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# Achieving sustainable cultivation of bananas

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**E-CHAPTER FROM THIS BOOK**



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# Overcoming the fertility crisis in bananas (*Musa* spp.)

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## 1 Introduction

Significant opportunities exist for improving edible bananas to tackle emerging productivity challenges due to the continuous spread of diseases and to improve nutritional/compositional qualities of the fruit through crop breeding. Hybridization, a means of generating genetic diversity for crop improvement is central to most crop-breeding schemes. However, the possibilities for hybridizations in bananas are hindered by several reproductive barriers. Edible bananas evolved from the seed-fertile diploid progenitors *Musa acuminata* and *Musa balbisiana*, but most cultivated varieties are triploids and (to a smaller extent) diploids, which are parthenocarpic and seedless (Simmonds, 1962; Brown et al., 2017). While seed production is important for breeding to ensure genetic recombination and provide viable progeny for recurrent selection, parthenocarpy and female sterility are paramount to ensure fruit edibility in

banana cultivars. Consequently, inter- or intra-ploidy crosses involving cultivated varieties, aiming towards their improvement, result in too few or no viable seeds leading to limited progeny hence constituting a 'fertility crisis'. This fertility crisis is unquestionably the biggest challenge for genetic improvement of banana.

Hybridizations in banana were first attempted in Trinidad in the 1920s aiming to introduce resistance genes from diploids to commercial triploids to create a *Fusarium* wilt resistant 'Gros Michel' (AAA) export dessert banana (Simmonds, 1966; Shepherd, 1974). From that time onwards banana breeding programs have mostly relied on conventional sexual hybridization involving crossing of triploid cultivars which, at most, have residual fertility with wild or cultivated fertile diploids to generate primary tetraploids. Subsequently fertile tetraploid plants are crossed with diploid hybrids to generate secondary triploids which are mostly sterile (Tenkouano and Swennen, 2004; Bakry et al., 2009). However, only a limited proportion of the wide diversity of *Musa* spp. has yielded viable hybrids in breeding programs while much of this diversity is still inaccessible for breeding. The majority of banana cultivars and hybrids have at most residual male and female fertility. Limited hybrid production also complicates the use of routine conventional breeding techniques and modern molecular techniques applicable to most seed producing crops. Banana breeders therefore tend to devote vastly more effort towards producing progeny rather than evaluating them (Tenkouano et al., 2011).

Several key reproductive steps in the life cycle of a flowering plant are responsible for adequate seed production. Pollination and subsequent fertilization of the ovules in the fruit are crucial processes in producing viable seeds. Knowledge of reproductive biology and breeding systems of bananas has progressed considerably although in-depth studies on flower development are limited to only a few banana cultivars (Fortescue and Turner, 2011) or *Musa* species (Kirchoff, 2017). Understanding the specific morphological, physiological and environmental factors surrounding the processes responsible for effective pollination, fertilization and viable seed production as well as identifying and overcoming possible barriers to these processes are essential for successful banana breeding.

This chapter discusses the key processes surrounding effective fertilization and viable seed production in relation to our current knowledge on banana-reproductive biology and the gaps to further our knowledge. Throughout the chapter we focus on possible limiting processes and provide insights on ways to overcome the fertility crisis to expand the possibilities for gene recombination through intra- and interspecific crosses.

## 2 Reproductive biology of banana

The reproductive biology of banana can be summarised as a series of processes (Fig. 1). The production of fertile seed can be derailed at numerous

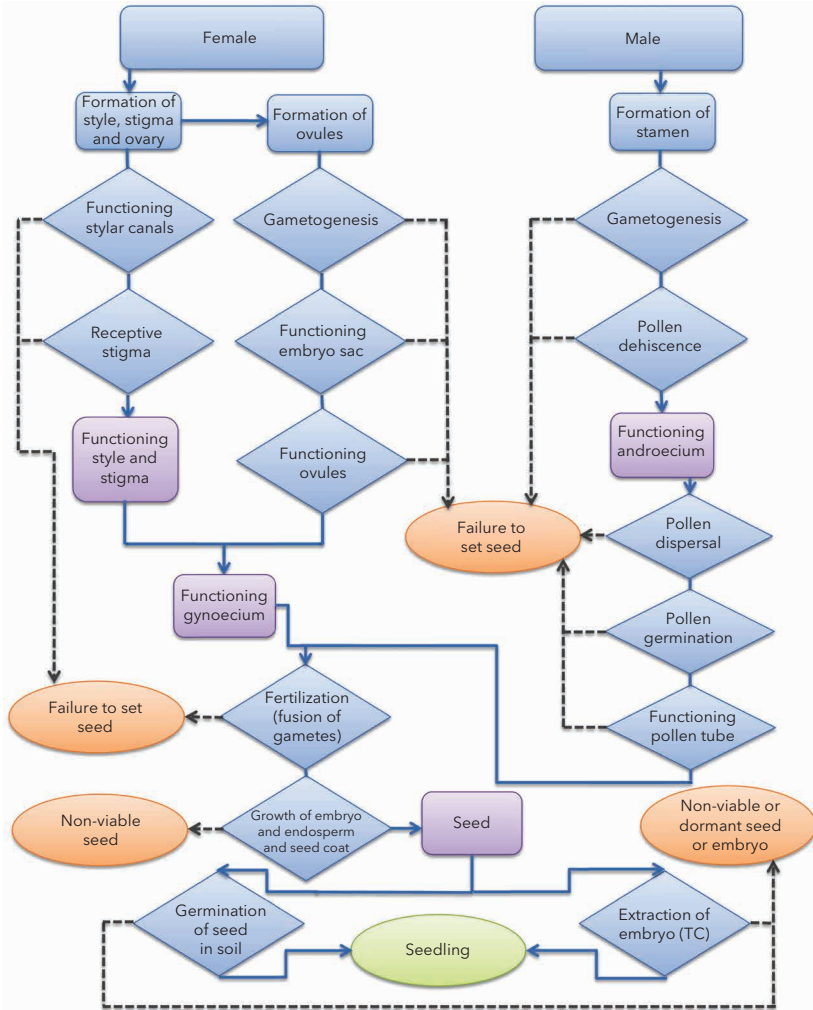
locations throughout the series due to genetic and environmental challenges. For a single gamete, failure at any 'decision' point (Fig. 1), in either the female or male series limits viable seed production. Some gametes will succeed and so the fusion of two fertile gametes may result in the production of a seed. The eventual seed set in an ovary will reflect to some degree the sequential success (or failure) of processes in the series. Furthermore, breeding schemes experience additional challenges at the level of seed and embryo germination which also needs to be addressed.

Historically the explanations for sterility emphasized meiotic errors arising from polyploidy, changes in chromosome structure, or inter (sub)species hybridity all leading to gametic sterility. However, research has revealed that there are more processes involved in sterility. For example, morphological errors occurring either before (multiple archesporia) or after gametogenesis (failure of embryo sac development) have been reported (Fortescue and Turner, 2005a). Subsequent errors such as pollen tube growth inhibition in the style or in the ovary, or failure of embryo development post fertilization have also been reported to contribute to infertility (Simmonds, 1962). Specific female sterility genes may also be involved (Sardos et al., 2016).

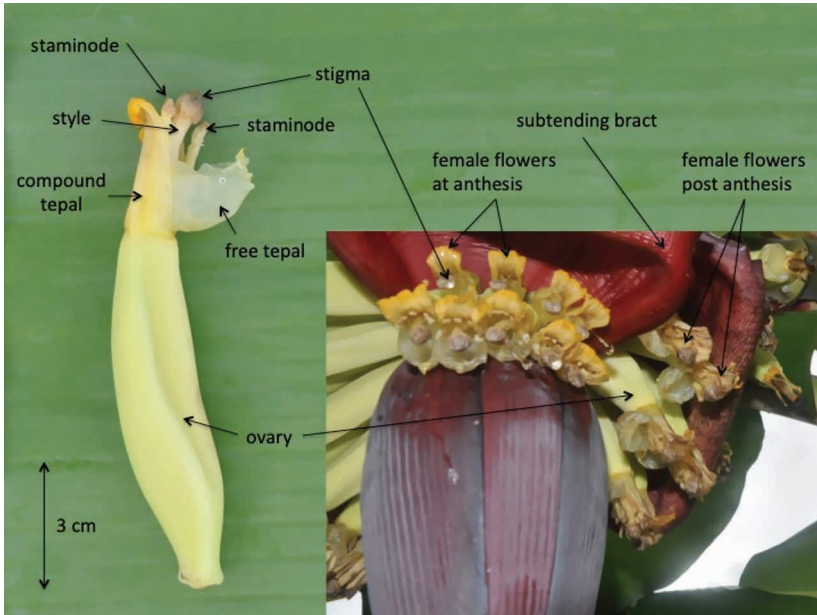
## **2.1 The inflorescence**

Bananas can be reproduced vegetatively through suckers and sexually through botanical seeds. The banana inflorescence is a thyrse with each node of flowers best described as a cincinnus (Turner and Gibbs, 2018). Most bananas are monoecious (female and male flowers produced on the same plant) and dichogamous (pistils [carpels] and stamens mature at different times), resulting in temporal and spatial separation of the two sexes within an inflorescence. The hands of female flowers are formed before the hands of male flowers, with each hand on the same floral axis and each subtended by a bract. Anthesis for each hand of flowers commences with their exposure upon lift of the subtending bract. Flowering time depends on cultivar and environmental conditions.

In the early stage of inflorescence development, the female and male flowers are morphologically almost indistinguishable, with gynoecial (pistils) and androecial (stamens) parts of the flower developing in both, although with subtle differences in the pattern and the size of organ development (Kirchoff, 2017). Ultimately, however, whether the mature flower is female or male is not due to small differences in the pattern of organogenesis but rather is concerned with the size and fertility of the gynoecium and androecium in the flowers (Kirchoff, 2017). By the time the developing inflorescence is about halfway up the pseudostem, the ovaries of female flowers have become longer than those of the male allowing female flowers to be distinguished from male



**Figure 1** Key pre- and post-fertilization processes in reproductive biology, leading to success or failure in producing a seedling. This diagram is conceptual, focusing on the processes around the successful fusion of a female gamete with a male gamete in a banana ovary. It does not represent the sequential development of female and then male flowers that occurs on a single inflorescence. The sequence flows from flower formation to seedling. It starts with the flower type and moves from formation of organs (blue rectangles) by the solid lines to the functional components (purple rectangles) needed for seedling production (green oval). The diamonds in each part of the sequence represent 'decision' points between each stage where events may fail (broken lines) and lead to unproductive end-points indicated by orange-coloured ovals. The outcome of these 'decision' points for an individual gamete or for a population of gametes is discussed in the accompanying text (Sections 2-6). Development of the inflorescence is described in Section 2.1. A key step in female reproductive biology is meiosis, which occurs about 5-7 days before the stigma becomes receptive; for the oldest female



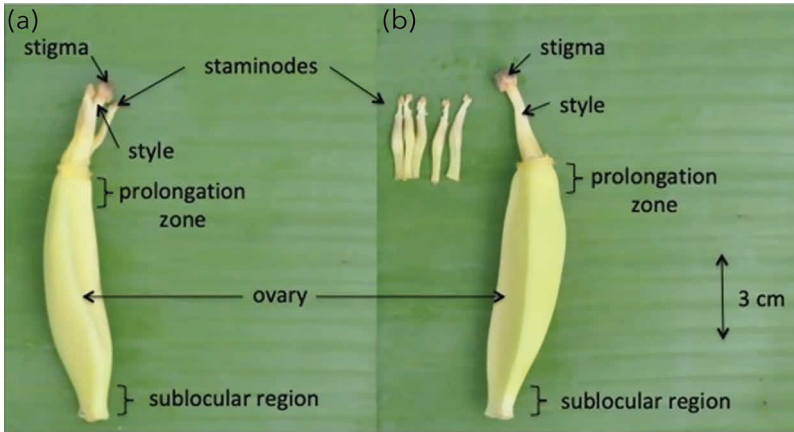
**Figure 2** A female flower of *Musa* (AAB) plantain 'Bobby Tannap', at anthesis showing its position within a hand beneath a subtending bract (inset) and parts of the individual flower. The ovary forms the fruit, the style and stigma are massive and the androecium is reduced to staminodes. The compound and free tepals enclose the style, stigma and staminodes. The 3 cm scale applies to the single fruit.

flowers (Stover and Simmonds, 1987). Functionally-female flowers, that is, fruit-forming flowers, typically have a non-functioning androecium, with less-well to poorly developed or absent staminodes and no pollen (Turner and Gibbs, 2018) (Figs 2 and 3). In male flowers, the ovary is much smaller and non-fruit forming, the style and stigma slender and the androecium morphologically well developed (Figs 4 and 5).

In some *Musa* species, hermaphrodite fruit-forming flowers with pollen-producing stamens capable of self-fertilization are found (Simmonds, 1966;

**Figure 1** (Continued)

flowers, this is about the time the bunch emerges from the pseudostem (Simmonds, 1962). Gametogenesis, including meiosis, is discussed in Section 2.2. Anthesis of female flowers occurs as or soon after the bract subtending the flower lifts (Section 3), at which time the stigma becomes receptive to pollen (Section 2.5.1). Following pollination and germination of pollen on the receptive stigma, it takes about 12 hours for the pollen tube to grow down the style; fertilization then occurs after which the zygote remains single-celled for about 4 weeks, although the endosperm begins development (Simmonds, 1962). For the style (2.3.1), pollen tube growth and pollen guidance, see Section 2.6. For pollen viability, see Section 2.5.2. For fertilization and seed set see Section 4 and for seed and embryo dormancy see Section 5.



