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PRELIMINARY INVESTIGATION OF THE CROSSING OF BAMBARA NUT (*VIGNA SUBTERRANEA* [L.] VERDC.)

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ABSTRACT
Effective crosses among selected parents are crucial for genetic analyses and for the breeding of crop plants. Bambara nut is an indigenous African legume with considerable genetic diversity useful for genetic enhancement of yield and quality traits through breeding. However, the crop has previously received limited research attention. This may be attributed to its extremely small flower size, its flower orientation, the delicate nature of the flower and it's mating system. The aim of this study was to establish a preliminary crossing protocol for Bambara nut for breeding and genetic studies. Controlled emasculation and pollination were performed using eight selected parents, using a diallel mating scheme under glasshouse conditions. Some successful crosses were achieved and F_1 seeds were recovered from the crosses of 211-40-1 x N211-2, N212-8 x 211-40-1 and M09-3 x 211-82-1.
Keywords: Bambara nut, emasculation, crossing, pollination, F_1 hybrids
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INTRODUCTION

Bambara nut is one of the most valuable grain legumes, native to Africa, which shares similar agro-ecology and growing environments with cowpea (Basu *et al.,* 2007). Bambara nut is a member of the Papilionaceae (Leguminosae) family, sub-family Papilionoideae (Fabaceae), genus *Vigna* and species *subterranea* (Fatokun *et al*., 1993). The species has two botanical varieties or sub-species: var. *spontanea* (the wild form) and var. *subterranea* (the cultivated form). Both are diploids with the chromosome number of 2n=22 (Frahm-Leliveld, 1953; Forni-Martins, 1986). The wild forms were found in 1909 in North-East of Nigeria, which supports the theory that the crop originated in West Africa (Dalziel, 1937). The crop spread to Asia and Latin America, probably through the slave trade, and is found in Sri-Lanka, Malaysia, Philippines and India, and Brazil (Rassel, 1960; Goli *et al*., 1997).

Bambara nut is an important source of dietary protein in sub-Saharan Africa, with protein levels of 16-25% (Brough *et al.,* 1993); carbohydrates and oil content is in the region of 55-72% and 6-7%, respectively (Suwanprasert *et al*., 2006). Fresh pods and seeds are eaten as a vegetable after boiling, like green peas. Dry seeds are roasted and eaten as a nutritionally balanced snack, while ground dry seeds are used to prepare many form of dishes such as Moi-moi, which is made from a steamed paste (Okpuzor *et al*., 2009). Bambara nut seed can be processed to make bread (Fetuga *et al*., 1975) and into vegetable milk similar to that made from soybean (Brough *et al.,* 1993). The paste can be fried in oil and be served as snack with porridge at breakfast. Bambara nut is a source of balanced food, and makes an important contribution to food security, and to reducing protein malnutrition in rural communities in Africa (Ouedraogo *et al.,* 2008). The crop combines the advantage of drought tolerance and some high level of resistance to insect pests and diseases (Obagwu, 2003). Bambara nut is versatile and can produce a moderate harvest in environments where other legumes such as nut fail to produce a crop (Linnemann and Azam-Ali, 1993). As a tropial legume, Bambara nut possesses the ability to fix atmospheric nitrogen through the activity of the symbiotic bacteria (*Bradyrhizobium* species) in root nodules.

Bambara nut shows wide genetic variation and is predominantly grown as landrace varieties, consisting of mixed seeds that display several morpho-types. The International Institute of Tropical Agriculture (IITA) based in Ibadan, Nigeria has the mandate for Bambara nut research and germplasm conservation. The Institute has collected and preserved over 2,000 accessions whose genetic diversity has not been adequately characterized to select for further genetic improvement in any breeding program (Massawe *et al*., 2005). However, several research reports (Ofori, 1996; Goli *et al*., 1997; Ntundu *et al*., 2006; Onwubiko *et al*., 2011) indicated that some of the Bambara nut landraces had been characterized for their morphological attributes. The reports noted that there was enough genetic variation to conduct strategic breeding (Massawe *et al*., 2005). Bambara nut is strictly a self-pollinating crop, bearing a perfect flower that stands on a short raceme attached to a long peduncle by the pedicle, alternately on stem nodes. The stamen, which is diadelphous, consists of 10 filaments that connect to the anthers on the tip carrying the pollen grains. The filaments are united into two sets: nine out of ten have their filaments fused, with one isolated vexillary stamen (Goli *et al*., 1997; Basu *et al*., 2007).

The stigma becomes receptive earlier and the anthers dehisce shortly before the flowers opens. The pollen grains of Bambara nut are trinucleate and short lived after anthesis. The flowers are cleistogamous, (i.e. the flowers are tightly enclosed by petals and sepals, and open only after pollination), and therefore pollination occurs immediately after the anthers dehisce. Fertilization takes place on the day of anthesis and after pollination (Linnemann, 1992).

