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Reproductive biology, breeding behaviour, emasculation, pollination techniques and hybridization in little millet (*Panicum sumatrense* L.)

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Abstract

Little millet belong to group of small millets and family Poaceae. Flower opening in this crop is chasmogamous type, in which pollination takes place prior to opening of flowers. Hence, self pollination highly predominates. So, to create variability, hybridization is prerequisite. Because of self pollination and non availability of male sterility, emasculation is necessary for crossing. There are many emasculation and crossing methods like contact method, hot water treatment, hand emasculation and USSR method which are used in this crop. But the disadvantage in these methods is damage to stigma this reduces the success rate of obtaining true F₁'s. To overcome all this problems of earlier methods, in the modified crossing 'SMUASB' recently utilized method. In this method, cold water (5-8 °C) sprayed on the panicle as a mechanical stimulator for opening of florets in male and female panicles. For emasculation, female panicle is gently washed in cold water. This will not affect stigma and its receptivity. All florets which are already fertilized and not opened immature florets are removed before pollination. Hence, success rate of obtaining true F₁s is more and less land and resources are required for evaluation of F₁'s. Using SMUASB method, we attempted crosses in Little millet and the success rate observed was 50 to 60%.

Keywords: Reproductive biology, breeding behaviour, emasculation and pollination techniques, hybridization, little millet

Introduction

Little millet is one of the coarse cereals consumed in the form of rice. It belongs to the family Poaceae and subfamily panicoideae. It is a self-pollinated crop with a chromosome number of $2n=4x=36$. Little millet is an important crop grown for food and feed in the tribal belt of Madhya Pradesh, Chhattisgarh, Gujarat, Maharashtra, Odisha and Andhra Pradesh in India. It is described as a quick growing, short (60 days) to long (160 days) duration cereal, which can withstand both drought and water logging (Doggett, 1989) [4]. It contains high amount of carbohydrates, proteins, minerals and vitamins, Because of their nutritional superiority it is also referred as nutricereals or nutrimillets. The protein has balanced amino acid profile and good source of methionine, cystine and lycine. Little millet can be grown in tropical and subtropical climates and it is well known for its drought tolerance and is considered as one of the least water demanding crop and it is suitable for delayed sowing, rain fed condition, drought tolerant, multiple and contingent cropping system. Compared to other small millets and staple food crops like rice and wheat, little millet contains fairly good amount of Iron and Ca. Crop improvement work carried out so far in these crops has thrown some success. In the recent past some improved cultivars were developed but have limited yield potential. Although considerable variability is available in the present germplasm collections, the same has not been utilized fully. The utilization of the available variability to develop new improved cultivars is possible by hybridization and selection in the segregating population. Hybridization is a cross between individuals from separate populations that differ in one or more heritable traits (Harrison, 1990) [9]. Hybridization can have immediate phenotypic consequences through the expression of hybrid vigour (Benjamin *et al*, 2017) [2]. Hybridization is needed for efficient utilization of available germplasm, generation of breeding material, to introgress the novel genes and to broadening the genetic base. In Little millet because of difficulties in artificial hybridization creation of variability is difficult.

For successful hybridization programme, understanding of the parameters that affect the duration of the flowering period, pollination behaviour and seed set is necessary for increasing the productivity and yield stability. The main problem associated with all small millets is the difficulty in emasculation due to the small size of florets.

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The main aspects related to floral structure, different emasculation and crossing methods, their disadvantages and to overcome problems associated with traditional methods of crossing, a new modified method (SMUASB) of crossing and its success rate and advantages in little millet is summarized here.

Constraints in hybridization and crossing techniques in Little millet

- This crop have small florets which are not amenable for easy hand emasculation and hybridization.
- For artificial hybridization, the knowledge of floral biology, a simple workable hybridization technique and suitable gene marker for identification of true F₁ are essential.
- In view of the problems in making crosses artificial, the task before the breeders is to search for simple and effective emasculation and pollination techniques in this crop.
- Though large amount of variability is available, to combine the desirable characters from these accessions in genotypes, the artificial hybridization has to be restored, for which a simple and effective method of emasculation and pollination is prerequisite. The study of floral biology will help in planning the most appropriate methods of emasculation and pollination.

