CHAPTER-6

SUMMARY AND CONCLUSIONS
The ubiquitously present glyoxalase system consists of two enzymes, glyoxalase I and glyoxalase II. These two enzymes act coordinately to convert hemithioacetal formed by the non enzymatic reaction between glutathione and methylglyoxal to lactic acid releasing the glutathione. Methylglyoxal is a cytotoxic compound and mainly synthesized as a by product of glycolysis, where it is synthesized from triose phosphates (DHAP and G3P). The close association of glyoxalase system with stress in plants was first reported by Espartheo et al (1995). They showed increased expression of glyoxalase I under stress. The elevated expression of glyoxalase I under different stresses was further shown by Veena et al (1999).

Glyoxalase II is also an important component of glyoxalase system and its activity has been reported in both cytosolic and mitochondrial fraction in plants. Other than expression analysis of glyoxalase II in different organs of Arabidopsis, there is hardly any report of this enzyme indicating its physiological function in plants. Recently, Singla-Pareek et al (2007) showed that the transgenic plants of Oryza sativa transformed with glyoxalase II gene from rice under a constitutive (CaMV 35S) promoter showed higher activity of glyoxalase II that increased further upon salt stress, reflecting the upregulation of endogenous glyoxalase II. Singla-Pareek et al (2003) had also shown that the overexpression of the gly I & the gly II gene together in tobacco confers improved salinity tolerance. These double transgenic lines of tobacco showed enhanced tolerance to salinity stress as compared to either of the lines transformed with a single gene (gly I or gly II) or the untransformed control plants.

In the present investigation the work was undertaken to compare the expression of the glyoxalase I gene under the influence of constitutive and inducible promoters in an oil seed crop plant, Brassica juncea. An attempt was also made to engineer both glyoxalase I & glyoxalase II genes together in this economically important plant for enhanced abiotic stress tolerance as reported for the model plant, tobacco. Apart from this, abiotic stress tolerant B. juncea transgenic plants were also developed by using the Cre-lox system for marker gene excision from the transgenic plants. The POSITECH system based on mannose selection was shown to be effective in B. juncea for the first time.
The highlights of the present investigation are as follows:

- *Brassica juncea* plants transformed with CaMV 35S-gly I were developed to study their response to salinity, heavy metal and drought stress. Constitutive expression of *glyoxalase I* gene showed enhanced tolerance of the transgenics for NaCl, ZnCl₂ & mannitol stress. There was no significant yield penalty with respect to plant biomass and seed set of the transgenic vs. the untransformed control plants.

- The regulation of *glyoxalase I* gene driven by the constitutive CaMV 35S promoter was also studied under non-stress conditions. It was found that the transgenics did not perform better under non-stress conditions than the untransformed control plants in terms of shoot & root length as well as seed production per plant. The data show that the *gly I* transgene was constitutively expressing even under non-stress conditions. This could be linked to the retarded growth of the transgenic plants in comparison to the untransformed control.

- The regulation of *glyoxalase I* gene driven by constitutive CaMV 35S promoter was also studied at transcript level under salt (NaCl), drought (mannitol) and heavy metal (ZnCl₂) stress conditions. Constitutive expression of *gly I* gene was observed in all the stress treated transgenic plants whereas no transcript could be detected in untransformed control plants under stress or non-stress condition.

- The transgensics of *B. juncea* overexpressing *glyoxalase I* gene under the control of CaMV 35S promoter showed delayed senescence of leaf discs at all the concentrations in each of the three cases (salt, heavy metal and MG) as compared to the leaf discs from the untransformed control plants which showed early bleaching. Measurement of the chlorophyll content of the leaf discs confirmed the phenotypic differences.

- Measurement of relative water content (RWC) of transgenic plants vs. untransformed control showed that the water retaining capacity of most transgenic lines was better than untransformed controls in both stress as well as non-stress conditions.

- The transgensics of *B. juncea* overexpressing *Bjgly I* under the control of a novel *Cestrum* leaf curling viral promoter were also generated. However, the two plants (T₀) overexpressing the *glyoxalase I* gene did not survive and hence no further studies could be carried out with these transgenic plants.

- Transgensics of *Brassica juncea* transformed with *rd29A-gly I* (where the *gly I* gene is driven by an inducible *rd29A* promoter) were also developed to study salinity, heavy
metal and drought stress tolerance. Induction of *glyoxalase 1* gene was observed under salt (NaCl), heavy metal (ZnCl₂) & drought (mannitol) stress in these transgenics. The morphology and growth pattern of transgenic plants was similar to the untransformed control plants. The biomass and seed set per plant was marginally better in the transgenic plants.

- The *glyoxalase 1* gene driven by the stress inducible *rd29A* promoter showed negligible induction of the *gly* gene under non-stress conditions which could save the metabolic energy of the cells for utilization in other developmental processes of the plant.

- The regulation of the *glyoxalase 1* gene driven by stress inducible *rd29A* promoter was also studied at the transcript level under salt (NaCl), drought (mannitol) and heavy metal (ZnCl₂) stress conditions. Induction of *gly* gene was observed in all the stress treated transgenic plants whereas no *gly* I mRNA was observed in untransformed control plants in stress or non-stress conditions. Maximum induction was observed in NaCl (200 mM) and ZnCl₂ (5 mM) treated transgenic plants followed by mannitol (200 mM) treated transgenic plants.

- The relative water content (RWC) for transgenic as well as untransformed control showed that the water retaining capacity was higher for most of the transgenic lines as compared to the untransformed controls in both stress as well as non-stress conditions.

