controlling photosynthesis rate in two wheat cultivars differing in drought resistance.

During leaf senescence, the assimilation of carbon is replaced by the catabolism of chlorophyll and macromolecules like proteins and membrane lipids; the
material accumulated is turned into exportable nutrients for the developing organs (Lim et al., 2007). Part of the energy required for the remobilization of the assimilates is obtained by a respiration increase (Hopkins et al., 2007). It is proposed that sugars and some growth regulators induce senescence (Jordi et al., 2000; Pourtau et al., 2006). During senescence, sugars have the following functions: they take part in the control of photosynthesis (Wingler et al., 1998, 2000, 2006) they are important elements in the source-sink relationships (Roitsch and Ehnea, 2000) and they induce and regulate the enzymes involved in the mobilization of nitrogen (Masclaux et al., 2000). Some of the enzymes of the amino acid metabolism like cytosolic glutamine synthetase take part in senescence (Buchanan-Wollaston et al., 2005). This enzyme participates in the conversion of amino acids into compounds that can be taken from the leaf; like glutamine, the main amino acid that is transportable during natural senescence (Feller and Fischer, 2007).

Plant growth regulators, including auxins, gibberellins, ethylene, abscisic acid, and cytokinins, are believed to play major roles in regulating senescence. Attention has focused on cytokinins that are key components of plant senescence (Singh et al., 1992; Gan and Amasino, 1995, 1996, 1997; Buchanan-Wollaston, 1997; Nam, 1997; Rivero et al., 2007, 2009). Cytokinins have a potent effect on plant physiology and environmental responses, and are intimately involved in the regulation of cell division, apical dominance, chloroplast development, anthocyanin production and maintenance of the source-sink relationship (Nagarajan et al., 1999). They promote accumulation of chlorophyll and conversion of etioplasts into chloroplasts, and delay leaf senescence. Their possible involvement in responses to adverse environmental conditions is also suggested (Hare et al., 1997, Brault and Maldiney, 1999).

During senescence the concentration of cytokinins diminishes, but their spraying or the over-expression of the ipt gene which encode the enzyme isopentenyldtransferase (IPT), under the control of the specific promoter of senescence SAG 12 (PSAG12-IPT), delay the catabolic changes of leaf senescence (McCabe et al., 2001; Lim et al., 2007). Despite the outstanding effect of cytokinins on delaying plant senescence, the mechanism of action is not well understood yet. The proposal is that during senescence cytokinins may be perceived by receptors associated with histidine kinases and the signal generated be translated through a cascade of
phosphorylations that finally stimulate target genes that are responsible for the delay program of leaf senescence (Lim et al., 2007). The use of cytokinins to delay senescence has potential uses such as to increase crop productivity, to prolong post-harvest storage, and to increase tolerance to stress (Lim et al., 2007). In cereals, cytokinins may be used in the manipulation of nutrient mobilization to improve yield (Rivero et al., 2009). Therefore, the present study is focused on the effect of cytokinin (BAP), on primary metabolism, during the delay of leaf senescence of wheat, with an emphasis on the photosynthesis and related physiological characteristics, accumulation of sugars and the activity of senescence related traits.

A better understanding of the mechanisms that enable wheat plants to adapt to drought stress and maintain growth, development, and productivity during stress periods affected by cytokinins would help in breeding for drought resistance and development of drought tolerant plants by delay in leaf senescence in wheat by transgenic approach.

4.1.3. Materials and Method

4.1.3.1. Plant material:

Two wheat varieties were selected C-306 (drought tolerant) and PBW 343 (drought sensitive), as suggested by breeders and procured from Division of Genetics, Indian Agricultural Research Institute, New Delhi.

Sowing was done in 30 cm earthen pots with clay loam soil and farmyard manure in 3:1 ratio during winter season and supplied with 60, 60 and 60 kg ha\(^{-1}\) of N, P, K, respectively, in the form of urea, single super phosphate and muriate of potash at the time of sowing. Remaining 60 kg N ha\(^{-1}\) was given after 25 days of sowing.

Plants were subjected to water stress after 55 days after sowing for providing water deficit stress condition during reproductive stage. Cytokinin treatment was given by spraying 6-Benzyl amino purine (BAP) 40 µM concentration at 40 DAS and 60 DAS.

Plant Sampling

The plants were sampled and observations were taken for growth, physiological, biochemical parameters and molecular study at pre-anthesis (67 DAS); anthesis (77 DAS) and post-anthesis stages (87 DAS) of the wheat plants described above. Three replications with five pots per replication were taken for each variety. Upper most fully expanded flag leaf was used for recording observation.
4.1.3.2. Relative water Content

Leaf relative water content (RWC) was estimated by recording the turgid weight of 0.5 g fresh leaf samples by keeping in water for 4 h, followed by drying in hot air oven till constant weight was achieved (Weatherley, 1950).

\[
RWC = \left[1 - \frac{(\text{Fresh wt.} - \text{Dry wt.})}{(\text{Turgid wt.} - \text{Dry wt.})}\right] \times 100
\]

4.1.3.3. Membrane stability index

Membrane stability index (MSI) was estimated as per Sairam et al. (1997). For estimation of membrane stability index 100 mg leaf material, in two sets, was taken in test tubes containing 10 ml of double distilled water. One set was heated at 40 °C for 30 min in a metabolic water bath, and the electrical conductivity of the solution was recorded on a conductivity bridge (C₁). Second set was boiled at 100 °C on a boiling water bath for 10 min, and its conductivity was measured on a conductivity bridge (C₂). Membrane stability index was calculated as:

\[
\text{MSI} = [1 - \left(\frac{C_1}{C_2}\right)] \times 100
\]

4.1.3.4. Total chlorophyll and carotenoid estimation

The procedure for estimation of chlorophyll content in plants is based on the absorption of light by chlorophyll extracts prepared by incubating the leaf tissues in DMSO (Dimethyl sulfoxide). DMSO renders plasmalemma permeable thereby, causing the leaching of the pigments (Hiscox andIsraelstam, 1979). The absorbance of the known volume of solution containing known quantity of leaf tissue at two respective, wavelengths (663 and 645) was determined for chlorophyll content and at 470 nm for total carotenoid contents. Chlorophyll content was estimated using the formula given by Arnon (1949). 50 mg fresh leaf samples (small discs) were added to the test tubes containing 10 ml DMSO. Tubes were kept in dark for 4 hrs at 65 °C. The absorbance was recorded at 663 and 645 nm using DMSO as blank and was expressed as mg g⁻¹ FW

- Chlorophyll ‘a’ = \((12.7 X \text{OD}_{663} - 2.69 X \text{OD}_{645}) X V/1000 X w\)
- Chlorophyll ‘b’ = \((22.9 X \text{OD}_{645} - 4.68 X \text{OD}_{663}) X V/1000 X w\)
- Total chlorophyll = \((20.2 X \text{OD}_{645} + 8.02 X \text{OD}_{663}) X V/1000 X w\)
- Total carotenoids = \((1000X \text{OD}_{470} - (3.29 x \text{Chl a}) - (104 x \text{Chl b}) / 198\)

Where,
OD\textsubscript{663} = Absorbance values at 663 nm
OD\textsubscript{645} = Absorbance values at 645 nm
OD\textsubscript{470} = Absorbance values at 470 nm
W = Weight of the sample in mg
V = Volume of the solvent used (ml)
Chl ‘a’ = (12.21 x OD\textsubscript{663}) – (2.81 x OD\textsubscript{645})
Chl ‘b’ = (20.13 x OD\textsubscript{645}) – (5.03 x OD\textsubscript{663})

### 4.1.3.5. Net Photosynthetic rate

Rate of photosynthesis was measured on leaves using portable Infrared Gas Analyzer (IRGA LI-6400 Model). The rate of photosynthesis was measured by operating the IRGA in the closed mode. The photosynthetic rate was determined at different growth stages in the upper most fully expanded leaf between 10 a.m and 11.30 a.m by providing artificial light source of light intensity 1000 µmol m\textsuperscript{-2} s\textsuperscript{-1}. The net photosynthetic rate was expressed as cm s\textsuperscript{-1}.

### 4.1.3.6. Stomatal conductance

The stomatal conductance was also measured using IRGA (LI-COR 6400). The stomatal conductance was expressed as µmoles m\textsuperscript{-2} s\textsuperscript{-1}.

### 4.1.3.7. Photochemical Efficiency

The photochemical efficiency was also measured using IRGA (LI-COR 6400). The photochemical efficiency was expressed as Fv/Fm ratio.

### 4.1.3.8. Total soluble sugars

**Extraction of sugars**

For sugar analysis, the sample was boiled and the supernatant decanted into a beaker. The extraction was repeated four times (three times with 20 ml of 80% (v/v) ethanol in water and finally with 20 ml of distilled water) by boiling the sample for 4-5 min and decanting the supernatant (McCready \textit{et al}., 1950). The combined sugar extract was made up with distilled water in a 100 ml volumetric flask.

**Clarification of the sugar extract**

For clarification, 50 ml aliquot of the above sugar extract was evaporated in a water bath, taking care not to let the liquid dry out completely. Subsequently, the sample was treated with 1 ml saturated solution of lead acetate to precipitate the
colloidal substances. It was then filtered into a 50 ml volumetric flask and made up to the volume. An aliquot of this solution was used for determining the total sugar by Anthrone reagent method **Determination of Total sugars**

One ml of sugar sample was taken and to this 4 ml solution of anthrone regent was added. The mixture is heated on a boiling water bath for 8 min followed by cooling. The optical density of green to dark green colour was read at 630 nm in UV-visible spectrophotometer (model Specord Bio-200, AnalytikJena, Germany). A blank and two freshly prepared glucose standards were also included with each set of samples.

**4.1.3.9. Estimation of starch content**

Starch content was also determined by Anthrone method (McCready *et al.*, 1950). The dry residue left after sugar extraction was powdered and 50 mg of it was hydrolyzed by boiling with 10 ml of 1N HCl for 30 minutes in a glycerine bath at 112-115 °C. After cooling, the samples were transferred into a 100 ml volumetric flask. The residue was repeatedly washed with distilled water until a negative test (iodine test) was obtained. The extract was collected and made to 100 ml. An aliquot of (0.5 ml) the above extract was made to 2.5 ml with distilled water. This was then mixed thoroughly with 10 ml of freshly prepared anthrone reagent (100 mg anthrone was dissolved in 100 ml chilled concentrated H₂SO₄), in a cold water bath. Subsequently, the tubes containing this mixture were kept in a boiling water bath for 15 minutes and then rapidly cooled in running tap water. Absorbance was measured at 620 nm. A reference standard curve was prepared using glucose in the range of 25-300 µg. Starch content was calculated by multiplying the glucose values (1OD = 600 µg/g dw ) with 0.9.

**4.1.3.10. Estimation of total carbon**

Estimation of carbon content was done by wet digestion following the modified Walkley-Black method (1934). The dried sample was oxidized with a mixture of potassium dichromate and concentrated sulphric acid using the heat of dilution of acid. The unused potassium dichromate was estimated by back titration with ferrous ammonium sulphate.
**Estimation of carbon:** 0.05 g of dried powdered sample was taken in a 500 ml conical flask to which 10 ml of 1N K$_2$Cr$_2$O$_7$ was added. The flask was gently swirled to disperse the sample in the reagent and was then placed on asbestos sheet. 20 ml of concentrated H$_2$SO$_4$ was rapidly added to it followed by immediate swirling of the flask. First gently until the sample and the reagent were mixed and then vigorously. The flask was swirled 2 or 3 more times and allowed to stand for 30 min. This was followed by addition of 200 ml of distilled water and 10 ml of orthophosphoric acid. After adding 1 ml of diphenylamine indicator the contents were titrated with ferrous ammonium sulphate solution till the colour changed from blue violet to green. A blank titration was also carried out (without sample). The carbon content was calculated using the following formula and was expressed in percentage.

\[
C\% = 10 \times (x - y) \times 0.003 \times 100/X \times A
\]

Where A is the weight of the sample, X is the volume (ml) of ferrous ammonium sulphate solution required for blank titration; y is the volume (ml) of ferrous ammonium sulphate needed for titration of the sample. The final carbon percentage was obtained by multiplying this carbon percent with a constant 1.3 as only 77 percent recovery was presumed from the oxidation of samples in this procedure.

**4.1.3.11. Gene expression study for selected enzymes/proteins by RT-PCR.**

Nucleotide sequences for candidate genes were obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/) was used to identify the homologs of candidate genes. For RT-PCR expression analysis and cloning of cDNAs, the following oligonucleotide primers were designed manually, and oligo quality (to avoid primer dimer, self dimer, etc.), GC % and Tm were analyzed by using Oligoanalyzer 3.0 tool (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/, Intergrated DNA Technologies, Coralville, IA 52241, USA).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Length (base s)</th>
<th>GC %</th>
<th>Tm (^°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
</table>
Isolation of Total RNA

Total RNA was extracted from flag leaf tissues of plants using RNA easy kit (Qiagen Inc., Chatsworth CA 91311, USA, Cat No: 749040) according to the manufacturer’s instruction.

RT-PCR expression analysis of target genes

Reverse transcriptase-polymerase chain reaction (RT-PCR) mixture using Qiagen One Step RT PCR Kit with gene specific forward and reverse primers was prepared as per the protocol given below:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity for 50 μl of reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease free water</td>
<td>21.0 μl</td>
</tr>
<tr>
<td>5X RT buffer</td>
<td>10.0 μl</td>
</tr>
<tr>
<td>(Tris Cl, KCl, (NH₄)₂SO₄, 13.5 mM MgCl₂, DTT; pH 8.7 at 20 °C)</td>
<td></td>
</tr>
<tr>
<td>dNTP Mix (10 mM each)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>Forward + Reverse primers (10 μM)</td>
<td>5.0 μl</td>
</tr>
<tr>
<td>RNA template (1 μg)</td>
<td>10.0 μl</td>
</tr>
<tr>
<td>RT Enzyme mix</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>50.0 μl</td>
</tr>
</tbody>
</table>

The above reaction mixture was prepared in 0.2 ml PCR tubes and reactions were conducted using QB 96 Thermal cycler (Quanta biotech, England), under the following conditions. Number of cycles was standardized as 25-27 cycles by
conducting semi quantitative RT-PCR, amplified products were not visible in susceptible cultivars when the number of cycles was less than 25.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcription</td>
<td>30 min 50 °C</td>
</tr>
<tr>
<td>Initial PCR activation step</td>
<td>15 min 95 °C</td>
</tr>
<tr>
<td>3-step cycling</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>1 min 94 °C</td>
</tr>
<tr>
<td>Annealing</td>
<td>1 min 57 °C</td>
</tr>
<tr>
<td>Extension</td>
<td>2 min 72 °C</td>
</tr>
<tr>
<td>Final extension</td>
<td>10 min 72 °C</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>27</td>
</tr>
</tbody>
</table>

Linear amplification for semi-quantitative RT-PCR was obtained with 27 cycles. To check the amplification, an aliquot of 5 μl of the reaction mixture was run on a 0.8 % (w/v) agarose gel stained with 0.5 μg ml⁻¹ ethidium bromide. 1 kb DNA ladder was included as a marker for size comparison of the amplified products. 1X TAE buffer was used to prepare the gel as well as for running buffer and electrophoresis was carried out at 5 V/cm. 6X loading dye was used to load the samples. The stained DNA products were photographed using Gel Documentation system.