Little millets breeding target traits/ objectives and improvement: Targeted traits in little millet for improvement in yield, fodder and quality traits, focus should be given to shoot fly resistance, non-lodging, days to maturity and bold grain size while selecting best donar parent. (Nandini *et al.* 2019)^[18].

In general, yield and parameters contributing to yield are the most targeted traits in small millets improvement. Therefore, selection for yield per se has been the major basis for improving productivity, but genotype × environment interactions highly influence these traits. Therefore, assessing yield stability across multiple environments and investigating physiological traits (such as harvest index, water use efficiency, etc.) associated with yield and adaptation are essential to target yield increase. Germplasm collections exhibit significant variation for various traits, including maturity duration that can be exploited to breed custom-made cultivars that fit into the different maturity groups: early, mid-late and late, depending on the location-specific requirements of soil, rainfall, temperature, humidity, day-length and cropping patterns. Short-duration varieties would be suitable for double/intensive cropping regions and medium-to long-duration varieties for single cropping season areas.

The main objective of the crossing is to create the variability and incorporate desirable traits such as high yielding, pest and disease resistance and important quality traits etc., in a single genotype and to widen the genetic base of the population for most effective selection.

Little millets production constraints

- Exploiting existing variability present in germplasm and hybridization-derived variations can support in breeding nutrients-dense cultivars in higher-yielding background.
- Small millets are well adapted to diverse climatic conditions and are less affected by major biotic and abiotic stresses. However, a few diseases and insect pests

are causing considerable yield loss, and therefore, breeding for cultivars resistant to diseases and pests is important.

- Grain and head smut and leaf spot diseases in little millet, Little millet are mostly damaged by shoot fly.
- Small millets are mostly grown as rainfed crops, and are considerably affected by drought due to the failure of monsoon. In addition to drought, lodging is major problem in all small millets, mainly due to their soft stalk, crop management and environmental factors.
- There is no direct estimate of yield loss in small millets due to lodging, but major cereals like rice and wheat have recorded up to 50% losses. It occurs due to the bending of plants at maturity due to higher panicle weight, soft stalk and weak anchorage roots.
- Lodging is genotype-dependent and influenced by the environment, therefore developing cultivars with improved lodging resistance is essential to minimize yield and quality losses.
- Another important trait for little millet is grain shattering. Significant yield losses occur due to grain shattering in small millets, therefore shattering-resistant/tolerant small millets will be critical to prevent shattering-induced yield losses.
- Special breeding traits to enhance the cultivation and consumption of small millets are developing machine harvestable cultivars, enhancing the nutritive value of grain and fodder to fetch high market value, developing cultivars suitable to make value-added products such as rice, flour, vermicelli, flakes, hot and cold extruded snacks, noodles and ready-to cook mixtures, shade-tolerant genotypes for orchards and agro-forestry, quick growing genotypes for inter-cropping and genotypes suitable for rice-fallows.
- Small millets have high nutritional potential, while their utilization is limited by the presence of antinutrients such as phytate, phenols, tannins, and enzyme inhibitors, and also a high amount of protease and amylase inhibitors that affect the digestibility of millets grains.
- Most grain nutrients in small millets are generally higher than the major cereals, however, large variability exists in germplasm, including for grain nutrients and antinutrients.

Floral Biology of Little millet

Little millet inflorescence is a panicle, contracted or thyriform and 15-45 cm long and 1-5 cm in wide (Seetharam *et al.*, 2003)^[25]. The spikelet is persistent and 2-3.5 mm long (Bor, 1960)^[3]. Panicle branches are scabrous and drooping at the time of maturity. Spikelets produce on unequal pedicels but solitary at the end of the branches. Each spikelet consists of two minute flowers. The lower one is sterile; the upper one is fertile or bisexual without rachilla extension. The lemma I and its palea encloses the staminate or sterile flower; lemma II and its palea encloses the fertile flower (Sundararaj and Thulasidas, 1976)^[27]. Spikelets are elliptical, dorsally compressed, and acute. It has three anthers about 1.5 mm in length. The glume reaching apex of florets, thinner than fertile lemma (Fig. 2); lower glume is ovate, 0.7-1.2 mm long, membranous, without keels, 1-3 veined. The lateral vein is absent in lower glume and its apex is acute. The upper glume is ovate and without keel but larger than lower glume. It has 11-15 veined (Nanda and Agrawal, 2008)^[17].

