Seed Set Patterns in East African Cooking Bananas are Asymmetric in Bunches and Fruits

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Seed set patterns in East African Cooking Bananas are asymmetric in bunches and fruits

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Abstract

Background: Low female fertility in bananas is the biggest hurdle for banana breeding. The aim of this study was to determine seed set patterns in East African cooking bananas EACBs to inform future decisions on a more targeted approach of increasing seed set and subsequently banana breeding efficiency. Matooke (AAA) and Mchare (AA) bananas are genetically distinct but belong to the same genetic complex, they referred to as EACBs. Seed set patterns in 'Enzirabahima' (AAA), 'Mshale' (AA) and 'Nshonowa' (AA) all with residual fertility were examined after hand pollination with a highly male fertile wild banana 'Calcutta 4' (AA).

Results: Seed set in 'Enzirabahima' is predominant in distal hands. Mchare cultivars have a slightly more even distribution of seeds in their hands compared to 'Enzirabahima.' There is a gradual increase in seed set from proximal to distal hands with a slight drop in the last hand. This pattern is more definite in 'Enzirabahima' and 'Mshale' while 'Nshonowa' has a somewhat inconsistent pattern. There is also a drop in seed set per 100 fruits per hand from small to larger bunches. However, larger bunches have a higher pollination success compared to smaller bunches. They therefor set more seed on 100 fruits per hand and per bunch basis if bunches without seed are accounted for. Pollination success rate increases from smaller to larger bunches of EACBs. Seed set is biased toward the distal third part of fruits of examined EACBs as well tetraploid Matooke hybrid '401K-1' (AAAA) and improved diploid 'Zebrina' GF (AA) that were used for comparison. In comparison, in the highly female fertile 'Calcutta 4,' seed set is along the entire length of the fruit.

Conclusion: Seed set bias in the distal hands and distal end of fruits suggests a systematic mechanism rather than a random occurrence. It is expected that this information will provide a foundation for increased crossbreeding efficiency in bananas.

Keywords: female fertility, banana breeding, Matooke and Mchare pollination, bunch size, pollination success

Background

Bananas (*Musa* ssp.) including plantains are the world's most popular fruit crop grown in about 137 tropical and subtropical countries on an estimated 5 million hectares of land (FAOSTAT, 2018) Though grown in the tropics and subtropics, their origin is traced back to Southeast Asian and Western Pacific regions where their wild relatives exist in natural forests (Robinson and Saúco, 2010). Bananas are also the only fruit crop that is a staple food for many farming communities in the tropics. Overall banana production (excluding plantains) was estimated at 125 million tons in 2017 (FAOSTAT, 2018) with only 14% exported (FAO, 2018) and the rest was locally consumed. Latin America and the Caribbean accounted for 86% of all exported bananas, Africa accounted for 4% and the rest came from Asia (FAO, 2018). The East African region produced close to 10 million tonnes of bananas in 2017 (FAOSTAT, 2018) with negligible export. The dominant banana type in the Great Lakes region of East Africa is the East African Highland Bananas (EAHBs) commonly known as Matooke (AAA) (Karamura et al., 2012). The Mchare subgroup (AA) which is genetically distinct from Matooke is common in Tanzania (Němečková et al., 2018); it is classified as a dessert banana by FAO. But Matooke and Mchare can be collectively referred to as East African Cooking Bananas (EACBs).

From the 1970s, banana production in East Africa experienced a drop attributed largely to a complex of diseases (fungal, bacterial, and viral), nematodes, and insect pests (Viljoen et al., 2017). Low production and productivity is also attributed to poor agronomic practices, abiotic stresses including poor edaphic factors and an imminent threat of climate change (Pillay and Tripathi, 2007; Ortiz and Swennen, 2014). Most banana breeding programs therefore focus on these production constraints when defining their breeding goals. The major stumbling block to crossbreeding in bananas is low seed set (Pillay and Tripathi, 2007). Ssebuliba *et al.* (2005) reported an average of 303 ovules per fruit in EAHBs, yet the seed set range was only 0-25 per bunch. This implies that an average sized EAHB bunch with 100 fruits could potentially yield over 30,000 seeds. A total of 78 EAHBs were screen in Uganda of which 37 were considered to be seed fertile (Ssebuliba *et al.,* 2005). On the other hand, Mchares have not been well characterized, especially for female fertility. The reasons for low seed set are a complex array of factors that have hindered efficient banana breeding. For the triploid bananas including the EAHBs, female sterility as a result of meiotic failures has been a major obstacle (Pillay and Tripathi, 2007). Some cultivars including those of the EAHBs are rendered "infertile" (Ssebuliba et al., 2006a) thus; improvement of such cultivars by conventional means is difficult.

Nevertheless, female sterility has also been observed among diploid bananas (Ray, 2002), suggesting factors beyond meiotic failures contribute to sterility. For example, successful pollination can be achieved when flowers of 'Gros Michel' bananas are pollinated between 7:00 am and 10:30 am (Shepherd, 1960b). To unveil the mystery of overcoming sterility in *Musa*, responsible factors have to be dealt with comprehensively to have a better perspective. In most analyses in *Musa*, seed set is considered on a bunch basis. However, bunch size for the same genotype varies depending on environmental and soil fertility factors (Fortescue and Turner, 2011). Intriguingly, analyses by Shepherd (1954) strongly suggested a relationship between bunch size and seed set with larger bunches setting more seed on a seed set per 100 fruits basis. Besides, significant differences in seed set for hand position have been observed in EAHBs and this was linked to stigma receptivity (Ssebuliba et al., 2006b). Distal hands were found to have more receptive stigmas than proximal hands thus the higher fertility in distal hands. In plantain, seed set also depends on hand position (Swennen and Vuylsteke, 1989) and there is a general tendency of seed set in the middle hands for both plantain and EAHBs. In addition, seed set in 'Gros Michel' was found to be predominant in the distal end of the fruit rather than the proximal end (Shepherd, 1954).

These observations have not been made on individual bunch size basis especially in EAHBs and there is a need to look into the behavior of seed set position in hands of different bunch sizes. Correlation coefficients between seed set and weather attributes are mostly less than 0.5 (Ssebuliba et al., 2009). This suggests that there are more factors that are yet to be identified and considered. Batte et al. (2019) also found no month effects for pollination success of EAHBs after 21 years of crossbreeding. This raises questions about the underlying causes of observations made and how understanding these factors can ultimately lead to overcoming the fertility crisis in *Musa.* The aim of this study was to investigate seed set patterns in hands of the bunch, number of fruits per hand in different bunch sizes, seed set position in the fruits as well as pollination success in relation to bunch size of EACBs. Seed set patterns were also compared for different pollination techniques used. The data generated will informs future decisions on a more targeted approach of increasing seed set in edible bananas and facilitating banana breeding.

Materials and methods

Banana genotypes used and study site

Musa (AAA group, Matooke subgroup) 'Enzirabahima' and *Musa* (AA group, Mchare subgroup) 'Mshale' and 'Nshonowa' with residual female fertility were used as female parents. *Musa* (AAA group, Matooke subgroup) 'Nakitembe' and *Musa* (AA group, Mchare subgroup) 'Mlelembo' were also pollinated along with the first three cultivars. Only one bunch of 'Nakitembe' set one seed while 'Mlelembo' set two seeds in the same bunch, over 200 bunches of each of these two cultivars were made. Because of the extreme low fertility, ‘Nakitembe’ and ‘Mlelembo’ were not considered for seed set patterns in EACBs. Matooke and Mchare banana groups are referred to as EACBs with reference to their utilization. A highly pollen fertile *Musa acuminata* ssp. *burmannicoides* (‘Calcutta 4’) wild banana was used as a male parent.

