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Application of Pollen Germination Media on Stigmas during Pollination Increases Seed Set in East African Highland Cooking Bananas (*Musa* spp.)

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Abstract: Seed set in East African Highland Cooking bananas (EAHBs) is extremely low and therefore hampers breeding. Pollen–pistil interaction is a key contributing factor. We assessed the effect of pollen germination media (PGM) on seed set in EAHBs. Five EAHB cultivars were pollinated with pollen from the wild banana 'Calcutta 4'. Glucose-based PGM sprayed on freshly emerged stigmas significantly increased seed set per 100 fruits per bunch. Increases were 73.5% in 'Enzirabahima', 39.9% in 'Mshale', and 302.4% in 'Nshonowa'. However, PGM did not increase seed set in the female sterile 'Mlelembo' and 'Nakitembe'. As larger bunches were more fertile, good field management practices are also recommended to get more seed to improve breeding efficiency.

Keywords: east african highland cooking bananas; pollination technique; seed set; breeding efficiency

1. Introduction

The East African Highland Cooking bananas (EAHBs) are an important food crop in the Great Lakes region of East Africa. They are composed of Matooke and Mchare banana types [1]. Their cultivation is however becoming difficult due to increasing pests and diseases that reduce yield [2–4]. Breeding resistant banana cultivars through crossbreeding is one of the most appropriate and cost-effective interventions [5–8]. However, conventional breeding of the EAHBs is impeded by low seed set [9,10]. Cultivated bananas are parthenocarpic and highly sterile. Conventional breeding programs have, nevertheless, screened landraces and selected those with residual fertility for genetic hybridization [10–14]. In Uganda, 37 out of 78 Matooke landraces are considered female fertile [9] yet their genetic variation is extremely low [15,16]. Quite often, the most preferred landraces are not included in crossing schemes because they have very poor seed set. For example, 'Nakitembe' and 'Mbwazirume' are among the most preferred Matooke landrace cultivars by end-users, but they do not set seed [9,10]. Hence, the use of seed fertile but less preferred Matooke landraces reduces the chance for acceptance of new hybrids by end-users [17]. There is the need to overcome sterility in the most preferred landraces, one approach being the use of new pollination techniques.

It is a common practice to perform hand pollinations in banana to generate seeds that are later germinated into new hybrids [18,19]. These pollinations are usually made either

on edible bananas types with residual female fertility or fertile diploids for purposes of male parent improvement. On an inflorescence, several female hands open over different days and are pollinated on a daily basis [20]. Flower opening in banana happens at about 5:00 p.m. and continues into the night [21,22], although hand pollinations are made after day break. After a series of pollinations at different times in the day on 'Gros Michel,' the highest seed set was obtained between 7:00 a.m. and 10:00 a.m. [20]. This range of pollination time has thus been widely adopted across banana breeding programs worldwide [22].

Immediately after pollination, flowers are bagged to avoid cross pollination from unwanted parents. Bagging ensures that pollen contamination by bats and insects or other animal activity is prevented [23]. The author in [20] experimented by keeping cotton bags on the pollinated bunches moist during the day by spraying with water, while controls were left unsprayed. The idea was to increase humidity around the freshly pollinated stigmas. Bunches from wetted bags only in the morning yielded the highest seed per bunch followed by wetting during the entire day, and controls gave the lowest seed set. However, the increments were not significant, and it was concluded that humidity alone cannot yield high seed set as observed in nature. The author in [20] also observed a delay of about an hour between pollination and pollen tube penetration on *Musa acuminata* wild types. Recently, we observed that more banana pollen germinates faster on Matooke stigmas treated with pollen germination media (PGM) compared to untreated stigmas [24]. The objective of the present study was therefore to assess the effect of pollination using PGM on seed set in selected EAHBs.

2. Materials and Methods

2.1. Field Site and Banana Germplasm

The experiment was conducted in Uganda at the National Agricultural Research Laboratories (NARL) in Kawanda located at 0°25′ N and 32°32′ E at an elevation of 1177 meters above sea level. The soils at NARL are sandy-loam with deep ferralitic clay type, and pH ranges from 5.5–6.0 [25]. Cultivars used as female parents were *Musa* (AAA group Matooke subgroup), 'Enzirabahima' and 'Nakitembe' as well as *Musa* (AA group subgroup Mchare) 'Mshale', 'Nshonowa', and 'Mlelembo'. The highly fertile wild banana *Musa acuminata* ssp. *burmannicoides* 'Calcutta 4' was used as the male pollen source.