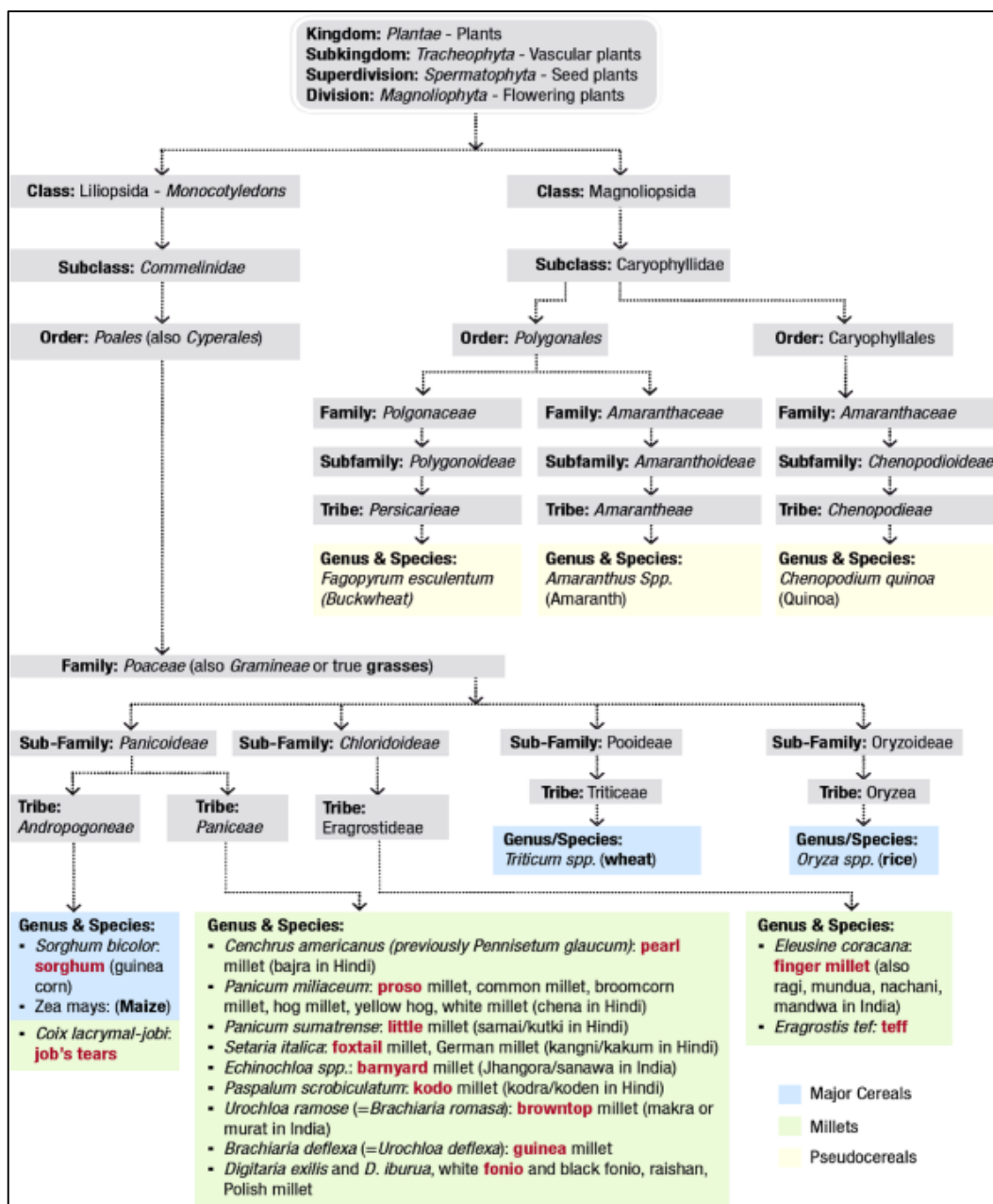
Anthesis and Pollination

The opening of the spikelets commence from the second or third day after the appearance of the panicle. The flowering progresses from the top to the bottom of the panicle. The maximum numbers of flowers open on sixth or seventh day. It takes about fortnight to complete the flowering in a panicle (Sundararaj and Thulasidas, 1976) [27]. The anthesis occurs between 9.30 to 10.30 a.m. (Jayaraman *et al.* 1997) [12]. The glumes open for a short while and self-pollination is the rule (Seetharam *et al.*, 2003) [25]. The whole process of the anthesis is very rapid and is completed within 2-5 min.

Emasculation and crossing techniques

The knowledge of correct technique for crossing and selfing in various crops allow the breeder to obtain the combination of characters which are desired. This needs skill and practice

before the worker can hope to accomplish best results. By recombining the alleles contributing for yield components like, tiller number, number of primary branches, number of secondary branches, number of grains per panicle and thousand grain weight. The removal of stamens or anthers or the killing of pollen grains of a flower without affecting in any way the female reproductive organ is known as emasculation. The purpose of emasculation is to prevent self-fertilization in the flowers of the female parent. In dioecious plants, male plants are removed, while in monoecious species the male flowers are removed to prevent self-pollination. But emasculation is essential in bisexual flowers. Naturally self-pollinated crops are shy pollinators with very poor movability to effect allogamy hence crossing is the technique where pollen from the desirable parent is dusted on the stigma of the seed parent.



Source: Vetriventhan *et al.* 2020; Nucleus, <https://doi.org/10.1007/s13237-020-00322-3>

Chart above indicates: Taxonomical classification of small millets and other major cereals and millets, and pseudocereals [Note: "Millet" is a common term to categorize small-seeded grasses that are often called dryland cereals. The

grasses most commonly referred to as millets are: Major millet (pearl millet) and Minor/ Small millets (finger millet, foxtail millet, proso millet, little millet, barnyard millet, kodo millet, browntop millet, fonio, teff and job's tears, and guinea

millet; and sometimes sorghum is categorized as a major millet - this is typically included in the common definition of millets in India but less so in other countries.

Different Crossing Methods used in Little Millet:

1. Contact method
2. Hand emasculation followed by pollination
3. Hot water treatment
4. USSR method

1. Contact method / Approach method