**Figure 3** A female flower of *Musa* (AAB) plantain 'Bobby Tannap', at anthesis with the tepals removed. (a) The gynoecium is intact with ovary, basal sublocular region, distal prolongation zone and massive style and stigma. Staminodes intact. (b) As in (a), but the five staminodes removed to reveal style and stigma. Scale applies to (a) and (b).

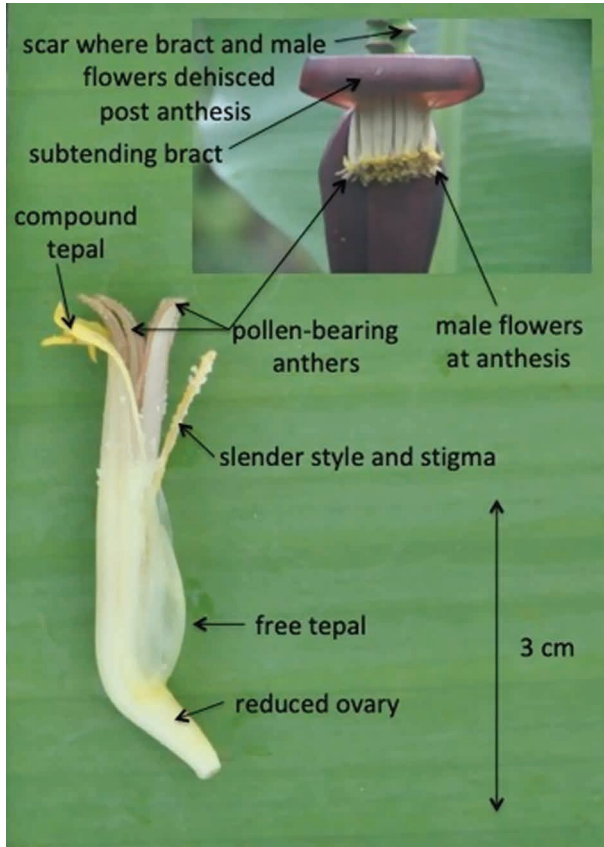
Argent, 1976). There are no reports of the occurrence of such flowers in edible cultivars. Moreover, there is no evidence of hermaphrodite fruit-forming flowers in *M. balbisiana* (BB), one of the foundation species for some of the edible bananas (Argent, 1976). This opens *M. balbisiana* to out-crossing, in contrast to hermaphrodite flowers which can self-fertilise. Functional stamens in fruit-forming flowers of wild species might increase selfing and limit species hybridisation (Shepherd, 1999).

## 2.2 Gametogenesis

Gametogenesis is the process by which gametes required for sexual reproduction are produced. A central part of the process is meiosis, a cell division mechanism whereby genetic material is exchanged in cross-overs between arms of paired homologous chromosomes, which then separate to form gametes (Harrison et al., 2010). Megaspores and pollen mother cells (2n) undergo meiosis to produce haploid cells (n). In flowering plants the cells formed by meiosis are the egg cell in the embryo sac in the case of female gametes and pollen in the case of male gametes. Meiosis involves two cell divisions and has a number of phases between each division – prophase, metaphase, anaphase and telophase.

### 2.2.1 Why is gametogenesis an issue in banana?

In banana hybrids, whether or not viable gametes are produced during gametogenesis depends in part on ploidy, and the interaction between the

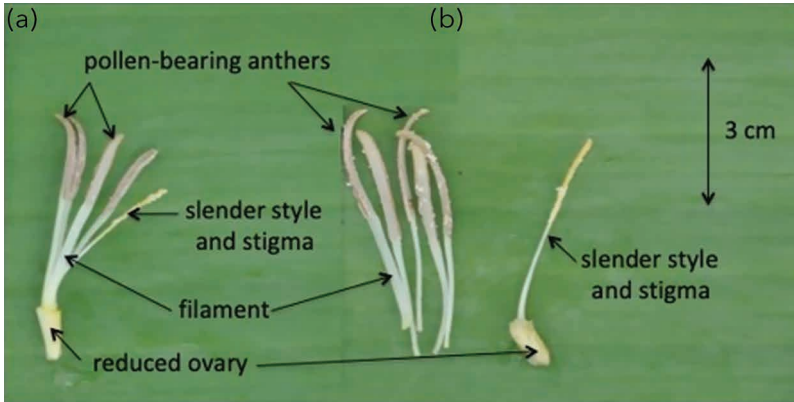


**Figure 4** A male flower of *Musa acuminata* ssp. *burmannica* 'Calcutta 4' (AA), at anthesis showing its position within a hand (inset), subtending bract and scars on the male peduncle from which preceding hands of male flowers have abscised post-anthesis. The gynoecium is greatly reduced, especially the ovary (compare with Figure 2), with a slender style and stigma. The androecium is well developed with large stamens and pollen-producing anthers. The 3 cm scale applies to the single male flower.

genomes, especially those arising from different species, such as *M. acuminata* and *M. balbisiana*. In addition, chromosome structural changes can contribute non-viable gametes resulting in seedless edible diploids (Dodds, 1943). These influences are expressed at the level of meiosis in ways such that Dodds and Simmonds (1946) concluded that failure can occur at any point during meiosis. Here we follow the association between fertility and unpaired chromosomes (univalents) for illustration.

*Musa* species follow the normal pattern of meiosis, but many edible bananas (triploids and diploids) have been selected by people over time for female sterility and parthenocarpic development of pulp. For triploids the

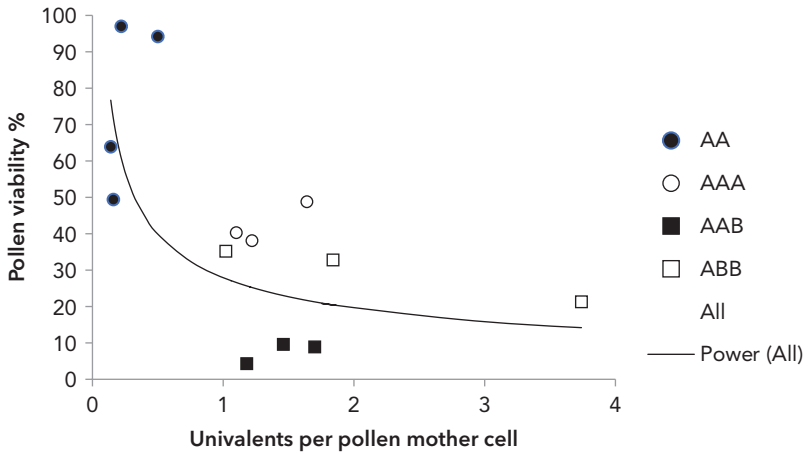




**Figure 5** The male flower of *Musa acuminata* ssp. *burmannica* 'Calcutta 4' (AA), with tepals removed to show (a) pollen bearing anthers and (b) the slender style and stigma separated from the androecium.

matching of pairs of chromosomes is incomplete or unbalanced, leading to infertility (Griffiths et al., 2015). For pairing of chromosomes in triploids in meiosis there are two possibilities: formation of trivalents or, bivalents + univalents. Bivalents are paired homologous chromosomes and univalents are unpaired chromosomes. In banana breeding, when a triploid female and diploid male are crossed, the segregation of the progeny into different ploidy levels allows inferences to be made about the successful fusion of female and male gametes (Wilson, 1946a). Experiments in Jamaica, in which the same diploid male parent was used, produced progeny of different ploidy from three different cultivars within the AAA genome (Wilson, 1946a). The progeny of 'Gros Michel' were mainly tetraploids, for 'Red' triploids dominated and for 'Cavendish' no progeny were produced. 'Bluggoe', an ABB cultivar tended to produce diploid progeny. The complexity of female and male fertility and their interaction in *Musa* has been long known (Cheesman and Dodds, 1942). An additional dimension is the species to species interactions between *acuminata* (A) and *balbisiana* (B) genomes in triploids and diploids (Jeridi et al., 2011).

In banana, studies of chromosome behaviour during meiosis have a long history (Cheesman, 1932) continuing to this day (e.g. Shepherd, 1999; Adeleke et al., 2004), and recently in the seminal work of Jeridi et al. (2011) and that of Šimoníková et al. (2019). Lack of pairing (asynapsis) or subsequent separation of pairs (desynapsis) may contribute to sterility. Asynapsis produces univalents that can be counted (Wilson, 1946b; Adeleke et al., 2004). Simmonds and Dodds (1949) found failure of pairing to be rare in seeded diploid *Musa* spp. In some genotypes many chromosomes show asynapsis but in others only one chromosome pair may be affected (Wilson, 1946b). The broader negative



**Figure 6** The relationship between the number of univalents per pollen mother cell and pollen viability in a range of diploid and triploid banana clones. For the equation  $y = -19.48\ln(x) + 38.54$ ,  $r^2 = 0.45$ . Data from Adeleke et al. (2004).

association between the number of univalents per pollen mother cell, for example, and pollen viability (determined by staining techniques) (Fig. 6) had some distinct components. Adeleke et al. (2004) counted univalents in 4 diploids and 9 triploids. The triploids had 1-2 univalents per pollen mother cell with pollen viability of 1-50%, compared with diploids with less than 0.5 univalents and pollen viability ranging from 60% to 97% (Fig. 6). Variation within these broad groupings of pollen viability showed that it was associated with factors other than just the number of univalents.

Under tropical conditions usually one or two hands of male flowers appear each day. For a seeded diploid meiosis in the pollen mother cells (prophase to young tetrads) is completed within the equivalent time taken for one or two male hands to open, that is one to two days (Dodds and Simmonds, 1946). From tetrads to pollen grains takes another 13 hands, that is, 13 days. In contrast, for a diploid hybrid with double restitution, tetraploid pollen was rapidly produced over 4 days because the two normal cell divisions in meiosis did not occur. In this case the stages of meiosis from first metaphase to tetrads were not detected. Shepherd (1999) described this as 'meiosis avoidance'. The tetraploid pollen grains once formed, quickly degenerated and were not detected when the hand of male flowers opened some two weeks later.

Another process giving rise to seedless fruit may occur through meiotic restitution which is the production of  $2n$  gametes (unreduced gametes) by incomplete cell division in meiosis. Male meiotic restitution can be induced through low temperatures as has been shown for *Arabidopsis* (Liu et al., 2018).

On the other hand, a possible role for temperature in the production of 2n female gametes in banana is associated with high temperatures, high solar radiation and low relative humidity (Ortiz and Vuylsteke, 1995a). The formation of unreduced 2n gametes under high temperatures was not considered a disadvantage but rather, a useful strategy for breeding tetraploid progeny from 2n gametes, avoiding some of the complexities of triploids (Ortiz and Vuylsteke, 1995a). Cool weather between archesporia (cells forming spores) and embryo sac development, the period during which meiosis occurs, was associated with ovule malformation (Fortescue and Turner, 2005a) suggesting that environmental conditions may be critical at this stage.

The proportion of mature embryo sacs found by Simmonds (1960) in three edible triploids varied from 2% to 36%. This low proportion arises from a combination of morphological abnormality, in the form of multiple archesporia, and from irregular meiosis during the development of female gametes. While we know that univalent and other meiotic errors are involved in sterility, predicting which ones might be important in any particular cross remains perilous.

### **2.3 The gynoecium**

The gynoecium (carpel) consists of the ovary, style and stigma (Fig. 2). The female flower of banana is normally made up of three carpels that fuse to form the ovary (Fahn et al., 1961). In *Musa* tri-carpellary ovaries are normal and bi-carpellary ovaries are formed infrequently according to Ram et al. (1962) who examined inflorescences grown in the tropics. Fahn et al. (1961) observed bi-carpellary ovaries, which they regarded as abnormal, and were associated with bunches flowering during May (late spring) in the northern hemisphere Mediterranean environment of Israel.

Each carpel has, among other flower parts, a terminal style and these fuse to form what then appears as a single style. The points of fusion of the styles form grooves along the exterior length of the composite style. Near its distal section each style bifurcates and this creates the 6 lobed stigma (Ram et al., 1962) consisting of stigmatic papillae. The lobes on the bi-furcated styles may appear as single lobes leading to the view of a three lobed stigma for the flower (Fig. 3).

We were unable to find in the published literature systematic studies of the anatomy of the banana style using transverse sections from the stigma through to the ovary. There are however numerous studies that focus on the ovary of female flowers and their ovules (eg. White, 1928; Dodds, 1945; Fortescue and Turner, 2005b,c), presumably because the ovary develops parthenocarpically to provide the edible fruit (e.g. White, 1928; Ram et al., 1962). Moreover, the formation of embryo sacs in ovules within the ovaries is an issue for fertility in edible banana cultivars because the frequency of normal embryo sacs is low

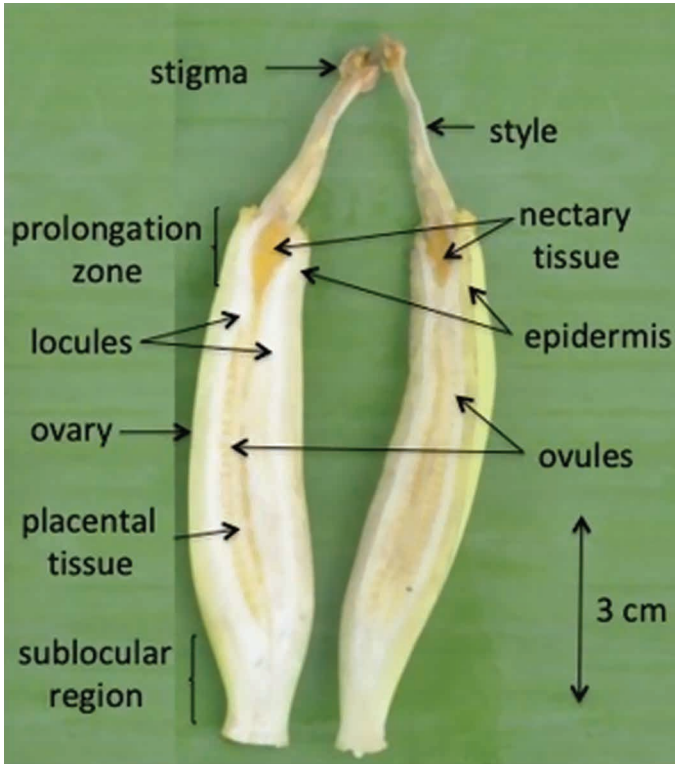
(Dodds, 1945; Shepherd, 1954). Even so, there are hundreds of ovules in an ovary and even with low frequency of normal embryo sacs, more seeds should be set than is currently the case.

