Development of novel interspecific fertile cytotype (4x) of *Pennisetum glaucum* x *Pennisetum purpureum* utilizing modified ploidy coupled with embryo rescue technique

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Key words: BxN Hybrid; Embryo rescue; Fertile; Modified ploidy; *Pennisetum* interspecific hybrids

Abstract
Interspecific hybrids of genus *Pennisetum* (*P. glaucum* x *P. purpureum*) is the one of the most popular man-made hybrid. It combines the unique features of both *P. glaucum* (Pearl millet; Bajra) and *P. purpureum* (Napier; Elephant grass) species, which makes it more resilient to harsh environments with superior fodder quality. Due to ploidy level variation among the parents, these hybrids are sterile and propagated vegetatively only. To overcome this, attempts were made in the present study by exploring the feasibility of novel tetraploid pearl millet (2n=4x=28; Tetra 1; INGR 09047) developed at IGFRI, as a female parent in crossing program involving different Napier genotypes as male parent. Due to limited crossability and hybrid necrosis issues among countless crosses (>1000), only 1% seed set was initially recorded that too in shriveled state and the developing embryos were aborted after 10-14 days of pollination and fertilization. To save these, embryo rescue technique was standardized and the developing embryos were dissected out aseptically and rescued after 8-10 days of pollination. Continuous crossing programme along with screening of large tissue culture raised nurseries resulted in development of a novel tetraploid seed producing BN hybrid (TBN-20-15) along with 14 novel sterile tetraploid BN hybrids. Presence of univalent chromosomes leads to sterility while proper pairing between parents of TBN-20-15 hybrid have significant effect on fertility. The fertile hybrid is able to produce >15,000-20,000 seeds throughout the year with 80-90% seed germination ability. Their hybridity was confirmed by morphology, molecular and cytogenetic studies. This fertile tetraploid BN hybrid (TBN-20-15) reported for the first time globally will be very helpful in easy and cost-effective dissemination of this highly potential forage crop to the farmer's field. It has the potential to be the game changer in biofuel production, grassland rejuvenation programs besides bridging the fodder demand supply deficit.

Introduction
Napier grass, popularly known as elephant grass, (*Pennisetum purpureum* Schum.) is one of the most important forage and bioenergy crop of tropical and subtropical climates. It is valued for its high biomass, perenniality and pest resistance. However, due to its undesirable quality attributes such as hard and fibrous stem, coarse leaf sheath and leaf blade along with serrated leaf margins, it is less preferred by livestock's. On contrary, its relative pearl millet (*Pennisetum glaucum* (L.)), has high palatability with desirable traits like high biomass quality, non-shattering seed nature and drought tolerance (Serraj et al. 2005). Sexual compatibility between both of these species favours development of interspecific hybrids (Burton 1944, Hanna et al. 1984). Since napier (2n = 4x = 28) is an allotetraploid with A'A'BB genomes and pearl millet is a diploid (2n = 2x = 14) species with AA genomes, this ploidy level difference resulted in development of triploid (2n = 3x = 21) interspecific hybrids with AA'B genomes. These hybrids proved to be superior to both the parents in yield and quality. These interspecific hybrids are generally more acceptable as forage plants than elephant grass (Jauhar, 1981). Keeping in view of its superior fodder value, it is also known as King's grass (Dowling et al. 2014). In India, it is popularly known as Bajra Napier (BN) hybrid or BN hybrid and worldwide as PMN hybrids. However, due to their triploid genome with irregular chromosomal segregation (Techio et al. 2006), these interspecific hybrids are sterile in nature, which causes major bottleneck for its widespread distribution. So, the only way of propagation of these interspecific F_1 hybrids is through rooted slips/stem cuttings i.e., vegetatively. But it adds cultivation cost by transporting the bulk rooted slips. It was found that the cost of transportation is much more than the cost of rooted slips. Therefore, high labour requirements and cost associated with establishment have limited the widespread use of this highly potential fodder cum bioenergy crop, especially in developing countries. Looking at its potential and popularity among farmers, several workers accomplished either *in vivo* or *in vitro* polyploidization with the perspective of restoring its fertility (Gildenhuys and Brix, 1964; Hanna, 1981; Gonzalez and Hanna, 1984; Hanna et al. 1984; Rajasekaran et al., 1986; Diz and Schank, 1993; Abreu et al. 2006; Campos et al. in 2009; Faleiro et al. 2016). However, till date no such seed producing hybrid is available. One of the major reasons