In this method, crossing is done by planting the suitable male parent adjacent to the female parent which has been prepared for the pollination. Male and female panicles are tied loosely. After pollination and fertilization have completed, both were separated, while selecting male parent, it should have morphological markers so that it is helpful in identifying true F1 (<http://agritech.tnau.ac.in>).

It is the easiest method of crossing followed in most of the self-pollinated crops. Success rate of obtaining true F1 is very less. Hardly 2-3% of true F1 can be observed. Large number of plant population has to be raised to select true F1. It needs more land and resources for evaluation.

2. Hand emasculation followed by pollination

In this method, flowers which would open on the next day were selected. Stamens or anthers were removed without affecting in any way the female reproductive organs. Hand emasculation is done in the evening and pollination is done in the next day morning hours. For pollination, male flowers which would open on the day are brought to the emasculated female flower. Then they are tied together and covered with butter paper bag. Natural cross pollination takes place in 2 to 5 days. Marker genes are utilized for identifying the true hybrid. (<http://agritech.tnau.ac.in>). This is the traditional method used in the self-pollinated crops. The major problem with this method was the lemma and palea are very tight and any attempt to open the flower prior to normal flower anthesis result in damage to the flower and no seed set. Another problem involved is length of time required for anthesis of the first flower on a panicle to the time of anthesis of the last floret on a panicle. This made it difficult to determine the proper time for emasculation prior to anthesis (Jasovskij, 1960). To overcome this problem Nelson, 1984 followed different technique for selection of flowers for emasculation. In this method, panicles were selected where the first florets are opened. The panicle was rubbed between the palms of the hands, causing the florets to begin to open. Florets were sprayed with room temperature water from an atomizer to keep the anthers wet so they would not dehisce until all the anthers were removed. When the florets stopped opening and all opened florets were emasculated, the floret which had not been emasculated were removed. That included the immature florets at the bottom and the previously fertilized florets at the top of the panicle. Best time for emasculation was between 8 AM to 9 AM. During this time florets opened at a rate that made it possible to emasculate them efficiently. Fertilization was done 15 minutes after emasculation. For pollination male parent were rubbed and allowed to open. Opened male florets were placed in a glassine bag, which was inverted over the emasculated panicle. This was left for five days for crossing and to preserve moisture. Advantage of this method is lemma and palea are not forced to open, but are allowed to open naturally. In this method disadvantage is that, while

