SUMMARY, CONCLUSIONS AND FUTURE PROSPECTS

Blackgram (Vigna mungo L. Hepper), is one of the important pulse crops which is grown as a source of income and nutrition to billions of people in South East Asia. It has very important role in human diet, as it contains vegetable proteins and supplement to cereal based diet. It contains about 26% protein and other minerals and vitamins. Besides, it is also used as nutritive fodder, especially for milch animals. Being a leguminous crop, it has all the essential nutrients which make it to turn in to a good fertilizer. With its ability to fix atmospheric nitrogen, it restores soil fertility as well. The production of blackgram is mostly confined to Asian countries, out of which India is the largest producer followed by Myanmar and Thailand. Despite being an important pulse crop, blackgram production is continuously decreasing resulting in protein-calorie malnutrition and escalation in its market price. So in order to meet the demand of increasing population, the yield is to be enhanced in the areas where it is exposed to numerous abiotic and biotic stresses. Major hurdle in achieving maximum production includes its susceptibility to several diseases (fungal and viral) and pests. Out of different constraints, viral diseases mainly yellow mosaic disease caused by mungbean yellow mosaic virus (MYMV) is the major threat for huge economical losses in the Indian subcontinent. The virus also infects other grain legumes, French bean, pigeon pea, soybean and mungbean. It can cause even 100% yield loss if infection occurs at the seedling stage. In India, the yield losses per annum due to MYMV in mungbean, blackgram and soybean have been estimated to be US $ 300 million. Eradication of the virus through chemical control of vector, avoidance of infection source and breeding for virus resistance has been proved to be inefficient. Therefore, the only option left is to use genetic engineering techniques to transfer desirable genes to fight against the virus. Pathogen-derived resistance (PDR) is a very effective genetic engineering approach to control plant viruses. PDR is a strategy in which resistance imparting genes are taken from the virus for which resistance is to be developed. Out of different viral genes used for PDR, invert repeat construct of MYMV-Vig rep gene to express ds RNA for RNA interference approach holds promise for developing geminivirus resistance. RNA interference is triggered by ds
RNA to suppress the target gene expression. Therefore, the present investigation was undertaken with the major objective to generate blackgram transgenic plants for MYMV resistance using Agrobacterium tumefaciens strain EHA 105 containing binary plasmid pGD3 harboring hp RNA of MYMV Vig-rep gene employing the optimized protocols. The results obtained in the present investigation are summarized as follow-

1. A fast, efficient and reproducible in vitro multiple shoot induction system from primary leaf petiole and cotyledonary node explants have been developed in blackgram. The factors affecting in vitro regeneration of multiple shoots were optimized. The genotype, age and size of the explant, type and concentration of cytokinin and combinations of cytokinin with auxins influenced the frequency of shoot formation and the number of shoots per explant. Explants cultured on MS basal medium produced a single or two shoots whereas those cultured on medium containing 6-benzylaminopurine induced multiple shoots. This indicates that cytokinin (BAP) is essential for multiple shoot induction. The age of explant also affects the multiple shoot forming response. The cotyledonary node and primary leaf explants excised from 4-d-old seedlings developed a maximum of 4.5 and 10 shoots per explant in 65% and 95% of the cultures respectively, on medium containing 1.0 μM BAP as a sole growth regulator. Genotype was found to exert a pronounced effect on the regeneration frequency as well as on the number of shoots per explant. Out of five cultivars checked, PS-1 produced the maximum number of shoots from both the explants. Shoots originated directly from the nodal regions and cut ends of the cotyledonary node and primary leaf petiole explants without callus formation.

2. The shoots regenerated from both explants were rooted on medium containing IBA (2.5 μM). Shoots with well-developed roots (plantlets) were established in soil where 80-100% of them survived and developed into morphological normal plants which subsequently produced flowers and pods with viable seeds.

3. Identification of an efficient selection agent and its suitable threshold concentration is a very important parameter for transformation system. The sensitivity of the primary leaf explants to selective agent, kanamycin was checked to determine its optimal concentration for the selection of transformed shoots. Kamamycin was
inhibitory at 70.0 mg l\(^{-1}\) for shoot organogenesis and 10.0 mg l\(^{-1}\) for root induction. It indicates that root organogenesis was more sensitive to kanamycin than shoot organogenesis. Kanamycin at 70.0 mg l\(^{-1}\) helps in the early identification of green transformed shoots as the shoots emerging from non-transformed cells were bleached (albino).

