CONCLUSION

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The threat of hunger once again looms large in the horizon. Food production has to be increased at a sustainable scale to feed the booming up population as well as to safeguard the environment for sustenance and better survival of the mankind. Self-sufficiency and self-reliance required to be restored to ensure house hold food security world wide. In this endeavour rice- the principal cereal crop especially in Asia where 92% of rice is grown and consumed has to be increased 60% more by 2025. The task is difficult and obviously challenging in which genetic improvement of existing varieties would be playing a key role.

Since green revolution (GR) in the sixties, significant strides have been made in improving rice productivity and thus a bulk increase in rice production world over could be discernible. The high yielding varieties, which were developed by the introgression of dwarfing (DGWG) gene constituted the most important component of the inputs in accomplishing GR. They helped tremendously to bring about radical changes in the productivity level mainly due to increased harvest index. Besides high yield, expansion of area under rice cultivation, improved management practices with the use of chemical fertilizer, pesticides, intensive efforts from private and government sectors, development of highly efficient National Agricultural Research Systems (NARS) in many of the major rice growing countries and most remarkably the marvelous contributions of the international organizations like International Rice Research Institute (IRRI), Philippines; West Africa Rice Development Association (WARDA), Cote d'Ivoire; Centro International de Agricultura Tropical (CIAT) , Colombia; etc. under the auspices of Consultative Group on International Agricultural Research (CGIAR) are worth mentioning. Internationally developed improved rice varieties were shared by rice growing countries through International Network for Germplasm Evaluation and Research (INGER), IRRI, Philippines and thus with the free exchange of germplasm/HYVs better varieties could spread world wide e.g. IR 36 developed at IRRI had once covered about one third of the rice growing area in the world.

However, this improvement, which was primarily achieved through the development of HYVs *en route* conventional plant breeding approaches seems to be

slowing down as evident from declining rice productivity data from major rice growing agro-ecosystems. Yield plateuing is discernible which has to be circumvented scientifically. It is mentionable that to achieve the yield of seventies, the same variety consumes more inputs nowadays which is discouraging to undertake cultivation of currently available many HYVs in many places.

Furthermore, the negative impact of GR on the growing environment is also being discernible. Soil fertility loss, fertilizer and pesticides abuse and bio-magnification of the pollutants, environmental pollution, genetic erosion due to monoculture of a few elite varieties, breaking down of insect and disease resistance pattern in released varieties, pest resurgence and soil contamination due to Stalinization and other abiotic stress influx (caused owing to major irrigation projects especially for the sustenance of irrigated ecosystems) demands more productive varieties with better stability to meet out ever increasing demands for sufficient food .

In this critical juncture to create a paradigm shift in rice productivity efforts have to be mounted systematically in which scientific intervention at the interface of conventional methods and modern biotechnological approaches would be more appropriate. In the present thesis major emphasis was made to develop an efficient *in vitro* culture protocol with optimization of culture environment through conducting a series of well thought experiments for somatic cell and tissue culture in rice, which is an indispensable requirement for genetic transformation to transfer foreign gene/s from homo/heterologous sources for genetic manipulation. It is mentionable that inspite of a long history of somatic tissue culture in rice, the state of art of this subject is still empirical. A large number of varieties belonging to three different race of *Oryza sativa* L. namely *indica, temperate japonica* and *tropical japonica* have been tested for their *in vitro* culture response. However, prescription of a generalized protocol is difficult since the races and even the genotypes within a race differ largely in *in vitro* culture response in respect of callus induction, callus health and plantlet regeneration. Similarly suitability of medium and appropriateness of exogenous plant hormones, which are the key factors for culture of somatic cells found to vary considerably as gleaned from a

wide variety of results. It has also been evident that other constituents of the culture medium like carbon sources, organic adjuvants and gelling agents hold profound influences in governing *in vitro* culture response. Keeping all these in account somatic tissue culture experiments were conducted systematically over a span of 4 years period and the results constitute the first chapter of the thesis.

