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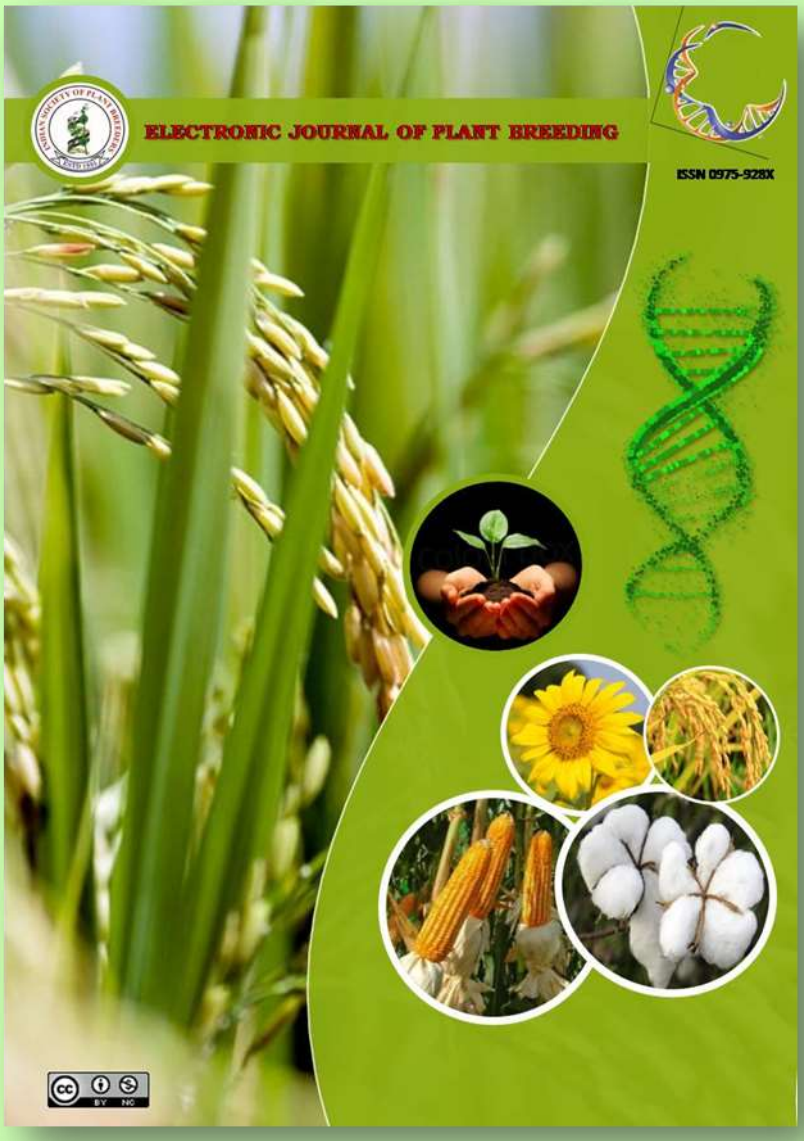

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Research Article

Modified crossing (SMUASB) method for artificial hybridization in proso millet (*Panicum miliaceum* L.) and Little millet (*Panicum sumatrense*)

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Abstract
Proso millet and Little millet belong to group of small millets and family poaceae. Flower opening in these crops are chasmogamous type, in which pollination takes place prior to opening of flowers. Hence, self pollination 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 these crops. But the disadvantage in these methods is damage to stigma this reduces the success rate of obtaining true F_1's. To overcome all these problems of earlier methods, in our present study, we proposed the modified crossing (SMUASB) method. In this method, cold water of 5-8^{0}C is 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 F_1s is more and less land and resources are required for evaluation of F_1·s. Using SMUASB method, we attempted crosses in Little millet and Proso millet and the success rate observed was 56% and 60% respectively.

Key Words
Chasmogamous, Emasculation, Hybridization, Little millet, Pollination, Proso millet, SMUASB method

Introduction

Small millets are group of 6 millets comprising of finger millet (*Eleusine coracana*), foxtail millet (*Setaria italica L.*), kodo millet (*Paspalum scrobiculatum*), little millet (*Panicum sumatrense)*, barnyard millet (*Echinochloa frumantacea*) and proso millet(*Panicum miliaceum*). These crops are having a long history of more than 5000 years and are known for their suitability to dry lands, hill and tribal agriculture, contributing to food, fodder and nutritional securities at farm and regional levels. They require small quantity of water, mature early and well suited for cultivation under scarcity conditions. They are considered as Nutria- cereals and are the source of food, feed and fodder. They are grown from sea level to mid hills right from Tamil Nadu in South to Uttarakhand in North, and Gujarat in the West to Arunachal Pradesh in the North East. (Rai *et al.,* 2008).