4. The inoculation and co-cultivation steps are crucial as the two different biological elements, i) plant explant and ii) *Agrobacterium*, shares the same space and conditions. Various factors affecting transformation efficiency, preculture of explants, bacterial concentration, bacterial inoculation, co-culture duration, use of acetosyringone and thiol compounds (L-cysteine and DTT) in co-cultivation medium and pH were optimized using the transient GUS activity. Bacterial concentration at \(10^7\) cells/ml, bacterial inoculation for 20 min. and co-cultivation for 3 days were found to be optimal. Co-cultivation in the presence of BAP (1.0 μM) and acetosyringone (100 μM) at pH 5.5 under light were effective in improving the transformation frequency. The primary leaf explants inoculated and co-cultured under optimized conditions showed GUS activity at regeneration site in 70% of the explants.

5. The addition of the thiol compounds, L-cysteine (5.0 mM) and DTT (1.5 mM) in co-cultivation medium reduced the browning of wounded tissue and improved the survival of explants on selection medium.

6. Using optimized plant regeneration and transformation protocols, further transformation experiments were carried out to achieve the main aim of the present study– the development of MYMV resistant blackgram transgenics, by the incorporation of hp RNA of MYMV-Vig rep gene in to the blackgram genome. However, the putative transgenic plants recovered from cotyledonary node on selection medium did not show any hybridization signal in Southern blot analysis. This may be attributed to the damage caused by mechanical injury to the regenerable cells which are present in limited number in the axil of cotyledons at the node.

7. Morphologically normal and fertile transgenic plants from primary leaf petiole explants inoculated with *A. tumefaciens* (carrying binary vector pGD3) have been regenerated for the first time. The agro-inoculated explants on selection medium (MSB + 1.0 μM BAP + 70 mg l\(^{-1}\) kanamycin + 500 mg l\(^{-1}\) cefotaxime) recovered
putatively transformed shoots which were rooted on MSB medium supplemented with IBA (2.5 μM) and kanamycin 10 mg l⁻¹. A second round of selection on kanamycin at rooting stage was effective in eliminating the escapants. The rooted shoots were successfully established in soil. The presence and integration of nptII gene into the blackgram genome was confirmed by PCR and Southern blot analysis. The transformation frequency was 1.3% and the total time required from inoculation of explants to establishment of plants in green house was approximately 2 months. Seeds were collected from T₀ transformed plants. The transgenes inheritance and expression in T₀ progeny was detected by PCR and Western blotting, respectively. The T₁ transgenic plants were further evaluated by agro-inoculation with partial dimers of MYMIV and whitefly transmission for their resistance against MYMIV. Under the green house conditions, transgenic plants did not show any symptoms of MYMV as compared to untransformed (control) plants. However, further analysis of the transgenic material for the presence of si RNA specific to transgene (rep) and decrease in accumulation of viral DNA while challenging the plants with MYMV will elucidate the potential of RNAi in MYMV resistance. The seeds collected from T₁ transgenic lines are a valuable material, for developing yellow mosaic disease resistant cultivar in Vigna mungo. Development of MYMV resistant blackgram transgenic plants will increase and stabilize its production and reduced the load of chemical insecticides on environment and farmers. Moreover, the present study will open the avenues for further research in the area of disease resistance by the use of RNA interference technology.

In conclusion, the present study demonstrates the potential of Agrobacterium-mediated transformation of primary leaf to develop blackgram transgenic plants resistant to yellow mosaic disease using hairpin technology. The transformation protocol is based on direct shoot regeneration from primary leaves of mature seeds as explants, which have the advantage of being easily available throughout the year and relatively free of microbial contamination. The primary leaf petiole explants produced the maximum number of shoots per explant, and the regeneration site (cut end of petiole) was completely exposed and easily accessible to Agrobacterium. An extra wounding treatment is not required as in cotyledonary node explants. The super
Summary, Conclusions and Future Prospects

A virulent strain of *Agrobacterium tumefaciens* EHA105 (pGD3) was able to transfer the transgenes into the regeneration-competent cells present at the cut end of 4-d-old primary leaf explants. Since the regeneration is direct, therefore, the chances of somaclonal variations are low. The use of super virulent *Agrobacterium* strain, thiol compounds during co-culture and a second round of selection at rooting stage were found to be critical for blackgram transformation. However, there is a scope to further improve the transformation frequency in order to develop a high throughput transformation system which is not only essential for introduction of desirable genes for the better yield and nutritional quality but also required to explore gene functions through reverse genetic techniques. The new tools of genomics, proteomics and metabolomics would allow better understanding of vital processes of this food legume for their improvement. In addition, for the sake of end user acceptance, the research should focus on (1) development of methods avoiding antibiotic or herbicide resistance genes as selectable marker or use of positive selectable markers such as phospho-mannose isomerase (*pmi*), xylose isomerase (*xyl A*) etc and (2) selecting genes for the desirable traits for the transfer and strategies for the seed distribution system, where the end user in the developing countries is benefited and not only industries in developed countries. Further, there is a need of a sustained coordinated research with long term funding and involvement of private sector for generation of transgenics in this tropical food legume so as to meet the daily protein requirement of millions of people of the developing countries.