Somatic embryogenesis ensures production of true to type plantlets, which is a prerequisite in the development of transgenic plants. Section of suitable variety that responds readily in respect of somatic embryogenesis and plantlet regeneration is one of the major components of *Agrobacterium*-mediated genetic transformation. To exploit the role of individual genotypes in governing tissue culture response in rice, nine varieties viz. IET 13856, IR 31851-6-6-3-3-2, Milyang 55, Taichung-Sen Yu, Quing Livan 1, BG 1639, Nanjing, C1136-3 and SIPI 681032 were selected. The *in vitro* culture response varies largely among the genotypes. Among the nine varieties assessed in the present experiment, IET 13856, IR 31856, IR 31851-6-6-3-3-2, Quing Livan 1 and C1136-3 were found to be the most responsive varieties in respect of callus induction and average number of plantlet regeneration. These four varieties were further selected for successive experiments of this thesis. Optimising the appropriate nutrient media composition for profuse callus induction, multiplication and regeneration into plantlets is also equally essential for genetic transformation. It is mentionable that nutritional requirements of different species vary largely even among the genotypes within a same sub species. So, manipulation of the basal media has led to the formulation of a number of tissue culture media. MS, B5, N6, AA, KPR-2, LS are commonly reported media being used extensively in rice tissue culture. With a view to pinpoint the maximum response that was exerted by the four selected promising varieties, four basal media viz. MS, N6, B5, LS were tested. Among the four basal media MS ensures best response like many observations made by the earlier investigators in rice.

Carbon source plays a key role in *in vitro* culture response. Its concentration is crucial since it bears profound influences in governing osmotic regulation. In the present study it was observed that 3% glucose and sucrose were equally effective in relation to

seed germination, callus induction and plantlet regeneration percentages. However, average number of plantlet regeneration per seed callus was maximum at 3% sucrose in all the varieties. Further experiments were undertaken to identify appropriate hormones and to optimize their level and combinations. The synthetic auxin 2,4-D displayed best response. A few other hormones viz. IAA, IBA, NAA, BAP and kinetin were also used in the callus induction, callus maintenance and regeneration media in three separate experiments. Results revealed that 2,4-D and 2,4,5-T in different combinations with hormones in CIM, 2,4,5-T along with hormones like 0.5 mg l^{-1} NAA, kinetin and BAP in CMM, 2 mgl^{-1} BAP+1 mgl^{-1} kinetin +1 mgl^{-1} NAA and 1 mgl^{-1} BAP + 2 mgl^{-1} kinetin + 0.5 mgl^{-1} NAA in RM showed optimal response in respect of plantlet regeneration per callus. Mean performance of Picloram was found to be discouraging in respect of average number of plantlet regeneration as compared to 2,4-D and 2,4,5-T. The addition of hormones in optimum dose could increase *in vitro* culture response when added to CIM. However, results showed that addition of hormones in CMM had no significant role rather induces extensive early rooting, which ultimately disturbed shootlet differentiation and consequently reduced the average number of plantlets per seed callus.

Many undefined components have been reported to have influence in promoting somatic embryos. Efforts were made to find out the effect of different adjuvants at varying concentrations in optimizing callus induction, plantlet regeneration and average number of plantlet per seed callus. All the varieties assessed in this experiment showed maximum plantlet regeneration when 10% coconut water was used in callus induction and plant regeneration media. Average number of plantlets were also found to be increased. Among the gelling agents agarose was found to be best for both callus induction as well as plantlet regeneration followed by agar. On an average IET 13856 showed maximum callus induction and plantlet regeneration in all the experiments. So, IET 13856 was choosen as an experimental variety for genetic transformation through *Agrobacterium* along with a few other varieties. It is mentionable that *Agrobacterium*-mediated genetic transformation is also genotypes dependent. It was reported earlier that most *in vitro* culture responsive rice varieties may not be equally amenable to

Agrobacterium. Sometimes poor culture responsive varieties produced maximum transformants.