Uguru *et al*. (2002) used cytogenetic analyses to understand the genetics of the floral system that can be employed to successfully cross Bambara nut. However, research reports indicated the difficulty of genetic analyses and breeding of Bambara nut using conventional manual crosses (Goli *et al*., 1997; Suwanprasert *et al*., 2006; Koné *et al*., 2007). During conventional breeding, controlled emasculation and pollination of flowers are essential to recover progenies for targeted selection. Factors hindering the emasculation and crossing procedures of Bambara nut are: its small flower size, its flower orientation, the delicate nature of the flower and its mating system (Myers, 1991). Despite the difficulties associated with crossing of the Bambara nut, efforts have been made to undertake controlled crosses, and segregating populations have been generated (Massawe *et al*., 2004; Suwanprasert *et al*., 2006; Basu *et al*., 2007). Management of the unavoidable variation in time-to-anthesis of different parental lines is critical for successful crossing, as reported by Suwanprasert *et al.* (2006) and Onwubiko *et al.* (2011). In addition, Oyiga and Uguru (2011) recommended the use of indole-3 acetic acid to enhance pollen germination. Suwanprasert *et al*. (2006) reported that the ideal emasculation time is between 3:00pm and 10:00pm, with successful crosses being made between 2:30 to 3:00am the next day. Onwubiko *et al*. (2011) suggested that pollination should be completed within 12 hours of emasculation, and that the blooming period ensues between 7:00am and 10:00am when pollination can be conducted. At the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), emasculation for crossing of nut, a related legume, is routinely carried out the between 1:30pm and 04:30pm, and pollination is conducted the following day between 6:00am and 08:00am (Nigam *et al*., 1990). In the case of cowpea Myers (1991) recommended that emasculation should be carried out in the evening between 4.00pm to 6.00pm, followed by pollination at 6.00am and 08.00am the next day when anthesis commences. These extreme differences in timing may be associated with the different environmental conditions under which the crosses were made, as well as genotypic and species differences.

Patel *et al*. (1935) showed that flowers in nut are blocked by bracts that make it difficult to get rid of unwanted flowers, which may result to selfing. A detailed, simple, step-by-step protocol is not available for making crosses in Bambara nut for effective genetic analyses and breeding. In the light of this limitation, the aim of this study was to establish a preliminary crossing protocol for Bambara nut for breeding and genetic studies.

MATERIALS AND METHODS

Selection of parents, planting and mating scheme

Selection of parents

Currently, seeds for Bambara nut production are available in the form of landraces, in which seed and plant morphology vary considerably. The present study used eight genotypes for the full diallel crosses (Table 1). The parents were kept true to type after rigorous selection with regards to source, uniform seed coat colour, and uniform seed eye and hilum patterns.

Planting

To facilitate crossing, 32 plastic pots of 5 litre capacity, filled with a composted pine bark medium, were assigned to each of the eight parents into which two seeds were planted. Out of the 32 pots allocated for each of the eight genotypes, four were designated as male parents, while 28 were designated as maternal parents. The seed was planted on the 7th of January, 2013 in the glasshouse kept under controlled temperatures and humidity. The day and night temperatures of the glasshouse were 25^{0}C and 18^{0}C, respectively, while relative humidity was kept at 70 to 80%. Within one week after germination, the seedlings were thinned to one plant per pot to allow sufficient growth, development and ease of accessing the flowers during crossing. Pots with growing plants were placed on tables high enough for convenience of crossing (Fig. 1F).

Mating scheme

Crosses were established following an 8 x 8 full-diallel mating scheme. Each parent was grown in at least four plastic pots. Planting of these pots was staggered at an interval of 10 days to ensure synchronized flowering among parents, and to allow for effective crosses. Depending on the accessions, flowering begins from 35 days after planting. Before starting the emasculation and pollination procedures, the first few flowers were removed for about three days; this was to encourage sequential flower production from both pollen and maternal parents.

Emasculation

Blooming of the Bambara nut flowers occurred for a brief period, about 1 to 2 hours before sunrise, depending on the temporal changes in the summer months between November and March. Usually, flowers destined to open the next day on the maternal parent(s) were prepared for the emasculation (the removal of filaments with immature anthers before self-pollination) and pollination (the transfer of pollen grains from a male parent onto the stigma of a female parent). At this stage the colour of the flower bud changes from green to pale yellow, during which time the stigma is receptive, but the anthers have not matured yet, and cannot deliver effective pollination and fertilization. On each day emasculation needs to begin between 4:30am and 5:00am and pollination needs to follow, between 8:30am and 9:00am. This approach is contrary to the procedures reported by Onwubiko *et al*., (2011) and Suwanprasert *et al*. (2006).