4.1.3.12. Statistical analyses

The data was analysed statistically using 3 factorial CRD (Biochemical analysis and growth parameters) and CD at 5% and ANOVA was calculated. The analysis was done using OPSTAT programme available online on CCS, HISAR web site.

4.1.4. Results

4.1.4.1. Relative Water Content

Relative water content (RWC) was measured at three different developmental stages of wheat i.e., pre-anthesis, anthesis and post-anthesis to assess the water status of the plants (Table 4.1.1; Fig 4.1.1). Under control conditions, wheat plants mean relative water content (%) was highest at pre-anthesis stage (67 DAS; 86.05) declined afterwards from anthesis (83.49) to post anthesis stages (76.42). Similarly, control
plants treated with cytokinin (6-Benzyl amino purine; BAP: 40 µM) also showed highest mean RWC at pre-anthesis (90.07) and followed the similar trend of decline in RWC from anthesis (87.57) to post-anthesis (78.87) as in control plants without cytokinin treatment. However, the mean RWC was declined around 20% at pre-anthesis stage under water deficit stress condition in comparison to control wheat plants. While, water deficit plants with cytokinin increased the mean RWC by 9% in comparison to water deficit plants without cytokinin. Similar trend were observed of cytokinin enhanced RWC under water deficit stress condition at anthesis as well as post anthesis stages in both the cultivar of wheat studied.

Cultivar differences were observed more under water deficit stress as compared to the control wheat plants affected by cytokinin. Control wheat plants of cultivar C-306 showed enhancement in RWC by around 3% treated by cytokinin as compared to 8% in water deficit plants treated with cytokinin at pre-anthesis stage. While, in PBW-343 it was 6% and 8.8%, respectively. However, higher differences in RWC were observed in wheat plants treated with cytokinin at later developmental stage of anthesis and post anthesis.

4.1.4.2. Membrane Stability Index

Membrane Stability Index (MSI) was also measured at three different developmental stages of wheat (Table 4.1.2; Fig 4.1.2). Under control condition, wheat plants mean MSI (%) was highest at pre-anthesis stage (86.55) and afterwards declined from anthesis (74.79) to post anthesis stages (73.17). Similarly, control plants treated with cytokinin showed highest mean MSI at pre-anthesis (87.81) and followed the similar trend of decline in mean MSI from anthesis (77.77) to post-anthesis (76.44). However, the mean MSI was declined around 18% at pre-anthesis stage under water deficit stress condition in comparison to control wheat plants. While, water deficit plants with cytokinin increased the mean MSI by 10% in comparison to water deficit plants without cytokinin. Similar trend were observed of cytokinin enhanced MSI under water deficit stress condition at anthesis as well as post anthesis stages in both the cultivar of wheat studied.

Control wheat plants of cultivar of C-306 showed no enhancement of MSI when treated with cytokinin while, water deficit stressed plants showed 8% increase in MSI when treated with cytokinin at pre-anthesis stage. In PBW-343 there is
increase of 3% in MSI when controlled plants are treated with cytokinin and 12% increase in plants treated with cytokinin under water stress condition.

4.1.4.3. Chlorophyll-a content

Chlorophyll-a content (mg g⁻¹dw) was estimated to assess the senescence behaviour of wheat plant under two water regimes (Table 4.1.3; Fig 4.1.3). Under control condition wheat plants mean chlorophyll-a content (mg g⁻¹dw) was highest at pre-anthesis stage (10.02) and afterwards dropped from anthesis (8.5) to post anthesis stages (6.94). Similarly, control plants treated with cytokinin also showed highest mean chlorophyll–a content at pre-anthesis (10.68) and followed the similar trend of decline in mean chlorophyll–a content from anthesis (8.86) to post-anthesis stages (8.04) as in control plants without cytokinin treatment. Cytokinin treatment enhanced the chlorophyll–a in both wheat cultivar at all three developmental stages under control condition. However, the mean chlorophyll–a content declined around 28% at pre-anthesis stage under water stress in comparison to control wheat plants. While, water deficit stress plants with cytokinin increased the chlorophyll-a content by 24% in comparison to water deficit plants without cytokinin. Similar trends were observed for cytokinin enhanced chlorophyll-a content under water deficit stress condition at anthesis as well as post anthesis stages in both the cultivar of wheat studied.

Cultivar differences were observed more under water deficit stress as compared to the control wheat plants affected by cytokinin. Control wheat plants of cultivar C-306 showed enhancement of chlorophyll-a content of around 5% treated by cytokinin as compared to 16% in water deficit plants treated with cytokinin at pre-anthesis stages. While, in PBW-343 it was 8% and 33%, respectively.

4.1.4.4. Chlorophyll-b content

Chlorophyll-b content (mg g⁻¹dw) was also estimated to assess the senescence behaviour of wheat plant under two water regimes (Table 4.1.4; Fig 4.1.4). Under control condition wheat plants mean chlorophyll-b content was highest at pre-anthesis stage (3.11) and afterwards declined from anthesis (2.09) to post anthesis stage (1.30). Similarly, control plants treated with cytokinin also showed highest mean chlorophyll-b content at pre-anthesis (3.22) and reduced and at post-anthesis (1.73). However, the mean chlorophyll-b content was declined around 32% at pre-anthesis stage under water stress in comparison to control wheat plants. While, water deficit plants with cytokinin increased the chlorophyll-b content by 16% in comparison to
water deficit plants without cytokinin. Similar trends were observed for cytokinin enhanced chlorophyll-b content under water deficit stress condition at anthesis as well as post anthesis stages.

Cultivar differences were also observed under water deficit stress. Control wheat plants of cultivar C-306 showed no enhancement in chlorophyll-b content by treatment with cytokinin where as 25% increase in chlorophyll -b content was observed in water deficit plants treated with cytokinin at pre-anthesis stages. While, in PBW-343 it was 9% and 14.5%, respectively.

4.1.4.5. Total Chlorophyll content

Total chlorophyll content (mg g⁻¹ dw) is one of the important trait of senescence was estimated under two water regimes in two cultivars differing in their tolerance to water deficit stress at reproductive stages of wheat (Table 4.1.5; Fig 4.1.5). Under control conditions wheat plants mean total chlorophyll content was highest at pre-anthesis stage (13.13) and afterwards declined from anthesis (10.59) to post anthesis stage (8.24). Similarly, control plants treated with cytokinin also showed highest mean total chlorophyll content at pre-anthesis (13.9) and followed the similar trend of decline in total chlorophyll content from anthesis (11.02) to post-anthesis (9.27) as in control plants without cytokinin treatment but rate of decline was little slow. However, the mean total chlorophyll content was declined around 6% at pre-anthesis stage under water stress in comparison to control wheat plants. While, water deficit plants with cytokinin increased the total chlorophyll content by 23% in comparison to water deficit plants without cytokinin. Similar trend were observed of cytokinin enhanced total chlorophyll content under water deficit stress condition at anthesis as well as post anthesis stages in both the cultivars of wheat studied.

Cultivar differences were observed more under water deficit stress as compared to the control wheat plants affected by cytokinin on total chlorophyll content. Control wheat plants cultivar C-306 treated by cytokinin showed enhancement of total chlorophyll content of around 4% as compared to 18% in water deficit plants treated with cytokinin at pre-anthesis stages. While, in PBW-343 it was 8% and 28%, respectively.

4.1.4.6. Chlorophyll a/b ratio

Chlorophyll a/b ratio was also measured at three different developmental stages of wheat (Table 4.1.6; Fig 4.1.6). Under control condition, wheat plants mean
chlorophyll a/b ratio at pre-anthesis stage was lowest (3.22), increased at anthesis (4.08) but it was highest at post-anthesis stage (5.33). Similarly, control plants treated with cytokinin showed mean chlorophyll a/b ratio lowest at pre-anthesis (3.31) which increases at anthesis (4.10) and at post-anthesis (4.36). However, the mean chlorophyll a/b ratio was dropped by 7% at pre-anthesis stage under water stress in comparison to control wheat plants. While, water deficit plants with cytokinin treatment showed increase in mean chlorophyll a/b ratio by 4% in comparison to water deficit plants without cytokinin. Similar trend were observed for cytokinin enhanced chlorophyll a/b ratio under water deficit stress condition at anthesis but at post anthesis stage reduction in chlorophyll a/b ratio was observed.

Cultivar differences were also observed under water deficit stress. Control wheat plants of cultivar C-306 showed increase in chlorophyll a/b ratio of around 5% treated by cytokinin and in water deficit plants treated with cytokinin decrease of 8% was reported at pre-anthesis stages. While, in PBW-343 there was no change under control condition where as in water deficit plants 18% increase in chlorophyll a/b ratio.

4.1.4.7 Carotenoid content

Carotenoid content (mg g\(^{-1}\) dw) was measured at three different developmental stages of wheat (Table 4.1.7; Fig 4.1.7). Under control condition wheat plants mean carotenoid content (mg g\(^{-1}\) dw) at pre-anthesis stage was 6.95 and afterwards it enhanced at anthesis to 8.08 but drop in carotenoid content was observed at post anthesis stage to 6.93. Similarly, control plants treated with cytokinin showed mean carotenoid content of 6.94 at pre-anthesis stage which increased at anthesis to 9.14 followed by decrease at post-anthesis to 7.51. However, the mean carotenoid content was declined only by 2% at pre-anthesis stage under water stress comparison to control wheat plants. While, in water deficit plants with cytokinin increased the mean carotenoid content by 8% in comparison to water deficit plants without cytokinin. Similar trend were observed for cytokinin enhanced carotenoid content under water deficit stress condition at anthesis as well as post anthesis stages in both the cultivar of wheat studied.

Cultivar differences were also observed under water deficit stress. Control wheat plants cultivar C-306 showed no enhancement in carotenoid content when
treated by cytokinin as compared to 7% rise in water deficit plants treated with cytokinin at pre-anthesis stages. While, in PBW-343 it was 8%.

4.1.4.8. Photosynthesis Rate

Photosynthesis rate (µmoles CO$_2$ m$^{-2}$ h$^{-1}$) was estimated to assess the CO$_2$ assimilation efficiency under different treatments at three different developmental stages of wheat (Table 4.1.8; Fig 4.1.8). Under control condition, wheat plants mean photosynthesis rate at pre-anthesis stage was 27.1 and it enhanced to 30.57 at anthesis but again reduced to 16.17 at post anthesis stage. Similarly, in control plants treated with cytokinin mean photosynthesis rate at pre-anthesis stage was 28.63 followed by the similar trend of increase in photosynthesis rate at anthesis 32.65 followed by decrease to 18.2 at post-anthesis as in control plants without cytokinin treatment. However, water deficit stress at pre-anthesis stage leads to decline in mean photosynthesis rate by 23% in comparison to control wheat plants. While, water deficit plants with cytokinin showed increase the mean photosynthesis rate by 6% in comparison to water deficit plants without cytokinin. Similar trends were observed for cytokinin enhanced photosynthesis rate under water deficit stress condition at anthesis as well as post anthesis stages. Cytokinin induced percentage enhancement in photosynthesis rate at post anthesis stages (25%) under water deficit stress was more in comparison to pre-anthesis and anthesis stages.

Cultivar differences were also observed under water deficit stress. Control wheat plants cultivar of C-306 showed enhancement in photosynthesis rate by 13% when treated by cytokinin as compared to 8% in water deficit plants treated with cytokinin at pre-anthesis stage. While, in PBW-343 it was 1% and 3%, respectively.

Rate of photosynthesis was high in sensitive cultivar (PBW-343) under control condition compare to tolerant cultivar (C-306) but decrease in mean photosynthesis rate was more in PBW-343 (40%) as compare to C-306 (22%) under water deficit stress.

4.1.4.9. Stomatal Conductance

Stomatal conductance (Cs; cm s$^{-1}$) was also measured at three different developmental stages of wheat (Table 4.1.9; Fig 4.1.9). Under control condition, wheat plants mean Cs at pre-anthesis stage was 0.26 increased to 0.35 at anthesis but during post anthesis stage mean Cs was reduced to 0.11. Similarly, in control plants treated with cytokinin showed mean Cs at pre-anthesis as 0.29 and followed the trend
of increase in mean Cs at anthesis to 0.42 and reduced to 0.15 at post-anthesis as in control plants without cytokinin treatment. However, the mean Cs declined around 48% at pre-anthesis stage under water deficit stress in comparison to control wheat plants. While, water deficit plants with cytokinin showed increase in the mean Cs by 15% in comparison to water deficit plants without cytokinin. Similar trend were observed for cytokinin enhanced Cs under water deficit stress condition at anthesis as well as post anthesis stages.

Cultivar differences were also observed under water deficit stress. Control wheat plants of cultivar C-306 showed enhancement in stomatal conductance by 2% when treated by cytokinin as compared to 30% in water deficit plants treated with cytokinin at pre-anthesis stage. While, in PBW-343 not much enhancement in stomatal conductance was observed under water stress and in control due to treatment of cytokinin.

4.1.4.10. Photochemical Efficiency (Fv/Fm)

Photochemical efficiency was also measured at three different developmental stages of wheat (Table 4.1.10; Fig 4.1.10). Under control condition mean photochemical efficiency at pre-anthesis stage was 0.74 at anthesis it enhanced to 0.76 but at post anthesis stage photochemical efficiency was again reduced to 0.64. Similarily, control plants treated with cytokinin also showed highest mean photochemical efficiency at pre-anthesis stage 0.76 followed by small decrease in photochemical efficiency at anthesis to 0.75 and it also reduced to 0.63 at post-anthesis as in control plants without cytokinin treatment. Mean photochemical efficiency was declined by 23% at pre-anthesis stage under water deficit stress in comparison to control wheat plants. While, water deficit plants on treatment with cytokinin showed increase in photochemical efficiency by 8% in comparison to water deficit plants without cytokinin. Similar trend were observed for cytokinin enhanced photochemical efficiency under water deficit stress condition at anthesis as well as post anthesis stages.