Each female parent was planted in a pollination block with 9 mats within columns and 22 mats within rows to yield a total of 198 mats. A spacing of 3 m between rows and 2 m between mats in columns was used which gave a density of 1,666 mats/ha. The male parent ‘Calcutta 4’ was planted in columns separating female parents. Improved diploid ‘Zebrina GF’ (AA) and tetraploid Matooke hybrid ‘401K-1’ (AAAA) from other pollination blocks were used for comparison with EACBs seed set patterns in fruits. The study was conducted at the National Agricultural Research Laboratories (NARL), Kawanda. NARL is located at latitude 0° 25’ N, longitude 32° 32’ E at an elevation of 1,177 m above sea level.

Pollination techniques and procedure

Different pollination techniques were used; these include, hand pollination as described by Vuylsteke *et al.* (1997) (technique 1 or control). Technique 1 was modified by applying pollen germination media (PGM) on stigmas after dusting pollen (technique 2). The third technique involved early pollination by forcing bracts open about a day before opening with PGM application on stigmas after dusting pollen. The third was explored between June 2016 and February 2018. The fourth and last technique was evening pollinations after normal bract opening with PGM application on stigmas. This was also explored between June 2016 and June 2017. These pollination techniques were performed on different bunches as they emerged. They were geared towards increasing seed set but the focus here is seed set patterns.

Fig 1. Procedure of early pollination (technique 3) on *Musa* (AA group subgroup Mchare) 'Nshonowa:' (**A**) Flower bract forced open and tepals being removed; (**B**) Tetals removed to expose stigmas for pollination and pollen applied; (**C**) PGM solution applied with hand sprayer (**D**) Flower bract returned in position (**E**) Inflorescence re-bagged and labeled. This process from A to E is repeated for pollination of next hand when it is ready. (**F**) Pollinated bunch left to mature in the open

Pollen germination media was applied with the aim of enhancing stigma receptivity for maximum pollen germination as demonstrated in our earlier work (Waniale et al., 2020). Using tap water, PGM was prepared using 30 g/L glucose as a substitute for sucrose along with other compounds as described by Nyine and Pillay (2007) and boric acid at 0.1 g/L. The PGM used for pollinations in 2016 was 30g/liter glucose and complete PGM in the rest of the pollinations. The PGM was applied to pollen dusted on stigmas in a fine mist using a hand spray pump before re-bagging (Figure 1). The three cultivars had varying days to full maturity, 'Enzirabahima' matured in 98 days, 'Mshale' in 131 days and 'Nshonowa' in 135 days. On the other hand, 'Nakitembe' and 'Mlelembo' matured in 96 and 130 days respectively.

Data handling

At full maturity when at least one finger started ripening, bunches were harvested, kept in a ripening room and seeds were hand extracted, washed, air-dried and counted. Total number of seeds per hand was recorded for each cultivar and each bunch. The number of fruits per hand was also recorded. For each cultivar, bunch sizes were categorized based on number of hands per bunch and percentage seed set was calculated for different hands and different bunch sizes as follows;

$$\frac{\text{Total number of seeds per hand position for a given bunch size category}}{\text{Total number of seeds for a given bunch size category}} X\ 100\%$$

Pollination success rate was calculated as follows;

$$\frac{\text{Total number of bunches with seed for a given bunch size category}}{\text{Total number of pollinated bunches for a given bunch size category}} X\ 100\%$$

Analysis of variance (ANOVA) on number of fruits per hand for different bunch sizes of the three cultivars was done using GenStat, 14th Edition (Payne et al., 2011). Fruits per hand were taken as treatment with total bunches as replicates. The number of hands in a bunch category was therefore the total number of treatments and different categories were analyzed separately. The ANOVA model used was as follows;

Fruits per hand = Mean + Treatment + Error

Seed set per bunch was adjusted to seed set per 100 fruits of each bunch for equal footing comparison of bunches with different total fruits and hands. The adjustment was made as follows;

$$\frac{\text{Total seed in a bunch}}{\text{Total number of fruits of that bunch}} X\ 100$$

To compare seed set of different hand positions on equal footing, average seed set per hand was adjusted to seed set per 100 fruits of that hand. Only bunches with seed were considered in the calculation. The adjustment was made for the three cultivars and all bunch sizes as follows;

$$\frac{\text{Average seed of hand position}}{\text{Average number of fruits of that hand position}} X\ 100$$

Where average seed of hand position was calculated as;

$$\frac{\text{Total seed of hand position}}{\text{Number of bunch with seed}}$$

Seed set per 100 fruits per hand was plotted against hand position for different bunch size categories of the same cultivar on the same plot.

Chi-square goodness of fit (X^2_{cal}) for seed set in hand positions was calculated as follows;

$$\sum \frac{(\text{Observed seed per hand} - \text{Expected seed per hand})^2}{\text{Expected seed per hand}}$$

Where;

Observed seed per hand is the number of seed in given hand position for all fertile bunches.

Expected seed per hand of a particular bunch size category and cultivar was calculated as;

$$\frac{\text{Average fruits per hand for that hand position}}{\text{Average fruits per bunch}} \text{ X Total seed}$$

Degrees of freedom for the Chi-square test are the number of hands in bunch size category minus one. Chi probability (X^2_{prob}) was taken as the right tailed Chi probability.

The position of seed set in the fruit pulp was observed by carefully splitting open pollinated ripe fruits of 'Mshale,' 'Nshonowa' and 'Enzirabahima' in comparison to the tetraploid Matooke hybrid '401K-1' (AAAA). Seed set positions of pollinated partially parthenocarpic improved diploid 'Zebrina GF' (AA) and in the non-parthenocarpic open pollinated wild banana 'Calcutta 4' (AA) were also observed for comparison with EACBs. Selected pictures of seed set position in fruits are presented.

Results

Percentage seed set per hand and fruits per hand

Irrespective of the pollination technique used, seed set pattern was similar within bunch size categories among the three female fertile EACBs. Data of different pollination techniques and same bunch size categories were therefore merged for the respective cultivars (Supplementary Tables 1, 2, and 3). For 'Enzirabahima,' maximal seed set was observed in the second last hand of the 4, 5, and 7 hand bunches (Table 1).

Table 1. Percentage seed set per hand and by number of hands per bunch in EACB

Cultivar	Bunches with seed	Bunch size (hands)	Total seed	Seed set per hand (%)									
				1	2	3	4	5	6	7	8	9	10
'Enzirabahima' (AAA)	6	4	27	3.7	22.2	44.4	29.6						
	23	5	89	0.0	4.5	28.1	34.8	32.6					
	43	6	243	1.6	10.7	15.2	30.5	22.6	19.3				
	34	7	210	0.0	5.2	15.2	14.8	25.7	27.1	11.9			
	7	8	28	0.0	10.7	10.7	32.1	7.1	21.4	10.7	7.1		
'Mshale' (AA)	2	4	42	11.9	38.1	31.0	19.0						
	31	5	610	5.7	17.2	24.1	24.6	28.4					
	47	6	961	5.5	8.3	19.7	22.8	24.2	19.5				
	29	7	1,193	10.9	18.3	17.5	17.5	15.8	10.7	9.3			
	4	8	67	0.0	7.5	13.4	9.0	28.4	26.9	10.4	4.5		
	1	10	66	6.1	43.9	19.7	12.1	12.1	6.1	0.0	0.0	0.0	0.0
'Nshonowa' (AA)	1	4	2	0.0	0.0	0.0	100.0						
	10	5	39	0.0	15.4	25.6	20.5	38.5					
	11	6	90	4.4	22.2	22.2	26.7	17.8	6.7				
	17	7	234	15.8	16.2	11.1	12.8	16.2	10.3	17.5			
	14	8	237	3.4	3.4	8.4	10.1	15.6	16.9	35.4	6.8		
	3	9	36	0.0	27.8	0.0	0.0	2.8	25.0	8.3	2.8	33.3	
	3	10	29	3.4	10.3	0.0	6.9	17.2	17.2	24.1	0.0	3.4	17.2