However, we found that it was more convenient to conduct both the emasculation and pollination steps on the same day. With our approach, a flower that is ready for emasculation is handled gently with the left hand using the thumb and the index finger. Using a pair of sharp scissors in the right hand a gentle cut is made, large enough to expose the stamens carrying the immature pollens, which is a cut of about 1/2 to 2/3 of the width of the unopened flower (Fig. 1A). Maximum care was taken to avoid damaging the flower in the process, because of the delicate nature of the flower bud. A cut was made from the side where the flower would be destined to open because the dorsal side contains the stamens. A pair of tweezers was used to gently pull out the cut the sepal and petal that enclose the stamen and pistil (Fig. 1B). The single and nine fused stamens were then shaved gently using tweezers, making sure that the stigma remains intact and undamaged. With care, the corolla, the standard and the stamens were removed at the same time. At this point the stigma is exposed and is ready for pollination.

A jeweler's loupe was used both during emasculation and pollination, in order to clearly see the small flower parts, and to ensure successful emasculation and pollination. To avoid contamination, 70% alcohol was used to clean both hands and all the tools used in making crosses at every step of the emasculation and pollination procedures between any two parents.

Pollination

Pollinations were carried out immediately after emasculation. The opening of flower buds begins at sun-rise, particularly on bright days. Pollination begins as the flowers open, typically from 5:30am until 9:00am. For pollination, a freshly opened flower was removed from the male parent as a source of pollen grains to be transferred to the stigma of the maternal parent. The anther sac was opened by tearing off the floral leaves (calyx, corolla and the wing). The anthers containing the pollen grains are squeezed out and placed onto the stigma of the maternal parent using a pollen brush. It was observed that flower size and the prevailing environmental condition affected pollen abundance. Therefore, at times up to 5 to 10 female flower were pollinated using one male flower. The keel top of the male flower was used to cap the stigma gently, to ensure pollen contact with the stigma.

Flowers that reach an advanced growth stage on the maternal parents but have not been hand-pollinated, and which are destined to open the next day were removed to avoid the development of any selfed seeds on maternal plants. The process also encouraged production of more flowers for future crosses. This activity was also practiced on the pollen parents to promote production of more flowers for use in forthcoming pollinations. Due to the small size of the Bambara nut flowers, pollinated flowers were covered to avoid uncontrolled pollinations. Each emasculated and pollinated flower was tagged and labelled by tying a string of thread at each node for proper identification of developing pod and for effective monitoring (Fig. 2 C).

Cross confirmation and management of hybrids

On completion of the crosses, maternal parents were routinely checked to remove any developing flower bud to exclude selfed seeds. This exercise continued for four weeks. Fertilized flowers (Figs. 1 and 2), were monitored until the pods were matured and harvested. During this period an insect problem was encountered specifically that black ants (*Monomorium minimum* [Buckley]) damaged some of the crossed flowers and developing pods on the maternal parents. An insecticide (cypermethrin) was sprayed to eliminate the problem. The F_1 pods were harvested and dried, put in separate envelopes and labelled according to crosses.

RESULTS AND DISCUSSION

Results of the attempted crosses are presented in Table 2 which showed that a total of 509 successful crosses were made that produced only 87 well developed F_1 pods representing 17% success. Genotypes N212-2 and 211-51 were better male parents which collectively produced higher F_1 pods of 21and 17 from 68 and 66 crosses, respectively when crossed with the other genotypes as female parents. On individual basis, N212-2 x 211-40-1 and 211-40-1 x N212-8 produced 8 well developed F_1 pods each as the highest (Table 2), followed by 211-51 x 211-40-1 and 608-2 x 211-40-1 where each produced 6 and 5 F_1 pods, respectively. The crossing technique described above was successful, although the numbers of hybrid seed generated were not sufficient for genetic analyses at the F_1 generation. However, the F_1 seed can be selfed and genetic analyses can be conducted on the F_2 or even the F_3 generations.

Success of crossing in the common nut has been shown to be influenced by the mishandling of flower buds by breeders or technicians, the prevailing environmental conditions and the genotypes involved (Nigam *et al.*, 1990). In this study all the genotypes were selected based on uniform seed morphology. There was no any prior information available on their agronomic attributes and nature of flowering.

From this study, cross-pollination of Bambara nut can be achieved by way of simultaneous emasculation and pollination on the same day, between 4:30am and 9:00am. F_1 hybrids were obtained from each of the cross combinations in the 8 x 8 diallel. In Thailand, Suwanprasert *et al.* (2006) carried out pollination of Bambara nut earlier in the morning at 2:30am and 3:30am but this may reflect the environmental differences between Thailand and the South Africa.

Despite the flower size being smaller than those of cowpea and nut, Bambara nut can be improved through conventional crossing techniques.