Matooke cultivars are widely grown throughout the Great lakes region of East Africa including Uganda, Rwanda, and Burundi. Mchare cultivars on the other hand are widely grown in Madagascar, Comoros islands, Pemba, and Zanzibar islands, around Mount Kilimanjaro and Mount Kenya to as far as a few spots in central Uganda [1,26]. Matooke and Mchare can be collectively referred to as EAHBs. Molecular data show that Matooke was not derived from Mchare, hence the two groups are genetically distinct [1].

'Enzirabahima' and 'Nakitembe' Matooke cultivars were selected as representatives for their relatively high and low seed fertility, respectively. On the other hand, 'Mshale', and 'Nshonowa' were selected as seed fertile and 'Mlelembo' as seed sterile parents in the Mchare subgroup. The selections in Matooke were made based on cultivar performance in previous crosses [9,10]. Fertility of Mchare is not well known thus selection was based on scanty data.

Each female parent was planted at a spacing of 3 m between rows and 2 m between plants in blocks of 9 rows with 22 mats. Single columns of 'Calcutta 4' were planted in between female parents. Agronomic management practices were optimal with the pollination blocks planted with manure and dressed with 80 g of NPK (17:17:17) per mat every six months. All pollinations were made between January 2016 and January 2019.

2.2. Pollen Germination Media Preparation and Pollination Procedure

Female flowers were covered with cotton bags or transparent polyethylene bags from shooting time until the last female hand was pollinated. Likewise, male buds to be used as pollen sources were also bagged with a cotton bag the day before pollination [27,28]. In

Matooke, flowers are said to be ready for pollination when bracts are halfway lifted and with stigmas appearing fresh with a creamish white color [28].

Hand pollination was done by excising freshly opened male flowers from the male bud and brushing anthers onto the stigmas. Bunches were rebagged and pollinations performed daily until the last female hand was pollinated [28,29]. The day after pollination of the last female hand, bunches were unbagged and tagged with the information of female and male parents as well as the initial pollination date [27].

The first PGM type used on 'Enzirabahima' and 'Mshale' in 2016 consisted of 30 g glucose dissolved in one liter of tap water. No bunches of 'Nshonowa' were ready for pollination in 2016. The rationale was to boost moisture and energy availability for pollen germination. Later pollinations were made with the second PGM type containing 30 g glucose, 0.25 g $MgSO_4.7H_2O$, 0.25 g KNO_3, 0.4 g $Ca(NO_3)_2$, and 0.1 g H_3BO_3 per liter [24]. Media with these ingredients are complete PGM that are typically used for in vitro pollen germination tests.

Control pollinations (without PGM) were performed as described by reference [30]. Pollination with PGM involved the same steps but were followed by misting the media via a hand spray pump at about 15–20 cm distance. Stigmas were wetted just enough with the PGM but not too much to avoid pollen being washed off from the stigmas (Figure 1). Each treatment was applied on a different bunch. Pollinations did not start at the same time in all the five cultivars as planting was staggered. Days to full maturity (from first pollination to harvest) varied with 'Enzirabahima', 'Nakitembe', 'Mshale', 'Nshonowa', and 'Mlelembo' maturing on average after 98, 96, 131, 135, and 130 days, respectively.

Figure 1. Procedure of pollination with pollen germination media to enhance stigma receptivity on *Musa* (AA group subgroup Mchare) 'Nshonowa': (**a**) male flowers rubbed on stigmas of the first hand to apply pollen; (**b**) spraying of pollen germination medium (PGM); (**c**) inflorescence re-bagged and labeled.

2.3. Seed Extraction and Data Collection

When some fruits started yellowing as a sign of full maturity, bunches were harvested and stored in a ripening room. Then, seeds were hand extracted from ripened fruit pulp, washed, air-dried, and counted. Number of fingers per bunch was also recorded and seed set was expressed on a 100 fruits per bunch basis as follows: (Total seed in a bunch)/(Total number of fruits) × 100. Pollination success was expressed as percentage of bunches with the seed of total pollinated bunches. Bunch sizes were categorized based on number

of hands per bunch per cultivar. In 'Enzirabahima', a few bunches were in the 8-hand category, hence merged with the 7-hand category. Likewise, 'Mshale' 4-hand and 5-hand categories were merged and 7, 8, 9, and 10-hand categories were also merged. In addition, in 'Nshonowa', 4-hand bunches were merged with the 5-hand bunch category while 9 and 10-hand bunch categories were merged with the 8-hand category.

In some months, a few bunches were pollinated with some missing data, especially in some bunch size categories. To address this, bi-monthly periods were analyzed. Over the entire study period, a bunch each of 'Nakitembe' and 'Mlelembo' yielded one and two seeds, respectively, after pollination with PGM treatment. They were, therefore, excluded from the analysis because of this very low seed set.