The 6 and 8-hand bunches had maximal seed set in the third last and fourth hand respectively. There was generally less seed set in the first two hands of 'Enzirabahima' with no seed in the first hands of 5, 7, and 8 hand bunches. There were no embryos in seeds from the 'Nakitembe' and 'Mlelembo' bunches the set one and two seeds per bunch respectively. There was seed set increase in 'Enzirabahima' after pollination with PGM. But the increase happened in the same fertile hand positions as of bunches pollinated without PGM (Supplementary Table 1).

In 'Mshale,' and 'Nshonowa,' on a percentage seed set basis, there was generally a more even seed set among middle hands with the proximal and distal hands having fewer seeds. Mchare cultivars had 7 hand bunches with relatively even seed set across all hands. Unlike 'Enzirabahima,' 5 hand bunches of Mchare were observed to set maximal number of seeds in last hand with female fruits. Similar results were obtained by Swennen and Vuylsteke (1989) for seed set in plantain (*Musa* AAB group), especially in cultivar 'Obino I`Ewai.' Smaller bunches had the highest seed set in the middle hands whereas larger bunches had a slight shift of maximal seed set to the distal hands (Table 1). Though fewer Mchare bunches were pollinated in comparison with 'Enzirabahima,' they had relatively higher pollination success (Supplementary Tables 1, 2, and 3). Generally, there was an increase in average seed set rates per 100 fruits as well as success rate from small to larger bunches in the three EACB cultivars (Supplementary Tables 1, 2, and 3).

The Mchare cultivars had a higher seed set range of 0 to 261 per bunch compared to the range in 'Enzirabahima' of 0 to 33 (Supplementary Table 1, 2, and 3). 'Mshale' had a higher seed set range of 0 to 261 per bunch than that of 'Nshonowa' with 0 to 85 seeds. The highest seed set per bunch in 'Mshale' was from a 7 hand bunch, from an 8 hand bunch in 'Nshonowa' while for

‘Enzirabahima,’ a 6 hand bunch had the highest. Strangely, there were hyper increases of seed set in a few bunches which could not be replicated in same size bunches of the same cultivars pollinated about the same time. This prompted an investigation of the features around the mats where such bunches grew. Interestingly, such mats were on pockets of poor soil with some pebbles.

There were significant differences (P<0.001) for number of fruits per hand among ‘Enzirabahima,’ ‘Mshale,’ and ‘Nshonowa’ bunches of different sizes (Table 2). Number of fruits in the last hand with female fruits of all different sized bunches evaluated were significantly lower than in other hands. The exception was 4-hand category of ‘Nshonowa’ which was non-significant even if there was a difference of 5 fruits between the proximal and distal hands. The number of fruits per hand, however, seemed not to account for highest seed set in the distal hands as there was a gradual decrease of fruits per hand from proximal to distal hands. With these differences in fruits per hand position, a better approach of comparing seed set between hands was a prerequisite.

Table 2. ANOVA for fruits per hand for different bunch sizes of ‘Enzirabahima,’ ‘Mshale,’ and ‘Nshonowa’

Cultivar	Bunch size (hands)	Number of bunches	Fruits per hand 1	2	3	4	5	6	7	8	F-pro
‘Enzirabahima’ (AAA)	4	39	12.3a	11.7a	10.9a	9.0b					<0.001
	5	107	14.3a	14.2a	13.1b	12.2c	10.0d				<0.001
	6	103	14.6ab	15.3a	14.1bc	13.5c	12.0d	8.5e			<0.001
	7	52	14.7ab	15.6a	15.0ab	14.2bc	13.2c	11.9d	7.3e		<0.001
	8	4	18.8a	18.8a	17.3ab	17.0ab	15.3b	15.3b	12.5c	8.5d	<0.001
‘Mshale’ (AA)	4	5	15.0a	14.2ab	13.0b	11.2c					<0.001
	5	63	16.1a	15.9a	14.9b	13.4c	11.6d				<0.001
	6	46	15.4a	15.2a	14.2ab	13.4bc	12.4c	11.1d			<0.001
	7	15	14.2a	14.4a	13.7ab	12.9ab	12.3ab	11.3bc	9.7c		0.004
	8	3	17.7a	17.7a	16.3ab	14.3bc	14.7abc	12.7cd	11.7cd	10.7d	0.002
‘Nshonowa’ (AA)	4	9	14.0a	12. 7a	11.1a	9.3a					0.143
	5	52	16.8a	16.2a	14.7b	13.4c	11.7d				<0.001
	6	35	17.1a	16.7ab	15.4bc	14.3cd	13.4d	11.1e			<0.001
	7	20	14.7a	14.7a	13.4ab	12.8bc	12.0bc	11.6cd	9.8d		<0.001
	8	12	15.9a	15.5a	14.8ab	14.3abc	13.6abc	12.5bc	11.5cd	9.3d	<0.001

Average seed set per 100 fruits per hand

In ‘Enzirabahima,’ seed set per 100 fruits per hand followed a hyperbolic pattern for all bunch sizes from proximal to distal hands (Figure 2). For all bunch sizes, proximal hands had less seed set which gradually increased in hands that followed and slightly dropped in last hand with female fruits. Small sized bunches had higher seed set per 100 fruits per hands which gradually reduced with increased bunch size. ‘Mshale’ had a similar pattern as ‘Enzirabahima’ with the exception of 7 hand bunch category which had the most seed in proximal hand (Figure 3). The 'Mshale' curve for 7 hand bunch category had highest averages partly because of a single bunch that had 261 seeds. For both 'Mshale' and 'Enzirabahima,' the 4 hand bunches experienced the biggest drop in seed set in the last hand. 'Nshonowa' did not have a consistent pattern as 5, 7, and 9 hand bunch categories had positive "a" coefficients of the quadratic equation (Figure 4 and Table 3). With the exception of 5 hand bunch category of 'Nshonowa,' bunches with 6 or less hands had high R^2 values (Table 3). These observations were as a result of few bunches used to calculate means especially for the 9 and 10 hand bunch categories which had three bunches with seed each. The 9 and 10 hands categories were therefore excluded from the plot (Figure 3). A chi-square test for goodness of fit revealed that observed number of seed was significantly different from expected number except in 'Mshale' of 4 hands per bunch category (Supplementary Table 4).