Small millet crops are viewed as important for health and wellness of people and can help in preventing many kinds of diseases related to modern life style including obesity, diabetes (Jones *et al.,* 2000; Jones, 2006). The total area under these crops during the year 2015-16 was 1.79 m. ha. with a production of 2.21 m. tons (www.aicrpsm.res.in)

Proso millet (*Panicum miliaceam* L.) is widely cultivated as a cereal crop across India, Nepal, western Burma, Srilanka, Pakistan and South East Asian countries. Proso millet is grown throughout India in more than half a million hectare mainly in the states of Tamil Nadu, Karnataka, Andhra Pradesh and Uttarakhand. It belongs to the family poaceae and chromosome number is 2n= 4x=36. It is a short duration crop and matures in 60-90 days after planting (Gupta and Gupta, 2007) and highly tolerant to heat and drought. It is preferred for extreme soil and climatic conditions as it yields reasonably well even in degraded soils under unfavorable weather conditions (Baltensperger, 2002). It has wide range of adaptation from hot summers in tropics to high altitude areas of the Himalaya, where crop growing season is short (Sahib, 1997). The protein content of proso millet is superior to rice and comparable to wheat (Gupta *et al.* 2010). The Proso milet is a rich source of essential amino acids namely leucine, isoleucine and methionine, hence the protein quality of proso millet is considered better than wheat (Kalinova and Moudry, 2006)

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,

Chattisgarh and Andhra Pradesh in India (Haider, 1997). It is described as a quick growing, short duration cereal, which can withstand both drought and water logging (Doggett, 1989). It contains high amount of carbohydrates, proteins, minerals and vitamins, Because of their nutritional superiority it is also referred as nutri cereals or nutri millets. 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.

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). Hybridization can have immediate phenotypic consequences through the expression of hybrid vigor (Benjamin *et al*, 2017). 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 and Proso 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 behavior 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. 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 Proso millet and Little millet is summarized here.

Constraints in hybridization and crossing techniques in Little millet and Proso millet

These crops 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 F_1 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 these crops. 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.

Floral biology of Proso millet and Little millet

Floral morphology of Proso millet

Proso millet inflorescence is a-drooping panicle, 10-45 cm long that may open or compact (Hulse *et al.*, 1980), primary branches spreading or ascending or appressed, terminating in a spikelet. The bristle below the spikelets is absent. The spikelets are generally solitary and about 0.5 cm long (Gowda *et al.,* 2003). Each spikelet has two glumes and two lemmas. The glumes are unequal in length; outer glume is short, while the inner glume is as long as the spikelet. Each lemma contains one floret. The floret in lower lemma is sterile without stamen; upper lemma is fertile and shorter than lower lemma (Fig.1). The palea of lower lemma (sterile floret) is very much reduced, while the palea of upper lemma (fertile floret) is well present (Seetharam *et al.,* 2003). It has three stamens; anthers are tan or amber or blackish or dark brown in colour. The ovary has bifid style and plumose stigmas (Nanda and Agarwal, 2008).

Anthesis and Pollination

Proso millet starts flowering from top to downward to the bottom of the panicle (Sundararaj and Thulasidas, 1976) .The timing of anthesis occurs between 10.00 to 12.00 noon (Jayaraman et al.,1997). It takes 12-15 days from the start of the anthesis of the first flower to the last floret on the panicle. In proso millet, the receptivity of stigma coincides with the shedding of pollen from anthers. Nelson (1984) observed that when the florets were open, the anthers were sticky and pollen did not shed. Within minutes after the opening of florets, the anthers dried out and begin to shed pollen. The florets remain open for 10-15 minutes (Zhuravel., 1956). The factors such as high temperatures, low humidity and bright sunlight promote the flowering. Flowering gets reduced on cloudy days. It can be stimulated by heating a panicle with lens. Proso millet (2n=36) is a self pollinated crop, but

natural cross –pollination may exceed 10% (Popov, 1946).

Floral morphology of Little millet

Little millet inflorescence is a panicle, contracted or thyrsiform and 15-45 cm long and 1-5 cm in wide (Seetharam et al., 2003). The spikelet is persistent and 2-3.5 mm long (Bor, 1960). 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). 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 alsao ovate and without keel but larger than lower glume. It has 11-15 veined (Nanda and Agrawal, 2008).