for this may the use of chromosome doubling agents, which leads to occurrence of somaclonal variations, mixoploidy, irregularities, genetic and epigenetic alterations. Thus, some alternative should be accessed and, in this direction, the successful development of fertile/seed producing hybrids will have great implications. Here we report a modified breeding strategy assisted with embryo rescue technique to develop first seed producing fertile tetraploid interspecific *Pennisetum* hybrid. Overall objectives of our studies were: (1) inclusion and evaluating feasibility of tetraploid (4x) *P. glaucum* as a female parent in crossing program; (2) understanding the growth dynamics of embryo and abortion pattern; (3) standardization of embryo rescue and plant regeneration protocol for *Pennisetum* hybrids; (4) morphological, cytological and molecular characterization of novel fertile seed producing hybrid. Thus this study envisage the overall story of modified breeding strategies to develop seed producing interspecific *Pennisetum* hybrids.

Methods

Plant material and experimental site

Experimental material consisted of 65 accessions of *P. purpureum* and one tetraploid *P. glaucum* accession. *P. purpureum* accessions were maintained at Central Research Farm of ICAR-Indian Grassland and Fodder Research Institute, Jhansi, India (latitude, 25.5114° N; longitude, 78.5337° E and 271 m altitude above sea level). Tetraploid *P. glaucum* (Tetra 1; IG 99-748) was developed at ICAR-IGFRI, Jhansi, India via colchiploidy from a male fertile line 81B procured from ICRISAT, India (Kaushal et al. 1999).

Modified breeding strategy for interspecific hybridization

Tetraploid *P. glaucum* was used as female parent to cross with three diverse *P. purpureum* accessions *viz.* N2, N3 and N27 to get normal/balanced genome fertile progeny. Hybridizations were carried out individually as described by Kaushal et al. 2003. We attempted a large number of crosses (>10000) with tetraploid *P. glaucum* but even then it failed to fetch fertile progeny and there were high rate of embryo abortion even after successful crossing with high pollen load. Microscopic studies showed that even after successful fertilization, the embryos were aborted after 10-14 days of pollination. To overcome this, embryo rescue technique was standardized and more number of napier genotypes (total 65 accessions) were crossed with Tetraploid *P. glaucum*.

Standardization of embryo rescue and in vitro plant regeneration

Initially embryo rescue technique was standardised using developing embryos of tetraploid pearl millet with different concentrations of NAA and Kinetin as described by Kaushal et al. 2003. Once standardized, the developing embryos of different tetraploid pearl millet and Napier crosses were dissected out aseptically and rescued after 8-10 days of pollination and cultured on MS medium supplemented with NAA and Kinetin. Germinated embryos were then hardened and acclimatized in controlled and net house facilities of Crop Improvement Division and then lateron transferred to Central Research Farm, ICAR-IGFRI, Jhansi, India.

Cytological validation of novel Pennisetum hybrids

Cytological studies were carried out on the various interspecific BxN hybrids produced. For meiotic studies, the young developing panicles at boot stage were collected and fixed in Carnoy's solution (3 ethanol: 1 glacial acetic acid) as described by Kaushal et al. 2008 and observed under Leica DM2000 microscope.

Molecular validation of novel Pennisetum hybrids

DNA was extracted from young leaf tissues of all the putative hybrids along with parents of different crosses using the CTAB method by Murray and Thomson (1980) and was quantified as per Rana et al. 2019. For hybridity determination, SSR primers that distinguished both the parents either with dominant or co-dominant inheritance were selected for molecular validation of hybridity. For PCR amplification and agarose gel electrophoresis, procedure as described by Rana et al. 2019 was followed.