### 2.3.1 The style

The morphology and anatomy of the style have been deduced from three sources. First, from the diploid species, *Musa velutina* and *M. ornata* and a triploid edible banana *Musa* (AAB) Silk 'Go Sai Yung' (Kirchoff, 1992). Secondly, from closely related genera, such as *Ensete* (Inta et al., 2015) and thirdly from members of the Zingiberales order, to which the Musaceae family belongs. The selected genera were *Strelitzia* (Kronstedt and Walles, 1986; Kronstedt et al., 1986) and *Canna* (Glinos and Cocucci, 2011).

Flowers made up of three fused carpels are a feature of all eight families within the Zingiberales (Specht et al., 2012). While evolutionary development of other floral parts such as the stigma, anthers, petals and sepals (Figs 2 and 3) differ across families according to interaction with pollinators, the architecture of the ovary and style (Figs 2 and 3) has remained largely unchanged (Specht et al., 2012). The three-carpelled flowers have a composite style formed by the fusion of the three styles. These surround an inner tube that forms the stylar canal or transmitting tract. The opening, or mouth, of the canal near the top of the composite style is clearly seen in *Ensete* (Inta et al., 2015). The transmitting tissue may have a three- or six-armed 'star' shape in transverse section, such as within the style of *Strelitzia reginae* (Kronstedt et al., 1986) or an elongated channel as in *Canna* (Glinos and Cocucci, 2011). The cells of the styles adjacent to the transmitting tissue are secretory and provide the nutrients and directional compounds to guide pollen tubes towards the ovary. Near the base of the style the transmitting tissue divides (from the view point of a pollen tube travelling towards the ovary) into three, one channel going to each locule of the ovary (*Strelitzia reginae* - Kronstedt and Walles, 1986; *Musa velutina* - Kirchoff, 1992). This partitioning is more obvious in the prolongation region of the ovary.

In *Strelitzia* the shape of the transverse section of transmitting tissue differs depending on the presence of pollen tubes. Empty stylar canals have a star shape but the tissue is able to expand to accommodate the pollen tubes and then has a more rounded appearance. Not all pollen tubes that enter the transmitting tissue make it to the base of the style and into the locules, as also observed in *Musa* (Dodds, 1945; Uma and Arun, 2016). The transmitting tissue may therefore act as a point of selection for pollen tubes. However, since many pollen tubes arrive within the locules (Fig. 7) but few ovules are fertilised, the pathway and guidance (Section 2.6.1) of pollen tubes from the stylar-conducting tissues to the ovules is a focus for future investigations.



**Figure 7** A longitudinal section of a female flower of *Musa* (AAB) plantain 'Bobby Tannap', showing distribution of ovules located centrally within locules and attached to the placental tissues. The placenta lies axially within the ovary between the sublocular region and the prolongation zone. The innermost section of the prolongation zone contains nectary tissue. The styler canal is centred within the style and separates into three channels distal to the nectary tissue. Within the prolongation zone the channels are placed between the nectary tissue and the epidermis. Each channel then enters a locule.

## 2.4 The androecium

Musaceae are grouped taxonomically within the Zingiberales by their androecium, which is described as non-petaloid with 5–6 stamens (Simmonds, 1962). Typically, the male flower of *Musa* species has five polliniferous stamens (Kirchoff, 1992), three in an outer stamen whorl and two in the inner whorl around the gynoecium, with the posterior stamen of the inner whorl absent (Kirchoff, 2017). The filaments are free and stiff, and the anthers are bilobed and tetrasporangiate. They dehisce via vertical slits and release their pollen toward the centre of the flower. This occurs just after the bract subtending each flower cluster rises (Stover and Simmonds, 1987) (Figs 4 and 5). It is assumed this androecial structure and behaviour applies to edible bananas,

except that in the male flowers of most edible bananas, the stamens bear little or no pollen.

### **2.4.1 Pollen production**

For a banana genotype to be used as a male parent in breeding, it must produce ample, viable pollen that can germinate and fertilize the ovule. If it fails to meet these criteria, it cannot be utilized even if it is a source for desirable traits to address breeding objectives (Fortescue and Turner, 2011). The young anther of a male fertile, diploid, *M. acuminata* (AA) can contain up to 2200 pollen mother cells, with up to 9000 microspores at maturity (Dodds, 1943). In contrast, the anthers of many, but not all edible diploids lack pollen as a consequence of meiotic errors arising from chromosomal structural changes leading to gametic sterility (Simmonds, 1962). Microspore numbers in mature anthers of four edible diploids was one hundredth of the numbers of pollen mother cells in the young anthers, with many mature anthers completely empty (Dodds, 1943). Triploid bananas also have little or no pollen, as a consequence of uneven chromosome pairing during meiosis (Stover and Simmonds, 1987).

Scarce pollen production at anthesis was observed in 28 out of 77 *Musa* landrace accessions (Dumpe and Ortiz, 1996), with about 60% of these landraces having less than moderate pollen production. Of these, all the AAA, AAB, ABB and AAAB genotypes and 40% of the AA genotypes exhibited less than moderate pollen production, confirming that a lack of pollen production is not confined to triploids. On the other hand, all five BB genotypes had moderate to abundant pollen production. The quantity of pollen produced at anthesis was positively associated with pollen viability ( $r = 0.61$ ,  $P \leq 0.01$ ), measured as % pollen stainability using acetocarmine (Dumpe and Ortiz, 1996). Not only the quality, but the quantity of pollen produced at anthesis by the male parent was critical for seed production, at least in some *Musa* accessions. For example, the genotype, *Musa* (AAA or AA Dochez et al., 2006) 'Marau', which produced a moderate amount of pollen with about 25% viability was sufficiently male fertile in crossing with female-fertile *Musa* (AAB) plantain 'Mbi Egome' for successful seed set, while genotype *Musa* (AA) 'Tuu Gia', which produced scarce pollen but with 75% viability, was not despite crossing with several female-fertile partners (Dumpe and Ortiz, 1996). Whether this was due merely to the number of viable pollen grains available for pollination is unclear. In any event, in seeking male parents for breeding, it is clear that identifying those accessions that are sufficiently male fertile is key. Using the criteria of pollen production and pollen stainability applied by Dumpe and Ortiz (1996), 30 of the 77 landrace accessions tested might be considered sufficiently fertile for use as male parents.

## **2.5 Anthesis**

At anthesis a flower is open and functional. For pollination to be successful the stigma must be receptive and pollen must be viable.

### **2.5.1 Stigma receptivity**

A stigma is said to be receptive when it provides favourable conditions to support pollen germination. Banana stigmas are six-lobed and when receptive, they are constantly wet with a sticky papillate surface. The sticky substance is thought to come from extracellular secretions that contain carbohydrates, enzymes, phenolic compounds and amino acids. The extracellular secretions interact with pollen for species recognition before pollen can germinate. Unfortunately, this area has not received a lot of attention in *Musa* to further understand the specific constituents and underlying biochemical reactions. The timing and duration of stigma receptivity determines the effective pollination period that ensures efficient fertilization and seed production from compatible genotypes.

Studies to determine stigma receptivity require assessing morphological changes on the surface of the stigma, carrying out pollen germination on the stigma and staining or testing for enzymatic activity (He et al., 2017). A more direct assessment of pollen germination, fertilization and seed set following controlled pollinations would be more precise. Uma and Arun (2016) studied seed set on 218 accessions from various banana groups and noted a correlation between the sticky secretion that accompanies stigma receptivity and seed set. Genotypes of the AAA-Cavendish group which had the lowest seed set were associated with lowest stickiness while all other banana groups which set more seeds had moderate to high stickiness. Similarly, they noted that production of stigmatic secretions lasted from 1 day (in AAB-plantains) to 4 days (in ABB-cooking bananas) implying that pollinations can be carried out over a period of 1-3 days for successful seed set. Furthermore, they observed a higher level of enzyme (peroxidase and catalase) activity in seed fertile cultivars than sterile cultivars with implications for stigma function.

Other research revealed four stages of stigma receptivity in banana and plantains (IITA, 1993; Ssebuliba et al., 2009), distinguished on the basis of shape, colour, and amount of mucilaginous mass on the stigma surface. Stage I stigmas have a smooth surface characterized by a conspicuous ridge and yellow- to bone-white coloured mucilaginous mass. Stage II stigmas develop a somewhat rounded shape with a cream coloured corrugated surface as a result of abundant mucilage. Stage III stigmas have a dark brown-coloured surface with black patches and less mucilage while stage IV stigmas have completely black surfaces with a few brown patches with no visible mucilage. In *Musa* (AAB) plantain, stages II and III were reported to be the most receptive in cultivars 'Bobby Tannap' and 'Obino l'Ewai' (IITA, 1993). On the other hand, stage III was reported to be the most receptive in *Musa* (AAA) East African Highland Banana

(EAHB) Matooke cultivars 'Bitambi,' 'Enzirabahima,' 'Kazirakwe,' 'Nakayonga,' and 'Nfuuka' (Ssebuliba et al., 2009). In addition to different stages of stigma receptivity, genetic differences and compatibility between parents are thought to also play a role in seed set in bananas.

Exploring the application of pollen germination and tube growth stimulation substances on the stigma before pollination may provide an opportunity to enhance seed set in bananas.

### **2.5.2 Pollen viability**

Depending on the pollen source used, the timing and method of application, seed yield from banana crosses varies between good to very poor seed with the latter having no embryos (Simmonds, 1952). Variation in seed set rates for various cross combinations calls for the need to evaluate pollen viability and genetic compatibility to identify potential fertile combinations of cultivars and hybrids for use in breeding. Pollen viability is crucial for the pollen-pistil interaction necessary to ensure fertilization and seed formation in flowering plants. To a limited extent, banana researchers have centred on pollen structure, quantification, viability, germination and growth of pollen tubes as components of breeding programs.

Pollen viability can be assessed using various methods such as staining with vital and nuclear dyes, *in vitro* and *in vivo* germination tests and direct assessment of final seed set (Heslop-Harrison et al., 1984). Ortiz et al. (1998) documented that pollen viability measured by staining did not correlate with seed set in triploid-diploid crosses implying that seed set in this case was affected by other factors in addition to pollen viability. Pollen viability assessment in banana has mainly involved staining with acetocarmine (Dumpe and Ortiz, 1996; Ssebuliba et al., 2008; Goigoux et al., 2013; Oselebe et al., 2014), Alexander (Fortescue and Turner, 2004) and 2,3,5-triphenyl tetrazolium chloride (Soares et al., 2008, 2015). *In vitro* germination tests using a sucrose solution and sucrose-based media has also been reported for bananas (Nyine and Pillay, 2007; Soares et al., 2015). Staining generally reported relatively higher viability values (possibly overestimating fertility) than germination tests. This discrepancy may be linked to inefficiencies of the pollen germination media to simulate stigma and pistil conditions, necessitating further optimization of germination media. This is clearly demonstrated by Nyine and Pillay (2007) who recorded a two-fold increase in pollen germination rates on diluted banana nectar compared with a sucrose solution.

In a broader context, pollen viability is affected by several factors including genotype, flowering time, developmental stage of the flower, temperature and relative humidity. Understanding the conditions that affect pollen viability will influence the practical aspects of optimizing pollen quantity and quality, collection and use in breeding schemes. However, most studies on pollen



viability have focused on the effect of genotype (Dumpe and Ortiz, 1996; Fortescue and Turner, 2004; Ssebuliba et al., 2008; Soares et al., 2008). Collectively, these studies indicate that banana cultivars and hybrids differ widely in their capacities to produce viable pollen either within or among genome groups. *Musa* species typically produce abundant viable pollen while cultivated diploids as well as diploid hybrids produce relatively more viable pollen than tetraploids and triploids. Low pollen production in triploids usually precludes their use as male parents.

Studies of environmental effects on pollen viability in bananas are scarce. Ortiz et al. (1998) observed a seasonal variation in pollen stainability for, *M. acuminata* ssp. *burmannica* 'Calcutta 4', *Musa* AA 'Galeo' and 'Pisang lilin' and a plantain-banana hybrid TMP2x1297-3 examined for a year in Nigeria. High pollen stainability was positively associated with high solar radiation, high temperature and high evaporation and negatively associated with high rainfall and high relative humidity. Soares et al. (2015) assessed pollen viability at different times of the day and two seasons of the year (summer and winter) in Brazil. They observed that *in vitro* germination rates and % viability from staining tests were highest at 8 am and lowest at 4 pm, and that pollen viability was higher in the summer than in the winter. Uma and Arun (2016) evaluated pollen viability following exposure to a range of temperatures (25–45°C for 1 hour) and noted a reduced germination rate (43–72%) for pollen exposed to temperatures over 35°C.

Nevertheless, pollen viability assessment is only indicative of fertility. Hence further studies should be targeted towards a functional analysis of *in vivo* pollen germination, pollen tube development and fertilization as a true reflection of pollen fertility for desirable genotypes.