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). The anthesis occurs between 9.30 to 10.30 a.m. (Jayaraman *et al.* 1997). The glumes open for a short while and self pollination is the rule (Seetharam *et al.*, 2003). 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. 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

Different emasculation and crossing methods used in little and proso millets

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 F_1 (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 F_1 is very less. Hardly 2-3% of true F_1can be observed. Large number of plant population has to be raised to select true F_1. 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 Proso millet. 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 emasculation 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 52^0 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) reported hot water emasculation of little millet was successful at 49^0 C for 8 – 10 minutes or 50^0 C for 5 minutes. Similarly, Primak and Jakovlev (1964) reported the hot water treatment at 50^0C for 5 minutes results in better seed set in Proso millet. 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).

Induced opening of the flower (USSR method)

The 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 re-bagged 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, hence named this method as SMUASB (Small millets, University of Agricultural Sciences, Bengaluru).

In Proso millet and 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 to 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^{0}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 ^{0}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 away 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 and Proso millet

Using SMUASB method, Crosses were attempted at Project Coordinating unit, small millets, University of Agricultural Sciences, Bengaluru in Little millet and Proso millet.

Little millet

Plant material: Female parent: JK-8, Male parent: Peddasame (Purple plant pigmentation)

Peddasame (Andhra local) is a high yielding desirable genotype. The main lacuna in this genotype is long duration which is not suitable for the cropping system in Karnataka. JK8 is an early, high tillering and iron rich variety. In order to develop short duration, high yielding and nutrient rich genotype we have crossed JK8 with Peddasame. Peddasame is used as a male parent, it is purple pigmented, while JK 8 is green colour and it is used as female parent. Purple pigmentation in Peddasame is used as marker for identification true F_1's.

Success rate: Three panicles were used for crossing. Panicles were emasculated and pollinated using SMUASB method. Seed harvested from female parent is planted for identification of true F_1' s. seeds harvested from each panicle were planted separately. In panicle 1, out of 30 F_1 plants planted 18 plants were identified as true F_1' s this shows 56% is the success rate. Similarly in panicle 2 and panicle 3, success rate was 53% and 55 % respectively. On an average 56% success was observed in little millet crossing using SMUASB method.

Confirmed successful crosses in Little millet

Crossing parents	Total number of panicles emasculated in female parent	No. of florets emasculated in each panicle female parent	No. of confirmed plants by morphological marker (Purple pigmentation)	Success rate (%)
JK 8 x Pedda same	3	Panicle 1: 30	18	60
		Panicle 2: 32	17	53
		Panicle 3: 27	15	55
Average success rate				**56**

Proso millet

Plant material: Female parent: GPUP 8, Male parent: CO 2 (Purple seed colour)

In Proso millet using SMUASB method crosses were attempted between GPUP 8 and CO2. GPUP 8 is a Proso millet variety wildly adopted in Andhra Pradesh, Tamil Nadu and Karnataka with long duration tall crop and gold colored bold grains and dense panicle but prone to lodging. CO2 is one of the oldest variety with high tillering capacity dwarf and early maturing variety with purple seeds. In order to develop, non lodging and high yielding types in Proso millet. CO2 is selected as male parent its purple seed colour is used as marker for identification true F_1's.

Success rate: Four panicles were emasculated and pollinated using SMUASB method. Seed harvested from female parent from each panicle is planted separately for identification of true F_1'. In panicle 1, out of 23 F_1 plants planted 14 plants were identified as true F_1' s this shows 60% is the success rate. Similarly in panicle 2, panicle 3 and panicle 4, success rate was 56%, 64% and 55 % respectively. On an average 60% success was observed in proso millet crossing using SMUASB method.

Confirmed successful crosses in Proso millet

Crossing parents	Total number of panicles emasculated in female parent	No. of florets emasculated in each panicle female parent	No. of confirmed plants by morphological marker (Purple seed colour)	Success rate (%)
GPUP 8 x CO2	4	Panicle 1: 23	14	60
		Panicle 2: 32	18	56
		Panicle 3: 28	18	64
		Panicle 4: 21	13	61
Average success rate				**60**

Advantages of modified crossing (SMUASB) method over other crossing techniques in Proso millet and 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 F_1'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 F_1. In case of contact method, large number of seeds obtained from female plant has to be tested for identification of true F_1's. Frequency of obtaining true F_1' 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 F_1's harvested from female plant has less number of seeds. So for evaluation and identification of true F_1s 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 F_1' 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 F_1's is more compared to other earlier methods. Less land and resources are required for evaluating F_1's in this method. Using SMUASB method we attempted crosses in little millet and proso millet. Success rate obtained was 56% in Little millet and 60% in case of Proso millet.