Cultivar differences were also observed more under water deficit stress. Control wheat plants of cultivar C-306 showed enhancement in photochemical efficiency only by 3% treated by cytokinin as compared to 1.3% in water deficit C-306 plants treated with cytokinin at pre-anthesis stage. While, in PBW-343 it was 0.5 % and 15%,
respectively, so we can say that cytokinin can induce the photochemical efficiency in sensitive variety.

4.1.4.11. Total sugar content

Total sugar (mg g\(^{-1}\)dw) was estimated to assess the carbon status of plants affected by cytokinin in two cultivars differing in their tolerance to water deficit stress at reproductive stages of wheat (Table 4.1.11; Fig 4.1.11). Under control condition, wheat plants mean total sugar was highest at pre-anthesis stage (125.38) and afterwards declined from anthesis (118.77) to post anthesis stage (99.32). However, control plants treated with cytokinin showed mean total sugar at pre-anthesis (135.51) and followed by increase in total sugar at anthesis to 141.57 and it again reduced to level of (113.09) during post-anthesis as in control plants without cytokinin treatment. However, under water deficit stress condition at pre-anthesis stage mean total sugar was declined by 41% in comparison to control wheat plants. While, water deficit plants with cytokinin increased the mean total sugar by 32% in comparison to water deficit plants without cytokinin. Cytokinin treatment enhanced the total sugar content in both wheat cultivar at all three developmental stages under control condition. Similar trend were observed for cytokinin enhanced total sugar under water deficit stress condition at anthesis as well as post anthesis.

Cultivar differences were observed more under water deficit stress as compared to the control wheat plants affected by cytokinin on total sugar content. Control wheat plants of cultivar C-306 showed enhancement of total sugar only by 6 % treated by cytokinin as compared to 24 % in water deficit C-306 plants treated with cytokinin at pre-anthesis stage. While, in PBW-343 it was 10 % and 42%, respectively.

4.1.4.12. Total Starch Content

Starch content (mg g\(^{-1}\)dw) was estimated to assess the carbon status of plants affected by cytokinin in two cultivars differing in their tolerance to water deficit stress at reproductive stages of wheat (Table 4.1.12; Fig 4.1.12). Under control condition, wheat plants mean starch content at pre-anthesis stage was 90.1 it increased at anthesis and has highest value (113.5) but reduction in starch content during post anthesis stage was observed (69.90). Similarly, in control plants treated with cytokinin showed mean starch content at pre- anthesis of 99.2 and followed the similar trend of increase in starch content at anthesis (123.7) and reduction in starch content at post anthesis (72.81) in control plants without cytokinin treatment. However, the mean
starch content was declined by 62% at pre-anthesis stage under water deficit stress in comparison to control wheat plants. While, under insufficient water condition plants treated with cytokinin showed increase in starch content by 69% in comparison to water deficit plants without cytokinin. Similar trend were observed for cytokinin enhanced starch content under water deficit stress condition at anthesis as well as post anthesis.

Cultivar differences were observed more under water deficit stress as compared to the control wheat plants affected by cytokinin on starch content. Control wheat plants of cultivar C-306 showed enhancement of starch content by 9.5% treated by cytokinin as compared to 38% in water deficit plants treated with cytokinin at pre-anthesis stage. While, in PBW-343 it was 11% and 100%, respectively.

4.1.4.13. Total carbon content

Total carbon (mg g\textsuperscript{-1} dw) was estimated to assess the carbon status of plants affected by cytokinin in two cultivars differing in their tolerance to water deficit stress at reproductive stages of wheat (Table 4.1.13; Fig 4.1.13). Under control condition, wheat plants mean total carbon at pre-anthesis stage was 37.28 and it highest at anthesis (45.7) and same carbon content was found at post anthesis stage (44.45). Similarly, in control plants treated with cytokinin mean total carbon was lowest at pre-anthesis stage (41) followed by increase in total carbon at anthesis (48.99) but it again reduced at post-anthesis (45.22) as in control plants without cytokinin treatment. However, the mean total carbon content was declined around 29% at pre-anthesis stage under water deficit stress in comparison to control wheat plants. While, water deficit plants with cytokinin increased the mean total carbon content by 57% in comparison to water deficit plants without cytokinin. Similar trend were observed for cytokinin enhanced total carbon content under water deficit stress condition at anthesis as well as post anthesis stages.

Cultivar differences were also observed more under water deficit stress. Control plants of cultivar C-306 showed enhancement of total carbon only by 11% treated by cytokinin as compared to 8% in water deficit plants treated with cytokinin at pre-anthesis stage. While, in PBW-343 it was 9% and 28%, respectively.

4.1.4.14. Rubisco SSU expression analysis

Expression analysis of smaller sub unit of rubisco was studied as it is more affected by water stress condition and also have regulatory role, at three stages of
wheat development (Plate 4.1.1.). The rubisco SSU expression was found highest at anthesis followed by post-anthesis and lowest at pre-anthesis stage in both the drought tolerant and sensitive cultivar of wheat.

Control plants of cultivar C-306 treated with cytokinin showed higher expression of rubisco SSU in comparison to plants without cytokinin treatment but under water stress condition there was no significant difference in expression were observed due to cytokinin treatment. Whereas, in sensitive cultivar (PBW-343) no significant effect was observed due to cytokinin treatment under control as well as water deficit stress condition at all the three developmental stages.

**4.1.4.15. COR Expression analysis**

Expression analysis of COR gene was studied at three stages of wheat development (Plate. 4.1.2). Highest expression of COR gene was found at post-anthesis and then decreased from anthesis to pre-anthesis stage in both drought tolerant and sensitive cultivar of wheat. Control plants of cultivar C-306 treated with cytokinin showed lower expression of COR gene at pre-anthesis and anthesis compared to plants without cytokinin treatment under control condition but expression of COR gene was high at post-anthesis stage but under water stress condition plants there was no significant difference in expression was observed due to treatment. Whereas, in sensitive cultivar PBW-343 treated with cytokinin showed no difference in expression at pre-anthesis and lower expression level of COR gene was reported at anthesis and post-anthesis stages compared to plants without cytokinin treatment

**4.1.4.16. DHN Expression analysis**

Expression analysis of DHN gene was studied at three stages of wheat development (Plate. 4.1.3). Water stress induced expression of DHN gene was observed in both cultivar compared to control condition at all three developmental stage. Lowest DHN gene expression was found at pre-anthesis stage followed by decrease in expression from anthesis to post-anthesis stages in both the drought tolerant and sensitive cultivar of wheat. No significant effect of cytokinin treatment was reported on both the cultivar under control and water stress condition.

**4.5. Discussion**
Most climate-change studies indicate an expansion of dry land areas on our planet. This increase in arid land and the world’s growing population will have a direct impact on water resources and water availability. Water scarcity and the concurrent high temperatures create the most significant limitations to crop productivity (Boyer, 1982). Our long-term objective is to generate transgenic plants that can survive severe drought episodes and that can grow under restricted water regimes with minimal yield losses. Here, we show that the over-expression of cytokinin (CKs) in terms of external spray of 6-Benzyl amino purine (BAP) on the two cultivar of wheat differing in their tolerance to drought under moisture stress conditions induced the synthesis of CKs in the plant and that the production of CKs contributed to the enhanced drought tolerance of the treated plants.

The drought resistant cultivar C-306 had a higher grain yield than PBW-343 under drought, and the reduction in grain yield under drought was smaller in C-306 than in PBW-343. The apparent drought resistance of C-306 was associated with the dry matter accumulation during grain filling, resulting from the continued increase in total shoot dry matter between anthesis (77 DAS) and maturity. On the contrary, the drought sensitive PBW-343 did not show any increase in total shoot dry matter between anthesis (77 DAS) and maturity under drought, leading to a lower grain yield. While, in both the cultivars cytokinin could able to enhance the grain yield in both control as well as moisture stressed plants, but in stressed plants the enhancement was significantly higher in comparison to control plants. When assimilates available from post-anthesis photosynthesis satisfy the requirements for grain filling in wheat, a high grain yield may be achieved (Flood et al., 1995, Ehdaie and Waines, 1996).

The flag leaves have a major role in determining wheat grain yield (Shimshi and Ephrat, 1975). Under drought stress, the photosynthesis of flag leaf was significantly higher in C-306 than in PBW-343 during grain filling (Fig. 4.1.8).

Photosynthetic capacity is determined by total leaf area as well as photosynthesis per unit surface area. Simpson (1968) reported that the area of the flag leaf was positively and highly correlated with grain yield of many cultivars of wheat. Under drought stress, the flag leaves in less irrigated treatment were shorter and narrower than those of normal irrigated wheat (Zhang et al., 1998). The flag leaf area of C-306 was more reduced than that of PBW-343 under drought. The area of flag
leaf in C-306 was reduced more than in PBW-343, C-306 also maintained a higher photosynthetic rate (Pn) in the flag leaf even under drought when photosynthesis in PBW-343 was lesser as compared to tolerant cultivar. This suggested that C-306 also had a higher photosynthetic rate per flag leaf than PBW-343. The early peak in photosynthesis of control plants in PBW-343 is similar to that observed in wheat and other species subjected to drought (Turner et al., 1978, Henson et al., 1989, Puech-Suanzes et al., 1989, Deng et al., 2000).

Under drought, stomatal closure and inhibition of chloroplast activity reduce photosynthesis (Farquhar and Sharkey, 1982). Stomatal closure increases the resistance to CO\(_2\) diffusion into the leaf. An inhibition of chloroplast activity at low leaf RWC decreases the capacity to fix CO\(_2\). This cannot be overcome by increasing the concentration of CO\(_2\) (Graan and Boyer, 1990). Decreasing leaf RWC caused a similar degree of reduction in stomatal conductance (gs) of flag leaf in C-306 and PBW-343 plants. These observations indicated that stomatal response to declining leaf RWC was similar in C-306 and PBW-343, and that any differences in drought stress inhibition of Pn between these wheat cultivars cannot be fully explained by altered stomatal responses to stress. The gs might play an important role in the high Pn under well watered or mild drought stress, but under severe drought stress the high Pn is related more to the maintenance of a higher capacity for mesophyll photosynthesis (Farquhar and Sharkey, 1982, Johnson et al., 1987, Rekika et al., 1998, Shangguan et al., 1999).

We conclude that under drought C-306 maintained higher Pn of the flag leaf during post-anthesis, which may account for the higher grain yield. This higher Pn of flag leaf in C-306 may be related to the greater chloroplast activity under low leaf RWC.

The final stage of leaf development is inevitably senescence with a decline in physiological activity. Senescence of flag leaves in two wheat cultivars grown in the field was characterized by Chl loss (leaf yellowing), decreases in the Chl a/b ratio, photosynthetic rate, stomatal conductance and photochemical efficiency. However, these photochemical and biochemical parameters did not show similar changing pattern and senescing behavior from emergence through senescence of flag leaves and there were some differences in these parameters between these two cultivars, which have different senescent appearance.
The well-known phenomenon in senescing leaves is the loss of Chl content. The degree of Chl loss of the wheat cultivars is strongly associated with light-saturated photosynthetic rate during grain filling, as well as being associated with differences in final grain yield (Reynolds et al., 2000). In this study, the Chl content in C-306 was higher than in PBW-343 during the flag leaf senescence, showing that process of leaf senescence in C-306 was delayed by cytokinins in both the cultivars studied. In combination with the longer duration of having a higher content of Chl at the late senescence, these findings may be partly responsible for the higher grain yield in C-306 than in PBW-343.

Selective loss of pigments denotes structural alterations in individual photosystems (Subhan and Murthy, 2001). The decrease in Chl a/b ratio during leaf senescence in stressed plants could be further confirmed in both the cultivars by the treatment of cytokinins. Chl a as the pigment of the reaction centres and Chl b as the antenna pigment play an important role in photosynthesis. The decrease at the red peak of around 680 nm, which was greater than that at the blue-violet peak of around 430 nm during leaf senescing, indicated that the senescence of photosynthetic core antenna was quicker than that of the LHC antenna system in the course of leaf senescence, which is consistent with the results reported by Wang et al., (2004). Gossauer and Engel (1996) stated that the conversion of Chl b to Chl a should precede Chl degradation in higher plants. Therefore, it is easy to conclude that the decreased Chl a/b ratio implied that the loss of Chl a may be much greater than Chl b conversion during leaf aging and could be protected by the cytokinin treatments.

As a useful measure of the two major classes of photosynthetic antennae (with Chl a only and Chl a and b), the change in this ratio can provide an insight into the stability of thylakoid membrane organization during leaf senescence (Subhan and Murthy, 2001). Strangely, it does not always change in a consistent direction, even within a species (Canfield et al., 1995), probably depending on senescence induced factors. Dark-induced senescence often results in an increased Chl a/b ratio (Fang et al., 1998), while natural leaf senescence leads to a deceased Chl a/b ratio. Based upon data from 16 field-grown wheat cultivars, Reynolds et al., (2000) reported a declined ratio with leaf aging, similar to the present result and other results showed an increase (Scheumann et al., 1999; Hikosaka and Terashima, 1996; Murchie et al., 2002; Kitajima and Hogan, 2003; Munne-Bosch and Penuelas, 2003). Lu et al. (2001)
reported a relatively stable value in 20 days after anthesis but then a marked increase up to harvest time in a wheat cultivar also grown in the field. Such a decrease in the Chl a/b ratio seems to be a conflict with the fact that Chl a is more or less stable during senescence, but chlorophyll b is almost labile (Thomas et al., 2002). One possible reason seems to be that there is a cycle of interconversion between Chl a and b that is particularly significant in senescence, because only pigments with the ‘‘a’’ configuration on pyrrole group B are recognized by the Chl degradation pathway (Metile et al., 1999). Thus, the relatively persistent Chl b may not be the same Chl b that was primarily localized in the antennae of pre-senescent leaves, but instead may be the product of unbalanced operation of the a – b interconversion cycle (Zavaleta-Mancera et al., 1999). Additionally, the chemical stability of Chl a and b may be different from their biological stability, that is, Chl a in living plant tissues may be less stable than Chl b, at least in aging wheat leaves.

In conclusion, all photosynthetic parameters were increased from emergence to full expansion, indicating that the photosynthetic apparatus was under functional maturation during this period. After full expansion the primary decline in photosynthesis was due to availability of Rubisco, being accompanied by a decrease in Chl content. Starting with a significantly declined Chl a/b ratio, the primary decline in photosynthesis of flag leaves was attributable not only to declined in Rubisco, but also to declined Fv/Fm ratio. Compared with the sensitive cultivar PBW-343, both the higher Chl content and the higher photosynthetic capacity of C-306 became apparent mainly at the late stage of senescence, which indicates that the rate of grain filling at the late stage might play an important role in determining the grain yield of wheat cultivars.