## 2.6 Pistil: pollen interaction

To reach an ovule within an ovary of banana a pollen grain and the tube arising from it has a journey consisting of several steps. The pollen needs to arrive at the stigma, germinate and produce a pollen tube. About an hour is needed for pollen to germinate and penetrate the stigma (Shepherd et al., 1987). The pollen tube then travels across/through the stigmatic tissue, into the transmitting tract of the style. After travelling down the style and through the prolongation section it enters the upper section of one of the locules of the ovary. Once in a locule it needs to locate an ovule and enter its micropyle. The stylar part of this journey (30–50 mm) takes about 12 h (Dodds, 1945). The whole journey may cover a distance from 50 mm to 100 mm or more and take about 18 h. These values represent rates of elongation of the pollen tube of 2.5–5.6 mm/h which are similar to the range of rates recorded by Shepherd et al. (1987) in Brazil. The sequential steps in this journey mean that stimulation or inhibition of the pollen or pollen tube can be exercised at a number of points (Fig. 7).

The style is essential for successful conventional breeding, but opinions about its significance in influencing female fertility vary. Dodds (1945) working in Trinidad, examined style function and found no inhibition of pollen tube growth in the styles of edible bananas. More pollen tubes arrived in the locules than were needed to fertilise the number of embryo sacs present. Even so, Dodds (1945) noted that most embryo-sacs do not receive pollen tubes. Shepherd et al. (1987) commented on good pollen tube growth in the styles of Cavendish (AAA) cultivars in Brazil, although these cultivars did not produce seeds when pollinated. Further experiments by Shepherd et al. (1987) suggested some difference between cultivars of the Pome group (AAB) in the rate of movement of pollen tubes along the style and in the number of pollen tubes within the style.

Uma and Arun (2016) working in Southern India, observed a lack of germination of pollen from *M. acuminata* ssp. *burmannica* 'Calcutta 4' and *Musa* (AA) 'Pisang lilin' on the stigmatic surface of *Musa* (AAA) Cavendish 'Grand Naine' but ample germination on the stigma of *Musa* (AAB) Silk 'Rasthali'. These observations on members of the Cavendish subgroup differ from those of Shepherd et al. (1987) who found no limitations to pollen germination on stigma of Cavendish bananas or movement through the style. Uma and Arun (2016) observed thinning of the pollen tube population within the style, thus reducing the number of pollen tubes likely to enter the ovary - a different observation from that of Dodds (1945). Soares et al. (2014) working in Brazil, observed necrosis in the prolongation region of the ovary in triploid cultivars (AAA and AAB), whether pollinated or not. In diploid cultivars no necrosis was observed. They thought necrosis at this location, between the style and the ovules, may interfere with pollen tube transfer at the point of entry into the locules of the ovary and consequently reduce fertilisation and seed fertility. Ssebuliba et al. (2005) working with *Musa* (AAA) EAHB found a negative association between seed set and style length. However, the residuals were very high and they concluded that style length, among other features of pistil anatomy were unlikely to be large contributors to seed fertility.

At present, observations differ about the importance of the stigmatic and stilar tissues in controlling the number of pollen tubes likely to arrive within locules of the ovary and so it is not possible to make general conclusions about points of control. Knowledge of the anatomy of the pathway and identification of the points of control, if any, are needed for a broader picture of pollen/pistil interactions in *Musa*.

### **2.6.1 Pollen guidance**

To form a seed following pollination, a pollen tube must move through the stigma and style, enter the locule, locate an ovule, enter the micropyle and

fertilise the embryo sac. An ovule needs only one pollen tube for fertilisation and so a mechanism for rejection of other pollen tubes is required once pollination has occurred. Mizuta and Higashiyama (2018) provide a very useful recent summary of the known mechanisms in flowering plants with emphasis on *Arabidopsis*. They refer to pre-ovular guidance from the stigma to the ovary and to ovular guidance that allows a single pollen tube to locate a single ovule within the ovary.

As far as is known, no studies of pollen guidance *per se* have been made in *Musa*, although failure of the guidance system could well contribute to poor seed set. There are a number of observations in the pollination of *Musa* flowers that seem consistent with a non-functional or partly functional pollen guidance system. For example, Shepherd (1960) observed two types of failure of pollen tubes moving towards the ovary. The first was failure to cross the stigma and was related to the perceived maturity of the style. The second was the failure of pollen tubes, once in the style, to reach the ovary. There were differences between female genotypes in the expression of these failures in pollen tube growth.

### **2.6.2 Distribution of seeds within a fruit**

Shepherd (1954) attributed the high concentration of seeds at the stylar end of the ovary of *Musa* (AAA) 'Gros Michel' to pollen tube 'growth factors'. In that case, 86% of the seeds in the fruit formed in the quarter nearest the stylar end. Shepherd observed considerable variation in the rate of pollen tube growth and concluded that when compared with the growth of pollen tubes in *Musa* species, pollen tube growth in styles of 'Gros Michel' was 'erratic'. However, more than half of the pollen tubes within the style grew sufficiently to reach the ovary.

In *Musa* species with a pollen guidance system intact the distribution of seeds is expected to be uniform along the length of the ovary, even in genotypes with long fruit such as *M. acuminata* ssp. *banksii* (Simmonds, 1953). Ovaries may be 9-15 cm in length in edible *Musa* (AAA) EAHB (Ssebuliba et al., 2005). The concentration of seeds at the stylar end in this sub-group may have a number of causes, including a non-functional pollen guidance system. In the absence of a guidance system, the growth of pollen tubes, all of which enter the locules from the stylar end, is likely to approximate a distribution where, with lack of direction, pollen tubes concentrate at the stylar end and few reach the base of the ovary. This is likely to be reflected in an exponential decline in seed number from the stylar end of the ovary to the base.

### **2.6.3 Quantifying seed distribution within fruit**

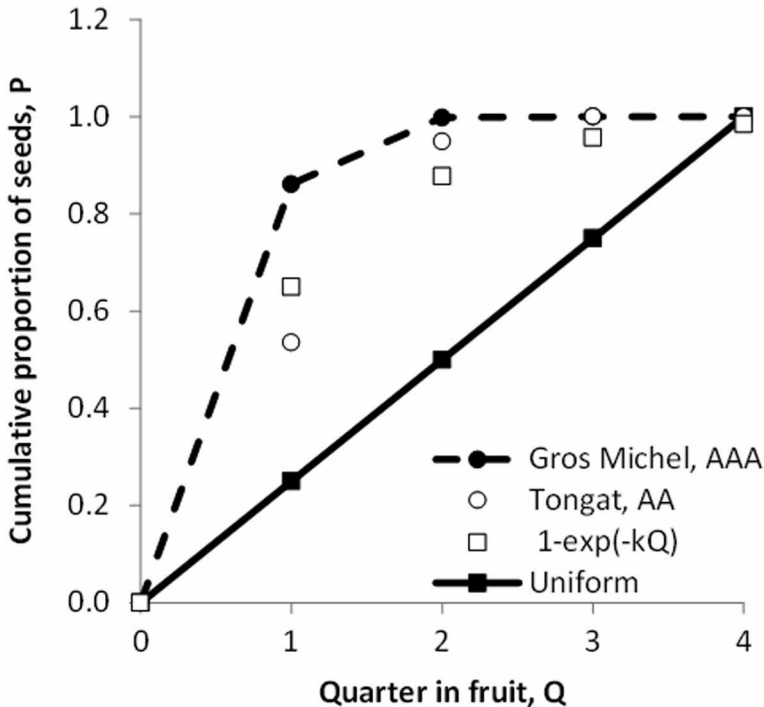
Shepherd (1960) presented data on the proportional distribution of seeds through four quarters of the fruit for a number of crosses of female diploid,

triploid and tetraploid clones with pollen from either *M. acuminata* ssp. *malaccensis* or ssp. *burmannica*. Seed set tended to be greatest at the stylar end of the ovary (Fig. 8). Here the cumulative distribution of seed from the stylar to the basal end of the ovary is compared against a theoretical uniform distribution of seed to estimate the functioning of a pollen tube guidance system. We assume that competent ovules are uniformly distributed along the ovary and that an abundance of pollen tubes enter the locule.

The function used to compare distributions is:

$$P = 1 - \exp(-kQ) \quad (1)$$

Where P is the cumulative proportion of seeds from the stylar end, Q is the quarter numbered from the stylar end and k is a coefficient of seed distribution within the ovary. A high value of k indicated a strong bias of seed distribution



**Figure 8** The cumulative proportional distribution of seed set in quarters from the stylar (1) to the basal end (4) of ovaries of 'Gros Michel' AAA (dashed line) and 'Tongat' AA clones. In the legend, Uniform (solid line) is a theoretical uniform distribution of seed throughout the quarters of the ovary. Points above the solid line show bias towards the stylar end of the fruit and below the solid line bias towards the basal end. The open squares are the fit for the equation  $1 - \exp(-kQ)$  for 'Tongat' with  $k = 1.05$ .

towards the stylar end as shown for 'Gros Michel' (AAA) where  $k = 1.9$  (Fig. 8). For 'Tongat' (AA), the concentration at the stylar end is reduced compared with 'Gros Michel' and  $k = 1.05$ .

### 3 Bract opening in banana

The opening of bracts that subtend hands of flowers on an inflorescence of bananas allows pollination of female flowers in *Musa* species and access for hand pollination in edible bananas. Matching pollination with maximum receptivity of the stigma increases the likelihood of success in generating seed for banana breeding, especially in edible cultivars that have low fertility (Simmonds, 1960). Time of stigma receptivity is short, less than a day and possibly only a few hours (IITA, 1993; Ssebuliba et al., 2006). In the practice of banana breeding, pollen is collected at dawn and female flowers pollinated during early to mid-morning (Shepherd, 1954; Rowe and Richardson, 1975; Swennen and Vuylsteke, 1993; Vuylsteke et al., 1993a,b). If flowers become available for pollination during the night, they may well have reduced receptivity by the time daylight arrives. Knowledge of the diurnal behaviour of bract lifting and curling and subsequent appearance of female and male flowers could lead to improved breeding efficiency.

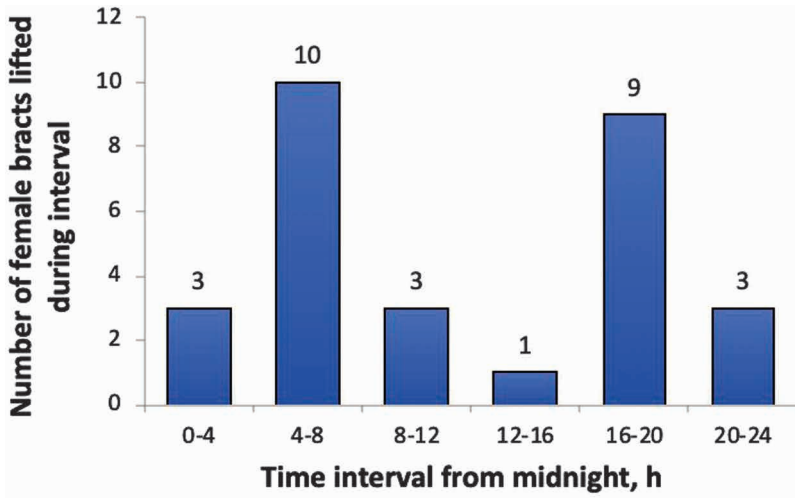
#### 3.1 The process of bract opening

In cultivars of *Musa* AA and AAA, bracts that subtend nodes of female flowers undergo two main events that expose flowers to pollinators at anthesis. The bract lifts away from the floral bud with the base of the bract as the fulcrum. Then, sometimes concurrently, there is an involute curling of the bract, starting from the tip, thereby exposing the flowers to pollinators. Once 'hands' of female flowers are opened the bracts dehisce. Unlike the bracts of female flowers, which always dehisce, for bracts subtending the transitional and male hands dehisce depends on the cultivar, with bracts of male flowers in some cultivars drying on the peduncle. Bracts usually lift, curl and dehisce in an acropetal sequence, first exposing the hands of female flowers and then the hands of male flowers on the inflorescence.

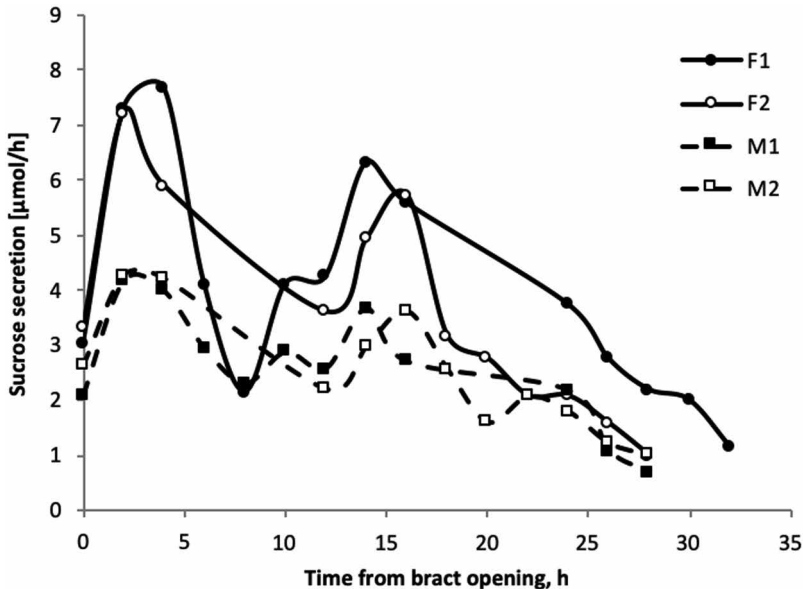
#### 3.2 Diurnal behaviour of bract opening

In Kunming, China (21°N), during October and November (autumn/early winter) most bracts of hands of female flowers of *Musa itinerans* open at night with a second major bract opening late in the afternoon (Liu et al., 2002) (Fig. 9).

Flowers of *M. itinerans* produced nectar in the 2–5 h after each set of bract openings and again 12 h later. At these times flowers were visited by either bats



**Figure 9** The number of female hands opening in each interval from midnight in *Musa itinerans* at Kunming, China. Data are for 18 inflorescences from Liu et al. (2002). Sunrise was at 7.15 am and day-length was 11.2 h.