- Comparison of transgenic *Brassica juncea* plants transformed with inducible *rd29A* & constitutive CaMV 35S promoter showed that the performance of former was better than the latter and also as compared to the untransformed control plants during stress as well as non-stress conditions. However, the CaMV 35S-gly 1 transformed plants performed better only under stress conditions in comparison to the untransformed controls. During non-stress condition constitutive expression of the *gly* I gene was not found to be beneficial.

- *Brassica juncea* plants transformed with *phsp-cre-npt II-lox-rd29A-gly 1* construct were used for marker excision studies. Heat shock treatment at 37°C followed by 40°C for 24 hrs each was found to be sufficient for the excision of the marker gene, *npt II* from the *Brassica juncea* genome, while retaining the gene of interest, the *gly* 1 gene.

- Two marker (*npt II*) free plants, abiotic stress tolerant transgenic were obtained after the heat shock treatment which set viable seeds under NaCl stress (200 mM).

- A POSITECH system with Phosphomannose Isomerase (*pmi*) or *manA* gene was used for the selection of transgenic *Brassica juncea* on selection medium containing
manose. Transformation efficiency of 4% was observed on mannose containing selection media.

- The Phosphomannose isomerase (pmi) gene regulation was studied at the transcript level which was found to be maximum in P-16 line. The PMI enzyme activity was also measured which ranged between 0.1 to 1.1 Umg⁻¹ of protein with maximum activity in P-16 line.

- Chlorophenol Red (CPR) Assay with the leaf discs of B. juncea transformed with pmi confirmed the PMI activity in transgenic plants which was evident by colour change (from orange to yellow) of mannose containing liquid medium. This colour change was observed due to change in the pH of the medium due to utilization of mannose as a carbon source by the transgenic plants. No change in colour was observed in the leaf discs of untransformed controls.

- An attempt was made to develop transgenic B. juncea containing both the gly I & the gly II genes together. For this a construct having Bjgly I from B. juncea & Osgly II from Oryza sativa under the influence of CaMV 35S promoter respectively was used to transform B. juncea. The T0 lines of B. juncea transformed with this CaMV 35S-gly I-gly II construct appeared stunted in comparison to the untransformed control plants.

- The CaMV 35S-gly I-gly II transgenic B. juncea overexpressing glyoxalase I & glyoxalase II genes under the control of CaMV 35S promoter were subjected to the leaf disc senescence assay. The leaf discs from the transgenic plants showed delayed senescence at all the concentrations in each of the three cases (salt, heavy metal and MG) while the leaf discs from the untransformed control plants showed early bleaching. Measurement of the chlorophyll content of the leaf discs confirmed the phenotypic differences.

- The plants transformed with CaMV 35S-gly I-gly II did not set viable seed. Therefore, no further studies could be carried out in the next generation.

- An alternative approach was used to develop double transgenics of B. juncea by cross breeding the lines of Brassica juncea overexpressing CaMV 35S-gly I gene as well as the salt inducible hsp-cre-npt II-lox-rod29A-gly I with those having the CaMV 35S-gly II gene. Further studies were conducted with double transgenics of B. juncea containing both hsp-cre-npt II-lox-rod29A-gly I as well as CaMV 35S-gly II genes. The double transgenics were healthy and set viable seed after the completion of their life cycle.
• Regulation of both \textit{gly I} & \textit{gly II} genes was studied at protein level and it was observed that the double transgenics overexpressed both the \textit{gly I} & \textit{gly II} genes and showed the presence of Gly I & Gly II proteins in Western blots on treatment with NaCl (200 mM). On the other hand only Gly I protein was formed in \textit{B. juncea} transformed with \textit{hsp-cre-npt II-lox-rd29A-gly I} and formation of only Gly II protein was observed in \textit{B. juncea} transformed with CaMV 35S-gly II. Neither Gly I nor Gly II protein was observed in untransformed control plants. The data on specific activity of Gly I & Gly II enzymes respectively corroborated these observations.

• Leaf disc senescence assay was carried out to compare the tolerance level of double transgenics vs single gene transgenics of \textit{gly I} & \textit{gly II} gene as well as untransformed control plants. The leaf discs from the double transgenic plants showed delayed senescence in comparison to the single gene transgenics (of either gene) followed by untransformed control at all the concentrations in each of the three cases (salt, heavy metal and MG). The leaf discs from the untransformed control plants showed early bleaching in comparison to all the transgenic plants. Amongst the \textit{gly I} & \textit{gly II} transgenic plants, leaf discs of \textit{gly II} transgenic plants bleached earlier than \textit{gly I} transgenics.

• Physiological data from all the three types of transgenics and untransformed control showed that the best output in terms of biomass and seed production per plant was obtained from \textit{B. juncea} transformed with \textit{hsp-cre-npt II-lox-rd29A-gly I} followed by double transgenics containing both \textit{gly I} & \textit{gly II} genes and then the transgenic plants having CaMV 35S-gly II in comparison to the untransformed control \textit{B. juncea} under non-stress condition.
Conclusion

The present study led to the development of transgenic *Brassica juncea* plants having the *glyoxalase I* gene under constitutive as well as inducible promoters. All the transgenics showed greater tolerance towards various abiotic stresses, e.g. salt, heavy metal and mannitol as compared to the untransformed controls. Abiotic stress tolerant, antibiotic marker free transgenics were developed using Cre-lox system. The use of a POSITECH system for selection has been successfully demonstrated in developing transgenic *Brassica juncea* for the first time. Double transgenics having the *gly I* and *gly II* gene expression were developed using the transgenic as well as cross-breeding approach. The latter showed enhanced tolerance to various stresses as compared to the transgenics having single transgene, *glyoxalase I* or *glyoxalase II*. Further studies are in progress to understand the mechanism of enhanced tolerance by overexpression of the two genes of the same pathway vs. the regulation of candidate genes under inducible promoter.