Various explants have been used to induce callus in rice by many investigators. Mature seed embryos may be made available at any time, however, constant efforts are being made to search out novel explants for establishment of efficient somatic tissue culture system in rice. Involving two promising *indica* varieties viz. Quing Livan 1 and IET 13856 were employed for root callus induction. Roots from *in vitro* growing seedlings of 4, 8 and 11 days were employed. Quing Livan 1 showed quick callus induction than IET 13856. Maximum callus induction per cultured root segment was observed in Quing Livan 1 in comparison to 8 days old seedlings, which were found to be superior than 4 and 11 days old seedlings. Induced calli in both the varieties when passaged through embryogenic induction medium (EIM) containing MS with 0.1% CH and 1 mg l^{-1} ABA, produced maximum regeneration in both the varieties. Where as, the calli which were directly tested for plantlet regeneration without passaging over EIM showed substantially less plantlet regeneration, which showed indispensability of EIM for optimum plantlet regeneration. Results gleaned fro the first experiment showed the more appropriateness of the 8 days old *in vitro* grown seedling for callus induction and plantlet regeneration involving root explants. Comparative assessment of root callus and mature seed embryo derived callus in another experiment revealed that the root calli induced in Quing Livan 1 was found to be better than IET 13856 in respect of average number of calli induced per root segment as well as plantlet regeneration. However, the performance was found to be inferior to mature seed derived calli in respect of plantlet regeneration. It was observed that the calli passaged through EIM in roots as well as somatic seed embryos displayed more plantlet regeneration. This shows ample scope of root callus in establishment of efficient somatic tissue culture in rice, which requires more elaborate experiments involving more varieties and variations in physical and chemical environments.

Transgenic plants in rice have been developed only very recently, though development of transformed calli in rice dates back to 1986. From a position of neglect,

now rice has acquired an important position in transgenic research and is being treated as model system for transgenic development in allied cereal crops also. Varying success in different gene transfer methods is discernible. Among the three races, transgenics were developed more in *japonica* rice probably because of there more amenability to *in vitro* culture system and major efforts were mounted on them since long back. However, since *indica* rice constitute the major varieties of the Asian countries, attention is being paid for the development of transgenics intensively in *indica* varieties too recently. Within a short spell of time, the success achieved is exciting and holds immense promise for the future work. Contrary to the expensive nature of microprojectile based transformation and pain staking and skill demanding protoplast based transformation systems, *Agrobacterium*-mediated genetic transformation offers more promise due to less cost and less skill intensiveness. To exploit the potential of *Agrobacterium*-mediated genetic transformation for the development of an optimised protocol to effect efficient gene transferred in *indica* rice an array of well orchestred experiments were undertaken. The results, which constitute the second chapter of the thesis, are highlighted briefly.

While evaluating transformation efficiency involving different *Agrobacterium* strains, the frequency was found to range from minimum 11.6% with EHA 105(1301) to 87.5% with LBA 4404 (pTOK 233). The *Agrobacterium* strain LBA 4404 (pTOK 233) showed deep GUS staining of the entire transformed calli that revealed that occurrence of full calli transformation. While in case of other *Agrobacterium* strain ¼ th to 2/3 rd calli were found to be stained. Results advocated the superiority of LBA 4404 (pTOK 233) in rice transformation over other strains.

Among rice varieties, extensive variation in transformation efficiency was observed. Scented rice displayed maximum transformation efficiency than elite high yielding varieties of Indian origin and elite lines of IRRI origin. Tarori Basmati was found to be more amenable to *Agrobacterium*-mediated genetic transformation between three Basmati varieties. Among the HYVs tested, IET 13856 showed good transformation efficiency. Preincubation of calli prior to transformation led to drastic reduction in the transformation efficiency in comparison to the control sets, in majority of the varieties.

This indicates that preincubation of embryogenic calli is not required to increase transformation efficiency rather it reduces the efficiency, so may be avoided in future works.

Among several other factors, transformation frequency was found to vary in future works among calli of different age groups. Results indicated that 60-90 days old calli showed full calli transformation as evidenced from complete deep blue GUS staining. It is also mentionable that the somatic embryos and proembryos were found to be good recipients of the foreign genes in *Agrobacterium*-mediated genetic transformation. On subsequent subculturing of calli on callus maintenance medium, profuse somatic embryos with high totipotency were developed, which was found to be less in 10 days old calli. This was probably the main reason of encountering less transformation frequency in 10 days old calli.