**Figure 10** The secretion of sucrose in nectar from flowers of *Musa itinerans* at Kunming, China, in the hours from bract opening. F1 and M1 refer to female and male flowers that commence opening in the early morning. F2 and M2 refer to female and male flowers that commence opening in the late evening. Data from Liu et al. (2002).

(evening) or birds (morning) (Liu et al., 2002). A high correlation between the volume of nectar, particularly the amount of sugars secreted in nectar, and the receptivity of the stigma to pollination might be expected (Farkas and Orosz-Kovacs, 2003; Roy et al., 2017). Two peaks of sucrose secretion after the bracts opened were identified using the data of Liu et al. (2002) (Fig. 10). Sucrose was available to pollinators 2–5 h after bract opening, whether they were bats (night) or birds (day). A second but broader peak of sucrose secretion occurred about 12 h later.

If bract opening of *M. itinerans* is characteristic of bananas, then bracts of edible cultivars also are likely to open during darkness, particularly around sunset and before sunrise. Maximum receptivity of the stigma, if linked to sugar secretion in nectar, would follow 2–5 h later. This provides some rationale for focussing pollination efforts in banana breeding schemes to the early hours after sunrise, but overlooks those flowers opening in the evening, which are likely to be past their optimum receptivity the following morning.

### 3.3 Monitoring bract opening in the glasshouse and field

Diurnal behaviour of bracts was monitored in movies of bract opening on (i) individual bunches of two *Musa* (AAA) Cavendish cultivars, 'Grand Nain' and 'Williams' in a glasshouse and *Musa basjoo* in a garden in Belgium (51°N) over several weeks from June to September 2015 (i.e. summer), and (ii) four *Musa* (AA) 'Mchare' cultivars, 'Akondro Mainty', 'Mchare Mlelembo', 'Kisukari Mchare' and 'Huti White', in the field at Arusha, Tanzania (3°S) in December 2015 and January 2016 (i.e. dry season). The analysis of the movies focussed on the beginning of involute curling and first visible flower and the lifting of the bracts.

The movies are accessible at:

<ftp://ftp.musabase.org/video/flowering/movie1.avi> (Grand Nain, Belgium, 3 June 2015)

<ftp://ftp.musabase.org/video/flowering/movie2.avi> (Williams, Belgium, 7 July 2015)

<ftp://ftp.musabase.org/video/flowering/movie3.avi> (Williams, Belgium, 28 September 2015)

<ftp://ftp.musabase.org/video/flowering/movie4.avi> (*Musa basjoo*, Belgium, 26 July 2015)

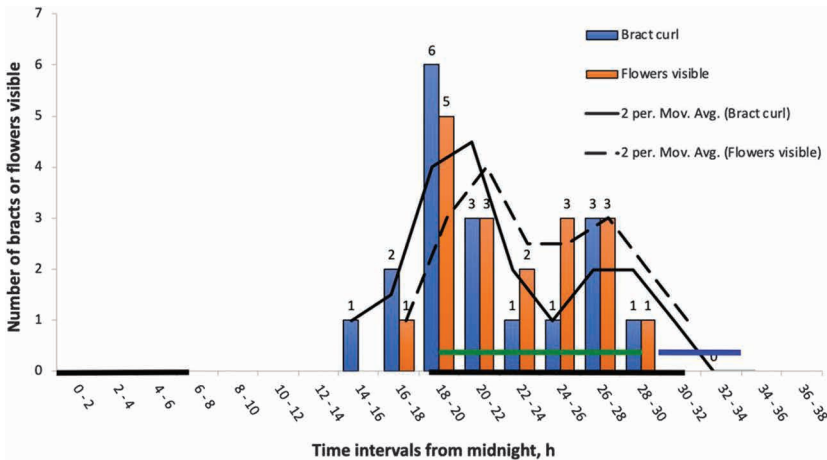
<ftp://ftp.musabase.org/video/flowering/movie5.avi> (Akondro Mainty, Tanzania, 21 December 2015)

<ftp://ftp.musabase.org/video/flowering/movie6.avi> (Mchare Mlelembo, Tanzania, 12 January 2016)

### 3.3.1 A link with sunrise and sunset?

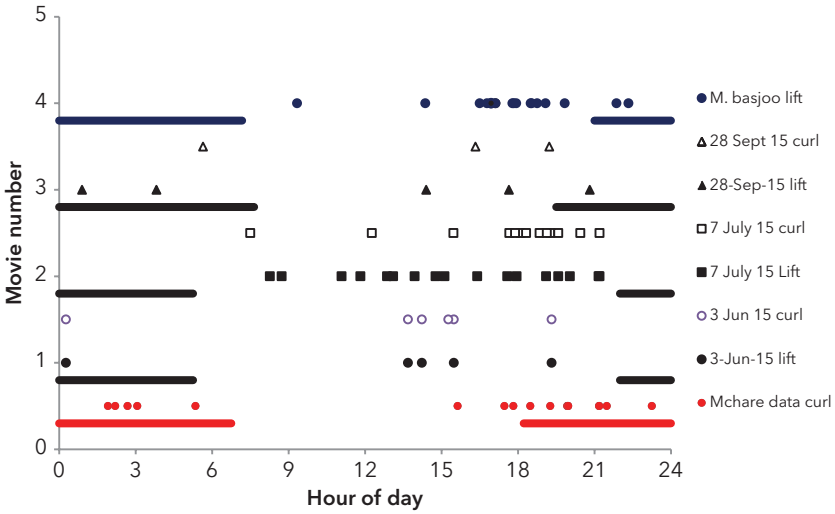
The pattern of bract curl for *Musa* AA Mchare cultivars at Arusha, Tanzania, (Fig. 11) was consistent with the bimodal distribution observed by Liu et al. (2002) for *Musa itinerans* at Kunming, China. This was not the case for bracts of Cavendish cultivars in the glasshouse (KUL) or *M. basjoo* in the garden in Belgium (Fig. 12). While there was no significant difference in the response to sunrise in the population of bracts of *Musa* AAA Cavendish and *M. basjoo* (Table 1) these differed from the field in Arusha in that during long days all bracts curled during the day and there was no clear bimodal distribution of bract curling.

Further analysis suggested that bract curling was linked to a fixed time after sunrise, with bracts responding to the change of light at dawn and beginning to curl between 9 h and 12 h later irrespective of where they grew at latitudes 3°S, 21°N or 51°N (Fig. 13). Thus, whether or not the bracts curl in the day or the night depends on the day-length of the location in which bunches grew. The short day (11.5 h) in the tropics meant that bracts began involute curling mostly during the evening and night, but in Belgium, during daylight in the long days of the summer (17 h) (Fig. 13). This phenomenon is consistent with a



**Figure 11** Beginning of ‘bract curl’ and ‘first visible flower’ in four ‘Mchare’ cultivars in the field at Arusha, Tanzania, over two days in December - January. Data were collected over two 24 h periods per cultivar and combined. Dark horizontal lines indicate night. Moving averages (2 per average) highlight the bimodal distribution. The horizontal green line indicates time over which moths visited open flowers. Horizontal blue line indicates time of intense visitation of flowers by bees, wasps, and flies. Time intervals are from midnight on day 1.



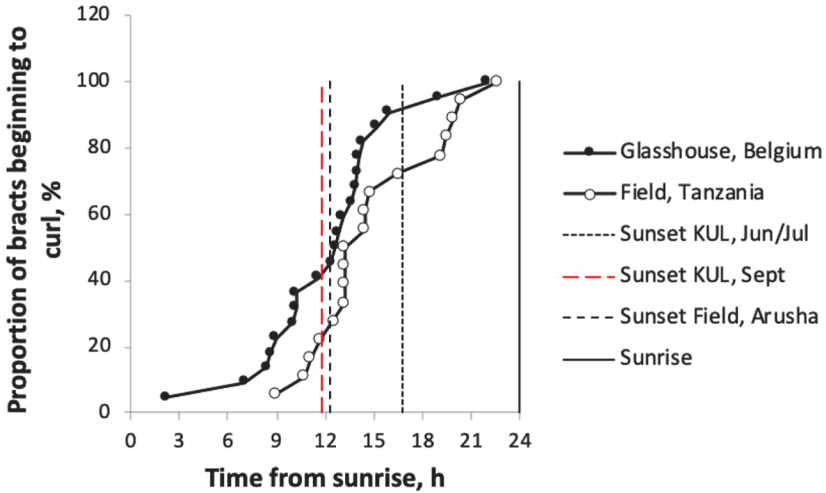


**Figure 12** The time of day when bracts began to either lift from the floral bud or curl from the tip for three occasions (movie number) in a glasshouse in Belgium. Movie 1 – male hands of ‘Grand Nain’, movies 2 and 3 – ‘Williams’ bunches. Movie 2 and 3 included female and male hands. The beginning of bract lift is shown in closed black symbols for movies 1, 2 and 3 and in dark blue symbols for *M. basjoo* bunches in the garden in Belgium. The beginning of bract curl is shown in open symbols for movies 1, 2 and 3, and for the ‘Mchare’ bunches in the field in Arusha. Horizontal black, red or blue lines show night time for each location and time of year. Hours are from midnight.

light signal received at dawn. However, a single light signal received at sunrise does not explain why in the field in Kunming, China (Liu et al., 2002), and in Arusha, Tanzania, bract opening or curling had a bimodal distribution during the hours of darkness. This may mean that two light signals are operating, one at sunrise and another at sunset. In each case curling of bracts begins about 9 h after the change in light regime, either from dark to light or the reverse. A light signal generated by a change from dark to light or the reverse seems likely, perhaps associated with a link between red light and ABA signalling (Lin and

**Table 1** A comparison of the time from sunrise to bract lift in populations of bracts in the glasshouse and garden in Belgium. Mean values are not significantly different as determined by Student t-test (P=0.129)

Time from sunrise to beginning of bract lift (h)	Mean	Standard error	Sample size (n)
Glasshouse, <i>Musa</i> AAA Cavendish cvs	12.29	0.88	22
Garden, <i>M. basjoo</i>	10.51	0.70	17



**Figure 13** The cumulative start of bract involute curling for the glasshouse and field data in relation to sunrise (time zero). The solid symbols are for bract curling of the glasshouse data. The open symbols are for bract curling of the field data. Three vertical lines indicate time of sunset for the glasshouse movies (Belgium), in June or July (dotted black) and in September (hatched black) and for the field in Arusha (red). A solid black vertical line indicates the following sunrise.

Tang, 2014) as ABA is often linked to leaf (a bract is a modified leaf) abscission (Patterson, 2001).

### 3.3.2 Season-dependent seed set in plantain

If pollination procedures in banana breeding are guided by clock time throughout the year (e.g. 6.30 am to 7.30 am), then the relationship between the time of pollination and sunrise (or the previous sunset) will change throughout the year, according to photoperiod. They are likely to be closest twice each year when the day-length is 12 h. Swennen et al. (1991) observed two seasonal peaks of seed production, repeated over two years, in plantain cultivars at Onne, Nigeria (Lat 5°N), one in February and another in September to October when pollination began about 1.0–1.5 h after sunrise. In February, 60–70 seeds were set per bunch while in September to October 20–30 seeds were set per bunch. At other times of the year there were greater displacements between the start of pollination and the time of sunrise and seed set varied from 2 to 15 seeds per bunch (Swennen et al., 1991). There is a need to explore the relationship between receptivity, hours after sunrise and clock time to see whether this is associated with any change in the efficiency of pollination.

### **3.3.3 Bract lift, curl, water relations and energy supply**

Observations on bract lift and curl in the glasshouse where three bracts lifted over three days, but curling of all three bracts occurred within an hour (Fig. 12, movie 2), suggested that while a signal at sunrise or sunset may result in bract curling 9–12 h later, bracts may not curl unless another condition is also met.

Flowering and pollination in plants requires energy to expose flowers to pollinators, excrete soluble carbohydrates, support pollen germination and movement. For lifting and curling of the bracts, energy is needed to move solutes from one part of the tissue to another so that there is a change of water distribution and cell turgor, providing a cantilever effect as bracts lift and curl. Exploring these relationships might shed some light on the variability in the sequence of bract lift and the readiness of the flower for pollination.

### **3.3.4 Bract opening and insect visitation**

Rutikanga et al. (2016) observed nectar production in *Musa* AAA EAH bananas, noting that the number of visits by insects was not associated with the amount of nectar, but with the presence of nectar. Given the presence of insects at the opening bracts in the movies taken in the field, nectar production in 'Mchare' cultivars is likely to be the attractant. Insects at the inflorescence may be associated with maximum receptivity of the stigma to pollen. For bunches in the field at Arusha, this could mean stigma were receptive throughout the night and in the first hour or so after dawn. Understanding the relationship between which hands of flowers were visited by insects at which times and bract lifting and curling might help to identify times of maximum receptivity.

Across genotypes, bract behaviour was consistent with bract opening in banana being an energy-dependent process with at least two requirements. First, sufficient energy to move solutes between tissues creating changes in water distribution to promote gradients of turgor required for the mechanical aspects of the cantilever action of lifting and curling. Secondly, a signal initiated by a change in light regime, dark to light (sunrise) or light to dark (sunset), perhaps associated with a link between red light and ABA signalling (Lin and Tang, 2014), allowing flower opening to occur at the same time as pollinators visit. These possible mechanisms have implications for manipulating bract opening to coordinate pollination and stigma receptivity in banana breeding. Clearly, further experimentation is needed to test the hypotheses arising from these observations, specifically: (1) whether bract curling is initiated in part by a change of light regime – from dark to light (sunrise) or light to dark (sunset), (2) whether bract curling has two separate requirements, a light signal and a supply of energy to undertake the mechanics of curling and (3) whether

maximum receptivity of the stigma is associated with nectar production and visiting insects may be indicative.

