Genetic and Cytoplasmic Diversification of Pearl Millet Hybrid Parents

KN Rai, Ranjana Bhattacharjee, RP Thakur, Rajan Sharma, CT Hash, Vincent Vadez, BIG Haussmann, MA Mgonja, HD Upadhyaya and Michael Blummel

1. Background and Rationale

Pearl millet (Pennisetum glaucum (L.) R. Br.) is an important cereal crop, grown on 26 million ha in the arid and semi-arid tropical regions of Africa and Asia, with India having the largest area of 10 m ha. It is a highly cross-pollinated crop with high degree of heterosis for grain yield, which can be exploited through hybrids and open-pollinated varieties (OPVs) as the two broad cultivar options. A commercially viable cytoplasmic-nuclear male sterility (CMS) system discovered in the USA and made available to pearl millet improvement programs in India laid the foundation for hybrid development in India in 1960s, which is the only significant pearl millet hybrid growing country in the world. Since then, extensive use of this CMS source in hybrid development, and search for new CMS sources was made at ICRISAT and elsewhere, mostly in India. Research during this period showed that single-cross hybrids have 25-30% grain yield advantage over OPVs. Also, during this period, there has been a gradual change in emphasis in Indian public sector towards hybrid development, and emergence of a large number of seed companies who have interest in hybrids only. Hybrids (to start with topcross and inter-population hybrids) hold promise for increasing the productivity of this crop in the African regions as well: there has been negligible hybrid research on this crop in these regions. A hybrid program, just initiated in the Western and Central Africa (WCA) region, is attempting to classify adapted landraces into heterotic groups for more targeted exploitation of genetic diversity in hybrid parents breeding and hybrid development.

The NARS and the private sector in India on one hand and ICRISAT on the other have a great deal of complementarities with respect to rapid and informed access to the global germplasm, research infrastructure, interdisciplinary integration, short-to-long term vision, product delivery perspectives, and linkages with various players on the germplasm use-cultivar adoption continuum. Taking these factors into account, ICRISAT-Patancheru has undertaken to emphasize more of the germplasm evaluation and introgression, development of improved breeding lines and parental lines of hybrids, and generation of scientific information related to enhanced breeding efficiency (all as IPGs), leaving development, testing and release of hybrids to the NARS and the private sector.

2. Principles and Strategies

In Asia region, development of hybrid parents has largely targeted relatively better-endowed environments, largely influenced by greater breeding efficiency (low CV and less crop failures), greater probability of producing high-yielding hybrids, which, by nature, are more responsive to improved management inputs, and the private sector and NARS thrust on hybrid testing and release for such environments (more consistent and reliable seed demand). Breeding parental lines with high grain yield potential has been the major selection criterion because (i) yield potential of seed parents is important from the viewpoint of seed production cost, and there is a large body of data showing that the correlation between the yield potential of lines per se and their general combining ability is either significant and positive, or non-significant. High levels of resistance to downy mildew has also remained a high priority trait as there have been epidemics of this disease on single-cross hybrids during the 1965-1990. Trait-based breeding for seed size, maturity, tillering ability, panicle size, and lodging resistance, etc. has been followed to develop
parental lines that can address a diverse range of agro-ecological environments and farmers preference. Emphasis in seed parents breeding has been for the dwarf-to-semi-dwarf height (important for quality seed production under high management) and mostly medium-height pollinators (important for producing hybrids of medium to medium-tall height where fodder has additional value for the farmers).

The focus so far has been on the development of breeding lines useful for grain hybrids, with increased yield and downy mildew (DM) resistance as the major criteria. Based on NARS needs and demand in the Asia region, a low-key forage research was initiated 3-4 years ago. Considering the high levels of its salinity tolerance, and the vast scale of salinity problem in Asia, research has also been recently initiated to identify germplasm and improved populations/breeding lines adapted to such environments. Supported by the HarvestPlus Challenge program, research has also been initiated to identify germplasm and improved populations/breeding lines with high levels of grain iron (Fe) and zinc (Zn), and further improved these traits through genetic enhancement.

