Crossability and Characterization of Interspecific Hybrids between Sexual *Pennisetum glaucum* (Pearl Millet) and a New Cytotype ($2n=56$) of Apomictic *P. squamulatum*

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Summary *Pennisetum squamulatum* is an important species belonging to tertiary gene pool of cultivated pearl millet as potential donor of several genes. Recently a new cytotype with $2n=56$ chromosomes has been identified. Crossability of this cytotype has been studied with diploid and induced tetraploid pearl millet. The hybrids produced with this novel cytotype were compared for their characteristics with the hybrids previously reported with widely reported $2n=54$ *P. squamulatum* cytotypes. Strong incompatibility barriers were observed between $2n=56$ cytotype with diploid pearl millet, and hybrids could not be obtained even after embryo rescue. Limited crossability was obtained with tetraploid pearl millet and 2 successful hybrids were produced. Their hybridity was confirmed by morphology, flowcytometry and cytology. The hybrids were highly fertile, perennial and segregated for mode of reproduction. Segregation for other traits confirmed heterozygous nature of *P. squamulatum* $2n=56$ cytotype. Cytological investigations suggested $2n=8x$ nature of this cytotype. Successful hybridization between pearl millet and this new cytotype shall be important for introgression of useful traits as well as to study inheritance of traits such as perenniality, apomixis and tolerance to stresses.

Key words Apomixis, Interspecific hybridization, Pearl millet, *Pennisetum*.

Pearl millet (*Pennisetum glaucum* L., Poaceae) is an important crop utilized both for feed and fodder purposes in semi arid tropics. Exotic germplasm in the genus *Pennisetum* comprises of primary, secondary and tertiary gene pools with many species possessing desirable genes for pearl millet improvement such as resistance to diseases, male sterility, improved fodder characteristics and apomixis (Hanna 1987, Jauhar and Hanna 1998). *P. squamulatum*, a wild relative of pearl millet belonging to tertiary gene pool, harbours many desirable traits for pearl millet improvement, including resistance to biotic and abiotic stresses, perenniality, and apomixis (Jauhar 1981, Hanna *et al.* 1989). This is an obligate aposporous species and has widely been used to study inheritance and transfer of genes controlling apomixis in pearl millet background, as well to produce high quality perennial fodder types in combination with pearl millet and napier grass (*P. purpureum*), in the form of tri-specific or double cross hybrids (Jauhar and Hanna 1998). Previous reports have characterized *P. squamulatum* with $2n=54$ chromosomes (Patil *et al.* 1961, SreeRangaswamy 1972) and its interspecific hybrids have been successfully produced with tetraploid pearl millet ($2n=4x=28$) (Dujardin and Hanna 1983, Marchais and Tostain 1997).

Recently, a $2n=56$ cytotype of *P. squamulatum* was identified, characterized by obligate apomictic mode of reproduction, perenniality and tolerance to ergot, smut, frost and drought under field conditions (Roy *et al.* 2003). The present investigation was aimed at studying the compatibility of this cytotype with diploid ($2n=14$) and tetraploid ($2n=28$) pearl millet, and cytogenetical char-

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acterization of the hybrids, as well as to make a comparison with previously reported hybrids involving $2n=54$ cytotypes.

Material and methods

Plant material: *P. squamulatum* accession MA, a cytotype with $2n=56$, hereafter called (*P. squamulatum* MA), was an indigenous collection maintained at Indian Grassland and Fodder Research Institute, India. Pearl millet diploid ($2n=2x=14$) accessions viz., 81A (a male sterile line) and 81B (maintainer of 81A) were procured from ICRISAT, India. Tetraploid pearl millet viz., Tetra1 ($2n=4x=28$), was an induced tetraploid of 81B (Kaushal *et al.* 1999) maintained by self-pollination.

Pollinations and Embryo culture: Hybridizations were carried out individually with both diploid and tetraploid pearl millet using them as female parent (*P. squamulatum* being apomictic). The female inflorescences were bagged with glycine bags at appropriate stage to avoid cross-pollination, and were dusted with freshly dehisced pollen collected from *P. squamulatum* during 0900–1100 h. In crosses involving diploid pearl millet, the developing embryos were dissected out aseptically and cultured on MS medium (Murashige and Skoog 1962) supplemented with NAA (1 mg/l) and Kinetin (0.5 mg/l), following embryo-cloning protocol (Kaushal *et al.* 2003). Observations were recorded for embryo germination, seedling growth and their establishment in the field. Embryos from 81A×81B, and Tetra1 self pollinations were used as control.