Table 1 Some of the seed characteristics of the Bambara nut genotypes used for the full diallel crosses

Name of genotype	ID number	Source	Seed coat colour	Eye pattern	Hilum colour	Seed size
ZIM 109-3	M 09-3	ZIM	Red	Plain	White	Medium
KN 211-2	N 211-2	KNG	Cream	Light-grey	White	Medium
PSC 211-51	211-51	CAPS	Black	Plain	White	Medium
ZM 6608-2	608-2	ZM	Brown	Plain	White	Medium
ZM 5712-3	712-3	ZM	White-cream	Plain	Chalk-white	Small
PSC 211-40-1	211-40-1	CAPS	Dark-brown	Plain	White	Small
KN 211-8	N 211-8	KNG	Cream-brown stripe	Light-brown thin	White	Medium
PSC 211-82-1	211-82-1	CAPS	Dark-brown black spots	Plain	White	Small

Legend on seed sources: ZIM =Department of Research and Specialist Services, Zimbabwe; **ZM** =The National Plant Genetic Resources Centre, Zambia; **KN** =Farmers' collection from Kano, Nigeria; **CAPS** =Capstone Seed Company, Howick, South Africa

Fig. 1 Processes of emasculation and pollination of Bambara nut: (A) cutting a flower bud; (B and C) removing the anthers of the flower bud; (D and E) introducing pollen grains from the paternal parent to the stigma of the maternal parent; (F) conducting cross-pollination in a glasshouse

Fig. 2 Monitoring of Bambara nut F1 hybrids: (A) pegs of developing pods of the F_1 hybrid seed, towards the tip of the peg; (B and C) showing well developed Bambara nut F_1 pods showing remains of dried feathery stigma of the crossed flowers.
Note: Remains of the dried feathery stigma are shown using the arrows on the developing pods (A), and on the well-developed pods (C), suggesting that the pods are derived from crosses; although this can only be confirmed when the F_1 seeds are phenotyped or genotyped.

Table 2 Number of successful crosses and F_1 pods harvested from 8 x 8 diallel crosses of Bambara nut

MALE	712-3		M09-3		N212-8		N211-2		608-2		211-51		211-40-1		211-82-1	
FEMALE	Successful Crosses	F_1 Pods	Successful Cross	F_1 Pods	Successful Cross	F_1 Pods	Successful Cross	F_1 Pods	Successful Cross	F_1 Pods	Successful Cross	F_1 Pods	Successful Cross	F_1 Pods	Successful Cross	F_1 Pods
712-3	**X**	**X**	12	0	14	0	9	1	14	2	21	2	21	3	2	0
M09-3	7	0	**X**	**X**	10	0	7	4	6	2	5	0	14	0	8	5
N212-8	9	0	12	2	**X**	**X**	4	0	9	1	12	5	32	8	7	3
608-2	20	3	7	2	12	1	12	5	**X**	**X**	9	3	0	0	3	2
211-51	8	1	8	0	9	0	6	0	7	0	**X**	**X**	8	2	7	1
211-40-1	9	3	12	0	12	0	14	8	12	5	12	6	**X**	**X**	14	1
211-82-1	4	0	10	0	5	0	16	3	13	2	7	1	0	0	**X**	**X**
N211-2	5	0	0	0	7	0	**X**	**X**	2	0	0	0	0	0	4	0
TOTAL	**62**	**7**	**61**	**4**	**69**	**1**	**68**	**21**	**63**	**12**	**66**	**17**	**75**	**13**	**45**	**12**

Key: Cells within columns marked '**X**' are selfs

CONCLUSION

In this study, a protocol for the cross-pollination of Bambara nut was developed, despite the delicate nature and small flower size of the crop which makes this process difficult. A key development was that the pollination step was conducted immediately after the emasculation step, which is contrary to protocols used to make crosses in cowpea and nut. In these protocols, emasculations are done the previous day and pollinations follow the next day. The protocol developed here will help breeders of Bambara nut to make crosses for genetic analyses and for breeding for the genetic enhancement of the crop. Relative to reports on the success of other crossing procedures used on nut and cowpea, and the crossing techniques used on Bambara nut previously, the improved protocol used here produced more F_1 seeds within the limited blooming period of Bambara nut, because both emasculation and pollination were carried out one after the other. Furthermore, the protocol could reduce the extent of flower damage from the interval between emasculation and pollination employed on nut, cowpea and Bambara nut, as reported by Nigam *et al*. (1990), Myers (1991) and Suwanprasert *et al*. (2006), respectively.

The limitation of this study was that few F_1 seeds were produced because of the difficult nature of crossing the Bambara nut flowers. Hence there is there is a need for more crosses using the same genotypes to obtain sufficient number of F_1 seeds that can be used for genetic analyses on the F_2 or F_3 generations.

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