6. CONCLUSIONS

- A review of literature has been presented on previous works done in this line. The review has been presented on heat induced physiological and biochemical changes in plants and of plants' response to heat injury, elevated temperature and plant disease interaction and role of PGPR in amelioration of heat stress and spot blotch.
- Experimental procedures and all the protocols related to present study has been presented.
- Preliminary screening of wheat cultivars was carried out to assess their basal thermo tolerance level. A total eight cultivars *viz.* C306, PBW343, PBW550, HT41, HT17, HT15, GY, and MW were selected for the study. Heat susceptibility index (HSI) of all the cultivars were calculated from % reduction in fresh weight and relative water content on exposure plants to 25^0C, 30^0C, 35^0C and 40^0C for 6 h and 12h. Leaf disc bioassay was also conducted to assess heat sensitivity of eight cultivars. Extent of changing colour of leaf discs from green to yellow when immersed in water at 40^0C for 12h was used as an indicator of effect of high temperature on leaf tissues. Comparative study of basal thermo tolerance in various cultivars has been done by observing changes in eight biomarkers for thermotolerance: relative water content, cell viability, chlorophyll pigment, electrolyte leakage, membrane lipid peroxidation, H_2O_2 content, total sugar and proline content upon exposure to 25^0C, 30^0C, 35^0C and 40^0C for 6 h and 12h. Finally from hierarchical cluster analysis two most heat susceptible cultivars PBW550 and HT41, having highest HSI were selected for PGPR application.
- Two PGPR strains previously isolated and identified by Chakraborty *et al.* (2013) *Bacillus safensis* (JX 660688) and *Ochrobactrum pseudogrignonense* (JX 660689) isolated from the wheat (*Triticum aestivum* L.) and blady grass (*Imperata cylindrica* L.) rhizosphere respectively were selected for amelioration of heat stress. Both PGPR could grow under elevated temperature conditions, but among the two, B. *safensis* could grow better at 40^0C and these two PGPR were able to retain their plant growth promoting attributes viz. phosphate solubilisation, protease production, siderophore production, chitinase, IAA producton, starch hydrolysis at 40^0C.
- *In vivo* plant growth promoting ability of these two strains was tested in wheat cultivars HT41 and PBW550 susceptible to elevated temperature. For this plant

height, root and shoot dry weight were tested in every 15 and 30 days of PGPR application. Result showed significant increase in plant height and dry weight in PGPR primed plants. Phosphate mobilization from soil to plant through root system was also increased in PGPR primed plants.

- After screening for growth promotion *Bacillus safensis* and *Ochrobactrum pseudogrignonense* were used for temperature stress alleviation at 40^0C. Pre treatment with PGPR lessened water loss, improved cell viability and increased thermo tolerance in HT 41 and PBW550 plants signifying a protective effect by these bacteria especially by *B.safensis* at 40^0C.
- Heat stress upto 12 hours at 40^0C significantly increased membrane lipid peroxidation, electrolyte leakage and produced ROS such as hydrogen peroxide and superoxide, in unprimed plants. *Bacillus safensis* and *Ochrobactrum pseudogrignonense* priming reduced heat induced oxidative stress in plants by reducing ROS (hydrogen peroxide and superoxide) generation and cell membrane injury.
- Total chlorophyll content was mainly affected by high temperature stress. Insignificant rise in chlorophyll content and chl. a/b ratio initially during heat treatments may be attributable to clustering of chloroplast within cell due to dehydration. With increase in exposure time total chlorophyll content reduced significantly in unprimed plants of both cultivars while in *B. safensis* and *O. pseudogrinonense* primed plants decline in chlorophyll content was much lesser over time. PGPR primimg also enhanced carotenoid accumulation thus protects chloloplast from photoinhibition during heat stress. Carotenoid quenches superoxide, singlet oxygen and peroxyl radicals, thus minimizes formation of ROS by receiving excess energy from the chlorophyll.
- TEM studies revealed high temperature distorted cell wall, mitochondria and chloroplast ultrastructure especially grana and thylakoid stacking and mitochondrial cristae with membrane damage leading to electrolyte leakage. Formation of large vacuoles within chloroplast and mitochondria can be observed in case of unprimed heat stressed plants exposed to 40^0C for 12 hours. PGPR priming particularly with B. *safensis* lessened chlorophyll reduction and also minimized heat induced ultrastructural damages in chloroplast which may be responsible for restoration of PSI and PSII system. PGPR primimg also reduced mitochondrial ultrastructural

abnormalities in leaf and accumulate greater amount of plastoglobules in chloroplasts during heat stress.