Pollen viability and stigma receptivity studies

Pollen fertility was estimated on the basis of stainability test. Freshly dehisced pollen grains were observed under Leica DM2000 micrsocope by staining with acetocarmine. The stigma receptivity was assessed using peroxidase test as described by Dafni and Maues (1998) with slight modification.

Results and Discussion

The present study was an attempt made for the first-time at ICAR-IGFRI, Jhansi, India to restore the fertility of BxN hybrid by exploring the feasibility of tetraploid pearl millet as a female parent in crossing program involving different Napier genotypes as male parent. Due to limited crossability and hybrid necrosis issues among countless crosses (>1000), only 1% seed set was initially recorded that too in shriveled state and the developing embryos were aborted after 10-14 days of pollination and fertilization. To save these, embryo rescue technique was standardized and the developing embryos were dissected out aseptically and rescued after 8-10 days of pollination and cultured on MS medium supplemented with NAA (1 mg/l) and Kinetin (0.5 mg/l). With this, seven interspecific hybrids were generated during 2017-18. Their hybridity was confirmed by morphology, flow cytometry and cytogenetic studies. Of these seven hybrids, two were not able to survive due to hybrid necrosis, while in rest five hybrids (TBN-18-1 to TBN-18-5), females were found to be fertile while male are still sterile due to the presence to cytoplasmic male sterility. Thus, five novel cytoplasmic male sterile (CMS) tetraploid BxN hybrids (TBN-18-1 to TBN-18-5) were developed during 2017-18. In order to restore the fertility of novel tetraploid BxN hybrids, these were crossed with 31 different Napier genotypes in hope of getting a restorer line. However, none of the cross was successful. Further, tetraploid male sterile pearl millet lines were also crossed with more number of Napier lines in hope of getting fertile hybrids. However, still sterile BxN hybrids were generated (TBN-19-6, TBN-20-7 to TBN-20-8) during 2018-19 and 2019-20. Alternatively, tetraploid maintainer pearl millet was crossed with different Napier lines to avoid any exchange of CMS traits. Interestingly, seven more tetraploid BxN hybrids were generated (TBN-20-9 to TBN-20-15) during 2020-21, however to our surprise, 6 are still sterile (TBN-20-9 to TBN-20-15) while one is fertile (TBN-20-15). This showed that it's not only CMS which governs the sterility but chromosomal pairing behavior between pearl millet and Napier chromosomes also have significant effect on fertility. Thus, continuous crossing programme along with screening of large tissue culture raised nurseries resulted in development of a novel tetraploid seed producing Bajra-Napier hybrid (TBN-20-15) along with 14 novel sterile tetraploid BN hybrids (TBN-18-1 to TBN-18-5, TBN-19-6, TBN-20-7 to TBN-20-8, TBN-20-9 to TBN-20-14). The fertile hybrid is able to produce more than 15,000-20,000 seeds throughout the year with 80-90% seed germination ability (Fig. 1). This novel tetraploid fertile Bajra-Napier hybrid (TBN-20-15) will be helpful in easy dissemination of this potential forage crop to the farmer's field and also for studying pairing behavior of pearl millet and Napier chromosomes.