### **3.4 Pollination in *Musa* species before bract opening**

Hermaphrodite flowers, which occur in some *Musa* species, have functional female and male gametes and can self-fertilise before the subtending bract opens (Simmonds, 1966). This feature influences the amount of out-crossing between *Musa* species and subspecies. For example, Argent (1976) discussed hybrids of *M. schizocarpa* and *M. acuminata* ssp. *banksii* observed in the field in Papua New Guinea. *M. schizocarpa* consistently had hermaphrodite flowers in basal hands but in *M. acuminata* ssp. *banksii* this character showed variability. He concluded that this difference meant *M. acuminata* ssp. *banksii* was the most likely female parent of observed hybrids. Shepherd (1999) described experimental crosses between *M. schizocarpa* and *M. acuminata* ssp. *banksii* in Jamaica. While the inheritance of hermaphrodite flowers was not recorded, in practice there was no need for emasculation of flowers for successful pollination to produce backcross seeds. In this case Shepherd (1999) assumed that in the hybrids anthers were either absent or rare.

Häkkinen and Hong (2007) recorded hermaphrodite flowers on *M. yunnanensis* and *M. acuminata* ssp. *chinensis* in Yunnan, China, with no evidence of hybrids nearby. This supported the view that the plants of each species had self-fertilised. For breeding purposes, these observations suggest that successful pollination of hermaphrodite banana flowers may occur before bracts open and therefore without pollinators. Stigma may be receptive before bracts open even in male sterile edible cultivars used as female parents in banana breeding. Clearly further observations and experimental research is required to improve our understanding of floral biology and pollination among cultivated as well as *Musa* species.

## **4 Fertilization and seed set**

In *Musa* there is little information about the journey of pollen tubes through the locules and their entry into ovules to prosecute fertilisation.

### **4.1 Pollination techniques**

In banana breeding, crossing is an essential component in the process to combine desirable traits in new hybrids. Generally, hybridizations can be carried out through open or hand pollinations. Hand pollination is the main practice of carrying out targeted crosses for banana (Brown et al., 2017), although open pollination relying on natural pollinating agents has been suggested in the

context of polycross breeding (Ortiz et al., 1995). Natural pollination in bananas is commonly carried out by bats, honey bees and birds (Ortiz and Crouch, 1997).

To make a controlled cross between two parents, unopened female and male flowers are covered with muslin bags before anthesis (Ortiz and Vuylsteke, 1995a; Ssebuliba et al., 2006). Bagging ensures that pollen contamination by bats and insects or other animal activity is prevented during pollination (Shepherd, 1954; Ray, 2002). In EAHB, flowers are ready for pollination when bracts are lifted away from the floral bud with an increasing angle from the tip of the bract and stigmas are exposed and appear fresh with a cream white color (Stage II and III) (Ssebuliba et al., 2006). After a series of pollinations at different times of the day on 'Gros Michel,' the highest seed set was obtained between 7 am and 10 am (Shepherd, 1954). This range of pollination time has thus been widely adopted without considering local and genetic variation across banana breeding programs with the understanding that pollen is normally shed in the morning and viability declines with time. Hand pollination is done by excising freshly exposed male flowers from the male bud and brushing sticky anthers onto stigmas of female flowers (Swennen and Vuylsteke, 1993). Pollinations are repeated daily on different hands on the same bunch for a period of 7–15 days depending on the number of hands present in the bunch (Ortiz and Vuylsteke, 1995a; Vuylsteke et al., 1997; Ssebuliba et al., 2006). The inflorescence usually bears 1–30 hands of fruit-forming female flowers followed by hands of non-fruit-forming flowers that might include 0–20 hands of transitional flowers and 100–300 male hands (White, 1928). These numbers depend on the genotype, environmental conditions and edaphic factors (Fortescue and Turner, 2011). On average, 1–3 female hands open over 24 h, often just after sunset or pre-dawn, and all open hands are pollinated (Shepherd, 1954). These values are approximate for a range of conditions and cultivars, and cannot be used to judge opening rates of female versus male hands.

The temporal and spatial separation of male and female flowers in bananas limit possibilities for self-fertilization between male and female flowers of the same inflorescence. But self-pollination is possible between plants of different cycles on the same mat (Fortescue and Turner, 2011). For breeding purposes, it is essential that by the time female flowers emerge from earmarked female parents, there is a ready source of pollen from male parents. To achieve this, male parents need to be planted earlier than female parents when establishing crossing blocks. Pollen preservation can also be explored to address the temporal and spatial separation of male and female flowers but protocols are yet to be established for pollen storage in bananas (Uma and Arun, 2016). Pollination can be carried out all year round depending on the availability of male and female flowers. Genotypes in crossing blocks may be planted at

different times to allow staggered flowering and ensure availability of male and female flowers.

## 4.2 Understanding seed set

Some of the first experiments regarding fertilization and seed set in banana were conducted by Dodds (1945), Dodds and Simmonds (1948) and Simmonds (1952). They realised that some *M. acuminata* clones yielded less seed than expected as a result of genotypic influence but sometimes had greater than expected seed set. Simmonds (1952) also found that some diploids when selfed yielded less seeds than when other pollen sources were used. This was the first indication of the need for genetic research and optimisation of pollination to attain maximum seed set in banana. These two observations suggest optimization at the level of the female parent as well as the pollen source. There was also an observation of what appears to be a significant pollen source and female parent interaction in a number of experiments (Simmonds, 1952; Swennen and Vuylsteke, 1989; Ssebuliba et al., 2009). These interactions seem to be strongest when crosses between genomic groups are made. For example, some crosses between plantain and *M. balbisiana* yield less seed compared to *M. acuminata* as a male parent. This can be attributed to pollen-stigma interactions which lead to extracellular secretions from both pollen and the stigma as discussed in Section 2.5.1. Efforts to get around these challenges are still limited and banana breeders rather avoid futile crosses and only focus on successful crosses. Alternatively, the number of bunches to be pollinated can be increased to boost seed numbers. Consequently, banana improvement programs are working with limited numbers of progeny and a narrow genetic base despite the vast untapped natural diversity within the *Musa* genus. Simmonds (1952) also observed that crosses between diploids in the *Eumusa* section with those in the *Rhodochlamys* section were futile.

After successful pollen germination, pollen tube growth inhibition was identified as the next challenge. This was reported in 'Gros Michel' by Shepherd (1954) who observed arrested or slowed normal pollen tube growth and the consequent development of a swelling at the pollen tube tip. This is typically gametophytic self-incompatibility but there is limited knowledge of its presence and mechanism of operation in *Musa*. In other crops such as citrus, high temperatures are thought to inactivate proteins responsible for gametophytic self-incompatibility (Kawano et al., 2016). Normal *in vivo* pollen growth is therefore realised when temperatures are between 15°C and 25°C depending on the cross (Distefano et al., 2012). This could explain why higher seed set is observed when pollinations are made during high temperatures, though optimum temperature ranges have not been fully researched in *Musa* spp. Conversely, Fortescue and Turner (2011), focussing on some of Shepherd's

observations, reported that pollen tube growth does not seem to be inhibited and more pollen tubes enter the ovary than the number of ovules. This suggests the uncertainty whether there are incompatibility systems at play. However, there is clear evidence that a necrosis forms at the tip of the ovary about a day after anthesis, especially in triploid edible bananas. This acts as a physical barrier preventing growth of pollen tubes into the ovary. It has also been observed that this necrosis develops much slower in diploid bananas (Soares et al., 2014). To optimise seed set, it is crucial that pollinations are made before this physical barrier develops.

It seems that seasonality plays a vital role in seed fertility in edible bananas but the link between season and seed fertility is complex and may involve a number of different pathways. Ortiz and Vuylsteke (1995a) reported a seasonal response in seed set, although this was variable across genotypes. Using 'Calcutta 4' as the male parent, low temperature, low radiation and high relative humidity favoured seed set in *Musa* (AAA) 'Yangambi KM 5' and in *Musa* (ABB) 'Bluggoe', peaking in August to September. Seed set in *Musa* (AAB) plantain cultivars had two peaks, February and September. High temperature, high solar radiation and low relative humidity, characteristic of February, favoured production of tetraploid seed in plantain cultivars, possibly linked to the production of  $2n$  gametes. These same conditions were associated with increased seed set in *Musa* (AAA) EAHB Matooke cultivars (Ssebuliba et al., 2009), although this seasonality was not apparent as determined by a long-term assessment of pollination success in a further study of Matooke cultivars (Batte et al., 2019). One has to keep in mind that the weather parameters of temperature, relative humidity and solar radiation are interrelated and the relative importance of each parameter is yet to be ascertained. For example, high temperature is positively correlated with high solar radiation and negatively correlated with relative humidity. Moreover, most  $r$  values reported by Ssebuliba et al. (2009) are less than 0.5, indicative of weak relationships suggesting the involvement of other factors.

### **4.3 Quantifying fertility in *Musa***

'Fertility' in *Musa* can have somewhat different meanings according to the scale adopted and whether ovules, pollen or seeds are considered. For broader studies in banana breeding, fertility is often expressed as the number of seeds per bunch. The more seeds the greater is the fertility. Seeds per bunch can also be used as a +/- comparison (either nil or at least one seed per bunch) to calculate the proportion of pollinated bunches containing at least one seed. This is considered a measure of 'pollination success' (Batte et al., 2019). In more detailed work with a focus on the biology of reproduction it is necessary to look

at more definitive measures of fertility, such as seeds per fruit (Shepherd, 1954), or better, seeds/1000 ovules.

Seeds per bunch is a widely used measure of fertility in banana breeding. It has a long history (Cheesman, 1932; Shepherd, 1954, 1960; Swennen and Vuylsteke, 1993) and has been used to measure the 'fertility' of a clone (Ssebuliba et al., 2005) and the ability of a cross to produce seeds (Uma et al., 2011). Seeds per bunch, as a measure, has a number of components. Because a seed is the product of a successful fertilisation of an ovule, seeds per bunch ( $S_b$ ) is made up of the number of seeds per thousand ovules ( $S_o$ ), the number of ovules per ovary  $O_f$ , the number of ovaries per hand ( $F_h$ ) and the number of female hands per bunch ( $H_b$ ):

$$S_b = (S_o/1000) \cdot O_f \cdot F_h \cdot H_b. \quad (2)$$

The number of seeds per thousand ovules is a measure of the fertility of ovules. The number of ovules per ovary is a measure of the capacity of the ovary to produce seed. The number of ovaries or fruits per hand and the number of hands per bunch are measures of the architecture of the inflorescence. A similar value of seeds per bunch in two different genotypes, or treatments, can only be achieved if either all other components of Eqn 2 are the same, which is very unlikely, or if there are compensatory changes in the components.

These compensatory changes may reflect differences in the architecture of the inflorescence, or, more importantly in this case, the capacity of the ovules to set seed. Thus, at best, for the purposes of reproductive biology, seeds per bunch as a measure of fertility, has limited appeal.

The number of ovules per flower ( $O_f$ ), also referred to as ovules per ovary, varies with genotype. Some examples are 130 ovules per flower in 'Calcutta 4' to >300 ovules in other diploids (Dodds, 1943). In triploid bananas, 260-350 ovules per ovary have been counted in genotypes of different clone sets of the *Musa* (AAA) EAHB (Ssebuliba et al., 2005). The major contributor to variation in the number of ovules per ovary is ovary length. From the data of Ssebuliba et al. (2005) for EAHB we can calculate the number of ovules per unit length of ovary, which ranges from 22 to 29 ovules per cm. Flowers per hand range from 5 to 20 and hands per bunch range from less than 5 to more than 17 in different plantain genotypes (Adheka et al., 2018).

The measure of fertility used in experimental studies needs to be considered in relation to the objectives of the study. Used alone, the number of seeds per bunch may be considered as a measure of success in breeding. However, it is unlikely to inform us of the reproductive strategies used to obtain seed set and so to increase breeding success for which seeds/1000 ovules is a more accurate measure.



## 5 Embryo dormancy: a cause for poor germination?

In addition to poor fertility and seed set a further hurdle faced by banana breeders is the low germination rates of seeds and even of cultured embryos. In general, germination of intact *Musa* seeds is variable and often not very successful. Both seed viability and seed dormancy can impact germination. In one study, less than half the seed harvested from 20 EAHB cultivars had an embryo (Ssebuliba et al., 2006), and so were viable, even before any impact of dormancy might be considered. Another example concerns the plantain breeding program in Onne, Nigeria, where only 1% of intact hybrid seeds germinated (Swennen et al., 1992). For plantain embryos extracted and cultured aseptically, the germination success rate was around 12% (Vuylsteke et al., 1990). So, for some seeds, embryo extraction and culture might have overcome a dormancy factor associated with the intact seed. However, the low success rate of embryo culture in this study raises the possibility of embryo dormancy.

Low germination of excised embryos *in vitro* in cultivars of *Musa* (AAA) EAHB (Ssebuliba et al., 2006) and other *Musa* hybrids (Bakry, 2008) is consistent with physiological dormancy of the embryo (embryo dormancy), although low germination of excised embryos is not always typical of *Musa* species. For example, more than 90% of embryos of *M. balbisiana* (BB), excised from fresh seeds (Bakry, 2008) and from seeds stored at room temperature for up to 18 months (Cox et al., 1960; Stotzky and Cox, 1962), developed into plantlets.

### 5.1 Is embryo dormancy heritable?

Breeding of bananas involves culturing the embryos of the seed of the hybrid progeny to grow new plants. This procedure overcomes limitations of using whole seed but the recovery of live plants from hybrid embryos undergoing culturing procedures is often low (Bakry, 2008), in the order of 20%-30% compared with 99% success for embryos of *M. balbisiana* (Bakry, 2008).