2.4. Data Analysis

Data were analyzed with the Genstat 19th edition to analyze variance (ANOVA). An unbalanced design was used as there were some missing data and because of an unequal number of bunches in bi-monthly periods. Predicted means are obtained since the unbalanced design accounts for unequal numbers and missing data. Coefficients of variation ranged from 150 to 277% and thus data transformation was necessary. Data transformations included: $Log_{10}(x + 1)$, $\sqrt{x}$, $\sqrt{(x + 0.5)}$, $\sqrt[3]{x}$, and $\sqrt[3]{(x + 0.5)}$; the latter gave the lowest coefficients of variation and hence was used in the analysis. Untransformed data were analyzed to obtain and present predicted means using unbalanced designs. In 'Mshale', bunch size was not significant; thus, data were re-analyzed without the smallest bunch size category of five hands to test the effect of small sized bunches. The two media types used on 'Enzirabahima' in 2016 and 2018 were compared with year of pollination, bunch size, and bimonthly periods as treatment factors. The control pollination technique was also compared between 2016 and 2018. Pollination success for the different bunch sizes was also compared with bunch size as treatments and pollination techniques as replicates. Pollination success between the two pollination techniques was compared in a paired *t*-test.

3. Results

There was no significant difference in seed set per 100 fruits per bunch in 'Enzirabahima' whether pollinated with 30 g/L glucose PGM in 2016 or complete PGM in 2018. Seed set per 100 fruits per bunch means for control pollination in the two years were also not significant (Table 1). Data for the two types of PGM were therefore merged for 'Enzirabahima' and 'Mshale'. However, seed set per 100 fruits per bunch in 2018 was numerically higher than that in 2016 for both pollination techniques. The F-probability value for comparison of the two media was less than that of the control comparison in 2016 and 2018 (Table 1). Reliable comparisons could not be made for 'Mshale' because only 15 bunches in total were pollinated in 2016.

Table 1. Mean seed set per 100 fruits per bunch after pollination with and without pollen germination media on 'Enzirabahima' in the years 2016 and 2018.

Year	N	PGM		N	Control	
		x	$\sqrt[3]{(x + 0.5)}$		x	$\sqrt[3]{(x + 0.5)}$
2016	43	1.8	1.1	44	1.3	1.0
2018	105	3.1	1.2	87	2.2	1.1
F-prob		0.559	0.250		0.498	0.953
CV (%)		208.5	50.2		234.4	46.9
SE		5.8	0.6		4.2	0.5

PGM—pollen germination media in 2016 is 30 g/L glucose and 2018 is complete PGM with 30 g/L glucose, N—number of bunches pollinated, x—seed set per 100 fruits, $\sqrt[3]{}$ (x + 0.5)—data transformation formula, CV (%)—percentage coefficient of variation, SE—standard error.

The two pollination techniques were significantly different for seed set per 100 fruits per bunch in 'Enzirabahima' and 'Nshonowa' but not in 'Mshale' (Table 2). However, when the least fertile bunch size of five hands of 'Mshale' was excluded from the analysis, there was significance. Bunch size and bi-monthly periods were significant in the three EAHBs (Table 2). Pollination with PGM increased seed set over the control (Table 3). The largest percentage seed set increase was in 'Nshonowa' and the least was observed in 'Mshale'. However, 'Mshale' had the highest seed set per 100 fruits per bunch means followed by 'Nshonowa' and finally 'Enzirabahima' (Table 3).

Table 2. Analysis of variance with mean squares of $\sqrt[3]{(x + 0.5)}$ transformed seed set data of East African Highland Cooking Bananas after pollination with and without pollen germination media.

Cultivar	'Enzirabahima' (N = 310)		'Mshale' (N = 181)		'Mshale'-5h (N = 106)		'Nshonowa' (N = 168)	
Change	d.f.	m.s.	d.f.	m.s.	d.f.	m.s.	d.f.	m.s.
Pol-Tech	1	1.19 *	1	1.17	1	5.63 *	1	1.67 *
Bunch size	3	1.11 *	2	10.54 ***	1	10.87 **	3	3.65 ***
Bi-monthly	12	0.55 *	9	5.69 ***	9	5.11 ***	7	1.49 ***
Residual	293	0.30	168	1.36	94	1.28	156	0.36
CV (%)		48.6		57.9		51.1		51.2

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, 'Mshale'-5h —'Mshale' without the 5-hand bunch category, N—number of bunches pollinated, d.f.—degrees of freedom, m.s.—mean squares, Pol-Tech—Pollination technique (pollination with and without pollen germination media), and CV (%)—percentage coefficient of variation.