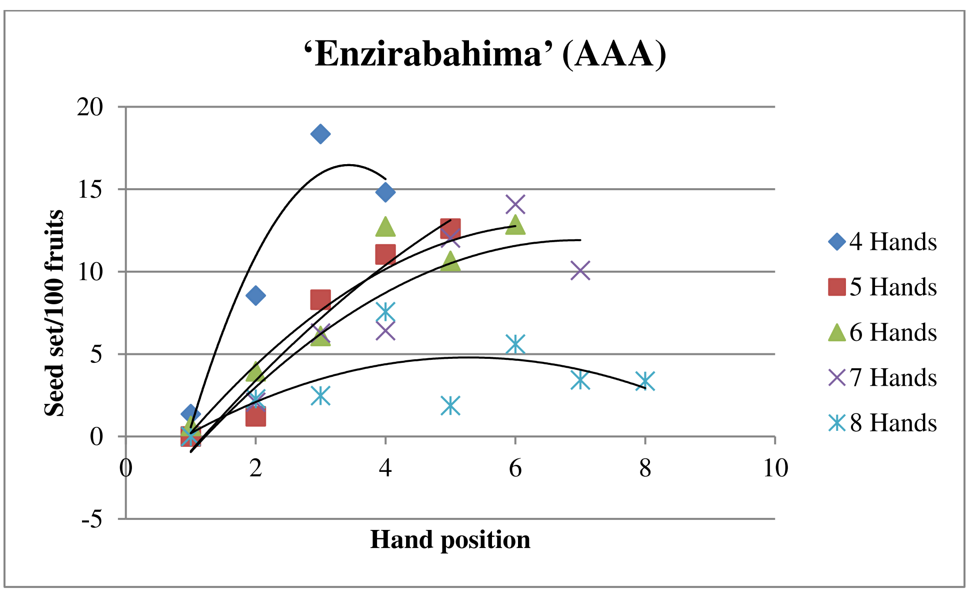

Fig 2. Seed set per 100 fruit per hand in different sized bunches of 'Enzirabahima'

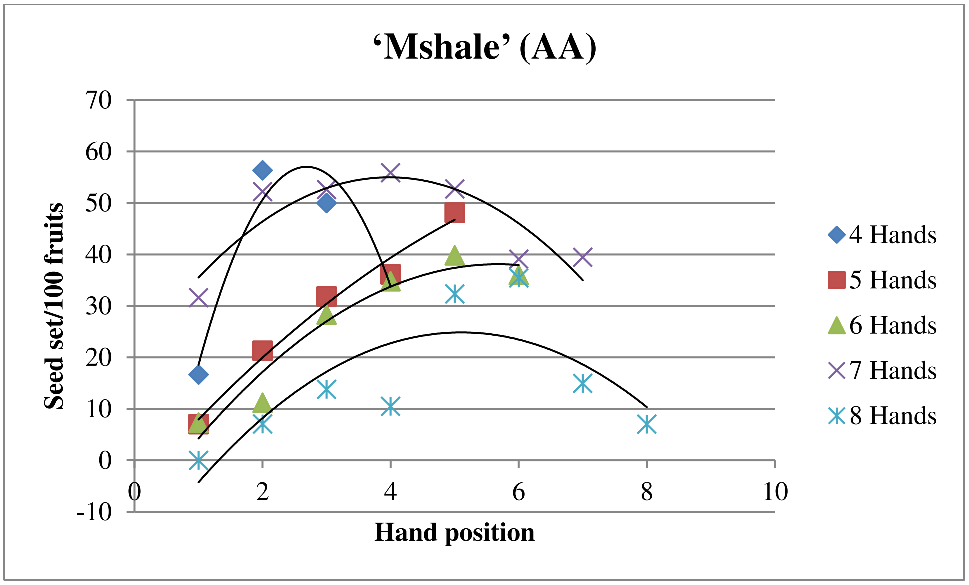

Fig 3. Seed set per 100 fruit per hand in different sized bunches of 'Mshale'

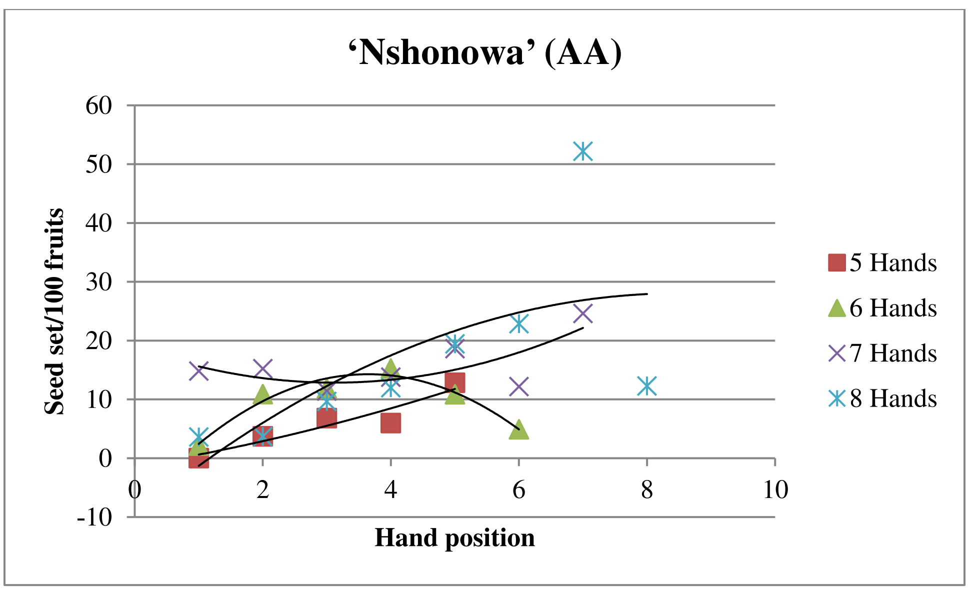

Fig 4. Seed set per 100 fruit per hand in different sized bunches of 'Nshonowa'

Table 3. Second order polynomial and R^2 values fitted for seed set per 100 fruits per hand

Cultivar	Bunch size (Hands)	Bunch number	a	b	C	R^2
'Enzirabahima' (AAA)	4	6	-2.68	18.43	-15.19	0.924
	5	23	-0.26	5.07	-5.70	0.943
	6	43	-0.40	5.33	-4.72	0.915
	7	34	-0.36	5.02	-5.61	0.880
	8	7	-0.25	2.65	-2.21	0.447
'Mshale' (AA)	4	2	-13.49	72.53	-40.47	0.922
	5	31	-0.77	14.34	-5.64	0.982
	6	47	-1.55	17.56	-11.77	0.939
	7	29	-2.19	17.46	20.25	0.773
	8	4	-1.73	17.66	-20.21	0.629
'Nshonowa' (AA)	5	10	0.17	1.78	-1.33	0.886
	6	11	-1.69	12.34	-8.22	0.948
	7	17	0.62	-3.84	18.84	0.529
	8	14	-0.52	8.86	-9.63	0.447
	9	3	0.94	-7.06	15.84	0.384
	10	3	-0.26	3.55	-2.31	0.193

Seed set position in fruits

For fruits which set seed among female fertile EACBs predominantly set seed in the distal one third of the fruit, especially when there were no more than 3 seeds (Figure 5c). The same pattern was observed in '401K-1' (AAAA) and 'Zebrina GF' (AA) that are used in breeding. In the event of multiple seed set in a fruit (more than 3), seeds were distributed in the distal half of the fruit (Figure 5b). This was observed mainly in Mchare. On the contrary, open pollinated wild banana 'Calcutta 4' set seed over the entire length of the fruit (Figure 5a).