Considering the relatively greater complexity of seed parents breeding, and much narrower genetic and cytoplasmic base of seed parents as compared to the restorer parents in all the organizations, much greater emphasis during the past 20 years was given to seed parents research and development. Based on the private sector and NARS demand, restorer parent research and development was also included, albeit as second priority, in hybrid parents research. Over the time, these researches have been conducted through expanding research partnerships involving the public sector institutes in India and abroad, and the private sector in India.

In the WCA region, heterotic hybrids will initially be used for the development of open-pollinated varieties (the major thrust area of the program), with the gradual shift to topercross hybrids. Development of male-sterile lines adapted to Sahelien zone will receive greater emphasis as compared to the Northern Soudanean zone. The hybrid program in the Eastern and southern Africa (ESA) region can exploit the male-sterile lines developed for Asia region. Population pollinators (to be used as male parents of topercross hybrids) will be developed from improved populations and elite landraces found adapted to these respective regions.

3. Activities and Achievements

3.1. CMS search and characterization: The entire hybrid development program has been (and still continues to be) based on the single CMS system (A1 system). This large-scale cytoplasmic uniformity exposes the hybrid seed industry to potential disease and insect-pest epidemics. Thus, efforts were made to assemble CMS sources developed elsewhere, initiate search for new CMS sources at ICRISAT, and characterize these for stability of male sterility, their maintainer frequency in the germplasm, character association, and their any possible impact on restorer line development. Isonuclear A-lines of five CMS sources assembled from other sources (A1 and A4 from USA, A2 and A3 from India, and A5 from France) and two CMS sources identified at ICRISAT (Aegp from an Early Gene pool, and A5 from a Large-seeded Gene pool) were developed in each of the three diverse genetic backgrounds (81B, 5054B and ICMB 88004). Initial evaluation of A-lines for pollen shedders (a measure of stability of male sterility) had shown that the A2 and A3 sources were most unstable. Further evaluation of A-lines with other CMS sources during the rainy, and mild and hot summer environments showed that A-lines with Aegp cytoplasm (0.0-0.1% pollen shedders, depending on the nuclear genetic backgrounds and the environments in which A-lines were evaluated), and those with the A4 cytoplasm (0.0-0.3% pollen shedders) were more stable than those with the A1 cytoplasm (0.0-2.5% pollen shedders).
The A-lines with the A₅ cytoplasm had no pollen shedders, implying that A₅ CMS system was the most stable.

Evaluation of male sterility of topcross hybrids developed from crosses between isonuclear A-lines with the A₁, A₄ and A₅ cytoplasm and several improved populations from Asia and Africa regions showed that the frequency of maintainers was highest for the A₅ system (85-98%), followed by A₄ system (mostly 25-50%), and it was the lowest for the A₁ CMS system (mostly 10-40%), thus providing the prospects of greatest genetic diversification of A-lines with the A₅ system, and then with the A₄ and the A₁ systems, in that order. Multi-environment evaluation of isonuclear hybrids showed that hybrids based on the A₄-system A-lines had about 95% of the grain yield of those based on the A₁-system A-lines; and hybrids based on the A₅-system A-lines had 99% of the grain yield of those based on the A₁-system A-lines. There has been no indication that the A-lines with the A₄ and A₅ cytoplasm are any more or any less susceptible to downy mildew (DM) than those with the A₁ cytoplasm, the latter shown in several studies not associated with DM susceptibility.