Recent studies revealed that monocots can be made amenable to *Agrobacterium* be using external sensors which activates vir genes. Role of phenolics in transformation was found to be indispensable to activate vir genes. They were found to facilated genetic transformation of plant cells through *Agrobacterium in natura.* The phenolics are externally added to the culture medium during genetic transformation. Results indicate that the acetosyringone alone was enough in increasing transformation frequency. Several other phenolics viz. ferulic acid, vanillin and ethyl vanillin were also used to replace acetosyringone, however, this results were found to be non appreciable.

Comparative assessment of somatic embryogenic calli in respect of *Agrobacterium*-mediated genetic transformation involving LBA 4404 (pTOK 233) revealed that androgenic calli were fully transformed whereas, mature seed derived calli showed 85% GUS expression. However, androgenic calli could not be regenerated into plantlets as they were found to be very sensitive to antibiotic wash and loss their totipotency due to repeated washing with cefotaxime, which was required to wash off excess Agrobacterial cells from putatively transformed calli after co-cultivation. In another experiment, calli derived from mature seeds and anthers were compared with

calli derived from coleoptiles and roots. It was observed that coleoptile and mature seed derived calli showed more or less similar transformation efficiencies while calli developed from roots results in very transformation frequency. Attempts were also made to develop putatively transformed plantlets in rice following the conditions found to be optimal for effecting maximum genetic transformation gleaned from earlier experiments in Chapter II. Only 84 putatively transformed plants could be developed. Molecular analyses through slot and Southern blot revealed that 14 plants were gus positive, 10 plants were hpt positive and only 8 plants were found to be both gus and hpt positive. While analysizing the band size among the 14 hpt plants, 9 were found to carrying expected 1.1kb size bands that indicates no alteration in the transgene size had occurred before integration to the rice genome. In *Agrobacterium*-mediated genetic transformation this advantage has well evident in many other cases.

Electroporation offers an easy to handle root for gene transfer. Mature seed derived calli from Pakistan Basmati was tested involving different combinations of voltage, capacitance and time constant. Based on the principle of providing a long pulse to make pores on the cell membrane for entry of transgene followed by a short pulse to seal the pores, electroporation technique supposed to be effective in dispensing foreign genes even in rice. However, optimization is a prerequisite in terms of voltage, capacitance and time constant with simultaneous standardization of electroporation buffer. Two sets of calli, one pretreated on ice for 10 minutes before and after electric repulses and another without cold shock was used in all the treatments. Results indicated that electric pulse set at 180 Vcm^{-1} (actual 186) and capacitance 1000μF (actual 791) produced maximum transformants in treated calli with set resistance of 600 μF (actual 50) and time constant of 86.3 and 79.4 m Sec as pulse I and pulse II, respectively. Electric pulse set at 190 Vcm^{-1} (actual 195) and capacitance 900μF (actual 689) with constant resistant 600μF (actual 50) showed electric sparking with zero transformation. The calli exposed of this set of treatments could not surviving even on antibiotic free medium. The actual combination of voltage and capacitance that would be tolerable plant cells reported to be crucial in executing electroporation experiments. In the control set 40% transformation efficiency was observed with the same set voltage capacitance

and time constant. This indicates null relationship of the transformation efficiency with pre and post cold treatment of calli. Anyhow the results gleaned from this experiment prospects ample scope of electroporation in effecting gene transfer of alien gene into rice system through electroporation. However, it required massive experimentation to optimize the entire protocol involving diverse genotypes, explants, physical and chemical environments so that a generalize protocol may be recommended.

Summary.

In nutshell, the result of the experiments in respect of *in vitro* culture response and plantlet regeneration as well as on optimization of *Agrobacterium*-mediated genetic transformation as well development of transgenic plants are found to be very much encouraging, which prospects ample scope of genetic transformation of *indica* rice. Varieties like IET 13856 may be used for transgenic development in future with more economical and agriculturally important genes through *Agrobacterium*-mediated genetic transformation with ease confidence, which may have far reaching consequences in the pearlance of agriculture.