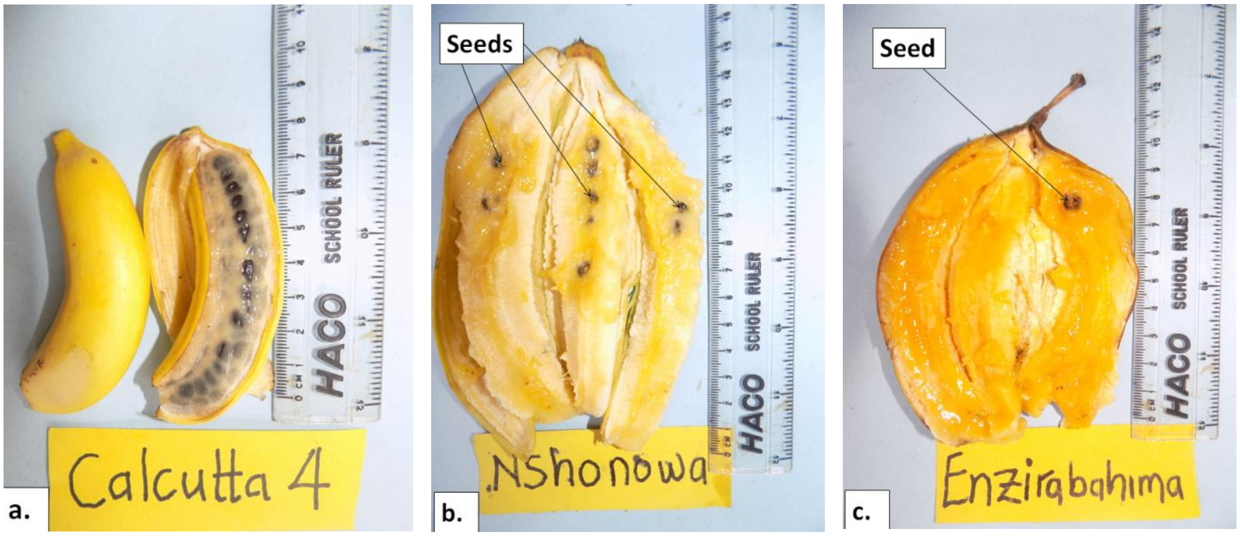

Fig 5. Seed set position in fruits as revealed after ripening among selected banana genotypes. a) Small intact and peeled fruits of non-parthenocarpic open pollinated wild banana ‘Calcutta 4’ with numerous evenly distributed seed. b) Multiple seed in the distal end of the fruit in the cultivar ‘Nshonowa’ pollinated with ‘Calcutta 4’. c) Single seed set in the distal end of ‘Enzirabahima’ pollinated with ‘Calcutta 4’.

Discussion

Getting an in depth understanding of seed set patterns in EACBs is certainly one of the measures that will help in designing experiments for increasing seed set in *Musa* spp. The end goal is to have a fast and an efficient banana breeding pipeline with better hybrids for many farmers in the tropics and subtropical regions globally. Here, there is a deliberate effort to understand the cause of sterility by looking at seed set patterns in relation of position of the hands, bunch size and position in the fruit in EACBs. In *Musa* spp. sterility has been understood to arise from developmental errors in the sporophyte, the gametophyte as well as pistil-pollen interactions. But more emphasis has been placed on understanding the gametophyte which is said to account most towards sterility (Fortescue and Turner, 2011). Meiotic errors as a result of chromosome mismatch were reported by Simmonds (1962) to cause embryo sac failure in sterile *Musa* spp. Fortescue and Turner (2005), however, found that about 10% of embryo sacs in ovules of triploid bananas were correctly positioned compared to 75% in *Musa acuminata* ssp. They also discovered that the presence of a B-genome increased the presence of an embryo sac to 96-100%. But these observations do not explain the fact that seed set in EACBs is biased toward distal hands and distal end of the fruit. There is also no documentation of a link between the presence and correct positioning of an embryo sac to seasonal influence of seed set in *Musa* spp. with residual fertility.

The observed bias of seed set in distal hands especially in Matooke has been attributed to the high stigma receptivity in distal hands (Ssebuliba et al., 2006b). But if this were entirely true, then the use of PGM on stigmas would have increased seed set in the proximal hands as well since all hands are pollinated. And since 'Enzirabahima' was reported to have fairly high stigma receptivity in all hands, seed set should have been distributed in all hands. Instead, seed set increase was observed in the same hand positions as of bunches pollinated without PGM. Use of PGM should have also resulted in seed set in 'Nakitembe' and 'Mlelembo' if stigma receptivity was a prime contributor of sterility in *Musa*. This implies that there are other factors which come into play after pollen has germinated on the stigmas in different hand positions. The necrosis formed in the prolongation zone of fruits after anthesis especially in triploids (Soares et al., 2014) could partly account for absence of seed set in proximal hands. This necrosis acts as a barrier to pollen tube growth. Shepherd (1960b) observed that pollen tube growth through the stigma in 'Gros Michel' looked normal, but was slowed or arrested and the tips developed a swelling resulting in pollen tubes not reaching the ovules. It may be worthwhile to investigate rates of necrosis formation in fruits of different hand positions to find a link to seed set if any.

In an attempt to avoid the necrosis formed soon after anthesis in the fruit prolongation zone, early pollinations were made (technique 3) but the technique did not increase seed set. Shepherd (1960a) speculated that physiologically immature stigmas may delay or prevent penetration by pollen tubes and this could have been the case with early pollination. Results in the present study suggest that application of PGM on stigmas enhanced pollen germination (Waniale et al., 2020). But this was most effective on stigmas presumed to be physiologically mature after natural flower opening as suggested by Shepherd (1960a). This observation may also suggest that arrest of pollen tube growth may be a biochemical rather than a physical process. It is supported by the fact that a necrosis is not formed in the prolongation fruit zone of diploids (Soares, et al. 2014). Pollination technique 3 was therefore discontinued in December 2016 since seed set was lower compared with the customary pollination technique described by Vuylsteke et al. (1997). The other plausible cause of low seed set with technique 3 could have been that the PGM did not have all components for *in vitro* pollen germination as 30g/L glucose was used. Evening pollinations were also discontinued in 2016 since they did not increase seed set. This resulted from reduced pollen viability with time of the day coupled with low humidity at the time of pollination between 5 and 6:30 pm. The rationale of evening pollination was to have flowers pollinated soon after opening as *Musa* flowers start opening from evening through the night. However, not all female flowers had fully opened at the time of evening pollination and fresh pollen could not be obtained. Fairly reliable comparisons were made between the control and technique 2 since a considerable number of bunches were fairly distributed in different months of year during the study period (Supplementary Tables 5, 6, and 7).

Ssebuliba et al. (2006b) found that bracts of distal female hands opened to a bigger angle than that of proximal hands. This phenomenon was linked to a response of stigma receptivity which increased from proximal to distal hands. But it might be that ovules in proximal hands and the proximal fruit end have higher abortion rates if pollen tubes reach them. Abortive ovules have been reported in other crops like hazelnut whereby some unigenes are upregulated and others down regulated in abortive ovules compared to developing ovules (Cheng et al., 2015). The gradual increase of seed set from proximal to distal hands could suggest a mechanism of ovule abortion rate in the same order. The most likely cause of this observation could be auxins in large amounts from the root tips that reach proximal hands first. Auxins induce formation of edible pulp in both seeded and non-seed bananas as well as partially parthenocarpic types (Simmonds, 1953; Simmonds, 1960). And in seeded bananas, synthetic auxins 4-CPA and 2,4,5-T have been reported to hinder seed development (Simmonds, 1960). Auxins therefore play a critical role in parthenocarpy as well as sterility.