The main effects of moisture and cultivar were highly significant for all the measured traits. The interaction between cultivar by moisture was also significant for all traits. A dramatic decline was observed in leaf RWC (Siddique, et al., 2000; Halder & Burrage, 2003; Basu et al., 2004). Detrimental effect of cell dehydration on physiological and biochemical reactions and consequently growth and productivity is well documented (Lawlor, 1995). Thus, an ability to maintain high RWC under stress conditions could be an adaptive feature. In the present study, resistant cultivars were able to maintain greater RWC (85-90%) compared to susceptible ones (70-75%). The stability of photosynthetic components could be attributed by maintenance of positive
leaf RWC under stress as a result of osmotic adjustment (Basu et al., 2004). In general, resistant cultivars performed higher Pn, gs and transpiration rate (Tr) than susceptible ones under either conditions. As observed for grain yield, water stress caused a significant reduction in Pn. Similar results have been reported by other investigators (Siddique et al., 2000, Stiller, et al., 2005; Ratnayaka & Kincaid, 2005). This appears to be a possible physiological mechanism by which drought can affect growth and productivity of crops such as wheat (Lawlor, 1995). While, addition of cytokinins could able to revive the photosynthesis and its components in both the cultivars and water regimes as also reported recently by Rivero et al., (2007) in ipt overexpressed tobacco transgenic plants.

Decline in leaf Pn under water stress was accompanied by decline in leaf RWC. Siddique et al., (2000) reported that the higher leaf water potential and relative water content of wheat cultivars were associated with a higher photosynthetic rate. Leaf dehydration can lead to turgor loss of guard cells causing passive stomatal closure, which in turn, would reduce gs and consequently supply of CO₂ to the fixation site. A remarkable decline in gs and Pn due to water stress implies to the importance of stomatal limitation to Pn under water stress in the examined cultivars. Although reduction in gs under water stress limits Pn, it may, on the other hand, reduce transpirational water loss which can be beneficial for plant under limited moisture supply. Compared to the susceptible one, resistant cultivar manifested a greater reduction in gs and Tr, but a smaller reduction in Pn under stress condition. Condon et al. (2002) explained that, the ratio of CO₂ assimilation rate to transpiration rate at the stomata may be one means of achieving greater yield per unit rainfall in dryland area. It has been hypothesized that any improvement in components of water use efficiency (WUE) would be expected to partially reduce the adverse effects of water stress. (Stiller et al., 2005).

It has been hypothesized that genotype which keep their stomata open under stress condition while maintaining adequate leaf RWC can be considered as suitable for dry region (Blum et al., 1981). In the present study, resistant cultivar had higher values of Tr and RWC indicating their greater ability to water uptake from the soil compared to susceptible ones.

Photosynthesis rate decline was significant at post-anthesis in both the cultivars at two water regimes in control plants; in contrast, the BAP treatment
delayed the degradation of photosynthetic proteins. Similar results were obtained in tobacco plants (*Nicotiana tabacum* L.) transformed with the *ipt* gene encoding the enzyme isopentenyltransferase (IPT), under the control of the promoter of a specific gene of senescence, SAG 12 (Jordi *et al*., 2000). In addition, BAP induced the accumulation of total sugars and starch at anthesis as well as at post-anthesis stages. The accumulation of these carbohydrates could be explained by the effect of cytokinins in the maintenance of CO₂ fixation during wheat leaf senescence (Wingler *et al*., 1998; Martinez-Gutierrez *et al*., 2008).

One of the strongest evidence of the useful effect of cytokinin spraying is the delay of leaf yellowing. The BAP treatment retained most of total chlorophyll in relation to the value of green pre senescent leaves (77 DAS), while the control plants without cytokinins preserved only little chlorophyll from pre-anthesis to post-anthesis. In the control, the amount of Chl *a* underwent a greater impact than that of Chl *b* since the Chl *a*: Chl *b* ratio diminished. In contrast, the leaves treated with BAP upheld values at higher rates, that is, during the delay of pigment degradation BAP maintained the proportion of Chl *a* and Chl *b*, a similar performance to that exhibited by the green leaf.

In the present study it was found that BAP induced the carotenoid synthesis during the anthesis stage; then the values were constant but significantly higher than in the control. A similar trend was reported by Zavaleta-Mancera *et al*., (2007) in fragments of wheat leaves incubated 6 d in BAP in the dark. On the contrary, under greenhouse conditions with natural light, the accessory pigments such as carotenoids increased during wheat natural senescence (Martinez-Gutierrez *et al*., 2008). In this same species, with intense light, accessory pigments increased their content (Lu *et al*., 2001). Cytokinins are likely to increase the synthesis of accessory pigments to protect the reaction centers of light and oxygen (Wingler *et al*., 2004; Vlckova *et al*., 2006).

Cytokinins induce the expression of photosynthetic genes, promote the synthesis of proteins and keep high levels of Rubisco during leaf senescence (Ookawa *et al*., 2004). In transgenic plants PSAG12-IPT of tobacco (*Nicotiana tabacum* L.) it was shown that soluble proteins (30 %) register better preservation levels than those of Rubisco (15 %) in senescent leaves (Jordi *et al*., 2000). In contrast, in the present study, the wheat leaves (*Triticum aestivum* L.) sprayed with BAP preserved a higher percentage of Rubisco SSU than of soluble proteins at all the stages of wheat
development; significantly in C-306 but not so significantly in PBW-343. This evidences the differences in the degradation of macromolecules between species and treatments. In this respect, Buchanan-Wollaston et al., (2005) document the results on the gene expression and the signaling pathways comparing natural senescence and the one induced with darkness, all of which showed both similarities and differences, including the presence of different channels in catabolic processes, especially in the mobilization of nitrogen and carbohydrates.

The results of this study indicated an increase in the content of total sugars and starch from 67 to 77 DAS, later decreased; however, in the control the levels were similar until 36 DAS. The control recorded similar concentrations of starch during senescence; in contrast plants sprayed with BAP registered a higher content of starch until 77 DAS, and then dropped. Differences were detected between the leaves with BAP and the control, at all the growth stages of wheat studied. In Nicotiana tabacum, Zea mays and Arabidopsis, soluble sugars increased during natural senescence (Wingler et al., 1998; Jongebloed et al., 2004; Pourtau et al., 2006). It is not yet clear what causes the accumulation of sugars in senescent leaves under stressed conditions despite a visible fall of photosynthetic activity (Wingler et al., 2006), nor in which cellular compartments sugars accumulate (Pourtau et al., 2006) contrary to our results where total sugars were less in stressed plants in both the studied cultivars. The increase of sugars is attributed to the breaking of starch or the preferential export of N₂ (Wingler et al., 1998).

The BAP external spraying promoted the accumulation of sugars. In this respect, Wingler et al., (1998) and assert that an increment of sugars is required to allow the delay of senescence in transgenic tobacco plants (PSAG12-IPT). Cytokinins regulate the expression of certain genes involved in the distribution of assimilates and in the regulation of the source-sink, like the expression of the extracellular invertase and hexose carriers that increase the use of carbohydrates to impede the start of catabolic processes (Balibrera-Lara et al., 2004; Guivarch et al., 2005).

During leaf senescence, the plasma membrane and the membranes of other organelles lose their permeability due to molecular modifications of the lipid bilayer, initially promoted by oxidative processes (Hopkins et al., 2007). But cytokinins delay this natural process. By increasing the activity of the antioxidant enzymes catalase and ascorbate peroxidase, cytoplasmatic and chloroplast membranes protect
themselves from the oxidative damage, evidenced by the reduced loss of electrolytes and the preservation of the chloroplast membrane structure (Zavaleta-Mancera et al., 2007). In the regreening process of *Nicotiana rustica* L, BAP promotes the reassembly of tilacoidal membranes in aged chloroplasts or gerontoplasts (Zavaleta-Mancera et al., 1999). The data of the present study also indicate that the release of electrolytes remained unchanged or lower in the leaves treated with BAP in both control and stressed plants in both the studied cultivars. But 87 DAS the release of electrolytes rose in the control and stressed plants, a value that reveals there is damage in the cytoplasmatic membrane. In segments of leaves of *Catharanthus roseus* L. the release of electrolytes remained constant for 10 d, but when they were incubated in BAP, significant changes occurred only in the last days of the treatment (Zhang et al., 2007).

The cytokinin BAP keeps chlorophyll concentrations longer, as well as total soluble proteins and Rubisco (SSU); it can also modify the photosynthesis and related activity, preserving a high content of sugars and reducing the loss of electrolytes. Understanding the delay of leaf senescence may lead to provide information for the implementation of technologies to address the senescence of fodder, green vegetables and ornamental foliage.

Creating water stress by stopping irrigation caused physiological damage in the leaves of the control and BAP treated plants; however, the treated plants exhibited better tolerance to the water stress, as demonstrated by the maintenance of higher Fv/Fm, Chl content, RWC, Pn, gs, and CK content, in flag leaves. The transgenic line has previously been reported to exhibit superior drought tolerance compared with the WT when plants were subjected to soil drying by withholding irrigation (Merewitz et al., 2010). The results, in combination with our study, demonstrated that expression of the *ipt* gene or external spray of cytokinins (BAP) in wheat crop was effective in improving plant tolerance to water stress.

The mechanisms of CK regulation of drought tolerance are not yet fully clear. It is known that CK stimulates stomatal opening, which could help with carbon absorption for photosynthesis, but may cause leaf desiccation under drought stress due to water loss (Chernyadev, 2005). More recently, it has been found that the timing of increased CK content, the form of CK present, and the balance of hormones may be more critical in determining stomatal responses during drought stress, particularly
with ipt plants (Pospisilova et al., 2000, 2005). In the current study, the flag leaves of
the treated plants had higher Pn, gs and E under water stress. The elevated CK may
have affected Pn through other mechanisms besides stomatal regulation. Similar
results were found in water-stressed treated plants stomatal conductance was largely
the same between ipt and WT plants (Rivero et al., 2009). They concluded that
maintenance of photosynthesis rates in ipt tobacco was possibly due to non-stomatal
traits such as increased photorespiration, which promotes photosynthesis under
drought stress by providing RUBP and other beneficial metabolites (Wingler et al.,
2000). This was evident to Rivero et al., (2009) by the increased level of transcripts
coding for enzymes in the photorespiration pathway, increased metabolites generated
by photorespiration, greater antioxidant content, and an increase in the CO2
compensation point in ipt plants compared with the WT. These mechanisms may also
be a factor in SAG12-ipt creeping bentgrass observed indirectly in as higher Pn in the
mature leaves of the transgenic plants, increases in chlorophyll content, and greater
Fv/Fm. In Kentucky bluegrass (Poa pratensis), higher values of WUE have been
associated with less wilting, greater TQ, and superior water relations under drought
stress (Ebdon and Kopp, 2004). In several cool season grasses, low D was associated
with higher instantaneous water use efficiency; plants with low D had higher water
potential, solute potential, and turgor pressure, exhibiting a better capacity for growth
under drought stress (Johnson and Basset, 1991). Thus, most likely the treated plants
with increased CK may enhance metabolic activities that may promote photosynthetic
activity without increasing stomatal conductance or transpiration rate, thereby
increasing water use efficiency, especially in flag leaves.

CK involvement in regulating Pn, gs, E, WUE, and D under drought stress
deserves additional investigation since increases in CK is not always associated with
increases in gs as previously proposed and some studies have shown a decrease in gs

Delay in leaf senescence is significant for plants to adapt to water stress, as the
first symptoms of senescence are seen as a loss of chlorophyll and chloroplasts as the
plant degrades leaf mesophyll cells for nutrient remobilization (Lim et al., 2007). The
results of this study show that the expression of the externally provided cytokinins in
wheat crop at highly detectable levels was sufficient to maintain levels of CK in flag
leaves and increase root CK of creeping bentgrass subjected to water stress. It may be
assumed that enough CK was produced to overcome degradation of the free forms of CK by cytokinin oxidases, which are up-regulated in the drought response in most plant organs (Vyroubalova et al., 2009).

In conclusion, cytokinin treated wheat plants resulted in increases in CK accumulation in the leaves and roots and in the overall plant tolerance to water stress. The physiological effects of treated plants on improving plant tolerance to water stress were reflected by an enhancement in RWC, photosynthetic characteristics, membrane stability, delay in leaf senescence, and maintenance of higher Rubisco and Lea proteins under water stress in wheat plants.

Future work will aim to identify the mechanisms associated with CK regulation of photosynthesis, and maintenance of chlorophyll in cytokinin treated wheat plants during water stress. In addition, the interaction of CK with other stress regulation hormones, such as ABA, auxin, and ethylene may deserve further investigation.
4.2 RESEARCH PAPER-II

Effect of cytokinin on nitrogen metabolism and yield attributes under water deficit stress condition in wheat (*Triticum aestivum* L.)
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4.2.1 Abstract

Drought, is the most significant factor restricting wheat production in the majority of agricultural fields of the world. A pot culture study was conducted to study the effect of exogenous application of cytokinin on two contrasting wheat cultivars, differing on the basis of their tolerance to drought under water deficit stress condition. The results suggested that water stress condition leads to decrease in activity of two main enzymes of nitrogen assimilation pathway that is nitrate reductase and glutamine synthetase. Cytokinin application enhanced the activity of both the enzymes across the cultivar and water regimes. Increase in level of total nitrogen and total protein contents was also found because of cytokinin in both the cultivars under two water regimes. Protease activity was found to be up regulated under water stress condition which led to degradation of protein while treated plants showed significant reduction in level of protease activity under stress condition. Yield related traits also showed significant decrease under water stress condition while cytokinin treated plants were observed with high in yield attributes and their components

*Key words:* Cytokinin, Nitrogen metabolism, BAP, Nitrate reductase, Glutamine synthetase, Protease.

4.2.2. Introduction

Drought stress is one of the most serious environmental factors that can severely limit plant growth, performance and productivity of agricultural crops and the predicted long-term effects of global warming include more frequent drought episodes in the future (Chaves and Oliveira, 2004). Development of adequate agricultural strategies to counteract this effect and minimize drought-related yield loss could be oriented towards emphasizing the urgent need to develop adaptive agricultural strategies for a changing environment. These ranges from changes in
traditional management and agronomic practices to the use of marker-assisted selection for the improvement of drought-related traits and the development of transgenic crops with enhanced tolerance of drought and improved water use efficiency that would minimize drought-related losses and ensure food production for a growing population.