Further, to characterize the novel BxN hybrids on the basis of cytology, meiotic studies of fifteen putative tetraploid BxN hybrids along with Tetra1 was studied. Tetra1 was a tetraploid *P. glaucum* line with chromosome number 2n = 4x = 28 (Fig. 2). Similarly, all the putative tetraploid BxN hybrids were analysed and validated as novel tetraploid BxN hybrid. The pollen grains of all putative hybrids were analyzed by acetocarmine stain and it was observed that all except TBN-20-15 hybrid were non-viable. The non-viability of pollen grains might be due to the formation of univalent chromosomes. Therefore, the reason for sterility and fertility between these hybrids was the chromosomal pairing behavior between *P. glaucum* and *P. purpureum* chromosomes. More univalent chromosomes leads to sterility as in case of TBN-20-13 while proper pairing between the parents of TBN-20-15 hybrid have significant effect on fertility (Fig. 2). Similarly, for hybridity determination at molecular level, a total 65 SSRs primers were screened in the parents and novel tetraploid BxN hybrids. Among them, 9 SSRs (BM 11, BM 6, BNM 20, BNM 23, BNM 15, NM6, BM 7, BM 12 and BM 14) showed the validation of either one parent or both parental bands in novel tetraploid hybrids. Two markers (BM 11 and BM 12) are *P. glaucum* specific. Whereas, rest seven markers showed the codominance pattern of inheritance for both Bajra and Napier (Fig. 3). These specific markers can be used in DNA fingerprinting, varietal identification and hybridity determination.

Conclusions and/or Implications

Feasibility of tetraploid *P. glaucum* as a female parent in crossing program involving different *P. purpureum* genotypes as male parent showed limited crossability with variation in abortion pattern. Different *P. purpureum* genotypes behaves differently as far as their crossing ability with tetraploid *P. glaucum* was concerned. Further, fertility of the interspecific hybrids was solely governed by pairing behaviour between *P. glaucum* and *P. purpureum* chromosomes. Interestingly, this fertile tetraploid BxN hybrid (TBN-20-15) reported for the first time globally will be very helpful in easy and cost-effective dissemination of this highly potential forage crop to the farmer's field. It has the potential to be the game changer in biofuel production, grassland rejuvenation programs besides bridging the fodder demand supply deficit.

Acknowledgements

The first author thanks Director, ICAR-Indian Grassland and Fodder Research Institute, Jhansi, Uttar Pradesh, India for providing necessary facilities and support throughout the execution of work. All the authors are thankful to Indian Council of Agricultural Research for providing funds for execution of the work.

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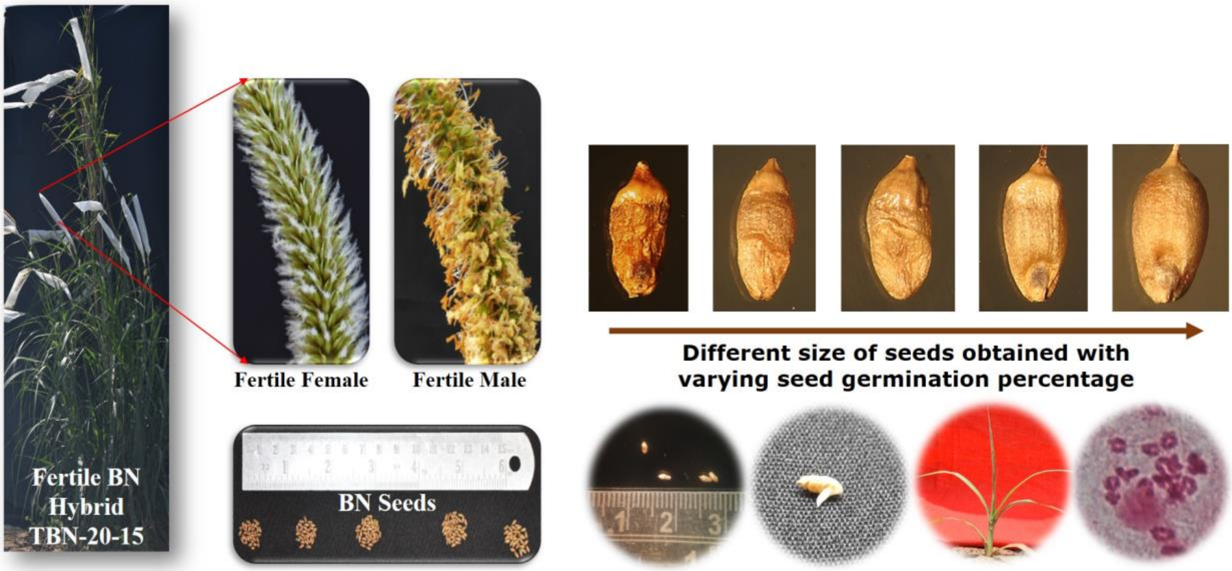