Cytological investigations: Spikelets from plants were fixed in glacial acetic acid-alcohol (1 : 3) at appropriate stage and pollen mother cells (PMCs) were observed under bright field microscope following anther squash preparations in 2% acetocarmine stain. Observations were recorded for total chromosome counts, pairing behaviour and pollen fertility. Pollen fertility was observed by stainability of pollen from freshly dehisced anthers in 1 : 1 acetocarmine-glycerol.

Embryo-sac analysis: Embryo sacs were analysed for discrimination between aposporous and sexual embryo sacs, following Young *et al.* (1979) with modifications. Enhanced treatment of 3–4 h was given at each step for better penetration and clearing in the samples under study, as compared to 2 h treatment. Additionally samples were kept overnight for second change of 100% ethanol and 100% methyl salicylate. Unpollinated spikelets were fixed in FAA (18 formaldehyde:1 acetic acid:1 70% alcohol) for atleast 48 h prior to anthesis. Gynoecia were dissected and passed through series of alcohol for dehydration and methyl salicylate for clearing. Cleared ovules were mounted in absolute methyl salicylate and observed under phase contrast microscope (Olympus CX40). Aposporous and sexual embryo sacs were discriminated on observations made on cleared ovules.

Flow cytometry: Parents and hybrids were subjected to flow cytometrical observations. Nuclei were isolated from leaf tissue by chopping with sharp razor blade, stained in DAPI (4',6-diamidino-2-phenylindole) (buffer pH 7.0 containing 100 mM Tris-HCl, 5 mM MgCl₂, 85 mM NaCl, 0.1% Triton×100 and 1 µg/ml DAPI) and observed with Partec Ploidy Analyzer PAI. Hybridity of progenies between Tetra1 and *P. squamulatum* MA was confirmed on the basis of flow cytometer histograms, by comparative measurement of the DNA quantity of parents and hybrids.

Results and discussion

*Crossability of $2n=56$ *P. squamulatum* with pearl millet*

Crossability of *P. squamulatum* MA was estimated by recovery of hybrid seeds/plantlet upon pollination with diploid as well as tetraploid pearl millet.

A. *Crossability with diploid pearl millet:* The crossability was almost nil between diploid pearl millet (male sterile as well as male fertile) and *P. squamulatum* MA. All pollinations produced several highly shriveled seeds. Even after following embryo rescue, no successful hybrid could be

recovered, out of more than 300 pollinations followed with embryo rescue of about 300 immature embryos (Table 1). Though 26 and 37% embryos from 81A and 81B crosses responded to culture conditions, only 1 plantlet from each cross was obtained, but none could survive in the field. All embryos that responded to culture, exhibited swelling and delayed germination, but soon showed retarded and abnormal growth and died. These results indicated the occurrence of strong pre- as well as post-zygotic barriers in this cross.

Abnormalities associated with development of these hybrid embryos rendered failure of rescue efforts, as well as seed formation under natural condition. High difference in chromosome number (and ploidy) of the 2 parents ($2n=14$ vs. 56) might be one of the cytological reasons impeding growth of pollen tubes, embryo and endosperm, thereby causing lethality. Hybridization events involving diploid pearl millet and $2n=54$ *P. squamulatum* had limited success in the past. The cross was characterized by strong pre- and post-fertilization barriers (Mohindra and Minocha 1991, Kaushal and Sidhu 2000). Severe embryo:endosperm developmental abnormalities have been studied in crosses between diploid pearl millet and $2n=54$ cytotypes of *P. squamulatum* (Marchais and Tostain 1997). Diploid pearl millet pollinated by *P. squamulatum* ($2n=54$) gave high frequency of proembryo surrounded by extensive undifferentiated starch free endosperm. Dujardin and Hanna (1985, 1989a) also reported highly restricted crossability between the two species, though crosses with male-fertile line yielded 0.08% hybrid plants, that contained $2n=27$ or 28 chromosomes, were highly male and female sterile.