Plants respond to water stress generally by synthesis of ABA, inhibition of photosynthesis and respiration, accumulation of osmotically active compounds, synthesis of protective proteins such as dehydrins and chaperones, adjusting sink/source allocation and senescence acceleration (Chaves and Oliveira, 2004). Suppression of drought-induced leaf senescence in transgenic tobacco plants, which accumulate cytokinins under stress, has been linked to enhanced expression of dehydrins and heat shock proteins and to increased drought tolerance (Rivero et al., 2007). Enhanced expression of genes coding for proteases is a common event both in senescence and under various environmental stresses (Martinez et al., 2007) which is necessary for reorganization of plant metabolism, remodeling of cell protein components, degradation of damaged or unnecessary proteins and nutrient remobilization (Feller, 2004; Grudkowska and Zagdanska, 2004). However, some experimental evidence suggests that proteases involved in drought response differ from those expressed specifically in senescence (Khanna-Chopra et al., 1999).

Senescence can be described as a nutrient remobilization process where mature leaves behave as source organs, providing both carbon and nitrogen organic molecules to the sink tissues, including young developing roots, leaves and seeds (Gan and Amasino, 1997; Roitsch and Ehness, 2000). In wheat (Triticum aestivum), 60-95% of the grain nitrogen (N) comes from the remobilization of N stored in roots and shoots before anthesis (Hirel et al., 2005), determining the grain protein concentration and the baking quality of flour. This remobilization is carried out by proteolytic enzymes that hydrolyze leaf proteins, releasing amino acids that may be transported to the ear (Vierstra, 1996). Degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) has been considered as a key component of the N-redistribution process since it constitutes the main plant reserve of N (Crafts-Brandner et al., 1998). However, the way in which the proteolysis and the export rate of amino acids are regulated is far from clear (Schwechheimer and Schwager, 2004; Barneix, 2007). Leaf senescence is controlled by many internal and external factors and can be
accelerated or delayed by alteration of these signals (Feller and Fischer, 1994). The external cues include stresses such as extreme temperatures, drought, ozone, nutrient deficiency, pathogen infection, wounding, and shading. Among these stresses, limited N availability is one of the main factors that induce senescence in crop leaves (Gan and Amasino, 1997; Crafts-Brandner et al., 1998).

Cytokinins are a group of plant growth regulators which play a role in many aspects of plant growth and development, such as cell division, photosynthesis, senescence, chloroplast development and assimilate partitioning (Binns, 1994). Despite the importance of cytokinins, this group of plant growth regulators is the least understood with respect to the mode of action (Balibrea Lara et al., 2004). The way by which physiological effects are evoked at the molecular level and, especially, how cytokinins regulate assimilate partitioning remains to be elucidated (Roitsch and Ehness, 2000). Transgenic tobacco (Nicotiana tabacum) plants expressing a cytokinin biosynthesis gene (isopentenyl transferase, ipt) under the control of the senescence-specific promoter SAG12 (PSAG12-IPT) have been used to avoid a direct interference with other aspects of normal plant development (Gan and Amasino, 1995). However, later studies have demonstrated that there are also indirect effects in these transgenic tobacco plants, and that the sink-source relation is modified, showing a preferential allocation of N to old senescing leaves and a reduced N accumulation in young leaves (Jordi et al., 2000; Cowan et al., 2005). Besides, only few reports have focused on the cytokinin-induced expression/activity of enzymes such as nitrate reductase (NR) and glutamine synthetase (Brenner et al., 2005).

Photosynthetic capacity is closely associated with leaf nitrogen (Mae, 1997; Llorens et al., 2003). The two key enzymes involved in assimilating intracellular ammonium into organic compounds are nitrate reductase (NR; EC 1.6.6.1) and glutamine synthetase (GS; EC 6.3.1.2), which also participate in photosynthesis and carbohydrate metabolism (Lam et al., 1996; Sibout and Guerrier, 1998). Although high N level enhances photosynthesis and delays senescence (Sinclair et al., 2000), generally, drought results in a decrease in leaf N content (Sinclair et al., 2000; Llorens et al., 2003), and NR and GS activities (Sibout and Guerrier, 1998; Baki et al., 2000). This is important because NR and GS can be associated with amino acid conversion (Lam et al., 1996), and amino acid composition might be altered due to drought, which could promote stress-resistance. Plant carbon and nitrogen assimilation are
coupled in plant metabolism and the limitation of photosynthesis and growth by the stress factor, such as drought might be associated with an alteration in nitrogen level and availability, aspect that has received little attention so far.

With this background, we would like to study the role of cytokinin (benzyl amino purine (BAP)), a synthetic cytokinin that has been widely used in plant physiology studies (Roitsch and Ehness, 2000), induced changes of nitrogen metabolism, its important enzymes and yield attributes under moisture deficit stress condition in wheat. Increase knowledge of the regulatory mechanisms controlling plant N economy is necessary for improving N use efficiency under water limited environment and for reducing excessive input of fertilizers, while maintaining an acceptable yield.

4.2.3. Materials and Methods

4.2.3.1. Plant material

Two wheat varieties were selected C-306 (drought tolerant) and PBW 343 (drought sensitive), as suggested by breeders and procured from Division of Genetics, Indian Agricultural Research Institute, New Delhi.

Sowing was done in 30 cm earthen pots with clay loam soil and farmyard manure in 3:1 ratio during winter season and supplied with 60, 60 and 60 kg ha\(^{-1}\) of N, P, K, respectively, in the form of urea, single super phosphate and muriate of potash at the time of sowing. Remaining 60 kg N ha\(^{-1}\) was given after 25 days of sowing.

Plants were subjected to water stress after 55 days after sowing for providing water deficit stress condition during reproductive stage. Cytokinin treatment was given by spraying benzyl amino purine (BAP) 40 µM concentration at 40 DAS and 60 DAS.

The plants were sampled and observations were taken for growth, physiological, biochemical parameters and molecular study at pre-anthesis (67 DAS); anthesis (77DAS) and post-anthesis stages (87 DAS) of the wheat plants described above. Three replications with five pots per replication were taken for each variety. Upper most fully expanded flag leaf was used for recording observation.

4.2.3.2. Relative water Content

Leaf relative water content (RWC) was estimated by recording the turgid weight of 0.5 g fresh leaf samples by keeping in water for 4 h, followed by drying in hot air oven till constant weight was achieved (Weatherley, 1950).
RWC = \([\text{Fresh wt.} – \text{Dry wt.}) / (\text{Turgid wt.} – \text{Dry wt.})]\) X 100

### 4.2.3.3. Plant Biomass

The plants were harvested at various growth stages and separated into stems and leaves then dried in an oven at 80 ºC for four hours and then at 60 ºC till constant dry weight was recorded. Dry weights were recorded and expressed as g plant\(^{-1}\).

### 4.2.3.4. Leaf area

The leaves were separated from the stem and cleaned thoroughly. All the green leaves from each replication (three plants per replication) were taken. Leaf area of flag leaf, main shoot leaves and total plant leaf area was measured using a standard leaf area meter (Model LiCOR 3100) and was expressed as cm\(^2\) plant\(^{-1}\).

### 4.2.3.5. Nitrate reductase activity (NR; EC 1.6.6.1)

Nitrate reductase activity was assayed following the method of klepper et al., (1971) as modified by Nair and Abrol (1973). Fully expanded flag leaves were harvested and immediately preserved in ice. Sampling was done at 1030 h IST on a bright and sunny day. The leaves were cleaned thoroughly and cut into small pieces of 2-3 cm each after the removal of the midrib. 0.3 gram of the cut leaf sample was taken in triplicate in ice cold infiltration medium containing: 3 ml of 0.1M Phosphate buffer (pH 7.5), 3 ml of 0.4 M potassium nitrate and 0.1 ml isopropanol. A blank was run simultaneously without any leaf tissue. The solution was infiltrated into the leaf tissues under vacuum for five minutes or till the tissues sank to the bottom of the tubes. All operations were performed on ice. These were then incubated at 33 ºC for 30 minutes in the water bath. The reaction was stopped by placing the tubes in the water bath at 70 ºC for 3-4 min.

Known volume of aliquot (0.2 ml) was taken in the fresh test tube and one ml each of sulphanilamide (1%) in 1N HCl and NEDD solution [N-(1-Naphthyl) ethylene diamine dihydrochloride solution (0.02%)] was added and colour was allowed to develop for 20 minutes. The final volume was made to 6 ml with distilled water. The absorbance was recorded at 540 nm using spectrophotometer (SPECORD 200). Zero was set using the blank without aliquot. The NR activity was calculated as the amount of nitrite formed from the standard curve of nitrite (1OD= 152.46 nmoles nitrite). The NR activity was expressed as µmoles NO\(_2^-\) formed g\(^{-1}\) dw h\(^{-1}\).

### 4.2.3.6. Glutamine synthetase (GS; EC 6.3.1.2)
The activity was assayed following the method of Mohanty and Fletcher (1980).

4.2.3.6.1 Extraction of protein

Flag leaf samples were collected in ice in the field as mentioned above for NR activity. After cleaning thoroughly the leaf samples were weighed (1g) and enzyme protein was extracted in the 6 ml ice cold extraction buffer. The extraction step was performed using chilled pestle and mortar in a tray filled with ice. Extract was centrifuged at 10,000 rpm for 20 min in a centrifuge (SIGMA 3K30). The supernatant was subsequently used for the assay of enzyme activity.

4.2.3.6.2 Assay of enzyme activity

The reaction mixture consisted of (0.1M Tris 1.25 ml, 10 μM hydroxyl amine 0.6 ml, 100 μM MgCl2 0.3 ml, 10 μM ATP 0.2 ml, enzyme extract 50 μl) except sodium glutamate, which is the substrate for the enzyme were added in the assay tubes along with 0.05 ml enzyme extract. All the procedures were done on ice. The tubes were incubated in water bath at 33 °C for 5 min. to let the solutions attain the desired temperature and then 0.6 ml of sodium glutamate (250 μM) was added to each of the tube. Blank contained all the reagents except sodium glutamate. After 15 min. of incubation reaction was stopped by adding 0.3 ml FeCl3 TCA. The brown precipitate was formed. The volume was made to 6 ml with distilled water and centrifuged at 16,000 rpm for 10 min. to remove the precipitate. The absorbance was recorded at 540 nm. The GS activity was calculated from the standard curve of γ-glutamyl hydroxamate as the amount of ferric γ-glutamyl hydroxamate formed (1OD=3.72 μmoles). The GS activity was expressed μmoleses γ-glutamyl hydroxamate formed g⁻¹ dw h⁻¹

4.2.3.7. Total soluble protein

4.2.3.7.1 Extraction of protein

The flag leaf samples were harvested. These were immediately wiped thoroughly and plunged in liquid nitrogen. The leaf tissue (100 mg) was powdered in liquid nitrogen and added to prewarmed extraction buffer (1:5). These were centrifuged at 16,000 rpm for 10 min. The supernatant was subsequently used for the assay of protein content.

4.2.3.7.2 Estimation of protein

The reaction mixture consisted of (0.1M Tris 1.25 ml, 10 μM hydroxyl amine 0.6 ml, 100 μM MgCl2 0.3 ml, 10 μM ATP 0.2 ml, enzyme extract 50 μl) except sodium glutamate, which is the substrate for the enzyme were added in the assay tubes along with 0.05 ml enzyme extract. All the procedures were done on ice. The tubes were incubated in water bath at 33 °C for 5 min. to let the solutions attain the desired temperature and then 0.6 ml of sodium glutamate (250 μM) was added to each of the tube. Blank contained all the reagents except sodium glutamate. After 15 min. of incubation reaction was stopped by adding 0.3 ml FeCl3 TCA. The brown precipitate was formed. The volume was made to 6 ml with distilled water and centrifuged at 16,000 rpm for 10 min. to remove the precipitate. The absorbance was recorded at 540 nm. The GS activity was calculated from the standard curve of γ-glutamyl hydroxamate as the amount of ferric γ-glutamyl hydroxamate formed (1OD=3.72 μmoles). The GS activity was expressed μmoleses γ-glutamyl hydroxamate formed g⁻¹ dw h⁻¹

4.2.3.7. Total soluble protein

4.2.3.7.1 Extraction of protein

The flag leaf samples were harvested. These were immediately wiped thoroughly and plunged in liquid nitrogen. The leaf tissue (100 mg) was powdered in liquid nitrogen and added to prewarmed extraction buffer (1:5). These were centrifuged at 16,000 rpm for 10 min. The supernatant was subsequently used for the assay of protein content.
4.2.3.8. Total protease activity

4.2.3.8.1 Extraction of protease

1.0 gm of leaf tissue was taken and homogenize the tissue sample. 10 ml of extraction buffer was added into the homogenate. If homogenate is crude then pass through 4 layers of muslin cloth. Centrifuge the filtrate at 25,000 g for 20 min at 4 °C. Take out the supernatant and use for the enzyme assay. To the 1.0 ml of the enzyme extract added 1.0 ml of 1 % casein in 0.2 M diphosphate buffer (pH=7) and 1.0 ml of 0.2 M of phosphate buffer (pH=7). Incubate the solution at 40 °C for 90 min. Stop the reaction after the end of incubation period by adding 1.0 ml of 20 % TCA. Keep the reaction vials in refrigerator for 30 min. Centrifuge the reaction mixture at 3000 g for 15 min. Take out the supernatant and use for the amino acid analysis. A control was also run along with sample.

4.2.3.8.2 Estimation of protease activity

0.5 ml of supernatant was added from the reaction mixture. Add 0.5 ml of 55% glycerol, 0.5 ml of ninhydrin solution. Make the volume to 6.0 ml by adding double distilled water. Boil for 20 min. Record the spectrophotometer reading at 570 nm. To calculate amount of amino acid formed due to protease activity, a standard curve was prepared by using 0.1 to 1.0 µM of glycine. The protease activity expressed as µM of amino acid produced per hour per gram F.W or per hour per gram dry weight.

4.2.3.9. Determination of total reduced nitrogen

100 g of finely powdered homogenous sample was weighed and put in a clear and dry digestion tube. To this 5.5 g of K$_2$SO$_4$ : CuSO$_4$ mixture was added and then 10 ml H$_2$SO$_4$ was also added. The sample was digested at 600 °C for 15 min and then at 450 °C for 1 h. When the samples were colourless tubes were taken out from the digestion unit.