Fig. 1 Novel seed producing fertile tetraploid BxN hybrid (TBN-20-15)

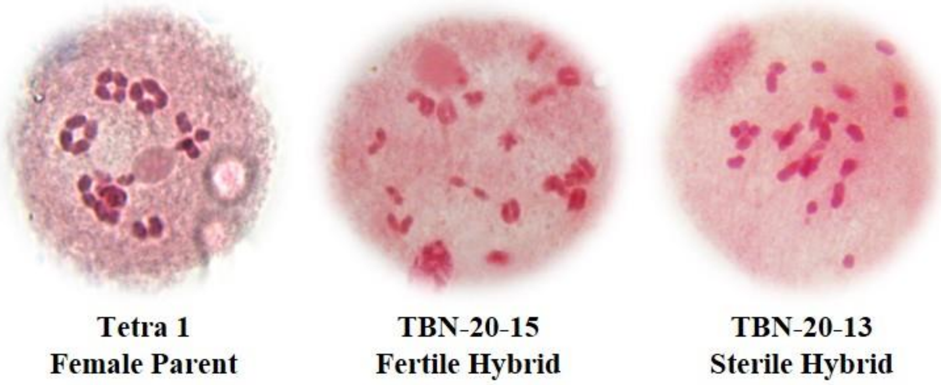

Fig. 2 Cytological differences among female parent, novel fertile and sterile interspecific BxN hybrids

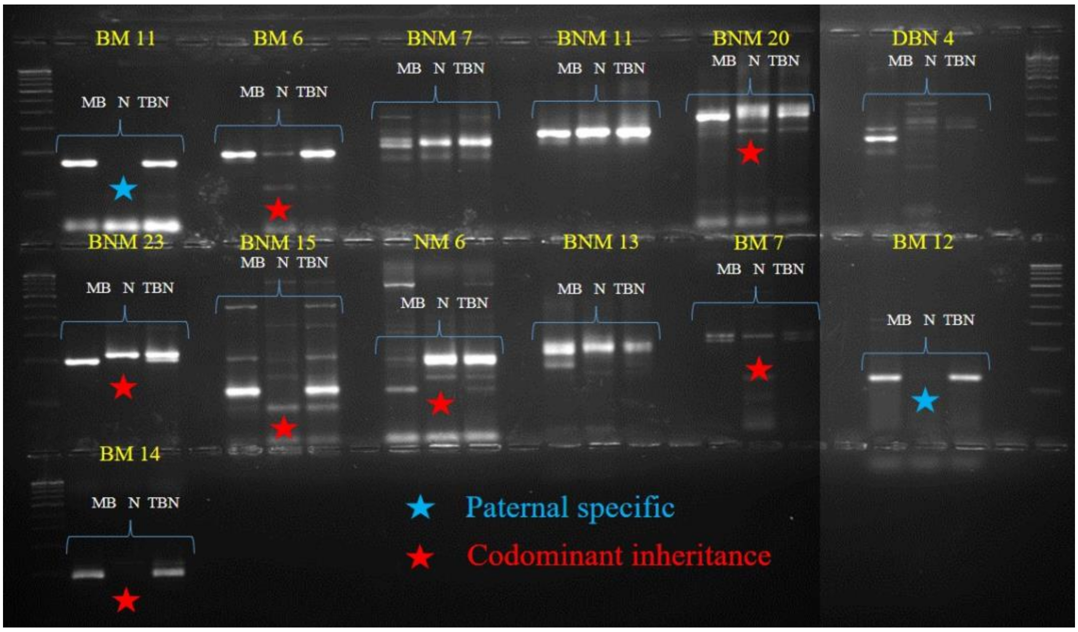

Fig. 3 Hybridity determination across Pearl Millet, Napier and novel tetraploid BN hybrids