The effect of weather on seed set in *Musa* spp. could be as a result of a drop in auxin levels with heat and/or moisture stress. This comes with high temperature, high solar radiation and low rainfall. These weather conditions correlate with increase in seed set in Matooke (Ssebuliba et al., 2009). This is likely to slow down evapo-transpiration pull as the plant tries to conserve moisture. Consequently, movement of materials including auxins does not reach the distal hands and fruit tips in adequate amounts. A reduced fruit circumference of 'Gros Michel' correlates with increased seed set (Shepherd, 1954) which can reflect reduced parthenocarpy. This theory also tends to fit the observed increase of seed set per 100 fruits with increase in bunch size; small bunches are saturated easily. A reduction in auxin levels during moisture stress could be caused by salicylic acid which is involved in response to both abiotic and biotic stress (Rivas-San Vicente and Plasencia 2011; An and Mou 2011). Salicylic acid and auxin signalling are mutually antagonist (An and Mou 2011) and producation of salicycic acid in response to moisture stress or heat stress could be responsible for reduced auxin levels thus reduced parthenocarpy. This may be linked to the sponteneous seed set increase observed from bunches on mats with poor soils which drain easily thus high salicylic acid production.

There were inconsistences in patterns of seed set in ‘Nshonowa’ as fewer bunches were available compared with the other two cultivars. Using few bunches with seed to calculate averages for ‘Nshonowa’ implied that a single bunch with high seed set affected the overall shape of the curve. It was also noticed that bunches with high seed set had uneven seed distribution among hands. Sudden seed increase was observed in hand position 7 of 8 hand bunch category. It strongly suggests that there are specific weather conditions for maximum seed set as hands were pollinated on different days with unique weather conditions. High morning temperatures are likely to overcome the issue of pollen tube growth arrest. In citrus (Kawano et al., 2016) and in apples (Yoder, et al. 2009), high temperatures were reported to overcome self-incompatibility and this it could be a similar issue in banana. This could have applied to all the three EACBs but use of many bunches evened out this effect in ‘Enzirabahima’ and ‘Mshale.’

Results in the current study revealed that the hands with the highest number of fruits do not necessary produce the highest number of seeds. This clearly suggests that there are other factors that are more important for seed set. Ideally, it is expected that different sized bunches have same pollination success and same seed set per 100 fruits. However, these observations suggest that bunch size has an influence on fertility based on differences of seed set per 100 fruits for different bunch sizes. Shepherd (1954) also observed an increase of seed set on 100 fruit basis from small to larger bunches of ‘Gros Michel’ as observed in EACBs. Bigger bunches are also more fertile than smaller bunches in terms of total seed and pollination success. The theory of auxins influencing fertility in different bunch sizes seems to fit this observation. Smaller bunches would be easily saturated by auxins compared with larger bunches. This leads to the low seed set (per 100 fruit basis) and low pollination success in smaller compared to larger bunches. However, there is higher seed set per 100 fruit per hand in smaller bunches if only bunches with seed are considered. But large bunches generally set more seed per hand as a result of higher pollination success compared to small bunches.

All these observations call for a slightly different approach to better understand sterility and use it profitably as it is a prerequisite in the final hybrids. It may be the right time for banana researchers to start looking in the direction of hormonal manipulations for increased seed set. But since segregation data suggests that parthenocarpy and sterility are independent (Simmonds, 1962), this would rule out auxins as the sole cause of sterility in *Musa* spp. A recent genome wide association study in *Musa* found parthenocarpy genes to be potentially linked to seedlessness. But the prime candidate gene was the gene orthologous to Histadine Kinase CKI1 (Sardos et al., 2016). Cytokinins have been reported to determine the fate of seed development (Jameson and Song, 2016) and they could be responsible for sterility in *Musa* even if there is successful fertilization. Non-parthenocarpic progeny that has sterile plants could therefore point to ovule abortion. This stems from a mutation in the gene orthologous to Histadine Kinase CKI1 that was linked to seedlessness by Sardos et al. (2016).

It could be possible that the same gene orthologous to Histadine Kinase CKI1 is responsible for production of poor seed and low embryo rescue rates in some parental combinations. Some edible banana genotypes have been rendered ‘infertile’ and this could be as a result of hyper production auxin individually or in combination with the gene orthologous to Histadine Kinase

CKI1 which has been linked to seedlessness. It could be that relative contribution these two factors along with other factors can result in high sterility as observed in 'Nakitembe' and 'Mlelembo.' Other factors such as fruit length are involved in banana fertility as demonstrated in the current study. In 'Calcutta 4,' seeds set covers the entire length of the fruit implying pollen tube growth covered an estimated distance of 10 cm. In 'Nshonowa,' pollen tube growth covered about 10 cm and about 7 cm of the fruit pulp had no seed. And in 'Enzirabahima,' pollen tubes cover a distance of about 5 cm with about 8 cm of pulp without seed. This suggests that pollen tube growth within the fruit is not the reason for a biased seed set towards the fruit tip as the distance covered in 'Enzirabahima' is shorter. With the same approach of auxin involvement in *Musa* fertility, it likely that auxins can covers a limited distance during moisture stress thus longer fingers set more seed. This explains the higher seed set observed in Mchare compared with Matooke in the current study. The drop in seed set from the last hand could also be as a result of reduced fruit length in the last hand. Fruits in the proximal hands are longer with bigger circumference which reduces towards the distal end of the bunch. This was reported in plantain and cooking banana types, distal hands have fruits with the least fruit length (Annor et al., 2016).

Conclusions

The results of this study demonstrated that small sized bunches of EACBs have low seed set and pollination success rates. Seed set in EACBs is mainly predominant in the distal hands starting in the mid-section of the bunch. Seed set in fruits is also skewed to the distal end with strong evidence that fruit length and bunch maturity period being involved in fertility. The study also showed that seed set patterns in hand positions are not influenced by stigma receptivity as earlier thought. There is a mechanism that prevents seed set in proximal hands that needs further experimentation to be understood. Auxins are likely to be one of the most important causes of sterility in *Musa* but they are not entirely responsible. A holistic approach will therefore be required to increase seed set and overcome sterility. Efforts have to be devoted to improving *in vivo* pollen germination and having agronomic practices that increase bunch size. In future, scientists may have to consider hormonal manipulations especially those that will reduce parthenocarpy. Overcoming sterility will ultimately broaden the parental base to include genotypes whose breeding potential is unknown. A selection criterion of parents has always included male and female fertility trait yet such parents are often non-parthenocarpic. Being able to use parents with parthenocarpy and inherent sterility in crosses will increase the number of parthenocarpic hybrids per cross thus a more efficient breeding pipeline.

Supplementary information

Abbreviations

EACBs: East African cooking bananas; EAHBs: East African Highland bananas; PGM: Pollen germination media; NARL: National Agricultural Research Laboratories; ANOVA: Analysis of variance; 4-CPA: 4-chlorophenoxy acetic acid; 2,4,5-T: (2,4,5-trichlorophenoxy) acetic acid

Declarations

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Authors' contributions

Conception of research idea and designing experiments: AW, RS, AKT. Performed experiments, analyzed data and wrote the paper: AW. Contributed materials: RT, JK, WKT. All authors read and commented on the paper before submission.