B. *Crossability with tetraploid pearl millet* ($2n=(4x)=28$): Numerous developing seeds were recovered when tetraploid pearl millet (Tetra 1) was pollinated with *P. squamulatum* MA, showing successful fertilization. However, since Tetra 1 was self-fertile, many seeds might have originated from self-pollination, and hence, the crossed seed set data was difficult to estimate. However, two categories of seeds were formed, *i.e.* well-filled seeds similar to Tetra 1 possibly arising from self-pollination, and secondly, relatively shriveled seeds that could have hybrid origin. These shriveled seeds were sown in the field in subsequent season. Out of a total of 318 established plants, 2 hybrids were recovered that showed distinct characters confirming their hybrid origin. These 2 hybrids were named as Hybrid H1 and H2 and characterized for their breeding and cytogenetic characteristics.

Hybridization with *P. squamulatum* ($2n=54$) cytotypes was also successful with tetraploid pearl millet (Dujardin and Hanna 1983, 1989a). Patil *et al.* (1961), reported hybrids using diploid pearl millet through, unreduced female gamete formation, which can be treated as equivalent to a gamete from tetraploid pearl millet. Marchais and Tostain (1997) produced fertile hybrids using tetraploid pearl millet and explained the successful hybridization events on the basis of endosperm balance number theory. Similar explanations were provided by Roche *et al.* (2001) in interspecific crosses involving *Pennisetum* species.

Characterization of hybrids

A. *Morphological characters*: The morphological characters of parents, hybrids as well as diploid pearl millet lines are represented in Table 2. The hybrids were intermediate to the parents, with preponderance of male-parent characters. Hybrids resembled *P. squamulatum* MA in tillering ability, perenniality, inflorescence characters (Fig. 1), shedding spiklets and short involucrel pedicels, while resembled female parent in leafiness and penicillate anther tips (more dense in H1 than H2). Hybrid H2 was more vigorous than H1. Segregation for certain characters, such as node colour and hairiness, violet stigma colour, tillering behaviour, and other traits observed in the hybrids suggests heterozygous nature of *P. squamulatum* MA, since Tetra 1 was homozygous being maintained by recurrent self-pollination. Some of these morphological characters were similar to hybrids reported between tetraploid pearl millet and $2n=54$ cytotypes of *P. squamulatum* (Dujardin and Hanna 1983, 1985, Marchais and Tostain 1997, Roche *et al.* 2001). These reports too suggested the heterozygous nature of *P. squamulatum*.

Table 1. Crossability of diploid and tetraploid pearl millet with *P. squamulatum* ($2n=56$)

Cross	No. pollinations	Embryo culture					Seed setting		
		Embryos cultured	Embryo responded	Embryos survived	Plantlets obtained	Plants established	Seeds obtained	Plants established	
81 A× <i>P. squamulatum</i> MA	198	208	55 (26.4%)	6	1	0	0	0	
81 B× <i>P. squamulatum</i> MA	108	115	43 (37.4%)	5	1	0	**	0	
Tetra 1× <i>P. squamulatum</i> MA	111	—	—	—	—	—	**	2	

** Numerous seeds obtained, including those arising from self-pollination, in addition to crossed ones, and hence figures not represented in text.

Table 2. Morphological characters of Pearl millet, *P. squamulatum* MA and their hybrids

S. No.	Plant	Plant height** ^a	Tiller no.	Spike length	Spike width	3rd leaf length	3rd leaf width	Flag leaf length	Flag leaf width	Stem girth	No. of nodes	Stigma colour
1	<i>P. glaucum</i> ($2n=14$)	92.3	4.7	22.3	2.4	44.7	2.7	22.0	2.5	1.0	4.7	White
2	<i>P. glaucum</i> ($2n=28$)	136.6	3.3	25.6	3.3	71.7	3.7	42.7	3.5	1.7	6.0	White
3	<i>P. squamulatum</i> MA ($2n=56$)	186.3	20	22.3	1.4	68.7	1.6	31.3	1.4	0.6	10.7	Violet
4	Hybrid H1	80	4	19.1	7.3	43.2	1.0	16.7	0.9	0.5	5	White
5	Hybrid H2	104	15	26.2	2.1	44.3	1.2	22.2	1.1	0.6	7	Violet

* all measurements in cm except for tiller number and number of nodes.

^a values represents mean of 3 replications for metric traits where ever applicable.