Table 3. Predicted mean seed set per 100 fruits per bunch of two pollination techniques on three East African Highland Cooking bananas.

Pollination Technique	'Enzirabahima'		'Mshale'		'Mshale'-5h		'Nshonowa'	
	N	x	N	x	N	x	N	x
Control	147	1.6	92	14.4	55	16.7	80	1.3
With PGM	163	2.8	89	20.1	51	29.2	88	5.0
% increase		73.5		39.9		74.4		302.4

'Mshale'-5h —'Mshale' without the 5-hand bunch size category, N—number of bunches pollinated, x—mean seed set per 100 fruits per bunch.

Bunches with more hands had significantly higher seed set per 100 fruits and pollination success (Table 4). 'Mshale' had the highest seed set per 100 fruits among the various bunch size categories followed by 'Nshonowa' and lastly 'Enzirabahima'. Again, pollination with PGM had a higher success rate compared to the control (paired *t*-test probability = 0.048, d.f. = 10). Even if the 5-hand bunches of 'Mshale' and 7-hand bunches of 'Nshonowa' had higher pollination success in the controls, they did not affect the overall result. The highest pollination success rates were with 'Mshale' bunches for the two pollination techniques.

Table 4. Bunches pollinated, pollination success, and predicted mean seed per 100 fruits using two pollination techniques on East African Highland Cooking bananas.

Cultivar	BS	Control		With PGM		Mean	
		N	Pol Suc	N	Pol Suc	Seed	Pol Suc
'Enzirabahima'	7	34	47.1	35	51.4	3.3	49.3a
	6	36	22.2	60	40.0	3.0	31.1b
	5	59	18.6	55	27.3	1.2	23.0bc
	4	18	11.1	13	23.1	1.7	17.1c
F-prob							0.013
'Mshale'	7	23	69.6	13	92.3	28.5	81.0
	6	32	59.4	38	71.1	16.6	65.3
	5	37	51.4	38	36.6	13.0	44.0
F-prob							0.213
'Nshonowa'	8	13	61.5	19	68.4	6.6	65.0a
	7	17	47.1	13	38.4	4.3	42.8b
	6	21	23.8	28	28.8	3.2	26.3c
	5	29	13.8	28	14.3	0.9	14.1c
F-prob							0.006

BS—Bunch size (number of hands in a bunch size category), N—Number of bunches pollinated, Pol Suc—pollination success (% of bunches with seed of total bunches pollinated), and Seed—Seed per 100 fruits.

Seed set per 100 fruits per bunch was also significantly variable in bi-monthly periods of the year. Irrespective of the year, January to February and March to April were the bi-monthly periods with the highest means for 'Enzirabahima' and 'Nshonowa' (Table 5). September to December, followed by January to February, was the best period for 'Mshale'. The periods with the highest seed set were not consistent across years. For example, January to February had 5.2 seeds per 100 fruits per bunch for 'Enzirabahima' in 2016 but 3.3 seeds per 100 fruits per bunch in 2018. These are statistically different means after data transformation. However, 'Nshonowa' had a relatively consistent number of seeds in the same period across the years. November to December and May to June had statistically the same number of seeds in 2017 and 2018. This was according to mean separations of transformed data. The periods with the least seed set were not consistent for the three EAHBs, but November to December and May to June periods were those with the lowest seed set.

Table 5. Predicted bi-monthly mean seed set per 100 fruits in East African Highland Cooking bananas after pollination with and without pollen germination media (30 g/L glucose and complete media).

'Enzirabahima'			'Mshale'			'Mshale'-5 h			'Nshonowa'		
Months	N	Mean	Months	N	Mean	Months	N	Mean	Months	N	Mean
Jan–Feb 16	10	5.2	Sep–Oct 16	5	78.5	Sep–Oct 16	3	115.9	Jan–Feb 18	20	14.7
Mar–Apr 16	12	4.8	Jan–Feb 18	13	53.7	Jan–Feb 18	13	55.0	Mar–Apr 18	15	6.1
Nov–Dec 18	39	4.0	Jul–Aug 16	6	53.6	Jul–Aug 16	5	44.4	Jul–Aug 18	27	3.4
Jan–Feb 18	28	3.3	Sep–Oct 18	25	22.3	Jan–Feb 19	4	24.6	Nov–Dec 17	15	2.2
Jul–Aug 18	41	2.4	Nov–Dec 16	4	19.5	Jul–Aug 18	21	20.4	Nov–Dec 18	28	1.1
Jan–Feb 19	31	2.3	Jul–Aug 18	40	15.4	Sep–Oct 18	10	13.9	Sep–Oct 18	31	0.9
Sep–Oct 18	34	2.0	Mar–Apr 18	11	12.1	Mar–Apr 18	11	13.6	May–Jun 18	25	0.6
May–Jun 18	42	1.7	Jan–Feb 19	17	10.7	Nov–Dec 16	2	11.8	May–Jun 17	7	0.1
May–Jun 16	16	1.3	May–Jun 18	31	9.0	May–Jun 18	25	9.1			
Mar–Apr 18	8	0.9	Nov–Dec 18	29	6.6	Nov–Dec 18	12	4.1			
Nov–Dec 16	4	0.5									
Sep–Oct 16	14	0.1									
Jul–Aug 16	31	0.0									