- Higher level of protein was observed in PGPR primed plants, especially *B. safensis* primed plants of both the cultivars as compared to control plant subjected to heat stress at 40^0C. Protein profiling showed appearance of new bands in the range of 20 kDa to 67 kDa in plants exposed to high temperature. Appearance of new stress responsive proteins along with expression of existing ones was more in PGPR primed plants; especially *B. safensis* primed plants as compared to untreated plants after heat treatment. Differential expression analysis of various HSP genes revealed that in unprimed PBW 550 plants transcript level of HSP 101C, HSP 90 and HSP 70 increased significantly in initial hours followed by sharp decrease during heat treatment. Whereas in *B. safensis* primed plants relative expression of HSP101 and HSP 90 steadily increased and higher transcript level was maintained overtime as compared to heat stressed unprimed plants. Similar trends were also observed in case of HSP 23.5 and HSP 17.8 HSP 26.3 and HSFA3. Relatively high expression of HSP101C, HSP 90 and small HSPs (HSP 23.5, HSP 17.8 and HSP 26.3) in PGPR primed plants all through heat treatment possibly provide better tolerance to heat injury.
- Production of ROS within plants tissues turns on different enzymatic and non-enzymatic signaling pathways during heat stress. Antioxidative enzymes contributing to stress signalling such as peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase, and superoxide reductase activity increased across all treatment during initial hours of heat stress at 40^0C. PGPR primimg maintain high level of antioxidative enzyme activity during heat stress. Among the two PGPR *B.safensis* was more effective in retaining high antioxidant activity at 40^0C. PGPR priming also increased accumulation of non enzymatic antioxidants total glutathione and ascorbic acid in PGPR primed especially in *B. safensis* treated plants. Ascorbic acid and total glutathione accumulation reached utmost level after 4 hours of exposure to 40^0C equally in unprimed and bacterized plants. Beyond 4th hour ascorbic acid content gradually declined in both unprimed and bacterized plants, although gradual reduction in ascorbic acid content was nominal in PGPR treated plants. Initial increase in ascorbic acid and total glutathione may add to glutathione

and ascorbate regeneration system ascorbate-glutathione cycle to accentuate APX activity.

- Total and O dihydroxy phenol significantly in all plants subjected to heat stress. However, in *B.safensis a*nd *O pseudogrignonense* primed plants maintained high level of total and O dihydroxy content in heat stressed plants. Enhanced expression of chalcone synthase (CHS) was also across all treatments during high temperature stress may give protection from heat injury. Relatively high expression of PAL gene in leaves of *B. safensis* primed plants in response to high temperature perhaps a part of acclimatory response where the enzyme induced the biosynthesis of other phenolics in the pathway.
- High temperature treatment significantly increased proline concentration in unprimed HT 41 and PBW550 as compared to PGPR primed plants of both the cultivars. RT PCR analysis indicated differential expression of proline biosynthesis related genes P5CS and PDH during temperature treatment might be responsible for different proline level in *B.safensis* primed and untreated plants. In both *B. safensis* and unprimed plants exposed to 40^0C transcript level of P5CS and P5CR increased with time. One interesting finding is that the PDH expression significantly increased with time in *B. safensis* primed plants in comparison untreated plants. Over expression of PDH genes in *B. safensis* primed plants turned out to be responsible for comparatively low level proline than untreated control plants.
- Polyamines biosynthesis related genes ADC1, ADC2 and SAMDC2 were found to be over expressed during heat stress in both unprimed and *B.safensis* primed plants exposed to heat stress. The first two genes regulate decarboxylation of arginine catalyzed by two isoform of arginine decarboxylase (ADC), followed by successive reactions which ultimately converts agmatine to Putrescine. In subsequent reactions aminopropyl groups are produced from S-adenosylmethionine (SAM) by SAM decarboxylase (SAMDC2) to alter putrescine to spermidine and subsequently spermine. ADC1 and SAMDC2 transcript level found to be relative high at 8^{th} and 12^{th} hours in unprimed plants in comparison to *B. safensis* primed plants.
- Seed bacterization facilitates accumulation of glycine betain and total sugar in plant tissues than unprimed plant during heat stress. High temperature increased glycine betain production in leaves for all treatments. It was observed that glycine betain and

total sugar content in wheat leaf attained much higher level with *B.safensis* than *O. pseudogrignonense* priming.