### 3.2 Cytoplasmic diversification of seed parent:

Although the seed parent development using the A₁ CMS system continues as most of the parental lines in the public and private sector breeding programs are not restorers of the A₄ and A₅ cytoplasm, there has been an increasing emphasis at ICRISAT on the utilization of the A₄ and A₅ CMS systems in seed parents developments, with a parallel effort on developing their restorers (see section 3.4). For instance, of the 99 designated A-lines developed till 2004, 62 are based on the A₁ cytoplasm, 33 on the A₄ cytoplasm, and only 4 on the A₅ cytoplasm, while of the 245 A/B pairs at the initial backcross stages, 83 backcross progenies had A₁ cytoplasm, 100 had A₄ cytoplasm, and 52 had A₅ cytoplasm. In addition, maintainers of the morphologically diverse 34 A₁-system A-lines were converted into A₄-system A-lines as well as into A₅-system A-lines. This provides a wide range of genetically diverse A-lines of these two new and the most stable CMS systems. These two new and highly stable CMS sources also provide opportunities to develop stable male sterile populations (as was demonstrated with the development of a male-sterile population using the A₄ CMS system and NCd₂ population as a test case) that can be used to breed inter-population grain hybrids (for Africa regions), and will be particularly useful for breeding forage hybrids. In the WCA region, all three CMS systems (A₁, A₄ and A₅) will be used for seed parent development.

### 3.3 Genetic diversification of seed parents:

Given that seed parents are stable for complete male sterility across seasons and sites, high grain yield and high levels of DM resistance with acceptable to high degree of lodging resistance are the basic requirements for all hybrid parents, especially seed parents. Breeders’ requirements for other traits such as maturity, tillering ability, plant height, panicle size, and grain size and color, however, vary considerably, depending on the agro-ecological conditions and farmers’ preference. Thus, a trait-based approach in breeding seed parents with high grain yield potential and high levels of DM resistance has been followed, resulting in the development of large diversity in seed parents. For instance, the flowering of the 99 of the designated A-lines ranges from 39 to 71 days, with 22 of these flowering in 39-45 days (useful for breeding early-maturing hybrids), and 73 flowering in 46-55 days (useful for breeding dual-purpose hybrids). The panicle length of these lines varies from 10 to 40 cm, panicle diameter from 15 to 40 mm, no. of panicles plant⁻¹ from 1-5 and 100-seed mass from 7 to 16 g. This large morphological diversity stems from focused selection for these traits. It must also reflect on large genetic diversity among these lines as 82 of these lines involve germplasm or composites in their parentage (3 from direct selection in the *iniasi* germplasm; 14 from direct selection in 7 composites; and 56 from crosses involving germplasm, and 9 involving composites in the crosses). Germplasm introgression for specific traits generated new variability. For instance, introgressed lines with large seed size (1000-seed mass of 18-20 g compared to mostly 10-12 g in
commercial parental lines) and long panicles (45-60 cm compared to mostly 20-25 cm in commercial parental lines) have been produced. In the WCA region, based on the information on heterotic pools, lines and populations with maintainer reaction will be used for seed parent development.

Characterization of the DM pathogen populations for virulence showed that there are at least five major pathotypes in India. While the collection of pathogen populations from new areas in India and their characterization for virulence diversity is a continuing process, evaluation of breeding lines at various stages of inbreeding and selection for two diverse and major pathotypes (one from Durgapura in the northern India, and the other one from Jalna in the central India), is followed by evaluation of candidate lines (lines having potential for designation and dissemination) for resistance to all the five pathotypes. All this screening is done through seedling inoculation under high disease pressure (generally >90% disease incidence in the susceptible checks) in the greenhouse. Those found highly resistant (<10% disease incidence) to at least two of the pathotypes are designated and disseminated as seed parents (5-9 A/B pairs every year). This breeding strategy has led to the development of several A-lines, most of which are resistant to 3-4 pathotypes, with some resistant to all five pathotypes.