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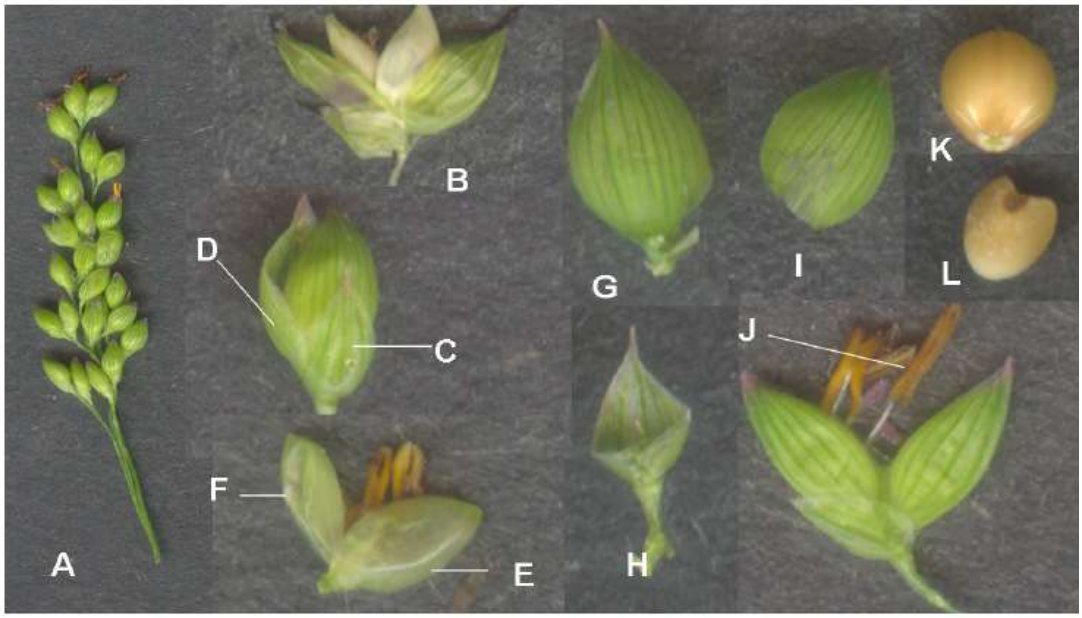

Fig.1 Proso millet inflorescence and its parts. (A) Inflorescence; **(B)** Opened spikelet; **(C)** Outer glume; **(D)** Inner glume; **(E)** Inner lemma; **(F)** Palea; **(G)** Inner glume; **(H)** Outer glume; **(I)**Upper lemma; **(J)** Anther; **(K)** Grain enclosed in lemma and palea; **(L)** Grain.

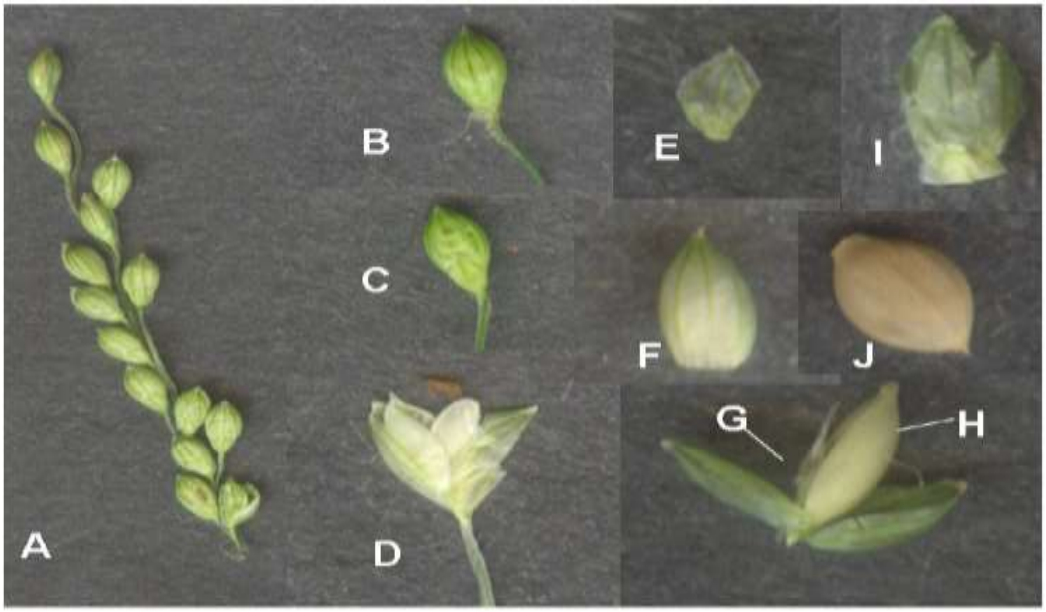

Fig. 2 Little millet inflorescence and its parts. (A) Inflorescence; **(B)** Spikelet; **(C)** Side view of spikelet; **(D)** Opened spikelet; **(E)** Outer glume; **(F)** First lemma; **(G)** Sterile floret; **(H)** Fertile floret; **(I)** Upper glume; **(J)** Grain enclosed in lemma and palea.