100 ml conical flask containing 4% solution of boric acid (10 ml) was taken. To this one or two drop of indicator solution was added which gave the pink colour. This solution was used to trap the ammonia liberated during distillation. For distilling the sample the digestion tube was placed in distillation unit and 50 ml water and 70 ml NaOH (40%) was added. After steam distillation, colour of the indicator dye changed to green as (NH$_4$)$_2$ SO$_4$ is trapped by boric acid. This was titrated against 0.01 N HCl until the pink colour
reappeared. Each digestion set had two control tubes. N per cent in the tissues and the N content per plant was calculated using the formula

\[ \text{Weight of sample} = 0.5 \text{ g} \]

\[ \text{Normality of HCl} = 0.1 \text{ N HCl} \]

\[ \text{Titration value (T.V.)} = \text{Sample titration value - Blank titration value} \]

\[ N = \frac{T.V \times \text{Normality of HCl} \times 1.4}{\text{wt. of oven dry sample (g)}} \]

### 4.2.3.10. Total protein content

The N% estimated in the shoot/leaf samples from different treatments were multiplied by factor 6.25 to get the total (crude) protein content (%).

### 4.2.3.11. Yield components

Following yield components were recorded at the time of final harvest number of tillers; number of grains per ear, grain yield per plant, 1000-grain weight (g)

### 4.2.3.12. Harvest Index

Plants were collected at the time of the harvest. Grains were separated and weighed (economic yield). The whole plant dry weight was measured as biological yield. The harvest index was calculated as the ratio of the economic yield to biological yield and was expressed as percentage (Gardner et al. 1985).

\[ HI = \left( \frac{\text{Economic yield}}{\text{Biological Yield}} \right) \times 100 \]

### 4.2.3.13 Statistical analyses

The data was analysed statistically using 3 factorial CRD (Biochemical analysis and growth parameters) and 2 factorial CRD (Yield attributes) design and CD at 5% and ANOVA was calculated. The analysis was done using OPSTAT programme available online on CCS, HISAR web site.

### 4.2.4. Results

#### 4.2.4.1. Total biomass
Total biomass (g plant\(^{-1}\)) was measured at three different developmental stages of wheat i.e., pre-anthesis, anthesis and post-anthesis (Table 4.2.4.1; Fig 4.2.4.1). Under control condition total biomass value is lowest at pre-anthesis stage (8.6) and afterwards increases at anthesis (23.06) but it again reduced at post-anthesis stage (22.64). Similarly, control plants treated with cytokinin lowest mean total biomass at pre-anthesis (9.0) and followed the trend of increase in mean total biomass at anthesis (25.8) and reduced afterwards post-anthesis (25.46) as in control plants without cytokinin treatment. Cytokinin treatment enhanced the total biomass in both wheat cultivar in all the three developmental stages under control condition. However, the mean total biomass was declined by 44 % at pre-anthesis stage under water deficit stress in comparison to control wheat plants. While, water deficit plants with cytokinin showed increase in mean total biomass by 20% in comparison to water deficit plants without cytokinin. Similar trend were observed for cytokinin enhanced total biomass under water deficit stress condition at anthesis as well as post anthesis stages.

Cultivar differences on total biomass were observed more under water deficit stress as compared to the control wheat plants affected by cytokinin. Control wheat plants of cultivar C-306 showed enhancement of total biomass by 8% treated by cytokinin as compared to 20% in water deficit plants treated with cytokinin at pre-anthesis stage. While, in PBW-343 it was 11% and 20%, respectively. The sensitive cultivar (PBW-343) was more responsive to cytokinin treatment as compared to tolerant cultivar (C-306) under water stress condition.

4.2.4.2. Total leaf area

Leaf area (cm\(^2\) plant\(^{-1}\)) was measured at three different developmental stages of wheat (Table 4.2.4.2; Fig 4.2.4.2). Under control condition total leaf area value was lowest at pre-anthesis stage (435.7) and afterwards increase at anthesis (619.7) and later decline at post anthesis stage (552.9). Similarly, control plants treated with cytokinin also showed highest mean leaf area at pre-anthesis (568.9) and followed the similar trend of increase in mean leaf area at anthesis (716.2) followed by decrease at post-anthesis (656.6) as in control plants without cytokinin treatment. However, the mean leaf area was declined by 50% at pre-anthesis stage under water deficit stress in comparison to control wheat plants. While, water deficit plants with cytokinin increased mean leaf area by almost 100% in comparison to water deficit plants
without cytokinin. Similar trend were observed of cytokinin enhanced leaf area under water deficit stress condition at anthesis as well as post anthesis stages.

Cultivar differences on leaf area were observed more under water deficit stress as compared to the control wheat plants affected by cytokinin. Control wheat plants of cultivar C-306 showed enhancement of leaf area of around 19\% treated by cytokinin as compared to 103\% in water deficit plants with cytokinin at pre-anthesis stage. While, in PBW-343 it was 41\% and 33\%, respectively.

4.2.4.3. Nitrate reductase activity

Nitrate reductase activity (\(\mu\)moles NO\(_2^–\) formed g\(^{-1}\)dw h\(^{-1}\)) is substrate inducible, highly sensitive to water stress and also induced by cytokinin was studied to know nitrogen status of the plants (Table 4.2.4.3; Fig 4.2.4.3). Under control condition mean NR activity of wheat plants was highest at pre-anthesis stage (19.25) and afterwards reduced from anthesis (15.67) to post anthesis stage (10.44). Similarly, control plants treated with cytokinin showed highest mean NR activity at pre-anthesis (22.48) and followed the similar trend of decline in NR activity from anthesis (18.29) to post-anthesis (13.31) as in control plants without cytokinin treatment. Cytokinin treatment enhanced the NR activity in both wheat cultivar at all the three developmental stages under control condition. However, water deficit stress condition at pre-anthesis stage leads to drop of 52\% in mean NR activity in comparison to control wheat plants. While, under water deficit stress condition plants treated with cytokinin showed enhancement of 42\% in mean NR activity in comparison to water deficit plants without cytokinin. Similar trend was observed for cytokinin enhanced NR activity under water deficit stress condition at anthesis as well as post anthesis stage in both cultivars of wheat.

Cultivar differences were observed more under water deficit stress as compared to the control wheat plants affected by cytokinin. Control wheat plant cultivar of C-306 showed enhancement of NR activity of around 25\% treated by cytokinin as compared to 49\% in water deficit plants treated with cytokinin at pre-anthesis stages. While, in PBW-343 it was 9\% and 34\%, respectively. Sensitive cultivar (PBW-343) was found more responsive to cytokinin treatment as compared to tolerant cultivar (C-306) under water stress condition.

4.2.4.4. Glutamine synthetase activity
Glutamine synthetase (GS) activity (µmoles γ-glutamyl hydroxymate g⁻¹ dw h⁻¹) was estimated to assess the nitrogen status of plants affected by cytokinin in two cultivars differing in their tolerance to water deficit stress at reproductive stages of wheat (Table 4.2.4.; Fig 4.2.4). Under control condition wheat plants mean GS activity at pre-anthesis stage was 7.66 and it increased up to anthesis to 12.56 but at post anthesis stage GS activity was again reduced to 6.33. Similarly, control plants treated with cytokinin showed mean GS activity of 8.24 at pre-anthesis and followed the similar trend of increase in mean GS activity at anthesis to 15.51 and it again reduced to 7.78 at post-anthesis as in control plants without cytokinin treatment. Cytokinin treatment enhanced the GS activity in both wheat cultivars at all the three developmental stages under control condition. However, the mean GS activity was declined by 22% at pre-anthesis stage under water deficit stress condition in comparison to control wheat plants. While, under insufficient water condition plants treated with cytokinin showed increase in GS activity by 24% in comparison to water deficit plants without cytokinin. Similar trend was observed for cytokinin enhanced GS activity under water deficit stress condition at anthesis as well as post anthesis in both the cultivars of wheat.

Cultivar differences were highly significant under water deficit stress as compared to the control wheat plants affected by cytokinin. Control wheat plants cultivar of C-306 showed enhancement of GS activity only by 10% treated by cytokinin as compared to 26% in water deficit plants treated with cytokinin at pre-anthesis stages. While, in PBW-343 it was 13% and 23%, respectively.

4.2.4.5. Total soluble protein

Total soluble protein (mg g⁻¹ dw) was estimated to assess the nitrogen status of plants under different treatment condition at three different developmental stages of wheat (Table 4.2.5; Fig 4.2.5). Under control condition wheat plants mean total soluble protein at pre-anthesis stage was 8.24 and it increased at anthesis to 9.56 but during post anthesis stage total soluble protein was again reduced to 6.77. Similarly, for control plants treated with cytokinin mean total soluble protein at pre-anthesis stage was 10.1 and followed the similar trend of increase in mean total soluble protein at anthesis to 11.39 and at post-anthesis it again reduced to 8.10 as in control plants without cytokinin treatment. Cytokinin treatment enhanced the total soluble protein in both wheat cultivars at all three developmental stages under control condition.
However, the mean total soluble protein was declined by 16% at pre-anthesis stage under water deficit stress condition in comparison to control wheat plants. While, water deficit plants with cytokinin increased the mean total soluble protein by 13% in comparison to water deficit plants without cytokinin. Similar trend was observed for cytokinin enhanced total soluble protein under water deficit stress condition at anthesis as well as post anthesis stages in both the cultivars of wheat studied.

Cultivar differences were also observed under water deficit stress. Control wheat plants of cultivar C-306 showed enhancement of total soluble protein by 22% treated by cytokinin as compared to 13% increase in water deficit plants treated with cytokinin at pre-anthesis stages. While, in PBW-343 it was 23% and 15%, respectively.

4.2.4.6. Total protease activity

Total protease activity (µmoles g⁻¹ dw h⁻¹) was also measured at three different developmental stages of wheat (Table 4.2.6; Fig 4.2.6). Under control condition wheat plants total protease activity value was lowest at pre-anthesis stage (612.34) and afterwards increase from anthesis (654.99) to post anthesis stage (817.41). Similarly, control plants treated with cytokinin also showed lowest mean total protease activity at pre-anthesis (533.39) and followed the trend of increase in mean total protease activity from anthesis (610.52) to post-anthesis (811.18) as in control plants without cytokinin treatment. Cytokinin treatment reduced the total protease activity in both wheat cultivar at all three developmental stages under control condition. However, the mean total protease activity was enhanced by 15% at pre-anthesis stage under water deficit stress in comparison to control wheat plants. While, water deficit plants with cytokinin showed decrease in total protease activity by 20% in comparison to water deficit plants without cytokinin. Similar trend was observed for cytokinin induced reduction in total protease activity under water deficit stress condition at anthesis as well as post anthesis stages in comparison to water deficit plants without cytokinin in both the cultivar of wheat.

Cultivar differences were observed under water deficit stress. Control wheat plants of cultivar C-306 showed reduction in total protease activity of around 8% treated by cytokinin as compared to 9% in water deficit plants treated with cytokinin at pre-anthesis stages. While, in PBW-343 it was 18% and 6%, respectively.

4.2.4.7. Total nitrogen content
Total nitrogen content (%) was measured to study effect of two water regimes at three different developmental stages of wheat (Table 4.2.7; Fig 4.2.7). Under control condition wheat plants mean total nitrogen content at pre-anthesis stage was 2.02 it increased further at anthesis (2.69) but reduction in total nitrogen content was observed during post anthesis stage (1.37). Similarly, control plants treated with cytokinin also showed mean total nitrogen content at pre-anthesis as 2.61 and followed the similar trend of increase in total nitrogen content at anthesis (3.10) and then again reduced at post anthesis stage (1.8) in control plants without cytokinin treatment. However, the mean total nitrogen was declined by 35% at pre-anthesis stage under water stress in comparison to control wheat plants. While, water deficit plants with cytokinin showed increase in the total nitrogen by 36% in comparison to water deficit plants without cytokinin. Similar trend was observed of cytokinin enhanced total nitrogen under water deficit stress condition at anthesis as well as post anthesis stages in both the cultivars of wheat.

Significant cultivar differences were also observed under water deficit stress. Control wheat plants of cultivar C-306 showed enhancement of total nitrogen by 37% treated by cytokinin as compared to 60% in water deficit plants treated with cytokinin at pre-anthesis stages. While, in PBW-343 it was 22% and 70%, respectively.

4.2.4.8. Total protein

Total protein content (%) was also measured at three different developmental stages of wheat (Table 4.2.8; Fig 4.2.8). Under control condition wheat plants, mean total protein content at pre-anthesis stage was 12.62, which increased at anthesis (16.80) but reduced later in starch during post anthesis stage (8.54). Similarly, in control plants treated with cytokinin also showed mean total nitrogen content at pre-anthesis of 19.35 and followed the similar trend of control plants without cytokinin treatment. However, the mean total protein content was declined by 28% at pre-anthesis stage under water stress comparison to control wheat plants. While, water deficit plants with cytokinin showed increase in total protein content by 30% in comparison to water deficit plants without cytokinin. Similar trend were observed of cytokinin enhanced total protein content under water deficit stress condition at anthesis as well as post anthesis stages.

Cultivar differences were also observed under water deficit stress. Control wheat plants cultivar of C-306 showed enhancement in total protein content of around
37% when treated by cytokinin as compared to 23% in water deficit plants treated with cytokinin at pre-anthesis stage. While, in PBW-343 it was 16% and 60%, respectively.

4.2.4.9. Number of tillers

Numbers of tillers were recorded at the time of harvest in wheat plants (Table 4.2.9; Fig. 4.2.9). Under control condition treated plants showed the highest mean number of tillers (11.17) as compared to control plant without cytokinin (10.16). While, there was decline of around 16% in number of tiller (8.5) under water deficit stressed plants as compared to control plants without cytokinin. In cytokinin treated plants 11% increased in number of tiller (9.5) were observed during water deficit conditions.

There was 16% increased in number of tiller in treated control plants while it was 11% increased in water deficit condition with cytokinin in tolerant C-306 cultivar of wheat. Similarly, in PBW-343 it was 3% and 12%, respectively.

4.2.4.10. Grain yield per plant

Grain yield per plant (gm) were also recorded at the time of harvest in wheat plants (Table 4.2.10; Fig 4.2.10). Under control condition treated plants showed the highest mean grain yield per plant (13.48) as compared to control plants without cytokinin (12.48). However, under water deficit condition there was fall of around 42% in grain yield per plant compared to control plants without cytokinin. In cytokinin treated plants 8% increased in grain yield per plant were observed during water deficit conditions.

There was 12% increased in grain yield per plant in treated control plants while it was 46% increased in water deficit condition with cytokinin in tolerant C-306 cultivar of wheat. Similarly, in PBW-343 it was 4% and 49%, respectively.