3.4 Restorer parent development: After a gap of 15 years, restorer parent research was revived in 2000, following the same approach as in seed parent, with respect to trait-based breeding for morphological diversity, high grain yield potential and high levels of DM resistance, followed by their designation and dissemination. For the first time in 2006, nine R-lines (5 A₁ and 4 A₄ restorers) were developed that flowered in 46-60 days, had 11-30 cm of panicle length, 130-200 cm plant height and 5.5-13.5 g of 1000-seed mass. These were highly resistant to at least two of the pathotypes, with eight of these resistant to four of the five pathotypes. R-lines with greater diversity will be developed and disseminated in the future as a large number of advanced breeding lines developed from diverse populations and OPVs bred in Asia and the southern Africa regions are currently being characterized for their male fertility restoration of the A₁ and A₄ CMS systems. Results of a bi-directional recurrent selection for male sterility and fertility restoration of the A₁ and A₄ CMS systems in two populations showed that 2-3 cycles of selection could convert almost any population into nearly complete maintainer and restorer versions, respectively, thus enhancing their value for maintainer and restorer line development. These results also apply equally well to the A₅ system. For the A₅ CMS system, a pedigree breeding program from the planned crosses has been initiated, and early-generations breeding lines have been developed. A backcross breeding procedure to breed restorer lines from the promising A₁-system restorers (otherwise maintainers of the A₄ and A₅ CMS systems) was developed. Using this breeding scheme, 4 lines from ICRISAT and 9 lines from the NARS were converted into their A₄ restorer versions, while 29 lines from ICRISAT and 10 lines from NARS were converted into their A₅ restorer versions. The backcross breeding produced not only restorers of these alternative CMS systems, but also showed the ease with which this can be accomplished. In WCA and ESA regions, populations producing high-yielding hybrids will be improved for restorer gene frequency by recurrent selection.

3.5 Potential parental lines of forage hybrids: Some of the male-sterile lines with dual-purpose characteristics (bred for grain hybrid) have been tested for the forage potential of their hybrids. The initial attempts led to the identification of ICMA 00999, which produced a high-yielding experimental topcross hybrid (ICMA 00999 x IP 17315). This hybrid gave 15-30% more dry forage yield than a released hybrid Proagro 1 at 80-d harvest across several trials. Subsequent experiments identified a forage type OPV that yielded as well as this experimental hybrid (13 t
3.6 Genetic enhancement for stress tolerance: Drought and soil salinity are the two widespread abiotic production constraints in pearl millet in most of the traditional pearl millet growing areas. Pearl millet is the most drought and salinity tolerant warm-season cereal. Recently, pearl millet has emerged as an important crop for summer cultivations in parts of Gujarat state of India where air temperatures during flowering can go as high as 46-48°C. Most of the hybrids fail to set any seed at that high temperatures, but a few set excellent seed, and have wider farmers acceptance. Conventional approach to breeding for drought tolerance in pearl millet has not been successful. Thus, a resort was made to molecular marker-assisted breeding for this trait, and the initial results look promising (see section 3.8).

Salinity-tolerance research showed large variability for tolerance at all stages, right from germination to grain filling. A standardized pot screening protocol was developed, some of the highly tolerant hybrid parental lines (ICMP 451, HTP 94/54, ICMB 95222, ICMB 0211), released OPVs (Raj 171 and GB 8735), an improved population (HHVBC Tall) and germplasm accessions (IP 22269, IP 36616, IP 6098) were identified. It was shown that salinity tolerance at the early vegetative stage was not correlated with salinity tolerance for grain and stover yield, there was highly significant and positive correlation between salinity tolerance for grain yield and stover yield ($r=0.89$), the correlation between biomass yield under salinity and salinity tolerance index was positive and highly significant ($r=0.92$), and that Na$^+$ concentration in the shoot was negatively correlated with early stage salinity tolerance ($r=-0.6$). These results have implications in breeding for salinity tolerance. A preliminary field screening at Jodhpur in the summer season showed most of the lines failing to set any seed, but individual plants in a few lines having 30-60% seed set at 46-48°C air temperatures during flowering.