- Elevated temperature affected seed quality in late planted unprimed and PGPR primed plants. Result revealed heat stress significantly reduced spike length, grain number and grain weight in unprimed heat stressed plants. Total protein and sugar content also reduced in heat stressed plants. Protein and starch content was relatively high in *B.safensis* primed plants in comparison to unprimed plants. Ultrastructural analysis of starch grains revealed presence of flat, deformed starch granules as compared to round, spherical shaped granules observed in control as well as *B.safensis* primed heat stressed plants. Aleuronic layer of *B. safensis* plants was less affected as compared to heat stressed control plants suggesting positive role of *B.safensis* priming in improving seed quality in plants under heat stress.
- Elevated temperature significantly increased the susceptibility of wheat plants to spot blotch causing pathogen *Bipolaris sorokiniana*. Maximum disease incidence was observed in the range of 35^0C to 38^0C and with relative humidity 70% and further rise in temperature from 38^0C to 40^0C reduce disease incidence. With the onset of disease electrolyte leakage, H_2O_2 accumulation and lipid peroxidation significantly increased over time in infected plants. High temperature during disease commencement changed membrane permeability in terms of electrolyte leakage and lipid peroxidation making the plants even more vulnerable to pathogen attack. Therefore dual application of heat stress and *B. sorokinana* intensified membrane damage many folds and altered antioxidative defense response.
- Potential of *B. safensis* and *O. pseudogrignonese* in induction of resistance was determined and their performance at normal and elevated temperature in terms of biochemical response of wheat plants against spot blotch causing pathogen, *B. sorokiniana* was compared.
- The antagonistic activity of *B. safensis* and *O. pseudogrignonese* against *B.sorokiniana* were prescreened *in vitro*. Both these PGPR effectively inhibit mycelia growth in soild media. Ethyl acetate fraction of cell free culture filtrate of *B. safensis* and *O. pseudogrignonese* were further characterised by GC/MS analysis. The analysis confirmed the presence of several compounds. 10-Octadecenoic acid (retention time 18.88 min) and Pyrollo pyrazine (retention time 25.858) were most

abundant in ethyle acetate fraction of *B. safensis* whereas in case of *O pseudogrignonense* Pyrollo pyrazine (retention time 25.858) was most abundant.

- ❖ PGPR priming significantly reduced disease severity in *B.sorokiniana* infected plants *in vivo*. However, at high temperature *B. safensis* was more effective in reducing disease severity in comparison to *O. pseudogrignonense.* Pre-treatment with the PGPR decreased the accumulation of H_2O_2, reduced lipid peroxidation in infected plants thus reduced electrolyte leakage and membrane damage protecting the plants from adverse consequences of oxidative injury. At high temperature PGPR, especially *O. pseudogrignonense* was slightly less effective in comparison to their performance at normal temperature.
- ❖ Total protein, phenol and O dihydroxy phenol significantly decreased over time in unprimed infected plants exposed to high temperature despite the fact that at normal temperature all these parameters increased in infected plants tissues across all treatment. However PGPR primimg were able to maintain comparatively high level of protein, phenol and O dihydroxy phenol content overtime in infected leaf tissues.
- ❖ Spot blotch significantly increased accumulation of proline and soluble sugar in infected plants. RT PCR analysis revealed *B.safensis* primimg significantly increase relative expression of P5CS gene in comparison to PDH and P5CR genes in infected plants; as a result proline accumulation increases drastically in PGPR primed infected plants pointing towards positive role of PGPR primimg in inducing hypersensitive response at elevated temperature.
- ❖ SDS PAGE analysis revealed expression of few new bands ranging from 10- 34 kDa in PGPR primed plant which were absent in case of unprimed infected plants both at normal and elevated temperature. RT PCR analysis of various HSPs revealed *B. safensis* seed priming significantly increased expression of HSP70 and HSP23.5, HSP26.3 and HsfA3. Whereas expression of theses HSPs remain more or less similar in unprimed healthy and infected plants during commencement of infection indicating role of these HSPs in *B. safensis* induced resistance spot blotch at high temperature.
- ❖ In addition to this, time course accumulation of defense enzymes such as chitinase, phenyl alanine ammonia lyase, peroxidase and β-1, 3 glucanase was determined following challenge inoculation with *B. sorokiniana.* Higher activities of CHT, POX, PAL, β-1, 3 GLU and accumulation of higher phenolic compounds were observed

heat susceptible wheat plants However, high temperature during disease commencement adversely affected of defense enzymes activity as well as protein, phenolics and osmolyte accumulation. Time course accumulation of all of these components during prolonged exposure at 38^0C decreased defense enzyme activity, phenol and osmolyte accumulation in infected plants which might possibly facilitated pathogen spread and cause susceptibility to the diseases. *B.safensis* and *O. pseudogrignonense* primimg maintained higher level enxzme activity and accumulate osmolyte and thus gave protection against *B. sorokiniana* even at high temperature. Results revealed *B. safensis* priming significantly increased PAL and CHS gene expression in infected plants especially during elevated temperature indicating greater amount of phenolics accumulation in *B. safensis* primed plants during disease commencement which helped plants to induced resistance against *B.sorokiniana.*