emasculating if damage occurs to the stigma results in no seed set.

3. Hot water treatment

Numerous investigators have used the hot water treatment for emasculation in an effort to get around the problem of manually removing the anthers from the florets (Keller, 1952). In this method panicles which likely to flower in next 2 to 3 days were selected and are immersed in hot water with 52 °C for 2 minutes. This was the best temperature and time as judged from the percentage of hybrid seed set (<http://agritech.tnau.ac.in>). Srivastav and Yadav (1972) ^[26] reported hot water emasculation of little millet was successful at 490 C for 8 – 10 minutes or 500 C for 5 minutes. Similarly, After emasculation using hot water for pollination, male parent that would open on the next day are tied to the emasculated female parent and covered by a butter paper. Limitations in this method are, necessary equipment is required to maintain correct temperature. Temperature will affect the stigma; this may result in relatively small amount of seed set.

4. USSR method

To overcome the problem found in hand emasculation and hot water treatment, to remove pollens, this modified method of crossing was proposed (Seetharam *et al.*, 1986) ^[23]. Induced opening of the flower (USSR method) has been successfully utilized in making and developing new cultivars, the details are provided below:

1. Florets are mechanically stimulated by gently massaging the panicle by hand.
2. Florets open within 2-3 minutes well before normal flowering.
3. To avoid anther bursting, dip in water at ambient temperature.
4. Thrash anthers from opened florets with fore fingers.
5. Clip off the unopened florets and retain the opened flowers.

Pollination method

Pollination was immediately done by positioning the emasculated female spike slightly below the male spike that was shedding the pollen and covering both spikes with a glassine bag. Pollen from the male spike showers down on the female spike affording good opportunity for fertilization. The spikes were shaken together for 2 days during the daily anthesis periods. On the third day of the pollination, the male spike was carefully removed and the female one was checked for any floret that may have developed later. Failure to remove such florets completely, often allowed them to develop and produce seed, which may be confused with the cross-fertilized ones. The female spike was rebagged and maintained until harvested at maturity. Disadvantage of this method is stigma may damage while massaging the panicles for mechanical stimulation to open the florets and also during removal of anthers from florets with fore fingers.

5. Modified crossing method (SMUASB method)

At Project Coordinating unit on Small Millets, University of Agricultural Sciences, GKVK, Bengaluru, introduced this modified method of crossing in Proso millet and Little millet during 2014-15, hence named this method as SMUASB (Small millets, University of Agricultural Sciences, Bengaluru). In Little millet lemma and palea are very tight

and any attempt to open the floret prior to normal anthesis results in damage to the flower and no seed set. In SMUASB method cold water is used as a mechanical stimulator for opening of florets. Emasculation In both Proso millet and Little millet flower opening starts from top to bottom of the panicle. Male and female parents are planted in the alternate rows for crossing. Best time for crossing is 8 AM 9 AM.

Details of emasculation is mentioned below:

- For emasculation, female plant panicle has to be selected in such a way that, first floret has opened on the panicle.
- Cold water of 5-8°C is sprayed on the panicle. This cold water spray stimulates the florets to open naturally, one hour earlier than naturally opened.
- When all the florets were opened, emasculation is done by dipping panicle in cold water, all the anthers were removed by washing the panicle in cold water.
- Florets which are not opened were removed. It includes immature florets at the bottom and previously fertilized florets at the top of the panicle.