3.7 Genetic enhancement for micronutrients: Research conducted under a HarvestPlus Challenge Program has identified high levels of grain iron (Fe) and zinc (Zn), mostly in breeding lines and populations (including some of the commercial OPVs and hybrid parents) with indica germplasm base. For instance, a commercial variety ICTP 8203, popular in Maharashtra state of India, has 78 mg kg$^{-1}$ Fe and 48 mg kg$^{-1}$ Zn.; and GB 8735 released in several African countries has 67 mg kg$^{-1}$ Fe and 52 mg kg$^{-1}$ Zn. Similarly, a highly drought tolerant and DM resistant seed parent 863B (female of three commercial hybrid) has 72 mg kg$^{-1}$ Fe and 53 mg kg$^{-1}$ Zn. It was also shown that there might exist large genetic variability for these micronutrients within populations as reflected in more than 2-fold variability detected among the progenies in six populations. For instance, the grain Fe content varied from 48 to 120 mg kg$^{-1}$ and Zn content from 36 to 90 mg kg$^{-1}$ among the progenies of ICTP 8203. Results from several experiments showed both micronutrients to be highly significantly and positively correlated ($r=0.75$), implying good prospects for simultaneous selection for both traits. Both micronutrients were also found to be predominantly under additive genetic control, with no evidence of hybrids exceeding the levels of parental lines with higher Fe and Zn contents, implying that to breed hybrid with high Fe and Zn contents will require the breeding of both parental lines with high levels of these micronutrients.

3.8 Biotech integration: Molecular marker-assisted breeding for DM resistance has been a high priority research area in pearl millet biotechnology, with an objective of pyramiding DM resistance genes in parental lines of commercial hybrids. Significant progress has been made as reflected in the identification of more than 55 QTL for resistance to 13 pathotypes (includes 6
major ones from India and 3 major ones from Mali). Development and release in 2006 of HHB 67-improved, a DM resistant version of the most widely cultivated and early-maturing hybrid HHB 67 (matures in 65 days and is cultivated on more than 0.5 million ha in parts of Haryana and Rajasthan states of India) is the greatest success story as it marks the release of the first public-bred cultivar of any crop developed following marker-assisted breeding in India. Gene pyramiding for DM resistance in several parental lines of commercial hybrids is underway. A similar biotech approach to breed for drought tolerance is at the proof-of-the-concept stage and the initial results look promising. For instance, a major QTL for panicle harvest index (a measure of drought tolerance), which contributes to improved grain set and grain filling, was identified. Backcross-derived lines with this QTL in the backgrounds of two sensitive lines have been found to have improved terminal drought tolerance, likely to arise, to a greater extent, from more profuse rooting in the deeper layers of the soils. For salinity tolerance and grain Fe and Zn, lines with large contrasts for these traits have been identified and development of mapping populations (one each for salinity tolerance and micronutrients) is underway.

4. Impacts

During the past 2-3 years, more than 70 hybrids (mostly from the private sector) have been reportedly grown on 5 million ha (about 50% of the total pearl millet area) in India. At least 60 of these hybrids are based on ICRISAT-bred A-lines, or on proprietary A-lines developed from improved lines bred at ICRISAT. This has made remarkable contribution to biodiversity (largest number of hybrids on-farm in pearl millet as compared to any coarse grain cereal), enhanced DM resistance (no DM epidemics in the last 15 years) and yield stability, and increased productivity (3-year mean grain yield increasing from 450 kg ha⁻¹ during 1970-1975 to 750 kg ha⁻¹ during 1982-84; 66% grain yield gain) for this hardy crop cultivated largely under most marginal environments with negligible external inputs. Since 1986 (when the first ICRISAT-bred hybrid was released) till 2004, 72 hybrids have been released in India, of which 43 hybrids (ie., 60%) are based on ICRISAT-bred A-lines. Of the 58 hybrids evaluated in the Initial Hybrid Trial of the All India Coordinated Pearl Millet Improvement Project (AICPMIP) during the 2005, 44 hybrids (ie., 75%) were based on ICRISAT-bred A-lines. During the three Scientists Field Days held in the recent past, we supplied 5217 breeding lines (2106 in 2000, 1312 in 2004, and 1799 in 2006) with a total of 22881 seed samples (12009 in 2000, 3318 in 2004 and 7554 in 2006) to more than 40 public and private sector research organizations. This has made substantial contribution to diversifying the genetic base of hybrid programs in India (and to a limited extent elsewhere).