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Availability of data and information

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Procedure of early pollination (technique 3) on Musa (AA group subgroup Mchare) ‘Nshonowa:’ (A) Flower bract forced open and tepals being removed; (B) Tetals removed to expose stigmas for pollination and pollen applied; (C) PGM solution applied with hand sprayer (D) Flower bract returned in position (E) Inflorescence re-bagged and labeled. This process from A to E is repeated for pollination of next hand when it is ready. (F) Pollinated bunch left to mature in the open

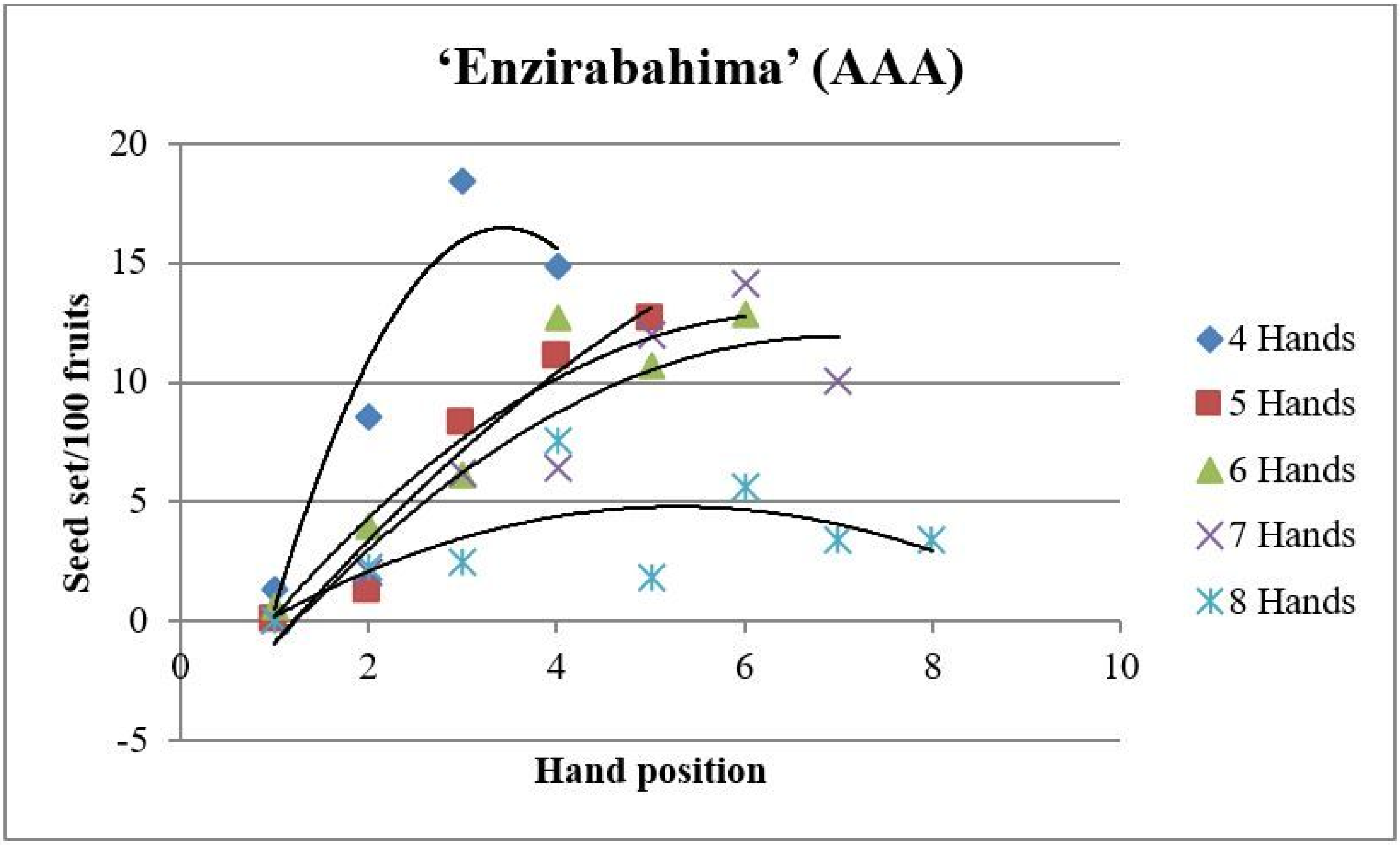

Figure 2

Seed set per 100 fruit per hand in different sized bunches of 'Enzirabahima'

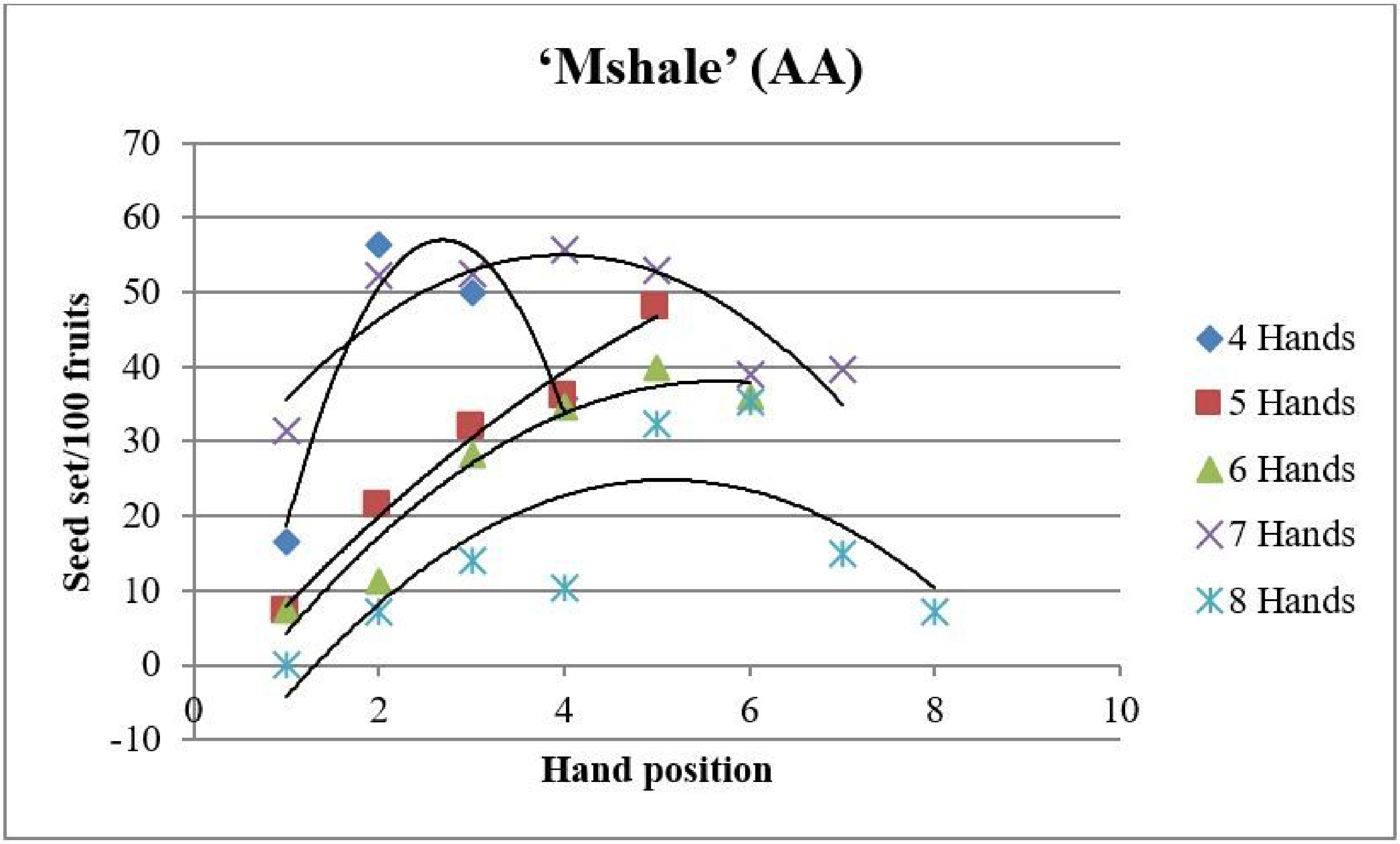

Figure 3

Seed set per 100 fruit per hand in different sized bunches of 'Mshale'