4.2.4.11. Grain numbers per panicle

Grain numbers per panicle were also recorded at the time of harvest in wheat plants (Table 4.2.11; Fig 4.2.11). Under control condition, treated plant showed same number of grain per panicle as in control plant without cytokinin (37). While, there was decrease of around 25% in grain number per panicle (28) under water deficit plants as compared to control plants without cytokinin. In cytokinin treated plants 21% increased in grain number per panicle (33) were observed during water deficit conditions.
There was no increased in grain number per panicle in treated control plants while there was 20% increased in water deficit condition with cytokinin in tolerant C-306 cultivar of wheat. While, in PBW-343 it was 2% and 22%, respectively.

4.2.4.12. 1000 Grain weight

1000 Grain weight (gm) was also recorded at the time of harvest in wheat plants (Table 4.2.12; Fig 4.2.12). Under control condition, treated plants showed highest 1000 grain weight (53.3) as compared to control plant without cytokinin (49.78). While, there was fall of around 22% in 1000 grain weight (38.73) under water deficit plants as compared to control plants without cytokinin. In cytokinin treated plants 17% increased in 1000 grain weight (45.57) were observed during water deficit conditions.

There was 12% increased in 1000 grain weight in treated control plants while it was 14% increased in water deficit condition with cytokinin in tolerant C-306 cultivar of wheat. Similarly, in PBW-343 it was 2% and 21%, respectively.

4.2.4.13. Harvest Index

Harvest index (HI) was also recorded to assess the partitioning efficiency of wheat plants at the time of harvest (Table 4.2.13; Fig 4.2.13). Under control condition, treated plants showed highest mean HI (53.1) as compared to control plants without cytokinin (52.43). While, there was decline of around 10% in mean HI (45.13) under water deficit plants as compared to control plants without cytokinin. In cytokinin treated plants 11% increased in mean HI were observed during water deficit conditions.

There was 7% increase in HI in treated control plants while it was 9% increase in water deficit condition with cytokinin in tolerant C-306 cultivar of wheat. While, in PBW-343 it was 3% and 12%, respectively.

4.2.5. Discussion

Drought is the most important factor limiting crop production in many parts of the world, especially in arid and semiarid regions (Boyer, 1982; Ewert et al., 2002; Shah and Paulsen, 2003), yet more land can become productive by using partial irrigation at strategic times during the growing season (Ghahraman and Sepaskhah, 1997). In many experiments, it has been found that wheat-leaf N concentration decreases with increasing soil drought, but modest soil drought is considered likely to enhance N mobilization from the leaf to the grain (Sinclair et al., 2000). However, the
total N-translocation amount decreases under water stress, because early leaf senescence enhanced by drought can lead to a decrease in leaf N levels (Seligman and Sinclair, 1995). The present results demonstrate that water deficit stress (RWC: Ca 70%) can decrease grain yield and leaf N concentration through adversely affecting N metabolism with early flag leaf senescence in both the tolerant and sensitive cultivars of wheat studied.

Leaf is an important N source for the developing grain after flowering (Guitman et al., 1991; Barneix et al., 1992), and photosynthetic rate and Rubisco activity increase with increasing leaf N concentration (Evans, 1983; Llorens et al., 2003; Xu and Zhou, 2005). Metabolic imbalance caused by water stress results in amino acid accumulation, decreased ATP and RuBP synthesis, and altered protein synthesis (Lawlor and Cornic, 2002). Drought can also have adverse effects on nitrate reductase (NR) activity (Hsiao, 1973; Huber and Kaiser, 1996; Baki et al., 2000; El-Komy et al., 2003; Burman et al., 2004), which is involved in carbohydrate metabolism (Solomonson and Barber, 1990). For example, high carbohydrate assimilation can increase nitrate uptake and nitrate reductase activity when the plant is growing on nitrate (Matt et al., 2001). Nitrate assimilation requires eight electrons to reduce nitrate to ammonia, which is mainly catalyzed by NR, and is thus the highest energy-consuming reaction of metabolism after CO₂ assimilation. Consequently, NR might play an important role in maintaining the balance between C and N (Champigny, 1995). A decrease in NR activity was positively correlated with a decrease in soluble-protein concentration and photosynthetic capacity under water stress (El-Komy et al., 2003; Burman et al., 2004).

Since NR is the first enzyme of the nitrate assimilation pathway, the increased NR activity observed in the BAP-treated plants indicates that BAP also modifies the N assimilatory capacity of plants. NR is transcriptionally induced by nitrate and is repressed by glutamate, cysteine, asparagines and malate (Masclaux et al., 2001; Stitt et al., 2002). Our finding strongly supports that NR is also induced by cytokinins.

In addition, glutamate is the main precursor of proline, which is considered to be involved in osmoregulation of plants subjected to drought (Venekamp, 1989). Enhanced plant growth has been closely correlated with higher nitrate levels and the higher activities of NR and glutamine synthetase (GS) (Lam et al., 1996; Chen et al., 2003). Moreover, in detached rice leaves, water stress decreases chlorophyll and
protein concentrations, and increases ammonia concentration due to the decreased GS activity, which in turn results in an enhancement of chlorophyll and protein loss (Tsai et al., 2003).

Glutamine synthetase activity is closely associated with protein hydrolysis (Tsai et al., 2003) and the ability of adapting to drought (Bianchi et al., 2002). In the present study, water deficit stress reduced the activities of the two enzymes (NR and GS) involved in N assimilation and increased the activity of proteases. Furthermore, the activities of NR and GS were positively and significantly correlated with the net photosynthetic rates, although that of proteases was negatively and significantly correlated with photosynthetic rates. Therefore, it was suggested that the N metabolism regulation could play an important role in the photosynthetic adaptation of wheat plants to water stress. In wheat seedlings, moderate soil-water stress can enhance antioxidant defense, but severe water stress or waterlogging causes loss of catalase activity (Keles and Oncel, 2002). In conclusion, the reduction in the activities of the two enzymes (NR and GS) involved in N assimilation and enhancement in the activities of proteases are involved in the decline of photosynthetic capacity, plant growth, and grain yield caused by water deficit stress.

Nitrogen concentration changes in wheat is associated with genotype and cultural practices (Papakosta and Gagianas, 1991). In the present study, leaf protein concentration ranged from 7% to 20%, and was higher for PBW-343 than C-306 and both the cultivars showed higher leaf protein percentage in cytokinin (BAP) treated plants, supporting that concept that leaf protein concentration mainly depends on genotype. However, changes in leaf N concentration and key enzyme activities for the two varieties were complex. This could have resulted from higher responses of the cytokinin treatment and different genotypes to drought.

Proteases play key roles in plants and contribute to the processing, maturation or destruction of specific sets of proteins in response to developmental or environmental factors (van der Hoorn, 2008). Reduced levels of protease activity in leaves of the drought resistant cultivar (C-306) suggested that suppressed expression of specific protease activity, accompanied only by slight changes in proteolytic activity, could be regarded as a prerequisite or at least as a marker for drought tolerance. This proteolytic activity was further declined by the treatment of cytokinins (BAP) across the cultivars and water regimes.
The increase in total protein concentration after BAP supply may be a consequence either of the redirection of the not-exported amino acids to the synthesis of protein or of an inhibition of protein degradation. In the previous work, we observed that the mRNA levels of the Rubisco smaller subunits were kept high by the addition of BAP in control as well as stressed plants, thus suggesting a continued synthesis of the enzyme. These results indicate that BAP is acting by inhibiting protein degradation and increasing the synthesis of proteins in stressed plants. Similarly, demonstrated that BAP promotes synthesis and inhibits degradation of proteins in tobacco leaf disk. Also, Ananieva and Ananiev (1999) showed that BAP stimulates protein and RNA synthesis in cotyledons of zucchini.

In the present study the results showed significant improvement in grain yield and harvest index of both the cultivars treated with cytokinins in control as well as stressed plants. This reflected towards the enhancement of total biomass, panicle number and leaf area of the treated plants. Similar kind of observation were founded (Shah, 2011) in cytokinin treated black cumin plants where they observed appreciable improvement in the yield parameters. They showed enhancement in number of capsules and seed yield because of sufficient availability of nutrient facilitated ample vegetative growth in treated plants, thus resulting in an increase in the number and size of reproductive sink. This is because of higher values for nitrate reductase, net photosynthetic rate, leaf protein content and dry mass, as compared to the control plants. Similarly, capsule number and seed yield per plant were elevated by 41 and 43% over the untreated control at harvest. The effect of kinetin on economic yield, though relatively mild, may be ascribed to the increased opportunity for formation of new buds (Bruinsma, 1977), and successful reversal of flower and fruit abscission (Nagel et al., 2001).

5 DISCUSSION

Comparative characterization of cultivars with different drought tolerance could be a useful approach to obtain information about drought response at biochemical level, assuming that higher drought tolerance is based largely on mechanisms at cellular level. Inhibition of photosynthesis is a typical response to
drought (Chaves and Oliveira, 2004) and may lead to nitrogen starvation. The reduced mobility of nitrogen in dry soil and reduced uptake of Nitrogen (N) by the roots cannot be ruled out especially under severe stress conditions. Nitrogen starvation under drought stress could result in increased proteolytic activity. This could compensate the effects of inhibited de novo synthesis of amino acids with an accelerated degradation of cell proteins and amino acid recycling (Feller, 2004). This process could resemble but may not be necessarily linked to natural senescence. Another possible function of proteolysis under severe drought could be to eliminate stress-damaged proteins.

Drought represents an excellent example of environmental stress that occurs in the field. In the present study, we observed that the adverse effects of drought on plant growth, leaf water status, CO₂ assimilation rate, photochemical efficiency, nitrogen assimilation level, and the cell membrane (as indicated by MSI) were significant, especially under drought. However, the treated (BAP) plants exhibited better tolerance to the water stress, as demonstrated by the maintenance of higher Fv/Fm, chlorophyll content, RWC, photosynthesis rate, stomatal conductance, and nitrogen content in flag leaves. The transgenic line has previously been reported to exhibit superior drought tolerance compared with the WT when plants were subjected to soil drying by withholding irrigation (Merewitz et al., 2010). The results, in combination with our study, demonstrated that expression of the ipt gene or external spray of cytokinins (BAP) in wheat crop was effective in improving plant tolerance to water stress.

In general, resistant cultivars performed higher photosynthesis, stomatal conductance and other photosynthetic attributes than susceptible ones under stress condition. As observed for grain yield, water stress caused a significant reduction in Pn. Similar results have been reported by other investigators (Ratnayaka and Kincaid, 2005). This appears to be a possible physiological mechanism by which drought can affect growth and productivity of crops such as wheat (Lawlor, 1995; Lawlor et al., 2002). However, addition of cytokinins could able to revive the photosynthesis and its components in both the cultivars and water regimes as also reported recently by Rivero et al., (2007) in ipt overexpressed tobacco transgenic plants. It has been hypothesized that genotype which keep their stomata open under stress condition while maintaining adequate leaf RWC can be considered as suitable for dry region
(Blum et al., 1981). In the present study, resistant cultivar had higher values of stomatal conductance that leads to higher value of transpiration and RWC indicating their greater ability to water uptake from the soil compared to susceptible ones.

Senescence of flag leaves in two wheat cultivars was characterized by chlorophyll loss, decreases in the chlorophyll a/b ratio, photosynthetic rate, stomatal conductance and photochemical efficiency. However, these photochemical and biochemical parameters did not show similar changing pattern and senescing behavior from emergence through senescence of flag leaves and there were some differences in these parameters between these two cultivars, which have different senescent appearance. The well-known phenomenon in senescing leaves is the loss of chlorophyll content. The degree of chlorophyll loss of the wheat cultivars is strongly associated with light-saturated photosynthetic rate during grain filling, as well as being associated with differences in final grain yield (Reynolds et al., 2000). In this study, the chlorophyll content in C-306 was higher than in PBW-343 during the flag leaf senescence, showing that process of leaf senescence in C-306 was delayed more by cytokinins in comparison to PBW-343. In combination with the longer duration of having a higher content of chlorophyll at the late senescence, these findings may be partly responsible for the higher grain yield and harvest index in C-306 than in PBW-343.

One of the strongest evidence of the useful effect of cytokinin spraying is the delay of leaf yellowing. The BAP treatment retained most of total chlorophyll in relation to the value of green presenescent leaves, while the control plants without cytokinins preserved only little chlorophyll from pre-anthesis to post-anthesis stages. In the control, the amount of chlorophyll a underwent a greater impact than that of chlorophyll b since the chlorophyll a: chlorophyll b ratio diminished. In contrast, the leaves treated with BAP upheld values at higher rates, that is, during the delay of pigment degradation BAP maintained the proportion of chlorophyll a and chlorophyll b, a similar performance to that exhibited by the green leaf.

In the present study it was found that BAP induced the carotenoid synthesis during all the three stages of wheat studied and significantly higher than in the control. A similar trend was reported by Zavaleta-Mancera et al., (2007) in fragments of wheat leaves incubated 6 d in BAP in the dark. On the contrary, under greenhouse conditions with natural light, the accessory pigments such as carotenoids increased
during wheat natural senescence (Martinez-Gutierrez et al., 2008). In this same species, with intense light, accessory pigments increased their content (Lu et al., 2001). Cytokinins are likely to increase the synthesis of accessory pigments to protect the reaction centers of light and oxygen (Vlekova et al., 2006).

Photosynthesis rate decline was significant at post-anthesis in both the cultivars at two water regimes, in control and stressed plants; in contrast, the BAP treatment delayed the degradation of photosynthetic proteins. Similar results were obtained in tobacco plants (Nicotiana tabacum L.) transformed with the ipt gene encoding the enzyme isopentenylationtransferase (IPT), under the control of the promoter of a specific gene of senescence, SAG 12 (Jordi et al., 2000). In addition, BAP induced the accumulation of total sugars and starch at anthesis as well as at post-anthesis stages. The accumulation of these carbohydrates could be explained by the effect of cytokinins in the maintenance of CO₂ fixation during wheat leaf senescence (Monakhova and Chernyadev, 2007; Martinez-Gutierrez et al., 2008). The BAP external spraying promoted the accumulation of sugars. In this respect, Wingler et al. (1998) and Jordi et al. (2000) assert that an increment of sugars is required to allow the delay of senescence in transgenic tobacco plants (PSAG12-IPT). Cytokinins regulate the expression of certain genes involved in the distribution of assimilates and in the regulation of the source-sink, like the expression of the extracellular invertase and hexose carriers that increase the use of carbohydrates to impede the start of catabolic processes (Balibrera-Lara et al., 2004; Guivarch et al., 2005).