Table 3. Cytological and reproductive characteristics of parents and hybrids

S. No.	Material	$2n$	Average chromosome configuration	Pollen fertility (%)	No. Embryo sacs studied	Embryo sac type	Multiple embryo sacs	Aborted embryo sacs	Selfed seed setting (%)
1	<i>P. glaucum</i> 81A	14	$6.9_{II}+0.2_{I_1}$	0	50	Sexual	0	1	0
2	<i>P. glaucum</i> 81B	14	$6.83_{II}+0.34_{I_1}$	98	50	Sexual	0	3	93
3	<i>P. glaucum</i> Tetra 1	28	$1.26_{IV}+0.13_{III}+11.17_{II}+0.2_{I_1}$	94	50	Sexual	3	4	88
4	<i>P. squamulatum</i> MA	56	$0.05_{VIII}+0.6_{VI}+1.76_{IV}+0.25_{III}+22.2_{II}$	85	50	Aposporous	39	11	18
5	Hybrid H1	42	$0.44_{IV}+0.8_{III}+17.36_{II}+3.12_{I_1}$	84	55	Sexual	3	7	37
6	Hybrid H2	42	$0.009_{V}+0.44_{IV}+0.34_{III}+19.4_{II}+0.37_{I_1}$	65	61	Aposporous	31	13	13

Fig. 1. Spike morphology of parents and hybrids. L- to R: Tetra 1, H1, H2, *P. squamulatum* MA.

Fig. 2. Flow cytometric histogram from leaf samples expressing hybridity. Peaks-1: Tetra 1, 2: hybrids, 3: *P. squamulatum* MA, 4: G2 of Tetra 1.

Fig. 3. Chromosomes at Diakinesis stage of meiosis I. A: tetraploid pearl millet Tetra 1, B: *P. squamulatum* MA, C: Hybrid H1, D: Hybrid H2.

B. Cytological characters: Hybrids were easily distinguishable on the basis of flowcytometric observations. Tetra 1 and *P. squamulatum* MA had different DNA content and thus hybrids were identified on the basis of intermediate DNA content, as revealed by the flowcytometric histograms (Fig. 2). *P. squamulatum* MA contained 1.4 times higher DNA content than Tetra 1. Hybrids exhibited histogram peaks at intermediate value, and thus offered a method for rapid identification of hybrids. Martel *et al.* (1997) reported polymorphism in DNA content between the species, with 2.36 and 4.77 pg DNA per haploid genome for pearl millet and *P. squamulatum* respectively. Flowcytometric identification of hybrids is believed to be a reliable tool when the parents differ in DNA content and has been widely utilized (Doležel and Bartoš 2005).

Summary of chromosome behaviour during meiosis in hybrids as well as parents is represented in Table 3. Both the hybrids exhibited $2n=42$ chromosomes, 14 derived from Tetra 1 pearl millet and 28 from *P. squamulatum* MA. Meiotic divisions were near normal in both the hybrids with occurrence of occasional multivalents, univalents and laggards. H1 exhibited 0–1 quadrivalents in contrast to 0–4 quadrivalents in H2. Rarely, single pentavalent was observed in H2, indicating higher forms of chromosomal associations in H2, whereas H1 was relatively more balanced. Presence of multivalents might be a cause for reduced relative pollen-fertility in H2.

Both hybrids showed very high frequency of bivalents (17.4 in H1 and 19.4 in H2) (Fig. 3).

Fig. 4. Embryo-sac studies in hybrids. A–B: Sexual embryo sacs showing antipodals in Hybrid H1, C–D: Aposporous embryo sac, antipodals absent, in Hybrid H2. A&C: whole ovule, B&D: magnified embryo sac.

Chromosomes were well marked for their male/female origin based on their size at early diakinesis. Previous cytological observations in hybrids between pearl millet and *P. squamulatum* ($2n=54$) cytotypes had certain differences with the present report. The chromosome number was reported 41–42, and with upto 16 bivalents by Patil *et al.* (1961) while Dujardin and Hanna (1983) observed upto 20 bivalents. Normal disjunction of chromosomes at anaphase I and II was observed in present study in contrast to the earlier reports of 18–23 disjunctions (Dujardin and Hanna 1983). In the present study, regular meiosis with upto 21 bivalents and their equal distribution during anaphase supports $2n=8x$ nature of *P. squamulatum* (with basic chromosome number 7-similar to pearl millet), as suggested by Roy *et al.* (2003). Formation of 14 bivalents by 28 chromosomes of *P. squamulatum* in present hybrids suggests its octoploid ($2n=8x$) nature. Twenty-one bivalents possibly had arisen from pairing between chromosomes of same species, *i.e.*, 7 bivalents originated from 14 glaucum chromosomes and 14 bivalents from 28 squamulatum chromosomes. Higher forms of chromosome associa-

tions were expected from pairing within *P. squamulatum* MA complement because of its $4x$ genome present in the hybrids. However, chromosome behaviour in advanced generations, such as F₂ or back cross (BC₁) derived from these crosses are required to confirm about the ploidy status of *P. squamulatum* MA.