Pollination

- Male parent is selected in such a way that, where first floret has opened.
- Cold water of 5-8 °C is sprayed on male panicle just as like in female panicle to open the florets and keep anthers wet.
- Immediately after male florets opened it is tied loosely around female parent panicle in such a way to allow proper aeration and pollination.
- Then water is sprayed on tied panicles to keep stigma and anthers in wet condition.
- Tied male and female panicles are covered with butter cover to avoid cross
- Pollination. As these crops are self-pollinated little amount of i.e. 3-5% cross pollination may occur.
- Tag the crossed panicle in the female parent for identification and collection of seeds from female parent. Tag should contain cross combination and date of crossing.

Application of SMUASB method for crossing in little millet: Using SMUASB method, Crosses were attempted in little millet.

Little millet Plant material

Female parent: GV-1,

Male parent: WV-126 (Purple plant stem pigmentation-Gujarat local) is a high yielding desirable genotype.

The main lacuna in this genotype is long duration which is not suitable for the cropping system. GV-1 is an early, high tillering and iron rich variety. In order to develop short duration, high yielding and nutrient rich genotype we have crossed GV-1 with WV-126. WV-126 is used as a male parent, it is purple pigmented, while GV-1 is green colour and it is used as female parent. Purple pigmentation is used as marker for identification true F1's.

Success rate

Three to four panicles were used for crossing. Panicles were emasculated and pollinated using SMUASB method. Seed harvested from female parent is planted for identification of true F1's. seeds harvested from each panicle were planted separately. In panicle 1, out of 40 F1 plants planted 20 plants were identified as true F1's this shows 50% is the success rate. Similarly in panicle 2 and panicle 3, success rate was 55% and 56% respectively. On an average 50% success was observed in little millet crossing using SMUASB method.

Table 1: Confirmed successful crosses in little millet attempted:

Sr. No.	Crosses	Objectives of the crossing programme
1.	GV-1 x WV 126	High yield and early to medium maturing, bold grain and lodging tolerant as well as shoot fly resistance.
2.	GV-2 x WV 126	
3.	GNV-3 x WV 126	

Advantages of modified crossing (SMUASB) method over other crossing techniques in little millet

Advantage of this SMUASB is, there is no occurrence of damage to the stigma while emasculation. This results in increased seed set and obtaining frequency of True F1's is more compared to the other traditional methods. Compared to the hand emasculation, it is less laborious and less time consuming. In hand emasculation, removing of pollen in each and every floret is laborious and technical skill is required. As the flowers of these two crops are small and technical expertise is required for emasculation. In modified crossing method just by spraying cold water florets are opened. Anthers are also easily removed by washing or dipping in water, hence it is a less laborious method.

Less land and resources are required for emasculation and identification of true F1. In case of contact method, large number of seeds obtained from female plant has to be tested for identification of true F1's. Frequency of obtaining true F1's is less. In case of modified method all unopened immature florets and already pollinated florets are removed. Only the florets where anthers are emasculated are kept for pollination. Hence, number of F1's harvested from female plant has less number of seeds. So for evaluation and identification of true F1s it needs less land and resources. Instead of using hand messaging as used in USSR method and hot water treatment for emasculation, cold water spray is used as mechanical stimulator for opening of florets in modified crossing method. This doesn't damage the stigma. Hence, success rate of obtaining true F1's is more.

Modified crossing (SMUASB) method of emasculation and crossing in Little millet and Proso millet is very advantageous method. This method overcomes the problems faced in contact method, hand emasculation, hot water method of emasculation and USSR method of crossing. In this method, success rate of obtaining true F1's is more compared to other earlier methods. Less land and resources are required for evaluating F1's in this method. Using SMUASB method we attempted crosses in little millet and proso millet. Success rate obtained was nearly 50% in Little millet.



Fig 1: Little millet inflorescence and its parts. (A) Inflorescence; (B) Spikelet; (C) Side view of spikelet; (D) Opened spikelet; (E) Outer glume; (F) First lemma; (G) Sterile floret; (H) Fertile floret; (I) Upper glume; (J) Grain enclosed in lemma and palea.



Fig 2: Modified crossing (SMUASB) technique followed in little millet

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