'Mshale'-5h —'Mshale' without the 5-hand bunch category, N—Number of bunches pollinated.

4. Discussion

The ability of pollen to germinate on the stigma depends on extracellular secretions that cover the stigmatic surface. These secretions typically contain carbohydrates, proteins, enzymes, phenolic compounds, and amino acids that primarily determine species recognition [30]. The lengthy pollen–pistil interactions of compatible crosses and negative outcomes of incompatible crosses can partly be modified through PGM application. Indeed, PGM stimulates pollen germination irrespective of the level of compatibility like it happens for in vitro pollen germination [24]. However, as observed in banana, pollen germination on the stigma is not the main barrier since several other factors operate in tandem to result in sterility [22]. For example, in the current study, PGM could not overcome sterility in 'Mlelembo' and 'Nakitembe'.

The application of PGM during pollination resulted in significantly more seed compared to the control. This increase was probably caused by enhanced pollen germination on the stigma [24]. The authors in [11,31] observed differences in seed set between landraces of plantain when pollinated with wild type bananas. The authors in [32] also observed significant differences in seed set between female parents. These observations strongly suggest variable pollen germination delays and/or pollen tube growth leading to a variable level of compatibility between crosses and thus variable seed set.

Seed set was the same whether pollinations were made with PGM containing only 30 g/L glucose in 2016 or complete media in 2018 in 'Enzirabahima'. The use of the customary control pollination technique also produced similar results in the two respective years. The F-probabilities for comparison of the control pollination in the two years and comparison of the two media were expected to be close. However, comparisons revealed that means of control pollination technique were much closer compared to means of pollination with two media types in 2016 and 2018. Since 2018 had a numerically higher mean, it implied that complete PGM in 2018 had a slight edge over 30 g/L PGM in 2016. Calcium ions play a key role in pollen germination and growth [33,34], and they can be sourced from plant tissues [35]. As expected, supplying only a glucose solution increased seed set, but results suggest that adding other components and calcium ions in the form of $Ca(NO_3)_2$ was slightly better. However, for simplicity, the two media were merged and analyzed as PGM specifically for 'Enzirabahima' and 'Mshale' based on comparison in 'Enzirabahima'.

Bunch size of cultivars depends on the soil fertility and environmental conditions [30,36]. Bunches are usually categorized according to number of hands, but large bunches have more fingers per hand compared to small bunches [20]. It was therefore reasonable to standardize seed set to seed per 100 fruits per bunch. After standardization, results revealed the positive effect of bunch size on fertility. Since large bunches set the highest seed, pollination blocks should be optimally managed to ensure the production of large bunch sizes. The underlying cause of low fertility in smaller bunches is unclear and this calls for further investigation.

Seed set in banana cultivars seems to be seasonal [9,11], but this is not always clear [10]; the current study demonstrates the influence of time or seasons on seed set. January to February favorable for seed set is characterized by high temperature and low rainfall while May to June is the reverse. Conditions of moisture stress and high temperatures are therefore favorable for seed set in *Musa* spp. [9,11]. To have a more accurate perspective of fertility in banana, the seed set has to be standardized to seed per 100 fruits or 1000 ovules per bunch. In addition, bunch size in terms of number of hands needs to be considered as this is not only related to field management but also the cultivar.

5. Conclusions

Banana breeding efficiency largely depends on number of seeds generated to produce a large offspring number for selection. This is currently done by expanding pollination blocks as seed set per bunch is very low. However, by using PGM in pollinations, seed set can be increased by 73.5% in 'Enzirabahima', 39.9% in 'Mshale', and 302.4% in 'Nshonowa'.

Hence, PGM should become standard practice in breeding bananas besides the optimal agronomic management of pollination blocks to ensure the production of large bunches. More research is needed to increase seed set in banana, because increases by PGM were still small given the high number of ovules present in a fruit. Use of PGM will therefore be part of other manipulations that will ultimately overcome sterility and realize full seed set potential in bananas.

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