C. Reproductive characters: Hybrids H1 and H2 exhibited 84% and 64% pollen stainability, respectively, which was comparatively higher than the hybrids previously reported with $2n=54$ cytotypes. The parents also showed 94% and 85% stainable pollen from pearl millet Tetra 1 and *P. squamulatum* MA, respectively. Anther dehiscence in the hybrids was normal and the timings matched with male parent (flowering under short day conditions, in late October). Pollen fertility in both the hybrids (H1 and H2) were comparatively higher than the hybrids previously reported with $2n=54$ cytotypes. Patil *et al.* (1961) reported highly male sterile hybrids, whereas Dujardin and Hanna (1983) observed male fertility ranging from 29–79%. Dujardin and Hanna (1983, 1989a) attributed fertility of hybrids to partial homology between the pearl millet A genome and one or more genomes of *P. squamulatum*, however, in present study, the balanced chromosome segregation in meiosis arising because of preferential pairing within glaucum and squamulatum genomes leading to high bivalent frequency might be additional cause for increased pollen fertility. Further, higher male fertility of Tetra 1 (94%) against low fertility of earlier reported tetraploid pearl millet (*e.g.*, *ca.* 80% as in Dujardin and Hanna 1989b), as well as higher female fertility/seed set in present studies (88%) as compared to 1–3% seed set on selfing in previously reported tetraploids (Dujardin and Hanna 1989b) might have contributed to increased fertility of hybrids in this study.

Female fertility in 2 hybrids varied because of mode of reproduction. H1 was sexual in mode of reproduction while H2 exhibited aposporous embryo-sac formation (Table 3, Fig. 4). Apomixis in *Pennisetum* genus is governed by *Panicum* type aposporous embryo sacs, which are 4-nucleated,

lack antipodals, and are clearly distinguishable from 8-nucleated sexual embryo sacs having antipodals (Young *et al.* 1979).

Pearl millet Tetral exhibited all sexual embryo-sacs with limited multiple embryo-sacs. *P. squamulatum* MA showed all aposporous embryo sacs with 78% ovules with multiple embryo-sacs. Hybrid H1 was sexual with only 5.5% ovules with multiple embryo-sacs, while H2 was aposporous with 41.9% multiple embryo-sacs. Segregation for mode of reproduction in hybrids further confirmed the heterozygous nature of *P. squamulatum* MA for this trait. Previously reported hybrids too exhibited differential segregation for mode of reproduction (Dujardin and Hanna 1983, Ozias-Akins *et al.* 2003). Embryo-sac abortion was higher in H1 (sexual type), similar to previous observations. Seed-setting upon selfing was higher in H1 (37%) as compared to H2 (13%).

Conclusions

Hybrids between tetraploid pearl millet and the new $2n=56$ cytotype of *P. squamulatum* have been successfully produced. Since *P. squamulatum* is considered as a potential donor for desirable traits for pearl millet improvement, the prospects of utilization of hybrids is high, not only with respect to introgression of desirable traits, but also to understand complex traits such as perenniality and apomixis. Genetic analysis involving *P. squamulatum* ($2n=54$) cytotypes revealed apomixis (apospory) under control of a complex locus designated as ASGR (Apospory Specific Genomic Region), localized on a single squamulatum chromosome, and characterized by hemizygosity and suppressed recombination (Ozias-Akins *et al.* 2003, Akiyama *et al.* 2006). Similar analyses involving $2n=56$ cytotype shall be important to identify the presence of same or different phenomenon as in $2n=54$ cytotypes. The additional genes localized on the 2 additional chromosomes and their interaction and comparison with the previously reported cytotypes shall be important, especially for economically important characters such as apomixis, perenniality, improved fodder traits and stress tolerance. For this, sufficient progeny between tetraploid pearl millet and *P. squamulatum* ($2n=56$) is required. We believe that the crossability could still be enhanced between these 2 species by using combination of techniques such as pollen-tube growth enhancers, auxin treatment to support embryo growth followed by *in vitro* embryo-culture where ever essential. Production and analysis of sufficient hybrids with $2n=56$ *P. squamulatum* cytotype shall be important to study genome relationships and evolution, as well as mapping, inheritance and introgression of traits such as apomixis, perenniality and tolerance to biotic and abiotic stress.

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