We hypothesised that embryos can express dormancy (D/d) and that this is inherited. We applied an elementary genetic analysis (Griffiths et al., 2000) to the data of Bakry (2008), because these data provided the parentage of the seeds and the success of embryos in culture.

We estimated the phenotypic ratio of the dormant:non-dormant character and then inferred possibilities of genetic composition (D/D, D/d, d/d) of parent(s) from the ratio. Simple Mendelian ratios were chosen in this exercise. We constructed a table indicating possible genotypes for dormancy for as many of the parents used by Bakry (2008) as possible (Table 2).

This provided some evidence that segregation of embryo dormancy in banana seed may follow Mendelian principles, although the patterns of

**Table 2** Summary table showing hypothesised make up of dominant (D) or recessive (d) dormancy gene(s) of parent

Genome constitution, cultivar/selection of parents	Dominant (D) / recessive (d)
BB, 'Lal Velchi'; 'Pisang Klutuk Wulung'	d/d
AA, 'Pisang Madu'; 'Calcutta 4'; 'Tha 052'; 'Long Tavoy'; 'SF265'	D/d
AAAA, 'Pisang lilin' autotetraploid (Bakry et al., 2007).	D/d, D/d

inheritance are likely to be complex. Initial explorations suggest that the B genome appears to be recessive (d/d) while the A genome could be D/d or other combinations, perhaps depending on the origins of the A genome.

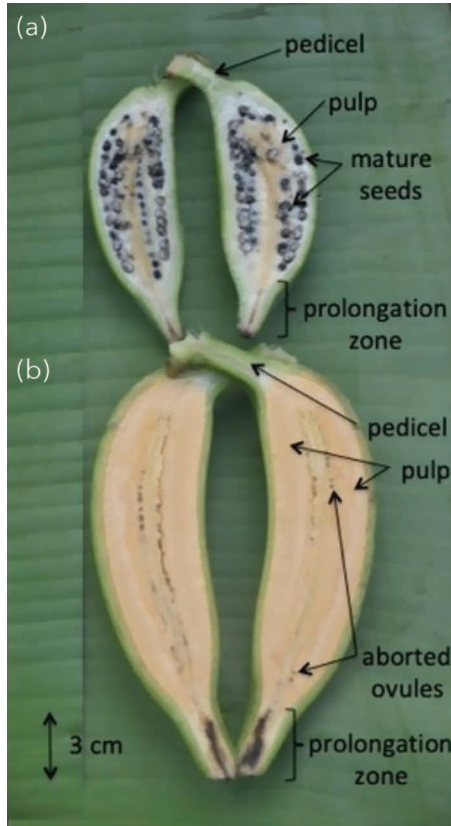
If it could be shown in a segregating population that embryo dormancy was inherited, then it could be selected against and increase the probability that cultured embryos will grow into plants. This would increase the efficiency of breeding by making more plants available for screening.

## 6 Parthenocarpy and sterility

Parthenocarpy is the growth of a fruit without fertilisation and seed development (Sinnott, 1960), or more broadly, producing fruit without seeds (Holmes, 1979). The broadly accepted hormonal mechanism of fruit formation and growth is that pollination itself produces hormones such as gibberellins and auxins, and the fertilised ovary subsequently produces hormones essential for fruit development. Fruit may be parthenocarpic without pollination or ovule fertilisation (vegetatively parthenocarpic), such as in banana, or it may be parthenocarpic because the embryo of the fertilised ovule aborts (stenospermocarpic), such as in sultana (*Vitis vinifera*), but the berry continues to grow (Atwell et al., 1999). While hormone mechanisms have dominated thinking about fruit growth, there are resource implications as well.

Pulp development in wild bananas is stimulated by seed development. Edible bananas are vegetatively parthenocarpic (Simmonds, 1962) and develop pulp autonomously from tissues on the ovary wall in the absence of pollination (Fig. 14). However, for edible bananas that set some seed, pulp development is more nuanced and will have a contribution not only from pulp that develops as a consequence of the autonomous process of vegetative parthenocarpy, but also from pulp that develops in association with seed set, if present (Simmonds, 1962). Partial parthenocarpy and partial sterility can be expressed in a single banana fruit at the same time. These observations contribute to the discussions in the literature about whether or not parthenocarpy is independent of sterility.

Sterility is the failure to form a seed and it has many causes. Female and/or male gametes may be defective because of faults in meiosis (Atwell et al., 1999) (see Section 2.2 on gametogenesis). Pollination and fertilisation are complex



**Figure 14** Longitudinal sections of mature fruit of a) seeded *Musa balbisiana* 'Tani' (BB) with mature seeds and a limited amount of pulp generated by the seeds, and b) parthenocarpic *Musa* (AAB) plantain 'Obino L'Ewai', with autonomous pulp originating from the ovary walls, and aborted ovules along the line of the placental tissue. In both fruits, flower parts have abscised, the prolongation zone shows necrotic tissues of the nectaries and the pedicel attaches the fruit to the female peduncle.

processes and failure in either may contribute to sterility in the absence of faulty meiosis. Edible banana clones generally do not form seeds, the latter reflecting in part their tendency to low pollen fertility (Simmonds, 1962). Provided with a fertile pollen donor, a low level of viable seed formation can occur (Shepherd, 1960). Some triploids, such as those from the commercially important *Musa* (AAA) Cavendish subgroup, are highly female sterile. Even in this highly female sterile sub-group, a few seeds have been obtained when very large numbers are crossed with pollen from Cavendish clones (Aguilar-Morán, 2013).

Simmonds (1962) warned of the confusion that has arisen in the literature from the use of the terms parthenocarpy and 'seedless' as if they are synonymous. He points out that a fruit in which pulp development has occurred can be

seedless due to the abortion of fertilised ovules, that is, the fruit is seedless but not parthenocarpic. Indeed, there is a wide range of behaviours in pulp development, from seed-dependent to autonomous, and it can be incorrect and confusing to assume that a seedless fruit is parthenocarpic. Simmonds (1953) urges that to avoid confusion, the term 'parthenocarpy' is used in a defined and limited way, as for vegetative parthenocarpy, and concludes that the term 'seedless' is an ambiguous description for 'a condition that has several different causes' (Simmonds, 1962).

### 6.1 The relationship between parthenocarpy and sterility

In banana breeding, the relationship, if any, between parthenocarpy and female sterility is important because if they are independent, then to increase seed set, the focus needs to be on managing sterility. On the other hand, if parthenocarpy increases sterility then this needs to be borne in mind, especially for cultivars that appear almost completely female sterile. The concern is that any increase in parthenocarpy may eliminate seed set, contributing to the 'fertility crisis' in banana breeding.

In the past a case has been made that parthenocarpy and sterility are independent, involve different tissues and organs and result from two separate but interrelated genetic systems (Dodds and Simmonds, 1948; Simmonds,

		→ Increasing parthenocarpy →		
		A1.	B1.	C1.
Increasing sterility ↓		Many seeds Pulp volume proportional to seed number	Many seeds Pulp volume more than expected from seed number	No genotypes found
		A2. Some or few seeds Pulp volume proportional to seed number	B2. Some or few seeds Pulp volume more than expected from seed number	C2. Some or few seeds Pulp volume much more than expected from seed number
		A3. No seeds No pulp	B3. No seeds Some pulp	C3. No seeds Pulp volume generated without seeds

**Figure 15** The phenotypic relationship between parthenocarpy (columns A -> C) and sterility (rows 1 -> 3) in different genotypes of bananas. A fertile genotype in A1 or A2 may show the behaviour of A3 if pollination fails or does not occur. Similarly, genotypes in B1 or B2 can appear as B3 and those in C2 can appear as C3. Based on Simmonds (1962).

1962). Parthenocarpy and sterility have a range of phenotypic expressions that give the appearance of a continuum (Fig. 15).

Simmonds (1962) concluded that the relationship between parthenocarpy and sterility in banana is not primarily causal, pointing out that failure of pollination and/or fertilization leads to sterility, but sterility does not lead to parthenocarpy (A3, Fig. 15). The logic behind his conclusion that parthenocarpy and sterility are independent systems is illustrated in Fig. 15. Simmonds (1962) acknowledged that this picture is not complete because there is no example of a highly parthenocarpic, highly fertile genotype (C1, Fig. 15). The absence of a genotype at C1 raises the possibility that a high level of parthenocarpy and a high level of fertility are not compatible. To take this possibility into account, Simmonds (1962) qualified his view on the independence of parthenocarpy and sterility with the statement that parthenocarpy might be responsible for failure to set viable seed, but if so, this effect is small. He commented that parthenocarpy and sterility are correlated in banana because 'they both have been subjected to human selection' (Simmonds, 1962).

Evidence from segregating populations indicated that parthenocarpy in banana is controlled by three independent but complementary dominant genes ( $P_1$ ), a major switch gene ( $P_1$ ) and two minor modifier genes ( $P_2$  and  $P_3$ ) (Dodds and Simmonds, 1948; Simmonds, 1953; Ortiz and Vuylsteke, 1995b). Seeded bananas, such as 'Calcutta 4' have double recessive genes for the  $P_1$  switch gene involved in parthenocarpy, and so show no parthenocarpy (Ortiz and Vuylsteke, 1995b).

Sardos et al. (2016) referenced these earlier works in a genome-wide association study of *Musa* that focussed on 'seedlessness'. They noted that, unlike parthenocarpy, which is driven by a major dominant and several minor genes, sterility in banana is due to structural and genetic factors and reiterated that it is independent of parthenocarpy, although likely to have been co-selected with parthenocarpy by humans.

Parthenocarpy and sterility are of interest in fruits other than banana. In tomato, the relationship between parthenocarpy and sterility appears to parallel that of bananas, with observations suggesting that parthenocarpy and sterility in tomato are independently functioning systems, but with the qualification that parthenocarpy can lower seed set. For example, in parthenocarpic tomatoes, sterility lies with the pollen, not because it is sterile, but because the gene responsible controls the release of pollen from the anthers so the ovules are not pollinated and seeds are not formed (Gorguet, 2007). This is an example of structural and genetic factors acting together resulting in sterility. Independently, parthenocarpy gene(s) disturb the 'hormonal balance' of the wall tissues resulting in the development of pulp. In a separate study, paclobutrazol, which blocks biosynthesis of gibberellin, promoted seed production in a parthenocarpic tomato resulting in the production of a few seeds (Ohkawa et al., 2012), linking a

change in the hormone balance associated with parthenocarpy with a change in sterility. Gorguet (2007) claimed that the *pat-1* gene, which is partly responsible for parthenocarpy in tomato, not only changed hormonal balance but disturbed meiosis in a proportion of female gametes. Takisawa et al. (2018) speculated that down-regulation of the *Pat-k* gene, which caused both parthenocarpy and low seed set in the parthenocarpic tomato cultivar MPK-1, could cause abnormal ovule formation, reducing the number of viable seeds formed.

### **6.1.1 How might parthenocarpy affect seed fertility?**

An early study of parthenocarpy and sterility using edible diploids of banana concluded that, while many edible diploids produce little viable pollen, parthenocarpy did not lead to male sterility because at least one of the five parthenocarpic diploids studied, 'Pisang lilin', had abundant viable pollen that successfully pollinated seeded diploids (Dodds, 1943). 'Pisang lilin' was, however, highly female sterile demonstrating a separation of female and male fertility. This phenomenon was also observed in the parthenocarpic offspring of crosses between the seeded (female) and edible (male) diploids (Dodds, 1943). In all these cases both parthenocarpy and female sterility were inherited through the pollen. Dodds (1943) concluded a common factor acting in parthenocarpy and female sterility, suggesting that the auxin responsible for parthenocarpy might disrupt ovule development. This conclusion was later discounted by Simmonds (1962) on the basis that parthenocarpy can occur in seeded fruits (B1, Fig. 15) and that growth factors other than auxin are involved in pulp-filling. A later study using hybrids of 'Pisang lilin' and seeded diploids found evidence suggesting genetic modifiers of parthenocarpy diminished female fertility (Dodds and Simmonds, 1948).

So, the question remains: is there a fertility crisis in banana because it is parthenocarpic? To recap, Simmonds (1962) gave an example of high sterility with nil parthenocarpy (A3, Fig. 15) consistent with this not being the case. However, he could find no example of a highly fertile, highly parthenocarpic genotype (C1, Fig. 15) suggesting that while sterility can occur in the absence of parthenocarpy, parthenocarpy *per se* might have a negative impact on viable seed set, via its influence on female fertility.

Might the interpretation of a relationship between parthenocarpy and sterility be confounded by the possibility of a 'common factor' influencing both? Knowledge about parthenocarpy and sterility in bananas is derived mainly from studying pollen production and seed set in a wide range of genotypes in crossing experiments. When examining their relationship across genotypes, sometimes the two appear correlated, either negatively (A1 to B2 to C3 in Fig. 15) or positively (A3 to B2 in Fig. 15) and sometimes there is no association at all (A1 to A3 or B1 to B3 in Fig. 15). A hypothesis to account for these

observations may indeed lie in a common factor that can affect the expression of parthenocarpy and sterility, but without any need for parthenocarpy to influence sterility or the reverse.

Whether parthenocarpy affects processes involved in viable seed sets will in part depend on:

- 1 Whether genes associated with parthenocarpy in banana influence or act on the expression of processes in the pathway to seed set. For example, genetic modifiers identified in diploid bananas by Dodds and Simmonds (1948), and the apparent influence of *pat*-genes linked with parthenocarpy on meiosis (Gorguet, 2007) and ovule formation (Takisawa et al., 2018) in tomato.
- 2 Whether proximate processes arising from the expression of parthenocarpy [autonomous pulp-filling] in the maternal parent negatively impact processes essential for viable seed set. For example, interaction and/or competition between pulp-filling and pollen-tube growth and guidance (Simmonds, 1962). For this to occur, pulp-filling processes would need to coincide with or precede seed set processes.