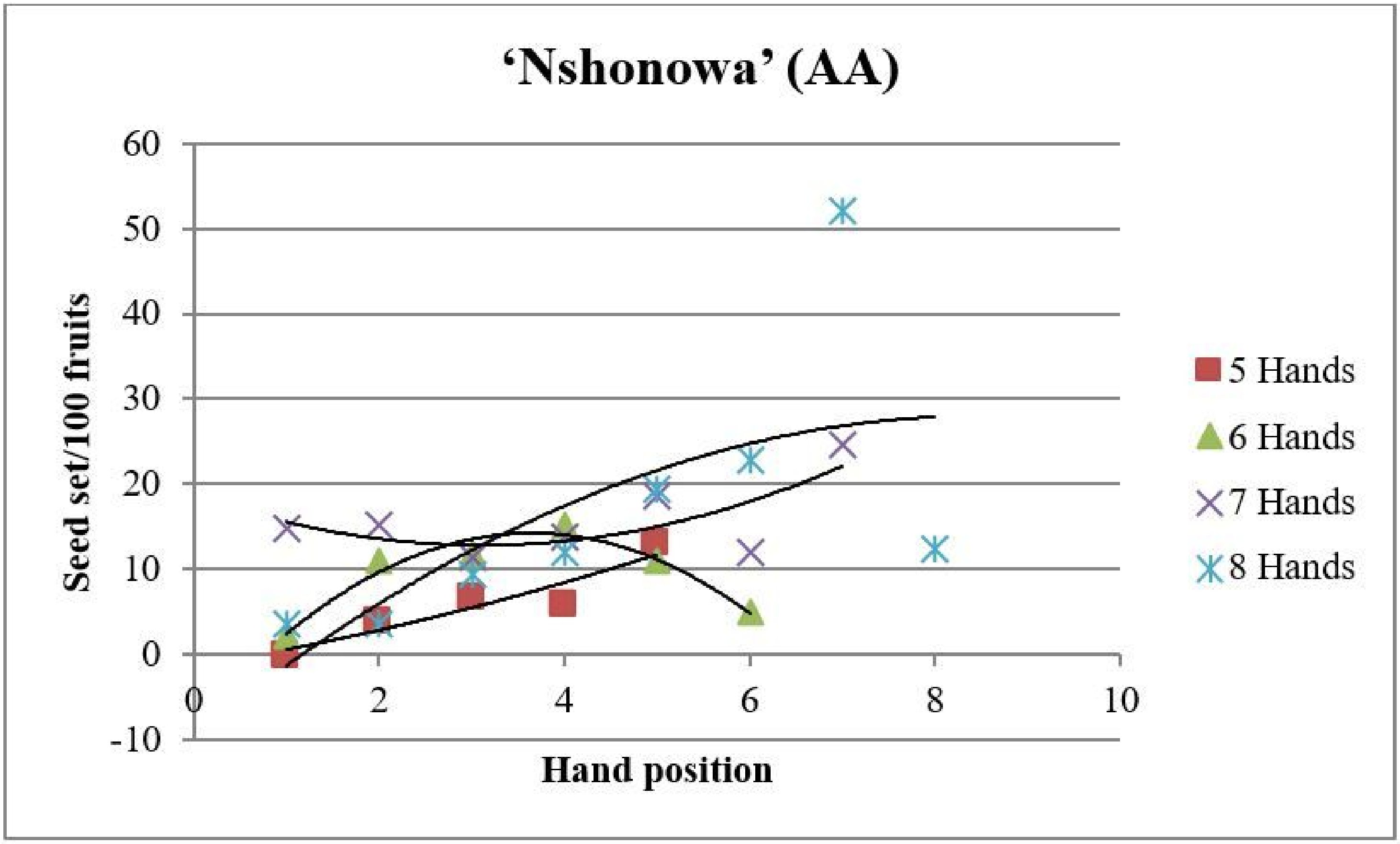

Figure 4

Seed set per 100 fruit per hand in different sized bunches of 'Nshonowa'

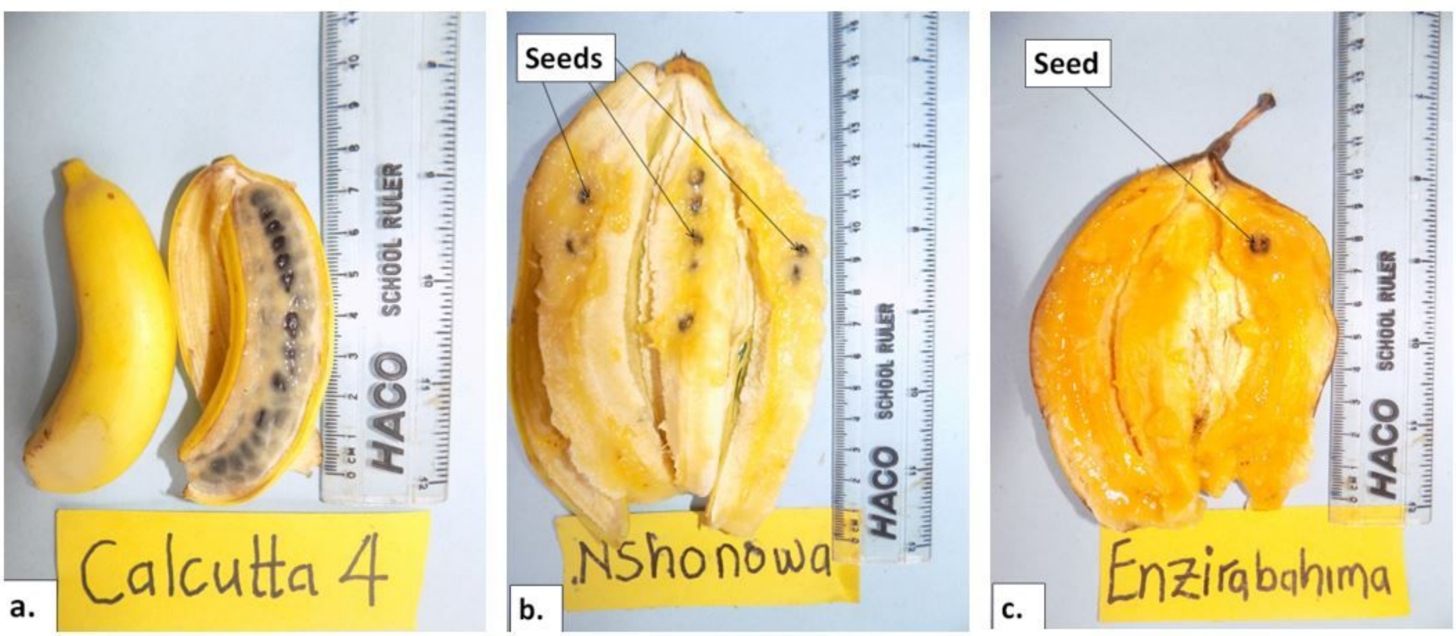

Figure 5

Seed set position in fruits as revealed after ripening among selected banana genotypes. a) Small intact and peeled fruits of non-parthenocarpic open pollinated wild banana ‘Calcutta 4’ with numerous evenly distributed seed. b) Multiple seed in the distal end of the fruit in the cultivar ‘Nshonowa’ pollinated with ‘Calcutta 4’. c) Single seed set in the distal end of ‘Enzirabahima’ pollinated with ‘Calcutta 4’.

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