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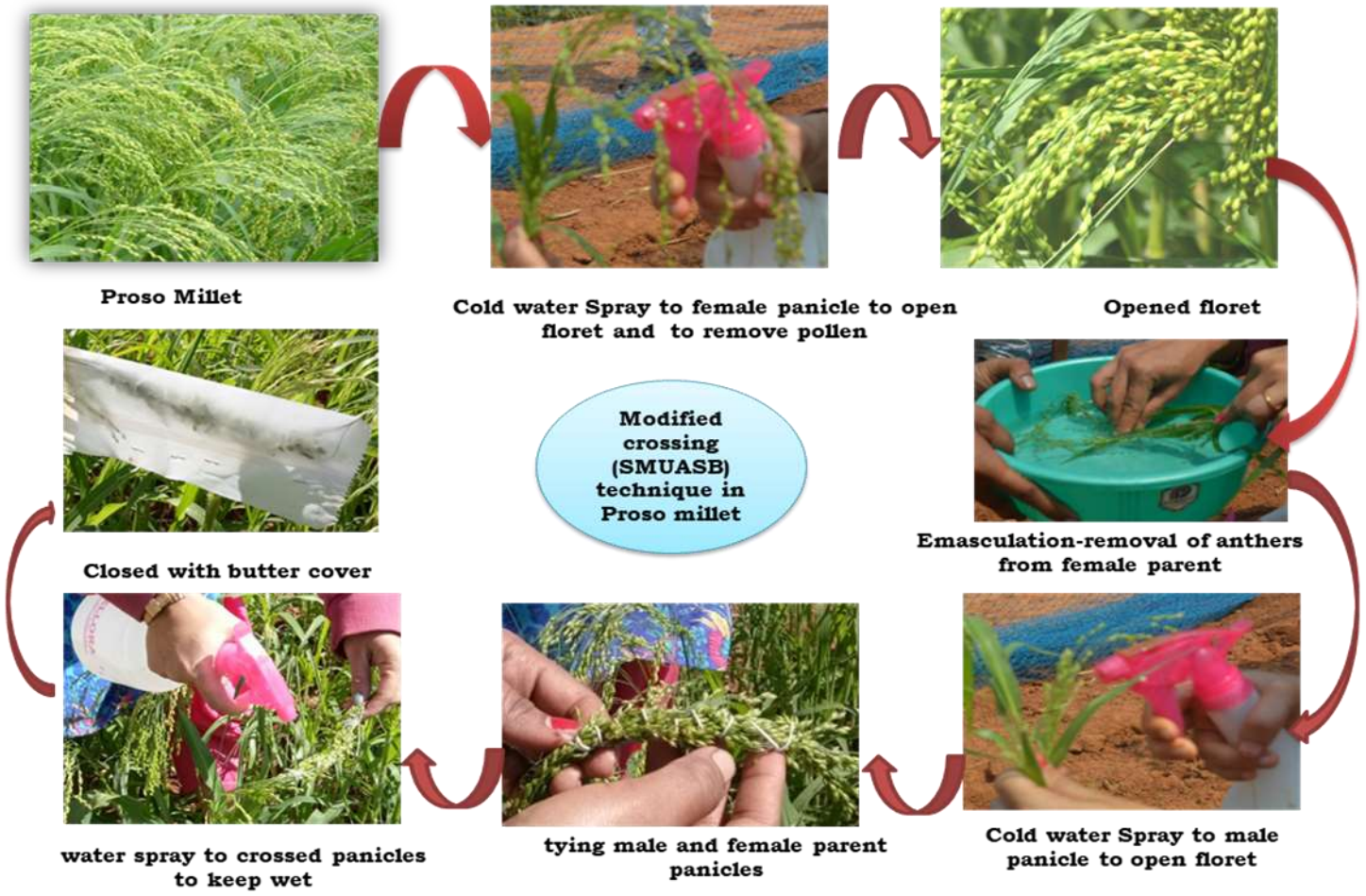

Fig. 1. Modified crossing (PCUASB) technique fallowed in Proso millet

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Fig. 2. Modified crossing (SMUASB) technique fallowed in Little millet

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