Genetic controls lie at the base of the phenological expressions of parthenocarpy and sterility. More investigations are needed to untangle their relationships in banana. We are left with the conclusion that in bananas parthenocarpy and sterility are independent, but the question of whether or not parthenocarpy increases sterility is still unresolved. We suggest that this is likely to be largely unimportant for breeding, except in the case of edible, highly female-sterile clones, where the high level of sterility might reflect the influence of parthenocarpy, and therefore some dependence between the processes. Although parthenocarpy and sterility are for the most part independent processes, an in-depth understanding of the genetics underlying both processes and their relationship is fundamental to improve seed set, particularly in edible, highly female sterile banana clones.

## **6.2 Parthenocarpy as an adaptive feature**

Picarella and Mazzucato (2019) described parthenocarpy as an adaptive feature, suggesting it could exert an advantage in multi-seeded fruit species under sub-optimal pollination regimes where too few seeds are formed to support fruit growth. In this circumstance, parthenocarpy would promote the development of an attractive fruit with which to spread the few seeds formed, so providing a selective advantage.

If the hypothesis of Picarella and Mazzucato (2019) is supported, then a corollary to be expected would be high viability and capacity for germination

in the few seeds per fruit. This seems not to be the situation in banana breeding programs based on current practices of seed germination. That parthenocarpy might depress viable seed set in banana seems at odds with the hypothesis of Picarella and Mazzucato (2019). Perhaps selection towards spread of viable seed contributes to adaptation in some plant species, but in bananas, co-selection of parthenocarpy and sterility by humans has reinforced parthenocarpy, favouring pulp-filling over production of viable seed. We suggest that female sterility is the issue of importance to banana breeders, not parthenocarpy, because at this stage, breeding programs need more seeds.

### **6.3 A gene linked to female sterility?**

The genome-wide association analysis conducted by Sardos et al. (2016) in bananas identified one strong candidate gene, a putative orthologous gene to histidine kinase CK11, as being associated with female sterility, based on work with *Arabidopsis*. In *Arabidopsis*, the gene histidine kinase CK11 is involved in gametophyte development, and, once silenced, resulted in female sterility (Deng et al., 2010). Subsequent analysis showed that the orthologous gene in banana was found on a region of the chromosome most significantly associated with 'seedlessness', strengthening its link to female sterility. Because of its association with sterility, it would be worthwhile looking at CK11 expression in experiments manipulating the environment, such as soil water supply, modification of the atmosphere around the bunch, pollination or not, and using edible banana clones, including those with a reputation for no seeds and those with some seeds. This would allow for examination of genetic and environmental components of sterility in banana, although issues with sampling of fruit or fruit components would require some thought.

## **7 Conclusion and future trends**

Banana breeding programs will need to play an increasing role in providing solutions to existing and emerging threats to production, such as the devastating threats of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) Tropical race 4 (TR4) and Black Leaf Streak caused by *Pseudocercospora fijiensis*. This will require the integration of conventional breeding techniques as well as modern molecular breeding tools for more efficient hybrid development, selection and delivery. The success of breeding using these techniques will depend on conducting recombinations focussed on target traits and generating sufficient progeny for selection, which is largely affected by varying levels of sterility. An increase in the number of possible recombinations to create large hybrid progenies to select from requires overcoming the obvious fertility challenges. While considerable efforts have been made in this area which underpins the progress made so far in banana breeding, much is still left to be done.



As summarized in Fig. 1, successful fertilization and seed production are affected by several factors including pollen quantity, viability, potency and compatibility of pollen-pistil interactions as well as the ability of the female gametophyte to be fertilised and developed into a viable seed. These processes are in turn affected by genotype and the environment. Therefore, a sound understanding of these processes in the context of the wide variability in bananas is critical. Most of the limited work on floral biology has focussed so far on *Musa* species forming a solid basis, but for the future this should be extended to edible cultivars frequently used in crossing schemes as sources of desirable traits, particularly including the triploid category where infertility is most obvious. This can lead to a more targeted approach where critical processes can be optimized to improve seed set and meet crossing objectives.

Shepherd (1954) first established the best time to pollinate (between 7 am and 10 am), which has so far been adopted to maximize pollen viability and stigma receptivity. However, there may be variations in bract opening times and sequence with different genotype and environment interactions. Moreover, the short window in which to pollinate limits the number of crosses that can be made in a day. Further studies on understanding bract opening may inform further optimization of pollination time. On-going work on the application of pollen germination medium to enhance stigma receptivity has shown promising results, giving rise to increased seed set, at least with the use of one male parent, with potential for further exploration. Application of growth regulators and other stimulating substances on the stigma to stimulate pollen germination and pollen tube growth (Wu et al., 2008) may also be explored.

Establishing pollination blocks in environments that favor seed set as described by Ortiz and Vuylsteke (1995a) in plantain and Ssebuliba et al. (2009) in EAHB 'Matooke' cultivars may be pursued and may need to be separated from progeny testing. Progeny testing requires an ideal growing environment and/or environments in which certain biotic and abiotic factors are present to provide sufficient selection pressure. Similarly using chemicals like boron which is well known to enhance pollen fertility and stigma receptivity (Peñaloza and Toloza, 2018) also needs to be explored.

Beyond the mechanical manipulations, Sardos et al. (2016) identified a putative gene associated with 'seedlessness' in *Musa*. The use of RNA-silencing technologies with applications in other crop improvement programs (Guo et al., 2016) is dependent on such findings and can also be applied to banana. Ultimately the challenge is that fertility is desirable for breeding while sterility is desirable in final products to ensure edibility. Further characterization of regulatory genes or sequences implicated in these processes, in combination with breeding strategies, are required before modern tools such as gene editing using CRISPR/Cas9 (Tripathi et al., 2019) can be of use. A thorough

understanding of the genes and processes involved in sterility and fertility is required to be able to effectively manipulate this using gene-editing techniques.

Hybridization and seed production are central to every crop breeding effort, and knowledge on these processes is unfortunately limited for bananas. In the era of modern molecular tools such as genomic selection and association genetics which hold considerable promise for banana (Nyine et al., 2017, 2019), multiple large progeny populations are required for their applications to increase breeding efficiency. Significant investments on advancing floral biology research in banana could revolutionize breeding efforts to deliver genetic gains in farmer's fields.

## 8 Where to look for further information

For a further detailed discussion on reproductive biology in *Musa*, see Fortescue and Turner (2011); for detailed anatomical studies of floral organogenesis in *M. velutina*, *M. ornata* and the triploid edible cultivar 'Go Sai Yung', see Kirchoff (1992) and Kirchoff (2017). For a functional approach to bunch formation including the occurrence of hermaphrodite flowers, see Turner and Gibbs (2018). For a description of the processes of parthenocarpy, Joldersma and Liu (2018) provide a recent review on the role of phytohormones in the induction of parthenocarpy, and the molecular and genetic basis of parthenocarpy. Mizuta and Higashiyama (2018) provide a very useful recent summary of the known mechanisms of pollen guidance. More information on banana flowers in general can be found on the ProMusa website, which is a platform for scientific discussions on banana (<https://www.promusa.org/Banana+flowers>). The Breeding Better Bananas project (<https://breedingbetterbananas.org/>), which seeks to improve banana breeding in Tanzania and Uganda, also provides information on current research to address the fertility crisis in bananas.

## 9 Glossary

**Androecium** the stamens, the male reproductive unit.

**Anther** the part of a stamen that produces pollen grains.

**Anthesis** the opening of a flower, or in *Musa* the lifting of the bracts on an emerged bunch.

**Archeporia** cells that form the spore mother cells, which give rise to the egg cell in the embryo sacs.

**Asynapsis** lack of pairing of chromosomes in meiosis.

**Autonomous** independent.

**Bract** a modified leaf that on the female and male peduncle subtends a number of flowers. It usually dehisces after anthesis.

**Bunch** a horticultural term for the inflorescence or thyrses, or for the female peduncle carrying the edible fruit.

**Carpel** the female reproductive unit or gynoecium. In *Musa* the ovary consists of three fused carpels.

**Cincinnus** a type of inflorescence, a scorpioid cyme. In *Musa*, commonly known as a 'hand' of fruit.

**Cultivar** a term that blends the words 'cultivated + variety'. It is a term for cultivated plants and does not apply to those plants that occur in the wild. Cultivars include hybrids that are either deliberate or accidental, and cultivated plants selected from wild populations and recognised as a separate entity from the wild plants. *Musa* cultivars arise from the intended actions of people. They are parthenocarpic with various degrees of residual fertility.

**Desynapsis** separation of paired chromosomes in meiosis.

**Dichogamous** pistils and stamens mature at different times, in the case of banana on different flowers.

**Dormancy** the failure of a viable embryo to germinate despite being in a suitable environment.

**Edible** a commonly used term for a banana ovary with pulp of parthenocarpic origin and without seeds. Edible bananas can have a residual fertility and when pollinated, can set seed, albeit in very low quantities.

**Embryo** the product of a fertilised egg sac.

**Embryo sac** the megaspore of flowering plants within the ovule.

**Energy-dependent** a biological process requiring metabolic energy to proceed.

**Fertilisation** the combination of female and male gametes to produce an embryo. The process takes place within an ovule.

**Fertility** the capacity to produce offspring through sexual processes.

**Flower** a blossom made up of the ovary, petals, sepals, pistil and stamens.

**Flowering** the emergence of an inflorescence (bunch) from the pseudostem.

**Gametogenesis** the process of formation of female or male gametes for sexual reproduction.

**Gynoecium (or Carpel)** the pistils, comprising the stigma, style and ovary, the female reproductive unit.

**Hand** a horticultural term describing a cincinnus of flowers subtended by a single bract.

**Hermaphrodite** a flower with functional female and male parts capable of self-fertilisation.

**Hybridity** a plant derived from a cross between two different parents and which may express sterility.

**Hybridization** the process of cross pollination of two parents to produce progeny.

**Infertility** unable to produce offspring by sexual means.

**Involute** curling inwards, as involute curling of a bract.

**Locule** a cavity. The ovary of *Musa* normally has three locules.

**Megaspore** the larger spore in flowering plants that gives rise to the female gamete.

**Meiosis** cell division where the homologous chromosome number is reduced from diploid to haploid.

**Microspore** cells arising from the pollen mother cells that mature into pollen grains.

**Monoecious** female and male flowers produced on the same inflorescence.

**Musa species** taxa of the genus *Musa* occurring in the wild that can be classified into the species or sub-species taxon. Propagated by seeds and vegetatively.

**Nectar** sugary secretion arising from tissues at the apex of the ovary. It collects in the perianth.

**Ovary** the organ of female flowers that become the fruit in edible cultivars. Contains ovules that when fertilised, subsequently become seed in the species of *Musa*.

**Parthenocarpy** the growth of a fruit in the absence of seeds. In *Musa*, the most obvious expression is the autonomous development of pulp in edible cultivars. Parthenocarpy is a quantitative trait.

**Parthenocarpy, vegetative** the growth of a fruit without the stimulation of pollination or ovule fertilisation, characteristic of edible bananas.

**Peduncle** the external part of the true stem of a banana plant normally with three sections: sterile, female and male.

**Pistil** the stigma, style and ovary; the gynoecium.

**Ploidy** a set of chromosomes, hence haploid (one set), diploid and triploid.

**Pollen mother cells** the larger spores giving rise to pollen grains.

**Pollen viability** a measure of the portion of a population of pollen grains that are living.

**Pollen tube** an outgrowth of a germinating pollen grain. It contains the male gametic material.

**Pollen tube guidance** the anatomical and physiological mechanisms by which pollen tubes move from the surface of the stigma through the style to the locule and subsequently to the ovules. It includes the exclusion of pollen tubes from fertilised ovules.

**Prolongation region** the closure of the distal end of the locules. The stylar end of the ovary.

**Receptivity** the stage in stigma development when pollen on the surface can germinate and the pollen tube can grow into the stigmatic surface.

**Restitution** failure of a cell division in meiosis producing  $2n$  gametes.

**Restitution, double** Failure of both cell divisions in meiosis, producing  $4n$  gametes. Meiosis avoidance.

**Scorpioid** an inflorescence with a curved axis.

**Seed** a mature fertilised ovule where the integuments form the seed coat that contains the embryo and endosperm. Also **botanical seeds**, to distinguish

them from vegetative material such as suckers, also called 'seed' – a horticultural term.

**Seedless, seedlessness** fruit without seed, not to be confused with parthenocarpy since the fruit of *Musa* species can be seedless if either not fertilised or fertilisation has failed, and in the absence of parthenocarpy.

**Staminodes** imperfectly formed stamens with no or few pollen that are likely to be infertile.

**Sterility** the inability of gametes to form zygotes, seedless. Sterility is a quantitative trait in *Musa*.

**Sterility, female** in *Musa*, a plant with ovules unable to produce seed although the flower has been pollinated with fertile pollen.

**Sterility, male** in *Musa*, a plant the pollen of which is unable to fertilise an ovule and form seed.

**Stigma** the tissue that surmounts the style. When receptive, pollen grains germinate on the stigma.

**Style** the slender connecting organ between the locule and the stigma. The style contains stylar canals.

**Sub-locular** the basal region of the ovary beneath the locule.

**Synapsis** pairing of chromosomes during meiosis.

**Transitional** if present, transitional flowers appear between the basal female flowers and the distal male flowers. Transitional flowers are characterised by reduced development of the gynoecium and androecium, but the non-functional ovaries are larger than in the male flowers. There are two types of transitional flowers: 'neuter' which are retained on the peduncle, and 'transitional' flowers that are functionally male and fall from the peduncle at after flowering.

**Univalents** unpaired chromosomes.

**Viability** a measure of the proportion of a population that is living.

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