The drought resistant cultivar C-306 had a higher grain yield than PBW-343 under drought, and the reduction in grain yield under drought was smaller in C-306 than in PBW-343. This apparent drought resistance of C-306 was associated with the dry matter accumulation during grain filling, resulting from the continued increase in total shoot dry matter between anthesis and maturity. On the contrary, the drought sensitive PBW-343 did not show any increase in total shoot dry matter between anthesis and maturity under drought, leading to a lower grain yield. While, in both the cultivars cytokinin could able to enhance the grain yield in both control as well as moisture stressed plants, but in stressed plants the enhancement was significantly higher in comparison to control plants. When assimilates available from post-anthesis photosynthesis satisfy the requirements for grain filling in wheat, a high grain yield may be achieved (Zhang et al., 1998, 2006).
Leaf is an important N source for the developing grain after flowering (Guitman et al., 1991; Barneix et al., 1992), and photosynthetic rate and Rubisco activity increase with increasing leaf N concentration (Evans, 1983; Llorens et al., 2003; Xu and Zhou, 2005). Metabolic imbalance caused by water stress results in amino acid accumulation, decreased ATP and RuBP synthesis, and altered protein synthesis (Lawlor and Cornic, 2002). Drought can also have adverse effects on nitrate reductase (NR) activity (Hsiao, 1973; Huber and Kaiser, 1996; Baki et al., 2000; El-Komy et al., 2003; Burman et al., 2004), which is involved in carbohydrate metabolism (Solomonson and Barber, 1990). For example, high carbohydrate assimilation can increase nitrate uptake and nitrate reductase activity when the plant is growing on nitrate (Matt et al. 2001). Nitrate assimilation requires eight electrons to reduce nitrate to ammonia, which is mainly catalyzed by NR, and is thus the highest energy-consuming reaction of metabolism after CO₂ assimilation. Consequently, NR might play an important role in maintaining the balance between C and N (Champigny, 1995). A decrease in NR activity was positively correlated with a decrease in soluble-protein concentration and photosynthetic capacity under water stress (El-Komy et al., 2003; Burman et al., 2004). Glutamine synthetase activity is closely associated with protein hydrolysis (Tsai et al., 2003) and the ability of adapting to drought (Bianchi et al., 2002). In the present study, water deficit stress reduced the activities of the two enzymes (NR and GS) involved in N assimilation and increased the activity of proteases. Furthermore, the activities of NR and GS were positively and significantly correlated with the net photosynthetic rates, although that of proteases was negatively and significantly correlated with photosynthetic rates. Therefore, it was suggested that the N metabolism regulation could play an important role in the photosynthetic adaptation of wheat plants to water stress. In wheat seedlings, moderate soil-water stress can enhance antioxidant defense, but severe water stress or water logging causes loss of catalase activity (Keles and Oncel, 2002).

Nitrogen-concentration change in wheat is associated with genotype and cultural practices (Papakosta and Gagianas, 1991). In the present study, leaf protein concentration ranged from 7% to 20%, and was higher for PBW-343 than C-306 and both the cultivars showed higher leaf protein percentage in cytokinin (BAP) treated plants, supporting that concept that leaf protein concentration mainly depends on genotype. However, changes in leaf N concentration and key enzyme activities for the
two varieties were complex. This could have resulted from higher responses of the cytokinin treatment and different genotypes to drought. Reduced levels of protease activity in leaves of the drought resistant cultivar (C-306) suggested that suppressed expression of specific protease activity, accompanied only by slight changes in proteolytic activity, could be regarded as a prerequisite or at least as a marker for drought tolerance. This proteolytic activity was further declined by the treatment of cytokinins (BAP) across the cultivars and water regimes.

The increase in total protein concentration after BAP supply may be a consequence either of the redirection of the not-exported amino acids to the synthesis of protein or of an inhibition of protein degradation. In the previous work, we observed that the mRNA levels of the Rubisco smaller subunits were kept high by the addition of BAP in control as well as stressed plants, thus suggesting a continued synthesis of the enzyme. These results indicate that BAP is acting by inhibiting protein degradation and increasing the synthesis of proteins in stressed plants. Similarly, demonstrated that BAP promotes synthesis and inhibits degradation of proteins in tobacco leaf disk. Also, Ananieva and Ananiev (1999) showed that BAP stimulates protein and RNA synthesis in cotyledons of zucchini.

In the present study the results showed significant improvement in grain yield and harvest index of both the cultivars treated with cytokinins in control as well as stressed plants. This reflected towards the enhancement of total biomass, panicle number and leaf area of the treated plants. Similar kind of observation were founded (Shah, 2011) in cytokinin treated black cumin plants where he shown appreciable improvement of the yield parameters.

Hence, these findings suggest the role of cytokinin at various stages of plant development in carbon and nitrogen metabolism. Cytokinin plays a significant role in plants at physiological, biochemical and molecular level. Cytokinin treatment was more effective under water stress condition. Sensitive cultivar (PBW-343) showed better response to cytokinin treatment as compared to moisture deficit stress tolerant cultivar (C-306). We concluded with the help of the present study by accumulating many evidences that it may be possible to enhance drought tolerance by delaying drought-induced leaf senescence through the synthesis of cytokinins.
5 DISCUSSION

Comparative characterization of cultivars with different drought tolerance could be a useful approach to obtain information about drought response at biochemical level, assuming that higher drought tolerance is based largely on mechanisms at cellular level. Inhibition of photosynthesis is a typical response to drought (Chaves and Oliveira, 2004) and may lead to nitrogen starvation. The reduced mobility of nitrogen in dry soil and reduced uptake of Nitrogen (N) by the roots cannot be ruled out especially under severe stress conditions. Nitrogen starvation under drought stress could result in increased proteolytic activity. This could compensate the effects of inhibited de novo synthesis of amino acids with an accelerated degradation of cell proteins and amino acid recycling (Feller, 2004). This process could resemble but may not be necessarily linked to natural senescence. Another possible function of proteolysis under severe drought could be to eliminate stress-damaged proteins.

Drought represents an excellent example of environmental stress that occurs in the field. In the present study, we observed that the adverse effects of drought on plant growth, leaf water status, CO₂ assimilation rate, photochemical efficiency, nitrogen assimilation level, and the cell membrane (as indicated by MSI) were significant, especially under drought. However, the treated (BAP) plants exhibited better tolerance to the water stress, as demonstrated by the maintenance of higher Fv/Fm, chlorophyll content, RWC, photosynthesis rate, stomatal conductance, and nitrogen content in flag leaves. The transgenic line has previously been reported to exhibit superior drought tolerance compared with the WT when plants were subjected to soil drying by withholding irrigation (Merewitz et al., 2010). The results, in combination with our study, demonstrated that expression of the ipt gene or external spray of cytokinins (BAP) in wheat crop was effective in improving plant tolerance to water stress.

In general, resistant cultivars performed higher photosynthesis, stomatal conductance and other photosynthetic attributes than susceptible ones under stress.
condition. As observed for grain yield, water stress caused a significant reduction in 
Pn. Similar results have been reported by other investigators (Ratnayaka and Kincaid, 2005). This appears to be a possible physiological mechanism by which drought can affect growth and productivity of crops such as wheat (Lawlor, 1995; Lawlor et al., 2002). However, addition of cytokinins could able to revive the photosynthesis and its components in both the cultivars and water regimes as also reported recently by Rivero et al., (2007) in ipt overexpressed tobacco transgenic plants. It has been hypothesized that genotype which keep their stomata open under stress condition while maintaining adequate leaf RWC can be considered as suitable for dry region (Blum et al., 1981). In the present study, resistant cultivar had higher values of stomatal conductance that leads to higher value of transpiration and RWC indicating their greater ability to water uptake from the soil compared to susceptible ones.

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Physiological basis of cytokinin induced drought tolerance in wheat  

(Triticum aestivum L.)

Wheat is one of the premier cereal crops of worldwide importance and India is second largest producer of wheat. Water stress is a worldwide problem, which is a severe threat for sustainable production of wheat. Water stress during wheat developmental stages limits production and its extent vary with stage at which plant encounters water stress. PGRs plays important role in plant response to water stress, cytokinin is important among them it can induce water stress tolerance by delaying leaf senescence. The present study was conducted to determine the effect of cytokinin treatment under two different water regimes (Control and water stressed) in two contrasting cultivars, water stress tolerant C-306 and water stress susceptible PBW-343.

In water stressed plants significant reduction were observed in traits related to photosynthesis which includes RWC, MSI, chlorophyll and carotenoid content, photosynthesis rate, stomatal conductance, photochemical efficiency, total sugar and total starch content. Cytokinin (BAP; 40 µM) treated plants were observed with higher activity of all the parameters studied in relation to photosynthesis and related traits in both the wheat cultivars under moisture deficit condition. Expression of Rubisco SSU was highest at anthesis stage but there was no significant effect due to cytokinin treatment under both water regimes. DHN and COR gene expression was more under water stress condition and expression was found to be increased on later developmental stages with no significant effect of cytokinin treatment.

Nitrogen metabolism in plants is severely affected by water stress. There was decrease in activity of enzymes of nitrogen assimilation pathway viz., nitrate reductase and glutamine synthetase which led to decrease in plant total nitrogen and total protein content. However, application of cytokinin was found effective under water stress to enhance activity of these enzyme and further increased leaf nitrogen and protein content in both cultivars. Total protease activity was enhanced significantly during water stress condition but it was decreased by application of cytokinin. Under water stress condition there was significant decrease in leaf area and total biomass of plants which increased by treatment of cytokinin in both wheat
cultivars. Yield related attributes also showed enhancement due to application of cytokinin in both the cultivars under water stress conditions. However, sensitive cultivars PBW-343 was found more responsive to cytokinin treatment under water stress condition, in comparison to drought tolerant cultivar C-306.
गेहूँ (ट्रिटिकम एस्ट्राइक्स एल.) में सायटोकाइनिन–प्रेरित सूखा–सहिष्णुता का पादप कार्यक्रिया आधार

सार

विश्वबर्म में महत्त्वपूर्ण खाद्यान्न फसलों में गेहूँ, एक मुख्य फसल है और भारत में गेहूँ का दूसरा सबसे बड़ा उत्पादक देश है। जल प्रतिबिंब एक विश्वव्यापी समस्या है जो गेहूँ के टिकाऊ उत्पादन हेतु एक गंभीर चुनौती है। गेहूँ की विकासशील अवस्थाओं के दौरान जल प्रतिबिंब के कारण उत्पादन कम हो जाता है और जिस विकास–अवस्था में पौधा जल प्रतिबिंब सहन करता है उससे के अनुसार उत्पादन पर भी प्रभाव पड़ता है। पौधों की जल प्रतिबिंब के प्रेरण्ट अनुक्रिया में पी.जी.आर.एस. की महत्त्वपूर्ण भूमिका होती है, इनमें से सायटोकाइनिन महत्त्वपूर्ण है क्योंकि यह पर्याय–प्रेरित तथा देरी कर जल प्रतिबिंब सहिष्णुता को प्रेरित कर सकता है। जल प्रतिबिंब सहिष्णुता सी–306 एवं जल प्रतिबिंब सुग्राही पी.बी.डब्लू–343, दो विपरीत प्रकृति की कृत्रिमप्रोपतावनियामों में, दो निम्न जल उपचार की परिस्थितियों (कंट्रोल एवं जल प्रतिबिंब सहित) में सायटोकाइनिन उपचार का प्रभाव ज्ञात करने हेतु प्रस्तुत अध्ययन किया गया।

जल प्रतिबिंब प्रस्ता पौधों में प्रकाशसंश्लेषण से संबंधित गुणों में महत्त्वपूर्ण रूप से कमी देखी गई जिनमें आर.डब्लू.सी., एम.एस.आई. हरितवर्ण एवं कैपिटोलियड अंश, प्रकाशसंश्लेषण दर, स्टोमेटा संबंधी चालकता, प्रकाश–रसायन दक्षता, कुल शरकरा एवं कुल स्टार्व अंश आते हैं। नमी जूनोता की आवश्यकता में गेहूँ की इन दोनों कृत्रिमप्रोपतावनियामों में, सायटोकाइनिन (पी.ए.पी.; 40 माइक्रॉं एम) से उपचारित पौधों में प्रकाशसंश्लेषण एवं संबंधी गुणों के सन्दर्भ में अध्ययन किए गए सभी प्राचीनों में अधिक सक्रियता पायी गई। आंशमिक संलग्न में एक्सप्रेसन ऑफ रुबिस्को एस.एस.यू. अधिकतम था किन्तु दोनों ग्राहक के जल अवस्था क्षेत्रों में सायटोकाइनिन उपचार के कारण कोई महत्त्वपूर्ण प्रभाव नहीं देखा गया। जल प्रतिबिंब युक्त अवस्था में डी.एच.एन. एवं सी. ओ.आर. जीन व्यजक अधिक था जिसका सायटोकाइनिन उपचार से अग्रभावत बाद की विकास संबंधी अवस्थाओं में बढ़ा देखा गया।

जल प्रतिबिंब के कारण पौधों में नाइट्रोजन का उपयोगकार हृदय रूप से प्रभावित होता है। नाइट्रोजन स्वागतकरण पाथवे के एन्जायमों यथा, नाइट्रेट रिडक्टेज एवं न्यूट्रोमाइन सिब्बेटिज, की सक्रियता में कमी पाई गई जिसके परिणामस्वरुप पादप–कुल नाइट्रोजन तथा कुल प्रोटीन अंश में कमी हो गई। वैसे इन एन्जायमों की सक्रियता को बढ़ाने में, जल प्रतिबिंबयुक्त अवस्थाओं में सायटोकाइनिन का अनुप्रयोग प्रभावी पाया गया तथा इसके अतिरिक्त इससे दोनों ही कृत्रिमप्रोपतावनियामों में पर्यास नाइट्रोजन एवं प्रोटीन अंश में बढ़ोतरी हुई। जल प्रतिबिंबयुक्त अवस्थाओं में कुल प्रोटिएज सक्रियता में बढ़ोतरी हुई किन्तु सायटोकाइनिन के अनुप्रयोग से
इसमें कमी हुई। जल प्रतिबल अवस्थाओं में अन्तर्गत पौधों के पर्यक्षेत्रकय एवं कुल जैव मात्रा में कमी देखी गई जिनमें सायटोकाइनिन उपचार द्वारा दोनों ही कृषिजोपजातियों में बढ़ोतरी देखी गई। जल प्रतिबल अवस्थाओं के अन्तर्गत गेहूं की दोनों ही कृषिजोपजातियों में सायटोकाइनिन के अनुप्रयोग से उपज संबंधी गुणों ने भी बढ़ोतरी दर्शायी। वैसे सूखा सहिष्णु कृषिजोपजाति सी-306 की तुलना में, जल प्रतिबल अवस्था के अन्तर्गत सायटोकाइनिन के उपचार से सुग्राही कृषिजोपजाति वी.बी.डब्ल्यू,-343 ने अधिक अनुक्रिया दर्शायी।