The pearl millet hybrid parents research at ICRISAT has also impacted on inter-institutional linkages, generating the synergy for accelerated and large-scale impacts. The most unique of these alliances is ICRISAT-Private Sector Pearl Millet Hybrid Research Consortium with 32 seed companies, a Research Foundation, and a Seed Corporation as its members, who contribute to this program not only by way of financial support, but also through their informed inputs to research orientation (based on changing farming system scenarios, and farmers preference), and impact assessment. The partnerships with the NARS, largely through ICAR-ICRISAT partnership projects (now focused on hybrid parents research) has become stronger over time, in terms of resource sharing, joint project development, human resource development, and documentation of research results. During the last 2-3 years, the research partnership has further grown, and it now includes International Center for Biosaline Agriculture, Dubai, UAE (for salinity tolerance research); Central Asian NARS and Mexico (for introduction of pearl millet for crop diversification, which will eventually evolve into hybrid development); ILRI (for forage research), and the National Institute of Nutrition, Hyderabad (for micronutrient research).
5. Future Directions

As the relative value of stover is on rise, the major thrust of ICRISAT’s pearl millet hybrid program for Asia will continue to be developing parental lines of dual-purpose hybrids with high grain and stover yields, and high levels of resistance to downy mildew, targeted for relatively better-endowed environments. It is these environments where there are opportunities for making continuing genetic gains in Asia (especially India). For increasing pearl millet productivity through hybrids in Africa, lessons learned from Asia should provide the guidance in terms of trait and geographical focus, hybrid cultivar options (topcross and inter-population hybrids to start with, which should evolve towards single-cross hybrids, with back up support from biotechnology). The success of hybrid program targeted to arid environments both in Asia and Africa would largely depend on the availability of reliable test locations and their number, available resources, and partnerships for harnessing complementary skills (all of them currently unsatisfactory both in Asia and Africa regions). However, development of early-maturing seed parents and restorers with greater likelihood of their use in hybrid development for arid regions will receive relatively great emphasis than in the past. Increasing use will be made of more stable CMS systems (A₄ and A₅) for seed parents development and for breeding of their restorers. Apart from their value in seed parents of grain hybrids, these CMS systems will be of exceptional values in breeding seed parents of forage hybrids. The basic germplasm for breeding parental lines of forage hybrid has been identified, which will now be used for hybrid parents development, with concurrent search for additional germplasm sources for introgression (as also will be the case for breeding parental lines of grain hybrids).

Greater use will be made of molecular marker-assisted breeding for DM resistance gene pyramiding in commercial and prospective hybrid parents. Also, greater use of the marker technology is likely to be made for characterization of the parental lines of hybrids, diversity analysis, and classification of improved breeding lines into heterotic gene pools. Search for new germplasm sources and breeding lines with higher levels of tolerance to soil salinity and high air temperature stress during flowering, and high levels of grain Fe and Zn will continue as will continue the genetic enhancement for these traits, following conventional breeding approaches. Increasing use, however, will be made of molecular marker-assisted breeding to address these issues. Genetic enhancement of drought tolerance will entirely be done through this approach as the conventional breeding has not yielded satisfactory results in the past.

While the research in the Asia region will be focused on developing improved breeding lines and parental lines of potential hybrids, those in the WCA and ESA regions will go a few steps further in breeding, testing and facilitating the release of hybrids developed in partnerships with NARS in the respective regions. Since the improved breeding lines and populations developed for Asia region are unadapted in the WCA region, the regional program has to develop its own parental lines and populations for use in hybrid development. In case of ESA region, the seed parents developed for the Asia region can be evaluated and those adapted to the region selected for use in hybrid development. However, the restorer populations for use as male parents of topcross hybrids have to be developed from improved OPVs and landraces found adapted in the region.

For additional information/clarification, contact Dr KN Rai (k.rai@cgiar.org)
6. Key References


